

THE UNIVERSITY OF MELBOURNE

DOCTORAL THESIS

**Application of Proanthocyanidin as Adjunctive
Treatment for Root Carious Lesions**

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Submitted in total fulfilment of the requirements of the degree of
Doctor of Philosophy

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July 2020

Abstract

In an aging world population, improvement in oral health awareness and behaviours has resulted in the progressively increasing retention of natural teeth in the geriatric population in developed countries. As a result, the number of root surfaces at risk of developing caries is increasing due to age-associated gingival recession and subsequent exposure of caries-prone cervical root dentine to the oral environment. Root carious lesions (RCLs) have a complex aetiology and can be challenging to treat, due to, in part, difficulties in restoring the structural composition of root dentine. Specifically, root dentine contains a considerable amount of organic materials which play a crucial role in the progression and treatment of root caries. Application of a naturally derived collagen cross-linking agent - proanthocyanidins (PA), which could cross-link and bio-modify the dentinal collagen, has been considered as a promising adjunct in RCL treatments. The aim of the thesis was to investigate the effects of PA used as an adjunctive treatment for RCLs. In addition, further evidence was provided regarding the efficacy of currently recommended treatments for RCLs in the laboratory setting.

Using an *in vitro* artificial RCL model, the effects of several frequently used fluoride (F) regimens on RCLs were investigated. It was observed that the application of silver diammine fluoride/potassium iodide (SDF/KI) improved the nano-mechanical properties and mineral density (MD) of demineralised root dentine. Both F varnishes and 5,000 ppm F toothpastes promoted the remineralisation of artificial RCLs and their treatment efficacy could be enhanced by combined application. The performance of different glass ionomer cements (GICs) such as resin-modified GIC (RMGIC), high viscosity GIC (HVGIC) and casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) modified low viscosity GIC when used as restorative materials for RCLs was partially dependent on the characteristics of the dentine substrate for bonding and the inherent formulations and properties of the GIC material itself.

When PA was used as an adjunct to the above-mentioned F agents, it had the potential in strengthening the demineralised dentine matrix and enhancing collagen-mineral interactions for improved uptake and distribution of remineralising ions, nano-mechanical properties and MD of demineralised root dentine in the subsurface areas. Furthermore, considering the acidic nature of PA in addition to its cross-linking capability and multiplicity of functional groups, PA was used as a dentine conditioner for GIC bonding. It was determined that PA conditioning

did not influence the bond strength of GICs to both sound and demineralised root dentine significantly in the short term. However, the interfacial interaction between GICs and PA-conditioned root dentine was compromised with an increased failure mode of interfacial debonding.

The results expounded in this thesis indicate the potential remineralisation-enhancing effects of PA when used as an adjunct to F regimens in non-invasive treatments of demineralised root dentine in the laboratory setting. However, using PA as a conditioner for GICs in the restorative treatment of demineralised root dentine did not show additional benefits for bonding in the short-term.

Declaration

This is to certify that:

- i) The thesis comprises only my original work towards the degree of Doctor of Philosophy except where acknowledged.
- ii) Due acknowledgement has been made in the text to all other material used.
- iii) The thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Jing Cai

Preface

The thesis research was carried out at the Melbourne Dental School, University of Melbourne, Australia. The ethical approval was obtained from the University of Melbourne Human Research Ethics Committee (HREC ID:164633.1).

This is a thesis with publications. Part of Chapter 1, Chapter 2 and Chapter 3 are author-accepted versions of manuscripts according to University of Melbourne guidelines. The corresponding published papers are included as Appendix 1.

- Chapter 1: Jing Cai, Joseph E.A. Palamara, David J. Manton, Michael F. Burrow. Status and progress of treatment methods for root caries in the last decade: a literature review. *Aust Dent J.* 2018;63(1):34-54. DOI: 10.1111/adj.12550.
- Chapter 1: Jing Cai, Joseph E.A. Palamara, Michael F. Burrow. Effects of Collagen Crosslinkers on Dentine: A Literature Review. *Calcif Tissue Int.* 2018;102(3):265-279. DOI: 10.1007/s00223-017-0343-7.
- Chapter 2: Jing Cai, Michael F. Burrow, David J. Manton, Yuka Tsuda, Eman G. Sobh, Joseph E.A. Palamara. Effects of silver diamine fluoride/potassium iodide on artificial root caries lesions with adjunctive application of proanthocyanidin. *Acta Biomater.* 2019;88:491-502. DOI: 10.1016/j.actbio.2019.02.020.
- Chapter 3: Jing Cai, Michael F. Burrow, David J. Manton, Rita Hardiman, Joseph E.A. Palamara. Remineralising effects of fluoride varnishes containing calcium phosphate on artificial root caries lesions with adjunctive application of proanthocyanidin. *Dent Mater.* 2021;37(1):143-157. DOI: 10.1016/j.dental.2020.10.021.
- Chapter 4: Jing Cai, Michael F. Burrow, David J. Manton, Joseph E.A. Palamara. Evaluation of adhesion and interfacial interaction of conventional GIC, RMGIC and CPP-ACP-modified GIC to sound and artificially induced caries-affected root dentine with proanthocyanidin pre-treatment (Submitted to *Journal of Dental Research*).

The candidate was the primary author on all manuscripts, and conceptualized the studies, implemented the work, analysed the data and wrote the first draft of the manuscripts under the supervision of Prof Michael F. Burrow, Prof David J. Manton and A/Prof Joseph E.A. Palamara. Ms Yuka Tsuda and Ms Eman G. Sobh contributed substantially to the design and implementation of the work in Chapter 2. Dr Rita Hardiman contributed heavily to the micro-CT analysis of the work in Chapter 3. All the other authors contributed their expertise from different aspects, revised all the manuscripts critically for important intellectual content and final approval of the version to be published.

No work was carried out by the candidate prior to enrolment, and no work has been submitted for other qualifications. No third-party editorial assistance was used for the formatting of this thesis.

Publications and Presentations

Publications

1. Cai J, Palamara J, Manton DJ, Burrow MF. Status and progress of treatment methods for root caries in the last decade: a literature review. *Aust Dent J.* 2018;63(1):34-54. DOI: 10.1111/adj.12550.
2. Cai J, Palamara JEA, Burrow MF. Effects of Collagen Crosslinkers on Dentine: A Literature Review. *Calcif Tissue Int.* 2018;102(3):265-279. DOI: 10.1007/s00223-017-0343-7.
3. Cai J, Burrow MF, Manton DJ, Tsuda Y, Sobh EG, Palamara JEA. Effects of silver diamine fluoride/potassium iodide on artificial root caries lesions with adjunctive application of proanthocyanidin. *Acta Biomater.* 2019;88:491-502. DOI: 10.1016/j.actbio.2019.02.020.
4. Cai J, Burrow MF, Manton DJ, Hardiman R, Palamara JEA. Remineralising effects of fluoride varnishes containing calcium phosphate on artificial root caries lesions with adjunctive application of proanthocyanidin. *Dent Mater.* 2021;37(1):143-157. DOI: 10.1016/j.dental.2020.10.021.

Presentations

1. Cai J, Burrow MF, Manton DJ, Palamara JEA. Effects of silver diamine fluoride/potassium iodide on artificial root caries lesions with adjunctive application of proanthocyanidin. Presented at International Association of Dental Research (IADR) General Session 2018 London, UK.
2. Cai J, Burrow MF, Manton DJ, Hardiman R, Palamara JEA. Remineralising effects of fluoride varnishes containing calcium phosphate on artificial root caries lesions with adjunctive application of proanthocyanidin. Presented at IADR-Asia Pacific Regional Session 2019 Brisbane, Australia.

Acknowledgements

First and foremost, I would like to thank my supervisors Associate Professor Joseph Palamara, Professor Michael Burrow and Professor David Manton. They have been not only great supervisors, but also mentors who give me endless and invaluable inspiration in my academic career in dentistry.

I am grateful to my principal supervisor, Associate Professor Joseph Palamara, for his unfaltering guidance and support throughout my whole projects. His knowledge, professionalism and enthusiasm in academia have greatly inspired me. I am always impressed by his excellent knowledge, advice, and insightful discussion. My great gratitude extends to my co-supervisor Professor Michael Burrow, who was once my principal supervisor before he moved to Hong Kong. He led and encouraged me to this challenging but rewarding research field from the beginning. His enormous guidance, support and encouragement never fail to give me the courage to face all the challenges throughout my Ph.D. journey. I am also deeply grateful for my co-supervisor Professor David Manton. His profound knowledge in cariology, timely response every time, excellent advice, endless support and help have contributed greatly to my projects.

I would like to acknowledge the professional assistance of Dr Rita Hardiman, Dr James Fernando, Mr David Stalder, Mr Roger Curtain and Ms Wurihan Wu during the implementation of my work and the help from Ms Carolyn Carter in Oral and Maxillofacial Surgeon Services in tooth collection. I would like to express my special gratitude to Ms Su Toulson, who not only provided me with professional technical assistance but also gave me tremendous psychological and tangible support during my stay in Melbourne.

I greatly value the friendship within the Ph.D. group in dental materials, thank Dr Chirayu Ruengrungsom, Dr Negin Alvanfroush, Dr Yuka Tsuda and Dr Eman Sobh for sharing all the happiness and sorrow with me throughout my research journey. I feel privileged to have the opportunity to conduct my Ph.D. program at the Melbourne Dental School and I wish to thank all the friends and colleagues here who have shared this journey with me. In no particular order, I would like to sincerely thank Dr Antonia Celentano, Dr Tami Yap, Dr Nidhi Medara, Dr

Acknowledgements

Nikki Liew, Dr Matthew Lim, Dr Elisabeth Sari, Dr Marwa Saudi, Dr Zhe Sun and Dr Yi Fan for their support and help.

I acknowledge the China Scholarship Council and University of Melbourne for providing me with financial support to pursue my doctoral degree; Australian Dental Research Foundation and Melbourne Dental School for the research funding to support my projects. I am also grateful to Prof Stuart Dashper for chairing my advisory committee and the administrative staff at the Melbourne Dental School, especially Ms Janelle Christie and Ms Cassie Kearns for their support.

Finally, I would like to extend my great appreciation to my parents who have made all my academic endeavours possible with their relentless support, love and encouragement; to my boyfriend, Dr Min Liu, for his caring and always being there for me, keeping me laughing and company; to my flatmate, Dr Ye Tian, an intelligent, easy-going and kind young lady, who has shared all the fantastic memories with me during these four years; and to all the friends from the community of Chinese doctoral students in Melbourne, who have made my overseas study full of wonderful moments.

Jing Cai

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List of Abbreviations

AAC	Acellular afibrillar cementum
ABR	Acid-base resistant
ACP	Amorphous calcium phosphate
AEFC	Acellular extrinsic fibre cementum
AFM	Atomic force microscopy
AmF	Amine fluoride
ANOVA	Analysis of variance
APF	Acidulated phosphate fluoride
ART	Atraumatic restorative treatment
Asp	Aspartic acid
BGP	Bone Gla (γ -carboxyglutamate) protein
BMP	Bone morphogenetic proteins
BSP	Bone sialoprotein
Ca	Calcium
Ca-P	Calcium-phosphate based compounds
CaF ₂	Calcium fluoride
CD14	Cluster of differentiation 14
CEJ	Cemento-enamel junction
CHX	Chlorhexidine
CIFC	Cellular intrinsic fibre cementum
CO ₂	Carbon dioxide
CPP	Casein phosphopeptide
CPP-ACFP	Casein phosphopeptide stabilised amorphous calcium fluoride phosphate
CPP-ACP	Casein phosphopeptide stabilised amorphous calcium phosphate
CPWV	Clinpro™ White Varnish
CSPS	Calcium sodium phosphosilicate

List of Abbreviations

CTX	C-terminal telopeptide
DD	Demineralised dentine
DDW	Distilled deionised water
DEJ	Dentino-enamel junction
deH-DHLNL	Dehydro-dihydroxylysinoonorleucine
deH-HLNL	Dehydro-hydroxylysinoonorleucine
DMAHDM	Dimethylaminohexadecyl methacrylate
DMFRS	Decayed, missing and filled root surfaces
DMFT	Decayed, missing and filled teeth
DMP1	Dentine matrix protein 1
DMSO	Dimethyl sulfoxide
DOM	Dentinal organic matrix
DPP	Dentine phosphophoryn
DSP	Dentine sialoprotein
DSPP	Dentine sialophosphoprotein
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ECM	Extracellular matrix
EDC	Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDC/NHS	1-ethyl-3-(3-dimethylaminopropyl carbodiimide/N-hydroxysuccinimide
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy dispersive X-ray spectroscopy
EGCG	Epigallocatechin gallate
Er, Cr:YSGG	Erbium, chromium:yttrium–scandium–gallium–garnet
FAp	Fluorapatite
FEL	Free electron laser

List of Abbreviations

FHAp	Fluorhydroxyapatite
FIB-SEM	Focused ion beam-scanning electron microscopy
FTIR	Fourier-transform infrared microscopy
FTP	Fluoride toothpaste
FV	Fluoride varnishes
GA	Glutaraldehyde
GAGs	Glycosaminoglycans
GIC	Glass ionomer cement
Gla	γ -carboxyglutamic acid
Glu	Glutamic acid
Gly	Glycine
GNP	Genipin
GSE	Grape seed extract
GV	Grayscale value
HAp	Hydroxyapatite
HEMA	2-Hydroxyethyl methacrylate
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HCl	Hydrochloric acid
HLA	Human leukocyte antigen
HLNL	Hydroxylysineonorleucine
HPN	Hesperidin
HVGIC	High-viscosity glass-ionomer cement
HYP	Hydroxyproline
ICTP	Type I collagen C-terminal cross-linked telopeptide
KI	Potassium iodide
MD	Mineral density
MDPB	12-methacryloyloxy dodecylpyridinium bromide
MEPE	Matrix extracellular phosphoglycoprotein

List of Abbreviations

Micro-CT	Micro-computed tomography
MID	Minimal intervention dentistry
MIR-FEL	Mid-infrared free-electron laser
MIV	MI Varnish TM
MMPs	Matrix metalloproteinases
MGP	Matrix Gla(γ -carboxyglutamate) protein
MSC	Mixed stratified cementum
MT-MMPs	Transmembrane-matrix metalloproteinases
NaF	Sodium fluoride
NaOCl	Sodium hypochlorite
NCP	Non-collagenous protein
NHS	N-hydroxysuccinimide
O ₃	Ozone
OCN	Osteocalcin
OCT	Optical coherence tomography
OHE	Oral hygiene education
OHI	Oral hygiene instruction
OPN	Osteopontin
PA	Proanthocyanidin
PAA	Polyacrylic acid
PAMAM	Poly (amidoamine)
PCR	Polymerase chain reaction
PG	Proteoglycan
PILP	Polymer-induced liquid precursor
PLM	Polarised light microscopy
PO ₄ ³⁻	Phosphate
ppm	Parts per million
PS-OCT	Polarisation-sensitive optical coherence tomography

List of Abbreviations

PTD	Peritubular dentine
PVPA	Poly(vinylphosphonic acid)
Pyr	Pyridinoline
RCI	Root Caries Index
RCLs	Root carious lesions
RCT	Randomised clinical trial
RDFS	Decayed and filled root surfaces
RGD	Arginine-Glycine-Aspartate
RH	Relative humidity
RMGIC	Resin-modified glass-ionomer cement
rhMMP	Recombinant human matrix metalloproteinase
ROI	Region of interest
SAED	Selected area electron diffraction
SBS	Shear bond strength
Ser	Phosphoserine
SD	Sound dentine
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SDF	Silver diammine fluoride
SDF/KI	Silver diammine fluoride/potassium iodide
SEM	Scanning Electron Microscopy
SIgA	Secretory immunoglobulin A
Sn	Tin
SnF ₂	Stannous fluoride
SPARC	Secreted protein acidic and rich in cysteine
spp.	Species
SPSS	Statistical package for the social sciences
SS-OCT	Swept-source optical coherence tomography
STMP	Sodium trimetaphosphate

List of Abbreviations

TA	Tannic acid
TCP	Tri-calcium phosphate
TEGDMA	Triethylene glycol dimethacrylate
TEM	Transmission electron microscopy
TiF ₄	Titanium fluoride
TIMP	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptors
TMR	Transverse microradiography
Tyr	Tyrosine
VOI	Volume of interest
w/v	Weight per volume
UVA	Ultraviolet light A
VHN	Vickers microhardness numbers
ZnF ₂	Zinc fluoride
2D	Two-dimensional
3D	Three-dimensional
β-TCP	β-tricalcium phosphate
μTBS	Microtensile bond strength
ΔZ	Integrated mineral loss (or gain)
%ΔZ	Relative mineral loss (or gain)

Chapter 1

Introduction

1.1 General introduction

With the demographic shift towards an aging population and improvement in oral health awareness and behaviour, the retention of natural teeth is progressively increasing in the elderly in developed countries - more elderly citizens with more teeth [1]. As a result, the number of root surfaces at potential risk of caries is increasing due to the age-associated gingival recession and the consequent exposure of caries-prone cervical root dentine [2]. Treatment of root caries is particularly challenging due to the difficulty in restoring the original structure of root dentine as well as the potentially complex clinical conditions in treatment procedures.

Dentine is a hierarchical bio-composite composed of a structured dentinal organic matrix impregnated with carbonated hydroxyapatite [3, 4]. The process of caries progression in dentine includes initial demineralisation caused by bacterial metabolite acidification and subsequent degradation of the demineralised organic matrix by bacteria- and host-derived matrix metalloproteinases (MMPs) and cysteine cathepsins [5]. The early stage of demineralisation can be reversed through the incorporation of component tooth mineral ions onto the remnant nano-crystallites, such as the application of fluoride (F) regimens which, once exposed to salivary ions, can deposit a 'CaF₂-like' material on tooth surfaces or substitute into tooth minerals through the dissolution/reprecipitation reaction and form an apatitic mineral (Ca₁₀(PO₄)₆(OH)_{2-x}F_x) of increased structural stability and lower solubility [6, 7]. In particular, high concentration F products such as 5,000 ppm F toothpastes (FTP), 22,600 ppm F varnishes (FV) and 38% SDF solution (44,800 ppm F) have been recommended to maintain the mineral balance in root carious lesions (RCLs) due to the increased solubility of smaller hydroxyapatite (HAp) crystallites and higher critical pH of root dentine than that of enamel [8].

However, limitations exist regarding the current high concentration F-mediated treatments for root caries. Firstly, the remineralisation efficacy of F depends on the availability of Ca²⁺ and PO₄³⁻, which primarily come from saliva and gingival crevicular fluid and may be decreased in elders due to hyposalivation caused by ageing and medications [9]. Secondly, despite the ability of remineralising agents in tilting the balance of demineralisation and remineralisation in dentine towards remineralisation, the quality of dentine remineralisation is dependent upon not only mineral gain but also the recovery of the sum of the root dentine characteristics which include the ultrastructure (especially the particular location of the mineral within the organic matrix of the tissue), mineral density (MD) and biomechanical properties [10-12]. Therefore,

preservation of the organic matrix of root dentine from degradation should also be considered in RCL treatments.

The restorative treatments for RCLs could be more challenging than that for enamel caries. Apart from the structural and compositional differences between enamel and root dentine, difficulties derived from the potentially complex clinical conditions in restorative procedures for RCLs exist. Specifically, RCLs can expand into subgingival areas, exposing difficulties in access and moisture control, especially for resin composite applications [13]. Moreover, hyposalivation resulting from ageing or medications in the geriatric population further increases the susceptibility of RCL development around restorations and on other root surfaces [14]. In addition, the tenet of minimally invasive dentistry has recommended the excavation of only non-remineralisable/denatured (necrotic) dentine during carious tissue removal whilst preserving the demineralised but remineralisable dentine which has undergone reversible changes in dentinal collagen cross-linkages [15, 16]. Carious dentine has lower tensile strength than sound dentine, resulting in decreased bond strength of restorations [17]. Moreover, demineralised dentinal collagen can collapse easily in the restorative process because of dehydration, thus limiting the infiltration of restorative materials into the dentinal matrix. The resultant exposed collagen is fragile and prone to proteolytic and chemical degradation, which deteriorates the quality of bonding interfaces in the long term [18, 19]. In this regard, the demineralised dentine organic matrix should be protected in the restorative treatments of RCLs.

Given the problems and challenges mentioned above, it is timely to explore effective and clinically applicable approaches for RCL treatments, especially with an emphasis on the preservation of the dentine organic matrix. In the following sections of Chapter 1, a literature review was performed to demonstrate the prevalence, incidence, aetiology, progression and current treatments of RCLs and identify the existing evidence of effects of various collagen cross-linking agents on dentine. Based on the review, three laboratory-based research projects presented in Chapter 2-4 were designed to provide further evidence on the efficacy of currently used treatments on RCLs in the laboratory setting. In particular, proanthocyanidin (PA), a naturally derived collagen cross-linking agent, was used as an adjunct to the F-mediated remineralisation as well as involved in the restorative procedures of GICs for RCL treatments to putatively strengthen the organic matrix in root dentine. Specifically, in Chapter 2, PA was used in conjunction with SDF/KI on RCLs. The interaction of SDF/KI with RCLs in the presence of PA was investigated from the morphology, birefringence, ion distribution, MD and

nano-mechanical properties of demineralised root dentine. In Chapter 3, PA was used in conjunction with FV and 5,000 ppm FTP for the remineralisation of RCLs. Their remineralising efficacy was evaluated in terms of the morphology, birefringence, ion uptake, MD and mechanical properties of root dentine before and after treatments. In Chapter 4, PA solution was used as a dentine conditioner for GICs (including high viscosity GIC (HVGIC), resin-modified GIC (RMGIC) and casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) modified low viscosity GIC) bonding on root dentine, with the hypothesis that PA conditioning putatively removed the smear layer and simultaneously strengthened the exposed dentinal collagen in demineralised root dentine before the placement of GIC restorations. Following the three research chapters, Chapter 5 gives a general discussion on the results and findings from the research projects presented in this thesis as well as provides some future directions in this research field.

1.2 Dental root structure and progression of root carious lesions

1.2.1 Structure of root dentine

Dentine is a hierarchical bio-composite material under multiple template-mediation. The basic building block of dentine is highly mineralised collagen fibrils (~50 – 100 nm diameter), where the tablet-shaped carbonated HAp crystallites (~5 nm thick, 70% by weight) are embedded within the gaps of the fibrils with an orientation crystallographic c-axis preferentially aligned along the long axis of the fibril. Proportionately large periodic mineralised collagen fibrils are densely staggered and overlapped, between which covalent intra- and inter-molecular cross-links are formed [4, 20-22]. The nanoscale interfacial interaction between collagen fibrils and the embedded mineral phase enable load transference between the organic and inorganic components, which endows on dentine a capacity of load bearing during mastication and recovery after deformation [23, 24].

1.2.1.1 The organic matrix of dentine and its functions related to mineralisation

Collagen

The extracellular matrix of dentine consists of collagen fibres that form a matrix for the deposition of plate-like crystals of carbonated HAp. Approximately 90% of the dentine matrix is composed of type I collagen with traces of type III and type V collagen [25]. Type I collagen is a heterotrimeric molecule composed of two $\alpha 1$ chains and one $\alpha 2$ chain. The chains are comprised of three domains, *i.e.*, the NH₂-terminal non-triple helical domain (N-telopeptide), the central triple helix and the COOH-terminal non-triple helical (C-telopeptide) domain [26].

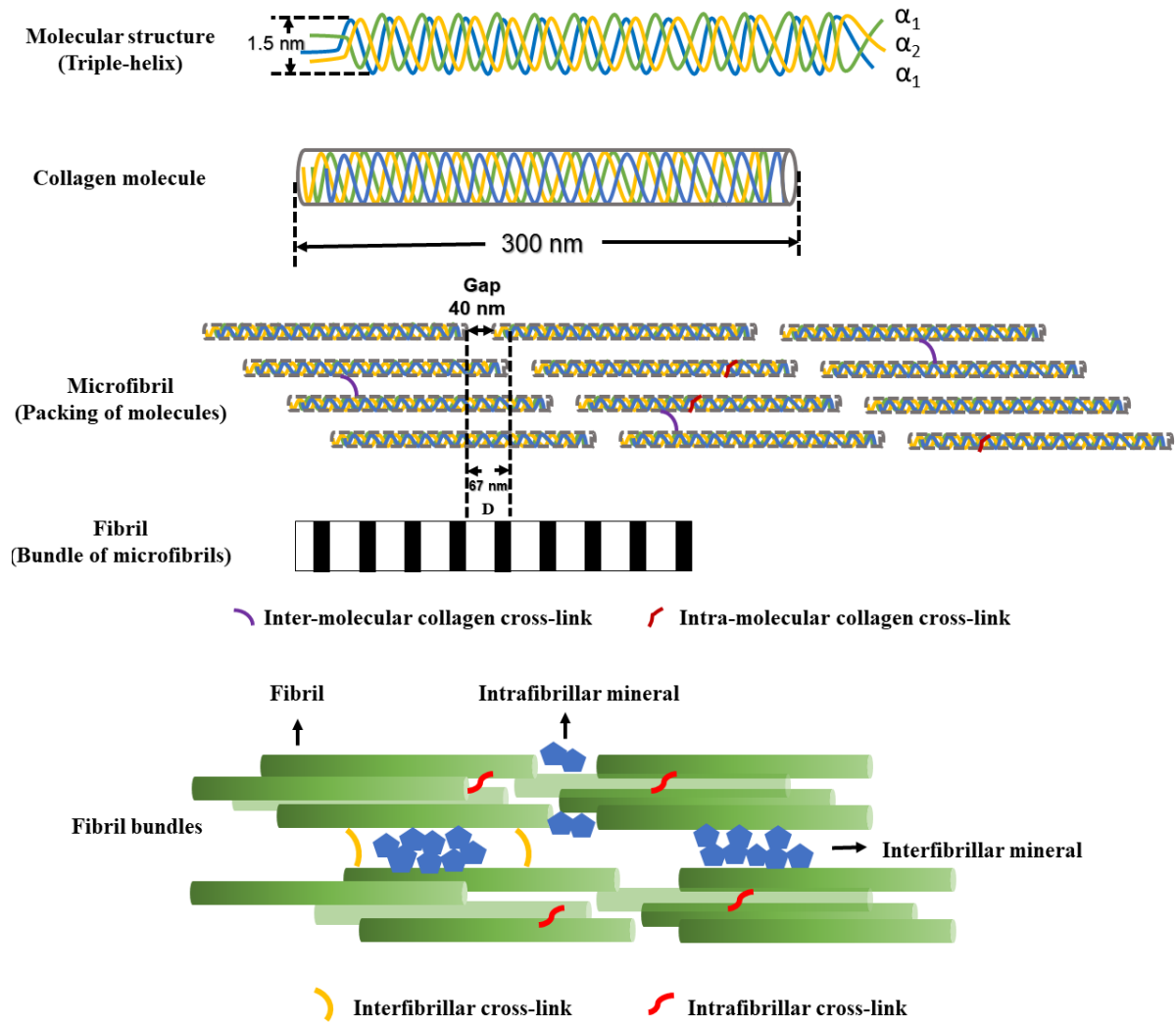


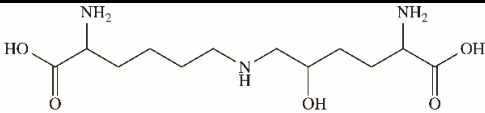
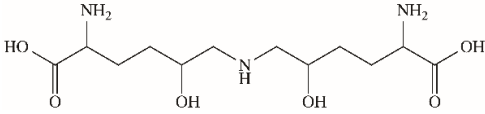
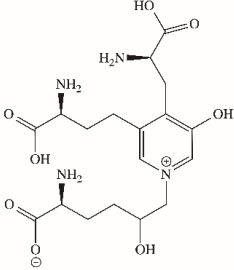
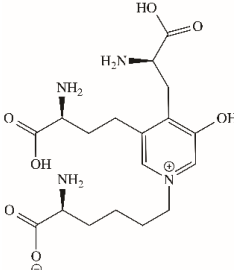
Figure 1.1 A schematic illustration of the hierarchical structure of mineralised collagen fibrils in dentine [27, 28].

Collagen units are self-assembled parallel in a quasi-hexagonal form to arrange the triple-helical polypeptides. The formed fibrils are staggered with adjacent fibrils in 67 nm periodicity, characterising by an overlapped zone of 27 nm and open spaces (holes or gaps) of 40 nm within collagen structures [29]. The open spaces are the primary sites for nucleation of mineral crystals within collagen (Figure 1.1). The fibres self-assembled from collagen molecules cross-linked with adjoining molecules by the lysine aldehyde pathway based on telopeptide lysine aldehyde and hydroxylysine aldehyde. These initial cross-links then undergo further condensation reactions, maturing into the stable complex. Dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL) constitute the major labile, reducible (immature) divalent cross-links in type I collagen. Pyridinoline (Pyr) and deoxy-pyridinoline (D-Pyr) are the major stable, non-reducible (mature) trivalent cross-links, which are formed by maturation of the

reducible divalent cross-links and contain a 3-hydroxypyridinium ring with three side chains that embody three hydroxylysine residues (Table 1.1; Figure 1.2) [30].

Cross-linking patterns in terms of types, density and molecular distribution are tissue specific, site dependent and reflect the physiological conditions of tissues. It was found that the ratios of DHLNL to HLNL and Pyr to D-Pyr were significantly higher in the root dentine than in the coronal dentine, presumably due to the differentially expressed enzymes during dentinogenesis [31]. Reducible cross-links mature to hydroxypyridinium residues, which are the major aldehyde-mediated cross-links of the dentinal collagen in adults, whereas significant levels of reducible cross-links remain throughout life [32].

Table 1.1 Types, chemical structure and location of cross-links in collagen.

Types of cross-links	Chemical structure	Location
Hydroxylysisonorleucine (HLNL)		Abundant in most collagens.
Dihydroxylysisonorleucine (DHLNL)		Mostly abundant in mineralised collagens.
Pyridinoline (Pyr)		Abundant in type I collagen of mineralised collagens and in type II collagen of cartilage and connective tissue.
Deoxy-pyridinoline (D-Pyr)		Mainly found in type I of mineralised collagen.

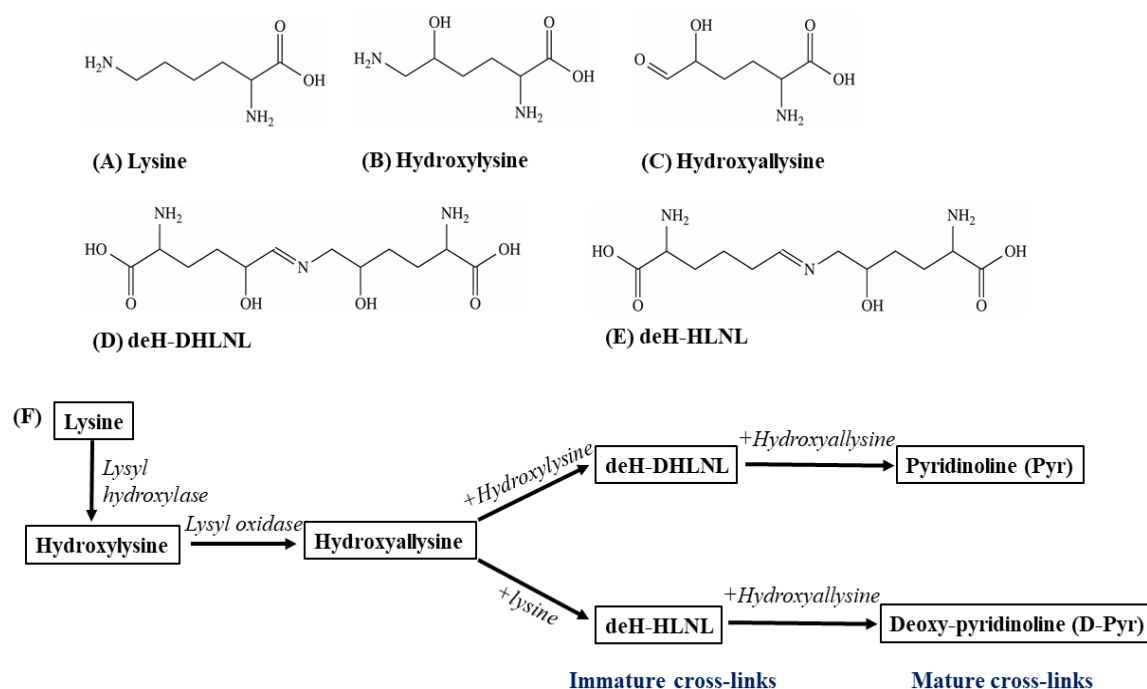


Figure 1.2 The chemical structure of (A) Lysine, (B) Hydroxylysine, (C) Hydroxyallysine, (D) Dehydro-dihydroxylysinonorleucine (deH-DHLNL) and (E) Dehydro-hydroxylysinonorleucine (deH-HLNL); (F) Diagram of the lysine aldehyde cross-linking pathway and formation of immature cross-links (deH-DHLNL and deH-HLNL) and mature cross-links (pyridinoline and deoxy-pyridinoline) [33].

Non-collagenous proteins

Whilst collagen fibrils (as the structural backbone) define the space and function for mineralisation, a variety of mineral interactive proteins termed non-collagenous proteins (NCPs) play active roles in regulating crystal growth and mediating the biomineralization process (Table 1.2). Non-collagenous proteins are synthesized and secreted by odontoblasts and detectable in pre-dentine and/or dentine [34]. Non-collagenous proteins constitute about 5-10% of the dentine matrix, and some of them are found exclusively in dentine, such as dentine sialophosphoprotein (DSPP) and dentine matrix protein 1 (DMP1). Dentine sialophosphoprotein is highly expressed in odontoblasts and transiently expressed in ameloblasts [35]. It is normally cleaved into two main products, namely, dentine phosphophoryn (DPP) from the DSPP C-terminal region and dentine sialoprotein (DSP) from the N-terminal [36].

Table 1.2 Classification of major non-collagenous proteins in dentine.

Classification	Name of non-collagenous proteins (NCPs)	Roles
Dentin-specific NCPs	Dentine sialophosphoprotein (DSPP)	Promote mineralisation
	Dentine matrix protein 1 (DMP1)	
Mineralised tissue-specific NCPs	Bone sialoprotein (BSP)	Promote mineral nucleation but act as a crystal growth inhibitor
	Osteocalcin (OCN; Bone Gla protein)	Bind calcium and regulate apatite formation
Other NCPs	Proteoglycan (PG)	Induce HAp formation
	Osteonectin	Initiate mineral deposition
	Osteopontin (OPN)	Promote or inhibit mineralisation depending on its level of phosphorylation
	Matrix extracellular phosphoglycoprotein (MEPE)	Inhibit mineralisation
	Bone morphogenetic proteins (BMP)	Growth factor
	Matrix Gla protein (MGP)	Bind calcium and regulate apatite formation

Dentine phosphophoryn accounts for 50% of NCPs. It is characterised by high contents of phosphoserine (45%-50%) and aspartic acid (Asp; 35%-38%), forming a structure of (Asp-Ser-Ser)_n [37]. It is a poly-anionic macromolecule with high affinity to Ca²⁺. Dentine phosphophoryn interacts with collagen molecules and forms molecularly staggered collagen-DPP aggregates in pre-dentine, near the collagen gap region where the initial mineral deposition is believed to begin [38]. Dentine sialoprotein is a sialic acid-rich (10%) glycosylated protein localised in odontoblasts and pulp cells, which might facilitate initiation of HAp formation along or inside the collagen fibril, leading to conversion of the pre-dentine to dentine at the mineralisation front [39].

Another dentine-specific NCP is DMP1, which is an acidic phosphorylated serine-rich (22%) phosphoprotein expressed by osteocytes. The phosphorylated DMP1 is able to induce the formation of parallel arrays of crystallites with their c-axes co-aligned [40]. Depending on its

state of phosphorylation, DMP1 can induce crystal nucleation, inhibit mineralization and affect crystal size in a concentration-dependent manner [41]. Especially, DMP1 was found to be localised in the peritubular dentine (PTD), an area lacking collagen fibrils, indicating involvement of DMP1 in mineralisation of PTD [42].

Some NCPs are found specifically in mineralised tissue, such as bone sialoprotein (BSP) and osteocalcin (OCN). Bone sialoprotein is a highly glycosylated and sulphated phosphoprotein, localised in odontoblasts and their processes. It contains an Arg-Gly-Asp (RGD) sequence near its COOH-terminal conferring to the protein cell binding properties and two-poly Glu sequences that are potential Ca^{2+} /HAp binding sites [43]. Therefore, BSP may mediate interactions between the matrix and cells and act as a nucleator for the formation of the initial apatite crystals. At later stages, as mineral grows on the collagen matrix, it acts as an inhibitor in directing the growth of crystals [44]. Osteocalcin, also termed bone Gla (γ -carboxyglutamate) protein (BGP), is a group of small proteins containing three Gla residues and one disulphide bridge synthesised by the odontoblasts prior to the formation of minerals. Being anionic, γ -carboxyglutamate may bind Ca^{2+} by its two closely arranged carboxyl groups, regulating apatite growth in dentine formation [45]. Another major group of Gla-containing proteins identified in dentine is matrix Gla protein (MGP), which contains 4 γ -carboxyglutamic acid (Gla) residues and three phosphoserines. Similarly, MGP has high affinity to Ca^{2+} /HAp through interaction with Gla residues [46].

Other major molecules synthesised and secreted by odontoblasts and found in ECM include proteoglycans (PGs), which belongs to the family of glycoproteins where a number of glycosaminoglycans (GAGs) side-chains bond covalently to a central protein core. Proteoglycans within dentine have a small molecular weight (below 100K) with only one or two GAG chains identified primarily as chondroitin-4-sulfate and chondroitin-6-sulfate. With substantial negative-charge density and relatively large hydrodynamic size, PGs bind electrostatically to cations such as Ca^{2+} , inducing HAp formation at physiological pH and ionic conditions.

Other NCPs involved in mineralisation include osteonectin, also known as SPARC (secreted protein acidic and rich in cysteine) which is a negatively charged acidic glycoprotein and binds both to HAp crystals and to type I collagen through different binding regions of the molecule. It would initiate mineral deposition and link mineral to matrix [47, 48]. Likewise, osteopontin

(OPN), also known as bone sialoprotein 1 (BSP-1), is a negatively charged acidic protein that contains a collagen-binding moiety. Depending on its level of phosphorylation and concentration, OPN has either an inhibitory or enhancing effect on HAp formation [49]. In addition, matrix extracellular phosphoglycoprotein (MEPE) is highly expressed in differentiated odontoblasts, which has an acidic Ser/Asp-rich motif located at the C-terminal, identified as a strong mineralisation inhibitor after enzymatic cleavage [50]. Growth factors such as bone morphogenetic proteins (BMP) act indirectly in mineral deposition by regulating the production of specific matrix molecules [51].

Matrix metalloproteinases and tissue inhibitors of metalloproteinases

Matrix metalloproteinases are a family of zinc and calcium-dependent endopeptidases trapped within the mineralised dentine matrix during the initial stages of odontogenesis, capable of processing and degrading various extracellular matrix (ECM) proteins [52]. Matrix metalloproteinases have a signal peptide sequence, one catalytic domain with a highly conserved zinc binding site and a hemopexin-like carboxy-terminal domain. Whilst MMPs are synthesised and mostly secreted as inactive pro-enzymes, they are activated when the pro-domain bridge with the catalytic zinc (the so-called ‘cysteine switch’) is disrupted by proteolytic cleavage by other members of MMPs, cysteine cathepsins, serine proteases, bacterial proteinases or chemical changes [53].

In general, vertebrate MMPs can be divided into collagenases, gelatinases, stromelysins, transmembrane MMPs (MT-MMPs) and others [54]. To date, the intact human dentine matrix has been reported to contain MMP-8 (collagenases), MMP-2 and MMP-9 (gelatinases), MMP-3 (stromelysin-1 or proteoglycanase) as well as MMP-20 (enamelysin) [55]. Matrix metalloproteinases are involved in various physiological processes in dentine, including maturation, formation and calcification of intra- and inter-tubular dentine as well as dentine caries progression. Matrix metalloproteinase expression and activity are regulated by the sectioning and activation of precursor zymogens, inhibition by tissue inhibitors of metalloproteinases (TIMPs) and interaction with specific ECM components. Under normal physiological conditions, MMPs are expressed only when needed for tissue remodelling, whilst aberrant expression leads to tissue destruction in pathological conditions, such as periodontitis and dental caries [56].

Another major family of proteases found in the dentine matrix is cysteine cathepsins, especially cysteine cathepsin B and cysteine cathepsin K. Unlike MMPs, which have the optimum functional activity in a neutral environment, cysteine cathepsins are active in a slightly acidic pH, generally between 5 and 6. Therefore, they could initiate, at an acidic pH, matrix degradation and activate host-derived MMPs by proteolytic cleavage [57, 58].

Tissue inhibitors of metalloproteinases are the major physiological inhibitors of MMPs. These inhibitors regulate MMP activity and therefore controls MMP-mediated ECM breakdown. There are four human TIMPs, all of which are low-molecular-weight secreted proteins that bind non-covalently and non-specifically to the active site of different MMPs [59]. Tissue inhibitors of metalloproteinase-1 and TIMP-2 have been found in human radicular dentine, co-localised with MMP-9 and MMP-2 respectively, with different concentrations and distribution patterns at different dentine depths [60].

1.2.1.2 Inorganics in root dentine

Minerals (*i.e.*, carbonated HAp) in root dentine appear as plate-like irregular particles that are embedded in collagen fibres. Compared to enamel, the HAp crystallite is smaller in root dentine and therefore is more prone to demineralisation in acidic conditions due to increased surface to volume ratios [61].

Variations in mineral platelet arrangements contribute to the anisotropy and stiffness in different locations of dentine. Specifically, the apatite in dentine is partitioned according to its location with respect to collagen fibrils - either within the fibrils (intrafibrillar) or between the fibrils (interfibrillar). Intrafibrillar minerals are mainly located in the gap regions within the fibrils and mostly arranged with their c-axes parallel to the collagen fibres of the matrix. Intrafibrillar minerals have a major contribution to the mechanical properties of dentine; whereas interfibrillar mineralisation is located in the spaces that separate the collagen fibrils, where the arrangement of crystals is possibly random [24]. The areas along the boundary of tubules, *i.e.*, PTD, are highly mineralised with a mineral content of greater than 90%, much greater (and therefore harder) than inter-tubular dentine (ITD) [62]. Peritubular dentine contains less collagen than the bulk of dentine and dissolves more readily than ITD in acid. The minerals in PTD are plate-shaped and would appear as thin needles in edge-on views. Moreover, PTD minerals are unoriented with no preferred crystal orientation in contrast to those in ITD where the c-axes of crystals are aligned with the collagen fibril axes [63]. Sclerotic

root dentine is formed during RCL development, where the crystals scattered in the lumen of dentinal tubules possibly appearing as rhombohedral, cuboidal, whitlockite (Mg-substituted β -tricalcium phosphate (TCP)) crystals with large particle size and may partially or completely obliterate the tubule lumens [64]. Small angle X-ray scattering studies revealed the smaller crystallite size in sclerotic dentine than that in normal dentine, contributing to the altered mechanical properties of transparent dentine [65].

1.2.2 Structure of cementum

Cementum is a calcified tissue covering the entire root surface and functions as a tooth-supporting device in concert with the principal periodontal fibres and alveolar bone. Two types of fibres are involved in cementum - extrinsic (Sharpey's) fibres secreted by fibroblasts and partly by cementoblasts and intrinsic fibres secreted by cementoblasts only. Based on the constituted cementocytes and fibres, cementum can be classified into acellular extrinsic fibre cementum (AEFC), cellular intrinsic fibre cementum (CIFIC) and mixed stratified cementum (MSC) [66]. Specifically, AEFC generally covers the cervical area of roots and contains densely packed extrinsic fibres connecting to the fibres in periodontal ligaments but no cementocytes. Thick AEFC shows several incremental lines. Cellular intrinsic fibre cementum covers the apical root surface and contains cementocytes as well as intrinsic and extrinsic fibres with various densities, functioning as either tooth support or tooth adaptation during tooth movement. Mixed stratified cementum is predominantly seen in the interradicular and apical regions of roots, which is partitioned by incremental lines. Mixed stratified cementum usually consists of stratified CIFIC with occasional appearance of AEFC. Other types of cementum include acellular afibrillar cementum (AAC) and intermediate cementum which is a highly calcified zone occupying the cemento-dentinal junction and contains enamel matrix proteins [67].

Early RCLs on cementum unexposed to the oral environment show slightly hypomineralised areas along the junctions between calcified layers of extrinsic (Sharpey's) and intrinsic collagen fibres as well as along incremental lines [68]. Demineralisation progresses along this area and ultimately becomes clefts, which might further traverse cementum and extend into the peripheral dentine [69]. However, once the cementum is exposed to the oral environment, it is lost quickly by abrasion, erosion or periodontal therapy, therefore RCLs are more frequently observed on dentine surfaces.

1.2.3 Initiation and progression of root carious lesions

The progression of RCLs can be divided into two successive stages, initially where acid produced by bacteria tilts the de- and remineralisation balance in the dentine towards demineralisation followed by breakdown of the demineralised organic matrix (Figure 1.3) [5].

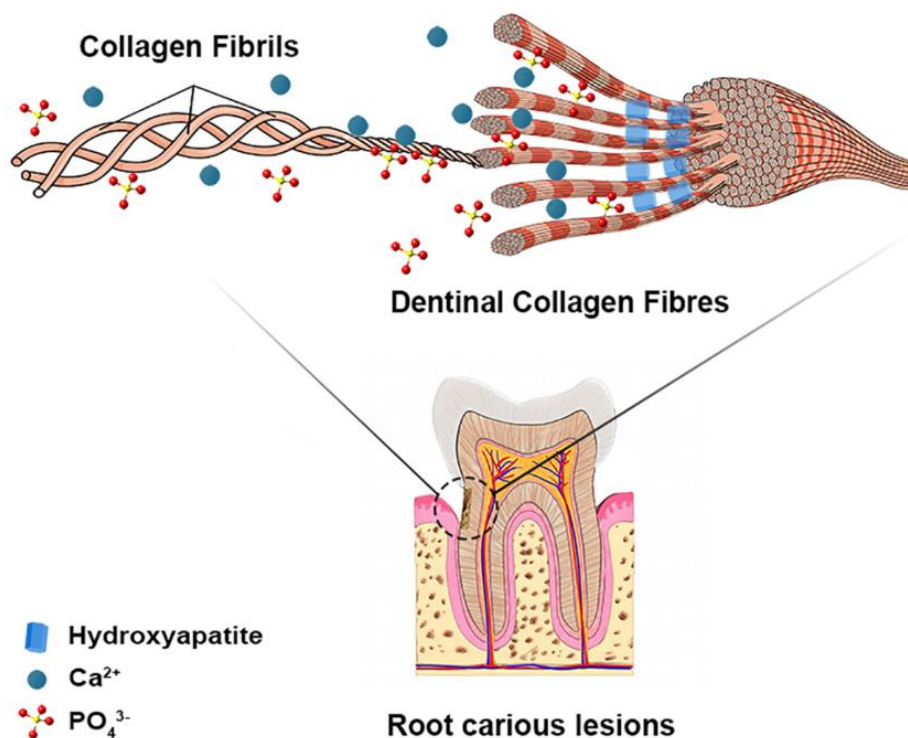


Figure 1.3 A schematic illustration of demineralisation and collagen degradation of root carious lesions [70].

1.2.3.1 Demineralisation and potential of remineralisation in dentine

In the early stages of lesion formation, minerals are dissolved by acids produced by bacteria. The PTD dissolves at a more rapid rate than ITD. Characterised by the MD difference, three major zones exist – a layer with fully demineralised collagen, a partially demineralised zone with minimally damaged organic matrices and the underlying normal dentine [71].

During the initial stage of demineralisation, the dentinal organic matrix is not structurally and biochemically different from that of sound dentine and therefore has the potential to act as a scaffold for mineral deposition [72]. However, highly purified collagen in an ionic solution of physiological concentrations does not confer a nucleating function. Also, heterogeneous precipitation of inter-fibrillar minerals on the dentine surface could not be regarded as true remineralization. Therefore, it was suggested that the collagen matrix only functions as a

passive template for mineral deposition. Given the anionic characteristics of NCPs and their affinity for cations such as Ca^{2+} , they might play a key role in the induction and regulation of mineral formation during biomineralisation [73]. Later studies demonstrated that whether NCPs exert promoting or inhibitory effects on mineralisation depends on their concentrations and chemical status [74]. Nevertheless, mineral induction by polyanions is a relatively non-specific process. Other regulating factors as well as compartmentalisation in tissues are of great significance for mineralisation.

1.2.3.2 Activation of matrix metalloproteinases and cathepsins and degradation of the organic matrix

Following dissolution of HAp, the collagenous network together with its associated macromolecules in dentine becomes exposed to the host-derived proteolytic enzymes such as MMPs which are localised in both dentine and saliva. Specifically, the acidic environment created by bacteria-derived acids causes conformational changes in the propeptide domain of MMPs and facilitates the cysteine switch that activates MMP activities [55]. Although the activated MMPs are stable at an acidic pH, neutralization of acids is achieved by the dentinal buffering capacity due to the dissolution of mineral phase or the salivary buffer system [55, 75, 76]. In addition, the phosphorylated proteins released from the collagen scaffold could interact with TIMP-inhibited MMPs within the carious lesion and activate them, promoting the degradation process [77]. The activated MMP-1, MMP-8 and MMP-13 degrade dentinal collagen, resulting in the release of $\frac{1}{4}$ to $\frac{3}{4}$ length peptides. These peptides lose the triple helical conformation and are further degraded by the gelatinases MMP-2 and MMP-9. Also, MMP-20 (enamelysin) which is incorporated into dentine during dentinogenesis may be released and activated during lesion progression. In addition, cysteine cathepsins in dentine are active under mildly acidogenic conditions and therefore initiate matrix degradation at low pH and activate host derived-latent MMPs by proteolytic cleavage [78, 79]

1.2.3.3 Stages and ultrastructural features of root carious lesions

The severity of RCLs can be categorised according to the index of Billings, where Grade I is an incipient lesion with no surface defects and needs remineralisation therapy; Grade II is a shallow lesion with surface defects < 0.5 mm in diameter and needs recontouring; Grade III, surface cavitation is present with surface defects > 0.5 mm in diameter, where restoration is needed; Grade IV is a deeply penetrating lesion with pulpal or root canal involvement (Figure 1.4) [80].

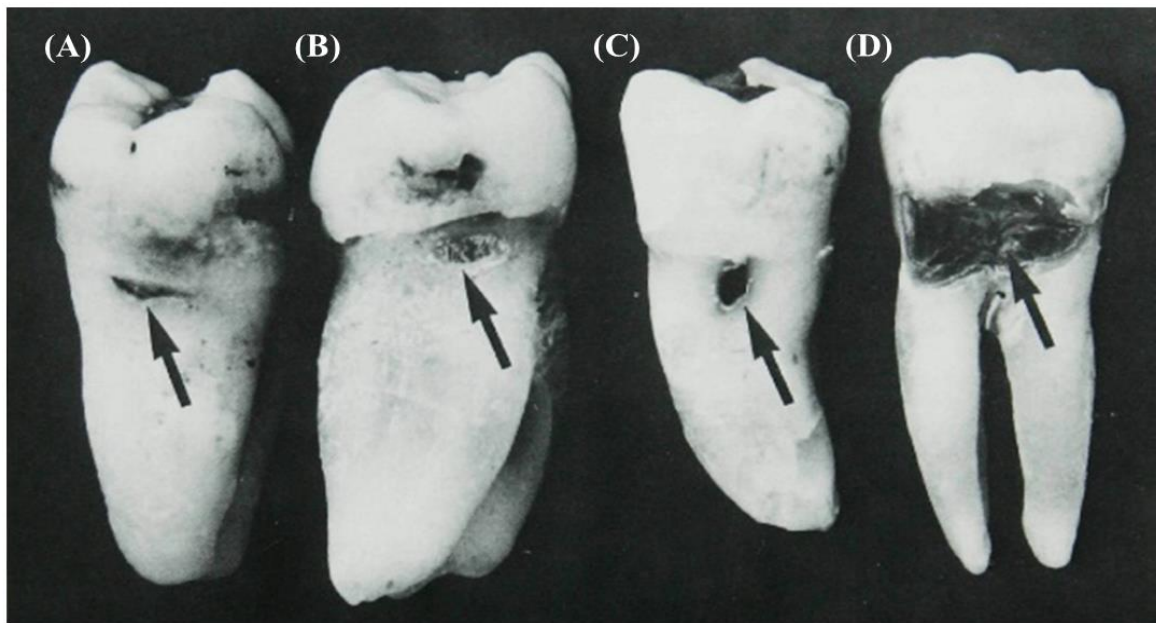


Figure 1.4 Severity classification of root surface dental caries: (A) Grade I (Incipient), (B) Grade II (Shallow), (C) Grade III (Cavitated), (D) Grade IV (Pulpal) [80].

The ultrastructural features of RCLs under microscopy vary from different lesion areas and stages of development. Normally, RCLs tend to spread laterally and gradually encircle the roots at the approximal surfaces [5]. The surface bacterial penetration zone appears brown in reflected light. Occasionally, a mineralised layer is evident beneath the surface at a depth of 10-20 μm , attributed to the reprecipitation of the dissolved and outward diffusing ions from inner dentine (Figure 1.5 A,B) [81]. The subsurface zone underneath appears transparent in reflected light which represents a zone of extended demineralisation. The dentinal tubules are less prominent within the lesion body and the course is disrupted in the deepest part of the lesion (Figure 1.5 C) [69]. The bacterial penetration zones have different modes of spreading around dentinal tubules – either penetrate following the dentinal tubules towards the root canal and spread into ITD or laterally spread at right angles to the dentinal tubules into demineralised ITD which leads to further penetration of microbes into the dentine (Figure 1.5 D) [81]. Higher magnification histopathology images indicate that the microorganisms are orientated parallel to collagen fibrils, then totally engulf dentinal tubules, and may become mineralised by irregular crystals, which is called the zone of tubular sclerosis (Figure 1.5 E). Initially, inner periphery tubules are partially occluded by needle-like crystals, where the zone of tubular sclerosis appears transparent in reflected light or dark in transmitted light. Later, the entire lumen is filled with fine granular crystals and a distinct zone is formed to separate the bacterial

penetration and advancing demineralisation. This zone limits the diffusion of acids and proteolytic enzymes to the deeper parts of dentine. However, tubular sclerosis can be absent in aggressive cariogenic challenges and there is no straight borderline between the two zones, as a rapid progression rate does not allow the dentinal tubules to react to the challenges causing carious lesions [64].

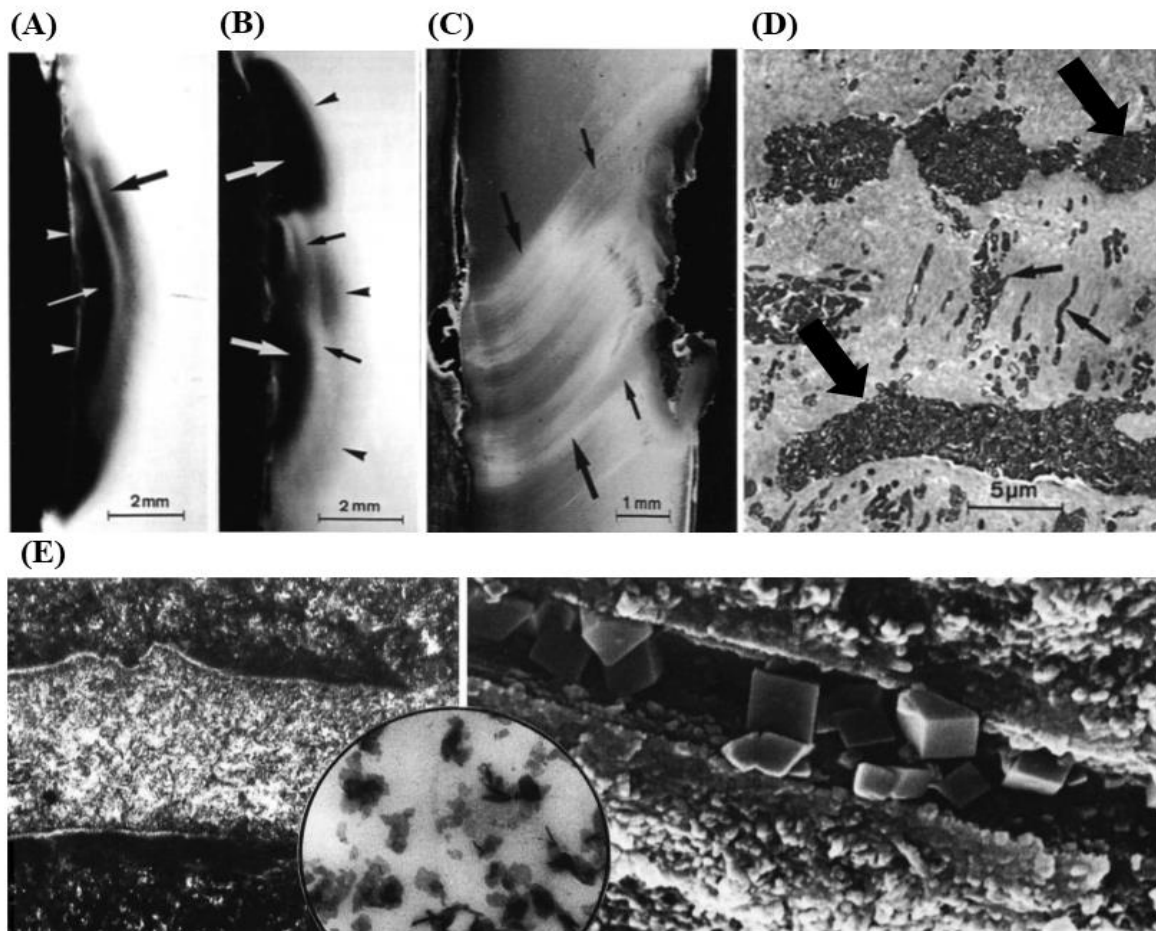


Figure 1.5 (A) Microradiographs of Grade II root carious lesions demonstrating demineralisation of dentine and occurrence of mineralised layers at the surface (arrowheads) and (B) in deeper parts of the lesions (arrows). (C) Longitudinal ground thin section through a Grade III cavitated root carious lesions, illustrating the s-shaped zones of tubular sclerosis (large arrows) and bacterial penetrations (small arrows). (D) Spreading of microorganisms along dentinal tubules (large arrows) or laterally into inter-tubular dentine (small arrows). (E) Ultrathin section showing accumulation of plate-like crystals (insert) in the sclerosed lumens of dentinal tubules and scanning electron micrograph demonstrating cuboidal and rhombohedral crystals in dentinal tubules [82].

1.3 Prevalence and incidence of root caries

Root caries has become a more common oral health problem in ageing populations who have retained more of their teeth, and subsequently epidemiological studies regarding root caries have increased in recent decades. Nevertheless, estimating the prevalence and incidence of root caries can be complicated because of the heterogeneity amongst studies regarding the selected population, lesion diagnostic criteria and indices used for the measurement of severity of carious lesions on root surfaces. Thus, the epidemiologic data regarding root caries may have limited generalisability and should be interpreted and compared across studies with caution.

The prevalence of root caries refers to the proportion of the population with root caries at a specific time. Wide ranges exist for the prevalence of root caries depending on the population selected in terms of its age distribution, co-morbidity conditions, geographic location, and the number of residual natural teeth. For instance, the prevalence of root caries amongst Australian elders (the majority aged 80-89) in Victorian nursing homes was 77.4%, whilst a younger age group (mean age of 69.1) of dentate European adults had a lower prevalence of 53.3% [83, 84]. The prevalence has been reported to increase with advancing age in Swedish elders who demonstrated higher prevalence of decayed root surfaces when more dentate individuals of higher ages were included in studies [85, 86]. In addition, the prevalence could vary due to different carious lesions assessment criteria used, which ranges from the root caries index to the percentage of decayed or decayed/filled teeth [87]. The prevalence of root caries in a group of community-dwelling Japanese elders mostly aged 60-69 was 53.3% when decayed/filled root lesions were included, whilst only 39.4% of subjects had unrestored carious root surfaces.

The incidence of root caries is the proportion of individuals who develop new RCLs during a specific period, which is normally determined from longitudinal data from either cohort studies or clinical trials. The length of follow-up in longitudinal studies is one of the factors that influence the estimation of incidence. Specifically, cumulative annualised root caries incidence significantly decreased in a longer follow-up time (9.4% for over 5 y) compared to a shorter time (32.95% for 2 y) because of the bias towards including relatively healthier elder adults in studies with a longer follow-up time [88]. Other sources of heterogeneity include baseline age, source of participants (random or volunteer) and baseline root caries parameters (*e.g.*, decayed and filled root surfaces (RDFS), available root surfaces at risk). The annual root caries incidence may also vary in different geographic locations and socio-demographic contexts -

10.1% in Ontario in Canada and 47.3% in Carlos Barbosa in south Brazil - reportedly industrialised and developing regions, respectively [89, 90].

Considering the substantial clinical and methodological heterogeneity amongst studies, it is, at this stage, difficult to produce definitive data on the global prevalence or incidence of root caries. Comparison of data retrieved from different studies is challenging. Future studies should be more precise in the parameters related to root caries measurements and populations selected thus improving homogeneity of studies and allowing for further comparison and meta-analysis.

1.4 Aetiology and risk factors for root caries

1.4.1 Microbial factors

The typical microbial composition of biofilm related to RCLs has a similarity to that of coronal carious lesions but is more variable. The compositions and physiological characteristics of the microflora are diverse at an individual level and at different stages of RCL progression [91]. Generally, three predominant bacterial species, namely *Lactobacillus spp.*, *Streptococcus spp.* and *Actinomyces spp.* are typically associated with RCLs. However, not all the species are required to be present for the disease to develop and their proportions can differ in different individuals and stages of RCL progression [92, 93]. Multiple species have been isolated from RCLs, indicating the complexity of overall bacterial diversity of root caries [94-96]. Advances in next-generation sequencing technologies have opened a new era in microbial ecology, enabling extensive analysis of microbial populations in a high-throughput and cost-effective manner. Hundreds of species or phylotypes have been isolated from RCLs including *Staphylococcus*, *Peptostreptococcus*, *Veillonella*, *Clostridium*, *Eubacterium*, *Bifidobacterium*, *Capnocytophaga* and *Candida* as well as proteolytic/amino acid-degrading bacteria such as *Prevotella*, *Propionibacterium* and *Fusobacterium spp.* The composition and diversity of the microbiome are shaped by selective pressures in oral environment imposed by dietary, saliva and the interaction between host and microbial species, thus providing ideal conditions for aciduric and acidogenic strains to establish themselves as the dominant species in the biofilm [5]. In this regard, Marsh's biofilm 'ecological hypothesis' has been widely recognised, which regards dental caries as a consequence of disruption in the homeostasis of commensal microbes [97]. Specifically, the process of RCLs is initiated by environmental changes caused by metabolic activities of acidogenic bacteria species, mostly mutans Streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*), *Lactobacilli spp.* and perhaps *Actinomyces spp.*, in the dental biofilm microbiota, whilst homeostatic mechanisms help maintain a balance of the oral

environment. Nevertheless, changes in the oral environment (*e.g.*, pH and fermentable carbohydrate intake) drive the selection of dental microbes towards favouring the changed conditions. When the homeostasis is disrupted by the aciduric population of oral microbes, mostly non-mutans streptococci species, carious lesions initiate on the root surface if the pathogenic biofilm is present for a sufficient period to allow sufficient net demineralisation. Furthermore, if the acidic conditions are not reversed, non-pathogenic microbes may be progressively replaced by stronger aciduric species whose metabolic activities further decrease the pH of the microenvironment and facilitate the loss of minerals and subsequent progression of lesions [5, 98, 99].

1.4.2 Biological factors

1.4.2.1 Saliva

Salivary function is one of the biological factors that determine caries susceptibility. Saliva performs multiple roles against caries progression dependent upon its components, flow rate, buffer capacity, antimicrobial activity and degree of saturation with respect to tooth minerals (impure HAp) [100]. Individuals suffering from impaired salivary function, mostly resulting from the intake of medication, chronic systemic diseases such as Sjögren's syndrome and sequelae of irradiation for cancer of the head and neck, exhibit increased caries experience [101, 102]. A longitudinal *in situ* study showed increased unstimulated saliva flow rate reduced carious lesion development in terms of decreased mineral loss and lesion depths. This was attributed to the remineralising, rinsing and clearing effects of saliva that relate to the protective effects against caries [103]. However, results from several clinical studies demonstrated no significant correlation between carious root surfaces and salivary flow rates [104, 105]. The inconsistent conclusions may be ascribed to the confounding salivary factors such as microbial counts which significantly influenced the risk of root caries in clinical trials and the small sample population. In addition, stimulated salivary flow rates were selected in clinical studies partially because of the difficulties in collecting measurable amounts of unstimulated saliva in the elderly. However, stimulated flow rates varied less than resting flow in response to the use of medications. Therefore, if resting flow could have been measured, the difference of caries incidence between subjects taking and those not taking hyposalivatory medications may have been greater [105]. In order to separate the effects of salivary constituents from flow rate, *in situ* caries models were employed to stratify and monitor the salivary composition variables. Salivary constituents, particularly Ca^{2+} , PO_4^{3-} , bicarbonate ions and proteins such as enzymes and mucins determined the pH, buffering and remineralising capacities as well as antimicrobial

effects of saliva. The quality of saliva could influence carious lesion development [106]. In addition, the salivary microbial counts were correlated with the occurrence of root caries which were higher in the elderly than in adults in general [107].

1.4.2.2 Gingival recession

Root carious lesions develop primarily on the tooth structures with tissue attachment loss. A higher prevalence rate of root caries was observed in subjects with gingival recession compared to recession-free subjects [108]. It is speculated that gingival recession leads to exposure of the vulnerable root surfaces to the oral environment, which is one of the predisposing factors contributing to root caries [109]. The causes of gingival recession in the elderly include mechanical trauma from excessive or incorrect methods of toothbrushing, periodontal disease and its treatment. People with a history of periodontitis and resective periodontal surgeries have a high risk of developing root caries [110]. However, definitive epidemiological evidence of the risk of root caries in patients with periodontal diseases or long-term periodontal treatment is still lacking. Additionally, the presence of concave root surfaces near the gingival margin and longitudinal grooves present in mesial and distal surfaces increase the difficulty of cleaning, which promotes bacterial biofilm accumulation on tissues and increases the possibility of RCL development [111].

1.4.2.3 Immune function

Immune molecules such as antimicrobial peptides, odontoblast Toll-like receptors (TLR), immunostimulatory proteins and immunoglobulins can react to caries-associated bacteria and mediate the homeostasis of the oral microbiota which has an impact on caries risk. Secretory immunoglobulin A (SIgA), the principal immunoglobulin isotype present in whole saliva, constitutes the main specific immune defence mechanism [112]. Secretory immunoglobulin A can interfere with bacterial adherence to host surfaces by blocking adhesins, inactivating bacterial enzymes by directing against glucosyltransferases of streptococci as well as acting synergistically with other innate immune factors. Therefore, SIgA levels in saliva are relevant to RCL initiation and progression [113]. Ageing affects the phenotype and functions of cells and secretory components of immune systems, leading to dysregulation of immune responses, which may increase caries susceptibility with increasing age. However, more research is required to better understand the relation between immune senescence and dental caries.

1.4.3 Behavioural and environmental factors

Root caries susceptibility is associated with people's behaviour. In the elderly population, there is a trend of changes in dietary habits towards favouring soft food rich in sugars. Subjects with root caries have more frequent intakes of fermentable carbohydrates than those without [114]. Individuals who use tobacco have higher risk of root caries, explained by the fact that tobacco users usually have relatively poorer oral hygiene and health habits as well as more advanced periodontal diseases resulting in more exposed root surfaces [115, 116].

In addition, the prevalence of root caries has been reported to exhibit socioeconomic inequality. Income-related inequality in dental service utilisation was identified in Europeans aged 50 y and older. Specifically, individuals with higher socioeconomic status had significantly higher access to dental service, especially more preventive dental visits compared to those with lower income and this was associated with the socioeconomic gradient in oral health [117]. Moreover, an adverse socio-economic environment reduces the chances of consuming a healthy diet and the likelihood of adopting oral health behaviours such as frequent tooth-brushing with fluoridated toothpaste, which in turn increases caries risk [118]. The living setting of elders is also related to root caries. Selection of study population, either independently living elders or those institutionalised, would have a great impact on the root caries prevalence, as institutionalised older adults may be more prone to caries due to lack of dexterity for conducting oral health behaviours, cognitive impairment and limited food types supplied in residential homes [83, 119, 120]. Lifelong residence in a water-fluoridated area and use of fluoridated toothpastes reduce the prevalence of root caries. This indicates frequent exposure to F should be an effective root caries prevention strategy [121].

1.4.4 Genetic susceptibility

Genetic variations of host factors may contribute to increased risk for dental caries. Twin studies, one of the most direct measures of analysing the contribution of inheritance to disease, suggest heredity plays an important role in the dental caries experience despite a smaller contribution than environmental factors [122, 123]. The advent of molecular biology and DNA sequence analysis leads to candidate gene approaches for genetic studies, where the major candidate gene categories are selected based on their known functions relevant to the risk factors that contribute to caries. Specifically, immune response genes which mediate the expression of proteins in immune response pathways in the caries process have been investigated; such as genes regulating human leukocyte antigen (HLA) types which are

associated with tooth development and genes regulating cluster of differentiation 14 (CD14) molecules which mediate innate immune responses to bacterial lipopolysaccharides that are of central importance for adherence of *S. mutans* to the tooth surfaces and contribute to the formation and structural integrity of the matrix of dental biofilms [124, 125]. Also, genes which control salivary secretions or components and salivary function have also been considered. In addition, genes associated with taste preferences and sugar metabolism have been of interest, as they may determine the frequency of individuals ingesting sugars which increases caries susceptibility [126]. However, current results regarding the association of candidate genes to caries experience are inconsistent and have been ascribed to inconsistent definitions of caries phenotype used in studies. Advances in molecular genetic tools have promoted the development of genome-wide linkage studies which identify regions in the genome likely to harbour genes that may influence caries risk. Although very few overlaps have been observed so far across existing studies due to different phenotype definitions for caries and population heterogeneity, some promising loci (genes) (*e.g.* 1p36.32 and 10p11.23) related to caries have been identified, which should be prioritised for future genetic studies for dental caries [126]. Future studies utilising the human genome sequence have the potential to improve understanding of the disease process.

1.5 Non-invasive treatments of root carious lesions

1.5.1 Fluoride formulations

The important role of F in caries prevention and control has been demonstrated by a range of evidence [127]. The anticaries effect of F is related to its ability to mediate the demineralisation-remineralisation balance in the oral environment by altering ionic saturation characteristics with respect to HAp. Specifically, F is able to adsorb and deposit as a ‘CaF₂-like’ material on teeth and biofilm, drive Ca²⁺ and PO₄³⁻ into the crystal lattice and form a more acid-resistant mineral phase—fluorhydroxyapatite (FHAp) with a lower ‘critical pH’ (the pH at which an equilibrium exists between mineral dissolution and precipitation) than HAp, thus tilting the mineral balance towards remineralisation [128]. In addition, at an acidic pH, F is able to enter the bacterial cell membranes and interfere with bacterial metabolism and acid production from glycolysis *via* a direct inhibition of intracellular glycolytic enzymes. Fluoride at levels as low as 0.1 mM at a pH value of 4 can cause complete arrest of glycolysis by intact cells of *S. mutans*, although the inhibition is reversible [129, 130]. Products containing F are the most extensively used agents for caries management, where different F formulations and delivery methods are chosen according to the purpose of F use and target groups of people. As

dentine is more caries-susceptible than enamel due to the difference in their composition and structure, effects of F on RCLs could be different from that on enamel carious lesions [131, 132].

1.5.1.1 High concentration fluoride toothpastes

Fluoridated toothpaste is a widespread form for topical F delivery and the major source of F in most communities where water fluoridation is not available [133, 134]. Standard fluoridated toothpaste (1,000-1,450 ppm F) can ‘heal’ non-cavitated carious lesions and the efficacy of F is enhanced in a dose-response manner [135]. Considering the progression of RCLs in the elder population is related to wider range of risk factors such as age and concurrent medical conditions as well as the higher critical pH and smaller HAp crystallites in root dentine than enamel, a high F content (5,000 ppm F) dentifrice has been recommended for elders who are considered with a high risk of RCL initiation. The beneficial effects of 5,000 ppm F on limiting RCL progression compared to regular 1,000-1,450 ppm F toothpastes were revealed in several laboratory and clinical studies [136-138], even though in some clinical trials, the effects of 5,000 ppm F toothpaste on RCLs can vary greatly when a visual-tactile diagnostic method is used to assess the severity and activity of RCLs, due to the subjectivity of this method [139]. Follow-up clinical trials with diverse clinical assessment/quantification methods for RCLs are necessary to confirm if the use of high concentration F toothpastes should be considered as the best practice for RCL management.

In addition, the effectiveness of fluoridated dentifrices is not only determined by F concentration, but also the frequency of use. Fluoride should be constantly available in the oral fluids and biofilms to maximise its effects. A positive correlation between the frequency of F exposure and reduction of root dentine demineralisation was shown *in situ* [140].

1.5.1.2 Fluoride toothpastes with functional additives

Calcium and phosphate-based systems

Fluoride is currently considered the gold standard therapeutic agent for managing early carious lesions, since it changes the ionic saturation characteristics of plaque fluid with respect to tooth minerals, pushing the balance towards precipitation. Fluoride deposits on tooth surfaces as a CaF_2 -like material or substitutes into dental hard tissues and forms an apatitic mineral - $\text{Ca}_5(\text{PO}_4)_3(\text{OH})_{1-x}\text{F}_x$ [141, 142]. However, the availability of Ca^{2+} is rate-limiting for remineralisation, where the low amount of free Ca^{2+} mainly from saliva and gingival crevicular

fluid limits the formation of CaF_2 -like deposits on tooth substrates which in turn depress the value of $\{\text{Ca}^{2+}\} \times \{\text{F}\}^2$ required for further precipitation [143]. Ageing and medication in elders can affect the flow and composition related to the buffering capacity of saliva, decreasing the availability of Ca^{2+} and PO_4^{3-} in saliva for remineralisation [107]. Therefore, bioavailable calcium and phosphate-based systems have been incorporated into fluoridated toothpastes to promote remineralisation [144].

Given that calcium-phosphate based compounds (Ca-P) normally have low water solubility and react with F when in solution, different techniques have been utilised to stabilise Ca-P in a bioavailable form and to keep it separated from F. Frequently used Ca-P in toothpastes include bovine milk-derived CPP-ACP (Recaldent™; Tooth Mousse Plus/MI Paste Plus, GC Corporation, Tokyo, Japan), un-stabilised amorphous calcium phosphate (ACP; Premier Dental Products; Plymouth Meeting, PA, USA), tri-calcium phosphate (TCP; Clinpro™ 5000, 3M ESPE, St Paul, MN, USA) and bioactive glass containing calcium sodium phosphosilicate (CSPS; NovaMin™, GlaxoSmithKline Consumer Healthcare, Weybridge, UK).

Specifically, in CPP-ACP, casein phosphopeptide binds to the (100) and (010) faces of apatite crystals and stabilises high concentrations of Ca^{2+} and PO_4^{3-} in a metastable solution together with F^- [145]. The casein phosphopeptide stabilised Ca-P can diffuse down a concentration gradient into subsurface areas in a bioavailable status and promote remineralisation of subsurface carious tissues [146-149]. It has also demonstrated an effect on preventing mineral loss of root dentine using an artificial RCL model [144]. Clinically, the combined use of CPP-ACP paste with topical F treatments could inhibit RCL progression in patients undergoing head and neck radiotherapy with hyposalivation [150].

The Enamelon™ technology (Premier Dental Products Co., PA, USA) is based on un-stabilised ACP, where a calcium salt and a phosphate salt are delivered separately. When the salts are mixed with saliva, they dissolve and release Ca^{2+} and PO_4^{3-} , producing an ion active product which leads to the precipitation of ACP [151]. Compared with a conventional dentifrice containing F, Enamelon™ toothpaste has been reported to reduce the incidence of RCLs significantly in a high-risk population of patients undergoing head and neck radiation therapy [152].

Functionalised β -TCP is prepared using a mechanochemical process, which prevents the reaction between Ca^{2+} and F^- by a protective fumaric acid barrier that breaks down in an aqueous environment (saliva) and releases Ca^{2+} and PO_4^{3-} . The presence of functionalised TCP in fluoridated toothpastes was shown to promote the formation of HAp on root dentine surfaces, however, it did not show additional inhibition of RCL development and progression *in vitro* [153, 154].

NovaMin[®] releases Ca^{2+} , Na^+ and PO_4^{3-} when introduced into the oral environment. The material interacts with oral fluids and results in the formation of a crystalline hydroxycarbonate apatite layer that is structurally and chemically similar to natural tooth minerals [155]. Compared to conventional F toothpastes, the additives of CSPA would putatively improve remineralisation of initial RCLs, although the remineralisation band formed on the tooth surface might partly result from the precipitation of the released ions, *i.e.*, Ca^{2+} , Na^+ and PO_4^{3-} , from CSPA due to its poor stability [156].

To summarise, current evidence regarding effects of toothpastes containing Ca-P on RCLs are mostly from laboratory studies, which should be interpreted with caution due to the absence of the complex oral environment and application mode of toothpastes. It is still controversial whether the addition of Ca-P into toothpaste has superior effects over F alone, as well as the relative remineralising efficacy of different calcium and phosphate-based systems.

Antimicrobial components in toothpastes

As F mainly reduces plaque cariogenicity by affecting bacterial metabolism but does not target the reduction of the amount of microbes in dental plaque directly - the major aetiological factor in conjunction with a diet rich in fermentable carbohydrates, antimicrobial components have been added into fluoridated dentifrices to complement their effects.

Being a non-fermentable sugar alcohol, xylitol inhibits glycolysis and enzyme activity in the metabolism and growth of *S. mutans* or induces the ecological transition towards less virulent strains of cariogenic bacteria [157]. However, a F dentifrice containing xylitol did not show additional effects on preventing demineralisation of root dentine *in vitro* compared to fluoridated dentifrice without xylitol [158]. Another clinical study on xylitol also indicated no significant reduction in caries increment when compared with a placebo [159]. However, further analysis suggested a tooth-surface specific caries-preventive effect of xylitol, where

xylitol appeared to be more effective for root (dentine) surface caries, but less so for coronal surface caries in a high-risk population, putatively because of more xylitol-sensitive strains of bacteria on root surfaces compared to coronal biofilm [157]. Consensus on anti-cariogenic effects of xylitol has not been reached, although xylitol exhibited superior caries-preventive effects to other polyols, such as sorbitol, by modifying the plaque flora (especially certain strains of *S. mutans*) in high caries risk populations [160].

In addition, factors that increase biofilm pH and therefore saturation with respect to tooth minerals can push the demineralisation-remineralisation balance towards remineralisation. Arginine, as a semi-essential amino acid, can be metabolized *via* the arginine deiminase system of oral bacteria and result in ammonia production which buffers plaque pH and inhibits tooth demineralisation. Fluoridated dentifrices containing 1.5% arginine exhibited significantly superior efficacy in arresting and reversing active RCLs than a matched F dentifrice in clinical studies [161, 162].

Moreover, antimicrobial agents, such as triclosan, which is a chlorinated aromatic compound that has phenolic functional groups with antibacterial properties, are added to F toothpaste. A three-year clinical study showed a significant reduction in RCLs in favour of triclosan-containing toothpaste compared to sodium fluoride (NaF) toothpastes and there were no serious safety concerns regarding the use of triclosan toothpastes in this three-year study [163]. Nevertheless, evidence of the beneficial effects of triclosan on reducing RCLs is still weak due to a lack of studies and concerns exist regarding the safety of triclosan formulated in toothpastes which has been removed from the market in many countries [164].

1.5.1.3 Bovine milk fluoridation

Bovine milk fluoridation is a convenient and cost-effective way of targeted F supplementation, which was first proposed in school-based programmes at a concentration of 5 ppm F for children. When used for managing RCLs, daily intake of milk supplemented with 5 ppm F showed a remineralising effect on RCLs in older adults, however, limitations existed in this clinical study due to the high dropout rate and large variations in the assessment of RCLs using electrical resistance values from an Electric Caries Monitor [165]. Moreover, participants were supplied with F toothpaste and thus it was difficult to distinguish the effects of F from either toothpaste or milk. A further study using an *in vitro* biofilm model indicated no significant additional effects in reducing root dentine demineralisation by adding 5 ppm F to bovine milk

[166]. Therefore, the anticaries effects of bovine milk fluoridation on root dentine remain inconclusive. Other components in milk, such as protein, fat and minerals especially Ca^{2+} and PO_4^{3-} , could influence the anticaries effect of fluoridated milk, because these components might serve as a physical barrier or buffer acids in RCL initiation and progression [166, 167].

1.5.1.4 Fluoride varnishes

Fluoride varnishes containing 22,600 ppm F have been recommended as a topical high concentration F delivery method for patients with a moderate- to high-risk of root caries development, however, clinical trials with large sample sizes are still required to confirm its caries preventive effects in this group of patients [168]. In addition, similar to the bioactive additives in F toothpastes (refer to 1.5.1.2), Ca-P remineralisation systems have also been incorporated into F varnishes to enhance the availability of Ca^{2+} and PO_4^{3-} for remineralisation. Commercialised F varnishes containing Ca-P remineralisation systems include MI Varnish™ containing CPP-ACP, Clinpro™ White Varnish containing TCP and Enamel Pro™ containing ACP. These varnishes release substantially more Ca^{2+} than F-only varnishes, potentially enhancing the remineralisation of carious tissues [169-171]. A few recent *in vitro* studies have indicated that F varnishes containing Ca-P could prevent demineralisation and enhance remineralisation of artificial RCLs. However, it is still controversial whether the addition of Ca-P into F varnishes has superior effects over F alone, as well as the relative efficacy of different Ca-P remineralisation systems in FV according to the different results from various studies [172-174]. In particular, there is a lack of published evidence of their effectiveness on RCLs.

1.5.1.5 Acidulated phosphate fluoride gel

Acidulated phosphate F (APF) gels generally contain 12,300 ppm F in a solution of orthophosphoric acid. The presence of high concentration F and phosphate suppress the dissolution of tooth minerals in low pH [175, 176]. The most rapid period for F uptake by enamel is within the first minute after APF application. This application time has also been recommended for root-surface caries prevention with APF according to the result of an *in vitro* study regarding demineralisation resistance of root surfaces in cariogenic challenges [177]. Root dentine demineralisation could be reduced to a larger extent when APF gel was used in combination with 1,100 ppm F dentifrice *in situ* [131]. This may be explained by the greater availability of loosely bound F from APF leading to deposition of ‘ CaF_2 -like’ minerals and formation of biological Ca-F bridges with the bacterial cell wall [143]. However, the combined

use of APF gel with 1,100 ppm F dentifrice was less effective than a 5,000 ppm F dentifrice in terms of reducing dentine demineralisation, although they were comparatively effective in enhancing remineralisation of RCLs, because a greater amount of CaF_2 from APF has been shown to form on carious dentine than on sound dentine [178].

1.5.1.6 Fluoride mouth rinses

Fluoride mouth rinses have often been used as an adjunctive preventive regimen to other F products such as the daily use of F toothpastes. The combined use of a F mouth rinse with fluoridated toothpastes reduced root surface caries incidence and reversed primary RCLs assessed by hardness and electrical resistance compared to the use of a placebo mouth rinse, especially in populations with high root caries susceptibility [179, 180]. Long-term compliance with the frequent daily use of a mouth rinse with supersaturated $\text{Ca}^{2+}/\text{PO}_4^{3-}$ with respect to the tooth minerals increased the concentration of Ca^{2+} and PO_4^{3-} in the saliva of patients with hyposalivation [181]. However, well-conducted RCTs are required to provide more evidence regarding the caries-preventive effects of F rinse on RCLs [8]. In addition, motivation and instruction of using mouth rinses for the elderly should be considered.

1.5.1.7 Fluoride bound to polyvalent metal and organic compounds

Localisation of F on tooth surfaces is enhanced when bound to polyvalent metal ions such as titanium and zinc, forming titanium fluoride (TiF_4) and zinc fluoride (ZnF_2) [182]. Titanium ions from TiF_4 may reduce demineralisation by binding with phosphate from tooth mineral and forming a glaze-like layer which is rich in Ti and F and considered to contain TiO_2 and organometallic complexes formed by Ti with the organic dentinal matrix [183]. The Ti-rich deposits on dentine surfaces after the application of TiF_4 varnish (24,500 ppm F, pH 1.0) are more acid-resistant than the deposits formed by NaF varnish (24,500 ppm F, pH 5.0) *in vitro* [184, 185]. However, the low pH of TiF_4 varnish or solution (around pH 2.0) may cause side-effects on other oral tissues and make the TiF_4 treatment agents unsuitable for self-administration by the patient. Whilst increasing the pH of the TiF_4 solution may decrease its efficacy, combined use of TiF_4 with other types of F salts has been proposed in order to raise the pH of TiF_4 and to minimise the possible deleterious effect [185, 186]. Although solution containing a combination of TiF_4 and NaF at a suitable pH (pH 4.4) showed comparable effects on inhibiting enamel erosion to TiF_4 varnish, its effects on dentine caries required further investigation [185]. In addition, acidic ZnF_2 (ZnF_2/HCl) exhibited inhibitory effects on dentine demineralisation and collagen degradation and might be a potential agent for root caries

prevention [187]. Likewise, stannous fluoride (SnF_2) showed a direct inhibition of MMP-2 and MMP-9 in dentine, thus potentially used for prevention of dental erosion and caries [188]. Although the polyvalent metal ions have demonstrated their unique advantages in conjunction with F, the persistence of their effects and their potential cytotoxic effects need further investigation.

Fluoride has also been bound to organic compounds such as organic amines, forming amine fluoride (AmF). Compared to inorganic F compounds, the amine (organic) component in AmF has an additional antimicrobial effect and a tensioactive property that allows accumulation of F close to the tooth surface [189, 190]. Toothpastes and mouth-rinses containing AmF have shown their potential in remineralising active soft and leathery primary RCLs in high caries risk populations [179].

1.5.2 Root surface sealants

Root surfaces become prone to rapid RCL progression if exposed to the oral environment as a result of gingival recession in a caries risk individual. In order to protect these susceptible surfaces, root surface sealants have been suggested to provide direct physical protection on these areas, thus preventing RCL initiation [191].

Fluoride-impregnated resin-based desensitising agents were first introduced to treat dentine hypersensitivity by penetration and subsequent precipitation of CaF_2 within the dentinal tubules, resulting in tubular occlusion [192]. Their application has been extended as an adjunctive option for RCL prevention. Infiltration of the agents into dentinal tubules and the released F enhanced the acid resistance of dentine during cariogenic challenges. Thus, a significant reduction of lesion depths was observed on sealant-protected root dentine *ex vivo*. Meanwhile, the polymerised resin layer can act as a physical barrier to acids whilst it is physically present [193, 194]. The effects of sealants on RCL prevention were evaluated *via* the changes in root surface texture and were comparable to that of chlorhexidine (CHX) varnishes. However, the result should be interpreted with caution, since the residual sealants on root surfaces might interfere with the assessment of surface texture, not to mention the subjectivity in texture evaluation [195]. Due to the limited evidence, no clinical recommendations regarding the use of sealants for root caries prevention can be made.

1.5.3 Antimicrobial agents

As dental caries is a biofilm-mediated disease of dental hard tissues, the application of antimicrobial agents could directly target the reduction of microbes in dental plaque.

1.5.3.1 Chlorhexidine

Chlorhexidine is a broad-spectrum antimicrobial agent, which is active against Gram-positive, Gram-negative, aerobic and anaerobic microorganisms, yeasts and fungi [196]. Selective cariogenic bacteria on root surfaces such as *Streptococci* and *Lactobacilli* were reported to have a high susceptibility to the antibacterial action of CHX [197]. However, a 0.2% CHX mouth-rinse (Corsodyl; GlaxoSmithKline, Zeist, The Netherlands) did not show additional protection of dentine against demineralisation compared to saline [198]. Indeed, CHX mouthwash is not suggested as a first-choice caries-protective agent as it possibly disturbs the oral micro-ecological balance. Varnish is another popular vehicle for the application of high concentration CHX. A CHX-thymol-based varnish (1% CHX; Cervitec, Ivoclar Vivadent, Liechtenstein) was reported to reduce the incidence and arrest the progression of RCLs amongst an institutionalised elderly cohort as a simple, quick and non-invasive method [199]. However, due to a small number of studies and the specific groups included in studies such as dry-mouth risk patients and frail institutionalised elder people, evidence for arresting RCLs in elderly subjects using CHX treatments is inconclusive [200].

1.5.3.2 Silver diammine fluoride

Silver diammine fluoride (SDF) contains ammonia and silver fluoride (AgF), where the ammonia ions combine with silver ions to produce a complex ion called the diammine-silver ion. The resultant complex of $\text{Ag}(\text{NH}_3)_2\text{F}$ is more stable than AgF [201]. Silver diammine fluoride has additional benefits in halting the caries process and simultaneously preventing the formation of new caries compared to other F-based caries preventive agents due to the antimicrobial effect of silver as well as its ability to be incorporated into the crystal structure of HAp and produce silver-containing HAp with a formula of $\text{Ca}_{10-x}\text{Ag}_x(\text{PO}_4)_6(\text{OH})_2$ [202, 203]. Thirty-eight percent SDF solution was not only effective in preventing new coronal carious lesions in primary teeth but also considered as a root caries preventive strategy which decreased the incidence of carious lesions on exposed root surfaces by at least 50% amongst community-dwelling elders in 12-month follow-up [204-206]. An annual SDF application was more effective than oral hygiene education (OHE) or oral hygiene instruction (OHI) and comparatively effective with other therapeutic interventions such as 1% CHX varnish or 5%

NaF varnish in RCL prevention over a 24-month period [207, 208]. In particular, for individuals at high risk of root caries, SDF is likely to be a cost-effective option for RCL management [209]. Since silver in the SDF solution can cause black staining, application of potassium iodide (KI) immediately after SDF to allow iodide ions to react with the excess silver ions and form a precipitate of silver iodide has been proposed, thus reducing staining. However, the long term effect of KI in reducing staining of arrested carious lesions is questionable [204]. In addition, there are concerns about KI reducing the beneficial effects of SDF, such as prevention of recurrent caries around restorations [210].

1.5.3.3 Ozone

Ozone (O₃), in a gaseous or aqueous phase, is a powerful antimicrobial agent due to its oxidant potential that induces destruction of cell walls and cytoplasmic membranes of bacteria and fungi [211]. Ozone therapy has also been used in dentistry, as it has strong bactericidal activity against bacteria in oral biofilms by oxidising the biomolecules that allow the microorganisms to survive and expand [212]. The antimicrobial effect of aqueous ozone or ozone gas on RCLs was initially assessed *in vitro*, where a reduction of microorganisms on root surfaces was shown [213, 214]. Further RCTs tested the efficacy of ozone for the management of ‘leathery’ RCLs, where ozone was delivered by a portable apparatus at a concentration of $2,100 \pm 200$ ppm for either 10 or 20 s. The treatment regime using ozone inhibited the lesion progression and led to re-hardening of some leathery RCLs in 6 to 18-month follow-up. However, methodological concerns exist within studies testing ozone therapy in terms of blinding methods, outcome measurement and sample sizes [215-217]. In addition, an arrested lesion could also show an leathery appearance, therefore it is questionable whether the ‘leathery’ RCLs selected in the studies was in an active or arrested stage [218]. The effects of ozone therapy would be minimal if the activity of the leathery lesions had already been arrested. Extensive clinical evidence for ozone treatment of RCLs is required before it can be supported.

1.5.3.4 Metal-based antimicrobial materials

Bioactive metals such as silver (mentioned in 1.5.3.2), zinc and tin have been an interesting alternative for the development of advanced antimicrobial agents [219]. Compared to antibiotics, bioactive metals possess a long-lasting antimicrobial effect and less bacterial resistance [220, 221]. Specifically, zinc has been reported to inhibit sugar transport, acid production and protease activity in microbial metabolism. Dentifrice incorporated zinc citrate could reduce plaque accumulation [222]. A novel product named KappazincTM (Bio-link

Australia Pty. Ltd.), which consists of natural milk-derived kappa-casein peptides (KappacinTM) formulated with zinc, was developed to provide antibacterial activity and inhibit the growth of oral pathogens, which may have some utility in dental caries prevention [223, 224]. In addition, tin ions showed inhibitory activity against a multispecies microbial biofilm and modulate the microbial composition. Moreover, a greater suppression of the supragingival acidogenic and aciduric bacteria was observed when SnF₂ was used in combination with CPP-ACP due to the prebiotic effect of the latter [225, 226]. Notwithstanding the antimicrobial effects of the above bioactive metals, most studies were conducted on enamel lesions and supragingival plaque, further research is warranted to explore their potential effects on root dentine caries.

1.6 Novel remineralisation strategies for non-invasive treatments of root carious lesions

1.6.1 Self-assembling Peptides

A commercially available self-assembling peptide P₁₁-4 (Ace-QQRFEWEFEQQ-NH₂) is involved in biomimetic mineralisation, building a three-dimensional (3D) bio-matrix for HAp formation [227]. Specifically, P₁₁-4 is a synthetic α -peptide that self-assembles into β -sheet amyloids and presents clusters of negative charges made up of four Glu-residues acting as potential Ca²⁺-binding sites and nucleation point on its surface [228].

When applied on enamel subsurface lesions, monomeric P₁₁-4 permeates through the pores of subsurface lesions and presumably forms a 3D matrix in the lesions for mineral deposition. Net mineral gain in the enamel carious lesions was observed with the presence of P₁₁-4 due to both increased remineralisation and inhibition of demineralisation. P₁₁-4 incubated in mineralisation solutions leads to nucleation of *de novo* HAp [227, 229]. However, the reactivity of P₁₁-4 is pH dependent [230]. Whilst under remineralising conditions, P₁₁-4 can be transformed into an elastomeric nematic gel and promote remineralisation; in an acidic environment, the peptide tends to change into a ‘flocculated state’ which is relatively unreactive and may even hamper the diffusion of remineralising ions into lesions [231]. The safety and clinical efficacy of P₁₁-4 for early carious lesion treatment were validated in clinical trials using a P₁₁-4 incorporated product named CurodontTM Repair (Credentis; Windisch, Switzerland), where the combination treatment of the P₁₁-4 product with F varnishes promoted remineralisation of initial enamel carious lesion compared to F alone. However, the sample size was relatively small and the diagnostic criteria used such as visual examinations were subjective in this clinical study [232]. Moreover, the acid-etching used before the application of P₁₁-4 may decrease the pH in the carious lesions, leading to flocculation of peptides and unreactive precipitates [230].

In addition, P₁₁-4 has a parallel nanofibrillar structure, which interacts with dentinal type I collagen and increases stiffness and proteolytic resistance of the dentine matrix, thus it could be considered for dentine remineralisation. However, it is unclear whether the peptides are susceptible to proteolytic degradation [233]. Further studies into the mechanisms of function of P₁₁-4 and clinical trials with a larger sample size and multiple validated diagnostic criteria are warranted to confirm the remineralisation efficacy of P₁₁-4.

1.6.2 Non-collagenous protein analogues and polymer-induced liquid precursor system

The majority of current clinical remineralisation approaches could only remineralise partially demineralised dentine, which relies on residual seed mineral crystals in dentine to achieve epitaxial deposition of Ca²⁺ and PO₄³⁻; termed ‘classical ion-based crystallisation’ [28]. However, following this approach, intrafibrillar mineralisation which determines the nanomechanical properties of dentine is more difficult to achieve compared to the extrafibrillar mineralisation occurring between adjacent collagen fibrils [234].

The natural mineralisation process in dentine is mediated by the dentinal organic matrix (mainly type I collagen) based on a ‘non-classical particle-based crystallisation’ concept [28]. In this process, a mesoscopic transformation is involved, where Ca²⁺/PO₄³⁻ are sequestered by highly anionic NCPs into prenucleation clusters (approximately 1 nm in diameter) which further aggregate into larger ACP nanoparticles (10-50 nm in diameter) which are highly hydrated and exhibit behaviour similar to that of liquid droplets [235, 236]. The nanoparticles penetrate into the intrafibrillar space of collagen which serves as a mineralisation template for the nucleation of meso-crystals at specific sites, and eventually form single apatite crystallites between collagen molecules [237, 238].

In this regard, the formation of amorphous nano-precursors and initiation of remineralisation at specific sites of dentinal collagen are considered crucial steps in dentine biomineralisation. In order to simulate this natural process, the polymer-induced liquid-precursor (PILP) system is proposed for biomimetic remineralisation of dentine by backfilling the demineralised dentine collagen with hydrated amorphous nano-precursors [239]. Specifically, the amorphous precursors are encapsulated by polyanionic polymers such as poly-L-aspartic acid and polyacrylic acid (PAA) which act as surfactants to prevent fluidic ACP nanoparticles from aggregating into larger particles [70,71]. The plastic properties of the precursors in a hydrated

status allow them to infiltrate into collagenous matrices, where polyphosphate-containing biomimetic analogues such as polyvinylphosphonic acid (PVPA), sodium trimetaphosphate (STMP) or sodium ascorbyl phosphate immobilise the amorphous precursors in specific sites of the collagen matrix and serve as a template for initiation of crystallisation and hierarchical mineralisation within intrafibrillar spaces, with concomitant extrafibrillar mineralisation between adjacent collagen fibrils [240, 241] (Figure 1.6). The PILP approach was reported to not only increase MD but also recover the mechanical properties of the demineralised dentine matrix *in vitro* [73].

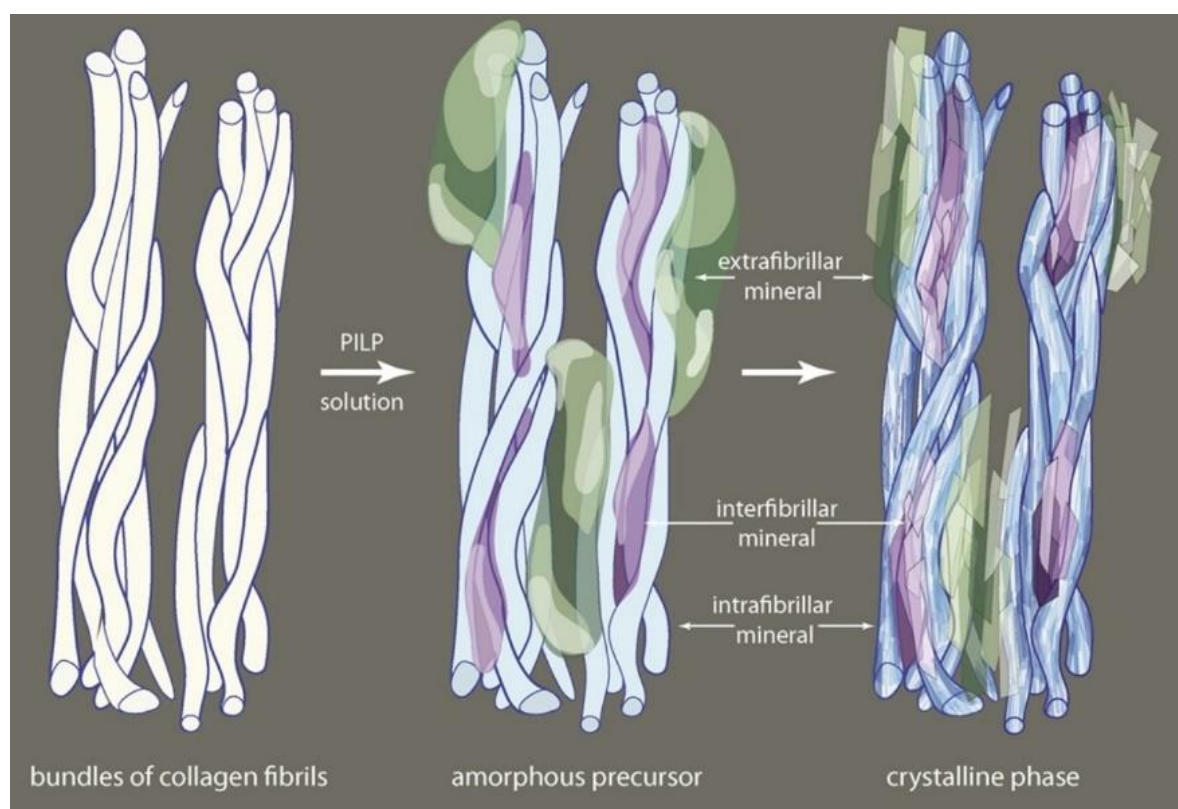


Figure 1.6 The schematic diagram of the polymer-induced liquid-precursor mineralisation process [242].

A well-defined steric structure is important for NCP analogues to regulate the biomineralisation process. Therefore, dendrimers, polymers of similar dimensional scales, topological architecture and biomimetic properties to proteins, become promising candidates to mimic natural NCPs in a remineralisation system. Poly (amidoamine) (PAMAM) dendrimer is the first dendrimer family for biomedical applications and is highly branched with reactive functional groups [243]. Furthermore, several types of PAMAM dendrimers, such as hydroxyl-terminated PAMAM (PAMAM-OH), carboxylic-terminated PAMAM (PAMAM-COOH) and amine-terminated PAMAM (PAMAM-NH₂) have been developed as nucleation templates to

sequester metastable ACP nano-precursors within the collagen matrix and induce remineralisation, especially intrafibrillar mineralisation [244-247]. Poly (amidoamine) has also been incorporated into bioactive multifunctional composites, which induced effective root dentine remineralisation in an acid challenge environment *in vitro* [248].

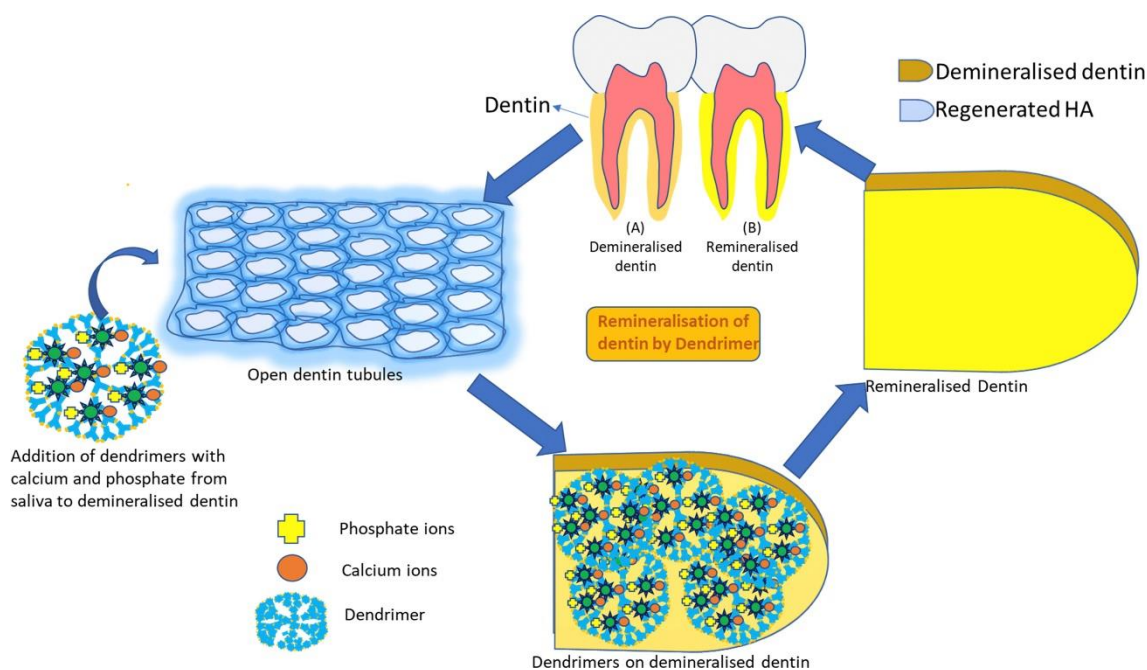


Figure 1.7 Remineralisation of demineralised dentine by dendrimers [249].

Other novel NCP analogues include phosphorylated chitosan (P-chi) [250], oligopeptide [251], glutamic acid [252]. However, biomimetic remineralisation methods in remineralising carious dentine were only performed *in vitro* and remain at a proof-of-concept stage. There is still a long way before these strategies can be used in clinical applications to benefit patients.

1.6.3 Collagen cross-linking agents

In root dentine, mineral crystallites are embedded within the organic dentinal matrix which plays an important role in the de-/remineralisation process. After partial dissolution of the mineral phase in dentine, the exposed dentinal collagen is fragile and easily attacked by bacterial and host-derived enzymes [5]. The irreversible destruction of dentinal collagen makes remineralisation difficult to achieve. Therefore, the stabilisation and strengthening of the dentinal organic matrix by collagen cross-linking agents may prevent demineralisation and promote remineralisation.

1.6.3.1 Synthetic chemical cross-linking agents

Glutaraldehyde

Glutaraldehyde (GA) contains two aldehyde groups which react with the amino groups of lysyl or hydroxyl polypeptide residues in collagen, forming reducible Schiff base cross-links within collagenous biomaterials [253]. Dentine exposed to GA has been observed to exhibit an irreversible increase in its stiffness and elastic modulus as well as improved resistance to collagenase degradation, with these effects from GA being concentration-dependent [254-257]. Moreover, by introducing carbonyl groups onto dentinal collagen, GA provided affinity sites for HAp nucleation and induced mineralisation of collagen [258]. Although GA has been widely recognised to cross-link collagen, it is also known for its cytotoxicity [259]. Therefore, further studies are required to achieve clinically applicable biocompatibility and safe application methods.

Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride

Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) is an imide-based zero-length cross-linking agent which does not add any extra atoms to the cross-linked products [260]. The mechanism of its cross-linking effect lies in the activation of the carboxylic acid groups of glutamic (Glu) and aspartic (Asp) acid residues in peptides *via* giving O-acylisourea groups. Often, N-hydroxysuccinimide (NHS) is added to the solution containing EDC to increase the number of cross-links introduced into the collagen matrix and prevent the hydrolysis of activated carboxyl groups [261]. Exposure to EDC resulted in a stiffer and more cross-linked collagen network, where the elastic modulus and thermal denaturation temperature of dentinal collagen were significantly increased whilst the degradation rate decreased even after 12-months of water storage [262]. Under a simulated dynamic physiological condition of mastication forces, less solubilised type I collagen C-terminal telopeptides (ICTP) and solubilised C-terminal telopeptides (CTX) were collected from the EDC-treated dentine than those without cross-linking [263]. Similar to GA, the cross-linking effect of EDC is concentration-dependent, but EDC has lower cytotoxicity than GA as the released urea derivative from EDC is easily rinsed from collagen, leaving no residual chemicals [264-266]. However, further clinical studies are required to test if EDC is non-toxic to pulpal tissues.

1.6.3.2 Naturally derived cross-linking agents

Considering the toxicity and instability over time of synthetic chemical cross-linking agents, plant-derived cross-linkers have been investigated extensively as an alternative [267]. Most plant-derived cross-linkers are polyphenolic flavonoids which contain a phenyl ring bearing a hydroxyl group with amphiphilic properties. This bi-functionality accounts for their ability to interact with collagen [268].

Proanthocyanidin-rich agents

Proanthocyanidin is a bioflavonoid polyphenol-rich agent with a chemical structure based on flavanols (also referred to as flavan-3-ols) (Figure 1.8 (A)) [269]. It is widely available in fruits, vegetables, nuts, seeds and flowers but its composition is highly diverse [270]. The activity of the PA extract is strongly related to the complexity of its structure, stereochemistry and the constituted bioactive compounds [27, 271, 272]. Specifically, grape seed extracts (GSE) have been identified as one of the most efficient PA-rich agents, containing various oligomeric PA such as catechin, epicatechin (EC), epigallocatechin (EGC) and epicatechin gallate (ECG) (Figure 1.8 (B-E)). Proanthocyanidin interacts with and stabilises dentinal collagen *via* covalent, ionic, hydrogen bonding and hydrophobic interactions [273]. The presence of galloyl moieties offers more hydroxyl groups for cross-linking [271].

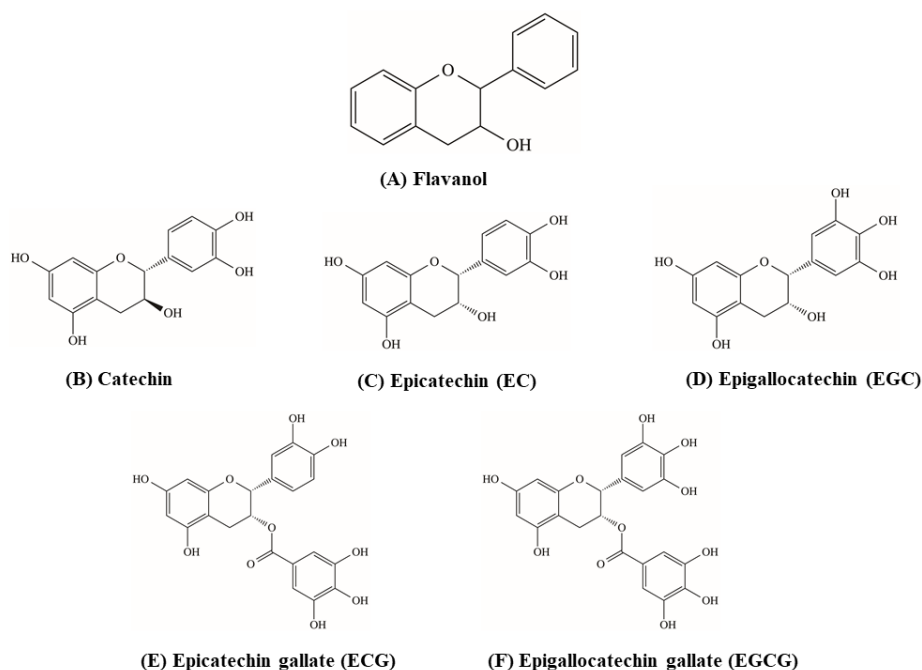


Figure 1.8 The chemical structure of (A) flavanol and (B, C, D, E, F) common monomeric flavanol present in oligomeric proanthocyanidins [274].

Early research on PA revealed its ability to improve the biomechanical properties of demineralised dentine, including ultimate tensile strength and modulus of elasticity [275, 276]. Moreover, PA can alter the biochemistry and hide cleavage sites of dentinal collagen, thus inhibiting the degradation activity of enzymes such as MMPs and cysteine cathepsins [257, 269, 277, 278].

In addition, the effect of PA-rich agents on RCL progression has been specifically noted. Proanthocyanidin inhibited lesion formation on root dentine surfaces in cariogenic bacterial incubation *in vitro* [98]. Under dynamic pH-cycling conditions (a procedure employed to simulate the dynamic variations in mineral deposition and pH values in the natural caries process), PA treatment significantly promoted mineral precipitation and increased the microhardness of carious dentine tissues. Its remineralisation mechanism was ascribed to the stabilisation of the dentine matrix by PA–collagen interaction as well as the formation of PA–calcium complexes [273]. Higher concentrations of PA reduced the treatment time without compromising its effects [279].

However, a clinical limitation of PA is discolouration of the treated tooth surface. In an attempt to address this issue, the extract mixture has been modified by lowering its molecular weight [280]. For instance, the monomeric PA, especially galloylated catechins such as epigallocatechin gallate (EGCG) extracted from green tea (*Camellia sinensis*) exhibited an even greater hydrogen bonding capacity than PA from GSE *in vitro* due to the presence of three vicinal phenolic hydroxyl groups from the galloyl moiety, thus more effective in cross-linking dentinal collagen fibrils and reducing proteolytic degradation [272, 281].

Other polyphenolic flavonoids

Hesperidin (HPN) is a glycoside flavonoid extracted from citrus fruits and belongs to the flavanone subgroup (Figure 1.9 (A)). The mechanism of interaction between flavanone and collagen fibrils has not been completely elucidated. However, as it is categorised as a phenolic flavonoid, it might be explained in a similar manner as the cross-linking effect of other flavonoids such as PA [281, 282]. Hesperidin has the potential to prevent dentinal collagen degradation against proteolytic enzymes [142].

Genipin (GNP) is a hydrolytic product of geniposide, which is extracted from the fruit of *Gardenia jasminoides* Ellis (Figure 1.9 (B)). Genipin reacts with primary amine groups within collagen molecules, forming cross-links [283]. Genipin treatment increased the resistance of collagen fibrils of bovine dentine against enzymatic digestion significantly in a concentration- and time-dependent manner [257]. However, its pigmentation impacts may lead to the staining of treated dentine surfaces, being a clinical limitation due to aesthetic concerns [283].

Tannic acid (TA) is a plant-derived polyphenol and consists of a complex mixture of polygalloyl glucose esters (Figure 1.9 (C)). The hydroxyl groups from TA connected with amide groups in collagen *via* hydrogen bonds, which significantly increased the mechanical properties of dentinal collagen and reduced enzymatic degradation [284].

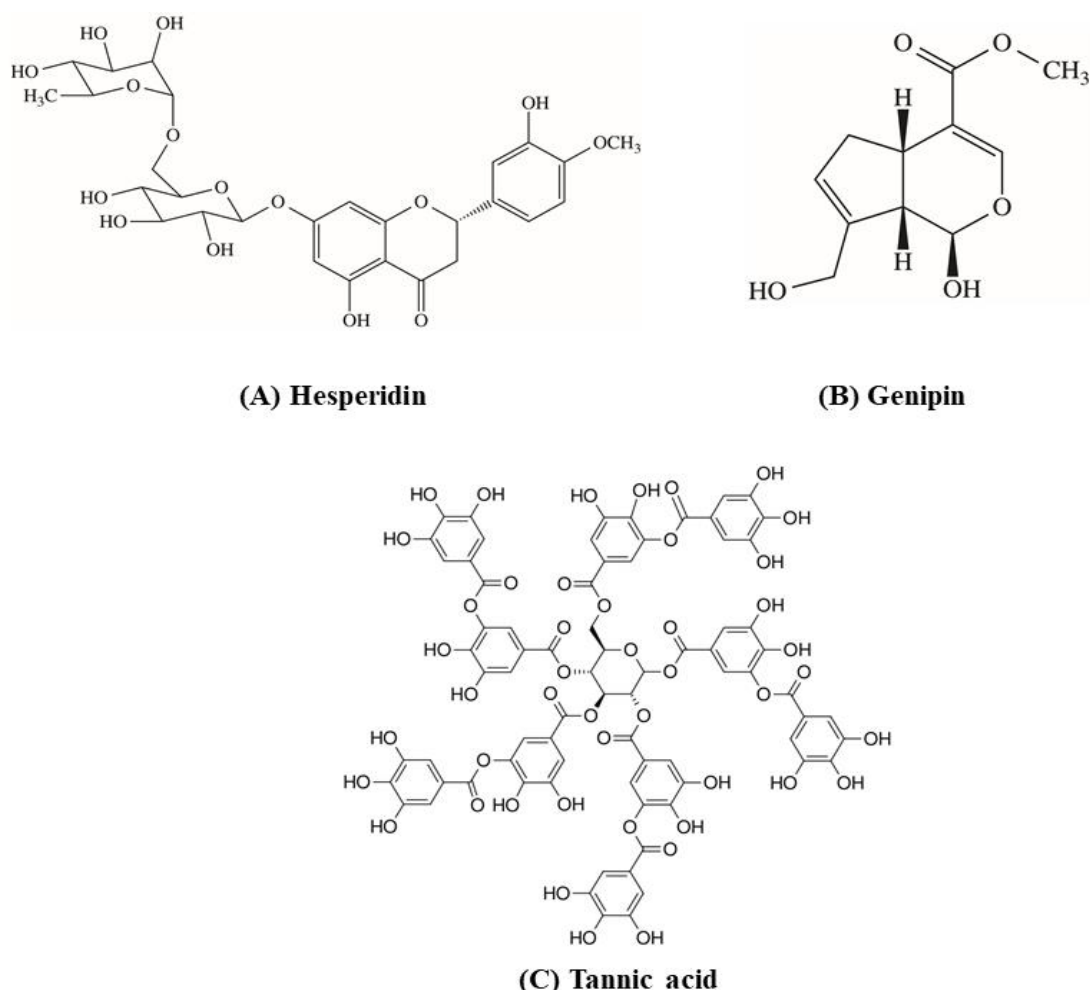


Figure 1.9 The chemical structure of polyphenolic flavonoids used for collagen cross-linking: (A) hesperidin, (B) genipin and (C) tannic acid [281].

1.6.3.3 Photo-oxidative cross-linking methods

Photo-oxidative cross-linking is a physical method which uses light exposure, especially ultraviolet to induce rapid and stable covalent cross-linking of collagen in the presence of singlet oxygen ($^1\text{O}_2$). Photosensitisers such as riboflavin (vitamin B₂) and rose Bengal are employed in this process to produce active singlet oxygen or other oxygen radicals such as hydroxyl radicals ($\text{HO}\cdot$) and superoxide anions (O^{2-}) after light activation. The singlet oxygen interacts with photo-oxidisable amino acid residues in a protein molecule to generate photo-oxidised products, which in turn react with residues in another protein to form cross-links [285, 286].

Riboflavin is one of the most frequently used photosensitisers for photodynamic cross-linking in dentine. Ultraviolet light A (UVA)-activated riboflavin increased the mechanical properties and biodegradation resistance of the dentinal collagen matrix as well as significantly inactivated endogenous proteases of dentine. A higher concentration could achieve a more clinically acceptable timeframe for application [287-289]. A recent study indicated UVA-activated riboflavin treatment increased the acid resistance of root dentine where significantly reduced mineral loss and lesion depths were observed after *in vitro* cariogenic challenges compared to that without treatment [290]. However, riboflavin at high concentration might lead to yellowish discolouration of dentine, in addition, there are concerns regarding the cytotoxic potential of riboflavin and clinical practicality of UVA light sources [291].

1.6.4 Enzyme inhibitors

Following the demineralisation caused by acids produced by oral micro-organisms, odontoblast or pulp-derived enzymes in dentine such as MMPs and cysteine cathepsins are activated and further degrade dentinal collagen. Therefore, the application of enzyme inhibitors is considered to be effective in inhibiting the degradation of demineralised dentinal matrices and RCL progression [292].

Other than the antimicrobial ability mentioned in 1.5.3.1, CHX could also act as an anti-proteolytic agent which reduces the activity of several host-derived proteases, such as MMP-2, -8 and -9, thus preventing the exposed collagen matrix from denaturation [293]. Chlorhexidine digluconate (0.12%) had therapeutic potential in controlling the progression of RCLs by inhibiting the proteolytic activity of carious root dentine [294]. Chlorhexidine is the

most used and widely recognised MMP inhibitors and it has often been used as a positive control to investigate the efficacy of other novel MMP inhibitors [295].

Several collagen cross-linking agents mentioned in 1.6.3.2, such as PA and EGCG, exhibited inhibitory effects against endogenous collagenases. Specifically, PA could effectively inactivate more than 90% of soluble recombinant MMP-2, MMP-8 and MMP-9 and around 75–90% cysteine cathepsins, higher than CHX [278]. In addition, treating dentine matrices with EGCG solution reduced collagen degradation by MMP and cysteine cathepsins, where a significantly decreased amount of the degraded collagen products such as ICTP and CTX fragments were released [296].

In addition, divalent metals such as Zn^{2+} can bind to specific sites of MMPs and thus inactivate the catalytic function of the enzymes [297]. Specifically, ZnF_2 agents demonstrated higher efficacy in inhibiting dentine demineralisation compared to other topical F agents such as NaF. This was attributed to the capability of Zn^{2+} in inhibiting the activity of collagenase enzymes and thus preventing collagen degradation [187, 298]. Similar effects have also been observed for iron which showed inhibitory effects on MMP-2 and -9 activities [299]. However, further studies should be conducted to examine the enzyme activities directly in order to eliminate other functions of these metals in preventing demineralisation.

1.7 Invasive treatments of root carious lesions

When the attempt to halt the progression of RCLs has failed and the lesion has advanced to frank cavitation, restorative treatment is required if the biofilm can no longer be disrupted and/or removed. Traditional restorative procedures based on the G.V. Black's concepts and surgical approaches without preventive actions can lead to recurrent caries and early failures of restorations, introducing the teeth to a repeat restoration cycle which is not cost effective [300]. Therefore, new management approaches have been proposed to protect dental hard tissues against caries and improve the longevity of restorations [301].

1.7.1 Minimally invasive methods for tissue removal

The conventional complete carious tissue excavation (nonselective removal to hard dentine) with rotary instrumentation can result in considerable removal of tooth structure and extensive tissue loss, which is now considered to be overly destructive in many instances [302]. Also, it is often not feasible for use in elders with physical or cognitive impairments as well as

domiciliary treatment for institutionalised elders when a portable dental unit with rotary instrumentation is not available [303]. Therefore, modified caries removal techniques, *i.e.*, selective removal of carious tissues, which advocates removing only non-remineralisable/denatured (necrotic) dental tissues whilst preserving the remineralisable and sound tissues, are considered for the restorative treatment of RCLs. However, clinical discrimination between these two layers of dentine tissues is difficult.

1.7.1.1 Atraumatic restorative treatment

Atraumatic restorative treatment (ART) is a minimal intervention care approach with the aim of preventing the development of carious lesions and restoring dentine carious lesions in a minimally invasive way. It is a lesion management concept that can be used for RCL treatments [304]. In ART, the necrotic carious dentine is removed with hand instruments whilst the remineralisable dentine is preserved. A restoration is then placed using an adhesive restorative material (usually GIC), which restores the cavity and seals any residual demineralised tissues simultaneously [305]. Without the use of rotary instrumentation, ART is more acceptable and accessible to the frail elderly or nursing home residents or in communities where access to a dental clinic is not possible [306].

The survival rates of single-surface ART restorations have been compared with that of conventional rotary technique. In general, more failures happened in ART restorations than those used rotary techniques, with the overall single-surface survival rate of 85.4% for ART and 90.9% for rotary techniques after 2 y. The survival rate of ART restorations could remain high in a longer period if ART was performed by experienced trained operators. However, compared to other tooth surfaces (*e.g.*, mesial/distal, buccal/lingual and occlusal/incisal surfaces), restorations on root surfaces have higher failure rates because of the difficulty in moisture control, the size and shape of cavities as well as the widespread nature of RCLs which tend to cover a larger surface area that is shallow rather than a distinct cavitation that occurs in other parts of a tooth. Thus, the survival rates of restorations on root surfaces were relatively lower with 61% for ART and 73% for the rotary technique [307-309]. In addition, failure of ART is associated with multiple factors including the restorative materials used, operator technique as well as the oral health status of the patient [310]. Despite limited evidence regarding the restoration survival, ART is useful for outreach dental services and warrants further investigation.

1.7.1.2 Tissue removal and surface modification with lasers

Application of laser technology for carious tissue removal and tooth surface modification has been an area of research interest. Absorption of laser irradiation causes morphological and chemical changes in dental hard tissues and possibly achieves hard tissue ablation depending on the wavelength, energy density and pulse duration of the laser irradiation [311-313]. Additionally, lasers were found to enhance F uptake into dentine, thus achieving a synergistic effect with F regimens on caries prevention of root dentine [314]. The frequently used active laser media for altering dental hard tissues include CO₂ lasers, free-electron lasers (FEL) and erbium, chromium: yttrium-scandium-gallium-garnet (Er, Cr: YSGG) lasers.

The CO₂ laser with a wavelength of 10.6 μm is highly absorbed by carbonated HAp. The thermal effects of irradiation potentially lead to ultrastructural and crystallographic changes in dentine minerals. Along with the ability to seal dentinal tubules, CO₂ laser irradiation increased the acid resistance of root dentine significantly [315, 316]. Low energy density levels (6-11 J/cm² for 10-20 s) of CO₂ laser irradiation caused a very small variation in pulp temperature that did not cause detectable thermal damage to pulp tissues [317]. Combination of CO₂ laser irradiation with F treatment would exert additive effects on reducing dentine demineralisation in acid challenge compared to CO₂ laser or F alone if suitable laser energy density and F levels were chosen [315, 318].

Carbonated HAp in dentine has intense absorption bands in the mid-infrared (MIR) region due to the presence of phosphate, carbonate and hydroxyl groups [319]. A tuneable mid-infrared free-electron laser (MIR-FEL) was used *in vitro* for carious dentine tissue modification towards well-recrystallised HAp-like materials. After modification, improved acid resistance and dentinal tubule sealing of root dentine were shown. Nevertheless, an in-depth explanation of the mechanism is required for the clinical application of FEL laser irradiation for dentine modification [319, 320].

The Er, Cr: YSGG laser is favoured in minimally invasive procedures, as it can be used for morphological and chemical modification of dentine surfaces and preparation of microcavities depending on its energy densities [312, 321]. Application of an Er, Cr: YSGG laser associated with 2% neutral NaF had a synergistic effect on increasing the acid resistance of human root dentine *in vitro* [322]. However, when an Er, Cr: YSGG laser was used for cavity preparation, the issue of marginal integrity of restorations on laser-prepared dentine was controversial.

Although the heat generated during laser irradiation might increase the Ca/P ratio in dentine due to the chemical changes in the molecular structure, resulting in more stable and less acid-soluble compounds which are less susceptible to acid attack and caries compared to that without irradiation, the presence of irregularities on the edge of the cavities and the difficulties in obtaining adequate marginal seal after carious tissue removal with lasers might affect restoration survival [323-325].

1.7.1.3 Chemo-mechanical methods of tissue removal

The chemo-mechanical method is a promising minimally invasive alternative for carious tissue removal by applying a solution to remove the outer ‘infected’ and non-remineralisable carious dentine and is often used to assist the manual removal of carious tissues [15]. Carisolv® gel (RLS Global AB, Gothenburg, Sweden) is one of the commercial chemo-mechanical agents composed of sodium hypochlorite and three amino acids (*i.e.*, glutamic acid, leucine and lysine) which are thoroughly mixed before use. After application, the positively and negatively charged groups on the amino acids become chlorinated. The chlorinated products interact with proteins in the denatured dentinal collagen and disrupt the collagen cross-linkage in the carious dentine matrix. The softened dentine is abraded by specially-designed hand instruments to leave a ‘hard, caries-free’ cavity [326]. Results from clinical trials showed that denatured dentine tissues could be effectively removed using the chemo-mechanical method and the one-year clinical performance of the restorations was comparable to that using a rotary removal method [327]. When Carisolv® was used in conjunction with ART in a geriatric population, the gel did not improve the survival rate of single-surface restoration of RCLs with GIC. Nevertheless, application of Carisolv® promoted removal of denaturalised dentine with manual instruments and reduced pain in this process [328].

1.7.2 Restorative Materials for root carious lesions

1.7.2.1 Glass ionomer cement

Glass ionomer cement is suitable for restoring RCLs due to its tolerance of moisture, ease of placement and ability to form chemical bonds to the tooth structure. Fluoride released from GIC promotes the conversion of HAp in dentine to the more acid-resistant fluorapatite (FAp), thus protecting root surfaces against cariogenic challenges [329]. The incidence of recurrent caries surrounding GIC restorations was reported to be significantly lower than resin composite or amalgam restorations [330, 331]. Despite lower flexural strength of GIC than resin

composite, this issue is not of central importance in the restoration of RCLs where load bearing is not paramount.

Modifications have been made in GIC composition to improve its mechanical properties as well as its ion-release ability to promote remineralisation. Specifically, restorations with high-viscosity GIC (HVGIC; Fuji IX GP; GC Corporation, Tokyo, Japan) on approximal RCLs achieved an average survival time of up to 74 months, supporting the use of GIC for restoration of RCLs in low stress-bearing areas [332]. Incorporation of CPP-ACP into GIC (Fuji VII EP; GC Corporation, Tokyo, Japan) protected dentine from acid demineralisation and inhibited marginal caries around restorations on root surfaces *in vitro* [333, 334]. Resin-modified GIC (RMGIC), which includes a free radical-polymerisable resin component, has superior mechanical properties than conventional GIC whilst retaining its advantage of anti-cariogenicity. Resin-modified GIC was reported to present a particularly strong effect on inhibiting recurrent caries formation around restorations compared to resin composite and poly-acid modified composites (compomer) [335]. Additionally, when RMGIC was used as a lining material in root caries treatment to protect the pulp from further bacterial invasion, it did not affect marginal integrity and retention of resin composite restorations over an 18-month period [336]. Moreover, adjunctive application of remineralising agents such as APF gel and SDF before the placement of GIC restorations may inhibit the progression of recurrent caries adjacent to restorations, thus extending the longevity of GIC restorations [337].

1.7.2.2 Resin composite

Resin composite has also been used for the restoration of RCLs, which does not require excessive removal of sound tooth structure when an adhesive system is used. However, good moisture control is essential, though difficult to achieve in subgingival areas, during the placement of resin composite to avoid failure of adhesive bonding. In addition, the lower permeability of root dentine than coronal dentine may reduce the infiltration capacity of hydrophilic resin into root dentine structure, leading to lower bond strength compared to resin composite on coronal dentine [338]. Due to its lack of anti-cariogenic properties, resin composite resulted in a higher rate of marginal caries than GIC in xerostomic patients who did not use a topical F supplement [339]. An alternative approach to address this problem is to modify the bonding systems to create therapeutic effects by incorporating antimicrobial and remineralising components. For instance, quaternary ammonium has a wide spectrum of antimicrobial activity, which is combined with a methacryloyl group to create an antimicrobial

monomer called 12-methacryloyloxy dodecylpyridiniumbromide (MDPB). Adhesives containing MDPB significantly reduced marginal lesion formation around resin composite restorations for RCLs *in vitro*, being a good candidate for restoring RCLs if further *in situ* and clinical studies are undertaken [340]. Other antimicrobial monomers incorporated into adhesive systems for root caries prevention include dimethylaminohexadecyl methacrylate (DMAHDM) [341]. However, concerns exist regarding the time of efficacy of these antibacterial dental monomers [342]. Except that no clinical evaluation of these monomers has been published, more robust *in vitro* studies with longer durations are in need to fully address concerns on survival of the monomer-incorporated restorations and persistence of their antibacterial activity.

1.7.2.3 Amalgam

Amalgam was routinely used to restore RCLs in the past [343]. Compared to other restorative materials, amalgam has advantages such as low-technique sensitivity, self-sealing margins due to corrosion products and good wear resistance. However, it lacks adhesive and therapeutic qualities. Fluoride-releasing amalgam was developed to confer amalgam with therapeutic effects, which decreased the incidence of recurrent caries when used for RCL restoration [344]. However, the amount of F released from this amalgam was small and the release was of short duration [14]. As amalgam requires excessive removal of tooth structure to obtain adequate mechanical retention form, it is not likely to be considered as a viable long-term option for restoration of RCLs in light of the concept of minimally invasive dentistry (MID) in cavity preparation which advocates that only the decomposed dentine needs to be removed from within the cavity whilst the potentially remineralisable dentine is remained [345]. In addition, application of dental amalgam has been phased down according to the Minamata Convention to protect the human health and the environment from anthropogenic releases of mercury and mercury compounds [346].

To date, there is insufficient evidence to recommend any specific restorative materials for cavitated RCLs, as all have limitations. In addition, selection of materials depends on the patients' compliance to adjunctive treatments such as oral hygiene, dietary control and frequent use of F products. There is an urgent need to evolve restorative materials and procedures to overcome the problems resulting from difficulty in moisture control, poor marginal adaptation and further development of marginal caries. Moreover, further well-controlled randomised clinical trials are warranted to guide practitioners to treat RCLs.

1.8 Laboratory studies of root carious lesions

1.8.1 Artificial root carious lesion models

Natural RCLs may vary in physiochemical properties such as the lesion dimension, MD and colour; thus, it is difficult to find sufficient similar lesions for laboratory studies. Artificial RCL models offer significant advantages over natural lesions in the laboratory setting for their reproducibility and consistency.

Demineralisation can be created by exposing tooth surfaces to solutions undersaturated with respect to HAp. Specifically, Van Dijk and colleagues presented a mathematical model to obtain artificial caries-like lesions in 1979, which was further developed by ten Cate to produce artificial enamel lesions [347, 348]. Later, Xie and colleagues used the same method to produce artificial RCLs [273]. Currently, the solutions used for producing artificial carious lesions have a pH ranging from 4.4 to 5.0 adjusted by acetate or lactate buffers which simulate the organic acids produced by cariogenic microorganisms and the demineralisation duration ranges from 16 h to 5 d [140, 349-351]. In addition, synthetic polymer gels such as Carbopol gels with PAA of high molecular weight have been used for the preparation of artificial RCLs. The gels protect the softened surface and produce subsurface lesions by mimicking ion diffusion in biofilms [352]. Despite the absence of bacteria and enzymes, the chemical model systems can create lesions with histological similarities (especially the mineral content and hardness) to natural RCLs of similar depths [353]. Nevertheless, carious lesions created with various chemical protocols have different characteristics that may influence the remineralisation efficacy of treatment agents [354].

In addition to the above static methods to create artificial lesions, pH-cycling models have been employed to mimic the dynamics of mineral loss and gain in carious lesion formation in the oral environment by exposing dental substrates to demineralising and remineralising solutions alternately. The exposure time and ionic concentrations in both solutions vary according to the desired outcomes. The duration of the whole pH-cycling process varies from 18 h to 14 d [144, 355-357].

However, the above chemical models are not able to reflect the role of microbes in lesion formation and the antimicrobial effect of certain treatments on RCLs. Therefore, biofilm models using microbial inoculation for biofilm growth on root dentine specimens for artificial

RCL formation have been developed. Specifically, highly acidogenic and aciduric bacteria such as *S. mutans* and *Lactobacilli* have been considered important in the aetiology of root caries and thus adopted as the microbial source for biofilm growth on root dentine slabs [358, 359]. Furthermore, a consortia plaque biofilm composed of four species, *i.e.*, *S. mutans*, *S. sobrinus*, *Lactobacilli naeslundii* and *Lactobacilli rhamnosus*, was constructed as a RCL model system [360, 361]. Although after incubation with these selected species of bacteria, the root dentine fragments showed alterations similar to that of RCLs, the complex dental biofilm which consists of hundreds of bacterial species is difficult to simulate in the laboratory setting [358, 360, 362].

In addition, the complexity of the oral environment has led to the development of sophisticated ‘artificial mouth’ systems which simulate the oral humidity, temperature and salivary constitution and flow [363]. Also, mixed salivary microbes were obtained from individuals to form a microcosm to inoculate the root samples in the artificial mouth with continuous or intermittent nutrients supplied to the biofilm [197]. Moreover, in *in situ* studies, root dentine specimens were placed into removable intraoral palatal appliances and therefore samples were colonised by the individual wearer’s own microbiome and placed in nearly the same conditions as in RCL development [140, 364-366]

To summarise, artificial RCL models are less costly, timely and reproducible approaches to evaluate the effects of treatments on RCLs *ex vivo*. Nevertheless, they cannot fully replicate the multifactorial nature of RCLs in the oral environment. Clinical trials are warranted with multiple participants and variable oral conditions to draw valid conclusions.

1.8.2 Characterisation of root carious lesions

1.8.2.1 Mineral density

Transverse microradiography (TMR) and micro-computed tomography (micro-CT) are the most frequently used techniques to determine the MD of dental hard tissues. Transverse microradiography is considered as the “gold standard” two-dimensional (2D) imaging method for determination of mineral loss or gain and lesion depths in experimental carious lesions, thus being used for the comparison and validation of other carious lesion quantification techniques [367]. Despite lower image resolution than TMR at present, micro-CT enables a non-destructive 3D analysis of MD of an entire specimen without specific time-consuming and destructive specimen preparation. When comparing the two methods, similar results of

integrated dentine mineral changes can be determined from TMR and micro-CT, whilst lesion depths measured from the two methods are inconsistent particularly in very deep or shallow lesions putatively because of the lower accuracy of micro-CT in delineation of outer boundaries of carious lesions [368].

1.8.2.2 Optical properties

Polarised light microscopy

Polarised light microscopy (PLM) analysis is a sensitive technique which can be used to show mineral changes in dental hard tissues by identification of its birefringence, being an effective approach to evaluate de-/remineralisation progression [369, 370]. In water, dentine birefringence is dominated by positive birefringence of the oriented collagen matrix which is greater than the similarly oriented but smaller intrinsic carbonate HAp crystals. During demineralisation, the mineral crystal loss causes a reduction in negative birefringence and enhances the resultant positive birefringence [10]. When mounted in quinoline solution, demineralised dentine is easily distinguished by showing a reversal sign of birefringence to sound dentine due to the ‘phenol reaction’ – a phenomenon occurs when the collagen fibres have lost some mineral phase and oriented adsorption of phenol molecules reverses the sign of birefringence [371]. The distinct birefringence between sound and demineralised dentine enables delineation of the outer surface contour of a lesion, thus enabling measurement of the lesion depth [70]. However, the quantitative relationship between changes in mineral content and birefringence has not been fully established, which limits the application of PLM in determining de-/remineralization of dentine quantitatively.

Optical coherence tomography

Optical coherence tomography (OCT) is an emerging tool utilising interferometric techniques to detect and assess carious lesions. Specifically, by measuring the echo time delay and magnitude of backscattered light, optical properties of the tooth structure are determined and interpreted into high-resolution, cross-sectional imaging of the hard tissues. This is a promising non-destructive and straightforward approach of diagnosing and detecting RCLs, which can potentially be used clinically. [372].

Swept-source OCT (SS-OCT) is one of the main OCT technologies used to obtain real-time morphological imaging of teeth and quantitatively estimate the progression of carious lesions. Lesion depths obtained from OCT have been reported to be comparable to that measured by

TMR. Moreover, there was a positive correlation between the integrated reflectivity (which increases with demineralisation progress) measured from OCT to the integrated mineral loss measured by TMR. Thus, it is possible to evaluate the MD of teeth based on OCT. However, the scattering property of dentine is associated with the orientations of dentinal collagen and HAp crystals. Variations in the scattering coefficient may influence the accuracy and reproducibility of MD estimation from OCT [373-375].

Taking advantage of the birefringent properties of dentine, polarisation-sensitive (PS)-OCT was developed to provide enhanced structural information where tooth demineralisation can be distinguished based on not only increased light scattering in porous demineralised tissues but also depolarisation of incident light. Similar to SS-OCT, PS-OCT images allow for non-destructive analysis of root dentine demineralisation, comparable to the well-established methods of TMR and PLM [376].

1.8.2.3 Morphological observation

The magnified structural features of teeth were first observed by optical microscopy whilst the advent of electron microscopy enables the visualisation of tooth structures in a micro- and nano-scale.

High-resolution transmission electron microscopy (TEM) techniques, occasionally combined with selected area electron diffraction (SAED) and microanalysis, enable site-specific investigation of dentine ultrastructure, crystallography as well as elemental analysis at a micro-fibrillar level. In particular, when TEM is used to observe the remineralisation process, it provides information on intra-fibrillar and extra-fibrillar apatite formation and the morphological interaction between minerals and collagen. The presence of HAp platelets within fibrils leads to banding patterns along the direction of periodicity of collagen shown as electron dense areas [377, 378]. However, there are concerns in TEM studies of biological tissues regarding the sample preparation as the biological specimens are exposed to the electron beam which may damage the samples, inducing artefactual results [379].

Electron images obtained from scanning electron microscopy (SEM), despite having lower resolution than TEM images, also demonstrate the dentine morphological changes in the de-/remineralisation process. Specifically, exposed and permeable dentinal tubule orifices are frequently present on demineralised dentine surfaces. At high magnification, the surface

morphology of active carious lesions could be porous and rough with collagen exposed and distributed in a disorganised manner as well as having sparsely distributed fibrils [380]. Remineralisation can induce the formation of apatite-like clusters on the dentine surface. Estimation of Ca/P molar ratios of the precipitates can be conducted if combined with microanalysis, allowing speculation of the components of the clusters [381]. In addition, observation of cross-sectional dentine surfaces could provide information on remineralisation of demineralised dentine collagen fibrils in dentinal tubules [382].

The development of focused-ion-beam-SEM (FIB-SEM) imaging in recent decades enables analysis of the 3D structure of teeth *via* reconstruction of consecutive high resolution 2D SEM photomicrographs reconstructed to a 3D image. When used in studies of carious lesions, FIB-SEM contributes to better understanding of the remineralisation mechanisms from a 3D observation and analysis [383]. Moreover, FIB-SEM is a useful tool for preparing TEM specimens of precisely selected areas [384].

1.8.2.4 Mechanical properties

Micro-indentation techniques such as microhardness testing are the most frequently used methods to measure the mechanical properties of dentine, where the hardness is determined from an applied load divided by the projected area of the indentation. However, the hydration status of the specimens is likely to influence the results and the large size of indentations with respect to the dentine microstructure is not able to provide information on site-specific mechanical properties of dentine which is an anisotropic biological tissue [385].

Application of ultra-micro indentation, nanoindentation and atomic force microscopy (AFM) which have a small length scale of indenters or probes offers the potential for high resolution imaging of hydrated samples and enables site-specific measurements [386-389]. Specifically, indentation using these techniques could be conducted on different dentine regions such as the inter-tubular or peri-tubular dentine which are microstructurally and compositionally heterogeneous and undergo different demineralisation progress, resulting in distinct mechanical properties [390]. Moreover, the site-specific elastic modulus and hardness values obtained could distinguish the remineralisation occurring in either intrafibrillar or interfibrillar spaces, providing an insight into the process and mechanism of remineralisation [11].

1.8.2.5 Chemical analysis using spectroscopic techniques

Raman and Fourier transform infrared (FTIR) spectroscopy are complementary vibrational spectroscopic techniques, utilising light scattering and absorption effects, respectively. The Raman and FTIR spectra obtained illustrate the characteristic bands of both inorganic and organic phases in dentine. The chemical features of dentine and mineral crystallinity distribution, collagen structure and cross-linkage alteration can be evaluated from the location, intensity and ratio of the corresponding bands [391]. Typical band assignments in Raman and FTIR spectra of dentine are shown in Table 1.3.

Table 1.3 Band assignments in Raman and Fourier transform infrared spectra of dentine [392-394].

Technique	Wavelength range (cm ⁻¹)	Assignment
Raman Spectroscopy	429-449	PO ₄ ³⁻ ν_2
	578-590	PO ₄ ³⁻ ν_4
	854	δ (CCH) aromatic (Pro, Tyr)
	875-880	ν (C-C) aromatic (Tyr, Hyp)
	961	PO ₄ ³⁻ ν_1
	1003-1005	ν (C-C) phenyl ring
	1031-1034	Pyridine ring
	1046	PO ₄ ³⁻ ν_3
	1071	B-type CO ₃ ²⁻ ν_1
	1103	A-type CO ₃ ²⁻ ν_1
	1243-1268	Amide III (ν (C-N) and δ (N-H))
	1447-1452	CH ₂ wag
	1670	Amide I (ν (C=O))
Infrared spectroscopy	2882-2986	ν (C-H) CH ₂
	470	PO ₄ ³⁻ ν_2
	568, 603	PO ₄ ³⁻ ν_4
	872	B-type CO ₃ ²⁻ ν_2
	897	A-type CO ₃ ²⁻ ν_2
	958-961	PO ₄ ³⁻ ν_1
	1003, 1013	HPO ₄ ²⁻ ν_3
	1032, 1046, 1087	PO ₄ ³⁻ ν_3
	1065-1103	CO ₃ ²⁻ ν_1
	1230-1238	Amide III
	1232	N-H deformation
	1235-1240	ν (C-N)
	1447-1452	δ (C-H) CH ₂

1540	Amide II
1542	δ (N-H)
1544	ν (C-N)
1544	$\text{CO}_3^{2-} \nu_3$
1630-1673	Amide I
3398	$\text{H}_2\text{O} \nu_1, \nu_2, \nu_3$

Note: ν : stretching mode; δ : bending mode.

In addition, the development of imaging/mapping function of Raman and FTIR micro-spectroscopy provides spatially resolved 2D spectroscopic images of a sample, which elucidate compositional and structural differences within the dentine substrate at a microscopic level [395]. Compared to conventional histological and microscopic methods, the micro-spectroscopy imaging technique is non-invasive, quantitative and less prone to human subjectivity [396].

1.8.2.6 Biological analysis of collagen degradation

Spectrophotometric measurement of hydroxyproline

The amount of collagen degradation of the exposed organic matrix after mineral loss is normally quantitatively determined by hydroxyproline (HYP) assay. Specifically, proline is thought to be confined almost exclusively to the connective tissue collagen and is present in the Y position of the Gly-X-Y repeating tripeptide [397]. Proline hydroxylation catalysed by prolyl hydroxylase results in the post-translational product of HYP [398]. Therefore, testing the amount HYP is determinant to quantify collagen hydrolysis. HYP assay has been used to evaluate the effect of collagen crosslinking agents on the inhibition of dentinal collagen degradation, where lower concentration of HYP release indicates the enhanced resistance of dentinal collagen to enzymatic degradation after cross-linking [399]. However, HYP is a non-specific collagen degradation marker, which is not informative on specific enzyme activity in dentinal collagen degradation.

Zymographic analysis of MMP activity

Several host-derived MMPs present within dentine or saliva are potentially activated during carious lesion development. Zymography, an electrophoretic method for measuring proteolytic activity, has been used as a simple, sensitive and quantifiable assay to analyse the expression of certain MMPs in carious lesion progression [400]. Specifically, samples containing enzymes

from dentine are firstly separated by molecular mass using the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) where the SDS gels are copolymerised using preferential proteolytic substrates of different MMPs. Although denatured during electrophoresis, the concentrated MMPs in the gel could be reactivated and digest the substrate, resulting in clear bands against the blue background of the intact substrate stained by Coomassie blue [401]. According to the different preferential substrates of MMPs, zymography is divided into gelatine zymography (used for MMP-2, MMP-9), casein zymography (used for MMP-3, MMP-7) and collagen zymography (used for MMP-1, MMP-8) [402]. In addition, the development of *in situ* zymography allows for exploration of the exact locations of enzymes in the demineralised dentine matrix and their activities in physiological and/or pathological conditions. For instance, zymographic analysis has been employed to investigate the effects of cross-linkers on inactivation of dentine MMPs, where a reduction in the MMP-2, -9 and -8 release was shown in cross-linker treated demineralised dentine samples [403]. Compared to the HYP assay, zymographic analysis provides information on the activity of specific enzymes during collagen degradation.

Application of the biological analysis techniques has enhanced the understanding of the role of collagen degradation and host-derived enzymes in the progression of RCLs and provide assistance in exploring novel treatment approaches which target the preservation of the organic materials in root dentine.

In summary, the characterisation techniques mentioned above evaluates the physicochemical or biological properties of root dentine from different aspects in the laboratory setting, multiple evaluation techniques should be employed to determine the overall characteristics of root dentine during RCL progression and the efficacy of various treatment approaches on RCLs.

1.9 Summary

To conclude, reviews of published literature has highlighted the effectiveness of high concentration F regimens as non-invasive approaches for RCL treatment by mediating the demineralisation and remineralisation balance in oral environment. As F does not target the reduction of dental plaque directly, adjunctive application of antimicrobial agents has also been proposed to modulate the oral biofilm. However, most studies regarding the effects of antimicrobial agents were conducted on coronal carious lesions with scant evidence on root surfaces which have a different microbial composition of biofilm. In addition, advanced

technologies have been utilised to incorporate other remineralising agents such as calcium phosphate-based systems into F products in an attempt to enhance their remineralisation efficacy. However, it is still controversial whether the addition of bioactive remineralising agents has superior effects over F alone; particularly as there is a lack of published evidence of their effectiveness on RCLs.

Moreover, the structural organisation of the dentine organic matrix and its role in the progression and treatment of RCLs has been highly recognised in recent decades. Accordingly, novel remineralisation strategies have been proposed to preserve the collagen matrix from degradation and promote remineralisation from a biomimetic manner. However, most biomimetic remineralisation approaches are still experimental procedures and not clinically applicable. Therefore, it is timely to find alternative approaches to preserve the dentinal organic matrix for RCL remineralisation.

With respect to the restorative treatments of RCLs, currently, there is insufficient evidence to recommend any specific materials for the restoration of cavitated RCLs. Moreover, involvement of the dentine organic matrix in bonding procedures poses more challenges and limitations in restorative treatments of RCLs. In this regard, further studies are warranted to evaluate and improve the performance of different restorative materials on RCLs, especially when bonded to the demineralised but remineralisable root dentine.

1.10 Aims and objectives

Following the review of the literature, further evidence is warranted to confirm the effectiveness and mechanisms of high concentration F regimens in RCL treatments, especially the potential additive or synergistic effects of combined F delivery methods. In addition, whether the addition of calcium phosphate-based bioactive compounds in F products would have complementary remineralising effects on RCLs needs further validation.

Moreover, in light of the emphasis on the preservation of the dentinal collagen in dentine remineralisation, application of the naturally derived collagen cross-linking agent – PA has raised great interest, as it could strengthen the structural organisation of the dentine organic matrix which acts as a scaffold to promote the remineralisation of RCLs. However, the treatment time and procedures of PA proposed in the existing laboratory research are not clinically applicable. Considering the different mechanisms of PA and F in dentine

remineralisation, it would be of interest to investigate the effects of PA when used as a bio-mediator to stabilise the dentine organic matrix with the adjunctive application of F agents in a clinically applicable time for RCL treatments.

In addition, amongst all the restorative materials for cavitated RCL restorations, GICs seem to be the promising choice due to their tolerance of moisture, chemical adhesion to dentine and anti-cariogenic properties, especially with the advent of different types of GICs with improved physicochemical and ion-release properties. However, their performance on RCLs is inconclusive, especially on remineralisable carious dentine where the demineralised organic matrix is involved in bonding. Although PA has been incorporated in the bonding procedure of resin composite to strengthen the dentinal collagen and stabilise the bonding interface, the effects of PA in GIC bonding on root dentine have not been explored.

Therefore, the overall aim of this thesis is to investigate the effects of PA, a collagen cross-linking agent, when used as an adjunctive treatment for RCLs. Further evidence regarding the efficacy of currently recommended treatments for RCLs in the laboratory setting where the number of experimental variables can be restricted will be provided.

Specifically, the objectives of this thesis are:

- 1) To investigate the effects of a PA-stabilised dentinal organic matrix on the efficacy of SDF/KI for RCL treatment.
- 2) To evaluate the remineralising effects of FV containing Ca-P remineralisation systems and 5,000 ppm FTP on RCLs and the potential effects of PA when used as an adjunct to FV and FTP on RCLs.
- 3) To evaluate the adhesion and interfacial interaction of HVGIC, RMGIC and CPP-ACP-modified GIC to sound and demineralised root dentine with PA conditioning.

Chapter 2

Effects of silver diammine fluoride/potassium iodide on artificial root caries lesions with adjunctive application of proanthocyanidin

2.1 Introduction

With the change in demography of society and improvement in oral health behaviour, the retention of natural teeth is progressively increasing in developed countries [1]. As a result, root caries, a cause of oral pain in the elderly, is expected to increase exponentially due to greater retention of their teeth and therefore cervical root surfaces being present, caused by age-related gingival recession [2, 8, 13, 404]. Treatment of carious root surfaces can be more challenging than those of coronal surfaces due to the complex pathological processes and the difficulty in restoring the root structure, composed of complex protein assemblies and organised mineral components of root dentine.

Dentine is an hierarchically bio-composite material under multiple template-mediation. The basic building block of dentine is highly mineralised collagen fibrils (~50-100 nm diameter), where the tablet-shaped carbonated apatite crystallites (~5 nm thick, 70% by weight) are embedded within the gaps of the fibrils with a c-axis orientation preferentially aligned along the long axis of the fibril. Proportionately massive periodic mineralised collagen fibrils are densely staggered and overlapped and form covalent intra- and inter-molecular cross-links [3, 4, 20, 405]. The nanoscale interfacial interaction between collagen fibrils and the embedded mineral phase enables load transference between the organic and inorganic components, which endows on dentine a capacity of load bearing during mastication and recovery after deformation [406, 407]. In a typical root caries process, firstly, exposure to biofilm-derived acid causes mineral to dissolve, leading to the exposure of cleavage sites of collagen fibres and activation of host-derived collagenases (*e.g.*, MMPs and cathepsins). The subsequent collagen proteolysis results in the breakage of high conformational cross-linkages, which contributes to further cleavage events by gelatinases/peptidases and accelerates subsequent mineral loss [5]. Consequently, demineralisation, together with structural damage of the collagen framework, causes localised destruction of dentine, which therefore impairs the biomechanics and biochemistry of the original dentine [408, 409].

The efficacy of currently recommended treatments, *e.g.*, 5,000 ppm NaF toothpastes, 22,600 ppm NaF varnish and 38% SDF solution (44,800 ppm F), highlights the efficacy of high concentration F in managing root caries [8, 13, 410]. The cariostatic mechanism of F mainly relies on the inhibition of demineralisation and enhancement of remineralisation [12]. Direct effects of F on preventing breakage of the collagen framework and de-activation of endogenous

proteases have not been demonstrated [411]. Thus, an incremental increase of MD alone cannot be regarded as an appropriate endpoint for assessing the effectiveness of the treatments with regard to improving dentine quality, which depends upon the sum total of the tissue characteristics including ultrastructure (especially the particular location of the mineral within the organic matrix of the tissue), MD and biomechanical characteristics [10, 412]. Failure to protect the organic structure of root dentine may compromise its biomechanical properties [413].

Given the pathological process of root caries, reducing enzyme activity and biomodification of dentine matrices are likely to protect the conformational dentinal organic matrix from degradation in root caries. A series of laboratory studies have reported the effects of MMP inhibitors in reducing the activity of MMPs in partially demineralised human dentine [414-418], whilst two studies have a direct observation on topical application of two MMP inhibitors, *i.e.*, non-antimicrobial chemically modified tetracyclines and zoledronate (a bisphosphate with MMP-inhibiting activity) in preventing carious lesion progression in rats [292, 419]. However, their inhibitory effects lack selectivity to certain enzymes and the effects may be limited to a certain time period. Thus, biomodification of dentine seems to be a promising approach which enhances the biomechanics and bio-stability of the dentine matrix. Amongst diverse biomodification strategies, application of cross-linking agents is of particular interest. The cross-linkers could induce multiple inter- and intra-molecular cross-links by interacting with various extracellular components of the dentine matrix [268]. As a result, the cross-linked organic matrix may modify or hide the regions in collagen fibrils vulnerable to collagenases and maintain the mineral phase in the gaps of collagen fibrils from further dissolution, thus improving the biomechanical and biochemical properties of the tissue [420].

Proanthocyanidin, a plant-derived cross-linking agent containing polyphenolic compounds, has been investigated extensively for its potential use in reparative caries therapies [421]. It not only interacts with proteins *via* covalent, ionic, hydrogen bonding or hydrophobic interaction, but also shows direct effects on non-collagenous protein such as proteoglycans [422] and inhibits the activity of endogenous proteases [278, 423]. Dentinal collagen treated with PA showed a significant improvement in mechanical and biological properties [256, 269]. Therefore, PA has the potential to stabilise the partially demineralised organic matrix and maintain the mineral phase in the stabilised collagen framework in its original arrangements,

which might be beneficial for ion uptake and reaction as well as the collagen-mineral phase interaction, thus leading to a higher MD and improved biomechanics.

In this study, SDF/KI was investigated as a regimen for root caries treatment, and PA was applied as a bio-mediator to protect the dentine organic matrix. An artificial root caries model was used to ensure the consistency of the results. The treated root dentine specimens were analysed regarding their mechanical, chemical and structural properties. Nano-indentation was used extensively to analyse the dynamic mechanical properties of dentine at a nanoscale. The viscoelastic behaviour of the treated dentine was systematically explored using a dynamic behaviour model. The microstructure and mineral uptake and distribution of SDF/KI with or without PA were also observed.

2.2 Materials and methods

2.2.1 Materials

Proanthocyanidin was obtained from the International Laboratory of USA (>95% oligomeric proanthocyanidins) and SDF/KI (Riva Star®; consisting of capsulated 30%-35% AgF and >60% ammonia solution and a separate capsulated saturated solution of potassium iodide) from SDI Limited (Bayswater, Australia). The demineralising solution was prepared using 50 mM acetic acid, 1.5 mM CaCl₂ and 0.9 mM KH₂PO₄ and adjusted to pH 5 with KOH. All the chemicals were purchased from local chemical suppliers.

2.2.2 Preparation of root dentine specimens

Non-carious human permanent molars were freshly extracted and collected in 0.1% thymol solution at 4 °C under the protocol approved by the Human Research Ethics Committee of the University of Melbourne. The crown and root apical portions were removed using a slow-speed diamond saw (Isomet, Buhler Ltd, IL, USA) under water irrigation. In order to determine the sample size (n), the mean values and standard deviations were estimated according to pilot study work. The effect size (f) in each measurement was calculated from the pilot study data following the Cohen's guidelines [424], in which for one-way analysis of variance (ANOVA) with n's equal, f was measured by:

$$f = \sqrt{\frac{\sum_{i=1}^k (\mu_i - \mu)^2}{k \cdot \sigma^2}}$$

Where μ_i = mean of Group i, μ = grand mean, σ^2 = variance within groups.

The minimum sample size was estimated based on a power of 0.8 with a confidence level of 95% and f calculated following the above equation using the G Power Version 3.1 program for the quantitative analysis [425]. In this regard, six specimens were used for cross-sectional microhardness test and nano-indentation respectively. Five samples (from those for cross-sectional microhardness testing) per group were prepared for surface microhardness testing, three samples for PLM observation, two specimens for micro-CT analysis and three for SEM examination. Considering the possible inconsistency of lesion depths, five additional samples were prepared in each treatment group. Thus, one hundred non-carious human permanent molar dentine blocks were prepared from buccal or lingual surfaces of the roots adjacent to the cemento-enamel junction (CEJ) and embedded in epoxy resin (KEP epoxy, Kemet International Ltd., Kent, UK). The tooth surfaces were polished using ascending grits (320, 600, 1200, 2500) of silicon carbide papers to expose the root dentine surface and the debris was removed by ultrasonic cleaning for 5 min in DDW. A layer of acid-resistant nail varnish was applied on the polished root surfaces to expose a window of 4×3 mm.

2.2.3 Treatment procedures

2.2.3.1 Demineralisation:

Each specimen was individually immersed in 20 mL of the buffered acidic demineralising solution (see 2.2.1) and stored at 37 °C for 5 d. The fresh demineralising solution was prepared and changed every 24 h. The protocol for demineralisation was modified from that previously reported [426-428].

2.2.3.2 Root dentine treatment

Root specimens were randomly assigned to the four treatment groups ($n = 25$ per group). Treatment agents and treatment times for each group are listed in Table 2.1. Specifically, the DDW group served as a negative control. The 6.5% w/v PA solution was prepared by adding PA powder into DDW and mixing thoroughly. The solution was applied to root dentine surfaces using a micro-brush, agitated for 15 s and left for 1 min. The excess solution was gently removed with a cotton pellet. This was followed by a further application of fresh PA solution. SDF/KI was applied according to the manufacturer's instructions. Specifically, SDF was applied to the treatment site using a micro-brush. Immediately after, a generous amount of KI was applied until no more white precipitate was formed on the treatment surface. For

samples in Group 4, 6.5 % PA was applied first in the same manner as for Group 2; the excess solution was removed with a cotton pellet; thereafter, SDF/KI was applied in the same manner as for Group 3. All specimens were blot dried and stored in DDW (20 mL for each sample) for 24 h before testing. Samples were sectioned longitudinally through the lesion centre and observed under the light microscope to evaluate the lesion depths. Samples with a lesion depth outside a range of $150 \pm 10 \mu\text{m}$ were excluded. The 20 samples that led to the lowest standard deviation of initial lesion depth values were selected in each group for analysis.

Table 2.1 Treatment agents and times in each group.

Gro up	Abbr eviation	Treatment agent	Treatment time	pH of the treatment agent
1	DDW	Distilled deionised water (DDW)	Agitation with a micro brush for 15 s and left for 1 min (two applications).	6.98 ± 0.00
2	PA	6.5% w/v proanthocyanidin (PA) dissolved in DDW	Agitation with a micro brush for 15 s and left for 1 min (two applications)	4.46 ± 0.01
3	SDF/ KI	SDF/KI (Riva Star®; consisting of 30%-35% silver fluoride, >60% ammonia solution and a saturated solution of potassium iodide; SDI limited, Bayswater, Australia)	Treatment time was determined according to the manufacturer's instruction. SDF was applied for approximately 30 s to cover the whole surface of the exposed dentine surface. KI was applied for another 30 s until no more white precipitate was formed.	SDF: 11 - 11.5 KI: 8 - 8.5 (measured with pH indicator strips (Merck, Darmstadt, Germany))
4	PA+S DF/KI	6.5% w/v PA dissolved in DDW; SDF/KI (as in Group SDF/KI)	15 s agitation and left for 1 min (two applications) for 6.5% PA (as in Group PA); thereafter, approximately 30 s for SDF and another 30 s for KI (as in Group SDF/KI).	PA: 4.46 ± 0.01 SDF: 11 - 11.5 KI: 8 - 8.5 (measured with pH indicator strips)

2.2.4 Analytical methods

2.2.4.1 Cross-sectional microhardness test

The longitudinally-sectioned samples were re-embedded in epoxy resin. The cross-sectional surface of each dentine block was polished on a water-cooled grinding and polishing unit (TegraPol 21, Struers, Copenhagen, Denmark) with abrasive papers (600, 1000, 1200, 2500,

4000 grit), followed by a 0.1 μm diamond suspension (Type K, Kemet International Ltd., Kent, UK). The debris on the surface was removed by ultrasonically cleaning for 2 min in DDW. Cross-sectional microhardness measurements perpendicular to the demineralised surface were performed below the surface at $\sim 5, 30, 60, 90, 120, 150 \mu\text{m}$ using Knoop hardness indentation (402 MVD, Wolpert Wilson Instrument, MA, USA) with a load of 10 gf ($98 \times 10^{-3} \text{ N}$) for 10 s dwell time at each testing point. For all samples, three indentations were performed at each depth on parallel tracks approximately 150 - 200 μm apart. Hardness was determined according to the following equation:

$$H = \frac{P}{A} \quad (1)$$

where P is the applied force and A is the contact area of the indentation which depends on the indenter geometry and the penetration depth [409]. To avoid variations of microhardness of different samples, an internal control of the sound area (150 μm from the surface) of each dentine block was used for comparison. Results were expressed as relative microhardness value [429].

2.2.4.2 Surface microhardness test

The indentations were made using a load of 10 gf ($98 \times 10^{-3} \text{ N}$) with 10 s dwell time using the Knoop hardness mode. Five samples were examined in each group and five indentations were performed on each specimen (near the four corners and at the centre of the exposed area) [430].

2.2.4.3 Nanoindentation test

Depth-sensing indentation techniques based on the load-displacement response of nanoindentation tests were used to obtain more comprehensive nano-mechanical properties of samples, which included hardness, elastic modulus and creep behaviour.

Nanoindentation experiments were performed on the longitudinal cross-sectional surface of specimens using a diamond pyramidal Berkovich tip attached to a quantitative nano-mechanical testing instrument (TI 950 TriboIndenter; Hysitron, Inc., MN, USA) interfaced with an AFM. During nanoindentation testing, the region of interest was presented as a flat surface to the indenter tip. The remainder of the specimen was covered with wet gauze. Fused quartz was used as the standard calibration material [431, 432]. Indenter load was 800 μN . The

nanoindentation test was performed with a step load followed by a hold period while monitoring the displacement of the indenter as a function of time. The holding periods were 10 s with a loading frequency of 200 Hz. Three sets of indentations (surface, subsurface and sound dentine area) were made on each specimen. Subsurface refers to the areas 20 μm below the surface. After executing the indentations, elastic moduli were calculated using the attached computer.

The displacement-time ($h'(t)$ - t) curves during the load-holding period were extracted. For viscoelastic materials such as dentine, creep deformation occurs under constant load. The viscoelasticity and creep behaviour of biological tissues could be analysed from this period [433].

The Burger's four-element model which connected Maxwell and Kelvin units in series were used to fit the $h'(t)$ - t curves obtained during the load-holding period. The following equation was used to describe the creep behaviours:

$$h'^2(t) = A_0 + A_1 (1 - e^{-t/\tau}) + A_2 t \quad (2)$$

where $h'(t)$ is the indentation depth (zero offset initial depth of penetration of indenter during maximum load held stage (creep)) during the load-holding period, the first term A_0 is the elastic deformation after the loading stage, the second term is the primary creep that reaches a steady state at buffer time τ , and A_1 is the primary creep co-efficient. The third term is the secondary creep and A_2 is the slope of the linear change [434].

The maximum penetration depth during creep ($h'_{\max}$) was calculated by subtracting the initial depth of penetration at the onset of the holding period against the total depth of penetration after creep at the maximum load.

According to previous studies [435, 436], the parameter of creep rate sensitivity (m) was obtained to provide a direct and simple comparison of the viscous flowability of dentine. It can be calculated from simple power-law relationship between stress (in nanoindentation test, accordingly, hardness) and applied strain rate:

$$H = K \dot{\epsilon}^m \quad (3)$$

where K is a material constant and m is the creep rate sensitivity. H is the measured dynamic hardness and $\dot{\epsilon}$ is the corresponding strain rate [435]. Thus, the creep rate sensitivity of dentine can be determined by fitting the experimental data and read from a logarithmic plot of the H - $\dot{\epsilon}$ curve (slope of the fitted curves).

The strain rate ($\dot{\epsilon}$) of nanoindentation can be extracted from the above fitted depth-time ($h'(t)$ - t) curve as the indenter displacement velocity divided by the penetration depth:

$$\dot{\epsilon} = \frac{h'}{h'(t)} \quad (4)$$

where $h' = \frac{dh'(t)}{dt}$ (5) is the indentation rate, which is the time derivative of the displacement during the hold period under constant load in the indentation depth-time ($h'(t)$ - t) curve; $h'(t)$ is the additional indenter penetration depth during creep at the timepoint of 't' and is calculated from the equation (2).

2.2.4.4 Polarised light microscopy and image analysis

Samples were sectioned longitudinally through the lesions (Minitom, Struers, Copenhagen, Denmark) with a low-speed water-cooled diamond saw to obtain 300 μm thick sections. Each specimen was further manually polished to a thickness of approximately 100 μm , mounted on a glass slide with DDW and covered with a clean coverslip [273]. Lesion depths were measured and changes in colour birefringence of the specimen were recorded using a first order red plate between crossed polarisers with transmitted light microscopy (DM2000, Leica Microsystems GmbH, Wetzlar, Germany) coupled to a camera (Leica EC3, Leica Microsystems GmbH, Wetzlar, Germany). The slides were visualised at original 200 $\times$ magnifications and the images were photographed and analysed by means of the software program Image J 1.38 $\times$ (National Institutes of Health, MD, USA).

2.2.4.5 Micro-CT analysis

Micro-CT analysis was performed to investigate how uptake of ions from SDF/KI would penetrate and distribute in demineralised (artificial carious) lesions. Specimens were scanned with a desktop X-ray micro-tomography system (Skyscan 1172, SkyScan N.V., Aartselaar,

Belgium) at a voltage of 80 kV and a current of 124 μ A. A 0.5 mm thick aluminium filter was placed in front of the detector to block low energy X-rays to limit the spectral bandwidth of X-rays. Prior to scanning, a new flat field reference was acquired for the dataset. The samples were rotated over 360° in a 0.2° rotation step and image pixel size of 1.95 μ m. The resulting 2D projected/transmission images (16 bit TIF) were reconstructed to a 3D object using the NRecon software interface based on a modified Feldkamp algorithm (NRecon, Version 1.4.2). Beam hardening artefact was corrected. All scanning was performed with identical parameters: file type of BMP, image width of 1760 pixels and height of 1740 pixels, average pixel size of 1.95 μ m, ring artefact correction of 15. Data were maintained as 16 bit TIF information.

For visualisation and analysis of 3D/volumetric data, Image J was used for selection of slices/regions of interest and 3D visualisation [380]. The reconstructed images were resliced to obtain slices that presented the whole lesion area. For each sample, 12 slices were randomly selected for analysis. Quantitative line profiles were evaluated across the lesions perpendicularly to the lesion surface and traversing into sound dentine. A paralleled line profile was drawn in the sound dentine area as a reference to determine the start and end of the lesion and as an internal control to avoid variations of original mineral intensities of sound dentine in different samples. In this regard, sound dentine area has a grayscale value (GV) percentage of approximately 100% whilst the air (blank area) of 0%. The low relative GV indicates a low value in MD. The grayscale change from 60 μ m outside the lesion surface to a depth of 150 μ m inside was determined. The integrated relative GV under the curve from the lesion surface to a depth of 150 μ m of sound dentine was calculated by the trapezoidal rule at 30 μ m increments [437].

2.2.4.6 SEM observation

To observe the morphology of the dentine surface and dentinal tubules, three dentine specimens were prepared for each treatment group. For each specimen, a groove was prepared on both the apical side and occlusal side of the lesion area approximately in the same plane using the Isomet diamond saw. Then, each specimen was split longitudinally along this plane into two halves to examine the cross sections of the dentine. The splitting operation was performed such that the cross-sectional surface was not influenced [438]. All dentine specimens were desiccated, Au-Pd alloy sputter-coated, and observed by environmental SEM (FEI Quanta ESEM, FEI, OR, USA) at 10.0 kV. Micrographs at magnifications of 70 $\times$, 2000 $\times$, 10000 $\times$ were captured.

2.2.5 Statistical analysis

Quantitative data were expressed as mean $\pm$ standard deviation. The assumptions of homogeneity of variance and normal distribution were checked by Levene's test and Shapiro-Wilks test, respectively. The surface hardness data were analysed by one-way ANOVA and Tukey's *post hoc* multiple comparisons. Statistical analysis of cross-sectional hardness and nanoindentation data was carried out by two-way ANOVA and Tukey's *post hoc* multiple comparisons. Integrated GV were analysed by Welch's ANOVA test followed by Dunnett's T3 *post hoc* multiple comparisons. A level of significance of $\alpha = 0.05$ was set in all statistical tests (SPSS Version 22.0, SPSS Inc., IL, USA).

2.3 Results

2.3.1 Surface and cross-sectional microhardness test

The surface and cross-sectional microhardness measurements of the demineralised (artificial caries) lesions from different treatment groups are presented in Table 2.2. Treatment with PA showed slightly lower surface hardness values (40.40 ± 5.78 MPa) than the control group (45.11 ± 3.02 MPa) but was not statistically significant ($P = 0.519$). Treatment of SDF/KI with or without the application of PA both significantly improved the sample surface hardness compared to the no treatment group ($P < 0.01$). However, there was no statistically significant difference between SDF/KI (59.23 ± 6.31 MPa) and PA+SDF/KI (62.96 ± 5.61 MPa) ($P = 0.692$) groups. The addition of PA resulted in a slightly larger incremental increase in the surface hardness, with 31.3% and 39.5% increases respectively for SDF/KI and PA+SDF/KI compared to the control group.

For the cross-sectional microhardness, generally, there were few comparative depths that were statistically different between control and PA groups except for the areas around 90 μm deep ($P = 0.013$). Although there was no significant difference between SDF/KI and PA+SDF/KI groups at all the depths, differences were present when hardness numbers were compared with the control group, respectively. Specifically, samples treated with SDF/KI showed statistically significantly greater microhardness values than the control group only in the surface area (~ 5 μm ; $P < 0.0001$), whilst there was no significant difference between SDF/KI and control groups from 30 μm to the lesion front (approximately 150 μm from the surface) ($P > 0.5$). However, when PA was used before SDF/KI application, significantly greater microhardness values were

observed up to 60 μm ($P < 0.01$) compared with the control group. At 90 μm , a statistical difference was observed between the PA+SDF/KI and PA groups ($P = 0.01$) whilst no difference between the PA+SDF/KI and control group was identified, putatively due to the limited penetration of the treatment agents.

Table 2.2 Longitudinal cross sectional and surface microhardness of the artificial carious lesions of different treatment groups.

Depth from surface (μm)	Longitudinal cross-sectional Knoop hardness numbers (relative to fraction values of sound dentine = 1)			
	Control	PA	SDF/KI	PA+ SDF/KI
~5	0.17 ± 0.04^A	0.16 ± 0.01^A	0.31 ± 0.03^B	0.37 ± 0.07^B
30	0.27 ± 0.04^A	0.19 ± 0.06^A	$0.35 \pm 0.04^{A,B}$	0.57 ± 0.26^B
60	$0.40 \pm 0.10^{A,B}$	0.28 ± 0.06^A	$0.53 \pm 0.08^{B,C}$	0.69 ± 0.16^C
90	0.72 ± 0.16^A	0.45 ± 0.11^B	$0.60 \pm 0.10^{A,B}$	0.73 ± 0.08^A
120	0.91 ± 0.12^A	0.80 ± 0.11^A	0.82 ± 0.13^A	0.88 ± 0.11^A
150	1 ^A	1 ^A	1 ^A	1 ^A
Surface Knoop hardness (MPa)				
	45.11 ± 3.02^A	40.40 ± 5.78^A	59.23 ± 6.31^B	62.96 ± 5.61^B

Values are means $\pm$ standard deviations ($n = 6$).

Uppercase superscript letters indicate statistically significant differences between treatments wo-way ANOVA, *post hoc* Tukey's test, $p < 0.05$).

2.3.2 Nanoindentation

2.3.2.1 Elastic modulus

An example of the nanoindentation image captured by AFM is shown in the supplementary information (Figure S2.2). The nanoindentation results of the elastic moduli of the artificial carious lesions after different treatments are presented in Figure 2.1 (A-B), which demonstrates the viscoelastic properties of dentine. The storage and loss of modulus is related to the ratio of the elastic and viscous components of a material to the stress, respectively. Demineralisation resulted in decreased elastic modulus in root dentine. Treatment with SDF/KI with or without PA both significantly improved the storage modulus in the surface area ($P < 0.0001$). Combined use of SDF/KI with PA had a superior effect on the subsurface area, with improvements in elastic moduli compared to all the other groups ($P < 0.0001$). Treatment with SDF/KI (with or

without PA) led to recovery of elastic moduli of the carious dentine to a degree that was greater than the original sound dentine. By contrast, treatment with PA significantly decreased the storage modulus of the subsurface dentine ($P < 0.0001$). Tan-delta, referred to as loss factor, is defined as the ratio of the moduli (loss modulus/storage modulus), which indicated the relative degree of energy dissipation or damping of a material. Results (Figure 2.1 C) confirmed the loss of elasticity of demineralised dentine and recovery of elastic modulus after SDF/KI treatment, with enhanced effects on subsurface area using PA as an adjunct.

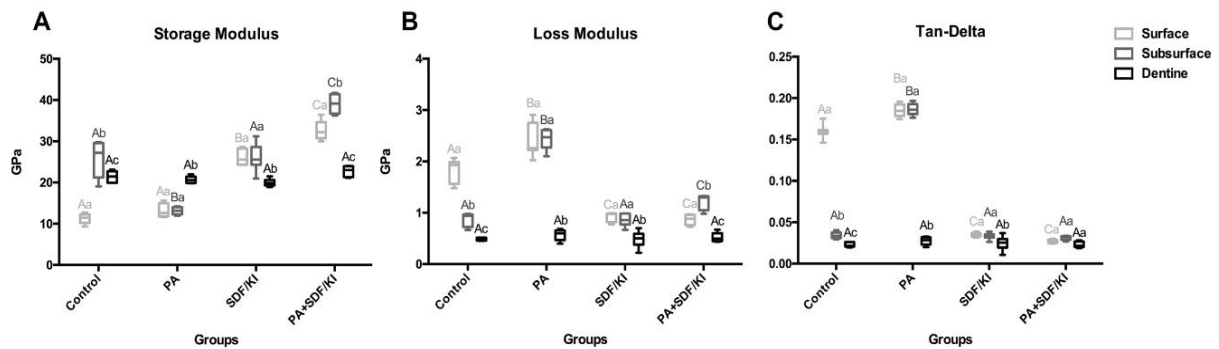


Figure 2.1. Comparison of the average dynamic moduli obtained by nanoindentation from different regions (surface, subsurface and sound dentine) of each group. Distinct superscript uppercase letters in the same grayscale indicate statistically significant differences between treatments and lowercase letters indicate statistically significant differences between depths with the same treatment.

2.3.2.2 Creep behaviour

The typical load displacement response for a single indent is illustrated in Figure 2.2 (A-C). Indentation responses are shown for surface, subsurface and sound dentine regions. The hold segment means specimens were indented with a constant maximum load, which could demonstrate the creep behaviour of a material.

The $h'(t)$ - t curve of each specimen (Figure 2.2(A1-C1)) was fitted using Eq. (2) and the fitted parameters were shown in the supplementary information (Table S2.1). From Table 2.3, the h'_{max} of sound dentine showed no statistically significant difference between samples from all groups. The demineralised surface presented a nearly 2.5-fold increase of h'_{max} than the sound dentine. After treatment with PA, a significantly higher h'_{max} was present in the subsurface area. Silver diammine fluoride/KI significantly decreased h'_{max} to half the value of the control

group in surface area, while no significant difference in subsurface area compared to the control group was observed. Adjunctive application of SDF/KI with PA distinctly preserved the resistance against creep deformation of the subsurface area (to a value not significantly different from sound dentine).

Log of strain rate vs. log of hardness was plotted (Figure S2.1). From Table 2.3, the creep rate sensitivity of sound dentine was $0.06 \sim 0.08$ in general. Demineralisation was associated with an increase in the creep rate sensitivity of dentine in the surface areas by nearly 3-fold (control surface vs. sound dentine: 0.18 ± 0.05 vs. 0.06 ± 0.01). Application of PA was further associated with a significant increase in the creep rate sensitivity around the subsurface area compared to the control group (PA vs. control = 0.15 ± 0.02 vs. 0.10 ± 0.02 ; $p = 0.011$). Application of SDF/KI individually decreased the creep rate sensitivity of demineralised dentine significantly only in the surface areas (SDF vs. control = 0.12 ± 0.02 vs. 0.18 ± 0.05 ; $p = 0.021$), whilst it increased the creep rate sensitivity significantly in the subsurface areas (SDF vs. control = 0.14 ± 0.03 vs. 0.10 ± 0.02 ; $p = 0.047$). The combined use of PA and SDF/KI reversed the creep rate sensitivity of demineralised dentine both in the surface and subsurface areas. Specifically, in the subsurface area, the creep rate sensitivity after PA+SDF/KI treatment decreased to a value not statistically different from that of sound dentine, which was different from SDF/KI treatment (Subsurface: SDF/KI vs. PA+SDF/KI = 0.14 ± 0.03 vs. 0.09 ± 0.02 ; $p = 0.043$).

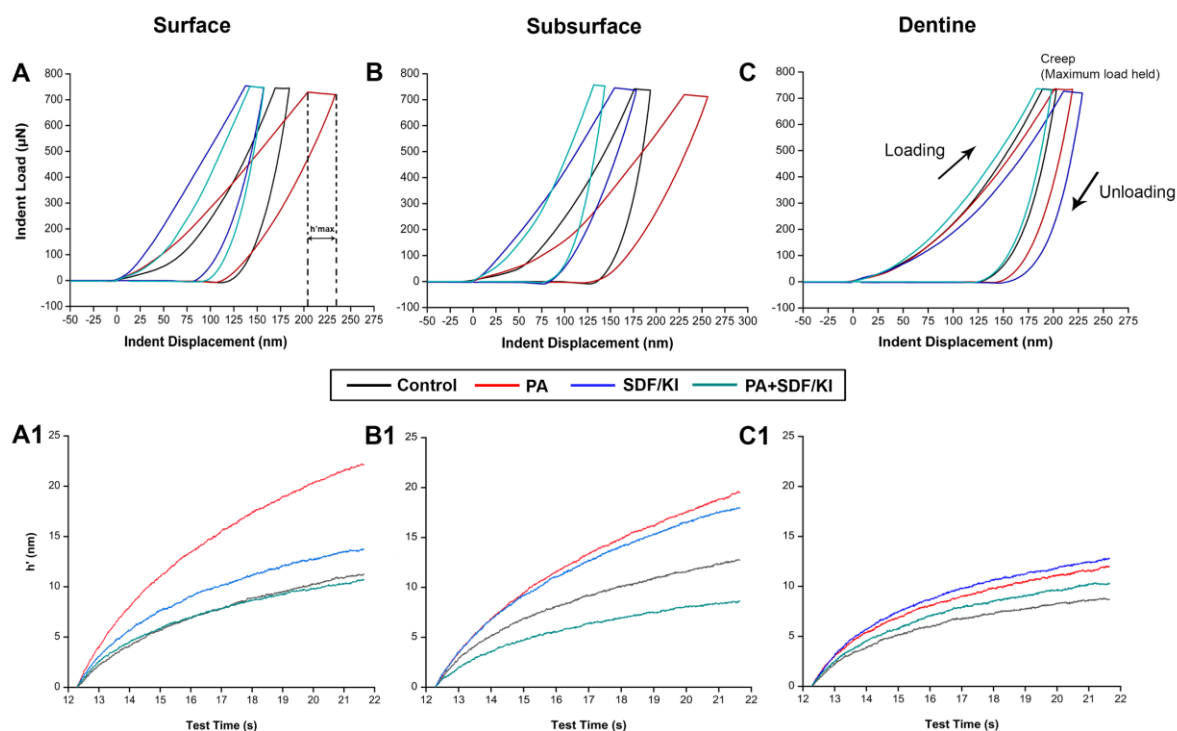


Figure 2.2. (A-C) Typical load displacement curves with a creep hold segment. (A1-C1) Zero offset initial depth of penetration of indenter during maximum load held stage (creep) against time (h' is the additional indenter displacement before unloading during creep. Indentation responses are shown for surface, subsurface and sound dentine areas after different treatments).

Table 2.3 Summary of the maximum penetration depth during creep ($h'_{\max}$) and creep rate sensitivity (m) of artificial root carious lesions after different treatments.

Area	$h'_{\max}$ (nm)			
	Control	PA	SDF/KI	PA+ SDF/KI
Surface	23.0 ± 2.4 ^{A,a}	19.4 ± 1.1 ^{B,a}	11.6 ± 0.7 ^{C,a}	9.2 ± 0.8 ^{C,a}
Subsurface	12.5 ± 2.0 ^{A,b}	19.0 ± 1.1 ^{B,a}	14.4 ± 1.2 ^{A,b}	7.8 ± 1.1 ^{C,a}
Dentine	9.3 ± 1.6 ^{A,c}	12.2 ± 1.2 ^{A,b}	11.5 ± 1.7 ^{A,a}	9.1 ± 0.8 ^{A,a}
Area	Creep rate sensitivity (m)			
	Control	PA	SDF/KI	PA+SDF/KI
Surface	0.18 ± 0.05 ^{A,a}	0.14 ± 0.02 ^{A,B,a}	0.12 ± 0.02 ^{B,C,a}	0.09 ± 0.01 ^{C,a}
Subsurface	0.10 ± 0.02 ^{A,b}	0.15 ± 0.02 ^{B,a}	0.14 ± 0.03 ^{B,a}	0.09 ± 0.02 ^{A,a}
Dentine	0.06 ± 0.01 ^{A,b}	0.08 ± 0.02 ^{A,b}	0.06 ± 0.01 ^{A,b}	0.06 ± 0.01 ^{A,a}

Values are means $\pm$ standard deviations (n = 6). Uppercase superscript letters indicate statistically significant differences between treatments and lowercase letters indicate statistically significant differences between depths (two-way ANOVA, *post hoc* Tukey's test, $p < 0.05$).

2.3.3 Polarised light microscopy and image analysis

Representative PLM micrographs of root fragment sections viewed in water from each group are presented in Figure 2.3(A-D). In the profile obtained from PLM, a baseline lesion after demineralisation was present in all samples. When imbibed with water, sound dentine showed positive birefringence. The staining of PA on dentine was observed along the tubule structure with precipitation on the surface, and the tubule structure was evident and clearly visible in PA treated groups. Silver diammine fluoride/KI treatment contributed to a homogeneous dark band. Proanthocyanidin followed by SDF/KI generated a stained band in the demineralised area with visible tubular structure.

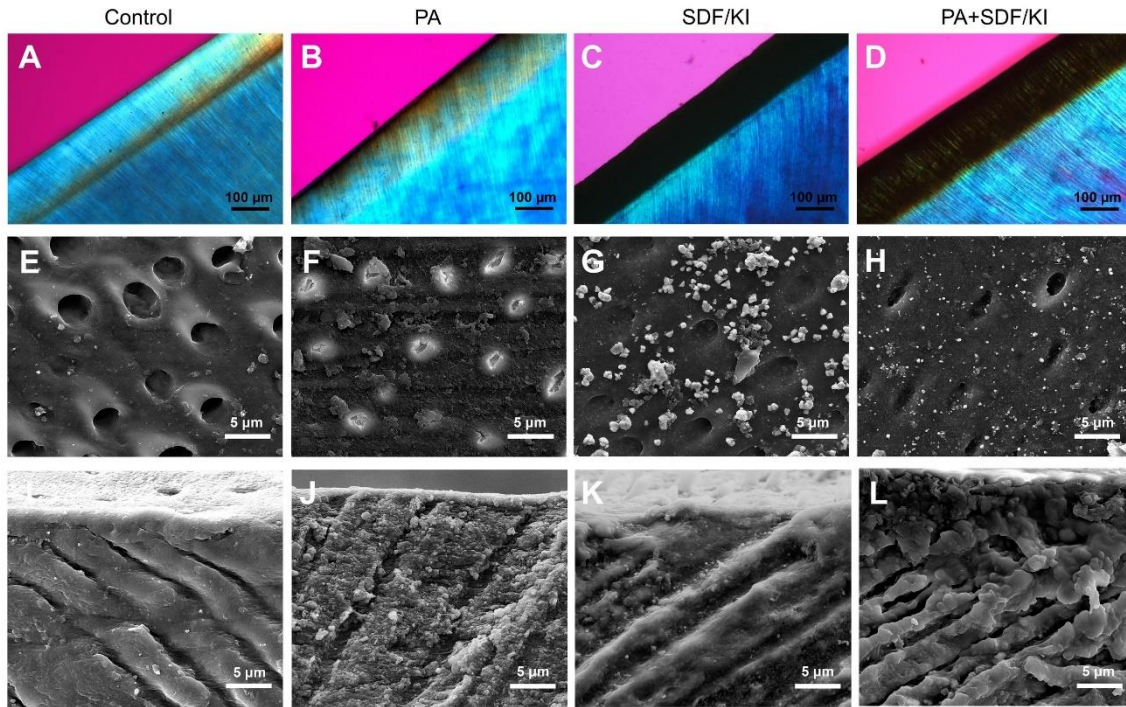


Figure 2.3 (A-D) Polarised light microscopy with a first-order red plate images of lesions after exposure to different treatments. Scanning electron microscopy images of (E-H) surface morphology and (I-L) longitudinal cross-sectional structure after treatments (For polarised light microscopy observation, specimens were viewed in water (refractive index 1.33)).

2.3.4 Micro-computed tomography

Three-dimensional reconstruction images (A-D) and typical cross-sectional slices (E-H) from each group are illustrated in Figure 2.4. The dentine surface showed a slightly bright area probably due to beam hardening effects. Typical subsurface lesion with decreased GV was present in the control and PA groups. After treatment with SDF/KI, a higher GV was present in the demineralised lesion area. With the adjunctive use of PA, the distribution of GV was more homogeneous.

The relative GV profiles of specimens are shown in Figure 2.5. After demineralisation, the profile indicated a typical subsurface lesion with a peak at the surface zone. Proanthocyanidin treatment had a similar profile. A significant increase in GV was observed when the specimens were treated with SDF/KI. Combined use of PA and SDI/KI enhanced the effect, as the relative GV had twice the value of sound dentine. The increase in GV was observed in a deeper area with smaller standard deviations, which indicated the adjunctive application of PA might

contribute to an improved ion uptake and homogenous distribution of the SDF/KI.

The integrated relative GV (areas under the curve) was determined from the lesion surface with 30 μm increments up to a depth of 150 μm of the lesion front (150 μm from the surface) (Table 2.4). Application of PA had no statistically significant effects on the integrated GV of the specimens. However, when the demineralised dentine was treated with SDF/KI, significantly higher integrated GV was observed around the 30 - 60 μm area compared to the control group ($P < 0.0001$), whilst the difference was not statistically significant around the 0 - 30 μm area. With combined use of PA and SDF/KI, a significant increment in integrated relative GV was observed from surface up to 90 μm ($P < 0.0001$).

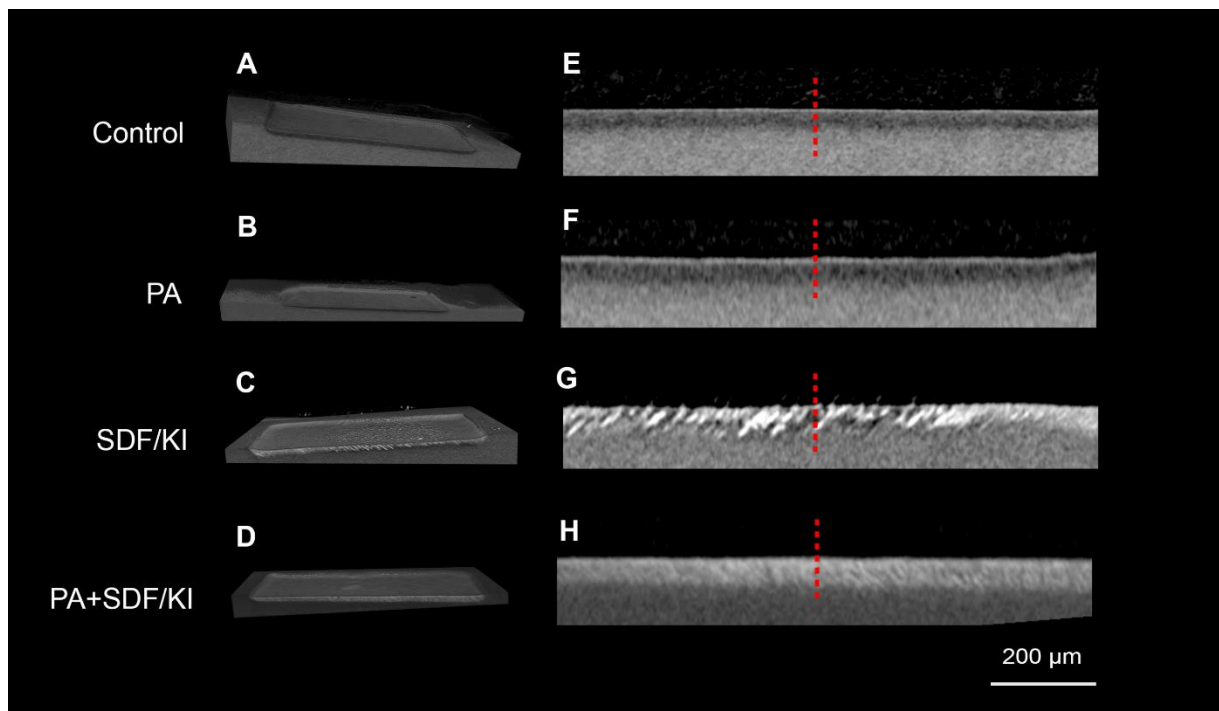


Figure 2.4 Three-dimensional construction of micro-CT scanning (A-D) and typical longitudinal cross-sectional slides (E-H) of samples with different treatments.

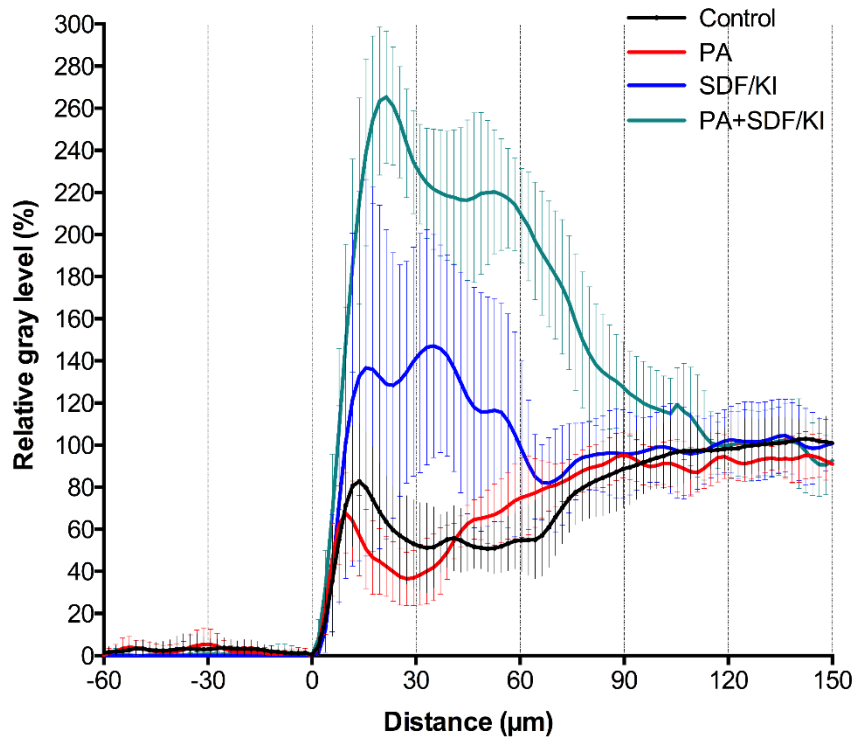


Figure 2.5 Analysis of the line profile (marked in red dotted lines in Figure 2.4) of relative grayscale value (%) in longitudinal cross-sectional slides from different groups.

Table 2.4 Integrated relative grayscale values from the lesion surface to sound dentine area with 30- μ m increments (GV% $\cdot\mu$ m).

Group	Depth increment (μ m)				
	0-30	30-60	60-90	90-120	120-150
Control	1620 $\pm$ 531 ^{a,b}	1544 $\pm$ 273 ^a	2018 $\pm$ 417 ^a	2796 $\pm$ 340 ^a	2761 $\pm$ 254 ^a
PA	1292 $\pm$ 341 ^a	1726 $\pm$ 305 ^a	2339 $\pm$ 277 ^a	2657 $\pm$ 254 ^a	2551 $\pm$ 173 ^a
SDF/KI	2882 $\pm$ 1420 ^b	3685 $\pm$ 818 ^b	2421 $\pm$ 396 ^a	2870 $\pm$ 415 ^{a,b}	2771 $\pm$ 419 ^a
PA+SDF/KI	5191 $\pm$ 726 ^c	6399 $\pm$ 724 ^c	4389 $\pm$ 688 ^b	3285 $\pm$ 333 ^b	2670 $\pm$ 357 ^a

Lowercase superscript letters indicate statistical significance among groups in each variable of each column (Welch's ANOVA, *post hoc* Dunnett's T3 test, $p < 0.05$).

2.3.5 Scanning electron microscopy observation

Typical SEM images of lesions in different groups are shown in Figure 2.3 (E-L). In the cross-sections, the demineralised organic portion was distinctly demarcated against the mineralised tissue (data not shown). The demineralised dentine specimens in the control group showed a surface with completely patent dentinal tubules with diameters around 3-4 μ m (Figure 2.3E).

Treatment with PA contributed to the formation of slit-shaped orifices of the dentinal tubules which were partially occluded (Figure 2.3F). For the SDF/KI treated groups, the SEM image showed clustering of minerals (Figure 2.3G) and when combined with PA, small discrete crystal formation (less than 1 μm) distributed over the dentine surface (Figure 2.3H). From the cross-sectional images, dense granular structures of spherical grains were found in the inter-tubular area of dentine treated with PA. A few small particles were distributed on the cross-sectional surface of dentine in the SDF/KI treated group.

2.4 Discussion

The present study evaluated the potential effects of the combined use of PA as a cross-linking agent with a high concentration F agent (SDF/KI) in the treatment of root caries in a laboratory-based study. The results indicated SDF/KI induced small discrete crystal formation that was distributed over the dentine surface and associated with an improvement in the mechanical properties (microhardness, elastic modulus and creep behaviour) of demineralised root dentine in the surface area 24 h after application, whilst the effects of SDF/KI in the subsurface areas were not significant. The ion uptake and distribution from SDF/KI could be observed up to 60 μm from the surface with a relatively heterogeneous distribution. Specimens biomodified with PA before application of SDF/KI seem to show some enhancement of the SDF/KI effects in the subsurface regions of the demineralised root dentine. Significantly improvement of the cross-sectional microhardness, elastic modulus and creep behaviour was all observed in both the surface and subsurface areas. The ion uptake from SDF/KI was found in deeper areas (up to 90 μm) with a more homogenous distribution observed in PA pre-treatment groups compared to those samples without the PA pre-treatment. The proposed treatment time of PA, which was two applications of 1 min each, is believed to be within a clinically acceptable time frame.

Chemical model systems have the possibility to simulate the physicochemical changes (*e.g.*, mineral content and hardness) of natural root caries for laboratory studies [427, 439]. The chemical models we used in the current study to create artificial root caries were based on previously published work [426-428]. With a controlled pH *via* buffering and composition of the demineralising solution, consistently reproducible subsurface lesions could be created. The artificial RCLs created in the current study were within the range of $150 \pm 10 \mu\text{m}$. The demineralisation solution, which diffused through the matrix and gradually dissolved the mineral components, significantly lowered the microhardness from the outer to the inner

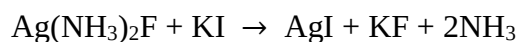
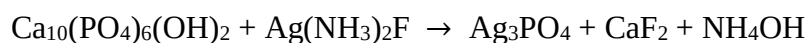
demineralised regions. Also, the low pH of the demineralisation solution is believed to alter the biochemical nature of intrapeptide and interpeptide cross-links resulting in decreased elastic modulus [440]. A significantly lower MD could be directly observed *via* micro-CT. Polarised light microscopy allowed for the identification of dentine's birefringence colour with the use of a first order red plate. In water, dentine is positively birefringent due to a superimposition of weak negative birefringence of oriented sub-microscopic dentinal crystals and stronger positively oriented birefringence of the collagen matrix [10]. As the demineralisation occurs, the strength of the positive birefringence increased by loss of the weak negative birefringent crystals.

Professional application of SDF has been widely acknowledged to be efficacious in arresting active dentinal caries in primary teeth as well as in the management of root caries in the elderly [411]. Application of KI after SDF was proposed as a method to limit the discoloration of teeth caused by SDF. The KI reacts with the free silver ions to form a white precipitate of silver iodide [441]. Nevertheless, its reliability and potential side-effects require further evaluation.

Results from the present study demonstrated that SDF/KI was able to interact with the demineralised root dentine area, resulting in a dark band in PLM, putatively because of the penetration of silver and F ions into sub-microscopic pores in the demineralised area. The ion uptake from SDF/KI contributed to an increase in GV in the demineralised dentine area that was approximately a two-fold increase compared to that of sound dentine.

The significant increase of GV after SDF/KI was observed up to a depth of 60 μm , which might indicate the effective penetration of silver ions into the chemically created artificial RCLs. This depth was generally similar to that observed in a laboratory-based *S. mutans* induced artificial caries model as well as in sound dentine in a previous study, which was recorded a depth of approximately 50 μm [442]. Silver diammine fluoride/KI was able to restore the mechanical properties of demineralised root dentine. Results from Knoop hardness test in this study demonstrated that the hardness recovery was limited to the surface area ($\sim 5\text{ }\mu\text{m}$ from the surface) compared to the control group. The application process was assumed to be diffusion-controlled because the active agents need to diffuse through the collagen structure of considerable thickness. The limited hardness recovery of SDF/KI might be due to the superficial barriers resulting from the reaction between silver and proteins in the outer area. The increase in microhardness is speculated to be a chemical reaction between HAp and SDF

leading to the deposition of silver salts, putatively including Ag_3PO_4 and AgI . The succinct chemical reaction between reactants and products is as follows:



The relatively insoluble silver phosphate and the subsequent precipitate of silver iodide might contribute to the hardening of the demineralised dentine [411, 443]. In this regard, hardening of SDF-arrested carious tissue is partly due to its reaction with silver in addition to F-mediated remineralisation [411, 444]. The application of SDF/KI is effective as a means of recovering the mechanical properties of the root dentine surface that has already undergone some demineralisation.

The introduction of F shifts the balance from demineralisation towards remineralisation or lesion arrest [445]. Nevertheless, root dentine has a more complex structure than enamel with more organic components (*i.e.*, dentinal organic matrix (DOM)). The treatment efficacy of the topical F agents strongly depends on the presence or absence of DOM. Specifically, it is believed that in the presence of DOM, the mechanism of action of topical chemicals is the uptake of remineralising ions in the underlying demineralised tissue, rather than surface precipitation – *i.e.*, ions diffuse through this structure and accumulate in the underlying partially demineralised tissue [446]. Effective remineralisation of root dentine requires the protection of both organic and inorganic components to ensure sufficient recovery of integrated characteristics of dentine, including its hierarchical structure, MD and interfacial interaction of the mineral within the organic matrix [10].

Although a number of research projects have focused on how crosslinking agents would protect the demineralised organic matrix [422, 447, 448], there are a lack of studies investigating whether the stabilised organic matrix would enhance mineral uptake and promote collagen-mineral interaction; and whether treatment strategies targeting both organic and inorganic components of dentine would contribute to a better treatment of root dentine caries. Results from two recent studies indicate that when PA was used with other topical remineralising agents such as pastes containing casein phosphopeptide-amorphous calcium fluoride phosphate (CPP-ACFP) and TCP, a synergistic effect on demineralised root dentine with a better

distribution of mineral precipitation throughout root dentine carious lesions was demonstrated [428, 449]. The present study took a further step on how PA, with the capability to stabilise the demineralised organic matrix, would help with the uptake and incorporation of remineralising ions and improve the properties of root dentine.

The results of the micro-CT demonstrated how the ions from the SDF/KI solutions penetrated and distributed in the demineralised carious lesions. Dentine is a porous biological composite material and the process of demineralisation created more microscopic pores in dentine tissues. Silver diammine fluoride, where F is present as complexes with silver, would expect to diffuse through the dentine [450]. The complexes would dissociate into silver and F ions as they diffused, reacted with and rebuilt the depleted dentine crystals. Also, dentine is permeable to silver ions *via* tubules because these ions have a particle size much smaller than the dentine tubule width [451]. Lambert-Beer's law predicts greater X-ray absorption in Ag than in Ca [187]. Thus, after the treatment with SDF/KI, silver was incorporated in dentinal tissues and manifested a higher GV in micro-CT images, even higher than the GV of sound dentine that is based on calcium phosphate salts. Moreover, when SDF/KI was used in conjunction with PA, a more significantly incremental increase in GV was achieved to a deeper area with a homogeneous distribution. The intermolecular cross-links of the collagen which were not irreversibly broken might be recovered after the application of PA. The cross-banded structure of the collagen fibres serves as a base to which ions can attach [452] and more interfibrillar space might be created for ion diffusion into the subsurface layer of demineralised dentine [279]. The rate of mineral deposition depends on the precipitation kinetics – the local factors such as pH, ionic concentrations, the presence of seed crystals or matrix and the surface area available for crystal growth [452]. In a well-constructed dentine matrix, the distribution of residual crystals was more homogeneous. The ions from treatment agents were not only precipitating on the superficial surface of dentine but also through ion diffusion. An evident structure of dentinal tubules from PLM images indicated PA's effects on the protection of the dentinal collagen.

The adjunctive application of PA appears to have a superior effect to SDF/KI alone on the nanomechanical properties (elastic modulus, nano-hardness and creep behaviour), especially in subsurface areas. The combined application achieved significantly greater hardness numbers than the control group at the same depths up to 60 μm . It has been reported that the use of a reducing agent such as TA from boiled tea with SDF could accelerate the deposition of silver

phosphate [453]. As a condensed tannin, PA might share a similar function for accelerated deposition of silver ions, where more reactants of silver compounds resulted in higher hardness number. The viscoelasticity of the collagen fibrils comes from the intermolecular cross-linking [454]. Polyphenols such as PA have strong molecular interactions with proteins which have a high proline content by the formation of hydrogen bonds between phenolic hydroxyl groups and carbonyl groups linked to proline amino groups of protein [455]. Also, ionic and covalent interaction of PA with the side-chains of collagen fibril molecules has been hypothesised [456]. Thus, pre-treatment with PA might induce more dentinal collagen crosslinks, which strengthens the collagen matrix, and helps increase the modulus of elasticity when used together with SDF/KI.

The creep behaviour of a material can be determined from nanoindentation testing with a load-holding stage, where the force and indenter displacement are recorded continuously as a function of time at a sub-micrometre spatial resolution [433]. Creep, which refers to continual deformation under constant load, is a characteristic of viscoelastic materials, such as dentine [436, 457]. Investigation into the creep behaviour of dentine provides an understanding of its ability to resist stress under functional loading conditions.

Three models, *i.e.*, the standard linear solid, Burgers and two dashpot Kelvin models, have been suggested to fit the $h'(t)$ - t curves during the load-holding stage of materials with viscoelastic properties [458]. Viscoelastic bio-composites such as dentine and bone have at least two phases, namely, linear elastic inorganic phase of HAp and inelastic organic phases of collagen and water [434, 435, 459]. It has been found that the Burgers model with four variable parameters was a better fit of $h'(t)$ - t curves of these materials, which was used in the current study to provide a numerical description of the nanoindentation creep behaviour of dentine [434]. Results showed that the Burgers model was a good fit to the $h'(t)$ - t curves of all specimens with R-squared > 0.99 (see supplementary information Table S2.1). From the curves, normalised creep deformation could be calculated. In order to investigate the creep behaviour in more detail, the parameter of creep rate sensitivity is calculated from stress (hardness) and strain-rate exponent, which reflects the flowability of a material and demonstrates how the rate of deformation of material changes in response to a change in the applied stress. In general, higher creep rate sensitivity would mean lower resistance of a material in response to mechanical stress [460].

The mechanical properties of dentine are strongly dependent on the interfacial interaction between collagen and the mineral phase [461]. It has been suggested that during loading, the HAp-collagen interface in dentine may undergo a sliding process, where the stress was transferred from the harder mineralised crystals to the softer organic matrix around them thus dissipating energy [407, 462]. Demineralisation may damage the HAp-collagen interface, therefore the demineralised specimens presented a higher maximum creep deformation ($h'_{\max}$) than the mineralised dentine, which is consistent with a previous study [463]. Also, significantly higher creep rate sensitivity (nearly 3-fold of the sound dentine) was observed, which indicated the weakened strain resistance of carious dentine. Treatment with PA significantly decreased the creep rate sensitivity of the demineralised surface putatively because more crosslinking was induced in the dentine matrix network structure, which increased the ability of collagen to transfer load therefore leading to increased creep resistance. Application of SDF/KI significantly increased the ability of dentine to resist against creep deformation in the surface area, whilst adjunctive application of PA and SDF/KI significantly increased the creep resistance of demineralised dentine to a value similar to the original sound dentine both in surface and subsurface areas. It was therefore speculated that the application of PA stabilised the organic matrix and protected its ability for stress redistribution. After introduction of minerals, a better organic-inorganic interaction was achieved, which contributes to improved creep behaviour.

The stabilised dentine organic matrix and improved organic-inorganic interaction are expected to be sustained under cariogenic (demineralising) conditions or collagenase exposure. Previous research has indicated that the chemical bonds introduced by PA were durable under hydrated conditions [422]. It was reported that improved biomechanical properties of PA-modified dentine could be sustained over time between 8 to 18 months [422, 447, 464]. Although not tested in the current study, PA has an inhibitory effect on MMPs and cysteines [278, 465] and can enhance dentinal collagen stability during pH-cycling with collagenase as a stressor [428] and such collagenase inhibition has been reported to be sustained after 6 months storage [466]. In addition, the silver compounds and CaF_2 -like materials deposited on the dentine after SDF/KI application were expected to have an antibacterial effect on cariogenic biofilms and act as a slow-release F reservoir respectively if a biofilm or pH-cycling model is used [411]. In the present study, when SDF/KI was applied in conjunction with PA, improved ion uptake was observed into deeper regions with a more homogenous distribution of ions. Therefore, the antibacterial and remineralising effects of SDF/KI are expected to be more extensive

throughout the lesion. The ultimate goal of the combined use of PA and SDF/KI would be to achieve remineralisation of RCLs, which should be quantitatively compared with classical remineralising agents such as NaF [467].

Of note, in this study, application of 6.5 w/v% PA solution on root dentine led to a decrease in the hardness and modulus of elasticity of the carious lesions. This seemed in contrast to most previous research where plant extracts with a high PA content could induce a significant increase in microhardness and modulus of elasticity [269, 448, 468]. However, in most of the previous experimental designs, the 6.5% w/v PA solution was adjusted to a neutral pH with KOH or NaOH, while in the present study, the 6.5% w/v PA solution was kept as its original pH. Pilot study work demonstrated the pH value of 6.5% w/v PA solution was 4.46 ± 0.01 and remained stable during a six-month storage period at both room temperature and 4 °C. Thus, application of acidic PA solution might further demineralise the dentine surface. A previous study by Tang and colleagues has revealed that weakly acidic grape seed extract solution could induce immediate reductions in microhardness values of dentine, while steady increases in hardness were shown following remineralisation [279]. Also, PA's ability to interact with proline-rich proteins is pH dependent, where the greatest precipitation was found to be near the isoelectric point of protein [469]. The active polyphenols in PA are more stable under acidic conditions. Adjustment of its pH could influence its stability and biological activities [279]. Therefore, to maintain the PA at its original pH might retain a higher biological activity, although a negative influence could be observed in the short term. On the other hand, the relatively low pH level of PA might accelerate dissolution of apatite, which provides more Ca^{2+} and consequently favours the precipitation of substances containing F after F treatment.

However, an issue of concern is that in the experimental process, treatment with SDF/KI after the application of PA might compromise the effect of KI, as the dentine surface was more prone to dark discoloration. At its unadjusted acidic pH, PA might behave as a weak reducing agent due to its phenolic groups, which accelerated the deposition of silver phosphate and silver oxide. The precipitation of silver compounds resulted in the instantaneous dark discoloration [453]. Precaution should be taken on the application of KI following SDF.

2.5 Conclusion

Within the limitations of this study, it was concluded that application of PA on artificial RCLs contributed to partial occlusion of dentinal tubules and it did not affect the mechanical properties and MD of the dentine ($P > 0.05$). Treatment of artificial RCLs with SDF/KI induced small discrete crystal formation distributed over the dentine surface and the ion uptake and distribution from SDF/KI was up to 60 μm . Silver diammine fluoride/KI application resulted in recovery of its nano-mechanical properties in the surface area. Adjunctive use of PA might enhance these effects, with a significant increase in surface and cross-sectional microhardness, elastic moduli and a recovery of creep behaviour of demineralised dentine with decreased creep deformation and increased creep rate sensitivity ($P < 0.05$), especially in the subsurface areas. Additional use of PA also contributed to ion uptake from SDF/KI into deeper areas (90 μm) with a more homogenous distribution throughout the lesions.

Acknowledgement

This work was supported by Australian Dental Research Foundation (ADRF) Nathan Cochrane Memorial Grant.

2.6 Supplementary information

Analysis of creep behaviour

The displacement-time ($h'(t)$ - t) curves during creep of all the specimens were fitted with the Burger's model. The corresponding parameters and adjusted R-squared of the fitted curves are listed in Table S2.1.

An example of calculation of creep rate sensitivity (m) is extrapolated as follow:

Indentation rate ($\dot{h}'$) was derived from the derivative of the fitted curves ($\dot{h}' = \frac{dh'(t)}{dt}$). The strain rate ($\dot{\epsilon}$) of a nanoindentation can be extracted from the above fitted depth-time ($h'(t)$ - t) curve as the indenter displacement velocity divided by the penetration depth at the timepoint 't':

$$\dot{\epsilon} = \frac{\dot{h}'}{h'(t)} = \frac{dh'(t)/dt}{h'(t)}$$

The dynamic hardness numbers (H) as a function of time (t) (the same timepoint as the strain rate) was directly obtained from nanoindentation test.

The log-log plot of hardness (H) versus indentation strain rate ($\dot{\epsilon}$) for demineralised dentine surface from the control group is shown in Figure S2.1. As $H = K\dot{\epsilon}^m$, the slope value of the plot is the obtained creep rate sensitivity (m). The parameters of slope (m) and intercept as well as the adjusted R-squared of the fitted curves are listed in Table S2.2.

Table S2.1 Parameters and adjusted R-squared of the fitted displacement-time ($h'(t)-t$) curves using the Burger's model.

	Parameters	A_0	A_1	τ	A_2	R^2
Control	Dentine	19.995	40324.340	5.422	0.154	0.984
		19.997	47182.738	5.608	0.154	0.999
		19.997	41013.349	5.125	0.133	0.999
		19.997	49920.300	5.926	0.154	0.999
		19.997	41782.751	5.066	0.154	0.999
	Subsurface	19.998	48546.141	6.168	0.154	0.999
		19.998	40709.496	5.873	0.183	0.999
		19.999	49450.320	7.184	0.183	0.999
		19.970	38681.088	6.321	0.100	0.999
		19.996	32912.099	7.176	0.183	0.999
	Surface	20.002	57470.797	10.698	0.183	0.999
		20.005	68922.944	8.037	0.183	0.999
		20.028	76190.195	7.658	0.183	0.999
		18.917	57158.849	8.399	0.115	0.999
		19.997	30415.986	5.978	0.183	0.999
		19.994	79555.630	6.979	0.140	0.999
		20.003	54203.815	7.658	0.140	0.999
PA	Dentine	19.972	49051.149	5.598	0.100	0.999
		19.972	49202.965	6.257	0.100	0.999
		19.971	42933.600	6.259	0.100	0.999
		19.973	51019.372	6.222	0.100	0.999
		19.972	48222.197	6.699	0.100	0.999
	Subsurface	19.973	67904.284	7.843	0.100	0.999
		19.972	55489.839	7.652	0.100	0.999
		19.974	67459.737	7.193	0.100	0.999
		19.972	51431.858	8.617	0.100	0.999
		19.973	57577.597	7.753	0.100	0.999
	Surface	19.973	60786.263	7.573	0.100	0.999
		19.974	68850.462	7.154	0.100	0.999

		19.975	71987.942	7.251	0.100	0.999
		19.973	59163.974	8.443	0.100	0.999
		19.974	66179.453	7.940	0.100	0.999
SDF/KI	Dentine	19.969	54671.572	5.304	0.099	0.999
		19.962	55114.696	5.392	0.099	0.999
		19.962	61029.314	5.779	0.099	0.999
		19.962	64442.090	6.127	0.099	0.999
		19.962	53670.309	5.699	0.099	0.999
	Subsurface	19.001	43153.233	7.0827	0.072	0.999
		19.001	45301.584	6.882	0.076	0.999
		20.001	38530.124	7.668	0.076	0.999
		20.001	34824.717	8.782	0.076	0.999
		20.001	33396.133	7.410	0.076	0.999
		19.997	31711.479	7.517	0.080	0.999
	Surface	20.001	25060.900	6.975	0.076	0.999
		20.003	29312.075	7.207	0.076	0.999
		19.001	35125.164	6.478	0.076	0.999
		20.001	33221.170	6.748	0.076	0.999
		20.001	26302.682	7.649	0.076	0.999
PA+SDF /KI	Dentine	20.001	42337.611	5.702	0.076	0.999
		20.001	37370.422	5.833	0.076	0.999
		20.002	40097.058	5.549	0.076	0.999
		20.002	40351.997	5.164	0.076	0.999
		20.001	41306.571	5.390	0.076	0.999
	Subsurface	19.005	19868.959	6.970	0.076	0.999
		19.001	19153.823	5.772	0.076	0.999
		19.002	21277.130	6.009	0.076	0.999
		19.007	19153.917	5.773	0.076	0.999
		19.004	16318.352	6.922	0.076	0.999
	Surface	20.004	24020.152	6.686	0.076	0.999
		20.004	23840.516	6.223	0.076	0.999
		20.003	26938.851	6.531	0.076	0.999

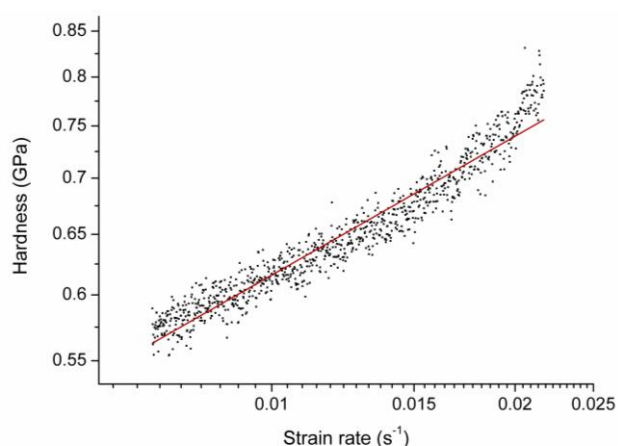
	20.006	25627.039	6.386	0.076	0.999
	20.005	22351.279	6.100	0.076	0.999

Table S2.2 Parameters and adjusted R-squared of double logarithmic plots of hardness (H) versus indentation strain rate ($\dot{\epsilon}$). The slope value is the obtained creep rate sensitivity (m).

Group	Region	Slope		Intercept		Statistics
		Value	Error	Value	Error	Adj. R-Square
Control	Surface	0.364	0.00234	0.319	0.0045	0.932
		0.195	0.00195	0.042	0.00387	0.914
		0.226	0.00225	0.201	0.00446	0.915
		0.141	0.00153	-0.098	0.00321	0.900
		0.173	0.00194	0.121	0.00396	0.894
		0.155	0.00179	0.035	0.0037	0.890
		0.115	0.00131	-0.175	0.00284	0.892
		0.162	0.00182	0.107	0.00377	0.894
	Subsurface	0.080	0.00136	-0.080	0.00309	0.787
		0.069	0.00135	-0.030	0.00311	0.738
		0.123	0.00184	0.028	0.00394	0.826
		0.086	0.00157	0.030	0.00353	0.760
		0.116	0.00192	0.188	0.00412	0.794
	Dentine	0.056	0.00131	-0.043	0.00313	0.663
		0.062	0.00119	-0.105	0.0028	0.743
		0.050	0.00118	-0.068	0.00289	0.659
		0.075	0.00129	-0.098	0.00297	0.784
		0.049	0.0012	-0.079	0.00295	0.636
PA	Surface	0.136	0.00173	-0.007	0.00364	0.869
		0.123	0.00139	-0.104	0.00299	0.892
		0.131	0.00154	-0.111	0.00329	0.885
		0.169	0.00203	0.087	0.00413	0.881
		0.152	0.00165	-0.009	0.00342	0.901
	Subsurface	0.149	0.00167	-0.034	0.00348	0.895
		0.137	0.00172	0.037	0.00362	0.871

		0.130	0.00153	-0.090	0.00321	0.886
		0.180	0.00204	0.177	0.00414	0.892
		0.147	0.00173	0.044	0.00363	0.885
	Dentine	0.058	0.00123	-0.123	0.00289	0.705
		0.083	0.00134	-0.062	0.00301	0.806
		0.079	0.00126	-0.095	0.00284	0.806
		0.096	0.00157	-0.023	0.00346	0.800
SDF/KI	Surface	0.117	0.00219	0.344	0.00474	0.753
		0.133	0.00218	0.307	0.00468	0.799
		0.093	0.0017	0.116	0.00377	0.760
		0.004	0.00185	0.176	0.00406	0.771
		0.149	0.00253	0.394	0.00533	0.788
	Subsurface	0.123	0.00175	0.114	0.00378	0.839
		0.110	0.00166	0.055	0.00361	0.824
		0.137	0.00209	0.203	0.00439	0.820
		0.189	0.00256	0.366	0.00517	0.853
		0.130	0.0021	0.269	0.00445	0.805
	Dentine	0.056	0.00114	-0.202	0.00275	0.716
		0.057	0.00111	-0.196	0.00265	0.739
		0.066	0.0012	-0.225	0.00279	0.766
		0.079	0.00131	-0.219	0.00298	0.796
		0.066	0.00122	-0.162	0.00286	0.756
PA+SDF/KI	Surface	0.089	0.00218	0.298	0.00477	0.642
		0.085	0.0021	0.275	0.00473	0.639
		0.105	0.00192	0.267	0.00424	0.766
		0.093	0.00221	0.259	0.00493	0.655
		0.095	0.00203	0.307	0.00459	0.705
	Subsurface	0.121	0.00272	0.546	0.0059	0.682
		0.099	0.00201	0.417	0.00466	0.728
		0.075	0.00201	0.302	0.00457	0.600
		0.073	0.00204	0.339	0.00473	0.602
		0.100	0.00254	0.417	0.00548	0.629

Dentine	0.060	0.00134	-0.051	0.00319	0.685
	0.050	0.00132	-0.058	0.0032	0.611
	0.066	0.00139	-0.021	0.00328	0.708
	0.074	0.00154	0.052	0.00356	0.714
	0.071	0.00136	-0.026	0.00316	0.746



Intercept value	Intercept Error	Slope value	Slope Error	Statistics Adj. R- Square
0.31854	0.0045	0.26452	0.00234	0.93154

Figure S2.1 An example of the double logarithmic plot of hardness (H) versus indentation strain rate ($\dot{\epsilon}$) of the demineralised dentine surface from the control group.

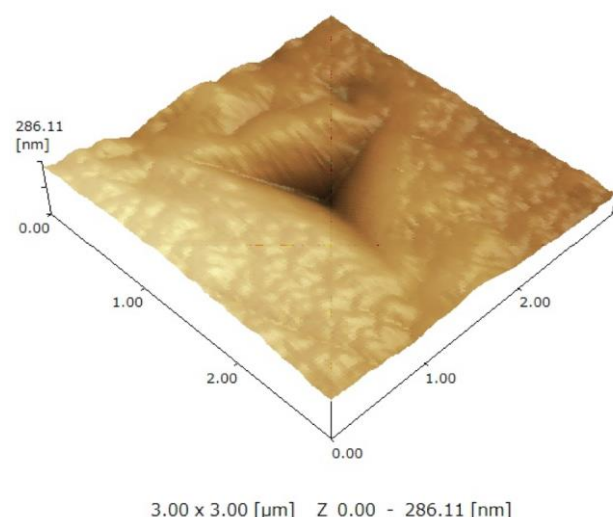


Figure S2.2 A three-dimensional atomic force microscopy image of a nanoindentation impression made on dentine.

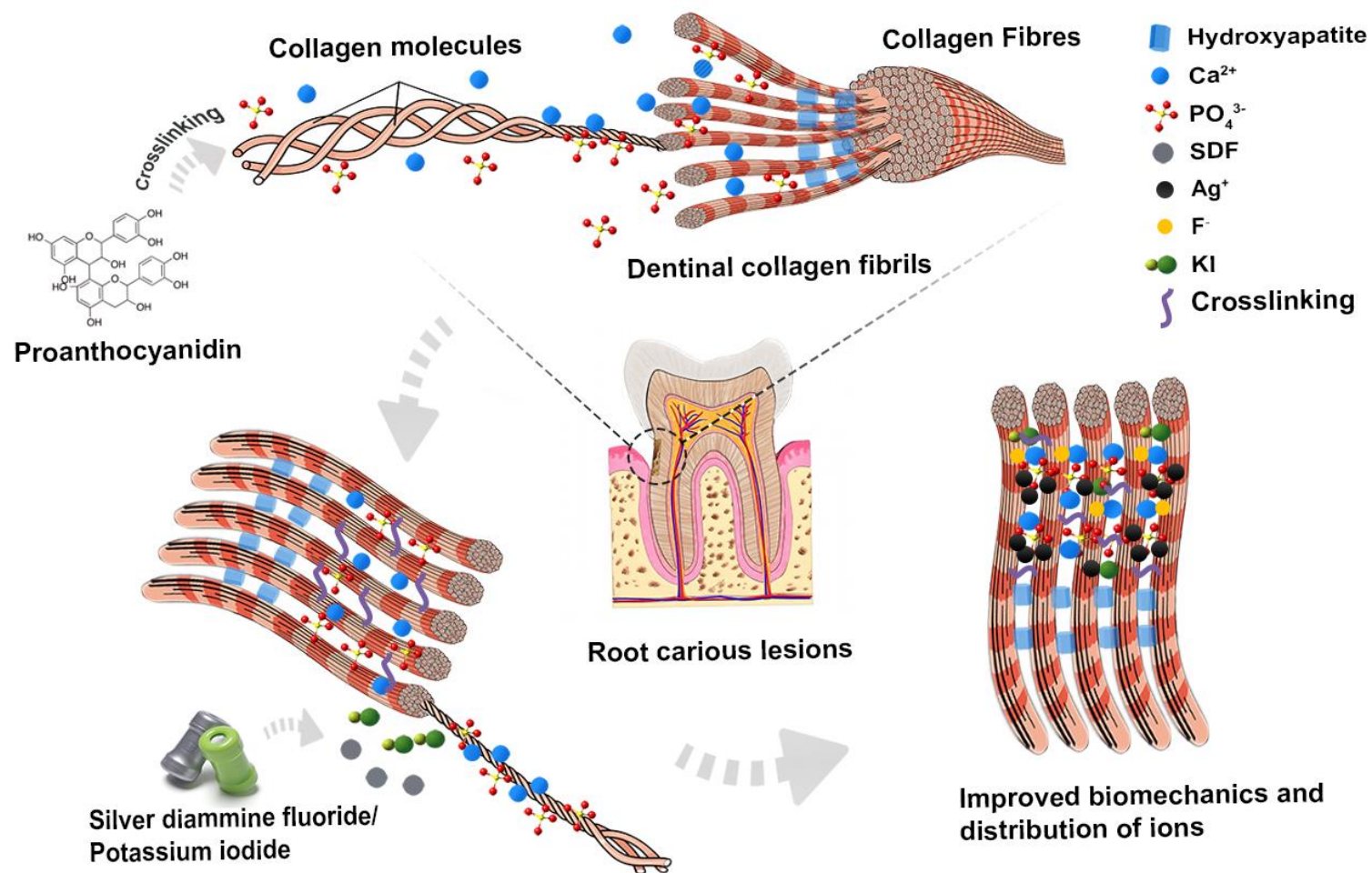


Figure 2.6 Graphical abstract of the study in Chapter 2.

Chapter 3

Remineralising effects of fluoride varnishes containing calcium phosphate on artificial root caries lesions with adjunctive application of proanthocyanidin

3.1 Introduction

Increasing tooth retention in aging populations in industrialised countries and age-related recession of gingival tissue exposing tooth root surfaces to the oral environment have contributed significantly to the increasing prevalence of root caries amongst the elderly [1, 88]. Current treatments mainly follow caries management philosophies established for enamel caries, which may not be equally efficacious for root dentine caries because of the different structures and compositions of enamel and dentine and the associated distinct aetiologies and disease progression of coronal and root caries [470].

Dentine is an hierarchically organised nanocrystalline composite structure formed by matrix-mediated mineralisation. The organic framework of dentine consists of parallel self-assembled 300 nm long collagen units composed of collagen fibrils of 67 nm periodicity staggered and overlapped along their long axes and stabilised by covalent inter- and intra-microfibrillar cross-linking [4, 471]. The gap spaces between the collagen molecular ends are the primary sites for nucleation and growth of the plate-like impure HAp crystals [20]. This structural and stereochemical matching between inorganic and organic materials confers dentine with specific mechanical properties and promotes synergy between matrix and mineral during the caries process [472]. Specifically, in the early stages of caries development, the pH decrease produced by metabolites of acidogenic bacteria tilts the balance of de- and re-mineralisation towards mineral dissolution - especially of the extrafibrillar minerals. Mineral loss affects the packing arrangement of the fibrils and activates the host proteases along with bacterial collagenase action, which leads to lysis of dentinal collagen. The loss of collagen periodicity and the shifts of inter-molecular cross-linkages of the molecules of dentinal collagen result in further loss of intrafibrillar minerals, which decreases the mechanical properties of dentine [473, 474].

Demineralisation can be reversed in its early stages through incorporation of component ions (Ca^{2+} , PO_4^{3-} and F^-) onto the remnant nano-crystallites [7]. Fluoride is currently considered the gold standard therapeutic agent for early carious lesions. It deposits on tooth surfaces as CaF_2 -like materials and substitutes into dental mineral through dissolution/re-precipitation reactions to form an apatitic mineral of increased structural stability - $\text{Ca}_5(\text{PO}_4)_3(\text{OH})_{1-x}\text{F}_x$ [6, 475]. Given that root dentine has a higher critical pH and smaller HAp crystallites than enamel, there is clinically validated consensus that high concentration F toothpastes and varnishes are recommended to maintain the mineral balance in RCLs which could be further enhanced by a combination of F delivery methods [7, 8]. In addition, the availability of Ca^{2+} and PO_4^{3-} (mainly

from saliva and gingival crevicular fluid) is rate limiting for remineralisation. Ageing and medication in elders could affect salivary flow and constituents and decrease the availability of Ca^{2+} and PO_4^{3-} for restoring MD of RCLs [107]. Therefore, bioavailable Ca-P has been incorporated and stabilised into F products to promote remineralisation [475]. Nevertheless, it is still controversial whether the addition of Ca-P has superior effects over F alone, as well as the relative efficacy of different Ca-P remineralisation systems [149, 172-174]. In addition, there is a lack of published evidence of their effectiveness on RCLs.

Whilst F treatments in combination with calcium and phosphate restore the mineral content of carious lesions, poorly remineralised tissue with majority of minerals occupying extrafibrillar space and decreased strength of collagen fibrils may not recover the overall functionality of dentine [472]. Increasing understanding of the hierarchical structure and biochemistry of dentine has promoted the advent of innovative biomimetic approaches for treatment of dentinal carious lesions. Specifically, the presence of dentinal collagen could putatively provide a template and exert spatial constraints to mediate spherulitic crystalline growth of minerals after infiltration of ionic clusters of Ca-P mineralisation precursors [476, 477]. Therefore, strategies for dentinal collagen biomodification to prevent degradation of the demineralised matrix and maintain an intact dentinal matrix have been explored to enhance the interaction of minerals within the collagen fibrils [268, 478]. Locally applied collagenase inhibitors such as CHX and Zn^{2+} could inhibit the activity of proteolytic enzymes during dentine demineralisation and reduce dentine matrix degradation, thus limiting lesion progression [294, 416, 479]. However, inhibitors might not affect all proteases involved in the degradation process. On the other hand, when affected by collagenase, the cross-links of dentinal organic matrix are partly shifted to precursors whilst the collagen matrix is not structurally and biochemically different from that of sound dentine, therefore, it has potential to revert to normal collagen fibres [480]. In this regard, induction of cross-linking might have the ability to recover the structure of dentinal collagen [481]. Especially, chemical cross-linking using agents such as GA and EDC/NHS are the most effective strategies to produce a uniform and high degree of cross-linking in dentine, however, their cytotoxicity may be a limitation for clinical applications [277, 448]. Biocompatible natural cross-linkers, mostly polyphenols, have received increasing attention in recent decades. Among them, PA, derived from grape seed extracts, holds great potential to cross-link dentinal collagen by covalent and ionic linkages as well as bridge-type hydrogen bonds and hydrophobic interaction [421]. Previous studies have reported that application of PA increased the mechanical properties of demineralised dentine and enhanced the

remineralisation of RCLs by stabilising collagen [273, 277]. Adjunctive application of PA with F/Ca/P showed promising results in improving mineral gain and mechanical properties and preventing proteolytic biodegradation [153, 449, 482]. Considering the potential beneficial effects of PA, we were motivated to investigate the strategy of application of remineralising agents with biomodification of dentinal collagen for RCL treatment.

In the present study, firstly, the remineralising effectiveness of MIV and CPWV on RCLs was investigated using laboratory simulated RCLs *ex vivo*. The effects of 5,000 ppm FTP were explored, also in combination with MIV or CPWV. Moreover, PA was used as an adjunct to MIV, CPWV and 5,000 ppm FTP and their combined effects on RCLs was examined in terms of birefringence, ion uptake, changes in MD and mechanical properties.

3.2 Materials and Methods

3.2.1 Materials

Materials used were proanthocyanidin (International Laboratory, CA, USA; containing >95% oligomeric proanthocyanidins); Clinpro™ White Varnish (CPWV; 5% NaF – 22,600 ppm F with TCP; 3M ESPE, MN, USA); MI Varnish (MIV; 5% NaF with RECALDENT™ (CPP-ACP); GC Corp., Tokyo, Japan); Clinpro™ 5,000 ppm FTP (3M ESPE, MN, USA) and Duraphat™ varnish (5% NaF – 22,600 ppm F; Colgate-Palmolive, NY, USA). The demineralising solution for root caries formation contained 50 mM acetic acid, 2.2 mM CaCl₂ and 2.2 mM KH₂PO₄, and the solution was adjusted to pH 5 with KOH. Treatment solutions in pH-cycling including a demineralisation solution prepared using 50 mM acetic acid, 1.5 mM CaCl₂ and 0.9 mM KH₂PO₄ and adjusted to pH 5 with KOH and a remineralisation solution prepared using 1.5 mM CaCl₂, 0.9 mM KH₂PO₄, 130 mM KCl and 50 mM HEPES buffer was adjusted to pH 7 with KOH. Details of the materials and the application methods are listed in Table 3.1.

Table 3.1 Materials used in the study in Chapter 3.

Material brand	Abbreviation	Manufacturer	Code (Lot#)	Therapeutic composition (w/w) ^[35]	Application instructions
MI Varnish TM	MIV	GC Corporation, Tokyo, Japan	1611141	5% sodium fluoride (NaF; 22,600 ppm fluoride), 1–5% CPP-ACP	1. Tooth surface cleaned and softly dried using the compressed air. 2. Stir the varnish in the unit dose container and apply a thin and uniform layer of MI varnish on tooth surface using a micro-brush
Clinpro TM White Varnish	CP WV	3M ESPE, MN, USA	N865382	5% NaF, 1-5% modified TCP	1. Tooth surface cleaned and softly dried using the compressed air. 2. Carefully mix the varnish with a micro-brush; apply the varnish evenly on the tooth surface.
Duraphat TM	5% NaF	Colgate®, NY, USA	70091	5% NaF	1. Tooth surface cleaned and softly dried using the compressed air. 2. Apply a thin layer to the dentine surface using a micro-brush.
Clinpro TM 5000 toothpaste	FTP	3M ESPE, MN, USA	131041	1.1% NaF	1. Brush the dentine specimen using a micro brush with the toothpaste for 3 min. 2. Rinse the specimens with distilled water.
Oligomeric proanthocyanidins (>95%)	PA	International Laboratory, CA, USA	781046	A collagen cross-linking agent containing polyphenolic compounds derived from grape seed extracts.	1. Tooth surface cleaned and softly dried using the compressed air. 2. Apply on the dentine surface using a micro-brush twice, 1 min each time.

3.2.2 Preparation of root dentine specimens

Extracted human permanent molars from individuals aged 20 to 50 were stored in 0.1% thymol solution at 4 °C under the protocol approved by the Human Research Ethics Committee of the

University of Melbourne. The teeth were cleaned with DDW to remove surface debris. Non-carious molars without obvious defects on the root surface were selected. The crown and root apical portions were removed using a slow-speed diamond saw (Isomet, Buhler Ltd, IL, USA) under water irrigation. The minimum sample size was estimated based on a power of 0.8 with a confidence level of 95% following the method in our previous study [482]. Six specimens per group were prepared for cross-sectional microhardness testing. Two specimens were chosen for micro-CT analysis, which afterwards were used for EDS analysis. Three samples in each group were prepared for PLM observation. One spare sample was prepared in each group. Therefore, 12 non-carious human permanent molar dentine blocks in each group were prepared from the buccal or lingual surfaces of the roots adjacent to the CEJ and embedded in epoxy resin (KEP epoxy, Kemet International Ltd., Kent, UK). The tooth surfaces were polished using ascending grits (320, 600, 1200, 2500) of silicon carbide papers to expose the root dentine surface and the debris was removed by ultrasonic cleaning for 5 min in DDW. A layer of acid-resistant nail varnish was applied on the polished root surfaces to expose a window of 3 × 4 mm for treatments.

3.2.3 Treatment procedure

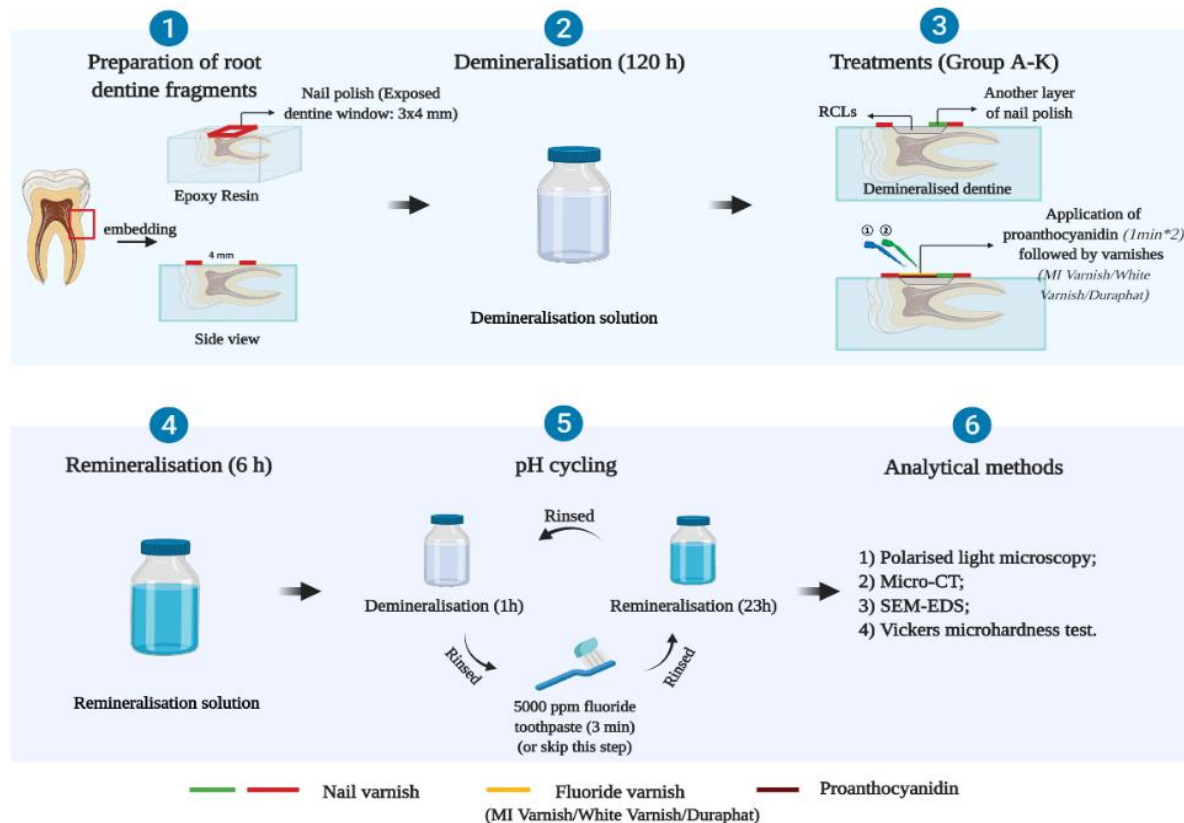


Figure 3.1 Schematic diagram of the treatment process in the study in Chapter 3.

3.2.3.1 Demineralisation

The treatment procedures are illustrated in Figure 3.1. The protocol for demineralisation was modified from that previously reported [153, 482]. Specifically, each specimen was immersed in 20 mL of the buffered acidic demineralising solution (see 3.2.1), which was prepared and changed every 24 h. After demineralisation for 120 h at 37 °C, another layer of acid-resistant nail varnish approximately 1 mm wide was applied onto the exposed surface to isolate part of the exposed demineralised dentine as a demineralised reference for comparison with the root dentine after remineralisation.

3.2.3.2. Treatments

Root dentine specimens were assigned randomly to the eleven treatment groups (n = 12 per group) (Figure 3.2). Specifically, the DDW group served as the negative control and Duraphat™ varnish as the positive control. The 6.5% w/v PA solution was prepared by adding PA powder into DDW and mixing thoroughly by pipetting up and down to ensure uniformity. The PA solution was applied to root dentine surfaces using a micro-brush and agitating for 1 min. The excess solution was removed gently with a cotton pellet. This was followed by a further application and agitation of fresh PA solution for 1 min. MIV or CPWV was applied according to the manufacturer's instructions (Table 3.1).

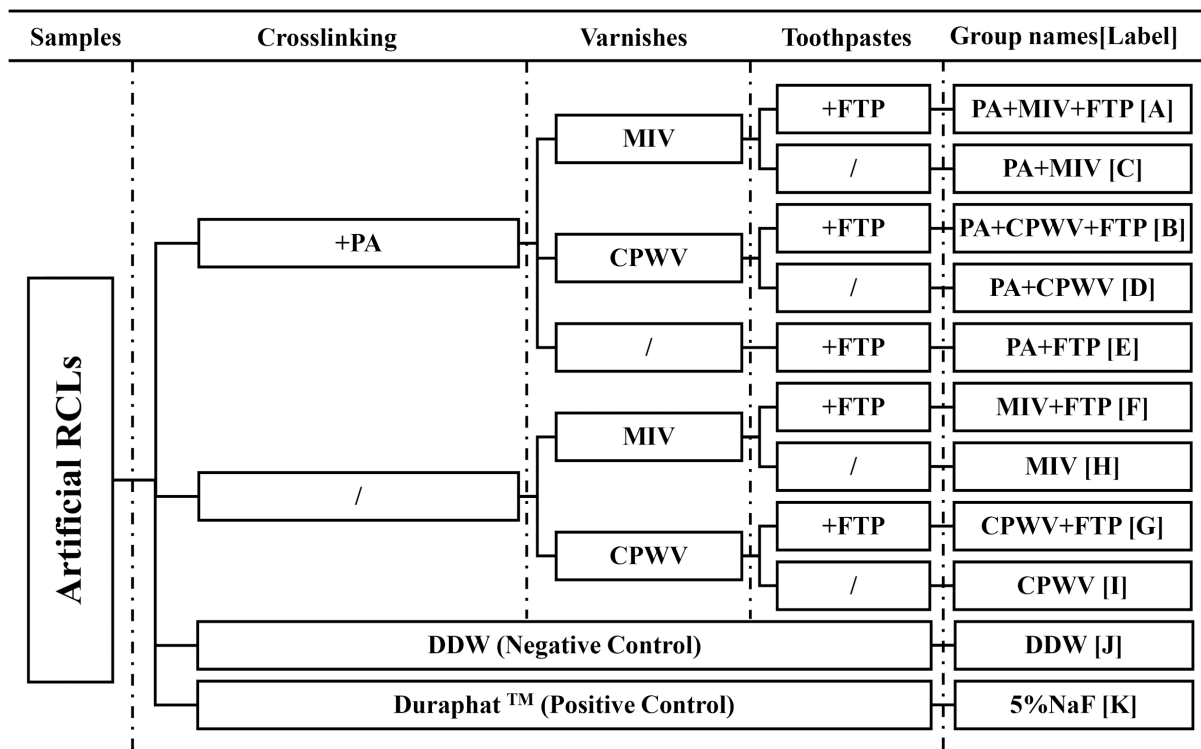


Figure 3.2 Treatments in each group and group labels.

3.2.3.3 pH-cycling

After treatment, all specimens were stored in a remineralisation solution (20 mL per sample) for 6 h without interference of the treated surface and then subjected to the pH-cycling process. Specifically, specimens were immersed in the demineralisation solution for 1 h and remineralisation solution for 23 h at 37 °C (see 3.2.1). Solutions were refreshed with each pH-cycle and the process lasted for 8 d. After each immersion in demineralisation solution for 1 h, the samples were cleaned gently using DDW and blot dried. For samples in Groups A-B and E-G, 5,000 ppm FTP was applied and brushed onto the exposed dentine surface with micro-brushes for 3 min, gently cleaned using DDW and blot dried before immersion in remineralisation solution. Samples in Groups C-D and H-K were gently cleaned with DDW without treatment with the FTP before immersion in the remineralisation solution. After pH-cycling, samples were cleaned ultrasonically in DDW for 5 min before analysis stored in a labelled container with damp gauze thereby creating a humidified environment before analysis.

3.2.4 Analytical methods

3.2.4.1. Polarised light microscopy

Three samples were randomly selected from each group, re-embedded in epoxy resin to protect the demineralised surfaces and sectioned longitudinally through the centre of the lesions with a low-speed water-cooled peripheral diamond saw (Minitom, Struers, Copenhagen, Denmark) to obtain 300 µm thick sections. During sectioning, the exposed demineralised dentine surfaces were covered with damp gauze to protect the demineralised surfaces from dehydration. Each specimen was further manually lapped to a thickness of approximately 100 µm. The sample was mounted on a glass slide with DDW and covered with a clean coverslip [49]. Changes in colour birefringence of the specimens were recorded using a first order red plate between crossed polarisers with transmitted light microscopy (DM2000, Leica Microsystems GmbH, Wetzlar, Germany) coupled to a camera (Leica EC3, Leica Microsystems GmbH, Wetzlar, Germany). The slides were visualised at original 200× magnification and the images were photographed and analysed by means of the software program Image J 1.38 × (National Institutes of Health, MD, USA).

3.2.4.2. Micro-CT analysis

Two dentine specimens randomly selected from one group were aligned in a line and fixed in a pipette tip for micro-CT scanning. High resolution Micro-CT analysis (Skyscan 1172, Skyscan N.V., Aartselaar, Belgium) was performed to investigate the changes of MD of the artificial RCLs post-treatment. The imaging settings were the following: voltage: 58 kV, beam current: 171 mA, filter: 0.5 mm aluminium, resolution: 2.1 μm and rotation: 360° in a 0.2° step. Prior to scanning, a new flat field reference was acquired. For MD calculation, two mineral cylinder phantoms of a similar size as the specimens with the MD value of 0.25 and 0.75 $\text{g}_{\text{HAP}}/\text{cm}^3$ respectively were scanned with the same imaging settings. The resulting 2D projected images were reconstructed using the NRecon software (Version 1.4.2, Skyscan N.V., Aartselaar, Belgium) interface based on a modified Feldkamp algorithm with the following parameter settings: smoothing: 3, ring artefact correction: 15 and beam hardening correction: 50%. After reconstruction, the reconstructed images were imported into Image J 1.38 × and resliced to obtain vertical 2D slices which showed cross-sectional surfaces of whole carious lesion areas [50]. The mean MD was estimated in a volume of interest (VOI) of 30×20×250 voxels (width×thickness×depth) ($30 \times 2.1 \times 20 \times 2.1 \times 250 \times 2.1 \mu\text{m}^3$) at each consecutive 2.1 μm in depth. The regions of interest (ROIs) were selected in dentine in a direction perpendicular to the lesion surface covering all lesion depths considering the difficulties in selecting ROIs exactly parallel to the lesion surface in a small root dentine segment. Otherwise large heterogeneity in mineral density values of each ROI would occur. An example of 2D image of ROI was shown in the red rectangular area in Figure 3.5 (I)). For each sample, three VOIs were selected randomly at the sites of the initial demineralised root dentine (covered by the second layer of nail polish), post-treatment (surface exposed for treatments) and sound dentine areas respectively.

The GVs were converted into MD values ($\text{g}_{\text{HAP}}/\text{cm}^3$) using a linear calibration curve based on the GVs obtained from the HAp phantoms (linear regression, $R^2 = 0.98 - 0.99$). To plot the linear calibration curve, GV measurements were taken by choosing a ROI at 10 different cross-sectional slices of the phantom cylinder. The mean of the 10 GVs were recorded as the GV of the phantom with specific MD (0.25 or 0.75 $\text{g}_{\text{HAP}}/\text{cm}^3$). The correlation coefficients and the regression equations of the phantom GV with their MD were calculated and plotted using GraphPad Prism Version 8.0.1 (GraphPad Software Inc. CA, USA).

For the normalisation of the MD profile in each group, the sound dentine area covered by nail polish was used to provide a superimposition reference of the original dentine surface (depth axis of 0 μm ; as shown in Figure 3.5 (I)). Thus, the MD data were reported with the same starting point regardless of the different depths of shrinkage of the demineralised area. Mineral loss (ΔZ) was the integrated mineral loss up to the lesion depth, which was calculated from the profiles of each sample by subtracting the area under the MD curve (demineralisation (ΔZ_d) or remineralisation (ΔZ_r)) from the area of sound dentine. Predominantly the observed mineral changes occurred within the first 250 μm so the mineral change from the original dentine surface to a depth of 250 μm was determined.

To evaluate the extent of remineralisation, relative changes in the mineral loss (or gain) ($\% \Delta Z$) were calculated as follows:

$$\% \Delta Z = \frac{\Delta Z_d - \Delta Z_r}{\Delta Z_d} \times 100$$

where ΔZ_d is the difference between the area under the curve (AUC) of the baseline demineralised root dentine and the sound dentine; ΔZ_r is the difference between the AUC of the dentine post-treatment and the sound dentine; and $\Delta Z_d - \Delta Z_r$ is equal to mineral gain (or loss) after treatments and pH-cycling (as shown in Figure 3.6 A).

In order to compare the mineral content changes in different layers of surface and subsurface, the relative mineral changes at 30 μm increments (a 40 μm increment for the last layer) from the dentine surface to a depth of 250 μm (*i.e.*, 0-30, 30-60, 60-90, 90-120, 120-150, 150-180, 180-210 and 210-250 μm) were determined and plotted in a 3D bar graph using OriginPro 2018 (OriginLab Corporation, MA, USA).

3.2.4.3 Scanning electron microscopy and energy-dispersive X-ray spectroscopy

Samples used for micro-CT analysis were polished again using 1000, 2500, 4000 SiC paper then 1 and 0.5 μm diamond suspensions; ultrasonically cleaned for 5 min and desiccated at room temperature overnight. The cross-sectional morphology of the carious lesion area of each sample was observed using SEM. Samples were not coated to avoid the influence caused by the peaks of the coating material and the uncontrolled deviations due to the difference in coating thickness in quantitative EDS analysis. The elemental analysis of Ca, P and F was

determined using the line scan mode of the EDS with a silicon drift detector (SDD) coupled to the SEM (Oxford instruments; voltage: 10.0 kV; working distance: 9.9-10.3 mm). The distance between each reading point was 1.2 μm and the live running time for each scan was 600 s. Assessments of the line scan were made in triplicate in three randomly chosen areas from the lesion surface to the sound dentine along a direction perpendicular to the dentine surface in the baseline demineralised area and remineralised area. A line scan was conducted on the sound dentine area as a comparison to show the elemental changes in un-demineralised dentine. The relative X-ray intensity from EDS spectra reflecting the elemental concentrations of Ca, P and F in demineralised and remineralised dentine regions from the line scan along the lesion depth were plotted and the atomic percentage of Ca, P and F was quantified by Aztec 3.3 software (Oxford Instruments, High Wycombe, UK) available in EDS. In particular, F was included from the periodic table manually for peak identification if it was not displayed automatically due to its low concentration in the specimens.

3.2.4.4. Cross-sectional microhardness test

Six longitudinally sectioned samples in each group were re-embedded in epoxy resin. The cross-sectional surface of each dentine block was polished on a water-cooled grinding and polishing unit (Tegra-Pol 21, Struers, Copenhagen, Denmark) with abrasive papers (600, 1200, 2500 and 4000 grit), followed by 1 and 0.5 μm diamond suspensions (Type K, Kemet International Ltd., Kent, UK). The debris on the surface was removed by ultrasonically cleaning for 5 min in DDW. Cross-sectional microhardness measurements perpendicular to the demineralised surface were performed below the surface at less than 15 μm to 200 μm with 10 μm intervals using Vickers hardness indentation (402 MVD, Wolpert Wilson Instrument, MA, USA) with a load of 10 gf ($98 \times 10^{-3}\text{N}$) for 10 s dwell time at each testing point. For all samples, indentations were performed both in demineralised and remineralised areas of the cross-sectional surface at each depth on parallel tracks approximately 150-200 μm apart both in the demineralised and remineralised cross-sectional surfaces. Hardness was determined according to the following equation:

$$H = \frac{P}{A}$$

where P is the applied force and A is the contact area of the indentation which depends on the indenter geometry and the penetration depth [14]. Measurements were discarded if cracks occurred on dentine during indentation. To avoid variations of microhardness of different

samples, an internal control of the sound area (250 μm from the surface) of each dentine block was used for comparison. Results were expressed as relative microhardness values [41].

3.2.5. Statistical analysis

Quantitative data were expressed as means $\pm$ standard deviation. The assumptions of homogeneity of variance and normal distribution were checked using Levene's and Shapiro-Wilks tests, respectively. Vickers microhardness numbers (VHN) data were subject to Student's paired t-test. The relative atomic percentage (At%) data from EDS were analysed by two-way ANOVA, *post hoc* Bonferroni test. Other statistical analysis was carried out by one-way ANOVA and *post hoc* Tukey's (equal standard deviation assumed) or Dunnett's T3 (equal standard deviation not assumed) multiple comparisons. A level of significance of $\alpha = 0.05$ was set in all statistical tests (SPSS Version 22.0; IBM, NY, USA).

3.3 Results

3.3.1 Polarised light microscopy

Polarised light microscopy images showed the changes in lesion depths and colour birefringence after de-/remineralisation. Specifically, the demineralised dentine showed different birefringence from the sound dentine area (Figure 3.3 A-K, left side of the white dotted line) and lesion depths were therefore easily measured from the demineralised part of all samples (Figure 3.3 A), which were shown to be $155.1 \pm 23.5 \mu\text{m}$ deep (supplementary information Table S3.1). A dark band on the dentine surface was observed in all PA-treated groups (Figure 3.3 A-B & E-G). Apart from the dark band, treatment with PA+FTP produced a change of birefringence below this band in the subsurface areas of demineralised dentine similar to sound dentine (Figure 3.3 E). After combined treatments using either MIV or CPWV with FTP, the birefringence of demineralised dentine was similar to sound dentine (Figure 3.3 C-D).

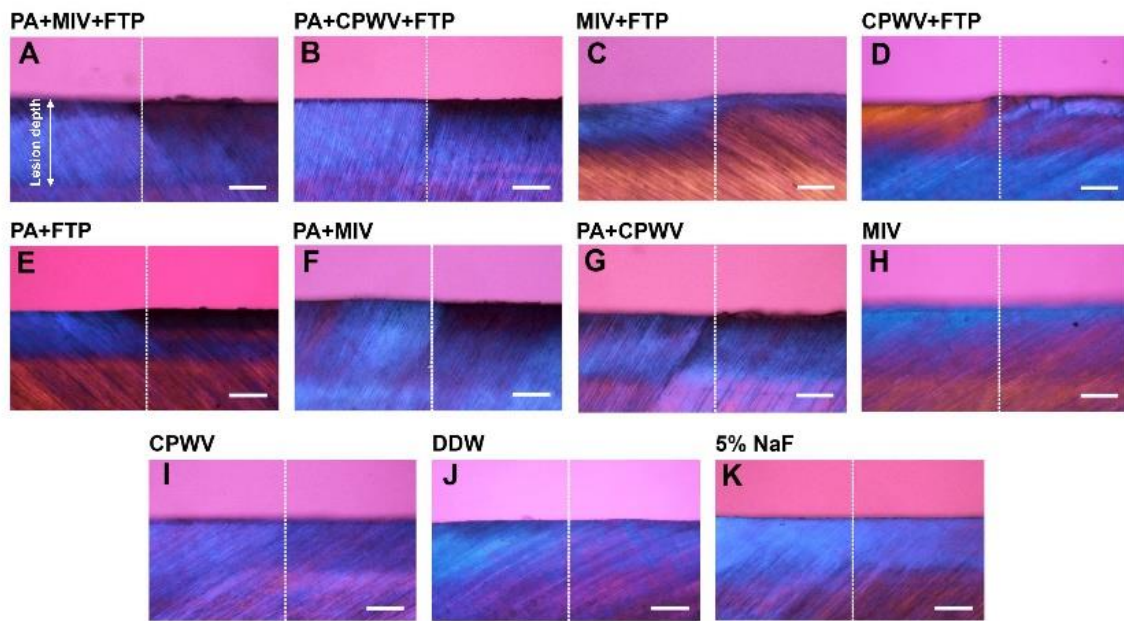


Figure 3.3 (A-K) Representative polarised light microscope images of specimens from all the groups viewed in water (refractive index 1.33) with a first-order red plate between crossed polarisers. In each image, on the left and right side of the white dotted line is the demineralised dentine and dentine after treatments and pH-cycling respectively; Bar scale = 100 μm .

3.3.2 Micro-computed tomography analysis

The GV of two phantoms were plotted against their MD (0.25 and 0.75 $\text{g}_{\text{HAP}}/\text{cm}^3$ respectively), which generated a scatter plot with a regression equation and a typical linear regression coefficient (R^2) of 0.9997 (Figure 3.4). The regression equation of the plot was used to calculate the MD of the specimens.

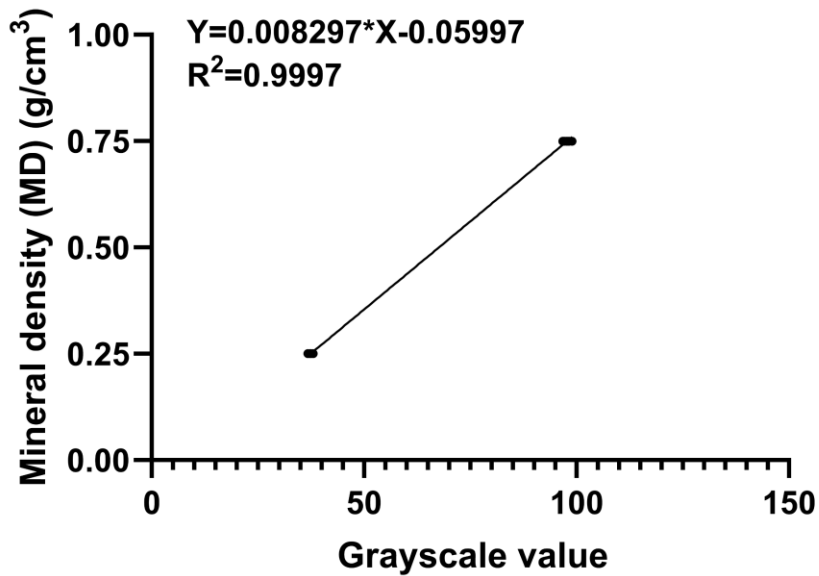


Figure 3.4 Scatter plot of grayscale values versus mineral density of the two standard phantoms with MD of 0.25 g_{HAP}/cm³ and 0.75 g_{HAP}/cm³ respectively.

Typical 2D micro-CT images showed de-/remineralised lesions and sound root dentine in each group (Figure 3.5 (II A-K)). Although specimens were embedded in epoxy resin to preserve the delicate demineralised structure at the uppermost surface during sectioning and polishing, dehydration was still unavoidable during this process. As such, shrinkage was observed in the de-/remineralised dentine areas with GV of approximately 0 in micro-CT images. In addition, although ring artefacts correction and flat-field corrections were applied in micro-CT reconstruction, ring artefacts can still be observed in some images (Figure 3.5 (II C-F)). Also, scanning was conducted in a very high resolution (2.1 µm) so that a slightly movement and dehydration during scanning could cause artefacts. Mineral density profiles against lesion depth of specimens in each group were plotted (Figure 3.6). The integrated mineral content changes throughout the lesions in all groups were calculated according to the MD profiles (Table 3.2).

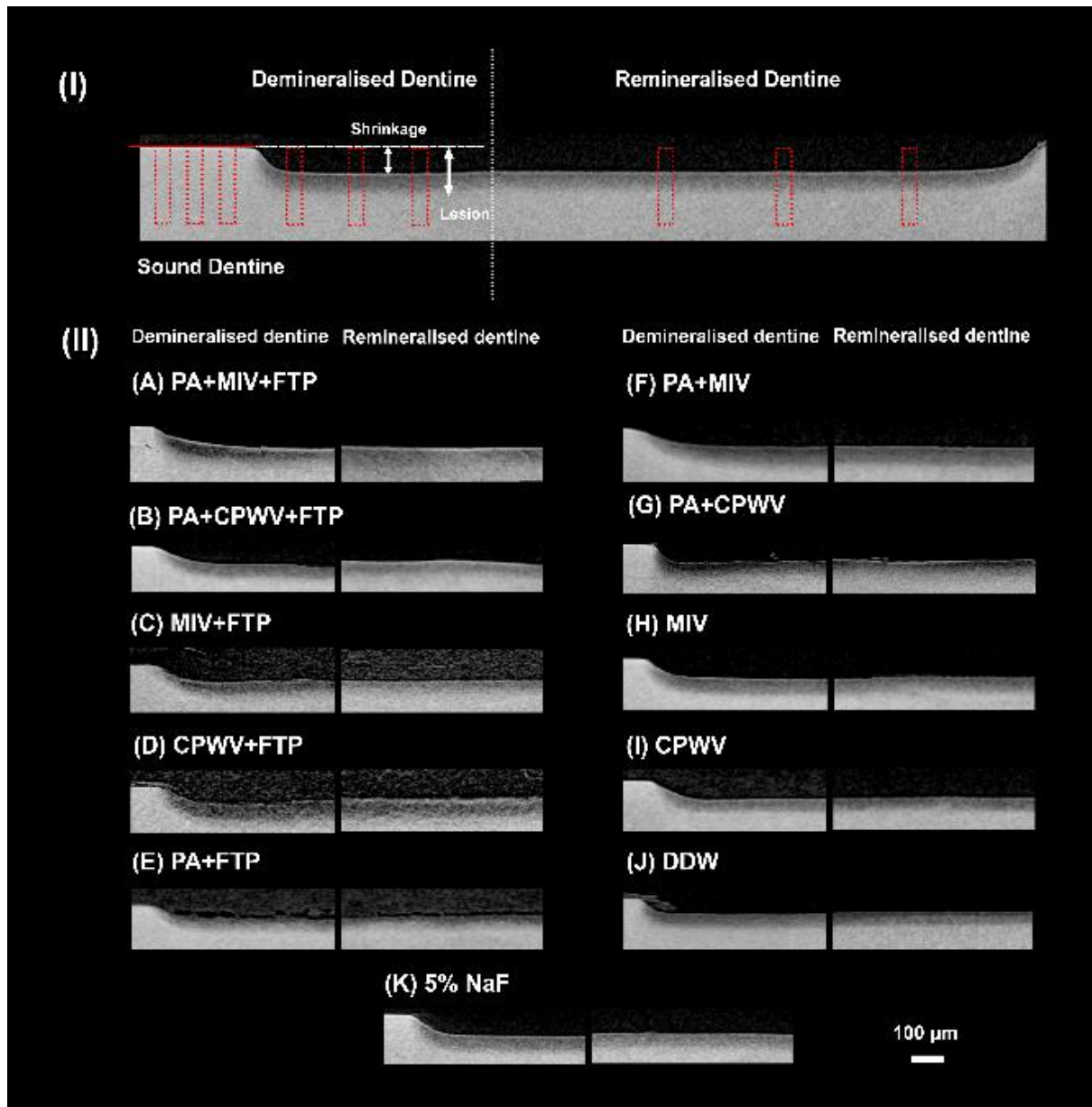


Figure 3.5 (I) An example of a two-dimensional (2D) cross-sectional slice of a specimen from micro-CT reconstruction. (II A-K) Representative 2D micro-CT slices of sound+demineralised and remineralised dentine from all the experimental groups. The red dotted boxes present the volume of interest (VOI) ($30 \times 2.1 \times 20 \times 2.1 \times 250 \times 2.1 \mu\text{m}^3$ (width $\times$ thickness $\times$ depth)) over which the mineral content profiles were plotted in each specimen. Three VOI were randomly chosen in sound, demineralised and remineralised dentine areas in each specimen. Shrinkage was observed on the lesion surface and root carious lesions showed a relatively lower grayscale than the sound dentine area.

Table 3.2 Integrated mineral content changes of initial dentine carious lesions after each experimental phase.

Groups ^a	ΔZd	ΔZr	Mineral gain (ΔZd-ΔZr)	ΔZ% ($\frac{\Delta Zd - \Delta Zr}{\Delta Zd} \times 100$)	Statistical analysis ^b
PA+MIV+FTP	127.39 ± 11.66	97.54 ± 7.64	29.85 ± 8.96	23.15 ± 5.56	
MIV+FTP	124.62 ± 4.61	99.65 ± 5.98	24.96 ± 2.42	20.09 ± 2.37	
PA+CPWV+FTP	152.76 ± 6.02	126.61 ± 7.33	26.15 ± 2.15	17.17 ± 1.89	
CPWV+FTP	151.09 ± 3.24	133.73 ± 6.85	17.36 ± 4.38	11.53 ± 3.01	
PA+ MIV	177.84 ± 11.89	159.90 ± 19.03	17.94 ± 8.35	10.38 ± 5.67	
PA+FTP	182.49 ± 2.71	163.87 ± 4.68	18.62 ± 3.73	10.21 ± 2.06	
PA+ CPWV	189.04 ± 2.84	169.98 ± 2.95	19.06 ± 1.93	10.08 ± 0.98	
CPWV	131.20 ± 5.48	118.99 ± 5.41	12.21 ± 3.04	9.29 ± 2.24	
MIV	142.73 ± 5.79	130.62 ± 6.94	12.12 ± 2.79	8.52 ± 2.01	
5%NaF	154.99 ± 4.90	142.77 ± 3.55	12.22 ± 2.03	7.86 ± 1.14	
DDW	131.33 ± 12.72	122.53 ± 14.66	8.80 ± 6.93	6.78 ± 5.40	

Values are mean ± standard deviation; ΔZ% is the relative mineral change (^aDescending order of mean ΔZ% ranks; ^bOne-way ANOVA, *post hoc* Dunnett's T3 test, α=0.05; mean values joined by a vertical line are not significantly different from each other).

The MD of sound dentine was estimated from the GV of all the sound dentine tested, which in the present study was $1.15 \pm 0.04 \text{ g}_{\text{HAP}}/\text{cm}^3$. Demineralisation was associated with a gradual mineral loss in the dentine subsurface with relatively lower GV than sound dentine (Figure 3.5 B, demineralised dentine). The convergence of the MD profiles of de-/re-mineralised and sound dentine part in each group demonstrated equivalence of MD in the test and control sides (Figure 3.6), indicating the untreated baseline demineralised halves were a valid self-control in each group.

Statistical analysis showed significant differences in mean mineral gain among groups after treatment and pH-cycling (Table 3.2). Specifically, results of DDW group indicated that the pH-cycling protocol used in the present study was a net-remineralising process, with total mineral gain of $8.80 \pm 6.93 \text{ g}_{\text{HAP}}/\text{cm}^3 \cdot \mu\text{m}$ and %ΔZ of $6.78 \pm 5.40\%$. All the post-treatments used in the present study were associated with mineral content gain throughout the RCLs, but the remineralisation amount varied. Mineral gain was partly from the surface precipitation promoted by remineralising agents, which was illustrated as a distinct peak in the mineral profile (Figure 3.6), and from the MD increase in the deepest part of RCLs. Duraphat™ varnish increased the RCL MD by $7.86 \pm 1.14\%$, slightly higher than pH-cycling treatment alone; both MIV and CPWV further increased the mineral content of RCLs. Adjunctive application of PA with either FV containing Ca-P or 5,000 ppm FTP showed no statistically significant

differences in MD change ($P>0.05$). Generally, MIV showed greater remineralisation effects than CPWV. Specifically, combined use of MIV with 5,000 ppm FTP showed superior remineralisation effects with $\% \Delta Z$ of $20.09 \pm 2.37\%$, significantly higher compared to CPWV with 5,000 ppm FTP ($11.53 \pm 3.01\%$) ($P<0.0001$). When PA was applied before the application of MIV or CPWV with FTP, the remineralisation efficacy of MIV and CPWV was significantly increased, with $\% \Delta Z$ of $23.15 \pm 5.56\%$ (PA+MIV+FTP) and $17.17 \pm 1.89\%$ (PA+CPWV+FTP), respectively ($P=0.0052$).

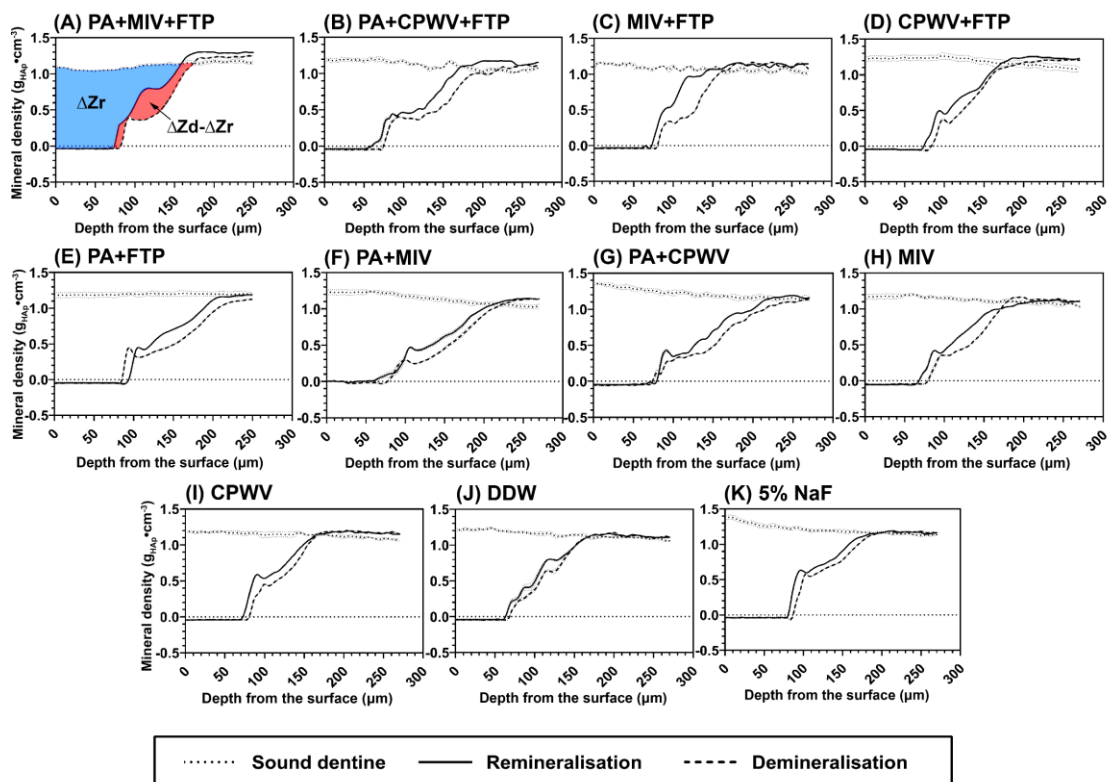


Figure 3.6 (A-K) Mineral density ($\text{g}_{\text{HAP}}/\text{cm}^3$) versus depth profiles of volume of interest in sound, demineralised and remineralised dentine areas in all the experimental groups. ΔZ was the integrated mineral loss up to the lesion depth, which was calculated from the profiles of each sample by subtracting the area under the mineral density curve (demineralisation (ΔZ_d) or remineralisation (ΔZ_r)) from the area of sound dentine. $\Delta Z_d - \Delta Z_r$ is equal to mineral gain (or loss) after treatments and pH-cycling.

Relative mineral changes post-treatment across each 30 μm increment from original dentine surface to 250 μm depth were analysed to demonstrate MD changes in different surface/subsurface layers of RCLs (Figure 3.7). Shrinkage was observed at 0-60 μm depth from

the original dentine surface and the mineral gain started from 60-90 μm depth in all samples. Using PA as an adjunct with F regimens (*i.e.*, MIV, CPWV and FTP) led to an incremental increase of mineral density in deeper subsurface areas (especially of 150-180 μm) compared to those without PA pre-treatment.

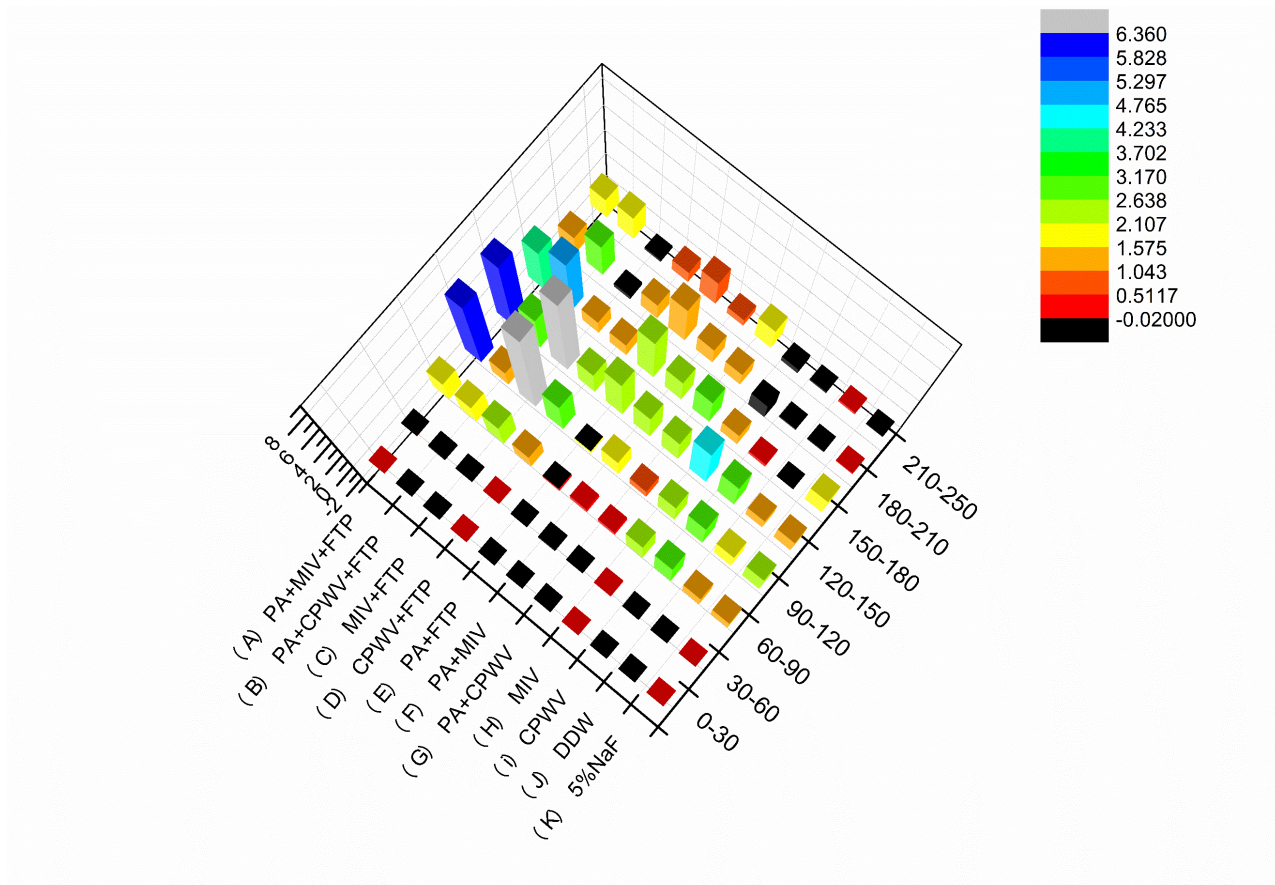


Figure 3.7 Three-dimensional bar charts of relative mineral changes in each group after pH-cycling from ~ 30 μm to 250 μm depth with 30 μm increments.

3.3.3 Scanning electron microscopy - energy-dispersive X-ray spectroscopy

Specimens were highly polished with 0.5 μm diamond suspension before measurements in order to minimise the geometric effects in EDS analysis. The line scan results of EDS revealed the elemental concentration and atomic percentage (At%) changes along the cross-sectional surface from the lesion surface to the sound dentine area. The line scan conducted on the sound dentine area which served as a comparison showed sharp decrease of elemental concentrations from the un-demineralised dentine into the epoxy resin (Supplementary Information Figure S3.1). Whilst in the demineralised section in each specimen, a gradual depletion of Ca and P (gradient of concentration of Ca and P) from the lesion base towards the dentine surface was

shown, which was possibly associated with decreased mineral content in artificial carious lesions (Figure 3.8 A-K, demineralised dentine). In the dentine section after treatment and pH cycling, the line scan demonstrated a slight increase of Ca, P and F concentration throughout the carious lesion area, although a depletion of Ca and P was still observed, indicating that the remineralisation process was not fully completed in RCLs.

From the semi-quantitative At% analysis, treatment with DDW did not result in significant changes in At% of Ca, P and F in the RCLs ($P > 0.05$) (Table 3.3). In other experimental groups, At% of Ca, P and F contents were selectively increased post-treatment compared to the demineralised dentine. Specifically, there was a significant increase of Ca and P concentration in the lesion surface when treated with PA+FTP (Figure 3.8 E). Exposure to MIV or CPWV together with PA and 5,000 ppm FTP significantly increased the concentrations and At% of Ca, P and F ($P < 0.05$), which might indicate their combined effects on ion uptake and distribution (Table 3.3). Whilst significant increase in F concentration throughout the RCLs was observed in all experimental groups (Table 3.3), less incremental increase of F concentration was present in all CPWV-treated groups (Figure 3.8 B, D, G, I) than MIV-treated groups (Figure 3.8 A, C, F, H), indicating the difference in the availability of F^- from varnishes which incorporated Ca-P using different technologies.

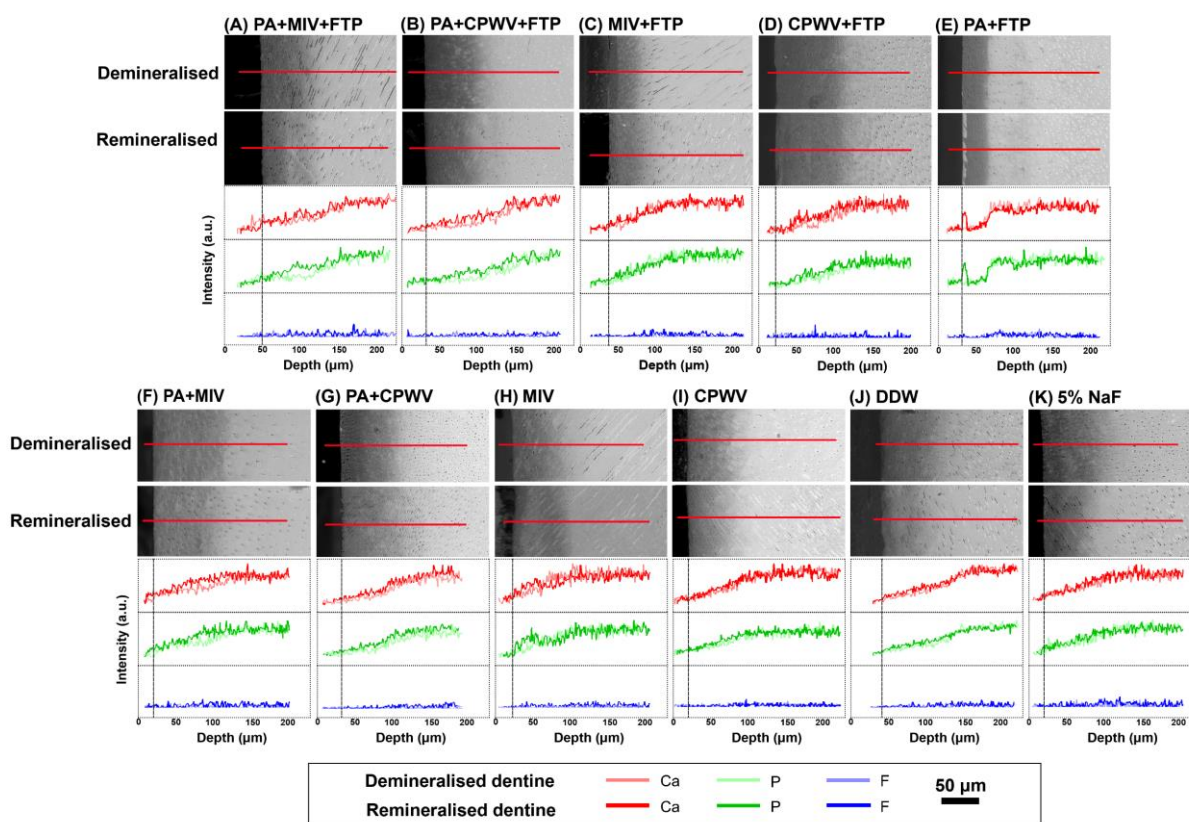


Figure 3.8 (A-K) Scanning electron microscopy images of cross-sectional surfaces of de-/remineralised dentine and the corresponding relative concentrations of calcium (Ca), phosphorus (P) and fluorine (F) analysed by the line scan mode of SEM-energy dispersive X-ray analysis.

Table 3.3 The relative atomic percentage (At%) of calcium, phosphorus and fluorine in demineralised and remineralised dentine analysed by energy dispersive X-ray microanalysis from line scan mode.

Groups	Atomic % (mean (standard deviation))					
	Ca		P		F	
	Demin	Remin	Demin	Remin	Demin	Remin
PA+MIV+FTP	10.80 (0.399)	12.72 (0.160)*	7.038 (0.207)	7.911 (0.042)*	0.436 (0.002)	1.031 (0.014)*
PA+CPWV+FTP	10.47 (0.210)	12.97 (0.261)*	6.729 (0.090)	8.272 (0.090)*	0.477 (0.015)	0.675 (0.045)*
MIV+FTP	10.89 (0.038)	12.03 (0.295)*	6.866 (0.090)	7.345 (0.109)*	0.438 (0.035)	1.013 (0.082)*
CPWV+FTP	10.54 (0.178)	10.50 (0.710)	6.903 (0.054)	6.934 (0.368)	0.488 (0.039)	0.747 (0.064)*
PA+FTP	11.03 (0.203)	10.30 (0.295)*	7.130 (0.093)	6.545 (0.063)*	0.483 (0.027)	0.780 (0.017)*
PA+MIV	10.24 (0.063)	11.43 (0.182)*	6.714 (0.012)	7.583 (0.032)*	0.450 (0.031)	1.413 (0.119)*
PA+CPWV	10.38 (0.252)	9.724 (0.378)*	6.470 (0.228)	6.274 (0.245)	0.407 (0.033)	0.898 (0.047)*
MIV	10.84 (0.244)	10.83 (0.316)	6.808 (0.144)	6.884 (0.232)	0.457 (0.006)	0.675 (0.014)*
CPWV	10.06 (0.190)	10.44 (0.113)	6.548 (0.235)	6.897 (0.115)*	0.513 (0.018)	0.602 (0.014)*
DDW	11.58 (0.378)	11.39 (0.569)	7.437 (0.174)	7.246 (0.333)	0.497 (0.084)	0.435 (0.046)
5%NaF	10.45 (0.158)	11.38 (0.231)*	6.653 (0.168)	7.126 (0.150)*	0.523 (0.078)	1.131 (0.073)*

The asterisks indicate statistically significant difference of At% between the demineralised and remineralised dentine in each group (two-way ANOVA, Bonferroni *post hoc* test, $\alpha=0.05$).

3.3.4 Cross-sectional Vickers microhardness

The cross-sectional VHN of RCLs were selectively increased by the treatment agents. The VHN of lesion areas reached normal dentine values at a depth of approximately 150 μm . The VHN of remineralised dentine was slightly increased at comparative depths of demineralised dentine from less than 15 μm to 100 μm in most groups ($p > 0.05$). Specifically, after treatment with Duraphat™, MIV or CPWV, the VHN of demineralised dentine did not significantly increase at all lesion depths (Figure 3.9 H, I and K). Combined use of MIV with PA increased VHN of demineralised dentine up to 20 μm (Figure 3.9 F) ($p < 0.05$). Significant increase in dentine surface (less than 15 μm depth areas) microhardness was observed after application of

MIV (Figure 3.9 C) and CPWV (Figure 3.9 D) followed by pH-cycling with 5,000 ppm FTP ($p < 0.05$). With adjunctive application of both PA and 5,000 ppm FTP, treatment with either MIV or CPWV was associated with increase of VHN in RCLs up to 30 μm depth ($p < 0.05$) (Figure 3.9 A-B).

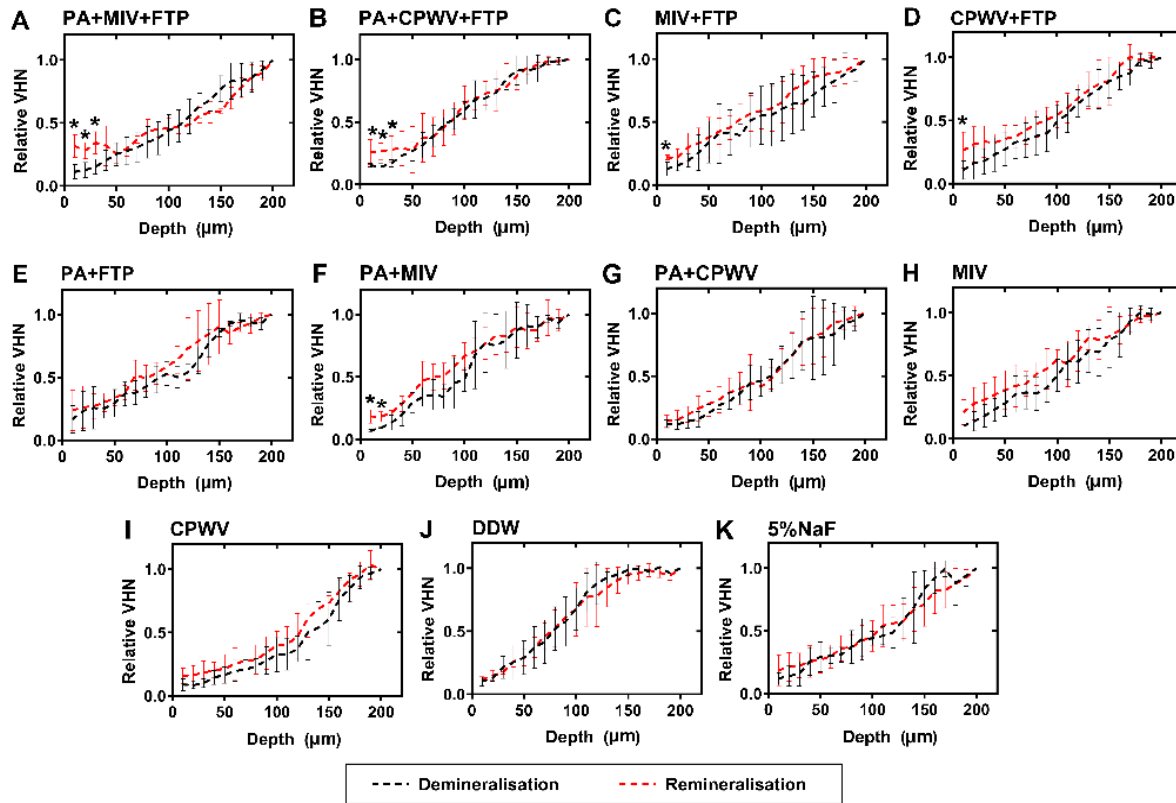


Figure 3.9 (A-K) Vickers microhardness numbers of the demineralised and remineralised regions of dentine from less than 15 μm to 200 μm depth from the lesion surface. Values are expressed as mean $\pm$ standard deviation. The asterisks indicate statistically significant difference of VHN between the demineralised and remineralised areas of a sample at the same depth (Student's t test, $\alpha=0.05$).

3.4 Discussion

In the present study, the remineralising effects of MIV, CPWV and high concentration (5,000 ppm) FTP on artificial RCLs were investigated. PA was used as a cross-linking agent which stabilised the demineralised dentinal collagen when used as an adjunct to remineralisation agents. The remineralising effects of varnishes containing F on RCLs were assessed after termination of the pH-cycling using PLM, micro-CT, Vickers microhardness testing as well as SEM-EDS. Results indicated that application of MIV or CPWV with 5,000 ppm FTP was able to incorporate Ca^{2+} , PO_4^{3-} and F^- into the RCLs, resulting in an increased integrated mineral

content throughout the lesion and increased surface microhardness (less than 15 μm) after 8 d of pH-cycling. The conjunctive application of PA with MIV, CPWV and FTP further enhanced uptake of remineralising ions, MD and cross-sectional microhardness recovery, especially in the subsurface region of RCLs. Nevertheless, further studies were warranted to investigate the clinical significance of the increase in mechanical properties and mineral content of RCLs after these treatments.

The *ex vivo* artificial RCL model created by a demineralising solution has high reproducibility under controlled conditions compared to natural RCLs. Nevertheless, root dentine is a material of structural anisotropy with nonidentical physicochemical characteristics in different regions because of various tubule densities and orientations and ratios of organic/inorganic components [483, 484]. In order to minimise these variations, half of the demineralised root dentine in each specimen was kept for comparison as baseline demineralisation. Under PLM, sound dentine is positively birefringent when imbibed in water due to a superimposition of strong positively oriented birefringence of the collagen matrix and weaker negative birefringence of oriented sub-microscopic dentinal mineral crystals. Demineralisation led to the putative loss of weak negative birefringent crystals, which relatively increase the strength of the positive birefringence. The subsurface lesion depths created were therefore easily recognised. The demineralisation solution diffused into the matrix and gradually dissolved the mineral components of the dentine leading to a gradient decrease of Ca and P concentration, gradually reducing MD as well as significantly lowering the microhardness from the lesion surface to the sound dentine area. Specifically, micro-CT profiles in this study show a demineralised shoulder in all samples, attributed to the different rates of demineralisation of intertubular and peritubular dentine [485].

Fluoride deposits a layer with relatively high MD on the RCL surface, which is reported to be a 'CaF₂-like' material [128, 486]. An increased concentration of F throughout the RCLs indicated that MIV and CPWV effectively introduced extrinsic F into the root carious lesion body. Fluoride is reported to be able to incorporate minerals into dentine through partial ionic substitution for hydroxyl ions in HAp and resulted in covalent Ca-F bonds and/or hydrogen OH-F bonds, thus potentially forming HAp, FHAp or FAp [6]. The increased MD suggested new minerals may have been formed which blocked the diffusion channels in the carious lesion and increased the acid resistance of the dentine. However, the unit cell volume of dentine HAp as well as the crystalline packing and mineral-matrix interactions may not have been

significantly altered, as hardness and birefringence showed no significant changes. Although the viscosity and adhesion of FV enables it to localise on the dentine surface, F penetration into dentine may be limited due to poor initial F release before the FV is lost from the tooth surface, which has been reported to be limited to 5 μm for Duraphat™ [487]. Nevertheless, MD and elemental concentration increase in the present study were observed in the subsurface areas, indicating that remnant FV ‘tags’ in dentine may release bioactive agents (F) over time.

Calcium phosphate salts normally have low water solubility and react readily with F when in solution, therefore, MIV and CPWV used in this study utilise different techniques to keep Ca-P in a bioavailable status and separate from F, enabling a diffusion concentration gradient to promote mineral formation in carious lesions. Specifically, MIV contains CPP-ACP which stabilises ionic calcium and phosphate in a metastable solution thus increasing the solubility of Ca-P. Casein phosphopeptides bind to the (100) and (010) faces of apatite crystals to form more thermodynamically stable crystals [488]. For CPWV, TCP was based on the crystalline technology which prevents a reaction between Ca^{2+} and F^- by a protective fumaric acid barrier that breaks down in an aqueous environment (saliva) releasing Ca^{2+} and PO_4^{3-} ions. Results from the present study indicated that MIV and CPWV introduced Ca^{2+} and PO_4^{3-} into the lesion body and an incremental increase of MD was achieved compared to the control, although MD differed between MIV and CPWV. Whilst both varnishes introduced F in demineralised dentine compared to the DDW treatment, comparatively lower incremental increase of F was detected in the RCLs treated with CPWV than MIV. This was consistent with previous studies in sound and demineralised enamel, where a varnish containing ACP promoted significantly more surface F deposition than the varnish containing functionalised TCP [489]. The reason may be ascribed to the addition of Ca-P that influenced the availability of F^- [169], as once Ca-P exposed to F in aqueous environment, they may reprecipitate quickly on the surface. Aggregation of CPP with Ca^{2+} and PO_4^{3-} forms clusters of ACP which prevents the precipitation of Ca^{2+} and PO_4^{3-} with F^- , enabling a high level release of available Ca^{2+} and PO_4^{3-} , whilst TCP, stabilised by solid state method might precipitate faster once exposed to aqueous environment, depleting ions from the lesion pores [445]. Moreover, the CPP-ACP complexes have a higher wettability than TCP and this improves the contact of CPP-ACP with tooth surfaces, forming a gradient to maintain a state of supersaturation with respect to HAp thus inhibiting the demineralisation of the underlying dentine. Nevertheless, both MIV and CPWV failed to improve the microhardness of RCLs.

The benefits of combined application of varnishes and toothpastes for the treatments of RCLs compared to the use of either product alone were also indicated, as either MIV or CPWV along with 5000 ppm FTP reversed the birefringence of demineralised dentine being similar to sound dentine, significantly improved the surface hardness of RCLs and the MD was further increased throughout the lesion. This was in accordance to the clinical recommendation for the use of FTP after MIV or CPWV to maintain the presence of F because varnishes may not persist for long in the oral environment due to mastication, oral hygiene procedures, soft tissue movement and salivary flow [490]. It has been speculated that the high concentration F from varnishes ‘pulsed’ after treatment with dentifrices, which might facilitate secondary nucleation of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_{1-x}\text{F}_x$ or other intermediate phases of calcium phosphate compounds before the formation of a thermodynamically more stable HAp, FHAp or FAp phase [491].

The mode of dentine remineralisation was revealed from the post-treatment micro-CT profiles as well as the PLM images of PA+FTP-treated group, where mineral gain originated both from precipitation of ions from remineralising agents and putatively dissolved mineral ions from dentine diffusing outward leading to reprecipitation. However, in varnish-treated groups, such ‘laminated zones’ were not as obvious, putatively because of the lower viscosity and comparatively greater solubility of FTP compared to varnishes leading to faster diffusion into the RCLs. The surface mineralised layer was not as prominent as what occurs in enamel remineralisation, although surface MD increase varied after different treatments, presumably because the outer layers of dentine had been exposed longer than the inner layers and affected more by acids. Therefore, the outer layers contained fewer residual crystals that could act as templates for remineralisation [492]. Also, the residual crystals may be blocked by the relatively large amount of demineralised organic matrix components, inhibiting their contact with the extrinsic remineralising ions. However, with less mineral loss, the inner zone at the base of the lesion was more readily remineralisable and fully recovered its properties at a rapid rate [240].

The concept of dentine biomineralisation recognises the critical roles of the collagen matrix in orchestrating mineralisation and provides new insights into novel approaches for dentine remineralisation. In light of this, PA, an exogenous collagen cross-linker, was used in the present study to strengthen the dentinal collagen matrix, which could aid the ability of remineralising ions to form mineralised matrix [481]. Application of PA tended to aggregate and cross-link the collagen fibrils and reduced interfibrillar spaces which would result in

decreased porosity of type I collagen, as suggested in the PLM images by the formation of the dark band in all PA-treated dentine. Although PA alone cannot enhance intrafibrillar mineralisation, the hydroxyphenyl groups in PA may act as ligands to bind Ca^{2+} [493]. The formation of calcium-PA complexes promotes mineral deposition and attracts more calcium phosphate onto the PA-modified collagen fibrils [279], as indicated by the increased At% of Ca, P and F and highest MD throughout the RCLs after pH-cycling compared to non-PA treatment methods. Especially, significant mineral content gain was achieved in the subsurface areas. Results in the present study indicated the active role of collagen for dentine remineralisation which follows a different mechanism from the classical crystallisation of enamel. On one hand, type I collagen in dentine could play an active role in intrafibrillar mineralisation by functioning as a template for attracting and controlling the infiltration of Ca-P mineralisation precursors and providing a spatial and hierarchical control for mineralisation occurring in a confined reaction environment [234]. Specifically, for intrafibrillar remineralisation which is the determinant for mechanical properties of dentine, the penetration and uptake of Ca^{2+} and PO_4^{3-} into dentine mainly relies on diffusion in the gap zones and intermolecular spaces of dentinal collagen *via* capillary forces and osmotic-regulated mechanisms as well as electroneutrality (CaHPO_4) [488, 494]. On the other hand, NCPs which mediate apatite nucleation need to be attached to a substrate (collagen matrix) to take effect [477].

Increased mineral content is not an appropriate endpoint of assessing the success or failure of the remineralisation effects of bioactive materials, as it was not necessarily translated into a recovery of mechanical properties. Whilst remineralisation may occur preferentially in the extra-fibrillar zone [495], the mineral content in the intrafibrillar compartments plays a more determinant role in the mechanical properties of dentine [496]. Results in the present study indicate that whilst combined use of MIV or CPWV with 5,000 ppm FTP significantly increased surface hardness (less than 15 μm depth), using PA as an adjunct significantly improved the subsurface hardness up to 30 μm . On one hand, application of PA cross-linked dentine collagen and provided a scaffold for mineral deposition into deeper lesion areas, which promoted newly formed minerals in the subsurface zone and contributed to an increase of VHN in deeper lesion areas. On the other hand, resistance of indentation force of dentine is strongly dependent on the interfacial interaction between collagen and the mineral phase [497]. Simultaneously strengthening the dentinal matrix when applying the remineralising agents might prevent the partially demineralised subsurface from further mineral loss and collagen

collapse as well as promote the formation of a more well-structured organic-inorganic interaction for stress redistribution during loading in microhardness testing. However, it was noted the hardness values had high variability. The size of the indentation depends highly on the organic and water content and degree of hydration of the tissue. Also, the orientation of the crystal platelets, *i.e.*, whether they are edge-on or face-on to the indenter influences the hardness, even though each indentation volume may include millions of crystals [483]. In an attempt to minimise the effect of the heterogeneity of dentine, the Vickers microhardness test which employed a symmetrical pyramid-shaped diamond indenter close to being isotropic was used in this study. Generally, the conversion of CaF_2 -like materials deposited on lesion surfaces to incorporated FAp for the recovery of hardness is a very slow process, which has been reported to take 2 - 4 weeks, at body temperature and neutral pH [240]. Therefore, in the present study, after 8 d pH-cycling, microhardness only selectively increased to a limited depth.

Several aspects in the present laboratory study were not the same as in clinical scenario which should be considered. Firstly, the time-frame of the present study was short (8 d pH-cycling) whilst partial remineralisation can take more than 200 days in the oral environment [445]. Significant differences between groups may have become apparent if the lesions had been followed for longer. In addition, only one demineralisation phase daily was used to simulate the oral environment, which meant results from the present study were more focused on remineralisation, whilst the reaction product formation during ‘upward and downward swings’ in pH and effects on demineralisation resistance might not be pronounced. Particularly, ion release from CPP-ACP could be significantly enhanced in an acidic environment [498]. Secondly, FVs were not removed before pH-cycling in order to protect the remineralising surface. It was observed in the experiment that ‘adhesive properties’ of the varnishes varied, thus varnishes with inferior adhesive properties may detach from the dentine surface and reduce the effectiveness, which partly contributed to the large range of some results [169]. Also, varnishes can release F into the surrounding medium in the oral environment to contact the areas not covered by varnishes during the application phase - not evaluated in the present study. Moreover, whilst the demineralisation solution used in the present study contributed to certain characteristics of baseline carious lesions, in the oral environment, root caries could vary due to the lesion activity status. Remineralising studies should be carried out in lesions with various degrees of MD and lesion depths, and the effects of saliva, dental plaque and enzymes should be considered in the de-/re-mineralisation process before conclusions regarding treatment efficacy of F regimens with collagen cross-linking agents can be drawn.

3.5 Conclusion

Within the limitations of this laboratory study, in a simulated net-remineralisation oral environment, application of DuraphatTM introduced F⁻ into RCLs and slightly increased the integrated mineral content. Fluoride varnishes containing Ca-P (CPWV and MIV) introduced F⁻ and possibly Ca²⁺ and PO₄³⁻ into RCLs and further increased MD but had no significant effects on microhardness. The surface (less than 15 µm) hardness of RCLs was significantly increased when using MIV or CPWV in conjunction with 5,000 ppm FTP and the mineral content throughout the artificial RCLs was increased. The birefringence of the test RCLs areas was mainly reversed to similar to sound dentine. Using PA as an adjunct with F regimens improved the MD significantly, especially in the subsurface areas. Pre-treating dentine with PA before application of CPWV or MIV along with 5,000 ppm FTP achieved the highest ion uptake and MD gain compared to the other treatment approaches. A significant increase of cross-sectional microhardness was observed up to 30 µm depth. Generally, MIV containing CPP-ACP was associated with a higher mineral content increase than CPWV containing TCP.

Acknowledgements

This work was supported by Australian Dental Research Foundation (ADRF) Nathan Cochrane Memorial Grant and ADRF grant No. 326-2018.

3.6 Supplementary information

Figure S3.1 Concentration of calcium (Ca), phosphorus (P) and fluorine (F) on the cross-sectional surface of sound dentine analysed by energy dispersive X-ray analysis.

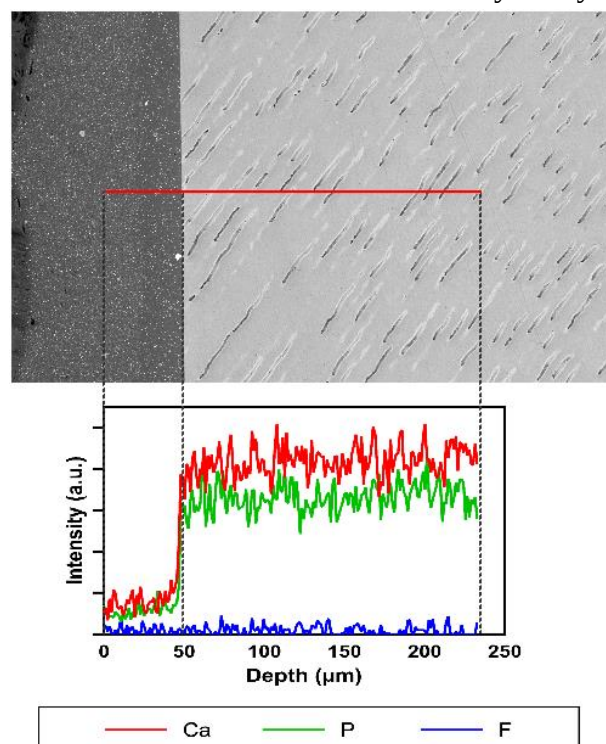


Table S3.1 Demineralised lesion depth measured under the polarised light microscopy.

Groups	Demineralised lesion depth (μm)		
	1	2	3
PA+MIV+FTP	127.2	160.4	149.3
PA+CPWV+FTP	136.6	132.5	140.3
MIV+FTP	197.2	123.9	162.1
CPWV+FTP	194.8	153.6	143.5
PA+FTP	170.8	157.8	141.3
PA+MIV	153.6	163.4	170.4
PA+CPWV	132.4	124.3	164.7
MIV	138	167	126.4
CPWV	139.6	135.6	217.8
DDW	185.2	174.6	168.2
5%NaF	127.2	160.4	149.3
Mean ± standard deviation	155.1 ± 23.5		

Table S3.2 Integrated mineral content changes of initial demineralised root dentine after each experimental phase from ~30 μm to 250 μm depth with 30 μm increments (a 40 μm increase in the last layer).

Depth	AUD (Demin)	AUD (Remin)	AUD (Sound)	ΔZd	ΔZr	Mineral gain ($\Delta\text{Zd}-\Delta\text{Zr}$)	Relative mineral gain (R%)
PA+MIV+FTP							
0-30	1.010	1.030	31.519	30.509	30.488	0.020	0.016
30-60	1.064	1.042	30.658	29.594	29.617	-0.023	-0.018
60-90	2.108	4.646	28.975	26.867	24.329	2.538	1.992
90-120	10.130	18.212	30.091	19.961	11.878	8.083	6.345
120-150	15.052	23.090	30.728	15.677	7.638	8.039	6.310
150-180	30.692	35.494	33.549	2.857	-1.945	4.802	3.769
180-210	33.465	35.424	31.783	-1.682	-3.641	1.959	1.538
210-250	46.738	48.850	44.090	-2.648	-4.760	2.111	1.657
PA+CPWV+FTP							
0-30	1.271	1.168	34.930	33.659	33.762	-0.103	-0.067
30-60	1.334	0.947	34.916	33.583	33.969	-0.387	-0.253
60-90	4.273	7.247	32.091	27.818	24.844	2.973	1.947
90-120	10.455	12.722	31.306	20.852	18.585	2.267	1.484
120-150	12.687	17.504	29.512	16.825	12.008	4.816	3.153
150-180	22.232	29.771	32.887	10.655	3.115	7.539	4.935
180-210	27.120	31.468	28.586	1.466	-2.882	4.348	2.846
210-250	40.687	43.763	39.239	-1.448	-4.525	3.077	2.014
MIV+FTP							
0-30	1.094	0.960	33.625	32.531	32.665	-0.134	-0.108
30-60	1.106	0.944	32.939	31.834	31.995	-0.161	-0.130
60-90	2.524	5.455	29.629	27.105	24.174	2.931	2.352
90-120	9.743	19.934	29.397	19.654	9.463	10.191	8.177
120-150	18.023	27.347	29.138	11.115	1.791	9.324	7.482
150-180	30.887	32.444	30.968	0.080	-1.476	1.556	1.249
180-210	31.260	30.850	28.870	-2.391	-1.981	-0.410	-0.329
210-250	42.650	42.436	39.972	-2.678	-2.464	-0.214	-0.172
CPWV+FTP							
0-30	1.282	1.307	36.074	34.791	34.766	0.025	0.017
30-60	1.438	1.444	36.240	34.802	34.796	0.006	0.004
60-90	1.497	3.667	33.722	32.225	30.056	2.170	1.436
90-120	9.928	14.587	34.063	24.135	19.476	4.658	3.083
120-150	17.756	21.059	33.047	15.291	11.988	3.303	2.186
150-180	30.302	32.097	34.364	4.062	2.267	1.796	1.189
180-210	31.565	33.798	31.328	-0.237	-2.470	2.233	1.478
210-250	45.325	46.823	42.204	-3.121	-4.619	1.498	0.991
PA+FTP							
0-30	1.240	1.164	35.701	34.461	34.537	-0.076	-0.042
30-60	1.277	1.146	35.754	34.477	34.609	-0.132	-0.072
60-90	4.235	6.869	32.244	28.009	25.375	2.634	1.443

90-120	13.339	16.784	31.659	18.320	14.875	3.445	1.888
120-150	21.122	23.262	30.837	9.716	7.575	2.141	1.173
150-180	32.195	32.154	32.922	0.726	0.768	-0.042	-0.023
180-210	31.534	31.301	30.298	-1.236	-1.003	-0.233	-0.128
210-250	42.148	42.552	41.604	-0.544	-0.948	0.404	0.221
PA+MIV							
0-30	0.197	0.144	36.069	35.873	35.926	-0.053	-0.030
30-60	0.556	0.152	36.182	35.625	36.030	-0.404	-0.227
60-90	1.532	2.385	33.170	31.638	30.785	0.853	0.480
90-120	7.244	10.572	31.904	24.660	21.332	3.328	1.871
120-150	9.553	14.174	31.105	21.551	16.931	4.620	2.598
150-180	17.324	21.265	32.441	15.117	11.177	3.941	2.216
180-210	24.059	26.768	29.263	5.204	2.496	2.709	1.523
210-250	40.854	42.001	39.791	-1.063	-2.209	1.146	0.645
PA+CPWV							
0-30	1.591	1.351	39.406	37.815	38.055	-0.240	-0.127
30-60	1.439	1.338	38.016	36.577	36.678	-0.101	-0.053
60-90	2.323	3.179	34.027	31.704	30.847	0.857	0.453
90-120	8.606	10.327	33.590	24.984	23.263	1.721	0.911
120-150	10.961	15.450	32.698	21.737	17.248	4.489	2.375
150-180	19.822	24.901	34.581	14.759	9.679	5.080	2.687
180-210	24.687	27.156	31.716	7.030	4.560	2.470	1.306
210-250	40.245	43.987	43.506	3.261	-0.481	3.743	1.980
MIV							
0-30	1.506	1.574	34.468	32.963	32.894	0.069	0.048
30-60	1.375	1.562	34.776	33.401	33.214	0.187	0.131
60-90	2.070	5.339	31.913	29.844	26.574	3.269	2.291
90-120	10.142	13.314	31.262	21.120	17.948	3.172	2.222
120-150	14.795	20.978	30.750	15.955	9.772	6.183	4.332
150-180	27.277	29.079	32.432	5.155	3.353	1.802	1.262
180-210	31.404	29.181	29.926	-1.478	0.745	-2.223	-1.558
210-250	42.728	42.078	40.583	-2.145	-1.495	-0.649	-0.455
CPWV							
0-30	1.257	1.221	34.708	33.451	33.487	-0.036	-0.027
30-60	1.189	1.181	34.637	33.448	33.456	-0.008	-0.006
60-90	2.394	6.082	31.726	29.332	25.643	3.689	2.812
90-120	12.451	16.078	31.595	19.144	15.517	3.627	2.764
120-150	18.986	22.756	31.093	12.107	8.338	3.770	2.873
150-180	30.859	31.305	31.967	1.108	0.663	0.445	0.339
180-210	34.482	34.287	32.494	-1.988	-1.793	-0.196	-0.149
210-250	44.654	44.438	41.700	-2.955	-2.739	-0.216	-0.165
DDW							
0-30	1.425	1.378	34.804	33.379	33.426	-0.047	-0.036
30-60	1.415	1.388	34.829	33.414	33.441	-0.027	-0.021
60-90	1.868	1.395	32.484	30.615	31.089	-0.474	-0.361
90-120	9.606	9.246	32.704	23.098	23.458	-0.360	-0.274
120-150	11.605	16.781	32.791	21.185	16.010	5.176	3.941
150-180	17.825	23.370	35.390	17.566	12.020	5.545	4.222
180-210	24.674	29.231	32.736	8.062	3.505	4.556	3.469

210-250	41.426	44.385	45.190	3.764	0.805	2.959	2.253
5%NaF							
0-30	1.087	1.114	39.797	38.710	38.683	0.027	0.018
30-60	1.077	1.096	37.345	36.268	36.249	0.019	0.012
60-90	1.246	3.193	33.697	32.451	30.504	1.947	1.256
90-120	13.971	17.305	32.893	18.922	15.589	3.334	2.151
120-150	18.914	21.338	32.533	13.619	11.195	2.424	1.564
150-180	27.990	30.922	34.526	6.536	3.604	2.932	1.892
180-210	31.526	31.724	31.774	0.248	0.051	0.197	0.127
210-250	44.335	44.440	43.232	1.103	1.207	0.104	0.095

Chapter 4

Evaluation of adhesion and interfacial interaction of HVGIC, RMGIC and CPP-ACP-modified GIC to sound and demineralised root dentine with proanthocyanidin pre-treatment

4.1 Introduction

Root caries is rapidly gaining wide attention as a potential public health problem because of improved tooth longevity, retention and age-associated gingival recession exposing caries-prone root surfaces to the oral environment and supra-gingival biofilm [13]. Restorative treatment of root caries has higher failure rates than that for coronal caries and is particularly challenging due to the more complicated nature of root dentine structure and composition as well as the potentially complex clinical conditions encountered for restorative procedures [499, 500].

Dentine is a hierarchically organised bio-composite composed of a structured organic matrix impregnated with matrix-mediated carbonated HAp [22]. Restorative materials applied to dentine can penetrate into and form complex nano-structural interactions with the dentinal organic matrix [20]. In particular, in light of the tenets of minimally invasive carious tissue removal, the bacterially contaminated and denatured dentine should be removed, whilst the demineralised but remineralisable dentine should be preserved [501]. Therefore, restorative materials are frequently placed on partially demineralised hard tissues and this may pose challenges in restorative treatments. Specifically, demineralised dentine (DD) has lower elastic modulus than sound dentine (SD), resulting in decreased bond strength of restorations [17]. Moreover, demineralised dentinal collagen collapses easily because of dehydration, imposing difficulties in the infiltration of restorative materials into the dentine matrix when a hybrid layer is needed to create the bonded interface. The resultant exposed collagen is fragile and prone to hydrolytic and chemical degradation in the long-term, thus causing a deterioration in the quality of the bond [18]. Nevertheless, the remineralisable dentine remains structurally intact and the dentinal collagen undergoes a reversible alteration with decreased intermolecular cross-links whilst it retains its amino acid composition [501, 502]. In this regard, application of cross-link promoting agents on dentinal collagen seems promising in reinforcing the dentinal collagen and thus enhancing the penetration of restorative materials into the dentine matrix for a more stable bonding interface and improved bond strength in restorative treatments.

A number of previous studies have indicated application of synthetic cross-linking agents such as GA and EDC/NHS on dentine was able to induce the formation of exogenous inter- and intra-molecular dentinal collagen cross-links. When pre-treating the dentine surface for bonding, synthetic cross-linking agents could strengthen the dentine matrix, thus reducing collagenase degradation and nano leakage in the bonding interface and improving bond

strengths. However, the clinical applicability of these synthetic cross-linkers may be limited because of their potential biotoxicity [503, 504]. Recently, biocompatible naturally-derived cross-linking agents such as PA have gained increasing interest for their ability to interact with and modify the physicochemical properties of dentinal collagen [421]. When involved in resin-dentine bonding, PA improved the mechanical properties and enzymatic resistance of the dentine matrix, contributing to a more stable interface between dentine and restorative materials [505, 506].

Advances in restorative materials, especially polymer-based resin materials in recent years, have tremendously improved restoration performance. However, application of resin composite would still be problematic in restoring root dentine caries [507]. Specifically, RCLs can extend into subgingival areas, thus creating difficulties in moisture control for resin application. Good isolation of bonding areas from oral fluids could be even more difficult to achieve in elderly patients who have limited mobility for cooperation during treatments [13]. In addition, the elder population frequently suffers from hyposalivation due to aging and medications, increasing the susceptibility for carious lesion development around restorations and on other root surfaces [14]. In this regard, GICs, with a tolerance to moisture, good chemical adhesion to tooth structure and anti-cariogenic properties, might be the material of choice for root caries restorations [508, 509]. In particular, the introduction of HVGIC and RMGIC endows GIC with higher mechanical strength and wear resistance in addition to the CPP-ACP-modified GIC that shows enhanced ion-releasing abilities for caries prevention especially in populations with increased caries risk [510-512].

Adhesion of GIC to dentine depends predominantly on the chemical bonding resulting from the initial hydrogen bonds gradually replaced by genuine ionic bonds formed between carboxylate groups ($-\text{COOH}$) in PAA molecules and Ca^{2+} in HAp as well as the following ion diffusion process between dentine and GIC forming an ion-exchange layer [513]. Moreover, the micromechanical interlocking arising from the cement tags within dentinal tubules or hybridising with carbonated HAp-coated dentinal collagen also contributes to the adhesion [514, 515]. Being hydrophilic, GIC is able to form durable adhesive bonds to dentine without additional pre-treatments of tooth surfaces [516]. However, in a restorative process, dental instrumentation prior to bonding often results in a 1-2 μm thick smear layer composed of a mineral phase and other debris embedded in denatured collagen attached to the underlying dentine surface. Bonding to this irregular contaminant results in a weak link at the dentine-GIC

interface [517, 518]. Therefore, dentine surface conditioning has been recommended before bonding to remove the smear layer. Polyacrylic acid is the most frequently used conditioner, which has a high molecular weight and contains a multiplicity of functional groups capable of hydrogen bonding with dentine [519]. However, PAA conditioning can lead to partially demineralised subsurface zones beneath the intermediate layer between dentine and GIC if aggressive protocols such as high concentrations or long application times are used [519]. With similar chemical characteristics to PAA, TA has also been used as a surface conditioner, which partially removes the smear layer and interacts with dentinal collagen [520]. Being a condensed tannin, PA are acidic in nature and able to dissolve dentine minerals [70]. Therefore, PA are potentially applicable as a dentine conditioner, that removes the smear layer and simultaneously cross-links the dentinal collagen. Particularly, when GIC is bonded to DD, PA conditioning could strengthen and stabilise the demineralised dentinal matrix which may potentially serve as a scaffold for the chemical interaction and cement penetration of GIC into the collagen network of the DD.

There has been no published study that has comprehensively evaluated and compared the bonding performance of different types of GIC to both sound and demineralised root dentine. In addition, despite improved adhesion when cross-linking agents were used for resin composite restorations in laboratory studies, the effects of a cross-linked dentinal matrix on GIC bonding has not been explored [521, 522]. Therefore, the aim of this study was to evaluate the adhesion and interfacial interaction with different conditioning regimes of three GICs - HVGIC, RMGIC and CPP-ACP-modified GIC, to sound and laboratory demineralised root dentine. In particular, the effect of PA used as a conditioner for GIC bonding on root dentine was investigated.

4.2 Materials and methods

4.2.1 Materials

Materials used were proanthocyanidin (International Laboratory, CA, USA; containing >95% oligomeric proanthocyanidins); HVGIC (Fuji IX GP EXTRA; GC Corp., Tokyo, Japan); light-cured RMGIC (Fuji II LC capsule shade A2; GC Corp., Tokyo, Japan); 3% CPP-ACP (RecaldentTM)-modified GIC (Fuji VII; GC Corp., Tokyo, Japan); Cavity Conditioner (GC Corp, Tokyo, Japan). The demineralising solution used to induce artificially demineralised root

dentine contained 50 mM acetic acid, 2.2 mM CaCl₂ and 2.2 mM KH₂PO₄, and the pH of the solution was adjusted to 5 with KOH. Details of the materials used are listed in Table 4.1.

Table 4.1 Materials used in the study in Chapter 4.

Materials	Classification	Manufacturer	Batch number (#)	Composition
Restorative materials				
Fuji II LC (FII)	Light-cured resin-modified glass ionomer cement (RMGIC)	GC Corporation, Tokyo, Japan	1802262	Powder: fluoro-alumino silicate glass. Liquid: PAA, HEMA, 2,2,4, trimethyl hexamethylene dicarbonate, TEGDMA.
Fuji VII EP (FVII)	CPP-ACP-modified GIC	GC Corporation, Tokyo, Japan	1803202	Powder: fluoro-alumino silicate glass, CPP-ACP. Liquid: PAA, polybasic carboxylic acid, distilled deionised water.
Fuji IX GP EXTRA (FIX)	High-viscosity GIC (HVGIC)	GC Corporation, Tokyo, Japan	1801231	Powder: aluminosilicate glass Liquid: PAA, distilled deionised water.
Conditioners				
Cavity Conditioner		GC Corporation, Tokyo, Japan	1702031	20% PAA with 3% aluminium chloride
6.5 w/v% PA		International Laboratory, CA, USA	781046	Oligomeric proanthocyanidins dissolved in DDW (pH=4.46 ± 0.01 measured by a pH metre (Eutech Instruments pH 700, Thermo Scientific, MA, USA)).
Abbreviations: CPP-ACP=casein phosphopeptide-amorphous calcium phosphate; HEMA=2-hydroxyethylmethacrylate; PAA=polyacrylic acid; TEGDMA=triethyleneglycoldimethacrylate.				

4.2.2 Preparation of root dentine specimens

Extracted human permanent molars from individuals aged 20 to 50 years were stored in 0.1% thymol solution at 4 °C under the protocol approved by the Human Research Ethics Committee of the University of Melbourne. The teeth were cleaned with DDW to remove surface debris. Non-carious molars without obvious defects on the root surface were selected. The crown and root apical portions were removed using a slow-speed diamond saw (Isomet, Buhler Ltd, IL, USA) under water irrigation. The remaining root portion was cut along the longitudinal axis to

obtain two dentine blocks from the cervical and middle parts of the root. Each block was then embedded in Type III dental stone (Yellowstone, Ainsworth Dental Co., NSW, Australia) using a cylindrical Teflon mould. After the setting of the dental stone, specimens were manually wet polished with 600-grit SiC paper to expose flat root dentine surfaces. Specimens were divided into two main groups: SD and DD. For the SD group, the smear layer was standardised by grinding with wet 600-grit SiC paper for 1 min. For the DD group, after the standard smear layer creation, each specimen was immersed in 20 mL of the demineralising solution (see 4.2.1) at 37 °C for 120 h. All specimens were rinsed thoroughly with DDW. From previous pilot study data, the depth of the demineralised layers created were approximately 150 to 180 µm.

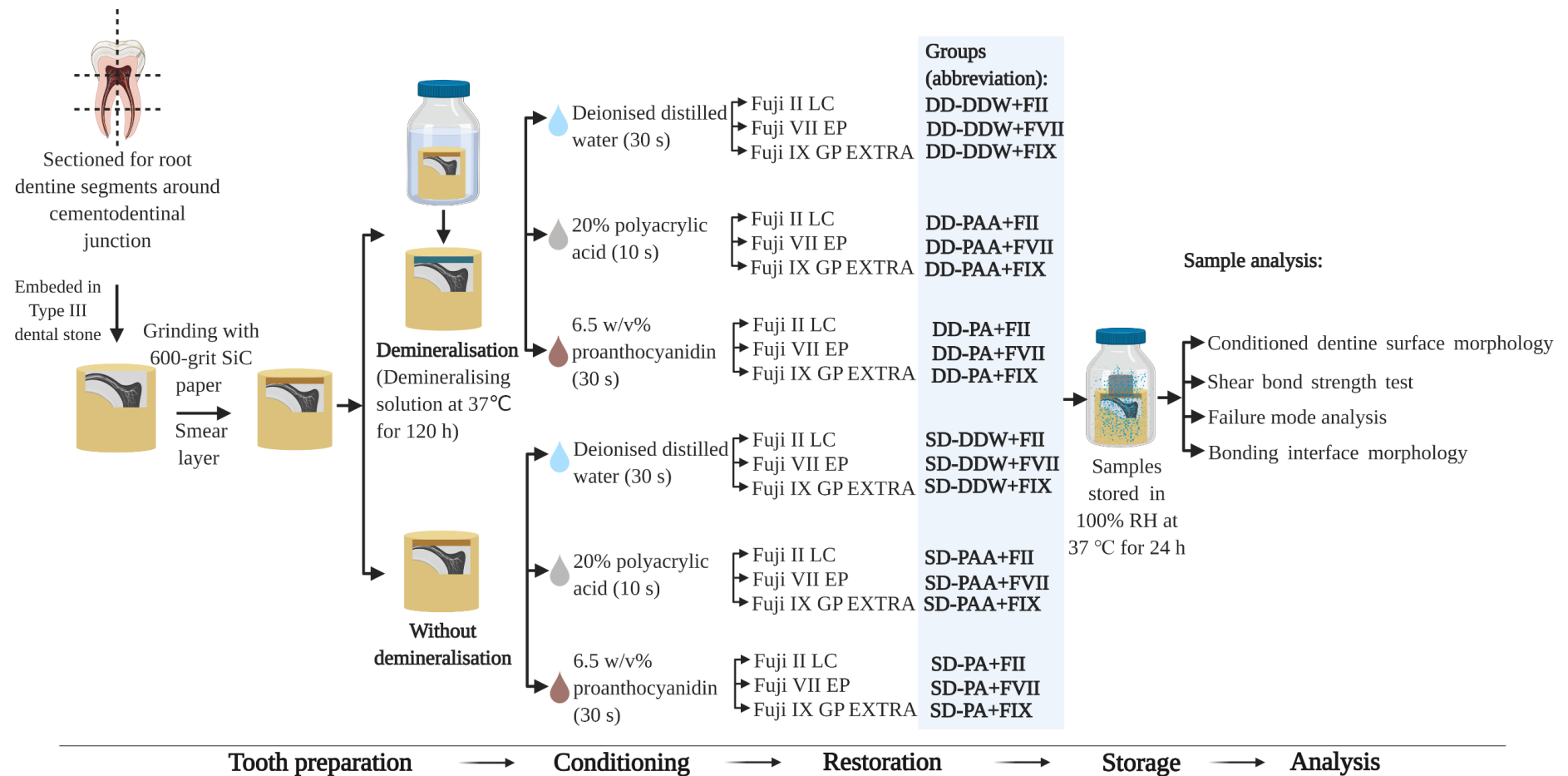


Figure 4.1 Schematic diagram of experimental design and groups in the study in Chapter 4.

4.2.3 Dentine pre-treatments and surface observation

Both SD and DD were divided into three groups which were conditioned with DDW, Cavity Conditioner or 6.5 w/v% PA solution, respectively (Table 4.1). Specifically, dentine treated with DDW had the DDW applied and agitated for 30 s using a micro brush which served as a negative control. Cavity Conditioner was applied to the dentine surface according to the manufacturer's instructions for 10 s using a disposable micro-brush and afterwards rinsed thoroughly with DDW and air-dried without desiccating the tooth. Proanthocyanidin solution was prepared by dissolving oligomeric PA in DDW and thoroughly mixed by pipetting up and down for more than 10 times to obtain a solution of 6.5 w/v% concentration. The 6.5 w/v% PA solution was applied onto the dentine surface with agitation for 30 s using a micro brush applicator, and then blot dried with a cotton pellet without rinsing.

To observe the conditioned-dentine surfaces, samples ($n = 2$ per group) were dehydrated using ascending concentrations of ethanol (30%, 50%, 75%, 95% and 100%, each 20 min), mounted on aluminium stubs with conductive tape, sputter-coated and imaged using scanning electron microscopy (SEM; Oxford instruments; voltage: 10.0 kV; working distance: 9.9-10.3 mm).

4.2.4 Shear bond strength testing

Dentine surfaces (either SD or DD) were conditioned in the same manner as in 4.2.3 and then divided into three subgroups according to the type of GICs used for bonding. Fifteen specimens of SD or DD with the same conditioner treatment (DDW, PAA or PA) were included in each subgroup. For Subgroup 1, Fuji II LC capsule (RMGIC) was mixed using a high-speed triturator (Ultramat 2; SDI Limited, VIC, Australia) for 10 s, applied to the dentine surface using a cylindrical mould (SDI limited, Victoria, Australia) 5 mm high and 3.5 mm diameter. The encapsulated GIC was syringed circumferentially from the periphery to the centre of the mould and then light-cured for 20 s using a high-density LED light curing unit (Radii-cal, SDI limited, VIC, Australia) at 1200 mW/cm² output. For Subgroup 2 and Subgroup 3, Fuji VII EP capsule (CPP-ACP-modified GIC) and Fuji IX GP EXTRA capsule (HVGIC) were mixed using the same high speed mixer for 10 s, applied to the dentine surface in the above-mentioned cylindrical mould, and then left for 6 min in 100% relative humidity (RH) at 37°C for setting without interference. Each bonded specimen was placed in a closed container separately with some damp tissues which have no direct contact with specimens in 100% RH at 37°C for 1 h for complete setting. After setting, DDW was added into the containers with damp tissues to a

level just below the bonding interface for specimen storage in 100% RH at 37 °C for 23 h before testing. The shear bond strength (SBS) testing was performed using a universal testing machine (Model 5544; INSTRON, MA, USA), where the shear force was applied at the junction between the GIC cylinder and the tooth surface using a loop wire parallel to the bonded interface. A crosshead speed of 0.5 mm/min was selected with a stroke length of 25 mm, peak force of 500 N. The SBS value is a function of stress distribution and concentration, determined by dividing the failure load by the total bonded area:

$$P = \frac{F}{\pi r^2}$$

where P is the SBS value, F is the load when debonding occurs and r is the radius of the cylindrical mould.

4.2.5 Failure mode analysis

After SBS testing, the de-bonded specimens were observed under a light microscope (DM2000, Leica Microsystems GmbH, Wetzlar, Germany) at 200× magnification, where failures were classified into one of the following three types, expressed as percentages:

- 1) Mixed: combinations of cohesive failure in GIC and interfacial failure along the dentine surface.
- 2) Interfacial: interfacial (adhesive) failure between dentine and GICs.
- 3) Cohesive: cohesive failure within the GIC.
- 4) Pre-test failure: specimens de-bonded before SBS testing.

4.2.6 Acid-base treatment technique

Specimens were conditioned and bonded in the same manner as in 4.2.4 except that GICs were bonded to root dentine surfaces directly without using the cylindrical mould. After storage in 100% RH at 37 °C for 24 h, bonded specimens were embedded in epoxy resin (KEP epoxy, Kemet International Ltd., Kent, UK), cured for 24 h and sectioned perpendicular to the bonding interface. The cross-sectioned surfaces were wet-polished with 1200-, 2400- and 4000-grit SiC paper and finished with 0.5 µm diamond suspensions (Type K, Kemet International Ltd., Kent, UK). Afterwards, the bonding interface was treated with 10% orthophosphoric acid for 10 s to remove the inorganic components of the dentine and 5% sodium hypochlorite (NaOCl) for 5 min to remove the organic components of the dentine, mounted on aluminium stubs, sputter-coated and observed using SEM.

4.2.7 Statistical analysis

The normal distribution of data was confirmed by the Shapiro-Walk test, and the equality of variances was assessed by the Levene's test. SBS data are expressed as the mean and standard deviation. Two-way ANOVA followed by Tukey's *post hoc* test was used to investigate the difference in SBS amongst specimens. Failure modes were analysed by the chi-square test. In all tests, statistical significance was set at $\alpha = 0.05$. Statistical analysis was conducted by using SPSS software (Version 22.0; IBM, NY, USA).

4.3 Results

4.3.1 Morphology of dentine surface with standard smear layer after conditioning

Alteration of the morphology of dentine surfaces after different conditioning was observed *via* SEM (Figure 4.2). Specifically, DDW-conditioned SD surfaces were grooved and covered with a homogeneous smear layer, where the dentinal tubules were occluded and just observable (Figure 4.2A). After PAA conditioning, the polishing grooves were markedly reduced, and PTD was observed. The dentinal tubules were opened to a certain extent with smear-plugs partially remaining (Figure 4.2B). PA conditioning reduced the polishing grooves slightly, and the dentinal tubules were observable to a considerable depth with smear-plugs (Figure 4.2C). Compared to SD, less smear layer was observed on DD surfaces, and dentinal tubules were opened widely with organic filaments being observed inside and dissolved PTD (Figure 4.2D). The DD surface was further demineralised with PAA conditioning, which exposed the dentinal collagen in the ITD areas (Figure 4.2E). The DD surface showed a regular and smoothed topography after PA conditioning (Figure 4.2F).

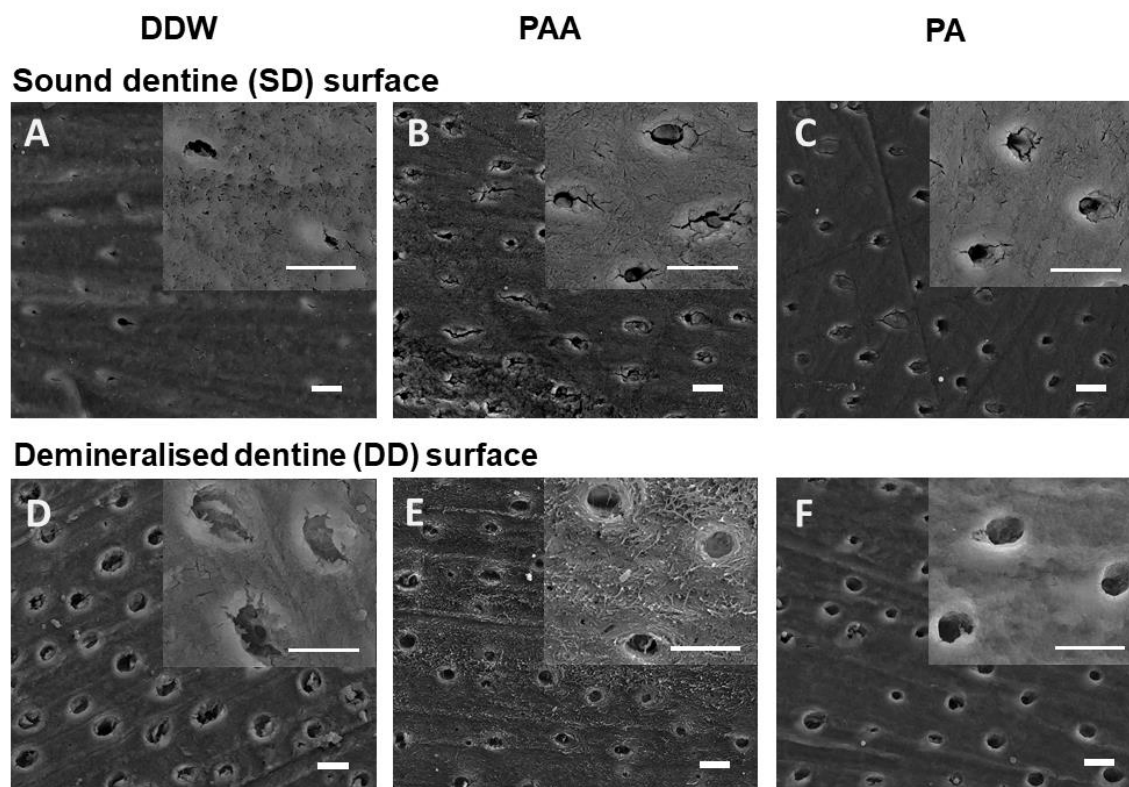


Figure 4.2 (A-F) Scanning electron microscopy images of sound and demineralised dentine surfaces with a standard smear layer after conditioning with distilled deionised water, polyacrylic acid or proanthocyanidin (Bar=5 μ m).

4.3.2 Shear bond strength testing

Shear bond strength tests were performed to evaluate the bond strength of three GICs on SD or DD with different conditioning. After 24 h storage in 100% RH, SD bonded with RMGIC showed the highest SBS value (8.44 ± 3.82 MPa) compared to that bonded to CPP-ACP-modified GIC (2.39 ± 0.94 MPa) or HVGIC (5.81 ± 1.92 MPa). Polyacrylic acid conditioning did not influence the SBS of GIC bonded to SD significantly ($P > 0.05$). Whilst PA conditioning did not influence the SBS of CPP-ACP-modified GIC and HVGIC bonded to SD ($P > 0.05$), it decreased the SBS of RMGIC to SD significantly compared to DDW conditioning ($P = 0.0013$).

The bond strength significantly decreased when GIC was placed on DD compared to SD except for CPP-ACP-modified GIC, which showed comparable bond strength to either SD or DD. Polyacrylic acid conditioning improved the bond strength of GIC on DD, especially for HVGIC where the SBS increased to a value comparable to that of HVGIC on SD. Using PA as a

conditioner did not influence the SBS significantly compared to DDW conditioning in all the three GICs tested, although a slight decrease in SBS was observed.

Table 4.2 Shear bond strength (MPa) values (mean $\pm$ standard deviation) of Fuji II LC, Fuji VII EP and Fuji IX GP EXTRA bonded to SD and DD with different conditioning after 24 h storage in 100% relative humidity.

Sub group	Dentine substrate	Treatment	SBS (mean $\pm$ standard deviation) (MPa)	Failure Mode (%) (Number of specimens)					P-value
				Interfacial	Mixed	Cohesive	Pre-test failure	Total	
Sub group 1		DDW+FII	8.44 $\pm$ 3.82 ^A	40.0 (6)	46.7 (7)	6.7 (1)	6.7 (1)	100 (15)	0.017
	SD	PAA+FII	9.31 $\pm$ 4.30 ^A	0 (0)	53.3 (8)	40.0 (6)	6.7 (1)	100 (15)	
		PA+FII	1.53 $\pm$ 1.82 ^B	33.3 (5)	53.3 (8)	0 (0)	13.3 (2)	100 (15)	
		DDW+FII	1.72 $\pm$ 0.72 ^B	40.0 (6)	33.3 (5)	0 (0)	26.7 (4)	100 (15)	0.660
	DD	PAA+FII	2.05 $\pm$ 2.18 ^B	60.0 (9)	20.0 (3)	0 (0)	20.0 (3)	100 (15)	
		PA+FII	0.71 $\pm$ 0.35 ^B	33.3 (5)	33.3 (5)	0 (0)	33.3 (5)	100 (15)	
Sub group 2		DDW+FVII	2.39 $\pm$ 0.94 ^{AB}	13.3 (2)	13.3 (2)	73.3 (11)	0 (0)	100 (15)	<0.001
	SD	PAA+ FVII	2.76 $\pm$ 1.22 ^A	0 (0)	26.7 (4)	73.3 (11)	0 (0)	100 (15)	
		PA+FVII	1.83 $\pm$ 0.75 ^{BD}	13.3 (2)	73.4 (11)	0 (0)	13.3 (2)	100 (15)	
	DD	DDW+FVII	1.95 $\pm$ 0.88 ^{ABE}	33.3 (5)	53.3 (8)	13.3 (2)	0 (0)	100 (15)	<0.001
		PAA+FVII	2.46 $\pm$ 1.04 ^{AB}	0 (0)	0 (0)	100 (15)	0 (0)	100 (15)	
		PA+FVII	1.23 $\pm$ 0.96 ^{CDE}	53.3 (8)	13.3 (2)	0 (0)	33.3 (5)	100 (15)	
Sub group 3	SD	DDW+FIX	5.81 $\pm$ 1.92 ^{AB}	0 (0)	53.3 (8)	46.6 (7)	0 (0)	100 (15)	<0.001
		PAA+FIX	5.47 $\pm$ 1.27 ^{AB}	13.3 (2)	13.3 (2)	73.3 (11)	0 (0)	100 (15)	
		PA+FIX	4.20 $\pm$ 1.94 ^{ACD}	46.6 (7)	53.3 (8)	0 (0)	0 (0)	100 (15)	
	DD	DDW+FIX	3.31 $\pm$ 2.64 ^{DE}	0 (0)	73.3 (11)	13.3 (2)	13.3 (2)	100 (15)	<0.001
		PAA+FIX	5.97 $\pm$ 1.61 ^B	0 (0)	13.3 (2)	86.6 (13)	0 (0)	100 (15)	
		PA+FIX	2.98 $\pm$ 1.44 ^{CE}	33.3 (5)	53.5 (8)	0 (0)	13.3 (2)	100 (15)	

Different superscript letters indicate statistically significant differences within a group using the same type of GIC (one-way ANOVA, Tukey's *post hoc* tests, $p < 0.05$). The P-value was analysed by the chi-square test and indicated the difference in the composition of failure modes due to different conditioning (n=15 per group).

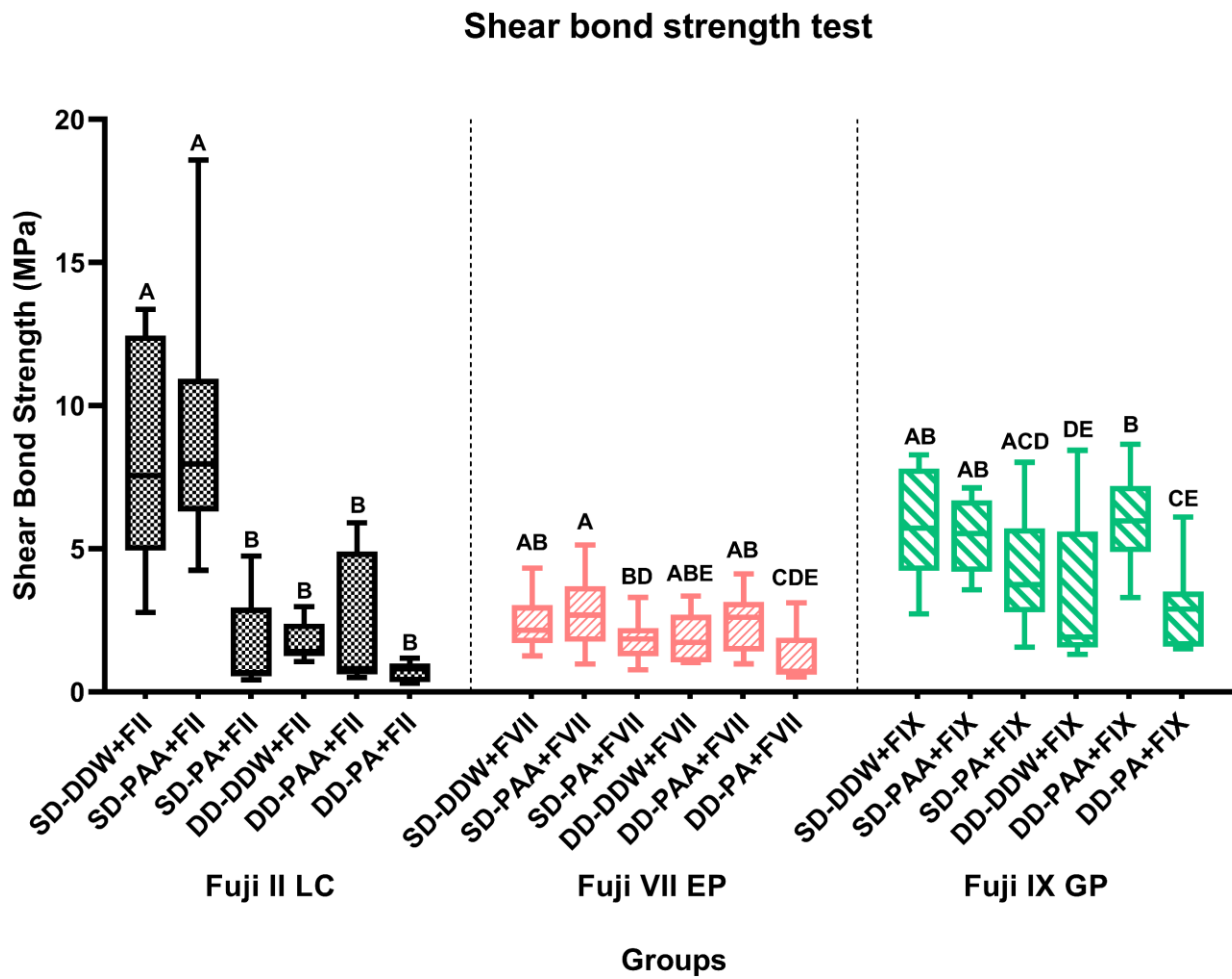


Figure 4.3 Box and whisker graphs of shear bond strength (MPa) values (mean $\pm$ standard deviation) of Fuji II LC, Fuji VII EP and Fuji IX GP EXTRA bonded to sound and demineralised dentine using different conditioners after 24 h storage in 100% relative humidity. Different superscript letters indicate statistically significant differences within a group using the same type of GIC (one-way ANOVA, Tukey's *post hoc* tests, $p < 0.05$).

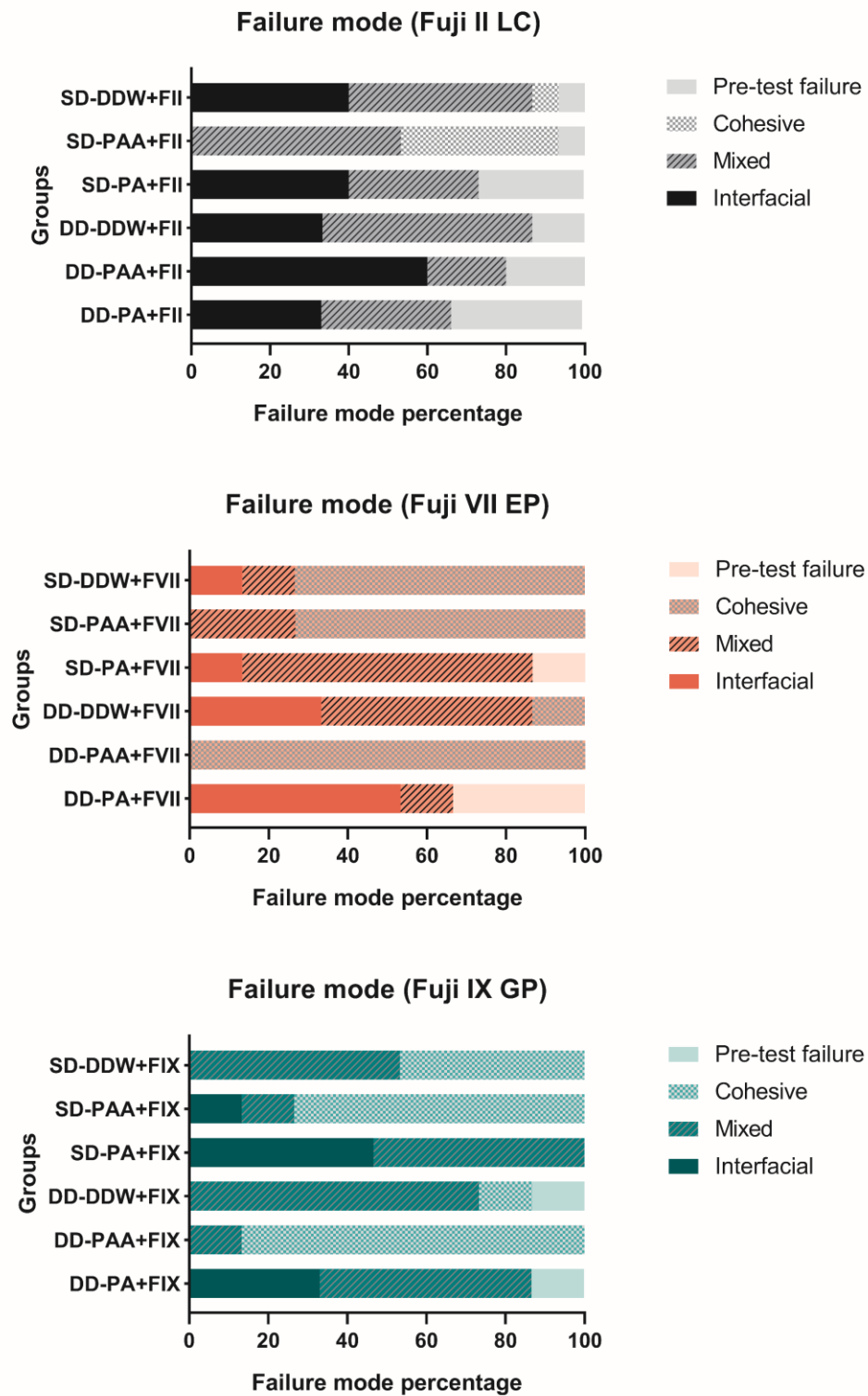


Figure 4.4 Failure modes (percentage) of specimens bonded with Fuji II LC, Fuji VII EP and Fuji IX GP EXTRA.

4.3.3 Failure modes

Different conditioning protocols affected the distribution of failure modes of GICs bonded either to SD or DD significantly according to the results of the chi-square statistical analysis except for the group of RMGIC bonded to DD.

Specifically, when the SD surface was conditioned with PAA, cohesive failures increased significantly in all types of GICs, indicating an enhanced interaction between GIC and dentine. However, after PA pre-treatment, no cohesive failures were observed whilst failures happened predominantly in mixed and interfacial modes or before testing, indicating a compromised dentine-GIC interaction. In addition, a comparatively higher proportion of cohesive failures was observed in CPP-ACP-modified GIC than the other two types of GICs.

When GIC was bonded to DD surfaces, the mixed failure mode was dominant for all three GICs. Polyacrylic acid conditioning increased the proportion of cohesive failures in CPP-ACP-modified GIC and HVGIC significantly compared to that without conditioning, indicating an improved dentine-GIC interaction, whilst PA conditioning increased the pre-test failure rate significantly in CPP-ACP-modified GIC and HVGIC and contributed to more interfacial failure modes. Both PAA and PA conditioning did not influence the distribution of failure modes of RMGIC when bonded to DD compared to that without conditioning.

4.3.4 Acid-base treatment technique

The bonding interfaces were treated with an acid-base technique where orthophosphoric acid and NaOCl were used to remove the inorganic and organic components on the sectioned longitudinal dentine surfaces, respectively. The micromorphology of the intermediate layer between dentine and GIC after the acid-base challenge was observed *via* SEM (Figure 4.5). Intimate adaptation between dentine and GIC was shown in most specimens except for an obvious gap between CPP-ACP-modified GIC and PA-conditioned SD. The gap putatively resulted from the dehydration and vacuum process during specimen preparation (Figure 4.5 H1).

An acid-base resistant (ABR) layer was shown in all SD samples. This layer was most pronounced between HVGIC and SD surfaces conditioned with PAA or PA, where a long thick funnel-shaped configuration of cement was shown, indicating good infiltration of cement into

the patent dentinal tubules. In addition, a structureless layer termed ‘absorption layer’ could be observed in the cement adjacent to dentine-GIC interface in some specimens (Figure 4.5 D1, E1) and this was related to the hydration during the bonding process [523].

Good adaptation was also observed in DD specimens, whilst ABR layers were not observed in some specimens. Specifically, ABR layers of different thicknesses with long thick funnel-shaped configuration of resin tags and intimate adaptation were observed between RMGIC and DD, indicating good cement infiltration into the patent dentinal tubules. In particular, PAA-treated samples (Figure 4.5 D2) showed a thicker cement infiltration zone into the dentine matrix than DDW-treated samples (Figure 4.5 A2). Thin cylindrical resin tags and a thinner interaction zone was observed after PA conditioning (Figure 4.5 G2). In addition, a thin ABR layer was also observed at the interface between CPP-ACP-modified GIC or HVGIC with PAA-conditioned DD.

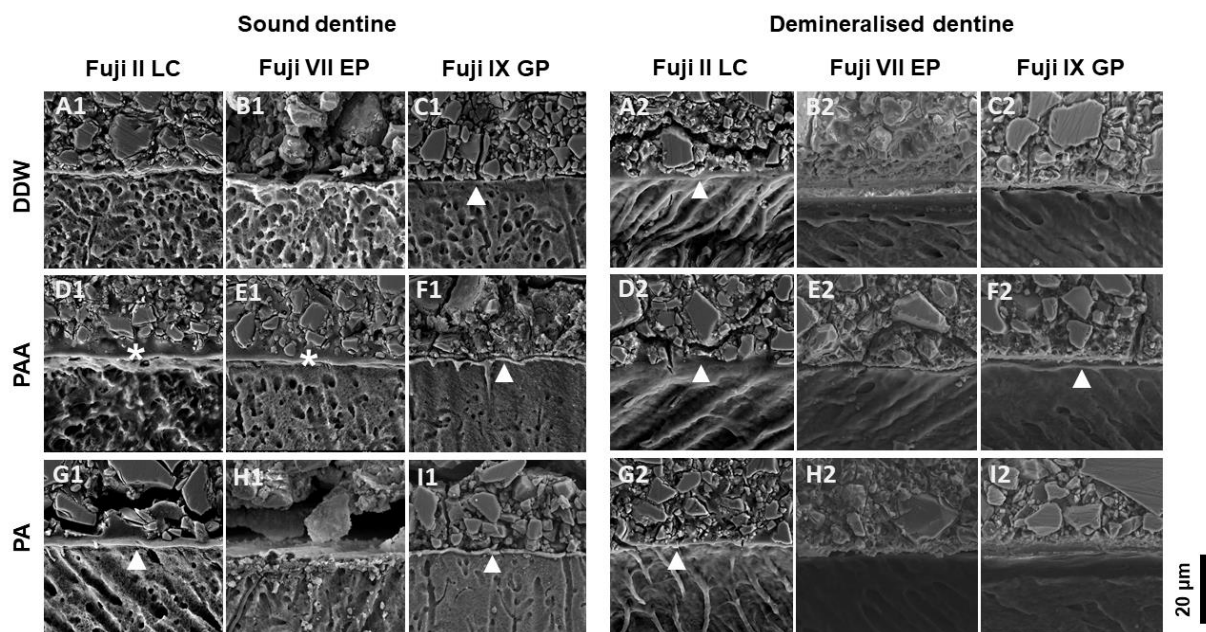


Figure 4.5 Scanning electron microscopy images of the bonding interface between Fuji II LC, Fuji VII EP or Fuji IX GP EXTRA and sound or demineralised dentine conditioned with distilled deionised water, polyacrylic acid or proanthocyanidin after acid-base challenge technique (Arrowheads: acid-base resistant layer; asterisks: adsorption layer).

4.5 Discussion

In this study, the bonding performance of RMGIC, HVGIC and CPP-ACP-modified GIC on both sound and demineralised root dentine was investigated as well as the dentine-GIC

interfacial interaction. Considering the variations in the inherent physicochemical properties of natural RCLs, demineralised root dentine was created using a demineralising solution to simulate the clinically remineralisable dentine of carious root dentine. The artificially created demineralised root dentine has high reproducibility under controlled conditions with similar degree of demineralisation and adequate flat surfaces for bonding, but it may not truly reflect a carious lesion.

When evaluating the performance of GIC restorations on teeth, adhesion is an important factor since it aids the retention of GIC restorations as well as reduces marginal leakage [514]. In the present study, adhesion of GIC was evaluated using SBS testing on specimens after 24 h storage in 100% RH, where the ion diffusion layer between dentine and GIC was not fully developed. The three GICs showed distinct bonding performance characteristics on root dentine due to their different formulations and inherent properties. Specifically, the dual bonding mechanisms of RMGIC including chemical bonding and micromechanical interlocking of the resin matrix resulted in higher bond strength than HVGIC and CPP-ACP-modified GIC relying mainly on chemical bonding interaction between carboxylate groups of polyalkenoic acid and calcium within HAp [524]. The lowest bond strength was observed in the CPP-ACP-modified GIC, which de-bonded from dentine predominantly in cohesive failures within the material itself. Therefore, the obtained SBS values of CPP-ACP-modified GIC did not reflect the true adhesive bond strength. The large proportion of cohesive failure modes of CPP-ACP-modified GIC may be related to its inherent low mechanical strength due to the incorporation of CPP-ACP that changes the powder compositions and the powder/liquid ratio in GIC [525]. In addition, previous work showed that low viscosity material was more readily causing air inclusions and increasing porosity during the rapid mechanical mixing process than high viscosity material (*e.g.* HVGIC) [526]. The resultant pores in low-viscosity CPP-ACP-modified GIC could act as a stress-concentration points that initiated fracture within the material itself [512]. With respect to bonding to carious dentine, typically, restorative materials show lower bond strength than bonding to SD, especially for resin-based materials [527]. Lower bond strength has also been observed in conventional GIC and RMGIC in previous work as well as in HVGIC in the present study [528, 529]. This reduction might be ascribed to the reduced mineral content and involvement of altered organic components in bonding that led to the formation of an irregular and weak hybrid-like layer between DD and GICs [529]. In addition, comparing the bonding performance of different GICs on carious dentine, most previous studies indicated higher bond strength of RMGIC than the traditional

GIC without resin monomer incorporation, whereas in the present study, RMGIC showed significantly lower SBS than HVGIC on demineralised root dentine [529, 530]. This distinction may be related to the degree of demineralisation and organic/inorganic components in the DD substrate which GIC was bonded to [499]. Specifically, naturally caries-affected dentine and artificial dentine carious lesions created by 0.2% Carbopol (pH = 5) were used in previous work, whilst a relatively more aggressive demineralisation solution which induced a great mineral loss from root dentine was used in the present study. The resultant demineralised root dentine of low mineral content along with an unsupported weak and collapsed collagen fibril network was not suitable for the chemical bonding and resin monomer penetration of RMGIC [528]. Moreover, the demineralisation process solubilised the mineral crystallites within dentine and replaced them with water [531]. The water-rich DD surface did not favour the uptake of hydrophobic monomers such as TEGDMA in RMGIC. Thus, a drastic decrease in the bond strength of RMGIC to DD was observed in the current study. However, CPP-ACP-modified GIC seemed to have comparable bonding capacity to either SD or DD from the current result, which is consistent with a previous study [532]. It was speculated that the lower viscosity of CPP-ACP-modified GIC allows easier cement penetration into lesion areas in addition to the enhanced release of Ca^{2+} , PO_4^{3-} and F^- , promoting the chemical reaction between GIC and dentine for bonding [512]. Therefore, CPP-ACP-modified GIC is applicable for carious root dentine where high load-bearing capacity is not required, although limited data regarding the performance of CPP-ACP-modified GIC on root dentine are available so far.

Reliable adhesion between GIC and dentine was predominantly dependent on the reciprocal diffusion and exchange of ions between tooth minerals and glass particles in GIC. The ion-exchange layer formed between dentine and GIC was reported to be resistant to acid-base treatments when orthophosphoric acid and NaOCl were used to remove the inorganic and organic components of dentine respectively [22]. In the present study, the ABR layer appeared in almost all bonded SD specimens. In particular, HVGIC was observed to penetrate into the dentinal tubules of SD, indicating the ability of the PAA component in HVGIC to disrupt the smear plugs, allowing infiltration of the cement into dentinal tubules. These morphologies were also observed in the study by Tanumiharja et al. who firstly introduced the concept of the ABR layer [22]. However, the interface formed between GIC and DD seemed to be more soluble to the acid-base challenge and was not clearly observed. A diffuse ABR layer has also been reported between naturally caries-affected dentine with GIC in previous work [532]. It was speculated that the mineral-deficient dentine whether naturally or artificially produced resulted

in a reduced chemical dentine-GIC interaction, rendering this layer more soluble to the attack of acid and base solutions. In addition, although the morphology and thickness of this ABR layer is an indication of the quality of dentine-GIC interaction, no consistent correlation between the bond strength and the thickness of ABR layers could be concluded [533]. Specifically, despite a lower SBS value when RMGIC was bonded to DD, the ABR layer between them was pronounced with greater thicknesses and resin tag lengths compared to that in SD, presumably because less smear layer and more exposed dentinal tubules with smear plugs on DD surface contributed to higher permeability to the polyelectrolytes formed with PAA and the resin monomers from RMGIC [24, 25]. However, despite the enhanced penetration, the incomplete infiltration of PAA polyelectrolytes of high molecular weight into the interfibrillar spaces within dentinal collagen might result in a weak link between RMGIC and DD [515]. The thicker ABR layer between RMGIC and DD is similar to the thicker hybrid layer formed between resin-based materials and carious dentine, where reduced bond strength has also been observed due to the lower tensile strength of carious tissues compared to SD [534].

In order to optimise the condition of dentine surfaces for bonding, application of conditioners was proposed to remove the proteinaceous debris of smear layer and provide a clean, easily wettable tooth surface for the placement of GIC restorations [535]. The aggressiveness and concentration of the conditioner as well as its application time were the determinants of conditioning efficacy with regard to its disturbance to the smear layer and dentinal tubules [520, 536]. In the present study, PAA and PA both acted as a mild conditioner on root dentine and they partially removed the smear layer but did not disturb the smear-plugs or overly widen the dentinal tubules in SD. For DD, PAA conditioning further demineralised ITD and exposed the collagen fibrils whilst PA smoothed the morphology of dentine surfaces without significant alterations, presumably due to a higher pH value of the PA solution (*ca.* 4.46) compared to PAA (*ca.* 1.9) [537]. The smoothed dentine surface topography after PA conditioning was attributed to either smearing or the formation of a layer of reaction products between dentinal collagen and PA, similar to the situation in TA conditioning in a previous study [520]. Nevertheless, PAA and PA are both high molecular weight substances containing a multiplicity of functional groups, *i.e.*, phenolic hydroxyls in PA and carboxylic acid hydroxyls in PAA. These functional groups are able to form a multiplicity of hydrogen bonds to dentine surfaces, potentially promoting wetting, cleaning and probably sorption of the conditioning agent on dentine surfaces [520].

Indeed, the conditioning regimens used in the present study influenced the bonding performance of GICs. Specifically, PAA conditioning did not have significant effects on the bond strength of GIC to SD. This result agrees with previous statements by Inuo et al. who indicated separate conditioning was not necessary since the PAA in GIC might be sufficiently acidic to exert a demineralising effect on dentine as well as the underlying collagen fibrils to achieve adequate bonding [538]. This phenomenon has also been observed in a recent *in vitro* study by Sauro et al. [539]. Nevertheless, PAA conditioning tended to improve the bonding performance of GICs on DD; it increased the bond strength of HVGIC on DD to a value comparable to that on SD. This promoting effect of PAA conditioning on carious dentine bonding has also been reported in a previous study on RMGIC and the reason was attributed to the removal of smear layer and partial demineralisation of the underlying dentine, increasing the surface area and micro porosities [528]. Indeed, from the current result, a thicker ABR layer with additional resin tags was observed when RMGIC was bonded to PAA-treated DD, indicating an improved penetration of the cement due to a more porous and permeable ITD substrate after PAA conditioning. Moreover, the aluminium chloride in Cavity Conditioner is believed to play a role in stabilisation of the demineralised collagen matrix [540]. The effect of PAA conditioning on GIC bonding was not fully demonstrated from SBS values due to large variations and the limited number of specimens in the present study. Analysing the changes in the proportion of failure modes could serve as a complementary approach to evaluate the effect of conditioning. Specifically, although PAA conditioning did not improve the SBS values significantly when GIC was bonded to SD, it altered the failure modes towards cohesive debonding, indicating a stronger bonding strength that surpassed the intrinsic cohesive strength of GICs. In addition, the increased proportion of cohesive failures of CPP-ACP-modified GIC and HVGIC bonded to PAA-conditioned DD also correlated with the increase in SBS values. Therefore, laboratory results from the present study agree with the recommendation of PAA conditioning prior to the application of GIC, especially in the situations where demineralised root dentine remains after carious tissue removal.

Considering the low tensile strength of the demineralised collagen fibrils that compromises the restoration bonding on DD, application of exogenous cross-linking agents such as PA would improve the elastic modulus of dentinal collagen which potentially serves as a scaffold for the interaction between dentine and restorative materials [534, 541]. Proanthocyanidin were used in the present study as a dentine conditioner which putatively removed the smear layer and

simultaneously cross-linked the demineralised dentinal collagen. From the current results, PA conditioning did not influence the bond strength of all three GICs to either SD or DD with the exception of the significantly reduced bond strength between RMGIC and SD. In light of this, the role of PA in GIC-dentine bonding seemed different from the situations when PA were involved in resin-dentine bonding, where PA strengthened the dentinal collagen within the hybrid layer and increased the bonding strength of resin composite to dentine in the laboratory setting [503, 542]. This could be ascribed to the different bonding mechanisms between GIC and resin adhesives. Specifically, the strengthened hybrid layer between resin and dentine was not only attributed to the biomodification of dentinal collagen by PA but also relied on the interaction between the catechol groups in PA and methacrylate groups in resins that chemically bridged dentinal collagen and resin [505, 506]. In addition, the interaction between dentine and GIC relies on the wetting of dentine surface aided by the hydrophilic nature of conditioners [520]. However, after the application of PA, the bound water in dentine displaced by PA-mediated collagen cross-linking altered the intrinsic water dynamics of the dentinal tissue. Whilst this water displacement facilitated the infiltration of hydrophobic resin monomers into dentine, the reduced surface hydrophilicity within the extracellular matrix led to inferior dentine-GIC contacts [543]. In addition, insoluble complexes could form from the reaction between PA and Ca^{2+} from the smear layer and precipitate on the dentine surface, interfering the bonding process [273]. Moreover, as reported previously, PA did not have significant cross-linking effects on un-demineralised dentine since the collagen fibrils were protected by the mineral phase and PA were not able to react with the dentinal collagen to form exogenous cross-links. Thus, effects of PA on SD-GIC bonding for a strengthened hybrid-like layer could not be expected [544]. Furthermore, despite the effects of PA on improving the durability of resin-dentine bonds in laboratory studies, recent clinical trials by de Souza et al. indicated no effects or even impairment of PA on the retention of resin composites in cervical areas of teeth due to the influence of PA on the resin free-radical polymerisation [545, 546]. This may also help explain the decreased bond strength between RMGIC and PA-conditioned SD. Nevertheless, a recent study from Atabek et al. showed 6.5% PA-rich grape seed extracts enhanced the bond strength of GIC on occlusal dentine specimens and this result is inconsistent with the current study. However, the application time of PA in Atabek's study was up to 10 min, which might improve the cross-linking efficacy of PA in a time-dependent manner, although the application time of 10 min is not clinically practical [547]. Additionally, the role of dentinal collagen on the adhesion of GIC has not been fully answered - although stronger bond strength of GIC to enamel than to dentine might suggest a greater importance of the

inorganic phase in teeth in GIC bonding, the amino and carboxylic acid groups in collagen have the possibility to interact with carboxylate groups in GIC [514]. Particularly at low pH, the positively charged dentinal collagen and negatively charged polyacid components of GIC might contribute to electrostatic attraction between collagen and GIC, promoting the GIC adhesion [548]. Thus, further investigations are warranted to unveil the role of dentinal collagen in GIC bonding.

Results from the present study would have clinical implications for the application of different GICs on root dentine, especially in situations where DD remains after carious tissue removal before the placement of GIC restorations. Further studies are warranted to investigate the long-term effects of different conditioning on GIC restorations as well as the ultrastructure of the dentine-GIC interface, especially the role of dentinal collagen in GIC bonding. Although PA conditioning compromised the dentine-GIC interaction in the present study, modification of constitutions and application procedures of the PA conditioner might optimise its effects on root dentine-GIC bonding.

4.6 Conclusions

The bond strength of different GICs used in this study on sound root dentine presented in order of decreasing strength was RMGIC, HVGIC, CPP-ACP-modified GIC. The bond strength decreased significantly when RMGIC and HVGIC were applied to demineralised root dentine, whilst CPP-ACP-modified GIC seemed to have comparable bonding capacity on either SD or DD. The acid-base resistant layer was more obviously observed when GIC was bonded to SD than to DD except for a thicker ABR layer with resin tags between RMGIC and DD. Using PAA as a conditioner enhanced the dentine-GIC interaction and particularly increased the bond strength of HVGIC on DD to a value comparable to that on SD and reduced the interfacial debonding failures. Conditioning dentine with PA, which is a collagen crosslinking agent, did not influence the bond strength of GIC to both SD and DD significantly in the short term with the exception of a significant decrease in the bond strength of RMGIC to SD. However, the interfacial interaction between GIC and PA-treated root dentine was compromised with increased interfacial debonding presumably because of the changes in hydrodynamics and surface cleanness of root dentine surface caused by PA cross-linking. Long-term adhesion tests are warranted to further clarify the effect of different conditioning approaches on the adhesion of GICs to both sound and demineralised root dentine.

Chapter 5

General discussion and conclusions

5.1 General discussion

5.1.1 Approaches to intervene in root caries processes

Within a laboratory setting, studies in Chapters 2-3 investigated the effects of PA when used as an adjunct to several non-invasive treatments including SDF, FV and 5,000 ppm FTP for RCLs. Root dentine specimens treated with the above-mentioned F agents without PA for collagen cross-linking served as a comparison group. The results from these comparison groups provide further information regarding the mechanisms and effectiveness of currently recommended high concentration F treatments for RCLs.

Specifically, the study in Chapter 2 gave insight into the interaction of SDF/KI with demineralised root dentine, where the released ions from SDF/KI were able to penetrate and distribute within the artificial RCLs up to a depth of 60 μm . The chemical reaction between SDF/KI and the remaining mineral in RCLs increased the MD and nano-mechanical properties of demineralised root dentine. Previous studies indicated the recovery of dentine hardness by SDF was mainly attributed to the F-mediated mineral formation [411], whilst the current results demonstrated that following SDF/KI treatment, the silver precipitates, including silver phosphate and silver iodide, also contributed to the recovery of MD, microhardness, elastic modulus and creep behaviour of carious dentine tissues. This finding should be considered in future studies regarding the effects of SDF on carious lesions especially in the period shortly after application of SDF/KI. In addition, the depth of the penetration of silver into dentine is not necessarily correlated with the depth of the microhardness increase.

Fluoridated dentifrices and varnishes are frequently used topical delivery methods for caries prevention. A high level of evidence has indicated the effectiveness of daily use of 5,000 ppm FTP in reducing active RCLs. However, a limited number of studies have demonstrated the effectiveness of FV in RCL treatments. Also, the efficacy of FV was evaluated *via* various criteria such as decayed, missing and filled root surfaces (DMFRS) and surface texture and this may lead to certain biases [8]. The results in Chapter 3 indicated the ability of FV containing Ca-P remineralisation systems and 5,000 ppm FTP to promote ion uptake and improve MD within demineralised root dentine. However, both FTP and FV failed to increase the mechanical properties of carious root dentine. Therefore, the effects of FV on root surfaces were different from those on enamel and primary teeth where FV was able to achieve a considerable uptake of F as well as improve the mechanical properties of primary enamel based

on outcomes of previous studies [549, 550]. In addition, although FTP has been reported to inhibit surface hardness decrease in root dentine in a concentration-dependent manner [551, 552], the improvement of mechanical properties of dentine is not only dependent on mineral content increase but also predominantly relies on intrafibrillar remineralisation which is hard to achieve *via* mineral deposition by F-based regimens [11]. Although a combined application of FV and FTP increased the microhardness of demineralised root dentine significantly, the increase was only observed at the dentine surface. This was putatively due to the abundant extrafibrillar mineral deposition along with the prolonged presence of F and more Ca^{2+} and PO_4^{3-} available enhancing the precipitation rate. Nevertheless, high concentration FTP could be recommended for use after professional application of FV in order to maintain the presence and concentration of F on tooth surfaces, especially in the oral environment where FV is easily removed due to mastication, oral hygiene procedures, soft tissue movement and salivary flow [490].

In addition, whilst the additive effect of Ca-P in FV possibly promoted the uptake of Ca^{2+} and PO_4^{3-} and further increased the MD of the artificial RCLs, it did not improve the mechanical properties of the demineralised root dentine (Chapter 3). Previous studies have indicated the efficacy of FV varies due to different forms of calcium phosphate-based delivery systems and was also influenced by the laboratory-based pH-cycling models used regarding the duration of demineralisation and remineralisation cycles [170, 173]. Therefore, it was speculated that the additional effects of FV containing calcium phosphate-based systems compared to F-only varnishes would vary depending on the different stages of caries progression as well as being influenced by the variations of the clinical oral environment of individuals. In order to maximise the remineralising potential of Ca-P, it has been suggested that the calcium phosphate-based remineralising agents could be considered for targeted delivery of Ca^{2+} which is rate limiting for remineralisation to proceed in specific locations of teeth at the right time [553].

With respect to the invasive treatments, GIC is one of the frequently used materials for restoration of RCLs. However, its performance in restoring RCLs is still inconclusive according to the limited number of available clinical and laboratory studies [500]. Results in Chapter 4 indicated the performance of GIC restorations on RCLs could be improved by pre-treating the root dentine substrate for bonding and selecting appropriate type of GIC according to the clinical circumstances. Generally, HVGIC and RMGIC have lower bond strengths when

bonded to demineralised root dentine compared to sound root dentine, whilst CPP-ACP-modified GIC showed comparable bond strengths to either sound or demineralised root dentine. However, CPP-ACP-modified GIC has the lowest bond strength to sound root dentine compared to HVGIC and RMGIC in the current results, therefore, CPP-ACP-modified GIC restorations should be limited to low stress-bearing situations and the potential for loss on root dentine in clinical situations may be greater than other GICs. In addition, the laboratory bond strength test results in Chapter 4 agree with the recommendations of using PAA as a dentine conditioner for GIC bonding, especially for the bonding of HVGIC to demineralised root dentine.

Although GIC might be the material of choice for RCL restorations due to moisture tolerance, good chemical adhesion to tooth structure and anti-cariogenic properties, results from the available clinical trials showed a relatively high failure rate of GIC restorations on RCLs [500]. However, the number of well-controlled studies is limited. Whilst the results in Chapter 4 demonstrated the bonding performance of GICs on both sound and demineralised root dentine, further studies are warranted to explore the potential problems such as retention loss, wear and inferior marginal integrity that lead to clinical GIC restoration failure. This is especially important in high caries risk groups such as elders with reduced saliva flow/xerostomia, often due to medications or head and neck irradiation in order to give comprehensive evidence-based recommendations for the use of GICs in treatment of RCLs.

5.1.2 Application of proanthocyanidin as an adjunct in root caries treatments

Although the above-mentioned F-mediated remineralising agents, *i.e.*, SDF/KI, FV and FTP, were introduced to arrest or remineralise RCLs, they generally deposit minerals superficially on root surfaces without promoting remineralisation of the deeper layers of carious dentine as the highly mineralised surface zone formed by F with available Ca^{2+} and PO_4^{3-} could act as an ionic diffusion barrier [554, 555]. The quality of dentine remineralisation depends on not only the increase of MD but also recovery of root dentine characteristics including the ultrastructure (especially the location of the mineral within the organic matrix) and biomechanical properties which predominantly rely on intrafibrillar remineralisation [10]. Therefore, preservation of the organic matrix in root dentine from degradation should be considered in root dentine remineralisation. A number of previous studies have revealed the effects of PA in bio-modifying and strengthening the dentine organic matrix by interacting with the collagen fibres

via covalent, ionic and hydrogen bonding as well as hydrophobic interactions [268, 277, 448]. The resultant stabilised dentinal organic matrix could potentially serve as a scaffold for mineral deposition and distribution throughout the RCLs after application of F regimens [153]. Studies reported in Chapters 2 and 3 demonstrated that using PA as an adjunct to high concentration F regimens promoted their effectiveness on RCLs. Specifically, the application of PA with SDF/KI enhanced ion uptake and penetration throughout RCLs as well as promoted the mineral-collagen interaction which resulted in an incremental increase of MD and nano-mechanical properties of demineralised root dentine. Furthermore, the combined use of PA with FV and 5,000 ppm FTP enhanced the RCL remineralisation in terms of improved ion uptake, MD and mechanical properties in both surface and subsurface areas. In this regard, non-invasive treatment strategies targeting both organic and inorganic components of root dentine are promising in enhancing remineralisation of RCLs. Results from Chapters 2 and 3 advanced the previous findings that additive effects were observed when calcium phosphate-based F toothpastes were used in combination with PA [153, 449]. However, in the previous studies, PA was mixed with pastes before application where the peptide cross-linking ability of PA may have an impact on the casein protein structure of CPP-ACP or CPP-ACFP contained in the toothpastes, thus compromising their efficacy [556]. Also, the treatment time was up to 2 h which is not clinically practical. Therefore, in the current studies, PA was used as a pre-treatment on demineralised root dentine to strengthen the dentine matrix before the application of remineralisation agents with a more clinically applicable treatment time and protocol.

With respect to the restorative treatments of RCLs, the development of adhesion science has laid more emphasis on the crucial role of collagen fibrils in the durability of restorations bonded to dentine, especially when bonded to demineralised dentine [557]. In Chapter 4, PA was used as a conditioner for GIC bonding in an attempt to remove the smear layer and simultaneously strengthen the dentinal collagen involved at the bonding interface. The bond strength of GIC to root dentine was not significantly influenced by PA conditioning whilst the dentine-GIC interaction was compromised. This result seemed inconsistent with the situations in resin-dentine bonding and what was expected from the findings in Chapters 2-3. Specifically, when PA was involved in resin-dentine bonding in several previous laboratory studies, the durability of resin restorations and the stability of the bonding interface were improved [503, 542, 558]. This advancement was attributed to the following reasons: firstly, PA bio-modified the dentine matrix and increased the collagen resistance to biodegradation in the hybrid layer; secondly,

the catechol groups available in PA could chemically bridge collagens and methacrylate groups in resins, enhancing the collagen-resin interaction in the bonding interface. Additionally, the bound water in dentine displaced by collagen cross-linking facilitated the infiltration of hydrophobic resin monomers [505, 506]. However, GICs bond to dentine *via* a different mechanism to resin composite and the bonding performance of GICs is determined by multiple factors. Whilst the interaction between dentine and GIC relies on the wetting of dentine surface aided by the hydrophilic nature of conditioners, additional collagen cross-links mediated by PA conditioning might reduce the dentine surface hydrophilicity, impairing the dentine-GIC contact [520, 559]. Moreover, due to the weak acidity of PA, it may dissolve the shattered HAP crystals in the smear layer, releasing Ca^{2+} which reacted with PA and formed insoluble complexes that precipitated on the dentine surface, interfering the bonding and the overall interaction of GIC to root dentine [273]. In addition, based on the findings in Chapters 2 and 3, a PA stabilised dentine matrix was expected to provide a scaffold to promote the reciprocal dentine-GIC ion exchange and distribution which is a determinant of GIC adhesion. Results in Chapter 4 demonstrated that when a PA solution was used as a dentine conditioner, the dentinal tubules were partially exposed with the smear layer removed. Further investigations are being conducted to investigate whether PA conditioning could enhance the penetration of GICs into demineralised root dentine.

5.1.3 Evaluation of dentine remineralisation using complementary evaluation techniques

The quality of root dentine remineralisation depends upon the sum of the tissue characteristics including ultrastructure, MD and biomechanical characteristics [10]. To characterise the properties of de-/remineralised root dentine from different aspects, complementary evaluation techniques including SEM, micro-CT, PLM, nanoindentation, microhardness test and EDS were used in the current studies in order to analyse the morphology, MD, birefringence, mechanical properties and chemical constitutions of root dentine during de-/remineralisation. Improvement in one aspect is not an appropriate endpoint to assess the overall quality of dentine remineralisation. Specifically, the results in Chapter 3 indicated that increased mineral content after exposure to F regimens was not necessarily translated into the recovery of mechanical properties. Moreover, the increase in the values of mechanical properties of carious dentine was not necessarily to a depth consistent with the MD improvement. Indeed, it has been revealed from a previous study that whilst remineralisation occurred preferentially in the extra-fibrillar zones leading to increased MD, the improvement of mechanical properties of

dentine mainly relied on the mineral formation in the intrafibrillar compartments [11]. On the other hand, however, correlations may be developed amongst the quantitative capabilities of different techniques. For instance, although the measurement of mineral loss and gain is best accomplished with micro-CT or TMR, MD can be extrapolated with cross-sectional microhardness values if standardised protocols are used for the transformation between them although accuracy can be compromised [430]. In addition, a relationship between MD and elastic modulus of teeth was also developed in a previous study where a high-resolution imaging system and high precision and accuracy of indentations were applied [560].

5.2 Future directions

Studies present in this thesis have advanced the knowledge of PA, a naturally derived collagen cross-linking agent, used as an adjunctive treatment for RCLs. The results give insight into how to potentially improve the current clinical treatments for root caries by protecting the dentinal organic matrix. Nevertheless, further validation of the findings is required in clinical settings.

The effects of PA used as an adjunctive treatment for RCLs demonstrated in the current studies are anticipated to be sustained under cariogenic conditions for a long period during remineralisation. Further research regarding the long-term effects of PA on RCLs is thus warranted. In addition, previous studies have revealed an inhibitory effect of PA on MMPs and cysteine cathepsins, reducing collagen degradation by the dentine proteases [153, 278, 465]. Therefore, it is of interest to further investigate the effects of PA on host-derived enzymes and collagen stability when used as an adjunct to F-mediated remineralisation regimens.

Moreover, the outcome from the studies presented in this thesis showed a significant increase in mechanical properties of carious root dentine in the subsurface areas when PA was applied topically in conjunction with F regimens. This improvement might be ascribed to a better mineral-collagen interaction that enabled efficient load transference and stress distribution between the organic and inorganic components of dentine [561]. Previous studies have indicated a more predominant role of intrafibrillar mineralisation in determining the mechanical properties of carious dentine [11]. In this regard, it would be sagacious to investigate the ultrastructural morphologies of carious root dentine to further understand the process and mechanism of PA-mediated biomimetic remineralisation. In particular, with the

development of the FIB-SEM technique, it is possible to identify a specific region in dentine specimens precisely and analyse the morphological interaction of collagen and minerals in root dentine by using TEM [383].

Additionally, even though using PA as a pre-treatment conditioner for root dentine reduced the interfacial interaction between dentine and GIC, the bonding performance was determined and influenced by multiple factors. Specifically, despite the fact that PA could strengthen the dentinal collagen, it may also alter the hydrodynamics and cleanliness of dentine substrates, thus influencing the bond strength. In addition, the bond strength of GIC was tested in specimens after 24 h of water-ageing whilst the ion-exchange between dentine and GIC as well as the F release from GICs for remineralisation can be sustained for a longer period of time [562]. Therefore, there is scope in the future to modify the conditioning protocol of PA and evaluate the bonding performance in the long term.

Furthermore, the solubility of PA and the solvents used for dissolving PA are of importance for the collagen cross-linking functionality of PA [398]. Currently, there is no standard process for extraction of PA-rich agents, for instance, the solvents used, which might affect the cross-linking efficacy and components of PA. Further research is required to fully elaborate the interaction mechanism between PA or its monomers with dentinal collagen, thus allowing the development of an application protocol of PA to optimise its cross-linking efficacy with clinically applicable treatment times and doses.

5.3 Conclusions

The effects of PA used as an adjunct to high concentration F agents and applied as a dentine conditioner for GICs in the treatment of RCLs were investigated in a laboratory setting in this thesis. Currently recommended high concentration F treatments such as SDF/KI, FV and 5,000 ppm FTP promoted the uptake of F^- and possibly Ca^{2+} and PO_4^{3-} , which reacted with the remnant apatite crystallites within RCLs. The F regimens improved MD and mechanical properties of carious root dentine. In particular, the combined use of F delivery approaches such as FV and 5,000 ppm FTP further enhanced the effectiveness of F treatments. When using PA as an adjunctive treatment for RCLs, the remineralisation efficacy of F agents was improved in terms of an incremental increase of ion uptake, MD, and mechanical properties of carious dentine in the subsurface areas. The bonding performance of GICs on RCLs depends

on the inherent formulations and properties of GICs and the characteristics of dentine substrates for bonding, where the bond strength of GICs on demineralised root dentine was generally lower than that on sound root dentine, except for CPP-ACP-modified GIC, which demonstrated comparable bonding performance on both sound and demineralised root dentine. Polyacrylic acid conditioning improved the interfacial interaction between GIC and demineralised root dentine. Using PA as a conditioner did not influence the bond strength of GIC on root dentine significantly whilst it compromised the dentine-GIC interaction with increased failure modes of interfacial debonding. Further research is warranted to validate these results and expand their relevance in clinical settings, which could provide a pathway for improvement in the clinical treatment of root caries.

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Appendix 1

Appendix 1 includes the published manuscripts from Chapter 1, Chapter 2 and Chapter 3.

Status and progress of treatment methods for root caries in the last decade: a literature review

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ABSTRACT

The aim of this literature review is to explore the treatment methods for root caries in laboratory and clinical research in the last decade. A systematic search of publications in PubMed and Web of Science databases was performed. The timespan was limited to the last 10 years and English language. Further retrieval was conducted using the search terms of specific therapies or treatments. Eighty-two articles were included in this systematic review and full texts were retrieved. Types of studies included laboratory studies and clinical trials. Therapeutic approaches for root caries without risk of pulp exposure can be categorized into non-invasive and restorative treatment. Non-invasive treatments which targeted different causative factors of root caries have been developed in the last decade. Accordingly, several artificial caries model systems have been proposed for the study of root caries in the laboratory. Carious tissue excavation techniques and restorative materials and procedures have been modified to improve the prognosis of invasive treatment. It is of importance to determine the most appropriate therapy for root caries and further clinical trials are needed to draw firm conclusions concerning the efficacy and consistency of the various treatment methods proposed.

Keywords: Dentine, geriatric dentistry, root caries, systematic review, treatment method.

Abbreviations: AmF = amine fluoride; APF = acidulated phosphate fluoride; ART = atraumatic restorative treatment; CaF₂ = calcium fluoride; CHX = chlorhexidine; CPP-ACP = casein phosphopeptide-amorphous calcium phosphate; Er, Cr:YSGG = erbium, chromium: yttrium–scandium–gallium–garnet; FEL = free electron laser; GIC = glass-ionomer cement; GSE = grape seed extract; HPN = hesperidin; KI = potassium iodide; MDPB = 12-methacryloyloxy dodecylpyridiniumbromide; MID = minimal intervention dentistry; MIR = mid-infrared; MMP = metalloproteinases; NaF = sodium fluoride; PO = pirocton olamine; RC = resin composite; RCL = root carious lesions; RCT = randomized clinical trial; RMGIC = resin-modified glass-ionomer cement; SDF = silver diammine fluoride; SnF₂ = stannous fluoride; TiF₄ = titanium fluoride.

(Accepted for publication 14 August 2017.)

INTRODUCTION

Elderly people make up an increasing share of the world's total population due to the dramatic increase in average life expectancy.¹ A larger proportion of elderly individuals now retain their teeth longer due to advances in oral health care and treatment.² Accordingly, the oral health problems of the elderly are shifting from complete tooth loss towards untreated carious lesions.³ In addition, gingival recession occurs frequently in elderly people and the exposed root surfaces increase the risk of root carious lesions (RCL; a carious lesion situated on the root surface) development and progression. Untreated RCL are now peaking later in life and root caries experience can be expected to continually increase in future years.⁴

Multiple risk factors contribute to the prevalence and progression of RCL in the elderly, such as impaired oral hygiene resulting from poor oral care behaviours, drug-induced salivary flow reduction, complex health status due to concomitant systemic diseases and frequent snacking at home.⁵ The success of treatment of RCL in this caries-susceptible group can be challenging, especially for those with limited mobility, inadequate financial resources or lack of dental health insurance.^{6,7} Also, traditional restoration placement may be hampered by difficulties in moisture control on root surfaces, as the RCL can extend subgingivally. With dentine as the only adherent substrate, the reliability of adhesion is also less predictable.⁸ Therefore, there is a continuing need for alternative techniques for RCL management.

Several reviews have critically evaluated the treatment methods for RCL from a specific aspect, such non-invasive approaches, therapeutic agents used in the treatment, as well as from a periodontal perspective.^{9–12} The validity of the treatment approaches proposed decades ago (e.g. 1100–1450 p.p.m. fluoride dentifrice) have been well established. Advances in knowledge regarding cariology and aetiological factors in recent years has given rise to novel approaches, with emphasis on the protection of the organic matrix in dentine and from the perspective of biomimetic remineralization, especially in laboratory studies.^{13,14} Also, the concept of minimal intervention dentistry (MID) has been emphasized if restorative treatment is necessary.¹⁵ Unlike previous research which mainly addressed restorative material options, recent studies have concentrated more on the improvement and modification of restorative materials as well as procedures in order to arrest the disease process and improve restoration longevity. As root caries is becoming an increasingly important oral health concern, research in this area has accelerated during the last decade. It is of significance to update previous knowledge and provide a general overview of developments in RCL management both from laboratory studies and clinical trials.

This literature review aims to categorize and critically evaluate the treatment methods for RCL over the past decade (from 2007 to the present) and provide recommendations for future research and clinical practise. Both laboratory studies and clinical trials are included and methods to model RCL *in vitro* are summarized.

MATERIALS AND METHODS

A database search was performed in PubMed and Web of Science for articles published in the last decade. The last search was made on 28 February 2017.

The search terms used for this review were as follows:

Initial retrieval

PubMed

((('dental caries' (MeSH Terms) OR ('dental' (All Fields) AND 'caries' (All Fields)) OR 'dental caries' (All Fields) OR 'caries' (All Fields)) OR carious (All Fields)) AND ('root' (All Fields))) AND ('therapy' (Subheading) OR 'therapy' (All Fields) OR 'treatment' (All Fields) OR 'therapeutics' (MeSH Terms) OR 'therapeutics' (All Fields)). Publication dates: within 10 years. Languages: English.

Web of Science

TOPIC = (caries OR carious) AND TOPIC = (root) AND TOPIC = (therapy OR treatment OR therapeutics) AND LANGUAGE: (English). Timespan = 2007–2017.

Further retrieval

PubMed

((('root' (All Fields)) AND ('dentin*' (MeSH Terms) OR 'dentin*' (All Fields)) AND ('dental caries' (MeSH Terms) OR 'dental' (All Fields) AND 'caries' (All Fields)) OR 'dental caries' (All Fields) OR 'caries' (All Fields)) AND ('restoration' (All Fields) OR 'amalgam' (All Fields) OR 'resin composite' (All Fields) OR 'glass-ionomer cement' (All Fields) OR 'glass-ionomer cements' (All Fields) OR 'remineralization' (All Fields) OR ('anti-bacterial agents' (Pharmacological Action) OR 'anti-bacterial agents' (MeSH Terms) OR ('anti-bacterial' (All Fields) AND 'agents' (All Fields)) OR 'anti-bacterial agents' (All Fields) OR 'antibacterial' (All Fields)) OR ('laser' (MeSH Terms) OR 'laser' (All Fields) OR 'lasers' (All Fields)) OR ('atraumatic restorative treatment' (All Fields) OR 'ART restoration' (All Fields) OR 'ART technique' OR 'ART') OR ('collagenases' (MeSH Terms) OR 'collagenases' (All Fields) OR 'collagenase' (All Fields)) OR ('fluorides' (MeSH Terms) OR 'fluorides' (All Fields) OR 'fluoride' (All Fields)). Publication dates: within 10 years. Languages: English.

Web of Science

TOPIC = ((caries OR carious OR dental caries) AND TOPIC = (root) AND TOPIC = (dentin) AND TOPIC = (fluoride OR laser OR restoration OR atraumatic restorative treatment OR ART OR antibacterial OR resin composite OR glass-ionomer)) AND LANGUAGE: (English). Timespan = 2007–2017. Cross-referencing was performed to identify further articles.

Study selection

The search was limited to English-language journals and papers published between 2007 and 2017. Studies selected met the following inclusion criteria:

Types of studies

Studies included in the present review were laboratory studies or clinical trials (level I or II). The following types of studies were excluded: reviews; case reports;

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conference abstracts; letters to the editor; and dissertations and theses.

Intervention

Either invasive or non-invasive treatments on RCL with no risk of pulp exposure were included. Deep RCL affecting dental pulp which involved endodontic treatment such as root canal therapy and pulp capping and studies concentrating on diagnostic methods or decision-making process for RCL treatment without assessment of efficacy were excluded.

Control

Negative (placebo treatment), positive (other intervention or frequently used standard therapy, e.g. conventional fluoride toothpaste).

Data extraction

Study type (clinical trial, laboratory study on human/bovine dentine or *in situ* trial), pharmaceutical agents or methods used for interventions, type of intervention (invasive or non-invasive approaches), type of control group, and assessment of intervention by measurement of outcomes which clearly indicated the effects of certain treatment (e.g. decayed-missing-filled root surface, root caries incidence, hardness and lesion depths).

RESULTS

In total, 1102 articles (808 in PubMed, 482 in Web of Science, with 188 duplicates) were identified by screening electronic databases. For further refinement, publications were limited to those from the scientific, peer-reviewed and English literature. Titles and abstracts of articles were read and the following papers were excluded: reviews; case reports; conference abstracts; letters to the editor; dissertation and theses; non-English articles; epidemiological studies, diagnostic methods or decision-making processes for root caries treatment without assessment of its efficacy; deep carious lesions with inflamed pulp and requiring pulp capping or endodontic treatment and research on root cementum. After screening, 60 papers remained for inclusion. Further retrieval was conducted using specific terms of treatment strategies. An additional 22 publications were identified (10 in Web of Science, 12 in PubMed). No articles were identified by cross-referencing. (The searching process was also undertaken to retrieve articles published between 1900 and 2006. The majority of the treatment methods in these papers have been reconfirmed

or updated in the research in recent years, thus with less novel insights. Therefore, only literature published during the last decade (2007–2017) were included).

A total of 82 of articles, including 46 laboratory studies, nine *in situ* experiments and 27 clinical trials were screened for full-text analysis. In the clinical trials, five studies were considered as level Ib randomized controlled trials (RCT),^{16–20} with adequate and appropriate description of random allocation sequence generation and allocation concealment as well as a reasonable sample size; the other studies were graded as level II evidence, with unclear or inadequate explanation of the randomization process or with a follow up of less than 80%.²¹ Laboratory studies were performed on either extracted bovine or human teeth. The nine *in situ* studies employed special palatal or buccal appliances to place the tooth specimens in patients' mouths for exposure to the oral environment.^{22–29} (Fig. 1).

Root caries manifests as a continuum of disease with increasing severity. The disease state could progress from an initial asymptomatic status to RCL leading to tooth destruction. When diagnosing RCL, it is of importance to distinguish between active and inactive/arrested lesions, thus determining the most favourable management strategies.

Treatment methods for RCL can be categorized into non-invasive approaches and restorative (surgical) treatment. The non-invasive approaches are generally based on several mechanisms and strategies listed in Table 1.

NON-INVASIVE MANAGEMENT OF RCL

Fluoride formulations

Thirty-one articles, which included 11 laboratory studies, six *in situ* studies and 14 clinical trials, investigated the application of fluoride-containing products such as dentifrices, varnishes, mouthwashes and milk, or an adjunctive use of fluoride with other 'active' components like triclosan,³⁰ arginine,^{19,31} casein phosphopeptide-amorphous calcium phosphate (CPP-ACP),³² calcium sodium phosphosilicate³³ or supersaturated mineralizing ions.³⁴ One laboratory experiment explored the role of hydroxyapatite nanoparticles on remineralization of artificial RCL.³⁵

Undisputed evidence has revealed that fluoride is effective in the prevention and treatment of carious lesions.³⁶ The anticaries effect of fluoride is related to its ability to alter ionic saturation characteristics with respect to tooth mineral and aid remineralization and prevent demineralization. Also, at high concentration it may interfere with bacterial metabolism and acid production. Fluoridated products are the

Review on treatment of root caries

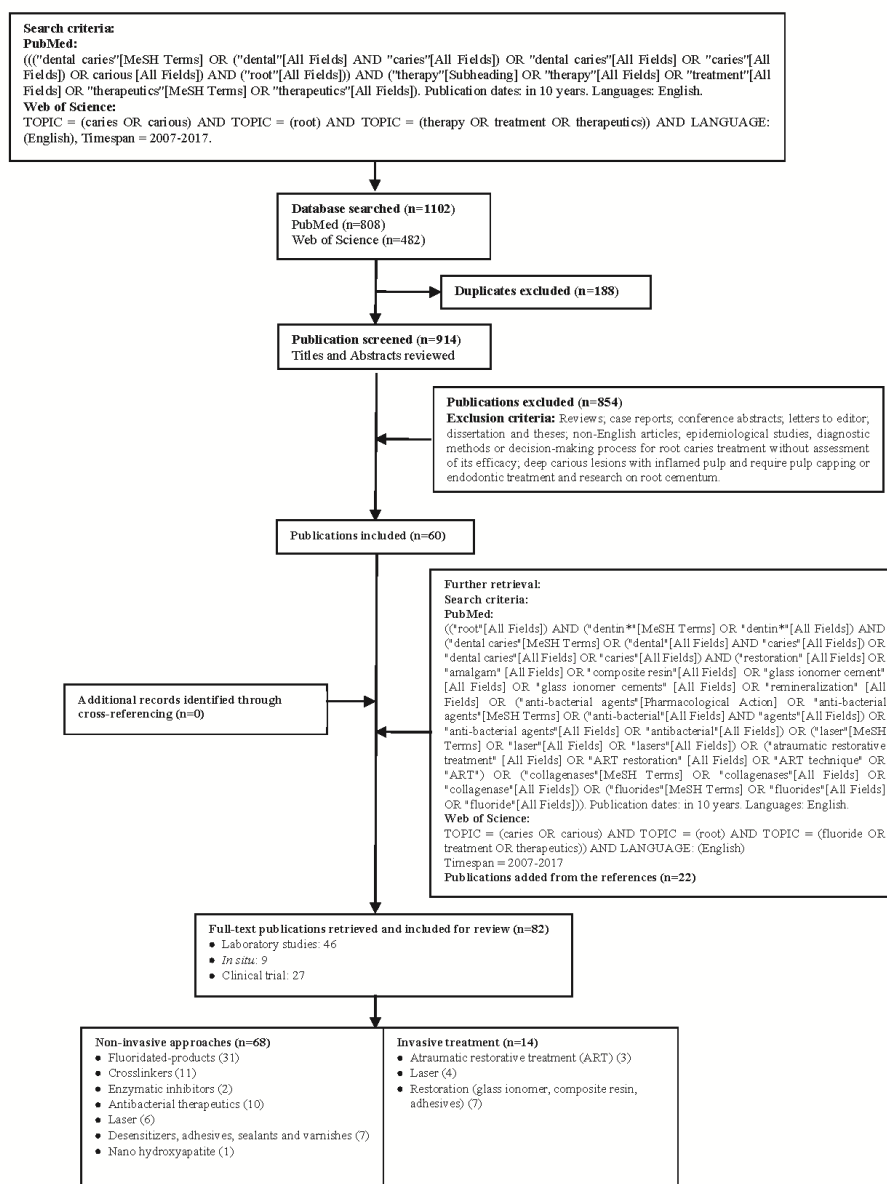


Fig. 1 Flowchart of search strategy

most widely described and most extensively used agents for carious lesion management. As dentine is more caries-susceptible than enamel due to the considerable difference in mineral composition, more

researchers have begun to investigate the effects of fluoride for dentine and RCL.^{28,37}

Toothpastes containing fluoride have been considered as the most rational topical fluoride delivery

Table 1. Categorization of non-invasive treatment for root caries

First author, Year	Intervention	Control	Type	Outcomes measured	Effects
Fluoride formulations Pettersson, 2007 ⁶⁴	1400 p.p.m. FD + 250 p.p.m. F mouthwash (Elmex® Sensitive Plus; Colgate-Palmolive, New York, NY, USA) twice a day	1400 p.p.m. FD + placebo mouthwash, twice a day	Clinical trial	ECM; changes in surface texture	Arrest and remineralization of caries lesions
Ekstrand, 2008 ⁵⁴	1450 p.p.m. FD twice a day + (professionally) 1450 p.p.m. FD (Colgate-Palmolive, UK) and Duraphat varnish once/month Carsolv® chemo-mechanical technique (Medteam, Svedalen, Sweden) + Duraphat 223% F varnish (Colgate-Palmolive, USA) Colgate® Total plus Whitening Toothpaste (0243% NaF, 03% triclosan in a silica base) (Colgate-Palmolive, USA) Hydroxyapatite nanoparticle 5 p.p.m. F-milk/probiotic bacteria	1450 p.p.m. FD; 5000 p.p.m. FD (Duraphat; Colgate-Palmolive, Manchester, UK); twice a day Duraphat 223% F varnish (Colgate-Palmolive, USA); SnF ₂ solution (8%) Colgate NaF toothpaste without triclosan 1000 p.p.m. F (NaF); DW Standard milk (placebo); milk with probiotic bacteria; 5 p.p.m. F milk 1450 p.p.m. FD (Colgate Ultra Cavity Protection; Colgate-Palmolive, UK) (professionally) 1450 p.p.m. FD (NaF, silica based); non-FD (calcium based)	Clinical trial Clinical trial Clinical trial Laboratory study Clinical trial Clinical trial	Number of RCL DMFS; DFRS as a percentage of exposed root surfaces (DFRS %); CFU in plaque from RCL Katz root caries index; mean RCL increment Knoop hardness test ECM; mean RCL increment Number of arrested/active RCL	
Fure, 2009 ⁵⁸	15% arginine, 1450 p.p.m. FD (sodium monofluorophosphate, calcium based) (Colgate-Palmolive, USA) 15% arginine, 1450 p.p.m. FD (sodium monofluorophosphate) (Colgate-Palmolive, Hamburg, Germany)	1450 p.p.m. FD (sodium monofluorophosphate)	Clinical trial	Hardness change; ECM	
Souza, 2013 ¹⁹	5000 p.p.m. FD (ClinPro 5000, 3M ESPE); CSPS toothpaste (ReNew; Sultan Healthcare, Hackensack, PA, USA); Prevident 5000 gel (Colgate Oral Pharmaceuticals, Canton, MA, USA)	DW	Clinical trial	Lesion size and distance from the gingival margin; texture change	
Goertsche, 2014 ⁴³	11% NaF (5000 p.p.m. F) FD (Duraphat; Colgate-Palmolive, Germany)	DW	Laboratory study	Mean depth of cavitation/ remineralization band/lesion	
Srinivasan, 2014 ²⁰	5% NaF varnish (Duraphat®; Colgate, Colgate-Palmolive, USA)	Standard regular FD (Odol-med 3, 1350 p.p.m. F; GlaxoSmithKline, Bruhl, Germany) A water-based gel without F as placebo (K-Y® Gel; McNEIL-PPC, Fort Washington, MD, USA) Conventional 1100 p.p.m. FD	Clinical trial	Hardness change	
Xin, 2016 ¹⁸			Clinical trial (patients with Sjögren's syndrome)	Mean DMFT, mean arrested root caries, mean oral Candida counts	
Papas, 2008 ³⁴				RCL increment per year	

(continued)

Table 1 continued

First author, year	Intervention	Control	Type	Outcomes measured	Effects
	1100 p.p.m. FD supplied with $\text{Ca}^{2+}/\text{PO}_4^{3-}$ (Enamelon Inc., Granbury, NJ, USA)		Clinical trial (patients with head and neck radiation)		Reduction of root caries incidence/inhibition of caries progression and demineralization
Singh, 2009 ⁴⁴	Compliant patients using 11% NaF with neutral supersaturated $\text{Ca}^{2+}/\text{PO}_4^{3-}$ mouthrinse (Caphasol; EUSA Pharma, Princeton, NJ, USA) at least once a day	Non-compliant patients using the same intervention less than once a day	Clinical trial (patients with xerostomia)	Risk for caries (surface at risk)	
Diamanti, 2010 ³³	Toothpaste slurries with 7.5% CSFS/1450 p.p.m. F/2800 p.p.m. F/5000 p.p.m. F	PD	Laboratory study	Knoop hardness test	
Vale, 2011 ²⁸	APF gel (12.3% F; Dentsply, Petropolis, Brazil) + 1100 p.p.m. FD	PD, 1100 p.p.m. FD, APF gel + PD	<i>In situ</i> trial	Surface hardness loss, integrated mineral loss, fluoride analysis in biofilm fluid and solids, determination of CaF_2 and FAp in dentine	
Giacaman, 2012 ⁵⁰	5 p.p.m. F bovine milk	0.9% NaCl, no F bovine milk; 0.05% NaF	Laboratory study (bovine)	Biofilm measurement, surface hardness loss	
Garcia-Godoy, 2013 ⁴⁷	Xylitol FD (1100 p.p.m. sodium monofluorophosphate, 0.020% xylitol; Fisher Scientific, Fair Lawn, NJ, USA)	No treatment; 1150 p.p.m. FD (Aquafresh® Advanced; GlaxoSmithKline, Moon Township, PA, USA); Experimental xylitol PD (0.45% xylitol; LMKao LLC, New York, NY, USA) Whole milk 35% fat	Laboratory study	Mean lesion depth	
Arnold, 2014 ⁵²	Whole milk 35% fat + 25 p.p.m./10 p.p.m. fluoride	PD	Laboratory study (bovine)	Lesion depth, elemental distribution	
Borelho, 2014 ²³	1100 p.p.m. FD (NaF, silica-based) (Colgate-Palmolive, São Bernardo do Campo, Brazil)	PD	Laboratory study (bovine)	Surface hardness loss	
Garcia-Godoy, 2014 ⁴¹	Denitricious 5000 FD (5000 p.p.m. F + xylitol; Research Based Professional Products, Aventura, FL, USA); PreviDent® 5000 dentifrice (5000 p.p.m. F; Colgate-Palmolive, USA); AIM® dentifrice (1500 p.p.m. F; Church & Dwight, Princeton, NJ, USA); Listerine® dentifrice (1300 p.p.m. F; Johnson & Johnson, Skillman, NJ, USA); Crest® Regular Paste (1500 p.p.m. F; Procter & Gamble, Cincinnati, OH, USA)	No treatment	Laboratory study	Mean lesion depth	
Arthur, 2015 ⁵⁵	1450 p.p.m. FD (twice a day) + 226 p.p.m. F mouthwash (Colgate Plax; Colgate-Palmolive, São Bernardo do Campo, Brazil) (once a day)	DW (three times a day); 1450 p.p.m. FD (Colgate Total) (twice a day); 1450 p.p.m. FD (three times a day)	Laboratory study (bovine)	Lesion depth, integrated mineral loss, Knoop hardness test	

(continued)

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Table 1 continued

First author, year	Intervention	Control	Type	Outcomes measured	Effects
Katsura, 2016 ²²	10% CPP-ACP paste (GC Corporation, Tokyo, Japan) with NaF toothpaste	NaF toothpaste alone, non-CPF-ACP	Clinical trial (patients with head and neck radiotherapy) Laboratory study (bovine)	Surface texture change	
Magalhaes, 2016 ⁶¹	TiF ₄ /NaF solution (500 p.p.m. F, pH 44); NaF varnish (24 500 p.p.m. F, pH 50); TiF ₄ varnish (24 500 p.p.m. F, pH 10); TiF ₄ varnish + TiF ₄ /NaF solution; NaF varnish + TiF ₄ /NaF solution	No treatment		Mineral loss; tooth erosion	
Nobrega, 2016 ²⁶	1100 p.p.m. FD (Colgate Palmolive, Brazil), once, twice or three times a day	PD	Laboratory study (bovine)	Surface hardness loss	
Thanatvarakorn, 2016 ⁶²	418% ZnF ₂ ·4H ₂ O in HCl (F = 9048 p.p.m., Zn = 156%, pH 2.7); 418% ZnF ₂ ·4H ₂ O (partially dissolved; F = 9048 p.p.m., Zn = 156%)	SDF (Saforide; Bee Brand Medico Dental, Osaka, Japan; 44 880 p.p.m. F, 255% Ag ⁺); Potassium fluoride (44 880 p.p.m. F, neutral pH); APF (NaF in 01 M H ₃ PO ₄ ; 9048 p.p.m. F, pH 3.8); no F treatment	Laboratory study (bovine)	Lesion depth, mineral loss, collagen degradation (erosion depth)	
Fernandez, 2017 ²⁵	APF gel (12 300 p.p.m. F, pH 3.6–3.9; DFL, Petropolis, Brazil) +1100 p.p.m. FD	PD; 5000 p.p.m. FD (NaF/silica, Colgate Palmolive, Brazil)	Laboratory study (bovine)	Cross sectional hardness; loosely/firmly bound fluoride concentration	
Commercially available fluoridated sealants used for root caries prevention Gernhardt, 2007 ²⁷	Sybaac Classic (Vivadent) and Xeno III (Dentsply) dentine bonding agents; Hyposen (lege artis, Frankfurt, Germany) desensitizer	No treatment	Laboratory study	Lesion depth	Reduction of root caries incidence/inhibition of caries progression and demineralization
Daneshmandi, 2008 ⁶⁶	(F-releasing adhesives) Reactmer Bond (Shofu Inc., Kyoto, Japan) and Shake One (Shofu)	(Non F-releasing adhesive) Hybrid Bond (Sun Medical, Moriyama, Japan)	Laboratory study (bovine)	Biofilm measurement	
Walter, 2008 ⁸³	(Adhesive systems) Gluma Comfort Bond + Desensitizer (Heraeus Kulzer); Gluma Comfort Bond (Heraeus Kulzer); Bond (Heraeus Kulzer); One-up Bond F (Tokuyama, Tokyo, Japan)	No treatment or etched with 37% phosphoric acid	Laboratory study (bovine)	Lesion depth	
Shiroya, 2012 ⁷⁰	F-releasing varnish containing surface reaction-type prereacted glass-ionomer (S-PRG) fillers (Shofu)	An S-PRG filler-free varnish	Laboratory study (bovine)	Mineral profiles, integrated mineral loss, lesion depth	
Sohn, 2012 ²⁷	(F-containing desensitizers) Clinpro XT Varnish (3M ESPS); Seal & Protect (Dentsply); Clearfil Protect Bond (Kuraray, Tokyo, Japan)	No treatment	Laboratory study	Lesion depth, calcium loss	
Oshima, 2015 ⁶⁸	MS Coat One (containing methacrylate-co-p-styrene sulfonic acid (MS polymer)	No treatment; 1% oxalic acid	Laboratory study (bovine)	Mineral loss	

(continued)

Table 1 continued

First author, year	Intervention	Control	Type	Outcomes measured	Effects
Miyajima, 2016 ⁶⁹	and 1% oxalic acid; MS Coat F (containing MS polymer, 1% oxalic acid and 3000 p.p.m. sodium fluoride) Calcium-fluoroaluminosilicate glass-based desensitizer (Nanoseal; Nippon Shika Yakuhin, Yamaguchi, Japan)	No treatment	Laboratory study	Mineral loss, lesion depth	
Cross-linkers Walter, 2008 ¹³⁴	0.5% proanthocyanidin (PA); 0.625% genipin	5% glutaraldehyde (GA)	Laboratory study (bovine)	Lesion depth; root surface colonization	Reducing demineralization and with the potential to promote remineralization
Xie, 2008 ⁷⁶	65% grape seed extract (GSE)	No treatment; 1000 p.p.m. F (NaF)	Laboratory study	Knoop hardness test; mineral precipitation profiles	
Hiraishi, 2011 ⁷⁴	10 p.p.m., 100 p.p.m., 1000 p.p.m., 10 000 p.p.m. hesperidin (Wako Pure Chemical Industries, Tokyo, Japan)	2000 p.p.m. CHX (Sigma-Aldrich, St Louis, MO, USA)	Laboratory study (bovine)	Collagen degradation (calcium release); lesion depth; mineral loss	
Pavan, 2011 ¹¹⁰	65% GSE + 1000 p.p.m. F	DW; 65% GSE; 1000 p.p.m. F	Laboratory study	Lesion depth; mineral loss; collagen degradation depth	
Benjamin, 2012 ⁷⁷	65% GSE	No treatment; Sodium monofluorophosphate (220 p.p.m.) with 0.05% calcium glycerophosphate or 0.5% calcium	Laboratory study	Relative optical density	
Guo, 2012 ⁷⁸	4000 mg/L <i>Galla chinensis</i> extract	glycerophosphate Artificial saliva; DW	Laboratory study (bovine)	Lesion depth	
Islam, 2012 ⁷⁵	0.5% hesperidin (Wako Pure Chemical Industries); 0.5% GSE (Gravimol® grape seed extract; Kikkoman, Chiba, Japan)	0.2% CHX (Sigma-Aldrich)	Laboratory study	Lesion depth, mineral loss	
Tang, 2013 ¹³⁵	5%, 10%, 15% GSE (acidic and neutralized)	DW	Laboratory study	Vickers hardness test	
Epasinghe, 2015 ⁸²	65% PA + Cpp-ACFP paste	65% PA; Cpp-ACP paste; CPP-ACFP paste; 1000 p.p.m. F; DW	Laboratory study	Lesion depth, mineral loss, Knoop hardness test	
Epasinghe, 2016 ⁸⁰	65% PA; 65% naringin; 65% quercetin	1000 p.p.m. F; DW	Laboratory study	Lesion depth, mineral loss, Knoop hardness test	
Epasinghe, 2017 ⁸¹	65% PA + tri-calcium phosphate (TCP) + F	65% PA; TCP + F; 1000 p.p.m. F; DW	Laboratory study	Lesion depth, mineral loss, Knoop hardness test	
MMP inhibitors Fukuda, 2009 ⁹⁴	0.042%; 0.083%; 0.167%; 0.330% pirocton olamine (PO)	0.07% NaF	Laboratory study (bovine)	Lesion depth, mineral loss	Inhibition of mineralization
Hannas, 2016 ⁷⁹	0.61% green tea extract; 0.12% or 0.004% CHX	PD; 1100 p.p.m. F	Laboratory study	Mineral loss	
Antimicrobial treatments Bacá, 2009 ⁹²	1% CHX – 1% thymol (Cervitac® varnish; Vivadent)	A placebo varnish	Clinical trial	Plaque index, gingival index, DF1, root caries index, oral	

(continued)

Table 1 continued

First author, year	Intervention	Control	Type	Outcomes measured	Effects
Bizhang, 2015 ²²	1% CHX, 1% thymol (Cervitec plus [®] , Ivoclar Vivadent); 10% F ⁻ (Bifluoride 10 [®] , VOCO, Cuxhaven, Germany)	No treatment	<i>In situ</i> trial	biofilms levels; RCL incidence; lesion size; changes in texture/colour	Arrest and remineralization of caries lesions
Zheng, 2011 ⁹¹	0.2% CHX (Corsodyl; SmithKline Beecham Consumer Healthcare, Maidenhead, UK); Listerine (IDS Manufacturing); Fluoride Listerine (IDS Manufacturing)	DW	Laboratory study	Integrated mineral loss, lesion depth	Antibacterial effects, prevention of caries
Rolland, 2011 ⁹³	Clearfil SE Bond (SE; Kuraray Medical; MDPB-containing resins as sealants)	Clearfil Protect Bond (PB; Kuraray Medical)	Laboratory study	Total CFU and percentage reduction in CFU	
Mei, 2013 ⁸⁹	0.12% CHX	DW	Laboratory study	CFU	
Tan, 2010 ⁸⁸	OHI + 38% SDF solution (Saforide; Toyo Seiyaku Kasei, Osaka, Japan)	OHI + 1% CHX varnish OHI + 38% SDF	Clinical trial	Mean number of RCL	Prevention and arrest of caries
Zhang, 2013 ¹⁶	38% SDF (Saforide; Toyo Seiyaku Kasei Co Ltd) + OHI + OHE	OHI + 38% SDF	Clinical trial	Exposed sound root surfaces; DFRS; arrested root surfaces; mean number of new/arrested root caries surfaces	
Li, 2016 ¹⁷	38% SDF solution (Saforide; Toyo Seiyaku Kasei); 38% SDF solution + KI solution (236 mol/L)	Placebo (soda water with a bitter flavour)	Clinical trial	Mean number of exposed sound root surface/DFRS/active	
Baysan, 2007 ⁹⁴	Ozone (HealOzone, Ka Vo Dental, Germany) + root sealant (Seal & Protect; Dentsply International, Surrey, UK)	No treatment control; ozone; root sealant	Clinical trial	rates of baseline active caries ECM, DIAGNOdent device, clinical assessment (colour, hardness, cavitation, dimensions, distance from the gingival margin, and severity index)	Reversal of leathery root caries
van Strip, 2008 ⁹⁰	0.2% CHX mouthrinse (Corsodyl; GlaxoSmithKline, the Netherlands)	Saline (0.9% NaCl) mouthrinse	<i>In situ</i> trial	Integrated mineral loss; lesion depth; lactic acid analyses	Prevention of demineralization
Lasers Heya, 2008 ⁹⁷	Mid-infrared Free Electron laser (90 or 97 μ m)	No treatment	Laboratory study	Morphological and chemical changes	Prevention of caries
de Souza-Zatoni, 2010 ⁹²	106 μ m CO ₂ laser (25 J/cm ² ; 40 J/cm ² ; 50 J/cm ² ; 60 J/cm ² per shot) (UM-L30; Union Medical Engineering, Yangju-si, Gyeonggi-do, Korea)	No treatment	Laboratory study	Mineral loss, morphology	
Parisotto, 2010 ¹³⁶	CO ₂ laser (106 μ m) (UM-L30; Union Medical Engineering Co) + Acidulated phosphate fluoride (APF) gel (12.3% F, pH = 36–39; DfI Industria E Comercio SA, Rio de Janeiro, Brazil)	No treatment; 12.3% APF gel; CO ₂ laser	Laboratory study (bovine)	Knoop hardness test	
Colucci, 2012 ²⁴	CO ₂ laser (106 μ m, 625 J/cm ²) (PC015D; Shanghai Jue Hua Laser Technology	No treatment; 12.3% APF gel; CO ₂ laser	<i>In situ</i> trial	Knoop hardness test	

(continued)

Table 1 continued

First author, year	Intervention	Control	Type	Outcomes measured	Effects
Esteves-Oliveira, 2011 ²⁹	Development, Shanghai, China) + 12.3% APF gel (Bioquant, São Paulo, SP, Brazil) CO ₂ laser (106 nm, 8 or 11 J/cm ²) (UM-L30; Union Medical Engineering, Gyeonggi-do, Korea) with or without 12.3% acidulated phosphate fluoride (APF) gel (DPL, Rio de Janeiro, Brazil) Er, Cr:YSGG laser (464 or 892 J/cm ²) (WaterLase Millennium TM ; Biolase Technologies Inc., San Clemente, CA, USA) with or without 2% neutral NaF gel (Colortana Maybelline, São Paulo, SP, Brazil) with or without water cooling (54 mL/min)	No treatment; APF gel	Laboratory study (bovine)	Calcium and phosphorous contents released in the de- and remineralization solution	
Geraldo, 2014 ¹⁰¹		No treatment; 2% NaF gel	Laboratory study	Knoop hardness test	

CFU = colony-forming units, CHX = chlorhexidine, CPP-ACP = casein phosphopeptide-amorphous calcium fluoride phosphate, CSPS = calcium sodium phosphosilicate, DFKS = decayed and filled root surfaces, DFT = decayed and filled teeth, DMFS = decayed, missed and filled surfaces, DMFT = decayed, missed and filled teeth, DW = distilled water, ECM = electrical caries monitor, Er, Cr:YSGG = erbium, chromium:yttrium-scandium-gallium-garnet, FAP = fluorapatite, FD = fluoride dentifrice, GCE = *Galla chinensis* extract, GSE = grape seed extract, NaF = sodium fluoride, OHE = oral hygiene education, OHI = oral hygiene instruction, PD = placebo dentifrice, RCL = root caries lesions, SnF₂ = stannous fluoride, TCP = tri-calcium phosphate, TiF₄ = titanium fluoride.

vehicle and are the major source of fluoride in some communities where water fluoridation is not available.³⁸ The effect of frequently-used standard fluoride (1000–1450 p.p.m.) toothpaste in reducing coronal caries experience has been well established, but its effect on dentine and RCL has not been extensively explored.³⁹ A 2-year follow-up study amongst community-dwelling elders using fluoride toothpaste showed a lower incidence of RCL and greater reversal of caries activity of non-cavitated carious lesions.⁴⁰ Evidence from several publications indicated a dose-response outcome of fluoride treatment. Specifically, a laboratory study showed that 5000 p.p.m. fluoride dentifrice was more effective for controlling RCL formation and progression than a fluoride concentration of 1300 or 1500 p.p.m. ($P < 0.05$)⁴¹. In addition, toothpastes with higher fluoride concentration (5000 and 2800 p.p.m.) improved the acid resistance of bovine root dentine more effectively than 1450 p.p.m. fluoride toothpaste.³³ The dose-dependent property was further confirmed in a clinical trial where 5000 p.p.m. fluoride toothpaste controlled RCL progression more efficiently among elders than the regular toothpastes of 1000–1450 p.p.m. fluoride.⁴² However, a large variability of the improvements was observed among participants with 5000 p.p.m. fluoride treatment in a multicentre clinical trial according to the standard deviation of surface hardness data.²⁰ Further to this, a pilot *in situ* study indicated that 1100 p.p.m. fluoride toothpaste was enough to reduce root dentine demineralization in a high cariogenic environment; however, the sample size was relatively small.²³ Follow-up clinical trials are necessary to confirm if use of higher fluoride concentration should be considered as the best practise for RCL management.

The effectiveness of fluoride dentifrices is not only determined by fluoride concentration but also the frequency of use. Fluoride should be constantly available in the oral fluids to maximize its effect. An *in situ* study demonstrated a positive correlation between the frequency of use and the reduction of root dentine demineralization, although no significant association was found between the frequency and the remineralization of existing carious tissue.²⁶

In order to improve the clinical effectiveness of fluoride dentifrices, especially in severe acidogenic conditions, new approaches have focused on incorporation of other active ingredients, especially calcium and phosphate to enhance existing salivary sources. Fluoride can reverse and arrest early dentine lesions by driving calcium and phosphate back into the lesion as low-solubility fluorapatite (Ca₅(PO₄)₃F). Together with soluble calcium and phosphate ions, fluoride further enhances the remineralization process. Calcium sodium phosphosilicate bioactive glass (NovaMin) has been added to a

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fluoride dentifrice (ReNew paste; Sultan Healthcare, Hackensack, PA, USA). When contact with water occurs, NovaMin dissolves and releases its inorganic calcium, sodium and phosphate ions that putatively initiate remineralization of the lesions, however, surface precipitation due to the poor stability of the ions is likely to be the major net activity.⁴³ Clinically, remineralizing toothpaste, supplied with fluoride, calcium and phosphate ions, was especially beneficial for patients after head and neck radiation or those with hyposalivation who have limited availability of calcium and phosphate ions in the reduced quantity of saliva. The toothpaste significantly lowered the incidence of RCL compared with the solely fluoride-containing dentifrice.^{34,44}

As fluoride does not directly target the reduction of dental plaque which is the major aetiological factor in conjunction with a diet rich in fermentable carbohydrates, components with caries-preventive benefits have been added to fluoride dentifrices to complement the effects. Being a non-fermentable sugar substitute, xylitol inhibits glycolysis and enzymes involved in the metabolism and growth of *Streptococcus mutans* or induces less virulent strains of cariogenic bacteria.⁴⁵ Specifically, a highly controlled RCT demonstrated that xylitol lozenges had a significantly pronounced caries-preventative effect on root surfaces compared with the sucralose-sweetened placebo lozenge, though not on coronal surfaces, probably because of the differences in composition and virulence between root and coronal biofilms related to anatomical factors.⁴⁶ However, no significant reduction in lesion depth formation was observed with the addition of xylitol into the fluoride dentifrice. This may be attributed to a lack of biofilm in the experimental physicochemical model used in this laboratory study where xylitol hardly showed any effect on bacterial metabolism.⁴⁷ In addition, factors that increase biofilm pH can modify caries susceptibility. Arginine, as a semi-essential amino acid, can be metabolized to ammonia that helps to neutralize plaque acids, thus reducing caries initiation. Fluoride dentifrices containing 1.5% arginine were shown to re-harden more RCL than a matched fluoride dentifrice, indicating the supplementary benefit to the established effects of fluoride dentifrice.^{19,31}

Antibacterial agents, such as triclosan, are added into fluoride toothpaste. Triclosan is a chlorinated aromatic compound that has functional groups of phenols with antibacterial properties. A 3-year clinical trial among adults revealed a significantly lower Katz Root Caries Index (expressed as a percentage of number of root surfaces with RCL / number of surfaces with gingival recession)⁴⁸ value in the triclosan group (Colgate Total; Colgate-Palmolive, New York, NY, USA) than an identical fluoride toothpaste without triclosan.³⁰

Methods of topical fluoride delivery have also been developed as mouthrinses, gels, milk additives and varnishes. Different fluoride formulations are chosen according to the purpose of fluoride use and target group of people. Bovine milk fluoridation is a convenient and cost-effective way of targeted fluoride supplementation, which was first proposed in school-based milk fluoridation programmes for children at a concentration of 5 p.p.m. F^- . Recently milk fluoridation has been considered for managing RCL. Daily intake of milk supplemented with 5 p.p.m. fluoride has been reported to have a reversing effect on RCL in older adults in a clinical trial. However, limitations existed regarding the high dropout rate, and methods of outcome measurement.⁴⁹ Also, the subjects were supplied with fluoride toothpaste which would most likely exert an additional effect. In a laboratory study using a *S. mutans* biofilm model with pH-cycling, 5 p.p.m. fluoridated bovine milk reduced decrease in surface hardness of dentine compared with milk only, although with no statistically significant difference.⁵⁰ Whether a 5 p.p.m. fluoride concentration in milk is enough to achieve an optimal anticaries effect on root dentine remains controversial. Moreover, other components in milk, such as protein, fat and other minerals may influence its protective effect, possibly because they serve as a physical barrier against the acids or buffer-related caries initiation and progression.^{50,51} A dose-dependent effect of fluoridated milk was reported.⁵²

Patients with hyposalivation or who have undergone radiotherapy in the head and neck region are at high risk for root caries because of the reduction in salivary flow rate. Topical fluoride application may be insufficient to control RCL in this group of people due to the decreased amount of intrinsic Ca^{2+} and PO_4^{3-} to drive remineralization. Use of varnish is able to deliver high concentrations of fluoride, which has been recommended among moderate- to high-risk patients. Topical 5% sodium fluoride (NaF) varnish (Colgate-Palmolive) has been tested in a group of participants with Sjögren's syndrome in a 24-month clinical trial. However, no clear evidence of its preventive effects in this group of patients was determined, probably attributable to an insufficient sample size and limited length of the study.¹⁸

An alternative approach to address the effects of reduced salivary flow amongst these groups of people uses calcium and phosphate ion supplementation along with fluoride, designed to mimic the supersaturation of those ions in normal saliva. Regular use of a supersaturated Ca^{2+}/PO_4^{3-} rinse in conjunction with commercially available 1.1% NaF showed its protective effect against caries progression in hyposalivating patients in a long-term study (up to 5 years).⁴⁴ In addition, Ca^{2+} and PO_4^{3-} at high concentrations can

be stabilized in a metastable solution using casein phosphopeptides (CPP) as the stabilizing agent, creating nanocomplexes (CPP-ACP).⁵³ The addition of 10% CPP-ACP paste to topical fluoride applications was observed to significantly reduce incidence of soft RCL in cancer patients subjected to head and neck radiotherapy.³²

The combined application of several fluoridated-products contributed to some additive effects for dentine caries prevention, which ensure a high fluoride concentration at oral surfaces or in saliva. Daily regular use of 1100-p.p.m. fluoride dentifrice combined with acidulated phosphate fluoride (APF) gel (12 300 p.p.m. F) application enhanced the availability of fluoride in biofilm fluid and significantly reduced the surface hardness loss and integrated mineral loss compared with the 1100-p.p.m. dentifrice alone when using *in situ* models.²⁸ However, the combined use of APF gel (12 300 p.p.m. F) with 1100-p.p.m. fluoride dentifrice or 22 600-p.p.m. fluoride varnish with 1450-p.p.m. fluoride dentifrice was not as comparatively effective as a higher 5000-p.p.m. fluoride dentifrice in inhibiting and repairing dentine caries lesions.^{25,54} Also, the sole use of fluoride dentifrice three times a day exhibited a higher caries-inhibiting effect than 'twice a day' even with a combined use of a fluoride mouthwash.⁵⁵ It was suggested that the treatment effect of fluoride could be not only ascribed to the fluoride component but also to the mode of application.¹²

Sodium fluoride and sodium monofluorophosphate are the most commonly used fluoride agents for toothpastes.^{39,56} Their mechanism of action is attributed to the precipitation of a low acid-resistant calcium fluoride (CaF₂)-like layer on the tooth surface as a 'mechanical barrier' or the bioavailability as plaque F reservoirs in the types of either mineral CaF₂ or biological/bacterial Ca-F (the reaction between fluoride and the intercellular or intracellular Ca 'bridge' forming Ca-F bonds at fixed bacterial sites).⁵⁷ However, it has been suggested that the fixation of fluoride would be enhanced if it is bound to a polyvalent metal such as titanium or tin, creating titanium fluoride (TiF₄) or stannous fluoride (SnF₂).^{58,59}

Titanium ions from TiF₄ may reduce demineralization by binding phosphate from apatite and forming a glaze-like layer on the tooth surface. The titanium-rich deposits were comparatively acid-resistant with a greater protective effect compared to NaF and SnF₂.⁶⁰ TiF₄ application in a low-concentration solution and high-concentration varnish were effective against root dentine erosion.⁶¹ Moreover, acidic zinc fluoride (ZnF₂/HCl) was investigated in laboratory research and exhibited inhibitory efficacy on dentine demineralization and collagen degradation.⁶² Although the polyvalent metal ions have

demonstrated their unique advantages in conjunction with fluoride, the sustainability of their effects and their cytotoxic effects need further investigation. Organic fluorides such as amine fluoride (AmF) compounds have also shown superior anticariogenic properties. The amine (organic) component has an antiplaque effect and a tensioactive property which allows accumulation of fluoride close to the tooth surface.⁶³ The results of a 1-year clinical trial indicated the potential of toothpaste and mouth-rinse containing AmF in remineralizing active soft and leathery primary RCL in an adult high caries risk population.⁶⁴

Commercially available fluoridated sealants used for root caries prevention

When root surfaces are exposed as a result of gingival recession, some areas may be prone to rapid RCL initiation. In this regard, root surface sealants such as desensitizers and adhesives, which contained effective anticaries components such as fluoride, chlorhexidine (CHX) and calcium-fluoroaluminosilicate, can be used on these susceptible areas to provide direct physical protection with a simple application, thus serving as an adjunctive option for the prevention of RCL. Their effects were assessed in seven papers.^{27,65-70}

Coating root surfaces with adhesive materials has been regarded as a promising procedure to prevent demineralization.⁷¹ A fluoride-releasing material was incorporated into adhesives (i.e. Giomer products)⁷² using a novel technique by pre-reacting acid-reactive glass containing fluoride with polyacrylic acid in the presence of water, which is presented as a stable glass-ionomer phase in fillers. Root dentine specimens coated with this adhesive showed decreased susceptibility for biofilm formation and increased acid resistance, thus protecting the root surface from carious lesion development.^{66,70}

Fluoride-containing resin-based desensitizing agents were first introduced to treat dentine hypersensitivity by penetration and precipitation of calcium fluoride within the dentinal tubules resulting in tubular occlusion.⁷³ They are currently used as an adjunctive option for the prevention of RCL, as they are able to enhance the chemical resistance of dentine to mineral dissolution against an acidic challenge and the polymerized resin layer is able to act as a physical barrier. Also, fluoride ions have been incorporated into a delivery carrier or vehicle, such as a polymer or a silicate glass, which exerted an additional benefit.⁶⁷⁻⁶⁹

Cross-linkers

Organic components of type I collagen fibrils are embedded with mineral crystallites in root dentine,

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and play an important role in the demineralization and remineralization process. After depletion of the mineral phase of dentine, the exposed collagen fibres are fragile and easily attacked by enzymes. The irreversible destruction makes remineralization difficult to achieve. Herein, attempts have been made to preserve and stabilize the dentine collagen matrix.

Several naturally-derived cross-linkers, such as hesperidin (HPN),^{74,75} grape seed extract,^{76,77} *Galla chinensis* extract,⁷⁸ green tea extract,⁷⁹ naringin and quercetin⁸⁰ have been applied on root dentine surfaces in laboratory studies. The role of cross-linking agents to decrease proteolysis in root caries have been extensively studied in 11 papers. However, the cross-linking agents have only been investigated in laboratory-based studies with no clinical trials so far.

Most of the natural cross-linkers are bioflavonoid polyphenol-rich agents with the chemical structure based on a phenyl ring bearing a hydroxyl group. The interaction of natural cross-linkers with the collagen fibres via covalent, ionic, hydrogen bonding or hydrophobic interactions stabilizes the collagen matrix. Significantly lower values of lesion depth and mineral loss during laboratory pH cycling indicated the cross-linkers' potential in inhibiting demineralization and promoting remineralization in natural RCL. Their effects could be enhanced when combined with other remineralizing agents, such as tri-calcium phosphate, casein phosphopeptide-amorphous calcium fluoride phosphate (CPP-ACFP) and fluoride in laboratory studies.^{81,82} Synthetic cross-linking agents have also been used, among which the most widely applied is 5% glutaraldehyde. It has been incorporated in commercial adhesive systems, such as iBond (Heraeus Kulzer, Hanau, Germany) and Gluma Comfort Bond + Desensitizer (Heraeus Kulzer). Reduced mean lesion depth was observed during pH cycling after treatment with the adhesives, indicating their potential to aid in RCL prevention.⁸³ However, no cross-linkers have been tested in clinical trials as yet and their mechanism in remineralization requires further investigation, not to mention the potential to stain the root dentine.

Collagenase inhibitor

A group of collagenolytic proteinases in dentine, for instance, matrix metalloproteinases (MMP) and cysteine cathepsins, participates in the caries process of dentine by degrading the denuded organic matrix after demineralization. Collagenase inhibitors are considered to be effective in decreasing RCL progression by suppressing enzyme activity. Application of MMP inhibitors in the prevention of dentine destruction has been identified in two laboratory studies, which include CHX, green tea extract and

a new active collagenase inhibitor, pirocton olamine (PO).^{79,84}

Chlorhexidine has been reported to inhibit the activity of several host-derived proteases, such as MMP-2, -8 and -9, thereby protecting the exposed collagen matrix from denaturation.⁸⁵ It is now often used as a positive control to test the efficacy of other MMP inhibitors. Several aforementioned cross-linking agents, such as proanthocyanidin, green tea extract and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide exhibited inhibitory effects against endogenous collagenases.^{79,86,87} Efforts are still made in seeking new active collagenase inhibitors, such as PO which has a large hydrophobic benzoic ring and alkyl chain that contributes to the interaction with enzymes.⁸⁴ However, most studies were undertaken on coronal dentine. Future studies should concentrate on providing direct evidence of changes of enzyme activity in root dentine after exposure to these collagenase inhibitors.

Antibacterial agents

As dental caries is a biofilm-mediated disease of dental hard tissues, the application of antibacterial agents has a direct effect on dentine caries. Antibacterial therapeutics in RCL have been well documented in 10 articles. The agents for the suppression of cariogenic microorganisms ranged from silver diamine fluoride (SDF; Ag(NH₃)₂F) to CHX.^{16,17,22,88–93} Application of ozone was mentioned in one study, which was stated to have the clinical potential to reverse RCL or inactivate most of the organisms on dentine.⁹⁴

Chlorhexidine acts as a broad-spectrum antimicrobial agent suppressing the growth of selected cariogenic bacteria (*Streptococci*, *Lactobacilli*, *Actinomyces*) in the oral biofilm on root surfaces.^{89,90} However, the available evidence supporting CHX as a caries-protective agent is inconclusive. An *in situ* study using a commercially available 0.2% CHX mouthrinse (Corsodyl; GlaxoSmithKline, Zeist, the Netherlands) failed to show greater protection of dentine against demineralization compared with saline. However, CHX mouthwash was shown to be more effective in inhibiting putative root-caries bacteria than Listerine (an antiseptic mouth rinse containing a combination of essential oils, e.g. menthol, thymol, methyl salicylate and eucalyptol) or Listerine with Fluoride (IDS Manufacturing, Pathum Thani, Thailand) in a laboratory study.⁹¹ Nevertheless, CHX mouthwash was not suggested as a first-choice caries-protective agent as it may disturb the oral micro-ecological balance and the real oral environment is different from the laboratory-established biofilm.⁹⁰ Cervitec (Ivoclar Vivadent, Liechtenstein), a CHX-thymol varnish (1% CHX), helped to arrest

the progression of established RCL and reduce the incidence of new lesions, being applied as a simple, quick and non-invasive method among institutionalized elderly.^{22,92}

Silver diammine fluoride has an intense antibacterial effect on multi-species cariogenic biofilms (such as *S. mutans* and *Actinomyces naeslundii*) on tooth surfaces due to the antimicrobial effect of silver.⁹⁵ Thirty-eight per cent SDF solution was not only effective in preventing new coronal caries in children, but has also been considered as a novel root caries preventative strategy among community-dwelling elders. The intervention of an annual SDF application was shown to be preferable to solely oral hygiene education or oral hygiene instruction (OHI) in preventing new RCL over a 24-month period.^{16,88} However, no significant difference was found in the effectiveness in preventing RCL between SDF and other therapeutic interventions such as 1% CHX varnish or 5% NaF varnish. Thus, these three agents have similar advantages when used in clinical and community programmes.⁸⁸ As silver ions in the SDF solution can cause black staining, potassium iodide (KI) can be applied immediately after the application of the SDF as the iodide ions will react with the excess silver ions and form a precipitate of silver iodide, thus reducing the staining. Results from a clinical trial showed that application of KI did not influence the effectiveness of SDF in arresting active root caries.¹⁷

Ozone (O₃) can act as a disinfectant and degrade to harmless, non-toxic and environmentally safe oxygen molecules. The antimicrobial effect of aqueous ozone or ozone gas was first assessed in a laboratory study.⁹⁶ There has been only one RCT testing the efficacy of ozone for the management of 'leathery' RCL in the last decade. In this 6-month follow-up study, a portable apparatus was used to deliver ozone at a concentration of 2100 p.p.m. ± 10% either for 10 or 20 s. The treatment regime using ozone dramatically reduced most of the microorganisms in leathery root caries and led to re-hardening of some of the non-cavitated leathery RCL. This improvement may be maintained when ozone was reapplied regularly.⁹⁴ However, methodological concerns exist within studies in ozone therapy in the aspects of blinding, standardized methods of outcome measurement, sample size and length of studies. Extensive clinical evidence for ozone application is needed before it can be supported.

Surface modification with lasers

Dentine surface modification, which is generally defined as morphological and chemical changes of dentine, may be beneficial for root caries resistance.⁹⁷

It is frequently conducted by the application of lasers. Laser technology is increasingly finding a useful place in dentistry, especially in management of carious lesions. The mineral of dentine, namely impure carbonated hydroxyapatite, has intense absorption bands in the mid-infrared (MIR) region due to phosphate, carbonate and hydroxyl groups in the crystal structure.^{97–99} Every laser has a gain medium to produce the laser light which can be a gas, a fluid or a solid state and the frequently-used ones for altering dental hard tissues include CO₂ laser, free electron laser (FEL) and erbium, chromium:yttrium–scandium–gallium–garnet (Er, Cr:YSGG) laser. Studies retrieved have focused on their effects as well as exploring the optimum energy density and wavelength, with three using a CO₂ laser, one using a MIR-FEL⁹⁷ and two using an Er, Cr:YSGG laser.^{100,101}

CO₂ laser

CO₂ laser, with a wavelength of 10.6 µm, is highly absorbed by carbonated hydroxyapatite, significantly reducing root surface demineralization.⁹⁹ The most frequently mentioned hypothesis for its mechanism is based on the thermal effects of irradiation leading to ultrastructural and crystallographic changes. Moreover, only a small variation was detected in the pulp temperature when irradiated with a CO₂ laser at an energy density of 6–11 J/cm² for 10–20 s, much less than the irreversible damage temperature change of 5.5 °C, indicating the safety of irradiation.^{24,99,102} Combination of fluoride sources and CO₂ laser irradiation achieved a synergistic effect in reducing demineralization on sound dentine in the laboratory. However, *in situ* situations are more complicated where no additive benefit was found by combining an APF gel with CO₂ laser irradiation in controlling RCL.²⁴ Nevertheless, a clinical case report demonstrated the potential of complementing the respective effectiveness of fluoride and CO₂ laser by combined application.¹⁰³

Free electron laser

A tuneable MIR-FEL has been proposed for surface modification of root dentine and the extent of modification by varying wavelengths and average power has been investigated macroscopically.¹⁰⁴ Irradiated with MIR-FEL of λ = 9.0 or 9.7 µm, dentine surfaces exhibited increased acid resistance.⁹⁷

Erbium, chromium:yttrium–scandium–gallium–garnet laser

An Er, Cr:YSGG laser with a wavelength of 2.78 µm is usually applied for cavity preparation due to the

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mechanism of ablation. Lasers with lower energy densities may not ablate the treated surface but change the tissue morphologically or chemically, thus being used for preventive purposes. This viewpoint was confirmed by an atomic force microscopy study that Er, Cr:YSGG laser at a low-energy density parameter can significantly alter the micro-topography of radicular dentine.¹⁰⁰ A significant synergistic effect of Er, Cr:YSGG laser associated with 2% neutral NaF application was demonstrated in increasing acid resistance of human root dentine.¹⁰¹

METHODS TO MODEL ROOT CARIES

Natural dentinal caries is complex with variations in width, depth, severity and colour; thus, it is difficult to find similar lesions for laboratory studies. An artificial caries model system offers significant advantages over natural lesions for its consistency and reproducibility in the research for non-invasive treatment.

Van Dijk *et al.* presented a mathematical model to obtain an artificially caries-like lesion in 1979, which was followed by ten Cate *et al.* to produce artificial enamel lesions.^{105,106} Later, Xie *et al.* used the same method to produce artificial RCL.⁷⁶ In detail, demineralization was carried out in solutions undersaturated with respect to hydroxyapatite, with a pH ranging 4.4–5.0, for a time ranging from 16 h²⁶ to 5 days.^{35,77,107} Accordingly, caries-like lesions were created with depths of 70–100 µm.^{35,76,77,80} Although without bacteria or enzymes present, the chemical model systems could create lesions with remarkable histological similarities (especially the mineral content and hardness) to natural RCL of similar depths.¹⁰⁸

However, as organic acids produced by cariogenic *Lactobacilli* are considered to play a major role in dentine demineralization, another artificial dentine caries model based on lactate buffers has been proposed. Different acids yielded lesions with different characteristics that may impact on strategies for remineralization.¹⁰⁹

In addition to the above static method to create artificial lesions, pH-cycling models have been employed to mimic the dynamics of mineral loss and gain in carious lesion formation in the oral environment by exposing dental substrates to both demineralizing and remineralizing solutions. The exposure time in both solutions and the concentrations of the ions can be varied according to the purpose of the study. The duration of the whole process varies from 18 h to 14 days.^{74,82,110,111} The demineralization periods were evenly divided into two 3-h demineralization periods to more accurately simulate the time per day that teeth in the oral cavity would be exposed to the cariogenic challenge associated with meals.⁴³

However, pH cycling is a chemical model that is not able to estimate the antimicrobial effect of treatment substances on lesion progression or regression. Therefore, biofilm models have been created by using microbial inoculation for biofilm growth on root dentine specimens that forms carious lesions. However, controversy exists regarding how the biofilm is predominantly formed and the microbial diversity. Specifically, highly acidogenic and aciduric bacteria such as *S. mutans* and *Lactobacilli casei* have been considered important in the aetiology of root caries and adopted as the microbial source for biofilm growth on root dentine slabs.^{55,112} However, there are other organisms involved in natural RCL development. Accordingly, a consortium plaque biofilm composed of four species, namely *S. mutans*, *Streptococcus sobrinus*, *Lactobacilli naeslundii* and *Lactobacilli rhamnosus*, was constructed as a root caries model system.^{91,113} Although after incubation, the root dentine fragments showed alterations similar to that of root caries, the complex dental biofilm which consists of hundreds of bacterial species is difficult to simulate in a laboratory. Therefore, laboratory root caries models are often based on consortium biofilms of selected species of bacteria.^{112–114}

The complexity of the oral environment led to the development of sophisticated ‘artificial mouth’ systems that simulate the oral environment humidity, temperature, and salivary characteristics and flow as well as providing a continuous or intermittent supply of nutrients to the biofilm.¹¹⁵ The sophisticated model was also used for root caries research by Mei *et al.*,⁸⁹ where mixed salivary bacteria from individuals were obtained to form a microcosm and used to inoculate the root samples in the artificial mouth.

Removable intraoral palatal appliances containing dentine specimens have been used in some *in situ* studies, which are worn by volunteers. In this case, the root dentine samples are placed in the oral environment with the patients’ own bacteria to colonize specimens.^{22,24,26,28,90}

Laboratory models are simple, low cost, fast and reproducible, and are indispensable for evaluating the effects of different treatment methods for RCL. However, they cannot fully replicate the multifactorial nature of root caries in the oral environment and clinical trials are still required among multiple patients and variable oral conditions to draw valid conclusions.

RESTORATIVE TREATMENT

When the attempt to halt the progression of an RCL has failed and the lesion has advanced to frank cavitation, restorative treatment is then required. Restoration of RCL condemns the tooth to a repeat

restoration cycle and therefore efforts are made to explore the MID approach. Also, challenges exist regarding new RCL formation associated with restorations as well as the failure rates of restorations.

Concerning restorative treatment for RCL, improvements have been made in the aspects such as novel techniques in carious tissue excavation and modification of restorative materials and procedures. Three clinical trials investigated the clinical outcomes of atraumatic restorative treatment (ART) restorations.^{29,116,117} Four studies analysed the efficacy of lasers (CO₂ and Er, Cr:YSGG) for carious tissue excavation.^{118–121} Modification of restorative materials (such as adhesives, glass-ionomer cement (GIC) and resin composite (RC)) or improvement in restorative procedure were investigated in seven laboratory studies.^{122–126}

Techniques in carious tissue excavation

The conventional carious tissue excavation technique with rotary instrumentation has often resulted in considerable removal of tooth structure and extensive tissue loss, which is now considered to be overly destructive.¹²⁷ Also, it is not so feasible for use in some elders with physical or cognitive disorders as well as institutionalized patients, as they may not be able to visit a dental office.

Atraumatic restorative treatment

In light of the prevalent concept of MID, ART is a potential restorative technique for RCL management. It removes the completely necrotic carious tooth tissue with hand instruments while conserving the partially demineralized dentine. A restoration is then placed using an adhesive restorative material (usually GIC), which restores the cavity and seals any susceptible surfaces simultaneously. This could be more acceptable and accessible to frail elderly or nursing home residents than 'conventional' dental treatment.¹²⁸

The survival rates of ART restorations have been compared with those of the conventional rotary technique for periods of 6 months to 2 years, where the majority (>80%) of restorations were placed on one root surface only. Although significantly more failures could be observed in the ART than in the rotary technique, partly due to material failure, the success and survival rate were still high.^{29,116} As the removal of softened demineralized dentine by hand instruments may present challenges to the clinician in the elderly, a chemomechanical method using Carisolv® gel (Medi Team Dental, Gothenburg, Sweden), which is composed of sodium hypochlorite and three amino acids – glutamic acid, leucine and lysine – and applied to

assist the removal of denatured dentine compensating for the limitations of manual removal. However, the use of Carisolv gel did not improve the success rate of GIC restorations used with ART technique in root caries of a geriatric population.¹¹⁷ Admittedly, the failure of ART is associated with many factors, which include the restorative material used, operator, technique as well as oral health status of the patient. Still, ART is useful for outreach dental services, and warrants further investigation.

Lasers

Laser systems can be used for carious tissue removal and cavity preparation, which has been an area of major research interest. The efficacy of lasers depends on numerous factors that include the wavelength characteristics, pulse energy and repetition rate. The Er, Cr:YSGG laser is favoured in minimally invasive procedures, especially for the preparation of microcavities, although the need for such restorations to be undertaken has been challenged recently.¹²⁹ A cavity prepared by Er, Cr:YSGG laser provided the lowest microleakage after GIC restoration compared with that prepared by a carbide bur at low speed or by hand excavator. A possible explanation is that the heat generated during laser irradiation increased the amount of calcium in the dentine substrate which in turn improved adhesion of the GIC.¹²⁴ However, the improvement in microleakage prevention was not observed in RC restorations.¹¹⁹

Improvement of restorative materials and procedures

Management of RCL with restorative materials such as GIC, RC and amalgam can be time-consuming and intractable because of the concomitant problems, such as difficulty in moisture control, poor marginal adaptation and proximity to the pulp, which can lead to leakage, new carious lesions at the restorative margins and finally restoration replacement or tooth failure. Hence, there is an urgent need to evolve restorative materials and procedures.

Glass-ionomer cement seems particularly suitable for restoring RCL. The major advantage of GIC is its ability for chemical bonding to tooth structure, acting as an F⁻ reservoir in the oral cavity and possessing acceptable aesthetic qualities and biocompatibility. It was shown to confer a preventive effect against cariogenic challenge by a mixed-species oral biofilm in an artificial mouth culture system.¹³⁰ Various modifications have been made in GIC composition to improve its properties, such as addition of CPP-ACP (Fuji VII EP; GC Corporation, Tokyo, Japan) or enhancing fluoride release (Fuji IX GP; GC Corporation).¹³¹ Specifically, approximal RCL were

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restored with Fuji IX GP a mean survival time of 74 months.^{125,132}

Resin composite has also been widely used for restoration of root surface caries as it does not require excessive removal of sound tooth structure. However, bacterial invasion (microleakage) could happen from the defective margin between RC and tooth structure if the environment continues to be cariogenic. An alternative means of clinical management to reduce this problem is to provide the restorative material with a therapeutic effect, such as incorporating antibacterial and remineralizing components into the adhesive systems. The monomer, 12-methacryloyloxy dodecylpyridiniumbromide (MDPB), was developed by combining a quaternary ammonium group and a methacryloyl group into an adhesive system. The quaternary ammonium component presents a wide spectrum of antibacterial activity. The MDPB-containing adhesive significantly reduced recurrent lesion formation around restorations, being a good candidate for restoring RCL.¹²² Other novel multifunctional adhesives have been developed in the laboratory by adding substances with different activities. However, no clinical evaluation has occurred.¹²⁶ In addition, in the cases of deep cavities where restorative treatment is required, the intensity of the pulpal response should be considered and the thus use of liners have been evaluated. Resin-modified GIC (RMGIC) has been used as a lining material to protect the pulp from further bacterial invasion. Its application did not affect marginal adaptation and retention of restorations over an 18-month period.¹²³

Another strategy proposed to optimize the longevity of restorations is to involve additional steps within the restorative procedure. A conjunctive use of 1.23% APF gel or 2.0% neutral NaF gel with GIC and RMGIC restorations inhibited the progression of caries-like lesions adjacent to restorations.¹³³ Additionally, CO₂ lasers have been considered for inhibition of recurrent caries adjacent to restorations. Irradiation of cavo-surface margins of the prepared root surface with a $\lambda = 10.6 \mu\text{m}$ CO₂ laser at densities of 5.0 and 6.0 J/cm² reduced the demineralization with the rates of 17.05% and 20.59%, respectively, around RC restorations.^{118,120} Its efficacy in GIC restorations was related to different GIC products used.¹²¹

CONCLUSION

In conclusion, this literature review summarized the various treatment approaches for RCL in the last decade. Non-invasive or invasive treatment methods were chosen according to the progression of RCL. Agents and substances applied in non-invasive methods involved fluoride-containing products, cross-linkers, collagenase inhibitors and antibacterial agents. These

agents mainly aimed at reducing demineralization, promoting remineralization and suppressing cariogenic microorganism growth. Dentine surface modification conducted by lasers increased resistance to RCL formation. Restorative treatment is necessary when RCL have advanced to frank cavitation with significant dental tissue loss. Improvements have been made regarding carious tissue excavation techniques and modifications of restorative materials and procedures. As invasive treatment with restorations is merely symptomatic in nature without dealing with the essential causative factors of dental caries. It is of importance to determine the most appropriate treatment for RCL. Clinical trials are urgently needed to draw firm conclusions concerning the efficacy of the various treatment methods described.

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REVIEW

Effects of Collagen Crosslinkers on Dentine: A Literature Review

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Received: 13 June 2017 / Accepted: 9 October 2017 / Published online: 20 October 2017
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Abstract This aim of this review is to explore the current research related to crosslinking agents used on dentine. A systematic search of publications in PubMed and Web of Science databases was performed. Further retrieval was conducted using the search terms of specific names of crosslinkers. Reviews, conference abstracts, dissertation and theses, non-English articles, studies of intrinsic crosslinking of dentine, studies of adhesives without specific crosslinker components, studies of crosslinker applications in other collagenous tissues or tooth-like structures and irrelevant studies were excluded. Manual screening was conducted on the bibliographies of remaining papers to identify other relevant articles. One hundred and one articles were included in this systematic review and full texts were retrieved. Both synthetic and naturally derived crosslinkers have been found to exhibit significant effects in biomodification of dentine via their multiple interactions with the dentine matrix. A stable matrix network or a durable hybrid layer in dentine bonding could be achieved, where the dentine collagen fibrils show improved biochemical and biomechanical properties and enzymatic biodegradation is reduced. Although no crosslinkers have been tested in clinical trials, extensive research has been conducted in laboratory studies to investigate their poten-

tial applicability for inhibition of demineralisation and/or promotion of remineralisation, caries prevention as well as improvement of bonding performance of adhesive systems. Further studies are needed to develop the feasibility for clinical use, reduce side effects as well as explore mechanisms of action and long-term effectiveness.

Keywords Crosslinkers · Dentine · Biomodification · Dental caries · Resin–dentine bonds

Abbreviations

CTX	C-terminal telopeptide
CHX	Chlorhexidine
DMSO	Dimethyl sulfoxide
EDC	Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
GA	Glutaraldehyde
GNP	Genipin
GSE	Grape seed extract
HEMA	2-Hydroxyethyl methacrylate
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPN	Hesperidin
ICTP	Type I collagen C-terminal cross-linked telopeptide
MES	2-(<i>N</i> -morpholino) ethanesulfonic acid
MMPs	Matrix metalloproteinases
NHS	<i>N</i> -hydroxysuccinimide
PA	Proanthocyanidin
TA	Tannic acid
UVA	Ultra violet light A
μTBS	Microtensile bond strength

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Introduction

Dentine is a complex mineralised tissue of which organic components are embedded within mineral crystallites. Fibrillar type I collagen accounts for 90% of the organic matrix [1]. The molecules of collagen assemble extracellularly to form a complex network of intra- and inter-microfibrillar crosslinks, and the deposition and dispersion of the mineral, namely hydroxyapatite, tend to be governed by the collagen matrix [2].

The crosslinking of collagen contributes to the tensile properties of the dentine matrix [3]. However, the collagen matrix can be degraded mechanically or biochemically due to physiological and pathological processes. Specifically, dental caries, a process of demineralisation, seriously depletes the mineral phase of dentine which leads to the collagen fibres becoming denuded. The exposed collagen fibres are fragile and easily attacked by the bacterially derived enzymes or endogenous proteases, leading to an irreversible destruction of the dentine [4]. Additionally, the stability and integrity of collagen fibrils within the hybrid layer is crucial for long-term bond durability in restorative dentistry [3]. In this regard, the stabilisation and strengthening of collagen fibrils has been considered as a potential approach for possibly restoring dentine damaged by dental caries and improving bond durability.

The formation of endogenous crosslinks contributes to the stiffness of the dentine collagen. With the application of exogenous collagen crosslinkers, additional formation of inter- and intra- molecular crosslinks of the collagen can be induced, thus strengthening the dentine matrix and decreasing the biodegradation rate [5]. In light of this, biomodification of dentine matrices with crosslinkers is regarded as a promising strategy for tissue repair, and specifically a new adjunct to manage dentine affected by caries.

The aim of this paper was to review the literature to understand the current status of the application of crosslinking agents on dentine, with particular emphasis on (1) categorisation of commonly used crosslinkers in dentistry; (2) specimen and crosslinker solution preparation; (3) methods to examine the effects of crosslinkers, and (4) perspective future work.

Methods and Materials

An electronic search was undertaken using the Internet databases: PubMed and Web of Science. The online searching was conducted using the keywords: 'crosslinker' or 'cross-linker' or 'crosslinking agent' or 'cross-linking' and 'dentin*'. Specific names of crosslinkers were later

included in the search keywords: 'glutaraldehyde', 'carbodiimide', 'proanthocyanidin', 'hesperidin', 'genipin', 'naringin', 'quercetin', 'epigallocatechin gallate', 'tannic acid' and 'riboflavin'.

Publications were limited to those of English language and from scientific, peer-reviewed literature. The last search was conducted on 1 December 2016. The following categories were excluded during the assessment process: reviews, conference abstracts, dissertation and theses, non-English articles, studies of endogenous crosslinking of dentine, studies of adhesives without a specific crosslinker component, studies of crosslinker application in other collagenous tissues or tooth-like structures and irrelevant studies. Crosslinker types and concentrations, specimen preparation, solvents to dissolve crosslinkers and methods to examine their effects were summarised and evaluated.

Results

The initial electronic search identified 250 potentially eligible articles (164 in PubMed, 181 in Web of Science, 95 articles were duplicates). After initial screening of titles and abstracts, 160 were excluded, leaving 90 papers for further analysis. Five references were identified from the bibliographies of the remaining papers. Afterwards, specific names of the crosslinkers used on dentine were included in the search terms. An additional 5 studies were identified. Finally, 101 publications remained for full-text analysis (Fig. 1).

Forty-nine articles [5–53] concentrated on the effects of crosslinkers on the biomodification of dentine which contributes to the stabilised dentine matrix with improved biomechanics and stability and reduced biodegradation. Among them, nine papers [45–53] gave an insight into their effects on inhibition of demineralisation and/or promotion of remineralisation as well as caries prevention. The other 51 studies [3, 4, 44, 54–101] mentioned their potential application in resin–dentine bonding.

Thirteen articles concentrated on the effects of glutaraldehyde (GA) or aqueous mixtures of glutaraldehyde and 2-hydroxyethyl methacrylate (HEMA) named Gluma on dentine [24, 27, 28, 43, 45, 46, 60, 69, 77, 83, 86, 91, 94]. Normally, GA or Gluma was more often used as a positive control to test the efficacy of other crosslinkers. Thirteen papers investigated the effects of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) on dentine with different concentrations and treatment times [11, 16, 30, 39, 40, 44, 56, 82, 87, 88, 95, 96, 98]. As for the naturally derived crosslinkers, 55 publications [4–6, 8–10, 12–14, 19, 20, 22, 23, 26, 29, 31, 36–38, 41, 42, 47–55, 57, 63–67, 70–76, 78–81, 84, 89, 90, 92, 99–102] investigated the effects of proanthocyanidin

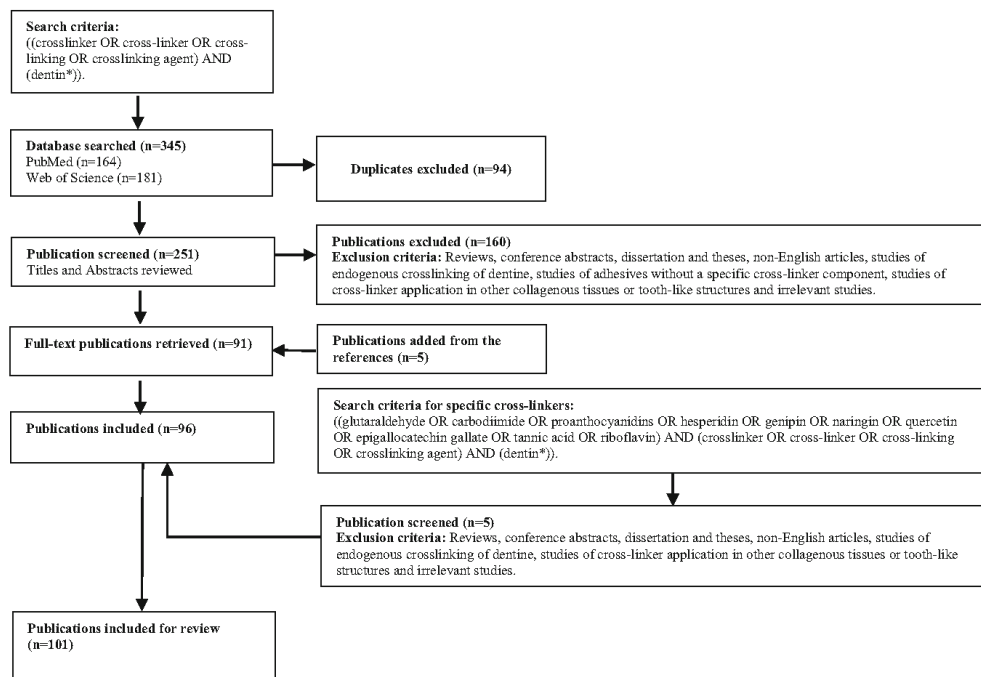


Fig. 1 Flowchart of the search strategy and results

(PA) or grape seed extract (GSE) on dentine, indicating it as a research focus. Among them, 6 articles compared the efficacy of PA from different sources, which ranged from cocoa seed, green tea, cinnamon and berries to curcumin [5, 12–14, 31, 33]. The primary source of PA was GSE. Other aspects investigated involved the concentration and treatment duration [23], solvents [14, 67], the pH to dissolve PA and its chemical composition [22, 26, 42]. Also, 2 studies applied PA in root canals to investigate how it impacted on endodontic treatment [75, 100]. Eleven papers investigated other categories of plant-derived crosslinking agents, including hesperidin [53, 73, 74], naringin, quercetin [48], epigallocatechin gallate (EGCG) [19, 41], tannic acid (TA) [7] and genipin (GNP) [9, 25, 51, 54]. A physical method, i.e. photo-oxidative, for crosslinking has been proposed and 13 articles explored the effects of a frequently used agent riboflavin [3, 17, 18, 21, 31, 33–35, 59, 61, 62, 68, 93]. Sixteen articles compared the effects of different crosslinkers, in which at least 3 types of crosslinkers were included [4, 6, 9, 19, 29, 31, 33, 51, 53–55, 70, 74, 76, 78, 93].

For the investigations on dentine collagen fibrils or hybrid layer, the dentine surface was typically

demineralised. Seventy-six studies used phosphoric acid as the demineralising agent, with 36 studies using concentrations varying from 32 to 37% and time ranging from 10 to 15 s, except for 2 studies that used 60 s [6, 59]. Twenty-nine studies reported the use of 10% phosphoric acid for 5–24 h to completely demineralise dentine. Ten studies used these two ranges of concentrations according to different outcomes measured [3, 18, 22, 44, 71, 77, 81, 82, 97, 98]. Nine studies used ethylenediaminetetraacetic acid (EDTA) at 17% or 0.5 M concentrations [21, 23–25, 27, 43, 75, 83, 88], except for 1 that used a concentration of 1 M [35]. Two studies used either phosphoric acid or EDTA according to different outcomes measured [19, 51]. Preparation of ‘artificial carious lesions’ by the pH-cycling procedure was reported in 4 studies [45, 47, 50, 52]. In bonding procedures, except for 5 studies that used a self-etching primer or respective bonding system etchant [7, 14, 73, 74, 80], most specimens were treated with 37% phosphoric acid to remove the dentine surface smear layer, which is consistent with the clinical application.

The majority of studies used sound or non-carious human coronal dentine, while 4 studies used human root

Table 1 Comparisons of effects of different crosslinkers on dentine

Author, year [Reference #]	Effects of crosslinkers	Outcomes measured	Comparisons			
			***	**	*	*
Bedran-Russo et al. [9]	Mechanical properties of dentine	Ultimate tensile strength (Demineralised dentine)	0.5% PA	0.625% GNP > 5% GA	–	–
Walter et al. [51]	Collagen stability	Hydroxyproline analysis (after collagenase degradation)	0.5% PA > 5% GA > 0.625% GNP	–	–	–
	Root caries prevention	Lesion depths	0.5% PA	5% GA > 0.625% GNP	–	–
Al-Anmar et al. [54]	Resin–dentine bond Strength	µTBS	6.5% GSE	5% GA	0.5% GNP	–
Islam et al. [53]	Collagen stability	Hydroxyproline analysis (of the remineralising solution after pH cycling)	0.5% HPN > 0.5% GSE	0.5% CHX	–	–
	Inhibition of demineralisation	Lesion depth, mineral loss	0.5% HPN	0.5% GSE > 0.5% CHX	–	–
Islam et al. [74]	Resin–dentine bond strength	µTBS	0.5% HPN	0.5% CHX > 0.5% GSE	–	–
		Microhardness of the bonded interface	0.5% HPN	0.5% CHX	0.5% GSE	–
Bedran-Russo et al. [6]	Mechanical properties of dentine	Elastic modulus of the bonded interface	6.5% GSE	5% GA > 0.3 M EDC	–	–
Hiraishi et al. [19]	Collagen stability	Hydroxyproline analysis (after collagenase degradation)	0.5% EGCG > 0.5% HPN > 0.5% PA	0.5% GA > 0.5% GNP	–	–
		Ultimate tensile strength	5% GA > 0.5% PA	0.5% HPN > 0.5% EGCG	0.5% GA > 0.5% GNP	–
Scheffel et al. [29]	Inhibition of dentine proteases	Inhibition of MMP activity	5% PA > 0.5 M EDC > 2% CHX	0.1 M EDC	–	–
Leme et al. [78]	Resin–dentine bond	Elastic modulus of the bonded interface	Adhesive- and ageing time-dependent	–	–	–
Seseogullari- Dirihan et al. [33]	Inhibition of dentine proteases	Hydrophilicity of the dentine matrix	6.5% PA > 5.75% EDC > 5% GA	0.1%, 0.5% riboflavin	–	–
Aydn et al. [55]	Resin–dentine bond	Loss of dry mass; solubilised telopeptides of collagen	5% GA; 5% GSE	GSE	EDC/NHS	–
Hass et al. [70]	Resin–dentine bond (in situ)	Collagen crosslinking	GA	–	–	–
		Micro-permeability	Adhesive-dependent	–	–	–
		Microhardness of the bonded interface	6.5% PA > 5% GA > 0.1% riboflavin	0.1% riboflavin	–	–
		µTBS	6.5% PA > 5% GA	5% GA > 0.1% riboflavin	–	–
		Inhibition of nanoleakage	6.5% PA	riboflavin	–	–

Table 1 continued

Author, year [Reference #]	Effects of crosslinkers	Outcomes measured	Comparisons	***	**	*
Hass et al. [4]	Resin–dentine bond	μ TBS	5% GA > 0.1% riboflavin > 6.5% PA (immediate); 6.5% PA > 0.1% riboflavin > 5% GA (18 months); 6.5% PA	–	–	–
Kim et al. [76]	Resin–dentine bond	Inhibition of nanoleakage	6.5% PA	–	0.1% riboflavin	5% GA
Sesegullari-Durhan et al. [31]	Inhibition of dentine proteases	Inhibition of MMP activity in the hybrid layer Inhibition of recurrent caries Inhibition of MMP activity	15% GSE > 2% CHX > 0.3 M EDC/0.12 M NHS 5% GSE (1 min)	–	5% GA 1% GSE; 1%, 5% GA (1 min)	0.1% riboflavin 0.1%, 0.5% riboflavin (1 min)
Venigalla et al. [93]	Resin–dentine bond	μ TBS	1%, 5% GSE (5 min)	–	0.1%, 0.5% riboflavin (5 min)	1%, 5% GA (5 min)
			0.1% riboflavin	–	1 M EDC	6.5% PA

***, **, * Positive effect (high, moderate, low), significant difference between groups ($p < 0.05$)

dentine [49, 52, 53, 88]. Two studies [69, 70] used human teeth with prepared cavities, while another 2 used natural non-cavitated carious lesions [6, 81]. However, 9 studies employed bovine incisors instead of human dentine [15, 25, 27, 35, 47, 51, 76, 86, 99]. In addition, 2 studies used tooth root canals for endodontic investigation [75, 100].

Discussion

The use of collagen crosslinkers has gained increasing popularity in dentistry in recent years. Crosslinking agents, either synthetic or naturally derived, cause additional molecular crosslinking on dentine collagen, thus enhancing its mechanical and structural stability. Apart from the crosslinking effect, some crosslinking agents possess an inhibitory effect on endogenous proteases, such as host-derived matrix metalloproteinases (MMPs) and cysteine cathepsins, which are responsible for degradation of type I collagen in dentine. Hence, biomodification of dentine matrices with collagen crosslinkers contributes to a stable matrix network and a durable hybrid layer in dentine bonding. The major types of crosslinkers used in the dental literature can be categorised as follows:

Synthetic Crosslinkers

Glutaraldehyde (GA)

GA is predominately used as a fixative that cross-links collagenous biomaterials. GA contains two aldehyde groups which are able to react with the amino groups of lysyl or hydrolysyl polypeptide residues in the collagen, thus forming reducible Schiff base crosslinks [27, 86].

Early work by Maciel et al. demonstrated that exposure to GA produced an irreversible increase in dentine collagen stiffness [24]. Then a commercialised GA-based desensitizer—Gluma (Heraeus Kulzer GmG, Germany)—which contains 5.0% GA, 35% HEMA and 60% water, has been tested in restorative procedures [69]. Inhibition of endogenous human dentine MMPs and improved bond stability were found in the Gluma-treated interfaces [28].

However, controversy arose about the influence of HEMA on the effects of GA in bonding. Later studies confirmed pure GA significantly improved the elastic modulus and resistance to bacterial collagenase degradation of dentine. Especially, higher concentrations up to 25% achieved a more rapid increase in elastic modulus and 10% led to an improved collagen degradation resistance compared with the commonly used concentration of 5% [10, 43, 51]. Used as a primer in restorative procedures, GA application produced significantly increased tensile

bond strengths regardless of the adhesive solvent (either acetone- or ethanol-based) and dentine conditions (dry or wet) [54, 60]. It was also found effective in bonding to 'caries-affected' dentine [81]. However, a limited treatment time or insufficient interaction of amino acid residues from dentine collagen may lead to unimproved adhesive behaviour [99]. A recent study revealed that GA pretreatment could also provide affinity sites for hydroxyapatite nucleation onto the collagen possibly due to the direct introduction of carbonyl groups, which could induce the mineralisation of collagen, thus being a potential strategy to promote the dentine remineralisation process [46].

Although GA has been widely acknowledged to enhance collagen stability by improving its mechanical properties and inhibiting collagen degradation in biological tissues, it is also known for its cytotoxicity [4]. Therefore, further studies are still needed to achieve clinically applicable biocompatibility and safe time-frames with respect to application time.

Ethyl-3-[3-dimethylaminopropyl] Carbodiimide Hydrochloride (EDC)

EDC is an imide-based zero-length crosslinking agent. The mechanism of its crosslinking effect lies in the activation of the carboxylic acid groups of glutamic and aspartic acid residues in peptides via giving *O*-acylisourea groups. Often, *N*-hydroxysuccinimide (NHS) is added to the EDC-containing solution to increase the number of crosslinks introduced to the collagen matrix and prevent the hydrolysis of activated carboxyl groups [56].

Exposure to EDC has been shown to significantly increase the elastic modulus of dentine and it decreased the degradation rate of collagen even after 12-month water storage. However, the treatment time is prolonged to as much as 1 h which was clinically impractical. Therefore, later studies aimed at shortening the application times [56].

A higher concentration of EDC was able to inactivate matrix-bound dentine proteinases and soluble recombinant human MMP-9 (rhMMP-9) with shorter treatment time such as 30–60 s [30]. The effect was observed to be concentration-dependent [39, 82]. As an indirect indicator of stability of collagen, the thermal denaturation temperature of the dentine collagen was examined after EDC treatment and the increase of temperature indicated a more resistant and cross-linked collagen network [11].

When EDC was involved in the hybrid layer in bonding procedures, the immediate bond strength of two total etch adhesives tested (Adper™ Scotchbond™ 1 XT (3 M ESPE, USA) and Optibond FL™ (Kerr, USA)) was not affected. EDC could completely inactivate gelatinases, which is believed to have preserved the bond strength even

after 1-year storage [82, 98]. Hydrolysis or incomplete resin infiltration could leave flaws in either the adhesive or hybrid layer, which may lead to resin–dentine bond failures under cyclic loading due to fatigue crack growth. EDC treatment significantly increased the resistance to fatigue crack growth of a bonded dentine specimens after 6-month storage [96].

In order to obtain more clinically reliable results, recent research has attempted to simulate the conditions involved in collagen degradation. Turco et al. simulated the dynamic physiological conditions of human mastication forces by subjecting dentine matrices to cyclic masticatory loads. Less amount of solubilised type I collagen C-terminal cross-linked telopeptides (ICTP) and solubilised C-terminal telopeptides (CTX) was obtained, indicating an inhibition of matrix degradation by MMPs and cysteine cathepsins. Hence, it is suggested that EDC has the potential in collagen protection even in this dynamic ageing with chewing simulation [40].

Compared to GA, EDC presents low cytotoxicity as the released urea derivative in the crosslinker is easily rinsed from the collagen, thus leaving no residual chemicals [98]. However, further in vivo studies are needed to test if EDC is non-toxic to pulpal tissues.

Naturally Derived Crosslinkers

Although synthetic agents have been used for crosslinking dentine collagen, their toxicity and instability over time have limited their use. Recently, plant-derived crosslinkers have been extensively studied and proven to be effective in enhancing the mechanical properties of dentine and controlling its biodegradation by inhibition of proteases. An even more remarkable aspect is that dentine biomodification by these naturally derived crosslinking agents has significant effects on caries prevention and dentine remineralisation [50, 52, 103]. Most of the plant-derived crosslinkers are polyphenolic flavonoids which contain a phenyl ring bearing a hydroxyl group with amphiphilic properties. This bi-functionality accounts for their ability to interact physically with proteins such as collagen.

Proanthocyanidin (PA)-Rich Agents

PA is a bioflavonoid polyphenol-rich agent with its chemical structure based on flavanols (also referred to as flavan-3-ols) (Fig. 2) [13]. It is widely available in fruits, vegetables, nuts, seeds and flowers but its composition is highly diverse [5]. The activity of the extracts is strongly related to the complexity of the structure, stereochemistry and contents of the bioactive compounds [15, 26, 33, 41, 42]. For instance, GSE was identified as

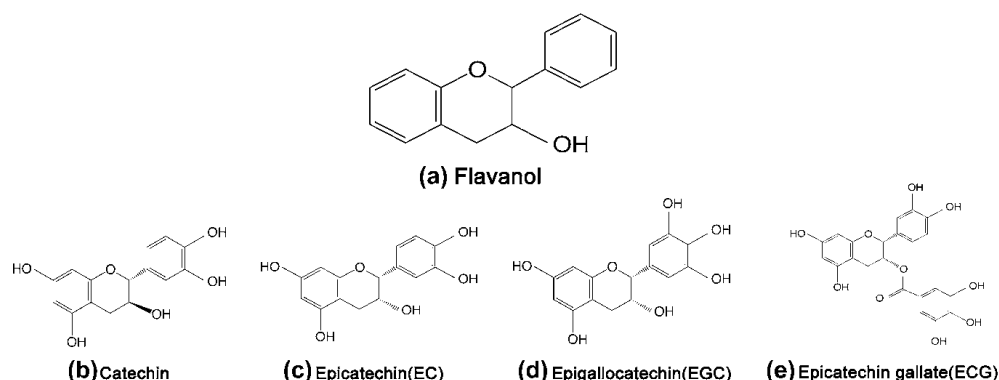


Fig. 2 a Chemical structure of flavan-3-ol (flavanol) and b, c, d, e common monomeric flavan-3-ol (flavanol) present in oligomeric proanthocyanidins

one of the most efficient PA-rich agents and the bioactive component in it was distinguished as galloylated oligomeric PA. The presence of a galloyl moiety offers more hydroxyl groups for crosslinking, leading to a better performance [26]. It is suggested that PA may interact with the collagen in dentine via covalent, ionic, hydrogen bonding or hydrophobic interactions, thus stabilising the exposed collagen matrix [52]. Also, by hiding cleavage sites on dentine collagen, PA exhibited inhibitory effects against proteases such as MMPs [13, 15].

Early work demonstrated PA's effects in improving the mechanical properties of demineralised dentine in terms of ultimate tensile strength and modulus of elasticity [9, 10]. Despite improved biomechanical properties, biomodification with PA affected the collagen biochemistry and proteoglycans—a major group of non-collagenous proteins in dentine [8]. Walter et al. confirmed PA's capability in decreasing collagenase degradation [51]. Further study demonstrated PA inhibited the activity of both collagenase (e.g. MMP-8) and gelatinases (MMP-2, MMP-9) [79]. It effectively inactivated more than 90% of soluble recombinant MMP-2, MMP-8 and MMP-9 and around 75–90% of a group of proteolytic enzymes in dentine called cysteine cathepsins [37]. The ability of PA to stabilise dentine collagen fibres against collagenolytic degradation could be achieved in 15–30 s [38].

Additionally, the effects of PA-rich agents on prevention of root dentine caries and remineralisation have been specifically noted. Incubation of PA-treated bovine root dentine with cariogenic bacteria resulted in the inhibition of root carious lesion formation [98]. Also, PA inhibited the development of recurrent caries around dentine-resin margins in a laboratory study [51, 76]. Under dynamic pH cycling conditions (a procedure employed to simulate the

dynamic variations in mineral deposition and pH values in the natural caries process), PA treatment significantly promoted remineralisation of artificial carious lesions. The results showed significantly thicker mineral precipitation and incremental increases in hardness of the remineralised lesions. Its mechanism was ascribed to the stabilisation of the dentine matrix by a PA–collagen interaction and the formation of PA–calcium complexes, which is different from the remineralisation by fluoride [47, 52]. Higher concentrations of PA succeeded in limiting the treatment time without compromising its effects [67, 102].

Biomodification of demineralised dentine with PA also resulted in a stronger hybrid layer in resin–dentine bonds, with increased elastic modulus and hardness, even after 6-month storage in Hank's buffered saline solution [63, 64]. Dentine surfaces pretreated with PA for 1 h resulted in increased bond strengths [54]. PA incorporation into a phosphoric acid solution rendered a collagen-stabilising etchant which stabilised collagen while etching dentine. Experimental primers containing PA impaired the enzymatic activity at the dentin–adhesive interface after cyclic loading and the activity of MMPs, which is comparable to the common protease inhibitor, namely chlorhexidine (CHX) [84]. Incorporating PA into an experimental adhesive showed no adverse effects on bond strength but effectively stabilised the interface and reduced degradation after long-term incubation in TESCA buffer (50 mM of N-Tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES), 0.36 mM of calcium chloride, pH 7.4) or collagenase [72, 92]. The mechanism may lie in the function of PA in impairment of the enzymatic activity at the dentine–adhesive interface and nanobiochemical changes of dentine matrices within the hybrid layer [63, 90]. However, negative results were also

reported in another study that showed no significant increase in durability of resin–dentine bonds when specimens were immersed in artificial saliva [66]. PA could even prevent complete collapse of acid-etched dentine collagen fibrils after air drying, ensuring sufficient space for resin infiltration [97].

The obvious drawback of PA is the discoloration of the treated surface. It is anticipated that this issue could be addressed by modification of the extract mixture, such as lowering its molecular weight fraction [22]. Monomeric catechins or epicatechins are typically present in PA. All catechins contain phenolic hydroxyl groups, which can interact with various proteins by forming multiple hydrogen bonds. Galloylated catechins like epigallocatechin gallate (EGCG) even exhibited a greater hydrogen bonding capacity due to the presence of three vicinal hydroxyl groups from the galloyl moiety [41]. PA extracted from green tea (*Camellia sinensis*) is rich in monomeric PA, such as EGCG [5]. This source of PA could significantly increase the bond durability to caries-affected dentine, where MMPs and cysteine cathepsins are highly activated [58]. Moreover, the application of a solution containing the main component of green tea—EGCG was effective in stabilising dentine collagen fibrils and improving its resistance to proteolytic degradation [19].

Other Polyphenolic Flavonoids

Besides PA, there are other polyphenolic flavonoids that have been applied to dentine. Tannic acid (TA) is a plant-derived polyphenol and consists of a complex mixture of polygalloyl glucose esters. Hydrogen bonds are able to form between the hydroxyl groups from TA and amide NH groups from collagen. TA treatment on dentine significantly increased its mechanical properties, reduced enzymatic degradation and further increased resin–dentine bond strengths [7].

Hesperidin (HPN)

HPN is a glycoside flavonoid extracted from citrus fruits, which belongs to the flavanone subgroup. The mechanism of interaction between flavanone and collagen fibrils has not been completely elucidated. However, as it is also categorised as a phenolic flavonoid, it might be explained in the same manner as the crosslinking effect of other flavonoids [19, 53]. The similar effects may also be found in naringin which is also categorised as flavanone. Studies showed the potential of HPN to prevent collagen degradation against proteolytic enzymes [74]. And 2% HPN-incorporated self-etch primer significantly improved the immediate and long-term (1-year storage in artificial saliva) microtensile bond strength (μ TBS).

Genipin (GNP)

GNP is a hydrolytic product of geniposide, which is extracted from the fruit of *Gardenia jasminoides* Ellis. GNP can react with primary amine groups within collagen molecules, thus forming crosslinks [25].

GNP has been tested on bovine dentine and shown to significantly increase the resistance of collagen fibrils against enzymatic digestion in a concentration- and time-dependent manner [25, 51]. Also, chemical modification of human dentine with GNP resulted in a significant increase in the ultimate tensile strength and increased bond strength in adhesive restorations [9]. However, its pigmentation impacts may lead to the staining of treated dentine surface, which presents a clinical limitation due to aesthetic concerns.

Riboflavin

Other than the aforementioned crosslinkers which increased crosslinking by chemical interactions, recent studies reported a physical method, i.e. photo-oxidative, for crosslinking. Riboflavin is a well-known crosslinker used in this method. By the absorption of ultraviolet light A (UVA light), riboflavin produces an excited or triplet state that produces reactive oxygen radicals, thus resulting in efficient physical crosslinking of a collagen network.

UVA-activated riboflavin was proven to be able to increase the mechanical properties of both demineralised and non-demineralised dentin [17, 21]. When included in an experimental primer to crosslink dentinal collagen, it increased immediate bond strength to dentine and decreased interfacial nanoleakage and MMP activity in aged specimens [3, 18, 68].

Although a higher concentration could achieve a more clinically acceptable time-frame for application, it led to more yellowish discoloration of dentine [18]. Also, there are concerns regarding the safety issues and clinical practicality of UVA light sources. The issue of the cytotoxic potential of riboflavin is still controversial.

Comparisons Among Different Crosslinkers

Both synthetic and naturally derived crosslinkers have been found to have significant effects in biomodification of dentine via their multiple interactions with the dentine matrix for an increment in inter- and intramolecular collagen crosslinks [9]. The outcomes could be seen in the aspects such as improved mechanical properties of the dentine matrix and reduced enzymatic biodegradation. Although no crosslinkers have been tested in clinical trials as yet, extensive research has been conducted in laboratory studies to investigate their potential applicability on

inhibition of demineralisation and/or promotion of remineralisation, caries prevention as well as improvement of bonding performance of adhesive systems.

The efficacy of crosslinkers is often material-dependent and their principle effects vary due to distinct mechanisms. General comparison of different crosslinkers was retrieved from the literature where at least three crosslinkers were involved and the results were summarised in Table 1.

Among the studies, 5% GA is frequently used as a positive control, due to its well-known crosslinking mechanism to dentine collagen. Additionally, 2% CHX, a classic non-specific MMP inhibitor, has been proven to be effective in maintaining the resin–dentine bond strength after *in vivo* ageing. Therefore, it is frequently employed as a positive control to test the other crosslinkers' potential in inhibiting the activity of endogenous MMPs in dentine [85].

Comparable concentrations of synthetic and naturally derived crosslinkers could achieve a similar improvement in dentine mechanical properties such as ultimate tensile strength and modulus of elasticity, though the efficacy may be varied [6, 9, 10, 19, 54]. The induction of exogenous collagen crosslinks significantly improves the enzymatic resistance of the collagen matrix. Specifically, dentine treated with plant-derived natural agents, such as PA, HPN and EGCG, is even more resistant to collagen degradation by bacterial collagenase than that treated with GA [19, 53]. However, the biochemical mechanism of these plant-derived crosslinkers has not been fully elucidated.

In addition, several synthetic and natural crosslinking agents have been discovered with the ability to inactivate dentine proteases. However, their efficacy varied according to different concentrations and treatment time. One percent and especially 5% GA and 5% GSE were found to be efficient in irreversibly inhibiting most of the MMPs and cathepsin K in short-term (14 days) as well as after 6-month storage, while the physical method of riboflavin/UVA light appeared to be less effective, probably due to the limited penetration of UVA light [32, 33]. Although endogenous enzyme, cathepsin K, in dentine appears to be more difficult for crosslinkers to inactivate than MMPs, polyphenol-rich agents are better inhibitors of cathepsin K than MMPs [33].

Synthetic crosslinkers usually have an effect on microorganisms as a disinfectant, which naturally derived crosslinkers are unlikely to have. However, plant-derived crosslinkers such as PA even exhibited a greater capability of caries prevention than GA when the crosslinker-treated dentine was incubated in a bacterial suspension [51]. More interestingly, PA primer exhibited greater inhibition on bacterial-induced recurrent caries development adjacent to the dentine–resin interface *in vitro* than the EDC and CHX counterparts [76]. The postulated mechanism of action was

initially attributed to the stabilised dentine tissue achieved by PA that prevented demineralisation and led to a tighter interfacial sealing against recurrent caries. However, recent research has demonstrated the potential anticariogenic properties of plant-derived natural products as well as their intervention in the metabolism or gene expression of cariogenic organisms [104]. The exact mechanism of their effects on caries prevention requires further investigations.

Depth of exogenous crosslinking induced by crosslinkers also varies. Crosslinking induced by the physical method of UVA light activation may not occur in deeper areas because of the limited absorption of UVA irradiation, though it is believed to induce faster crosslinking through immediate production of free radicals by photo-oxidation [93]. The chemical crosslinker, GA, promotes rapid crosslinking on the tissue surface that generated a barrier impeding the further diffusion into the tissue bulk.

Considering their potential in increasing the strength of collagen fibrils and inactivating dentine proteases, crosslinkers have been included into bonding protocols. Most research used crosslinking agents as a separate pre-treatment step and showed their effectiveness in improving the μ TBS both in short- and long-term storage in water, artificial saliva or collagenase. Afterwards, the fractured interfaces were examined and the fracture would more likely occur in the adhesive layer or composite instead of in dentine or at the bottom of hybrid layer after crosslinker treatment, indicating a shift of the weakest sites in the bonded complex [105]. Regarding the treatment time, some research has shown it to be too long for clinical use (1 h or more), while others were feasible with clinically relevant time periods of less than 1 min.

In order to avoid extra steps and reduce time, crosslinkers have been incorporated into dentine bonding agents. Incorporation of riboflavin, GA and EDC in adhesive restorative agents was shown to contribute to intact interfacial integrity of resin–dentine bonding and increased collagen biodegradation resistance within the adhesive/dentine interface [29, 61, 94]. However, this effect may depend on certain combinations of dentine primers and luting materials [4, 57, 89, 99]. The long-term effects are determined by several aspects that included degree of conversion of the resins, MMP activity within the dentine–bonding interface [54, 70, 99]. Although it has been reported that macromolecular naturally derived crosslinkers, such as PA, have the potential to reduce the polymerisation efficacy of resins, this may depend on the concentration of the agents as well as how they are incorporated into the adhesive system. The degree of conversion of the adhesives could be controlled within a range acceptable for clinical use [66, 101]. Studies have confirmed a more pronounced effect of PA-containing

phosphoric acid etching in reduction of nanoleakage in resin–dentine bond [71].

The advantages of PA are manifested when it is incorporated into the primers or etchants of etch-and-rinse bonding systems. Traditional chemical collagen crosslinkers such as GA or EDC are incompatible with phosphoric acid in etch-and-rinse systems because the amines within them were protonated at low pH and not applicable for covalent bonding, while PA, based on a non-covalent mechanism, is anticipated to remain active at such a low pH [70, 80]. It has been reported that PA was less sensitive to its pH environment compared with other crosslinkers such as GA, EDC and GNP [36]. Nevertheless, the optimum interaction of PA with proline-rich proteins is pH-dependent, where the greatest precipitation was found to be near the isoelectric point of protein. This has been ascribed to the decreased electrostatic repulsion of protein molecules at this pH [7].

The most superior advantage of naturally derived crosslinkers is their biocompatibility. Synthetic crosslinkers are often associated with a high cytotoxic potential which is less acceptable for clinical use [70]. However, a clinical limitation of naturally derived crosslinkers is due to the staining of the dentine collagen, leading to the discoloration of the tooth [51].

Solvents to Dissolve the Crosslinkers

The category of solvents to dissolve crosslinkers plays a key role in their efficacy. Water, ethanol and acetone are the most commonly used solvents to dissolve crosslinkers in dental bonding systems. Although distilled water is frequently used to dissolve crosslinkers, solvents with ethanol or acetone contribute to different dielectric constants of the media, thus influencing the aqueous solubility behaviour of the crosslinkers.

Phenolic compounds extracted from different plants vary in the amount of monomeric, oligomeric and polymeric PA. The variation in chemical structure and composition of the complex mixture leads to a different solubility behaviour. The solvent used in the extraction and manufacturing process of PA-rich extracts has a profound influence on the solubility. Specifically, it is said that an acetone–water mixture is the best extraction solvent both for monomeric and oligomeric and polymeric PA from grape seed [14]. All tested solvent systems (ethanol–water, acetone–water and neat water) for PA showed increased stiffness values of dentine collagen when compared to control groups, while the effects were stronger in neat water. However, the study by Liu et al. reported ethanol as a preferable solvent for PA-based preconditioners as it decreased the dielectric constant of the media and stimulated PA–collagen interaction [20]. Later study confirmed

GSE in 5% ethanol/95% water gave higher resin–dentine bond strengths compared to 100% water or ethanol [97]. The inconsistent result may be due to the different extraction process of PA in these studies. In addition, PA from cocoa extract treatment resulted in higher values of stiffness when an ethanol–water solution was used, followed by acetone–water solution [12, 14].

When PA is used as the pretreatment solution in resin–dentine bonds, the solvent used could also impact on the performance of different adhesive systems. The reason may lie in the discrepancy of the solvent used to dissolve PA and the solvent in the bonding agents [67].

Some plant-derived crosslinkers like HPN and EGCG are hard to be dissolved in the commonly used solvents. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer or a mixture of 50/50 ethanol/water has been used to dissolve EGCG [22, 41]. Attempts have been made to dissolve HPN firstly in 100% dimethyl sulfoxide (DMSO) and then diluted by distilled water to the final tested concentration [19].

The crosslinking efficacy of EDC in water, ethanol and acetone was also compared and the results suggested an enhanced potential by using acetone as a solvent as it helps stabilise the activated carboxyl groups in dentine collagen for crosslinking [16]. EDC delivered in media of distilled water, 2-(*N*-morpholino) ethanesulfonic acid (MES) or ethanol increased the modulus of elasticity of dentine without a significant difference [56].

Specimen Preparation

In order to expose dentine for treatment, the root and crown enamel are removed from the tooth. The obtained dentine blocks are then cut or ground into different types of samples such as beams, discs, slabs or powder for different measurements. In some studies, bovine instead of human dentine was used because of its size and easy access for specimen preparation and analysis.

The majority of recent studies of crosslinkers have been undertaken using coronal dentine. However, the increasing prevalence of root caries has resulted in more research on root dentine fragments to investigate if crosslinkers could be a promising agent in non-invasive root caries therapy [52, 74]. Also, dentine of various conditions, e.g. ‘caries-infected’, ‘caries-affected’ or bleached, was used to test the effects of crosslinkers on altered forms of dentine [91].

Dentine is a highly organised biological composite with high density of crosslinks. The effect of collagen crosslinking agents could hardly be observed if dentine was kept undemineralised [9]. Therefore, dentine specimens used for crosslinker testing are often demineralised to expose the type I collagen matrix or create caries-like lesions. Phosphoric acid is the most commonly used agent

to denature the dentine collagen matrix. A high concentration between 35 and 37% with a 10–15 s treatment time was often used. A moderate phosphoric acid concentration such as 10 or 15% was also used on dentine in some studies to reach an optimal pH for dentinal MMP activation and demineralise a sufficient amount of the dentine mineral. However, longer times as much as 5–24 h may be required for treatment [31, 34, 97]. Thirty-five percent phosphoric acid was used to remove the smear layer if a bonding procedure was involved, which is in accordance with clinical practice.

Some researchers are concerned about the potential denaturing effect of phosphoric acid on the dentine collagen matrix. The EDTA-etching approach became an alternative to remove all minerals, leaving matrix-bound proteases on the dentin matrix [35, 43].

The pH-cycling procedure has been used to simulate the dynamic variations in pH and minerals as occur in the natural caries process [47, 49, 50, 52]. Involvement of pH-cycling would provide an insight into the effects of crosslinkers on inhibition of demineralisation or/and promotion of remineralisation [6]. In addition, ‘artificial root caries’ was induced by storing root dentine fragments in a demineralisation solution, and thus the potential effects of crosslinkers on carious lesions could be investigated. However, this chemical model excluded the vital role of bacteria in lesion formation. Therefore, Walter et al. incubated treated-root dentine in culture tubes containing cariogenic bacteria to simulate a more natural carious environment [51]. By use of specific devices, an *in situ* study was conducted in a cariogenic oral environment of patients, though the duration was limited [70]. In this regard, the effects of crosslinkers on caries prevention or dentine-bond duration in a cariogenic oral environment were able to be investigated.

Methods to Examine the Effects of Crosslinkers on Dentine

Crosslinkers interact with the dentine matrix collagen and alter its stability, structure and stiffness [9, 10]. Mechanical properties of the collagen fibrils, including modulus of elasticity, hardness and ultimate tensile strength, are usually measured before and after treatment. Despite the bulk mechanical properties, recent studies aim at assessing the properties of dentine in a more site-specific and precise way. Thus, nano-mechanical testing such as nanoindentation has been recommended [17]. Through precision, this measurement is limited to submicrometre surface depth and provides the information of elastic recovery of dentine samples.

Mineral content is changed after treatment and various analytical methods have been employed to elucidate the

degree of mineral gain or loss. Transverse microradiography, for example, has been employed to evaluate lesion depth; in addition, mineral loss could be further analysed as mineral profile against lesion depth, which is regarded as a gold standard [49, 50, 53].

To further illustrate how the crosslinkers interact with dentine, spectroscopic analysis methods such as micro-Raman and Fourier transform infrared spectroscopy (FTIR) were employed to detect the formation of chemical bonds between the dentine matrix and crosslinkers [15, 22].

The micromorphological changes of the collagen matrices could be observed by electron microscopic analysis. High-resolution micrographs either by scanning or transmission electron microscopy provide qualitative information regarding the pattern and degree of mineralisation that may be achieved [17, 62, 68].

Improved biomechanical properties of the dentine matrix result in increased resin–dentine bond strengths [7]. The crosslinkers bring the stiffness of the collagen matrix closer to that of adhesive resin in the hybrid layer. Microtensile bond strength testing is frequently used as a measure of bonding efficacy. Afterwards, the debonded interfaces are observed using scanning electron microscopy (SEM) to determine the failure mode, which could be visually classified as failure at the interface, in composite and in adhesive [67]. It serves as a powerful indication of bond stability. One drawback is that the surface preparation techniques during SEM observation may adversely affect the morphological detail of the specimens. Confocal Raman spectroscopy has become popular for its capability to provide information on chemical compositional changes and detect the information from the sub-surface by adjusting the focusing positions [62, 94].

The collapse of the hybrid layer in the bonding process may be due to the gelatinolytic and collagenolytic activities induced by oral bacteria or the endogenous proteolytic enzymes. Assessing the liberation of hydroxyproline and weight loss indirectly indicates the effects of crosslinkers on degradation [41, 68, 77, 95]. Also, the activity of MMPs and cysteine cathepsins are frequently measured to reveal the inhibitory effects of crosslinkers on endogenous collagenases. In order to obtain a more specific action of proteases, analysis of ICTP and CTX by the MMP-induced collagen degradation was performed [11, 40]. In addition, zymographic analysis has recently become popular, which provides a direct insight into enzyme activity, even in an *in situ* way to obtain precise localisation of the MMP activity within the hybrid layer [82, 98].

Nanoleakage reveals the nanometre-sized defective locations at the bonding interface and serves as an indicator of bonding degradation over time. The degree of infiltrated tracer silver grains is often observed by light microscopy or

SEM, thus giving information of crosslinkers' effects in interfacial nanoleakage [29, 65, 70].

Perspectives

Situation Simulation

Firstly, the major limitation of current studies is that the majority were performed in laboratories so that the challenging conditions of the oral environment such as pH, occlusal loading and thermal stress are barely reproduced. Recent research has aimed at simulating the possible parameters involved in the degradation of collagen. Validated methods are still needed to create physiological conditions with an optimum pH for endogenous enzymes in dentine matrices, thus making the results more adequate for degradation of demineralised dentine. Still, even in situ models cannot substitute for randomised controlled clinical trials, so the effects of crosslinkers are still worth further clinical investigation.

Long-Term Function

Many studies attempted to prolong the time for degradation test (up to 1 year), while further research is still required to clarify the long-term function of crosslinkers regarding inactivation of endogenous proteases and preservation of the resin–dentine interface.

Clinical Feasibility

Further studies are also needed to find the optimal formulation and application time of crosslinking solutions to reinforce the dentine collagen network as well as stabilise the adhesive interface over time. Reduced pretreatment time with higher concentrations sometimes goes hand in hand with increased side effects—the notorious ones are cytotoxicity and discolouration. More research should be undertaken to obtain a more precise knowledge of the mechanism how crosslinkers interact with dentine collagen and systematically evaluate effective constituents. Also, using several crosslinkers simultaneously and use of controlled drug-delivery systems could also be considered as alternatives.

Though direct incorporation of crosslinkers into bonding systems decreases the complexity of the bonding procedure, factors such as the optimum concentration, formulation and avoiding the interference of conversion and polymerisation of an adhesive also needs to be taken into consideration to ensure its effectiveness. Additionally, some crosslinkers are product-dependent so that future

studies are also needed to validate the effectiveness in association with different bonding systems.

Side Effects

Excessive crosslinking may also compromise the integrity of the dentine matrix and negatively affect the ultimate tensile strength of the dentine, so the degree and range of crosslinking should be controlled, which requires further studies. The cytotoxicity potential of synthetic crosslinkers or discolouration of nature-derived ones should be considered.

Mechanism

In addition, some inconsistent results were drawn probably due to the distinct physical conditions or different sources of crosslinkers. More research should concentrate on the mechanism of how the crosslinkers interact with the dentine matrix or hybrid layer.

A variety of testing methods have been proven to be powerful predictors in evaluating the effects of crosslinkers; however, further characterisation should also employ multiple techniques to warrant the precision of the results. For instance, nano-testing is necessary in the future to understand the how crosslinkers interact with dentine at the nanostructural level.

Conclusion

In conclusion, within the limitations of this review, both synthetic and naturally derived crosslinkers have been found to have significant effects on stabilising the dentine matrix network or providing a durable hybrid layer in dentine bonding, though the mechanism of how they crosslink collagen varies. Extensive research has been conducted in laboratory studies using different methods to investigate their role in improving biochemical and biomechanical properties of dentine collagen fibrils and reduced enzymatic biodegradation, indicating their potential applicability for inhibition of demineralisation and/or promotion of remineralisation, caries prevention as well as improvement of bonding performance of adhesive systems. Further studies are still required to establish the clinical feasibility of crosslinkers, reduce their side effects as well as explore the mechanism of action and long-term effectiveness.

Compliance with Ethical Standards

Conflict of interest J. Cai, J. E. A. Palamara and M. F. Burrow declare that they have no conflict of interest.

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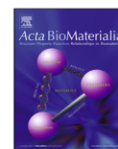
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Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Effects of silver diamine fluoride/potassium iodide on artificial root caries lesions with adjunctive application of proanthocyanidin

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ARTICLE INFO

Article history:

Received 31 October 2018

Received in revised form 29 January 2019

Accepted 14 February 2019

Available online 15 February 2019

Keywords:

Proanthocyanidin

Silver diamine fluoride

Potassium iodide

Mechanical properties

Dentinal organic matrix

Root caries

ABSTRACT

Treatment of carious root surfaces remains challenging due to the complex pathological processes and difficulty in restoring the original structure of root dentine. Current treatments targeting the de-/re-mineralisation processes are not entirely satisfactory in terms of the protection of the dentinal organic matrix and the highly organised structure of dentine. In this *in vitro* study, a cross-linking agent – proanthocyanidin (PA) was used in conjunction with a fluoride-based treatment – silver diamine fluoride/potassium iodide (SDF/KI) to putatively stabilise the organic dentinal framework as well as strengthen the collagen-mineral phase interaction. The effectiveness of this strategy was evaluated 24 h after application in terms of the distribution of ion uptake and microstructure of dentine after treatment as well as analysis of the nano-mechanical properties using a dynamic behaviour model. Results showed that individual use of SDF/KI significantly improved the surface microhardness and integrated mineral density (Z) up to 60 µm depth and the recovery of creep behaviour of demineralised dentine in the surface area compared to that treated with deionised distilled water (DDW). The combined treatment of PA and SDF/KI achieved a more homogenous mineral distribution throughout the lesions than SDF/KI alone; a more significant incremental increase in surface microhardness and Z was observed. Specifically, a superior effect on the subsurface area occurred with PA + SDF/KI, with significant improvements in microhardness, elastic modulus and recovery of creep behaviour of the demineralised dentine. Application of SDF/KI induced small discrete crystal formation distributed over the dentine surface and PA contributed to the formation of slit-shaped orifices of the dentinal tubules that were partially occluded.

Statement of Significance

Demographic transitions and improved oral health behaviour have resulted in increased tooth retention in elderly people. As a consequence, the risk of root dentine caries is increasing due to the age-associated gingival recession and the related frequent exposure of cervical root dentine. Root caries is difficult to repair because of the complex aetiology and dentine structure. The recovery of dentine quality depends not only on reincorporation of minerals but also an intact dentinal organic matrix and the organic-inorganic interfacial structure, which contribute to the biomechanics of dentine. With the capability of dentine modification, cross-linking agents were applied with a fluoride regimen, which improved its treatment efficacy of root caries regarding the distribution of ion uptake and recovery of dentine biomechanics.

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1. Introduction

With the change in demography of society and improvement in oral health behaviour, the retention of natural teeth is progressively increasing in developed countries [1]. As a result, root caries,

the major cause of oral pain in the elderly, is expected to increase exponentially due to more teeth and therefore cervical root surfaces being present, caused by age-related gingival recession [2–5]. Treatment of carious root surfaces can be more challenging than those of coronal surfaces due to the complex pathological processes and the difficulty in restoring the root structure, composed of the complex protein assemblies and organised mineral components of root dentine.

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Dentine is an hierarchically bio-composite material under multiple template-mediation. The basic building block of dentine is highly mineralised collagen fibrils (~50–100 nm diameter), where the tablet-shaped dahllite crystallites (~5 nm thick, 70% by weight) are embedded within the gaps of the fibrils with an orientation preferentially aligned along the long axis of the fibril. Proportionately massive periodic mineralised collagen fibrils are densely staggered and overlapped and form covalent intra- and inter-molecular cross-links [6–9]. The nanoscale interfacial interaction between collagen fibrils and the embedded mineral phase enables load transference between the organic and inorganic components, which endows on dentine a capacity of load bearing during mastication and recovery after deformation [10,11]. In a typical root caries process, firstly, exposure to biofilm-derived acid causes mineral to dissolve, leading to the exposure of cleavage sites of collagen fibres and activation of host-derived collagenases (e.g. matrix metalloproteinases (MMPs) and cathepsins). The subsequent collagen proteolysis results in the breakage of high conformational cross-linkages, which contributes to further cleavage events by gelatinases/peptidases and accelerates subsequent mineral loss [12]. Consequently, demineralisation, together with structural damage of the collagen framework, causes localised destruction of dentine, which therefore impairs the biomechanics and biochemistry of the original dentine [13,14].

The efficacy of currently recommended treatments, e.g. 5 000 ppm fluoride toothpastes, 22 600 ppm fluoride varnish and 38% silver diamine fluoride solution (44 800 ppm fluoride), highlights the efficacy of high concentration fluoride in managing root caries [3,4,15]. The cariostatic mechanism of fluoride mainly relies on the inhibition of demineralisation and enhancement of remineralisation [16]. Direct effects of fluoride on preventing breakage of the collagen framework and de-activation of endogenous proteases have not been demonstrated [17]. Thus, an incremental increase of mineral density alone cannot be regarded as an appropriate endpoint for assessing the effectiveness of the treatments with regard to improving dentine quality, which depends upon the sum total of the tissue characteristics including ultrastructure (especially the particular location of the mineral within the organic matrix of the tissue), mineral density and biomechanical characteristics [18,19]. Failure to protect the organic structure of root dentine may compromise its biomechanical properties [20].

Given the pathological process of root caries, reducing enzyme activity and biomodification of dentine matrices are likely to protect the conformational dentinal organic matrix from degradation in root caries. A series of laboratory studies have reported the effects of MMP inhibitors in reducing the activity of MMPs in partially demineralised human dentine [21–25], whilst two studies have a direct observation on topical application of two MMP inhibitors, i.e. non-antimicrobial chemically modified tetracyclines and zoledronate (a bisphosphonate with MMP-inhibiting activity) in preventing carious lesion progression in rats [26,27]. However, their inhibitory effects lack selectivity to certain enzymes and the effects may be limited to a certain time period. Thus, biomodification of dentine seems to be a promising approach which enhances the biomechanics and bio-stability of the dentine matrix. Amongst diverse biomodification strategies, application of cross-linking agents is of particular interest. The cross-linkers could induce multiple inter- and intra-molecular cross-links by interacting with various extracellular components of the dentine matrix [28]. As a result, the cross-linked organic matrix may modify or hide the regions in collagen fibrils vulnerable to collagenases and maintain the mineral phase in the gaps of collagen fibrils from further dissolution, thus improving the biomechanical and biochemical properties of the tissue [29].

Proanthocyanidin (PA), a plant-derived cross-linking agent containing polyphenolic compounds, has been investigated exten-

sively for its potential use in reparative caries therapies [30]. It not only interacts with proteins via covalent, ionic, hydrogen bonding or hydrophobic interaction, but also shows direct effects on non-collagenous protein such as proteoglycans [31] and inhibits the activity of endogenous proteases [32,33]. Dentine collagen treated with PA showed a significant improvement in mechanical and biological properties [34,35]. Therefore, PA has the potential to stabilise the partially demineralised organic matrix and maintain the mineral phase in the stabilised collagen framework in its original arrangements, which might be beneficial for ion uptake and reaction as well as the collagen-mineral phase interaction, thus leading to a higher mineral density and improved biomechanics.

In this study, SDF/KI was investigated as a regimen for root caries treatment, and PA was applied as a bio-mediator to protect the dentine organic matrix. An artificial root caries model was used to ensure the consistency of the results. The treated root dentine specimens were analysed regarding their mechanical, chemical and structural properties. Especially, nano-indentation was used extensively to analyse the dynamic mechanical properties of dentine at a nano-scale. The viscoelastic behaviour of the treated dentine was systematically explored using a dynamic behaviour model. The microstructure and mineral uptake and distribution of SDF/KI with or without PA were also observed.

2. Materials and methods

2.1. Materials

Proanthocyanidin was obtained from the International Laboratory of USA (>95% oligomeric proanthocyanidins) and SDF/KI (Riva Star®; consisting of 30%–35% silver fluoride, >60% ammonia solution and a saturated solution of potassium iodide) from SDI Limited (Bayswater, Australia). The demineralising solution was prepared using 50 mmol/L acetic acid, 1.5 mmol/L CaCl₂ and 0.9 mmol/L KH₂PO₄ and adjusted to pH 5 with KOH. All the chemicals were purchased from local chemical suppliers.

2.2. Preparation of root dentine specimens

Non-carious human permanent molars were freshly extracted and collected in 0.1% thymol solution at 4 °C under the protocol approved by the Human Research Ethics Committee of the University of Melbourne. The crown and root apical portions were removed using a slow-speed diamond saw (Isomet, Buhler Ltd, IL, USA) under water irrigation. In order to determine the sample size (*n*), the mean values and standard deviations were estimated according to pilot study work. The effect size (*f*) in each measurement was calculated from the pilot study data following the Cohen's guidelines [36], in which for one-way analysis of variance (ANOVA) with *n*'s equal, *f* was measured by:

$$f = \sqrt{\frac{\sum_{i=1}^k (\mu_i - \mu)^2}{k \cdot \sigma^2}}$$

Where μ_i = mean of Group *i*, μ = grand mean, σ^2 = variance within groups.

The minimum sample size was estimated based on a power of 0.8 with a confidence level of 95% and *f* calculated following the above equation using the G Power Version 3.1 program for the quantitative analysis (see Supplementary Information Table A.3) [37]. In this regard, six specimens were used for cross-sectional microhardness test and nano-indentation respectively. Five samples (from those for cross-sectional microhardness testing) per group were prepared for surface microhardness testing, three sam-

ples for polarised light microscopy (PLM) observation, two specimens for micro-CT analysis and three for scanning electron microscopy (SEM) examination. Considering the possible inconsistency of lesion depths, five additional samples were prepared in each treatment group. Thus, one hundred non-carious human permanent molar dentine blocks were prepared from buccal or lingual surfaces of the roots adjacent to the cemento-enamel junction (CEJ) and embedded in epoxy resin (KEP epoxy, Kemet International Ltd., Kent, UK). The tooth surfaces were polished using ascending grits (320, 600, 1200, 2500) of silicon carbide papers to expose the root dentine surface and the debris was removed by ultrasonic cleaning for 5 min in DDW. A layer of acid-resistant nail varnish was applied on the polished root surfaces to expose a window of 4×3 mm.

2.3. Treatment procedures

2.3.1. Demineralisation

Each specimen was individually immersed in 20 mL of the buffered acidic demineralising solution (see 2.1) and stored at 37 °C for 5 d. The fresh demineralising solution was prepared and changed every 24 h. The protocol for demineralisation was modified from that previously reported [38–40].

2.3.2. Root dentine treatment

Root specimens were randomly assigned to the four treatment groups ($n = 25$ per group). Treatment agents and treatment times for each group are listed in Table 1.

Specifically, the DDW group served as a negative control. The 6.5% w/v PA solution was prepared by adding PA powder into DDW and mixing thoroughly. The solution was applied to root dentine surface using a micro brush, agitated for 15 s and left for 1 min. The excess solution was gently removed with a cotton pellet. This was followed by a further application of fresh PA solution. SDF/KI was applied according to the manufacturer's instructions. Specifically, SDF was applied to the treatment site using a micro-brush. Immediately after, a generous amount of KI was applied until no more white precipitate was formed on the treatment surface. For samples in Group 4, 6.5% PA was applied first in the same manner as for Group 2; the excess solution was removed with a cotton pellet; thereafter, SDF/KI was applied in the same manner as for Group 3. All specimens were blot dried and stored in DDW (20 mL for each sample) for 24 h before testing. Samples were sectioned longitudinally through the lesion centre and observed under the light microscope to evaluate the lesion depths. Samples with a

lesion depth outside a range of 150 ± 10 µm were excluded. The 20 most suitable samples in each group were selected for analysis.

2.4. Analytical methods

2.4.1. Cross-sectional microhardness test

The longitudinally-sectioned samples were re-embedded in epoxy resin. The cross-sectional surface of each dentine block was polished on a water-cooled grinding and polishing unit (Tegra-Pol 21, Struers, Copenhagen, Denmark) with abrasive papers (600, 1000, 1200, 2500, 4000 grit), followed by a 0.1 µm diamond suspension (Type K, Kemet International Ltd., Kent, UK). The debris on the surface was removed by ultrasonically cleaning for 2 min in DDW. Cross-sectional microhardness measurements perpendicular to the demineralised surface were performed below the surface at ~5, 30, 60, 90, 120, 150 µm using Knoop hardness indentation (402 MVD, Wolpert Wilson Instrument, MA, USA) with a load of 10 gf (98×10^{-3} N) for 10 s dwell time at each testing point. For all samples, three indentations were performed at each depth on parallel tracks approximately 150–200 µm apart. Hardness was determined according to the following equation:

$$H = \frac{P}{A} \quad (1)$$

where P is the applied force and A is the contact area of the indentation which depends on the indenter geometry and the penetration depth [14]. To avoid variations of microhardness of different samples, an internal control of the sound area (150 µm from the surface) of each dentine block was used for comparison. Results were expressed as relative microhardness value [41].

2.4.2. Surface microhardness test

The indentations were made using a load of 10 gf (98×10^{-3} N) with 10 s dwell time using the Knoop hardness mode. Five samples were examined in each group and five indentations were performed on each specimen (near the four corners and at the centre of the exposed area) [42].

2.4.3. Nanoindentation test

Depth-sensing indentation techniques based on the load-displacement response of nanoindentation tests were used to obtain more comprehensive nano-mechanical properties of samples, which included hardness, elastic modulus and creep behaviour.

Nanoindentation experiments were performed on the longitudinal cross-sectional surface of specimens using a diamond pyramidal Berkovich tip attached to a quantitative nano-mechanical

Table 1
Treatment agents and times in each group.

Group	Abbreviation	Treatment agent	Treatment time	pH of the treatment agent
1	DDW	Distilled deionised water (DDW)	Agitation with a micro brush for 15 s and left for 1 min (two applications)	pH = 6.98 ± 0.00
2	PA	6.5% w/v proanthocyanidin (PA) dissolved in DDW	Agitation with a micro brush for 15 s and left for 1 min (two applications)	pH = 4.46 ± 0.01
3	SDF/KI	SDF/KI (Riva Star®; consisting of 30–35% silver fluoride, >60% ammonia solution and a saturated solution of potassium iodide; SDI limited, Bayswater, Australia)	Treatment time was determined according to the manufacturer's instruction. SDF was applied for approximately 30 s to cover the whole surface of the exposed dentine surface. KI was applied for another 30 s until no more white precipitate was formed	SDF: 11–11.5; KI: 8–8.5. (measured with pH indicator strips (Merck, Darmstadt, Germany))
4	PA + SDF/KI	6.5% w/v PA dissolved in DDW; SDF/KI (as in Group SDF/KI)	15 s agitation and left for 1 min (two applications) for 6.5% PA (as in Group PA); thereafter, approximately 30 s for SDF and another 30 s for KI (as in Group SDF/KI)	6.5% w/v PA: pH = 4.46 ± 0.01 ; SDF: 11–11.5; KI: 8–8.5 (measured with pH indicator strips)

testing instrument (TI 950 TriboIndenter; Hysitron, Inc., MN, USA) interfaced with an atomic force microscope (AFM). Fused-quartz was used as the standard calibration material [43,44]. Indenter load was 800 μN . The nanoindentation test was performed with a step load followed by a hold period while monitoring the displacement of the indenter as a function of time. The holding periods were 10 s with a loading frequency of 200 Hz. Three sets of indentations (surface, subsurface and sound dentine area) were made on each specimen. Subsurface refers to the areas 20 μm below the surface. After executing the indentations, elastic moduli were calculated using the attached computer.

The displacement–time (h - t) curves during the load-holding period were extracted. For viscoelastic materials such as dentine, creep deformation occurs under constant load. The viscoelasticity and creep behaviour of biological tissues could be analysed from this period [45].

Burger's four-element model which connected Maxwell and Kelvin units in series were used to fit the h - t curves obtained during the load-holding period. The following equation was used to describe the creep behaviours:

$$h^2(t) = A_0 + A_1(1 - e^{-t/\tau}) + A_2t \quad (2)$$

where $h(t)$ is the indentation depth, the first term A_0 is the elastic deformation after the loading stage, the second term is the primary creep that reaches a steady state at buffer time τ , and A_1 is the primary creep co-efficient. The third term is the secondary creep and A_2 is the slope of the linear change [46].

The maximum penetration depth during creep (h'_{max}) was calculated by subtracting the initial depth of penetration at the onset of the holding period against the total depth of penetration after creep at the maximum load.

According to previous studies [47,48], the parameter of creep rate sensitivity (m) was obtained to provide a direct and simple comparison of the viscous flowability of dentine. It can be calculated from simple power-law relationship between stress (in nanoindentation test, accordingly, hardness) and applied strain rate:

$$H = K\dot{\epsilon}^m \quad (3)$$

where K is a material constant and m is the creep rate sensitivity. H is the measured dynamic hardness and $\dot{\epsilon}$ is the corresponding strain rate [47]. Thus, the m value of the dentine can be determined by fitting the experimental data and read from a logarithmic plot of the H - $\dot{\epsilon}$ curve (slope of the fitted curves).

The strain rate of nanoindentation can be extracted from the above fitted depth-time (h - t) curve as the indenter displacement velocity divided by the penetration depth:

$$\dot{\epsilon} = \frac{\dot{h}}{h(t)} \quad (4)$$

where $\dot{h} = \frac{dh(t)}{dt}$ (5) is the indentation rate, which is the time derivative of the displacement during the hold period under constant load in the indentation depth-time (h - t) curve.

2.4.4. Polarised light microscopy and image analysis

Samples were sectioned longitudinally through the lesions (Minitom, Struers, Copenhagen, Denmark) with a low-speed water-cooled diamond saw to obtain 300 μm thick sections. Each specimen was further manually polished to a thickness of approximately 100 μm , mounted on a glass slide with DDW and covered with a clean coverslip [49]. Lesion depths were measured and changes in colour birefringence of the specimen were recorded using a first order red plate between crossed polarisers with transmitted light microscopy (DM2000, Leica Microsystems GmbH, Wetzlar, Germany) coupled to a camera (Leica EC3, Leica

Microsystems GmbH, Wetzlar, Germany). The slides were visualised at original 200 $\times$ magnifications and the images were photographed and analysed by means of the software program Image J 1.38 $\times$ (National Institutes of Health, MD, USA).

2.4.5. Micro-CT analysis

Micro-CT analysis was performed to investigate how uptake of ions from SDF/KI would penetrate and distribute in demineralised (artificial carious) lesions. Specimens were scanned with a desktop X-ray micro tomography system (Skyscan 1172, SkyScan N.V., Aartselaar, Belgium) at a voltage of 80 kV and a current of 124 μA . A 0.5 mm thick aluminium filter was placed in front of the detector to block low energy X-rays to limit the spectral bandwidth of X-rays. Prior to scanning, a new flat field reference was acquired for the dataset. The sample was rotated over 360 $^\circ$ in a 0.2 $^\circ$ rotation step and image pixel size of 1.95 μm . The resulting 2D projected/transmission images (16 bit TIF) were reconstructed to a 3D object using the NRecon software interface based on a modified Feldkamp algorithm (NRecon, Version 1.4.2). Beam hardening artefact was corrected. All scanning was performed with identical parameters: file type of BMP, image width of 1760 pixels and height of 1740 pixels, average pixel size of 1.95 μm , ring artefact correction of 15. Data were maintained as 16 bit TIF information.

For visualisation and analysis of three-dimensional/volumetric data, Image J was used for selection of slices/regions of interest and 3D visualisation [50]. The reconstructed images were resliced to obtain slices that presented the whole lesion area. For each sample, 12 slices were randomly selected for analysis. Quantitative line profiles were evaluated across the lesions perpendicularly to the lesion surface and traversing into sound dentine. A paralleled line profile was drawn in the sound dentine area as a reference to determine the start and end of the lesion and as an internal control to avoid variations of original mineral intensities of sound dentine in different samples. In this regard, sound dentine area has a grayscale value (GV) percentage of approximately 100% whilst the air (blank area) of 0%. The low relative GV indicates a low value in mineral density. The grayscale change from 60 μm outside the lesion surface to a depth of 150 μm inside was determined. The integrated relative GV under the curve from the lesion surface to a depth of 150 μm of sound dentine was calculated by the trapezoidal rule at 30 μm increments [51].

2.4.6. SEM observation

To observe the morphology of the dentine surface and dentinal tubules, three dentine specimens were prepared for each treatment group. For each specimen, a groove was prepared on both the apical side and occlusal side of the lesion area approximately in the same plane using the Isomet diamond saw. Then, each specimen was split longitudinally along this plane into two halves to examine the cross sections of the dentine. The splitting operation was performed such that the cross-sectional surface was not influenced [52]. All dentine specimens were desiccated, Au-Pd alloy sputter-coated, and observed by environmental scanning electron microscopy (FEI Quanta ESEM, FEI, OR, USA) at 10.0 kV. Micrographs at magnifications of 70 $\times$, 2000 $\times$, 10000 $\times$ were captured.

2.5. Statistical analysis

Quantitative data were expressed as means $\pm$ standard deviation. The assumptions of homogeneity of variance and normal distribution were checked by Levene's test and Shapiro-Wilks test, respectively. The surface hardness data were analysed by one-way ANOVA and Tukey's *post hoc* multiple comparisons. Statistical analysis of cross-sectional hardness and nanoindentation data was carried out by two-way ANOVA and Tukey's *post hoc* multiple comparisons. Integrated GV were analysed by Welch's ANOVA test

followed by Dunnett's T3 *post hoc* multiple comparisons. A level of significance of $\alpha = 0.05$ was set in all statistical tests (SPSS Version 22.0, SPSS Inc., IL, USA).

3. Results

3.1. Surface and cross-sectional microhardness test

The surface and cross-sectional microhardness measurements of the demineralised (artificial caries) lesions from different treatment groups are presented in Table 2. Treatment with PA showed slightly lower surface hardness values (40.40 ± 5.78 MPa) than the control group (45.11 ± 3.02 MPa) but was not statistically significant ($P = 0.519$). Treatment of SDF/KI with or without the application of PA both significantly improved the sample surface hardness compared to the no treatment group ($P < 0.01$). However, there was no statistically significant difference between SDF/KI (59.23 ± 6.31 MPa) and PA + SDF/KI (62.96 ± 5.61 MPa) ($P = 0.692$) groups. The addition of PA resulted in a slightly larger incremental increase in the surface hardness, with 31.3% and 39.5% increases respectively for SDF/KI and PA + SDF/KI compared to the control group.

For the cross-sectional microhardness, generally, there were few comparative depths that were statistically different between control and PA groups except for the areas around $90 \mu\text{m}$ deep ($P = 0.013$). Although there was no significant difference between SDF/KI and PA + SDF/KI groups at all the depths, a difference was shown when hardness numbers were compared with the control group respectively. Specifically, samples treated with SDF/KI showed statistically significantly greater microhardness values than the control group only in the surface area ($\sim 5 \mu\text{m}$; $P < 0.0001$), whilst there was no significant difference between SDF/KI and control groups from $30 \mu\text{m}$ to the lesion front (approximately $150 \mu\text{m}$ from the surface) ($P > 0.5$). However, when PA was used before SDF/KI application, significantly greater microhardness values were observed up to $60 \mu\text{m}$ ($P < 0.01$) compared with the control group. At $90 \mu\text{m}$, a statistical difference was observed between the PA + SDF/KI and PA groups ($P = 0.01$) whilst no difference between the PA + SDF/KI and control group was identified, putatively due to the limited penetration of the treatment agents.

3.2. Nanoindentation

3.2.1. Elastic moduli

An example of the nanoindentation image captured by AFM was shown in the Supplementary Information (Fig. A.2). The nanoindentation results of the elastic moduli of the artificial caries lesions after different treatments are presented in Fig. 1 (A–B), which demonstrates the viscoelastic properties of dentine. The storage

and loss of modulus is related to the ratio of the elastic and viscous components of a material to the stress, respectively. Demineralisation resulted in decreased elastic modulus in root dentine. Treatment with SDF/KI with or without PA both significantly improved the storage modulus in the surface area ($P < 0.0001$). Combined use of SDF/KI with PA had a superior effect on the subsurface area, with improvements in elastic moduli compared to all the other groups ($P < 0.0001$). Treatment with SDF/KI (with or without PA) led to recovery of elastic moduli of the carious dentine to a degree that was greater than the original sound dentine. By contrast, treatment with PA significantly decreased the storage modulus of the subsurface dentine ($P < 0.0001$). Tan-delta, referred to as loss factor, is defined as the ratio of the moduli (loss modulus/storage modulus), which indicated the relative degree of energy dissipation or damping of a material. Results (Fig. 1C) confirmed the loss of elasticity of demineralised dentine and recovery of elastic modulus after SDF/KI treatment, with enhanced effects on subsurface area using PA as an adjunct.

3.2.2. Creep behaviour

The typical load displacement response for a single indent is illustrated in Fig. 2(A–C). Indentation responses are shown for surface, subsurface and sound dentine regions. The hold segment means specimens were indented with a constant maximum load, which could demonstrate the creep behaviour of a material.

The h - t curve of each specimen (Fig. 2(A1–C1)) was fitted using Eq. (2) and the fitted parameters were shown in the Supplementary Information (Table A.1). From Table 3, the h'_{max} of sound dentine showed no statistically significant difference between samples from all groups. The demineralised surface presented a nearly 2.5-fold increase of h'_{max} than the sound dentine. After treatment with PA, a significantly higher h'_{max} was present in the subsurface area. SDF/KI significantly decreased h'_{max} to half the value of the control group in surface area, while no significant difference in subsurface area compared to the control group was observed. Adjunctive application of SDF/KI with PA distinctly preserved the resistance against creep deformation of the subsurface area (to a value not significantly different from sound dentine).

Log of strain rate vs. log of hardness was plotted (Fig. A.1). From Table 3, the m value of sound dentine was 0.06 – 0.08 in general. Demineralisation was associated with an increase in the creep rate sensitivity of dentine in the surface areas by nearly 3-fold (control surface vs. sound dentine: 0.18 ± 0.05 vs. 0.06 ± 0.01). Application of PA was further associated with a significant increase in the creep rate sensitivity around the subsurface area compared to the control group (PA vs. control = 0.15 ± 0.02 vs. 0.10 ± 0.02 ; $p = 0.011$). Application of SDF/KI individually decreased the creep rate sensitivity of demineralised dentine significantly only in the surface areas (SDF vs. control = 0.12 ± 0.02 vs. 0.18 ± 0.05 ; $p = 0.021$), whilst it

Table 2

Longitudinal cross sectional and surface microhardness of the artificial caries lesions of different treatment groups. Uppercase superscript letters indicate statistically significant differences between treatments.

Depth from surface (μm)	Longitudinal cross-sectional Knoop hardness numbers (relative to fraction values of sound dentine = 1)			
	Control	PA	SDF/KI	PA + SDF/KI
~ 5	0.17 ± 0.04^A	0.16 ± 0.01^A	0.31 ± 0.03^B	0.37 ± 0.07^B
30	0.27 ± 0.04^A	0.19 ± 0.06^A	0.35 ± 0.04^{AB}	0.57 ± 0.26^B
60	0.40 ± 0.10^{AB}	0.28 ± 0.06^A	0.53 ± 0.08^{BC}	0.69 ± 0.16^C
90	0.72 ± 0.16^A	0.45 ± 0.11^B	0.60 ± 0.10^{AB}	0.73 ± 0.08^A
120	0.91 ± 0.12^A	0.80 ± 0.11^A	0.82 ± 0.13^A	0.88 ± 0.11^A
150	1^A	1^A	1^A	1^A
Surface Knoop hardness (MPa)	45.11 ± 3.02^A	40.40 ± 5.78^A	59.23 ± 6.31^B	62.96 ± 5.61^B

Values are means $\pm$ standard deviations ($n = 6$). Uppercase superscript letters indicate statistically significant differences between treatments (two-way ANOVA, *post hoc* Tukey's test, $p < 0.05$).

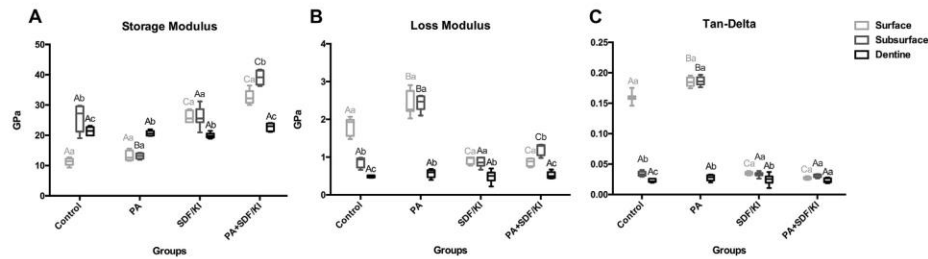


Fig. 1. Comparison of the average dynamic elastic moduli (loss, storage and complex moduli) obtained by nanoindentation from different regions (surface, subsurface and sound dentine) of each group. Distinct superscript uppercase letters in the same grayscale indicate statistically significant differences between treatments and lowercase letters indicate statistically significant differences between depths with the same treatment.

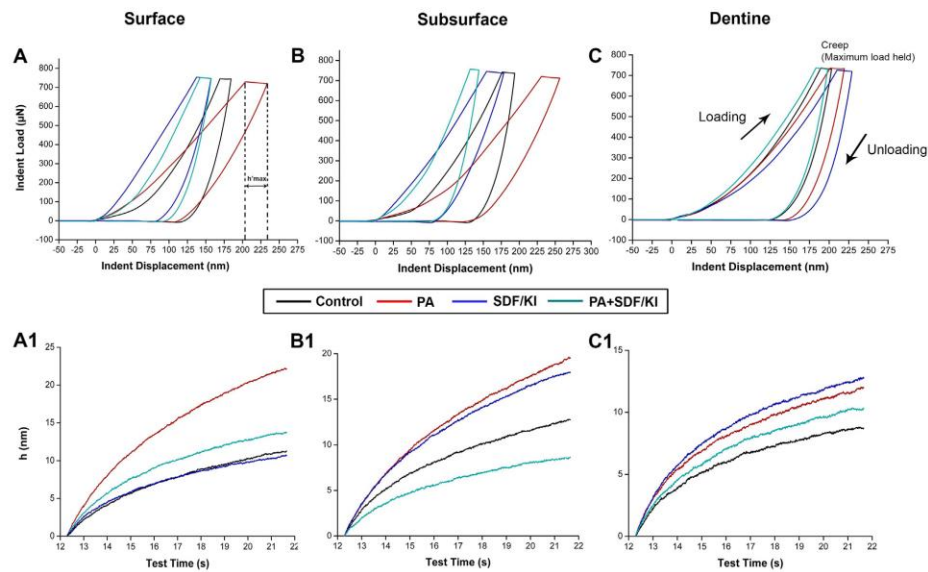


Fig. 2. (A–C) Typical load displacement curves with a creep hold segment. H: the additional indenter displacement before unloading during creep. Indentation responses are shown for surface, subsurface and sound dentine areas and results from different treatments. (A1–C1) Zero offset initial depth of penetration of indenter during maximum load held stage (creep) against time (h–t).

Table 3

Summary of the maximum penetration depth during creep (h_{max}) and creep rate sensitivity (m). Uppercase superscript letters indicate statistically significant differences between treatments and lowercase letters indicate statistically significant differences between depths.

Area	H_{max} (nm)			
	Control	PA	SDF/KI	PA + SDF/KI
Surface	23.0 ± 2.4 ^{A,a}	19.4 ± 1.1 ^{B,a}	11.6 ± 0.7 ^{C,a}	9.2 ± 0.8 ^{C,a}
Subsurface	12.5 ± 2.0 ^{A,b}	19.0 ± 1.1 ^{B,a}	14.4 ± 1.2 ^{A,b}	7.8 ± 1.1 ^{C,a}
Dentine	9.3 ± 1.6 ^{A,c}	12.2 ± 1.2 ^{A,b}	11.5 ± 1.7 ^{A,a}	9.1 ± 0.8 ^{A,a}
Area	Creep rate sensitivity (m)			
	Control	PA	SDF/KI	PA + SDF/KI
Surface	0.18 ± 0.05 ^{A,a}	0.14 ± 0.02 ^{A,B,a}	0.12 ± 0.02 ^{B,C,a}	0.09 ± 0.01 ^{C,a}
Subsurface	0.10 ± 0.02 ^{A,b}	0.15 ± 0.02 ^{B,a}	0.14 ± 0.03 ^{B,a}	0.09 ± 0.02 ^{A,a}
Dentine	0.06 ± 0.01 ^{A,b}	0.08 ± 0.02 ^{A,b}	0.06 ± 0.01 ^{A,b}	0.06 ± 0.01 ^{A,a}

Values are means ± standard deviations (n = 6). Uppercase superscript letters indicate statistically significant differences between treatments and lowercase letters indicate statistically significant differences between depths (two-way ANOVA, post hoc Tukey's test, p < 0.05).

increased the creep rate sensitivity significantly in the subsurface areas (SDF vs. control = 0.14 ± 0.03 vs. 0.10 ± 0.02 ; $p = 0.047$). The combined use of PA and SDF/KI reversed the creep rate sensitivity of demineralised dentine both in the surface and subsurface areas. Specifically, in the subsurface area, the creep rate sensitivity after PA + SDF/KI treatment decreased to a value not statistically different from that of sound dentine, which was different from SDF/KI treatment (Subsurface: SDF/KI vs. PA + SDF/KI = 0.14 ± 0.03 vs. 0.09 ± 0.02 ; $p = 0.043$).

3.3. Polarised light microscopy and image analysis

Representative PLM micrographs of root fragment sections viewed in water from each group are presented in Fig. 3(A–D). In the profile obtained from PLM, a baseline lesion after demineralisation was present in all samples. When imbibed with water, sound dentine showed positive birefringence. The staining of PA on dentine was observed along the tubule structure with precipitation on the surface, and the tubule structure was evident and clearly visible in PA treated groups. SDF/KI treatment contributed to a homogeneous dark band. PA followed by SDF/KI generated a stained band in the demineralised area with visible tubular structure.

3.4. Micro-CT

3D reconstruction images (A–D) and typical cross-sectional slices (E–H) from each group are illustrated in Fig. 4. The dentine surface showed a slightly bright area probably due to beam hardening effects. Typical subsurface lesion with decreased GV was present in the control and PA groups. After treatment with SDF/KI, a higher GV was present in the demineralised lesion area. With the adjunctive use of PA, the distribution of GV was more homogeneous.

The relative GV profiles of specimens are shown in Fig. 5. After demineralisation, the profile indicated a typical subsurface lesion with a peak at the surface zone. PA treatment had a similar profile. A significant increase in GV was observed when the specimens were treated with SDF/KI. Combined use of PA and SDF/KI enhanced the effect, as the relative GV had twice the value of sound dentine. The increase in GV was observed in a deeper area with smaller standard deviation, which indicated the adjunctive application of PA might contribute to an improved ion uptake and homogenous distribution of the SDF/KI.

The integrated relative GV (areas under the curve) was determined from the lesion surface with $30 \mu\text{m}$ increments up to a depth of $150 \mu\text{m}$ of the lesion front ($150 \mu\text{m}$ from the surface) (Table 4). Application of PA had no statistically significant effects on the integrated GV of the specimens. However, when the demineralised dentine was treated with SDF/KI, significantly higher integrated GV was observed around the $30\text{--}60 \mu\text{m}$ area compared to the control group ($P < 0.0001$), whilst the difference was not statistically significant around the $0\text{--}30 \mu\text{m}$ area. With combined use of PA and SDF/KI, a significant increment in integrated relative GV was observed from surface up to $90 \mu\text{m}$ ($P < 0.0001$).

3.5. SEM observation

Fig. 3 (E–L) presents typical SEM images of lesions in different groups. In the cross-sections, the demineralised organic portion was distinctly demarcated against the mineralised tissue (data not shown). The demineralised dentine specimens in the control group showed a surface with completely patent dentinal tubules with diameters around $3\text{--}4 \mu\text{m}$ (Fig. 3E). Treatment with PA contributed to the formation of slit-shaped orifices of the dentinal tubules which were partially occluded (Fig. 3F). For the SDF/KI treated groups, the SEM image showed clustering of minerals (Fig. 3G) and when combined with PA, small discrete crystal for-

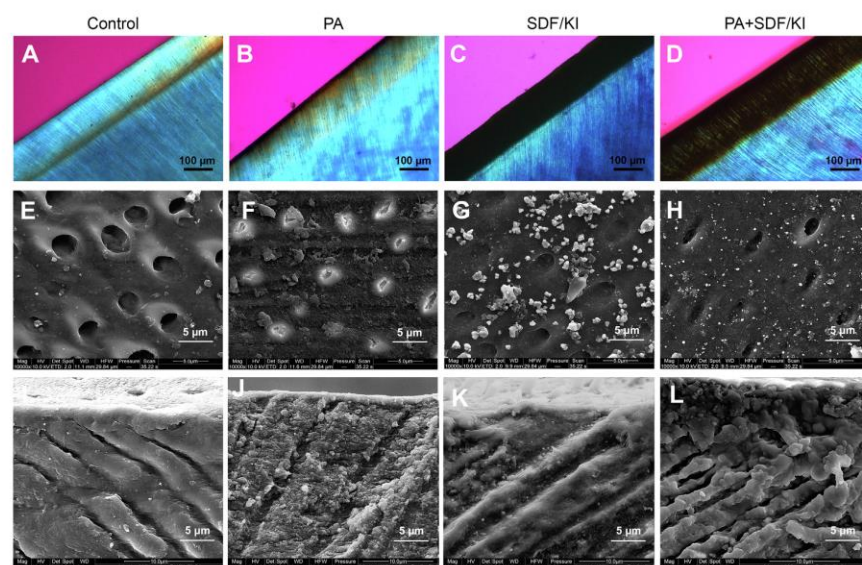


Fig. 3. (A–D) Polarised light microscopy with a first-order red plate images of lesions after exposure to different treatments. Specimens were viewed in water (refractive index 1.33); SEM images of (E–H) surface morphology and (I–L) longitudinal cross-sectional structure after treatments.

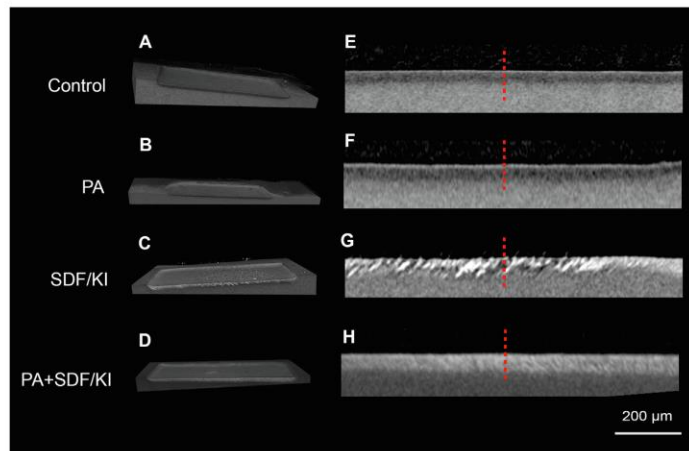


Fig. 4. 3D construction of micro-CT scanning (A–D) and typical longitudinal crosssectional slides (E–H) from samples with different treatments.

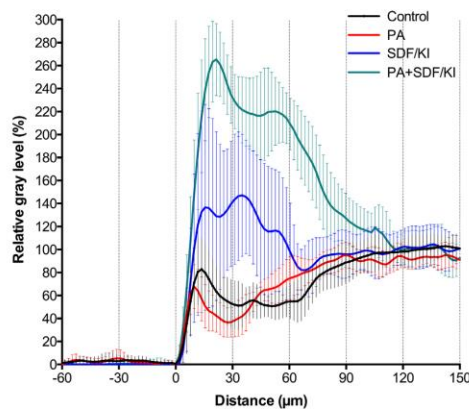


Fig. 5. Analysis of the line profile (marked in red dotted lines in Fig. 4) of grayscale value (%) in longitudinal cross-sectional slides from different groups.

mation ($<1 \mu\text{m}$) distributed over the dentine surface (Fig. 3H). From the cross-sectional images, dense granular structures of spherical grains were found in the inter-tubular area of dentine treated with PA. A few small particles were distributed on the cross-sectional surface of dentine in the SDF/KI treated group.

4. Discussion

The present study evaluated the potential effects of the combined use of PA as a cross-linking agent with a high concentration fluoride agent (SDF/KI) in the treatment of root caries in a laboratory-based study. The results indicated SDF/KI induced small discrete crystal formation that was distributed over the dentine surface and associated with an improvement in the mechanical properties (microhardness, elastic modulus and creep behaviour) of demineralised root dentine in the surface area 24 h after application, whilst the effects of SDF/KI in the subsurface areas were not significant. The ion uptake and distribution from SDF/KI could be observed up to $60 \mu\text{m}$ from the surface with a relatively heterogeneous distribution. Specimens biomodified with PA before application of SDF/KI seem to show some enhancement of the SDF/KI effects in the subsurface regions of the demineralised root dentine. Significant improvement of the cross-sectional microhardness, elastic modulus and creep behaviour were all observed in both the surface and subsurface areas. The ion uptake from SDF/KI was found in deeper areas (up to $90 \mu\text{m}$) with a more homogenous distribution observed in PA pre-treatment groups compared to those samples without the PA pre-treatment. The proposed treatment time of PA, which was two applications of 1 min each is believed to be within a clinically acceptable time frame.

Chemical model systems have the possibility to simulate the physicochemical changes (e.g. mineral content and hardness) of natural root caries for laboratory studies [39,53]. The chemical models we used in the current study to create artificial root caries were based on previously published work [38–40]. With a

Table 4

Integrated relative grayscale value (GV) (areas under the curve) from the lesion surface to sound dentine area with $30\text{-}\mu\text{m}$ increments ($\text{GV}\%\cdot\mu\text{m}$). Lowercase superscript letters indicate statistical significance among groups in each variable of each column.

Group	Depth increment (μm)				
	0–30	30–60	60–90	90–120	120–150
Control	$1620 \pm 531^{\text{a,b}}$	$1544 \pm 273^{\text{a}}$	$2018 \pm 417^{\text{a}}$	$2796 \pm 340^{\text{a,b}}$	$2761 \pm 254^{\text{a}}$
PA	$1292 \pm 341^{\text{a}}$	$1726 \pm 305^{\text{a}}$	$2339 \pm 277^{\text{a}}$	$2657 \pm 254^{\text{a}}$	$2551 \pm 173^{\text{a}}$
SDF/KI	$2882 \pm 1420^{\text{b}}$	$3685 \pm 818^{\text{b}}$	$2421 \pm 396^{\text{a}}$	$2870 \pm 415^{\text{a}}$	$2771 \pm 419^{\text{a}}$
PA + SDF/KI	$5191 \pm 726^{\text{c}}$	$6399 \pm 724^{\text{c}}$	$4389 \pm 688^{\text{b}}$	$3285 \pm 333^{\text{b}}$	$2670 \pm 357^{\text{a}}$

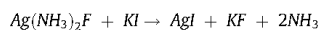
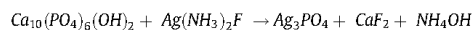
Lowercase superscript letters indicate statistical significance among groups in each variable of each column (Welch's ANOVA, *post hoc* Dunnett's T3 test, $p < 0.05$).

controlled pH via buffering and composition of the demineralising solution, consistently reproducible subsurface lesions could be created. The artificial root caries lesions created in the current study were within the range of $150 \pm 10 \mu\text{m}$. The demineralisation solution, which diffused through the matrix and gradually dissolved the mineral components, significantly lowered the microhardness from the outer to the inner demineralised regions. Also, the low pH of the demineralisation solution is believed to alter the biochemical nature of intrapeptide and interpeptide cross-links resulting in decreased elastic modulus [54]. A significantly lower mineral density could be directly observed via micro-CT. Polarised light microscopy allowed for the identification of dentine's birefringence colour with the use of a first order red plate. In water, dentine is positively birefringent due to a superimposition of weak negative birefringence of oriented submicroscopic dentinal crystals and stronger positively oriented birefringence of the collagen matrix [18]. As the demineralisation occurs, the strength of the positive birefringence increased by loss of the weak negative birefringent crystals.

Professional application of SDF has been widely acknowledged to be efficacious in arresting active dentinal caries in primary teeth as well as in the management of root caries in the elderly [17]. Application of KI after SDF was proposed as a method to solve the discoloration of teeth caused by SDF. The KI reacts with the free silver ions to form a white precipitate of silver iodide [55]. Nevertheless, its reliability and potential side-effects require further evaluation.

Results from the present study demonstrated that SDF/KI was able to interact with the demineralised root dentine area, resulting in a dark band in PLM, putatively because the penetration of silver and fluoride ions interfered with the orientation of sub-microscopic pores in the demineralised area. The ion uptake from SDF/KI contributed to an increase in GV in the demineralised dentine area that was approximately a two-fold increase compared to that of sound dentine.

The significant increase of GV after SDF/KI was observed up to a depth of $60 \mu\text{m}$, which might indicate the effective penetration of silver ions into the chemically-created artificial root carious lesions. This depth was generally similar to that observed in a laboratory-based *S. mutans* induced artificial caries model as well as in sound dentine in a previous study, which was recorded a depth of approximately $50 \mu\text{m}$ [56]. SDF/KI was able to restore the mechanical properties of demineralised root dentine. Results from Knoop hardness test in this study demonstrated that the hardness recovery was limited to the surface area ($\sim 5 \mu\text{m}$ from the surface) compared to the control group. The application process was assumed to be diffusion-controlled because the active agents need to diffuse through the collagen structure of considerable thickness. The limited hardness recovery of SDF/KI might be due to the superficial barriers resulting from the reaction between silver and proteins in the outer area. The increase in microhardness is speculated to be a chemical reaction between HAP and SDF leading to the deposition of silver salts, putatively including Ag_3PO_4 and AgI. The succinct chemical reaction between reactants and products is as follows:



The relatively insoluble silver phosphate and the subsequent precipitate of silver iodide might contribute to the hardening of the demineralised dentine [17,57]. In this regard, hardening of SDF-arrested carious tissue is partly due to its reaction with silver in addition to fluoride-mediated remineralisation [17,58]. The

application of SDF/KI is effective as a means of recovering the mechanical properties of root dentine surface that has already undergone some demineralisation.

The introduction of fluoride shifts the balance from demineralisation to remineralisation or lesion arrest [59]. Nevertheless, root dentine has a more complex structure than enamel with more organic components (*i.e.* dentinal organic matrix (DOM)). The treatment efficacy of the topical fluoride agents strongly depends on the presence or absence of DOM. Specifically, it is believed that in the presence of DOM, the mechanism of action of topical chemicals is the uptake of remineralising ions in the underlying demineralised tissue, rather than surface precipitation – *i.e.* ions diffuse through this structure and accumulate in the underlying partially demineralised tissue [60]. Effective remineralisation of root dentine requires the protection of both organic and inorganic components to ensure sufficient recovery of integrated characteristics of dentine, including its hierarchical structure, mineral density and interfacial interaction of the mineral within the organic matrix [18].

Although a number of research projects have focused on how crosslinking agents would protect the demineralised organic matrix [31,61,62], there are a lack of studies investigating whether the stabilised organic matrix would enhance mineral uptake and promote collagen-mineral interaction; and whether treatment strategies targeting both organic and inorganic components of dentine would contribute to a better treatment of root dentine caries. Results from two recent studies indicate that when PA was used with other topical remineralising agents such as pastes containing casein phosphopeptide-amorphous calcium fluoride phosphate (CPP-ACFP) and tri-calcium phosphate (TCP), a synergistic effect on demineralised root dentine with a better distribution of mineral precipitation throughout root dentine carious lesions was demonstrated [40,63]. The present study took a further step on how PA, with the capability to stabilise the demineralised organic matrix, would help with the uptake and incorporation of remineralising ions and improve the properties of root dentine.

The results of the micro-CT demonstrated how the ions from the SDF/KI solutions penetrated and distributed in the demineralised carious lesions. Dentine is a porous biological composite material and the process of demineralization created more microscopic pores in dentine tissues. Silver diamine fluoride, where fluoride is present as complexes with silver, would expect to diffuse through the dentine [64]. The complexes would dissociate into silver and fluoride ions as they diffused, react with and rebuild the depleted dentine crystals. Also, dentine is permeable to silver ions via tubules because these ions have a particle size much smaller than the dentine tubule width [65]. Lambert-Beer's law predicts greater X-ray absorption in Ag than in Ca [66]. Thus, after the treatment with SDF/KI, silver was incorporated in dentinal tissues and manifested a higher GV in micro-CT images, even higher than the GV of sound dentine that is based on calcium phosphate salts. Moreover, when SDF/KI was used in conjunction with PA, a more significantly incremental increase in GV was achieved to a deeper area with a homogeneous distribution. The intermolecular cross-links of the collagen which were not irreversibly broken might be recovered after the application of PA. The cross-banded structure of the collagen fibres serves as a base to which ions can attach [67] and more interfibrillar space might be created for ion diffusion into the subsurface layer of demineralised dentine [68]. The rate of mineral deposition depends on the precipitation kinetics – the local factors such as pH, ionic concentrations, the presence of seed crystals or matrix and the surface area available for crystal growth [67]. In a well-constructed dentine matrix, the distribution of residual crystals was more homogeneous. The ions from treatment agents were not only precipitating on the superficial surface of dentine but also through ion migration. An evident structure of

dentinal tubules from PLM images indicated PA's effects on the protection of the dentine collagen.

The adjunctive application of PA appears to have a superior effect to SDF/KI alone on the nanomechanical properties (elastic modulus, nano-hardness and creep behaviour), especially in sub-surface areas. The combined application achieved significantly greater hardness numbers than the control group at the same depths up to 60 μm . It has been reported that the use of a reducing agent such as tannic acid from boiled tea with SDF could accelerate the deposition of silver phosphate [69]. As a condensed tannin, PA might share a similar function for accelerated deposition of silver ions, where more reactants of silver compounds resulted in higher hardness number. The viscoelasticity of the collagen fibrils comes from the intermolecular cross-linking [70]. Polyphenols such as PA have strong molecular interactions with proteins which have a high proline content by the formation of hydrogen bonds between phenolic hydroxyl groups and carbonyl groups linked to proline amino groups of protein [71]. Also, ionic and covalent interaction of PA with the side-chains of collagen fibril molecules has been hypothesised [72]. Thus, pre-treatment with PA might induce more dentinal collagen crosslinks, which strengthens the collagen matrix, and helps increase the modulus of elasticity when used together with SDF/KI.

The creep behaviour of a material can be determined from nanoindentation test with a load-holding stage, where the force and indenter displacement are recorded continuously as a function of time at a sub-micrometre spatial resolution [45]. Creep, which refers to continual deformation under constant load, is a characteristic of viscoelastic materials, such as dentine [48,73]. Investigation into the creep behaviour of dentine provides an understanding of its ability to resist stress under functional loading conditions.

Three models, *i.e.* the standard linear solid, Burgers and two dashpot Kelvin models, have been suggested to fit the h - t curves during the load-holding stage of materials with viscoelastic properties [74]. Viscoelastic bio-composites such as dentine and bone have at least two phases, namely, linear elastic inorganic phase of hydroxyapatite and inelastic organic phases of collagen and water [46,47,75]. It has been found that the Burgers model with four variable parameters was a better fit of h - t curves of these materials, which was used in the current study to provide a numerical description of the nanoindentation creep behaviour of dentine [46]. Results showed that the Burgers model was a good fit to the h - t curves of all specimens with R-squared > 0.99 (see [Supplementary Information Table A.1](#)). From the curves, normalised creep deformation could be calculated. In order to investigate the creep behaviour in more detail, the parameter of creep rate sensitivity (m) is calculated from stress (hardness) and strain-rate exponent, which reflects the flowability of a material and demonstrates how the rate of deformation of material changes in response to a change in the applied stress. In general, higher creep rate sensitivity would mean lower resistance of a material in response to mechanical stress [76].

The mechanical properties of dentine are strongly dependent on the interfacial interaction between collagen and the mineral phase [77]. It has been suggested that during loading, the HAP-collagen interface in dentine may undergo a sliding process, where the stress was transferred from the harder mineralised crystals to the softer organic matrix around them thus dissipating energy [11,78]. Demineralisation may damage the HAP-collagen interface, therefore the demineralised specimens presented a higher maximum creep deformation (h'_{max}) than the mineralised dentine, which is consistent with a previous study [79]. Also, significantly higher creep rate sensitivity (nearly 3-fold of the sound dentine) was observed, which indicated the weakened strain resistance of carious dentine. PA treatment significantly decreased the creep rate sensitivity of demineralised surface putatively because more

crosslinking was induced in the dentine matrix network structure, which increased the ability of collagen to transfer load therefore leading to increased creep resistance. Application of SDF/KI significantly increased the ability of dentine to resist against creep deformation in the surface area, whilst adjunctive application of PA and SDF/KI significantly increased the creep resistance of demineralised dentine to a value similar to the original sound dentine both in surface and subsurface areas. It was therefore speculated that the application of PA stabilised the organic matrix, which protected its ability for stress redistribution, and after introduction of minerals, a better organic-inorganic interaction was achieved, which contributes to improved creep behaviour.

The stabilised dentine organic matrix and improved organic-inorganic interaction are expected to be sustained under cariogenic (demineralising) conditions or collagenase exposure. Previous research has indicated that the chemical bonds introduced by PA were durable under hydrated conditions [31]. It was reported that improved biomechanical properties of PA-modified dentine could be sustained over time between 8 and 18 months [31,61,80]. Although not tested in the current study, PA has an inhibitory effect on MMPs and cysteines [32,81] and can enhance dentinal collagen stability during pH-cycling with collagenase as a stressor [40] and such collagenase inhibition has been reported to be sustained after 6 months storage [82]. In addition, the silver compounds and CaF_2 -like materials deposited on the dentine after SDF/KI application were expected to have an antibacterial effect on cariogenic biofilms and act as a slow-release fluoride reservoir respectively if a biofilm or pH-cycling model is used [17]. In the present study, when SDF/KI was applied in conjunction with PA, improved ion uptake was observed into deeper regions with a more homogenous distribution of ions. Therefore, the antibacterial and remineralising effects of SDF/KI are expected to be more extensive throughout the lesion. The ultimate goal of the combined use of PA and SDF/KI would be to achieve remineralisation of the root carious lesions, which should be quantitatively compared with classical remineralising agents such as NaF [83].

Of note, application of 6.5 w/v% PA solution on root dentine in this study led to a decrease in the hardness and modulus of elasticity of the carious lesions. This seemed in contrast to most previous research where plant extracts with a high PA content could induce a significant increase in microhardness and modulus of elasticity [34,62,84]. However, in most of the previous experimental designs, the 6.5% w/v PA solution was adjusted to a neutral pH with KOH or NaOH, while in the present study, the 6.5% w/v PA solution was kept as its original pH. Pilot study work demonstrated the pH value of 6.5% w/v PA solution was 4.46 ± 0.01 and remained stable during a six-month storage period at both room temperature and 4 °C. Thus, application of acidic PA solution might further demineralise the dentine surface. A previous study by Tang and colleagues has revealed that weakly acidic grape seed extract solution could induce immediate reductions in microhardness values of dentine, while steady increases in hardness were shown following remineralisation [68]. Also, PA's ability to interact with proline-rich proteins is pH dependent, where the greatest precipitation was found to be near the isoelectric point of protein [85]. The active polyphenols in PA are more stable under acidic conditions. Adjustment of its pH could influence its stability and biological activities [68]. Therefore, to maintain the PA at its original pH might retain a higher biological activity, although a negative influence could be observed in the short term. On the other hand, the relatively low pH level of PA might accelerate dissolution of apatite, which provides more free calcium ions and consequently favours the precipitation of fluoride-containing material after fluoride treatment.

However, an issue of concern is that in the experimental process, treatment with SDF/KI after the application of PA might compromise the effect of KI, as the dentine surface was more prone to

black discoloration. At its unadjusted acidic pH, PA might behave as a weak reducing agent due to its phenolic groups, which accelerated the deposition of silver phosphate and silver oxide. The precipitation of silver compounds resulted in the instantaneous black discoloration [69]. Precaution should be taken on the application of KI following SDF.

5. Conclusion

Within the limitations of this study, it was concluded that application of PA on artificial root carious lesions contributed to partial occlusion of dentinal tubules and it did not affect the mechanical properties and mineral density of the dentine ($P > 0.05$). Treatment of artificial root caries with SDF/KI induced small discrete crystal formation distributed over the dentine surface and the ion uptake and distribution from SDF/KI was up to 60 μm . SDF/KI application resulted in recovery of its nano-mechanical properties in the surface area. Adjunctive use of PA might enhance these effects, with significant increase in surface and cross-sectional microhardness, elastic moduli and a recovery of creep behaviour of demineralised dentine with decreased creep deformation and increased creep rate sensitivity ($P < 0.05$), especially in the subsurface areas. Additional use of PA also contributed to ion uptake from SDF/KI into deeper areas (90 μm) with a more homogenous distribution throughout the lesions.

Acknowledgements

This work was supported by Australian Dental Research Foundation (ADRF) Nathan Cochrane Memorial Grant. J.C. was supported by the China Scholarship Council - University of Melbourne PhD Scholarship. The authors would like to thank staffs in Showa University for their help in the nanoindentation testing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.actbio.2019.02.020>.

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Remineralising effects of fluoride varnishes containing calcium phosphate on artificial root caries lesions with adjunctive application of proanthocyanidin

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ARTICLE INFO

Keywords:
proanthocyanidin
CPP-ACP
TCP
fluoride varnish
dental organic matrix
root caries

ABSTRACT

Objectives. To evaluate the remineralising effects of fluoride (F) varnishes containing bioavailable calcium-phosphate compound (Ca-P) based remineralisation systems and 5000 ppm F toothpaste (FTP) on root caries lesions (RCLs) and the potential effects of proanthocyanidin (PA) for the treatments of RCLs when used as an adjunct to F regimens.

Methods. Demineralised root dentine and a pH-cycling model were used to mimic RCLs and the oral environment. Remineralising effects of MI Varnish™ (MIV) containing casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) and Clinpro™ White Varnish (CPWV) containing tri-calcium phosphate (TCP) along with FTP and PA were evaluated regarding the birefringence, elemental composition, mechanical properties and mineral density of remineralised dentine with Duraphat™ as a comparison.

Results. MIV, CPWV and Duraphat™ promoted the incorporation of F into RCLs and increased mineral density but did not change microhardness of root dentine significantly. Surface microhardness increased significantly when MIV or CPWV was used with 5000 ppm FTP. Application of PA with F regimens significantly increased subsurface mineral density. When PA was applied with MIV or CPWV along with FTP, the highest ion uptake and relative mineral gain (%ΔZ) was achieved, and significant increase of microhardness was up to 30 μm depth. Generally, MIV was associated with a higher mineral content gain than CPWV.

Significance. Treatment of carious root surfaces remains challenging due to the complex pathological processes and difficulty in restoring the highly organised structure of root dentine. Treatment strategies targeting both remineralisation and preservation of the dental organic matrix have the potential to improve the fluoride-mediated remineralisation approaches.

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<https://doi.org/10.1016/j.dental.2020.10.021>

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1. Introduction

Increasing tooth retention in ageing populations in industrialised countries and the age-related recession of gingival tissues exposing tooth root surfaces to the oral environment have contributed significantly to the increasing prevalence of root caries amongst the elderly [1,2]. Current treatments following caries management philosophies established for enamel caries may not be equally efficacious for root dentine caries because of the different structures and compositions of enamel and dentine and the associated distinct aetiologies and disease progression of coronal and root caries [3].

Dentine is an hierarchically organised nanocrystalline composite structure formed by matrix-mediated mineralisation. The organic framework of dentine consists of parallel self-assembled 300 nm long collagen units composed of collagen fibrils of 67 nm periodicity staggered and overlapped along their long axes and stabilised by covalent inter- and intra-microfibrillar cross-linking [4,5]. The gap spaces between the collagen molecular ends are the primary sites for nucleation and growth of the plate-like impure hydroxyapatite crystals [6]. This structural and stereochemical matching between inorganic and organic materials confers dentine with specific mechanical properties and promotes synergy between matrix and mineral during the caries process [7]. Specifically, in the early stages of caries development, the pH decrease produced by metabolites of acidogenic bacteria tilts the balance of de- and re-mineralisation towards mineral dissolution - especially of the extrafibrillar minerals. Mineral loss affects the packing arrangement of the fibrils and activates the host proteases along with bacterial collagenase action, leading to lysis of dentinal collagen. The loss of collagen periodicity and the shifts of inter-molecular cross-linkages of the molecules of dentinal collagen result in further loss of intrafibrillar minerals, decreasing the mechanical properties of dentine [8,9].

Demineralisation can be reversed in its early stages through incorporation of component ions (calcium, phosphate and fluoride) onto the remnant nano-crystallites [10]. Fluoride (F) is currently considered the gold standard therapeutic agent for early carious lesions. It deposits on tooth surfaces as CaF_2 -like materials and substitutes into dental mineral through dissolution/re-precipitation reactions to form an apatitic mineral of increased structural stability - $\text{Ca}_5(\text{PO}_4)_3(\text{OH})_{1-x}\text{F}_x$ [11,12]. Given that root dentine has a higher critical pH and smaller impure hydroxyapatite crystallites than enamel, there is clinically validated consensus that high concentration F toothpastes (FTP) and F varnishes are recommended to maintain the mineral balance in root caries lesions (RCLs) and the efficacy of F treatments could be further enhanced by a combination of F delivery methods [10,13]. In addition, the availability of calcium and phosphate (mainly from saliva and gingival crevicular fluid) is rate limiting for remineralisation. Ageing and medication in elders could affect salivary flow and constituents and decrease the availability of calcium and phosphate for restoring mineral density of RCLs [14]. Therefore, bioavailable calcium-phosphate compound (Ca-P) based remineralisation systems have been incorporated and stabilised into F products to promote remineralisation [11]. Nevertheless, it is still controversial whether the addition

of Ca-P has superior effects over F alone, as well as the relative efficacy of different Ca-P based remineralisation systems [15–18]. In particular, there is a lack of published evidence of their effectiveness on RCLs.

Whilst F treatments in combination with calcium and phosphate restore the mineral content of carious lesions, poorly remineralised tissue with majority of minerals occupying extrafibrillar space and decreased strength of collagen fibrils may not recover the overall functionality of dentine [7]. Increasing understanding of the hierarchical structure and biochemistry of dentine has promoted the advent of innovative biomimetic approaches for treatment of dentine carious lesions. Specifically, the presence of dentinal collagen could putatively provide a template and exert spatial constraints to mediate spherulitic crystalline growth of minerals after infiltration of ionic clusters of mineralisation precursors containing Ca-P [19,20]. Therefore, strategies for dentinal collagen biomodification to prevent degradation of the demineralised matrix have been explored to enhance the interaction of minerals within the collagen fibrils [21,22]. Locally applied collagenase inhibitors such as chlorhexidine and Zn^{2+} could inhibit the activity of proteolytic enzymes during dentine demineralisation and reduce dentine matrix degradation, thus limiting lesion progression [23–25]. However, inhibitors might not affect all proteases involved in the degradation process. On the other hand, when affected by collagenase, the cross-links of dentinal organic matrix are partially shifted to precursors whilst the collagen matrix is not structurally and biochemically different from that of sound dentine, therefore, it has the potential to revert to normal collagen fibres [26]. In this regard, induction of cross-linking might have the ability to recover the structure of dentinal collagen [27]. Particularly, chemical cross-linking using agents such as glutaraldehyde and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide (EDC/NHS) are the most effective strategies to produce a uniform and high degree of cross-linking in dentine, however, their cytotoxicity may be a limitation for clinical applications [28,29]. Biocompatible natural cross-linkers, mostly polyphenols, have received increasing attention in recent decades. Amongst them, proanthocyanidin (PA), derived from grape seed extracts, holds great potential to cross-link dentinal collagen by covalent and ionic linkages as well as hydrogen bonds and hydrophobic interaction [30]. Previous studies have reported that application of PA increased the mechanical properties of demineralised dentine and enhanced the remineralisation of RCLs by stabilising collagen [28,31]. Adjunctive application of PA with F/Ca/P showed promising results in improving mineral gain and mechanical properties as well as preventing proteolytic biodegradation in dentine [32–34]. Considering the potential beneficial effects of PA, we were motivated to investigate the strategy of application of remineralising agents along with biomodification of dentinal collagen for the treatment of RCLs.

In the present study, firstly, the remineralising efficacy of MIV and CPWV on RCLs was investigated using laboratory simulated RCLs *ex vivo*. The effects of 5000 ppm FTP were explored, also in combination with MIV or CPWV. Moreover, PA was used as an adjunct to MIV, CPWV and 5000 ppm FTP and their combined effects on RCLs was examined in terms

of birefringence, elemental composition, mineral density and mechanical properties of the treated root dentine.

2. Materials and Methods

2.1. Materials

Materials used were proanthocyanidin (International Laboratory, CA, USA; containing >95% oligomeric proanthocyanidins); Clinpro™ White Varnish (CPWV; 5% NaF – 22,600 ppm F with TCP; 3 M ESPE, MN, USA); MI Varnish (MIV; 5% NaF – 22,600 ppm F with RECALDENT™ (CPP-ACP); GC Corp., Tokyo, Japan); Clinpro™ 5000 ppm FTP (3 M ESPE, MN, USA) and Duraphat™ varnish (5% NaF – 22,600 ppm F; Colgate-Palmolive, NY, USA). The demineralising solution for RCL formation contained 50 mmol/L acetic acid, 2.2 mmol/L CaCl₂ and 2.2 mmol/L KH₂PO₄, and the solution was adjusted to pH 5 with KOH. Treatment solutions in pH-cycling included a demineralisation solution prepared using 50 mmol/L acetic acid, 1.5 mmol/L CaCl₂ and 0.9 mmol/L KH₂PO₄ and adjusted to pH 5 with KOH and a remineralisation solution prepared using 1.5 mmol/L CaCl₂, 0.9 mmol/L KH₂PO₄, 130 mmol/L KCl and 50 mmol/L HEPES buffer and adjusted to pH 7 with KOH. Details of the materials and the application methods are listed in Table 1.

2.2. Preparation of root dentine specimens

Extracted human permanent molars from individuals aged 20 to 50 were stored in 0.1% thymol solution at 4 °C under the protocol approved by the Human Research Ethics Committee of the University of Melbourne. The teeth were cleaned with deionised distilled water (DDW) to remove surface debris. Non-carious molars without obvious defects on the root surface were selected. The crown and root apical portions were removed using a slow-speed diamond saw (Isomet, Buhler Ltd, IL, USA) under water irrigation. The minimum sample size was estimated based on a power of 0.8 with a confidence level of 95% following the method in our previous study [34]. Six specimens per group were prepared for cross-sectional microhardness testing, two for micro-computed tomography (micro-CT) analysis and afterwards energy-dispersive X-ray spectroscopy (EDS) analysis and three for polarised light microscopy observation. One spare sample was prepared in each group. Therefore, 12 dentine blocks in each group were prepared from the buccal or lingual surfaces of the roots adjacent to the cemento-enamel junction (CEJ) and embedded in epoxy resin (KEP epoxy, Kemet International Ltd., Kent, UK). The tooth surfaces were polished using ascending grits (320, 600, 1200, 2500) of silicon carbide (SiC) papers to expose the root dentine surface and the debris was removed by ultrasonic cleaning for 5 min in DDW. A layer of acid-resistant nail varnish was applied on the polished root surfaces to expose a window of 3 × 4 mm for treatments.

2.3. Treatment procedure

2.3.1. Demineralisation

The whole treatment procedure is illustrated in Fig. 1. The protocol for demineralisation was modified from that previously

reported [32,34]. Specifically, each specimen was immersed in 20 mL of the buffered acidic demineralising solution (see 2.1), which was prepared and changed every 24 h. After demineralisation for 120 h at 37 °C, another layer of acid-resistant nail varnish approximately 1 mm wide was applied onto the exposed dentine surface to isolate part of the exposed demineralised dentine as a demineralisation baseline for comparison.

2.3.2. Treatments

Root dentine specimens were assigned randomly to the 11 treatment groups (n = 12 per group) (Fig. 2). Specifically, the DDW group served as the negative control and Duraphat™ varnish as the positive control. The 6.5% w/v PA solution was prepared by adding PA powder into DDW and mixing thoroughly by pipetting up and down to ensure uniformity. The PA solution was applied to root dentine surfaces using a micro-brush and agitating for 1 min. The excess solution was removed gently with a cotton pellet. This was followed by a further application and agitation of fresh PA solution for 1 min. MIV or CPWV was applied according to the manufacturer's instructions (Table 1).

2.3.3. pH-cycling

After treatment, all specimens were stored in a remineralisation solution (20 mL per sample) for 6 h without interference of the treated surface and then subjected to the pH-cycling process. Specifically, specimens were immersed in the demineralisation solution (see 2.1) for 1 h and remineralisation solution (see 2.1) for 23 h at 37 °C. Solutions were refreshed with each pH-cycle and the process lasted for 8 d. After each immersion in demineralisation solution for 1 h, the samples were cleaned gently using DDW and blot dried. For samples in Groups A-B, E-G, 5000 ppm FTP was applied and brushed onto the exposed dentine surface with a micro-brush for 3 min, gently cleaned using DDW and blot dried before immersion in remineralisation solution. Samples in Group C-D, H-K were gently cleaned with DDW without brushing with FTP before immersion in the remineralisation solution. After pH-cycling, samples were cleaned ultrasonically in DDW for 5 min and stored in a labelled container with damp gauze thereby creating a humidified environment before analysis.

2.4. Analytical methods

2.4.1. Polarised light microscopy

Three samples were randomly selected from each group, re-embedded in epoxy resin to protect the demineralised surfaces and sectioned longitudinally through the centre of the lesions with a low-speed water-cooled diamond saw to obtain 300 µm thick sections. During sectioning, the exposed demineralised dentine was covered with damp gauze to protect the demineralised dentine from dehydration. Each specimen was further manually lapped to a thickness of approximately 100 µm. The sample was mounted on a glass slide with DDW and covered with a clean coverslip. The slides were visualised at original 200× magnification and the birefringence was recorded using a first order red plate between crossed polarisers with transmitted light microscopy (DM2000, Leica Microsystems GmbH, Wetzlar, Germany) coupled to a camera

Table 1 – Materials used in this study.

Material brand	Abbreviation	Manufacturer	Code (Lot#)	Therapeutic composition (w/w) [35]	Application instructions
MI Varnish™	MIV	GC Corporation, Tokyo, Japan	1611141	5% sodium fluoride (NaF; 22,600 ppm F), 1–5% CPP-ACP	1. Tooth surface cleaned and softly dried using the compressed air. 2. Stir the varnish in the unit dose container and apply a thin and uniform layer of MI varnish on tooth surface using a micro-brush.
Clinpro™ White Varnish	CPWV	3 M ESPE, MN, USA	N865382	5% NaF (22,600 ppm F), 1–5% modified TCP	1. Tooth surface cleaned and softly dried using the compressed air. 2. Carefully mix the varnish with a micro-brush; apply the varnish evenly on the tooth surface.
Duraphat™	5%NaF	Colgate®, NY, USA	70091	5% NaF (22,600 ppm F)	1. Tooth surface cleaned and softly dried using the compressed air. 2. Apply a thin layer to the dentine surface using a micro-brush.
Clinpro™ 5000 toothpaste	FTP	3 M ESPE, MN, USA	131041	1.1% NaF (5,000 ppm F)	1. Brush the dentine specimen using a micro brush with the toothpaste for 3 min. 2. Rinse the specimens with deionised distilled water.
Oligomeric proanthocyanidins PA (>95%)		International Laboratory, CA, USA	781046	A collagen cross-linking agent containing polyphenolic compounds derived from grape seed extracts.	1. Tooth surface cleaned and softly dried using the compressed air. 2. Apply on the dentine surface using a micro-brush and agitate for 1 min (two applications).

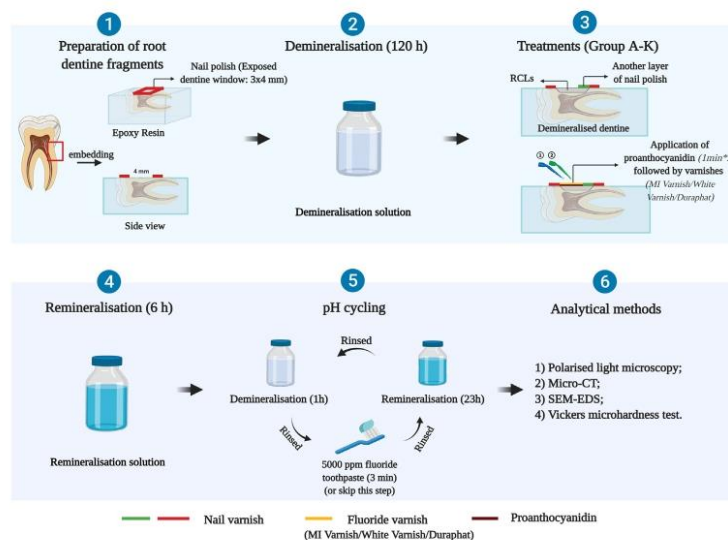


Fig. 1 – Schematic diagram of the treatment process.

(Leica EC3, Leica Microsystems GmbH, Wetzlar, Germany). The images were photographed and analysed by means of the software program ImageJ 1.38 × (National Institutes of Health, MD, USA).

2.4.2. Micro-CT analysis

Two dentine specimens randomly selected from one group were aligned in a line and fixed in a pipette tip for micro-CT scanning where a scout scan mode was used. The den-

Samples	Crosslinking	Varnishes	Toothpastes	Group names[Label]
Artificial RCLs	+PA	MIV	+FTP	PA+MIV+FTP [A]
			/	PA+MIV [C]
		CPWV	+FTP	PA+CPWV+FTP [B]
			/	PA+CPWV [D]
		/	+FTP	PA+FTP [E]
			/	PA+FTP [F]
	/	MIV	+FTP	MIV+FTP [G]
		/	/	MIV [H]
		CPWV	+FTP	CPWV+FTP [I]
		/	/	CPWV [J]
	DDW (Negative Control)			DDW [K]
	Duraphat™ (Positive Control)			5%NaF [L]

Fig. 2 – Treatments in each experimental test group and group names and labels.

tine samples were dehydrated overnight before scanning to avoid misalignment caused by shrinkage during the long-time scanning. The imaging settings for high resolution Micro-CT analysis (Skyscan 1172, Skyscan N.V., Aartselaar, Belgium) were as follows: voltage: 58 kV, beam current: 171 mA, resolution: 2.1 μm and rotation: 360° in a 0.2° step. A 0.508 mm aluminium filter was used to reduce the beam-hardening artifacts. Prior to each scanning, a new flat field reference was acquired. For calculation of mineral density, two mineral cylinder phantoms of a similar size as the specimens with the mineral density value of 0.25 and 0.75 $\text{g}_{\text{HAP}}/\text{cm}^3$ respectively were scanned with the same imaging settings. The resulting 2D projected images were reconstructed using the NRecon software (Version 1.4.2, Skyscan N.V., Aartselaar, Belgium) interface based on a modified Feldkamp algorithm with the following parameter settings: smoothing: 3, ring artefact correction: 15 and beam hardening correction: 50% (see Supplementary Information on beam hardening settings). After reconstruction, the reconstructed images were imported into Image J 1.38 x and resliced to obtain vertical two-dimensional slices which showed cross-sectional surfaces of whole carious lesion areas.

The mean mineral density was estimated in a volume of interest (VOI) of $30 \times 20 \times 250$ voxels (width $\times$ thickness $\times$ depth) ($30 \times 2.1 \times 20 \times 2.1 \times 250 \times 2.1 \mu\text{m}^3$) at each consecutive 2.1 μm in depth. The regions of interest (ROIs) were selected in dentine in a direction perpendicular to the lesion surface covering all lesion depths given the difficulties in selecting ROIs exactly parallel to the lesion surface in a small root dentine segment. Otherwise large heterogeneity in mineral density values of each ROI would occur. An example of a 2D image of ROI was shown in the red rectangular area in Fig. 4 (I). For each sample, three VOIs were selected randomly at the regions of demineralised dentine (covered by the second layer of nail polish), remineralised dentine (demineralised dentine exposed for treatments) and sound dentine respectively.

For the normalisation of the mineral density profile in each group, the sound dentine region covered by nail polish was used to provide a superimposition reference of the original

dentine surface (depth axis of 0 μm ; as shown in Fig. 4 (I)). Thus, the mineral density data were reported with the same starting point irrespective of the depths of shrinkage in the demineralised dentine regions. The mineral loss (ΔZ) across the lesion depth was calculated from the mineral density profile of each sample by subtracting the area under the mineral density curve of demineralised or remineralised dentine from the area of sound dentine. Mineral density changes predominantly occurred within the first 250 μm so the mineral loss (or gain) from the original dentine surface to a depth of 250 μm was determined.

To evaluate the extent of remineralisation, the relative mineral gain (% ΔZ) after remineralisation was calculated as follows:

$$\% \Delta Z = \frac{\Delta Z_d - \Delta Z_r}{\Delta Z_d} \times 100$$

where ΔZ_d is the difference between the area under the curve (AUC) of mineral density profiles of baseline demineralised root dentine and sound dentine; ΔZ_r is the difference between the AUC of mineral density profiles of remineralised dentine and sound dentine; and $\Delta Z_d - \Delta Z_r$ is equal to the mineral gain during treatments and pH-cycling (as shown in Fig. 5A).

In order to compare % ΔZ during treatments and pH-cycling in different layers of dentine surface and subsurface, % ΔZ at 30 μm increments (a 40 μm increment for the last layer) from the dentine surface to a depth of 250 μm (i.e. 0-30, 30-60, 60-90, 90-120, 120-150, 150-180, 180-210 and 210-250 μm) was determined and plotted in a 3D bar graph using OriginPro 2018 (OriginLab Corporation, MA, USA).

2.4.3. Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS)

Samples used for micro-CT analysis were polished again using 2500 and 4000 SiC paper then 1 and 0.5 μm diamond suspensions in order to reduce the geometric effects on quantitative analysis by EDS; ultrasonically cleaned for 5 min to remove the polishing debris and desiccated at room temperature overnight. The cross-sectional morphology of each sample was observed by using SEM. Samples were not coated to avoid

the influence caused by the peaks of the coating material and the uncontrolled deviations due to the difference in coating thickness in quantitative EDS analysis [35]. The elemental analysis of Ca, P and F were determined using the line scan mode of EDS with a silicon drift detector (SDD) coupled to the SEM (Oxford instruments; voltage: 10.0 kV; working distance: 9.9–10.3 mm). The distance between each reading point was 1.2 μm and the live running time for each scan was 600 s to ensure sufficient counts for peak identification. Assessments of the line scan were made in triplicate in three randomly chosen areas from the lesion surface to the sound dentine along a direction perpendicular to the dentine surface in the demineralised and remineralised dentine regions. A line scan was conducted on the sound dentine area as a comparison to show the elemental changes in un-demineralised dentine. The relative X-ray intensity from EDS spectra reflecting the elemental concentrations of Ca, P and F in dentine was plotted against the lesion depth and the atomic percentage of Ca, P and F was quantified by Aztec 3.3 software (Oxford Instruments, High Wycombe, UK) available in EDS. In particular, F was included from the periodic table manually for peak identification if it was not displayed automatically due to its low concentration in the specimens.

2.4.4. Cross-sectional microhardness test

Six longitudinally sectioned samples in each group were re-embedded in epoxy resin and polished on a water-cooled grinding and polishing unit (Tegra-Pol 21, Struers, Copenhagen, Denmark) with abrasive papers (600, 1200, 2500 and 4000 grit), followed by 1 and 0.5 μm diamond suspensions (Type K, Kemet International Ltd., Kent, UK). The debris on the surface was removed by ultrasonically cleaning for 5 min in DDW. Cross-sectional microhardness measurements perpendicular to the demineralised surface were performed below the surface at less than 15 μm , 20 μm , 30 μm , 40 μm ... to 200 μm depth with 10 μm interval using Vickers hardness indentation (402 MVD, Wolpert Wilson Instrument, MA, USA) with a load of 10 gf ($98 \times 10^{-3}\text{N}$) for 10 s dwell time at each testing point. For all samples, indentations were performed on the cross-sectional dentine surface at each depth on parallel tracks approximately 150–200 μm apart both in the demineralised and remineralised dentine regions. Hardness was determined according to the following equation:

$$H = \frac{P}{A}$$

where P is the applied force and A is the contact area of the indentation which depends on the indenter geometry and the penetration depth [9]. Measurements were discarded if cracks occurred on dentine during indentation. To avoid variations of microhardness of different samples, an internal control of the sound area (250 μm depth from the surface) of each dentine block was used for comparison. Results were expressed as relative microhardness values [36].

2.5. Statistical analysis

Quantitative data were expressed as mean $\pm$ standard deviation (SD). The assumptions of homogeneity of variance and normal distribution were checked using Levene's and Shapiro-

Wilks tests, respectively. Vickers microhardness numbers data were subject to Student's paired t-test. The relative atomic percentage (At%) data from EDS were analysed by two-way ANOVA followed by *post hoc* Bonferroni test. Other statistical analysis was carried out by one-way ANOVA and *post hoc* Tukey's (equal SD assumed) or Dunnett's T3 (equal SD not assumed) multiple comparisons. A level of significance of $\alpha = 0.05$ was set in all statistical tests (SPSS Version 22.0; IBM, NY, USA).

3. Results

3.1. Polarised light microscopy

Images of polarised light microscopy demonstrated the lesion depths and changes of colour birefringence which indicated the mineral content changes qualitatively during de-/remineralisation [37]. Specifically, demineralised dentine showed different birefringence from sound dentine (Fig. 3 A–K, left side of the white dotted lines). Therefore, the average lesion depth of demineralised dentine lesions ($155.1 \pm 23.5 \mu\text{m}$) was determined from all the samples tested ($n = 3$ in each group) (Fig. 3 A; Table 2). A dark band on the dentine surface was observed in all PA-treated groups (Fig. 3 A–B & E–G). Treatment with PA + FTP produced a change of birefringence below this band in the subsurface areas of demineralised dentine, similar to sound dentine (Fig. 3 E). After treatments using either MIV or CPWV with FTP, the birefringence of demineralised dentine was reversed being similar to sound dentine (Fig. 3 C–D).

3.2. Micro-CT analysis

Typical 2D micro-CT images showed de-/remineralised and sound root dentine in each group (Fig. 4 (I A–K)). Shrinkage resulting from sample dehydration prior to scanning was observed in the de-/remineralised dentine regions with a grayscale value of approximately 0. Although ring artefacts corrections and flat-field corrections were applied in micro-CT reconstruction, ring artefacts can still be observed in some images (Fig. 4 (II C–E)). In addition, the influence of beam-hardening phenomenon was not eliminated, which led to the emergence of dark-streak artifacts in the reconstructed regions close to the lesion surface and might result in non-linear relationship in the conversion between X-ray attenuation and grayscale values, where the attenuation was more underestimated with increasing grayscale values [38]. Therefore, the absolute grayscale values, especially in the lesion surface areas, should be interpreted with caution.

The regression equation of the scatter plot (Fig. S2) of grayscale values versus mineral density of the two standard phantoms was used to convert the grayscales of specimens to mineral density. The mineral density profiles of specimens in each group were plotted against lesion depths (Fig. 5) and therefore the integrated mineral content throughout the lesions could be calculated according to the mineral density profiles (Table 3).

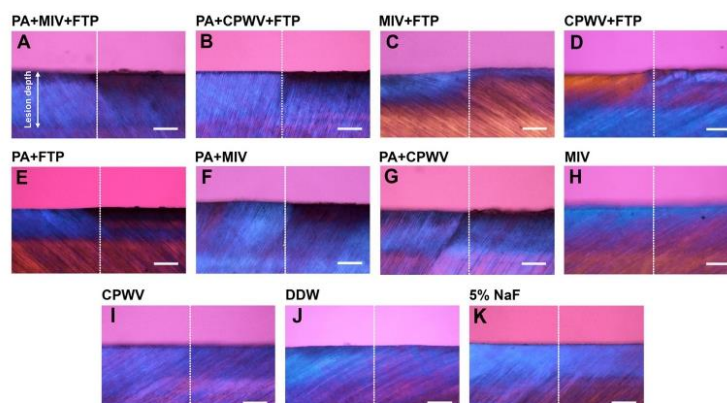


Fig. 3 – (A–K) Representative polarised light microscopy images of specimens from all the groups viewed in water (refractive index 1.33) with a first-order red plate between crossed polarisers. In each image, on the left and right side of the white dotted line is the demineralised dentine and dentine after treatments and pH-cycling respectively; Bar scale = 100 μm . The white double arrow illustrates the lesion depth.

Table 2 – Demineralised lesion depths measured under the polarised light microscopy.

Groups	Demineralised lesion depth (μm)		
	1	2	3
PA + MIV + FTP	127.2	160.4	149.3
PA + CPWV + FTP	136.6	132.5	140.3
MIV + FTP	197.2	123.9	162.1
CPWV + FTP	194.8	153.6	143.5
PA + FTP	170.8	157.8	141.3
PA + MIV	153.6	163.4	170.4
PA + CPWV	132.4	124.3	164.7
MIV	138	167	126.4
CPWV	139.6	135.6	217.8
DDW	185.2	174.6	168.2
5% NaF	127.2	160.4	149.3
Mean $\pm$ SD		155.1 $\pm$ 23.5	

Table 3 – Integrated mineral content changes of initial demineralised root dentine after each experimental phase.

Groups ^a	ΔZd	ΔZr	Mineral gain ($\Delta\text{Zd} - \Delta\text{Zr}$)	$\Delta\text{Z}\%$ ($= \frac{\Delta\text{Zd} - \Delta\text{Zr}}{\Delta\text{Zd}} \times 100$)	Statistical analysis ^b
PA + MIV + FTP	127.39 $\pm$ 11.66	97.54 $\pm$ 7.64	29.85 $\pm$ 8.96	23.15 $\pm$ 5.56	A
MIV + FTP	124.62 $\pm$ 4.61	99.65 $\pm$ 5.98	24.96 $\pm$ 2.42	20.09 $\pm$ 2.37	A, B
PA + CPWV + FTP	152.76 $\pm$ 6.02	126.61 $\pm$ 7.33	26.15 $\pm$ 2.15	17.17 $\pm$ 1.89	B
CPWV + FTP	151.09 $\pm$ 3.24	133.73 $\pm$ 6.85	17.36 $\pm$ 4.38	11.53 $\pm$ 3.01	C
PA + MIV	177.84 $\pm$ 11.89	159.90 $\pm$ 19.03	17.94 $\pm$ 8.35	10.38 $\pm$ 5.67	C, D
PA + FTP	182.49 $\pm$ 2.71	163.87 $\pm$ 4.68	18.62 $\pm$ 3.73	10.21 $\pm$ 2.06	C, D
PA + CPWV	189.04 $\pm$ 2.84	169.98 $\pm$ 2.95	19.06 $\pm$ 1.93	10.08 $\pm$ 0.98	C, D, E
CPWV	131.20 $\pm$ 5.48	118.99 $\pm$ 5.41	12.21 $\pm$ 3.04	9.29 $\pm$ 2.24	C, D, E
MIV	142.73 $\pm$ 5.79	130.62 $\pm$ 6.94	12.12 $\pm$ 2.79	8.52 $\pm$ 2.01	D, E, F
5%NaF	154.99 $\pm$ 4.90	142.77 $\pm$ 3.55	12.22 $\pm$ 2.03	7.86 $\pm$ 1.14	E, F
DDW	131.33 $\pm$ 12.72	122.53 $\pm$ 14.66	8.80 $\pm$ 6.93	6.78 $\pm$ 5.40	F

Values are mean $\pm$ standard deviations (SD); $\Delta\text{Z}\%$ is the relative mineral gain during treatments and pH-cycling.

^a Descending order of mean $\Delta\text{Z}\%$ ranks.

^b One-way ANOVA, post hoc Dunnett's T3 test, $\alpha = 0.05$; different uppercase letters indicate statistically significant differences between $\Delta\text{Z}\%$ values.

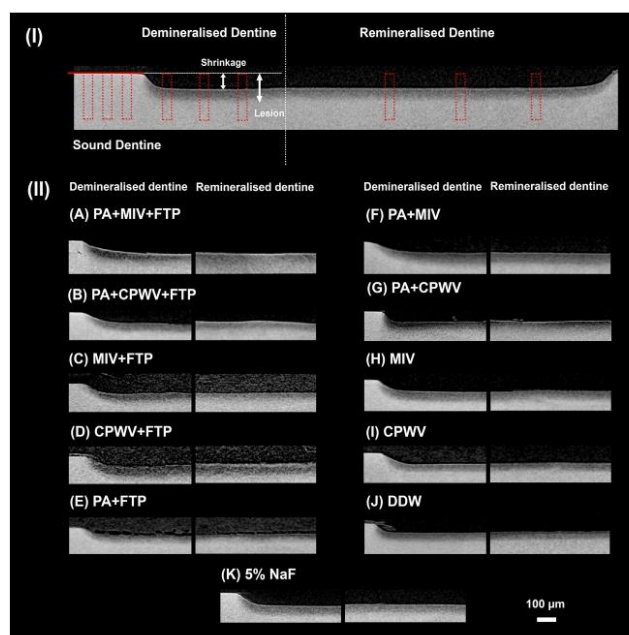


Fig. 4 – (I) An example of a two-dimensional (2D) cross-sectional slice of a specimen from micro-CT reconstruction. The red dotted boxes present the volume of interest (VOI) ($30 \times 2.1 \times 20 \times 2.1 \times 250 \times 2.1 \mu\text{m}^3$ (width $\times$ thickness $\times$ depth)) over which the mineral density profiles were plotted in each specimen. Three VOIs were randomly chosen in sound, demineralised and remineralised dentine regions in each specimen. Shrinkage due to dehydration was observed in the lesion surface areas and demineralised dentine showed relatively lower grayscale values than sound dentine. (II A–K) Representative 2D micro-CT slices of sound + demineralised and remineralised dentine from all the experimental groups.

The mineral density of sound dentine ($1.15 \pm 0.04 \text{ g}_{\text{HAP}}/\text{cm}^3$) was estimated from the grayscale values of all the sound dentine tested. Demineralisation was associated with a gradual mineral loss in the dentine subsurface with relatively lower grayscale values than sound dentine (Fig. 4 (II), demineralised dentine). The convergence of the mineral density profiles of de-/re-mineralised and sound dentine regions in each group demonstrated equivalence of mineral density in the test and control sides (Fig. 5), indicating the untreated baseline demineralised dentine were a valid self-control in each group. Compared to the method of scanning the specimens after demineralisation to obtain the baseline comparison, it was more time-efficient to use the approach in the present study to obtain the mineral density values of demineralised, remineralised and sound dentine regions for comparison simultaneously and could reduce the inaccuracy from misalignment and different conditions in each scan. However, the mineral content of adjacent demineralised regions may not be exactly the same (a slight divergence of the mineral density profiles of sound dentine) because of the structural and componential anisotropy of root dentine.

Statistical analysis showed significant differences in $\%\Delta Z$ after different treatments followed by pH-cycling (Table 3). Specifically, results of DDW group indicated the pH-cycling protocol used in the present study was a net-remineralising process with $\%\Delta Z$ of $6.78 \pm 5.40\%$. All the post-treatments used in the present study were associated with mineral gain throughout RCLs, but the remineralisation amount varied. Duraphat™ increased the mineral content of RCLs by $7.86 \pm 1.14\%$, slightly higher than pH-cycling treatment alone; both MIV and CPWV further increased the mineral content of RCLs. Adjunctive application of PA with MIV, CPWV or 5000 ppm FTP showed no statistically significant difference in mineral content changes ($P > 0.05$). Generally, MIV showed greater remineralisation effects than CPWV. Specifically, combined use of MIV with 5000 ppm FTP achieved significantly higher $\%\Delta Z$ compared to CPWV with 5000 ppm FTP ($P < 0.0001$). When PA was applied before the application of MIV or CPWV with FTP, the remineralisation efficacy of MIV or CPWV was significantly increased.

Relative mineral content changes post-treatment across each $30 \mu\text{m}$ increment from the original dentine surface to $250 \mu\text{m}$ depth were analysed to demonstrate the mineral con-

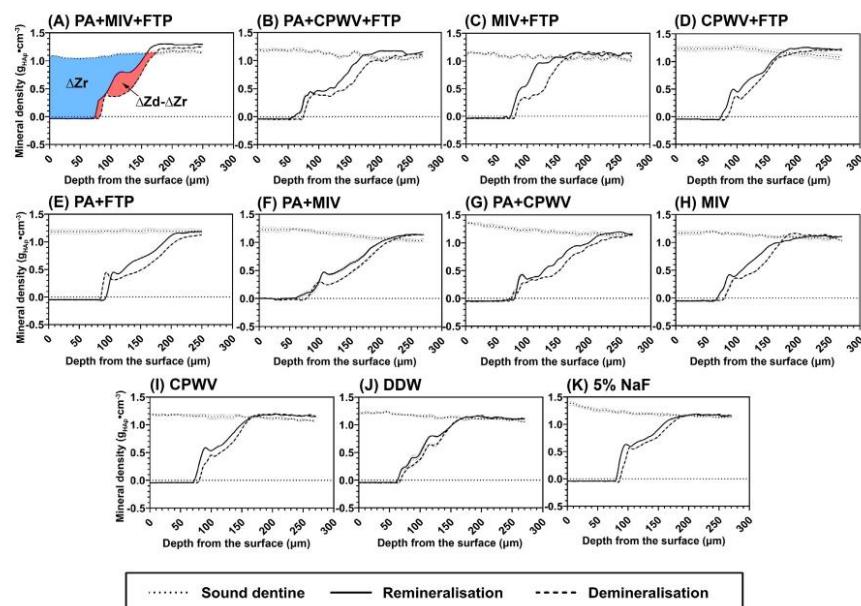


Fig. 5 – (A–K) Mineral density (g_{HAP}/cm^3) versus lesion depth profiles of VOIs in sound, demineralised and remineralised dentine regions in all the experimental groups. ΔZ was the integrated mineral loss across the lesion depth and was calculated from the mineral density profiles by subtracting the area under the curve (AUC) of demineralised or remineralised dentine from AUC of sound dentine. $\Delta Z_d - \Delta Z_r$ is equal to mineral gain during treatments and pH-cycling.

tent in different surface/subsurface layers of RCLs (Fig. 6; Table S1). Shrinkage was observed at 0–60 μm depth from the original dentine surface and the mineral gain started from 60–90 μm depth in all samples. Using PA as an adjunct with F regimens (i.e. MIV, CPWV and FTP) led to an incremental increase of mineral density in deeper subsurface areas (especially of 150–180 μm) compared to those without PA pre-treatment.

3.3. Scanning electron microscopy (SEM) - energy-dispersive X-ray spectroscopy (EDS)

The EDS analysis has the advantage of detecting all elements simultaneously with high efficiency. However, compared to the high spectral resolution wavelength-dispersive spectrometer (WDS), EDS is less accurate in determining elements with low atomic number and low concentration [39]. Therefore, quantitative data obtained by EDS should be interpreted with caution. The X-ray intensity in the EDS spectra with line scan mode reflected the elemental concentration changes along the cross-sectional dentine surface from the lesion surface to the sound dentine area. The line scan conducted on the sound dentine area which served as a comparison showed a sharp decrease of elemental concentrations from dentine into the epoxy resin (Fig. S1), whilst in the demineralised dentine regions, a gradient of elemental (Ca and P) concentrations was shown from the lesion base towards the lesion surface (Fig. 7 A–K, demineralised dentine). In the remineralised den-

tine regions, the line scan demonstrated a slight increase of Ca, P and F concentrations throughout the artificial RCL area (Fig. 7 A–K, remineralised dentine).

From the semi-quantitative analysis (Table 4), treatment with DDW did not result in significant changes in the atomic percentage (At%) of Ca, P and F in RCLs ($P > 0.05$). The At% of Ca, P and F was increased after remineralisation in some groups compared to the demineralised dentine. Particularly, there was an increase of Ca and P in dentine surface areas when treated with PA + FTP (Fig. 7 E), although At% of Ca and P throughout RCLs was significantly decreased (Table 4). Exposure to MIV or CPWV with PA and 5000 ppm FTP increased the At% of Ca, P and F significantly after pH-cycling ($P < 0.05$) and this might indicate their combined effects on ion uptake and distribution (Fig. 7 A–B; Table 4). Whilst a significant increase in At% of F throughout RCLs was observed in all experimental groups (Table 4), a less incremental increase of At% of F was present in CPWV involved groups (Fig. 7 B, D, G, I) than MIV involved groups (Fig. 7 A, C, F, H), indicating the difference in the availability of F- from varnishes incorporating Ca-P using different technologies.

3.4. Cross-sectional Vickers microhardness testing

The cross-sectional Vickers microhardness numbers (VHN) of dentine were slightly increased after treatments at comparative depths of demineralised dentine from less than 15 μm to

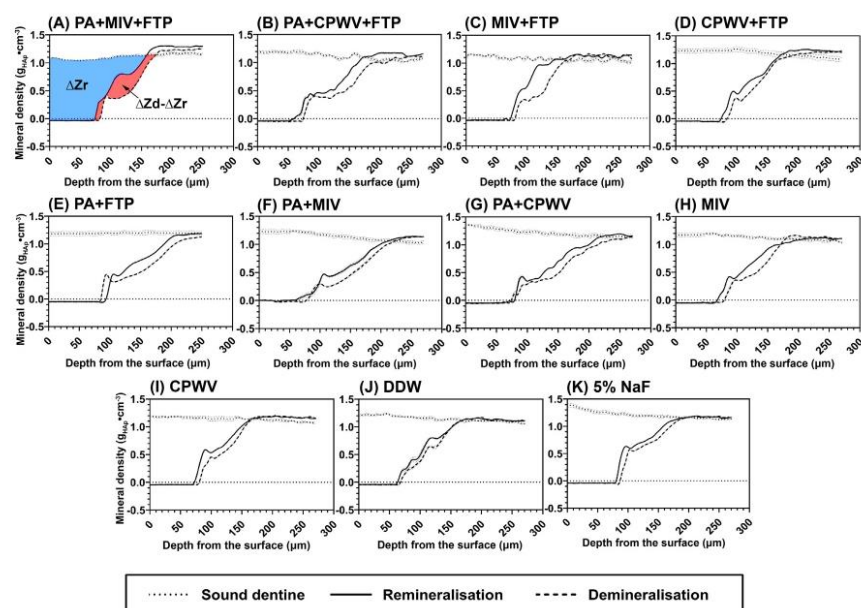


Fig. 5 – (A–K) Mineral density (g_{HAP}/cm^3) versus lesion depth profiles of VOIs in sound, demineralised and remineralised dentine regions in all the experimental groups. ΔZ was the integrated mineral loss across the lesion depth and was calculated from the mineral density profiles by subtracting the area under the curve (AUC) of demineralised or remineralised dentine from AUC of sound dentine. $\Delta Z_d - \Delta Z_r$ is equal to mineral gain during treatments and pH-cycling.

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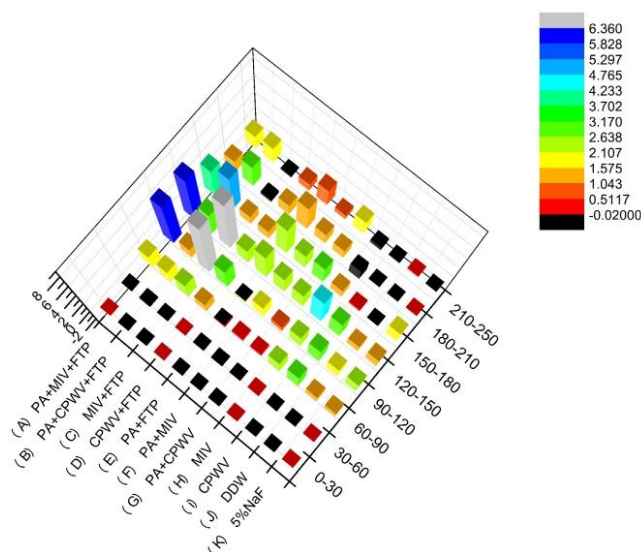


Fig. 6 – Three-dimensional (3D) bar charts of relative mineral content changes in each group after pH-cycling from $\sim 30 \mu\text{m}$ to $250 \mu\text{m}$ depth with $30 \mu\text{m}$ increments (a $40 \mu\text{m}$ increment in the deepest layer of demineralised dentine).

Table 4 – The relative atomic percentage (At%) of calcium (Ca), phosphorus (P) and fluorine (F) in demineralised and remineralised dentine regions analysed by energy dispersive X-ray microanalysis (EDS) with a line scan mode.

Groups	Atomic percentage (At%) (Mean (SD))					
	Ca		P		F	
	Demin	Remin	Demin	Remin	Demin	Remin
PA + MIV + FTP	10.80 (0.399)	12.72 (0.160)*	7.038 (0.207)	7.911 (0.042)*	0.436 (0.002)	1.031 (0.014)*
PA + CPWV + FTP	10.47 (0.210)	12.97 (0.261)*	6.729 (0.090)	8.272 (0.090)*	0.477 (0.015)	0.675 (0.045)*
MIV + FTP	10.89 (0.038)	12.03 (0.295)*	6.866 (0.090)	7.345 (0.109)*	0.438 (0.035)	1.013 (0.082)*
CPWV + FTP	10.54 (0.178)	10.50 (0.710)	6.903 (0.054)	6.934 (0.368)	0.488 (0.039)	0.747 (0.064)*
PA + FTP	11.03 (0.203)	10.30 (0.295)*	7.130 (0.093)	6.545 (0.063)*	0.483 (0.027)	0.780 (0.017)*
PA + MIV	10.24 (0.063)	11.43 (0.182)*	6.714 (0.012)	7.583 (0.032)*	0.450 (0.031)	1.413 (0.119)*
PA + CPWV	10.38 (0.252)	9.724 (0.378)	6.470 (0.228)	6.274 (0.245)	0.407 (0.033)	0.898 (0.047)*
MIV	10.84 (0.244)	10.83 (0.316)	6.808 (0.144)	6.884 (0.232)	0.457 (0.006)	0.675 (0.014)*
CPWV	10.06 (0.190)	10.44 (0.113)	6.548 (0.235)	6.897 (0.115)*	0.513 (0.018)	0.602 (0.014)*
DDW	11.58 (0.378)	11.39 (0.569)	7.437 (0.174)	7.246 (0.333)	0.497 (0.084)	0.435 (0.046)
5%NaF	10.45 (0.158)	11.38 (0.231)*	6.653 (0.168)	7.126 (0.150)*	0.523 (0.078)	1.131 (0.073)*

The asterisks indicate statistically significant difference of At% between the demineralised and remineralised dentine in each group (two-way ANOVA, Bonferroni post hoc test, $\alpha = 0.05$).

$100 \mu\text{m}$ in most groups although mostly without significant difference ($p > 0.05$). After treatment with DuraphatTM, MIV or CPWV, the VHN of demineralised dentine did not increase significantly at all lesion depths (Fig. 8 H, I and K). Combined use of MIV with PA increased VHN of demineralised dentine up to $20 \mu\text{m}$ (Fig. 8 F) ($p < 0.05$). A significant increase in dentine surface ($\sim 15 \mu\text{m}$ depth) microhardness was observed after application of MIV (Fig. 8 C) and CPWV (Fig. 8 D) followed by pH-cycling with 5000 ppm FTP ($p < 0.05$). With adjunctive application of both PA and 5000 ppm FTP, treatment with either MIV

or CPWV was associated with an increase of VHN in RCLs up to $30 \mu\text{m}$ depth ($p < 0.05$) (Fig. 8 A-B).

4. Discussion

In the present study, the remineralising effects of MIV, CPWV and high concentration (5000 ppm) FTP on artificial RCLs were investigated. PA was used as an adjunct to F regimens to putatively stabilise the demineralised dentinal collagen. The ex vivo artificial RCL model created by the deminer-

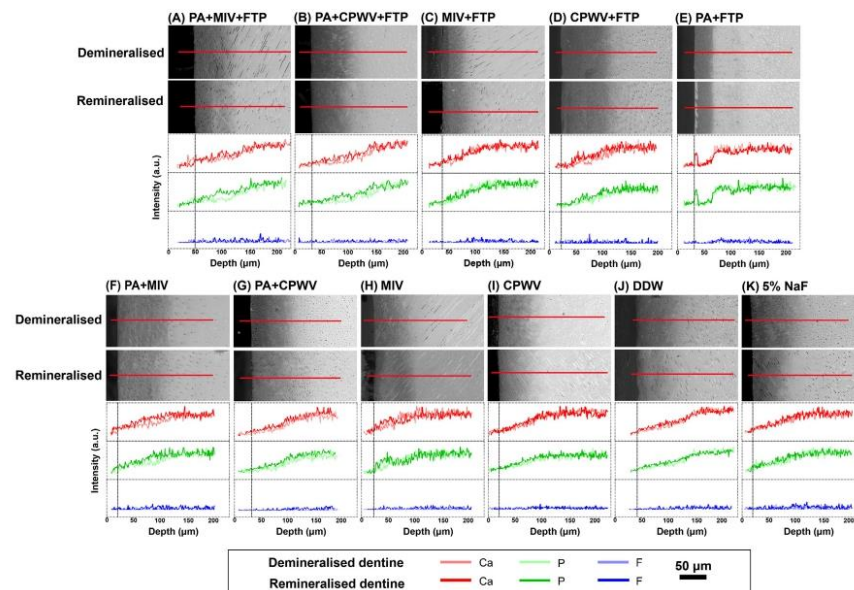


Fig. 7 – (A–K) Scanning electron microscopy (SEM) images of cross-sectional surfaces of de-/remineralised dentine and the corresponding relative concentrations of calcium (Ca), phosphorus (P) and fluorine (F) analysed by the line scan mode of SEM-energy dispersive X-ray analysis (SEM-EDS).

alising solution has high reproducibility under controlled conditions compared to natural RCLs. Nevertheless, root dentine is a material of structural anisotropy with nonidentical physicochemical characteristics in different regions and in order to minimise these variations, part of the demineralised root dentine in each specimen was kept as baseline demineralisation for comparison [40,41]. The demineralisation process led to the putative loss of dentine mineral crystals with weak negative birefringence, thus increasing the strength of positive birefringence relatively. The demineralisation solution diffused into the organic matrix and gradually dissolved the mineral components of dentine, leading to a gradient concentration decrease of Ca and P. The mineral content and microhardness were gradually reduced from the lesion surface to the sound dentine area. Specifically, micro-CT profiles in this study demonstrate a demineralised shoulder in all samples, attributed to the different rates of demineralisation of intertubular and peritubular dentine [42].

Application of varnishes and toothpastes containing F promoted the deposition of a layer with relatively high mineral density on RCL surfaces and achieved an increased concentration of F throughout RCLs. This was consistent with previous reports that F was able to incorporate minerals into dentine through partial ionic substitution for hydroxyl ions in hydroxyapatite and resulted in covalent Ca-F bonds and/or hydrogen O-H...F bonds, thus potentially forming hydroxyapatite, flu-

orhydroxyapatite or fluorapatite [12]. However, the unit cell volume of dentine hydroxyapatite as well as the crystalline packing and mineral-matrix interactions may have not been altered, as there were no significant changes in microhardness of dentine after treatments with FTP or Duraphat™.

MIV and CPWV used in this study utilise different techniques to keep the Ca-P based remineralisation systems in a bioavailable status and separated from F, enabling a concentration gradient for ion diffusion into demineralised dentine. Specifically, in CPP-ACP, which is contained in MIV, CPP binds to the (100) and (010) faces of apatite crystals and stabilises ionic calcium and phosphate in a metastable solution that increases the solubility of Ca-P [43]. TCP in CPWV is based on the crystalline technology which prevents the reaction between Ca^{2+} and F^- by a protective fumaric acid barrier that breaks down in an aqueous environment (saliva) releasing Ca^{2+} and PO_4^{3-} . Results from the present study indicated that comparatively lower incremental increases of At% of F and mineral content were detected in RCLs treated with CPWV than MIV due to the addition of Ca-P that influences the availability of F^- , as once Ca^{2+} and PO_4^{3-} were exposed to F^- in an aqueous environment, they may reprecipitate quickly on the dentine surface [44]. Formation of ACP clusters by the aggregation of CPP with Ca^{2+} and PO_4^{3-} prevents their precipitation with F^- , enabling a high-level release of available Ca^{2+} and PO_4^{3-} . Whilst in TCP, Ca-P was stabilised by the solid state method and might precipitate faster once exposed

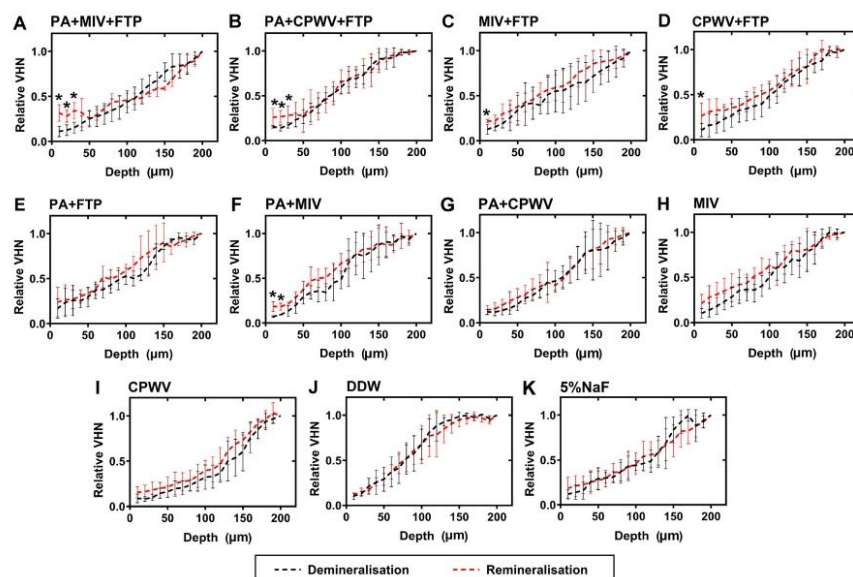


Fig. 8 – (A–K) Vickers microhardness numbers (VHN) of demineralised and remineralised dentine from less than 15 μm to 200 μm depth from the lesion surface with 10 μm interval (i.e. $\sim 15 \mu\text{m}$, 20 μm , 30 μm , 40 μm , ... to 200 μm). Values were expressed as mean $\pm$ standard deviation (SD). The asterisks indicate statistically significant difference of VHN between the demineralised and remineralised dentine regions of a sample at the same depth (Student's *t* test, $\alpha = 0.05$).

to the aqueous environment, depleting remineralising ions from lesion pores [45]. Moreover, CPP-ACP complexes have a higher wettability than TCP and thus have a better contact with tooth surfaces. The CPP-ACP complexes attached to dentine surfaces form a concentration gradient to maintain a state of supersaturation with respect to hydroxyapatite, therefore inhibiting the demineralisation of underlying dentine. Nevertheless, both MIV and CPWV failed to improve the microhardness of artificial RCLs. In this regard, compared to enamel, remineralisation of root dentine by MIV was more difficult to achieve in term of recovery of hardness especially in the sub-surface areas due to the difficulty in achieving intrafibrillar mineralisation which determines the mechanical properties of dentine and relies upon the particular location and interaction of minerals within the dentinal organic matrix [46,47].

The benefits of combined application of F varnishes and toothpastes compared to the use of either product alone for the treatments of RCLs were also indicated. Specifically, application of MIV or CPWV along with 5000 ppm FTP reversed the birefringence of demineralised dentine being similar to sound dentine and significantly improved the surface hardness of artificial RCLs; the mineral density was further increased throughout the lesions. This was in accordance to the clinical recommendation for the use of FTP after F varnishes to maintain the presence of F, as F varnishes may not persist for long in the oral environment due to mastication, oral hygiene procedures, soft tissue movement and salivary flow [48]. It has been speculated that the high concentration F from var-

nishes 'pulsed' after treatment with dentifrices and this might facilitate the secondary nucleation of $\text{Ca}_5(\text{PO}_4)_3(\text{OH})_{1-x}\text{F}_x$ or other intermediate phases of Ca-P before the formation of the more thermodynamically stable hydroxyapatite, fluorhydroxyapatite or fluorapatite phase [49].

As indicated by the micro-CT profiles, mineral gain in demineralised root dentine after treatments originated from the precipitation promoted by remineralising agents and the reprecipitation of the dissolved mineral ions from inner dentine moving outward. Particularly, the surface mineralised layer on root dentine was not prominent presumably because the outer layers of dentine had been exposed longer and affected more by acids than the inner layers. Therefore, the outer layers contained fewer residual crystals that could act as nuclei for remineralisation [50]. Also, the residual crystals may be blocked by the relatively large amount of demineralised organic matrix components, inhibiting their contact with the extrinsic remineralising ions. With less mineral loss, the inner zone at the base of the lesion was more readily remineralisable and fully recovering its properties at a rapid rate [51]. In addition, the absolute mineral density values by micro-CT analysis obtained in this study should be interpreted with caution. Specifically, samples were dehydrated before micro-CT scanning and the shrinkage caused by the dehydration could lead to an underestimation of the absolute values of total mineral loss calculated from the mineral density profiles. Nevertheless, there was a constant linear relationship between the results of mineral loss of dehydrated dentine samples accord-

ing to micro-CT profiles and the real mineral loss evaluated by chemical analysis [52]. Therefore, comparisons of the values of $\% \Delta Z$ ($= \frac{\Delta Z_d - \Delta Z_c}{\Delta Z_d}$) amongst different groups would not be influenced by dehydration.

The concept of dentine biomineralisation recognises the critical roles of the collagen matrix in orchestrating mineralisation and provides new insights into novel approaches for dentine remineralisation. In light of this, PA, an exogenous collagen cross-linker, was used in the present study to strengthen the dentinal collagen matrix, presumably promoting the formation of a mineralised matrix [27]. Application of PA tended to aggregate and cross-link the collagen fibrils and reduced interfibrillar spaces which would decrease the porosity of type I collagen, as suggested in the polarised light microscopy images by the formation of a dark band in all PA-treated dentine. Although PA alone cannot enhance intrafibrillar mineralisation, the hydroxyphenyl groups in PA may act as ligands to bind Ca^{2+} [53]. The formation of calcium-PA complexes could promote mineral deposition and attract more calcium phosphate onto the PA-modified collagen fibrils, as indicated by the increased At% of Ca and P as well as the improved $\% \Delta Z$ throughout the artificial RCLs compared to treatments without PA [54]. In particular, significant $\% \Delta Z$ was achieved in the subsurface areas. Results in the present study indicated the active role of collagen as a template for attracting and controlling the infiltration of Ca-P, as revealed in previous studies, via capillary forces, osmotic-regulated mechanisms as well as electroneutrality (CaHPO_4) for dentine remineralisation, following a different mechanism from the classical crystallisation of enamel [43,55].

Increased mineral content is not an appropriate endpoint of assessing the success or failure of the remineralisation effects of bioactive materials, as it was not necessarily translated into a recovery of mechanical properties. Whilst remineralisation may occur preferentially in the extra-fibrillar zone, the mineral content in the intrafibrillar compartments plays a more determinant role in the mechanical properties of dentine [56,57]. Results in the present study indicated that whilst combined use of MIV or CPWV along with 5000 ppm FTP significantly increased surface (less than 15 μm depth) microhardness, using PA as an adjunct significantly improved the subsurface microhardness up to 30 μm . It was speculated that simultaneously strengthening the dentinal matrix whilst applying the remineralising agents might prevent the partially demineralised subsurface from further mineral loss and collagen collapse as well as promote the formation of a more well-structured organic-inorganic interaction for stress redistribution during loading in microhardness testing. However, conversion of the CaF_2 -like materials deposited on lesion surfaces to fluorhydroxyapatite incorporated in dentine for the recovery of hardness is a very slow process [51]. Therefore, in the present study, after 8-day pH-cycling, microhardness of demineralised dentine only increased to a limited depth.

Of note, several aspects should be considered which may influence the remineralising efficacy of the treatments used in the current study. The pH-cycling model used included only one demineralisation phase daily, therefore the reaction products formed during pH changes and their effects on demineralisation resistance might not be pronounced. In

particular, ion release from CPP-ACP could be significantly enhanced in an acidic environment [58]. Also, varnishes were not removed before pH-cycling in order to protect the remineralising surface, therefore varnishes with inferior 'adhesive properties' may detach from the dentine surface more easily, resulting in reduced effectiveness - this may also contribute partly to the large deviations of some results (e.g. microhardness) [44]. In addition, varnishes containing F can release F- into the surrounding medium in the oral environment and to the tooth surfaces that are not covered by varnishes. This effect of F varnishes was not evaluated in the present study. Moreover, the effects of saliva, dental plaque and enzymes and the different characteristics of natural RCLs compared to demineralised root dentine should be considered before conclusions regarding the treatment efficacy of F regimens along with collagen cross-linking agents on RCLs can be drawn.

5. Conclusion

Within the limitations of this laboratory study, in a simulated net-remineralisation oral environment, application of Duraphat™, CPWV and MIV introduced F- into artificial RCLs and slightly increased the mineral content but had no significant effects on microhardness of demineralised root dentine. When MIV or CPWV was applied in conjunction with 5000 ppm FTP, the birefringence of the demineralised dentine was reversed being similar to sound dentine and the surface (less than 15 μm) microhardness of artificial RCLs was significantly improved in addition to increased mineral content throughout the lesions. Using PA as an adjunct to F regimens improved the mineral density of demineralised root dentine significantly, especially in the subsurface areas. Pre-treating dentine with PA before application of MIV or CPWV along with 5000 ppm FTP achieved the highest ion uptake of Ca^{2+} , PO_4^{3-} and F- compared to the other treatment approaches; a significant increase of cross-sectional microhardness of demineralised dentine was observed up to 30 μm depth. Generally, MIV containing CPP-ACP was associated with a higher mineral content increase than CPWV containing TCP.

Declarations of interest

None.

Acknowledgements

This work was supported by Australian Dental Research Foundation (ADRF) Nathan Cochrane Memorial Grant and ADRF grant No. 326-2018. Jing Cai was supported by the China Scholarship Council - University of Melbourne Research Scholarship. The authors appreciate the Bio21 Advanced Microscopy Facility and Ms Su Toulson for the assistance with SEM-EDS analysis.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.dental.2020.10.021>.

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Publication: Acta Biomaterialia

Publisher: Elsevier

Date: 1 April 2019

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Publication: Journal of Dental Research

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The potential of dendrimer in delivery of therapeutics for dentistry

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Publication: Heliyon

Publisher: Elsevier

Date: October 2019

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