Title: Surface-bound collagen 4 is significantly more stable than collagen 1

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Abstract

Collagen 1 (C1) is commonly used to improve biological responses to implant surfaces. Here the stability of C1 was compared with collagen 4 (C4) on a mixed macrodiol polyurethane, both adsorbed and covalently bound via acetaldehyde glow discharge polymerisation and reductive amination. Substrate specimens were incubated in solutions of C1 and C4. The strength of conjugation was tested by incubation in 8 M urea followed by enzyme linked immunosorbent assays to measure residual C1 and C4. The basal lamina protein, laminin-332 (L332) was superimposed via adsorption on C4-treated specimens. Keratinocytes were grown on untreated, C1-treated, C4-treated, and C4 + L332-treated specimens, followed by measurement of cell area, proliferation, and focal adhesion density. Adsorbed C4 was shown to be significantly more stable than C1 and covalent conjugation conferred even greater stability, with no degradation of C4 over twenty days in 8 M urea. Cell growth was similar for C1 and C4, with no additional benefit conferred by superimposition of L332. The greater resistance of C4 to degradation may be consequent to cysteine residues and disulphide bonds in its non-collagenous domains. The use of C4 on implants, rather than C1, may improve their long-term stability in tissues.

Key Words

Surface analysis; Collagen; Cell adhesion; Interfaces; Immobilisation

Introduction

As part of research into creating a robust skin-material interface ¹, techniques for optimising the attachment of collagen and epidermal cells (keratinocytes) to surfaces were explored. There are at least 16 types of collagen ², with over 80% of the collagen in the body consisting of types 1, 2, and 3, which are fibrillar in structure. Collagen 4 (C4), in contrast, forms two-dimensional reticula. Collagen improves the adhesion, proliferation, and differentiation of many cell types in culture ³; it improves biological responses to implanted materials and has many diverse biomedical applications ⁴⁻⁶. Collagen 1 (C1) is the mostly widely used protein for the surface optimisation of materials in clinical practice ^{4,7}. In addition, constructs made primarily from C1 have been used in the engineering of cartilage, bone, nerve, and skin.

Mammalian skin consists of epidermis, whose main cell type is the keratinocyte, acting as a waterproof barrier to infection; and the dermis, which houses skin appendages, providing metabolic and mechanical support for the overlying epidermis. The basal lamina is a layer of extracellular matrix (ECM) secreted by epidermal cells, on which the epidermis sits. The basal lamina is divided into two layers, the lamina lucida, which consists of laminin-332 (L332), entactins, and dystroglycans; and the lamina densa, which consists of C4 and perlecan ⁸.

Urea in high concentrations degrades proteins by disrupting non-covalent bonds in proteins ⁹ via mechanisms that are not fully elucidated ¹⁰. It is extremely difficult to model the effects of wound enzymes *in vitro*. While urea in high concentration may not cause proteolysis in the manner of the proteolytic and oxidative enzymes found in wounds, it enables an approximate determination of protein stability and resistance to degradation. To address the problem of degradation *in vivo*, collagen

commonly is covalently conjugated to materials to improve stability¹¹⁻¹⁶ and to minimise non-specific adsorption of proteins, a problem exemplified by the role of glycosaminoglycans in the failure of tooth implants¹⁷. To further improve stability, collagen commonly is cross-linked after attachment to materials by physical or chemical means. The former includes photo-oxidation, dehydrothermal treatments (DHT), and ultraviolet irradiation, all of which avoid introducing cytotoxic chemical residuals¹⁸. However, these physical treatments are unable to cross-link to the degree required for tissue implants¹⁹ and chemical cross-linking is preferred in most situations. Commonly used chemical cross-linking methods involve the use of dialdehydes, diisocyanates, azides, and carbodiimides²⁰ that act by cross-linking amino acids between adjacent polypeptide chains. Given that protein functionality is decreased in direct proportion to the degree of cross-linking, it follows that excessive cross-linking impairs cellular attachment and proliferation^{15,21,22}. Also, chemical cross-linking can result in residual chemical species that cause cellular toxicity²³⁻²⁶. Ideally, an alternative to cross-linking is required, aimed at increasing the stability of collagen on materials to improve implant stability in living tissues.

Cells attach to the ECM via focal adhesions. The connection between focal adhesions and the ECM involves integrins. These transmembrane receptors bind to surface-bound proteins via short amino acid sequences, such as RGD²⁷ [Arginine-Glycine-Aspartic acid] (found in fibronectin, laminin, and vitronectin), or DGEA²⁸ [Asp-Gly- Glutamic acid - Alanine] (found in C1), and to the cyanogen bromide fragment CB3²⁹ (found in C4). Integrin-mediated binding to collagen is significantly dependent on GFOGER [Gly- Phenylalanine -Hydroxyproline -Gly-Glu-Arg], found in all collagens, including C1 and C4³⁰. The intracellular part of integrin binds to the

cytoskeleton via adapter proteins (talin, paxillin, alpha-actinin, filamin, vinculin, and tensin). The integrin-adapter protein–cytoskeleton complex forms the essence of the focal adhesion³¹. In addition to their anchoring role, integrins transduce signals internally in response to the chemical composition of the ECM, altering transcription and consequently cell cycle, shape, and motility³².

A mixed macrodiol polyurethane, Elast-Eon™ was chosen for *in vitro* and *in vivo* research on the skin-material interface¹ because it exhibits the physical and mechanical performance of polyurethanes along with excellent stability *in vivo* imparted by a silicone component. It is among the most biostable of all polyurethane-based materials and as such has been used for long-term tissue implantation^{33,34}.

As an emblematic biostable polymer, it also was a suitable choice for vitrobiological experimentation. Mixed macrodiol polyurethanes such as Elast-Eon™ have low intrinsic surface bioactivity and require techniques such as surface functionalisation and the conjugation of proteins to improve biological responses.

It was hypothesised that both adsorbed and covalently conjugated C4 would show increased stability on Elast-Eon™ compared to C1. In addition, it was hypothesised that L332 superimposed on surfaces already treated with C4 would lead to increased keratinocyte attachment and proliferation.

Materials and Methods

Substrates

Elast-Eon™ (kindly gifted by Biometrics, Salt Lake City, UT 84119, United States) was laser cut (Advanced Cutting Technology, Melbourne, Australia) into 5 mm x 5

mm x 2 mm blocks or into 5 mm and 8 mm diameter discs (0.15 mm thickness). The blocks were used for collagen-binding studies and the discs were used to measure cell attachment, proliferation, and the formation of focal adhesions between the cells and the materials surface. Each block was designed to stand on one 2 mm x 5 mm edge, thereby exposing five faces to chemical modification, both in the reactor and in solution. To enable orientation, each disc was marked with an "L" on the opposite surface to the chemically treated side. After functionalisation, all material samples were sonicated in purified water with 2% phosphate-free detergent (Tween 20™, Sigma-Aldrich) for two 20' periods, sonicated in purified water for two 10' periods, sonicated in ethanol (Sigma-Aldrich) for two 20' periods, sonicated in purified water for a further 10', and then dried in laminar-flow air at 20°C.

Collagen conjugation

Glow discharge polymerisation

Surface functionalisation by glow discharge polymerisation of acetaldehyde (Aapp) was performed in a custom-built reactor, described in detail elsewhere³⁵. Previous research on reactor parameters by the principal investigator³⁶ led to the frequency being set at 125 kHz, power at 20 W, pressure at 0.3 mbar, and time at 60 s. Specimens were placed on the reactor's lower electrode. Care was taken to avoid oxygen contamination of specimens: Prior to glow discharge polymerisation, the reactor was evacuated via large and fine gauge conduits to a base pressure below 0.001 mbar. This pressure was maintained for periods of 30-60' to evaporate any volatile agents in the system. A maximum leak rate of 0.01 mbar/minute was permitted. Subsequently, monomer was introduced to the system for 90 s, after

which the vessel was evacuated again until a pressure of less than 0.001 mbar was obtained. Glow discharge then was initiated within a commercial reactor (Rochester N.Y. HPG-2 High Frequency Plasma Generator). After deposition, the reactor was evacuated to under 0.001 mbar before venting with air.

Functionalisation and collagen coating of Elast-Eon™ blocks and discs

Following Aapp glow discharge polymerisation, Elast-Eon™ blocks were removed and placed into C1 solution (rat tail collagen, Sigma-Aldrich, Catalogue #C7661) or C4 solution (Collagen Type IV, Chemicon (Millipore), Catalogue #CC076) solution for 2 h at room temperature (rt), at a concentration of 4 µg/ml in PBS in individual wells containing 180 µl of collagen solution, in 96 well TCPS plates, in such a way that five sides of the Elast-Eon™ blocks were exposed. Subsequently sodium cyanoborohydride (Sigma-Aldrich, reagent grade, 95%, CAS Number 25895-60-7), in an excess concentration of 20 mg/ml, was added to cause reductive amination. After incubating with sodium cyanoborohydride for 10 h, specimens were washed with PBS five times.

Following Aapp functionalisation, Elast-Eon™ discs were and placed into a 50 ml conical tube containing 20 ml of C1 solution or C4 solution for 2 h at rt, at a concentration of 4 µg/ml in PBS. Subsequently 400 mg of sodium cyanoborohydride was added to the tube for a final concentration of 20 mg/ml, followed by five washes with PBS.

Measurement of C1 and C4 on functionalised and untreated control Elast-Eon™ blocks by ELISA

The amount of collagen 1 and 4 present on Elast-Eon™ blocks was assessed by two-step ELISA. Prior to addition of the primary antibody, specimens were washed with 0.05% Tween 20™ in PBS (pH 7.4), washed twice with PBS, and then blocked with 1% bovine serum albumin (BSA) ('BovoStar', Bovogen) in PBS (pH = 7.4) for 2 h. Primary antibodies used were mouse anti-collagen 1 (Sigma-Aldrich, Product #C2456), diluted 1:160000 and rabbit anti-human 4 polyclonal Antibody (Chemicon, Product #AB748), diluted 1:5000, in 1% BSA in PBS. Specimens were incubated in primary antibody for 2 h at rt. This was followed by three washes as previously described. Secondary antibodies used were anti-mouse IgG, HRP-linked antibody (Cell Signaling Technology, Product #7076), diluted 1:8000, and anti-rabbit IgG, HRP-linked (Cell Signaling Technology, Product #7074), diluted to 1:1000 in 1% BSA in PBS, and incubated for 2 h at rt. This was followed by three washes as previously described. Specimens then were incubated in 200 µl of HRP substrate reagent (R&D Systems, Catalog #D999) for 25'. Substrate reaction was stopped with 100 µl of 1M sulphuric acid. 100 µl of resulting solution was transferred to 96-well plate and colour change measured in a Tecan Genios Pro microplate reader at a wavelength of 450 nm. Three samples were used for each data point. 68% confidence interval error bars are included in all charts, representing one standard deviation either side of the mean.

Measurement of C1 and C4 on Elast-Eon™ blocks by urea elution and ELISA

Elast-Eon™ blocks were Aapp-functionalised using reactor parameters of 20 W, 0.3 mbar, 60 s, and 125 kHz, followed by incubation in collagen solution. Standard curves showed saturated binding on Elast-Eon™ for both collagen 1 and 4 at 2

$\mu\text{g}/\text{m}$, so an in-excess solution of $4 \mu\text{g}/\text{ml}$ was used. Untreated, Aapp-functionalised alone, and specimens treated with Aapp and sodium cyanoborohydride were incubated in solutions of either C1 or 4. Specimens then were incubated in 8 M urea for periods of up to 5 days (C1) and 20 days (C4). Early urea elution experiments with these collagens suggested these time frames. Specimens then were washed five times with PBS. Two-step ELISA was used to measure residual collagen. Three samples were used for each ELISA data point. 95% confidence interval error bars are included in all charts, representing one standard deviation either side of the mean.

Measurement of C4 produced by cells on Elast-Eon™ discs over 15 days

To assess the rate and amount of deposition of C4 by HaCaT keratinocytes, untreated ("U") Elast-Eon™ discs were seeded with HaCaT and maintained for 1, 2, 5, 10, and 15 days. Control Elast-Eon™ discs were incubated in cell-free media and maintained for 15 days. The 8 mm diameter Elast-Eon™ disc controls were: untreated discs without cells, untreated discs with adsorbed C4 ("C4"), and Aapp-treated discs with covalently bound C4. Discs were pre-incubated in 1000 μl of media for two days at 37°C to allow for dissipation of air bubbles, then seeded with HaCaT at a concentration of 8×10^4 cells in 500 μl of medium (RPMI 1640 Medium, GlutaMAX™, Product #61870, Gibco, Invitrogen, Life Technologies, Mulgrave, Australia) with 1% penicillin and streptomycin and 10% foetal calf serum (Invitrogen). After 1, 2, 5, 10, and 15 days of incubation at 37°C in a humidified atmosphere supplied with 5% CO_2 in air, media was aspirated, discs transferred to clean well and adherent cells were trypsinised for 60', followed by three washes with PBS, and then

incubated in PBS at 37°C until 15 days of total incubation time was reached. At the 15-day time-point, all specimens including controls, were washed 3x with PBS and quantitative measurement of C4 produced by cells was performed by routine ELISA as described above. Triplicates of each data point were used, with data being expressed as an average, with error bars indicating 95% confidence intervals.

Measurement of cell area and focal adhesion density on Elast-Eon™ discs

HaCaT were seeded on 8 mm diameter Elast-Eon™ discs of each of type (untreated (U), treated with C1 (U + C1), treated with C4 (U + C4), treated with Aapp + C1 (Aapp + C1), treated with Aapp + C4 (Aapp + C4), and treated with Aapp + C4 + 10 µg/ml laminin-332 (Abcam, product # ab14509) (Aapp + C4 + L332) in individual wells of 48-well TCPS culture plate containing 500 µl of cell culture medium (RPMI medium (RPMI 1640 Medium, GlutaMAX™, Product # 61870, Gibco, Invitrogen, Life Technologies, Mulgrave, Australia) supplemented with 1% penicillin and streptomycin and 10% foetal calf serum (Product #10082147, Invitrogen, Mulgrave, Australia) and incubated at 37°C in a humidified atmosphere supplied with 5% CO₂ in air. For cell area and focal adhesion vinculin assessment, one Elast-Eon™ disc of each type was incubated for 4 h with 4×10^4 cells per well of a 48-well TCPS culture plate.

For cell area assessment, discs with adherent cells were washed 3x with PBS and fixed with 4% formaldehyde (Sigma-Aldrich) in PBS for 10', followed by 3 washes with PBS. Cells then were dehydrated in ascending series of ethanol (Sigma-Aldrich) concentrations of 10%, 30%, 50%, and 70% for 10' each. The area of cells in each of 10 fields 500 x 500 µm was measured by SEM (Philips XL30

Field-emission Scanning Electron Microscope). Only singlets (single cells) were measured, with doublets ignored to minimise the effect of cell cycle stage. Cells were traced individually with a computer mouse and analysed for cell area using ImageJ software downloaded from NIH (National Institutes of Health, Bethesda, Maryland, U.S.). Statistical analysis of keratinocyte cell area on untreated and protein-treated Elast-Eon™ discs was performed by one-way ANOVA with Bonferroni post-hoc analysis for multiple groups.

Focal adhesions were labelled using routine immunofluorescent techniques with a vinculin antibody. Discs with adherent cells were washed 3x with PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl₂; pH 6.9) (this buffer is known to improve the signal intensity of stained cellular antigens). Cells then were fixed in 3.7% paraformaldehyde (Sigma-Aldrich, product #P6148) in PHEM buffer for 10' at . Cells were oxidised in 4.2 mM sodium periodate (Sigma-Aldrich, product #311448) in PBS for 30' at 4°C, and rinsed 3x in PHEM buffer. Discs then were inverted on a 50 µl drop of 10 mM fluorescein-5-thiosemicarbazide (Sigma-Aldrich, product #46985) that had been filtered with 0.45 µm syringe filter, and incubated for 2 h at rt to stain the cell membrane, and washed 3x in PHEM buffer. Cells were permeabilised with 1% Triton X-100 (Sigma-Aldrich, Catalogue No. T8787) in PHEM for 15' at rt. The vinculin in focal adhesions, as opposed to the diffusible cellular fraction, is resistant to Triton-X-100 extraction³⁷. Cells were washed 3x in PHEM buffer and treated with 2 mg/ml sodium borohydride (Sigma-Aldrich, Catalogue No. 213462) in PHEM for 10' at rt to reduce background fluorescence, and rinsed 3x with PHEM buffer. Specimens then were blocked with 1% BSA in PBS for 12 h. Vinculin in focal adhesions was labelled with monoclonal anti-vinculin antibody (mouse IgG1 isotype)

(Sigma-Aldrich Product No. V4505) diluted to 1:400 in 1% BSA in PBS, for 2 h, followed by three washes with PHEM buffer. Discs with adherent cells were inverted on a 50 μ l drop of secondary antibody, Alexa Fluor® 594 Goat Anti-Mouse IgG 1:400 (Catalogue No A24921) 1:400 in 1% BSA in PBS, for 2 h, followed by 3 washes with PHEM buffer. Discs with labelled cells were placed on a microscope slide, mounted in 1:1 glycerol: PBS solution and covered with a glass coverslip. For each Elast-Eon™ type, six randomly selected single cells were selected. Cells were viewed using 100x objective lens in oil immersion fitted to a Laser Confocal Microscope (Leica SP2 AOBS). The outer boundary of the cell membrane, as visualised with fluorescein-5-thiosemicarbazide staining, was traced individually with a computer mouse and analysed for cell area using ImageJ software. The total area of focal adhesions as assessed by anti-Vinculin immunofluorescence was analysed in the same manner and expressed as a proportion of the total cell area. Statistical analysis of focal adhesion formation of keratinocytes on untreated and collagen-treated discs was performed by one-way ANOVA with Tukey post-hoc analysis for multiple groups.

Measurement of cell proliferation on Elast-Eon™ discs

The proliferation/growth of cells was measured on each of the Elast-Eon™ disc treatments using a standard MTS assay conducted daily over 5 days. Elast-Eon™ discs were each 8 mm in diameter, U, U + C1, U + C4, Aapp + C1, Aapp + C4, and Aapp + C4 + L332. HaCaT were seeded on all discs held in individual wells in a 48-well TCPS plate at a density of 1×10^4 cells/well for day 1, 5×10^3 cells/well for day 2, and 2×10^3 cells/well for days 3-5 of incubation. Triplicate wells were established for

each Elast-Eon™ treatment, and cell proliferation measured by MTS assay at 1, 2, 3, 4, and 5 days. The MTS assay used solutions of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (CellTiter 96® AQueous Assay, Promega, Product #G1112) and an electron coupling reagent (phenazine methosulfate) PMS (Sigma-Aldrich, Product#P9625). After incubation for 1 and 2 days, proliferation was assessed on U, U + C1, U + C4, Aapp + C1, Aapp + C4, Aapp + C4 + L332 specimens. Because of the expense of L332 and in light of the lack of additional benefit observed for incubation periods of 1 or 2 days, L332 studies extending to 5 days were not performed. After incubation with HaCaT, discs were washed 3x with phenol red free media (RPMI) (Invitrogen, product #11835) at 37°C, leaving 300 µl of RPMI in each well. Discs then were transferred to fresh wells and 100 µl of MTS/PMS solution was added to each well. Specimens were incubated in the MTS/PMS solution for 1 h at 37°C. After trituration three times to mix the solution, 50 µl of MTS/PMS solution was transferred from each well to a 96-well TCPS plate and colour change was measured in a Tecan Genios Pro microplate reader at a wavelength of 490 nm. Three samples were used for each data point. 95% confidence interval error bars are included, representing one standard deviation either side of the mean.

Results

Retention of C1 and C4 on Elast-Eon™ measured by urea elution and ELISA

The amount of collagen 1 and 4 on functionalised Elast-Eon™ and control Elast-Eon™ blocks was measured with ELISA following urea elution. Outcomes presented

in Figure 3 showed that that the C1 adsorbed to the control non-functionalised Elast-Eon™ blocks was eluted completely by day 1 while 92% of the C1 covalently conjugated to Elast-Eon™ was retained on day 1 with levels decreasing slowly to 39% on day 5. Outcomes presented in Figure 4 showed that that the C4 adsorbed to the control non-functionalised Elast-Eon™ blocks was reduced by 59% of its initial concentration after 20 days while the amount of C4 that was covalently bound to Elast-Eon™ blocks remained steady with no evidence of elution over the 20-day period.

C4 produced by cells on Elast-Eon™ discs over 15 days

The amount of C4 produced by cells adherent to non-functionalised Elast-Eon™ discs was measured over 15 days by ELISA. Data presented in Figure 5 showed that the amount of C4 increased on Elast-Eon™ discs until ten days and then plateaued. C4 deposited by HaCaT did not reach the levels of exogenously applied C4.

Area and focal adhesion density of cells on Elast-Eon™

The data presented in Figure 6 revealed significant difference in cell area between controls and Elast-Eon™ surfaces with any type of collagen treatment, whether the C1 or C4 was conjugated or adsorbed. The addition of L332 to C4 treated surfaces made no difference. The average area of cells on control Elast-Eon™ was 238 μm^2 and the average cell area on collagen-treated Elast-Eon™ was 516 μm^2 (Figure A and B). There was approximately 40x greater density of focal adhesions on protein-treated discs compared to control discs with 5/6 control discs showing no vinculin immunofluorescence (Figure C and D). Taken together, this data showed that collagen increased both cell spread and formation of focal

adhesions compared with untreated surfaces, but the method used to coat collagen onto surfaces and the type of collagen used made no difference to the outcome.

Proliferation of cells on Elast-Eon™

Data presented in Figure 7 revealed that collagen coating, whether adsorbed or covalently conjugated, improved the kinetics of cell proliferation compared with equivalent surfaces without any collagen coating, over both the 2-day and 5-day assays. At 5 days, there were five times as many cells on protein-treated discs compared to the untreated discs. The superimposition of L332 made no difference.

Discussion

C4 was shown to be significantly more stable than C1 and the covalent binding of collagen following acetaldehyde surface functionalisation conferred additional stability, with covalently bound C4 showing no degradation over twenty days in 8 M urea.

All collagens consist of three polypeptide chains (two identical alpha 1 chains and one alpha 2 chain), bound together by hydrogen bonds³⁸, that together form the characteristic triple helix collagenous domain. In C4, but absent in C1, the triple helix is surrounded by non-collagenous (NC) domains that appear to define tissue specificity and interactions with other proteins³⁹⁻⁴¹. C4 consists of six homologous polypeptide chains. Each chain has a 7 S domain at the amino terminus, a long collagenous domain, and an NC domain at the carboxyl terminus. The C4 NC domain is comprised of two subdomains consisting of cysteine residues. Intrachain disulphide bonds between the cysteine residues form triangular structures, from

which loops project that clamp the subdomains tightly together⁴²⁻⁴⁵. The observed additional stability of C4 compared to C1 may be consequent to the cysteine residues and disulphide bonds in the NC domains of C4.

It is possible that some collagen was present after incubation with concentrated urea but was sufficiently denatured (loss of quaternary, tertiary structure, and secondary structure) or disaggregated as to be unrecognisable by the primary antibody. However, urea in high concentrations is known to disrupt non-covalent bonds in proteins⁹ and both the collagenous and non-collagenous domains of C1 and C4 involve covalent bonds between residues. The bond variable being tested in these experiments was between C1 or C4 and the copolymer substrate; bond strength was shown clearly by ELISA to increase significantly with Aapp and sodium cyanoborohydride treatment (covalent conjugation). While the most likely explanation for the ELISA findings is covalent bonding, if urea-induced denaturation or disaggregation was the cause of the detected differences, then protection against denaturation or disaggregation may still be a useful finding. For completeness, in future it may be worthwhile considering elution experiments with concentrated urea in the presence of a reducing agent to minimise the possibility of disaggregation. In addition, future experiments may benefit from spectroscopic surface analysis to assess for protein signals after a period of incubation in urea. For example, it may be possible to mirror the slow decrease in covalently bound C1 between days 1 and 5 via XPS (X-ray photoelectron spectroscopy) analysis of nitrogen signalling. Any such experimentation would need to account for errors in peak intensity measurements inherent to XPS, reported at 10-15%⁴⁶, possibly reducing the sensitivity of XPS measurement of collagen content compared with ELISA. In addition, to assess

biological responses to retained protein, it would be worthwhile measuring cellular adhesion and proliferation on materials following a period of incubation in urea.

Currently, when exogenous collagen is used to optimise implants, it is C1^{6,7,47}.

While C4 is expensive, the data herein showed that only 2 µg/ml was required to saturate surfaces. In addition, recombinant sources of human collagen are becoming more readily available^{48,49} and inexpensive recombinant human C4 may be available in the near future^{50,51}. The use of C4 rather than C1 could obviate the need for cross-linking and may improve the stability of tissue implants.

Epidermal cells (keratinocytes) have been reported by others to attach and differentiate preferentially on C4, compared to collagen 1-3⁵². The research herein showed no significant differences in vitrobiological responses of keratinocytes to C1 compared to C4, whether adsorbed or covalently bound.

A number of previous studies showed that L332⁵³⁻⁵⁶ or the laminin sequence (TWYKIAFQRNRK)⁵⁷ improved epithelial attachment to materials. However, the research herein indicated that superimposing L332 on surfaces already saturated with C4 conferred no additional benefit, suggesting that biological responses to materials may be optimised by saturation with one protein only.

Discoidin domain receptors (DDR) are anchored in cell membranes and recognise collagens as their ligands⁵⁸. DDRs consist of two distinct subsets, DDR1 and DDR2. Following attachment to collagen, DDRs undergo tyrosine autophosphorylation, leading to a variety of cell-collagen interactions, altering cell adhesion and migration⁵⁹. Both DDR1 and DDR2 bind to, and are activated by, fibrillar collagens (C1, 2, 3, and 5)^{60,61}. C4 is known to activate DDR1 but not DDR2

^{58,62,63}. The results reported herein indicate no differences for keratinocyte attachment or proliferation on surfaces treated with C1 compared with C4 suggesting that, at least for HaCaT keratinocytes, any differences in DDR activation are insignificant. In addition, these results suggest that any differences in integrin-mediated adhesion to DGEA compared to CB3 are insignificant compared to integrin-mediated binding to GFOGER. DDRs are known to bind to the native triple helix of collagens and not to individual collagen alpha-chains or denatured collagens ^{58,62}. Similarly, deglycosylated or degraded collagen cannot support DDR activation ^{58,64,65} suggesting the mechanisms via which DDR-collagen interactions are influenced negatively by protein cross-linking.

Basement membranes separate epithelium (found in skin, respiratory tract, gastrointestinal tract, etc.), mesothelium (found in the pleural cavity, peritoneal cavity, pericardial cavity, etc.) and endothelium (found in blood vessels, lymph vessels, etc.) from underlying connective tissue ⁶⁶. Hence, attached C4 may improve cellular responses to materials implanted in tissues with basement membranes, such as skin, blood vessels (coronary artery stents, vascular grafts, heart valves), and corneal grafts. In addition, C4 may improve long-term tissue responses to implants not closely associated with basement membranes, such as joint replacements, catheters, and polymer mesh (e.g. for hernia repairs), acting as a generic surface protein that is particularly resistant to degradation. Non-specific methods for improving cellular responses to implanted materials are increasingly used, such as coatings of omega 3, hyaluronate, and titanium ^{67,68}. C4 coatings ultimately may prove to be more positively bioactive than non-specific methods and, given the

results reported herein, may retain positive bioactivity for extended periods of time and possibly indefinitely.

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Disclosure

A patent has been applied for in Australia, the U.S., and Europe, on a skin-material interfacing device that includes surface optimisation with collagen 4.

Figures

Figure 1: Acetaldehyde glow discharge polymerisation (image courtesy of CSIRO, with permission).

Figure 2: Covalent bonding of collagen to surface aldehyde groups. After deposition of surface aldehyde groups, collagen was added (represented by an amine group), forming imines ($-\text{CH}=\text{N}-$ bonds). The imines were reduced to stable amines ($-\text{CH}_2\text{-NH}-$) by the addition sodium cyanoborohydride.

Figure 3: Urea elution of C1 from Elast-Eon™ blocks over 5 days. There is improved retention of covalently bound C1 compared with the rapid and complete loss of C1 from non-functionalised control.

Figure 3: Urea elution of C4 from Elast-Eon™ blocks over 20 days. There was a steady loss of adsorbed C4 while covalently bound C4 showed high retention that persisted over 20 days.

Figure 4: C4 deposited on Elast-Eon™ discs by keratinocytes compared to exogenously applied C4. C4 was deposited with increasing duration of incubation with cells, plateauing at 10-15 days. C4 deposited by cells did not reach the density of exogenously applied C4.

Figure 5 A, B, C, and D: (A) SEM images of keratinocytes on Elast-Eon™ and (B) average cell area in 10 SEM fields at 4 h. (C) Confocal microscope images of focal adhesion vinculin immunofluorescence at 4 h and (D) average vinculin density per cell at 4 h. Significant differences only were seen between control (untreated) and treated Elast-Eon™ discs. The area and vinculin density of cells were similar on all

treated discs, regardless of the type of protein or the mechanism of attachment (adsorption or covalently bound).

Figure 7 A and B: Keratinocyte proliferation on Elast-Eon™ discs at 2 and 5 days.

Significant differences were seen only for “untreated” discs compared to any type of protein treatment.

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Figure 1: Vacuum plasma acetaldehyde functionalisation (image courtesy of CSIRO, with permission).

85x44mm (300 x 300 DPI)

Accepted

Figure 2: Covalent bonding of collagen to surface aldehyde groups. After deposition of surface aldehyde groups, collagen was added (represented by an amine group), forming imines (-CH=N- bonds). The imines were reduced to stable amines (-CH₂-NH-) by the addition sodium cyanoborohydride.

38x8mm (300 x 300 DPI)

Accepted Article

Figure 3: Urea elution of C1 from Elast-Eon™ blocks over 5 days. There is improved retention of covalently bound C1 compared with the rapid and complete loss of C1 from non-functionalised control.

90x46mm (300 x 300 DPI)

Accepted

Figure 4: Urea elution of C4 from Elast-Eon™ blocks over 20 days. There was a steady loss of adsorbed C4 while covalently bound C4 showed high retention that persisted over 20 days.

102x62mm (300 x 300 DPI)

Accepte

C4 on Elast-Eon™ discs

Figure 5: C4 deposited on Elast-Eon™ discs by keratinocytes compared to exogenously applied C4. C4 was deposited with increasing duration of incubation with cells, plateauing at 10-15 days. C4 deposited by cells did not reach the density of exogenously applied C4.

112x74mm (300 x 300 DPI)

Accepted

99x52mm (300 x 300 DPI)

Accepted

B Average cell area

108x74mm (300 x 300 DPI)

Accept

362x223mm (300 x 300 DPI)

Accepte

Figure 6 A, B, C, and D: (A) SEM images of keratinocytes on Elast-Eon™ and (B) average cell area in 10 SEM fields at 4 h. (C) Confocal microscope images of focal adhesion vinculin immunofluorescence at 4 h and (D) average vinculin density per cell at 4 h. Significant differences only were seen between control (untreated) and treated Elast-Eon™ discs. The area and vinculin density of cells were similar on all treated discs, regardless of the type of protein or the mechanism of attachment (adsorption or covalently bound).

121x99mm (300 x 300 DPI)

ACC

97x59mm (300 x 300 DPI)

Accepte

B 5 days proliferation

Figure 7 A and B: Keratinocyte proliferation on Elast-Eon™ discs at 2 and 5 days. Significant differences were seen only for “untreated” discs compared to any type of protein treatment.

96x61mm (300 x 300 DPI)

Accepted

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