

Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:

Sun, Zhe

Title:

The development of a biodegradable nano-particle vaccine delivery system

Date:

2019

Persistent Link:

<https://hdl.handle.net/11343/235531>

Terms and Conditions:

Terms and Conditions: Copyright in works deposited in Minerva Access is retained by the copyright owner. The work may not be altered without permission from the copyright owner. Readers may only download, print and save electronic copies of whole works for their own personal non-commercial use. Any use that exceeds these limits requires permission from the copyright owner. Attribution is essential when quoting or paraphrasing from these works.

The development of a biodegradable nano- particle vaccine delivery system

Zhe Sun

ORCID: 0000-0001-7000-0848

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

November 2019

**Melbourne Dental School
Department of Medicine, Dentistry and Health Sciences
The University of Melbourne**

Abstract

Despite the recent advances in cancer treatment, this disease continues to pose a serious threat to public health. Nanoparticle-based delivery platforms have been shown to be a promising strategy for cancer immunotherapy. Therapeutic cancer vaccines are designed specifically to elicit a potent adaptive immune response, giving rise to the specific eradication of cancer cells. Among the different synthetic nanoparticle carriers currently available calcium phosphate nanoparticles (CaP NPs) are the most promising potential vaccine transporters, and have attracted increasing attention during the past decade. In this thesis, the synthesis of a novel CaP NP vaccine is described and the further investigation of their effects on immune cells is examined.

In this study, a novel citrate chelation method for CaP NP synthesis was developed to obtain well-defined, homogeneous NPs. The method of synthesis is innovative, convenient, inexpensive and, most significantly, consistent and reproducible. Additionally, this study is the first investigation to describe the effect of different types of calcium and phosphate salts on NP synthesis. It was found that various sizes of CaP NPs can be obtained by using different calcium and phosphate salts to synthesise the nanoparticles.

To further develop the formulation of the nanoparticle vaccine, a layer-by-layer approach to vaccine synthesis was utilised. All experimental parameters of the layer-by-layer approach of nanoparticle formulation were optimised during synthesis to avoid NP aggregation, and to increase NP size stability. Significantly, it was found that cross-linking the protein antigen on the NP surface enhanced salt stability and greatly reduced the host-plasma protein adsorption on to the NPs. Moreover, the composition of the protein corona identified by mass spectrometry showed the major component of bound protein was albumin.

To study the impact of NP size on the interactions between NPs and host cells three sizes (170 nm, 260 nm and 360 nm) of rod-like shaped NPs were used *in vitro*, and the effects on epithelial cells and macrophages were observed. This study demonstrated that the three sizes of NPs used in this study can efficiently bind to epithelial cells and migrate through the epithelial barrier, and that they induce a cytokine profile from epithelial cells favouring the recruitment of further immune cells. Moreover, the three sizes of cross-linked

CaP-PEI-OVA NPs are phagocytosed by RAW 264.7 cells in a dose-dependent manner. Significantly, large CaP NPs induced significantly stronger cell-binding, cellular-uptake, phagocytosis, NF- κ B activation, cytokine secretion, and inflammatory cell surface marker expression at the highest NP to cell ratio than the smaller nanoparticles.

Finally, a new potential adjuvant with favourable chemical properties for use in vaccine applications was designed and synthesised. A TLR2 ligand, Pam₂KK₄CG, was synthesised and conjugated to three sizes of calcium phosphate OVA NPs. The effect of these functionalised NPs on macrophages was compared to commercially available Pam₃CSK₄ and Palmitoyl alone. It was shown that the particles were biocompatible with macrophages, and that they were phagocytosed by RAW 264.7 cells in a dose-dependent manner. Additionally, the functionalised NPs significantly increased the expression of cell surface markers (CD40, CD80, and MHC II) in RAW 264.7 cells. Finally, the expression of IL-1 β , IL-10, IL-12 and TNF- α was greatly enhanced by Pam₂KK₄CG lipopeptide treatment as compared to CaP-OVA NPs alone.

Taken together, the results presented in this thesis provide an in-depth understanding of the immune response of epithelial cells and macrophages to a synthesised CaP NPs vaccine. Because these cells are vital for the induction of the adaptive immune responses, further work arising from the results of this study may make a significant contribution to the development of therapeutic vaccines for the treatment of cancer.

Declaration

This is to certify that:

- (i) This thesis comprises only my original work towards the PhD, except where indicated in the preface;
- (ii) Due acknowledgement has been made in the text to all other material used;
- (iii) This thesis is fewer than 100,000 words in length, exclusive of figures, tables, maps, bibliographies and appendices.

Zhe Sun

Preface

All work herein has been carried out by the candidate, Zhe Sun, except the scanning electron microscopy, helium ion microscopy, X-ray diffraction, confocal microscopy and peptide synthesis. Contributions by fellow collaborators are detailed below:

Chapter 2:

- The scanning electron microscopy for calcium phosphate nanoparticles was conducted by Dr Yu-Yen Chen (Melbourne Dental School, University of Melbourne)
- The helium ion microscopy for calcium phosphate nanoparticles were carried out by Babak Nasr (Materials Characterization and Fabrication Platform, University of Melbourne)
- The X-ray diffraction for calcium phosphate nanoparticles was conducted by Materials Characterisation and Fabrication Platform (University of Melbourne).

Chapter 4:

- The confocal microscopy for calcium phosphate nanoparticles were carried out by Paul Brannon (Materials Characterization and Fabrication Platform, University of Melbourne)

Chapter 5:

- The Pam₂KK₄CG lipopeptide was synthesised by Wenyi Li (Melbourne Dental School, University of Melbourne)

Publications

Li, W., **Sun, Z.**, O'Brien-Simpson, N. M., Otvos, L., Reynolds, E. C., Hossain, M. A., Wade, J. D. (2017). The Effect of Selective D- or N α -Methyl Arginine Substitution on the Activity of the Proline-Rich Antimicrobial Peptide, Chex1-Arg20. *Frontiers in Chemistry*, 5, 1.

Conferences

Zhe S *et al*, The effect of the rod-like calcium phosphate nanoparticles on cellular uptake and cell function, 30th Annual Conference of the European Society for Biomaterials conference, Dresden, Germany, 2019

Zhe S *et al*, The immunological effect of the calcium phosphate nanoparticles on oral epithelial cells and macrophages, 5th Annual MDHS ECR Network Symposium, Melbourne, Australia, 2019

Zhe S *et al*, Preparation of calcium phosphate nanoparticles by reverse microemulsion route with narrow size distribution, ICONN 2018 international conference on nanoscience and nanotechnology conference, Wollongong, Australia, 2018

Scholarships

Australian Postgraduate Awards

Melbourne University International Travel Scholarship

Rowden White Scholarship

Melbourne University Local Travel Scholarship

Oral Health Cooperative Research Center (CRC) Travel Scholarship

Melbourne Dental School Research Scholarship

Oral Health Cooperative Research Center (CRC) Top-up Scholarship

Melbourne International Research Scholarship

Melbourne International Fee Remission Scholarship

Acknowledgements

First and foremost, I would like to express my deepest appreciation and gratitude to my supervisor, Prof. Neil O'Brien-Simpson, for accepting me into his group and guiding me through my PhD studies. With Neil's guidance, inspiration and support, I have been well-trained, which allows me to grow as a young research scientist. Your contribution to my PhD has been enormous.

I am also grateful for my co-supervisors, Prof. Eric Reynolds, Prof. Michael McCullough, Dr Jason Lenzo and Dr James Holden, for all that you have done for me throughout my PhD studies. My profound thanks to Prof. Eric Reynolds, for giving me the opportunity to undertake my PhD at the Melbourne Dental School among such amazing people. I would also like to express my sincerest gratitude to Prof. Michael McCullough for his suggestions and participation in some of my PhD meetings. Many thanks to Dr Jason Lenzo and Dr James Holden for co-supervising my PhD project. Thanks for teaching me everything about immunology and helping me about flow cytometry analysis. I am so grateful for their guidance through all of experiments.

Furthermore, I gratefully thank Dr Dhana Govind Gorasia for assistance with protein studies and research discussions, Dr Yu Yen Chen for help with imaging, David Stanton for teaching and assistance in calcium and phosphate concentration analysis. Thanks Dr Nacho Insua for your support and help for in the nanoparticle field and kind suggestions. Additionally, I would like to thank Dr Wenyi Li for peptide synthesis and encouragement throughout my PhD. Thanks to William Singleton and Dr Rebecca Orth for giving me valuable suggestions for my thesis.

Many thanks also go to the staff from the Oral Health CRC and Bio21: Dr Jacqueline Heath, Dr Lianyi Zhang, Dina Chen, Assoc. Prof. Glen Scholz, Dr Alexis Gonzalez, Yi Yuan, Dr Peiyan Shen, and Deanne Catmull. Thank you for the technical support and your guidance that has make my research possible.

Special thanks to all my friends: Tao Huang, Yi Fan, Jiamin, May, Hong, Lin, Caroline, Sayeed, Claire and Sally. Thank you for making my PhD a fun and memorable experience.

Most importantly I would like to deeply thank my mom, dad, brother and sister-in-law. Your love, support and encouragement were always deeply appreciated. You built a strong foundation for me to achieve my aims. Thanks you for being on call whenever I needed help and for believing in me.

Last but not least, I would like to thank to Supeng Zhang, your support means more than you can imagine. Your helped me cope during the hard times and celebrate during the good times. Thank you for supporting me through my years of studies.

Thanks to all of you who have made my PhD study such a rewarding, fulfilling and exciting journey.

Table of Contents

Abstract	i
Declaration	iii
Preface	iv
Publications	v
Acknowledgements	vi
Table of Contents.....	viii
List of Figures	xiv
List of Tables.....	xix
Abbreviations.....	xxii
Chapter 1.....	1
General Introduction.....	1
1.1 Nanotechnology in vaccine delivery system.....	2
1.1.1 Polymeric nanoparticles	4
1.1.2 Lipid nanoparticles.....	7
1.1.3 Gold nanoparticles	8
1.1.4 Silica nanoparticles	9
1.2 The immune system and vaccination	10
1.2.1 Innate immunity	12
1.2.2 Adaptive immunity.....	12
1.2.3 Antigen-presenting cells (APCs).....	13
1.2.4 T cells (CD4 ⁺ and CD8 ⁺ T lymphocytes).....	13
1.2.4.1 T cell development and differentiation.....	13
1.2.4.2 Antigen recognition by CD4 ⁺ and CD8 ⁺ T cells	14
1.2.4.3 Induction of effector cytotoxic T cell responses	15
1.2.5 Vaccine adjuvants, delivery system and TLR ligands	16
1.2.6 CTL vaccines for oral cancer	18

1.3 Calcium phosphate nanoparticles (CaP NPs).....	20
1.3.1 Synthesis of CaP NPs.....	21
1.3.2 Properties of CaP NPs	22
1.3.2.1 Safety, biocompatibility and stability.....	22
1.3.2.2 CaP NPs pH-dependent solubility	23
1.3.2.3 Ease of surface modification	24
1.3.2.4 High adjuvanticity	25
1.3.3 Strategies for antigen loading onto CaP NPs	25
1.3.4 CaP NPs uptake and immunogenicity.....	27
1.3.4.1 CaP NPs cellular uptake	27
1.3.4.2 Targeting the innate immune system.....	28
1.3.4.3 Targeting the adaptive immune system.....	30
1.4 Summary.....	33
1.5 Hypothesis and aims of the thesis	37
Chapter 2	38
Development, evaluation and optimisation of a method for calcium phosphate nanoparticle synthesis with defined sizes and narrow polydispersity index.....	38
2.1 Introduction.....	39
2.2 Materials and Methods.....	43
2.2.1 Conventional precipitation synthesis method	43
2.2.2 Microemulsion synthesis method.....	43
2.2.3 Calcium citrate chelation method.....	45
2.2.4 CaP NPs characterisation	46
2.2.5 Compositional analysis of CaP NPs.....	47
2.2.6 Statistical analysis	48
2.3 Results	49
2.3.1 Conventional precipitation synthesis method	49

2.3.2 Microemulsion synthesis method.....	51
2.3.3 Citrate chelation synthesis method.....	55
2.3.3.1 Optimisation of citrate chelation method	55
2.3.3.2 Effect of different calcium salts and phosphate salts on CaP NPs synthesis	59
2.3.3.3 Effect of constant pH (8.5) on CaP NPs synthesis	62
2.3.3.4 Characterisation of the CaP NPs	65
2.3.3.5 Fourier transform infra-red spectroscopy (FTIR).....	66
2.3.3.6 EM images.....	67
2.3.3.7 X-ray diffraction.....	68
2.3.3.8 Process scale-up of the calcium citrate chelation method	69
2.4 Discussion	71
2.5 Conclusion	75
2.6 Supplementary Information	76
Chapter 3.....	82
Optimisation of antigen loading of calcium phosphate nanoparticles.....	82
3.1 Introduction.....	83
3.2 Materials and Methods.....	85
3.2.1 Preparation of the CaP NPs.....	85
3.2.2 Preparation of OVA-loaded CaP NPs via encapsulation	85
3.2.3 SDS-PAGE analysis	85
3.2.4 Synthesis of stable, positively charged CaP NPs	86
3.2.5 Optimisation of OVA adsorption onto CaP-PEI NPs.....	87
3.2.6 Optimisation of cross-linking OVA on CaP NPs.....	87
3.2.7 Cell culture and cell cytotoxicity assay.....	88
3.2.8 The size stability of nanoparticles vaccine in serum containing media	89
3.2.9 Identification of serum proteins absorbed onto CaP-PEI-OVA NPs	89

3.2.10 Statistical analysis	90
3.3 Results	91
3.3.1 Ovalbumin encapsulation into CaP NPs	91
3.3.2 Absorption of OVA onto CaP NPs using a layer-by-layer approach.....	92
3.3.3 The different molecular weight of PEI adsorption onto CaP NPs	95
3.3.4 Size stability of various molecular weight of PEI coated CaP NPs	97
3.3.5 CaP-PEI NPs cytotoxicity	97
3.3.6 Optimisation of OVA adsorption	99
3.3.7 Second layer of PEI.....	102
3.3.8 Cross-linking OVA on the surface of CaP-PEI NPs	103
3.3.9 Optimal conjugation buffer for the BS ³ conjugation	106
3.3.10 SDS-PAGE and mass spectrometry analyse OVA cross-linking	107
3.3.11 Evaluation of the protein corona from cell culture media and serum on cross-linked CaP-PEI-OVA NPs	109
3.3.12 Optimised method for stable protein antigen coated CaP NPs	114
3.4 Discussion	117
3.5 Conclusion	121
3.6 Supplementary Information	122
Chapter 4.....	126
Evaluation of the immunological effects of different sizes of CaP NPs vaccine on oral epithelial cells and macrophages	126
4.1 Introduction.....	127
4.2 Materials and Methods.....	130
4.2.1 Preparation and characterisation of cross-linked CaP-OVA NPs of different sizes	130
4.2.2 Cell cultures and growth conditions.....	130
4.2.3 Cytotoxicity of nanoparticles	130
4.2.4 Nanoparticle OKF6 cell migration assay	131

4.2.5 Nanoparticle cell binding assays by flow cytometry	131
4.2.6 Cellular uptake of nanoparticles by confocal imaging.....	132
4.2.7 Nanoparticle phagocytosis assays by flow cytometry.....	132
4.2.8 NF- κ B activation assay with RAW-Blue cells.....	133
4.2.9 Cell surface marker production by flow cytometry	133
4.2.10 Cytokine production assays with OKF6 cells and RAW cells.....	134
4.2.11 Statistical analysis	134
4.3 Results	135
4.3.1 Characterisation of different sizes of cross-linked CaP-OVA NPs.....	135
4.3.2 Nanoparticle cytotoxicity	137
4.3.3 Binding of nanoparticles to oral epithelial cells.....	141
4.3.4 Nanoparticle migration across epithelial cell monolayers	143
4.3.5 Nanoparticle uptake by confocal imaging.....	146
4.3.6 Nanoparticles induction of inflammatory cytokines by oral epithelial cells	147
4.3.7 Phagocytosis of nanoparticles by macrophages.....	150
4.3.8 Nanoparticle activation of NF- κ B in RAW-Blue cells and cell surface marker production	152
4.3.9 Nanoparticle induced cytokine production in RAW 264.7 cells.....	154
4.4 Discussion	157
4.5 Conclusion	162
4.6 Supplementary Information	163
Chapter 5.....	165
Co-encapsulation of antigen ovalbumin and TLR2 ligand in calcium phosphate nanoparticles leads to macrophages maturation and activation.....	165
5.1 Introduction.....	166
5.2 Materials and Methods.....	168
5.2.1 Cell cultures and growth conditions.....	168
5.2.2 Pam ₂ KK ₄ CG peptide synthesis	168

5.2.3 Preparation and characterisation of TLR2 ligand functionalised CaP-PEI-OVA NPs	169
5.2.4 TLR2 activation assay with HEK293-TLR2 reporter cell line	170
5.2.5 NF- κ B activation assay with RAW-Blue cells.....	170
5.2.6 Cytotoxicity of TLR2 ligand functionalised CaP NPs	171
5.2.7 Phagocytosis of TLR2 ligand functionalised CaP NPs by RAW 264.7 cells	171
5.2.8 Cell surface marker production by flow cytometry	171
5.2.9 Cytokine production assays with RAW cells.....	172
5.2.10 Statistical analysis	172
5.3 Results	175
5.3.1 Characterisation of TLR2 ligand functionalised CaP-PEI-OVA NPs	175
5.3.2 TLR2 activation in HEK293-TLR2 reporter cell line and NF- κ B activation assay in RAW-Blue cells	176
5.3.3 Cytotoxicity of Pam ₂ KK ₄ CG functionalised nanoparticles	180
5.3.4 Pam ₂ KK ₄ CG functionalised nanoparticles phagocytosis by macrophages..	181
5.3.5 Cell surface marker production.....	181
5.3.6 Pam ₂ KK ₄ CG functionalised nanoparticles induce cytokine production in RAW 264.7 cells	186
5.4 Discussion	188
5.5 Conclusion	192
5.6 Supplementary Information	193
Chapter 6.....	195
General Discussion	195
References	201

List of Figures

Figure 1. 1: The advantages of NP used as platform for vaccine applications.....	4
Figure 1. 2: Different nanoparticle-based vaccine delivery systems.....	4
Figure 1. 3: The components of the immune system.....	11
Figure 1. 4: The desired immune response following vaccination.....	11
Figure 1. 5: Induction of effector T cell responses needs three signals.	15
Figure 1. 6: Different types of Toll-like receptors, antigen presentation and the elicited immune response.	18
Figure 1. 7: Schematic representation of CaP NPs functions (outer ring, blue), surface functionalisation methods (left sector, green) and moieties (right sector, orange).....	21
Figure 1. 8: The release process of siRNA entrapped in lipid-coated CaP NPs in the endosome.....	24
Figure 1. 9: Strategies of antigen interaction with CaP NPs through conjugation, encapsulation, adsorption or simple mixing.....	27
Figure 1. 10: A triple-shell calcium phosphate nanoparticles loaded an antigen and a TLR ligand (CpG or poly (I:C)) for cellular uptake.	32
Figure 2. 1: Schematic diagram of the the conventional precipitation method for the formation of CaP NPs.....	43
Figure 2. 2: Schematic diagram of the preparation of CaP NPs by the microemulsion method.	44
Figure 2. 3: Schematic diagram of the preparation of CaP NPs by the calcium citrate chelation method.	46
Figure 2. 4: Characterisation of particles synthesised by conventional precipitation method.	50
Figure 2. 5: Characterisation results of nanoparticles (A) correlation curve, (B) size distribution, (C) zeta potential (D) TEM image and (E) SEM image (F) size stability (G) reproducibility from microemulsion method.....	54
Figure 2. 6: Photo-image of CaCl ₂ plus a chelating agent 1 hour after addition of NaH ₂ PO ₄ : (A) EDTA; (B) Glycine; (C) Citric acid; (D) L-glutamic acid; (E) Iminodiacetic acid and (F) MilliQ water at pH 8 and room temperature.	56
Figure 2. 7: Photograph of various molar ratios of CaCl ₂ to citric acid: A (1:1.4); B (1:1.5); C (1:1.6); D (1:1.7); E (1:1.8) after 1 hour following addition of NaH ₂ PO ₄ at room temperature pH 8.5.	57

Figure 2. 8: Effect of reaction time on particle size and polydispersity index as determined by DLS.....	58
Figure 2. 9: Characterisation results of nanoparticles (A) reproducibility from citrate chelation method, (B) size stability, (C) correlation curve, (D) size distribution, (E) zeta potential, (F) TEM image and (G) SEM image.....	59
Figure 2. 10: The particle size and correlation curve of eight combinations at 40 °C for 6 h reaction time by citrate chelation method.....	61
Figure 2. 11: The pH change of eight combinations at 40 °C for 6 h reaction time and drift pH 8.5 by citrate chelation method.....	62
Figure 2. 12: The particle size and correlation curve of eight combinations at 40 °C for 6 h reaction time and constant pH 8.5 by citrate chelation method.....	64
Figure 2. 13: FTIR spectra of the CaP NPs in the presence (A) or absence (B) of citrate acid (CaCl ₂ , NaH ₂ PO ₄) by citrate chelation method. The two patterns have been normalised.	67
Figure 2. 14: SEM (A, B, C) and HIM (D, E, F) images of rod-like CaP NPs synthesised by citrate chelation method.....	68
Figure 2. 15: X-ray diffraction pattern of various sizes of CaP NPs synthesised by citrate chelation method.	69
Figure 2. 16: Photograph of the 600 mL reaction synthesis scale; pre-heating (A) and post-heating (B).	70
Supplementary Figure 2. 1: Electron dispersion spectroscopy (EDS) microanalysis of OVA-loaded CaP NPs synthesised by microemulsion method. (A) SEM image, (B) EDS spectrum, (C) elemental analysis.....	76
Supplementary Figure 2. 2: Different molar ratios of CaCl ₂ to IDA: A (1:15); B (1:20); C (1:25); D (1:30) and NaH ₂ PO ₄ at room temperature.	76
Supplementary Figure 2. 3: Different molar ratios of CaCl ₂ to citric acid to IDA: A (1:0.5:15); B (1:0.5:20); C (1:0.5:25); D (1:0.5:30); E (1:0.5:35) and NaH ₂ PO ₄ at room temperature.	77
Supplementary Figure 2. 4: Different molar ratios of CaCl ₂ to citric acid to IDA: A (1:1:10); B (1:1:12); C (1:1:15); D (1:1:18) and NaH ₂ PO ₄ at room temperature.....	77
Supplementary Figure 2. 5: Different molar ratios of CaCl ₂ to L-glutamic acid: A (1:15); B (1:20); C (1:25); D (1:30); E (1:35); F (1:40); G (1:50); H (1:60); I (1:70); J (1:80) and NaH ₂ PO ₄ at room temperature.	78

Supplementary Figure 2. 6: Different molar ratios of CaCl ₂ to citric acid to L-glutamic acid: A (1:1:10); B (1:1:20); C (1:1:30); D (1:1:40); E (1:1:50) and NaH ₂ PO ₄ at room temperature.....	79
Supplementary Figure 2. 7: FTIR spectra of the CaP NPs synthesised in different combinations at drifting pH 8.5 by citrate chelation method.	80
Supplementary Figure 2. 8: FTIR spectra of the CaP NPs synthesised in different combinations at constant pH 8.5 by citrate chelation method.....	81
Figure 3. 1: (A) Effects of different loading methods on OVA loading efficiency. (B) The SDS-PAGE analysis of the standard curve and OVA-loaded CaP NPs.....	92
Figure 3. 2: The characterisation of CaP NPs with different molecular weight of PEI in MilliQ water.	97
Figure 3. 3: The toxicity of CaP NPs with different molecular weight of PEI on HEK (A, B) and H4IIE cells (C, D). Data are expressed as mean ± SD of at least 3 biological replicates; error bars show the SDs.	98
Figure 3. 4: Effect of OVA concentration on particles. (A) size (B) PDI (C) zeta potential (D) SDS-PAGE. Data are expressed as mean ± SD of at least 3 biological replicates; error bars show the SDs.	99
Figure 3. 5: SDS-PAGE of CaP-PEI-OVA NPs in the various solutions for 6 h at room temperature.....	100
Figure 3. 6: Characterisation of the second layer of PEI adsorbed on CaP-PEI-OVA NPs.	102
Figure 3. 7: SDS-PAGE analyses of the different molar ratio of cross-linkers to OVA. (A) different molar ratio of GA/OVA; (B) different molar ratio of BS ³ /OVA; (C) different molar ratio of DMTMM/OVA.	104
Figure 3. 8: CaP-PEI-OVA NPs in different conjugation buffer after 6 h.	106
Figure 3. 9: The SDS-PAGE analysis of non-cross-linked and cross-linked CaP-PEI-OVA NPs.....	107
Figure 3. 10: Size stability of unquenched and quenched CaP-PEI-OVA NPs in MilliQ water. Data are expressed as mean ± SD of at least 3 biological replicates; error bars show the SDs.....	109
Figure 3. 11: Size and correlation curve of non-cross-linked and cross-linked CaP-PEI-OVA NPs in various media measured by DLS.	111

Figure 3. 12: SDS-PAGE of protein corona comes from non-cross-linked or cross-linked CaP-PEI-OVA NPs incubated with various media for 6 h at room temperature.	112
Figure 3. 13: FTIR spectra of the LbL CaP NPs. (A) PEI, (B) OVA, (C) CaP NPs, (D) CaP-PEI NPs, (E) CaP-PEI-OVA NPs. The three patterns have been normalised.	113
Figure 3. 14: Established protocol of the preparation of cross-linked CaP-PEI-OVA NPs.	114
Figure 3. 15: HIM images of cross-linked CaP-PEI-OVA NPs synthesised using layer-by-layer method.	116
Figure 4. 1: Characterisation of different sizes of CaP-OVA NPs.	136
Figure 4. 2: Cell viability and toxicity effects of CaP-OVA NPs.	138
Figure 4. 3: Cell viability and toxicity effects of CaP-PEI-OVA NP components.	139
Figure 4. 4: Cytotoxic effects of NP-associated PEI.	140
Figure 4. 5: Binding of cross-linked CaP-OVA NPs to the oral epithelial cells (OKF6).	142
Figure 4. 6: Migration of CaP-OVA NPs through cellular monolayer.	144
Figure 4. 7: Localisation of CaP-OVA NPs during cellular migration.	145
Figure 4. 8: Confocal microscopy of internalised CaP-OVA NPs.	146
Figure 4. 9: OKF6 cytokines induced by CaP-OVA NPs for 6 h.	148
Figure 4. 10: OKF6 cytokines induced by CaP-OVA NPs for 24 h.	149
Figure 4. 11: Effects of NP size on macrophage phagocytosis.	151
Figure 4. 12: NF- κ B activation by CaP-OVA NPs.	153
Figure 4. 13: Induction of RAW 264.7 cytokines by CaP-OVA NPs for 6 h.	155
Figure 4. 14: Induction of RAW 264.7 cytokines by CaP-OVA NPs for 24 h.	156
Supplementary Figure 4. 1: Confocal microscopy of internalised CaP-OVA NPs for 1 and 3 h.	163
Supplementary Figure 4. 2: Confocal microscopy of internalised CaP-OVA NPs at NP to cell ratio of 1,000:1 and 10,000:1 for 3 h.	164
Figure 5. 1: Schematic representation of the preparation of TLR2 ligand functionalised nanoparticles.	173
Figure 5. 2: Chemical structures of Pam molecules.	174
Figure 5. 3: Different numbers of palmitoyl (Pam) chains functionalised nanoparticles activates TLR2 ligand in HEK293-TLR2 reporter cell line at increasing nanoparticle to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1).	178

Figure 5. 4: Different numbers of palmitoyl (Pam) chains functionalised nanoparticles activates NF- κ B in RAW-Blue cells at increasing nanoparticle to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1).....	179
Figure 5. 5: Cell toxicity by MTS assay (A) and LDH assay (B) on RAW 264.7 cells incubated with different NP to cell ratios of Pam ₂ KK ₄ CG functionalised nanoparticles for 90 minutes.	180
Figure 5. 6: Phagocytosis of Pam ₂ KK ₄ CG functionalised nanoparticles by the macrophages (RAW 264.7).	182
Figure 5. 7: Pam ₂ KK ₄ CG functionalised nanoparticles induction of CD40 expression in RAW 264.7 cells at various NP to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1).	183
Figure 5. 8: Pam ₂ KK ₄ CG functionalised nanoparticles induction of CD86 expression in RAW 264.7 cells at various NP to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1).	184
Figure 5. 9: Pam ₂ KK ₄ CG functionalised nanoparticles induction of MHC II expression in RAW 264.7 cells at various NP to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1).	185
Figure 5. 10: Pam ₂ KK ₄ CG functionalised nanoparticles induce cytokine release in RAW 264.7 cells.....	187
Supplementary Figure 5. 1: HPLC profile of Pam ₂ KK ₄ CG (dipalmitoyl-KK ₄ CG)....	193
Supplementary Figure 5. 2: Pam ₂ KK ₄ CG functionalised nanoparticles induce cytokine release in RAW 264.7 cells.	194

List of Tables

Table 1. 1: Comparison of main methods for synthesis CaP NPs.....	22
Table 1. 2: The recent research of CaP NPs in vaccine applications.	35
Table 2. 1: Various phases of CaP NPs compounds.....	42
Table 2. 2: Comparison of rapid and dropwise addition obtained particles by conventional precipitation method (n = 3). Data are expressed as mean $\pm$ SD of at least 3 biological replicates.....	51
Table 2. 3: Effect of the various parameters on CaP NPs from microemulsion method (n = 3). Data are expressed as mean $\pm$ SD of at least 3 biological replicates.	52
Table 2. 4: Particle size and polydispersity index (PDI) of precipitated particles. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.	56
Table 2. 5: Effect of different molar ratios of CaCl ₂ to citric acid on CaP NPs at 40 °C for 6 h. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.	57
Table 2. 6: Effect of different calcium salts and phosphate salts on CaP NPs synthesis. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.....	60
Table 2. 7: NP characteristics of CaP NPs produced using different calcium salts and phosphate salts and the citrate chelation method at constant pH 8.5. Data are expressed as mean $\pm$ SD of at least 3 biological replicates of 3 replicates.	63
Table 2. 8: Percent of calcium and phosphate converted to nanoparticles and the Ca/P ratio of CaP NPs synthesised under constant pH 8.5 and drifting pH 8.5 by citrate chelation method.	66
Table 2. 9: Characteristics of the prepared CaP NPs by the citrate chelation method, by a small volume (15 mL) and large volume (600 mL) synthesis scale. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.	70
Table 2. 10: Summary of various size range synthesised by different material and pH via citrate chelation method.....	75
Supplementary Table 2. 1: Effect of different molar ratios of calcium: citric acid: IDA on CaP NPs. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.....	78
Supplementary Table 2. 2: Effect of different molar ratios of calcium: citric acid: L- glutamic acid on CaP NPs. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.....	79

Supplementary Table 2. 3: Comparison of DLS and SEM images on CaP NPs by citrate chelation method at drifting pH 8.5. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.....	80
Table 3. 1: Characterisation of OVA-loaded CaP NPs at different loading methods. Data are expressed as mean $\pm$ SD of at least 3 biological replicates; error bars show the SDs...	91
Table 3. 2: Influence of different concentration of amino acids on CaP NPs size and zeta potential. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.....	93
Table 3. 3: Effect of different nanoparticle dilution factor on CaP NPs size and zeta potential. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.....	94
Table 3. 4: Effect of various incubation time on particle size and zeta potential. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.	94
Table 3. 5: Effect of different molecular weight of PEI on CaP NPs size and zeta potential. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.....	96
Table 3. 6: The size stability of CaP-PEI-OVA NPs in different media for 6 h. Data are expressed as mean $\pm$ SD of at least 3 biological replicates; $\pm$ indicates the SDs.	101
Table 3. 7: Different cross-linkers and their structures.	103
Table 3. 8: Characterisation of the different molar ratio of cross-linkers to OVA. Data are expressed as mean $\pm$ SD of at least 3 biological replicates; $\pm$ indicates the SDs. ..	105
Table 3. 9: Determination of the optimal concentration of conjugation buffer for cross-linking OVA on nanoparticles.....	107
Table 3. 10: Identification of cross-linked peptides by mass spectrometry.	108
Table 3. 11: Zeta potential of cross-linked and non-cross-linked CaP-PEI-OVA NPs in various media. Data are expressed as mean $\pm$ SD of at least 3 biological replicates; $\pm$ indicates the SDs.	110
Table 3. 12: Characterisation of different sizes of cross-linked CaP-PEI-OVA NPs. Data are expressed as mean $\pm$ SD of at least 3 biological replicates; $\pm$ indicates the SDs. ..	115
Supplementary Table 3. 1: Identification of bound protein on the nanoparticle surface by mass spectrometry. The protein corona comes from non-cross-linked NPs incubated with cell culture media for 6 h at room temperature.....	122
Supplementary Table 3. 2: Identification of bound protein on the nanoparticle surface by mass spectrometry. The protein corona comes from non-cross-linked NPs incubated with SBF buffer with 55% human serum for 6 h at room temperature.	123

Supplementary Table 3. 3: Identification of bound protein on the nanoparticle surface by mass spectrometry. The protein corona comes from cross-linked NPs incubated with SBF buffer with 55% human serum for 6 h at room temperature.	125
Table 4. 1: Physical characterisation of different sizes of cross-linked CaP-OVA NPs..	136
Table 5. 1: Properties of palmitoyl functionalisation of CaP-PEI-OVA NPs.....	176

Abbreviations

AAS	Atomic absorption spectrophotometry
ACP	Amorphous calcium phosphate
Arg	Arginine
APC	Antigen presenting cell
ASC	Apoptosis-associated
Au NPs	Gold nanoparticles
BCR	B cell receptor
BMDCs	Bone marrow dendritic cells
BPE	Bovine pituitary extract
BSA	Bovine serum albumin
BS ³	Bis(sulfosuccinimidyl)suberate
CaP NPs	Calcium phosphate nanoparticles
CD	Circular dichroism
CFA	Complete Freund's adjuvant
CpG	Cytosine and guanine triphosphate motifs
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DIPEA	N,N-Diisopropylethylamine
DLS	Dynamic light scattering
DMTMM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DTNB	Dithionitrobenzoic acid
DTNP	2,2'-Dithiobis(5-nitropyridine)
DTT	Dithiothreitol

DMF	Dimethylformamide
EDTA	Ethylenediaminetetraacetic acid
EDS	Electron dispersion spectroscopy microanalysis
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ESI-MS	Electrospray mass spectrometry
FACS	Flow cytometry staining buffer
FasL	Fas ligand
FBS	Fetal bovine serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
FLH	<i>Fissurella latimarginata</i>
FTIR	Fourier transform infra-red spectroscopy
GA	Glutaraldehyde
HA	Hemagglutinin
HAP	Hydroxyapatite
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBV	Hepatitis B virus
HBs Ag	Hepatitis B surface antigen
HEL	Hen egg lysozyme
HEK	Human embryonic kidney
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HEV-71	Human enterovirus-71 virus
H4IIE	Rat hepatoma cell lines
HIM	Helium ion microscopy
HPV	Human papillomavirus
HSV-2	Herpes simplex virus type 2

IDA	Iminodiacetic acid
IL	Interluekin
IFA	Incomplete Freund's adjuvant
IFN	Interferon
Ig	Immunoglobulin
IV	Intravenous
IM	Intramuscular
JNK	c-Jun N-terminal kinase
KLH	Keyhole limpet hemocyanin
KIF	Kinesin family member
LbL	Layer-by-layer
LCP	Lipid-calcium-phosphate
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
Lys	Lysine
MCP	Monocyte chemoattractant protein
MCPM	Monocalcium phosphate monohydrate
MFI	Mean fluoresence intensity
MHC	Major histocompatibility complex
MSNs	Mesoporous silica nanoparticles
MW	Molecular weight
NDV	Newcastle disease viruses
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	Succinimidyl ester
NLR	NOD-like receptor
NK	natural killer
OKF6	Oral epithelial cells
OVA	Ovalbumin

OSCC	Oral squamous cell carcinoma
PAMAM	Poly(amidoamine)
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PBM	Peripheral blood monocytes
PBMC	Peripheral blood mononucleated cell
PDI	Polydispersity index
PEI	Polyethylenimine
PEG	Poly (ethylene glycol)
PI	Isoelectric point
PLA	Poly-dl-lactide
PLGA	Poly(lactic-co-glycolic acid)
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
RES	Reticuloendothelial system
RHS	Reticulohistocytic system
RNA	Ribonucleic acid
R-PE	R-phycoerythrin
RP-HPLC	Reversed-phase high-performance liquid chromatography
RT	Room temperature
SBF	Simulated body fluid
SC	Subcutaneous
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEAP	Secreted embryonic alkaline phosphatase
SEM	Scanning electron microscopy
TAA	Tumour-associated antigen
TCP	Tricalcium phosphate
TCR	T cell receptor

TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
TGF- β	Transforming growth factor- β (TGF- β) siRNA
TIPS	Triisopropylsilane
Th	T helper cell
TGF- β	Transforming growth factor-beta
TRAIL	TNF-related apoptosis-inducing ligand
TMT	Tumour metastasis targeting
TNF- α	Tumor necrosis factor-alpha
TLR	Toll-like receptor
V/V	Volume per volume
XRD	X-ray diffraction

Chapter 1

General Introduction

1.1 Nanotechnology in vaccine delivery system

The development of vaccines was one of the most promising and cost-effective ways to protect people from infectious diseases and cancers, leading to the elimination of many diseases [1-3]. Since Edward Jenner discovered the first vaccine (derived from the *Orthopoxvirus* cowpox species) to trigger protective immune responses against smallpox (*Vaccinia virus*) in 1796, significant progress has been made to save millions of lives [4]. Additionally, vaccination has shown tremendous potential in the field of cancer immunotherapy through stimulating the immune system [5]. There are two kinds of cancer vaccines; prophylactic (preventive) and therapeutic (treatment). The former induces a humoral immune response to protect patients from tumour development, while the latter attempts to treat an existing tumour by eliciting a potent cell-mediated immune response against tumour-associated antigens (TAA) [6]. Therapeutic cancer vaccines have been highlighted as a promising strategy to treat cancer patients by eliciting a potent adaptive immune response, giving rise to the eradication of cancer cells [6, 7]. These vaccines aim to use the patient's own immune system to recognise and kill tumour cells [6, 8]. Compared with conventional treatment strategies, these approaches have several advantages. These vaccines are designed specifically to kill cancer cells, with minimal harm to normal cells, and could stimulate systemic anti-tumour immunity. Additionally, a long-lasting memory immune response could be induced, providing continuous protection from cancer recurring [7, 9].

Despite the potential of treatment vaccines, most clinical research is still at an early stage, and mainly concentrating on the safety, availability, and immunogenicity of these vaccines. Currently there are only a few therapeutic cancer vaccines that have been approved by the U.S. Food and Drug Administration (FDA) [10-13]. The main drawback of current cancer vaccines is that they are unable to induce a sufficient immune response against the cancer cells [7]. Current research includes virus-modified tumour vaccines, dendritic cell-based vaccines, DNA vaccines, protein vaccines, and peptide-based vaccines, as well as combinations of these strategies [14-16]. Peptide-based vaccines are among the most commonly used strategies in cancer vaccination [17]. Classical vaccines are derived from a variety of sources including live-attenuated, inactivated, subunit, and conjugate vaccines [18]. These vaccines are not ideal for cancer vaccines as they generally do not have the specificity required to distinguish between normal host cells and cancerous host cells

[19]. Peptide-based vaccines, however, are specifically designed to deliver histocompatibility complex (MHC) class I restricted peptide epitopes, (derived from shared TAAs), with the aim to activate cancer-specific CD8⁺ T cells [17]. This specificity has proven peptide-based vaccines as having great potential compared to classical vaccines [20]. However, these methods are limited by the fact that the antigen itself is unable to cross the cell membrane [21], and because peptide antigen can be easily degraded by endogenous proteases. To meet these challenges, nanotechnology can be used to develop techniques that can deliver antigens to the desired sites [22].

In the last two decades the use of nanoparticle-based vaccines has emerged as a very promising strategy in vaccine development [19, 23]. Nanoparticles (NPs) are synthesised solid cores (particles) with a diameter ranging from 1 nm to 1,000 nm, and have been shown to have significant adjuvant effects as vaccine carriers [24-28]. Tumour antigens, targeting ligands, TLR ligands, and small molecule drugs can be loaded on to NPs for therapeutic applications (Figure 1.1) [29]. Because of their relatively small size, NPs can penetrate host-cell walls [30]. Additionally, NPs are easily taken up by antigen-presenting cells (APCs) through endocytosis, and consequently can induce both systemic and mucosal immunity [31]. Antigenic material can be enveloped within NPs in order to protect the antigenic cargo from degradation at the site of vaccination (which may result in only short-lived, localised immune response), allowing the antigen to be delivered to immune cells such as APCs and subsequently leading to a long-term immune response [28]. Various types of NPs have been used for vaccination (Figure 1.2), and the most widely studied include polymeric nanoparticles, lipid nanoparticles, inorganic nanoparticles (calcium phosphate, silicate, gold), and their applications in immunotherapy have been widely reviewed in the published literature [32-38].

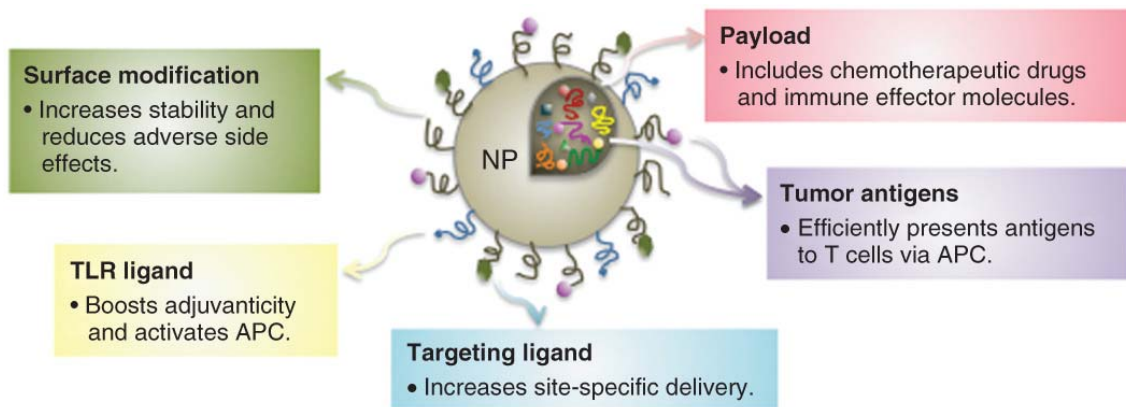


Figure 1. 1: The advantages of NP used as platform for vaccine applications.

Targeting ligands, TLR ligands, tumour antigen, and immune effector molecules can be load on to NPs for therapeutic applications. NP, nanoparticle; APC, antigen-presenting cells; TLR, toll-like receptor. Adapted with permission from ref [29]. Copyright (2014) Wiley Interdiscip Rev Nanomed Nanobiotechnol.

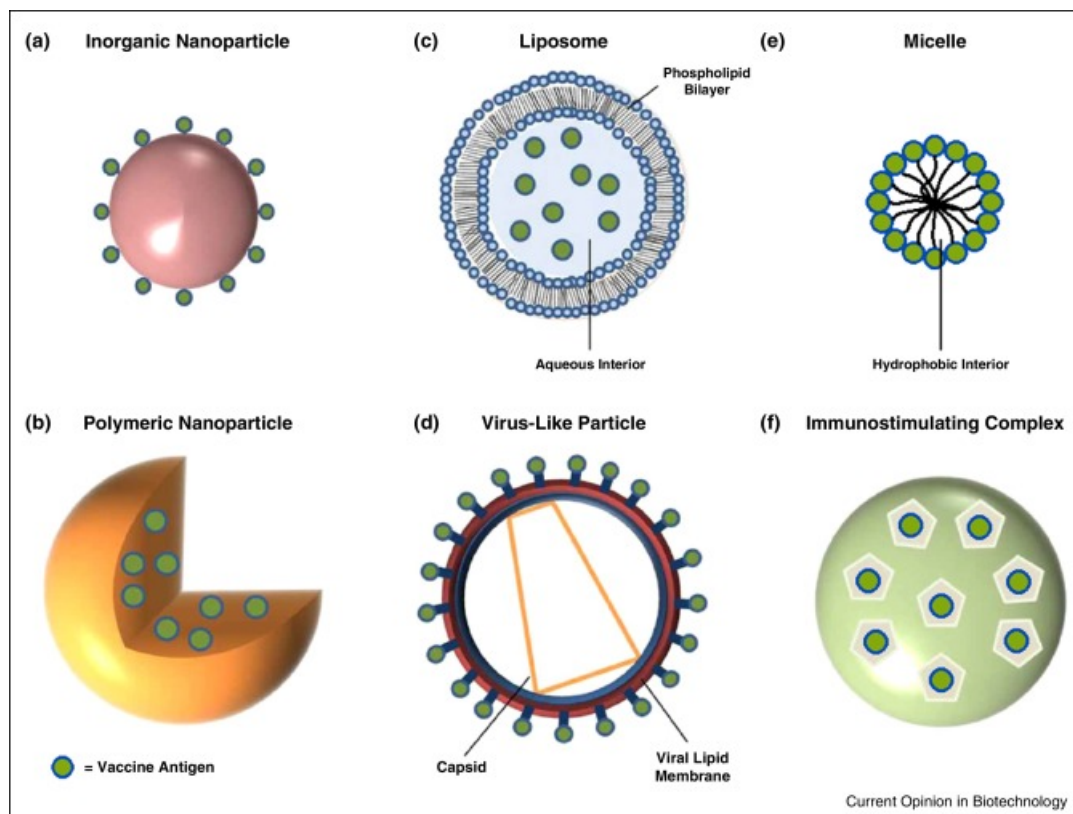


Figure 1. 2: Different nanoparticle-based vaccine delivery systems.

(a) inorganic NPs, (b) polymetric NPs, (c) liposome, (d) virus-like NPs, (e) micelle and (f) immunostimulating complex. Adapted with permission from ref [25]. Copyright (2013) Frontiers In Cellular And Infection Microbiology.

1.1.1 Polymeric nanoparticles

Polymeric NPs are attractive as vaccine carrier platforms due to their adjuvant qualities. Generally, they are biodegradable, water-soluble, non-toxic, and relatively economical to

produce [28, 39]. Additionally, cationic polymers could produce more compounds that are more stable, enabling them to withstand cellular trafficking [28]. Both synthetic and natural polymers can be used to form polymeric NPs, such as chitosan, poly (lactic-co-glycolic acid) (PLGA), poly (lactic acid) (PLA), poly(D,L-lactide-co-glycolide) (PLG) and polysaccharides [40-42]. These particles could deliver antigens to specific cells for sustained antigen release [43]. The antigen loading capacity and the speed of polymer biodegradation can be controlled by changing NP properties such as shape, size, charge, hydrophobicity, polymer composition and concentration [44]. Polymeric NPs are able to enhance the vaccine efficacy as vaccine adjuvants against cancers and infectious diseases and have been investigated for various biomedical applications [24, 45, 46].

Compared with other polymeric NPs, PLGA NPs are the most extensively investigated NPs as vaccine carriers largely because they have previously been approved by FDA and licensed for human use in medical applications such as bone implants and sutures [44, 47]. It has been reported that PLGA NPs loaded with indocyanine green (ICG) and a toll-like-receptor-7 agonist combined with photothermal therapy can elicit a greater anti-tumour immunity compared to traditional adjuvants [48]. In a comparative research report of cationic liposomes and PLGA NPs it was shown that when compared with Montanide ISA 51- and squalene-based emulsions, synthetic long peptide-loaded cationic liposomes and PLGA NPs were induced greater production of T cells *in vivo*, demonstrating that these two PLGA delivery systems are strong candidates for cancer immunotherapy [49]. In another study, Zuo and co-workers developed a tumour vaccine that consisted of Dermatophagoides protein 1 (Der p1) encapsulated in PLGA NPs that was able to notably inhibit the growth of Lewis lung cancer cells in a mouse model by activating the generation of a Th1 cytokine (IFN- γ and IL-4) [50]. Similarly, in a mouse model of disease (diphtheria and tetanus) when the antigens diphtheria and tetanus toxoids (DTaP) were adsorbed into PLGA NPs and co-delivered with TLR7 ligand, there was enhanced production of IgG and IgG2a antibodies, suggesting that PLG NPs is a potent adjuvant for vaccine formulation [51]. Several modifications of PLGA NPs have been explored in order to improve the effectiveness of NPs as a vaccine. When mannose-functionalised PLGA NPs designed to target melanoma cancer it was found that PLGA NPs with a diameter of 150 nm encapsulating MHC class I- or class II-restricted melanoma antigens and toll-like receptor (TLR) ligands (Poly (I:C) and CpG) elicited the highest tumour growth delay [52]. When Hu *et al.* conjugated higher concentrations of cholesterol to

PLGA NPs it was demonstrated that the vaccine had better-controlled antigen release, higher uptake by dendritic cells, and increased antigen stability than lower concentrations of cholesterol [53]. Despite these advantages, PLGA NPs have some considerable limitations. PLGA NPs vaccine has a short half-life as they are often degrade rapidly, thus losing immunogenicity and effectiveness. Immunogenicity loss during storage has adverse effects on the encapsulated protein antigen that makes the vaccine less effective [28]. The vaccine may require more boosting injections because the boosted immune responses are shorter without long-term protection. Future efforts should focus on these disadvantages to optimise PLGA NPs [24, 28].

Another polymer that has been used in the development of NP vaccine delivery systems is PLA. Similar to PLGA nanoparticles, PLA NPs are biodegradable, non-toxic, and biocompatible, and PLA has also been approved by the US FDA for use in biomedical applications [47]. Research has indicated that PLA NPs could significantly improve vaccine efficacy [54, 55]. Pavot *et al.* synthesised PLA NPs (200 nm) containing Gag p24 HIV-1 antigen, carrying pattern recognition receptor (PRR) domains (Nod)-like receptors 1 and 2. Results showed that PLA NPs were effectively taken up by dendritic cells (DCs) and that there was increased production of pro-inflammatory cytokines (IL-6, IL-1 β , TNF α , IFN γ and IFN α). Furthermore, as compared to Alum [56] (a commonly used vaccine adjuvant derived from aluminium salts), the NPs gave rise to a 100-fold increase of the antibody response [54]. Other researchers have used cationic polymer (including chitosan, chitosan chloride, and polyethylenimine) coated PLA microspheres with conjugated viral Hepatitis B antigen (HBsAg) to elicit robust humoral and cell-mediated immune responses. HBsAg adsorbed on PLA microspheres significantly increased antigen uptake and the expression of CD86, MHC I, and MHC II and IL-1 β , IL-6, TNF- α , and IL-12 production in macrophages [55]. Interestingly, the same group also determined that the route of vaccine administration has an influence on the efficacy of PLA NP vaccines; intramuscular administration was found to induce both a stronger humoral and cell-mediated immune response than that which followed subcutaneous vaccination [57]. Although PLA NPs showed the potential of acting as an efficient vaccine adjuvant, it has been shown that they are susceptible to deterioration under conditions such as excessive heat, sonication, in the presence of organic solvents, and freezing, which may lead to serious aggregation or degradation of antigens [47]. In addition, the acidic monomers produced by the degradation of polymers can result in degradation of the tertiary NP

structure. PLA NPs can be strengthened through adding stabilised chemicals and surfactants or by optimising synthesis methods [47]. The greater limitations of PLA NPs as vaccine candidates include low encapsulation efficiency and insufficient drug loading capacity, which need to be addressed for PLA NPs to be used in the near future [58].

Chitosan is a natural cationic polymer that is derived from chitin [59]. It is inexpensive, easy to manufacture, of biological origin, with high biocompatibility and biodegradation. These properties have facilitated the development of chitosan as a vaccine carrier over the last decade [60]. In addition, chitosan has proven to be suitable for mucosal vaccine delivery and can improve mucosal immune response [46]. Zhao and co-workers designed a chitosan loaded NP vaccine that incorporated the Newcastle disease viruses (NDV) through an ionic cross-linking strategy. They demonstrated that chitosan NPs were safe and inexpensive, compared with commercially attenuated NDV vaccine, as well as exhibiting better and quicker cellular immunity (increased IFN- γ production), humoral immunity (increased IgG production) and mucosal-immunity (increased IgA production). The production of IgA, which is a major immunoglobulin secreted by B lymphocytes, occurs primarily in mucosa-associated lymphoid tissue, and the increased IgA production following chitosan exposure indicates that chitosan can be effective as a mucosal vaccine adjuvant [61]. However, one of the major drawbacks of chitosan is that it has weak solubility in aqueous solutions, being only soluble in acidic organic solvents ($\text{pH} < 6.5$), which limits its application in medical research [60]. Chitosan has not yet been used in human studies as an adjuvant, nor has it been approved for human use, and currently there are no commercialised chitosan products on the market [62].

1.1.2 Lipid nanoparticles

Lipid carriers have been intensively explored as drug vectors in the last few decades [63, 64]. In recent years research into their potential use as vaccine carriers have markedly increased [65]. Liposomes, which are vesicular structures composed of lipid bilayers and an aqueous inner component, are the most commonly used lipid NPs [66]. Liposomes have many advantages over other vaccine delivery systems as they have good biocompatibility, are able to enclose various agents, have versatility, and plasticity [66]. Lipid NPs have a great deal of flexibility in terms of their synthesis, and this flexibility enables for modification of the lipid composition for a variety of properties such as size, charge,

and to design a liposome that can entrap either a lipophilic component or hydrophilic antigen [44]. A recent study applied lipid NPs as immunomodulatory adjuvants where, combined with a TLR9 agonist, induced significantly CD4⁺ and CD8⁺ T cell responses to Ovalbumin (OVA), suggesting that lipid NPs could act as a potent subunit vaccine carriers [67]. Another study demonstrated that, in a mouse cancer model, modified lipid NPs incorporating a tumour metastasis targeting (TMT) peptide greatly inhibited tumour metastasis progression, and lengthened the survival time of mice, showing the potential for a lipid NP vaccine to prevent tumour metastasis [68]. In order to optimise the characteristics of NPs, researchers have also synthesised lipid-polymer nanoparticles that consist of a polymeric core and a lipid shell. These lipid-polymer nanoparticles enhanced cellular uptake by DCs and protected antigens from elimination during circulation [69]. Despite these advantages, lipid carriers are not stable when they come into contact with serum both *in vitro* and *in vivo*. Upon contact the liposomes can quickly leak encapsulated molecules (such as antigens) before being captured by APCs, thus limiting their use in vaccine delivery [66]. Taken together, lipid NPs have better cellular uptake, and the ability to elicit strong humoral and cell-mediated immune response [70]. For the successful application of lipid NPs in vaccine delivery, further studies on solving their instability at physiological conditions need to be performed.

1.1.3 Gold nanoparticles

Gold nanoparticles (Au NPs) are also suggested as vaccine candidates as their surface can be highly modified with diverse ligands, they are biocompatible, physiologically stable, their size and shape can be easily controlled, and because they are easy to synthesis [71]. In vaccine studies Au NPs are often conjugated with protein/peptide antigens or carbohydrates in order to determine the interactions between Au NPs and leukocyte cell-membrane receptors [72]. In one study, Au NPs were coated with ovalbumin (OVA) that had been conjugated with a peptide antigen. In a mouse tumour model, the coated Au NPs induced strong antigen-specific immune responses, which resulted in significant anti-tumour activity and prolonged survival time. Notably, this anti-tumour response occurred without the need for additional adjuvants, suggesting Au NPs are competent peptide vaccine delivery systems in vaccination [73]. A recent study has shown that the size and shape of Au NPs can affect the immunogenicity of vaccine compounds. Niikura and co-workers found that 40 nm spherical Au NPs coated with West Nile virus (Au NP-Es)

elicited a high antibody titre, and was twice as effective as rod-shaped Au NP-Es induced in terms of antibody titre [74]. It has also been shown that different Au NPs shapes can activate different cytokine pathways; rod-shaped Au NPs significant induced production of interleukin-1 β (IL-1 β) and interleukin-18 (IL-18), while spherical Au NPs and Cube-shaped Au NPs both greatly promoted inflammatory cytokine production (TNF- α , IL-6, IL-12) [72]. However, there are several disadvantages that limit the potential use of Au NPs as a cancer therapy. They are non-porous and non-biodegradable, and thus they are not suitable for the time-release of small molecules [75]. In addition, although the Au NPs are considered safe, the repeated use of Au NPs may result in bioaccumulation, which might have long term effects that are yet to be determined [24]. More knowledge concerning Au NPs adjuvant efficacy is also required because Au NPs are attractive vaccine carriers.

1.1.4 Silica nanoparticles

Other inorganic nanoparticles such as silica have also been considered for vaccine carrier development [76]. Despite polymeric and lipid NPs showing significant promise as vaccine delivery systems, these nanoparticles are not stable and are easily degraded during interstitial transit [32]. After the polymer matrix has been hydrolysed, the encapsulated or adsorbed molecules (such as antigens or drugs) is released within hours of administration [32]. This is one of the major obstacles to vaccine development. For this reason silica NPs have drawn considerable attention in recent years as a possible solution to antigen leakage and NP instability [76]. In addition, it is relatively easy to control the size, shape, and structure of silica NPs. Silica also has excellent chemical stability, biocompatibility, and can be easily modified through surface functionalization. These characteristics make silica NPs highly promising vehicles for protein, gene and drug delivery [76]. Currently, various studies have applied silica NPs in biomedical research, there are lots of publications that describe the use of silica NPs as antigen delivery systems [77-80]. Of these reports it has been reported that silica NPs could reinforce the immune response as a vaccine adjuvant and as delivery vehicles [81]. It has been recently reported that smaller-sized silica NPs (30 nm) show a greater adjuvant influence and stronger T helper (Th)1, Th2, and Th17 immunity than larger-sized silica NPs (100, and 1,000 nm) [81]. It has also been reported that silica NPs can enhance mucosal and systemic immunity, and can act as a carrier for the oral vaccine to prevent many infectious diseases (such as hepatitis

and influenza) [82]. As a result, silica NPs are attractive as vaccine carriers, and more information concerning their adjuvant efficacy is required.

In addition, there still have many other types of nanoparticles such as carbon, VLPs (virus like particles), immunostimulating complex and dendrimers. Many literatures have summarised their characteristics and demonstrated that they have the ability to induce antibody and cytokine responses [25, 83-85]. In summary, when nanoparticles are used as vaccine delivery system are able to enhance vaccine efficacy against infectious disease and cancers [33, 84, 86]. However, there still remains some potential problems that require further research to overcome. First, the instability of biomaterials at physiological conditions is a concern for some medical applications [87]. Additionally, the potential of adverse local inflammatory reactions induced by nanoparticle vaccines is a potential issue for some materials that needs to be further investigated. Additionally, the biodegradability and solubility of the materials can be a major problem for some nanomaterials [25] Consequently, further research is needed covering the general issues as well as issues for specific nanomaterials in the development of effective and safe nanoparticle-based materials for vaccine applications.

1.2 The immune system and vaccination

The immune system is composed of the innate and the adaptive immune response (Figure 1.3) [88]. Vaccination aims to stimulate the adaptive immune response for effective and long-term immunogenicity [33]. For this to occur, vaccines need to be initially recognised by the host defence system that triggers an immune response [25] (Figure 1.4).

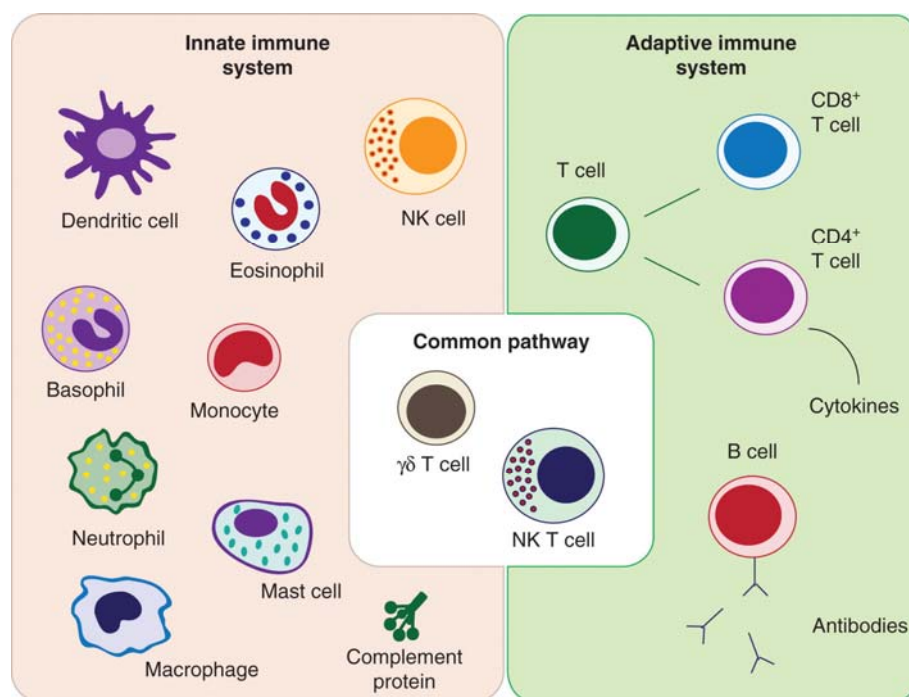


Figure 1. 3: The components of the immune system.

The immune system is composed of the innate and the adaptive immune response. The innate immunity is the first line of defence that fights against any pathogen in a nonspecific manner. After infection by a pathogen (bacteria, viruses and fungi), the innate immune system would activate quickly within a few hours. The adaptive immune system takes several days to provide long-lasting protection. The adaptive immunity is composed of humoral immunity and cell-mediated immunity. Macrophages and dendritic cells are antigen-presenting cells that play an important in bridging innate and adaptive immune responses. Adapted with permission from ref [89]. Copyright (2015) Expert Opin Drug Deliv.

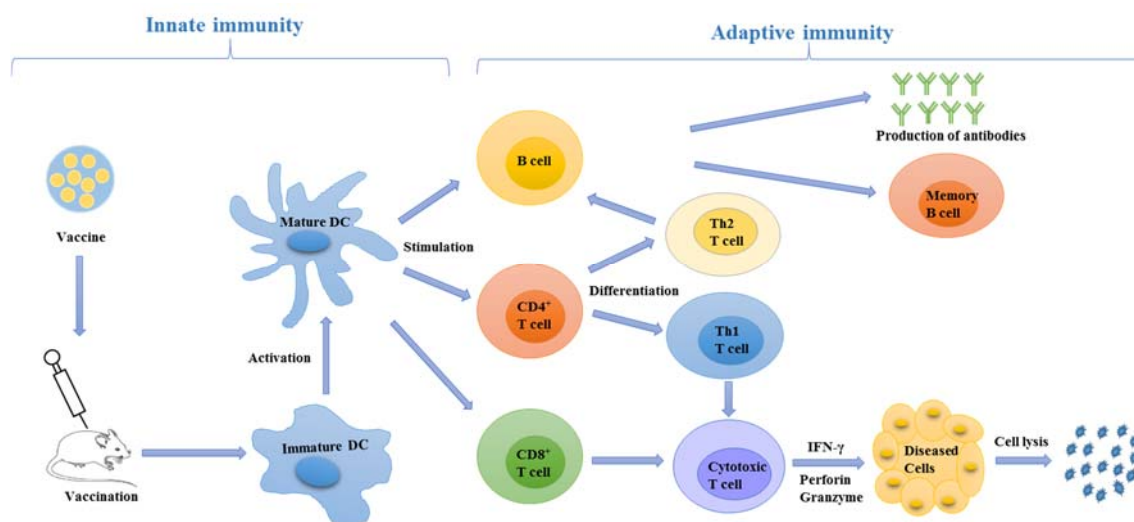


Figure 1. 4: The desired immune response following vaccination.

The aim of vaccination is to stimulate the body's immune system to cure infections and diseases and prevent them from spreading. Immunity are composed of innate and adaptive immunity. The former is regarded as the first line that fights against any pathogen and nonspecific. In the meantime, its function is closely associated with adaptive immunity. Because DC are the component of the innate immune system, which uptake and present antigen to B and T cells, leading to stimulation and activation of humoral and cellular immune responses, respectively. Humoral immunity is the aspect of immunity that secrete antibodies to the foreign antigens, while cell-mediated immunity includes CD4⁺ and CD8⁺ T cells. CD4⁺ T cells could differentiate into Th1 cells (drive cellular immunity) and Th2 cells (support humoral immunity). CD8⁺ T cells could

differentiate into cytotoxic T cells, which are able to directly kill cancer and infected cells. DC: dendritic cell; Th: T helper cell; IFN- γ : Interferon-gamma.

1.2.1 Innate immunity

The innate immune response is regarded as the first line of defence that, when triggered, responds to pathogens in a nonspecific manner [90]. After damage caused by a pathogen (bacteria, viruses and fungi), the innate immune system activates within a few minutes [91]. These responses to invading pathogens are mediated by cellular effectors (e.g. macrophages, neutrophils, DCs, and natural killer (NK) cells) and other soluble factors (such as the complement cascade proteins) [90]. Macrophages are phagocytic cells that can both circulate or reside in tissues. They play a central role in presenting antigens, mediating inflammatory processes by clearing pathogens and apoptotic cells [90, 92]. Macrophages encountering foreign antigens in the tissues are primed to secrete cytokines and chemokines that mediate the activation of adaptive immune responses [93]. This process, inflammation, plays a crucial role in the elimination of pathogens [94]. APCs and mucosal/oral epithelial cells express PRRs, such as Toll-like receptors (TLRs) [95], which are involved in inducing and enhancing the innate and adaptive immune responses [95, 96]. TLRs are a group of cell surface or intracellular transmembrane proteins that recognise microbial components called pathogen-associated molecular patterns (PAMPs) to initiate an immune response [97]. They are able to identify microbial components from viruses, bacteria, and fungi [98]. TLRs are known to facilitate the recognition of pathogens by immune cells, resulting in the increased expression of pro-inflammatory cytokines, chemokines and co-stimulatory molecules such as CD80, 86 and 40, leading to the production of robust innate immunity [95]. Innate immunity makes a significant contribution to induce adaptive immune response activation [94].

1.2.2 Adaptive immunity

The adaptive immune system takes several days to provide long-lasting protection [88]. Adaptive immunity is composed of humoral immunity and cell-mediated immunity, both of which is essential for the complete elimination of pathogens [88, 99]. Humoral immunity is the aspect of the immune system that secretes antibodies to the foreign antigens [100]. B lymphocytes produce antibodies such as IgA, IgG, IgE, IgM and IgD and provide

protection against extracellular antigens [101]. The cell-mediated immunity mainly includes $CD4^+$ and $CD8^+$ T cells, which are activated by APCs. $CD4^+$ T cells can differentiate into Th1 cells (drive cellular immunity) and Th2 cells (support humoral immunity) [102]. Th1 cells play a significant role in the elimination of intracellular pathogens and are involved in organ-specific autoimmunity, while Th2 cells trigger an immune response to extracellular parasites and associate with the induction and persistence of asthma and other allergic diseases [103]. $CD8^+$ T cells play a significant role in combatting cancer and intracellular infections and are able to directly kill cancer and infected cells [100].

1.2.3 Antigen-presenting cells (APCs)

Antigen-presenting cells (APCs) are a component of the innate immune system that uptake and present antigen to B and T cells, leading in turn to stimulation and activation of humoral and cellular immune responses, respectively [104]. DCs, B cells and macrophages can all act as APCs. The most robust and professional APCs are DCs, which play a crucial role in both the innate and adaptive immunity. DCs serve as a modulator between innate and adaptive immunity, triggering T cells activation and differentiation [93]. Through the PRR, DCs can recognise a variety of antigens, which induces DCs maturation and proliferation [95]. Moreover, antigens can be captured and processed by DCs and presented to naïve T cells as peptides bound on the MHC, which then stimulates a T cell response [105].

1.2.4 T cells ($CD4^+$ and $CD8^+$ T lymphocytes)

1.2.4.1 T cell development and differentiation

The activation and maturation of $CD4^+$ and $CD8^+$ T lymphocytes is influenced by several factors, including epigenetic modification, interactions between cell to cell, intracellular transcription factors, signals by intracellular cytokines, a family of E26 transformation proteins and the level of receptor expression [106-108].

T cells derive from bone marrow and the thymus [103, 108], and go through a series of selective maturation steps, including positive (ensures T cell could induce potent immunity to foreign antigens) and negative pressures (to protect against T cell self-tolerance)

that lead to single-positive thymocytes, which express CD4 or CD8. The positive selection determines if a T cell will become a CD4⁺ T cell or a CD8⁺ T cell. These T cells remain in the thymus medulla during maturation before being released to localise in peripheral lymphoid organs [103, 106, 108, 109].

Under the stimulation of several cytokines such as IL-2, 4, 6, 12, 21, 23, TGF- β and IFN- γ , the naïve CD4⁺ T cells further differentiate into various subtypes of T lymphocytes, including the helper T-helper (Th) 1, 2, 9, 17 cells and the regulatory T cell (Treg) [103]. These populations play a pivotal role in coordinating and regulating immune responses and can enhance the induction of cytotoxic T lymphocytes (CTL) responses and increase the generation of antibodies. For example, IFN- γ secreted by Th1 cells can initiate cellular immune responses and increase IgG2 antibody generation [103, 108]. Th2 cells can produce IL-4, 5, 10, which in turn mediate B cells activation and enhance the generation of IgE and IgG1 antibodies [100]. In the presence of IL-12, 18 and IFN- γ , naïve CD8⁺ T cells could differentiate into CTLs, which contribute to protecting the body from infectious disease progression and cancer [108, 110]. While the majority of CTLs are degraded after infection resolution a small population will further progress into effector memory CD8⁺ T cells, which have a central role in the function of immune memory [106].

1.2.4.2 Antigen recognition by CD4⁺ and CD8⁺ T cells

APCs mature during their migration to secondary lymphoid organs, these cells are then capable of activating naïve T cells (CD4⁺ and CD8⁺) by present antigens on their surface as peptide/MHC-class I/II complexes [111]. Antigen, T cell receptor (TCR) interaction, and co-stimulatory signals are essential for naïve T cells stimulation [103, 108]. The co-stimulatory signals released by CD28 on T cells bind to the CD80/86 on DCs. This results in naïve T cells proliferation and differentiation into effector cells [103, 108]. Extracellular antigens are usually presented by MHC-class II molecules on DCs, which can then lead to CD4⁺ T cells activation [103]. Cytosolic antigens presented by MHC-class I molecules on DC give rise to the activation of CD8⁺ T cells (cytotoxic T cells). Additionally, antigens released after lysis of infected cells can be captured by bystander DCs and presented to MHC-class I molecules to CD8 T cells, which is termed antigen cross-presentation, helps to induce cell-mediated immune responses, which is vital to generate an effective CTL-based vaccine [112, 113].

1.2.4.3 Induction of effector cytotoxic T cell responses

The induction of a cytotoxic lymphocyte (CTL) response is essential for the eradication of cancers as well as viral and some bacterial infections [110]. A CTL response can be divided into 4 phases, which includes effector, contraction, immunological memory and a quick recall response [114]. DCs present antigen in the context of MHC class I to CD8⁺ T cells, which is crucial for the activation of naïve and memory CD8⁺ T cells [115]. There are three signals for the CD8⁺ T cell to develop an optimal CTL response during this process. The first signal is elicited by the TCR/peptide-MHC class I interactions. The second signal comes from several co-stimulatory receptors/ligands, which are expressed by the activated DC and CD8⁺ T cell. The third signal is delivered via IL-12 or type I interferons (IFN) or through inflammatory signals from TLR ligands, finally leading to the required CTL response [114, 116] (Figure 1.5). It is generally believed that for the induction of effective long-lived CD8⁺ T cells, CD4⁺ T cell help is essential for APCs activation and the resulting production of IL-2 and IFN- γ [117, 118]. Furthermore, CD4⁺ T cells also help CD8⁺ T cells maintain and infiltrate at a tumour site by rendering the tumour environment permissive [118]. As a result, activation of both CD4⁺ and CD8⁺ T cell responses are essential to induce an effective anti-tumour immune response [119].

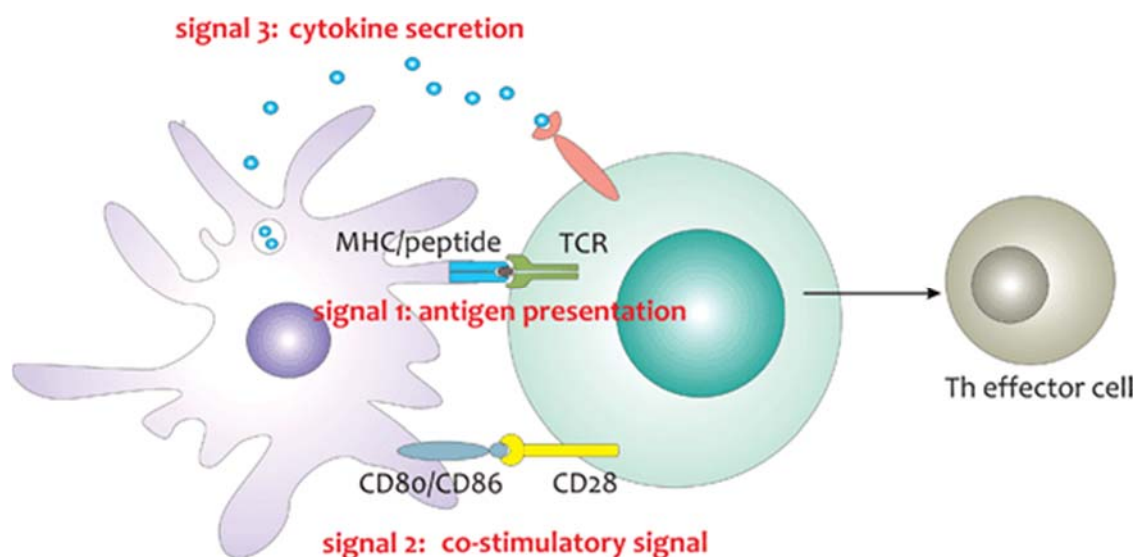


Figure 1. 5: Induction of effector T cell responses needs three signals.

The first signal is elicited by the TCR/peptide-MHC complexes interactions. The second signal comes from co-stimulatory receptors/ligands, which are expressed by the activated DC and T cell. The third signal is the production of inflammatory cytokines. Adapted with permission from ref [116]. Copyright (2011) Chemical Society Reviews.

As previously described, CTLs are a primary immune cell associated with killing cancer cells, giving rise to the eradication of a tumour [110]. The two major mechanisms that are involved in this process are via granule exocytosis (perforin and granzymes), or by the induction by death ligands/death receptor system [120]. Activation of CTLs relies on the interaction of a tumour antigen (processed by APCs and presented as a peptide) bound to an MHC class I molecule with TCR–CD3 complex, along with costimulatory signals (CD28-CD80/86). Once the CTL response is stimulated, granules are quickly secreted by the microtubule-organising centre to the presynaptic membrane [120]. Granules then fuse with the plasma membrane and release perforin and granzymes, resulting in target cell death. For the death ligands/death receptor system, after CTL activation, the expression of death ligands on the CTLs cell surface such as FasL (Fas ligand) or TRAIL (TNF-related apoptosis-inducing ligand) would increase, which can destroy susceptible cancer cells by interaction with death receptors [120, 121].

1.2.5 Vaccine adjuvants, delivery system and TLR ligands

Vaccine adjuvants, delivery systems and TLR ligands are considered three important components for an effective vaccine [122]. Nonliving vaccine antigens, particularly recombinant subunit vaccines, are usually weakly immunogenic [123]. Natural/synthetic molecules such as aluminium mineral salts act as immune-stimulatory adjuvants that induce the desired potent immune responses [124]. An adjuvant is used as a component of the vaccine that can boost the immunogenicity of vaccine antigens [125], while a delivery system ensures optimal delivery of antigen and adjuvant cargo to the desired site or immune cell [19]. Vaccine adjuvant and delivery systems are not mutually exclusive, and can both act to prevent the antigen from degradation while it is carried to the desired site [19]. Appropriate and optimised adjuvants play a central role in vaccine design as they can overcome immunosuppression and help induce a strong immune response [126].

At present, only a few adjuvants (such as mineral salts, aluminium salts (alum), calcium phosphate and MF59, AS03 and AS04) have been widely approved for human use [127]. These adjuvants are effective at inducing humoral immune response but with the exception of calcium phosphate materials have a poor ability to stimulate a cellular immune response, which is critical for viral and tumour eradication [19, 125]. They also with the

exception of calcium phosphate have several drawbacks such as severe local tissue irritation, longer inflammatory response and induction of allergic responses [128]. Consequently, there is an urgent need for a novel delivery vehicle and adjuvant to increase cancer vaccine immunogenicity.

The induction of a robust and specific CTL response by vaccination is still a challenging goal in cancer immunotherapy as cancers cells typically induce immunosuppression of T cells via a number of pathways [110]. A key factor in enhancing the efficacy of a vaccine is to increase its binding to host cells and to deliver the antigen cargo to the endocytic pathway of APCs, thus initiating a strong immune response [33]. Toll-like Receptors (TLRs) are a group of transmembrane protein complexes that recognise microbial components called pathogen-associated molecular patterns (PAMPs) in order to initiate an immune response [97]. The use of TLR agonists as adjuvants has gained increased interest due to their ability to improve the efficacy and immunogenicity of vaccines formulations [39, 45, 51, 129-134]. Pattern recognition receptors (PRRs), such as TLRs, are expressed on APCs (such as dendritic cells and macrophages) and mucosal/oral epithelial cells. PRRs play a significant role in inducing and enhancing both innate and adaptive immune responses [134]. TLRs facilitate the recognition of pathogens by immune cells, resulting in increased antigen uptake and processing, expression of pro-inflammatory cytokines, chemokines, and co-stimulatory molecules such as CD80, 86 and 40, leading to the induction of a robust innate and adaptive immune response [135, 136]. Several different TLR ligands have been investigated for use as vaccine adjuvants, including ligands for TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 [137-139]. The selection of the ligand often depends on the disease being targeted [140]. Figure 1.6 shows different types of TLRs, antigen presentation and the elicited immune response. One of the main challenges in cancer immunotherapy is the difficulty to abrogate the immunosuppressive tumour environment. However, immunosuppression can be overcome by co-immunising an antigen with a TLR ligand, with the benefit of combining mechanisms of up-regulating the production of co-stimulatory molecules, and induction of pro-inflammatory cytokines [141]. Ligation of TLRs 2, 3, 4, 7 and 9 enhances DC activation and antigen phagocytosis, which in turn improves CTL priming [142, 143].

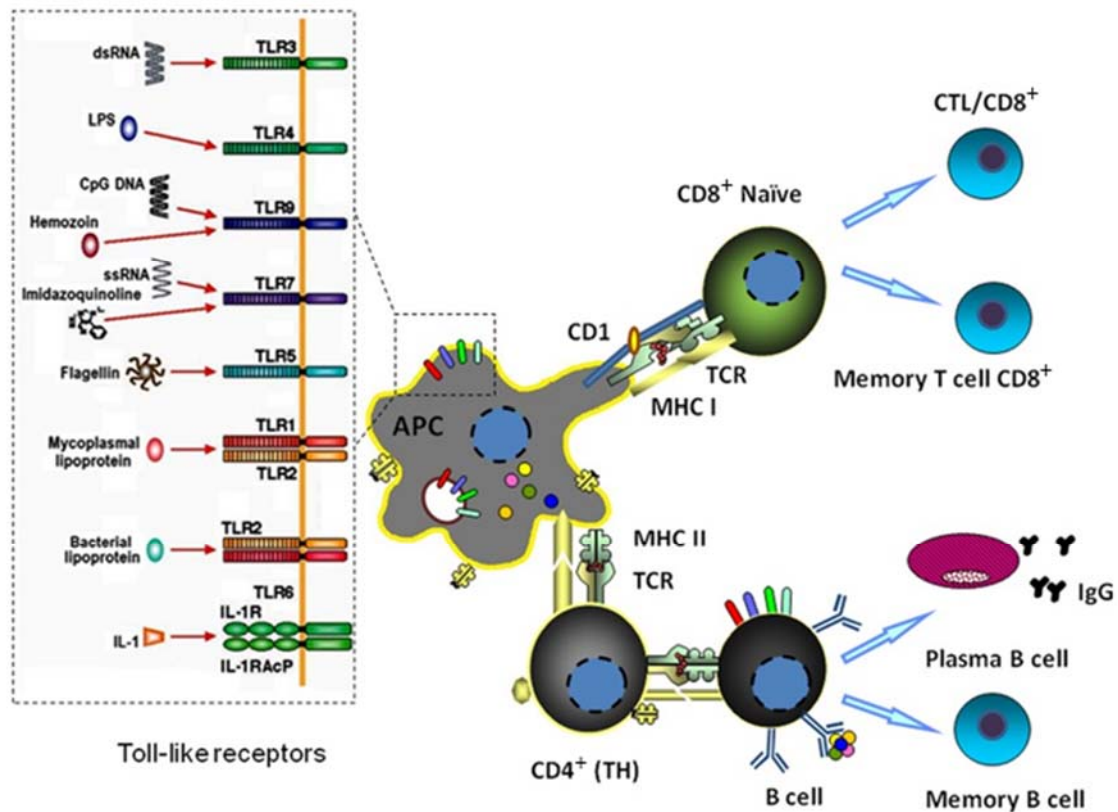


Figure 1. 6: Different types of Toll-like receptors, antigen presentation and the elicited immune response. LPS, lipopolysaccharide; IL-1, interleukin-1; TLR, toll-like receptor; APC, antigen-presenting cell; MHC, major histocompatibility complex; TCR, T cell receptor; CTL, cytotoxic T lymphocyte. Adapted with permission from ref [125]. Copyright (2014) Apmis.

1.2.6 CTL vaccines for oral cancer

Oral cancer, particularly oral squamous cell carcinoma (OSCC), are amongst the most common cancers thought the world [144]. In the United States in 2016, it was estimated that 48,330 new cases of OSCC were be diagnosed, and that approximately 10,000 people died from these diseases [145]. Despite advances in the diagnosis, operative techniques, chemotherapy and radiotherapy, five-year survival rates (roughly 50%) have not improved over the past three decades [146]. Consequently there is a great need for novel therapeutic strategies. Therapeutic cancer vaccines are considered to be promising strategies to improve disease outcome by eliciting a potent adaptive immune response, giving rise to the eradication of cancer cells [6].

Currently, several therapeutic peptide vaccines are under investigation for oral cancer [147-149]. While it is now well established that the high-risk human papillomavirus (HPV) is highly associated with oral cancer, it is widely accepted that HPV 16 is the

major contributor for this cancer [150, 151]. The oncoproteins E6 and E7 are encoded by HPV 16, and thus immunotherapy targeting E6 and E7 may offer an effective way to prevent and treat HPV-related diseases [152]. More recently, Domingos-Pereira *et al.* used a synthesised HPV E7-long-peptide therapeutic vaccine with an encapsulated TLR9 agonist CpG, combined with carboplatin/paclitaxel (C + P) chemotherapy, to target HPV-associated malignancies in a mouse model of disease. It was reported that compared to any of the dual treatments, the vaccine significantly increased mouse survival rate. It was also shown that the addition of CpG greatly leads to increased E7-specific CD8 T cells [147]. Ye *et al.* reported that, in a OSCC model, a HPV-16mE6Delta/mE7/TBhsp70Delta fusion-protein vaccine not only suppressed tumour growth, but also elicited tumour death and provided protection against OSCC [153].

The immunotherapy potential of protein used in combination with adjuvants has been proposed as a new way to treat oral carcinoma. For example, the use of Mollusk Hemocyanins in conjunction with vaccine adjuvants (such as alum, AddaVax, and QS-21) in OSCC has been investigated [148]. Results from this study revealed that the hemocyanins together with QS-21 had high immunogenicity, which was reflected by a potent, specific humoral response (IgG2a antibodies) against OSCC and a sustained cellular response. The keyhole limpet hemocyanin (KLH) and *Fissurella latimarginata* (FLH)-QS-21 formulations have demonstrated decreased tumour development and higher overall survival [148].

Some of these vaccines have previously been used in clinical trials. A survivin-derived peptide vaccine in phase I clinical trial has demonstrated the induction of an effective CTL response, and consequently has therapeutic potential for advanced or recurrent oral cancer patients [149]. Survivin is an inhibitor of apoptosis that reinforces cell cycle progression and enhances angiogenesis. While it is rarely detectable in normal tissues, overexpression of survivin can be found in approximately 80% of OSCC, and consequently, it is often used as a tumour antigen for oral cancer [154, 155]. Recently, the Kinesin family member 11 (KIF11) has been identified as a potential therapeutic target for oral cancer. KIF11 is a motor protein that essential to establish a bipolar spindle in cell division, and high expression of KIF11 has been closely associated with poor prognosis in oral cancer patients [156]. MAGED4B is a melanoma antigen that promotes cell proliferation and migration that overexpresses in OSCC and high expression. Research into MAGED4B

has led to the identification of immunogenic MAGED4B peptides for potential oral cancer vaccine targets. The results demonstrated that the vaccine induced anti-tumour immune responses [157]. These findings demonstrate that peptide-based vaccines are a promising treatment for OSCC patients.

Although there have been recent advances in the development of novel therapies including anti-tumour vaccines, these results to date have not yet been transferred into clinical use and thus have not increased the survival rate of OSCC patients. As a result, further investigations need to be made to establish an effective immunotherapy.

1.3 Calcium phosphate nanoparticles (CaP NPs)

Of the different synthetic antigen delivery carriers currently available, calcium phosphate nanoparticles (CaP NPs) are one of the most promising vaccine transporters and have attracted increasing attention during the past decade [158, 159]. CaP is a natural component of the human body (bone and teeth) [158]. The biomedical research involving CaP increased in the 1970s [158] and CaP have been studied in a number of applications such as DNA/gene silencing [160, 161], drug delivery [162, 163], protein/peptide delivery [30, 31, 132, 164], dental implant and restorations materials [165-167], imaging [168, 169], bone tissue engineering [170, 171] and achieved significant outcomes (Figure 1.7). CaP NPs are non-toxic, biodegradable, cost-effective, and have pH-dependent solubility [159]. Most importantly, they provide protection to the antigen cargo from premature enzymatic and proteolytic degradation [84] and prevent elimination by the body [128, 172]. Further, they can be functionalised with various molecular adjuvants to enhance active cell targeting and consequently the efficacy of vaccines [131, 173-175].

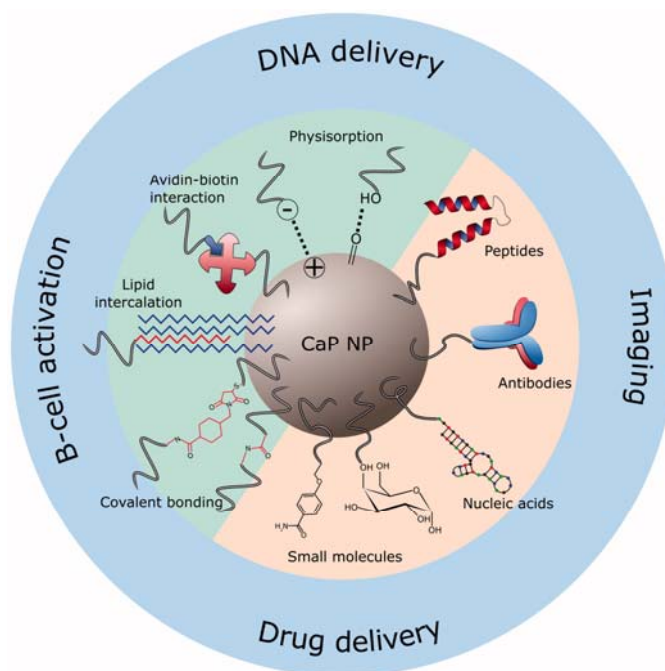


Figure 1. 7: Schematic representation of CaP NPs functions (outer ring, blue), surface functionalisation methods (left sector, green) and moieties (right sector, orange).
Adapted with permission from ref [176]. Copyright (2018) Drug Dev Ind Pharm.

1.3.1 Synthesis of CaP NPs

Current strategies of CaP NPs preparation generate various sizes and shapes. Synthesis methodologies can be divided into the dry method [177, 178] and wet chemical routes [179, 180]. Dry methods mainly includes solid-state [177] and mechanochemical [178] techniques, while wet methods includes chemical precipitation [179, 181, 182], sol-gel [180], microemulsion [183-186], hydrothermal [187, 188], sonochemical [189], hydrolysis [190] and microwave methods [191]. Comparison of main methods for synthesis CaP NPs is summarised in Table 1.1. Various kinds of CaP NPs can be obtained via the aforementioned manufacturing methods, such as particle size (10 nm to 10 μ m), size distribution (from narrow to broad), shape (from spherical to rod-shaped), crystallinities (low to high) and compositions (all CaP NPs forms).

Table 1. 1: Comparison of main methods for synthesis CaP NPs.

Method of Preparation	Synthesis complexity	Synthesis time	Size	Size distribution	Shape	Crystallinity	Cost	Production	Ref.
Solid-state synthesis	Simple	Days	Nano to micron	Very broad	Diverse	Very high	Low	High	[177]
Mechano-chemical method	Simple	Days	Nano to micron	Very broad	Diverse	Very high	Low	High	[178]
Co-precipitation	Very simple	Minutes	Nano to micron	Very broad	Diverse	Usually low	Low	High	[179, 181]
Hydrolysis method	Simple	Days	Nano to micron	Very broad	Diverse	Variable	High	High	[190]
Sol-gel method	Simple	Hours	Nano	Narrow	Diverse	Variable	High	Low	[180]
Hydrothermal	Simple	Days	Nano to micron	Broad	Usually needle-like	High	High	High	[187, 188]
Emulsion	Complex	Days	Nano	Very Narrow	Diverse	Low	High	Low	[183, 184]
Sonochemical method	Simple	Hours	Nano	Narrow	Diverse	Variable	Low	Low	[189]
Microwave-assisted method	Simple	Hours	Nano to micron	Broad	Diverse	Variable	Low	Low	[191]
Combustion	Simple	Minutes	Nano to micron	Very broad	Diverse	Variable	Low	Low	[192]
Pyrolysis	Relatively simple	Hours	Nano to micron	Very broad	Diverse	High	Low	High	[193]

1.3.2 Properties of CaP NPs

1.3.2.1 Safety, biocompatibility and stability

As calcium phosphate is a natural component of the human body, and CaP NPs are reported to have no side effects as a vaccine adjuvant [194-196]. It has also been approved by the FDA, and that they are safe and bioresorbable [197]. A phase I study revealed that

when administered subcutaneously in healthy volunteers, CaP was safe and no toxic effects were found [32]. Viswanathan *et al.* demonstrated that CaP NPs are suitable for biological applications as CaP NPs did not show any significant cytotoxicity on peripheral blood mononucleated cells (PBMCs) by MTT assay [196]. According to one study, CaP NPs elicited nearly no inflammation at the injection site, no local tissue irritation and induced no allergic reaction [195]. CaP is stable both *in vitro* and *in vivo*, especially when the ratio of Ca/P is similar to HAP ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, Ca/P = 1.67), which is a naturally occurring calcium phosphate [198]. Their storage stability is excellent as they are very poor microbial substrates [128].

CaP NPs have excellent biocompatibility compared to other NPs because they mimic naturally occurring calcium phosphate found in the human body such as teeth (enamel, dentine), tendons and bones. They are well-tolerated and would not be considered as foreign materials by the immune system. CaP NPs biocompatibility is associated with the surface charge of the particle. Chen *et al.* reported that positive charged HAP has better biocompatibility to negatively charged HAP on MC3T3-E1 cell lines (osteoblast) based on MTT and LDH assay results [198]. Regarding biodegradability, CaP could be quickly captured by HeLa cells (human epithelial cervical cancer cells) within a few hours [199] and dissolved in lysosomes [200]. As a result, the calcium ions are easily pumped out of the cell [201].

1.3.2.2 CaP NPs pH-dependent solubility

CaP has pH-dependent solubility, and they are stable at human blood pH (7.4) and more alkaline environments but easily dissolved at low pH (< 5.0). A low pH is often found in tumour zones, endosome and lysosome, making CaP more suitable delivery systems and used for human body application [15]. As shown in Figure 1.8, there are four steps involved in CaP NP cargo release. First, CaP NPs are endocytosed by the cell and entered the endosome. Then in the low pH environment of the endosome the CaP NPs dissolve and disassemble. Third, the release of Ca^{2+} and PO_4^{3-} ions from the dissolved NPs give rise to high osmotic pressure, causing the endosome to expand. Finally, the endosome ruptures, releasing the cargo, Ca^{2+} , and PO_4^{3-} ions into the cytoplasm. This mechanism has been used to deliver siRNA to cells [202].

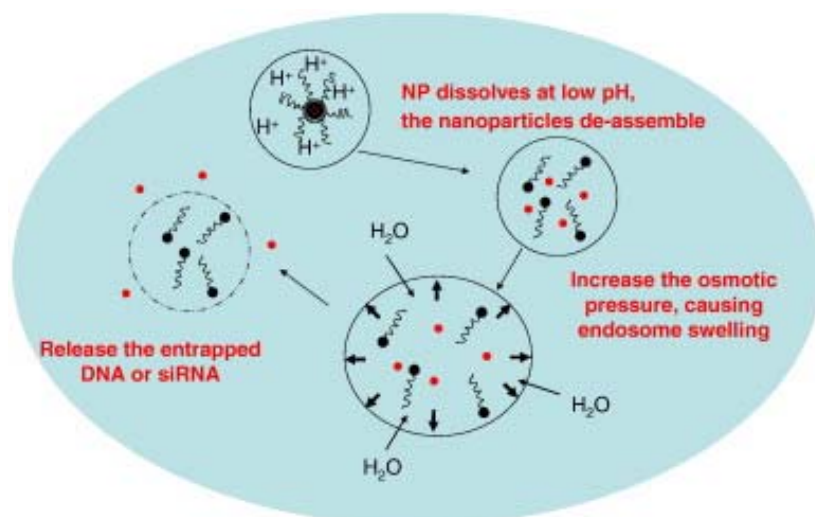


Figure 1. 8: The release process of siRNA entrapped in lipid-coated CaP NPs in the endosome.

There are four steps involved in CaP NP cargo release. First, CaP NPs are endocytosed by the cell and entered the endosome. Then in the low pH environment of the endosome the CaP NPs dissolve and disassemble. Third, the release of Ca^{2+} and PO_4^{3-} ions from the dissolved NPs give rise to high osmotic pressure, causing the endosome to expand. Finally, the endosome ruptures, releasing the cargo, Ca^{2+} , and PO_4^{3-} ions into the cyto-plasm. Adapted with permission from ref [202]. Copyright (2010) J Control Release.

1.3.2.3 Ease of surface modification

It is well known that physicochemical properties such as size, shape and surface charge have a crucial role in the efficacy of vaccines [33]. Additionally, the control of NP size and shape is essential to vaccine formulations. As previously described, numerous synthetic protocols for CaP NPs have been developed to regulate the size and shape precisely [203]. For example, by increasing the number of amino groups on the surface of the Poly(amidoamine) (PAMAM) dendrimers, the size of CaP NPs decreased gradually from 82, 77, 48 to 38 nm [204]. Another example of this is that size and shape could be changed by modifying the pH and reaction temperature [205].

Ease of surface modification makes CaP NPs suitable for vaccine applications, and they can load both hydrophobic and hydrophilic molecules [128]. The surface of HAP possesses positively charged C (Ca^{2+}) and negatively charged P (PO_4^{3-}) sites that provide binding sites for stabilisation as well as bio-conjugation to agents such as polymers [206]. For instance, polyethyleneimine (PEI) and poly (ethylene glycol) (PEG) could be added to the surface of CaP NPs to increase stabilisation in the physiological environment [30, 207]. Furthermore, the addition of agents on the NP surface such as peptide/protein, drugs, DNA/siRNA, or toll-like receptors (TLR) ligands during the manufacturing process enhances the immunological effects required for the vaccine [208]. CaP can also be prepared

in a multi-shell system to protect biomolecules inside from degradation and increase transfection efficiency [164].

1.3.2.4 High adjuvanticity

Compared with conventional adjuvants such as aluminium (alum) salts, CaP NPs have higher adjuvanticity and greater biological affinity to biological materials such as proteins, cells and enzymes [209]. Calcium phosphate materials have been reported to induce a higher immunoglobulin G1 (IgG1) production after the administration of tetanus and pertussis *in vivo* [210]. In comparison with alum, calcium phosphate promoted a higher level of antibody response to Bothrops asper snake venom [211]. CaP NPs was more potent compared to alum to facilitate a high production of IgG2a and neutralising antibody against Epstein-Barr (EBV) infections [195]. In addition, CaP NPs can be an efficient mucosal adjuvant. He *et al.* reported that herpes simplex virus type 2 (HSV-2) vaccine using CaP NPs as an adjuvant significantly elicited potent mucosal and systemic immunity when administered intravaginally and intranasally *in vivo* than Alum [212]. Another study by Saeed showed human Enterovirus-71 vaccine encapsulated CaP NPs enhanced the antibody responses (IgA & IgG) in rabbits [213]. This indicates that CaP NPs has great potential to be used as a vaccine adjuvant [195].

1.3.3 Strategies for antigen loading onto CaP NPs

In order for NPs to act as effective vectors for the delivery of antigens to the desired site, a strong interaction between NPs and the antigen is required. In general, there are four approaches for the attachment of antigen to NPs: adsorption; encapsulation; mix and chemical conjugation (Figure 1.9) [33]. Adsorption and encapsulation are the most common methods used for antigen loaded onto CaP NPs and is normally achieved by charge attraction and hydrophobic interaction. However, the antigen adsorbed onto the surface of NPs in this way is a considerably weak interaction (as compared to other antigen loading methods), which can lead to a burst release of antigen from NPs *in vivo* [214]. Electrostatic attraction between antigen and CaP NPs plays the most important role in surface adsorption. For example, the adsorption of bovine serum albumin (BSA) on HAP arises from electrostatic interaction between Ca^{2+} and PO_4^{3-} anions of HAP with the COO^- and NH_4^+ cations of BSA protein [206, 215-217]. Other examples can be found in ovalbumin (OVA) and lysozyme [218, 219].

Encapsulation and conjugation can be an appropriate alternative to achieve steady antigen release. Encapsulation can be achieved by antigens mixed with chemical reagents and solvents during NP synthesis route, resulting in interaction that is strong, similar to that of chemical conjugation. This method can enhance antigen transfection efficiency as the antigen cannot release until the dissolution of NPs, thus delivering the antigen at the desired site. This is reported by Dasgupta *et al.* where BSA (here used as a model for protein loading) with Ca^{2+} solution during synthesis to achieve controlled release of protein [220]. Similarly, Li *et al.* encapsulated siRNA in PO_4^{3-} aqueous solution before the manufacture of CaP NPs as a systemic siRNA delivery in cancer therapy [221].

Chemical conjugation has also been used to incorporate antigenic molecules such as proteins and peptides that are covalently linked to the surface of NP [222]. Phosphate groups can be conjugated to amine-containing molecules by carbodiimide-mediated reactions [223]. One research group selected CaP binding peptides and inserted these peptides on CaP NPs surface, demonstrating a novel route for vaccine applications [224]. Ramachandran *et al.* modified the surface of CaP NPs through conjugation with PEG for oral insulin delivery, leading to increased stability [207]. Kozlova *et al.* synthesised CaP NPs coated with a silica shell that was covalently functionalized by thiol/amino groups or salinization, leading to stable molecules/NP construct [30]. Antigen-conjugated NPs are a strong candidate for mucosal immunisation as the antigen is protected by the NP cross-linked amino system [102]. CaP NPs act as an immune potentiator so that the vaccine formulations can be achieved by a mixture of antigen and NPs. Conjugation between antigens, adjuvants and NPs may give better immunity in comparison to a mixed solution, further improving the immunogenicity of the vaccine [225, 226]. Hen Egg Lysozyme (HEL) conjugated with CaP as well as TLR 9 ligand elicited a significantly higher humoral immune response than non-conjugated NPs [131]. Many studies have also shown that modified peptides or antibodies chemically conjugated to HAP in bone tissue engineering [227, 228] and drug delivery [229, 230]. For example, Hydroxyapatite encapsulated anticancer drug copper bis(8-hydroxyquinoline) has controlled drug release and 98.67% of encapsulation efficiency [231]. However, the use of NPs as vaccine candidates has attracted little attention in the published literature, indicating that further investigation of the use of NPs as vaccines would be advantageous [232, 233].

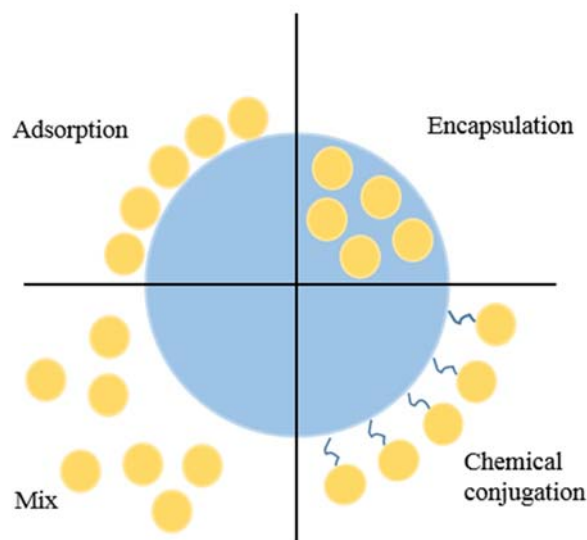


Figure 1. 9: Strategies of antigen interaction with CaP NPs through conjugation, encapsulation, adsorption or simple mixing.

1.3.4 CaP NPs uptake and immunogenicity

1.3.4.1 CaP NPs cellular uptake

The effective uptake of NPs by DCs is crucial to induce a protective immune response. By using CaP NPs as adjuvants and antigen carriers, the efficiency of the delivery antigen to DCs is greatly enhanced compared with antigen alone [212]. As previously stated, the capabilities of NPs uptake is influenced by particle size, shape, surface charge, and composition. Elucidating the effect of these parameters is essential for the development of effective, novel NP vaccines.

It has been widely accepted that the size of NPs plays a critical role in vaccine immunogenicity [234-237]. Foge *et al.* have shown that size is the most critical factor that affects NP uptake [234]. At a size range of 20 to 100 nm, NPs can easily enter lymph vessels [238]. Once localised in the lymph nodes NP-released antigens can directly activate B cells and be effectively phagocytosed by DCs, resulting in the induction of both long-lasting cell-mediated and humoral immunity. Larger NPs (~ 200 nm) can be efficiently taken up by cells (via endocytosis or micropinocytosis) before migration to the lymph nodes and inducing a robust immune response [238]. *In vitro* studies have suggested that NPs of a size below 500 nm are optimal for DC uptake [239]. Researchers have also

reported that compared with micro-sized CaP particles (size range), nano-sized CaP particles elicited higher antibody responses and that they significantly inhibited the generation of human gastric cancer cells [208, 240, 241]. Additionally, nano-sized particles can easily penetrate cells and induce higher immune responses [237]. Similarly, a recent study compared a smaller or larger sized range of CaP NPs, and the size range between 100 to 400 nm could elicit significantly stronger antibody responses [234].

The immunological effects induced by NPs is strongly influenced by their surface charge. It has been reported that positively charged HAP are more readily taken up by cells than those with a negative or neutral charge [198]. This may be due to the negatively-charged cell membrane, and thus the NPs and cell are electrostatically attracted to each other [198]. This study also reported that positively charged HAPs have the strongest enhancement for osteoblast cells (MC3T3-E1) proliferation and viability compared to negatively charged HAPs [198]. In addition to surface charge, particle shape plays a significant role in the stimulation of immune responses [234, 242, 243]. Ramesh *et al.* observed that knife-shaped CaP NPs are more stimulatory than both plate-like and spherical NPs [242]. Rod-shaped HAPs generated greater IL-1 β production *in vitro* than spherical NPs, but induced similar humoral immunity *in vivo* [234]. Cell adhesion and proliferation may also be influenced by the composition of NPs. Hu *et al.* observed that compared with ACPs, more bone marrow mesenchymal stem cells adsorb and proliferate on HAP film and that cellular differentiation was significantly promoted by HAP [243].

1.3.4.2 Targeting the innate immune system

The innate immune system composed of various cells and proteins that play a significant role in the early recognition and subsequent pro-inflammatory immune response, and the role of the innate immune system in promoting adaptive immunity [96]. It is also well established that when the innate immune response is triggered by microbial pathogens it can induce and recruit the cellular components of the adaptive immune system and eventual long-lasting adaptive immunity [244]. Because of these immune mechanisms, the innate immune system is crucial in vaccine formulations. Generally, pattern-recognition receptors (PRRs) on the surface of APCs (particularly DCs) recognise microbial infections, which results in the induction of inflammatory responses by the innate host defences and the eventual induction of adaptive immune responses [245]. In the last decade,

the adjuvant effect of CaP NPs in the innate immune system has been investigated [246]. An India-based research group demonstrated that *Labeo rohita* H. S-layer protein (of *Aeromonas hydrophila*) loaded CaP elicited a robust innate immunity in the fish model via parenteral immunisation [247]. This study included several experiments such as respiratory burst mechanisms and myeloperoxidase activity and found that antigen adsorbed on CaP were significantly higher compared with negative control groups [247]. Matesanz *et al.* reported that BSA loaded nano-sized CaP produced an early stimulation of the innate immune response in mice. It was reported that macrophage proliferation and phagocytic activity were greatly decreased, while the generation of inflammatory cytokines such as IL-6, TNF- α was strongly enhanced [248]. Additionally, Tai *et al.* found that β -tricalcium phosphate (β -TCP) may also have an immunostimulatory effect on macrophages. Mice injected with β -TCP NPs elicited extensive migration of mouse monocyte/macrophage cells to the injection site, and that it significantly enhanced the production of macrophage inflammatory protein 1 alpha (MIP-1 α), suggesting a novel role for β -TCP in vaccine application [249].

As mentioned previously, the innate immune system depends on the recognition of pathogen-associated molecular patterns (PAMPs) by PRRs such as TLRs. TLR activation can elicit inflammatory cytokines production, enhance macrophage phagocytosis activity, and reinforce antigen presentation to induce adaptive immunity [98, 250]. Consequently, the TLR agonists in vaccine development have been widely investigated in preclinical studies [251]. Sokolova *et al.* evaluated the adjuvant effect of CaP when encapsulated TLR ligands CpG and poly (I:C) in a mouse model. They showed that functionalized NPs elicited high levels of the innate inflammatory cytokines IL-12p70 and TNF- α , which resulted in DCs maturation and CD4⁺ T cell activation rapidly [174]. Similarly, other researchers have developed a flagellin-functionalized CaP NPs and studied the immunostimulatory effect on the innate immunity both *in vitro* and *in vivo* in mice [252]. Flagellin is a PAMP that can be recognised by TLR5 on the host cell surface [253]. Compared to free flagellin, flagellin-functionalized CaP NPs produced a high-level production of the proinflammatory cytokines IL-1 β on bone marrow-derived macrophages [246]. These findings suggest that CaP NPs could effectively activate the innate immune system with or without the help of TLR ligands.

1.3.4.3 Targeting the adaptive immune system

1.3.4.3.1 Effects of CaP NPs on humoral immunity

The capability of vaccines to induce an active humoral immunity plays an essential role in the elimination of microbial infections. Some earlier studies have shown that CaP can elicit stronger and longer-lived antibody responses (IgG, IgG1) when compared to traditional adjuvants such as aluminium salts [195, 219, 254]. Behera and Swain reported that administration of CaP NPs loaded with *Labeo rohita* H. S-layer protein elicited high B cell antibody response against viral infection in the fish model [247]. Viswanathan *et al.* developed a Newcastle disease virus (avian avulavirus 1)-conjugated CaP NP vaccine and conducted trials in chickens [197]. They observed that the vaccine induced higher and earlier humoral immunity than live commercial vaccines [196]. A significant humoral immune response was also found in a guinea pig model of disease [255]. In a recent study, Temchura *et al.* developed and tested CaP NPs functionalized with Hen Egg Lysozyme (HEL), which effectively targeted and activated B cells *in vitro* and *in vivo* using BCR-transgenic B-cells from SW-HEL mice as a model. These NPs significantly increased the expression of B cell activation markers and were 100 times more effective in the maturation of B cells than soluble antigen protein [256]. Similar studies have shown that nano-sized CaP coated with an inactivated human enterovirus-71 virus (HEV-71) led to a higher antibody response than the unadsorbed vaccine alone or micro-sized CaP NPs in rabbits via intradermal injection [208]. Moreover, Chua *et al.* developed a novel vaccine that used OVA as a model antigen and chitosan and HAP as the delivery vehicles. After immunisation with a single dose of vaccine, a strong antibody response was induced in mice, which persisted for at least twelve months [257]. Additionally, Zilker *et al.* optimised the administration route of CaP-HEL NPs without additional adjuvants and demonstrated that intramuscular immunisation elicited a higher antibody response in comparison to the monovalent antigen. In this report, the authors also compared different types of TLR ligands for CaP-HEL, and reported that TLR ligands loaded with CaP-HEL gave higher IgG antibody levels than when CaP-HEL was used without TLR ligands. This study also demonstrated that CaP-HEL NPs with various TLR ligands allowed modulating the IgG response, and the production of mucosal IgA antibodies. Such a vaccine could theoretically induce strong humoral immunity without the help of Th2 cells, which are known to influence the differentiation of B cells to generate IgG and IgE [131]. Taken as a whole, these data demonstrate that CaP NPs loaded with antigens and TLR ligands are

promising vaccine candidates for effective induction and modulation of the antibody response.

1.3.4.3.2 Effects of CaP NPs on T cell-mediated immunity

A potent T cell immune response is indispensable to clearing and recovering from infectious disease. There are multiple studies that have demonstrated that CaP NPs are able to activate T cell responses against viral and bacterial infections [173, 258, 259]. A novel vaccine has been developed for use in preclinical trials, and it has been reported that immunisation with malaria antigen (merozoite surface protein-1₁₉, MSP-1₁₉) in HAP could elicit a vigorous cellular response (IL-2 and IFN- γ) in mice [254]. Nanoparticles can be surface-functionalized with antigenic moieties to enhance activation of immune cells. For example, CaP loaded with TLR9 ligand (CpG) as well as the antigen hemagglutinin (HA) could induce DCs maturation and significantly triggered CD4⁺ T cell proliferation [174]. The same group in 2013 showed that immunisation of mice with functionalized CaP NPs can be effectively taken up by DCs as well as inducing high production of CD4⁺ and CD8⁺ effector T cells [260]. Others have reported that CaP loaded with model antigen ovalbumin (OVA), combined with a fusion protein induced a robust and long-lasting CD8⁺ T cell response in mice that were characterised by the high production of IFN- γ and TNF- α [259]. It has also been shown that magnesium (Mg) and zinc (Zn) loaded with HAP NPs could effectively promote Th1 and Th2 immunity *in vitro*, and a considerable amount of type 1 and 2 cytokines was observed in the RPMI 1640 media from bone marrow dendritic cells (BMDCs), suggesting these particles can be used as human adjuvants [261]. Furthermore, in a recent study, CaP NPs encapsulating CpG combined with viral peptides from murine leukaemia Friend retrovirus (FV) were shown strongly enhanced anti-viral immunity [262].

It is interesting to note that different kinds of adjuvant can strongly affect T-cell immunity. Knuschke *et al.* compared CpG and poly (I:C) functionalized NPs with CaP NPs *in vitro*, and observed that CpG loaded with CaP NPs elicited a more robust T cell-mediated immunity [263]. In another study, CaP NPs encapsulating both CpG and poly (I:C) ligands and the antigen hemagglutinin were found to elicit DC maturation and T cell activation significantly [258] (Figure 1.10). Sokolova *et al.* demonstrated CaP NPs functionalised with TLR ligand poly (I:C) results in a pronounced immunostimulation to liver cells both

in vitro and *in vivo* [132]. DNA vaccines have also been delivered by using CaP as an adjuvant [264]. Joyappa *et al.* used CaP to carry the foot and mouth disease virus P1-3CD gene in mouse and guinea pig disease models. This work demonstrated CaP was a potent adjuvant in DNA vaccine, as well as inducing a strong cell-mediated immune response [255].

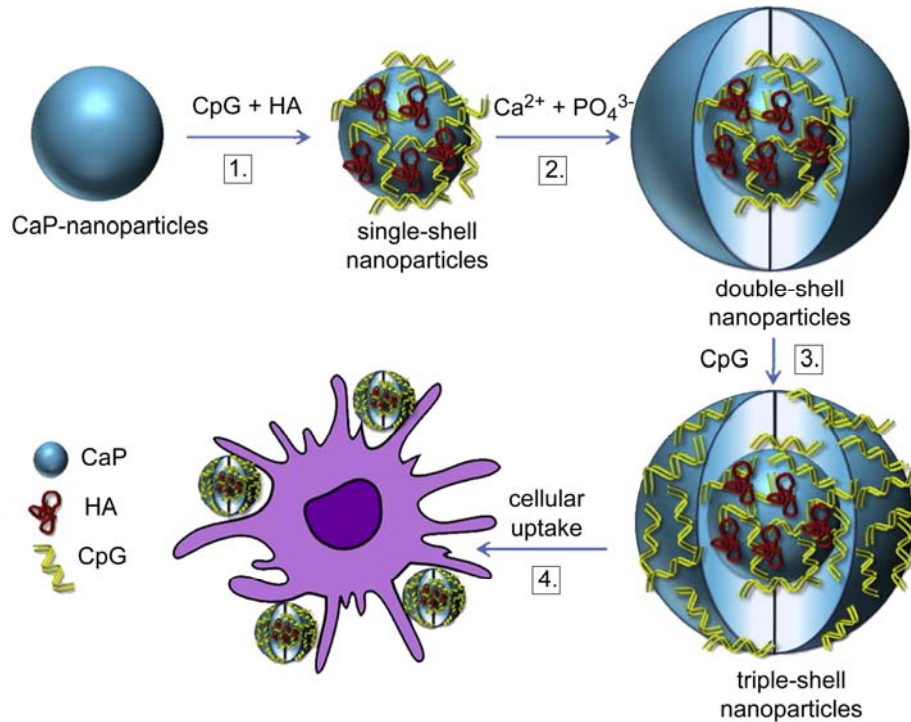


Figure 1. 10: A triple-shell calcium phosphate nanoparticles loaded an antigen and a TLR ligand (CpG or poly (I:C)) for cellular uptake.

The first step is the functionalisation of the CaP NPs with CpG and HA to form single-shell nanoparticles. Then, calcium and phosphate solution was added to form double-shell nanoparticles. CpG or poly (I:C) was added next to form triple-shell nanoparticles. Finally, CaP NPs were uptake by dendritic cells. CaP, calcium phosphate; HA, hemagglutinin. Adapted with permission from ref [258]. Copyright (2010) Biomaterials.

In the case of cancer vaccines, the potential of CaP as a vaccine carrier and adjuvant has also been explored [175, 265]. The induction of a potent CTL response is crucial for the eradication of cancer cells and prevent cancer recurrence [2, 14]. Xu *et al.* have used a mouse model of cancer to study the effect of NPs on CTL responses. In this model, female mice were subject to the induction of melanoma. Prior to the induction of the disease, mice were immunised with either free melanoma antigen (Tyrosinase-related protein 2, p-Trp2) with free TLR 9 ligand (CpG), or NPs loaded with lipid-calcium-phosphate (LCP) NPs containing p-Trp2 peptide and TLR 9 ligand [173]. Mice vaccinated with the Trp2-TLR ligand NPS that inhibit tumour growth and its lung metastasis, exhibited a robust

and specific CTL response *in vivo* and generated significant production of IFN- γ , indicating that CaP is a promising peptide vaccine delivery system for cancer immunotherapy [173]. However, this vaccine was less effective in a late-stage melanoma, despite the fact that the CTL response was consistent. In order to completely modify the suppressive tumour microenvironment, and to achieve a more effective therapeutic outcome, this group subsequently designed a novel LCP delivered Transforming Growth Factor- β (TGF- β) siRNA to contribute to tumour immunosuppression. Through silencing and decreased levels of TGF- β in cells, this vaccine resulted in greatly inhibited tumour growth and significantly increased the levels of CD8⁺ T cells compared with vaccine treatment alone [175]. The anti-tumour effect on advanced stage melanoma has also been enhanced by a combination of chemotherapeutic agents and LCP [265]. Another type of CaP, β -TCP, has also shown anti-tumour activity *in vivo* and have immune-stimulatory effects on immune cells [266]. Naito *et al.* reported that in an experimental xenograft tumour model in mice the growth rate of tumour TCP group was greatly lower than the control group and that macrophages might play a role in tumour growth inhibition [266]. To fully understand the biological effects of β -TCP in-depth, Tai *et al.* studied the *in vivo* and *in vitro* effect of β -TCP in C57bl/6 mice and a macrophage cell lines (J774A.1 cells), respectively. They found that vaccination with β -TCP elicited substantial migration of abundant neutrophils to the injection area in immunocompetent B6 mice. Additionally, β -TCP elicited upregulation of surface marker (CD86) expression and secretion of TNF- α and MIP-1 α in mouse monocyte/macrophage cells [249].

1.4 Summary

Although there has been significant research on the use of CaP NPs in the field of vaccine development (Table 1.2), they currently have several critical problems which prevent their clinical usage [267]. Low antigen loading capacity and rapid aggregation are major issues of CaP NPs [268]. For *in vivo* use of CaP NPs, it is necessary to synthesis CaP NPs of uniform size to avoid batch-to-batch variation [268]. However, the reproducibility of the synthesis of non-aggregated CaP NPs with uniform size and shape remains a significant challenge [268]. Similarly, it is challenging to predict release kinetics in the normal physiological environment as there can be the premature release of the antigen cargo from NPs at physiological pH. Since the application of CaP NPs in vaccine development is a relatively new concept, and that has not been investigated in great detail, further studies

on how manipulating the physical properties of CaP NPs to influence their pharmacokinetics, bio-distribution, and targeting are needed. To fully explore the potential of CaP NPs as antigenic carriers more research is required to address these issues [15, 42, 62].

In conclusion, the interaction between CaP NPs and the immune system has received a high level of interest in the last two decades. They can be used as an effective vaccine adjuvant and delivery vehicles for vaccine formulations to achieve immunity, especially in mucosal vaccination. The immunological effects of CaP NPs can be influenced by their physicochemical properties such as size, shape and surface charge. Although there are some difficulties that need to be addressed for NPs to be used as vaccine vehicles, it is clear that there is a rare opportunity to pursue the use of CaP NPs as a promising new platform in immunotherapy.

1 **Table 1. 2: The recent research of CaP NPs in vaccine applications.**

Molecule coupled to CaP NPs	Antigen/disease	Synthesis method	Size (SEM/TEM)/Shape/surfaces charge/phase	Adjuvant	Model animal vaccination site	Immunological outcomes	Reference
Protein	Herpes simplex virus type 2 (HSV-2) and Epstein-Barr virus (EBV) infections	Chemical precipitation	< 1000 nm	None	Mice Intra-peritoneal	Robust adjuvant, Elicited greater production of IgG2a antibody and neutralising antibodies.	[47]
Protein	Herpes simplex virus type 2 (HSV-2)	Chemical precipitation	< 1200 nm	None	Mice Intravaginal and intranasal	Potent mucosal adjuvant for viral infections Induced high mucosal IgA and IgG antibodies.	[58]
Protein	Malarial merozoite surface protein-1(19) (MSP-1(19))	Chemical precipitation	784 ± 5.8 nm/spherical/ -19.2 ± 1.2 mV/HAP	None	Mice Subcutaneous	Induced a strong IgG, IgG2a antibodies Significant amount of IFN- γ and IL-2 in mice.	[85]
Gene	Foot and mouth disease (FMD)	Chemical precipitation	50-100 nm/spherical	None	Mice and guinea pigs Intra-peritoneal	Robust cell-mediated and humoral immunity were elicited.	[86]
Peptide	Hemagglutinin/ Viral infection	Chemical precipitation	150 nm/ Spherical/-25 mV	CpG, poly (I:C)	Mice	CaP can be effectively uptake by DCs. Induced innate inflammatory cytokines IL-12p70 and TNF-α Strong stimulation of antigen-specific CD4 ⁺ T cells	[95]
Gene	Fab094/Rabies	Chemical precipitation	260 nm	None	Mice	Induced Fab094 neutralizing antibodies.	[96]
Protein	<i>Labeo rohita</i> H. S-layer protein (of <i>Aeromonas hydrophila</i>)	Commercial CaP NPs	200nm/spherical/ 34.81 ± 3 mV	None	Fish Intra-peritoneal	Induced both innate and cell-mediated immunity	[80]
Protein	a dendritic cell-specific antibody (CD11c)	Chemical precipitation	< 120 nm/spherical/ +15 ± 9 mV	None	Mice	CaP NPs effectively uptaken by cells.	[55]

Protein	OVA	Chemical precipitation	350 ± 22.5 nm/-12.93 ± 1.02 mV/ HAP	None	Mice Intradermal	High antibody titers (IgG and IgG1).	[67]
Peptide	Tyrosinase-related protein 2 (Trp2)/ Melanoma	Micro-emulsion	30 nm/15 mV	CpG	Mice Subcutaneous	Induced inhibition of tumour growth Elicited high IFN- γ production.	[20]
Peptide	Hemagglutinin/Viral infection	Chemical precipitation	250 nm	CpG	Mice Intraperitoneal and intranasal	Rapidly uptake by DCs. High numbers of IFN- γ -producing effector CD4 ⁺ and CD8 ⁺ T cells.	[88]
Protein	Newcastle disease	Continuous flow	< 100 nm/Spherical/+8 mV	None	Chicken Intramuscular	Chicken immunized with these particles elicited higher and earlier antibody responses.	[46]
Protein	Model protein	Chemical precipitation	60-100 nm/Spherical/+10 mV	Flagellin	Mice Intra-peritoneal	Induced the production of the proinflammatory cytokines IL-8, IL-1 β and IL-6.	[84]
Peptide	CD8 ⁺ or CD4 ⁺ T cell epitope peptides/(GagL85–93 or Env gp70123–141) of Friend retrovirus (FV)	Chemical precipitation	120 nm/Spherical/-21 mV	CpG	Mice Subcutaneous	Elicited a greatly efficient T cell-mediated immune response (CD4 ⁺ and CD8 ⁺ T cells).	[74]
Protein	Hen Egg Lysozyme (HEL)	Chemical precipitation	100-170 nm/Spherical/-21 ± 4 mV	None	Mice	Activation of antigen-specific B-cells response.	[57]
None	None	Chemical precipitation	5.373 ± 0.342 nm/ β -TCP	None	Mice Subcutaneous	Elicited an immune-stimulatory effect.	[82]
Protein	OVA	Chemical precipitation	75 $\pm$ 12 nm	None	Mice Subcutaneous	Robust CD8 ⁺ T cells response.	[89]
Protein	BSA	Chemical precipitation	< 50 nm/HAP	None	None	Early activation of innate response Increased production of IL-6, TNF- α .	[81]
Peptide	Tyrosinase-related protein 2 (Trp2)/ Melanoma	Micro-emulsion	30 nm/15 mV	CpG	Mice Subcutaneous	30 nm CaP elicited a potent T cell immunity in advanced cancer by silencing TGF- β in cancer cells.	[23]

Inactivated virus	Human enterovirus-71 virus (HEV-71)	Commercial CaP NPs	30 nm/905 nm	None	Rabbits Intradermal, Intramuscular	Nano-sized particles showed safer and greater antibody response through the intradermal route	[76]
Peptide	Tyrosinase-related protein 2 (Trp2)/Melanoma	Micro-emulsion	30 nm/Spherical/-15 mV	CpG	Mice Intra-peritoneal	Co-delivery with methyl-2-cyano-3, 12-dioxooleana-1, 9(11)-dien-28-oate (CDDO-Me) Significantly inhibited tumour growth Increased CTL infiltration in mice.	[93]
Protein	Hen Egg Lysozyme (HEL)	Chemical precipitation	40-125 nm/Spherical/+20 mV	CpG, Poly(I:C), R848, Flagellin	Mice Intramuscular, Subcutaneous, Intravenous	Effectively induced B cell activation Significantly humoral immunes <i>in vivo</i> .	[21]
Peptide	CD8 ⁺ or CD4 ⁺ T cell epitope peptides/(GagL85–93 or Env gp70123–141) of Friend retrovirus (FV)	Chemical precipitation	120 nm/Spherical/-21 mV	CpG	Mice Subcutaneous	Rapidly CD8 ⁺ T cell activation Greatly decreased the viral loads, Enhanced anti-viral immunity.	[91]
mRNA	mRNA/melanoma	Micro-emulsion	45 nm/0 mV	TRP2	Mice Intra-peritoneal	Significant antigen-specific cytotoxic T cell response and humoral immune response Inhibited the melanoma growth.	[97]
Protein	Viral infection	Chemical precipitation	275 nm/20 mV	Poly (I:C)	Mice Intra-peritoneal	The production of IFN- α/β , TNF- α , IL-6 and IP-10 in hepatocytes, NPCs and LSECs greatly upregulated.	[98]
siRNA	siRNA/hepatocellular carcinoma	Micro-emulsion	76 $\pm$ 3.9 nm/12 mV	None	Mice Intra-peritoneal	Significant inhibition of tumour growth.	[99]

1.5 Hypothesis and aims of the thesis

Hypothesis:

Calcium phosphate nanoparticles that contain antigens and molecular adjuvants can be used for vaccine delivery and to stimulate immune cells.

The overall aim of this study is to develop a biodegradable nanoparticle vaccine delivery system. To achieve this aim, several objectives need to be addressed:

- (1) To develop an efficient and reproducible method for the synthesis of various sizes of calcium phosphate nanoparticles.
- (2) To optimise the functionalisation of CaP NPs vaccine by using the layer-by-layer approach.
- (3) To evaluate the immunological effects of different sizes of CaP NPs vaccine on oral epithelial cells and macrophages.
- (4) To investigate TLR2 ligand loaded calcium phosphate nanoparticles induce macrophages maturation and activation.

Chapter 2

Development, evaluation and optimisation of a method for calcium phosphate nanoparticle synthesis with defined sizes and narrow polydispersity index

2.1 Introduction

In recent years, there has been growing interest in using nanoparticles as vaccine carriers and adjuvants [25, 71]. Adjuvants are used as a component of the vaccine to boost the immunogenicity to the antigen cargo [125]. Appropriate and optimised adjuvants play a central role in vaccine formulations as they can help induce a strong immune response and overcome immunosuppression [126]. To date, only a few adjuvants (such as the mineral adjuvants aluminium salts (alum), calcium phosphate and the oil-based adjuvants MF59, AS03 and AS04) have been approved for human use [127]. These adjuvants are effective at inducing humoral immune response [269]. However, with the exception of calcium phosphate, they have poor ability to stimulate a cellular immune response, which is critical for viral and tumour eradication [125]. Further, the oil-based adjuvants have several drawbacks and side effects such as severe local tissue irritation, longer inflammatory response and induction of allergic responses [128].

The utilisation of mineral adjuvants in the field of vaccine research has a long established efficacy [270]. These mineral adjuvants include aluminium hydroxide, aluminium phosphate and calcium phosphate [271]. In the mid-20th century, both aluminium and calcium-based adjuvants were initially used in a range of vaccine formulations [270, 272, 273]. However, aluminium-based formulations became a favoured adjuvant in the late 1980's [270]. Although widely used aluminium-based adjuvants have come under scrutiny due to reported side effects such as inducing autoimmunity, utero toxicity and low biodegradability [274, 275]. Calcium phosphate adjuvants, first reported by Relyveld and his colleagues in 1964 [276], have been used for many years as a vaccine adjuvant [277]. Calcium phosphate (CaP) adjuvant has been used in diphtheria, pertussis, tetanus and poliomyelitis vaccines in France [272, 273, 278]. Several recent papers have highlighted that CaP material should be considered as a replacement of alum salts in vaccine formulations [279, 280]. Major advantages of CaP materials is that they are non-toxic, biodegradable, production cost is low, and have pH-dependent solubility and can be produced as nanoparticles (NPs) [159]. Additionally, CaP adjuvants have been shown to provide protection to the antigen cargo from premature enzymatic and proteolytic degradation [84] and prevent elimination by the reticuloendothelial system (RES) [172]. Another advantage of CaP materials is that they can be functionalised with various molecular adjuvants to enhance active cell targeting and vaccine efficacy [131, 173, 175]. Due to these properties,

CaP nanoparticles have recently attracted attention in many biomedical applications such as DNA/gene silencing [160, 161], drug delivery [162, 163], protein/peptide delivery [30, 31, 132, 164], dental implant materials [165, 166], imaging [168, 169], bone tissue engineering [170, 171].

Despite the many advantages for the use of CaP nanoparticles as vaccine carriers/adjuvants, they currently have several major synthesis hurdles which have prevented their widespread use [267]. A major issue in the use of CaP NPs is reproducibly synthesising non-aggregated CaP NPs with homogeneous size and shape. In addition, the protein antigen loading obtained in many studies using CaP materials is low. CaP NPs have been shown to rapidly aggregate in aqueous solutions, which results in uncontrolled biological outcomes [268]. Thus the major challenge in the clinical use of CaP is that it is necessary to synthesis CaP NPs of uniform size, stable in aqueous solution and production is reproducible [268].

Current strategies of CaP NPs preparation usually generate various sizes, shapes and formulations. Synthesis methodologies can be divided into the dry [177, 178] and wet chemical routes [179, 180]. Dry methods mainly includes solid-state [177] and mechanochemical [178] techniques, while wet methods includes chemical precipitation [179, 181], sol-gel [180], microemulsion [183-186], hydrothermal [187, 188], sonochemical [189], and hydrolysis methods [190]. Depending on the manufacturing process CaP NPs can be obtained with a variety of physical properties, such as differing particle size (10 nm to 10 μ m), size distribution (from narrow to broad), shape (from spherical to rod-shaped), crystallinities (low to high) and composition (Ca:P ratio).

Among the various dry and wet methods, the chemical precipitation strategy is the most widely used to produce CaP NPs in academic and industry fields for vaccine formulations [164, 238, 252, 258]. With this approach, CaP NPs are produced by typically combining aqueous calcium chloride with sodium phosphate either via a drop-wise or a rapid addition method [159, 281]. To prevent CaP NP aggregation sodium citrate is added as a particle stabiliser [281]. The advantages of the precipitation method are low cost, rapid production, the reaction is carried out under normal pressure and temperature and ease of scale-up from laboratory to industrial scale for vaccine applications. Recently, the use of the microemulsion method has attracted more attention by academic researchers due to

its particular advantage in synthesising NPs of well-defined size and narrow size distribution [282-284]. An advantage of the microemulsion method is that the growth and nucleation of CaP NPs is constrained in the defined micelles in the microemulsion system, thus producing CaP NPs with defined sizes, narrow polydispersity with minimal aggregation [203]. Microemulsion routes are currently considered the most efficient way to reduce the aggregation of particles and to control particle size. Additionally, this method does not require high-temperature conditions during synthesis, thus making it an appropriate strategy to load a vaccine antigen by encapsulation.

An intrinsic property of CaP materials is that they have a variety of forms and phases due to the humidity, temperature and the presence of impurities in a reaction. Depending on the calcium to phosphate ratio in the CaP material, which can range from 0.5:1 to 2:1 there are eight defined CaP compositions (Table 2.1). Four of these: hydroxyapatite (HAP), amorphous calcium phosphate (ACP), tricalcium phosphate (TCP) and monocalcium phosphate monohydrate (MCPM) can and are being used in biomedical applications because of their superior stability in physiological environments [158]. HAP is the most physiologically stable and has been widely used in biomedical engineering applications [201, 209].

For CaP NPs to be considered as a viable mineral adjuvant and a replacement for alum adjuvant, for today's commercial needs there needs to be a standardised method that is able to be tuned to produce CaP NPs of a desired and defined size. This method must also result in CaP NPs having no post-synthesis aggregation, be highly stable, have very low polydispersity and can be scaled up for commercial use. In this Chapter the precipitation and microemulsion methods are explored along with a novel chelation precipitation method to produce CaP NPs with the above desired characteristics.

Table 2. 1: Various phases of CaP NPs compounds.

Ca/P ratio	Compound	Abbreviation	Formula	Density (g/cm ³)
0.5	Monocalcium phosphate monohydrate/ Monocalcium phosphate anhydrous	MCPM/MCPA	$\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}/$ $\text{Ca}(\text{H}_2\text{PO}_4)_2$	2.23/2.57
1.0	Dicalcium phosphate dehydrate, mineral brushite/ Dicalcium phosphate anhydrous, mineral monetite	DCPD/DCPA	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}/\text{CaHPO}_4$	2.3/2.93
1.33	Octacalcium phosphate	OCP	$\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$	2.67
1.5	α -Tricalcium phosphate/ β -Tricalcium phosphate	α -TCP/ β -TCP	$\alpha\text{-Ca}_3(\text{PO}_4)_2/\beta\text{-Ca}_3(\text{PO}_4)_2$	2.86/3.07
1.2-2.2	Amorphous calcium phosphate	ACP	$\text{Ca}_x\text{H}_y(\text{PO}_4)_z \cdot n\text{H}_2\text{O}$, $n = 3-4.5$; 15–20% H_2O	2.67
1.5-1.67	Calcium-deficient hydroxyapatite	CDHA	$\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_2$ $-x$ ($0 < x < 1$)	---
1.67	Hydroxyapatite	HA	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	3.16
2.0	Tetracalcium phosphate	TTCP	$\text{Ca}_4(\text{PO}_4)_2\text{O}$	3.05

2.2 Materials and Methods

2.2.1 Conventional precipitation synthesis method

Unless otherwise stated, MilliQ water purified through a 0.22 μm filter (Merck-Millipore, Victoria, Australia) was used throughout the synthesis of CaP NPs.

The pH of all solutions was adjusted using a 30% v/v ammonia solution (1 M, $\text{NH}_3\cdot\text{H}_2\text{O}$, Chem-Supply, Victoria, Australia) to pH 9. Reactions were conducted at RT and the molar ratio of $\text{Ca}^{2+}/\text{PO}_4^{3-}$ ions was 1.67:1 to allow for the formation of hydroxyapatite. The CaP NPs were fabricated by either rapid or drop wise (1 drop s^{-1}) addition of (0.4 mL) a solution of 18 mM calcium chloride (CaCl_2 , Univar, pH 9.0, NSW, Australia) to 0.4 mL of 10.8 mM disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$, Univar, 99%, pH 9.0, NSW, Australia). The reaction pH was maintained at 9.0 by addition of ammonia solution (1 M, $\text{NH}_3\cdot\text{H}_2\text{O}$, Chem-Supply, 30% v/v, Victoria, Australia). After 10 min, the reaction was stopped by harvesting the CaP NPs by centrifugation ($7,000 \times g$, 15 min, RT). The CaP NP pellet was washed 3 times in MilliQ water and suspended in MilliQ water and stored at 4 $^\circ\text{C}$. Figure 2.1 shows the schematic diagram for the preparation of calcium phosphate nanoparticles by the conventional precipitation method.

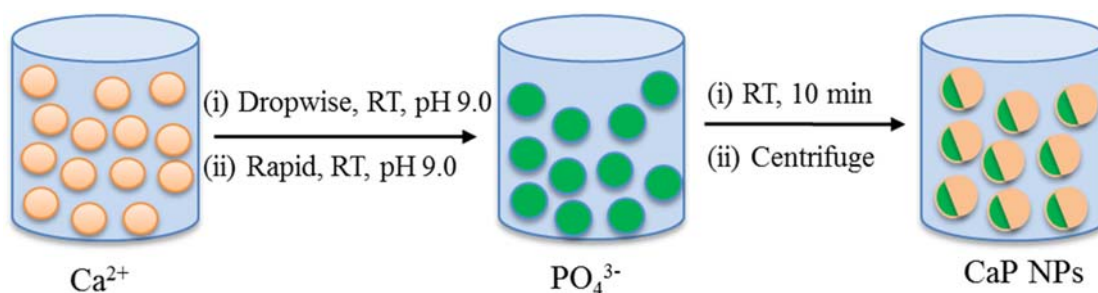


Figure 2. 1: Schematic diagram of the the conventional precipitation method for the formation of CaP NPs.

2.2.2 Microemulsion synthesis method

The microemulsion method was conducted as previously described [221] with the following modifications. Triton X-114 (Sigma-Aldrich, NSW, Australia) was used as the surfactant and a co-surfactant of hexane (C_6H_{14} , Sigma-Aldrich, 95%, NSW, Australia) and cyclohexane (C_6H_{12} , Sigma-Aldrich, 99.5%, NSW, Australia) as the oil phase. Figure 2.2 shows the schematic diagram for the preparation of calcium phosphate nanoparticles by the microemulsion

method. Briefly, a solution of 417.5 mM calcium chloride (CaCl_2 , Univar, 99%, NSW, Australia) and a solution of 250 mM disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, Univar, 99%, NSW, Australia) were prepared separately in MilliQ water and pH adjusted using 1 M ammonia ($\text{NH}_3 \cdot \text{H}_2\text{O}$, Chem-Supply, 30%, Victoria, Australia) to pH 10.5. The molar ratio of $\text{Ca}^{2+}/\text{PO}_4^{3-}$ was 1.67:1 to allow for the formation of hydroxyapatite. To 9 mL of cyclohexane/hexane/Triton X-114 (4:2:3 v/v) solution, 150 μL of CaCl_2 solution was added and the mixture stirred using a stir/hot plate at setting 1 ((IEC model (2093-001), NSW, Australia)) overnight to form a reverse water-in-oil microemulsion (emulsion A). In a separate 9 mL cyclohexane/hexane/Triton X-114 (4:2:3 v/v) solution, 150 μL of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ solution was added and the mixture stirred using a stir/hot plate at setting 1 overnight to form a microemulsion (emulsion B). Following the formation of the microemulsions, emulsion B was added dropwise (1 drop s^{-1}) to emulsion A under continuous stirring (setting 1) at RT to form a combined emulsion C. After complete addition of emulsion B the reaction was stirred for 15 min, after which 125 μL of 15.8 mM aqueous sodium citrate (Sigma-Aldrich, 99.5%, NSW, Australia) was added dropwise (about 1 drop s^{-1}) to the emulsion C. After addition of sodium citrate the reaction was incubated for 15 min after which 20 mL of absolute ethanol (Chem-Supply, 99.9%, Victoria, Australia) was rapidly added to break the emulsion system. The dispersed emulsion was stirred for 10 min (RT) after which the CaP NPs were harvested by centrifugation ($4,500 \times g$, 15 min, RT). The CaP NP pellets were washed 3 times ((ethanol (99.9% v/v), $4,500 \times g$, 15 min, RT)). Following the final wash, the CaP NP pellet was dispersed in MilliQ water and stored at 4°C .

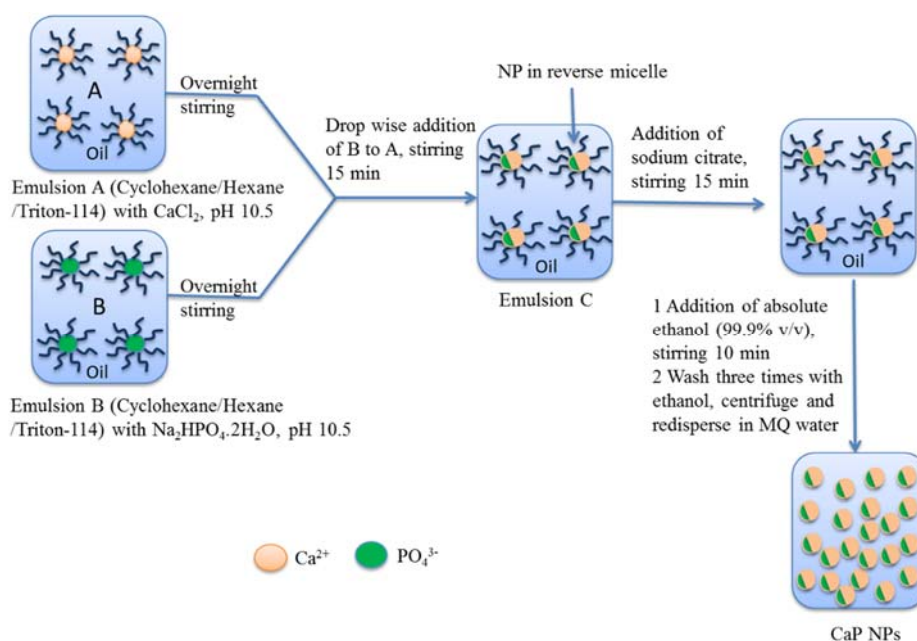


Figure 2. 2: Schematic diagram of the preparation of CaP NPs by the microemulsion method.

2.2.3 Calcium citrate chelation method

CaP NPs were prepared using a chelated-calcium precipitation method, as outlined in Figure 2.3. All solutions had their pH adjusted to 9 using 1 M ammonia ($\text{NH}_3\cdot\text{H}_2\text{O}$, Chem-Supply, 30% v/v, Victoria, Australia), and the molar ratio of $\text{Ca}^{2+}/\text{PO}_4^{3-}$ was 1.67:1 to allow for the formation of hydroxyapatite. To an aqueous solution (5 mL) of 0.05 M calcium chloride (CaCl_2 , Univar, 99%, NSW, Australia) or calcium nitrate ($\text{Ca}(\text{NO}_3)_2\cdot 4\text{H}_2\text{O}$, Univar, 99%, NSW, Australia), 5 mL of 0.085 M citric acid ($\text{C}_6\text{H}_8\text{O}_7$, MP Biomedical, 99.5%, NSW, Australia) was added, and the reaction incubated (1 h, stirring speed setting 1 ((IEC model (2093-001), NSW, Australia)), RT). Following incubation, 5 mL aqueous solution of 0.03 M monosodium phosphate (NaH_2PO_4 , Univar, 99%, NSW, Australia) or disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$, Univar, 99%, NSW, Australia) or ammonium phosphate monobasic ($\text{NH}_4\text{H}_2\text{PO}_4$, Sigma-Aldrich, 98%, NSW, Australia) or ammonium phosphate dibasic ($(\text{NH}_4)_2\text{HPO}_4$, Sigma-Aldrich, 98%, NSW, Australia) was immediately added to the calcium-citric acid solution, and the mixture stirred at setting 1 ((IEC model (2093-001), NSW, Australia)) for 10 min at RT. Following incubation, the temperature was increased and maintained at 40 °C for 6 h under stirring (setting 1) after which the reaction was cooled to RT, and the CaP NPs were harvested by centrifugation ($10,000 \times g$, 5 min, 4 °C, Avanti J-25I Centrifuge, Beckman Coulter, NSW, Australia). The CaP NP pellet was resuspended and washed three times with MilliQ water. The CaP NP pellet was finally resuspended in MilliQ water and sonicated for 1 min using a probe sonicator (Cole-Parmer, probe dimensions: L 150 mm; D 5 mm, at 25% amplitude, pulse on for 1 s and pulse off for 1 s). The particle preparations were stored at 4 °C.

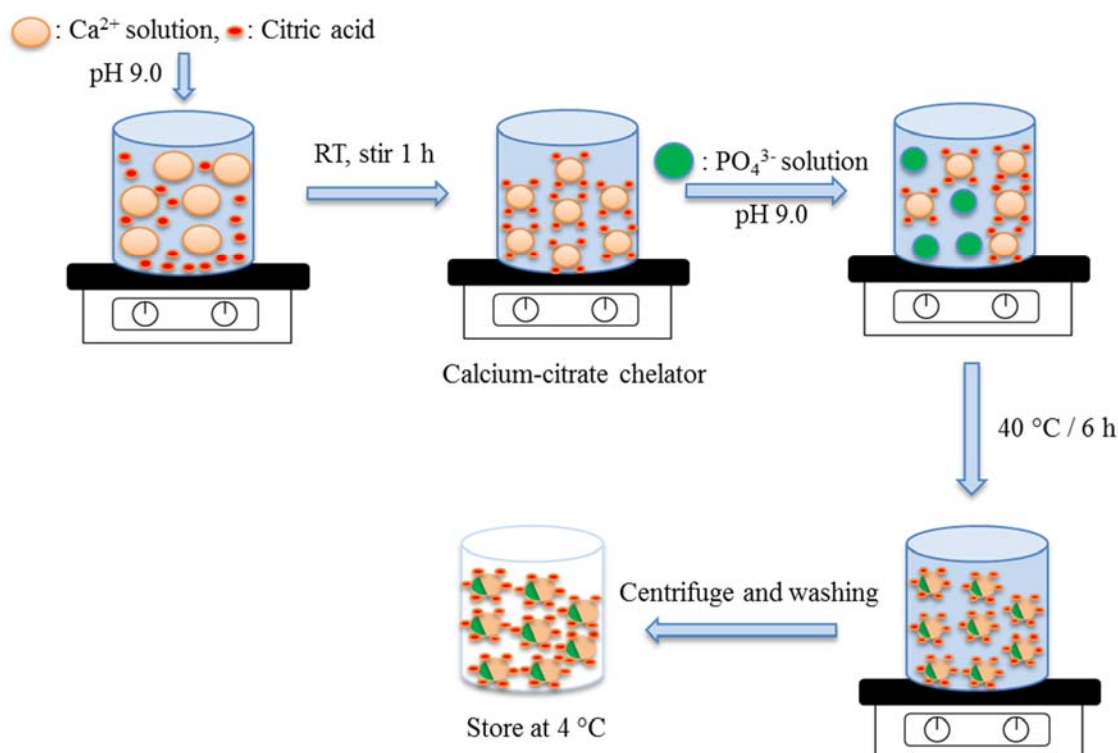


Figure 2. 3: Schematic diagram of the preparation of CaP NPs by the calcium citrate chelation method.

2.2.4 CaP NPs characterisation

The zeta potential, hydrodynamic diameter, and size distributions (polydispersity index, PDI) were determined by dynamic light scattering (DLS, Malvern Zetasizer, ATA Scientific Vic Australia) [285]. CaP NPs were diluted in MilliQ water for all measurements. The measurements were performed at 25 °C with a wavelength of 532 nm. All measurements were performed in triplicate.

The morphology and diffraction pattern of the CaP NPs was determined by transmission electron microscopy (TEM, FEI Tecnai G2 F30 instrument, FEI Company, American) imaging. CaP NPs were prepared in MilliQ water at 1 mg/mL for measurements. 30 μL of the diluted sample solution containing nanoparticles was aliquoted on a carbon-coated copper grid (300 mesh, Quantifoil) and air dried. The dried nanoparticles were then examined by a transmission electron microscopy with an acceleration voltage of 200 kV. Image J software was used to analyse images.

Scanning electron microscopy (SEM, Philips XL30 FEG, Eindhoven, Netherlands) was used to examine the average particle size of CaP NPs and observe particle shape at an acceleration

voltage of 5 kV. The CaP NPs sample was dispersed in MilliQ water (1 mg/mL), and 30 μ L of the sample was loaded onto a carbon-coated copper grid (300 mesh, Quantifoil) and air dried. Measurements were performed on the dried samples. Electron dispersion spectroscopy (EDS) microanalysis was used to analyse the elemental composition of CaP NPs.

The CaP particle size and surface morphology was also examined by helium ion microscopy (HIM, Zeiss, Orion Nanofab, Australia). The CaP NPs sample was dispersed in MilliQ water (1 mg/mL), and 30 μ L of the sample was aliquoted onto a carbon-coated copper grid (300 mesh, Quantifoil) and air dried. The dried nanoparticles were then examined by the HIM microscopy, with a beam current of 3.2 pA, an acceleration voltage of 32 kV and dwell time 100 μ s.

2.2.5 Compositional analysis of CaP NPs

Fourier transform infrared spectroscopy (FTIR) was carried out for structural analysis of nanoparticles, and to confirm the presence of OH and PO₄ functional groups on NPs. For the preparation of CaP NPs, 1 mg/mL of CaP NPs in MilliQ water were lyophilised (CHRIST, Quantum, Australia). The lyophilised samples were analysed with a scanning time for each sample of 64 seconds. FTIR spectra were collected on a Varian 7000 FTIR (Varian, CA) within the range of 4000 - 400 cm^{-1} , at a resolution of 4 cm^{-1} . The spectrum was analysed with respect to the standard IR series data.

The conversion rate as a percentage of calcium and phosphate in the solutions before and after synthesis and the proportion of calcium and phosphate in the nanoparticles was measured by atomic absorption spectrophotometry (Varian-AA240, Varian, Inc., NSW, Australia) for calcium concentration and the malachite green phosphate assay (Sigma, NSW, Australia) used to determine the phosphate concentration. After the preparation of CaP NPs the synthesised CaP NPs were centrifuged at $10,000 \times g$ for 5 min at 4 $^{\circ}\text{C}$ (Avanti J-25I Centrifuge, Beckman Coulter, NSW, Australia), and the unreacted calcium and phosphate ions in the supernatant were collected and measured. The phosphate concentration was determined by a colourimetric method with malachite green dye according to the manufacturer's instructions (Sigma, NSW, Australia). Atomic absorption spectrophotometry was used to measure calcium concentration into the solution. To determine the percent conversion, the reaction efficiency was calculated as the difference between the initial calcium or phosphate amount added to the solutions, and the measured unreacted calcium or phosphate amount, using the formula $(E\%) = (\text{total calcium}$

or phosphate added – unreacted calcium or phosphate / total calcium or phosphate added) $\times$ 100%.

In order to determine the phase of CaP NPs, X-ray diffraction (XRD) was conducted. The XRD analysis of lyophilised CaP NPs was conducted using a diffractometer (Bruker, D8, Bruker, Germany) with Cu K α radiation ($\lambda = 1.5408 \text{ \AA}$). Samples were analysed by the Materials Characterisation and Fabrication Platform (University of Melbourne, VIC, Australia).

2.2.6 Statistical analysis

GraphPad Prism 5 software was used to analyse data in experiments. One-way ANOVA and student T-test were used to determine P and a $P < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 Conventional precipitation synthesis method

The conventional precipitation method was used to study the efficiency of preparing low polydispersity particles using either the rapid or dropwise addition of a calcium chloride solution to a solution of disodium hydrogen phosphate.

The rapid addition method resulted in nanoparticles with an average diameter of 330 ± 120 nm, a PDI of 0.863 ± 0.12 and a zeta potential of -16.7 ± 0.05 mV. Figure 2.4 shows the correlation curve and particle intensity distribution of the CaP NPs and indicates that there are several distinct particle populations. SEM image analysis confirmed a heterogeneous NP population and showed the particles had a cuboidal shape (Figure 2.4D). Further, CaP NPs synthesis showed significant batch to batch variation (Figure 2.4E and Table 2.2).

To address the high PDI and size variation from different reactions the dropwise method was evaluated. However, this method also resulted in CaP NPs with high PDIs and significant batch to batch variation in size (Table 2.2).

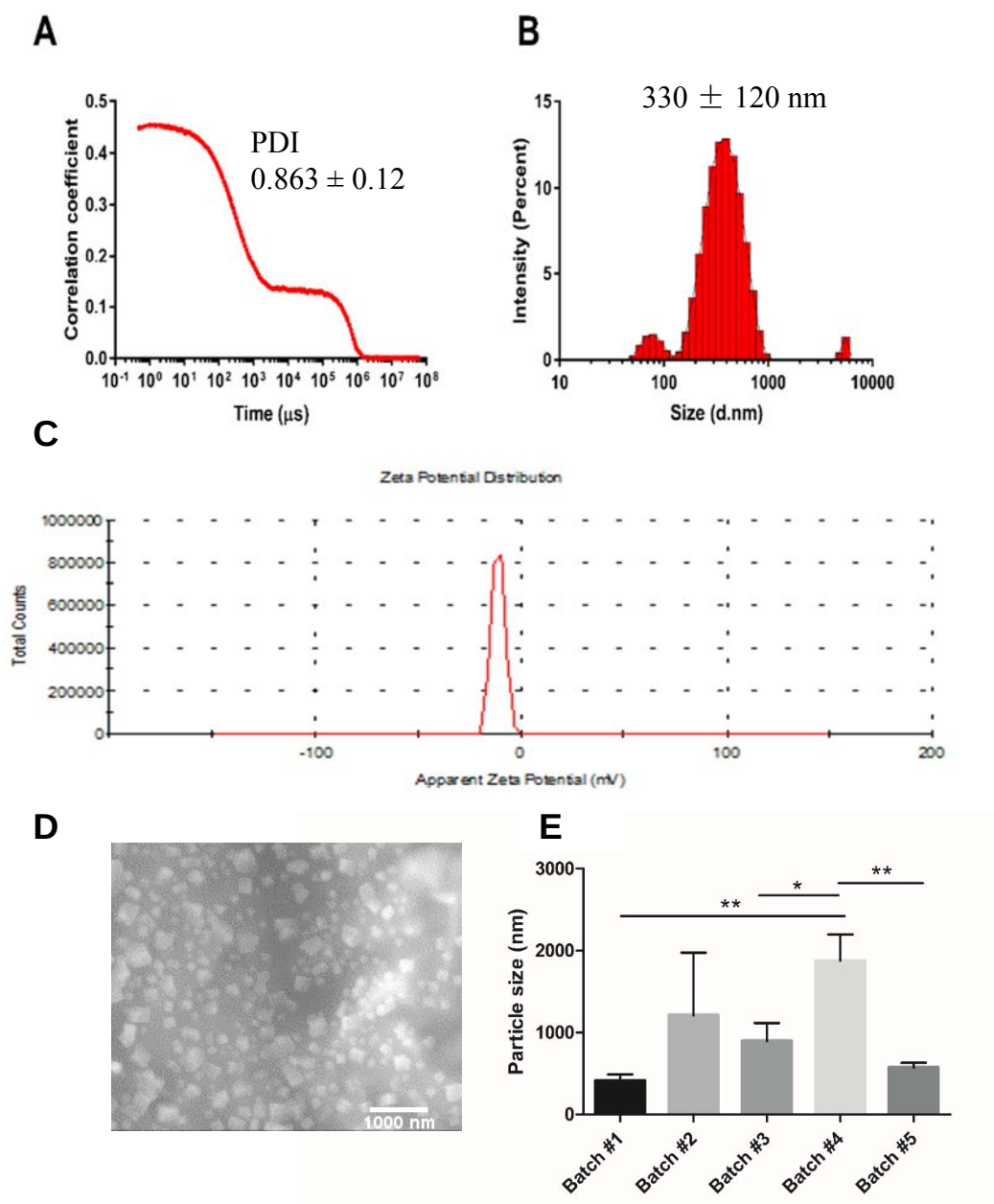


Figure 2. 4: Characterisation of particles synthesised by conventional precipitation method.

(A) correlation curve, (B) size distribution, (C) zeta potential, (D) SEM image and (E) synthesis reproducibility. Data are expressed as mean $\pm$ SD of at least 3 biological replicates. * P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001 were considered statistically significant.

Table 2. 2: Comparison of rapid and dropwise addition obtained particles by conventional precipitation method (n = 3). Data are expressed as mean $\pm$ SD of at least 3 biological replicates.

Group	Size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
Rapid (Batch #1)	410 $\pm$ 78	0.46 $\pm$ 0.15	-16.0 $\pm$ 0.09
Rapid (Batch #2)	1212 $\pm$ 765	0.75 $\pm$ 0.22	-15.1 $\pm$ 0.15
Rapid (Batch #3)	895 $\pm$ 221	0.68 $\pm$ 0.23	-14.7 $\pm$ 0.08
Rapid (Batch #4)	1876 $\pm$ 322	0.86 $\pm$ 0.12	-16.7 $\pm$ 0.05
Rapid (Batch #5)	574 $\pm$ 56	0.57 $\pm$ 0.19	-14.3 $\pm$ 0.10
Dropwise (Batch #1)	598 $\pm$ 97	0.66 $\pm$ 0.20	-14.9 $\pm$ 0.03
Dropwise (Batch #2)	356 $\pm$ 107	0.60 $\pm$ 0.19	-13.8 $\pm$ 0.10
Dropwise (Batch #3)	501 $\pm$ 111	0.72 $\pm$ 0.17	-14.0 $\pm$ 0.11
Dropwise (Batch #4)	486 $\pm$ 179	0.79 $\pm$ 0.16	-12.7 $\pm$ 0.12
Dropwise (Batch #5)	309 $\pm$ 85	0.62 $\pm$ 0.21	-13.4 $\pm$ 0.09

2.3.2 Microemulsion synthesis method

The microemulsion method was investigated to produce CaP NPs with a low PDI. Several parameters in the microemulsion method are known to affect particle size. These are reaction time, surfactant, water to surfactant ratio (W_0), centrifuge speed and sonication time. Each of these parameters were investigated to produce an optimised method. Following the co-mixing of emulsion A and B the reaction time was varied (15, 60, 120, 180 min) and the resultant particles analysed by DLS. Table 2.3 shows that reaction times greater than 15 mins produced particles under 100 nm but with a high PDI. Only the 15 min reaction time produced particles with a low PDI of 0.30 ± 0.06 . Using the 15 min reaction time, the effect of Triton X-114 and Tween-20 as compared to Triton X-100 was investigated. Triton X-114 produced an NP of 124 ± 8.4 nm with a low PDI of 0.15 ± 0.04 . Although Triton X-100 and Tween-20 produced NPs of different sizes the PDI was higher than Triton X-114 (Table 2.3). Using Triton X-114 and a reaction time of 15 mins, the effect of water to surfactant ratio (W_0) was investigated (Table 2.3). As can be seen in Table 2.3 increasing W_0 results in an increase in particle size. Each of the particles had a similar low PDI ranging from 0.35 to 0.41. Finally it can be seen that harvesting NPs at 4,500 g and a 30 min sonication time resulted in particles with lower PDIs (Table 2.3).

Table 2. 3: Effect of the various parameters on CaP NPs from microemulsion method (n = 3). Data are expressed as mean $\pm$ SD of at least 3 biological replicates.

Category	Parameter	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
Emulsion incubation (min)	15	130 $\pm$ 26	0.30 $\pm$ 0.06	-35 $\pm$ 0.7
	60	85 $\pm$ 28	0.82 $\pm$ 0.15	-5.9 $\pm$ 1.4
	120	13 $\pm$ 3.5	0.64 $\pm$ 0.08	-20.1 $\pm$ 2.2
	180	20 $\pm$ 0.6	0.69 $\pm$ 0.08	-13.2 $\pm$ 3.3
Surfactant	Triton X-114	124 $\pm$ 8.4	0.15 $\pm$ 0.04	-31.1 $\pm$ 0.3
	Triton X-100	198 $\pm$ 34	0.74 $\pm$ 0.07	-5.8 $\pm$ 0.1
	Tween-20	68 $\pm$ 39	0.77 $\pm$ 0.18	-7.5 $\pm$ 0.4
Ratio of water to surfactant	1:1	130 $\pm$ 26	0.35 $\pm$ 0.06	-32 $\pm$ 2.3
	2:1	652 $\pm$ 143	0.41 $\pm$ 0.10	-15.7 $\pm$ 3.7
	4:1	1247 $\pm$ 205	0.37 $\pm$ 0.11	-9.57 $\pm$ 2.2
Centrifuge speed (g)	4,500	147 $\pm$ 36	0.34 $\pm$ 0.15	-32 $\pm$ 1.3
	12,000	218 $\pm$ 62	0.45 $\pm$ 0.17	-34 $\pm$ 2.3
Sonication time (min)	30	177 $\pm$ 26	0.10 $\pm$ 0.06	-35 $\pm$ 0.7
	60	100 $\pm$ 42	0.65 $\pm$ 0.13	-30.7 $\pm$ 0.9

Using the optimised conditions (15 min reaction time, Triton X-114, Wo of 1:1, 4,500 g and 30 min sonication) the reproducibility of the method was investigated (Figure 2.5). The first experiment produced particles with an average diameter of 124 $\pm$ 10 nm and a low PDI of 0.15 $\pm$ 0.04 and zeta potential of -31.1 $\pm$ 0.3 mV (Figure 2.5A, B and C). Image analysis of the NPs by TEM and SEM confirmed the DLS data with a medium NP size of 105 $\pm$ 22 nm and that the NPs were spherical (Figure 2.5D and E). As indicated by the zeta potential the particles were found to be stable in MilliQ water for up to 28 days with no evidence of aggregation (Figure 2.5F).

Figure 2.5G shows that the microemulsion method has significant batch to batch variation with an average particle size varying from 123 $\pm$ 4 to 515 $\pm$ 30 nm. Interestingly, the PDI of each batch was low ranging from 0.12 $\pm$ 0.05 to 0.27 $\pm$ 0.08. Further, EDS analysis of the NPs found

that the Ca^{2+} and PO_4^{3-} ions were detected with a relative atomic percentage of 0.16 and 0.22, respectively, resulting in a Ca/P molar ratio of 0.73:1. This is considerably lower than the initial Ca/P molar ratio of 1.67:1 and indicates the NPs are dicalcium phosphate anhydrous (DCPA) (Supplementary Figure 2.1). Using atomic absorption spectrophotometry and the malachite green colourimetric assay to measure Ca^{2+} and PO_4^{3-} ions respectively. The rate of conversion of Ca^{2+} and PO_4^{3-} ions into CaP NPs was determined by evaluating the levels of each ion in the pre and post reaction solutions. The result showed 1.3% Ca^{2+} and 2.2% PO_4^{3-} of the starting material converted to CaP NPs, indicating this method has a low reaction efficiency.

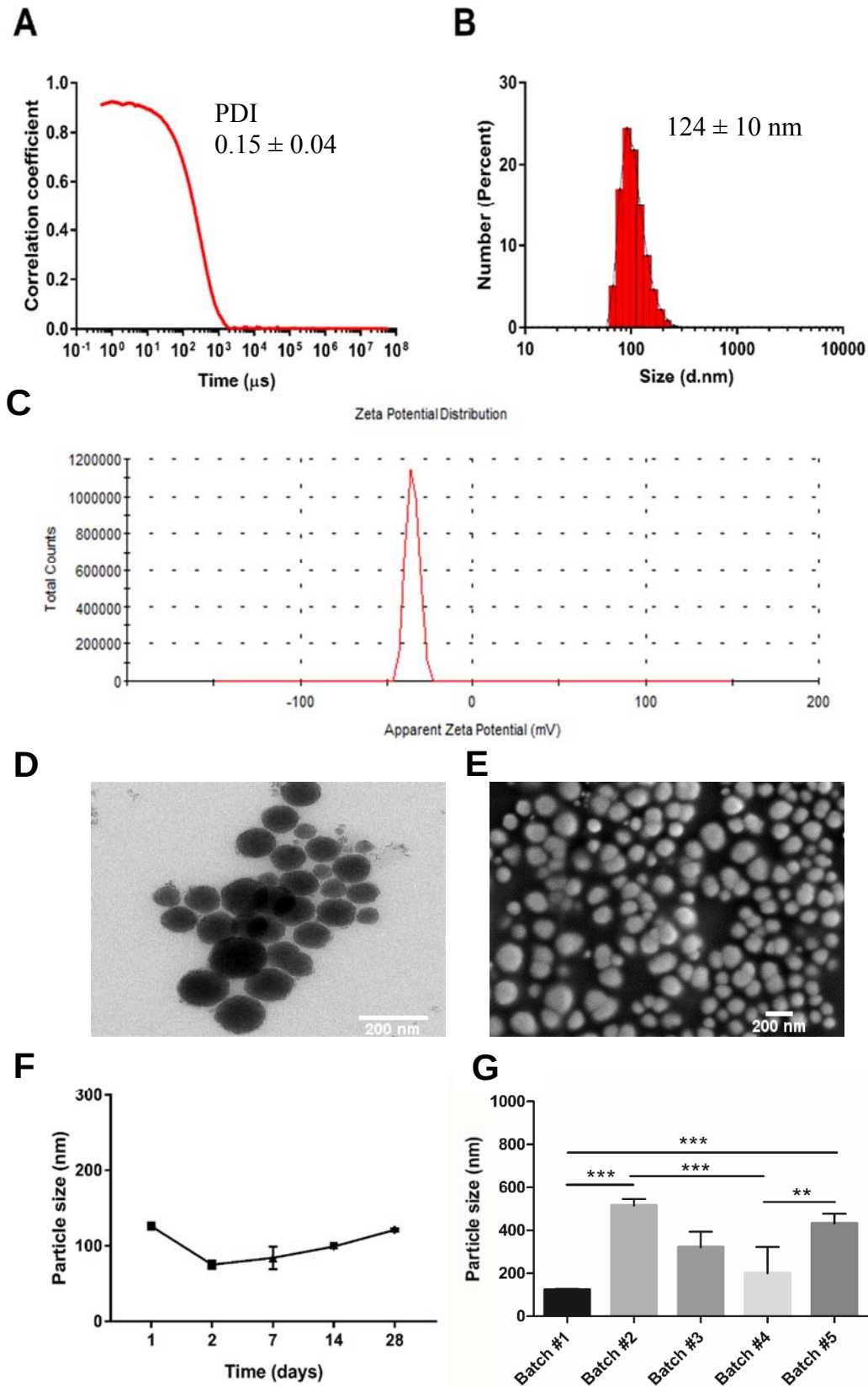


Figure 2. 5: Characterisation results of nanoparticles (A) correlation curve, (B) size distribution, (C) zeta potential (D) TEM image and (E) SEM image (F) size stability (G) reproducibility from microemulsion method.

Data are expressed as mean $\pm$ SD of at least 3 biological replicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ were considered statistically significant.

2.3.3 Citrate chelation synthesis method

2.3.3.1 Optimisation of citrate chelation method

A common feature of the traditional precipitation and microemulsion method is that initial particle nucleation is poorly controlled resulting particles of varying sizes between batches and/or high PDI. It was hypothesised that if particle nucleation could be inhibited at one time, the resultant particles would have a uniform size, low PDI and this could be repeated. By adding a moiety to chelate calcium, the phosphate solution could be added without the initiation of nucleation and the precipitation of CaP NPs. Heating the chelated-calcium and phosphate solution would result in destabilising the calcium-chelate and initiating nucleation under a controlled parameter.

Five Ca^{2+} chelators (EDTA, glycine, citric acid, L-glutamic acid and iminodiacetic acid) were initially investigated at a calcium to chelator ratio of 1:1.5. Figure 2.6 shows that on addition of the phosphate solution only EDTA and citric acid prevented precipitation at room temperature and at a solution of pH 8, with no observable particles detected by DLS (Table 2.4). All of the other chelators resulted in large particles with high PDI. It was found that increasing either glycine or glutamic acid to a calcium to chelator of 1:40 did not prevent precipitation (data not shown). Increase the ratio of calcium:iminodiacetic acid (IDA) to 1:30 was found to prevent precipitation at room temperature (Supplementary Figures 2.2). Heating the 1:30 calcium:IDA phosphate solutions resulted in precipitation of CaP NPs at 45 °C respectively producing particles of 898 ± 309 nm with a high PDI of 0.52 ± 0.38 . Interestingly, heating the calcium-EDTA/phosphate solution up to 95 °C did not result in CaP NPs precipitation, and lower calcium:EDTA ratios did not prevent precipitation, thus this approach was abandoned.

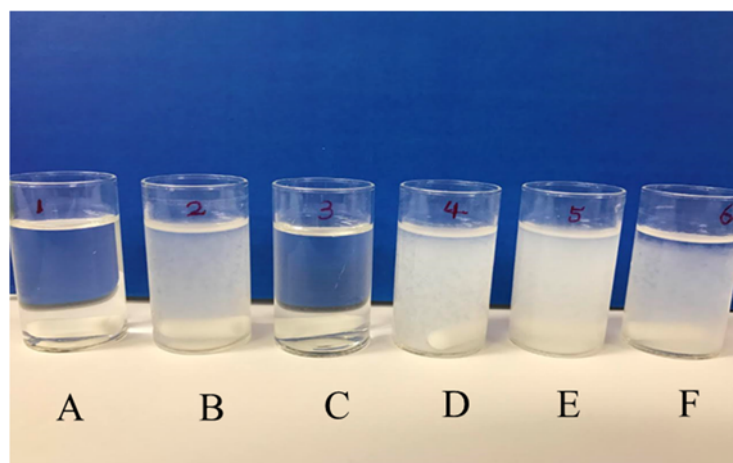


Figure 2. 6: Photo-image of CaCl_2 plus a chelating agent 1 hour after addition of NaH_2PO_4 : (A) EDTA; (B) Glycine; (C) Citric acid; (D) L-glutamic acid; (E) Iminodiacetic acid and (F) MilliQ water at pH 8 and room temperature.

Table 2. 4: Particle size and polydispersity index (PDI) of precipitated particles. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.

Sample	Size (nm)	PDI
EDTA	0	0
Glycine	2161 ± 562	0.79 ± 0.23
Citric acid	0	0
L-glutamic acid	2472 ± 369	0.67 ± 0.26
Iminodiacetic acid	3601 ± 523	0.74 ± 0.21
MilliQ water	1889 ± 237	0.69 ± 0.24

It was noted that at a calcium:citrate ratio of 1:1.5 there was occasionally precipitation at room temperature. However, increasing the ratio to 1:1.7 and pH of the solutions to 8.5 resulted in stable solutions at room temperature. Heating the calcium:citrate (1:1.7) and phosphate solution resulted in precipitation of CaP NPs at 40 °C (Table 2.5). The 1:1.7 calcium to citrate ratio produced particles with the lowest PDI as compared to reactions using lower calcium:citrate ratios (Table 2.5). Interestingly, a calcium:citrate ratio of 1:1.8 did not result in the precipitation of CaP NPs at 40 °C over 6 hours (Table 2.5). A known phenomenon with calcium phosphate particles is that once formed they stabilise over time. To investigate this aliquots from the cal-

cium:citrate (1:1.7) / phosphate solution were taken hourly and particle size and PDI determined. Figure 2.8 shows that the particle size and PDI decreased from 1 to 4 hours and plateaued at 5 and 6 hours.

In a series of experiments to investigate the effect of mixtures of chelating agents (citric acid, L-glutamic acid and iminodiacetic acid) had on NP size and PDI were conducted. The Supplementary Figures 2.2, 2.3, 2.4, 2.5 and 2.6 and Supplementary Tables 2.1 and 2.2 show that mixtures of chelators produced particles larger than a calcium: citric ratio of 1:1.7 and high PDIs.

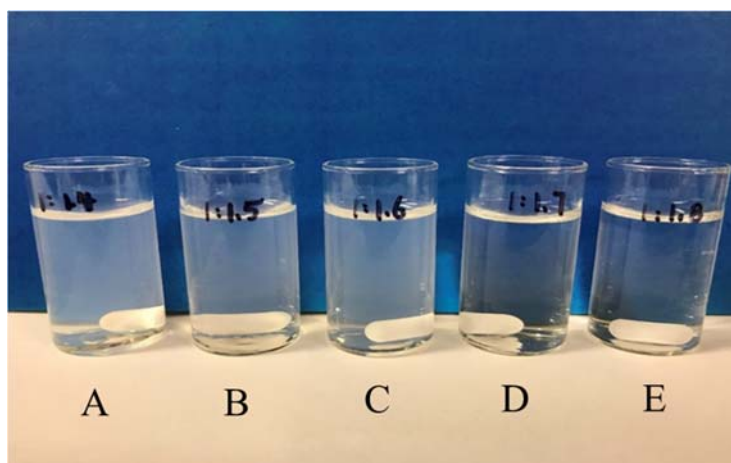


Figure 2. 7: Photograph of various molar ratios of CaCl_2 to citric acid: A (1:1.4); B (1:1.5); C (1:1.6); D (1:1.7); E (1:1.8) after 1 hour following addition of NaH_2PO_4 at room temperature pH 8.5.

Table 2. 5: Effect of different molar ratios of CaCl_2 to citric acid on CaP NPs at 40 °C for 6 h. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.

Ratio of calcium/citric acid	Particle Size (nm)	PDI
1:1.5	62 ± 18	0.39 ± 0.20
1:1.6	92 ± 39	0.32 ± 0.14
1:1.7	185 ± 26	0.14 ± 0.03
1:1.8	-	-

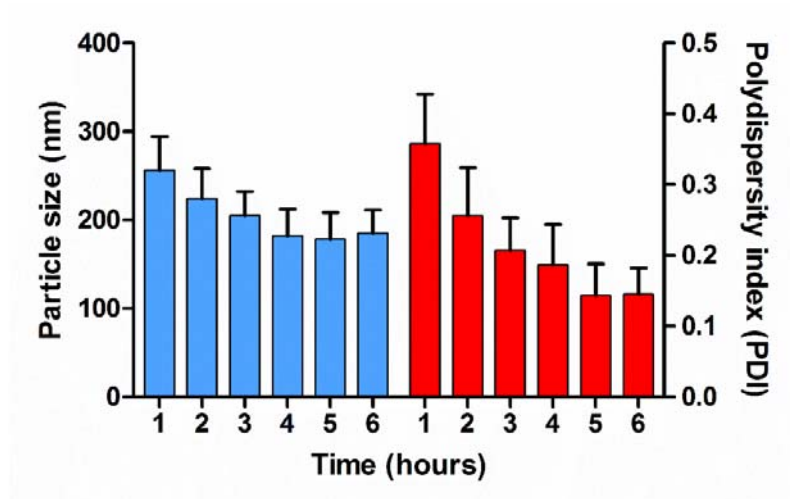


Figure 2. 8: Effect of reaction time on particle size and polydispersity index as determined by DLS. Data are expressed as mean $\pm$ SD of at least 3 biological replicates, blue bars = particle size, red bars = PDI.

Using the optimised reaction conditions of a calcium-citrate ratio of 1:1.7, reaction pH 8.5, time 6 h and precipitation temperature of 40 °C, the variation in particle size and PDI for different batches was investigated. As can be seen in Figure 2.9A, the calcium-citrate method produced particles with similar size (range from 156 ± 43 to 190 ± 32 nm) and low PDI (range from 0.09 ± 0.03 to 0.17 ± 0.07) across all five batches. Each batch was stable in MilliQ water for 32 days (Figure 2.9B) and the DLS results showed a smooth and exponential decay function and an average particle size of 185 ± 26 nm (Figure 2.9C). The morphologies of the CaP NPs synthesised by citrate chelation method were visualised by TEM and SEM and show that the CaP NPs have a rod-like shaped structure (Figure 2.9F and Figure 2.9G).

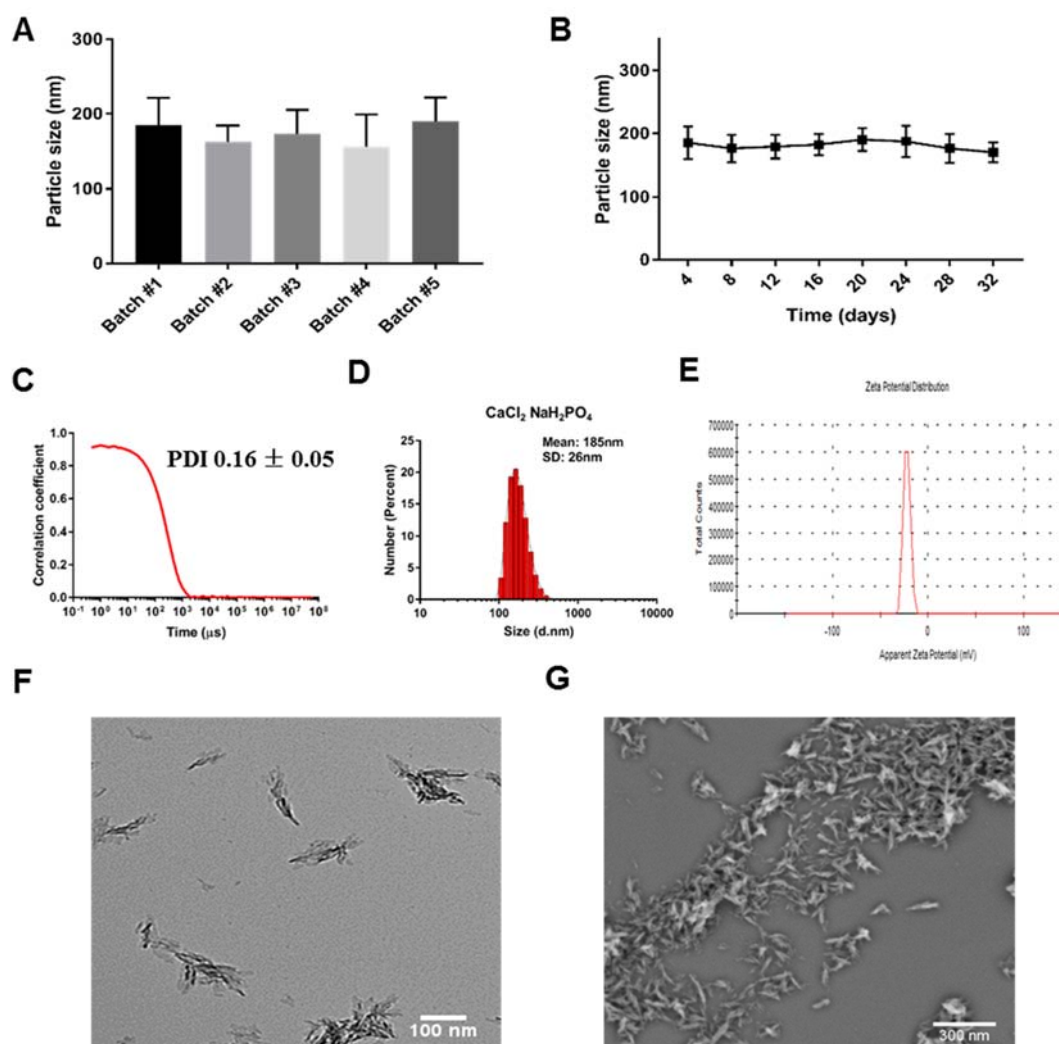


Figure 2. 9: Characterisation results of nanoparticles (A) reproducibility from citrate chelation method, (B) size stability, (C) correlation curve, (D) size distribution, (E) zeta potential, (F) TEM image and (G) SEM image.

Data are expressed as mean $\pm$ SD of at least 3 biological replicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ were considered statistically significant.

2.3.3.2 Effect of different calcium salts and phosphate salts on CaP NPs synthesis

In the preparation of CaP NPs, typically two calcium salts (calcium chloride and calcium nitrate) and four phosphate salts (monosodium phosphate, disodium hydrogen phosphate, ammonium phosphate monobasic and ammonium phosphate dibasic) have been used as starting materials. Using the optimised calcium-citrate chelation method for the synthesis of CaP NPs the effect of the different salts on CaP formulation and size was investigated. As can be seen from the Figure 2.10 and Table 2.6, each calcium salt and phosphate salt combination resulted in CaP NPs of distinct sizes with low PDI's (range from 60 ± 17 to 338 ± 13 nm). Interestingly, all of

the CaP NPs produced using calcium chloride were around 50% larger than CaP NPs synthesised using calcium nitrate (Table 2.6). Further, CaP NP's synthesis using monosodium phosphate were 50% smaller than CaP NPs synthesised using disodium hydrogen phosphate (Table 2.6). Ammonium phosphate monobasic produced CaP NPs that were 40% smaller than CaP NPs produced using ammonium phosphate dibasic (Table 2.6). All of the CaP NPs had a large and similar negative zeta potentials (Table 2.6).

Table 2. 6: Effect of different calcium salts and phosphate salts on CaP NPs synthesis. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.

Calcium salts	Phosphate salts	Size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
CaCl ₂	NaH ₂ PO ₄	185 $\pm$ 26	0.18 $\pm$ 0.05	-20.93 $\pm$ 0.33
CaCl ₂	Na ₂ HPO ₄ .2H ₂ O	338 $\pm$ 13	0.08 $\pm$ 0.05	-23.06 $\pm$ 1.56
CaCl ₂	NH ₄ H ₂ PO ₄	126 $\pm$ 45	0.27 $\pm$ 0.03	-24.80 $\pm$ 1.08
CaCl ₂	(NH ₄) ₂ HPO ₄	264 $\pm$ 59	0.23 $\pm$ 0.04	-24.63 $\pm$ 0.91
Ca(NO ₃) ₂ .4H ₂ O	NaH ₂ PO ₄	99 $\pm$ 24	0.23 $\pm$ 0.16	-24.86 $\pm$ 1.65
Ca(NO ₃) ₂ .4H ₂ O	Na ₂ HPO ₄ .2H ₂ O	173 $\pm$ 46	0.27 $\pm$ 0.02	-22.56 $\pm$ 0.51
Ca(NO ₃) ₂ .4H ₂ O	NH ₄ H ₂ PO ₄	60 $\pm$ 17	0.19 $\pm$ 0.05	-21.10 $\pm$ 1.02
Ca(NO ₃) ₂ .4H ₂ O	(NH ₄) ₂ HPO ₄	89 $\pm$ 36	0.22 $\pm$ 0.05	-26.50 $\pm$ 1.43

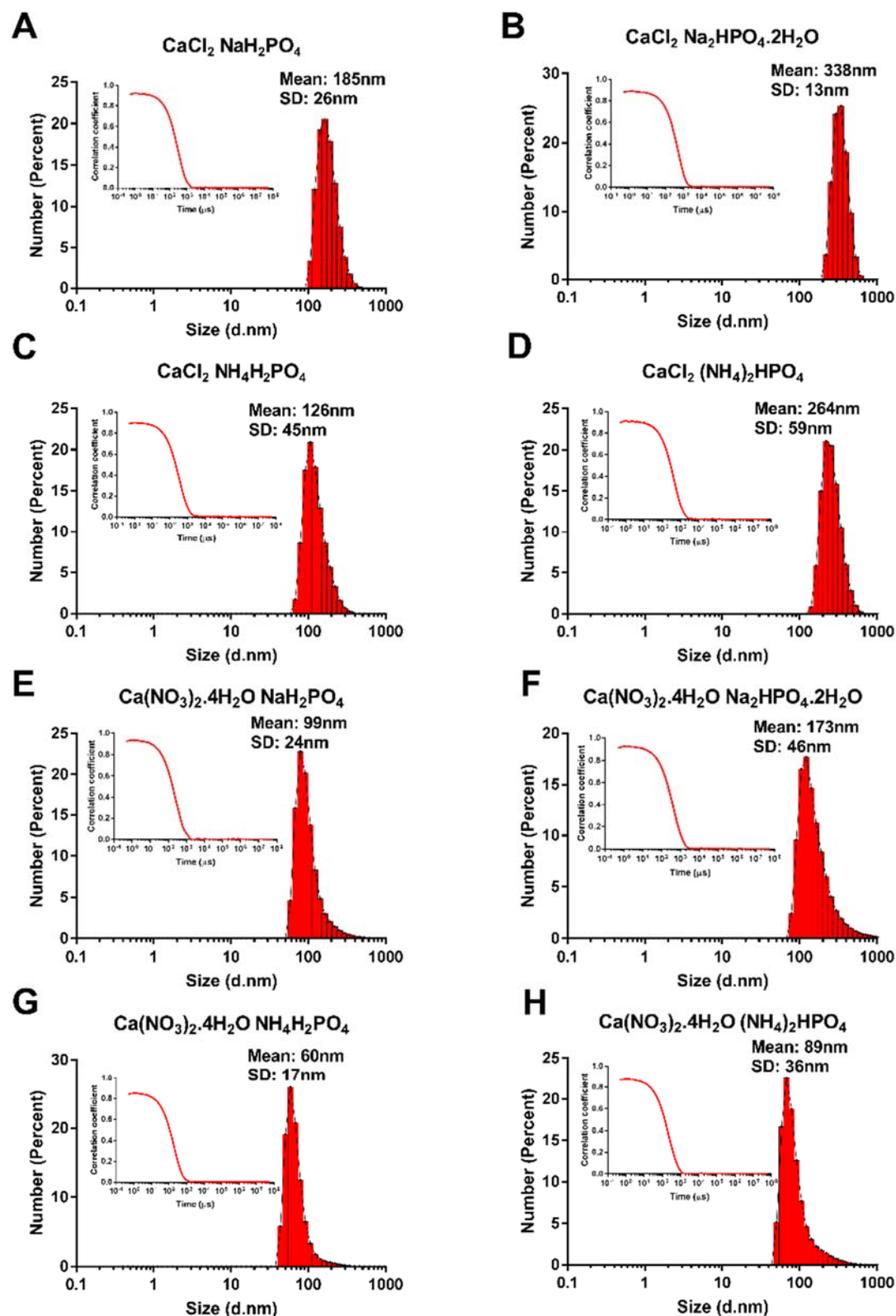


Figure 2. 10: The particle size and correlation curve of eight combinations at 40 °C for 6 h reaction time by citrate chelation method.

Data are expressed as mean $\pm$ SD of at least 3 biological replicates. (A) $\text{CaCl}_2 + \text{NaH}_2\text{PO}_4$, (B) $\text{CaCl}_2 + \text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, (C) $\text{CaCl}_2 + \text{NH}_4\text{H}_2\text{PO}_4$, (D) $\text{CaCl}_2 + (\text{NH}_4)_2\text{HPO}_4$, (E) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{NaH}_2\text{PO}_4$, (F) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, (G) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{NH}_4\text{H}_2\text{PO}_4$, (H) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + (\text{NH}_4)_2\text{HPO}_4$.

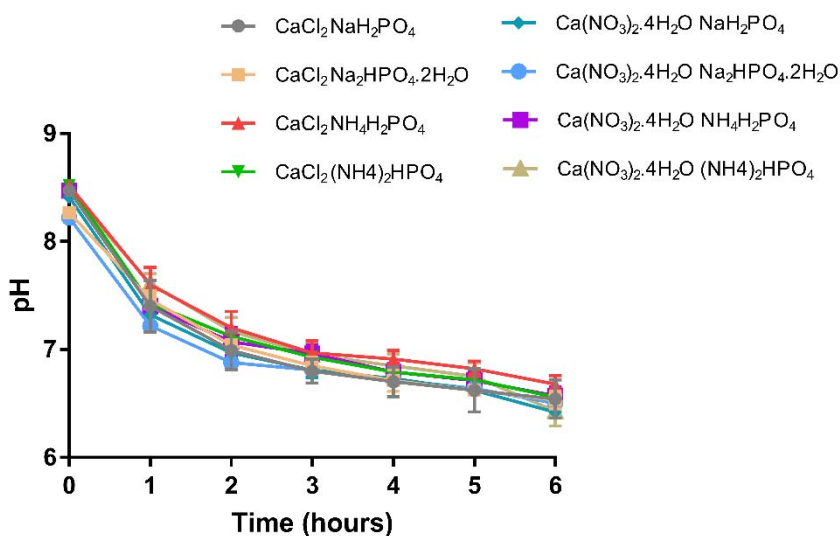


Figure 2. 11: The pH change of eight combinations at 40 °C for 6 h reaction time and drift pH 8.5 by citrate chelation method.

Data are expressed as mean $\pm$ SD of at least 3 biological replicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ were considered statistically significant.

Interestingly, monitoring the pH of the citrate chelation method over the course of the reaction showed that the solution pH from all reactions decreased from 8.5 to approximately 6.5 over the 6 hour period (Figure 2.11). This ‘drifting’ pH is commonly reported in CaP NP synthesis and the effect of a constant pH of 8.5 over the course of the reaction for each calcium salt and phosphate salt combination was investigated.

2.3.3.3 Effect of constant pH (8.5) on CaP NPs synthesis

For each calcium salt and phosphate salt combination the pH was maintained at pH 8.5 over the 6 h reaction time by addition of 1 M ammonia solution. Figure 2.12 and Table 2.7 shows that each calcium salt and phosphate salt combination produced CaP NPs that were larger than those produced by the ‘drifting’ pH method and all NPs had low PDI’s (range from 0.18 ± 0.09 to 0.26 ± 0.19) and a large negative zeta potential. Similar to ‘drifting’ pH, CaP NPs synthesised with calcium chloride and ammonium phosphate mono or di-basic were around 50% larger than these synthesised with calcium nitrate. There was a small, $< 25\%$, difference in size of particles synthesised using sodium phosphate salts except for calcium chloride where the use of disodium phosphate produce NPs that were 50% larger than those produced using monosodium phosphate salt.

Table 2. 7: NP characteristics of CaP NPs produced using different calcium salts and phosphate salts and the citrate chelation method at constant pH 8.5. Data are expressed as mean $\pm$ SD of at least 3 biological replicates of 3 replicates.

Calcium salts	Phosphate salts	Size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
CaCl ₂	NaH ₂ PO ₄	246 $\pm$ 6	0.18 $\pm$ 0.09	-27.95 $\pm$ 0.25
CaCl ₂	Na ₂ HPO ₄ .2H ₂ O	526 $\pm$ 30	0.21 $\pm$ 0.08	-23.45 $\pm$ 0.25
CaCl ₂	NH ₄ H ₂ PO ₄	397 $\pm$ 80	0.23 $\pm$ 0.06	-32.5 $\pm$ 0.1
CaCl ₂	(NH ₄) ₂ HPO ₄	425 $\pm$ 112	0.25 $\pm$ 0.06	-30.15 $\pm$ 1.55
Ca(NO ₃) ₂ .4H ₂ O	NaH ₂ PO ₄	360 $\pm$ 130	0.26 $\pm$ 0.19	-32.75 $\pm$ 0.85
Ca(NO ₃) ₂ .4H ₂ O	Na ₂ HPO ₄ .2H ₂ O	488 $\pm$ 99	0.20 $\pm$ 0.07	-26 $\pm$ 0.5
Ca(NO ₃) ₂ .4H ₂ O	NH ₄ H ₂ PO ₄	197 $\pm$ 110	0.22 $\pm$ 0.03	-31.2 $\pm$ 0.1
Ca(NO ₃) ₂ .4H ₂ O	(NH ₄) ₂ HPO ₄	232 $\pm$ 16	0.23 $\pm$ 0.02	-28.8 $\pm$ 0.5

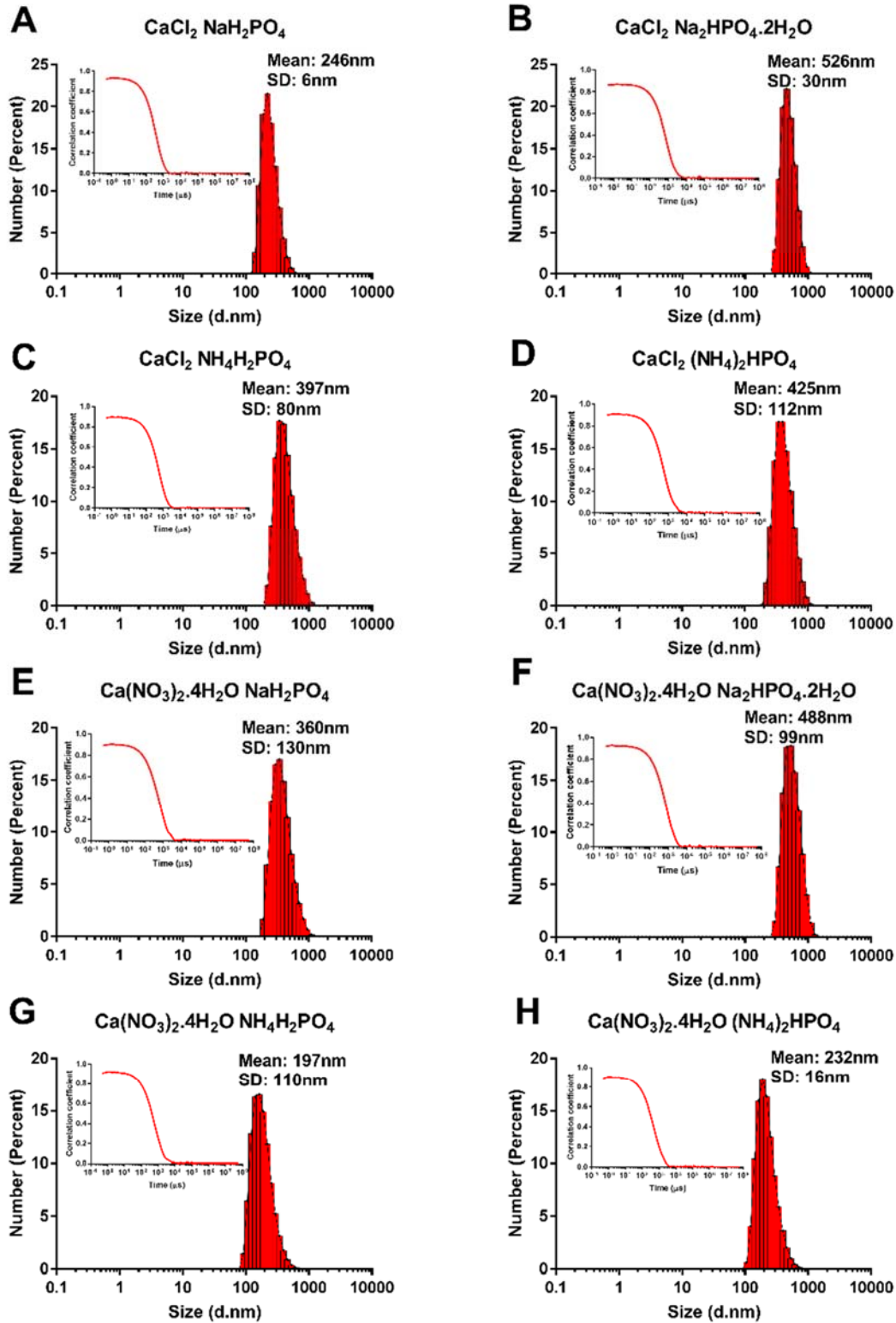


Figure 2. 12: The particle size and correlation curve of eight combinations at 40 °C for 6 h reaction time and constant pH 8.5 by citrate chelation method.

Data are expressed as mean $\pm$ SD of at least 3 biological replicates. (A) $\text{CaCl}_2 + \text{NaH}_2\text{PO}_4$, (B) $\text{CaCl}_2 + \text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, (C) $\text{CaCl}_2 + \text{NH}_4\text{H}_2\text{PO}_4$, (D) $\text{CaCl}_2 + (\text{NH}_4)_2\text{HPO}_4$, (E) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{NaH}_2\text{PO}_4$, (F) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, (G) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{NH}_4\text{H}_2\text{PO}_4$, (H) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + (\text{NH}_4)_2\text{HPO}_4$.

2.3.3.4 Characterisation of the CaP NPs

The efficiency of synthesis was determined by the percentage of ions remaining in the reaction supernatant following NP synthesis compared to the starting solutions. The free calcium ions and phosphate ions in the respective supernatants were analysed by atomic absorption spectrophotometry (AAS) and colourimetric assays, respectively. As can be seen in Table 2.8 the percentage of Ca^{2+} and PO_4^{3-} ions converted into CaP NPs was approximately 95% to 70% in the constant and drifting pH conditions (Table 2.8). The calculated calcium to phosphate ratios based on the amounts converted show that CaP NPs synthesised with CaCl_2 had a Ca/P ratio consistent with being hydroxyapatite (Ca/P ratio 1.67:1) using the constant and ‘drifting’ pH method. CaP NPs synthesised using $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and a constant pH also had a hydroxyapatite formula ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), however, with ‘drifting’ pH, the Ca/P ratio was consistent with being calcium deficient hydroxyapatite (Ca/P ratio 1.5-1.67:1).

Table 2. 8: Percent of calcium and phosphate converted to nanoparticles and the Ca/P ratio of CaP NPs synthesised under constant pH 8.5 and drifting pH 8.5 by citrate chelation method.

Group		Constant pH 8.5			Drifting pH 8.5		
		Ca ²⁺ converted ^a	PO ₄ ³⁻ converted ^a	Ca/P ratio	Ca ²⁺ converted ^a	PO ₄ ³⁻ converted ^a	Ca/P ratio
	NaH ₂ PO ₄	93.89%	94.75%	1.65	76.5%	77.8%	1.64
CaCl ₂ +	Na ₂ HPO ₄ ·2H ₂ O	93.83%	93.9%	1.67	70.2%	73.15%	1.60
	NH ₄ H ₂ PO ₄	92.99%	93.9%	1.65	79.1%	79.6%	1.66
	(NH ₄) ₂ HPO ₄	92.03%	93.55%	1.64	71.8%	75.2%	1.59
	NaH ₂ PO ₄	89.94%	93.5%	1.61	74.2%	80.2%	1.55
Ca(NO ₃) ₂ ·4H ₂ O +	Na ₂ HPO ₄ ·2H ₂ O	92.28%	91.5%	1.68	76.5%	80.46%	1.59
	NH ₄ H ₂ PO ₄	94.19%	90.35%	1.74	71.2%	76.1%	1.56
	(NH ₄) ₂ HPO ₄	93.95%	87.1%	1.80	68.0%	76.15%	1.50

^a percent of ion (calcium or phosphate) converted into nanoparticles.

2.3.3.5 Fourier transform infra-red spectroscopy (FTIR)

Fourier transform infra-red spectroscopy (FTIR) was carried out for structural analysis of the CaP NPs and to confirm the presence of OH and PO₄ functional groups in the NPs. Figure 2.13 shows the FTIR spectra of the CaP NPs in the presence or absence of citric acid. As can be seen from the Figure 2.13, the CaP NPs produced a FTIR spectrum with the main vibrational bands at 1022, 953, 603, 557 and 455 cm⁻¹ which correspond to PO₄³⁻ characteristics of a tetrahedral apatite structure.

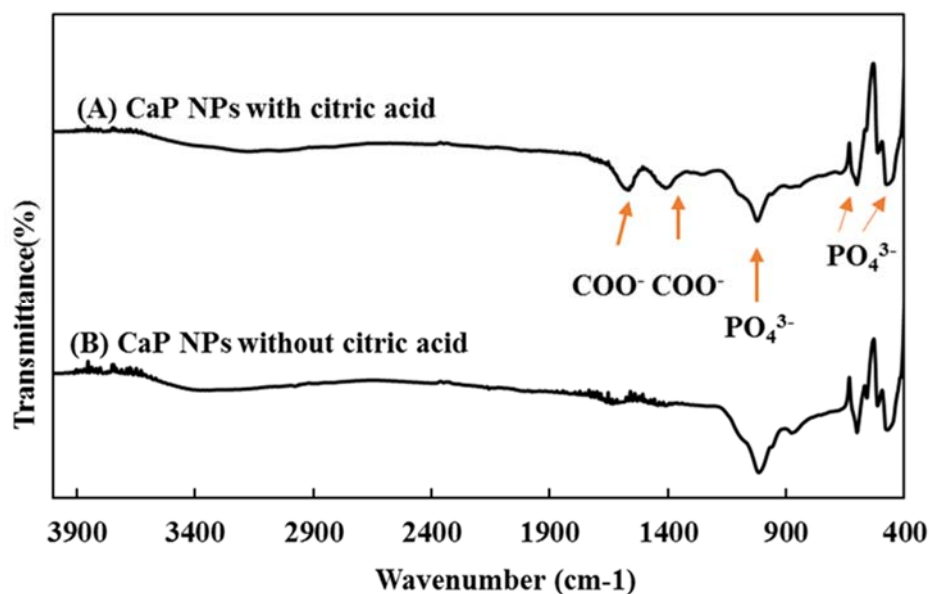


Figure 2. 13: FTIR spectra of the CaP NPs in the presence (A) or absence (B) of citrate acid (CaCl_2 , NaH_2PO_4) by citrate chelation method. The two patterns have been normalised.

Two strong peaks at 1571 and 1408 cm^{-1} for CaP NPs with citric acid can be assigned to citrate carboxylate groups COO^- (Figure 2.13A). Compared with CaP NPs prepared in the absence of citric acid (Figure 2.13B), the spectra results confirmed that citric ions were adsorbed onto the particle surface.

The particles prepared by the eight combinations at drifting/constant pH 8.5 were also characterised by FTIR (Supplementary Figure 2.7 and 2.8) and show the same PO_4^{3-} and COO^- vibrational band patterns for each CaP NPs prepared by drifting or constant pH 8.5. Overall, all the NPs prepared by using different calcium salts, phosphate salts and drifting or constant pH are likely to have similar CaP phase.

2.3.3.6 EM images

The morphologies of the CaP NPs were visualised by SEM and HIM (Figure 2.14), which shows that the CaP NPs from the smallest to the largest have a rod-like shape. Using $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{NH}_4\text{H}_2\text{PO}_4$, the obtained CaP NPs were much shorter and thinner compared to $\text{CaCl}_2 + \text{NaH}_2\text{PO}_4$ (Figure 2.14A and D), while the NPs were longer and broader from the combination of $\text{CaCl}_2 + \text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (Figure 2.14C and F).

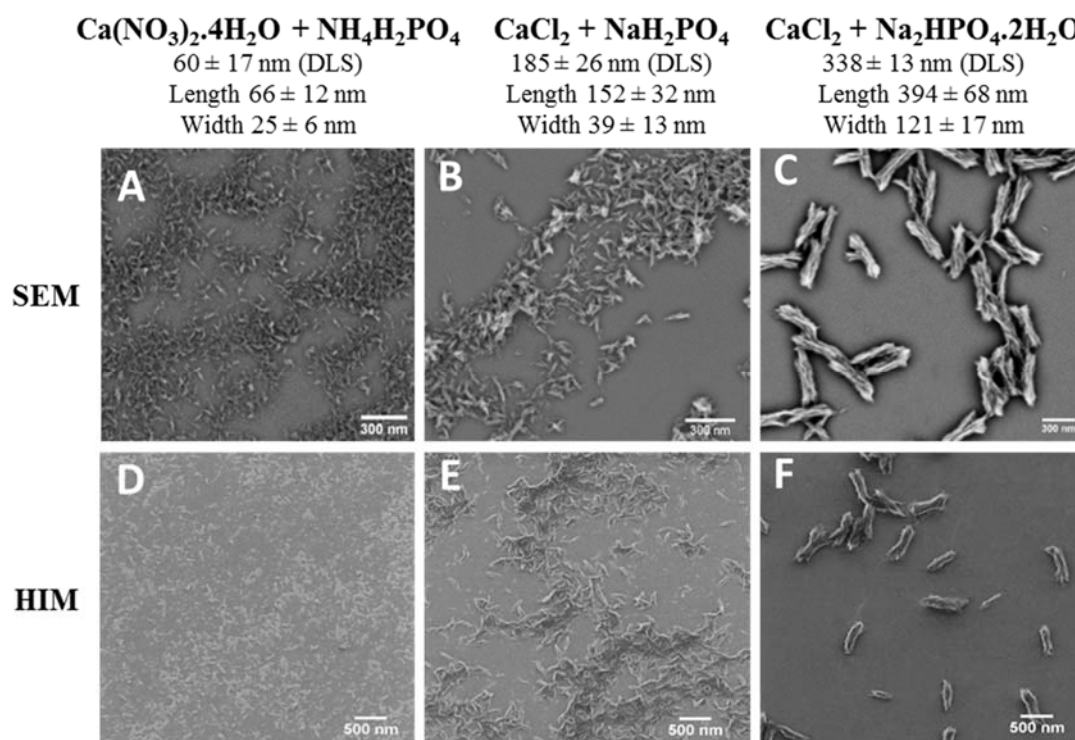


Figure 2. 14: SEM (A, B, C) and HIM (D, E, F) images of rod-like CaP NPs synthesised by citrate chelation method.

CaP NPs produced using $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{NH}_4\text{H}_2\text{PO}_4$ (A, D), $\text{CaCl}_2 + \text{NaH}_2\text{PO}_4$ (B, E), $\text{CaCl}_2 + \text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (C, F).

2.3.3.7 X-ray diffraction

X-ray diffraction was used to identify the types of chemicals compounds are present in the synthesised CaP NPs. To understand the phase of CaP NPs, an X-ray measurement was used to determine X-ray diffraction (XRD). The X-ray diffraction measurement can give several diffraction broad peaks, which can be assigned to CaP crystalline phase. The crystalline phase of different sizes of CaP NPs was identified by XRD. The diffraction peaks in X-ray spectra coincided with standard hydroxyapatite XRD pattern [219, 286, 287] and confirmed at the peak at $2\theta = 25.5^\circ, 31.5^\circ, 31.8^\circ$ and 32.5° for each CaP NP, indicating that these nanoparticles were hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (Figure 2.15) [288]. The samples did not show any other phase from the X-ray pattern.

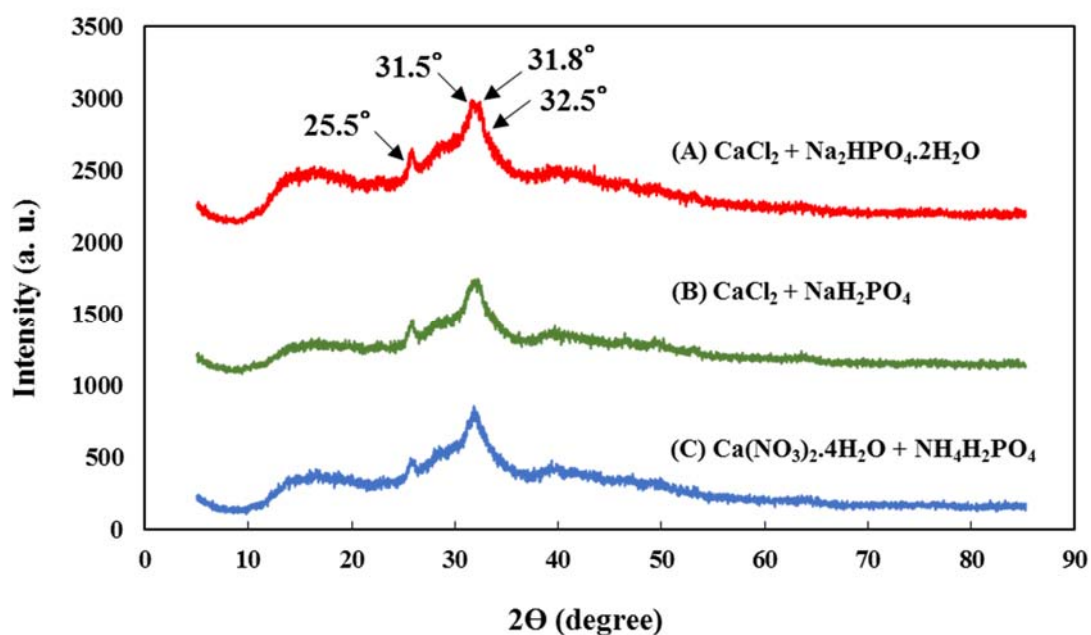
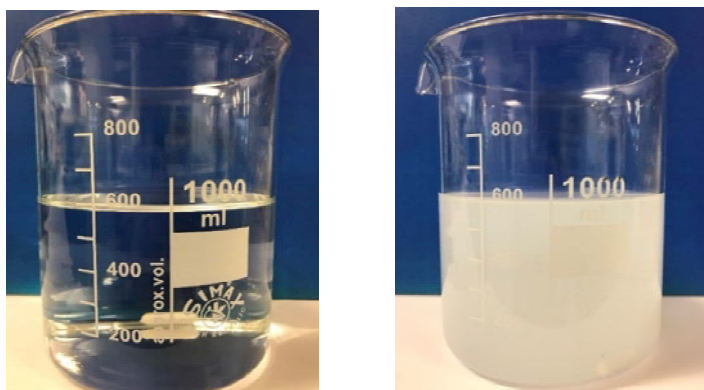


Figure 2. 15: X-ray diffraction pattern of various sizes of CaP NPs synthesised by citrate chelation method. CaP NPs produced using CaCl₂ + Na₂HPO₄·2H₂O (A), CaCl₂ + NaH₂PO₄ (B), Ca(NO₃)₂·4H₂O + NH₄H₂PO₄ (C). The three patterns have been normalised.

2.3.3.8 Process scale-up of the calcium citrate chelation method

An important part of nanoparticle synthesis for biomedical applications is that the process when scaled-up results in the production of the same material. The optimised calcium-citrate chelation method reaction volume was increased from 15 mL to 600 mL, a 40 fold increase as a proof of concept that the method would work on a large scale. Figure 2.16 shows that the scaled-up reaction produced CaP precipitate and Table 2.9 shows that the size, PDI and zeta potential of CaP NPs produced using ‘drifting’ pH and CaCl₂ + NaH₂PO₄ salts were not significantly different from the CaP NPs produced at a small scale. The batch to batch variation in particle diameter from different scales of volume is about ± 20%.



(A) Photographs of mixing reagents at room temperature

(B) Photographs of mixing reagents at 40 °C after 6 h

Figure 2. 16: Photograph of the 600 mL reaction synthesis scale; pre-heating (A) and post-heating (B).

Table 2. 9: Characteristics of the prepared CaP NPs by the citrate chelation method, by a small volume (15 mL) and large volume (600 mL) synthesis scale. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.

Parameters	Small volume (15 mL)	Large volume (600 mL)
Diameter (SEM and HIM, nm)	162 $\pm$ 32	185 $\pm$ 25
Diameter (DLS, nm)	159 $\pm$ 21	174 $\pm$ 19
Polydispersity Index (PDI)	0.12 $\pm$ 0.06	0.16 $\pm$ 0.03
Zeta potential (mV)	-18.7 $\pm$ 2.6	-20.1 $\pm$ 1.5
Molar Ca/P ratio	1.64	1.72
Batch-to-batch variation	$\pm$ 20 %	$\pm$ 14 %

2.4 Discussion

Calcium phosphate materials hold great promise as delivery vehicles for vaccines and/or drugs [176, 289]. They are biocompatible, production cost is low, and have pH-dependent solubility and can be produced as nanoparticles [159, 289]. However, current synthesis methods have limited CaP NPs use because of issues around reproducibility, aggregation and yield [290]. Here, a novel, efficient and reproducible synthesis method to produce CaP NPs of a defined size, with a low PDI, and can be easily scaled-up was developed.

CaP NPs were synthesised and compared by three different routes: a conventional precipitation method, a microemulsion method, and a citrate chelation method. Although conventional precipitation method is quick, cost-effective and widely used, this strategy is unsuitable to be used in vaccine studies [291] due to the severe aggregation, irregular size, shape and high polydispersity.

The inverse microemulsion method has the potential to mitigate the issues of the precipitation method as the water-in-oil emulsion is achieved by nano-sized water droplets that are dispersed in a continuous oil phase. The droplets are surrounded by surfactant and adsorbed on the surface of NPs are not only inhibiting particle aggregation but acting as a soft template to regulate the size and shape of NPs [292-294]. The synthesis of CaP NPs by this method is influenced by several process parameters, such as forming microemulsion and sonication time, centrifuge speed, pH, type of surfactant and the ratio of water to surfactant. Among these factors, the type of surfactant and the ratio of aqueous to surfactant play a critical role in particle synthesis and microemulsion droplet size.

Recently, Guo *et al.* [295] manufactured CaP NPs in the water-in-oil emulsion system using mixed surfactants, Triton X-100 and Tween-80. Their study demonstrates that the microemulsion route can significantly decrease particle size and degree of aggregation compared to the conventional precipitation method. Guo *et al.* also suggests that various size (10 - 90 nm) and shape of CaP NPs could be obtained by changing the weight ratio of Triton X-100 and Tween-80 [295]. Their study showed that the morphology and size of CaP NPs can be controlled by modifying different synthesis parameters. Similarly, Sing *et al.* [292] reported that the size, shape and crystallinity of CaP NPs could be controlled by the cationic surfactant Aliquot 336. By decreasing the concentration of Aliquot 336, the size of the particle can decrease from 129

to 47 nm and the shape of particle change from rod-like to spherical particles. Additionally, they demonstrated that an increase of Aliquot 336 concentration would give rise to high crystallinity of the CaP NPs [292]. Three different surfactants were used in the present study, which includes Triton X-100, Triton X-114 and Tween-20. Based on the DLS results, all three surfactants tested can be used to obtain nanosized particles, of which Triton X-114 resulted in the lowest PDI. It indicates that Triton X-114 plays an essential role in CaP NPs synthesis. Sun *et al.* investigated the influence of Triton X-114 on particle formulation [296]. First, the addition of surfactant reinforces the homogeneous mixing of the materials in the emulsion system. Furthermore, Triton X-114 can prevent excess particle aggregation after nanoparticles are formed. Most importantly, it can act as a soft template to regulate particle growth and nucleation, these effects of Triton X-114 may explain why the use of Triton X-114 resulted in CaP NPs with the lowest PDI.

In the microemulsion method, the molar ratio of water to surfactant (W_0) is a crucial factor in controlling the particle size [297, 298]. As shown in this study, the higher value of W_0 leads to the larger water droplets, which is consistent with the findings in this study and those reported by García *et al.* and Granata *et al.* [186, 297]. García and co-workers reported that the size of hydroxyapatite particles can increase as the value of W_0 increases. Similarly, Granata *et al.* demonstrated that the size of manganese carbonate nanoparticles increased from 10 nm to 100 nm by adjusting the water to surfactant molar ratio of 5 - 7.5 using the microemulsion method [297].

Although the microemulsion method resulted in nano-sized particles, the method had poor reproducibility with different reactions producing different sized NPs. Furthermore, large amounts of organic solvents such as cyclohexane would be required during synthesis and this limits its use to academic research [203]. Further, cyclohexane would need to be completely removed from the CaP NPs as it has acute neurotoxicity [299, 300]. Moreover, the yield is low, which in turn means it is more difficult to scale up for *in vivo* and clinical studies.

As previously mentioned, one of common features of the conventional precipitation and microemulsion method is that initial particle nucleation is poorly controlled. It was predicted that if particle nucleation could be initiated at one time, the produced nanoparticles may have a uniform size and low PDI. By chelating calcium with citric acid the phosphate solution could be added without the typical precipitation of CaP NPs, and heating the chelated-calcium and

phosphate solution would lead to destabilising the calcium-chelate and inducing nucleation of particles [301, 302]. To generate stable calcium chelator complexes, various molar ratios of CaCl_2 to chelators were investigated. Different chelation of organic compounds was used to control nucleation and particle growth. The results in this study demonstrate that only EDTA and citric acid can form stable calcium chelator complexes at room temperature. However, calcium-EDTA complexes did not result in CaP NPs precipitation even at 95 °C and this approach was abandoned. After mixing with calcium and citric acid solutions, the synthesis solution can form very stable calcium citrate complexes and would inhibit the precipitation of calcium and phosphate ions [303]. Another advantage of citric acid is that it is non-toxic and is a common food additive. With these considerations, citric acid is considered an attractive chelating agent in biomedical applications [304]. It has been reported that the solubility of CaP NPs can decrease with increasing temperature [301]. As shown in this study, nanosized CaP NPs can be obtained with citrate chelation method with a calcium phosphate phase typical of hydroxyapatite. This is in accordance with a previous publication demonstrating that nanosized CaP NPs produced from the mixtures of calcium/citrate/phosphate solutions were the hydroxyapatite, no matter using conventional heating or microwave heating [302]. Similarly, Tan *et al.* demonstrated that water-dispersible hydroxyapatite was obtained by using calcium nitrate, sodium citrate and triammonium phosphate as calcium salt, chelator and phosphate salt [305]. Again, this observation is consistent with the recent findings by Snihirova and co-workers who found that through heating the mixtures of calcium nitrate, citric acid and ammonia phosphate solution at 37 °C for 24 h, hydroxyapatite microparticles were produced [306].

This study demonstrates that the TEM, SEM and HIM images showed CaP particles are rod-like shaped. This finding is consistent with a recent paper that citrate chelation method resulted in rod-like shaped hydroxyapatite (length: 300–400 nm, width: 40–60 nm) [305]. Conversely, several other investigations have shown different shaped CaP NPs can be obtained through citrate-chelation method. Martins *et al.* demonstrated that the shape of CaP NPs was ranging from a micro-sized bundle-like to a nanosized needle-like shape [301]. Previous studies have also found that by using citric acid as a stabiliser the obtained hydroxyapatite microparticles were bowtie-shaped [306]. These discrepancies may be explained by the pH of reactions between these studies, as in one study Martins *et al.* demonstrated that small changes in solution pH from 7.4 to 8.5 resulted in various shaped CaP NPs [301]. The formed CaP particles were rod-like shaped for all of the calcium and phosphate salt combinations used and were directly

used as vaccine carriers because of good size range and reproducibility. It has been reported that for hydroxyapatite, size instead of shape is the most important factor that affects vaccine efficacy [234].

An important aspect of NP synthesis is the stability of the formulation and the surface charge of nanoparticles determined by zeta potential gives an indication of the stability of NPs in the suspension [285, 307]. Generally the relationship of the NP size stability with zeta potential values is classified as: $\pm 0 - 10$ mV, highly unstable; $\pm 10 - 20$ mV, relatively stable; $\pm 20 - 30$ mV, moderate stable; $> \pm 30$ mV highly stable [285, 307]. The zeta potential of CaP NPs prepared by citrate chelation method was within the moderately stable range of $-20 - 30$ mV. The conventional precipitation method produced CaP NPs with relative stability, while the micro-emulsion method produced particles with high stability. The negative charge associated with all CaP NPs would be due to the citrate ions adsorbed on the particle surface [221, 308]. Although the citrate chelation method produced NP with moderate stability as determined by zeta potential, the NPs were found not to aggregate over 30 days indicating they were highly stable. Interestingly, silica particles are shown to be extremely stable but have a zeta potential of 0 mV [309], thus it is important to conduct time stability studies as zeta potential is only a guide to NP emulsion stability.

In CaP NPs synthesis, typically two calcium salts (calcium chloride and calcium nitrate) and four phosphate salts (monosodium phosphate, disodium hydrogen phosphate, ammonium phosphate monobasic and ammonium phosphate dibasic) are used as starting materials. Using the optimised citrate chelation method for the synthesis of CaP NPs the influence of the different salts on CaP formulation and size was first investigated. While several reports used different calcium and phosphate salts for the CaP NPs synthesis [301, 302, 305, 306], there was no paper compared the effect of these had on NP size and zeta potential. Here, current study analyses eight combinations of calcium and phosphate to determine the optimal conditions for CaP NP synthesis. Each calcium salt and phosphate salt combination resulted in CaP NPs of distinct sizes with low PDI's. This is probably because different salts would have an effect on nanoparticle nucleation rate and reaction speed during synthesis [310-312]. All the formed CaP NPs displayed the same phase, indicating that they might be all the hydroxyapatite. Additionally, the values of zeta potential did not change with different combinations. The reason for this may be due to the similar negative surface charge by the same concentration of citrate ions.

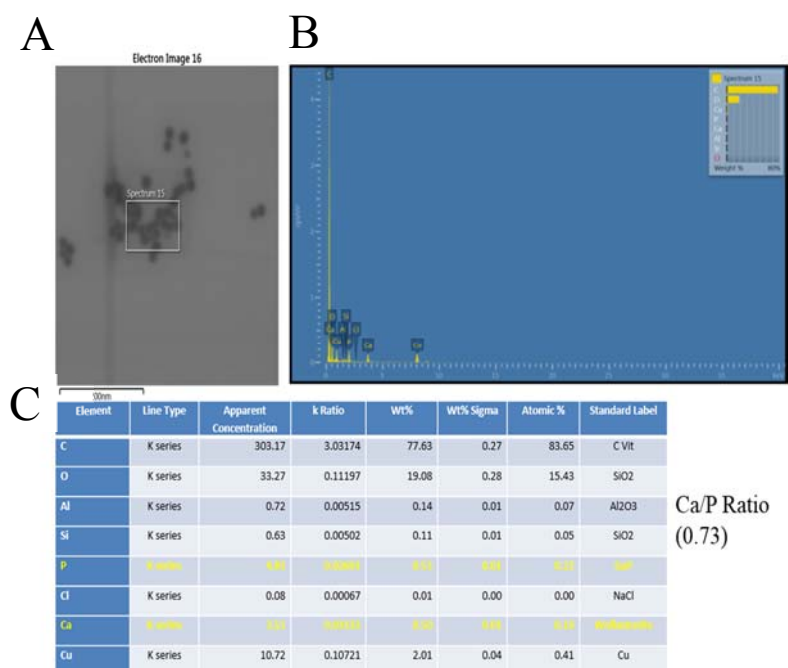
2.5 Conclusion

In conclusion, a novel CaP NPs preparation method has been developed, which used citric acid as a chelating agent to prevent initiation of nucleation at room temperature. Raising the temperature to 40°C resulted in nucleation of calcium and phosphate at a specific point leading to CaP NPs of a defined size which was reproducible. This method displayed advantages over the conventional precipitation method and microemulsion method, in that the method of synthesis was able to control the size of NPs, it is straight forward, highly reproducible and scalable. Furthermore, with this strategy, various size of particles (50 ~ 600 nm) can be obtained by using different calcium or phosphate salts alone with having a drifting or constant pH (Table 2.10). The influence of different types of calcium and phosphate sources on the formation of CaP NPs is a unique finding. Various sizes of homogenous nanoparticles with different calcium and phosphate sources can be obtained. Overall, in this chapter, a novel citrate chelating method was developed, which can be a valuable, reliable and effective strategy in nanoparticle synthesis.

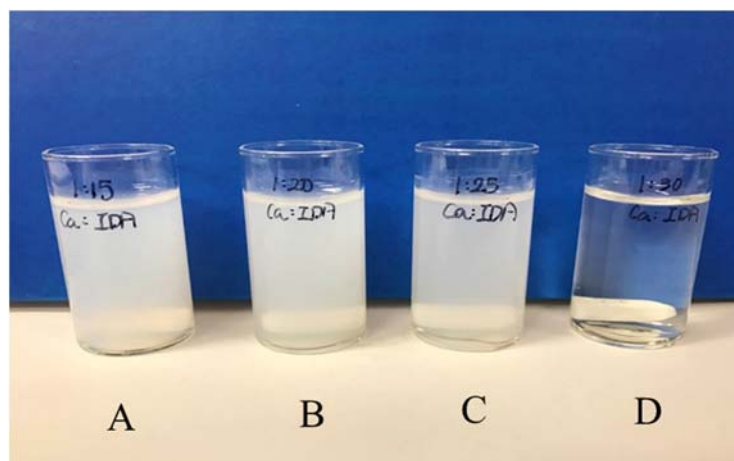
Table 2. 10: Summary of various size range synthesised by different material and pH via citrate chelation method.

Nanoparticle size range (nm)	pH and starting material
50 ~ 100	Drifting pH 8.5 $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{NaH}_2\text{PO}_4$; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{NH}_4\text{H}_2\text{PO}_4$; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + (\text{NH}_4)_2\text{HPO}_4$
100 ~ 200	Drifting pH 8.5 $\text{CaCl}_2 + \text{NH}_4\text{H}_2\text{PO}_4$; $\text{CaCl}_2 + \text{NaH}_2\text{PO}_4$; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{Na}_2\text{PHO}_4 \cdot 2\text{H}_2\text{O}$ Constant pH 8.5 $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{NH}_4\text{H}_2\text{PO}_4$
200 ~ 300	Drifting pH 8.5 $\text{CaCl}_2 + (\text{NH}_4)_2\text{HPO}_4$ Constant pH 8.5 $\text{CaCl}_2 + \text{NaH}_2\text{PO}_4$; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + (\text{NH}_4)_2\text{HPO}_4$
300 ~ 400	Drifting pH 8.5 $\text{CaCl}_2 + \text{Na}_2\text{PHO}_4 \cdot 2\text{H}_2\text{O}$ Constant pH 8.5 $\text{CaCl}_2 + \text{NH}_4\text{H}_2\text{PO}_4$; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{NaH}_2\text{PO}_4$
400 ~ 500	Constant pH 8.5 $\text{CaCl}_2 + (\text{NH}_4)_2\text{HPO}_4$; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{Na}_2\text{PHO}_4 \cdot 2\text{H}_2\text{O}$
500 ~ 600	Constant pH 8.5 $\text{CaCl}_2 + \text{Na}_2\text{PHO}_4 \cdot 2\text{H}_2\text{O}$

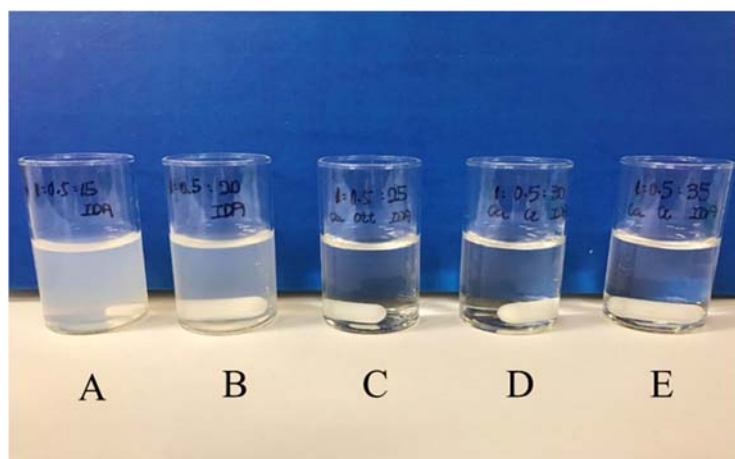
2.6 Supplementary Information



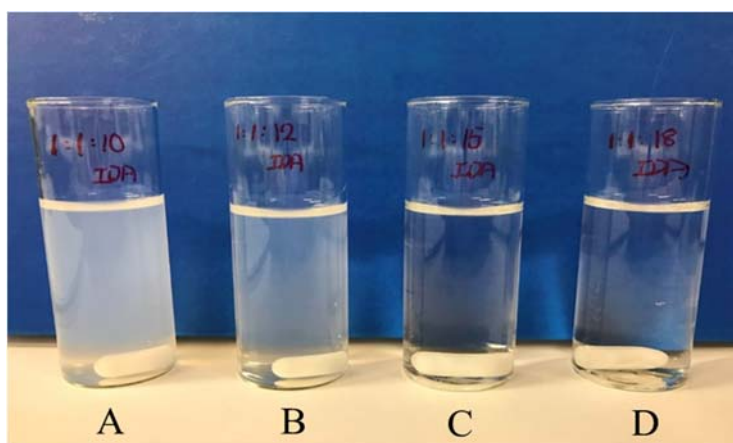
Supplementary Figure 2. 1: Electron dispersion spectroscopy (EDS) microanalysis of OVA-loaded CaP NPs synthesised by microemulsion method. (A) SEM image, (B) EDS spectrum, (C) elemental analysis.



Supplementary Figure 2. 2: Different molar ratios of CaCl₂ to IDA: A (1:15); B (1:20); C (1:25); D (1:30) and NaH₂PO₄ at room temperature.



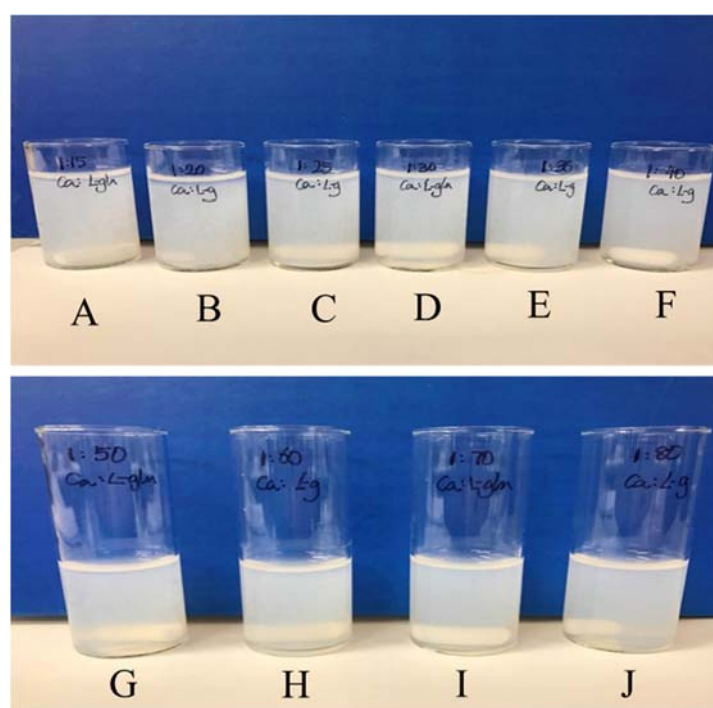
Supplementary Figure 2. 3: Different molar ratios of CaCl₂ to citric acid to IDA: A (1:0.5:15); B (1:0.5:20); C (1:0.5:25); D (1:0.5:30); E (1:0.5:35) and NaH₂PO₄ at room temperature.



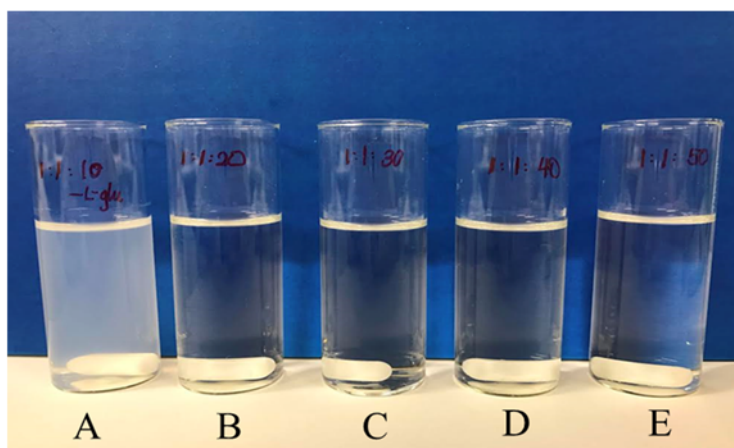
Supplementary Figure 2. 4: Different molar ratios of CaCl₂ to citric acid to IDA: A (1:1:10); B (1:1:12); C (1:1:15); D (1:1:18) and NaH₂PO₄ at room temperature.

Supplementary Table 2. 1: Effect of different molar ratios of calcium: citric acid: IDA on CaP NPs. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.

Group	Reaction time (min)	Particle size (nm)	Zeta potential (mV)	PDI
calcium: IDA = 1:30	30	568 $\pm$ 277	-6.4 $\pm$ 1.4	0.87 $\pm$ 0.26
	60	898 $\pm$ 309	-6.4 $\pm$ 1.4	0.52 $\pm$ 0.38
calcium: citric acid: IDA = 1:0.5:25	30	1267 $\pm$ 924	-10.1 $\pm$ 0.97	0.62 $\pm$ 0.34
	60	1063 $\pm$ 827	-10.1 $\pm$ 0.97	0.78 $\pm$ 0.28
calcium: citric acid: IDA = 1:1:15	30	1133 $\pm$ 565	-15.3 $\pm$ 0.78	0.25 $\pm$ 0.21
	60	728 $\pm$ 106	-15.3 $\pm$ 0.78	0.16 $\pm$ 0.08



Supplementary Figure 2. 5: Different molar ratios of CaCl₂ to L-glutamic acid: A (1:15); B (1:20); C (1:25); D (1:30); E (1:35); F (1:40); G (1:50); H (1:60); I (1:70); J (1:80) and NaH₂PO₄ at room temperature.



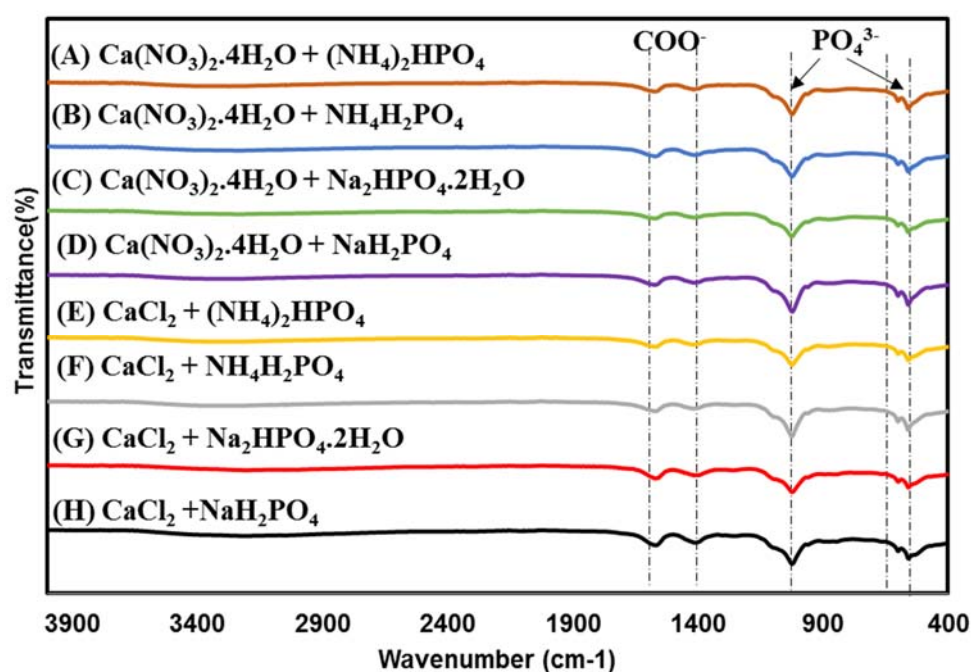
Supplementary Figure 2. 6: Different molar ratios of CaCl_2 to citric acid to L-glutamic acid: A (1:1:10); B (1:1:20); C (1:1:30); D (1:1:40); E (1:1:50) and NaH_2PO_4 at room temperature.

Supplementary Table 2. 2: Effect of different molar ratios of calcium: citric acid: L-glutamic acid on CaP NPs. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.

Group	Reaction time (min)	Particle size (nm)	Zeta potential (mV)	PDI
1:1:10	30	2667 $\pm$ 856	-17.1 $\pm$ 0.86	0.59 $\pm$ 0.28
	60	4257 $\pm$ 675	-13.5 $\pm$ 0.73	0.69 $\pm$ 0.30
1:1:20	30	1325 $\pm$ 437	-13.3 $\pm$ 0.67	0.47 $\pm$ 0.23
	60	865 $\pm$ 397	-11.7 $\pm$ 0.69	0.67 $\pm$ 0.19

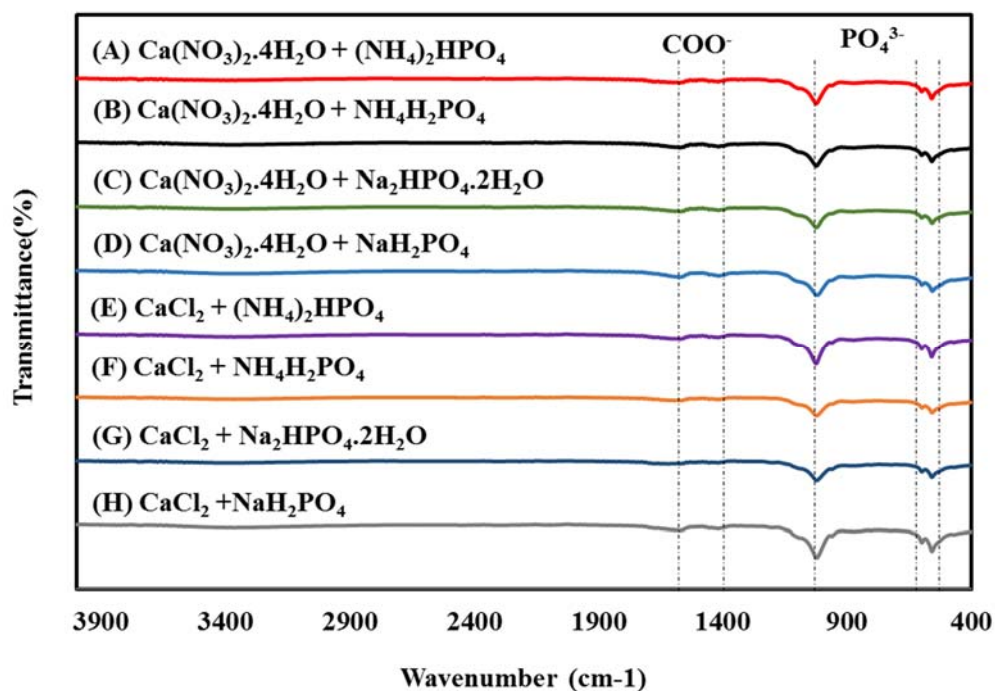
Supplementary Table 2. 3: Comparison of DLS and SEM images on CaP NPs by citrate chelation method at drifting pH 8.5. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.

Group	DLS number distributions	SEM analysis	
		Length	Width
Ca(NO ₃) ₂ ·4H ₂ O, NH ₄ H ₂ PO ₄	67 $\pm$ 15	66 $\pm$ 12	25 $\pm$ 6
CaCl ₂ , NaH ₂ PO ₄	159 $\pm$ 21	152 $\pm$ 32	39 $\pm$ 7
CaCl ₂ , Na ₂ HPO ₄ ·2H ₂ O	335 $\pm$ 45	434 $\pm$ 68	121 $\pm$ 17



Supplementary Figure 2. 7: FTIR spectra of the CaP NPs synthesised in different combinations at drifting pH 8.5 by citrate chelation method.

(A) Ca(NO₃)₂·4H₂O + (NH₄)₂HPO₄, (B) Ca(NO₃)₂·4H₂O + NH₄H₂PO₄, (C) Ca(NO₃)₂·4H₂O + Na₂HPO₄·2H₂O, (D) Ca(NO₃)₂·4H₂O + NaH₂PO₄, (E) CaCl₂ + (NH₄)₂HPO₄, (F) CaCl₂ + NH₄H₂PO₄, (G) CaCl₂ + Na₂HPO₄·2H₂O, (H) CaCl₂ + NaH₂PO₄. The eight patterns have been normalised.



Supplementary Figure 2. 8: FTIR spectra of the CaP NPs synthesised in different combinations at constant pH 8.5 by citrate chelation method.

(A) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + (\text{NH}_4)_2\text{HPO}_4$, (B) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{NH}_4\text{H}_2\text{PO}_4$, (C) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, (D) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O} + \text{NaH}_2\text{PO}_4$, (E) $\text{CaCl}_2 + (\text{NH}_4)_2\text{HPO}_4$, (F) $\text{CaCl}_2 + \text{NH}_4\text{H}_2\text{PO}_4$, (G) $\text{CaCl}_2 + \text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, (H) $\text{CaCl}_2 + \text{NaH}_2\text{PO}_4$. The eight patterns have been normalised.

Chapter 3

Optimisation of antigen loading of calcium phosphate nanoparticles

3.1 Introduction

For nanoparticles (NPs) to be effective carriers and delivery vehicles of antigens to a desired site, there needs to be a strong interaction between NPs and the antigen. In general, there are four approaches of antigen attachment to NPs: adsorption; encapsulation; mix and chemical conjugation [33]. Adsorption and encapsulation are the most common methods used to load antigen onto CaP NPs and is normally achieved by charge attraction and hydrophobic interaction [214]. Electrostatic attraction between antigen and CaP NPs plays the most important role in surface adsorption. For example, the adsorption of bovine serum albumin (BSA) on hydroxyapatite (HAP) arises from electrostatic interaction between Ca^{2+} and PO_4^{3-} anions of HAP with the COO^- and NH_4^+ cations of BSA protein [206, 215-217]. Chemical conjugation has also been used to incorporate antigenic molecules such as proteins and peptides via a covalent linker to the surface of NP [222]. Ramachandran *et al.* modified the surface of CaP NPs through conjugation with PEG by activating the phosphate group using carbodiimide chemistry for oral insulin delivery, leading to increased stability [207]. Kozlova *et al.* synthesised CaP NPs coated with a silica shell that were covalently functionalised by thiol/amino groups or salinisation, leading to stable molecules/NP construct [30]. Antigen-conjugated NPs are thought to be strong candidates for mucosal immunisation as the antigen is protected to some degree from degradation by being cross-linked on and to the NP surface [102].

Layer-by-layer (LbL) technology offers a promising way to formulate multilayered thin films and coatings on a nanometer scale that has been widely applied in several biomedical fields, such as drug delivery systems and nano bioreactors [313-318]. The type of polymer used in an LbL approach is dependent on the core nanoparticle size, shape, and charge. Typically, polymers, proteins, or small molecules are assembled by LbL using electrostatic interaction [314]. Recently, the LbL approach has been widely used in the delivery of siRNA [315, 319], DNA [313], protein [30, 320, 321], and peptides [322]. During LbL approach several parameters, including the type of polymer, polymer concentration, the ion strength of the solution, and the protein cross-linking, need to be optimised to avoid nanoparticle aggregation in aqueous and media solutions [323]. Although the use of LbL technology has been investigated in many studies [313, 316, 323, 324], there remains significant issues during the formulation process that lead to the aggregation of NPs, thus a specific LbL strategy needs to be optimised for each application.

Several cationic nanoparticles, formulated from amino acids, polylysine, chitosan, amine-dendrimers and polyethyleneimine (PEI), have been extensively studied as protein delivery systems [325]. Among these polymers, PEI is widely used as it is readily soluble in water, is cost-effective, and has both high transfection efficiency and cellular uptake efficiency. A disadvantage of PEI is that it has a degree of cytotoxicity and can cause severe problems for clinical applications, it has been reported that PEI with lower molecular weights incorporated into the delivery vehicle or NP can reduce or eliminate cell toxicity [326].

Typically an electrostatic interaction does not result in a highly stable bond between the antigen protein and the nanoparticle, and this may lead to protein detachment from the nanoparticle over time or if the surrounding environment changes, such as with injection into an animal or human [33, 214]. Other factors that would affect NP-cell interactions are the size of the NP, and the formation of a protein corona on the nanoparticle surface when the NP vaccine is exposed to body fluids. It is reported that the NP protein corona forms immediately after the particle is suspended in a protein containing medium which may hinder the NP biological activity [327]. Thus, it is necessary to have a better understanding of plasma protein adsorption on the particle surface, as these proteins may affect NP-cell interactions.

In this study the LbL approach of nanoparticle formulation was used and optimised to avoid aggregation, and to enhance stability. Additionally, the effects of various commonly used media and serum on particle size and zeta potential and protein corona were evaluated. Chicken egg ovalbumin (OVA) was used as a model antigen to investigate the LbL strategy. The investigations in this chapter will provide valuable knowledge regarding the effect of environmental influences on the synthesis of nanomaterials. Moreover, this LbL approach offers significant potential as a useful template for protein delivery and vaccine generation.

3.2 Materials and Methods

3.2.1 Preparation of the CaP NPs

The citrate chelation synthesis method for CaP NPs was performed as described in Chapter 2. Unless otherwise stated, MilliQ water was purified through a 0.22 μm filter (Merck-Millipore, Victoria, Australia) was used to prepare chemical solutions and to disperse CaP NPs. All nanoparticle analyses were conducted at room temperature.

3.2.2 Preparation of OVA-loaded CaP NPs via encapsulation

Ovalbumin (OVA, lyophilised powder, 98%, Sigma-Aldrich, NSW, Australia) encapsulation into CaP NPs was performed using the citrate-chelation method; where by OVA (1.2 mg/mL) was mixed with CaCl_2 (0.05 M, pH = 9.0) solution only, or OVA (0.6 mg/mL) was mixed with both the CaCl_2 (0.05 M, pH = 9.0) and NaH_2PO_4 (0.03 M, pH = 9.0), respectively, or OVA (1.2 mg/mL) was mixed with NaH_2PO_4 (0.03 M, pH = 9.0) solution only and the formation of CaP-OVA NPs conducted using the process described in Chapter 2. The efficiency of OVA encapsulation in CaP NPs was determined by firstly pelleting the CaP-OVA NPs by centrifugation ($10,000 \times g$, 5 min, 4 $^\circ\text{C}$) in an ultracentrifuge (Avanti J-25I Centrifuge, Beckman Coulter, NSW, Australia). The collected NPs were then washed and resuspended in 2 mL MilliQ water. The protein loading efficiency was determined by placing OVA-loaded particles in HCl (1 M, pH = 3.0, 1 h), and the released protein in solution was measured by the BCA protein assay kit according to the manufacturer's instructions (BCA assay kit, Thermo Fisher Scientific, NSW, Australia). The protein loading efficiency was quantified as (OVA in the nanoparticles/total protein added) $\times$ 100%. A standard curve using OVA was used to calculate OVA concentration in the NPs (concentration range 0 ~ 2000 $\mu\text{g/mL}$, $R^2 = 0.99$).

3.2.3 SDS-PAGE analysis

OVA-loaded CaP NPs were evaluated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). To examine OVA in CaP NPs, the NPs were dissolved in HCl (1 M, pH = 3.0, 1 h) to release OVA protein. The extracted protein was added to loading dye ((200 mM Tris-HCl (pH 6.8, Sigma-Aldrich, NSW, Australia), 400 mM dithiothreitol (DTT, Invitrogen, USA), 8% (w/v) sodium dodecyl sulfate (SDS, Sigma-Aldrich, NSW, Australia), 0.4% (w/v) bromophenol blue (Sigma-Aldrich, NSW, Australia), 40% (v/v) glycerol (PubChem, USA)) and were heated at 90 $^\circ\text{C}$ for 10 min. The samples were then electrophoresed

on 4 ~ 12% NuPAGE Bis-Tris pre-cast gel using MOPS running buffer (Invitrogen, USA) for 50 min at 180 V. The gel was stained using Coomassie blue G-250 staining (Thermo Fisher Scientific, NSW, Australia). The protein bands were analysed using ImageQuant software (GE Healthcare, CA). Band detection was conducted manually. All experiments were performed in triplicate.

3.2.4 Synthesis of stable, positively charged CaP NPs

To reverse the surface charge of CaP NPs produced via the citrate chelation method, a variety of amino acids L-lysine, L-arginine, L-lysine ethyl ester, and L-arginine ethyl ester and PEI polymers were used and their effect on both particle size and zeta potential determined. To aqueous L-lysine, L-arginine, lysine ethyl ester and arginine ethyl ester at various concentrations (6.25, 12.50, 25.00, 50.00 mM), pH adjusted to 8.0 by an ammonia solution ($\text{NH}_3\cdot\text{H}_2\text{O}$, Chem-Supply, 30%). Synthesised CaP NPs (3 mg/mL) were added dropwise to an equal volume of the amino acid. The CaP NPs/amino acid solutions were incubated under agitation (1 h, RT) using a stir/hot plate at setting 1 ((IEC model (2093-001), NSW, Australia)) then centrifuged ($10,000 \times g$, 5 min, 4 °C). The particles were washed three times and dispersed in MilliQ water, then characterised by DLS as described in Chapter 2. All amino acids were supplied by Sigma-Aldrich (NSW, Australia).

In another approach to reverse the surface charge of CaP NPs, different molecular weights (MWs) of polyethyleneimine (PEI, Sigma-Aldrich, NSW, Australia) were used. The following PEI polymers were used; Linear PEI (MW = 0.423 kDa, 1.8 kDa, 2.5 kDa, 5.0 kDa, 10.0 kDa), Branched PEI (MW = 25.0 kDa) (Sigma-Aldrich, NSW, Australia), Branched PEI (MW = 10.0 kDa) (Polysciences, CA). Various concentration of PEI polymers were added dropwise to an equal volume of CaP NPs (3 mg/mL). The CaP NPs/PEI solutions were incubated under agitation at setting 1 (1 h, RT) and the NPs collected by centrifugation ($10,000 \times g$, 5 min, 4 °C). After washing with MilliQ water ($3 \times$, $10,000 \times g$, 5 min, 4 °C) to remove any unbounded PEI, the particles were dispersed in MilliQ water and characterised by DLS as described in Chapter 2.

3.2.5 Optimisation of OVA adsorption onto CaP-PEI NPs

OVA was adsorbed onto the surface of CaP-PEI NPs by electrostatic interaction. Various concentrations of OVA (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 10.0, 12.0, 14.0, 16.0 mg/mL) in MilliQ water (pH 6.5) were used to optimise adsorption. To obtain monodispersed NPs, equal volumes of NP suspensions (1.5 mg/mL) were added dropwise to OVA under constant agitation using a stir/hot plate at setting 1 ((IEC model (2093-001), NSW, Australia)) and incubated for 30 min at room temperature. The NPs were then pelleted by centrifugation ($10,000 \times g$, 5 min, 4 °C) and dispersed in MilliQ water and size, PDI and zeta potential determined as described in Chapter 2.

To study protein loading efficiency and capacity, OVA-loaded particles were dissolved in HCl (1 M, pH = 3.0, 1 h), and the released protein was measured by the BCA protein assay kit according to the manufacturer's instructions. The protein loading efficiency was quantified as (OVA in the nanoparticles/total protein added) $\times$ 100%.

3.2.6 Optimisation of cross-linking OVA on CaP NPs

Electrostatic adsorbed OVA on the CaP-PEI NPs were cross-linked using either GA (glutaraldehyde solution 50 wt. % in H₂O, Sigma, NSW, Australia), or DMTMM (4-(4,6-dimethoxy-1,3,5-triazine-2-yl)-4-methylmorpholinium chloride, Sigma, NSW, Australia), or bis(sulfosuccinimidyl)suberate (BS³, Thermo Fisher Scientific, NSW, Australia).

In order to produce GA and DMTMM cross-linking, the CaP-PEI-OVA NPs were redispersed in 200 mM sodium phosphate buffer (pH 7.5) by centrifugation ($10,000 \times g$, 5 min, 4 °C). Different molar ratios of GA/OVA (0.11, 0.33) or DMTMM/OVA (2.7, 4.2) were added to the nanoparticle suspension (0.75 mg/mL) with under constant stirring with a magnetic stirrer at 300 rpm, then incubated at RT for 2 h with constant shaking. After incubation, the nanoparticles were harvested by centrifugation ($10,000 \times g$, 5 min, 4 °C), washed three times with MilliQ water and dispersed in MilliQ water. The nanoparticle size, PDI and zeta potential was determined as described in Chapter 2.

For the BS³ cross-linking, CaP-PEI-OVA NPs (0.75 mg/mL) were suspended in 5 mM borate buffer (500 μ L, pH 8.5) followed by the addition of 10 μ L of freshly prepared 50 mM BS³ in MilliQ water. The solution was then incubated under constant stirring with a magnetic stirrer

at 300 rpm (30 min, RT). After incubation, the reaction was quenched by adding 250 μ L 150 mM glycine (pH 7.5, Sigma-Aldrich, NSW, Australia) to a final concentration of 50 mM glycine and incubated at RT for 15 minutes. The nanoparticles were harvested by centrifugation ($10,000 \times g$, 5 min, 4 °C). The collected NPs were washed three times with MilliQ water and dispersed in MilliQ water. The nanoparticle size, PDI and zeta potential was determined as described in Chapter 2.

To validate the chemical cross-linking with the cross-linked CaP-PEI-OVA NPs were analysed by mass spectrometry as previously reported [328]. Briefly, the cross-linked CaP-PEI-OVA NPs pellets were resuspended in PBS containing 10 mM DTT and incubated at 37 °C for 1 h. The solution was then alkylated with 25 mM iodoacetamide (30 min, RT, no light). After incubation, 25 mM NH_4HCO_3 was used to dilute the solution to a final 1 M urea concentration. The solutions were digested with trypsin (10 ng/ μ L) at 37 °C overnight. Millipore μ C18 Zip-tips (Sigma-Aldrich, NSW, Australia) were used to desalt and concentrate the digested peptides according to the manufacturer's instructions. Orbitrap LC-MS was used to analyse the eluted samples. The cross-link search was conducted using pLink V 1.23, which downloaded from <http://pfind.ict.ac.cn/software/pLink> (Fan SB, 2015 Curr Protoc Bioinformatics) [329] with the following settings. Cross-linker = BS^3 , Enzyme = trypsin, spectra format = mgf, missed cleavages = 3, spectra type = HCD, MS tolerance = 5 ppm, variable modification = oxidation_M, fixed modification = carbamidomethyl_C.

3.2.7 Cell culture and cell cytotoxicity assay

Human embryonic kidney (HEK) and rat hepatoma (H4IIE) cell lines were used to test nanoparticle cytotoxicity. The HEK and H4IIE cells were cultured in complete media ((Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine)) under 5% CO_2 at 37 °C. DMEM, FBS, penicillin/streptomycin, L-glutamine, trypsin were obtained from Life Technologies. Cell cytotoxicity was measured by the lactate dehydrogenase (LDH, Promega, NSW, Australia) and CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega, NSW, Australia) methods (performed according to the manufacturer's directions). HEK and H4IIE cells were seeded in 96 well plates at a concentration of 5×10^3 cells per well in 200 μ L of complete DMEM. After overnight incubation to attain 80 ~ 90% confluency, the supernatant was removed and replaced with 200 μ L complete media containing nanoparticles and incubated

for 90 min at 37 °C. After treatment, 50 µL of media from each well was transferred to a 96 well plate and measured for LDH. For the MTS assays, MTS was diluted in 100 µL fresh cell media and added to the cells after removing NPs in suspension, then assessed by MTS kits according to the manufacturer's protocol.

3.2.8 The size stability of nanoparticles vaccine in serum containing media

Non-cross-linked and cross-linked CaP-PEI-OVA NPs were resuspended in either DMEM (Dulbecco's Modified Eagle's Medium, 10% FBS), SBF (Simulated Body Fluid with 55% human serum) or PBS buffer (Sigma, D8537, NSW, Australia) and incubated at RT for 6 hours. After incubation, the nanoparticles were harvested by centrifugation ($10,000 \times g$, 5 min, 4 °C) and washed three times (MilliQ water) and dispersed in MilliQ water. The nanoparticle size, PDI and zeta potential was determined as described in Chapter 2. Human serum was obtained from healthy individuals from Melbourne Dental School. Blood from the donor was centrifuged ($800 \times g$, 5 min, 25 °C) and discard blood cells. The supernatant plasma was stored at - 80 °C.

3.2.9 Identification of serum proteins absorbed onto CaP-PEI-OVA NPs

For protein corona analysis, non-cross-linked and cross-linked CaP-PEI-OVA NPs were incubated with cell culture media (DMEM with 10% FBS) or simulated body fluid (SBF) buffer with 55% human serum (6 h, RT). The preparation of SBF buffer was used as previously published [330, 331]. The NPs were harvested by centrifugation ($10,000 \times g$, 5 min, 4 °C) and washed three times (MilliQ water) and were dispersed in MilliQ water by sonication for 1 min by a probe sonicator (Cole-Parmer, at 25% amplitude, pulse on for 1 s and pulse off for 1 s).

The proteins adsorbed on the particle surface were analysed by SDS-PAGE and mass spectrometry. SDS-PAGE method was performed as described in section 3.2.3. Mass spectrometry was used. Tryptic in-gel digested samples were analysed by mass spectrometry as previously described [328, 332, 333]. Briefly, protein gel bands were excised, then washed in 50 mM NH_4HCO_3 /ethanol 1:1 solution. After reduction with DTT and alkylation with iodoacetamide, in-gel digestion by trypsin was conducted at 37 °C overnight using trypsin (10 ng/µL) (Promega, NSW, Australia). The tryptic peptides were analysed using LTQ orbitrap Elite mass

spectrometer with a nano ESI source interfaced with an ultimate 3000 RSLC nano-HPLC (Go-rasia, DG 2016, Plos Pathogens) [334]. Protein identification was analysed by Mascot MS/MS ions search using the following settings: Enzyme = trypsin, protein database = Swiss-Prot, fixed modification = carbamidomethyl (Cys), MS/MS tolerance = 0.2 Da, variable modification = Oxidation (Met), MS tolerance = 10 ppm, missed cleavages = 1.

3.2.10 Statistical analysis

GraphPad Prism 5 software was used to analyse data in experiments. One-way ANOVA and student T-test were used to determine P and a $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Ovalbumin encapsulation into CaP NPs

Initially, encapsulation of OVA into CaP NPs was attempted using the citrate-chelation method. OVA was either incorporated in the calcium salt buffer, or phosphate salt buffer or distributed equally in both buffers. As shown in Table 3.1, the formation of CaP NPs loaded with OVA under different buffer conditions did not result in a significant change in the size of the NPs as compared to non-OVA loaded CaP NPs ($P > 0.05$). Furthermore, the PDI of OVA-loaded particles were similar to non-OVA loaded CaP NPs ($P > 0.05$). However, the zeta potential of OVA-loaded CaP NPs were higher as compared to non-OVA loaded CaP NPs ($P < 0.01$).

Table 3. 1: Characterisation of OVA-loaded CaP NPs at different loading methods. Data are expressed as mean $\pm$ SD of at least 3 biological replicates; error bars show the SDs.

Group	Size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
Without OVA loading	185 ± 26	0.13 ± 0.02	-20.25 ± 0.55
OVA + Calcium chloride	186 ± 17	0.18 ± 0.02	-26.3 ± 0.68
OVA + Calcium chloride and sodium phosphate	196 ± 12	0.23 ± 0.06	-24.6 ± 0.69
OVA + Sodium phosphate	188 ± 19	0.16 ± 0.03	-26.9 ± 0.65

As shown in Figure 3.1A, the loading efficiency of OVA was dependent on the buffer was initially dissolved in. The loading efficiency of these three methods were $0.75 \pm 0.16\%$, (calcium chloride), $1.19 \pm 0.10\%$ (calcium chloride and sodium phosphate) and $1.34 \pm 0.13\%$ (sodium phosphate) respectively, indicating that OVA loading was significantly less when OVA was dissolved in calcium chloride initially than in the other buffers.

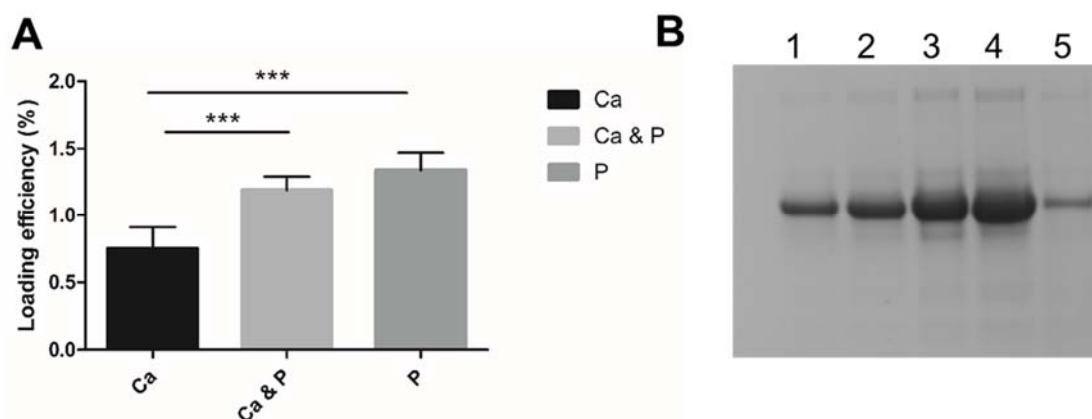


Figure 3. 1: (A) Effects of different loading methods on OVA loading efficiency. (B) The SDS-PAGE analysis of the standard curve and OVA-loaded CaP NPs.

Lane 1: pure OVA (5 μ g); lane 2: pure OVA (10 μ g); lane 3: pure OVA (15 μ g); Lane 4 (20 μ g) and 5: OVA released from CaP NPs. Data represent the mean loading efficiency (%) of three replicates; error bars show the SDs. *** = points were found to be significantly different ($p < 0.001$) from the calcium chloride loading buffer.

To confirm the loading efficiency 150 μ g of OVA loaded using both buffers to form the CaP-OVA NPs was loaded onto an SDS-PAGE gel with known amounts of free OVA protein as a set of standards (Figure 3.1B). According to the band intensity measured from the gel using ImageQuant software, a standard curve (range from 5 to 20 μ g) was created with an $R^2 = 0.97$ (Figure 3.1B). Figure 3.1B shows that the amount of OVA encapsulated in CaP NPs is 2.3 μ g, which corresponds to an OVA loading efficiency of 1.6%.

3.3.2 Absorption of OVA onto CaP NPs using a layer-by-layer approach

To improve OVA loading efficiency, a layer-by-layer approach was adopted. Initially, amino acids (L-lysine, L-arginine, L-lysine ethyl ester and L-arginine ethyl ester) were used to reverse the CaP NPs charge from negative to positive. Amino acid concentration of 6.25 mM, 12.5 mM, 25 mM and 50 mM at pH 8 (L-lysine, L-arginine, L-lysine ethyl ester and L-arginine ethyl ester) were incubated with CaP NPs. For all of the amino acids tested there was an increase in particle size and PDI with increasing concentration of amino acid with only a slight decrease in CaP NPs surface charge as compared to the starting CaP NP (Table 3.2). From these results it can be surmised that an increasing concentration of amino acids during preparation additionally leads to NPs aggregation.

Table 3. 2: Influence of different concentration of amino acids on CaP NPs size and zeta potential. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.

Amino acid	Amino acid concentration (mM)	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
	0.00	185 $\pm$ 26	0.12 $\pm$ 0.06	-20.0 $\pm$ 3
L-arginine	6.25	335 $\pm$ 65	0.33 $\pm$ 0.08	-18.3 $\pm$ 0.43
	12.50	362 $\pm$ 39	0.29 $\pm$ 0.10	-18.4 $\pm$ 0.25
	25.00	389 $\pm$ 67	0.35 $\pm$ 0.07	-17.9 $\pm$ 0.55
	50.00	989 $\pm$ 378	0.70 $\pm$ 0.22	-17.4 $\pm$ 0.46
L-lysine	6.25	275 $\pm$ 65	0.21 $\pm$ 0.10	-17.1 $\pm$ 0.60
	12.50	333 $\pm$ 54	0.32 $\pm$ 0.05	-16.03 $\pm$ 0.45
	25.00	896 $\pm$ 350	0.69 $\pm$ 0.23	-15.7 $\pm$ 0.58
	50.00	1425 $\pm$ 323	0.75 $\pm$ 0.16	-16.5 $\pm$ 0.38
L-arginine ethyl ester	6.25	375 $\pm$ 50	0.33 $\pm$ 0.06	-17.3 $\pm$ 0.21
	12.50	328 $\pm$ 53	0.34 $\pm$ 0.09	-18.8 $\pm$ 0.05
	25.00	340 $\pm$ 135	0.30 $\pm$ 0.15	-18.7 $\pm$ 0.49
	50.00	902 $\pm$ 490	0.69 $\pm$ 0.30	-17.7 $\pm$ 0.21
L-lysine ethyl ester	6.25	286 $\pm$ 101	0.18 $\pm$ 0.06	-17.5 $\pm$ 0.50
	12.50	319 $\pm$ 53	0.31 $\pm$ 0.08	-18.03 $\pm$ 0.49
	25.00	819 $\pm$ 410	0.65 $\pm$ 0.33	-15.7 $\pm$ 0.31
	50.00	1321 $\pm$ 267	0.79 $\pm$ 0.15	-17.0 $\pm$ 0.21

To investigate if decreasing CaP NP concentration affected NP aggregation, lower concentrations of NP were tested. Lowering CaP NP concentration did alter the CaP NP surface charge from approximately -20 mV to -5.82 mV for all amino acids tested (Table 3.3). However, this resulted in significant aggregation as indicated by increase in particle size and PDI (Table 3.3). Consequently, in the subsequent studies, a particle dilution factor of 1:2 was chosen to study the incubation time effect (Table 3.4). A significant increase in NP size was observed with increasing the incubation time with the CaP NPs forming aggregates 3-5 μ m (Table 3.4).

Table 3. 3: Effect of different nanoparticle dilution factor on CaP NPs size and zeta potential. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.

Amino acid (500 mM)	Particle dilution factor	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
L-lysine	0	185 $\pm$ 26	0.12 $\pm$ 0.06	-20.0 $\pm$ 3.1
	1:2	1398 $\pm$ 101	0.21 $\pm$ 0.10	-11.3 $\pm$ 0.32
	1:4	1659 $\pm$ 133	0.16 $\pm$ 0.06	-7.73 $\pm$ 0.11
	1:8	1582 $\pm$ 57	0.11 $\pm$ 0.05	-6.18 $\pm$ 0.75
L-lysine ethyl ester	1:2	1740 $\pm$ 64	0.36 $\pm$ 0.22	-11.8 $\pm$ 0.25
	1:4	1763 $\pm$ 25	0.33 $\pm$ 0.02	-8.32 $\pm$ 0.40
	1:8	1649 $\pm$ 185	0.86 $\pm$ 0.15	-5.82 $\pm$ 0.38
L-arginine	1:2	1596 $\pm$ 55	0.47 $\pm$ 0.07	-11.4 $\pm$ 0.87
	1:4	1178 $\pm$ 15	0.30 $\pm$ 0.02	-10.1 $\pm$ 0.76
	1:8	1555 $\pm$ 321	0.55 $\pm$ 0.32	-7.5 $\pm$ 0.27
L-arginine ethyl ester	1:2	1723 $\pm$ 103	0.35 $\pm$ 0.06	-11.8 $\pm$ 0.45
	1:4	1436 $\pm$ 329	0.57 $\pm$ 0.31	-8.1 $\pm$ 0.66
	1:8	1970 $\pm$ 922	0.45 $\pm$ 0.40	-7.44 $\pm$ 0.45

Table 3. 4: Effect of various incubation time on particle size and zeta potential. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.

Amino acid (500 mM)	Incubation time	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
L-lysine	0 h	185 $\pm$ 26	0.12 $\pm$ 0.06	-20.0 $\pm$ 3.1
	2 h	1398 $\pm$ 101	0.21 $\pm$ 0.10	-11.3 $\pm$ 0.32
	4 h	3282 $\pm$ 533	0.38 $\pm$ 0.06	-12.9 $\pm$ 1.39
	18 h	3307 $\pm$ 588	0.37 $\pm$ 0.12	-14.2 $\pm$ 0.81
L-lysine ethyl ester	2 h	1740 $\pm$ 64	0.36 $\pm$ 0.22	-11.8 $\pm$ 0.25
	4 h	2931 $\pm$ 685	0.78 $\pm$ 0.01	-12.1 $\pm$ 0.78
	18 h	4157 $\pm$ 309	0.27 $\pm$ 0.22	-8.15 $\pm$ 0.56
L-arginine	2 h	1596 $\pm$ 55	0.47 $\pm$ 0.07	-11.4 $\pm$ 0.87
	4 h	3132 $\pm$ 112	0.23 $\pm$ 0.08	-16.8 $\pm$ 0.41
	18 h	4959 $\pm$ 911	0.51 $\pm$ 0.01	-13.5 $\pm$ 1.16
L-arginine ethyl ester	2 h	1723 $\pm$ 103	0.35 $\pm$ 0.06	-11.8 $\pm$ 0.45
	4 h	3212 $\pm$ 353	0.44 $\pm$ 0.16	-13.0 $\pm$ 0.99
	18 h	3381 $\pm$ 171	0.54 $\pm$ 0.18	-13.5 $\pm$ 2.99

3.3.3 The different molecular weight of PEI adsorption onto CaP NPs

The use of a polycationic material such as polyethyleneimine (PEI) was used to investigate if a polymer rather than a monomer (amino acids) would reverse CaP NPs surface charge without inducing aggregation. Different molecular weight (MW) of PEI (0.423 kDa, 1.8 kDa, 2.5 kDa, 5.0 kDa, 10.0 kDa and 25.0 kDa) were used in this study. As can be seen in Table 3.5, the CaP NP surface charge (zeta potential) could be increased to positive in a concentration-dependent manner by all MWs of PEI concentration with the exception of 0.423 kDa linear PEI which also resulted in CaP NP aggregation. Although both 1.8 kDa and 2.5 kDa linear PEI resulted in CaP NPs with a positive charge, they also resulted in significant aggregation. As can be seen in Table 3.5, increasing the concentration of 5 kDa linear, 10 kDa linear, 10 kDa branched and 25 kDa branched PEI to 6 mg/mL, 4 mg/mL, 2 mg/mL, 1 mg/mL respectively, resulted in CaP NPs with a positive surface charge and similar size and PDI as the non-coated CaP NP.

Table 3. 5: Effect of different molecular weight of PEI on CaP NPs size and zeta potential. Data are expressed as mean $\pm$ SD of at least 3 biological replicates.

Type of PEI	PEI concentration (mg/mL)	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
0.423 kDa Linear	0.00	185 $\pm$ 26	0.12 $\pm$ 0.06	-20.0 $\pm$ 3.1
	2.00	1798 $\pm$ 361	0.25 $\pm$ 0.07	-13.9 $\pm$ 0.7
	4.00	1575 $\pm$ 59	0.38 $\pm$ 0.08	-8.26 $\pm$ 0.05
	6.00	1453 $\pm$ 65	0.25 $\pm$ 0.04	-6.19 $\pm$ 0.27
	12.00	1011 $\pm$ 180	0.46 $\pm$ 0.09	-4.23 $\pm$ 0.05
1.8 kDa Linear	0.250	2948 $\pm$ 651	0.37 $\pm$ 0.23	15.53 $\pm$ 0.12
	0.500	2581 $\pm$ 124	0.37 $\pm$ 0.06	19.63 $\pm$ 0.18
	1.000	1098 $\pm$ 286	0.67 $\pm$ 0.46	17.5 $\pm$ 0.12
	6.00	978 $\pm$ 96	0.31 $\pm$ 0.15	27.2 $\pm$ 0.37
2.5 kDa Linear	2.00	654 $\pm$ 185	0.71 $\pm$ 0.21	18.1 $\pm$ 0.78
	4.00	584 $\pm$ 163	0.45 $\pm$ 0.17	19.0 $\pm$ 0.57
	6.00	515 $\pm$ 105	0.67 $\pm$ 0.18	19.5 $\pm$ 0.69
	10.00	384 $\pm$ 111	0.42 $\pm$ 0.07	25.5 $\pm$ 0.22
5 kDa Linear	1.00	211 $\pm$ 27	0.40 $\pm$ 0.04	17.6 $\pm$ 0.37
	2.00	203 $\pm$ 14	0.18 $\pm$ 0.09	19.0 $\pm$ 0.16
	4.00	195 $\pm$ 18	0.13 $\pm$ 0.06	20.9 $\pm$ 0.52
	6.00	189 $\pm$ 18	0.09 $\pm$ 0.04	23.5 $\pm$ 0.81
10 kDa Linear	1.00	879 $\pm$ 231	0.75 $\pm$ 0.25	14.6 $\pm$ 0.37
	2.00	509 $\pm$ 177	0.45 $\pm$ 0.05	19.6 $\pm$ 0.35
	4.00	214 $\pm$ 10	0.23 $\pm$ 0.04	22.8 $\pm$ 0.65
	6.00	211 $\pm$ 12	0.16 $\pm$ 0.10	26.9 $\pm$ 0.32
10 kDa Branched	0.125	1254 $\pm$ 235	0.91 $\pm$ 0.07	14.5 $\pm$ 0.12
	0.25	1224 $\pm$ 124	0.31 $\pm$ 0.19	16.5 $\pm$ 0.25
	0.50	493 $\pm$ 84	0.27 $\pm$ 0.15	17.2 $\pm$ 0.50
	1.00	321 $\pm$ 87	0.31 $\pm$ 0.11	21.3 $\pm$ 0.18
	2.00	205 $\pm$ 27	0.14 $\pm$ 0.08	22.5 $\pm$ 0.61
25 kDa Branched	0.125	1136 $\pm$ 128	0.54 $\pm$ 0.12	10.86 $\pm$ 0.27
	0.25	1072 $\pm$ 112	0.36 $\pm$ 0.13	16.71 $\pm$ 0.20
	0.50	870 $\pm$ 108	0.32 $\pm$ 0.14	20.51 $\pm$ 0.43
	1.00	194 $\pm$ 35	0.27 $\pm$ 0.02	22.13 $\pm$ 0.47
	2.00	189 $\pm$ 25	0.23 $\pm$ 0.04	25.36 $\pm$ 0.28

3.3.4 Size stability of various molecular weight of PEI coated CaP NPs

The emulsion stability of CaP-PEI NPs prepared using the 5 kDa linear, 10 kDa linear, 10 kDa branched, and 25 kDa branched PEI were investigated over 5 days using DLS. After two days the 5 kDa linear and 10 kDa linear CaP-PEI NPs formed aggregate, as seen by an increase in size, and the PDI of particles became polydisperse and were observed to sediment (Figure 3.2A, B and D). However, both the 10 kDa branched and 25 kDa branched PEI-coated CaP NPs were stable at RT, with no change in NP size or PDI or observable sedimentation over the five day period (Figure 3.2).

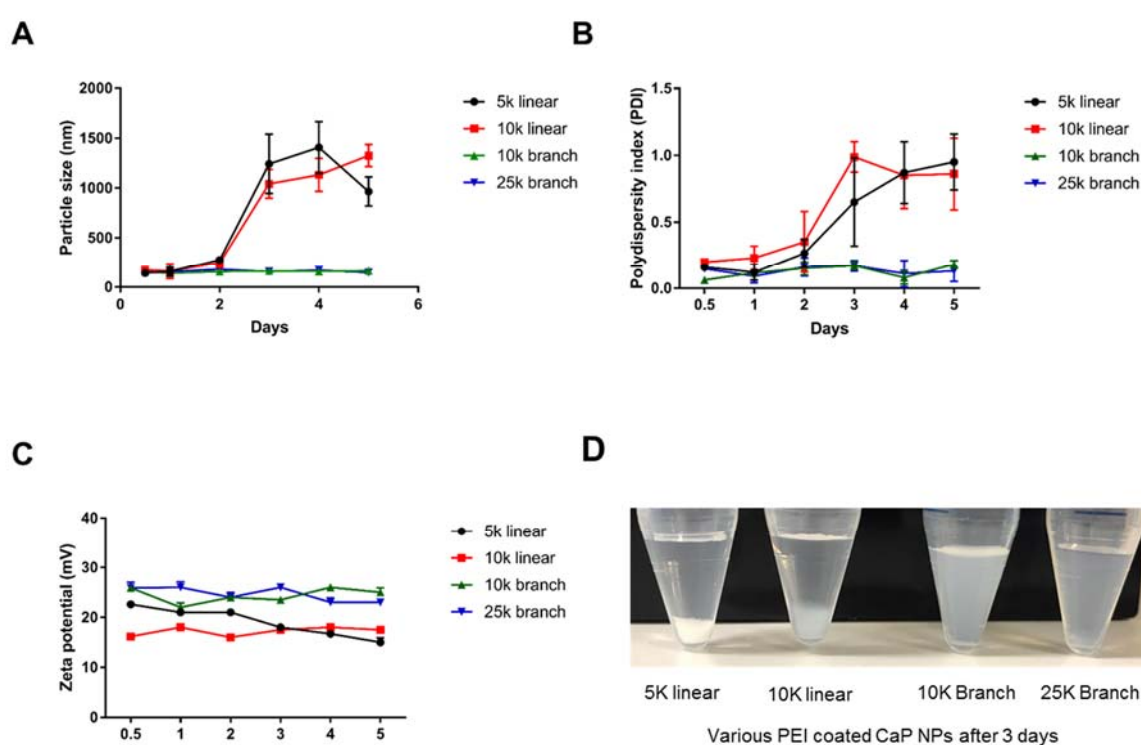


Figure 3. 2: The characterisation of CaP NPs with different molecular weight of PEI in MilliQ water.

(A) size (B) PDI (C) zeta potential (D) photo-image. Data are expressed as mean $\pm$ SD of at least 3 biological replicates; error bars show the SDs.

3.3.5 CaP-PEI NPs cytotoxicity

For the potential use NPs in *in vivo* applications, the biological safety of NPs must be taken into consideration. The toxicity of PEI coated NPs was quantified by MTS (a cell proliferation assay) and LDH (lactate dehydrogenase, and indicator of cellular toxicity) assays (Figure 3.3). The HEK and H4IIE cell monolayers were incubated with PEI coated CaP NPs at NP to cell ratios of 5,000:1, 10,000:1, 15,000:1 and 20,000:1. 5 kDa linear PEI, 10 kDa linear PEI, 10

kDa branched PEI, and 25 kDa branched PEI were used in these experiments as they were previously shown to both modify the charge of nanoparticles and prevent NP aggregation over 24 hours. MTT and LDH results indicated that 5 kDa linear PEI, 10 kDa linear PEI, 10 kDa branched PEI had no toxic effects on HEK or H4IIE cells across all NP:Cell ratios (Figure 3.3). In contrast, 25 kDa branched PEI exhibited slight toxicity on both cell types, but only at the highest NP:Cell ratio, as compared to untreated cells. Based on the size stability and CaP-PEI NPs cytotoxicity, the 10 kDa branched PEI was chosen as the most suitable polymer for formulating positively charged NPs for the subsequent studies.

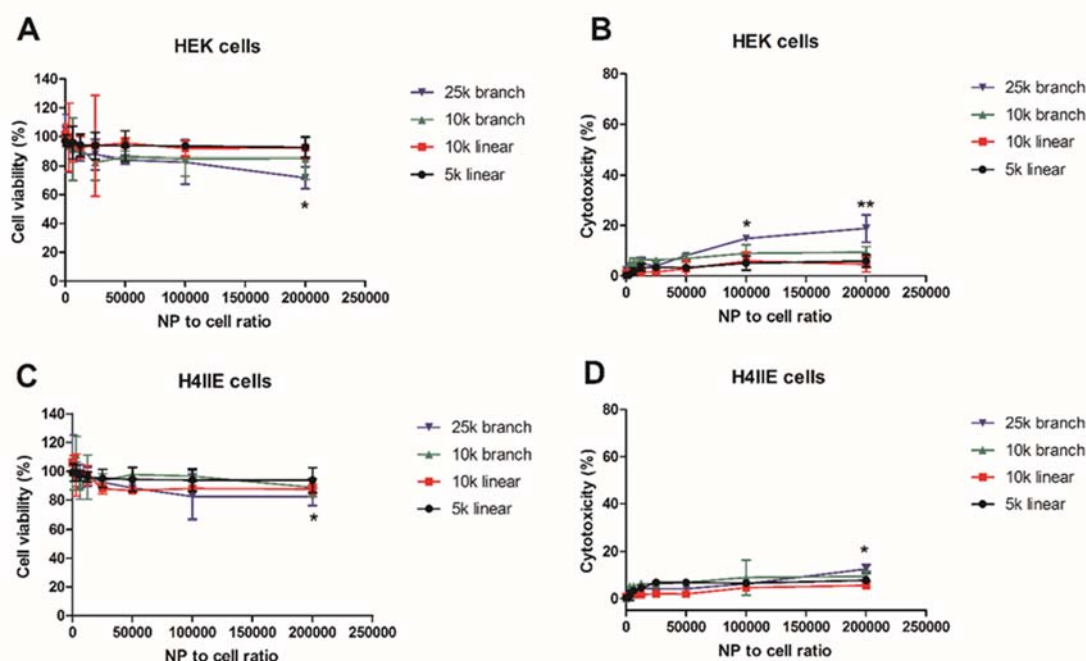


Figure 3. 3: The toxicity of CaP NPs with different molecular weight of PEI on HEK (A, B) and H4IIE cells (C, D). Data are expressed as mean $\pm$ SD of at least 3 biological replicates; error bars show the SDs.

3.3.6 Optimisation of OVA adsorption

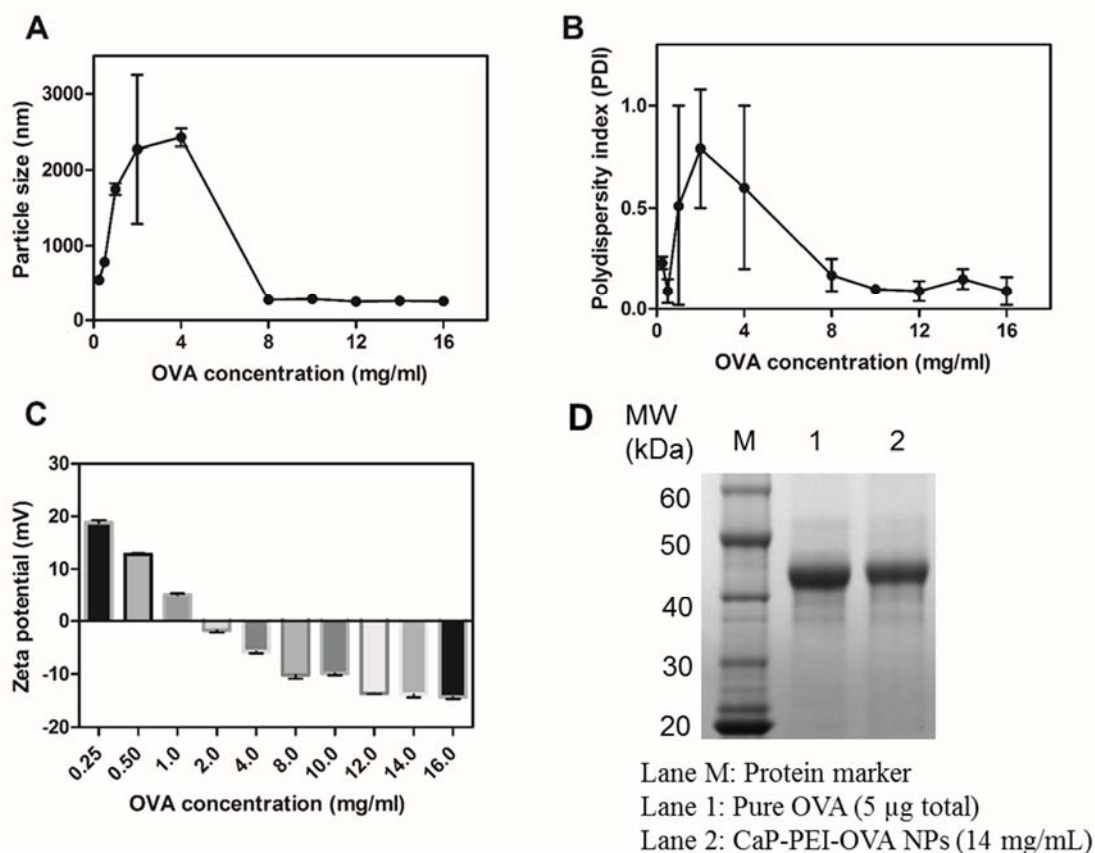


Figure 3. 4: Effect of OVA concentration on particles. (A) size (B) PDI (C) zeta potential (D) SDS-PAGE. Data are expressed as mean $\pm$ SD of at least 3 biological replicates; error bars show the SDs.

To investigate the optimal conditions for OVA adsorption on the surface of CaP-PEI NPs, a series of OVA concentrations were tested. CaP-PEI NPs were added dropwise to various OVA concentrations under constant agitation and incubated for 30 min. The CaP-PEI-OVA NPs were then pelleted by centrifugation and were dispersed in MilliQ water and characterised by DLS. Figure 3.4 shows that at an OVA concentration of 4 mg/mL and less the NPs formed aggregates with high PDI's. The particle size (273 ± 11 nm) and PDI (0.17 ± 0.08) remained low after incubation with 8 mg/mL or greater of OVA (Figure 3.4A and B), and that the charge of NPs became negative from 2 mg/mL of OVA (-1.74 ± 0.39 mV) (Figure 3.4C). With increasing OVA concentration there was an increase in the negative surface charge of the NPs which plateaued from 12 mg/mL. The amount of OVA loaded onto particles prepared using 10, 12, 14 and 16 mg/mL was determined using the BCA method after removal of the CaP core with HCl, resulting in 1.06×10^{-14} g/NP, 2.91×10^{-14} g/NP, 2.87×10^{-14} g/NP and 2.80×10^{-14} g/NP, respectively. This indicates that the NPs were saturated with OVA at 14 mg/mL and above.

Based on the size, PDI and zeta potential, the concentration of OVA (14 mg/mL) was selected for adsorption on NPs. CaP-PEI-OVA NPs synthesised using 14 mg/mL OVA were used in an SDS-PAGE to confirm protein loading. Figure 3.4D demonstrates that OVA was absorbed onto CaP-PEI-OVA NPs.

To indicate the properties of NPs *in vivo*, the size stability of CaP-PEI-OVA NPs was evaluated in various biological media. The DLS results demonstrated that the particles were stable in MilliQ water without any aggregation (Table 3.6). However, when the particles were dispersed in PBS buffer the particle size increased significantly from 259 ± 24 nm to 4625 ± 335 nm, indicating particle aggregation. The size of CaP-PEI-OVA NPs remained unchanged in DMEM and SBF buffer with 55% human serum after six hours (Table 3.6). However, there was a decrease in the values of zeta potential. Consequently, SDS-PAGE was conducted to investigate protein corona and the loss of OVA compared to starting NPs. Figure 3.5 shows that NP-OVA adsorbed a number of serum proteins from the cell culture media and serum, which displaced absorbed OVA. In an attempt to reduce the protein corona from serum and culture media, additional layers of PEI were investigated.

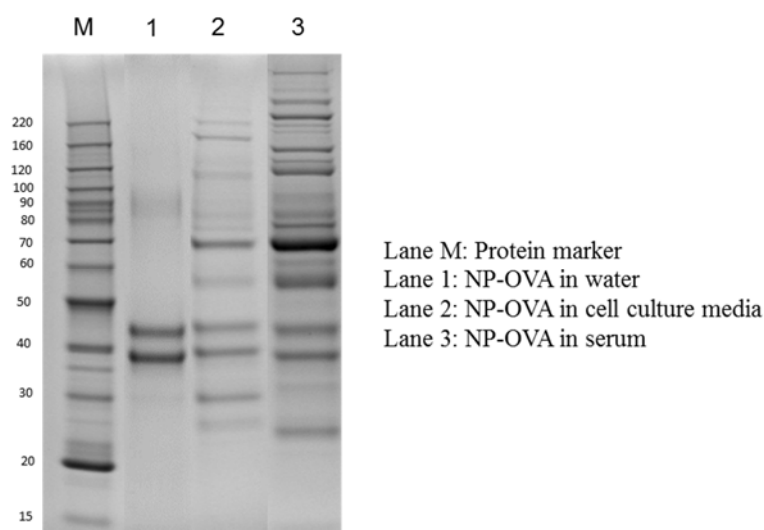


Figure 3. 5: SDS-PAGE of CaP-PEI-OVA NPs in the various solutions for 6 h at room temperature.

Table 3. 6: The size stability of CaP-PEI-OVA NPs in different media for 6 h. Data are expressed as mean $\pm$ SD of at least 3 biological replicates; $\pm$ indicates the SDs.

Media	NP size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
MilliQ water	259 $\pm$ 24	0.15 $\pm$ 0.05	-13.8 $\pm$ 0.54
PBS buffer	4625 $\pm$ 335	0.58 $\pm$ 0.38	-3.41 $\pm$ 0.60
DMEM with 10% FBS	265 $\pm$ 28	0.22 $\pm$ 0.08	-2.56 $\pm$ 0.32
SBF buffer with 55% human serum	277 $\pm$ 32	0.29 $\pm$ 0.09	-2.08 $\pm$ 0.42

3.3.7 Second layer of PEI

To coat the second layer of PEI, the CaP-PEI-OVA NPs were mixed with different concentrations of 10 kDa branched PEI. The solution was incubated under constant agitation (1 h), then centrifuged and dispersed in MilliQ water. Figure 3.6 shows after the addition of a second layer of PEI, the particle size and PDI remained unchanged from 12 mg/mL PEI (Figure 3.6A). At this concentration, the particle became positively charged at $+25 \pm 1.25$ mV (Figure 3.6B). The protein content of the NPs was quantified by the BCA protein assay kit and SDS-PAGE and Figure 3.6C and D demonstrate that the OVA was decreased by more than 90% by the addition of PEI. As a result, cross-linking of OVA on the particle surface was investigated to avoid detachment and replacement by serum proteins was performed.

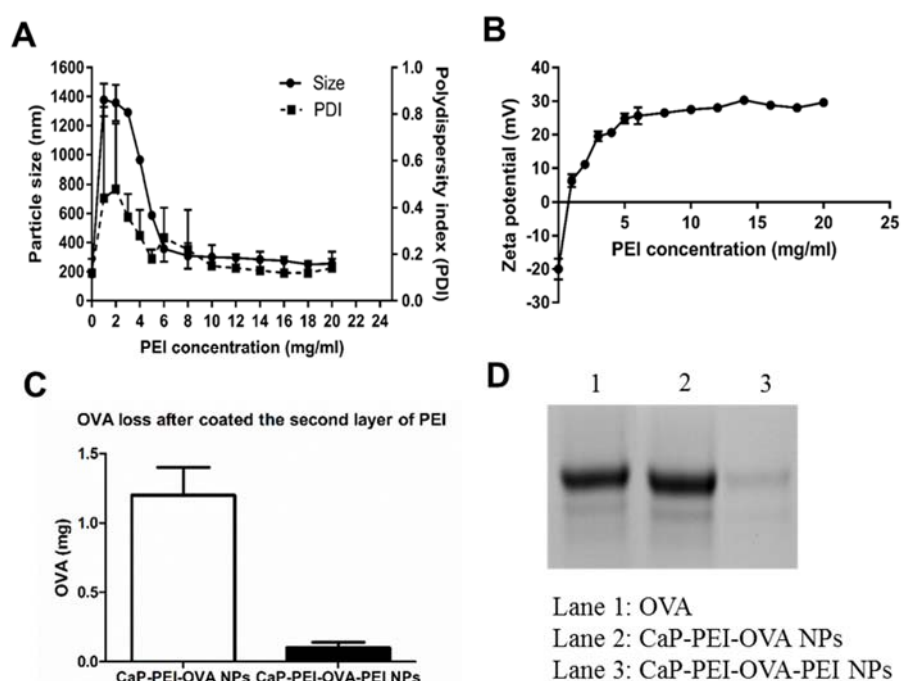


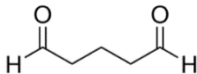
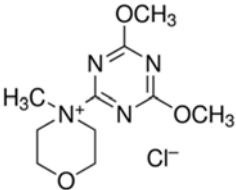
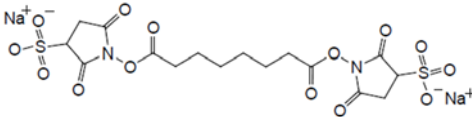
Figure 3. 6: Characterisation of the second layer of PEI adsorbed on CaP-PEI-OVA NPs.

Particle size and PDI (A), zeta potential (B), BCA protein assay results (C), SDS-PAGE (D). Data are expressed as mean $\pm$ SD of at least 3 biological replicates; error bars show the SDs. The used second layer PEI concentration is 14 mg/mL.

3.3.8 Cross-linking OVA on the surface of CaP-PEI NPs

OVA has a number of amino acids (20 lysines, 10 tyrosine, 6 cysteines, 14 aspartic acids, and 33 glutamic acids) that are suitable for conjugation and cross-linking. Three cross-linking agents glutaraldehyde, DMTMM and BS³ were used (Table 3.7). Figure 3.7 shows that at all concentrations of GA and BS³ resulted in stable cross-linked OVA, as there was no OVA bands detected by SDS-PAGE (Figure 3.7A and B). However, DMTMM resulted in no cross-linking (Figure 3.7C).

Table 3. 7: Different cross-linkers and their structures.

Cross-linkers	Structure	Reactive groups
GA (Glutaraldehyde)		Aldehydic groups
DMTMM (4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride)		Carboxylic acid
BS ³ (bis[sulfosuccinimidyl] suberate)		Sulfo-NHS ester

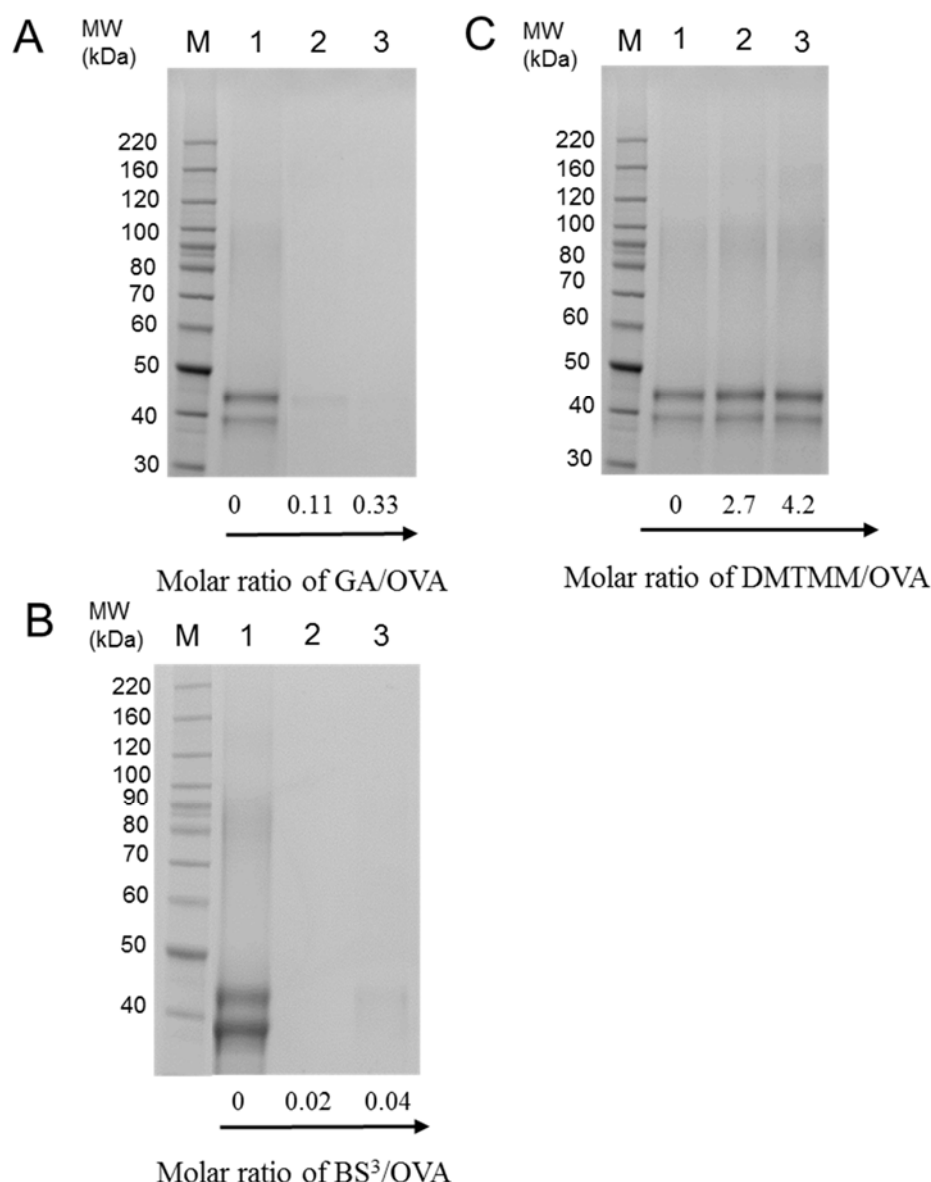


Figure 3. 7: SDS-PAGE analyses of the different molar ratio of cross-linkers to OVA. (A) different molar ratio of GA/OVA; (B) different molar ratio of BS³/OVA; (C) different molar ratio of DMTMM/OVA.

Although GA and BS³ successfully cross-linked OVA, cross-linking itself may lead to an increase in NP size and PDI. When the molar ratio of GA/OVA was 0.11 and 0.33, the particle size increased to $1,312 \pm 115$ nm and $1,709 \pm 425$ nm, respectively. The PDI was 0.87 ± 0.10 and 0.78 ± 0.19 , respectively (Table 3.8). In comparison, the particle size was smaller after BS³ cross-linking, which was 430 ± 35 nm when the molar ratio of BS³/OVA was 0.02. Considering the particle size and PDI, BS³ was chosen as an OVA cross-linker for further optimisation and processing for the purpose of decreasing the particle size.

Table 3. 8: Characterisation of the different molar ratio of cross-linkers to OVA. Data are expressed as mean $\pm$ SD of at least 3 biological replicates; $\pm$ indicates the SDs.

Group	Size (nm)	PDI
Non-cross-linked NPs in water	259 $\pm$ 24	0.12 $\pm$ 0.07
Molar ratio of GA/OVA at 0.11	1312 $\pm$ 115	0.87 $\pm$ 0.10
Molar ratio of GA/OVA at 0.33	1709 $\pm$ 425	0.78 $\pm$ 0.19
Molar ratio of BS ³ /OVA at 0.02	430 $\pm$ 35	0.21 $\pm$ 0.06
Molar ratio of BS ³ /OVA at 0.04	398 $\pm$ 39	0.26 $\pm$ 0.05

3.3.9 Optimal conjugation buffer for the BS³ conjugation

When conjugation was attempted according to the manufacturer's instructions (BS³, Thermo Fisher Scientific), there a number of BS³ conjugation buffer options there being 100 mM sodium phosphate, 0.15 M NaCl, 20 mM HEPES and 50 mM borate. The suggested molar ratios of these buffers and a 5 and 10 fold molar reaction were used to evaluate conjugation of OVA on the surface of CaP NPs.

Figure 3.8 shows all 10 fold molar reduced buffers resulted in a stable suspension, with the starting and 5 fold molar reduced buffer resulting in aggregation. These visual observations were confirmed by DLS (Table 3.9), with 10 fold buffers having the smallest aggregation and PDI's. 5 mM borate buffer resulted in same size and PDI as compared to the non-cross-linked NPs. Based on these data 5 mM borate buffer was chosen as the OVA conjugation buffer for further experimentation.

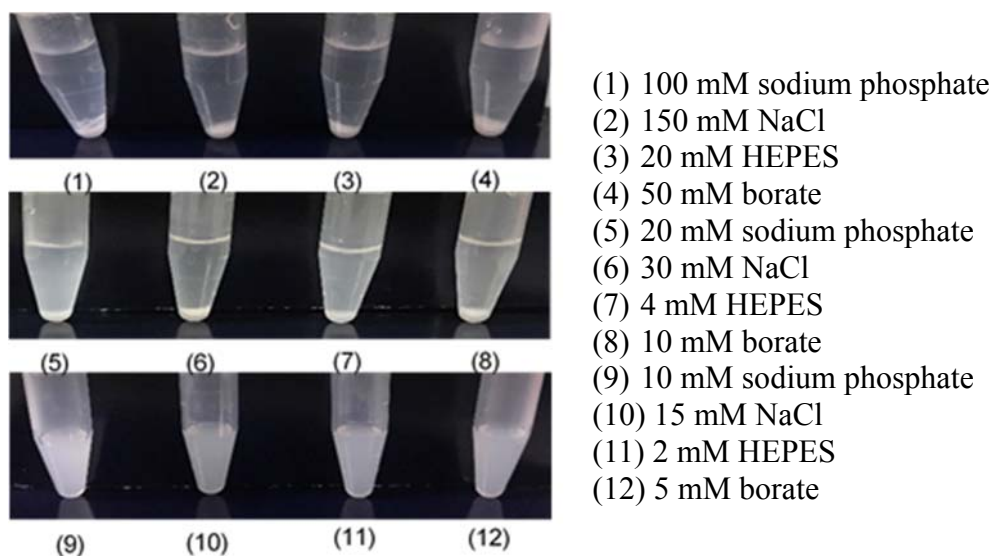


Figure 3. 8: CaP-PEI-OVA NPs in different conjugation buffer after 6 h.

Table 3. 9: Determination of the optimal concentration of conjugation buffer for cross-linking OVA on nanoparticles.

The non-cross-linked CaP-PEI-OVA NPs were incubated with various concentration of conjugation buffer for 6 h, and the particle size and zeta potential were measured by DLS. Data represent the mean of three replicates; $\pm$ indicates the SDs.

	Conjugation buffer	Size (nm)	Polydispersity index (PDI)
	MilliQ water	259 $\pm$ 24	0.12 $\pm$ 0.07
1	100 mM sodium phosphate	3202 $\pm$ 523	0.87 $\pm$ 0.37
2	150 mM NaCl	2965 $\pm$ 367	0.69 $\pm$ 0.25
3	20 mM HEPES	2513 $\pm$ 657	0.62 $\pm$ 0.31
4	50 mM borate	1840 $\pm$ 276	0.75 $\pm$ 0.24
5	20 mM sodium phosphate	2097 $\pm$ 217	0.65 $\pm$ 0.32
6	30 mM NaCl	2273 $\pm$ 365	0.78 $\pm$ 0.28
7	4 mM HEPES	1620 $\pm$ 749	0.68 $\pm$ 0.37
8	10 mM borate	1553 $\pm$ 311	0.72 $\pm$ 0.28
9	10 mM sodium phosphate	411 $\pm$ 88	0.43 $\pm$ 0.14
10	15 mM NaCl	677 $\pm$ 153	0.32 $\pm$ 0.23
11	2 mM HEPES	526 $\pm$ 65	0.45 $\pm$ 0.11
12	5 mM borate	255 $\pm$ 16	0.12 $\pm$ 0.03

3.3.10 SDS-PAGE and mass spectrometry analyse OVA cross-linking

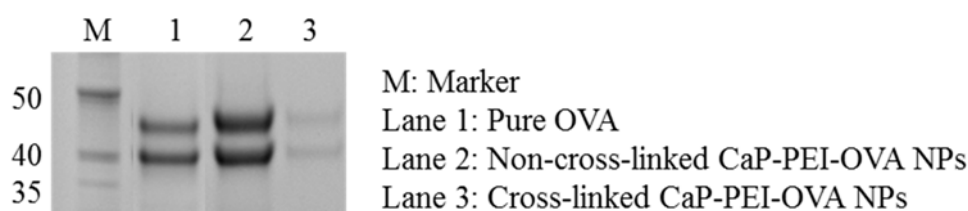


Figure 3. 9: The SDS-PAGE analysis of non-cross-linked and cross-linked CaP-PEI-OVA NPs.

As can be seen from the SDS-PAGE (Figure 3.9), pure OVA and non-cross-linked CaP-PEI-OVA NPs all show a protein band at 42.3 kDa. However, for the cross-linked CaP-PEI-OVA NPs, there was a faint band on the gel, suggesting the 5 mM borate buffer was suitable for conjugation and OVA was successfully cross-linked. In order to further confirm OVA was cross-linked by BS³, the cross-linked CaP-PEI-OVA NPs were digested with trypsin, and cross-linked peptides identified by LC-MS/MS. PLINK software was used to analyse the MS data to

identify cross-linked peptides. The results from mass spectrometry identified 29 cross-linked peptides of OVA (Table 3.10).

Table 3. 10: Identification of cross-linked peptides by mass spectrometry.

Number of cross- linked peptides	Cross-linked peptides	E-value	Mass error (ppm)
1	²⁷⁹ IKVYLPR ^{285_286} MKMEEK ²⁹¹	7.68E-08	-0.042834
2	⁵² TQINKVVR ^{59_286} MKMEEK ²⁹¹	2.92E-10	0.636502
3	²⁷⁷ KIKVYLPR ^{284_52} TQINKVVR ⁵⁹	3.28E-12	0.927388
4	²⁷⁹ IKVYLPR ^{285_279} IKVYLPR ²⁸⁵	1.15E-04	1.23394
5	²²⁰ VASMASEKMK ^{229_279} KIKVYLPR ²⁸⁵	2.55E-05	0.169555
6	¹⁸⁸ AFKDEDTQAMPFR ^{200_52} TQINKVVR ⁵⁹	1.68E-06	0.917232
7	²⁷⁷ KIKVYLPR ^{284_279} IKVYLPR ²⁸⁵	6.15E-08	1.309315
8	⁵² TQINKVVR ^{59_279} IKVYLPR ²⁸⁵	4.79E-09	1.622621
9	¹⁸⁸ AFKDEDTQAMPFR ^{200_279} IKVYLPR ²⁸⁵	9.72E-10	1.921089
10	¹⁸⁸ AFKDEDTQAMPFR ^{200_286} MKMEEK ²⁹¹	1.07E-04	0.40311
11	¹⁸⁸ AFKDEDTQAMPFR ^{200_277} KIKVYLPR ²⁸⁴	3.86E-10	1.928441
12	⁵² TQINKVVR ^{59_52} TQINKVVR ⁵⁹	1.03E-07	1.335134
13	²²⁰ VASMASEKMK ^{229_279} IKVYLPR ²⁸⁵	4.09E-06	1.159852
14	⁵² TQINKVVR ^{59_52} TQINKVVR ⁵⁹	2.14E-04	1.029619
15	⁵² TQINKVVR ^{59_286} MKMEEK ²⁹¹	1.07E-01	-0.05643
16	²⁰¹ VTEQESKPVQMMYQIGLFR ^{219_279} KIK- VYLPR ²⁸⁵	1.38E-05	1.409808
17	²²⁰ VASMASEKMK ^{229_52} TQINKVVR ⁵⁹	1.00E+00	0.225184
18	²⁰¹ VTEQESKPVQMMYQIGLFR ^{219_220} VASMA- SEKMK ²²⁹	2.83E-05	1.259934
19	²⁷⁹ IKVYLPRMK ^{287_279} IKVYLPR ²⁸⁵	1.80E-05	2.145591
20	²²⁰ VASMASEKMK ^{229_52} TQINKVVR ⁵⁹	1.24E-02	1.402131
21	²⁷² KIKVYLPR ^{284_52} TQINKVVR ⁵⁹	4.99E-01	-0.893046
22	¹¹² YPILPEYLQCVKELYR ^{127_52} TQINKVVR ⁵⁹	1.48E-01	0.362324
23	²²⁰ VASMASEKMK ^{229_52} TQINKVVR ⁵⁹	1.18E-05	1.048751
24	²⁷⁹ KIKVYLPR ^{285_286} MKMEEK ²⁹¹	1.64E-04	0.924623
25	²⁰¹ VTEQESKPVQMMYQIGLFRVASMASEK ^{227_286} MKMEEK ²⁹¹	6.65E-02	-0.42939
26	²²⁰ VASMASEKMK ^{229_277} KIKVYLPR ²⁸⁴	3.65E-06	1.929082
27	²²⁰ VASMASEKMK ^{229_220} VASMASEKMK ²²⁹	6.91E-01	3.82499
28	²²⁰ VASMASEKMK ^{229_279} IKVYLPR ²⁸⁵	1.00E+00	0.366435
29	²⁷⁹ KIKVYLPR ^{285_52} TQINKVVR ⁵⁹	2.78E-02	1.50144

After BS³ cross-linking it was noted that upon storage, the CaP NPs quickly aggregated (Figure 3.10). However, this was prevented by quenching unreacted BS³ by adding 150 mM glycine. This is supported by the resulting size stability of the quenched CaP-PEI-OVA NPs in MilliQ water (Figure 3.10). The DLS data showed the size of unquenched CaP-PEI-OVA NPs in MilliQ water increased from 256 ± 22 nm to 1296 ± 256 nm after four days, while the size of the quenched CaP-PEI-OVA NPs remained stable in MilliQ water. Taken together, for CaP-PEI-OVA NPs cross-linking, BS³ was used as a cross-linker, 5 mM borate buffer was the most suitable conjugation buffer, and quenching with 150 mM glycine to prevent NP aggregation.

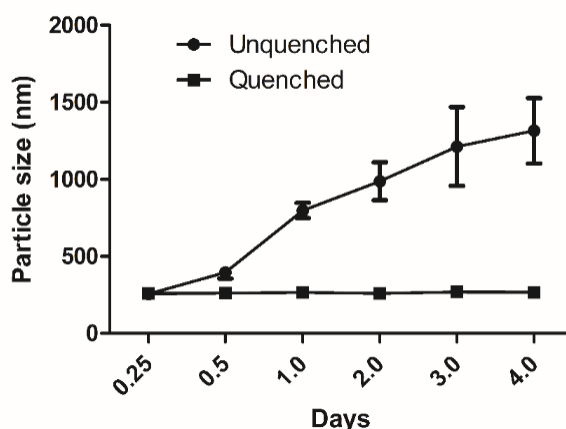


Figure 3. 10: Size stability of unquenched and quenched CaP-PEI-OVA NPs in MilliQ water. Data are expressed as mean $\pm$ SD of at least 3 biological replicates; error bars show the SDs.

3.3.11 Evaluation of the protein corona from cell culture media and serum on cross-linked CaP-PEI-OVA NPs

To investigate the interaction of CaP-PEI-OVA NPs with cell culture media and serum, cross-linked, and non-cross-linked CaP-PEI-OVA NPs were dispersed in various media, and particle size and zeta potential were measured by DLS. Figure 3.11 showed when cross-linked, and non-cross-linked CaP-PEI-OVA NPs were suspended in MilliQ water, the sizes of NPs showed no difference ($P > 0.05$) (Figure 3.11A and B). Very little difference was observed from the non-cross-linked and cross-linked NPs in cell culture media and SBF buffer with 55% human serum (Figure 3.11C, D, E and F). However, when non-cross-linked NPs were dispersed in PBS buffer, the particle size increased from 259 ± 24 nm to 4625 ± 335 nm, with a high PDI of 0.623 ± 0.31 (Figure 3.11A and G). However, cross-linked CaP-PEI-OVA NPs did not aggregate in PBS and had a similar particle size as compared to particles in MilliQ water (261 ± 20 nm).

Table 3. 11: Zeta potential of cross-linked and non-cross-linked CaP-PEI-OVA NPs in various media. Data are expressed as mean $\pm$ SD of at least 3 biological replicates; $\pm$ indicates the SDs.

Type of NPs	Media	Size (nm)	Zeta potential (mV)
Non-cross-linked NPs	MilliQ water	259 $\pm$ 24	-13.8 $\pm$ 0.54
	Cell culture media	269 $\pm$ 25	-0.29 $\pm$ 0.03
	SBF buffer with 55% human serum	279 $\pm$ 26	-2.08 $\pm$ 1.0
	PBS buffer	4625 $\pm$ 335	-3.41 $\pm$ 0.60
Cross-linked NPs	MilliQ water	261 $\pm$ 20	-17.5 $\pm$ 1.2
	Cell culture media	275 $\pm$ 35	-7.22 $\pm$ 0.93
	SBF buffer with 55% human serum	289 $\pm$ 28	-10.3 $\pm$ 0.65
	PBS buffer	297 $\pm$ 32	-9.73 $\pm$ 0.39

Furthermore, incubation in cell culture media, SBF buffer with 55% human serum or PBS altered the particle surface charge (Table 3.11). The surface charge of non-cross-linked NPs was negative (-13.8 $\pm$ 0.54 mV) in water and less negative in cell culture media (-0.29 $\pm$ 0.03 mV), human serum (-2.08 $\pm$ 1.0 mV) and PBS buffer (-3.41 $\pm$ 0.60 mV). The surface charge of cross-linked NPs was negative (-17.5 $\pm$ 1.2 mV) in water and less negative in cell culture media (-7.22 $\pm$ 0.93 mV), human serum (-10.3 $\pm$ 0.65 mV) and PBS buffer (-9.73 $\pm$ 0.39 mV).

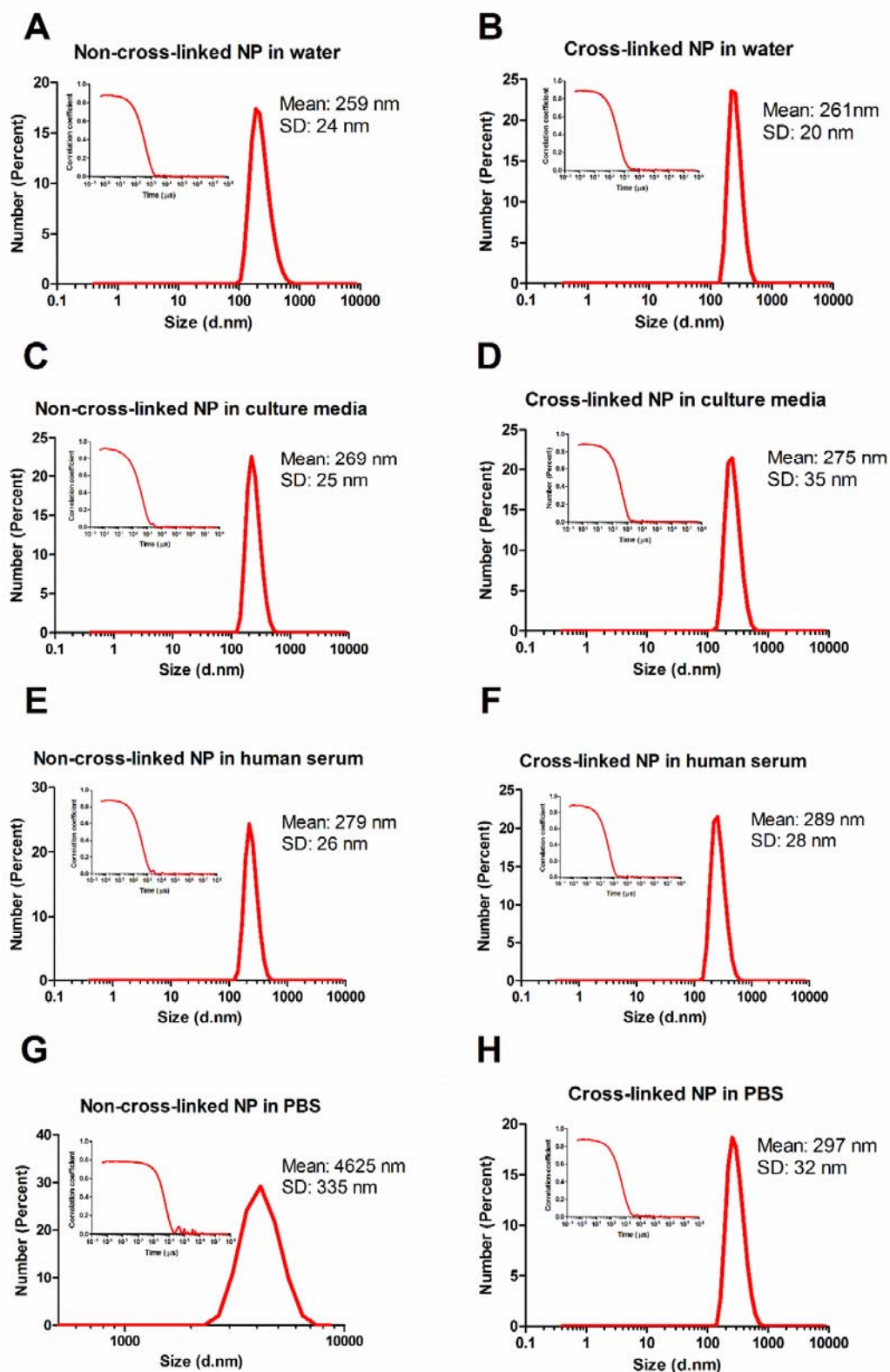


Figure 3. 11: Size and correlation curve of non-cross-linked and cross-linked CaP-PEI-OVA NPs in various media measured by DLS.

(A, B) non-cross-linked and cross-linked NPs in MilliQ water; (C, D) non-cross-linked or cross-linked NPs in cell culture media; (E, F) non-cross-linked or cross-linked NPs in SBF with 55% serum; (G, H) non-cross-linked or cross-linked NPs in PBS.

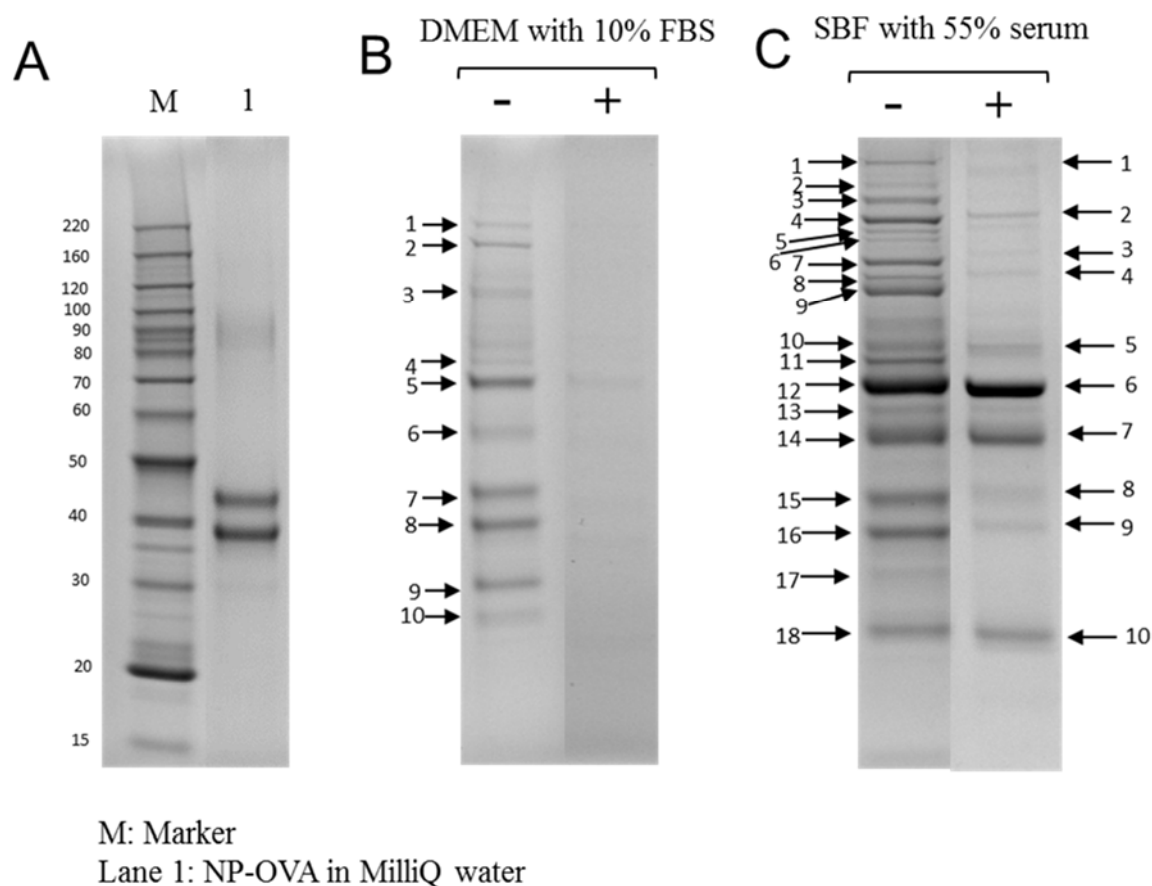


Figure 3.12: SDS-PAGE of protein corona comes from non-cross-linked or cross-linked CaP-PEI-OVA NPs incubated with various media for 6 h at room temperature.

(A) Non-cross-linked NP-OVA in MilliQ water; (B) non-cross-linked (-) or cross-linked (+) CaP-PEI-OVA NPs incubated with cell culture media; (C) non-cross-linked (-) or cross-linked (+) CaP-PEI-OVA NPs incubated with SBF with 55% serum. The arrow indicates the serum protein adsorbed on NPs.

Non-cross-linked and cross-linked CaP-PEI-OVA NPs then were subjected to SDS-PAGE, trypsin digestion and the protein bands identified by mass spectrometry. Cross-linked NPs resulted in substantially fewer serum proteins absorbed as compared to non-cross-linked NPs (Figure 3.12). No observable protein bands were found in cross-linked CaP-PEI-OVA NPs incubated with cell culture media, 10 bands was found in non-cross-linked NPs (Figure 3.12B). In addition, when non-cross-linked or cross-linked CaP-PEI-OVA NPs were incubated with SBF with 55% serum, the identified protein bands were 18 and 10, respectively (Figure 3.12C). The identified adsorbed coronal proteins are shown in Supplementary Table S3.1, Table S3.2 and Table S3.3. Serum albumin was found to be the major protein in the corona from both FBS and SBF. Apolipoprotein, fibrinogen, and complement C3 were also identified in the corona of NPs, which are common proteins present in the blood. Various other proteins were found bound to non-cross-linked NPs, unlike the protein found in the cross-linked NPs following

incubation with cell culture media, where there were very few faint bands visible on the gel (Figure 3.12).

FTIR was carried out for structural analysis of the NPs and to confirm the presence of PEI and OVA functional groups in the CaP NPs. Figure 3.13 shows the FTIR spectra of the layer by layer synthesised CaP-PEI-OVA NPs. PEI showed the characteristic peaks at $1,660\text{ cm}^{-1}$ for NH_2 vibration (Figure 3.13A). CaP-PEI NPs had the increased signals at $1,660\text{ cm}^{-1}$ correspondings to the NH_2 vibration of PEI, which in turn is caused by the absorption of PEI on the particle surface during preparation (Figure 3.13A and D). FTIR is also an effective way to analyse protein structure [335]. The secondary structure analysis of OVA on CaP NPs is shown in Figure 3.14B. The FTIR spectra of the CaP-PEI-OVA NPs confirms the presence of OVA in the nanoparticles (Figure 3.13B and E). The peaks at [336] $1,654\text{ cm}^{-1}$ corresponds to amide I C-O stretching mode, $1,540\text{ cm}^{-1}$ corresponds to amide II N-H bending mode, and $1,397\text{ cm}^{-1}$ corresponds to amide III C-N stretching mode. Amide I, amide II, and amide III peaks can be observed from Figure 3.13E after adsorption of OVA on particles, indicating CaP-PEI NPs were coated with OVA.

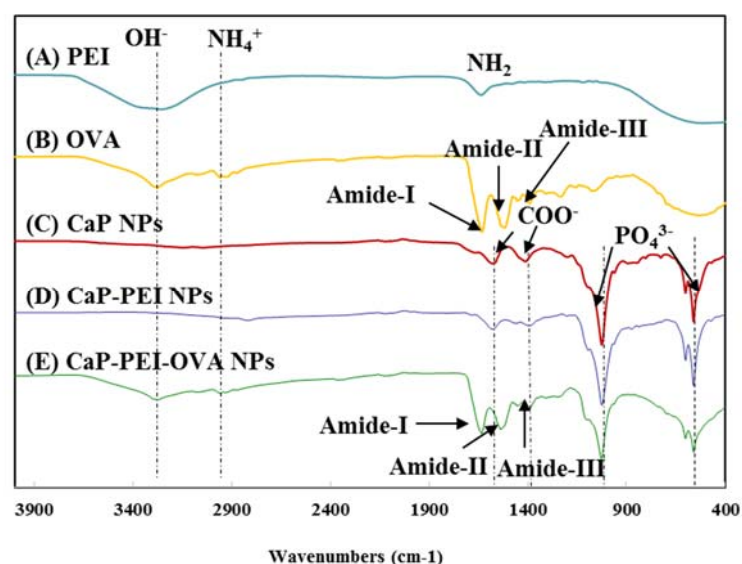


Figure 3. 13: FTIR spectra of the LbL CaP NPs. (A) PEI, (B) OVA, (C) CaP NPs, (D) CaP-PEI NPs, (E) CaP-PEI-OVA NPs. The three patterns have been normalised.

3.3.12 Optimised method for stable protein antigen coated CaP NPs

The optimised method for coating CaP NP with a protein antigen using LbL approach is shown in Figure 3.14 whereby, particles were initially coated with PEI, followed by adsorption of OVA, and then the OVA was cross-linked by BS³, and quenched by glycine. This approach was then applied to other CaP NP's.

The particle size, size distribution, and surface charge of different cross-linked CaP-PEI-OVA NPs were characterised by DLS. Table 3.12 summarises the physical properties of three different sizes of CaP nanoparticle formulations after the addition of PEI, OVA and treatment with BS³ and glycine quenching. The size of each particle increased after each coating step and there was little change in particle size after cross-linking in comparison to the non-cross-linked NPs. The PDI of all samples remained below 0.2. A reversal of charge was identified following each coating step (CaP NPs: negative charge; PEI: positive charge; OVA: negative charge), suggesting the PEI and OVA were successfully adsorbed onto each NPs. According to BCA protein assay results, the OVA loading efficiency was $8.5 \pm 1.2\%$. HIM images show the characteristic morphology of synthesised nanoparticles by the LbL approach. HIM images demonstrate that all cross-linked CaP-PEI-OVA NPs were rod-like shaped, uniform and monodispersed (Figure 3.15).

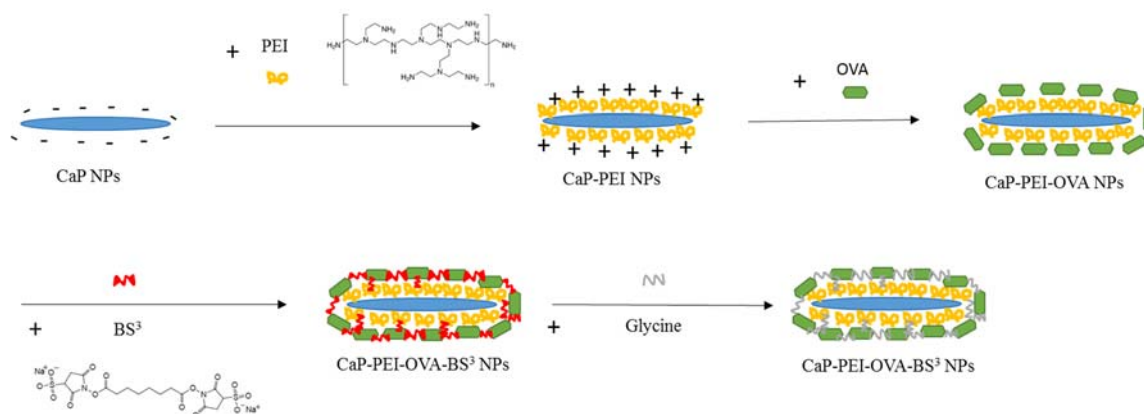


Figure 3. 14: Established protocol of the preparation of cross-linked CaP-PEI-OVA NPs.

The charge of CaP NPs was reversed to positive by PEI. Next, OVA was added to CaP-PEI NPs by electrostatic interaction. Finally, OVA on the NP surface was cross-linked by BS³, and quenched by glycine. Abbreviations: CaP NPs, calcium phosphate nanoparticles; PEI, polyethyleneimine; OVA, OVA; BS³ (bis[sulfosuccinimidyl]suberate).

Table 3. 12: Characterisation of different sizes of cross-linked CaP-PEI-OVA NPs. Data are expressed as mean $\pm$ SD of at least 3 biological replicates; $\pm$ indicates the SDs.

Layer-by-layer	Ca(NO ₃) ₂ .4H ₂ O, NH ₄ H ₂ PO ₄			CaCl ₂ , NaH ₂ PO ₄			CaCl ₂ , Na ₂ HPO ₄ .2H ₂ O		
	Size (nm)	PDI	Zeta potential (mV)	Size (nm)	PDI	Zeta potential (mV)	Size (nm)	PDI	Zeta potential (mV)
CaP NPs	75 $\pm$ 15	0.18 $\pm$ 0.05	-26.50 $\pm$ 1.43	165 $\pm$ 21	0.12 $\pm$ 0.06	-18.7 $\pm$ 2.6	335 $\pm$ 45	0.16 $\pm$ 0.04	-23.06 $\pm$ 1.56
CaP-PEI NPs	125 $\pm$ 15	0.19 $\pm$ 0.08	27.0 $\pm$ 0.92	189 $\pm$ 57	0.14 $\pm$ 0.03	22.5 $\pm$ 0.61	368 $\pm$ 21	0.08 $\pm$ 0.09	25.0 $\pm$ 0.73
CaP-PEI-OVA NPs	173 $\pm$ 15	0.12 $\pm$ 0.05	-12.7 $\pm$ 0.28	259 $\pm$ 24	0.12 $\pm$ 0.07	-13.8 $\pm$ 0.54	371 $\pm$ 25	0.15 $\pm$ 0.09	-11.08 $\pm$ 0.77
CaP-PEI-OVA-BS ³ NPs	174 $\pm$ 10	0.09 $\pm$ 0.04	-16.7 $\pm$ 0.6	261 $\pm$ 20	0.11 $\pm$ 0.07	-17.5 $\pm$ 1.2	364 $\pm$ 21	0.09 $\pm$ 0.07	-18.3 $\pm$ 0.8

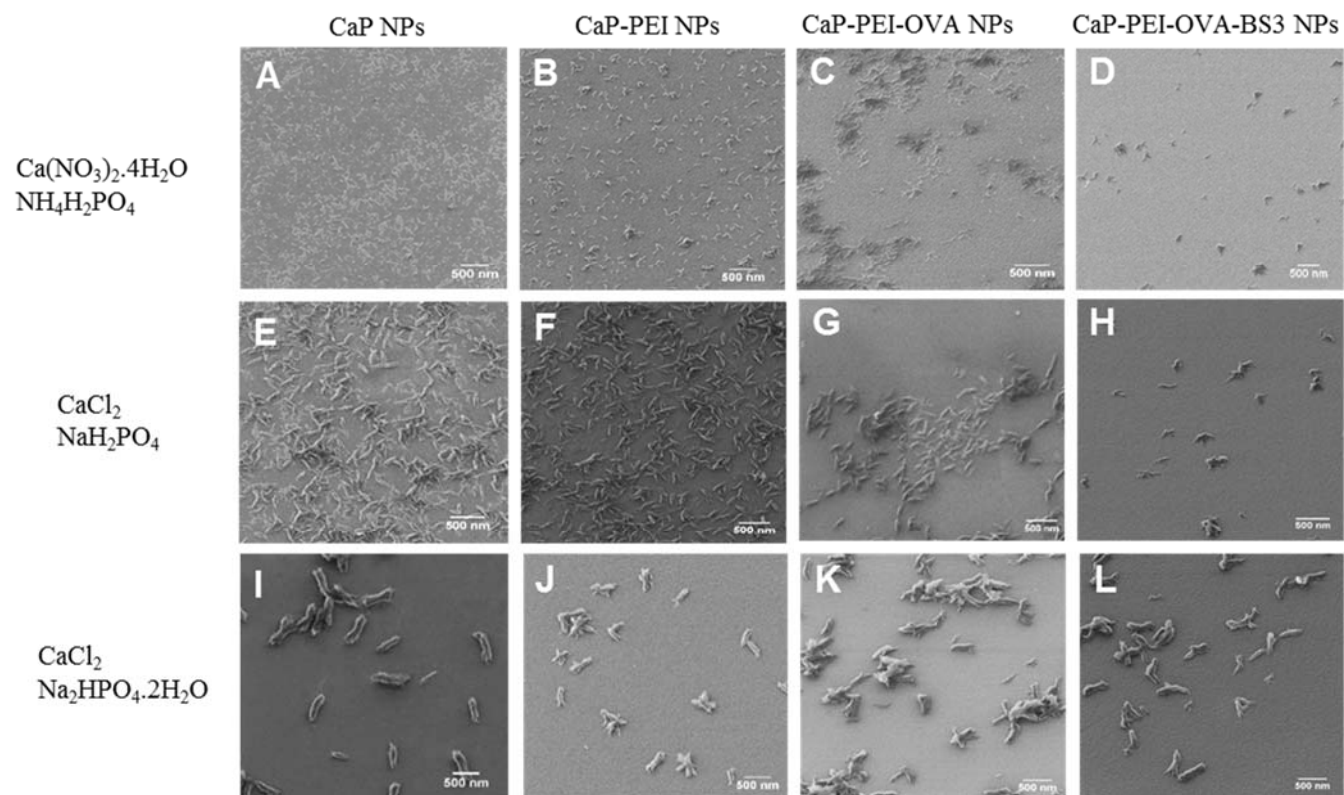


Figure 3. 15: HIM images of cross-linked CaP-PEI-OVA NPs synthesised using layer-by-layer method.
 (A, B, C, D) Ca(NO₃)₂·4H₂O + NH₄H₂PO₄, (E, F, G, H) CaCl₂ + NaH₂PO₄, (I, J, K, L) CaCl₂ + Na₂HPO₄·2H₂O.

3.4 Discussion

The incorporation of a model protein into nanoparticles is a fundamental aspect of NPs vaccine development. With the aim to load OVA into CaP NPs, initially, encapsulation method was used. However, the loading efficiency of OVA using encapsulation in our study was very low, which is consistent with previous studies [173, 219]. A recent study, collaborating our data, reported that only 4% of OVA was loaded onto sugar coated CaP NPs when the initial OVA concentration was 1 mg/mL [219]. Because of OVA loading efficiency was extremely low in the present study, modification of the charge of citrate-coated CaP NPs was explored as a means of increasing the protein loading efficiency by a layer-by-layer approach.

Layer-by-layer (LbL) technology offers a promising way to formulate multilayered thin films and coatings on a nanometer scale that has been widely applied in several biomedical fields [313-318]. Typically, the LbL method uses using electrostatic interactions to assemble NPs with layers of biological molecule on them [314]. A number of authors have found that simple amino acids can act as a stabilising agent to coat the NPs [337-340]. It has commonly been suggested that amino acids can bind to the NP surface as a means to avoid bulk NP aggregation [337]. For example, glutamic acid and histidine have been used as a capping agent to synthesise 10-15 nm gold nanochains [338]. Arginine coated hydroxyapatite was positively charged and demonstrated had higher protein adsorption [339, 340]. Similarly, amino acid derivatives would adsorb to the nanoparticle surface to change the surface charge of hydroxyapatite [341]. As a result, in the current study the amino acids L-lysine, L-arginine, L-lysine ethyl ester and L-arginine ethyl ester were tested as stabilisers to modify the charge of NPs. Although amino acids did reduce the negative charge of the CaP NPs, they all induced significant aggregation. This aggregation is likely to be caused by slow and incomplete replacement of citrate ions on the particle surface, resulting in an unequal surface charge, which is an agreement with the literature [337, 342]. Sethi *et al.* showed the arginine partially replaced with citrate ions on the Au NPs surface resulted in aggregation [342]. Another study reported that the addition of arginine resulted in the incomplete substitution of citrate-capped NPs and branched linear chains of citrate-coated Au NPs [337]. Thus it is likely that amino acids were unable to entirely displace the citrate ions on the particle surface, so the partially negatively and positively CaP NPs resulted in bridging flocculation and aggregates [343].

Another method of coating NPs and change surface charge rapidly is to use polymers. Polyethylenimine (PEI) is a commonly used polymer for many years in protein [55] and gene delivery [313, 326, 344] as it has a high number of positive charges. PEI is a synthetic, positively charged polymer that can enhance cell uptake and the delivery of agents to the desired sites [345]. PEI has a high positive charge density at physiological pH, and permits the condensation of negatively charged protein into dense particles by electrostatic interaction. Nevertheless, the widespread application of PEI *in vivo* is limited by its cytotoxicity [344]. The current study showed 5 kDa linear, 10 kDa linear, 10 kDa branched, and 25 kDa branched PEI could fully modify the charge of particles. However, linear PEI of 5 kDa and 10 kDa were unable to stabilise NP emulsion for more than 3 days whereas the branched PEI which have high repulsive forces avoided nanoparticle bridging and aggregation [346].

As the biosafety of a delivery system for vaccine application must be taken into consideration, the cytotoxicity of CaP-PEI NPs was assessed. It is reported that high molecular weight PEI has significant cell toxicity [87, 345, 347]. In an effort to reduce cytotoxicity of PEI, researchers have previously focused on the use of low molecular weight form of PEI in preference to high molecular one. A recent study reported that when 1.8 kDa PEI was used at a concentration of 120 µg/mL, MTT assays showed the cell viability to be more than 85%, whereas the cell viability was less than 25% when the 25 kDa form of PEI was used at the same concentration. PEI with a higher molecular weight (25 kDa) shows high cytotoxicity and transfection efficiency, whereas PEI with a lower molecular weight (1.8 kDa) shows low cytotoxicity and transfection efficiency [326]. In contrast, a recent paper reported that the 25 kDa PEI coated particles did not have significant toxicity to DCs within the concentration range of 12.5-100 µg/mL [320]. PEI with low molecular weight (1.8 kDa) has been investigated to form 1.8 kDa PEI/citric acid/DNA complexes and was shown to be non-toxic for *in vitro* and *in vivo* DNA delivery [289, 326]. In the present study, no toxicity was found on HEK and H4IIE cells, with the exception of 25 kDa PEI. This decreased cytotoxicity is likely to be due to the contribution of the protein corona. It is reported that protein corona could reduce the toxicity of PEI capped nanoparticles [313]. Another possibility is the low PEI concentration, and full removal of any unreacted PEI during formulation [87, 313, 326, 345].

It is known that NPs may have severe aggregation when subjected to biological fluids. As a result, the size stability of non-cross-linked CaP-PEI-OVA NPs was evaluated in various cell culture media. The size of the particle was stable in MilliQ water without any aggregation,

indicating the colloidal stability of CaP-PEI-OVA NPs. However, the particle size became unstable in PBS. This is likely to be due to the various types of salts and has high ionic strength in PBS, and that these salts change particle surface structure and surface charge and can lead to severe aggregation [348-350]. The non-cross-linked CaP-PEI-OVA NPs showed excellent size stability in cell DMEM with 10% FBS and SBF buffer with 55% human serum, suggesting that serum proteins improve the size stability of nanoparticles. This is also in agreement with previous literature [320, 348]. Chen *et al.* showed that Fe₂O₃ nanoparticles had serious aggregation in media without FCS, whereas in RPMI media with 10% FCS the particle size remained unchanged [351]. However, this study found that the size of the particle slightly increased when the non-cross-linked and cross-linked NPs suspended in cell culture media and SBF buffer with 55% human serum. This may be due to the formation of protein corona in these media increased the hydrodynamic diameter of CaP-PEI-OVA NPs. FCS and serum contain a number of proteins and lipids such as lipoproteins and glycoproteins, and these proteins might improve the stability of particles. This is likely to be due to being incorporated into the protein corona, which contributes to inhibiting further aggregation of particles [313, 352, 353]. Furthermore, previous research has found that FBS could suppress the CaP NPs mineralisation development and phase transformation, which impedes particle aggregation [354]. The size stability of cross-linked CaP-PEI-OVA NPs was also observed in various media. Similar to non-cross-linked nanoparticles, they were stable in aqueous solution, cell culture media, and SBF buffer with 55% serum. Most importantly, cross-linking of OVA can significantly enhance the stability of NPs in the salt buffer, which could be observed from the size of cross-linked NPs. The cross-linked complexes not only show increased storage stability but also exhibit stability in various applications [355]. Also, cross-linked CaP NPs have significantly fewer protein bands compared to non-cross-linked NPs in cell culture media and human serum. This indicates that the cross-linked NPs reduced the plasma protein adsorption protein in the culture media and human serum, which facilitate NP application *in vivo* investigations [356]. Protein binding can change the size and surface charge of NPs, which affect the overall biodistribution of NPs throughout the body [357]. Some serum proteins such as IgG are reported to facilitate NP phagocytosis and removal by systemic circulation through macrophages of the reticuloendothelial system (RES) [358, 359]. It is clear from these results that the cross-linked CaP NPs can decrease the amount of protein binding, and thus that it is suitable for vaccine applications.

Taken together, the findings of this investigation demonstrate that the LbL approach and then cross-linking method is an effective, safe and promising way to formulate a nanoparticle vaccine.

3.5 Conclusion

In summary, an efficient LbL procedure to synthesise a protein vaccine using calcium phosphate nanoparticles was successfully developed. This chapter investigated the effect of several preparation parameters on nanoparticle vaccine size, PDI and zeta potential by independent experiments. Furthermore, the interaction between CaP NPs with human serum: effects of cross-link on plasma protein binding was also examined. The results show that the synthesis method is reproducible, and straightforward and the cross-linked CaP-PEI-OVA NPs enhanced salt stability and greatly reduced the plasma protein adsorption. These results provide important information about the types of protein adsorbed onto particles and therefore give deeper insight into initial biological interactions between NPs and blood. These data highlight the importance of cross-linking protein in improving particle stability. Therefore, this LbL method shows great potential in protein delivery, which could be used for therapeutic applications.

3.6 Supplementary Information

Supplementary Table 3. 1: Identification of bound protein on the nanoparticle surface by mass spectrometry. The protein corona comes from non-cross-linked NPs incubated with cell culture media for 6 h at room temperature.

Normalised concentration indicates the intensity of each band as a percentage of all band intensities.

Band No. (Normalised conc. %)	Protein Name	Swisprot Accession No.	Mascot Score	Number of Pep- tides
1 (2.06%)	Alpha-2-macroglobulin	A2MG BOVIN	1342	88
	Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1 BOVIN	1305	79
	Serum albumin	ALBU BOVIN	943	70
	Ovalbumin	OVAL CHICK	869	41
	Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3 BOVIN	592	42
	Coagulation factor V	FA5 BOVIN	469	32
	Complement C3	CO3 BOVIN	434	43
2 (4.60%)	Alpha-2-macroglobulin	A2MG BOVIN	5666	342
	Ovalbumin	OVAL CHICK	935	43
	Serum albumin	ALBU BOVIN	933	62
3 (4.64%)	Alpha-2-macroglobulin	A2MG BOVIN	2144	132
	Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3 BOVIN	1596	97
	Ovalbumin	OVAL CHICK	1173	48
	Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1 BOVIN	947	66
	Complement component C6	CO6 BOVIN	789	47
	Complement C3	CO3 BOVIN	731	66
	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4 BOVIN	473	50
	Phosphatidylinositol-glycan-specific phospholipase D	PHLD BOVIN	254	19
	Apolipoprotein A-I	APOA1 BOVIN	242	15
4 (3.33%)	Prothrombin	THRB BOVIN	219	10
	Serum albumin	ALBU BOVIN	1508	94
	Ovalbumin	OVAL CHICK	1360	69
5 (17.42%)	Serotransferrin	TRFE BOVIN	1029	71
	Serum albumin	ALBU BOVIN	6710	407
	Complement C3	CO3 BOVIN	2426	116
6 (8.23%)	Ovalbumin	OVAL CHICK	1748	55
	Alpha-2-macroglobulin	A2MG BOVIN	1491	74
	Complement component C9	BOVIN	1313	70
7 (18.11%)	Serum albumin	ALBU BOVIN	1166	93
	Ovalbumin	OVAL CHICK	821	41
	Alpha-2-HS-glycoprotein	FETUA BOVIN	451	25
	Antithrombin-III	ANT3 BOVIN	412	34
8 (22.4%)	Ovalbumin	OVAL CHICK	5045	200
9 (13.59%)	Complement C3	CO3 BOVIN	5527	227
			1829	106
10 (5.59%)	Ovalbumin	OVAL CHICK	2382	99
	Adiponectin	ADIPO BOVIN	1234	100
	Apolipoprotein A-I	APOA1 BOVIN	1404	105
	Ovalbumin	OVAL CHICK	1292	65

The band's number was shown in Figure 3.15 A.

Supplementary Table 3. 2: Identification of bound protein on the nanoparticle surface by mass spectrometry. The protein corona comes from non-cross-linked NPs incubated with SBF buffer with 55% human serum for 6 h at room temperature.

Normalised concentration indicates the intensity of each band as a percentage of all band intensities.

Band No. (Normalised conc. %)	Protein Name	Swisprot Accession No.	Mascot Score	Number of Pep- tides
1 (2.98%)	Apolipoprotein B-100	APOB Human	9113	540
	Fibronectin	FINC Human	2452	122
2 (1.25%)	Apolipoprotein B-100	APOB Human	3611	260
	Fibronectin	FINC Human	2269	114
3 (1.96%)	Fibronectin	FINC Human	5557	316
4 (5.31%)	Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1 Human	4403	191
	Inter-alpha-trypsin inhibitor heavy chain H2	ITIH2 Human	3396	202
5 (0.80%)	Alpha-2-macroglobulin	A2MG Human	3519	215
6 (0.77%)	Complement factor H	CFAH Human	3018	243
	Apolipoprotein B-100	APOB Human	2524	173
	Serum albumin	ALBU Human	901	62
7 (4.86%)	Ceruloplasmin	CERU Human	4674	213
	Apolipoprotein B-100	APOB Human	2250	176
	Complement C3	CO3 Human	1924	121
	Ovalbumin	OVAL Chick	1802	70
8 (2.49%)	Complement C3	CO3 Human	5729	313
	Complement C5	CO5 Human	1162	72
	Serum albumin	ALBU Chick	852	47
	Complement C4-B	CO4B Human	747	43
9 (7.88%)	Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3 Human	4256	196
	Inter-alpha-trypsin inhibitor heavy chain H2	ITIH2 Human	3027	175
	Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1 Human	2606	103
	Complement C3	CO3 Human	1329	85
	Protein AMBP	AMBP Human	1096	40
	Serum albumin	ALBU Human	1029	53
10 (3.28%)	Ovalbumin	OVAL Chick	2828	101
	Serotransferrin	TRFE Human	2252	108
	Immunoglobulin heavy constant mu	IGHM Human	2013	109
	Complement C4-B	CO4B Human	1849	82
	Prothrombin	THRB Human	1330	50
	Serum albumin	ALBU Human	776	55
	Immunoglobulin gamma-1 heavy chain	IGG1 Human	446	22
	Coagulation factor XIII A chain Insulin-like	F13A Human	439	28
	Growth factor-binding protein complex acid labile subunit	ALS Human	365	22
	Coagulation factor	VFA5 Human	356	35
11 (3.43%)	Complement C4-A	CO4A Human	4954	251
	Complement C3	CO3 Human	4594	217
	Ovalbumin	OVAL Chick	2116	75
	Serum albumin	ALBU Human	1412	86
	Hyaluronan-binding protein 2	HABP2 Human	1308	83
	Prothrombin	THRB Human	128411	43
	Immunoglobulin heavy constant mu	IGHM Human	70	62
	C4b-binding protein alpha chain	C4BPA Human	1165	64
	Complement component C9	CO9 Human	555	35
	Complement C5	CO5 Human	532	34
	Coagulation factor XIII A chain	F13A Human	474	28
	Vitronectin	VTNC Human	422	24
	Vitamin K-dependent protein S	PROS Human	422	17
12 (22.3%)	Serum albumin	ALBU Human	9627	430
	Complement C3	CO3 Human	5365	222

	Ovalbumin	OVAL Chick	1663	49
	Complement C4-A	CO4A Human	1204	50
	Vitronectin	VTNC Human	697	37
	Complement component C9	CO9 Human	637	30
	Hyaluronan-binding protein 2	HABP2 Human	539	41
	Heparin cofactor 2	HEP2 Human	361	31
13 (3.98%)	Serum albumin	ALBU Human	1688	107
	Immunoglobulin heavy constant alpha 1	IGHA1 Human	1095	61
	Vitronectin	VTNC Human	829	74
	Complement C3	CO3 Human	816	83
	Alpha-1-antichymotrypsin	AACT Human	777	32
	Ovalbumin	OVAL Chick	608	29
	Angiotensinogen	ANGT Human	434	13
14 (11.98%)	Serum albumin	ALBU Human	2025	99
	Immunoglobulin gamma-1 heavy chain	IGG1 Human	1734	91
	Alpha-1-antitrypsin	A1AT Human	1603	82
	Ovalbumin	OVAL Chick	975	34
	Antithrombin-III	ANT3 Human	933	54
	Alpha-1-antichymotrypsin	AACT Human	920	39
	Complement C3	CO3 Human	893	53
	Complement C4-B	CO4B Human	890	41
	Fibrinogen beta chain	FIBB Human	462	24
	Vitamin D-binding protein	VTDB Human	291	21
	Kininogen-1	KNG1 Human	277	19
15 (8.33%)	Ovalbumin	OVAL Chick	7136	245
	Complement C3	CO3 Human	2231	134
	Haptoglobin	HPT Human	991	59
	Serum paraoxonase/arylesterase 1	PON1 Human	671	24
	Apolipoprotein A-IV	APOA4 Human	574	41
16 (9.31%)	Ovalbumin	OVAL Chick	5792	240
	Complement C3	CO3 Human	2879	154
	Serum albumin	ALBU Human	1139	72
	Complement C4-A	CO4A Human	895	59
	Prothrombin	THRB Human	822	27
	Haptoglobin Serum	HPT Human	625	37
	Paraoxonase/arylesterase 1	PON1 Human	374	12
	CD5 antigen-like	CD5L Human	365	21
	Immunoglobulin gamma-1 heavy chain	IGG1 Human	340	30
	Clusterin	CLUS Human	317	22
17 (1.80%)	Ovalbumin	OVAL Chick	1952	89
	Prothrombin	THRB Human	1528	93
	Serum albumin	ALBU Chick	953	68
	Complement C4-A	CO4A Human	892	77
	Complement C3	CO3 Human	693	61
	Protein AMBP	AMBP Human	529	27
	Complement C1q subcomponent subunit B	C1QB Human	362	17
18 (7.24%)	Immunoglobulin kappa light chain	IGK Human	2118	69
	Ovalbumin	OVAL Chick	1425	50
	Complement C4-A	CO4A Human	672	54
	Serum albumin	ALBU Human	615	49
	Serum amyloid P-component	SAMP Human	449	25

The band's number was shown in Figure 3.15 B.

Supplementary Table 3. 3: Identification of bound protein on the nanoparticle surface by mass spectrometry. The protein corona comes from cross-linked NPs incubated with SBF buffer with 55% human serum for 6 h at room temperature.

Normalised concentration indicates the intensity of each band as a percentage of all band intensities.

Band No. (Normalised conc. %)	Protein Name	Swisprot Accession No.	Mascot Score	Number of Pep- tides
1 (1.4%)	Alpha-2-macroglobulin	A2MG Human	1921	118
	Complement factor H	CFAH Human	905	52
	Fibronectin	FITC Human	798	53
2 (3.5%)	Serum albumin	ALBU Human	1010	45
	Ceruloplasmin	CERU Human	733	28
	Alpha-2-macroglobulin	A2MG Human	638	49
3 (1.5%)	Complement C3	CO3 Human	1252	68
	Serum albumin	ALBU Human	740	38
	Alpha-2-macroglobulin	A2MG Human	701	50
4 (2.2%)	Serotransferrin	TRFE Human	1598	86
	Serum albumin	ALBU Human	953	56
	Immunoglobulin heavy constant mu	IGHM Human	899	50
5 (6.2%)	Serum albumin	ALBU Human	6264	344
	Complement C3	CO3 Human	1674	72
6 (45.7%)	Serum albumin	ALBU Human	2388	111
	Immunoglobulin gamma-1 heavy chain	IGG1 Human	1827	103
	Alpha-1-antitrypsin	A1AT Human	1048	63
7 (19.5%)	Serum albumin	ALBU Human	769	45
	Immunoglobulin gamma-1 heavy chain	IGG1 Human	535	27
	Serotransferrin	TRFE Human	763	39
8 (2.5%)	Serum albumin	ALBU Human	1484	82
	Complement C3	CO3 Human	1276	57
	Serotransferrin	TRFE Human	763	39
9 (3.1%)	Serum albumin	ALBU Human	1524	65
	Immunoglobulin kappa light chain	IGK Human	1461	47
10 (14%)	Serum albumin	ALBU Human	745	41
	Immunoglobulin kappa constant	IGKC Human	287	13

The band's number was shown in Figure 3.15 B.

Chapter 4

Evaluation of the immunological effects of different sizes of CaP NPs vaccine on oral epithelial cells and macrophages

4.1 Introduction

Currently, most of the licensed vaccines are parenterally administered, and the common injection routes include intravenous (IV), subcutaneous (SC), and intramuscular (IM) [360]. While these vaccinations confer systemic immunity, many infections and squamous cell carcinoma (e.g. oral cancer) are mucosally initiated. Although mucosal vaccines have been investigated for many years, relatively few mucosal vaccines have been approved for commercial use [361]. One of the challenges associated with mucosally delivered vaccines is the mucosal epithelium itself. Epithelial cells perform a critical role in the body, and are the first line of defence against any pathogen in a non-specific manner [362]. These mucosal epithelial cells play an essential role in host-pathogen interaction and significantly affect the type of immunity induced by the host [104]. However, due to their biological function epithelial cells form a tight monolayer, which can prevent the administration of some vaccines and drugs [363, 364]. As large molecules, such as proteins, cannot penetrate the cell wall nor effectively pass through tight junctions. As a result, an efficient delivery system for mucosal vaccines would have significant value and is generating considerable research interest [365].

Over the last two decades, nanoparticle-based vaccines have emerged as a very promising strategy for vaccine development [33, 83]. Nanoparticles (NPs) are synthesised as solid cores with a diameter range from 1 to 1,000 nm, which have shown significant adjuvant effects as antigen carriers [366]. Various types of NPs have been used for vaccination, and the most widely studied NPs include polymeric nanoparticles, lipid nanoparticles, carbon nanotubes, and inorganic nanoparticles (calcium phosphate, silicate, gold) [32-37, 71]. Among these different types of nanoparticles calcium phosphate nanoparticles (CaP NPs) have attracted increasing attention. CaP NPs are non-toxic, biodegradable, cost-effective, and have pH-dependent solubility. Most importantly, they protect the antigen cargo from degradation and elimination, and deliver it to immune cells [128]. They can also be functionalised with various molecular adjuvants to enhance active immune cell targeting and thus the efficacy of a vaccine [30, 340]. Due to these properties, CaP NPs have been studied in a number of biomedical applications such as DNA/gene silencing, drug delivery, protein/peptide delivery, and dental implant materials [237, 238, 340, 367].

The successful mucosal delivery of a nanoparticle vaccine greatly relies on their interactions with epithelial cells and antigen-presenting cells (APCs). Several studies have reported that

different physiological properties such as size, surface charge, shape, and surface coating on NPs have significant effects on NP uptake and biological responses [74, 198, 199, 234, 239, 345, 368]. It has been shown that the interaction between NPs and immune cells is highly dependent on size. In particular, NP size has been demonstrated to be the most critical factor in influencing nanoparticle vaccine efficacy [74, 234, 239, 313, 369-373]. Kulkarni *et al.* reported that the size of the particle correlates with cellular uptake and NP biodistribution [368]. Considering how vital CaP NP size is for biomedical applications, the effect of NP size on immunological responses is gaining increased research. NPs between 20-100 nm can easily enter lymph vessels through the subcapsular sinus [374]. After migrating to the lymph nodes the released antigens could directly activate B cells, and be effectively phagocytosed by dendritic cells, resulting in the induction of long-lasting cell-mediated and humoral immunity. Larger NPs of up to 200 nm are generally taken up at the injection site by phagocytic cells via endocytosis or micropinocytosis which then migrate to the draining lymph nodes and initiate an immune response [238]. A recent study found that at an optimal size range of 100 nm to 400 nm could elicit substantially stronger antibody responses than either smaller or larger particles [234]. Together, these results emphasise the importance of synthesising NPs of a specific, uniform size to induce a robust immune response against the delivered antigen.

Compared to the effects of NP particle size, there are comparatively fewer investigations that have focused on the influence of nanoparticle shape on the host immune response. Rod-like shaped gold NPs have been shown to have a greater cellular uptake by macrophages as compared to star and triangle-shaped NPs [375]. Furthermore, the most effective shape for cellular uptake by APCs was determined to be rod-shaped gold NPs, as compared to spherical and cube-shaped [74]. Rod-shaped hydroxyapatite, compared with sphere-shaped hydroxyapatite, elicited stronger inflammasome-dependent IL-1 β production *in vitro*, indicating greater cellular uptake. Interestingly, both rod- and star-shaped NPs induced a similar level of antibody response *in vivo* [234]. Although the interaction of spherical CaP NPs and cells has been extensively investigated [132, 159, 161, 258], little is known about the cellular interaction with rod-shaped CaP NPs.

The experiments described in this chapter are designed to investigate the ability of rod-like crystals of CaP NPs to bind to epithelial cells and migrate through epithelial cell layers. These experiments also explored the effect of different NP sizes on the immunological response *in vitro*. Three sizes of CaP NPs, 170 nm, 260 nm and 360 nm in diameter, were chosen as they

all fall within the biologically active size window previously reported [234]. Moreover, these NP formulations have a narrow size distribution that is suitable for biomedical applications. OVA was used as a model antigen for the NP formulation, which is widely used in the development of novel vaccine systems [81, 222, 320]. The interactions between three sizes of rod-like shaped NPs and epithelial cells and macrophages were observed. Further experiments on the effects of these nanoparticles included cytotoxicity, host-cell binding, migration, phagocytosis, uptake, NF- κ B activation, cytokine secretion, and cell surface marker expression. These experiments were performed on oral epithelial cells (OKF6) and a mouse macrophage cell line (RAW 264.7).

4.2 Materials and Methods

4.2.1 Preparation and characterisation of cross-linked CaP-OVA NPs of different sizes

Different sizes of cross-linked CaP-OVA NPs were synthesised using the layer-by-layer method. The combination of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ + $\text{NH}_4\text{H}_2\text{PO}_4$, CaCl_2 + NaH_2PO_4 or CaCl_2 + $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ were chosen to formulate CaP NPs cores of different sizes. The cross-linked particle suspensions were stored at 4 °C for future characterisation. The cross-linked CaP-OVA NPs were characterised by Dynamic Light Scattering (DLS) for particle size, size distribution and zeta potential. HIM (Helium ion microscopy) was used to observe NPs size and shape. The characterisation technique is described in detail in Chapter 2 and 3. The BCA protein assay kit (Thermo Fisher Scientific, SA, Australia) was used to measure ovalbumin concentration according to the manufacturer's instructions. The number of NPs were counted by NanoSight NS300 (Malvern). All experiments were carried out in triplicate to ensure reproducibility.

4.2.2 Cell cultures and growth conditions

All cells were obtained from the Melbourne Dental School culture tissue culture collection. Human embryonic kidney cells (HEK), rat hepatoma cell line (H4IIE), macrophage cell line RAW 264.7, and RAW-Blue cells were cultured with complete media (Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine) under 5% v/v CO_2 at 37 °C. In addition, RAW-Blue cells were incubated with 200 µg/ml zeocin (Invivogen, USA). Human oral keratinocytes (OKF6) were grown in keratinocyte serum-free medium supplemented with 25 µg/ml bovine pituitary extract (BPE), 0.4 ng/ml human epidermal growth factor (EGF), 0.4 mM CaCl_2 , and 1% penicillin/streptomycin (KSF media). The OKF6 cells were cultured under 5% v/v CO_2 at 37 °C. Complete media was obtained from Life Technologies, NSW, Australia.

4.2.3 Cytotoxicity of nanoparticles

Cell cytotoxicity was measured by the lactate dehydrogenase (LDH, Promega, NSW, Australia) and CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega, NSW, Australia) method (performed according to the manufacturer's instructions). HEK, H4IIE and OKF6 cells were seeded into 96 well plates at a cell density of 5×10^3 cells per well in 200 µL complete media. After overnight incubation (5% v/v CO_2 , 37 °C), the cells had reached 70 -

80% confluency. The media was replaced with 150 μ L fresh media containing NPs at different NP to cell ratios or calcium/phosphate and incubated for 90 minutes (5% v/v CO₂, 37 °C). After incubation, 50 μ L of media was transferred to a new 96 well plate and measured for LDH. For the MTS assays, MTS was diluted in 100 μ L fresh cell media and added to the cells after removing NPs in suspension, then assessed by MTS kits according to the manufacturer's protocol.

4.2.4 Nanoparticle OKF6 cell migration assay

A 6.5 mm Transwell® with 3.0 μ m Sterile Pore Polycarbonate Membrane Insert (Corning, VIC, Australia) was used to perform the NP migration assay and OKF6 cells were grown in the top well of a cell culture membrane at a cell density of 2×10^5 cells/200 μ L in complete media. After overnight incubation (5% v/v CO₂, 37 °C), the cells were 95 - 100% confluent. To demonstrate cell confluency, the same number of cells were also grown in 96 well plates (the base of which is the same surface area as the transwell membrane) and imaged by EVOS Flouid Imager (Life Technologies, NSW, Australia). The next day the media in the upper chamber was replaced with 200 μ L fresh media containing 2×10^9 cross-linked CaP-OVA NPs with different sizes. The lower chamber was replaced with 600 μ L fresh media. Following various incubation time (15, 30, 60, 120 and 180 min), the media from the upper chamber and lower chamber were collected. The ability of NP to migrate across the cell monolayer was determined by the number of NPs in the lower chamber. The percentage of migration was calculated by the number of NPs in the lower chamber divided by the total NPs added. The number of NPs was counted using the NanoSight NS300 (Malvern). The media without NPs were used as a negative control.

4.2.5 Nanoparticle cell binding assays by flow cytometry

Different sizes of cross-linked CaP-OVA NPs were fluorescently labelled using FITC (Invitrogen, USA) according to the manufacturer's instructions. OKF6 cells were grown in 24 well plates at a cell density of 4×10^5 cells in 200 μ L KSF media. The number of NPs were counted by NanoSight NS300 (Malvern). OKF6 cells were incubated with three sizes of NPs in 200 μ L complete media at NP to cell ratios of 10:1, 100:1, 1,000:1 and 10,000:1 and incubated (1 h, 5% v/v CO₂, 37 °C). After incubation, the cells were washed with FACS buffer ((PBS + 1% BSA + 0.1% Sodium Azide (NaN₃)) three times to remove unassociated nanoparticles and then removed from the plates by trypsin. The collected cell suspensions were centrifuged ($800 \times g$,

5 min, RT). The cell pellet was resuspended in FACS buffer, and the level of cell binding was quantified by flow cytometry (BD LSRFortessa X-20, BD Biosciences, NSW, Australia). The OKF6 cells without NPs were used as a negative control.

4.2.6 Cellular uptake of nanoparticles by confocal imaging

OKF6 cells were seeded into 8-well Lab-Tek II chamber #1.5 German Coverglass System (Thermo Fisher Scientific, SA, Australia) at a cell density of 2×10^5 cells/200 μ L. After overnight incubation (5% v/v CO₂, 37 °C), the cells were 95 - 100% confluent. The number of NPs were counted by NanoSight NS300 (Malvern). Cross-linked CaP-OVA NPs (170 nm, 260 nm, or 360 nm) in 200 μ L KSF media were added to OKF6 cells at NP to cell ratios of 1,000:1 and 10,000:1 and incubated for 1 and 3 h (5% v/v CO₂, 37 °C). NPs were pre-labelled with the green fluorescent marker, Atto 488 (Sigma). Following the incubation period, the cell media was removed and the cells washed three times with PBS to remove free NPs. The cell membrane was stained using FM Lipophilic Styryl Dyes (15 μ g/mL, Thermo Fisher Scientific, SA, Australia) for 10 min at 4 °C and DAPI was used to stain the nuclei (1 μ g/mL, Thermo Fisher Scientific, SA, Australia, 10 min, 4 °C). Finally, the cells were fixed with 4% paraformaldehyde for 10 min at 4 °C. The uptake of NPs by cells was examined by fluorescence microscopy using the Nikon A1R+ (Nikon Instruments, NSW, Australia) and the images were analysed by focus stacking.

4.2.7 Nanoparticle phagocytosis assays by flow cytometry

Cross-linked CaP-OVA NPs were fluorescently labelled using pHrodo Red (Thermo Fisher Scientific, SA, Australia) according to the manufacturer's instructions. Briefly, 2 mg/mL CaP-OVA NPs resuspended in 100 μ L 0.1 M sodium bicarbonate buffer (pH 8.5), 10 μ L pHrodo Red (1 mg/mL) then added to the NP solution and incubated for 1 h at RT (no light). After incubation, the CaP-OVA NPs were harvested by centrifugation ($10,000 \times g$, 5 min, RT) and washed 3 times with MilliQ water. Following the final wash, the CaP-OVA NPs pellet was dispersed in MilliQ water and stored at 4 °C. The number of NPs were counted by NanoSight NS300 (Malvern). Macrophage RAW 264.7 cells were resuspended at a density of 2.5×10^5 cells/200 μ L in complete media. These cells were incubated with three sizes of NPs in 200 μ L complete media at NP to cell ratios of 10:1, 100:1, 1000:1 and 10,000:1 and incubated (1 h, 5% v/v CO₂, 37 °C). After incubation, the cells were washed with FACS buffer three times and

resuspended in 200 μ L FACS buffer. The amount of phagocytosis was quantified by flow cytometry (BD LSRFortessa X-20, BD Biosciences, NSW, Australia). RAW 264.7 cells in complete media without NPs were used as the negative control.

4.2.8 NF- κ B activation assay with RAW-Blue cells

RAW-Blue cells (Invivogen, USA) were seeded into 96 well plates at a concentration of 1×10^5 cells per well in 200 μ L complete DMEM. After overnight incubation (5% v/v CO₂, 37 °C), different sizes of cross-linked CaP-OVA NPs in 200 μ L complete media were added to the RAW-Blue cells at NP to cell ratios of 10:1, 100:1, 1,000:1 and 10,000:1 and incubated for 24 h (5% v/v CO₂, 37 °C). The number of NPs were counted by NanoSight NS300 (Malvern). Following the incubation period, 50 μ L of media was taken from the cultures and transferred to a new 96 well plate and added to 150 μ L Quanti-Blue (Invivogen, USA). The plate was incubated for 2 h (5% v/v CO₂, 37 °C), and the optical density was measured at 620 nm on a spectrophotometer to determine secreted embryonic alkaline phosphatase (SEAP) levels. RAW-Blue cells incubated in complete media without NPs were used as the negative control and complete media containing Lipopolysaccharide (LPS, 10 μ g/mL, Sigma-Aldrich, NSW, Australia) was used as a positive control.

4.2.9 Cell surface marker production by flow cytometry

RAW 264.7 cells were seeded into 24 well plates at a cell density of 2×10^5 cells per well in 200 μ L complete media. The number of NPs were counted by NanoSight NS300 (Malvern). After overnight incubation (5% v/v CO₂, 37 °C), different sizes of cross-linked CaP-OVA NPs in 200 μ L complete media were added to cells at NP to cell ratios of 10:1, 100:1, 1,000:1 and 10,000:1, and incubated (24 h, 5% v/v CO₂, 37 °C). The cells were washed with FACS buffer and blocked for 20 min on ice with rat anti-mouse CD16/CD32 antibody (BD Pharmingen, US) to prevent Fc-receptor binding of antibodies. The cells were then incubated with the following antibodies (20 min, 0 °C): PE-Cy™7 rat anti-mouse CD86; APC rat anti-mouse CD40; APC MHC class II (I-A/I-E) monoclonal antibody. These antibodies were all purchased from BD Bioscience (NSW, Australia). The level of CD40, CD80, and MHC II expression were quantified by flow cytometry (BD LSRFortessa X-20, BD Biosciences, NSW, Australia). The data was exported and FlowJo software (Three Star) was used for data analysis. RAW 264.7 cells

incubated in complete media without NPs were used as the negative control and complete media containing Lipopolysaccharide (LPS, 10 µg/mL, Sigma-Aldrich, NSW, Australia) was used as a positive control. All experiments were performed in triplicate.

4.2.10 Cytokine production assays with OKF6 cells and RAW cells

OKF6 cells and RAW 264.7 cells were seeded separately into 24 well plates at a cell density of 2×10^5 cells per well in 200 µL complete media. The number of NPs were counted by NanoSight NS300 (Malvern). After overnight incubation (5% v/v CO₂, 37 °C), cross-linked CaP-OVA NPs (170 nm, 260 nm, or 360 nm) in 200 µL complete media were added to cells at NP to cell ratios of 10:1, 100:1, 1,000:1 and 10,000:1 and incubated for 6 and 24 h (5% v/v CO₂, 37 °C). The supernatant was collected by centrifugation (800 × g, 5 min, RT) after incubation to remove any cells and NPs. Cells incubated in complete media without NPs were used as a negative control. The level of cytokines ((IL-1β, IL-6, IL-8, IL-10, IL-12, TNF-α and monocyte chemoattractant protein (MCP)-1)) in the supernatant was determined by ELISA (BD Biosciences, NSW, Australia) according to the manufacturer's instructions.

4.2.11 Statistical analysis

GraphPad Prism 5 software was used to analyse data in experiments. One-way ANOVA and student T-test were used to determine *P* and a *P* < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Characterisation of different sizes of cross-linked CaP-OVA NPs

The repeatable synthesis and characterisation of uniform, monodispersed nanoparticles is essential for their use as vaccine carriers. Three different sizes of cross-linked CaP-OVA NPs were prepared using the layer-by-layer approach. The CaP NPs cores were synthesised by the citrate chelating method, and then a layer of PEI was added to give the particle surface a positive charge. After coating with the model antigen ovalbumin, the nanoparticles were cross-linked using BS³ and quenched with glycine as described in Chapter 3.

The particle size, size distribution and surface charge of different sizes of cross-linked CaP-OVA NPs were characterised by DLS. Table 4.1 summarises the physical properties of different sizes of nanoparticle formulations. The hydrodynamic diameters of three different sizes of NPs (small (S), medium (M), and large (L)) as measured by DLS were 174 ± 10 , 261 ± 20 and 364 ± 21 nm and termed CaP-OVA-S, CaP-OVA-M and CaP-OVA-L, respectively (Table 4.1, Figure 4.1A). The PDI value of the three samples was low (0.08 - 0.11), indicating a homogeneous and monodispersed NP population. All CaP-OVA-S, CaP-OVA-M and CaP-OVA-L NPs were negatively charged, with surface charges of -16.7 ± 0.6 , -17.5 ± 1.2 and -18.3 ± 0.8 mV in water. Nanoparticles were counted using the NanoSight NS300, and the BCA protein assay was used to measure the protein content. Based on the total number of nanoparticles and protein content, the protein per nanoparticles was calculated for each nanoparticle (Table 4.1). The amount of OVA-loaded onto each nanoparticle was different for the three sizes of nanoparticles. The smallest sized NPs (174 ± 10 nm) loaded the lowest amount of OVA per particle (0.72×10^{-15} g), and the largest NPs (364 ± 21 nm) loaded the highest amount of OVA per particle (2.16×10^{-15} g).

Helium ion microscopy (HIM) was used to investigate the shape and confirm the size of the nanoparticles. All of the nanoparticles demonstrated a rod-like and uniform morphology (Figure 4.1B). The consistency of the shape of NPs remains the same between the CaP NP core and following OVA cross-linked across all NP sizes; small-sized NPs (Figure 4.1B (a) and 4.1B (d)), medium-sized NPs (4.1B (b) and 4.1B (e)), and large-sized NPs (4.1B (c) and 4.1B (f)). Furthermore, the HIM images show that nanoparticles are monodispersed, which is consistent with polydispersity data (Table 4.1). The length of nanoparticles was analysed by Image J software and corresponded to the DLS results (Figure 4.1 and Table 4.1).

Table 4. 1: Physical characterisation of different sizes of cross-linked CaP-OVA NPs.

Sample	Size (nm)	Polydispersity index (PDI)	Zeta potential (mV)	OVA per NP (g)
CaP-OVA-S	174 ± 10	0.09 ± 0.04	-16.7 ± 0.6	0.72×10^{-15}
CaP-OVA-M	261 ± 20	0.11 ± 0.07	-17.5 ± 1.2	1.96×10^{-15}
CaP-OVA-L	364 ± 21	0.08 ± 0.07	-18.3 ± 0.8	2.16×10^{-15}

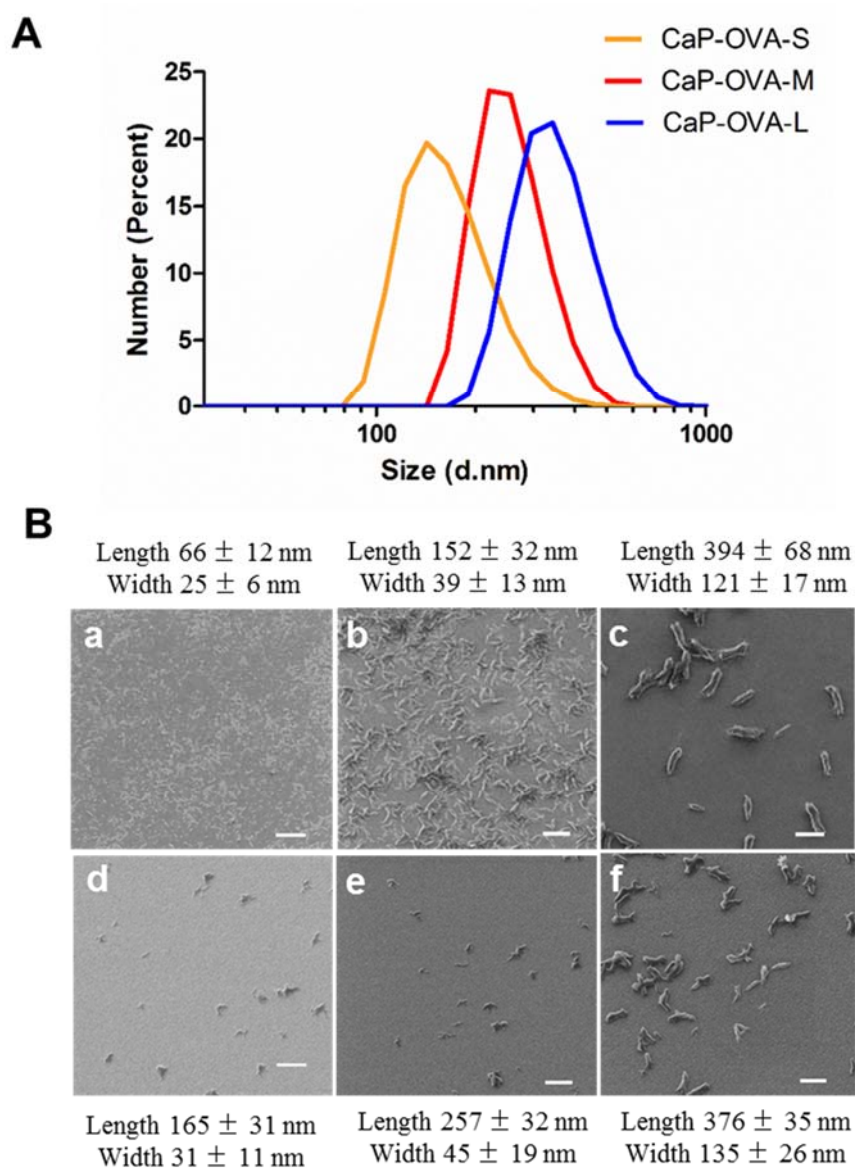


Figure 4. 1: Characterisation of different sizes of CaP-OVA NPs.

(A) The hydrodynamic diameters of different sizes of cross-linked CaP-OVA NPs; (B) HIM image of different sizes of cross-linked CaP-OVA NPs before (CaP NPs core) and after OVA coating (cross-linked CaP-OVA NPs). (a) CaP-S (b) CaP-M (c) CaP-L (d) CaP-OVA-S (e) CaP-OVA-M (f) CaP-OVA-L. Scale bar = 500 nm.

4.3.2 Nanoparticle cytotoxicity

The biocompatibility of a nanoparticle delivery system is crucial for its application as a vaccine carrier. In this study, the cytotoxicity of cross-linked CaP-OVA NPs (S, M and L) were initially evaluated by MTS (cell viability) and LDH (cytotoxicity) assays on HEK, H4IIE, and OKF6 cells (Figure 4.2).

All size of nanoparticle did not display significant cytotoxicity on HEK cells, except at the highest nanoparticle concentration (2.0×10^{10} per well) (Figures 4.2A and B). At this NP concentration both CaP-OVA-M and CaP-OVA-L induced a significant loss of cell viability ($P < 0.05$ and $P < 0.01$, respectively) (Figures 4.2A), and a significant increase in cytotoxicity ($P < 0.001$) (Figure 4.2B) compared to cells in media alone (negative control). Although significant, the increase in cell death or loss of cell viability was $< 10\%$ as compared to the negative control. Each CaP NP (S, M and L nanoparticles), when used at concentrations above 5.0×10^9 per well induced significant decrease in cell viability ($P < 0.05 - 0.01$) (Figure 4.2C) and increased toxicity ($P < 0.05 - 0.001$) (Figure 4.2D) of H4IIE cells ($< 30\%$), as compared to the negative control. For the OKF6 cells none of the CaP-OVA NPs at any concentration tested were found to be cytotoxic (Figures 4.2E and F).

In order to determine the mechanisms that lead to the slight cellular toxicity of NPs towards HEK and H4IIE cells, these cells were treated with different concentrations of calcium and phosphate ions (to match the level in CaP NPs), naked NPs, or PEI coated CaP (CaP-PEI NPs) (Figure 4.3). Calcium and phosphate ions at the concentration tested were found to not be cytotoxic towards HEK or H4IIE cells (Figure 4.3). Similarly, naked NPs were not cytotoxic towards HEK and H4IIE cells (Figure 4.4A and B).

A CaP-PEI-L concentration of 2.0×10^{10} nanoparticles per 10^5 cells induced a significant but small increase in the cytotoxicity of HEK cells as compared to the negative control ($P < 0.001$) (Figure 4.4C). Additionally, high concentrations of CaP-PEI-S (2.5×10^9 to 2.0×10^{10} nanoparticles per 1×10^5 cells) induced a small but significantly higher toxicity of H4IIE cells as compared to the negative control ($P < 0.05 - 0.001$) (Figure 4.4D). CaP-PEI-M and CaP-PEI-L NPs also induced small but significant toxicity on H4IIE cells at 2.0×10^{10} nanoparticles per 10^5 cells compared to the negative control ($P < 0.01$) (Figure 4.4D).

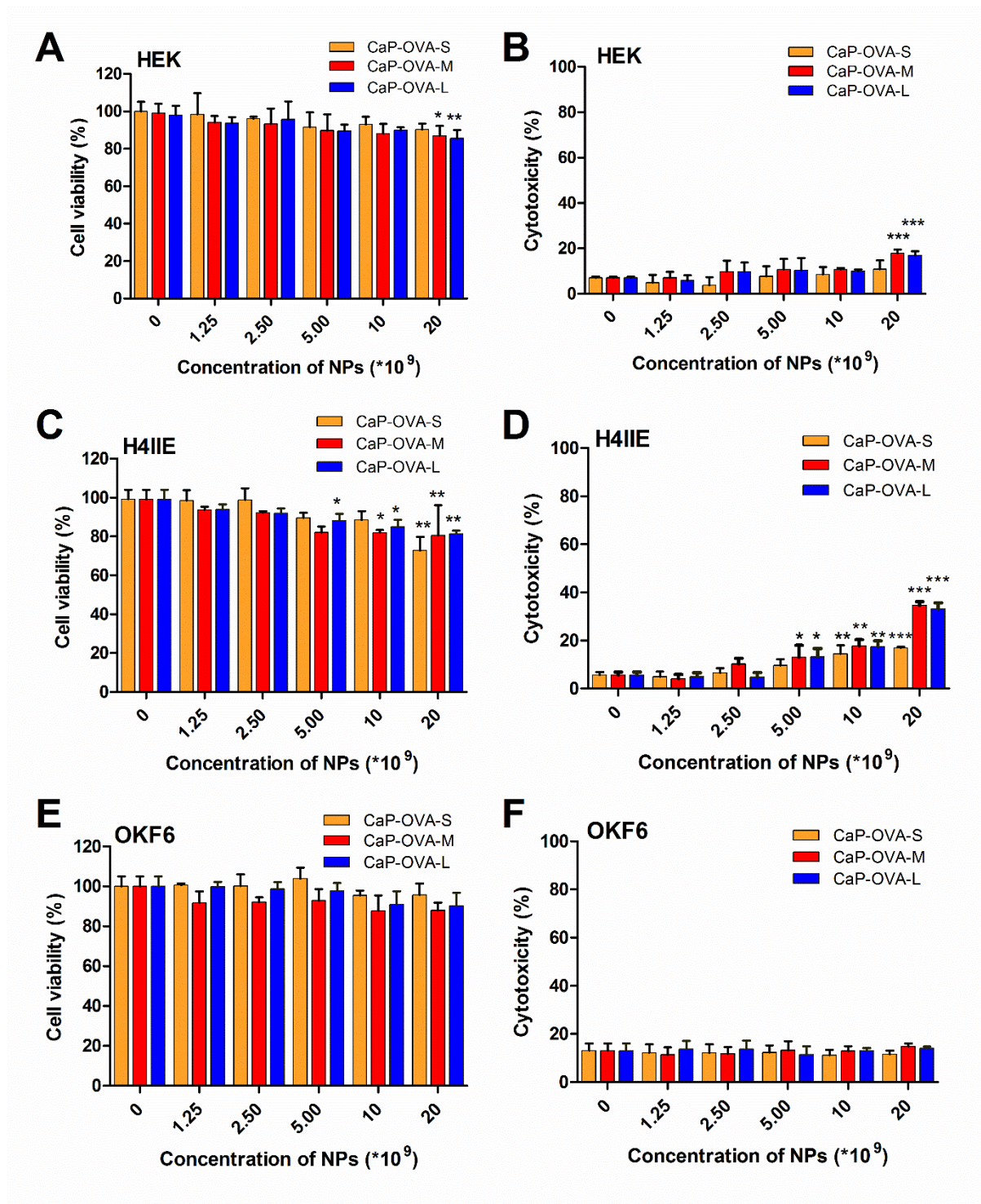


Figure 4. 2: Cell viability and toxicity effects of CaP-OVA NPs.

Cell viability was determined by MTS assays (A, C, E), and cytotoxicity was determined by LDH assays (B, D, F) in HEK, H4IIE, and OKF6 cells exposed to different concentrations of three sizes of cross-linked CaP-OVA NPs for 90 minutes. Data are expressed as mean $\pm$ SD. Fresh media were used as the negative control. The negative control is the cells alone. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

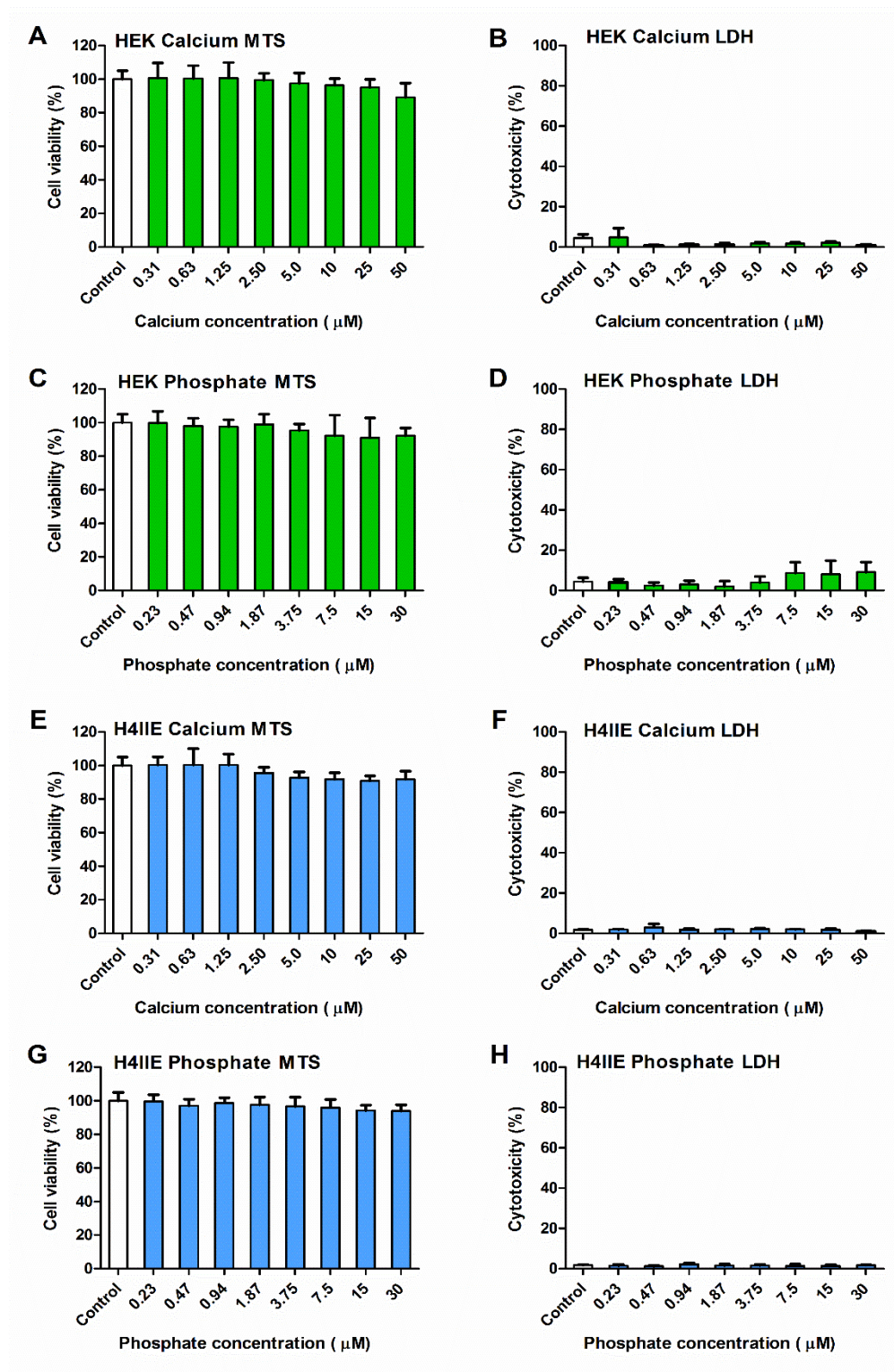


Figure 4. 3: Cell viability and toxicity effects of CaP-PEI-OVA NP components.

Cell viability was determined toxicity by MTS assays (A, C, E, G), and cytotoxicity was determined by LDH assays (B, D, F, H) on HEK and H4IIE cells exposed to different concentrations of calcium and phosphate for 90 min. Data are expressed as mean $\pm$ SD. Assays were blanked by media alone. Untreated cells were used as the negative control. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

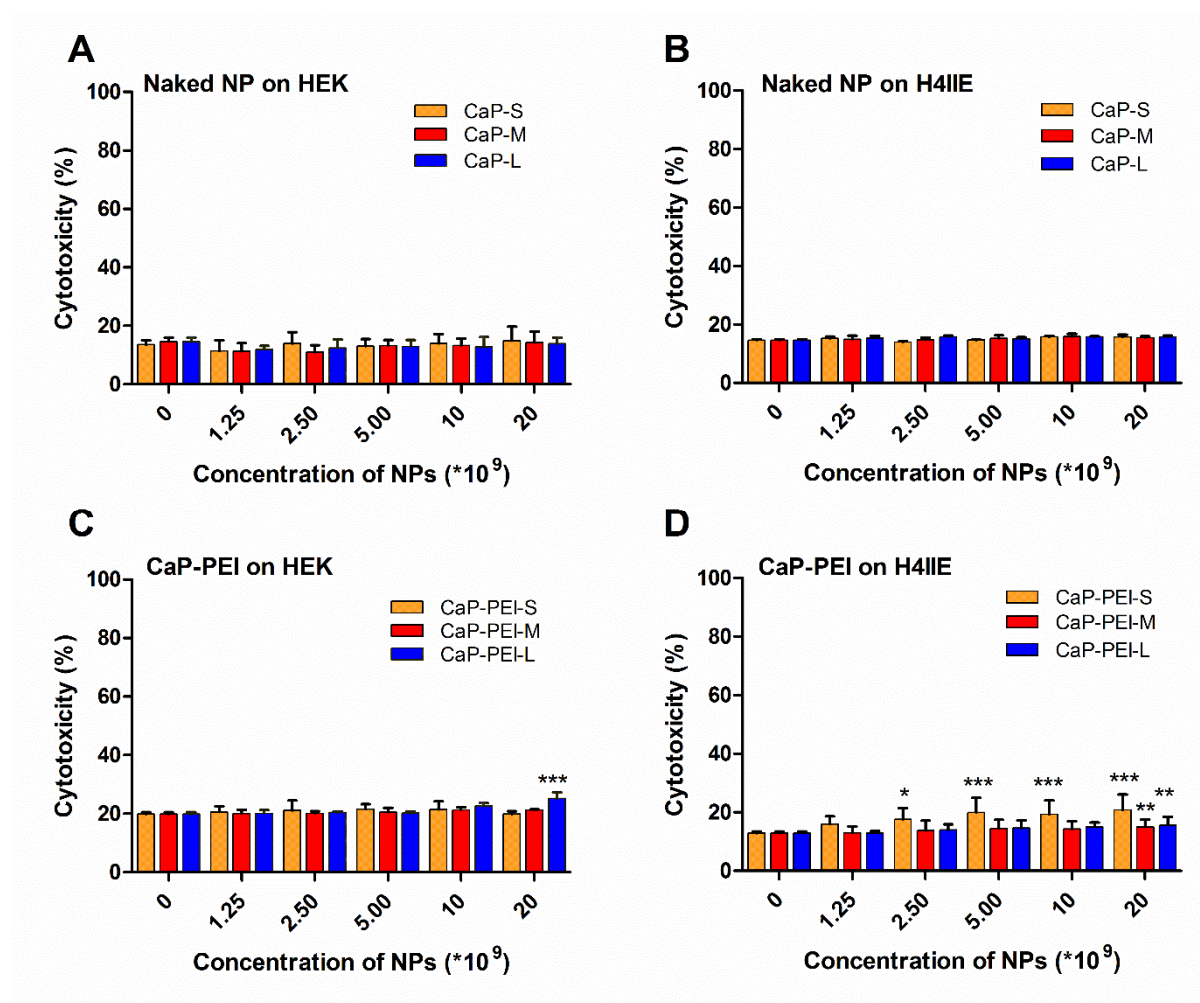


Figure 4. 4: Cytotoxic effects of NP-associated PEI.

Cell toxicity by LDH assay on HEK and H4IIE cells exposed to three sizes of naked CaP NPs and CaP-PEI NPs for 90 min. Data are expressed as mean $\pm$ SD. The blank is the culture media alone. Untreated cells were used as a negative control. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

4.3.3 Binding of nanoparticles to oral epithelial cells

In order to investigate the binding of the three sizes of CaP-OVA NPs to oral epithelial cells, nanoparticles were labelled with FITC and the level of binding was determined by flow cytometry. The CaP-OVA NPs (S, M and L) all bound to OKF6 cells in a dose-dependent manner (Figure 4.5), as indicated by both the percentage of cells bound with NPs and mean fluorescence intensity (MFI: the amount of NPs/cell) increase. CaP-OVA-M and CaP-OVA-L NPs showed a greater affinity for binding to OKF6 cells compared to CaP-OVA-S NPs at the NP to cell ratio of 100:1, reflected by the increase in the binding percentages (Figure 4.5B, $P < 0.0001$). A significant difference was also observed at the NP to cell ratio of 1,000:1 between CaP-OVA-S and CaP-OVA-M NPs, where a higher percentage of OKF6 cells were CaP-OVA-M positive (Figure 4.5B, $P < 0.05$). No significant difference was found in the binding of CaP-OVA-M and CaP-OVA-L NPs at this concentration to OKF6 cells (Figure 4.5B). Furthermore, no cell binding difference was observed among S, M and L nanoparticles at NP to cell ratio of 10,000:1 (Figure 4.5B), indicating binding saturation of the assay at this high NP concentration. However, in terms of MFI (number of NPs per cell), at an NP to cell ratio of 10,000:1 there were more CaP-OVA-L NPs per cell than CaP-OVA-M which was higher than cells incubated with CaP-OVA-S (Figure 4.5C).

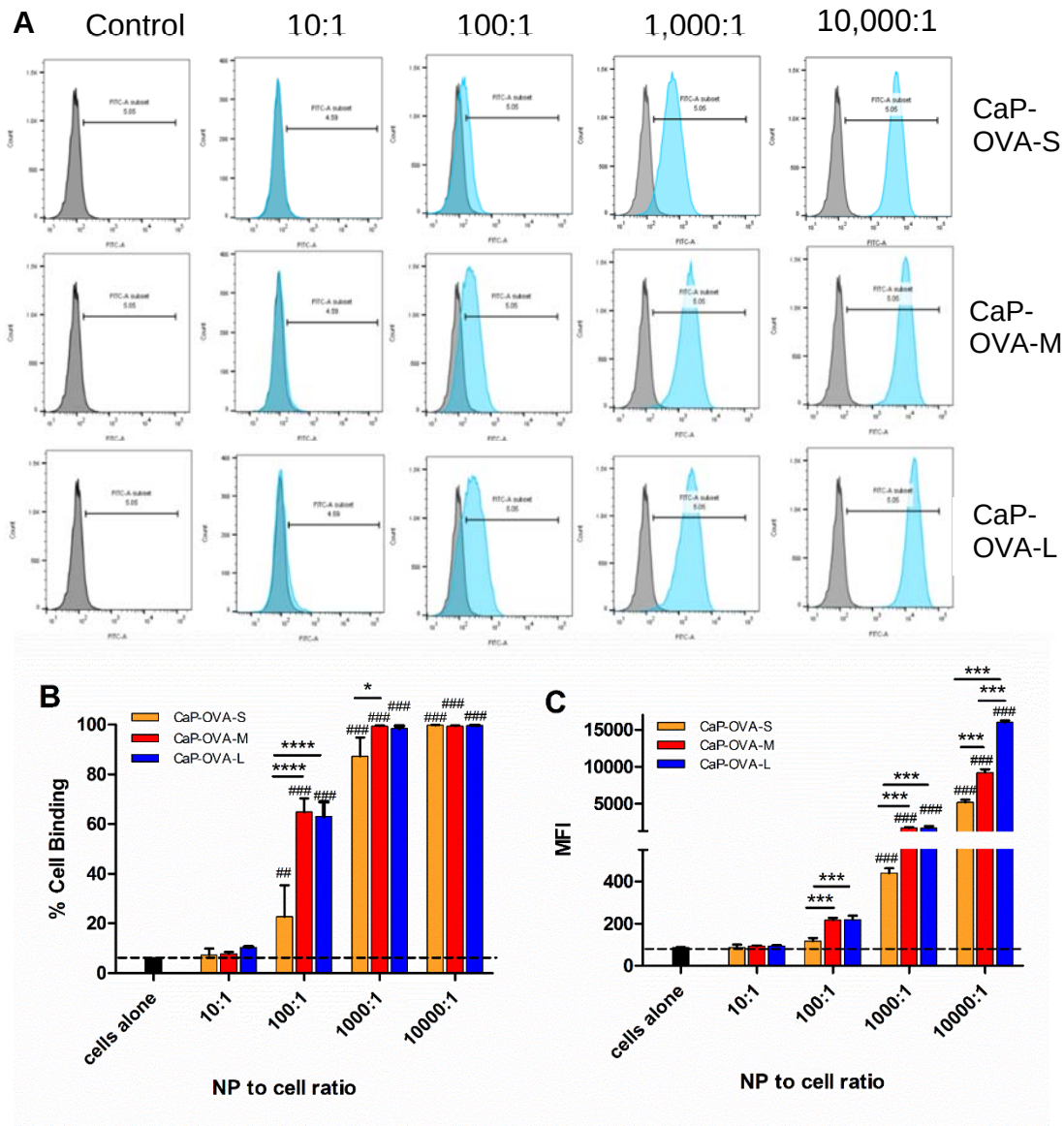


Figure 4. 5: Binding of cross-linked CaP-OVA NPs to the oral epithelial cells (OKF6).

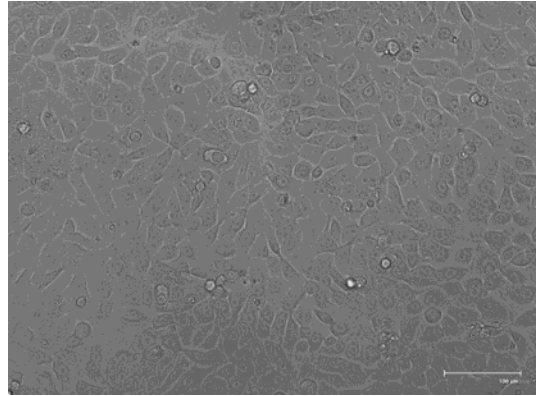
Nanoparticles were labelled with FITC to determine their ability to bind to OKF6 cells at increasing nanoparticle to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1) for 1 h. Nanoparticles bound to OKF6 cells was determined by an increase in FITC fluorescence on OKF6 cells (A). Binding properties were determined by both the cell binding percentage (B), which indicates the percentage of OKF6 cells with bound nanoparticles. The capacity of NP binding was also shown by the mean fluorescence intensity (MFI), which is equivalent to an increase in nanoparticles per cell (C). Data are expressed as mean $\pm$ SD. $\#P < 0.05$, $\#\#P < 0.01$, and $\###P < 0.001$ were considered statistically significant when compared between treatment groups and cells alone. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ was considered statistically significant when compared among the three sizes of particles.

4.3.4 Nanoparticle migration across epithelial cell monolayers

As nanoparticle migration across the epithelial cell barrier is one of the first important steps towards inducing an immune response, the ability of CaP-OVA NPs (S, M and L) to migrate across a cell monolayer was examined. OKF6 cells were grown in 96 well plates and imaged by an EVOS Flouid Imager to confirm confluency (Figure 4.6A). Initial nanoparticle migration studies demonstrated that all sizes of CaP-OVA NPs could migrate across the epithelial barrier in a time-dependent manner, with all S, M and L NPs shown to have commenced crossing the cell monolayer by 30 min (Figure 4.6B). The percentage of migrated CaP-OVA NPs of all sizes increased with increasing incubation time (Figure 4.6B). The CaP-OVA-S NPs transmigrated across the cell monolayer more efficiently (in terms of migration percentage) when compared to CaP-OVA-L at all time points tested. Additionally, incubation with CaP-OVA-S resulted in significantly higher migration percentage when compared to CaP-OVA-M after 3, 12 and 24 h incubation ($P < 0.01$) (Figure 4.6B). CaP-OVA-M was found at 12 and 24 hours to have a higher migration percentage as compared to CaP-OVA-L (Figure 4.6B).

To further investigate the localisation of NPs, the percentage of non-migrated nanoparticles in the upper chamber and NP associated with cells and migrated NP in the lower chamber was further investigated. The percentage of all migrated CaP-OVA NPs increased with increasing incubation time (Figure 4.7A, B and C). Correspondingly, the percentage of all nanoparticles remaining in the upper chamber decreased with longer incubation time (Figure 4.7A, B and C). When analysing NPs associated with the cell monolayer the binding percentage increased with the size of the nanoparticle, which corresponds to the previous NP to OKF6 cell binding data (Figure 4.5). CaP-OVA-S predominately migrated, with low cell association. CaP-OVA-M was approximately 50% migration with 50% cell associated. CaP-OVA-L NPs did have a proportion that transmigrated but were predominately cell associated (Figure 4.7).

A



B

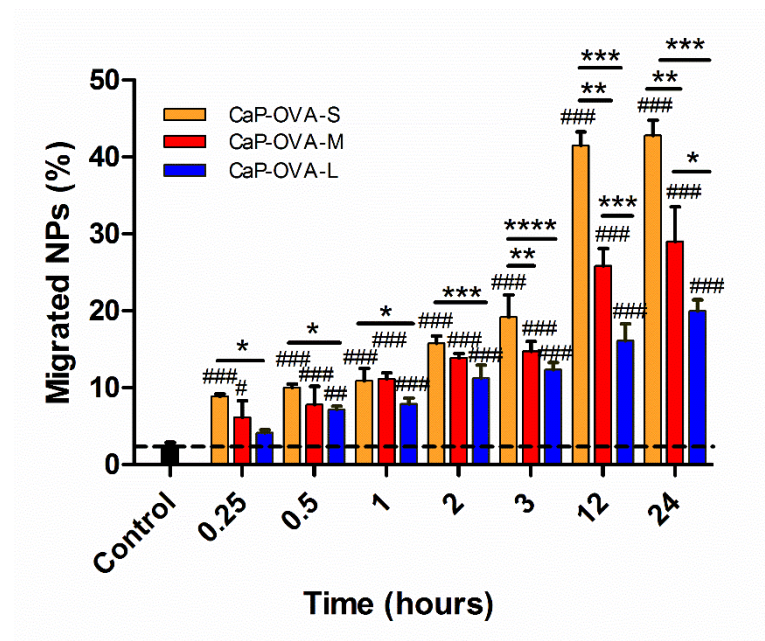


Figure 4. 6: Migration of CaP-OVA NPs through cellular monolayer.

In vitro NP migration assay in OKF6 cells exposed to three sizes of cross-linked CaP-OVA NPs from 0.25 to 24 h. Data is expressed as mean $\pm$ SD. [#] $P < 0.05$, ^{##} $P < 0.01$, and ^{###} $P < 0.001$ were considered statistically significant when compared between treatment groups and cells alone. $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered statistically significant when compared among the three sizes of particles.

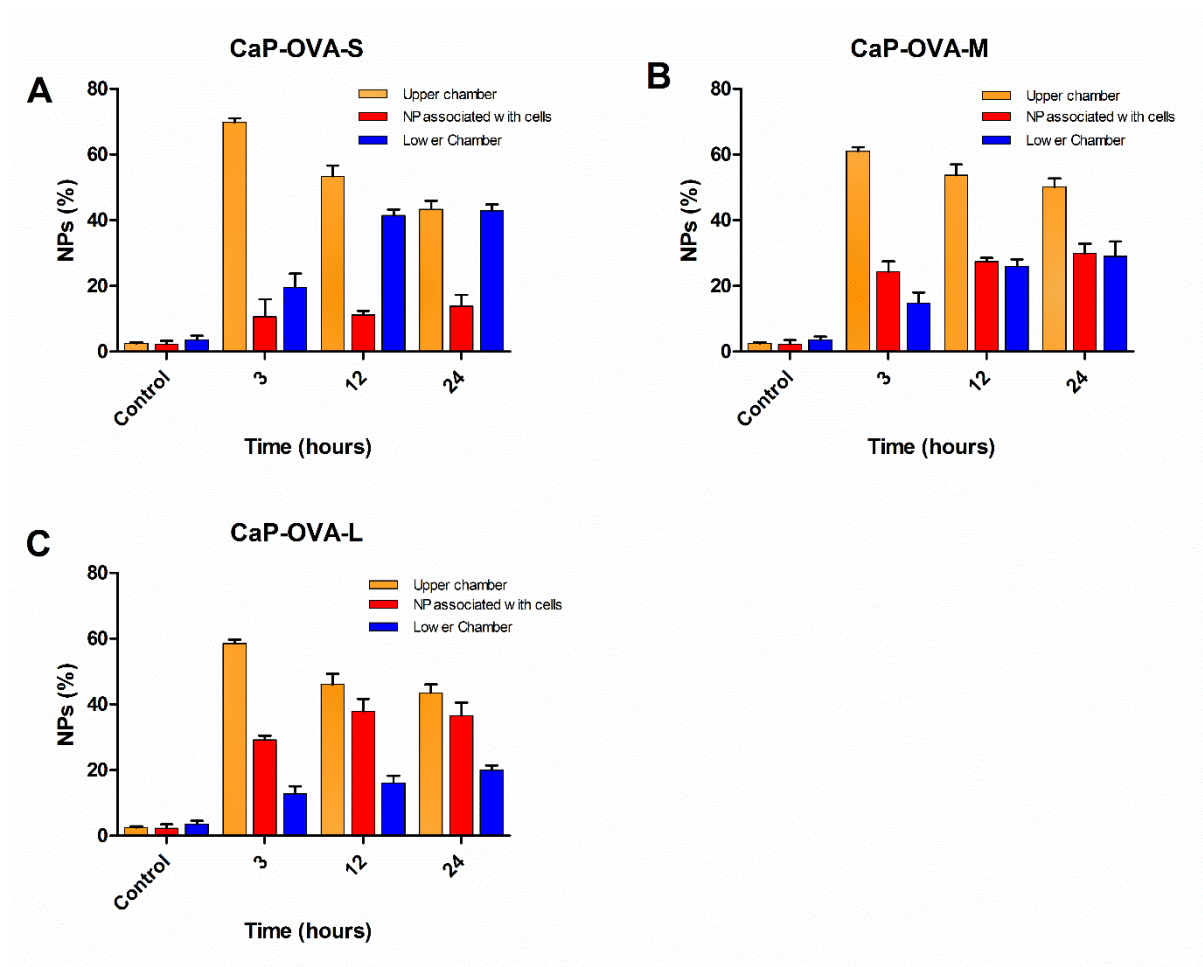


Figure 4. 7: Localisation of CaP-OVA NPs during cellular migration.

In vitro NP migration assay in OKF6 cells exposed to three sizes of cross-linked CaP-OVA NPs from 3 to 24 h. Data is expressed as mean $\pm$ SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were considered statistically significant when compared between treatment groups and cells alone. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant when compared among the three sizes of particles.

4.3.5 Nanoparticle uptake by confocal imaging

Confocal microscopy was used to study the uptake of nanoparticles by OKF6 cells by the visualisation of intracellular nanoparticles. The S, M and L nanoparticles were labelled with a fluorescence dye (FITC) and incubated with OKF6 cells for 1 and 3 h at two NP to cell ratios (1,000:1 and 10,000:1). After incubation of OKF6 cells for 1 and 3 h at two NP to cell ratios, internalised NP fluorescence was observed for CaP-OVA S, M and L (Supplementary Figure 4.1 and 4.2). Size, time and dose-dependent increases in internal NP fluorescence were apparent, with higher numbers of CaP-OVA-L NPs internalised than CaP-OVA-S and CaP-OVA-M at 1 and 3 h. Additionally, higher numbers of NPs were internalised with an increase in incubation time from 1 to 3 h and with an increase in dose from 1,000:1 to 10,000:1 (Supplementary Figure 4.1 and 4.2). The Z-stacking images composited from the data at 3 h incubation with 1,000:1 or 10,000:1 (Figure 4.8) shows CaP-OVA-L NPs were internalised more than CaP-OVA-M which was more than CaP-OVA-S, which corroborates the migration data (Figure 4.7). This indicates that the NPs were efficiently taken up by the cells, and that they were internalised within the OKF6 cells, rather than binding on the cell surface.

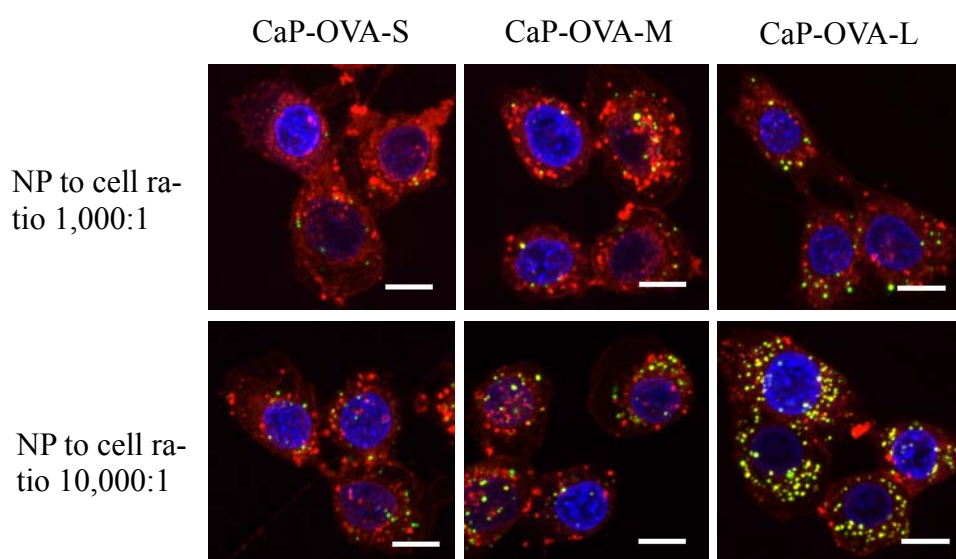


Figure 4. 8: Confocal microscopy of internalised CaP-OVA NPs.

Cellular uptake of three sizes of nanoparticles in OKF6 cells at NP to cell ratio of 1,000:1 and 10,000:1 for 3 h. Nanoparticle (green) were pre-labelled with Atto 488, plasma membrane (red) were stained with FM dyes, and nuclei (blue) were stained with DAPI. Scale bar = 10 μ m.

4.3.6 Nanoparticles induction of inflammatory cytokines by oral epithelial cells

OKF6 cells were incubated with CaP-OVA NPs (S, M and L) for 6 and 24 h, and the supernatant was collected and the secretion of IL-1 β , IL-6, IL-8, IL-10, TNF- α and monocyte chemoattractant protein (MCP)-1 by were determined by ELISA (Figure 4.9 and Figure 4.10). After incubation with nanoparticles for 6 h, at NP to cell ratio of 10,000:1, CaP-OVA-S and CaP-OVA-M NPs induced a significant IL-1 β and IL-8 secretion compared to the negative control ($P < 0.05$) (Figure 4.9A and C). Only OKF6 cells treated with CaP-OVA-S NPs elicited a significant IL-1 β and IL-8 response at the NP to cell ratio of 1,000:1 as compared to the negative control ($P < 0.05 - 0.01$) (Figure 4.9A and C). In addition, a significant difference in IL-8 production was observed between CaP-OVA-S and CaP-OVA-L at the highest NP to cell ratio ($P < 0.05$). None of the nanoparticles was observed to induce IL-6, IL-10, TNF- α and MCP-1 secretion in OKF6 cells compared to control cells (Figure 4.9).

OKF6 cells incubation (24 h) with CaP-OVA-S and CaP-OVA-M NPs induced significantly higher concentrations of IL-1 β and IL-8 at NP:cell ratios of 1,000:1 and 10,000:1 as compared to the negative control ($P < 0.01$) (Figure 4.10A and C). CaP-OVA-L at 1,000:1 and 10,000:1 NP to cell ratio induced a significantly higher IL-8 secretion as compared to the negative control (Figure 4.10C). CaP-OVA-S, CaP-OVA-M and CaP-OVA-L NPs treatment did not induce any significant IL-6 and IL-10, TNF- α or MCP-1 cytokine production (Figure 4.10B, D, E and F). No significant difference in the induction of cytokines levels was observed between the different sized CaP-OVA NPs (Figure 4.10).

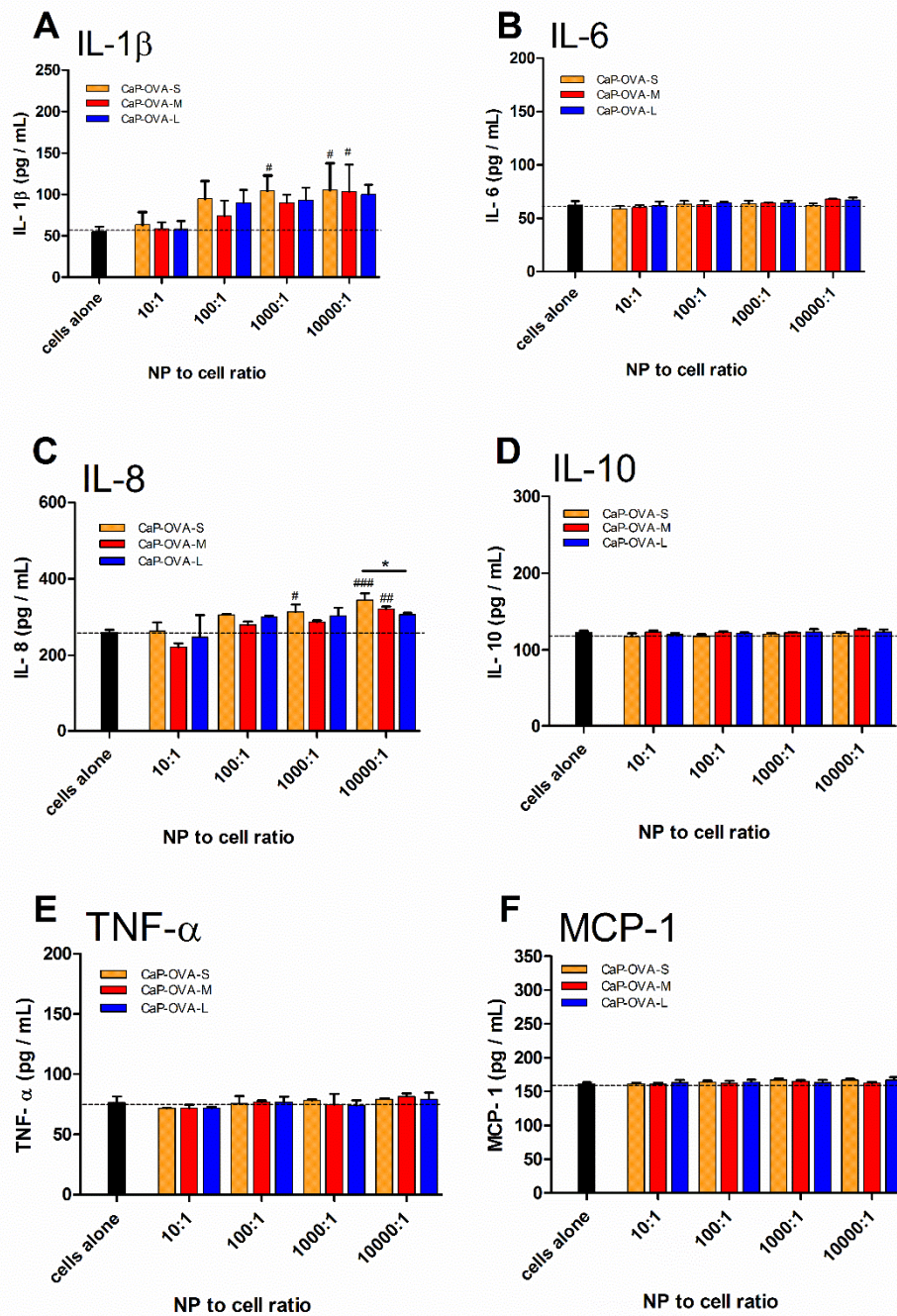


Figure 4. 9: OKF6 cytokines induced by CaP-OVA NPs for 6 h.

Three sizes of cross-linked CaP-OVA NPs induce inflammatory cytokine production in OKF6 cells. Cytokine production in OKF6 cells was measured following 6 h incubation of three sizes of cross-linked CaP-OVA NPs at various NP to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1). IL-1 β (A), IL-6 (B), IL-8 (C), IL-10 (D), TNF- α (E) and MCP-1 (F) were detected by ELISA. Data are expressed as mean $\pm$ SD. # P < 0.05, ## P < 0.01, and ### P < 0.001 were considered statistically significant when compared between treatment groups and cells alone. * P < 0.05, ** P < 0.01, and *** P < 0.001 were considered statistically significant when compared among the three sizes of particles.

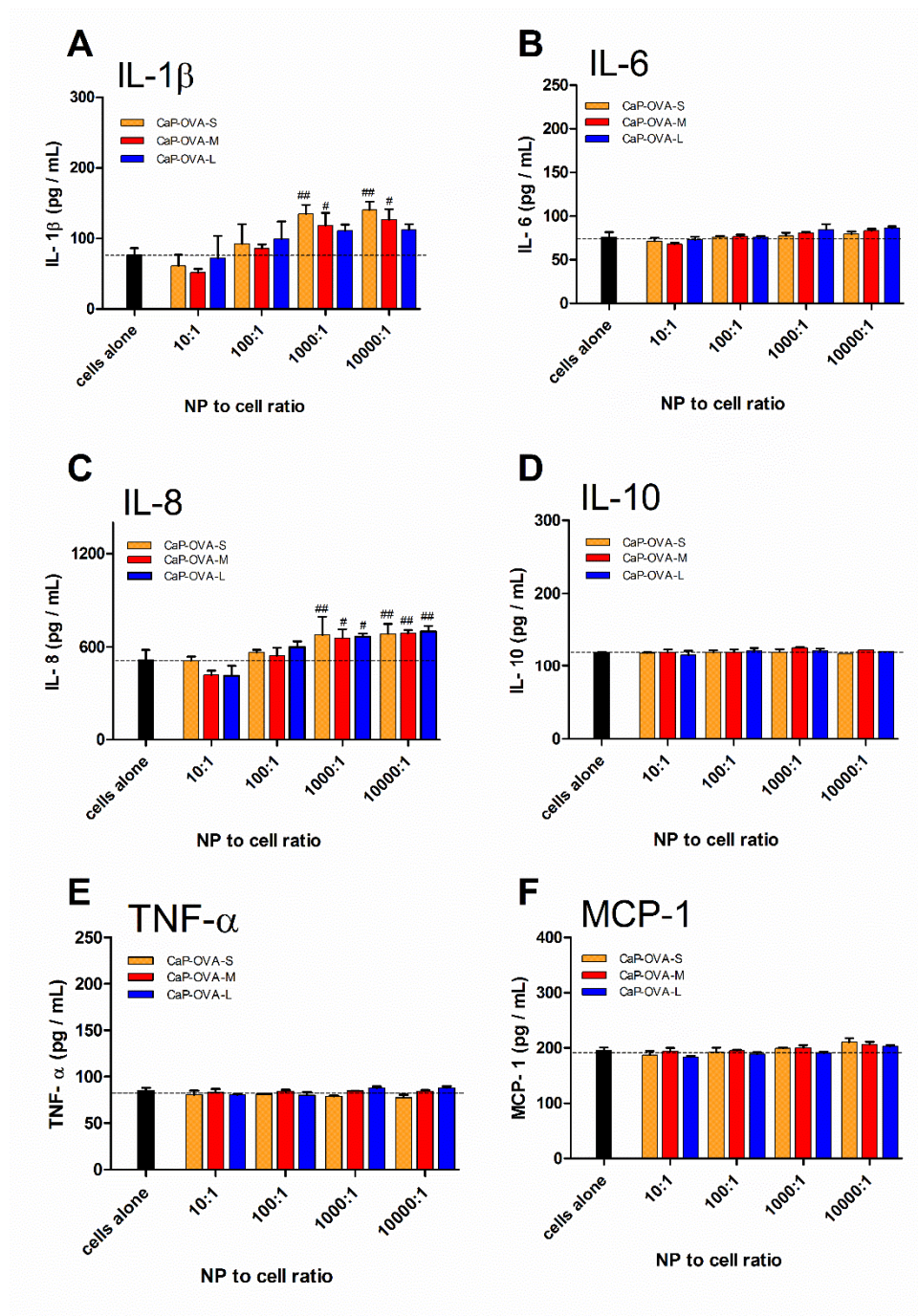


Figure 4. 10: OKF6 cytokines induced by CaP-OVA NPs for 24 h.

Three sizes of cross-linked CaP-OVA NPs induce inflammatory cytokine production in OKF6 cells. Cytokine production in OKF6 cells was measured following 24 h incubation of three sizes of cross-linked CaP-OVA NPs at various NP to cell ratios (10:1, 100:1, 1000:1 and 10000:1). IL-1 β (A), IL-6 (B), IL-8 (C), IL-10 (D), TNF- α (E) and MCP-1 (F) were detected by ELISA. Data are expressed as mean $\pm$ SD. [#] P < 0.05, ^{##} P < 0.01, and ^{###} P < 0.001 were considered statistically significant when compared between treatment groups and cells alone. ^{*} P < 0.05, ^{**} P < 0.01, and ^{***} P < 0.001 were considered statistically significant when compared among the three sizes of particles.

4.3.7 Phagocytosis of nanoparticles by macrophages

The uptake of vaccines by professional phagocytes such as macrophages and dendritic cells is a crucial first step in the induction of a protective immune response. In order to measure the phagocytosis of the CaP-OVA NPs (S, M and L) by macrophages, nanoparticles were labelled with pHrodo red (a pH-sensitive fluorescence dye), and phagocytosis of nanoparticles was determined by flow cytometry. Each of the CaP-OVA NPs (S, M and L) were phagocytosed by RAW 264.7 cells in a dose-dependent manner after 1 h incubation (Figure 4.11A), as indicated by an increase in both percentages of fluorescent cells (Figure 4.11B) and mean fluorescence intensity (MFI) ($P < 0.001$) (Figure 4.11C). In terms of phagocytosis percentage, all sizes of CaP-OVA NPs were found to be phagocytosed by RAW 264.7 cells to a significantly higher amount at NP to cell ratios of 100:1, 1,000:1 and 10,000:1, as compared to the negative control ($P < 0.001$) (Figure 4.11B). Likewise, analysis of the MFI demonstrated that all sizes of CaP-OVA NPs were significantly phagocytosed by RAW 264.7 at NP:cell ratios of 100:1, 1,000:1 and 10,000:1, as compared to the negative control (Figure 4.11C).

It was observed that NP size had an effect on phagocytosis of NPs; CaP-OVA-L NPs were phagocytosed to a significantly greater extent than CaP-OVA-M and CaP-OVA-S at NP:cell ratios of 100:1, 1,000:1 and 10,000:1 in terms of percent of RAW cells positive for NPs and the amount of NPs per cell (Figure 4.11B and 4.11C, respectively). However, no significant difference was found between CaP-OVA-S and CaP-OVA-M at the lowest NP to cell ratio (10:1).

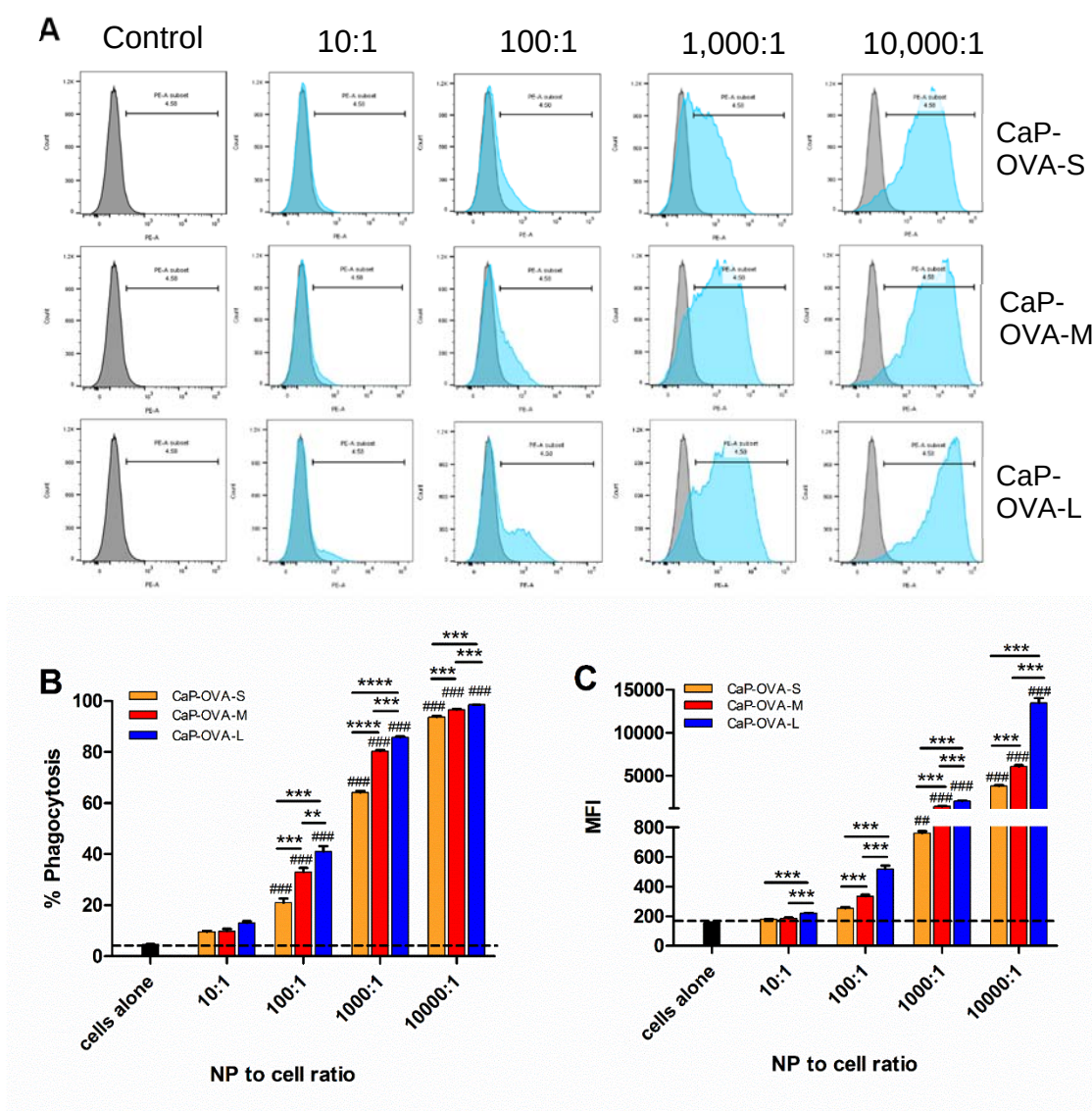


Figure 4. 11: Effects of NP size on macrophage phagocytosis.

Phagocytosis of three sizes of cross-linked CaP-OVA NPs by the macrophages (RAW 264.7). Nanoparticles were labelled with pHrodo red and studied their phagocytosis by RAW 264.7 cells at increasing nanoparticle to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1). Cells and nanoparticles were incubated for 1 h, and the level of phagocytosis was determined by flow cytometry. Nanoparticles are able to phagocytosis by RAW 264.7 cells as measured by an increase in pHrodo red fluorescence (A). The level of phagocytosis was determined by both the cell phagocytosis percentage (B), which indicates the percentage of RAW 264.7 cells with at least one phagocytised nanoparticles. In addition, the level of phagocytosis also showed by the mean fluorescence intensity (MFI), which is relative to the number of phagocytised nanoparticles (C). Data are expressed as mean $\pm$ SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were considered statistically significant when compared between treatment groups and cells alone. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ was considered statistically significant when compared among the three sizes of particles.

4.3.8 Nanoparticle activation of NF- κ B in RAW-Blue cells and cell surface marker production

The nuclear factor NF- κ B signalling pathway is the means by which immune cells transduce the detection of foreign materials into the production of various cytokines and chemokines. To determine if nanoparticle binding would induce NF- κ B activation, RAW-blue cells were incubated with S, M and L CaP-OVA NPs for 24 h, after which the level of SEAP was determined by using Quanti-Blue. As shown in Figure 4.12A, S, M, and L nanoparticles induced significantly stronger NF- κ B activation only at very high NP to cell ratios, as compared to the negative control ($P < 0.001$, 10,000:1). At this ratio, CaP-OVA-S induced significant less NF- κ B activation than CaP-OVA-M and CaP-OVA-L ($P < 0.001$), while no difference was observed between CaP-OVA-M and CaP-OVA-L.

A further cellular consequence of NF- κ B phosphorylation and translocation is the upregulation of cell surface markers that allow the macrophages to interact with other cells of the host immune system. To investigate whether the phagocytosis of NPs altered the degree of macrophage maturation, the level of co-stimulatory molecules (CD40, CD80, and MHC II) were analysed by flow cytometry. It was found that at the highest NP to cell ratio of 10,000:1, there was a significant difference for all measured co-stimulatory molecules after treatment with all S, M and L CaP-OVA NPs, as compared to the negative control (10,000:1, Figure 4.12B, C and D). CaP-OVA-S induced significant less co-stimulatory molecules expression than CaP-OVA-M and CaP-OVA-L ($P < 0.05 - 0.001$), while no difference was observed between CaP-OVA-M and CaP-OVA-L the NP to cell ratio of 10,000:1 (Figure 4.12B, C and D).

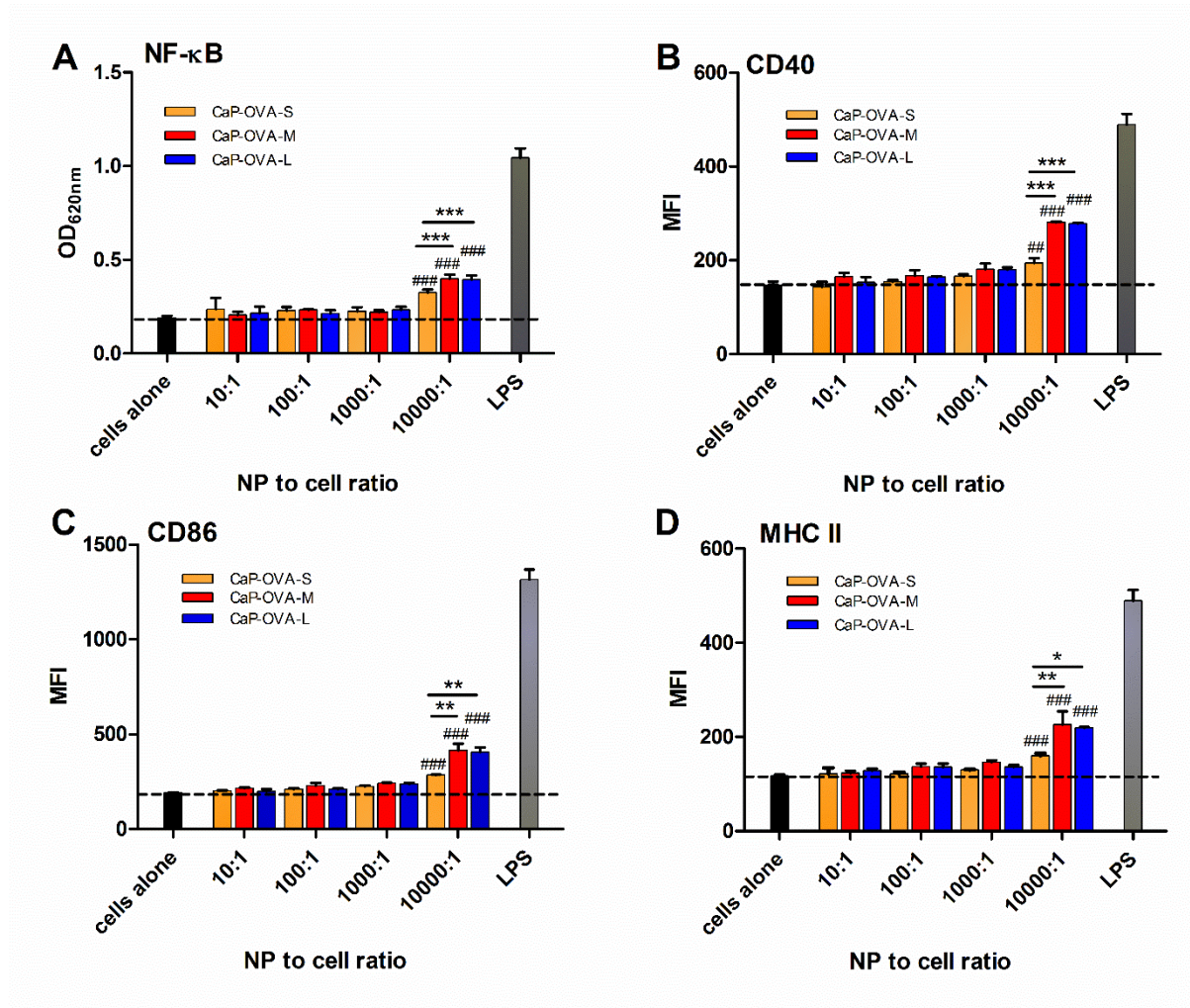


Figure 4. 12: NF-κB activation by CaP-OVA NPs.

Three sizes of cross-linked CaP-OVA NPs activate NF-κB in RAW-Blue cells and induce enhanced macrophage maturation in RAW 264.7 cells at various NP to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1). Data is expressed as mean $\pm$ SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were considered statistically significant when compared between treatment groups and cells alone. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant when compared among the three sizes of particles.

4.3.9 Nanoparticle induced cytokine production in RAW 264.7 cells

To investigate whether CaP-OVA S, M and L were able to induce a cytokine response in macrophages, RAW 264.7 cells were incubated with S, M and L CaP-OVA NPs for 6 and 24 h (Figure 4.13 and Figure 4.14). After incubation the supernatant was collected and analysed for IL-1 β , IL-6, IL-10, IL-12 and TNF- α by ELISA.

After incubation with CaP-OVA-S for 6 h no significant difference was observed in the production of IL-1 β , IL-6, IL-10 and IL-12 by RAW 264.7 cells at all NP to cell ratios compared to cells alone. However, all of the NP to cell ratios except 10:1, CaP-OVA-S induced a significant TNF- α response ($P < 0.05$) (Figure 4.13). CaP-OVA-M produced IL-1 β , IL-6, IL-12 and TNF- α response in RAW 264.7 cells at the highest NP to cell ratio ($P < 0.05$), but did not produce significant levels of IL-10. CaP-OVA-L was found to induce significant production of each cytokine at the highest NP to cell ratio (10,000:1). CaP-OVA-L induced significantly more IL-10 and TNF- α secretion compared with CaP-OVA-S and CaP-OVA-M at the NP to cell ratio of 10,000:1; and induced significantly more IL-10 and IL-12 secretion than CaP-OVA-S at the ratio of 100:1 ($P < 0.05$) (Figure 4.13).

Following incubation for 24 h, a significantly stronger production of IL-1 β , IL-6, IL-10, IL-12 and TNF- α was found for CaP-OVA-M and CaP-OVA-L at the highest NP to cell ratio, as compared to the negative control ($P < 0.05$) (Figure 4.14). Furthermore, at an NP to cell ratio of 1,000:1, significant levels of IL-6, IL-12 and TNF- α were induced by CaP-OVA-M and CaP-OVA-L, as compared to the negative control ($P < 0.05$). Only CaP-OVA-L elicited significant production of IL-10 at the NP to cell ratio of 1,000:1, as compared to the negative control ($P < 0.05$). CaP-OVA-S induced significant production of IL-6, IL-12 and TNF- α at the highest ratio, as compared to the negative control ($P < 0.05$). Additionally, TNF- α was induced at significantly higher amounts in response to NPs at cell ratios of 100:1, 1,000:1 and 10,000:1 for all particles, as compared to the negative control ($P < 0.05$) (Figure 4.14E). It was found that CaP-OVA-L induced significant higher amounts of IL-6, IL-10 and TNF- α compared with CaP-OVA-S and CaP-OVA-M at the highest NP to cell ratio (10,000:1). Furthermore, a significant of IL-6 production at the ratios of 100:1 and 1,000:1 was observed between CaP-OVA-L and CaP-OVA-M NPs ($P < 0.05$) (Figure 4.14B).

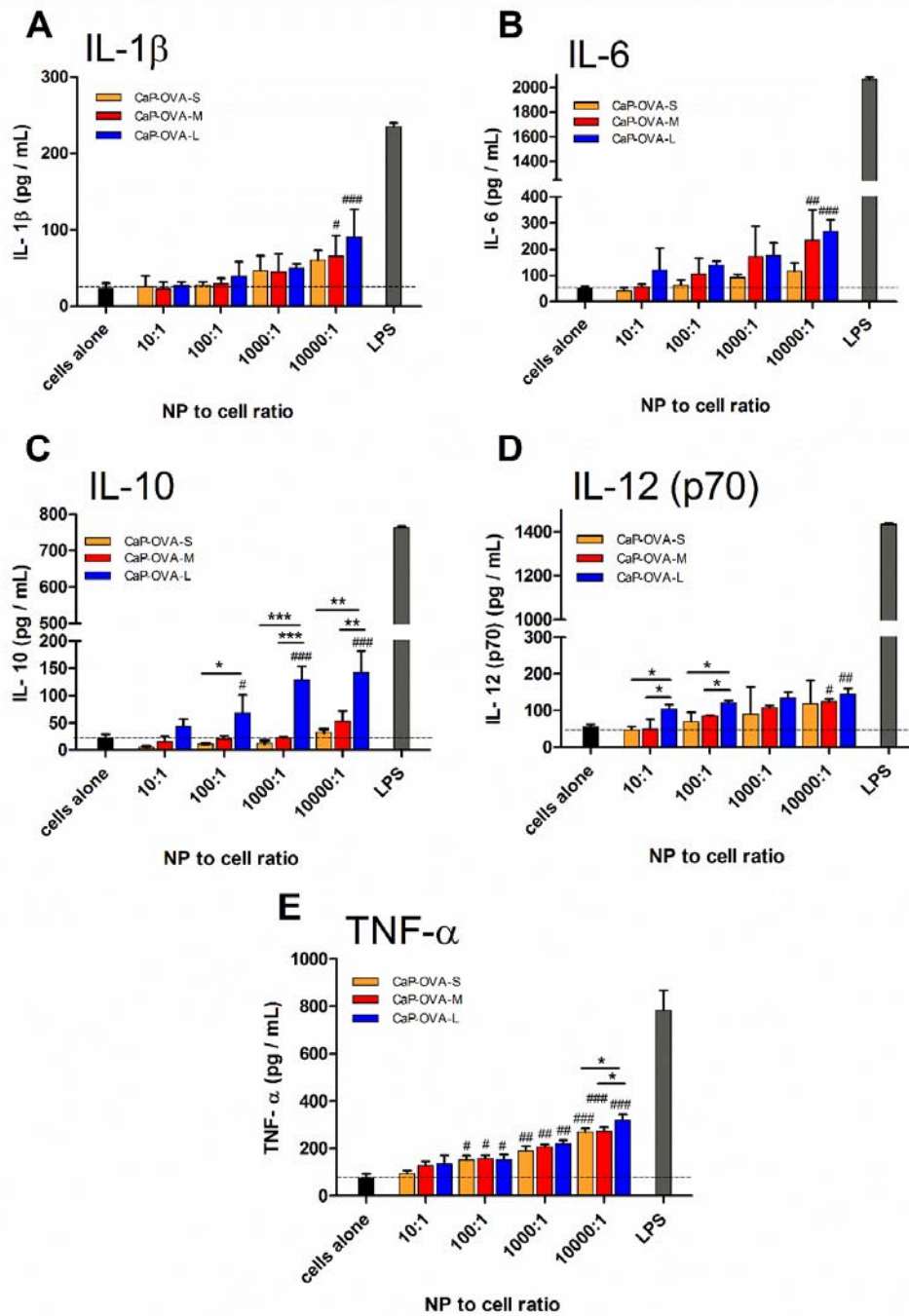


Figure 4. 13: Induction of RAW 264.7 cytokines by CaP-OVA NPs for 6 h.

Three sizes of cross-linked CaP-OVA NPs induce cytokine release in RAW 264.7 cells. Cytokine production in RAW 264.7 cells was measured following 6 h incubation of three sizes of cross-linked CaP-OVA NPs at various NP to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1). IL-1 β (A), IL-6 (B), IL-10 (C), IL-12 (D) and TNF- α (E) were detected by ELISA assays. Data are expressed as mean $\pm$ SD. # P < 0.05, ## P < 0.01, and ### P < 0.001 were considered statistically significant when compared between treatment groups and cells alone. * P < 0.05, ** P < 0.01, and *** P < 0.001 were considered statistically significant when compared among the three sizes of particles.

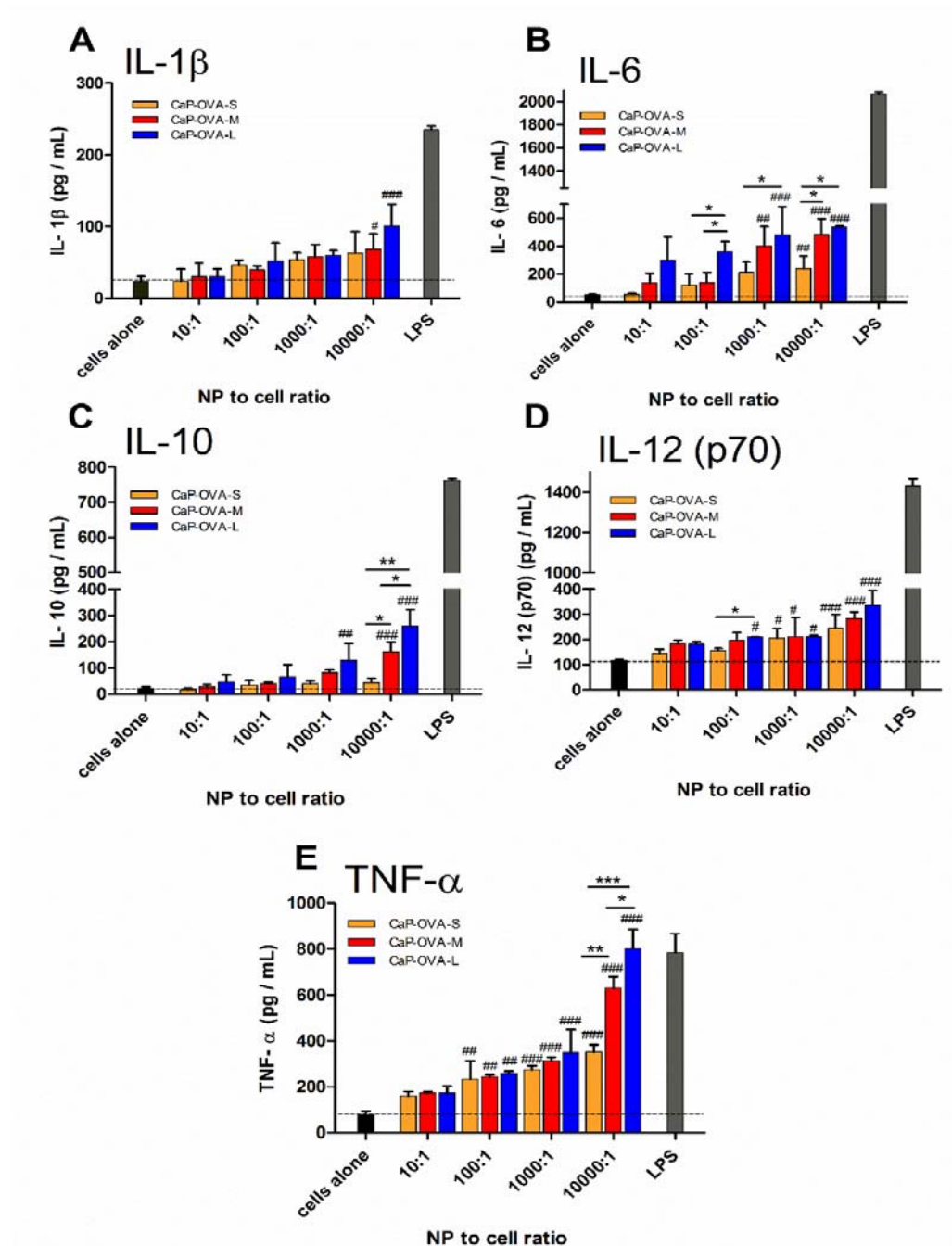


Figure 4. 14: Induction of RAW 264.7 cytokines by CaP-OVA NPs for 24 h.

Three sizes of cross-linked CaP-OVA NPs induce cytokine release in RAW 264.7 cells. Cytokine production in RAW 264.7 cells was measured following 24 h incubation of three sizes of cross-linked CaP-OVA NPs at various NP to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1). IL-1 β (A), IL-6 (B), IL-10 (C), IL-12 (D) and TNF- α (E) were detected by ELISA assays. Data are expressed as mean $\pm$ SD. # P < 0.05, ## P < 0.01, and ### P < 0.001 were considered statistically significant when compared between treatment groups and cells alone. * P < 0.05, ** P < 0.01, and *** P < 0.001 were considered statistically significant when compared among the three sizes of particles.

4.4 Discussion

The aims of this investigation were to compare three sizes of nanoparticles and to explore the induction of an immune response. The number of nanoparticles were normalised in each experiment, allowing for direct comparisons of the immunological effects induced by nanoparticles of different sizes. This study demonstrates that there are significant differences in the immunological effects induced by the three sizes of nanoparticles investigated, particularly at high nanoparticle to cell concentrations. Overall, it was found that the largest size nanoparticles, CaP-OVA-L (364 ± 21 nm), had the strongest ability to bind to host cells and were more readily taken up and phagocytosed by both epithelial cells and macrophages.

The toxicity of three sizes of cross-linked CaP-OVA NPs on mammalian cells was determined by MTS and LDH assays. The results of these assays showed that none of the nanoparticles (regardless of size) induced significant cytotoxicity on OKF6 cells or HEK cells. However, slight toxicity was observed in H4IIE cells at higher nanoparticle to cell ratios. According to ISO 10993-5 standards [376], any compound that decreases cell viability below 70% is defined as toxic. None of the CaP-OVA NPs induced this loss in the level of cell viability. Consequently, the nanoparticles can be considered as non-toxic. In order to determine which component of CaP-PEI-OVA nanoparticles was responsible for the small amount of cytotoxicity observed, HEK, H4IIE, and OKF6 cells were exposed to different concentrations of calcium ions, phosphate ions, 'naked' nanoparticles or nanoparticles with a positive polymer (PEI). It was found that CaP-PEI NPs displayed cytotoxicity, indicating that the PEI was the main source of the cytotoxicity. Indeed, several groups have shown PEI is toxic to many cell types. Xia *et al.* demonstrated that mesoporous Silica NPs coated with the 25 kD PEI elicited decreased cell viability in RAW 264.7 cell line [345]. When the polymeric gene delivery carriers with 25 kD PEI incubated with HeLa cells for 24 h, the cell viability reduced by 40 – 90% [377].

The first interactions that nanoparticles have with the host is when they interact with the cell membrane. All sizes of nanoparticles in this study demonstrated excellent binding ability on oral epithelial cells in both a size- and dose-dependent manner. Cross-linked CaP-OVA-M and CaP-OVA-L showed a greater affinity for binding OKF6 cells compared to CaP-OVA-S at an NP to cell ratio of 100:1. Conversely, several other investigations have shown nano-sized particles bound better to epithelial cells than micro-particles. McClean *et al.*, through a

combination of techniques such as fluorescence microscopy, found that poly-dl-lactide (PLA) nanoparticles adsorbed better on Caco-2 monolayers than microparticles [378]. In a similar study, 100 nm polylactic polyglycolic acid copolymer (PLGA) NPs were shown to have a 2.7-fold higher uptake than 1,000 nm by Caco-2 cells [379]. It is possible that differences in the composition and shape of the NPs, in addition to different concentrations, used may explain these variations.

The ability of nanoparticles to migrate across the cell monolayer is a crucial factor for the successful mucosal delivery of a nanoparticle vaccine. In this study, all three sizes of nanoparticles were shown to migrate through epithelial layers significantly in a time-dependent manner. Additionally, incubation with CaP-OVA-S resulted in significantly higher migration percentage when compared to CaP-OVA-L at all incubation times. This finding is in contrast to a recent paper that compared 5 nm and 40 nm gold nanoparticles, which demonstrated that migration efficiency is highly dependent on NP surface properties, rather than on NP size [380]. These differences may be a consequence of both the different surface properties between metallic NPs and CaP NPs, and NP size, as the gold NPs (5 nm and 40 nm) was significantly smaller than CaP NPs used in this study.

While effective migration through the epithelial cell layer is desirable for a mucosally delivered vaccine, NPs must be bound and phagocytosed by cells of the immune system in order to induce an immune response [381]. Cellular uptake is one of the crucial steps which determines the therapeutic effectiveness of nanoparticle vaccine [380]. In this study confocal microscopy was used to demonstrate that the nanoparticles were efficiently transported into cells. This phenomenon has been previously confirmed with the use of calcium phosphate/polyethyleneimine/R-phycoerythrin (CaP/PEI/R-PE) NPs, as phycobilliprotein phycoerythrin can be easily taken up by human epithelial cervical cancer cells [365]. The phycobilliprotein phycoerythrin is a multimeric protein that can fluoresce, and consequently can be used in immunofluorescence assays [382]. Several studies have suggested that nanoparticles that are less than 500 nm in size are ideal for phagocytosis by APCs [378, 383]. The results from confocal microscopy assays performed in this study correlate with the flow cytometry phagocytosis assays. The effect of size on nanoparticle uptake is contentious. He *et al.* found that in comparison with 157 nm polymeric nanoparticles, larger 300 nm nanoparticles result in a 1.1-fold increase in uptake by macrophages [235]. Conversely, Lu *et al.* found that for a size range of

30 to 280 nm of Mesoporous silica nanoparticles (MSNs), 50 nm has the highest uptake efficiency [384]. Previous studies have also found that 456 nm polymeric nanoparticles are quickly taken up by murine macrophages, and that 149 nm nanoparticles are easily taken up by non-phagocytic cells such as HEK 293 cells, indicating each cell type has a preferred size of particle for uptake [235, 385], and some cells, such as primary human umbilical vein endothelial cells, only prefer to take up larger (560 nm and 1010 nm) nanoparticles [386].

While binding to immune cells and subsequent phagocytosis are important in initial interactions with host immune cells, the downstream signalling cascades are required to initiate and sustain an immune response to the delivered antigen. The NF- κ B signalling pathway serves as a central mediator of the inflammatory response, and plays a vital role in the regulation of the innate and adaptive immune responses [387, 388]. Relatively few studies using murine or human cell lines have investigated the influence of CaP NPs on NF- κ B activation have been published [389, 390]. However, it has been reported that nanoparticles induced downregulation of NF- κ B activity. It has been reported that using sphere-shaped nanoparticles resulted in inhibition of NF- κ B activation [389]. This is in contrast with the results of this study, where it was demonstrated that the phosphorylation of NF- κ B activation was significantly increased at the highest NP to cell ratio (10,000:1) for all sizes of nanoparticles (rod-shaped). These discrepancies may be explained by the shape of NPs between these studies, as in another study Grandjean-Laquerriere *et al.* demonstrated that 300 nm rod-shaped hydroxyapatite particles increased NF- κ B activation and TNF- α production on macrophages [391].

A consequence of NF- κ B binding is translocation into the cell nucleus, where they initiate transcription of cytokines [392]. An increase in the concentration of proinflammatory cytokines can be considered an indicator of nanoparticle immunomodulatory effects. In this study, the ability of the rod-like CaP NPs to activate inflammatory responses in OKF6 cells, and subsequently produce inflammatory cytokines, was measured by various immunoassays. It was demonstrated that none of the nanoparticles induced IL-6, IL-10, TNF- α , or MCP-1 secretion in OKF6 cells, and that CaP-OVA-S and CaP-OVA-M induced a small amount of IL-8 and IL-1 β secretion. These results are consistent with a previous paper that showed smaller particles are capable of eliciting strong inflammatory responses than larger particles of the same chemical composition [393]. Yang *et al.* also reported that smaller (5 nm) silver NPs induced more IL-1 β than larger (28 nm) silver NPs [394]. It is well established that IL-8 is a chemokine that can stimulate neutrophils and granulocytes, and attracts them to the site of

inflammation [395]. IL-1 β plays a significant role in the induction of innate immunity [394]. Taken together, the increase of IL-1 β and IL-8 concentrations after exposure to the CaP-OVA NPs suggests that immune cells such as neutrophils and monocytes can be recruited to the site of infection, potentially resulting in the initiation of an immune response to the vectored antigen.

This study demonstrates that the following exposure to CaP-OVA NPs, epithelial cells produce a selection of cytokines that are associated with the recruitment of immune cells to the site of infection. Resident tissue macrophages are considered primary cell types to encounter and process foreign material such as particles, while dendritic cells (DC) are generally considered professional APCs [396, 397]. In the current study, RAW 264.7 cells were used as model macrophages to quantify the phagocytosis of nanoparticles. In order to process the engulfed particles and initiate a robust immune response, it is necessary for phagosome to mature into a phagolysosome with a low internal pH (< 5.0) [398]. This acidic environment assists in the degradation/digestion of pathogens, and leads to the presentation of processed antigens to cognate T cells [397, 399]. By labelling nanoparticles with a pH-sensitive dye, it can be determined if nanoparticles are cell-associated, cytoplasmic, or phagocytosed by flow cytometry. pHrodo Red dye increases fluorescence intensity under low pH conditions and has been extensively used to study phagocytosis [400, 401]. The three sizes of CaP-OVA NPs were phagocytosed by RAW 264.7 cells in a dose-dependent manner. This correlates to the confocal microscopy results, suggesting that the internalised fluorescent particles observed by confocal microscopy were internalised within a phagosome. This is in accordance with a previous publication demonstrating that hydroxyapatite crystals phagocytosed by human polymorphonuclear leucocytes in a time-dependent manner [402]. It was also observed that RAW 264.7 cells had a greater affinity for the CaP-OVA-L in terms of phagocytosis. Again, this observation is consistent with the recent findings by Shah and co-workers who found that larger particles (< 0.5 μ m) are more easily phagocytosed by macrophages, and smaller particles (20 - 200 nm) were more easily phagocytosed by dendritic cells [73].

The ability of a nanoparticle vaccine to elicit APC maturation is crucial for the induction of effective immune responses [396]. Immature macrophages also function as antigen capturing cells, while mature macrophages can act as APCs after exposure to the appropriate cytokines [403]. After capturing the antigens, macrophages will degrade the antigen into peptides and then present the peptide-major histocompatibility complex (MHC) complex to T cells to elicit

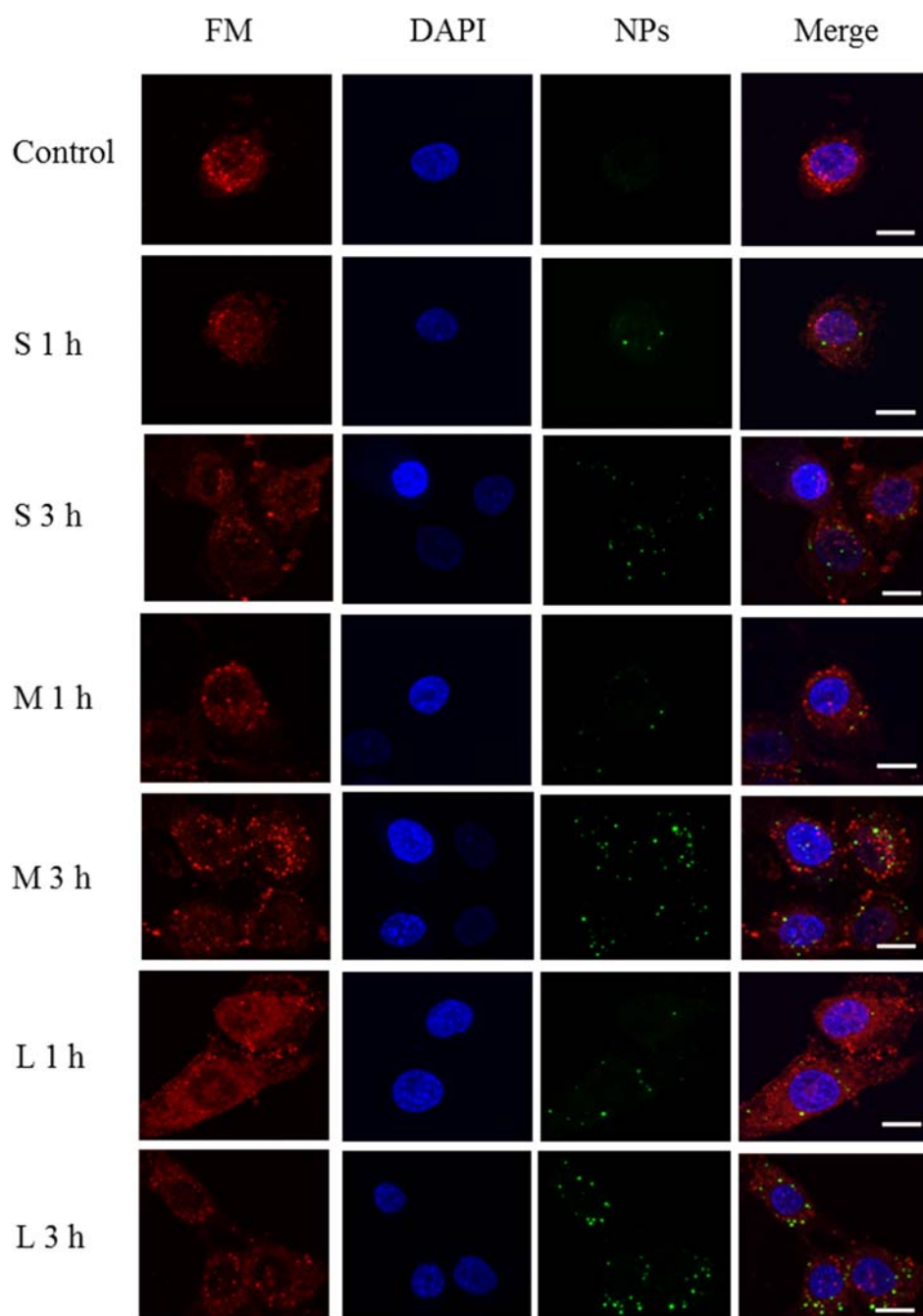
immune responses [404]. The upregulation of antigen presentation markers such as MHC-II and co-stimulatory marker expression markers expression, such as CD40 and CD86, is a strong indicator of the degree of APC maturation [405]. All sizes of nanoparticles tested were found to induce significant cell surface marker expression (CD40, CD80, and MHC II) at the highest NP dose, indicating that CaP-OVA NPs have the capability to induce macrophage maturation and thus may elicit an immune response against the vectored antigen.

It is known that many cytokines can be induced when the APCs are stimulated by nanoparticles [251]. Interleukin 1 β (IL-1 β) has a pivotal role in the activation of natural killer cells (NK) for effective priming of cytotoxic T cells (CTLs) [406]. IL-6 plays an important role in CD4⁺ [407] and CD8⁺ [408] T cell differentiation, and the presence of IL-10 in primary responses also lead to an increased number of CD8⁺ T cells [409]. IL-12 is an important bridge between innate and adaptive immunity, and is essential for activating the full range of macrophages processing abilities. IL-12 can stimulate CD8⁺ T cell activation and also promotes more extended conjugation events between APCs and CD8⁺ T cell [410]. Additionally, TNF- α has been shown to play a pivotal role in the proliferation, activation and differentiation of macrophages [411]. All of these cytokines contribute to effective and potent T cell responses. Strong production of IL-6, IL-12 and TNF- α by RAW 264.7 macrophages was found for all sizes of nanoparticles. The limited production of IL-1 β under these conditions may be due to the requirement of caspase-1 inflammasome activation for secretion [412]. Additionally, as there is no ASC (an apoptosis-associated, speck-like protein containing a CARD domain) adaptor expression on raw cells, IL-1 β production is limited [85]. In this study, CaP-OVA-L NPs induced significantly more NF- κ B activation, cell surface marker expression (CD40, CD80, and MHC II), and cytokine production (IL-6, IL-10, TNF- α) than CaP-OVA-S and CaP-OVA-M NPs. These findings are in agreement with previous studies on human peripheral blood mononuclear cells that have found that 32 μ m and 40 μ m particulate beta-tricalciumphosphate particles can cause a high degree of cytokine production (IL-1 β , IL-8 and TNF- α), and that smaller particles (1 μ m) induce very little [413]. This may be attributable to the adsorbance properties that larger sized particles provide, which allow more protein to be absorbed. Additionally, an increased preference for phagocytosis of larger particles would lead to increased immune responses.

4.5 Conclusion

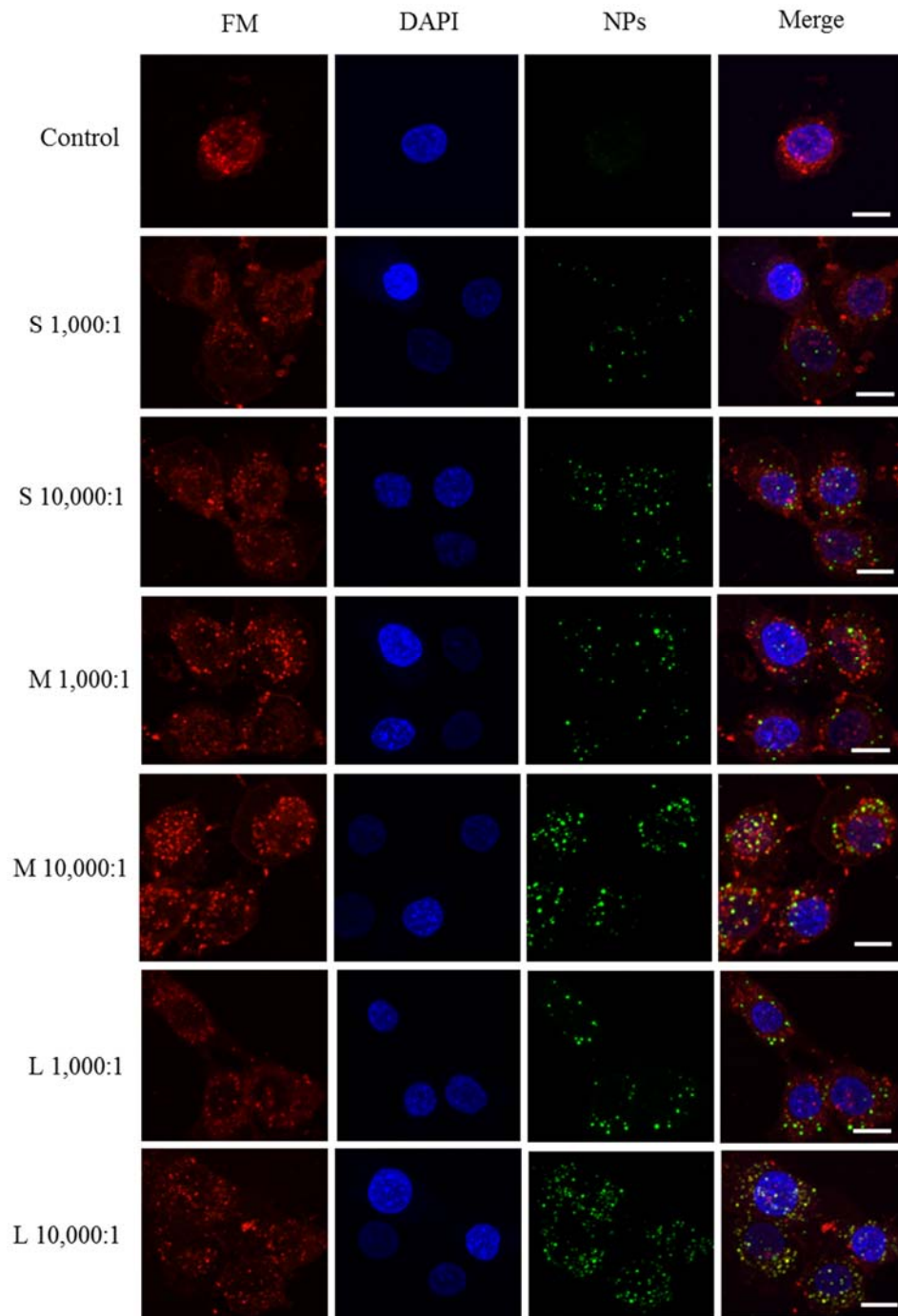
The present study has investigated the immunological effects of different sizes of CaP NPs vaccine on oral epithelial cells and macrophages. It was shown that the three sizes of NPs were biocompatible and non-toxic towards HEK, H4IIE and OKF6 cells. It was further demonstrated that all sizes of NPs can efficiently bind to and migrate through the epithelial barriers, and that they induced a specific and significant IL-1 β and IL-8 secretion, but no TNF- α , IL-6, IL-10 and MCP-1 response as compared to the negative control from epithelial cells. These data also highlight that the three sizes of cross-linked CaP-OVA NPs can be phagocytosed by RAW 264.7 cells in a dose-dependent manner. Finally, of the CaP-OVA nanoparticles CaP-OVA-L NPs induced significantly stronger cell-binding, cellular-uptake, phagocytosis, NF- κ B activation, cytokine secretion, and inflammatory cell surface marker expression at the highest NP to cell ratio than the smaller nanoparticles. The CaP-OVA-S NPs transmigrated across the mucosal monolayer significantly more efficiently than CaP-OVA-L at all time points tested. These data suggest that CaP NPs could be considered as safe vehicles for vaccine applications. This study provides a better understanding of how rod-like nanoparticles interact with epithelial cells and macrophages, demonstrating that rod-like CaP NPs have a promising potential as a platform for protein delivery in vaccine therapy.

4.6 Supplementary Information



Supplementary Figure 4. 1: Confocal microscopy of internalised CaP-OVA NPs for 1 and 3 h.

Cellular uptake of three sizes of nanoparticles in OKF6 cells at NP to cell ratio of 1,000:1 for 1 and 3 h. Nanoparticle (green) were pre-labelled with Atto 488, plasma membrane (red) were stained with FM dyes, and nuclei (blue) were stained with DAPI. Scale bar = 10 μ m.



Supplementary Figure 4. 2: Confocal microscopy of internalised CaP-OVA NPs at NP to cell ratio of 1,000:1 and 10,000:1 for 3 h.

Cellular uptake of three sizes of nanoparticles in OKF6 cells at NP to cell ratio of 1,000:1 and 10,000:1 for 3 h. Nanoparticle (green) were pre-labelled with Atto 488, plasma membrane (red) were stained with FM dyes, and nuclei (blue) were stained with DAPI. Scale bar = 10 μ m.

Chapter 5

Co-encapsulation of antigen ovalbumin and TLR2 ligand in calcium phosphate nanoparticles leads to macrophages maturation and activation

5.1 Introduction

Significant advancements have been made recently in the development of sub-unit recombinant protein and peptide-based vaccines for the prevention of infectious diseases and cancer, with several formulations under clinical phase of development [414-416]. In developing a sub-unit based vaccine, it is well known that generally a protein or peptide delivered alone, often has poor immunogenicity and requires potent adjuvants such as toll-like receptor (TLR) ligands, co-stimulatory molecules, and cytokines in the vaccine formulation to induce the desired immunity [52, 417, 418]. TLRs are a group of transmembrane protein complexes that recognise microbial components called pathogen-associated molecular patterns (PAMPs) in order to initiate an immune response [97]. The use of TLR agonists as adjuvants has gained increased interest due to their ability to improve the efficacy and immunogenicity of vaccine formulations [39, 45, 51, 129-134]. Pattern recognition receptors (PRRs), such as TLRs are expressed on APCs (such as dendritic cells and macrophages) and mucosal/oral epithelial cells. PRRs play a significant role in inducing and enhancing both innate and adaptive immune responses [134]. TLRs facilitate the recognition of pathogens by immune cells, resulting in increased antigen uptake and processing, expression of pro-inflammatory cytokines, chemokines, and co-stimulatory molecules such as CD80, 86 and 40, leading to the induction of a robust innate and adaptive immune response [135, 136].

Several different TLR ligands have been investigated for use as vaccine adjuvants, including ligands for TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 [137-139]. The selection of the ligand often depends on the disease being targeted [140]. TLR2 is a transmembrane protein, often found as a dimer with TLR1 or TLR6, and is expressed on the surface of immune cells such as macrophages [419]. TLR2 activation mediates a broad range of immune responses to many bacterial species by recognising triacylated lipoproteins and diacylated lipoproteins in Gram-negative and Gram-positive bacteria, respectively [420]. Administration of lipoproteins has been used in several experimental vaccine formulations in order to modulate the induced immune response [421-425]. A synthetic TLR2 ligand was used as a cancer vaccine adjuvant, eliciting increased cell adhesion and inhibited tumour growth *in vivo* [421]. Reiser *et al.* reported that a naturally occurring TLR2 ligand enhanced antigen cross-presentation and antigen-presenting cell trafficking [422]. It has been demonstrated that a TLR2 ligand peptide, Pam₃CSK₄ conjugated to a synthetic antigenic peptide significantly increased DC maturation and the induction of an effective CD8⁺ T cell response [423]. Similarly, Azuma *et al.* found

that TLR2-agonist lipopeptides enhanced DC maturation and NK cell activation [424]. The inclusion of TLR ligands has also resulted in increased NF- κ B activation and promoted the release of inflammatory cytokines in human mast cell line LAD2 cells [425]. These findings suggest that the TLR2 ligand may be a promising adjuvant for vaccine formulation.

At present, the most commonly used commercially available TLR2 ligands are Pam₂CSK₄ and Pam₃CSK₄, containing two or three palmitoyl (Pam) chains, respectively [426]. Previous reports have shown that the inclusion of Pam₃CSK₄ as a component of synthetic virus-like particles resulted in an increased humoral and cell-mediated immune response *in vivo* [129, 427]. In addition, Pam₃CSK₄ fused to Ovalbumin increased antigen presentation through TLR2 signalling and enhanced effector memory-like CD8⁺ T cells after immunisation [428]. Pam₂CSK₄ has also been reported to induce NF- κ B activation with the corresponding production of several cytokines (such as IL-8, IL-10 and IFN- γ) in murine and human cells [429, 430]. Additionally, Zeng *et al.* showed vaccines incorporating dipalmitoyl-S-glycerol cysteine (Pam₂Cys) could effectively induce maturation of DCs evidenced by upregulation of MHC class II molecules [431]. Pam₂CSK₄ has also been shown to induce a preferentially Th2 (protective) skewed immune response and antibody-dependent immunity [134]. These findings indicate that Pam₂CSK₄ and Pam₃CSK₄ have excellent potential as adjuvants for vaccine studies.

In this chapter, a new potential adjuvant with favourable chemical properties for use in vaccine applications was explored. A novel TLR2 ligand, Pam₂KK₄CG, was synthesised and conjugated to three sizes of calcium phosphate OVA NPs. The effect of these functionalised NPs on macrophages was compared to commercially available Pam₃CSK₄ and Palmitoyl alone. Several assays were used to characterise the immunostimulatory effects of these ligands conjugated to particles, including NPs toxicity, phagocytosis, HEK TLR2 and macrophage NF- κ B activation, as well as cell surface marker expression and cytokine production by macrophages.

5.2 Materials and Methods

5.2.1 Cell cultures and growth conditions

All cells were obtained from the Melbourne Dental School tissue culture collection. Macrophage cell line RAW 264.7, human embryonic kidney293 (HEK293) cells, RAW-Blue cells were cultured with Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine under 5% v/v CO₂ at 37 °C. Also, HEK293 cells were incubated with 100 µg/mL Zeocin, 30 µg/mL Blasticidin S and 200 µg/mL Hygromycin B. RAW-Blue cells were incubated with 200 µg/mL Zeocin. DMEM, keratinocyte serum-free medium, FBS, penicillin/streptomycin, L-glutamine, trypsin was obtained from Life Technologies, NSW Australia. The antibiotics (Zeocin, blasticidin S and hygromycin B) for and HEK293 and RAW-Blue cells were purchased from Invivogen, USA.

5.2.2 Pam₂KK₄CG peptide synthesis

The Pam₂KK₄CG synthesis was carried out on a CEM Liberty microwave-assisted synthesiser using RAM-resin by Fmoc/tBu solid-phase methods [432-435]. Standard Fmoc-chemistry was used throughout with a 4-fold molar excess of the 9-Fluorenylmethoxycarbonyl (Fmoc)-L-amino acids (GL Biochem, Shanghai, China) in the presence of 4-fold 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, GL Biochem, Shanghai, China) and 8-fold N,N-Diisopropylethylamine (DIPEA, Auspep, Melbourne, Australia). The *N*-terminal palm chain was functionalised with palmitic acid N-hydroxysuccinimide ester (Sigma, NSW, Australia) for the dimeric palmitic fatty chains. The peptides were cleaved from the solid support resin with trifluoroacetic acid (TFA, Auspep, Melbourne, Australia) in the presence of triisopropylsilane (TIPS, Sigma, NSW, Australia) as a scavenger (ratio 95:5), and 2,2'-Dithiobis(5-nitropyridine) (DTNP, Sigma, NSW, Australia) as the alternative protection group for C-terminal cysteine (2 h, RT). After filtration to remove the resin, the filtrate was concentrated under a stream of nitrogen, and the peptide products were precipitated in ice-cold diethyl ether and washed three times. The Pam₂KK₄CG-NH₂ peptide in buffer A (0.1% TFA MilliQ water) was then purified by reversed-phase high-performance liquid chromatography (RP-HPLC) with the gradient 0% to 100% of buffer B (0.1% TFA 90% acetonitrile) for 40 min using a C4 column (3.6u C, 150 × 4.6 mm, Aeris, USA). The final products were characterised by both RP-HPLC and electrospray mass spectrometry (ESI-MS). Rink Amide resin was purchased from GL Biochem (Shanghai, China). Dimethylformamide (DMF) was obtained from Auspep

(Melbourne, Australia). Piperidine was obtained from Sigma (NSW, Australia).

5.2.3 Preparation and characterisation of TLR2 ligand functionalised CaP-PEI-OVA NPs

Different sizes of cross-linked CaP-PEI-OVA NPs were synthesised using the layer-by-layer method. The combination of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ + $\text{NH}_4\text{H}_2\text{PO}_4$, CaCl_2 + NaH_2PO_4 , or CaCl_2 + $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ were chosen to produce CaP NPs cores of different sizes. Three different sizes of NPs small (S, 174 ± 10 nm), medium (M, 261 ± 20 nm), and large (L, 364 ± 21 nm) were each selected for encapsulation by Pam1, Pam2, and Pam3.

To formulate Pam1 functionalised NPs, 400 μL of the CaP-PEI-OVA NPs suspension (2 mg/mL) was harvested by centrifugation ($10,000 \times g$, 5 min, 4 °C) and resuspended in 50 μL 25 mM sodium bicarbonate (pH = 8). Then, 5 μL of Palmitic acid N-hydroxysuccinimide ester (2 mg/mL in DMSO) was added to the NPs solution and incubated (1 h, 37 °C). After incubation, the NPs were washed with MilliQ water three times to remove any unreacted Pam1.

To formulate Pam2 (dipalmitoyl-KKKKKKC(dithio(5-nitropyridine))G-NH₂) functionalised NPs, 400 μL of the CaP-PEI-OVA NPs suspension (2 mg/mL) was harvested by centrifugation ($10,000 \times g$, 5 min, 4 °C) and resuspended in 1.8 mL 22 mM sodium bicarbonate (pH = 8). Then, 20 μL Pam2 (2 mg/mL in DMSO) was added to the NP solution and incubated (1 h, 37 °C). After incubation, the NPs were washed with MilliQ water for three times to remove any unreacted Pam2 peptide.

To formulate Pam3 (tripalmitoyl-cysteinyl-seryl-(lysyl)₃-lysine (P₃CSK₄) functionalised NPs, 400 μL of the CaP-PEI-OVA NPs suspension (2 mg/mL) was harvested by centrifugation ($10,000 \times g$, 5 min, 4 °C) and resuspended in 100 μL MilliQ water. DIPEA (1.741 mL, 10 mmol, Sigma, NSW, Australia) was added to 1 mL HBTU (5 mmol in DMF), and the solution was adjusted to 5 mL with DMF. After mixing, 10 μL of the HBTU/DIPEA solution was added to 40 μL 2 mg/mL Pam₃CSK₄ (1.3 mM, Invivogen, USA). After 2 minutes, the 50 μL of activated Pam₃CSK₄ solution was added to 100 μL of the NP suspension and incubated (2 h, RT). The NPs were then washed with MilliQ water three times to remove any unreacted Pam₃CSK₄ and organic solvents. The TLR2 ligand functionalised NPs were suspended in MilliQ water and stored at 4 °C for future characterisation.

The TLR2 ligand functionalised NPs were characterised by Dynamic Light Scattering (DLS) for particle size, size distribution, and zeta potential. The characterisation technique is described in detail in Chapter 2. The BCA protein assay kit (Thermo Fisher Scientific, SA, Australia) was used to measure ovalbumin concentrations. The number of NPs were counted by NanoSight NS300 (Malvern). All experiments were carried out in triplicate.

5.2.4 TLR2 activation assay with HEK293-TLR2 reporter cell line

HEK293 TLR2 reporter cells (Invivogen, USA) were seeded into 96 well plates at a cell density of 1×10^5 cells per well. After overnight incubation, TLR2 ligand functionalised NPs (S, M, and L) in 200 μ L complete media were added to the HEK293 TLR2 receptor cells at NP to cell ratios of 10:1, 100:1, 1,000:1 and 10,000:1, and incubated for 24 h (5% v/v CO₂, 37 °C). Following the incubation period, 50 μ L media was transferred to a new 96 well plate and added to 150 μ L Quanti-Blue (Invivogen, USA). The plate was incubated for 2 h (5% v/v CO₂, 37 °C), and the optical density was measured at 620 nm on a spectrophotometer. HEK293 TLR2 reporter cells in complete media without NPs were used as the negative control. Pam₃CSK₄ (Invivogen, USA, 500 ng/mL) was used as a positive control.

5.2.5 NF- κ B activation assay with RAW-Blue cells

RAW-Blue cells (Invivogen, USA) were seeded into 96 well plates at a cell density of 1×10^5 cells per well in 200 μ L complete DMEM. After overnight incubation (5% v/v CO₂, 37 °C), TLR2 ligand functionalised NPs (S, M, and L) in 200 μ L complete DMEM were added to the RAW-Blue cells at NP to cell ratios of 10:1, 100:1, 1,000:1 and 10,000:1 and incubated for 24 h (5% v/v CO₂, 37 °C). Following the incubation period, 50 μ L of media was taken from the cultures and transferred to a new 96 well plate and added to 150 μ L Quanti-Blue (Invivogen, USA). The plate was incubated for 2 h (5% v/v CO₂, 37 °C), and the optical density was measured at 620 nm on a spectrophotometer to determine the secreted alkaline phosphatase (SEAP) levels. RAW-Blue cells in complete media without NPs were used as the negative control. Complete media containing Lipopolysaccharide (LPS, 10 μ g/mL, Sigma-Aldrich, NSW, Australia) was used as a positive control.

5.2.6 Cytotoxicity of TLR2 ligand functionalised CaP NPs

Cell cytotoxicity was measured by the lactate dehydrogenase (LDH, Promega, NSW, Australia) and CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega, NSW, Australia) methods (performed according to the manufacturer's instructions). RAW 264.7 cells were seeded into 96 well plates at a cell density of 5×10^3 cells per well in 200 μ L complete DMEM. After overnight incubation (5% v/v CO₂, 37 °C), the cells had reached 70 - 80% confluency. The media was replaced with 150 μ L fresh media containing NPs at different NP to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1) and incubated for 90 min (5% v/v CO₂, 37 °C). After incubation, 50 μ L of media was transferred to a new 96 well plate and measured for LDH. For the MTS assays, MTS was diluted in 100 μ L fresh cell media and added to the cells after removing NPs in suspension, then assessed by MTS kits according to the manufacturer's protocol.

5.2.7 Phagocytosis of TLR2 ligand functionalised CaP NPs by RAW 264.7 cells

Different sizes of TLR2 ligand functionalised NPs were fluorescently labelled using pHrodo Red (Thermo Fisher Scientific, SA, Australia) according to the manufacturer's instructions. RAW 264.7 cells were resuspended at a density of 2.5×10^5 cells/200 μ L. These cells were incubated with NPs (S, M, and L) at NP to cell ratios of 10:1, 100:1, 1,000:1 and 10,000:1, and incubated (1 h, 5% v/v CO₂, 37 °C). After incubation, the cells were washed with flow cytometry staining buffer (FACS, PBS + 1% BSA + 0.1% Sodium Azide (NaN₃)) three times and resuspended in 200 μ L FACS buffer. The amount of phagocytosis was quantified by flow cytometry (BD LSRFortessa X-20, BD Biosciences, NSW, Australia). RAW 264.7 cells in complete media without NPs were used as the negative control.

5.2.8 Cell surface marker production by flow cytometry

RAW 264.7 cells were seeded into 24 well plates at a cell density of 2×10^5 cells per well. After overnight incubation (5% v/v CO₂, 37 °C), TLR2 ligand functionalised NPs (S, M, and L) in 200 μ L complete media were added to cells at NP to cell ratios of 10:1, 100:1, 1,000:1 and 10,000:1, and incubated (24 h, 5% v/v CO₂, 37 °C). The cells were washed with FACS buffer and blocked for 20 min on ice with rat anti-mouse CD16/CD32 antibody (BD Pharmingen, CA) to prevent Fc-receptor binding of antibodies. The cells were then incubated

with the following antibodies (20 min, 0 °C): PE-CyTM7 rat anti-mouse CD86; APC rat anti-mouse CD40; APC MHC class II (I-A/I-E) monoclonal antibody. These antibodies were all purchased from BD Bioscience (NSW, Australia). The level of CD40, CD80, and MHC II expression was quantified by flow cytometry (BD LSRFortessa X-20, BD Biosciences, NSW, Australia). The data was exported and FlowJo software (Three Star) was used for data analysis. RAW 264.7 cells incubated in complete media without NPs were used as the negative control and complete media containing Lipopolysaccharide (LPS, 10 µg/mL, Sigma-Aldrich, NSW, Australia) was used as a positive control. All experiments were performed in triplicate.

5.2.9 Cytokine production assays with RAW cells

RAW 264.7 cells were seeded into 24 well plates at a cell density of 2×10^5 cells per well. After overnight incubation (5% v/v CO₂, 37 °C), TLR2 ligand functionalised NPs (S, M, and L) in 200 µL complete media were added to cells at NP to cell ratios of 10:1, 100:1, 1,000:1 and 10,000:1 and incubated for 6 and 24 h (5% v/v CO₂, 37 °C). The supernatant was collected by centrifugation ($800 \times g$, 5 min, RT) after incubation to remove remaining cells and NPs. RAW 264.7 cells in complete media without NPs were used as the negative control. Complete media containing LPS (10 µg/mL) was used as a positive control. The cytokine levels (IL-1 β , IL-6, IL-10, IL-12 and TNF- α) in the supernatant were determined by ELISA kits (BD Biosciences, NSW, Australia) according to the manufacturer's instructions.

5.2.10 Statistical analysis

GraphPad Prism 5 software was used to analyse data in experiments. One-way ANOVA and student T-test were used to determine *P* and a *P* < 0.05 was considered statistically significant.

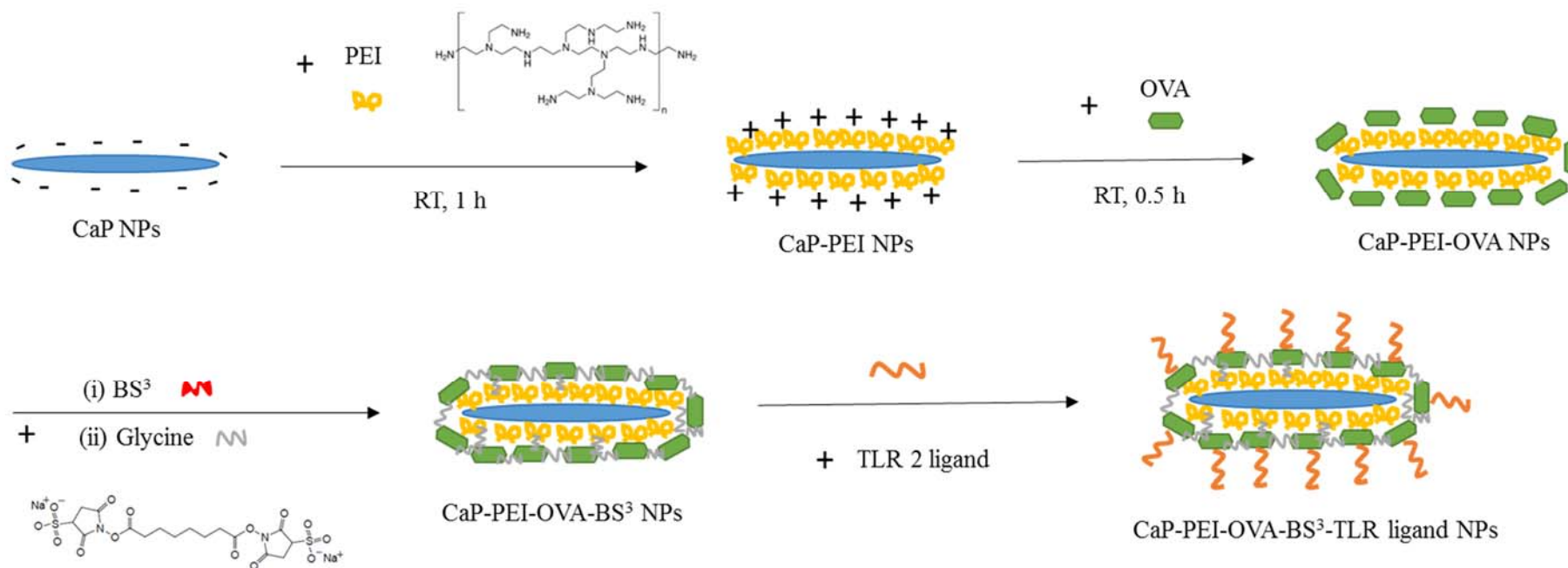


Figure 5. 1: Schematic representation of the preparation of TLR2 ligand functionalised nanoparticles.

The charge of CaP NPs was reversed by addition of PEI. Next, OVA was added to CaP-PEI NPs by electrostatic interaction. OVA on the NP surface was then crosslinked by BS³. Finally, TLR2 ligand was bound to the surface of nanoparticles. Abbreviations: CaP NPs, calcium phosphate nanoparticles; PEI, polyethylenimine; OVA, ovalbumin; BS³ (bis[sulfosuccinimidyl] suberate), TLR2 ligand.

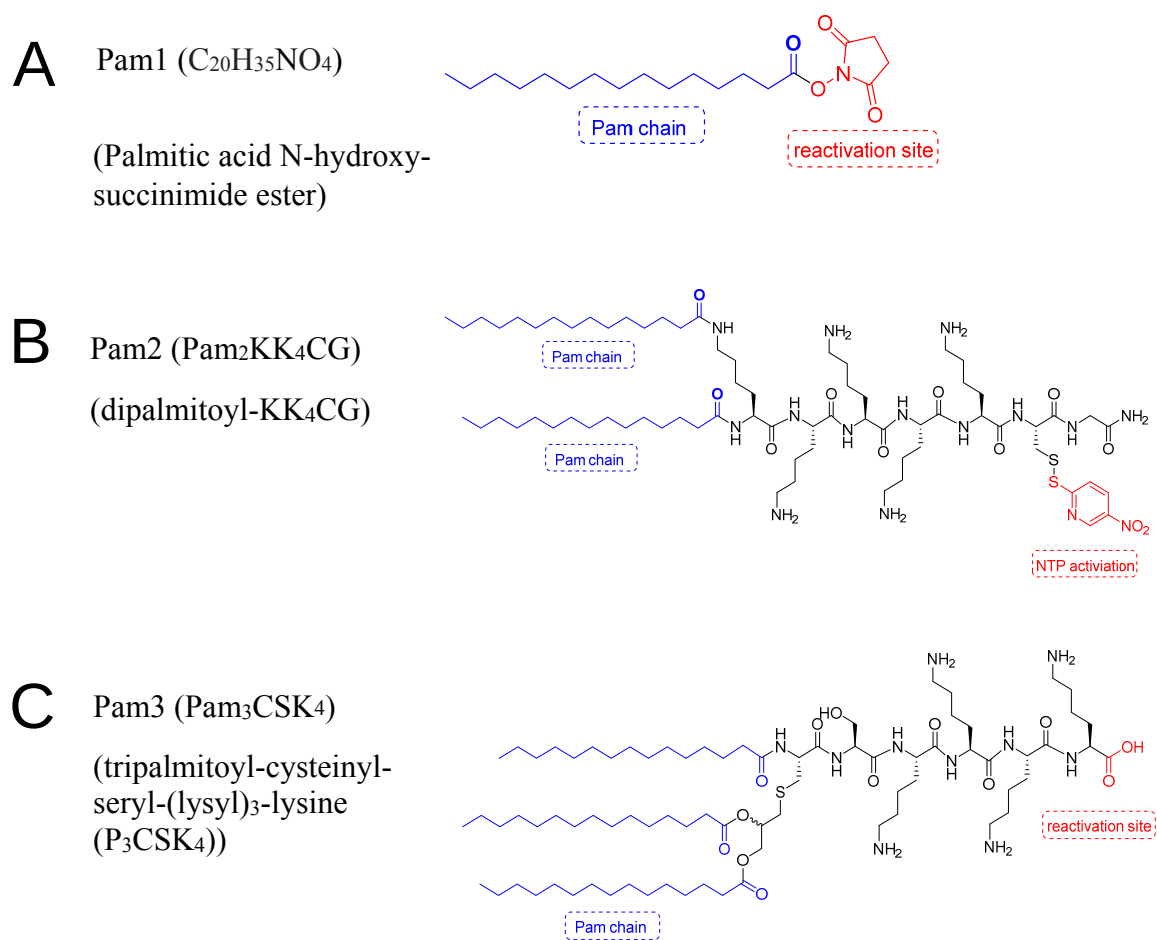


Figure 5. 2: Chemical structures of Pam molecules.

Palmitic acid N-hydroxysuccinimide ester (A, Pam1, $C_{20}H_{35}NO_4$), Pam_2KK_4CG (B, Pam2, dipalmitoyl- KK_4CG), Pam_3CSK_4 (C, Pam3, (tripalmitoyl-cysteinyl-seryl-(lysyl) $_3$ -lysine (P_3CSK_4)).

5.3 Results

5.3.1 Characterisation of TLR2 ligand functionalised CaP-PEI-OVA NPs

Three different sizes of TLR2 ligand functionalised CaP-PEI-OVA NPs were prepared by the layer-by-layer approach. The CaP NPs core was synthesised by the citrate chelating method, followed by coating the NPs with PEI to produce particles with a positive surface charge. CaP-PEI NPs were then coated with the model antigen, ovalbumin, the NPs were cross-linked using BS³. Finally, the TLR2 ligand (Pam1, or Pam2 or Pam3) was added to the surface of NPs by chemical conjugation. A schematic of the preparation method is shown in Figure 5.1. During this study, three different TLR2 ligands were investigated, which varied by the number of palmitoyl (Pam) chains they possessed; Palmitic acid N-hydroxysuccinimide ester (Pam1), Pam₂KK₄CG (Pam2), and Pam₃CSK₄ (Pam3). The structure of these chemicals is shown in Figure 5.2. The purified peptide Pam₂KK₄CG was characterised by using reverse-phase high performance liquid chromatography (RP-HPLC) (Supplementary Figure 5.1).

The particle size, size distribution and surface charge of different sizes of TLR2 ligand functionalised NPs were characterised by DLS. Table 5.1 summarises the physical properties arising from different sizes and numbers of Pam chains on functionalised NP formulation. The particle size for each addition of Pam1, Pam2 or Pam3 on to either CaP-OVA-S, M or L resulted in a slight increase in NP size that was not significantly different from the non-functionalised particles (Table 5.1). The PDI value of these samples was relatively low (0.05 to 0.14), which suggests that the NPs are homogeneous and monodispersed. All three sizes of different types of Pam chains functionalised NPs were positively charged. As Pam2 and Pam3 have 4 lysines, these Pam functionalised NPs have higher positive charge than Pam1. The number of functionalised NPs were counted using the NanoSight NS300, and the results showed both the Pam 1 and Pam 2 functionalised NPs have good yield, Pam 3 functionalised NPs had low yield (data not shown).

Table 5. 1: Properties of palmitoyl functionalisation of CaP-PEI-OVA NPs.

The characterisation of different numbers of palmitoyl (Pam) chains functionalised CaP-PEI-OVA NPs as determined by DLS. The different size formulations are represented by S (small), M (medium), and L (large).

Sample	Size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
CaP-OVA-S	174 ± 10	0.09 ± 0.04	-16.7 ± 0.6
CaP-OVA-Pam1-S	189 ± 23	0.12 ± 0.05	+3.9 ± 0.6
CaP-OVA-Pam2-S	201 ± 37	0.14 ± 0.09	+29 ± 1.1
CaP-OVA-Pam3-S	171 ± 19	0.09 ± 0.02	+20 ± 2.4
CaP-OVA-M	261 ± 20	0.11 ± 0.07	-17.5 ± 1.2
CaP-OVA-Pam1-M	279 ± 19	0.05 ± 0.02	+5.5 ± 0.8
CaP-OVA-Pam2-M	283 ± 10	0.08 ± 0.02	+28.2 ± 0.4
CaP-OVA-Pam3-M	268 ± 27	0.11 ± 0.06	+21 ± 1.5
CaP-OVA-L	364 ± 21	0.08 ± 0.07	-18.3 ± 0.8
CaP-OVA-Pam1-L	388 ± 25	0.13 ± 0.09	+5.1 ± 0.7
CaP-OVA-Pam2-L	362 ± 19	0.12 ± 0.08	+28.0 ± 0.3
CaP-OVA-Pam3-L	365 ± 30	0.13 ± 0.07	+21 ± 1.8

5.3.2 TLR2 activation in HEK293-TLR2 reporter cell line and NF-κB activation assay in RAW-Blue cells

Specific activation of TLR2 and NF-κB by TLR2 ligand functionalised particles was determined using HEK293 TLR2 and RAW-Blue reporter cells. HEK293-TLR2 cells are stably transfected with both a TLR2 encoding plasmid and the SEAP reporter system, and therefore will only respond to TLR2 activation. RAW-Blue cells are derived from mouse macrophages, and stably express an NF-κB-inducible SEAP reporter construct that responds to any NF-κB phosphorylation. To investigate whether TLR2 ligand modified particles can induce TLR2 activation and NF-κB phosphorylation, HEK293-TLR2 cells and RAW-Blue cells were incubated with three sizes of TLR2 ligand functionalised NPs for 24 h, and the level of SEAP was determined by using Quanti-Blue.

Results showed that HEK-Blue TLR2 cells were not activated by any of the small Pam1 particles as compared to CaP-OVA-S control particles (Figure 5.3A). HEK-Blue TLR2 cells were activated by the medium and large sizes of Pam1 modified particles only at the highest NP to

cell ratio (10,000:1, Figure 5.3B, C). However, TLR2 expressing cells were activated by Pam2 modified particles at the two highest concentrations of 1,000:1 and 10,000:1 (Figure 5.3D, E and F), and were strongly activated by Pam3 modified particles at all the NP to cell ratios (Figure 5.3G, H and I).

With the RAW-blue macrophage cells, all three sizes of particles conjugated with Pam1 (Figure 5.4A, B and C) and Pam2 (Figure 5.4D, E and F) induced significant NF- κ B activation at the higher NP to cell ratios of 1,000:1 and 10,000:1. Moreover, all three sizes of Pam3 functionalised NPs induced significant phosphorylation of NF- κ B in RAW-Blue cells at all NP to cell ratios tested (Figure 5.4G, H and I). As the Pam3 had low yield and good activity, Pam2 had good yield and relatively good activity, Pam1 had good yield but low activity, Pam2 was selected for further study from this point.

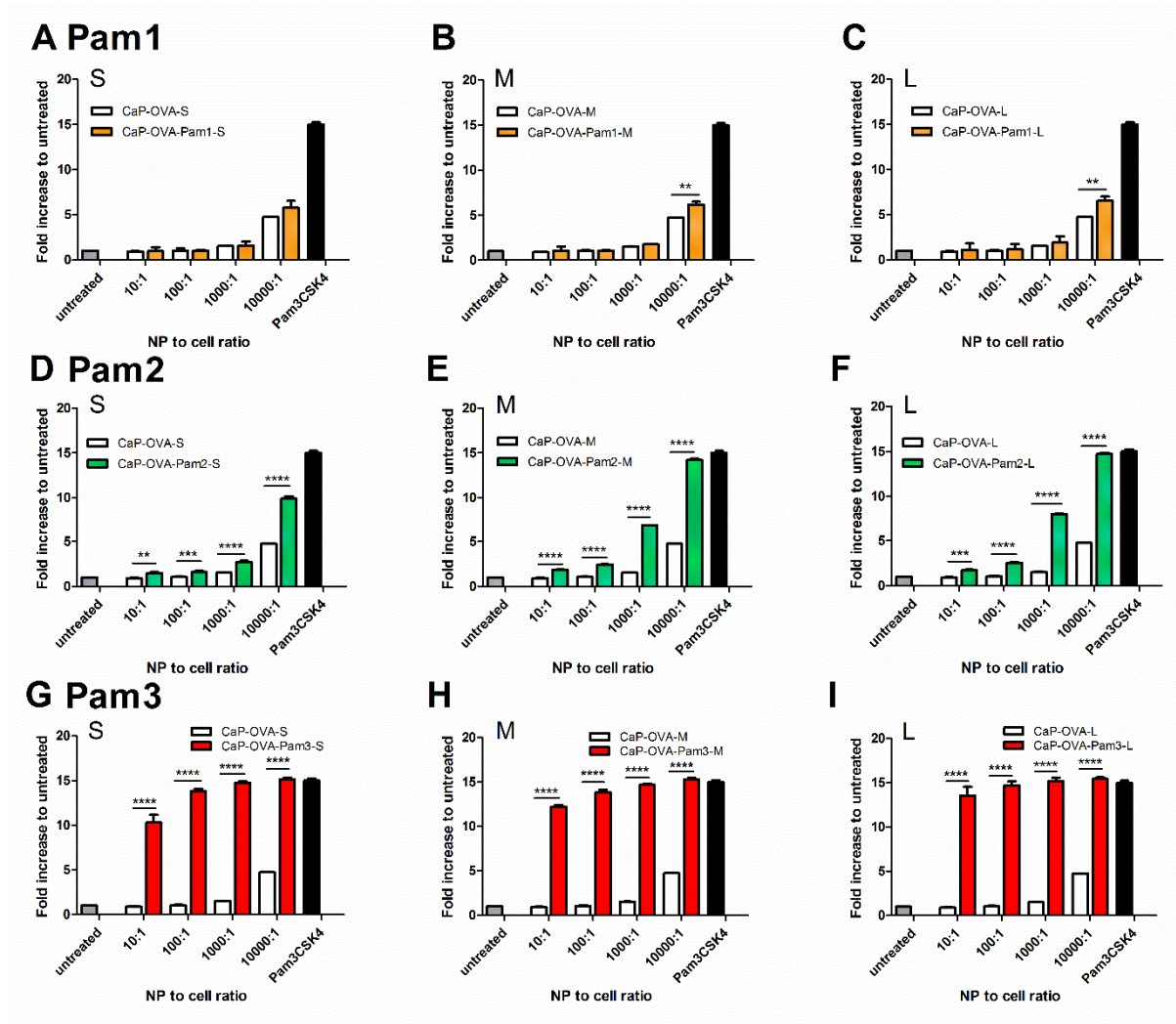


Figure 5. 3: Different numbers of palmitoyl (Pam) chains functionalised nanoparticles activates TLR2 lig- and in HEK293-TLR2 reporter cell line at increasing nanoparticle to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ were considered statistically significant.

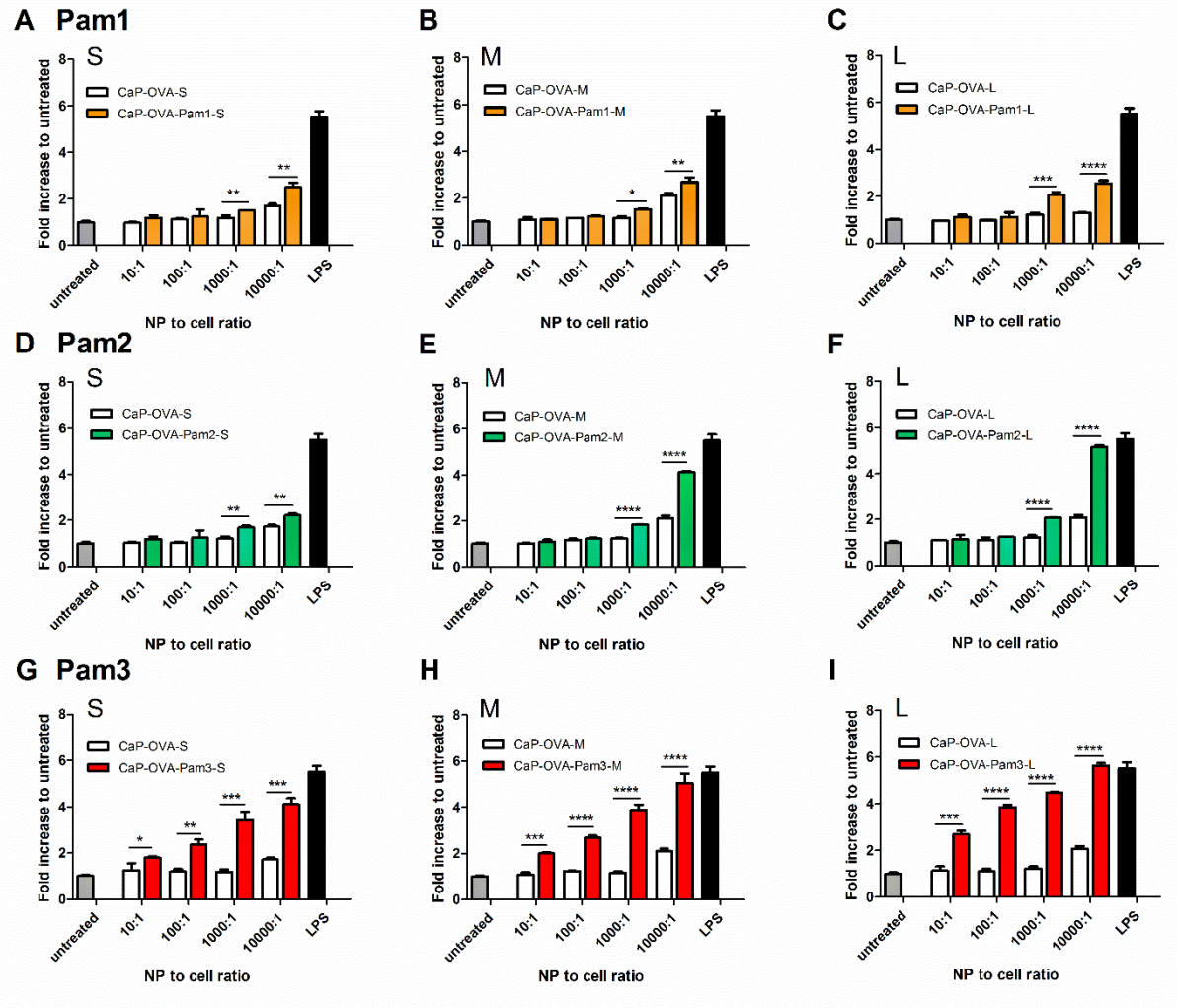


Figure 5. 4: Different numbers of palmitoyl (Pam) chains functionalised nanoparticles activates NF- κ B in RAW-Blue cells at increasing nanoparticle to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ were considered statistically significant.

5.3.3 Cytotoxicity of Pam₂KK₄CG functionalised nanoparticles

To investigate the cytotoxicity of Pam₂ functionalised NPs, these NPs were incubated with RAW 264.7 cells for 90 min, and the cell viability was determined by MTS and LDH assay. The three sizes of NPs (S, M, and L) had no significant ($P \geq 0.05$) cytotoxic effect on RAW 264.7 cells at all NP to cell ratios after 90 min incubation (Figure 5.5).

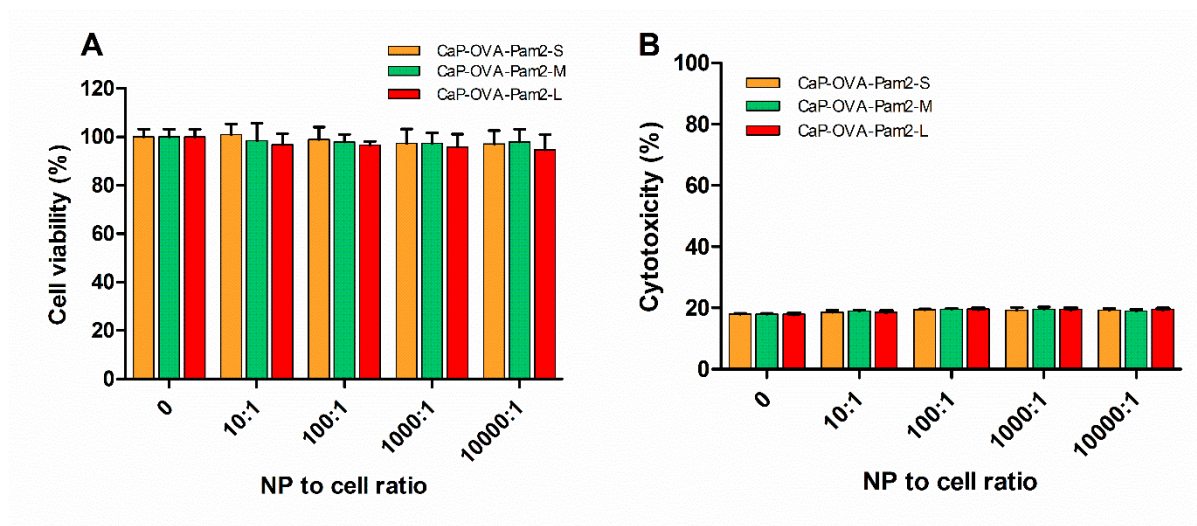


Figure 5. 5: Cell toxicity by MTS assay (A) and LDH assay (B) on RAW 264.7 cells incubated with different NP to cell ratios of Pam₂KK₄CG functionalised nanoparticles for 90 minutes.

Data is expressed as mean $\pm$ SD. The blank is the culture media alone. The negative control are cells alone. There were no statistically significant differences between these data, ($P \geq 0.05$).

5.3.4 Pam₂KK₄CG functionalised nanoparticles phagocytosis by macrophages

The uptake of vaccines by professional phagocytes such as macrophages and dendritic cells is a crucial first step in the induction of an immune response. To determine whether Pam2 functionalised NPs are phagocytosed by macrophages, NPs were labelled with pHrodo red (a pH-sensitive fluorescence dye), and the level of phagocytosis was determined by flow cytometry. The three sizes of Pam2 functionalised NPs (S, M, and L) were phagocytosed by RAW 264.7 cells in a dose-dependent manner, as indicated by an increase in the percentage of fluorescence observed (Figure 5.6A, B, C and D). In comparison with CaP-OVA, Pam2 functionalised CaP-OVA had significantly enhanced phagocytosis for S, M, and L particles at all NP to cell ratios (Figure 5.6B, C and D).

5.3.5 Cell surface marker production

A further consequence of NF- κ B phosphorylation and translocation is the upregulation of cell surface markers that allow macrophages to interact with other cells of the host immune system. To determine the level of co-stimulatory marker upregulation induced by Pam2 functionalised particles, RAW 264.7 cells were incubated with Pam2 functionalised NPs or CaP-PEI-OVA NPs for 24 h. The upregulation of antigen presentation markers, MHC-II and co-stimulatory markers: CD40 and CD86, were analysed by flow cytometry. There was no significant difference in the induced expression of CD40, CD86 and MHC II observed between CaP-OVA-Pam2-S and CaP-OVA-Pam2-M and the corresponding control CaP-PEI-OVA NPs at cell ratios of 10:1 and 100:1 (Figure 5.7B and C, Figure 5.8B and C, and Figure 5.9B and C). However, at NP to cell ratios of 1,000:1 and 10,000:1, there was a significant upregulation of CD40, CD86 and MHC II expression after treatment with CaP-OVA-Pam2-S and CaP-OVA-Pam2-M (Figure 5.7B and C, Figure 5.8B and C, and Figure 5.9B and C). Further, all co-stimulatory molecules were significantly upregulated in response to CaP-OVA-Pam2-L as compared to CaP-PEI-OVA NPs controls at all tested concentrations (Figure 5.7D, Figure 5.8D, and Figure 5.9D).

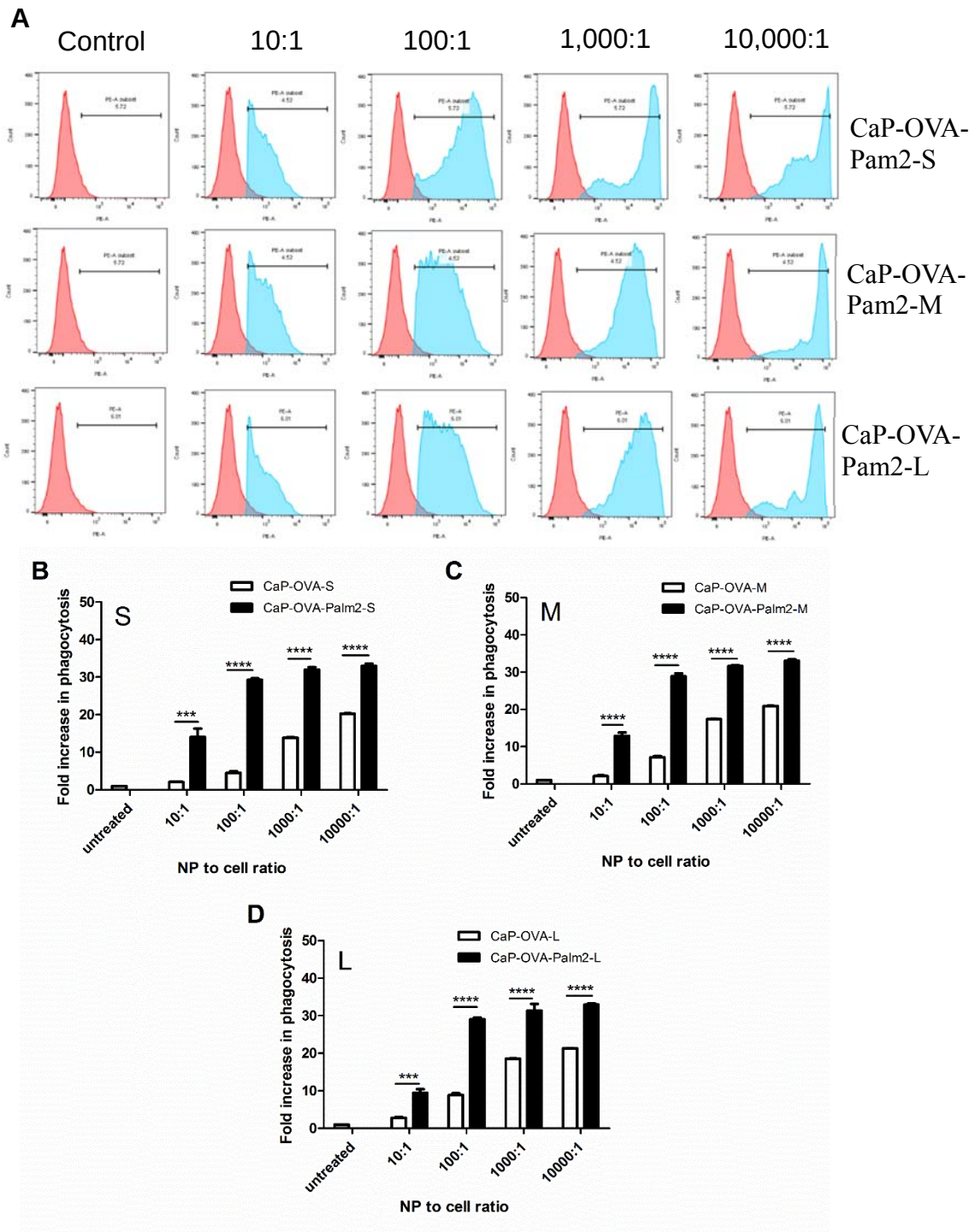


Figure 5. 6: Phagocytosis of Pam₂KK₄CG functionalised nanoparticles by the macrophages (RAW 264.7). Nanoparticles were labelled with pHrodo red and examined their phagocytosis by RAW 264.7 cells at increasing nanoparticle to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1). Cells and nanoparticles were incubated for 1 h, and the degree of phagocytosis was determined by flow cytometry. (A) Phagocytosis was demonstrated by an increase in red fluorescence (PE-A), the presence of Pam2 on NPs induced an increase in phagocytosis for S (B), M (C), and L (D) NPs. The level of phagocytosis was determined by the cell phagocytosis percentage. Data are expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ were considered statistically significant.

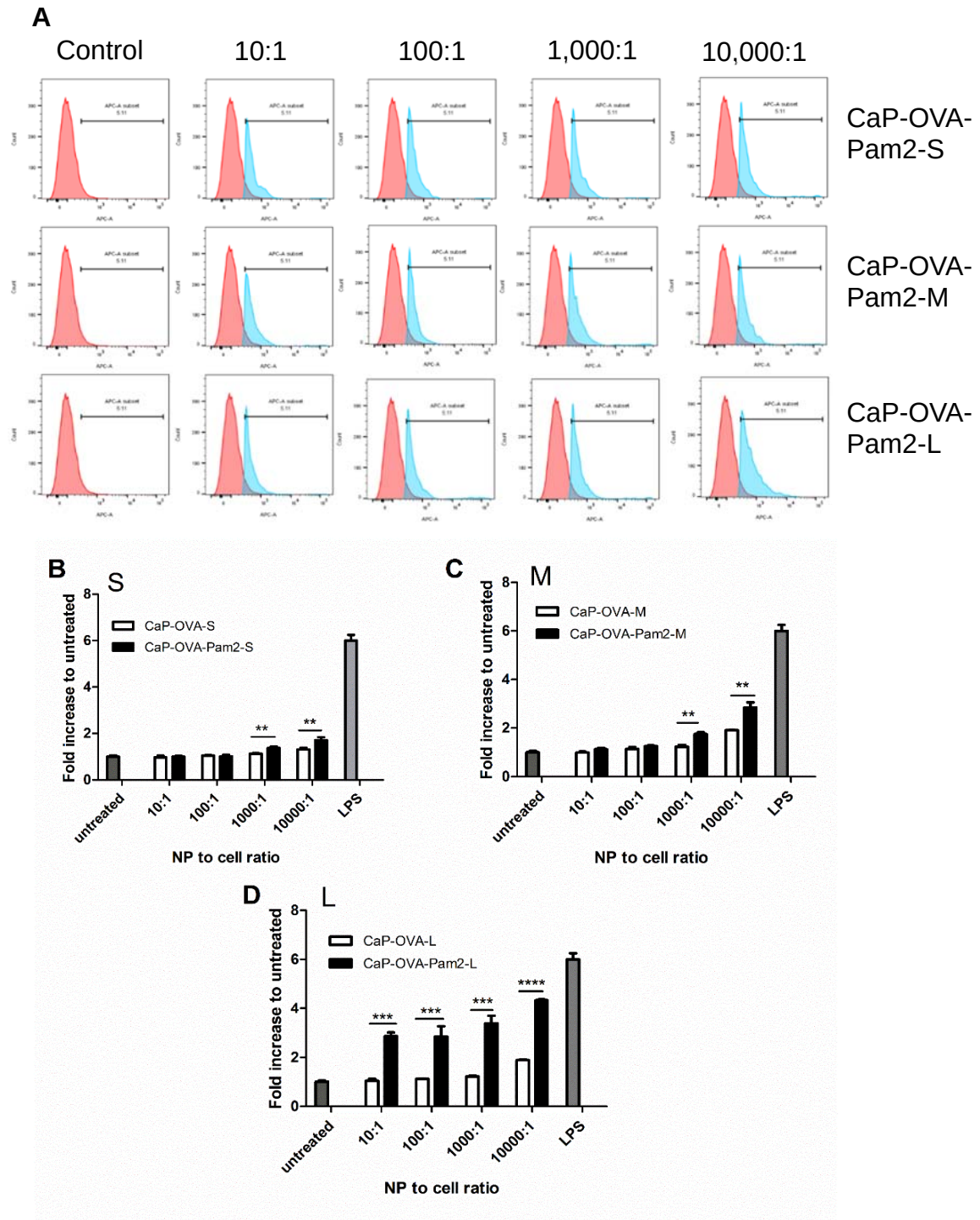


Figure 5. 7: Pam₂KK₄CG functionalised nanoparticles induction of CD40 expression in RAW 264.7 cells at various NP to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1).

(A) Flow cytometry histograms of CD40 expression of RAW 264.7 cells; (B) Fold increase compared to untreated cells of RAW 264.7 cells CD40 expression induced by CaP-OVA-S and CaP-OVA-Pam2-S NPs incubation; (C) Fold increase compared to untreated cells of RAW 264.7 cells CD40 expression induced by CaP-OVA-M and CaP-OVA-Pam2-M NPs incubation; (D) Fold increase compared to untreated cells of RAW 264.7 cells CD40 expression induced by CaP-OVA-L and CaP-OVA-Pam2-L NPs incubation. Data are expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ were considered statistically significant.

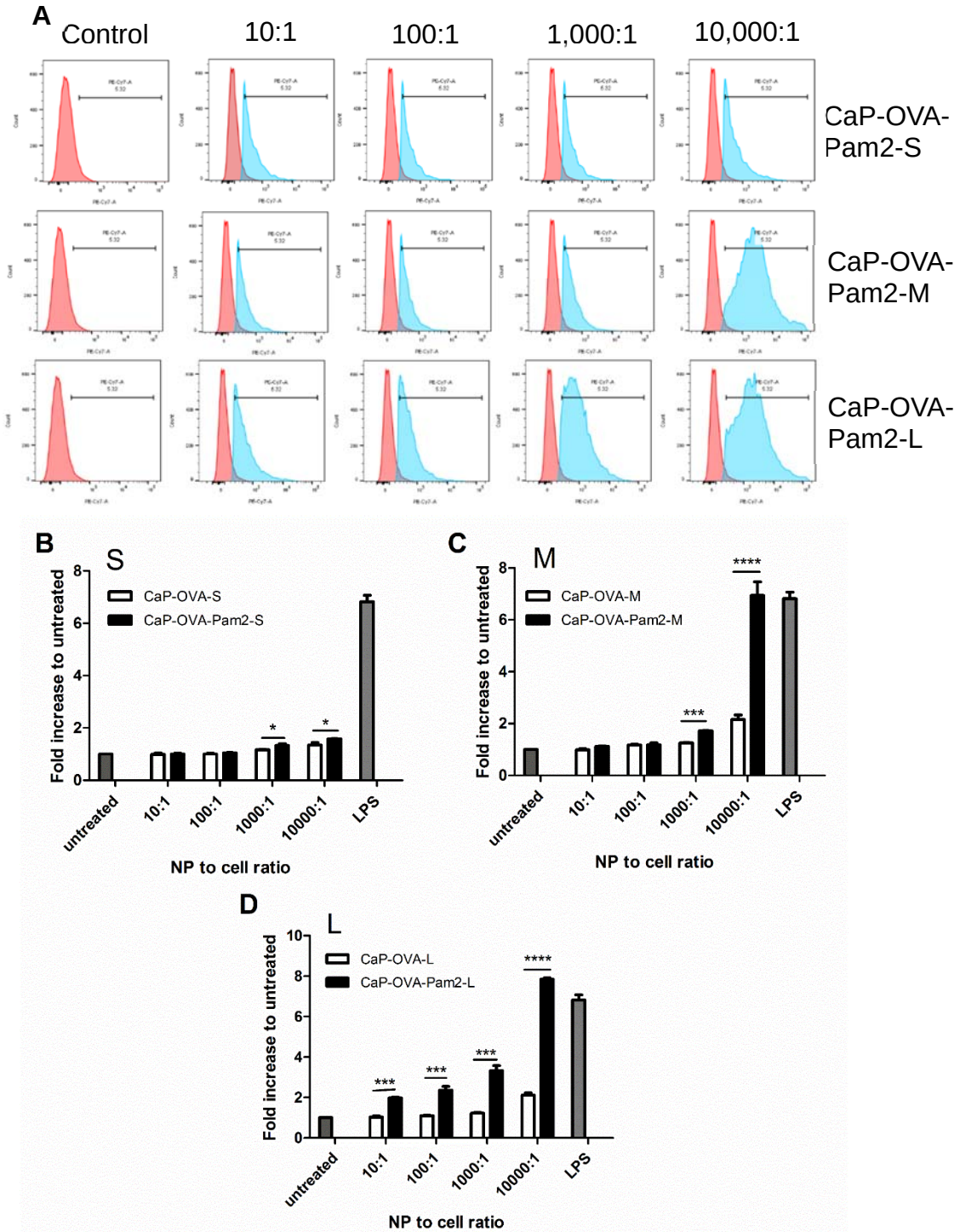


Figure 5. 8: Pam₂KK₄CG functionalised nanoparticles induction of CD86 expression in RAW 264.7 cells at various NP to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1).

(A) Flow cytometry histograms of CD86 expression of RAW 264.7 cells; (B) Fold increase compared to untreated cells of RAW 264.7 cells CD86 expression induced by CaP-OVA-S and CaP-OVA-Pam2-S NPs incubation; (C) Fold increase compared to untreated cells of RAW 264.7 cells CD86 expression induced by CaP-OVA-M and CaP-OVA-Pam2-M NPs incubation; (D) Fold increase compared to untreated cells of RAW 264.7 cells CD86 expression induced by CaP-OVA-L and CaP-OVA-Pam2-L NPs incubation. Data are expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ were considered statistically significant.

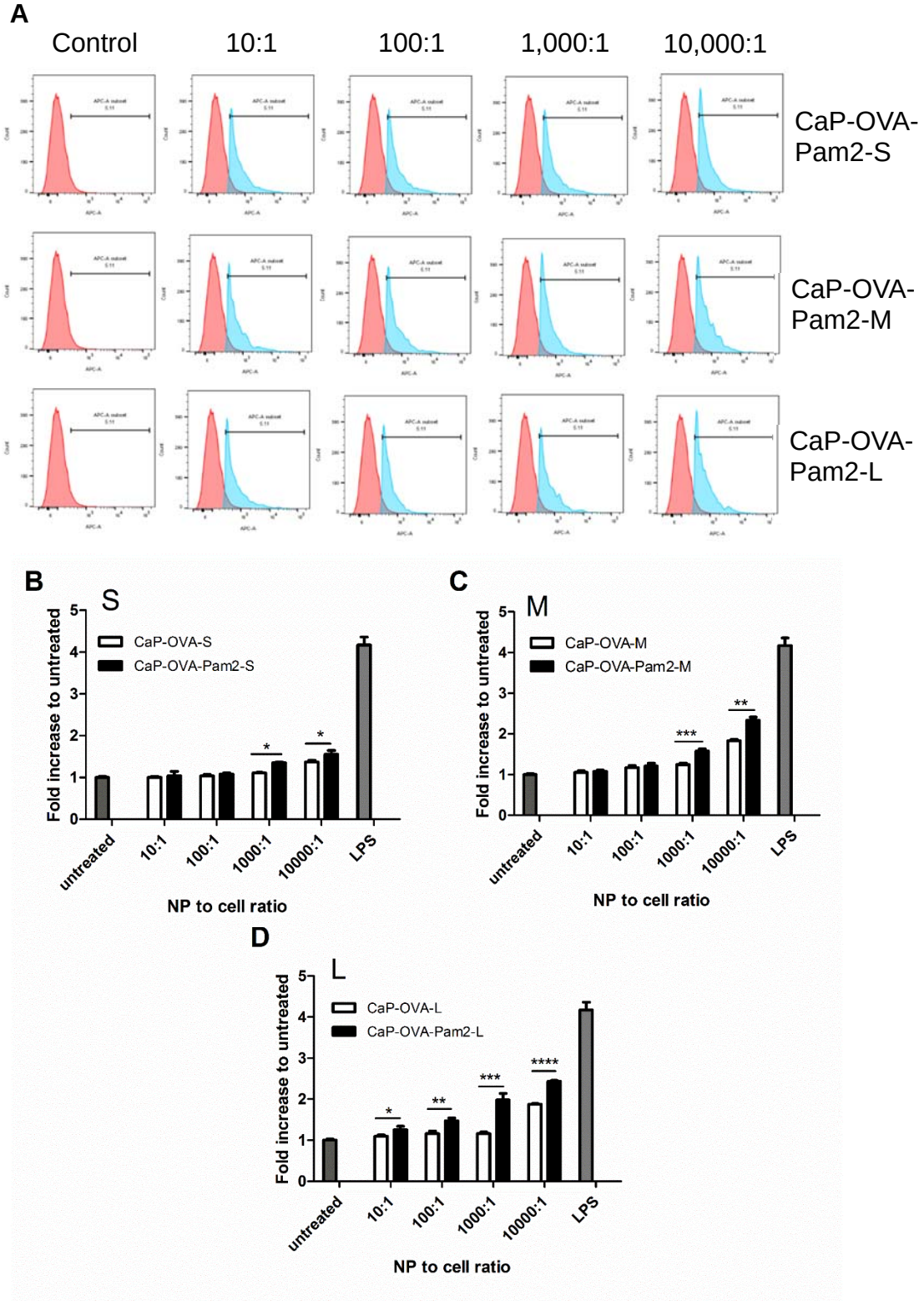


Figure 5. 9: Pam₂KK₄CG functionalised nanoparticles induction of MHC II expression in RAW 264.7 cells at various NP to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1).

(A) Flow cytometry histograms of MHC II expression of RAW 264.7 cells; (B) Fold increase compared to untreated cells of RAW 264.7 cells MHC II expression induced by CaP-OVA-S and CaP-OVA-Pam2-S NPs incubation; (C) Fold increase compared to untreated cells of RAW 264.7 cells MHC II expression induced by CaP-OVA-M and CaP-OVA-Pam2-M NPs incubation; (D) Fold increase compared to untreated cells of RAW 264.7 cells MHC II expression induced by CaP-OVA-L and CaP-OVA-Pam2-L NPs incubation. Data are expressed as mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001 were considered statistically significant.

5.3.6 Pam₂KK₄CG functionalised nanoparticles induce cytokine production in RAW 264.7 cells

One function of phosphorylated NF- κ B is to cross the nuclear membrane and bind to response elements on the host DNA to upregulate various cytokines and chemokines. In order to measure whether there were differences in the upregulation of multiple cytokines between Pam2 functionalised NPs and CaP-PEI-OVA NPs, RAW 264.7 cells were incubated with three sizes of Pam2 functionalised NPs and the corresponding control CaP-PEI-OVA NPs for 6 h (Supplementary Figure 5.2) and 24 h (Figure 5.10), after which the supernatant was collected and analysed by ELISA for the level of IL-1 β , IL-6, IL-10, IL-12, and TNF- α .

After incubation with the Pam2 functionalised particles for 6 h, no significant change in cytokine production was observed at the lowest NP to cell ratio (Supplementary Figure 5.2). CaP-OVA-Pam2-S resulted in a significant increase in IL-1 β , IL-10, IL-12 and TNF- α secretion compared to CaP-OVA-S from RAW 264.7 cells at the highest NP to cell ratio of 10,000:1 (Supplementary Figure 5.2A, G, J and M). CaP-OVA-Pam2-M also induced the secretion of these cytokines (IL-1 β , IL-10, IL-12 and TNF- α) but at high NP to cell ratios after 6 h (Supplementary Figure 5.2B, H, K and N). CaP-OVA-Pam2-L induced secretion of each cytokine tested at high NP to cell ratios of 1,000:1 and 10,000:1. Further, IL-12 and TNF- α were induced at a 100:1 NP to cell ratio (Supplementary Figure 5.2C, F, I, L and O). Incubation of RAW 264.7 cells with each of the palmitoyl functionalised particles for 24 h resulted in the same pattern of cytokine expression induced by each CaP-OVA NP at 6 h, except the amount of each cytokine was increased (Figure 5.10).

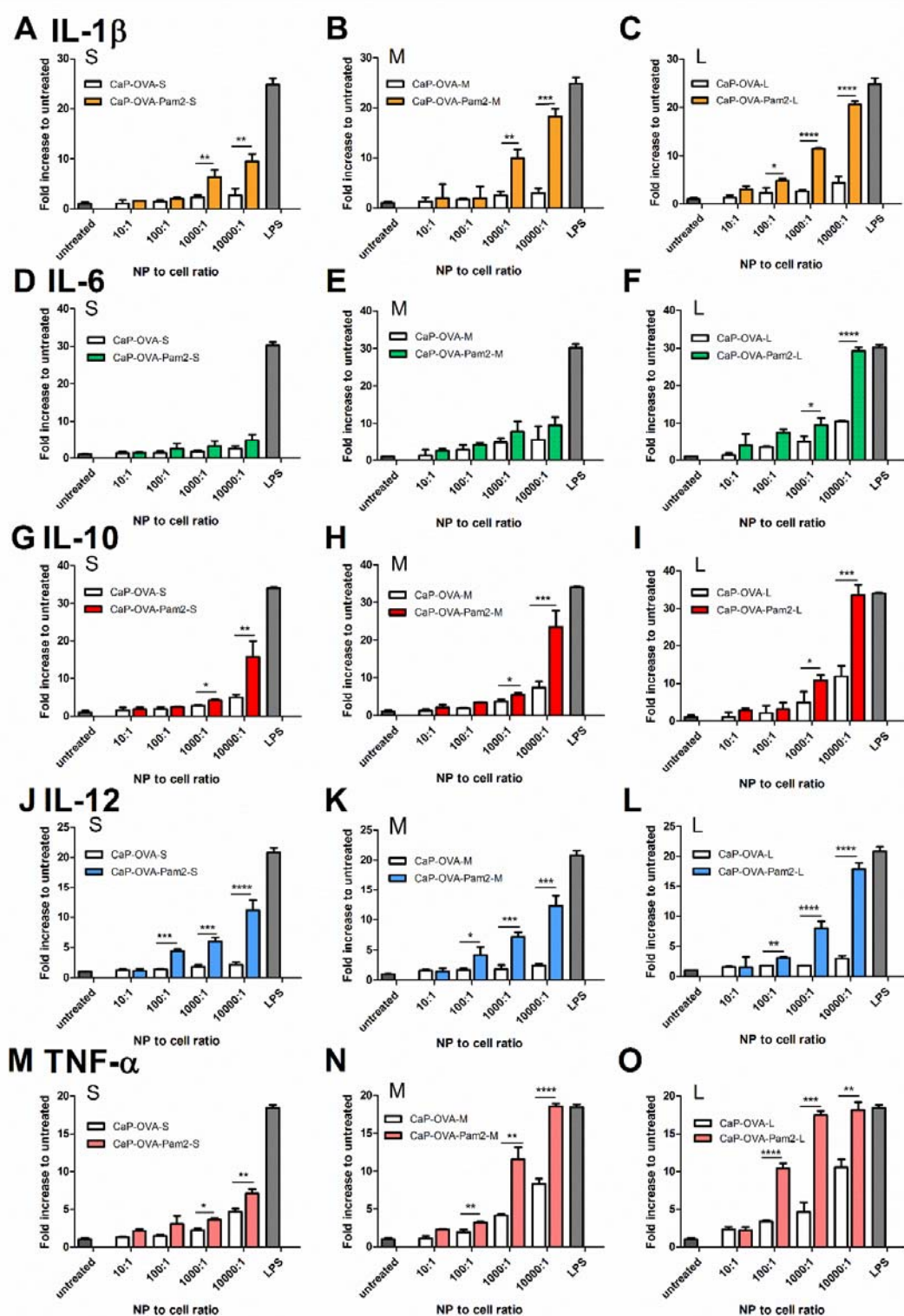


Figure 5. 10: Pam₂KK₄CG functionalised nanoparticles induce cytokine release in RAW 264.7 cells.

Cytokine production in RAW 264.7 cells was measured following 24 h incubation of three sizes of TLR2 ligand functionalised nanoparticles at various NP to cell ratios (10:1, 100:1, 1000:1 and 10000:1). IL-1 β (A, B, C), IL-6 (D, E, F), IL-10 (G, H, I), IL-12 (J, K, L), and TNF- α (M, N, O) were detected by ELISA assays. Data are expressed as mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001 were considered statistically significant.

5.4 Discussion

The addition of ligands for TLR2 has been shown to have great potential to be used as an adjuvant in vaccine applications [436-438]. The aim of this investigation was to synthesise a novel TLR2 peptide with improved binding chemistry and to determine how it contributes to macrophage activation. In this study, the surface functionalisation of CaP-PEI-OVA NPs with palmitoyl groups; Pam2 and Pam3, in particular, were found to induce the activation of macrophages. The addition of CaP-OVA-Pam2 to macrophages increased NF- κ B activation, phagocytosis, cell surface marker expression and cytokine secretion compared to the CaP-PEI-OVA NPs.

All three sizes of Pam₂KK₄CG functionalised NPs were positively charged, indicating the successful TLR ligand functionalisation of NPs which were previously negatively charged. Due to the multiple lysines from the Pam2 or Pam3 peptide, the net charge of Pam₂KK₄CG or Pam3 conjugate would likely be positively charged [439]. Furthermore, all the palmitoyl functionalised NPs displayed low polydispersity and high emulsion stability.

In this chapter, the effect of Palmitoyl (Pam1), Pam₂KK₄CG (Pam2), and Pam₃CSK₄ (Pam3) loaded particles of various sizes on NF- κ B phosphorylation following TLR2 ligation was compared to determine the optimal combination of number of acyl chains and size of the particle. Surface functionalisation with a single palmitoyl chain resulted in the lowest TLR2 activation of all the NP formulations [440-442]. This is consistent with studies investigating the crystal structure of both the TLR2-1 and TLR2-6 heterodimers interacting with Pam₂CSK₄ and Pam₃CSK₄, respectively. The hydrophobic lipid-binding pockets of the TLR molecules are essential for binding the acyl chains of the TLR2 ligands. However, the N-terminal cysteine, and to a lesser extent serine, also interacts with TLR dimers via strong hydrogen bonds, further stabilising the dimer and increasing signal transduction. The low TLR2 activation observed in this study in response to surface functionalisation with a single palmitoyl may reflect the inability of a single acyl chain to fully engage the binding pocket of TLR2, analogous to penta- and tetra-acylated lipid A engagement with TLR4.

Surface functionalisation with Pam₂KK₄CG and Pam₃CSK₄ significantly increased TLR2 activation at all tested doses as compared to CaP-OVA NPs alone. It has been reported that the synthetic diacylated lipopeptide Pam₂CSK₄ is an agonist of TLR2/TLR6 heterodimer [134,

443, 444], while the synthetic triacylated lipopeptide Pam₃CSK₄ is an agonist of TLR2/TLR1 heterodimer [134, 418]. The crystal structure of both the TLR2/1 and TLR2/6 heterodimers with their respective triacylated and diacylated ligands revealed that the TLR2 binding pocket can accommodate two acyl chains [445, 446]. The TLR1 monomer of the TLR1/2 dimer provides a second binding pocket for the third acyl chain of the Pam₃CSK₄ molecule. However, the TLR6 monomer of the TLR1/6 dimer lacks any binding pockets, providing close association and dimerisation in the absence of a third acyl chain not present in Pam₂CSK₄ [447].

The results of the TLR2 and NF- κ B activation assays show that Pam₂KK₄CG induces a slightly stronger TLR2 activation compared to Pam₁ and weaker TLR-2 activation than Pam₃CSK₄. Despite the stronger immune stimulation of macrophages observed from Pam₃CSK₄, commercialised Pam₃CSK₄ has no orthogonal reactive group for ligation to proteins thus it hard to use and may be why the yield was lower. This would lead to a less defined orientation of the Pam₃CSK₄ molecules on the surface of the functionalised NPs. To allow for a defined attachment of the Pam molecules to the surface of the NPs, a novel TLR2 ligand was developed that can be site-specifically conjugated with cysteine in a protein via a disulphide bond. Compared to Pam₃CSK₄, Pam₂KK₄CG has only two palmitoyl chains and thus results in less hydrophobicity and higher water solubility. By comparison to Pam₃, Pam₂KK₄CG not only has a site-specific reaction but also has a relatively high degree of immune stimulation, indicating Pam₂ could be used as a novel TLR2 ligand to conjugate to particles.

For the development of successful vaccines delivery systems, it is important to develop a safe adjuvant which enhances immunity while minimising damaging effects to the host. The Pam₂ functionalised NPs did not have any cytotoxic effects on macrophages at tested NP concentrations, indicating that these NPs may be safe and biocompatible adjuvants.

When the immune system encounters pathogenic bacteria and eukaryotic pathogens, the process of phagocytosis is a central mechanism in antigen processing and presentation [448, 449]. The phagocytosis of NPs by macrophages is a major mechanism for NP uptake [397] and is an important step in the maturation and activation of immune cells. In particular, macrophages are thought to be the primary cells that recognise, internalise, distribute nanomaterials and mediate biological immunological responses [397]. To induce a robust immune response, it is necessary for the phagosome containing engulfed NPs to develop into a phagolysosome, which has a low pH (< 5.0) environment [398]. This acidic environment assists in the destruction of pathogens

and presentation of processed antigens to cognate T cells [397, 399]. CaP NPs dissolve under acidic pH conditions and the subsequent release of their antigenic cargo into the mature phagolysosome and is suggested to be an ideal attribute in a vaccine carrier [128]. By labelling NPs with a pH-sensitive dye, it is possible to distinguish between phagocytosed NPs, cytoplasmic NPs and cell-associated NPs by flow cytometry [448]. pHrodo Red dye increases fluorescence intensity under low pH conditions and has been extensively used to study phagocytosis [400, 401]. The results demonstrated that Pam₂KK₄CG functionalised particles were phagocytosed significantly better than particles without the TLR ligand functionalisation. Although there are no previous experimental observations regarding the increased uptake of TLR2-functionalised NPs, this observation is consistent with the recent findings by Shen *et al.* who determined that TLR2 receptors enhance the phagocytosis of *Listeria monocytogenes* by macrophages [450] through the c-Jun N-terminal kinase (JNK) signalling pathway [451]. Further, Fatehchand *et al.* [452] reported that the TLR2 agonist Pam₂CSK₄ enhances human peripheral blood monocytes phagocytosis.

Immature macrophages function as antigen capturing and sentinel cells while mature macrophages, expressing T cell co-stimulatory molecules, act as antigen-presenting cells [403]. After capturing the antigens, macrophages will degrade the antigen into peptides and then present the peptide-major histocompatibility complex (MHC) complex to T cells to elicit immune responses [404]. The maturation of macrophages is closely related to the upregulation of antigen presentation markers such as MHC-II and certain co-stimulatory marker expression, such as CD40 and CD86 [453]. In the experiments conducted in this study, the expression of CD40, CD86 and MHC II were significantly enhanced by functionalisation with the Pam₂KK₄CG peptide. Similar to these results, Yamazaki *et al.* demonstrated that the Pam₂CSK₄ lipopeptides significantly increased the expression of CD40 and CD86 on spleen DCs *in vivo* when delivered as free TLRs [406]. Additionally, the combination of TLR2 and TLR7 elicited significant expression of CD80 and CD86 on DCs [129]. These studies showed that this induced upregulation of CD40, CD86 and MHC II resulted in a strong activation of T cells [129, 406], may suggest that Pam₂KK₄CG used for surface functionalisation which does induce macrophage expression of CD40, CD86 and MHC II may then activate naïve bystander T cells and potentially induce an immune response to the carrier antigen.

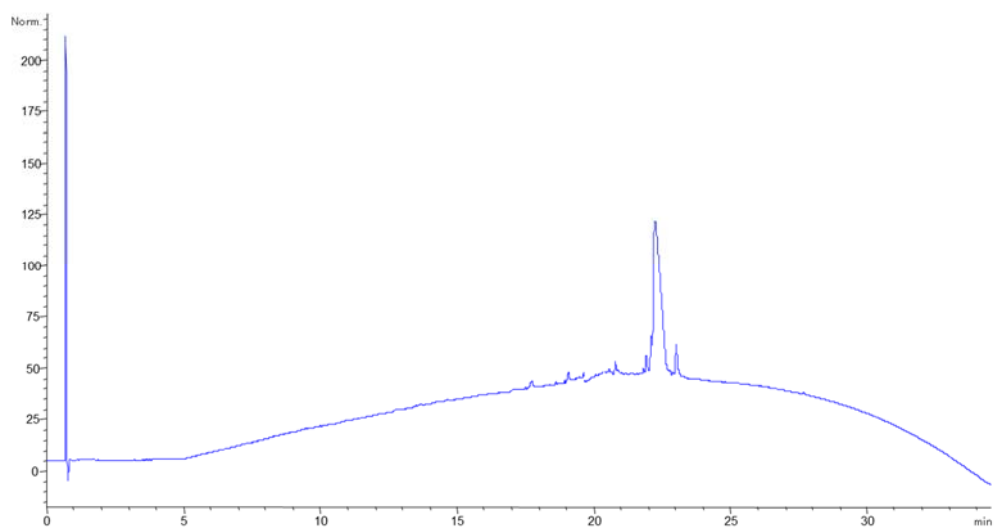
It is known that, in addition to upregulation of co-stimulatory molecules, many proinflammatory cytokines are induced when APCs are stimulated by TLR ligands [454]. The data from

this study showed that incubation with Pam₂KK₄CG functionalised particles resulted in significantly higher IL-1 β , IL-6, IL-10, IL-12 and TNF- α secretion than CaP-OVA NPs. All of these cytokines are known to contribute to effective and potent T cell responses: interleukin 1 β (IL-1 β) has a pivotal role in the activation of natural killer cells (NK) for effective priming of cytotoxic T cells (CTLs) [406]; IL-6 also plays an important role in CD4⁺ [407] and CD8⁺ [408] T cell differentiation; the presence of IL-10 in primary responses leads to an increased number of CD8⁺ T cells [409]; IL-12 is an important bridge between innate and adaptive immunity by stimulating CD8⁺ and CD4⁺ T cell activation but promoting a more extended conjugation events between APCs and T cells [410]; TNF- α has been shown to play a pivotal role in the proliferation, activation and differentiation of macrophages [411]. The induction of cytokines by Pam₂KK₄CG functionalised NPs is consistent with a report by Chen *et al.*, which showed that the TLR2 ligand Pam₃CSK₄ significantly enhances TNF- α , IL-1 β , IL-10, and IL-6 expression in neutrophils [417]. It has also been observed that Pam₂CSK₄ lipopeptides can induce significant IL-10 secretion in DCs [455]. A similar study showed that TLR2-primed DCs produced more IL-10 cytokines when compared with naïve DCs [456]. Heuking and his colleagues demonstrated that Pam₃CSK₄ functionalised DNA nanoparticles significantly enhanced IL-8 and TNF- α secretion in the human bronchial epithelium [457]. These studies have demonstrated that Pam₂CSK₄ can influence the regulation of cytokines and leads to increased levels of proinflammatory cytokines. Based on the cytokine production induced by Pam₂KK₄CG functionalised NPs, it is likely that delivery of vaccine antigens *in vivo* would recruit DCs, NK cells and macrophages to the site of injection, and induce DCs and macrophage migration to the draining lymph nodes where they could potentially stimulate an effective CD4⁺ and CD8⁺ T cell response against the delivered antigen.

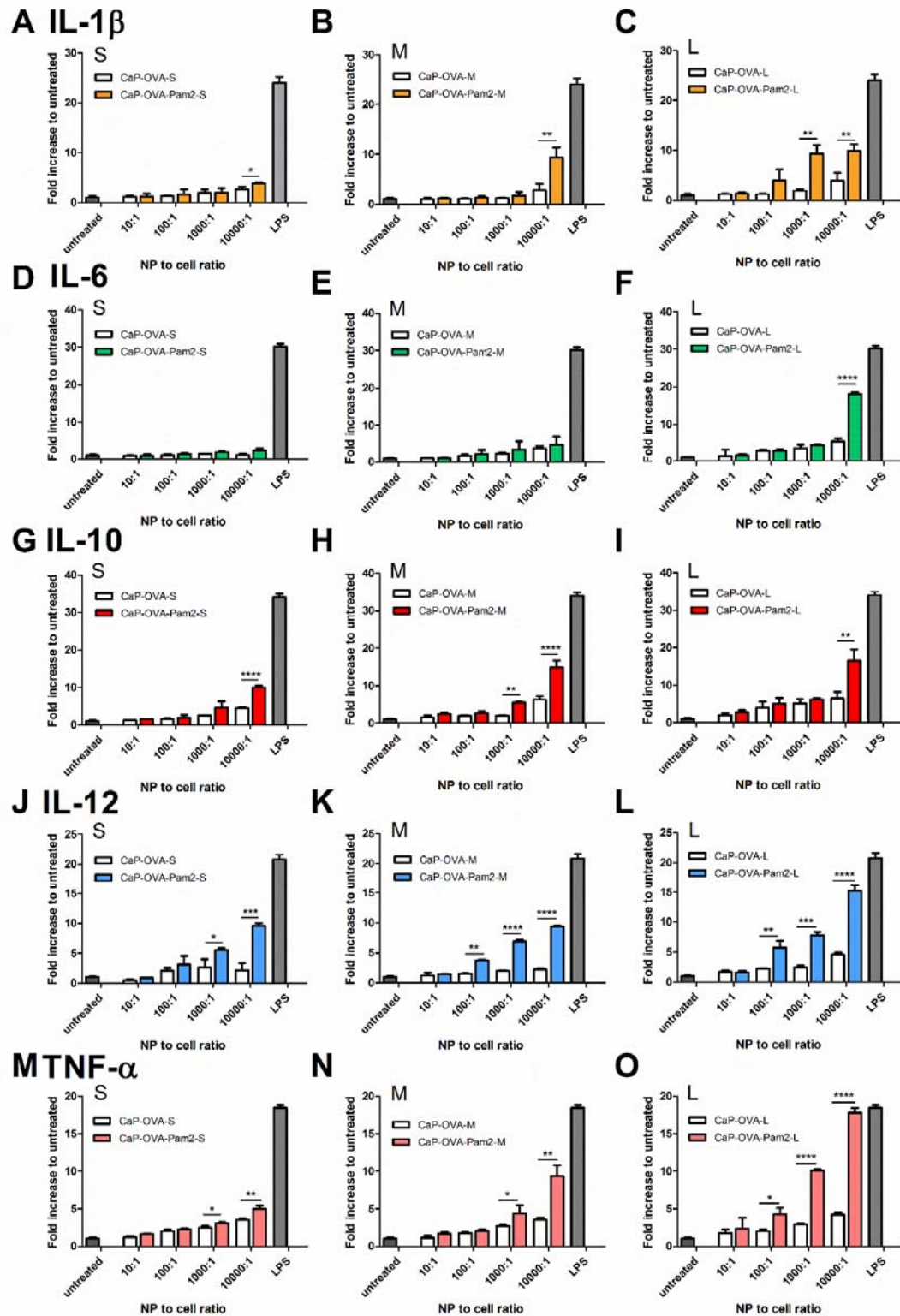
5.5 Conclusion

This study has demonstrated that palmitoyl functionalised NPs induce a stronger cytokine response, induction of NF- κ B activation and are more readily phagocytosed than non-functionalised NPs. The palmitoyl functionalised NPs were demonstrated to be biocompatible and non-toxic to macrophages *in vitro*. As a functionalising ligand Pam2 was found to have better yield and conjugation chemistry than Pam3, which is more suitable for NP production. Taken together, this chapter shows that functionalisation of NP vaccines with Pam₂KK₄CG peptide may be highly effective in the induction of an immune response in vaccines applications.

5.6 Supplementary Information



Supplementary Figure 5. 1: HPLC profile of Pam₂KK₄CG (dipalmitoyl-KK₄CG).



Supplementary Figure 5. 2: Pam₂KK₄CG functionalised nanoparticles induce cytokine release in RAW 264.7 cells.

Cytokine production in RAW 264.7 cells was measured following 6 h incubation of three sizes of TLR2 ligand functionalised nanoparticles at various NP to cell ratios (10:1, 100:1, 1,000:1 and 10,000:1). IL-1 β (A, B, C), IL-6 (D, E, F), IL-10 (G, H, I), IL-12 (J, K, L), and TNF- α (M, N, O) were detected by ELISA assays. Data are expressed as mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001 were considered statistically significant.

Chapter 6

General Discussion

In the last two decades the use of nanoparticle-based delivery of antigens has emerged as a very promising strategy for vaccine development [25, 32]. Nanoparticles (NPs) are synthesised as solid cores with a diameter range from 1 to 1,000 nm, which have shown significant adjuvant effects as antigen carriers [366]. Among these different types of nanoparticles calcium phosphate nanoparticles (CaP NPs) have attracted increasing attention and have been investigated in a number of biomedical applications [158, 159, 176, 289]. Despite the advances of CaP NPs in the field of antigen delivery of vaccines, they currently have several critical problems which prevent their clinical usage [267]. The rapid growth (increasing size) during post-preparation is still the major issue of CaP NPs preparation. Unstabilised CaP NPs have a tendency to aggregate and form large particles [458]. Additionally, although the interaction of spherical CaP NPs and cells has been extensively investigated [258], little is known about the cellular interaction with rod-shaped CaP NPs. Consequently, in this thesis, a biodegradable CaP NPs vaccine delivery system was developed for novel vaccine design. This was done by developing a reproducible method for the synthesis of various sizes of CaP NPs, including the optimisation of functionalisation of CaP NPs vaccine by using a layer-by-layer synthesis. Additionally, the immunological effects of different sizes of CaP NPs vaccine on oral epithelial cells and macrophages were evaluated, including the effects of TLR2 ligand loaded CaP NPs on the induction of macrophages maturation and activation.

Here, a novel effective and convenient CaP NPs preparation protocol has been established that display great advantages over previously published methods, such as microemulsion [183-186], hydrothermal [187, 188], sonochemical [189], hydrolysis [190] and microwave methods [191]. Unlike other methods of CaP NP synthesis, the protocols described here are innovative, very effective, quick, low cost of production, and, most importantly, is consistent and reproducible. Furthermore, with the synthesis method described here various sizes of particles (50 ~ 600 nm) can be obtained from different starting materials (such as different calcium and phosphate sources) and pH value (drifting and constant of pH 8.5). It was determined that altering the calcium and phosphate sources used to synthesise CaP NPs resulted in CaP NPs of various sizes. To our best knowledge, this investigation is the first to find the influence of different calcium and phosphate salts on NP synthesis. The establishment of the citrate chelation protocol allowed us to accurately synthesise different sizes of nanoparticles. This methodology will significantly contribute to future CaP NPs research and have huge applications for future investigations involving CaP NPs.

After developing the citrate chelation method to synthesise the CaP NPs core, further investigations into the formulations of nanoparticle vaccines were performed. On the basis of the results arising from these experiments an efficient layer-by-layer procedure to synthesise a pH-sensitive protein vaccine using CaP NPs was successfully developed. The effect of several preparation parameters (various amino acids, types and concentrations of PEI, OVA concentration, and cross-linking) on the synthesis of CaP NPs such as size, PDI, and zeta potential were determined. Furthermore, the adsorption of plasma protein onto the surface of the CaP NPs after exposure to human serum was also examined. In this study we found that the major protein corona component identified by mass spectrometry was albumin. This is in accord with a recent investigation where serum albumin was the most protein adsorbed on gold nanoparticles (nanorods and nanostars) *in vivo* [459]. Significantly, the cross-linked vaccine demonstrated enhanced salt stability and reduced the plasma protein adsorption in both culture media and human serum, which facilitated NP application *in vivo* investigations [356]. This effect was due to the tendency of protein binding to change the size and surface charge of NPs, which in turn affects the overall biodistribution of NPs throughout the body [357]. It is clear from these results that the cross-linked CaP NPs can decrease the amount of protein binding, enhancing their stability, and thus that it is suitable for vaccine applications. This supports the finding that cross-linked hydrogel platforms resulted in reduced nonspecific adhesion on serum [460]. The results demonstrate that the vaccine synthesis method is reproducible, and straightforward. This strategy enables the production of a well-defined and homogeneously dispersed nanoparticle vaccine.

To further study the immunogenicity of the nanoparticle vaccine, the effects of different sized CaP NPs vaccine on oral epithelial cells and macrophages was assessed. It was shown that the three sizes of NPs were biocompatible with HEK and OKF6 cells. All sizes of NPs examined were able to efficiently bind to and migrate through epithelial cell layers, and that they all induced cytokine responses from epithelial cells. These data also demonstrated that all sizes of cross-linked CaP-PEI-OVA NPs studied can be phagocytosed by RAW 264.7 cells in a dose-dependent manner. These immunological effects suggest that CaP NPs vaccine are likely to induce strong immune responses. Finally, of the CaP-OVA nanoparticles, CaP-OVA-L NPs induced significantly stronger cell-binding, cellular-uptake, phagocytosis, NF- κ B activation, cytokine secretion, and inflammatory cell surface marker expression at the highest NP to cell ratio than the smaller nanoparticles used in this study. These findings are in agreement with previous studies that have suggested nanoparticles that are less than 500 nm in size are ideal

for phagocytosis by APCs [48, 53]. He *et al.* found that in comparison with 157 nm polymeric nanoparticles, larger 300 nm nanoparticles result in a 1.1-fold increase in uptake by macrophages [54]. A previous study reported that smaller-sized silver nanoparticles (22 nm, 42 nm, and 71 nm) significantly increased TGF- β production in serum than 323 nm NPs [461]. However, in this study 364 nm CaP-OVA NPs induced significantly more IL-6, IL-10 and TNF- α secretion than 261 nm and 174 nm NPs at the highest NP to cell ratio. This may be attributable to the adsorbance properties that larger sized nanoparticles provide, which allows more protein to be absorbed specially due to an increased surface area. Additionally, the increased preference for phagocytosis of larger particles would lead to increased immune responses. These data suggest that CaP NPs could be considered as minimally toxic vehicles for vaccine applications. This study provides a better understanding of how rod-like nanoparticles interact with epithelial cells and macrophages, demonstrating that rod-like CaP NPs have a promising potential as a platform for protein delivery in vaccine therapy. Knowledge of these interactions with immune cells is crucial for synthesis optimal vaccine formulations for protein antigen delivery.

The investigation of the immunological impacts of CaP NPs vaccine indicate that CaP NPs vaccine have the potential to induce macrophage activation. The use of a TLR2 ligand with CaP NPs vaccine to potentially enhance immune responses was also studied. A TLR2 ligand Pam₂KK₄CG lipopeptide was synthesised and was shown to be able to be successfully loaded onto calcium phosphate OVA nanoparticles. The particles were demonstrated to be biocompatible with macrophages *in vitro*. In addition, the particles were phagocytosed by RAW 264.7 cells in a dose-dependent manner. The expression of cell surface markers (CD40, CD80, and MHC II) in RAW 264.7 cells were significantly increased as compared to CaP-OVA NPs alone. Similar to these results, Yamazaki *et al.* demonstrated that the Pam₂CSK₄ lipopeptides significantly increased the expression of CD40 and CD86 on spleen DCs *in vivo* when delivered as free TLRs [455]. Additionally, the combination of TLR2 and TLR7 elicited significant expression of CD80 and CD86 on DCs [129]. Finally, Pam₂KK₄CG lipopeptide treatment enhanced the expression of IL-1 β , IL-10, IL-12 and TNF- α . This is consistent with a report by Chen *et al.*, which showed that the TLR2 ligand Pam₃CSK₄ significantly enhances TNF- α , IL-1 β , IL-10, and IL-6 expression in neutrophils [417]. These studies have demonstrated that TLR2 ligand can influence the regulation of cytokine production and subsequently leads to increased concentrations of proinflammatory cytokines. Based on the cytokine production profile observed after exposure of macrophages to Pam₂KK₄CG functionalised NPs, it is likely that delivery of vaccine antigens *in vivo* would recruit NK cells and T lymphocytes to the site of

injection, and induce macrophages to migrate to the draining lymph nodes where they could potentially stimulate an effective CD4⁺ and CD8⁺ T cell response against the delivered antigen. While further *in vivo* experimentation is needed to confirm this hypothesis, this report presents the findings that the Pam₂KK₄CG peptide is highly effective in the induction of phagocytosis, NF-κB activation, cell marker expression and cytokine production, indicating the Pam₂KK₄CG peptide may be a highly effective adjuvant for use in vaccines applications. This information would help us to understand better the interaction between TLR2 ligand encapsulated particles and macrophages. Future studies could also focus on other antigen presenting cells, such as dendritic cells. Although, the immune stimulatory effects of CaP NPs were demonstrated in a series of *in vitro* cell-based experiments, indicating that they may have efficacy *in vivo*, a limitation in the current study is an animal experiment to confirm adjuvanticity, however, these studies are currently on-going.

The work described in this thesis has led to many possible further research opportunities. Further investigations into the immunological effects of CaP NP vaccine delivery could be performed. Several studies have demonstrated the importance of TLR ligands on immune cells activation [132, 258, 428]. More recently, Zilker *et al.* formulated a Hen Egg Lysozyme (HEL)-conjugated CaP NPs vaccine encapsulated with different TLR ligands (CpG, poly I:C, Flagellin, and R848) to target B cells. Results showed that CaP-HEL-Flagellin nanoparticle immunisations elicited significantly greater mucosal responses than CaP-HEL-PolyI:C and CaP-HEL-R848 nanoparticles, indicating that different TLR ligands can modulate the strength of the humoral immune response [131]. Given that the protein antigen contains various amino groups, which are suitable for conjugation, it would be interesting to load other TLR ligands to the current CaP nanoparticle system and to compare which TLR combinations enhance the CaP NPs vaccine to induce a robust immune response in immune cells.

In order to eradicate cancer cells, it is essential for a nanoparticle cancer vaccine to induce CD4 and CD8 T cells activation during vaccination [462]. A previous study reported that CaP NPs encapsulating both CpG and poly (I:C) ligands and the antigen hemagglutinin were found to elicit strong proliferation of CD4⁺ T cells [258]. Knuschke *et al.* compared CpG and poly (I:C) functionalized NPs with CaP NPs *in vitro*, and observed that CpG loaded with CaP NPs elicited a more robust T cell-mediated immunity [260]. Further research into the impacts of functionalised CaP NPs vaccine conjugated cancer antigen and TLR ligands on T cells on the resultant CTL responses both in *in vitro* and *in vivo* is a promising field. Ongoing studies of CaP NPs

vaccine may result in the development of more robust nanoparticle vaccines that will serve as significant platforms against various types of cancer. Additionally, combination nanoparticle vaccine immunotherapy with checkpoint blockade antibodies has been shown improve the anti-tumour effect [463], and further investigations using the CaP NPs described here in combination therapy could be explored. CaP NPs have been used as a vaccine vehicle in a number of investigations such as foot and mouth disease [255], rabies [464], and herpes [212]. Further studies utilising CaP NPs could be applied other infectious diseases.

In addition to the nanoparticle vaccine system, the systematic scalable CaP NPs fabrication technique presented in this thesis enables this protocol has immediate applicability in a wide number of fields. CaP is a safe, biodegradable material that is naturally present in the body. As such, it is less likely to induce any significant health risks to the patients [465]. Consequently, CaP could be used as a carrier for drug and protein/peptide for therapeutic investigations. Recently, Li *et al.* reported an antimicrobial peptide (AMP), Chex1-Arg20. The results demonstrated that this peptide had antibacterial activity against the Gram-negative bacterium *Klebsiella pneumoniae* [466]. Hence, CaP NPs conjugated Chex1-Arg20 AMP peptides could be a possible way to treat infectious diseases caused by bacteria, which will be a promising follow-up to the investigations reported in this thesis.

Overall, the research presented in this thesis provides insight into the nanoparticle vaccine design, which has not yet been well developed in the literature. As a result, the findings from these investigations have the significant effect of informing and guiding the next generation of safe and effective nanoparticle vaccine design and may have major implications in cancer vaccine development.

References

1. Purcell, A.W., J. McCluskey, and J. Rossjohn, *More than one reason to rethink the use of peptides in vaccine design*. Nat Rev Drug Discov, 2007. **6**(5): p. 404-14.
2. Rosenberg, S.A., J.C. Yang, and N.P. Restifo, *Cancer immunotherapy: moving beyond current vaccines*. Nature Medicine, 2004. **10**(9): p. 909-915.
3. Kaech, S.M., E.J. Wherry, and R. Ahmed, *VACCINES: EFFECTOR AND MEMORY T-CELL DIFFERENTIATION: IMPLICATIONS FOR VACCINE DEVELOPMENT*. Nature Reviews Immunology, 2002. **2**(4): p. 251.
4. Riedel, S., *Edward Jenner and the history of smallpox and vaccination*. Proc (Bayl Univ Med Cent), 2005. **18**(1): p. 21-5.
5. Zhu, G., et al., *Efficient Nanovaccine Delivery in Cancer Immunotherapy*. ACS Nano, 2017. **11**(3): p. 2387-2392.
6. Wierzbicka, M., et al., *HPV vaccination in head and neck HPV-related pathologies*. Otolaryngol Pol, 2014. **68**(4): p. 157-73.
7. Emens, L.A., *Cancer vaccines: on the threshold of success*. Expert Opin Emerg Drugs, 2008. **13**(2): p. 295-308.
8. Huang, P., et al., *Nano-, micro-, and macroscale drug delivery systems for cancer immunotherapy*. Acta Biomater, 2019. **85**: p. 1-26.
9. Krishnamachari, Y., et al., *Nanoparticle Delivery Systems in Cancer Vaccines*. Pharmaceutical Research, 2011. **28**(2): p. 215-236.
10. Whang, S.N., M. Filippova, and P. Duerksen-Hughes, *Recent Progress in Therapeutic Treatments and Screening Strategies for the Prevention and Treatment of HPV-Associated Head and Neck Cancer*. Viruses, 2015. **7**(9): p. 5040-65.
11. Cheever, M.A. and C.S. Higano, *PROVENGE (Sipuleucel-T) in prostate cancer: the first FDA-approved therapeutic cancer vaccine*. Clin Cancer Res, 2011. **17**(11): p. 3520-6.
12. Mougel, A., M. Terme, and C. Tanchot, *Therapeutic Cancer Vaccine and Combinations With Antiangiogenic Therapies and Immune Checkpoint Blockade*. Frontiers in Immunology, 2019. **10**(467).
13. Lopes, A., G. Vandermeulen, and V. Pr  at, *Cancer DNA vaccines: current preclinical and clinical developments and future perspectives*. Journal of Experimental & Clinical Cancer Research, 2019. **38**(1): p. 146.
14. Thomas, S. and G.C. Prendergast, *Cancer Vaccines: A Brief Overview*. Methods Mol Biol, 2016. **1403**: p. 755-61.
15. Maeng, H.M. and J.A. Berzofsky, *Strategies for developing and optimizing cancer vaccines*. F1000Res, 2019. **8**.
16. Hollingsworth, R.E. and K. Jansen, *Turning the corner on therapeutic cancer vaccines*. NPJ Vaccines, 2019. **4**: p. 7.
17. Skwarczynski, M. and I. Toth, *Peptide-based subunit nanovaccines*. Curr Drug Deliv, 2011. **8**(3): p. 282-9.
18. Lee, N.H., et al., *A review of vaccine development and research for industry animals in Korea*. Clin Exp Vaccine Res, 2012. **1**(1): p. 18-34.
19. Skwarczynski, M. and I. Toth, *Peptide-based synthetic vaccines*. Chemical Science, 2016. **7**(2): p. 842-854.
20. Skwarczynski, M. and I. Toth, *Recent advances in peptide-based subunit nanovaccines*. Nanomedicine (Lond), 2014. **9**(17): p. 2657-69.

21. Fu, A., et al., *Promises and pitfalls of intracellular delivery of proteins*. Bioconjug Chem, 2014. **25**(9): p. 1602-8.
22. Sokolova, V., et al., *Calcium phosphate nanoparticles as versatile carrier for small and large molecules across cell membranes*. Journal of Nanoparticle Research, 2012. **14**(6): p. 1-10.
23. Marasini, N., M. Skwarczynski, and I. Toth, *Oral delivery of nanoparticle-based vaccines*. Expert Rev Vaccines, 2014. **13**(11): p. 1361-76.
24. Sun, B. and T. Xia, *Nanomaterial-based vaccine adjuvants*. Journal of Materials Chemistry B, 2016. **4**(33): p. 5496-5509.
25. Gregory, A.E., R. Titball, and D. Williamson, *Vaccine delivery using nanoparticles*. Frontiers In Cellular And Infection Microbiology, 2013. **3**: p. 13-13.
26. Peek, L.J., C.R. Middaugh, and C. Berkland, *Nanotechnology in vaccine delivery*. Advanced Drug Delivery Reviews, 2008. **60**(8): p. 915-928.
27. Hamburg, M., *Science and regulation. FDA's approach to regulation of products of nanotechnology*. Science, 2012. **336**(6079): p. 299-300.
28. Bolhassani, A., et al., *Polymeric nanoparticles: potent vectors for vaccine delivery targeting cancer and infectious diseases*. Hum Vaccin Immunother, 2014. **10**(2): p. 321-32.
29. Sheen, M.R., et al., *Stimulating antitumor immunity with nanoparticles*. Wiley Interdiscip Rev Nanomed Nanobiotechnol, 2014. **6**(5): p. 496-505.
30. Kozlova, D., et al., *Cell targeting by antibody-functionalized calcium phosphate nanoparticles*. Journal of Materials Chemistry, 2012. **22**(2): p. 396-404.
31. Rotan, O., et al., *Uptake of the proteins HTRA1 and HTRA2 by cells mediated by calcium phosphate nanoparticles*. Beilstein J Nanotechnol, 2017. **8**: p. 381-393.
32. Peek, L.J., C.R. Middaugh, and C. Berkland, *Nanotechnology in vaccine delivery*. Adv Drug Deliv Rev, 2008. **60**(8): p. 915-28.
33. Zhao, L., et al., *Nanoparticle vaccines*. Vaccine, 2014. **32**(3): p. 327-37.
34. Park, Y.M., et al., *Nanoparticle-based vaccine delivery for cancer immunotherapy*. Immune Netw, 2013. **13**(5): p. 177-83.
35. Smith, D.M., J.K. Simon, and J.R. Baker, Jr., *Applications of nanotechnology for immunology*. Nat Rev Immunol, 2013. **13**(8): p. 592-605.
36. Irvine, D.J., et al., *Synthetic Nanoparticles for Vaccines and Immunotherapy*. Chem Rev, 2015. **115**(19): p. 11109-46.
37. Zhu, M., R. Wang, and G. Nie, *Applications of nanomaterials as vaccine adjuvants*. Hum Vaccin Immunother, 2014. **10**(9): p. 2761-74.
38. Torres-Sangiao, E., A.M. Holban, and M.C. Gestal, *Advanced Nanobiomaterials: Vaccines, Diagnosis and Treatment of Infectious Diseases*. Molecules, 2016. **21**(7).
39. Gutjahr, A., et al., *Biodegradable Polymeric Nanoparticles-Based Vaccine Adjuvants for Lymph Nodes Targeting*. Vaccines (Basel), 2016. **4**(4).
40. Yue, H. and G. Ma, *Polymeric micro/nanoparticles: Particle design and potential vaccine delivery applications*. Vaccine, 2015. **33**(44): p. 5927-5936.
41. Wallis, J., D.P. Shenton, and R.C. Carlisle, *Novel approaches for the design, delivery and administration of vaccine technologies*. Clinical & Experimental Immunology, 2019. **196**(2): p. 189-204.
42. Ali, O.A., et al., *Vaccines Combined with Immune Checkpoint Antibodies Promote Cytotoxic T-cell Activity and Tumor Eradication*. Cancer Immunology Research, 2016. **4**(2): p. 95-100.
43. Walters, A.A., et al., *Next generation vaccines: single-dose encapsulated vaccines for improved global immunisation coverage and efficacy*. Journal of Pharmacy and Pharmacology, 2015. **67**(3): p. 400-408.

44. Vartak, A. and S. Sucheck, *Recent Advances in Subunit Vaccine Carriers*. Vaccines, 2016. **4**(2): p. 12.
45. Rahimian, S., et al., *Polymeric nanoparticles for co-delivery of synthetic long peptide antigen and poly IC as therapeutic cancer vaccine formulation*. Journal of Controlled Release, 2015. **203**: p. 16-22.
46. Li, D., et al., *Advances and Potential Applications of Chitosan Nanoparticles as a Delivery Carrier for the Mucosal Immunity of Vaccine*. Curr Drug Deliv, 2017. **14**(1): p. 27-35.
47. Pavot, V., et al., *Poly(lactic acid) and poly(lactic-co-glycolic acid) particles as versatile carrier platforms for vaccine delivery*. Nanomedicine (Lond), 2014. **9**(17): p. 2703-18.
48. Chen, Q., et al., *Photothermal therapy with immune-adjuvant nanoparticles together with checkpoint blockade for effective cancer immunotherapy*. Nat Commun, 2016. **7**: p. 13193.
49. Varypataki, E.M., et al., *Synthetic long peptide-based vaccine formulations for induction of cell mediated immunity: A comparative study of cationic liposomes and PLGA nanoparticles*. Journal of Controlled Release, 2016. **226**: p. 98-106.
50. Zuo, J., et al., *PLGA-Der pI Vaccine Inhibited Tumor Growth in a Murine Model of Lung Cancer*. Arch Med Res, 2015.
51. Bruno, C., et al., *The preparation and characterization of PLG nanoparticles with an entrapped synthetic TLR7 agonist and their preclinical evaluation as adjuvant for an adsorbed DTaP vaccine*. European Journal of Pharmaceutics and Biopharmaceutics, 2016. **105**: p. 1-8.
52. Silva, J.M., et al., *In vivo delivery of peptides and Toll-like receptor ligands by mannose-functionalized polymeric nanoparticles induces prophylactic and therapeutic anti-tumor immune responses in a melanoma model*. J Control Release, 2015. **198**: p. 91-103.
53. Hu, Y., et al., *Engineering the lipid layer of lipid-PLGA hybrid nanoparticles for enhanced in vitro cellular uptake and improved stability*. Acta Biomaterialia, 2015. **28**: p. 149-159.
54. Pavot, V., et al., *Encapsulation of Nod1 and Nod2 receptor ligands into poly(lactic acid) nanoparticles potentiates their immune properties*. Journal of Controlled Release, 2013. **167**(1): p. 60-67.
55. Chen, X., et al., *Enhanced humoral and cell-mediated immune responses generated by cationic polymer-coated PLA microspheres with adsorbed HBsAg*. Mol Pharm, 2014. **11**(6): p. 1772-84.
56. Kool, M., et al., *Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells*. J Exp Med, 2008. **205**(4): p. 869-82.
57. Chen, X., et al., *Polycation-decorated PLA microspheres induce robust immune responses via commonly used parenteral administration routes*. International Immunopharmacology, 2014. **23**(2): p. 592-602.
58. Lee, B.K., Y. Yun, and K. Park, *PLA micro- and nano-particles*. Adv Drug Deliv Rev, 2016.
59. Gan, Q. and T. Wang, *Chitosan nanoparticle as protein delivery carrier--systematic examination of fabrication conditions for efficient loading and release*. Colloids Surf B Biointerfaces, 2007. **59**(1): p. 24-34.
60. Vasiliev, Y.M., *Chitosan-based vaccine adjuvants: incomplete characterization complicates preclinical and clinical evaluation*. Expert Rev Vaccines, 2015. **14**(1): p. 37-53.

61. Zhao, K., et al., *Biological evaluation of N-2-hydroxypropyl trimethyl ammonium chloride chitosan as a carrier for the delivery of live Newcastle disease vaccine*. Carbohydrate Polymers, 2016. **149**: p. 28-39.
62. Andersen, T., et al., *Pectosomes and Chitosomes as Delivery Systems for Metronidazole: The One-Pot Preparation Method*. Pharmaceutics, 2013. **5**(3): p. 445-456.
63. Das, S. and A. Chaudhury, *Recent advances in lipid nanoparticle formulations with solid matrix for oral drug delivery*. AAPS PharmSciTech, 2011. **12**(1): p. 62-76.
64. Almeida, A.J. and E. Souto, *Solid lipid nanoparticles as a drug delivery system for peptides and proteins*. Advanced Drug Delivery Reviews, 2007. **59**(6): p. 478-490.
65. Reichmuth, A.M., et al., *mRNA vaccine delivery using lipid nanoparticles*. Ther Deliv, 2016. **7**(5): p. 319-34.
66. De Serrano, L.O. and D.J. Burkhart, *Liposomal vaccine formulations as prophylactic agents: design considerations for modern vaccines*. J Nanobiotechnology, 2017. **15**(1): p. 83.
67. Swaminathan, G., et al., *A novel lipid nanoparticle adjuvant significantly enhances B cell and T cell responses to sub-unit vaccine antigens*. Vaccine, 2016. **34**(1): p. 110-9.
68. Wang, Z., et al., *A specific peptide ligand-modified lipid nanoparticle carrier for the inhibition of tumor metastasis growth*. Biomaterials, 2013. **34**(3): p. 756-64.
69. Hu, Y., et al., *Engineering the lipid layer of lipid-PLGA hybrid nanoparticles for enhanced in vitro cellular uptake and improved stability*. Acta Biomater, 2015. **28**: p. 149-59.
70. Schwendener, R.A., *Liposomes as vaccine delivery systems: a review of the recent advances*. Ther Adv Vaccines, 2014. **2**(6): p. 159-82.
71. Smith, J.D., L.D. Morton, and B.D. Ulery, *Nanoparticles as synthetic vaccines*. Current Opinion in Biotechnology, 2015. **34**: p. 217-224.
72. Cao-Milan, R. and L.M. Liz-Marzan, *Gold nanoparticle conjugates: recent advances toward clinical applications*. Expert Opin Drug Deliv, 2014. **11**(5): p. 741-52.
73. Almeida, J.P., et al., *In vivo gold nanoparticle delivery of peptide vaccine induces anti-tumor immune response in prophylactic and therapeutic tumor models*. Small, 2015. **11**(12): p. 1453-9.
74. Niikura, K., et al., *Gold nanoparticles as a vaccine platform: influence of size and shape on immunological responses in vitro and in vivo*. ACS Nano, 2013. **7**(5): p. 3926-38.
75. Almeida, J.P., E.R. Figueroa, and R.A. Drezek, *Gold nanoparticle mediated cancer immunotherapy*. Nanomedicine, 2014. **10**(3): p. 503-14.
76. Mody, K.T., et al., *Mesoporous silica nanoparticles as antigen carriers and adjuvants for vaccine delivery*. Nanoscale, 2013. **5**(12): p. 5167-79.
77. Malachin, G., et al., *Combined Action of Human Commensal Bacteria and Amorphous Silica Nanoparticles on the Viability and Immune Responses of Dendritic Cells*. Clin Vaccine Immunol, 2017. **24**(10).
78. Cha, B.G., J.H. Jeong, and J. Kim, *Extra-Large Pore Mesoporous Silica Nanoparticles Enabling Co-Delivery of High Amounts of Protein Antigen and Toll-like Receptor 9 Agonist for Enhanced Cancer Vaccine Efficacy*. ACS Central Science, 2018. **4**(4): p. 484-492.
79. Lu, Y., et al., *Glutathione-depletion mesoporous organosilica nanoparticles as a self-adjuvant and Co-delivery platform for enhanced cancer immunotherapy*. Biomaterials, 2018. **175**: p. 82-92.
80. Yang, Y., et al., *Multi-shelled Dendritic Mesoporous Organosilica Hollow Spheres: Roles of Composition and Architecture in Cancer Immunotherapy*. Angewandte Chemie International Edition, 2017. **56**(29): p. 8446-8450.

81. Toda, T. and S. Yoshino, *Enhancement of ovalbumin-specific Th1, Th2, and Th17 immune responses by amorphous silica nanoparticles*. Int J Immunopathol Pharmacol, 2016.
82. Wang, T., et al., *Enhanced mucosal and systemic immune responses obtained by porous silica nanoparticles used as an oral vaccine adjuvant: Effect of silica architecture on immunological properties*. International Journal of Pharmaceutics, 2012. **436**(1–2): p. 351-358.
83. Pati, R., M. Shevtsov, and A. Sonawane, *Nanoparticle Vaccines Against Infectious Diseases*. Front Immunol, 2018. **9**: p. 2224.
84. Salem, A.K., *Nanoparticles in vaccine delivery*. Aaps j, 2015. **17**(2): p. 289-91.
85. Wen, R., et al., *Nanoparticle systems for cancer vaccine*. Nanomedicine (Lond), 2019. **14**(5): p. 627-648.
86. Mahapatro, A. and D.K. Singh, *Biodegradable nanoparticles are excellent vehicle for site directed in-vivo delivery of drugs and vaccines*. J Nanobiotechnology, 2011. **9**: p. 55.
87. Zhan, Z., et al., *Improved Gene Transfer with Functionalized Hollow Mesoporous Silica Nanoparticles of Reduced Cytotoxicity*. Materials (Basel), 2017. **10**(7).
88. Yamauchi, T. and T. Moroishi, *Hippo Pathway in Mammalian Adaptive Immune System*. Cells (2073-4409), 2019. **8**(5): p. 398.
89. Oliveira, C., et al., *Recent advances in characterization of nonviral vectors for delivery of nucleic acids: impact on their biological performance*. Expert Opin Drug Deliv, 2015. **12**(1): p. 27-39.
90. Medzhitov, R. and C. Janeway, *Innate Immunity*. New England Journal of Medicine, 2000. **343**(5): p. 338-344.
91. Janeway, C.A. and R. Medzhitov, *INNATE IMMUNE RECOGNITION*. Annual Review of Immunology, 2002. **20**(1): p. 197.
92. Dacoba, T.G., et al., *Modulating the immune system through nanotechnology*. Semin Immunol, 2017. **34**: p. 78-102.
93. Cronkite, D.A. and T.M. Strutt, *The Regulation of Inflammation by Innate and Adaptive Lymphocytes*. J Immunol Res, 2018. **2018**: p. 1467538.
94. Hoebe, K., E. Janssen, and B. Beutler, *The interface between innate and adaptive immunity*. Nature Immunology, 2004. **5**(10): p. 971-974.
95. Kumar, H., T. Kawai, and S. Akira, *Toll-like receptors and innate immunity*. Biochemical and Biophysical Research Communications, 2009. **388**(4): p. 621-625.
96. Iwasaki, A. and R. Medzhitov, *Regulation of adaptive immunity by the innate immune system*. Science, 2010. **327**(5963): p. 291-5.
97. Akira, S., K. Takeda, and T. Kaisho, *Toll-like receptors: critical proteins linking innate and acquired immunity*. Nat Immunol, 2001. **2**(8): p. 675-80.
98. Moresco, E.M.Y., D. LaVine, and B. Beutler, *Toll-like receptors*. Current Biology, 2011. **21**(13): p. R488-R493.
99. Bonilla, F.A. and H.C. Oettgen, *Adaptive immunity*. J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S33-40.
100. Sahdev, P., L.J. Ochyl, and J.J. Moon, *Biomaterials for nanoparticle vaccine delivery systems*. Pharm Res, 2014. **31**(10): p. 2563-82.
101. Merlo, L.M.F. and L. Mandik-Nayak, *Chapter 3 - Adaptive Immunity: B Cells and Antibodies*, in *Cancer Immunotherapy (Second Edition)*, G.C. Prendergast and E.M. Jaffee, Editors. 2013, Academic Press: San Diego. p. 25-40.
102. Navarro-Tovar, G., G. Palestino, and S. Rosales-Mendoza, *An overview on the role of silica-based materials in vaccine development*. Expert Rev Vaccines, 2016: p. 1-14.

103. Luckheeram, R.V., et al., *CD4+T Cells: Differentiation and Functions*. Clinical & Developmental Immunology, 2012: p. 1-12.
104. Fritz, J.H., et al., *Innate immune recognition at the epithelial barrier drives adaptive immunity: APCs take the back seat*. Trends Immunol, 2008. **29**(1): p. 41-9.
105. Yoo, S. and S.J. Ha, *Generation of Tolerogenic Dendritic Cells and Their Therapeutic Applications*. Immune Netw, 2016. **16**(1): p. 52-60.
106. Liu, M., et al., *Role of Ets Proteins in Development, Differentiation, and Function of T-Cell Subsets*. Med Res Rev, 2016. **36**(2): p. 193-220.
107. Capece, T. and M. Kim, *The Role of Lymphatic Niches in T Cell Differentiation*. Mol Cells, 2016. **39**(7): p. 515-23.
108. Weninger, W., N. Manjunath, and U.H. von Andrian, *Migration and differentiation of CD8+ T cells*. Immunol Rev, 2002. **186**: p. 221-33.
109. Kurd, N. and E.A. Robey, *T-cell selection in the thymus: a spatial and temporal perspective*. Immunol Rev, 2016. **271**(1): p. 114-26.
110. Butterfield, L.H., *Cancer vaccines*. Bmj, 2015. **350**: p. h988.
111. Zhang, N. and M.J. Bevan, *CD8(+) T cells: foot soldiers of the immune system*. Immunity, 2011. **35**(2): p. 161-8.
112. Vartak, A. and S.J. Suchek, *Recent Advances in Subunit Vaccine Carriers*. Vaccines (Basel), 2016. **4**(2).
113. Cruz, F.M., et al., *The Biology and Underlying Mechanisms of Cross-Presentation of Exogenous Antigens on MHC-I Molecules*. Annu Rev Immunol, 2017. **35**: p. 149-176.
114. Williams, M.A. and M.J. Bevan, *Effector and Memory CTL Differentiation*. Annual Review of Immunology, 2007. **25**(1): p. 171-192.
115. Jung, S., et al., *In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens*. Immunity, 2002. **17**(2): p. 211-20.
116. De Koker, S., et al., *Designing polymeric particles for antigen delivery*. Chemical Society Reviews, 2011. **40**(1): p. 320-339.
117. Wang, J.C. and A.M. Livingstone, *Cutting edge: CD4+ T cell help can be essential for primary CD8+ T cell responses in vivo*. J Immunol, 2003. **171**(12): p. 6339-43.
118. Wong, S.B., R. Bos, and L.A. Sherman, *Tumor-specific CD4+ T cells render the tumor environment permissive for infiltration by low-avidity CD8+ T cells*. J Immunol, 2008. **180**(5): p. 3122-31.
119. Kajihara, M., et al., *Advances in inducing adaptive immunity using cell-based cancer vaccines: Clinical applications in pancreatic cancer*. World J Gastroenterol, 2016. **22**(18): p. 4446-58.
120. Martinez-Lostao, L., A. Anel, and J. Pardo, *How Do Cytotoxic Lymphocytes Kill Cancer Cells?* Clin Cancer Res, 2015. **21**(22): p. 5047-56.
121. O'Connell, J., et al., *The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand*. J Exp Med, 1996. **184**(3): p. 1075-82.
122. Malyala, P., D.T. O'Hagan, and M. Singh, *Enhancing the therapeutic efficacy of CpG oligonucleotides using biodegradable microparticles*. Adv Drug Deliv Rev, 2009. **61**(3): p. 218-25.
123. Mohan, T., P. Verma, and D.N. Rao, *Novel adjuvants & delivery vehicles for vaccines development: a road ahead*. Indian J Med Res, 2013. **138**(5): p. 779-95.
124. Egli, A., et al., *Vaccine adjuvants--understanding molecular mechanisms to improve vaccines*. Swiss Med Wkly, 2014. **144**: p. w13940.
125. Garcia, A. and J.B. De Sanctis, *An overview of adjuvant formulations and delivery systems*. Apmis, 2014. **122**(4): p. 257-67.

126. Kano, Y., et al., *Combined adjuvants of poly(I:C) plus LAG-3-Ig improve antitumor effects of tumor-specific T cells, preventing their exhaustion*. *Cancer Sci*, 2016. **107**(4): p. 398-406.
127. Del Giudice, G., R. Rappuoli, and A.M. Didierlaurent, *Correlates of adjuvanticity: A review on adjuvants in licensed vaccines*. *Seminars in Immunology*, 2018. **39**: p. 14-21.
128. Sharma, S., et al., *An insight into functionalized calcium based inorganic nanomaterials in biomedicine: Trends and transitions*. *Colloids and Surfaces B: Biointerfaces*, 2015. **133**: p. 120-139.
129. Gutjahr, A., et al., *Cutting Edge: A Dual TLR2 and TLR7 Ligand Induces Highly Potent Humoral and Cell-Mediated Immune Responses*. *J Immunol*, 2017. **198**(11): p. 4205-4209.
130. Hansen, J., *Adjuvants for Vaccines Against Cancer: An Investigation of CAF-based Adjuvants Incorporating TLR Ligands for CTL-induction Against Cancer : PhD Thesis*. 2011: Faculty of Science, University of Copenhagen.
131. Zilker, C., et al., *Nanoparticle-based B-cell targeting vaccines: Tailoring of humoral immune responses by functionalization with different TLR-ligands*. *Nanomedicine*, 2016.
132. Sokolova, V., et al., *Delivery of the TLR ligand poly(I:C) to liver cells in vitro and in vivo by calcium phosphate nanoparticles leads to a pronounced immunostimulation*. *Acta Biomaterialia*, 2017. **64**: p. 401-410.
133. Kasturi, S.P., et al., *Adjuvanting a Simian Immunodeficiency Virus Vaccine with Toll-Like Receptor Ligands Encapsulated in Nanoparticles Induces Persistent Antibody Responses and Enhanced Protection in TRIM5alpha Restrictive Macaques*. *J Virol*, 2017. **91**(4).
134. Halliday, A., et al., *The TLR2/6 ligand PAM2CSK4 is a Th2 polarizing adjuvant in Leishmania major and Brugia malayi murine vaccine models*. *Parasit Vectors*, 2016. **9**: p. 96.
135. Temizoz, B., E. Kuroda, and K.J. Ishii, *Vaccine adjuvants as potential cancer immunotherapeutics*. *Int Immunol*, 2016. **28**(7): p. 329-38.
136. Blander, J.M. and R. Medzhitov, *Toll-dependent selection of microbial antigens for presentation by dendritic cells*. *Nature*, 2006. **440**(7085): p. 808-12.
137. Duthie, M.S., et al., *Use of defined TLR ligands as adjuvants within human vaccines*. *Immunol Rev*, 2011. **239**(1): p. 178-96.
138. Coffman, R.L., A. Sher, and R.A. Seder, *Vaccine Adjuvants: Putting Innate Immunity to Work*. *Immunity*, 2010. **33**(4): p. 492-503.
139. Kawai, T. and S. Akira, *Signaling to NF-kappaB by Toll-like receptors*. *Trends Mol Med*, 2007. **13**(11): p. 460-9.
140. Kaur, A., et al., *An efficient and scalable synthesis of potent TLR2 agonistic PAM2CSK4*. *RSC Advances*, 2018. **8**(18): p. 9587-9596.
141. Dewitte, H., et al., *Nanoparticle design to induce tumor immunity and challenge the suppressive tumor microenvironment*. *Nano Today*, 2014. **9**(6): p. 743-758.
142. Watts, C., M.A. West, and R. Zaru, *TLR signalling regulated antigen presentation in dendritic cells*. *Current Opinion in Immunology*, 2010. **22**(1): p. 124-130.
143. Johnson, S., et al., *Selected Toll-like receptor ligands and viruses promote helper-independent cytotoxic T cell priming by upregulating CD40L on dendritic cells*. *Immunity*, 2009. **30**(2): p. 218-27.
144. Kumar, M., et al., *Oral cancer: Etiology and risk factors: A review*. *Journal of Cancer Research & Therapeutics*, 2016. **12**(2): p. 458-463.

145. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2016*. CA: A Cancer Journal For Clinicians, 2016. **66**(1): p. 7-30.
146. Rivera, C., *Essentials of oral cancer*. Int J Clin Exp Pathol, 2015. **8**(9): p. 11884-94.
147. Domingos-Pereira, S., et al., *Carboplatin/paclitaxel, E7-vaccination and intravaginal CpG as tri-therapy towards efficient regression of genital HPV16 tumors*. J Immunother Cancer, 2019. **7**(1): p. 122.
148. Mora Roman, J.J., et al., *Immunotherapeutic Potential of Mollusk Hemocyanins in Combination with Human Vaccine Adjuvants in Murine Models of Oral Cancer*. J Immunol Res, 2019. **2019**: p. 7076942.
149. Miyazaki, A., et al., *Phase I clinical trial of survivin-derived peptide vaccine therapy for patients with advanced or recurrent oral cancer*. Cancer Sci, 2011. **102**(2): p. 324-9.
150. Yete, S., W. D'Souza, and D. Saranath, *High-Risk Human Papillomavirus in Oral Cancer: Clinical Implications*. Oncology, 2018. **94**(3): p. 133-141.
151. Sritippoph, T., P. Chotjumlong, and A. Iamaroon, *Roles of Human Papillomaviruses and p16 in Oral Cancer*. Asian Pac J Cancer Prev, 2015. **16**(15): p. 6193-200.
152. Yang, M.C., et al., *Buccal injection of synthetic HPV long peptide vaccine induces local and systemic antigen-specific CD8⁺ T-cell immune responses and antitumor effects without adjuvant*. Cell Biosci, 2016. **6**: p. 17.
153. Ye, D., et al., *Inhibitory Effect of the HPV-16mE6Delta/mE7/TBhsp70Delta Vaccine on Oral Squamous Cell Carcinoma*. The American Journal of the Medical Sciences, 2013. **345**(5): p. 380-384.
154. Lo Muzio, L., et al., *Survivin expression in oral squamous cell carcinoma*. British Journal of Cancer, 2003. **89**(12): p. 2244-2248.
155. Altieri, D.C., *Validating survivin as a cancer therapeutic target*. Nat Rev Cancer, 2003. **3**(1): p. 46-54.
156. Daigo, K., et al., *Characterization of KIF11 as a novel prognostic biomarker and therapeutic target for oral cancer*. Int J Oncol, 2018. **52**(1): p. 155-165.
157. Lim, K.P., et al., *Identification of immunogenic MAGED4B peptides for vaccine development in oral cancer immunotherapy*. Hum Vaccin Immunother, 2014. **10**(11): p. 3214-23.
158. Habraken, W., et al., *Calcium phosphates in biomedical applications: materials for the future?* Materials Today, 2016. **19**(2): p. 69-87.
159. Lin, Y., et al., *Calcium phosphate nanoparticles as a new generation vaccine adjuvant*. Expert Rev Vaccines, 2017. **16**(9): p. 895-906.
160. Lee, M.S., et al., *Target-specific delivery of siRNA by stabilized calcium phosphate nanoparticles using dopa-hyaluronic acid conjugate*. Journal of Controlled Release, 2014. **192**: p. 122-130.
161. Tang, J., et al., *Preparation of optimized lipid-coated calcium phosphate nanoparticles for enhanced in vitro gene delivery to breast cancer cells*. Journal of Materials Chemistry B, 2015. **3**(33): p. 6805-6812.
162. Qi, C., et al., *Highly stable amorphous calcium phosphate porous nanospheres: microwave-assisted rapid synthesis using ATP as phosphorus source and stabilizer, and their application in anticancer drug delivery*. Chemistry, 2013. **19**(3): p. 981-7.
163. Jun, W., et al., *Recent advances of calcium phosphate nanoparticles for controlled drug delivery*. Mini Rev Med Chem, 2013. **13**(10): p. 1501-7.
164. Temchura, V.V., et al., *Targeting and activation of antigen-specific B-cells by calcium phosphate nanoparticles loaded with protein antigen*. Biomaterials, 2014. **35**(23): p. 6098-105.

165. Wu, J., et al., *Development of novel self-healing and antibacterial dental composite containing calcium phosphate nanoparticles*. Journal of Dentistry, 2015. **43**(3): p. 317-326.
166. Zhang, L., et al., *Novel rechargeable calcium phosphate dental nanocomposite*. Dental Materials, 2016. **32**(2): p. 285-293.
167. Xu, H.H.K., et al., *Nanocomposite containing amorphous calcium phosphate nanoparticles for caries inhibition*. Dental Materials, 2011. **27**(8): p. 762-769.
168. Haedicke, K., et al., *Multifunctional calcium phosphate nanoparticles for combining near-infrared fluorescence imaging and photodynamic therapy*. Acta Biomaterialia, 2015. **14**: p. 197-207.
169. Zhang, M., et al., *Precise synthesis of unique polydopamine/mesoporous calcium phosphate hollow Janus nanoparticles for imaging-guided chemo-photothermal synergistic therapy*. Chemical Science, 2017. **8**(12): p. 8067-8077.
170. Kim, M.H., et al., *Injectable methylcellulose hydrogel containing calcium phosphate nanoparticles for bone regeneration*. International Journal of Biological Macromolecules, 2018. **109**: p. 57-64.
171. Liang, K., et al., *Poly (amido amine) dendrimer and dental adhesive with calcium phosphate nanoparticles remineralized dentin in lactic acid*. J Biomed Mater Res B Appl Biomater, 2018. **106**(6): p. 2414-2424.
172. Posadas, I., S. Monteagudo, and V. Cena, *Nanoparticles for brain-specific drug and genetic material delivery, imaging and diagnosis*. Nanomedicine (Lond), 2016. **11**(7): p. 833-49.
173. Xu, Z., et al., *Multifunctional nanoparticles co-delivering Trp2 peptide and CpG adjuvant induce potent cytotoxic T-lymphocyte response against melanoma and its lung metastasis*. Journal of Controlled Release, 2013. **172**(1): p. 259-265.
174. Sokolova, V., et al., *The use of calcium phosphate nanoparticles encapsulating Toll-like receptor ligands and the antigen hemagglutinin to induce dendritic cell maturation and T cell activation*. Biomaterials, 2010. **31**(21): p. 5627-33.
175. Xu, Z., et al., *Nanoparticle-delivered transforming growth factor-beta siRNA enhances vaccination against advanced melanoma by modifying tumor microenvironment*. ACS Nano, 2014. **8**(4): p. 3636-45.
176. Degli Esposti, L., et al., *Calcium phosphate-based nanosystems for advanced targeted nanomedicine*. Drug Dev Ind Pharm, 2018. **44**(8): p. 1223-1238.
177. Zhang, H.G. and Q. Zhu, *Preparation of fluoride-substituted hydroxyapatite by a molten salt synthesis route*. Journal of Materials Science: Materials in Medicine, 2006. **17**(8): p. 691-695.
178. Fathi, M.H. and E. Mohammadi Zahrani, *Mechanical alloying synthesis and bioactivity evaluation of nanocrystalline fluoridated hydroxyapatite*. Journal of Crystal Growth, 2009. **311**(5): p. 1392-1403.
179. Gentile, P., et al., *Process Optimisation to Control the Physico-Chemical Characteristics of Biomimetic Nanoscale Hydroxyapatites Prepared Using Wet Chemical Precipitation*. Materials (1996-1944), 2015. **8**(5): p. 2297-2310.
180. Sidane, D., et al., *Morphological and Mechanical Properties of Hydroxyapatite Bilayer Coatings Deposited on 316L SS by Sol–Gel Method*. Metallurgical and Materials Transactions B, 2015. **46**(5): p. 2340-2347.
181. Andrade, F.A.C., et al., *Preparation, characterization and antibacterial properties of silver nanoparticles–hydroxyapatite composites by a simple and eco-friendly method*. Ceramics International, 2016. **42**(2, Part A): p. 2271-2280.

182. Banik, M. and T. Basu, *Calcium phosphate nanoparticles: a study of their synthesis, characterization and mode of interaction with salmon testis DNA*. Dalton Trans, 2014. **43**(8): p. 3244-59.
183. Yang, Y., et al., *Systemic delivery of siRNA via LCP nanoparticle efficiently inhibits lung metastasis*. Mol Ther, 2012. **20**(3): p. 609-15.
184. Hou, S., et al., *A calcium phosphate nanoparticle-based biocarrier for efficient cellular delivery of antisense oligodeoxynucleotides*. ACS Appl Mater Interfaces, 2013. **5**(3): p. 1131-6.
185. Saha, S.K., et al., *Synthesis of nanocrystalline hydroxyapatite using surfactant template systems: Role of templates in controlling morphology*. Materials Science and Engineering: C, 2009. **29**(7): p. 2294-2301.
186. García, C., C. García, and C. Paucar, *Controlling morphology of hydroxyapatite nanoparticles through hydrothermal microemulsion chemical synthesis*. Inorganic Chemistry Communications, 2012. **20**: p. 90-92.
187. Jin, X., et al., *Hydrothermal synthesis of hydroxyapatite nanorods in the presence of sodium citrate and its aqueous colloidal stability evaluation in neutral pH*. Journal of Colloid and Interface Science, 2015. **443**: p. 125-130.
188. Sarath Chandra, V., et al., *Synthesis of magnetic hydroxyapatite by hydrothermal-microwave technique: Dielectric, protein adsorption, blood compatibility and drug release studies*. Ceramics International, 2015. **41**(10, Part A): p. 13153-13163.
189. Utara, S. and J. Klinkaewnarong, *Sonochemical synthesis of nano-hydroxyapatite using natural rubber latex as a templating agent*. Ceramics International, 2015. **41**(10, Part B): p. 14860-14867.
190. Yang, H. and Y. Wang, *Morphology control of hydroxyapatite microcrystals: Synergistic effects of citrate and CTAB*. Materials Science and Engineering: C, 2016. **62**: p. 160-165.
191. Hassan, M.N., et al., *Microwave-assisted preparation of Nano-hydroxyapatite for bone substitutes*. Ceramics International, 2016. **42**(3): p. 3725-3744.
192. Kavitha, M., et al., *Optimization of process parameters for solution combustion synthesis of Strontium substituted Hydroxyapatite nanocrystals using Design of Experiments approach*. Powder Technology, 2015. **271**: p. 167-181.
193. Cho, J.S., J.C. Lee, and S.H. Rhee, *Effect of precursor concentration and spray pyrolysis temperature upon hydroxyapatite particle size and density*. J Biomed Mater Res B Appl Biomater, 2016. **104**(2): p. 422-30.
194. Goto, N., et al., *Studies on the toxicities of aluminium hydroxide and calcium phosphate as immunological adjuvants for vaccines*. Vaccine, 1993. **11**(9): p. 914-8.
195. He, Q., et al., *Calcium phosphate nanoparticle adjuvant*. Clin Diagn Lab Immunol, 2000. **7**(6): p. 899-903.
196. Viswanathan, K., V.P. Gopinath, and G.D. Raj, *Formulation of Newcastle disease virus coupled calcium phosphate nanoparticles: an effective strategy for ocular delivery to chicken*. Colloids Surf B Biointerfaces, 2014. **116**: p. 9-16.
197. Dorozhkin, S.V., *Calcium Phosphates and Human Beings*. Journal of Chemical Education, 2006. **83**(5): p. 713-719.
198. Chen, L., et al., *The role of surface charge on the uptake and biocompatibility of hydroxyapatite nanoparticles with osteoblast cells*. Nanotechnology, 2011. **22**(10): p. 105708.
199. Sokolova, V., et al., *Mechanism of the uptake of cationic and anionic calcium phosphate nanoparticles by cells*. Acta Biomater, 2013. **9**(7): p. 7527-35.

200. Liu, Z., et al., *Calcium phosphate nanoparticles primarily induce cell necrosis through lysosomal rupture: the origination of material cytotoxicity*. Journal of Materials Chemistry B, 2014. **2**(22): p. 3480-3489.
201. Dorozhkin, S.V. and M. Epple, *Biological and medical significance of calcium phosphates*. Angew Chem Int Ed Engl, 2002. **41**(17): p. 3130-46.
202. Li, J., et al., *Biodegradable calcium phosphate nanoparticle with lipid coating for systemic siRNA delivery*. J Control Release, 2010. **142**(3): p. 416-21.
203. Sadat-Shojai, M., et al., *Synthesis methods for nanosized hydroxyapatite with diverse structures*. Acta Biomater, 2013. **9**(8): p. 7591-621.
204. Yan, S.-J., et al., *Effect of anionic PAMAM with amido groups starburst dendrimers on the crystallization of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ by hydrothermal method*. Materials Chemistry and Physics, 2006. **99**(1): p. 164-169.
205. Wang, Y., et al., *Surfactant-assisted synthesis of hydroxyapatite particles*. Materials Letters, 2006. **60**(27): p. 3227-3231.
206. Kandori, K., et al., *Effects of heat treatment of calcium hydroxyapatite particles on the protein adsorption behavior*. J Phys Chem B, 2009. **113**(31): p. 11016-22.
207. Ramachandran, R., W. Paul, and C.P. Sharma, *Synthesis and characterization of PEGylated calcium phosphate nanoparticles for oral insulin delivery*. J Biomed Mater Res B Appl Biomater, 2009. **88**(1): p. 41-8.
208. Saeed, M.I., et al., *Systemic antibody response to nano-size calcium phosphate biocompatible adjuvant adsorbed HEV-71 killed vaccine*. Clin Exp Vaccine Res, 2015. **4**(1): p. 88-98.
209. Kalita, S.J., A. Bhardwaj, and H.A. Bhatt, *Nanocrystalline calcium phosphate ceramics in biomedical engineering*. Materials Science and Engineering: C, 2007. **27**(3): p. 441-449.
210. Relyveld, E.H., *Preparation and use of calcium phosphate adsorbed vaccines*. Dev Biol Stand, 1986. **65**: p. 131-6.
211. Olmedo, H., et al., *Comparison of the adjuvant activity of aluminum hydroxide and calcium phosphate on the antibody response towards Bothrops asper snake venom*. J Immunotoxicol, 2014. **11**(1): p. 44-9.
212. He, Q., et al., *Calcium phosphate nanoparticles induce mucosal immunity and protection against herpes simplex virus type 2*. Clin Diagn Lab Immunol, 2002. **9**(5): p. 1021-4.
213. Saeed, M.I., et al., *Development of enhanced antibody response toward dual delivery of nano-adjuvant adsorbed human Enterovirus-71 vaccine encapsulated carrier*. Hum Vaccin Immunother, 2015. **11**(10): p. 2414-24.
214. Kamaly, N., et al., *Degradable Controlled-Release Polymers and Polymeric Nanoparticles: Mechanisms of Controlling Drug Release*. Chem Rev, 2016. **116**(4): p. 2602-63.
215. Swain, S.K. and D. Sarkar, *Study of BSA protein adsorption/release on hydroxyapatite nanoparticles*. Applied Surface Science, 2013. **286**: p. 99-103.
216. Dasgupta, S., A. Bandyopadhyay, and S. Bose, *Reverse micelle-mediated synthesis of calcium phosphate nanocarriers for controlled release of bovine serum albumin*. Acta Biomaterialia, 2009. **5**(8): p. 3112-3121.
217. Boonsongrit, Y., et al., *Controlled release of bovine serum albumin from hydroxyapatite microspheres for protein delivery system*. Materials Science and Engineering: B, 2008. **148**(1-3): p. 162-165.
218. Ma, Y., et al., *Biomimetic mineralization of nano-sized, needle-like hydroxyapatite with ultrahigh capacity for lysozyme adsorption*. Materials Science and Engineering: C, 2016. **68**: p. 551-556.

219. Sahdev, P., et al., *Calcium phosphate nanoparticles for transcutaneous vaccine delivery*. J Biomed Nanotechnol, 2013. **9**(1): p. 132-41.
220. Dasgupta, S., et al., *Zn- and Mg-doped hydroxyapatite nanoparticles for controlled release of protein*. Langmuir, 2010. **26**(7): p. 4958-64.
221. Li, J., et al., *Biodegradable calcium phosphate nanoparticle with lipid coating for systemic siRNA delivery*. Journal of Controlled Release, 2010. **142**(3): p. 416-421.
222. Slutter, B., et al., *Conjugation of ovalbumin to trimethyl chitosan improves immunogenicity of the antigen*. J Control Release, 2010. **143**(2): p. 207-14.
223. Staros, J.V., R.W. Wright, and D.M. Swingle, *Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions*. Analytical Biochemistry, 1986. **156**(1): p. 220-222.
224. Chiu, D., et al., *Biom mineralization and size control of stable calcium phosphate core-protein shell nanoparticles: potential for vaccine applications*. Bioconjug Chem, 2012. **23**(3): p. 610-7.
225. Zhao, Z., et al., *A nanoparticle-based nicotine vaccine and the influence of particle size on its immunogenicity and efficacy*. Nanomedicine, 2017. **13**(2): p. 443-454.
226. Nembrini, C., et al., *Nanoparticle conjugation of antigen enhances cytotoxic T-cell responses in pulmonary vaccination*. Proc Natl Acad Sci U S A, 2011. **108**(44): p. E989-97.
227. Ribeiro, C.A., et al., *Electrochemical preparation and characterization of PNIPAM-HAp scaffolds for bone tissue engineering*. Mater Sci Eng C Mater Biol Appl, 2017. **81**: p. 156-166.
228. Kemence, N. and N. Bolgen, *Gelatin- and hydroxyapatite-based cryogels for bone tissue engineering: synthesis, characterization, in vitro and in vivo biocompatibility*. J Tissue Eng Regen Med, 2017. **11**(1): p. 20-33.
229. Weerasuriya, D.R.K., W. Wijesinghe, and R.M.G. Rajapakse, *Encapsulation of anticancer drug copper bis(8-hydroxyquinoline) in hydroxyapatite for pH-sensitive targeted delivery and slow release*. Mater Sci Eng C Mater Biol Appl, 2017. **71**: p. 206-213.
230. Mondal, S., S.V. Dorozhkin, and U. Pal, *Recent progress on fabrication and drug delivery applications of nanostructured hydroxyapatite*. Wiley Interdiscip Rev Nanomed Nanobiotechnol, 2018. **10**(4): p. e1504.
231. Weerasuriya, D.R.K., W.P.S.L. Wijesinghe, and R.M.G. Rajapakse, *Encapsulation of anticancer drug copper bis(8-hydroxyquinoline) in hydroxyapatite for pH-sensitive targeted delivery and slow release*. Materials Science and Engineering: C, 2017. **71**: p. 206-213.
232. Murphy, M.B., et al., *Synthesis and in vitro hydroxyapatite binding of peptides conjugated to calcium-binding moieties*. Biomacromolecules, 2007. **8**(7): p. 2237-43.
233. Iafisco, M., et al., *Conjugation of hydroxyapatite nanocrystals with human immunoglobulin G for nanomedical applications*. Colloids Surf B Biointerfaces, 2012. **90**: p. 1-7.
234. Hayashi, M., et al., *Optimization of physiological properties of hydroxyapatite as a vaccine adjuvant*. Vaccine, 2016. **34**: p. 306-312.
235. He, C., et al., *Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles*. Biomaterials, 2010. **31**(13): p. 3657-3666.
236. Knuschke, T., et al., *Prophylactic and therapeutic vaccination with a nanoparticle-based peptide vaccine induces efficient protective immunity during acute and chronic retroviral infection*. Nanomedicine: Nanotechnology, Biology and Medicine, 2014. **10**(8): p. 1787-1798.

237. Sharma, S., et al., *An insight into functionalized calcium based inorganic nanomaterials in biomedicine: Trends and transitions*. Colloids Surf B Biointerfaces, 2015. **133**: p. 120-39.
238. Knuschke, T., et al., *Prophylactic and therapeutic vaccination with a nanoparticle-based peptide vaccine induces efficient protective immunity during acute and chronic retroviral infection*. Nanomedicine, 2014. **10**(8): p. 1787-98.
239. Foged, C., et al., *Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model*. Int J Pharm, 2005. **298**(2): p. 315-22.
240. Hou, C.-H., et al., *The in vivo performance of biomagnetic hydroxyapatite nanoparticles in cancer hyperthermia therapy*. Biomaterials, 2009. **30**(23–24): p. 3956-3960.
241. Li, J., et al., *Effect of nano- and micro-hydroxyapatite/chitosan-gelatin network film on human gastric cancer cells*. Materials Letters, 2008. **62**(17–18): p. 3220-3223.
242. Ramesh, M., et al., *Effects of the physico-chemical nature of two biomimetic crystals on the innate immune response*. International Immunopharmacology, 2007. **7**(13): p. 1617-1629.
243. Hu, Q., et al., *Effect of crystallinity of calcium phosphate nanoparticles on adhesion, proliferation, and differentiation of bone marrow mesenchymal stem cells*. JOURNAL OF MATERIALS CHEMISTRY, 2007. **17**(44): p. 4690-4698.
244. Medzhitov, R., *Recognition of microorganisms and activation of the immune response*. Nature, 2007. **449**(7164): p. 819-26.
245. Iwasaki, A. and R. Medzhitov, *Control of adaptive immunity by the innate immune system*. Nat Immunol, 2015. **16**(4): p. 343-53.
246. Kozlova, D., et al., *Calcium phosphate nanoparticles show an effective activation of the innate immune response in vitro and in vivo after functionalization with flagellin*. Virologica Sinica, 2014. **29**(1): p. 33-39.
247. Behera, T. and P. Swain, *Antigen adsorbed calcium phosphate nanoparticles stimulate both innate and adaptive immune response in fish, Labeo rohita H*. Cell Immunol, 2011. **271**(2): p. 350-9.
248. Matesanz, M.C., et al., *Early in vitro response of macrophages and T lymphocytes to nanocrystalline hydroxyapatites*. Journal of Colloid and Interface Science, 2014. **416**: p. 59-66.
249. Tai, S., et al., *Characterization of beta-tricalcium phosphate as a novel immunomodulator*. International Immunopharmacology, 2014. **19**(1): p. 45-51.
250. Krutzik, S.R., et al., *TLR activation triggers the rapid differentiation of monocytes into macrophages and dendritic cells*. Nat Med, 2005. **11**(6): p. 653-60.
251. Luo, Y.H., L.W. Chang, and P. Lin, *Metal-Based Nanoparticles and the Immune System: Activation, Inflammation, and Potential Applications*. Biomed Res Int, 2015. **2015**: p. 143720.
252. Kozlova, D., et al., *Calcium phosphate nanoparticles show an effective activation of the innate immune response in vitro and in vivo after functionalization with flagellin*. Virol Sin, 2014. **29**(1): p. 33-9.
253. Hayashi, F., et al., *The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5*. Nature, 2001. **410**(6832): p. 1099-103.
254. Goyal, A.K., et al., *Development of self-assembled nanoceramic carrier construct(s) for vaccine delivery*. J Biomater Appl, 2009. **24**(1): p. 65-84.
255. Joyappa, D.H., et al., *Calcium phosphate nanoparticle prepared with foot and mouth disease virus P1-3CD gene construct protects mice and guinea pigs against the challenge virus*. Veterinary Microbiology, 2009. **139**(1–2): p. 58-66.

256. Temchura, V.V., et al., *Targeting and activation of antigen-specific B-cells by calcium phosphate nanoparticles loaded with protein antigen*. Biomaterials, 2014. **35**(23): p. 6098-6105.
257. Chua, B.Y., et al., *A single dose biodegradable vaccine depot that induces persistently high levels of antibody over a year*. Biomaterials, 2015. **53**: p. 50-7.
258. Sokolova, V., et al., *The use of calcium phosphate nanoparticles encapsulating Toll-like receptor ligands and the antigen hemagglutinin to induce dendritic cell maturation and T cell activation*. Biomaterials, 2010. **31**(21): p. 5627-5633.
259. Zhou, W., et al., *Just-in-time vaccines: Biom mineralized calcium phosphate core-immunogen shell nanoparticles induce long-lasting CD8+ T cell responses in mice*. Nanomedicine: Nanotechnology, Biology and Medicine, 2014. **10**(3): p. 571-578.
260. Knuschke, T., et al., *Immunization with biodegradable nanoparticles efficiently induces cellular immunity and protects against influenza virus infection*. J Immunol, 2013. **190**(12): p. 6221-9.
261. Wang, X., et al., *Rod-shaped and substituted hydroxyapatite nanoparticles stimulating type 1 and 2 cytokine secretion*. Colloids and Surfaces B: Biointerfaces, 2016. **139**: p. 10-16.
262. Knuschke, T., et al., *Combination of nanoparticle-based therapeutic vaccination and transient ablation of regulatory T cells enhances anti-viral immunity during chronic retroviral infection*. Retrovirology, 2016. **13**: p. 24.
263. Knuschke, T., M. Epple, and A.M. Westendorf, *The type of adjuvant strongly influences the T-cell response during nanoparticle-based immunization*. Hum Vaccin Immunother, 2014. **10**(1): p. 164-9.
264. Bisht, S., et al., *pDNA loaded calcium phosphate nanoparticles: highly efficient non-viral vector for gene delivery*. International Journal of Pharmaceutics, 2005. **288**(1): p. 157-168.
265. Zhao, Y., et al., *Nanoparticle delivery of CDDO-Me remodels the tumor microenvironment and enhances vaccine therapy for melanoma*. Biomaterials, 2015. **68**: p. 54-66.
266. Naito, Y., et al., *Locally applied TCP inhibits tumor growth via possible activation of macrophages*. J Biomed Mater Res A, 2010. **92**(2): p. 542-7.
267. Tsiourvas, D., et al., *Controlling the Formation of Hydroxyapatite Nanorods with Dendrimers*. Journal of the American Ceramic Society, 2011. **94**(7): p. 2023-2029.
268. Huang, X., et al., *Characterization of Calcium Phosphate Nanoparticles Based on a PEGylated Chelator for Gene Delivery*. ACS Appl Mater Interfaces, 2017. **9**(12): p. 10435-10445.
269. Wilson-Welder, J.H., et al., *Vaccine adjuvants: Current challenges and future approaches*. Journal of Pharmaceutical Sciences, 2009. **98**(4): p. 1278-1316.
270. Gupta, R.K., *Aluminum compounds as vaccine adjuvants*. Advanced Drug Delivery Reviews, 1998. **32**(3): p. 155-172.
271. Lindblad, E.B. and L. Duroux, *Chapter 18 - Mineral Adjuvants**The present chapter is an updated version of the chapter "Mineral Adjuvants," published in Immunopotentiators in Modern Vaccines, p. 217–233. Ed. Virgil Schijns & Derek O'Hagan, Elsevier Science Publishers (2005), in Immunopotentiators in Modern Vaccines (Second Edition), V.E.J.C. Schijns and D.T. O'Hagan, Editors. 2017, Academic Press. p. 347-375.*
272. Coursaget, P., et al., *Simultaneous administration of diphtheria-tetanus-pertussis-polio and hepatitis B vaccines in a simplified immunization program: immune response to diphtheria toxoid, tetanus toxoid, pertussis, and hepatitis B surface antigen*. Infection and Immunity, 1986. **51**(3): p. 784-787.

273. Goto, N., et al., *Local tissue irritating effects and adjuvant activities of calcium phosphate and aluminium hydroxide with different physical properties*. Vaccine, 1997. **15**(12): p. 1364-1371.
274. Tomljenovic, L. and C.A. Shaw, *Mechanisms of aluminum adjuvant toxicity and autoimmunity in pediatric populations*. Lupus, 2012. **21**(2): p. 223-30.
275. Tomljenovic, L. and C.A. Shaw, *Aluminum vaccine adjuvants: are they safe?* Curr Med Chem, 2011. **18**(17): p. 2630-7.
276. Relyveld, E.H., E. Henocq, and M. Raynaud, *[STUDY OF THE ANTIDIPHThERIA VACCINATION OF ALLERGIC SUBJECTS WITH A PURE ANATOXIN ABSORBED ON CALCIUM PHOSPHATE]*. Bull World Health Organ, 1964. **30**: p. 321-5.
277. Relyveld, E.H., et al., *Calcium phosphate adjuvanted allergens*. Ann Allergy, 1985. **54**(6): p. 521-9.
278. Gupta, R.K. and G.R. Siber, *Adjuvants for human vaccines—current status, problems and future prospects*. Vaccine, 1995. **13**(14): p. 1263-1276.
279. Sesardic, D., S. Rijpkema, and B.P. Patel, *New adjuvants: EU regulatory developments*. Expert Rev Vaccines, 2007. **6**(5): p. 849-61.
280. Issa, A.M., et al., *Evaluation of the Effects of Aluminum Phosphate and Calcium Phosphate Nanoparticles as Adjuvants in Vaccinated Mice*. International Journal of Chemical Engineering and Applications, 2014. **5**(5): p. 367-373.
281. Banik, M. and T. Basu, *Calcium phosphate nanoparticles: a study of their synthesis, characterization and mode of interaction with salmon testis DNA*. Dalton Transactions, 2014. **43**(8): p. 3244-3259.
282. Kester, M., et al., *Calcium phosphate nanocomposite particles for in vitro imaging and encapsulated chemotherapeutic drug delivery to cancer cells*. Nano Lett, 2008. **8**(12): p. 4116-21.
283. Li, J., Y. Yang, and L. Huang, *Calcium phosphate nanoparticles with an asymmetric lipid bilayer coating for siRNA delivery to the tumor*. Journal of Controlled Release, 2012. **158**(1): p. 108-114.
284. Cengiz, B., et al., *Synthesis and characterization of hydroxyapatite nanoparticles*. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2008. **322**(1): p. 29-33.
285. Bhattacharjee, S., *DLS and zeta potential – What they are and what they are not?* Journal of Controlled Release, 2016. **235**: p. 337-351.
286. Sunil, B.R., et al., *Role of biomineralization on the degradation of fine grained AZ31 magnesium alloy processed by groove pressing*. Mater Sci Eng C Mater Biol Appl, 2013. **33**(3): p. 1607-15.
287. Zeimaran, E., et al., *Hydrothermal synthesis and characterisation of bioactive glass-ceramic nanorods*. Journal of Non-Crystalline Solids, 2016. **443**: p. 118-124.
288. Shimabayashi, S., et al., *Formation of Hydroxyapatite in the Presence of Phosphorylated and Sulfated Polymer in an Aqueous Phase*, in *Mineral Scale Formation and Inhibition*, Z. Amjad, Editor. 1995, Springer US: Boston, MA. p. 157-167.
289. Cai, Y. and R. Tang, *Calcium phosphate nanoparticles in biomineralization and biomaterials*. Journal of Materials Chemistry, 2008. **18**(32): p. 3775-3787.
290. Alves Cardoso, D., J.A. Jansen, and S.C. Leeuwenburgh, *Synthesis and application of nanostructured calcium phosphate ceramics for bone regeneration*. J Biomed Mater Res B Appl Biomater, 2012. **100**(8): p. 2316-26.
291. Roy, I., et al., *Calcium phosphate nanoparticles as novel non-viral vectors for targeted gene delivery*. International Journal of Pharmaceutics, 2003. **250**(1): p. 25-33.

292. Singh, S., et al., *Synthesis of nanocrystalline calcium phosphate in microemulsion—effect of nature of surfactants*. Journal of Colloid and Interface Science, 2008. **319**(1): p. 322-329.
293. Lin, K., et al., *Hydrothermal microemulsion synthesis of stoichiometric single crystal hydroxyapatite nanorods with mono-dispersion and narrow-size distribution*. Materials Letters, 2007. **61**(8): p. 1683-1687.
294. Zhang, W., X. Qiao, and J. Chen, *Synthesis of nanosilver colloidal particles in water/oil microemulsion*. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2007. **299**(1): p. 22-28.
295. Guo, G., et al., *Preparation of hydroxyapatite nanoparticles by reverse microemulsion*. Ceramics International, 2005. **31**(6): p. 869-872.
296. Sun, J., L. Gao, and M. Iwasa, *Noncovalent attachment of oxide nanoparticles onto carbon nanotubes using water-in-oil microemulsions*. Chem Commun (Camb), 2004(7): p. 832-3.
297. Granata, G., et al., *Effect of surfactant/water ratio and reagents' concentration on size distribution of manganese carbonate nanoparticles synthesized by microemulsion mediated route*. Applied Surface Science, 2015. **331**: p. 463-471.
298. Arriagada, F.J. and K. Osseo-Asare, *Synthesis of Nanosize Silica in a Nonionic Water-in-Oil Microemulsion: Effects of the Water/Surfactant Molar Ratio and Ammonia Concentration*. Journal of Colloid and Interface Science, 1999. **211**(2): p. 210-220.
299. Kimura, E.T., D.M. Ebert, and P.W. Dodge, *Acute toxicity and limits of solvent residue for sixteen organic solvents*. Toxicology and Applied Pharmacology, 1971. **19**(4): p. 699-704.
300. Malley, L.A., et al., *Subchronic toxicity of cyclohexane in rats and mice by inhalation exposure*. Drug Chem Toxicol, 2000. **23**(4): p. 513-37.
301. Martins, M.A., et al., *Hydroxyapatite micro- and nanoparticles: Nucleation and growth mechanisms in the presence of citrate species*. Journal of Colloid And Interface Science, 2008. **318**: p. 210-216.
302. López-Macipe, A., J. Gómez-Morales, and R. Rodríguez-Clemente, *Nanosized Hydroxyapatite Precipitation from Homogeneous Calcium/Citrate/Phosphate Solutions Using Microwave and Conventional Heating*. Advanced Materials, 1998. **10**(1): p. 49.
303. Han, Y., et al., *Synthesis and sintering of nanocrystalline hydroxyapatite powders by citric acid sol–gel combustion method*. Materials Research Bulletin, 2004. **39**(1): p. 25-32.
304. Ambrosio, L., C. Demitri, and A. Sannino, *2 - Superabsorbent cellulose-based hydrogels for biomedical applications*, in *Biomedical Hydrogels*, S. Rimmer, Editor. 2011, Woodhead Publishing. p. 25-50.
305. Tan, J., M. Chen, and J. Xia, *Water-dispersible hydroxyapatite nanorods synthesized by a facile method*. Applied Surface Science, 2009. **255**(21): p. 8774-8779.
306. Snihirova, D., et al., *Hydroxyapatite Microparticles as Feedback-Active Reservoirs of Corrosion Inhibitors*. ACS Applied Materials & Interfaces, 2010. **2**(11): p. 3011-3022.
307. Patel, V.R. and Y.K. Agrawal, *Nanosuspension: An approach to enhance solubility of drugs*. J Adv Pharm Technol Res, 2011. **2**(2): p. 81-7.
308. Rani, M., et al., *Understanding the mechanism of replacement of citrate from the surface of gold nanoparticles by amino acids: a theoretical and experimental investigation and their biological application*. RSC Advances, 2016. **6**(21): p. 17373-17383.
309. Huang, T., et al., *Engineering highly effective antimicrobial selenium nanoparticles through control of particle size*. Nanoscale, 2019. **11**(31): p. 14937-14951.

310. Thanh, N.T., N. Maclean, and S. Mahiddine, *Mechanisms of nucleation and growth of nanoparticles in solution*. Chem Rev, 2014. **114**(15): p. 7610-30.
311. Kumari, M., et al., *Physico-Chemical Condition Optimization during Biosynthesis lead to development of Improved and Catalytically Efficient Gold Nano Particles*. Sci Rep, 2016. **6**: p. 27575.
312. Polte, J., *Fundamental growth principles of colloidal metal nanoparticles – a new perspective*. CrystEngComm, 2015. **17**(36): p. 6809-6830.
313. Elbakry, A., et al., *Layer-by-layer coated gold nanoparticles: size-dependent delivery of DNA into cells*. Small, 2012. **8**(24): p. 3847-56.
314. Ai, H., S.A. Jones, and Y.M. Lvov, *Biomedical applications of electrostatic layer-by-layer nano-assembly of polymers, enzymes, and nanoparticles*. Cell Biochem Biophys, 2003. **39**(1): p. 23-43.
315. Lee, M.Y., et al., *Target-specific gene silencing of layer-by-layer assembled gold-cysteamine/siRNA/PEI/HA nanocomplex*. ACS Nano, 2011. **5**(8): p. 6138-47.
316. Powell, T.J., et al., *Synthetic nanoparticle vaccines produced by layer-by-layer assembly of artificial biofilms induce potent protective T-cell and antibody responses in vivo*. Vaccine, 2011. **29**: p. 558-569.
317. Wang, Y., A.S. Angelatos, and F. Caruso, *Template Synthesis of Nanostructured Materials via Layer-by-Layer Assembly*. Chemistry of Materials, 2008. **20**(3): p. 848-858.
318. Su, X., et al., *Layer-by-Layer-Assembled Multilayer Films for Transcutaneous Drug and Vaccine Delivery*. ACS Nano, 2009. **3**(11): p. 3719-3729.
319. Li, Y., et al., *Multifunctional selenium nanoparticles as carriers of HSP70 siRNA to induce apoptosis of HepG2 cells*. Int J Nanomedicine, 2016. **11**: p. 3065-76.
320. Xiang, J., et al., *Antigen-Loaded Upconversion Nanoparticles for Dendritic Cell Stimulation, Tracking, and Vaccination in Dendritic Cell-Based Immunotherapy*. ACS Nano, 2015. **9**(6): p. 6401-11.
321. Lvov, Y., et al., *Assembly of Multicomponent Protein Films by Means of Electrostatic Layer-by-Layer Adsorption*. Journal of the American Chemical Society, 1995. **117**(22): p. 6117-6123.
322. Gormley, A.J., et al., *Layer-by-Layer Self-Assembly of Polymer Films and Capsules through Coiled-Coil Peptides*. Chemistry of Materials, 2015. **27**(16): p. 5820-5824.
323. Schneider, G. and G. Decher, *Functional core/shell nanoparticles via layer-by-layer assembly. investigation of the experimental parameters for controlling particle aggregation and for enhancing dispersion stability*. Langmuir, 2008. **24**(5): p. 1778-89.
324. Guo, S., et al., *Enhanced gene delivery and siRNA silencing by gold nanoparticles coated with charge-reversal polyelectrolyte*. ACS Nano, 2010. **4**(9): p. 5505-11.
325. Samal, S.K., et al., *Cationic polymers and their therapeutic potential*. Chemical Society Reviews, 2012. **41**(21): p. 7147-7194.
326. Giron-Gonzalez, M.D., et al., *Polyelectrolyte Complexes of Low Molecular Weight PEI and Citric Acid as Efficient and Nontoxic Vectors for in Vitro and in Vivo Gene Delivery*. Bioconjug Chem, 2016. **27**(3): p. 549-61.
327. Lynch, I. and K.A. Dawson, *Protein-nanoparticle interactions*. Nano Today, 2008. **3**(1): p. 40-47.
328. Gorasia, D.G., et al., *Structural Insights into the PorK and PorN Components of the Porphyromonas gingivalis Type IX Secretion System*. PLoS Pathog, 2016. **12**(8): p. e1005820.
329. Yang, B., et al., *Identification of cross-linked peptides from complex samples*. Nat Methods, 2012. **9**(9): p. 904-6.

330. Oyane, A., et al., *Preparation and assessment of revised simulated body fluids*. J Biomed Mater Res A, 2003. **65**(2): p. 188-95.
331. Kokubo, T. and H. Takadama, *How useful is SBF in predicting in vivo bone bioactivity?* Biomaterials, 2006. **27**(15): p. 2907-2915.
332. Glew, M.D., et al., *PG0026 is the C-terminal signal peptidase of a novel secretion system of Porphyromonas gingivalis*. J Biol Chem, 2012. **287**(29): p. 24605-17.
333. Veith, P.D., et al., *Porphyromonas gingivalis outer membrane vesicles exclusively contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors*. J Proteome Res, 2014. **13**(5): p. 2420-32.
334. Gorasia, D.G., et al., *Porphyromonas gingivalis Type IX Secretion Substrates Are Cleaved and Modified by a Sortase-Like Mechanism*. PLoS Pathog, 2015. **11**(9): p. e1005152.
335. Arrondo, J.L.R., et al., *Quantitative studies of the structure of proteins in solution by fourier-transform infrared spectroscopy*. Progress in Biophysics and Molecular Biology, 1993. **59**(1): p. 23-56.
336. Liu, T.Y., et al., *On the study of BSA-loaded calcium-deficient hydroxyapatite nano-carriers for controlled drug delivery*. J Control Release, 2005. **107**(1): p. 112-21.
337. Sethi, M. and M.R. Knecht, *Experimental studies on the interactions between Au nanoparticles and amino acids: bio-based formation of branched linear chains*. ACS Appl Mater Interfaces, 2009. **1**(6): p. 1270-8.
338. Polavarapu, L. and Q.H. Xu, *A single-step synthesis of gold nanochains using an amino acid as a capping agent and characterization of their optical properties*. Nanotechnology, 2008. **19**(7): p. 075601.
339. Lee, W.H., et al., *Modulating protein adsorption onto hydroxyapatite particles using different amino acid treatments*. J R Soc Interface, 2012. **9**(70): p. 918-27.
340. Bakan, F., et al., *Synthesis and characterization of amino acid-functionalized calcium phosphate nanoparticles for siRNA delivery*. Colloids Surf B Biointerfaces, 2017. **158**: p. 175-181.
341. Ozhukil Kollath, V., et al., *A Modular Approach To Study Protein Adsorption on Surface Modified Hydroxyapatite*. Chemistry, 2015. **21**(29): p. 10497-505.
342. Sethi, M. and M.R. Knecht, *Understanding the mechanism of amino acid-based Au nanoparticle chain formation*. Langmuir, 2010. **26**(12): p. 9860-74.
343. Hiemenz, P.C., *Principles of colloid and surface chemistry*. 1986: M. Dekker.
344. Zakeri, A., et al., *Polyethylenimine-based nanocarriers in co-delivery of drug and gene: a developing horizon*. Nano Rev Exp, 2018. **9**(1): p. 1488497.
345. Xia, T., et al., *Polyethyleneimine coating enhances the cellular uptake of mesoporous silica nanoparticles and allows safe delivery of siRNA and DNA constructs*. ACS Nano, 2009. **3**(10): p. 3273-86.
346. Al-Khatib, O., et al., *Adsorption of polyelectrolytes onto the oppositely charged surface of tubular J-aggregates of a cyanine dye*. Colloid and Polymer Science, 2019. **297**(5): p. 729-739.
347. Florea, B.I., et al., *Transfection efficiency and toxicity of polyethylenimine in differentiated Calu-3 and nondifferentiated COS-1 cell cultures*. AAPS PharmSci, 2002. **4**(3): p. E12.
348. Zeng, B., H. Shi, and Y. Liu, *A versatile pH-responsive platform for intracellular protein delivery using calcium phosphate nanoparticles*. Journal of Materials Chemistry B, 2015. **3**(47): p. 9115-9121.
349. Wang, X., et al., *Use of coated silver nanoparticles to understand the relationship of particle dissolution and bioavailability to cell and lung toxicological potential*. Small, 2014. **10**(2): p. 385-98.

350. Pavlin, M. and V.B. Bregar, *STABILITY OF NANOPARTICLE SUSPENSIONS IN DIFFERENT BIOLOGICALLY RELEVANT MEDIA*. Digest Journal of Nanomaterials & Biostructures (DJNB), 2012. **7**(4): p. 1389-1400.
351. Chen, Z.P., et al., *Preparation and characterization of water-soluble monodisperse magnetic iron oxide nanoparticles via surface double-exchange with DMSA*. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2008. **316**(1): p. 210-216.
352. Casals, E., et al., *Time evolution of the nanoparticle protein corona*. ACS Nano, 2010. **4**(7): p. 3623-32.
353. Maiorano, G., et al., *Effects of cell culture media on the dynamic formation of protein-nanoparticle complexes and influence on the cellular response*. ACS Nano, 2010. **4**(12): p. 7481-91.
354. Liu, P., et al., *Role of fetal bovine serum in the prevention of calcification in biological fluids*. Journal of Crystal Growth, 2008. **310**(22): p. 4672-4675.
355. Govardhan, C.P., *Crosslinking of enzymes for improved stability and performance*. Current Opinion in Biotechnology, 1999. **10**(4): p. 331-335.
356. Faraasen, S., et al., *Ligand-specific targeting of microspheres to phagocytes by surface modification with poly(L-lysine)-grafted poly(ethylene glycol) conjugate*. Pharm Res, 2003. **20**(2): p. 237-46.
357. Aggarwal, P., et al., *Nanoparticle interaction with plasma proteins as it relates to particle biodistribution, biocompatibility and therapeutic efficacy*. Adv Drug Deliv Rev, 2009. **61**(6): p. 428-37.
358. Camner, P., et al., *Experimental and calculated parameters on particle phagocytosis by alveolar macrophages*. J Appl Physiol (1985), 2002. **92**(6): p. 2608-16.
359. Goppert, T.M. and R.H. Muller, *Adsorption kinetics of plasma proteins on solid lipid nanoparticles for drug targeting*. Int J Pharm, 2005. **302**(1-2): p. 172-86.
360. Zhang, L., W. Wang, and S. Wang, *Effect of vaccine administration modality on immunogenicity and efficacy*. Expert Rev Vaccines, 2015. **14**(11): p. 1509-23.
361. Takahashi, I., et al., *New horizon of mucosal immunity and vaccines*. Current Opinion in Immunology, 2009. **21**(3): p. 352-358.
362. Cartiera, M.S., et al., *The uptake and intracellular fate of PLGA nanoparticles in epithelial cells*. Biomaterials, 2009. **30**(14): p. 2790-2798.
363. des Rieux, A., et al., *Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach*. Journal of Controlled Release, 2006. **116**(1): p. 1-27.
364. Prego, C., et al., *Transmucosal macromolecular drug delivery*. Journal of Controlled Release, 2005. **101**(1): p. 151-162.
365. Kopp, M., et al., *Delivery of the autofluorescent protein R-phycoerythrin by calcium phosphate nanoparticles into four different eukaryotic cell lines (HeLa, HEK293T, MG-63, MC3T3): Highly efficient, but leading to endolysosomal proteolysis in HeLa and MC3T3 cells*. PLoS One, 2017. **12**(6): p. e0178260.
366. Jeevanandam, J., et al., *Review on nanoparticles and nanostructured materials: history, sources, toxicity and regulations*. Beilstein J Nanotechnol, 2018. **9**: p. 1050-1074.
367. Bohner, M., et al., *Synthesis of spherical calcium phosphate particles for dental and orthopedic applications*. Biomater, 2013. **3**(2).
368. Kulkarni, S.A. and S.S. Feng, *Effects of particle size and surface modification on cellular uptake and biodistribution of polymeric nanoparticles for drug delivery*. Pharm Res, 2013. **30**(10): p. 2512-22.
369. Cai, Y., et al., *Role of hydroxyapatite nanoparticle size in bone cell proliferation*. Journal of Materials Chemistry, 2007. **17**(36): p. 3780-3787.

370. Chikaura, H., et al., *Effect of particle size on biological response by human monocyte-derived macrophages*. Biosurface and Biotribology, 2016. **2**(1): p. 18-25.
371. Cooley, M., et al., *Influence of particle size and shape on their margination and wall-adhesion: implications in drug delivery vehicle design across nano-to-micro scale*. Nanoscale, 2018. **10**(32): p. 15350-15364.
372. Chen, S., et al., *Influence of particle size on the in vivo potency of lipid nanoparticle formulations of siRNA*. Journal of Controlled Release, 2016. **235**: p. 236-244.
373. Cui, X., et al., *Correlation of particle properties with cytotoxicity and cellular uptake of hydroxyapatite nanoparticles in human gastric cancer cells*. Materials Science and Engineering: C, 2016. **67**: p. 453-460.
374. Manolova, V., et al., *Nanoparticles target distinct dendritic cell populations according to their size*. Eur J Immunol, 2008. **38**(5): p. 1404-13.
375. Xie, X., et al., *The Effect of shape on Cellular Uptake of Gold Nanoparticles in the forms of Stars, Rods, and Triangles*. Sci Rep, 2017. **7**(1): p. 3827.
376. ISO, P., *10993-5: 2009 Biological Evaluation of Medical Devices—Part 5: Tests for In Vitro Cytotoxicity*. ISO: Geneva, Switzerland, 2009.
377. Forrest, M.L., J.T. Koerber, and D.W. Pack, *A Degradable Polyethylenimine Derivative with Low Toxicity for Highly Efficient Gene Delivery*. Bioconjugate Chemistry, 2003. **14**(5): p. 934-940.
378. McClean, S., et al., *Binding and uptake of biodegradable poly-dl-lactide micro- and nanoparticles in intestinal epithelia*. European Journal of Pharmaceutical Sciences, 1998. **6**(2): p. 153-163.
379. Desai, M.P., et al., *The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent*. Pharm Res, 1997. **14**(11): p. 1568-73.
380. Pitchaimani, A., et al., *Impact of cell adhesion and migration on nanoparticle uptake and cellular toxicity*. Toxicology in Vitro, 2017. **43**: p. 29-39.
381. Zhang, Y., et al., *Zeta potential: a surface electrical characteristic to probe the interaction of nanoparticles with normal and cancer human breast epithelial cells*. Biomed Microdevices, 2008. **10**(2): p. 321-8.
382. Ficner, R. and R. Huber, *Refined crystal structure of phycoerythrin from Porphyridium cruentum at 0.23-nm resolution and localization of the gamma subunit*. Eur J Biochem, 1993. **218**(1): p. 103-6.
383. Foged, C., et al., *Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model*. International Journal of Pharmaceutics, 2005. **298**(2): p. 315-322.
384. Lu, F., et al., *Size effect on cell uptake in well-suspended, uniform mesoporous silica nanoparticles*. Small, 2009. **5**(12): p. 1408-13.
385. Lunov, O., et al., *Differential uptake of functionalized polystyrene nanoparticles by human macrophages and a monocytic cell line*. ACS Nano, 2011. **5**(3): p. 1657-69.
386. Zauner, W., N.A. Farrow, and A.M.R. Haines, *In vitro uptake of polystyrene microspheres: effect of particle size, cell line and cell density*. Journal of Controlled Release, 2001. **71**(1): p. 39-51.
387. Christian, F., E.L. Smith, and R.J. Carmody, *The Regulation of NF-kappaB Subunits by Phosphorylation*. Cells, 2016. **5**(1).
388. Liu, T., et al., *NF-κB signaling in inflammation*. Signal Transduction And Targeted Therapy, 2017. **2**: p. 17023.
389. Duan, W. and H. Li, *Combination of NF-kB targeted siRNA and methotrexate in a hybrid nanocarrier towards the effective treatment in rheumatoid arthritis*. J Nanobiotechnology, 2018. **16**(1): p. 58.

390. Pazar, B., et al., *Basic calcium phosphate crystals induce monocyte/macrophage IL-1 β secretion through the NLRP3 inflammasome in vitro*. J Immunol, 2011. **186**(4): p. 2495-502.
391. Grandjean-Laquerriere, A., et al., *Involvement of toll-like receptor 4 in the inflammatory reaction induced by hydroxyapatite particles*. Biomaterials, 2007. **28**(3): p. 400-404.
392. Elsabahy, M. and K.L. Wooley, *Cytokines as biomarkers of nanoparticle immunotoxicity*. Chem Soc Rev, 2013. **42**(12): p. 5552-76.
393. Oberdorster, G., E. Oberdorster, and J. Oberdorster, *Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles*. Environ Health Perspect, 2005. **113**(7): p. 823-39.
394. Yang, E.-J., et al., *Inflammasome formation and IL-1 β release by human blood monocytes in response to silver nanoparticles*. Biomaterials, 2012. **33**(28): p. 6858-6867.
395. Struyf, S., et al., *Chemokines synergize in the recruitment of circulating neutrophils into inflamed tissue*. Eur J Immunol, 2005. **35**(5): p. 1583-91.
396. Kang, S., et al., *Effects of gold nanoparticle-based vaccine size on lymph node delivery and cytotoxic T-lymphocyte responses*. Journal of Controlled Release, 2017. **256**: p. 56-67.
397. Gustafson, H.H., et al., *Nanoparticle Uptake: The Phagocyte Problem*. Nano Today, 2015. **10**(4): p. 487-510.
398. Watts, C., *Phagosome Neutrality in Host Defense*. Cell, 2006. **126**(1): p. 17-19.
399. Foote, J.R., et al., *Variations in the Phagosomal Environment of Human Neutrophils and Mononuclear Phagocyte Subsets*. Front Immunol, 2019. **10**: p. 188.
400. Koh, E., et al., *Exosome-SIRP α , a CD47 blockade increases cancer cell phagocytosis*. Biomaterials, 2017. **121**: p. 121-129.
401. Miksa, M., et al., *A novel method to determine the engulfment of apoptotic cells by macrophages using pHrodo succinimidyl ester*. Journal of Immunological Methods, 2009. **342**(1): p. 71-77.
402. Maurer, K.H. and H.R. Schumacher, *Hydroxyapatite phagocytosis by human polymorphonuclear leucocytes*. Ann Rheum Dis, 1979. **38**(1): p. 84-8.
403. Makela, S.M., et al., *Multiple signaling pathways contribute to synergistic TLR ligand-dependent cytokine gene expression in human monocyte-derived macrophages and dendritic cells*. J Leukoc Biol, 2009. **85**(4): p. 664-72.
404. Silva, J.M., et al., *Immune system targeting by biodegradable nanoparticles for cancer vaccines*. J Control Release, 2013. **168**(2): p. 179-99.
405. de Vries, I.J., et al., *Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients*. Clin Cancer Res, 2003. **9**(14): p. 5091-100.
406. Gustafsson, K., et al., *Recruitment and activation of natural killer cells in vitro by a human dendritic cell vaccine*. Cancer Res, 2008. **68**(14): p. 5965-71.
407. Dienz, O. and M. Rincon, *The effects of IL-6 on CD4 T cell responses*. Clin Immunol, 2009. **130**(1): p. 27-33.
408. Yang, R., et al., *IL-6 promotes the differentiation of a subset of naive CD8 $^{+}$ T cells into IL-21-producing B helper CD8 $^{+}$ T cells*. J Exp Med, 2016. **213**(11): p. 2281-2291.
409. Kang, S.S. and P.M. Allen, *Priming in the presence of IL-10 results in direct enhancement of CD8 $^{+}$ T cell primary responses and inhibition of secondary responses*. J Immunol, 2005. **174**(9): p. 5382-9.
410. Henry, C.J., et al., *IL-12 produced by dendritic cells augments CD8 $^{+}$ T cell activation through the production of the chemokines CCL1 and CCL17*. J Immunol, 2008. **181**(12): p. 8576-84.

411. Parameswaran, N. and S. Patial, *Tumor necrosis factor-alpha signaling in macrophages*. Crit Rev Eukaryot Gene Expr, 2010. **20**(2): p. 87-103.
412. Franchi, L., et al., *The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis*. Nature Immunology, 2009. **10**(3): p. 241-247.
413. Lange, T., et al., *Size dependent induction of proinflammatory cytokines and cytotoxicity of particulate beta-tricalciumphosphate in vitro*. Biomaterials, 2011. **32**(17): p. 4067-75.
414. Obara, W., et al., *Phase I clinical trial of cell division associated 1 (CDCA1) peptide vaccination for castration resistant prostate cancer*. Cancer Sci, 2017. **108**(7): p. 1452-1457.
415. Miyazawa, M., et al., *Phase II clinical trial using novel peptide cocktail vaccine as a postoperative adjuvant treatment for surgically resected pancreatic cancer patients*. Int J Cancer, 2017. **140**(4): p. 973-982.
416. Migliorini, D., et al., *Phase I/II trial testing safety and immunogenicity of the multi-peptide IMA950/poly-ICLC vaccine in newly diagnosed adult malignant astrocytoma patients*. Neuro Oncol, 2019.
417. Chen, Y., et al., *TLR2 agonist Pam3CSK4 enhances the antibacterial functions of GM-CSF induced neutrophils to methicillin-resistant Staphylococcus aureus*. Microb Pathog, 2019. **130**: p. 204-212.
418. Spohn, R., et al., *Synthetic lipopeptide adjuvants and Toll-like receptor 2--structure-activity relationships*. Vaccine, 2004. **22**(19): p. 2494-9.
419. Basto, A.P. and A. Leita, *Targeting TLR2 for vaccine development*. J Immunol Res, 2014. **2014**: p. 619410.
420. Omueti, K.O., et al., *Domain exchange between human toll-like receptors 1 and 6 reveals a region required for lipopeptide discrimination*. J Biol Chem, 2005. **280**(44): p. 36616-25.
421. Akazawa, T., et al., *Adjuvant engineering for cancer immunotherapy: Development of a synthetic TLR2 ligand with increased cell adhesion*. Cancer Sci, 2010. **101**(7): p. 1596-603.
422. Reiser, M.L., et al., *The TLR2 Binding Neisserial Porin PorB Enhances Antigen Presenting Cell Trafficking and Cross-presentation*. Sci Rep, 2017. **7**(1): p. 736.
423. Khan, S., et al., *Chirality of TLR-2 ligand Pam3CysSK4 in fully synthetic peptide conjugates critically influences the induction of specific CD8+ T-cells*. Molecular Immunology, 2009. **46**(6): p. 1084-1091.
424. Azuma, M., et al., *The peptide sequence of diacyl lipopeptides determines dendritic cell TLR2-mediated NK activation*. PLoS One, 2010. **5**(9).
425. Jin, M., et al., *Toll-like receptor 2-mediated MAPKs and NF-kappaB activation requires the GNAO1-dependent pathway in human mast cells*. Integr Biol (Camb), 2016. **8**(9): p. 968-75.
426. Hamley, I.W., et al., *Toll-like receptor agonist lipopeptides self-assemble into distinct nanostructures*. Chem Commun (Camb), 2014. **50**(100): p. 15948-51.
427. Ghasparian, A., et al., *Engineered synthetic virus-like particles and their use in vaccine delivery*. Chembiochem, 2011. **12**(1): p. 100-9.
428. Wu, C.C., et al., *A Toll-like receptor 2 agonist-fused antigen enhanced antitumor immunity by increasing antigen presentation and the CD8 memory T cells population*. Oncotarget, 2016. **7**(21): p. 30804-19.
429. Kimbrell, M.R., et al., *Comparison of the immunostimulatory and proinflammatory activities of candidate Gram-positive endotoxins, lipoteichoic acid, peptidoglycan, and lipopeptides, in murine and human cells*. Immunol Lett, 2008. **118**(2): p. 132-41.

430. Noh, S.Y., et al., *Lipoteichoic acid from Lactobacillus plantarum inhibits Pam2CSK4-induced IL-8 production in human intestinal epithelial cells*. Molecular Immunology, 2015. **64**(1): p. 183-189.
431. Zeng, W., et al., *Highly immunogenic and totally synthetic lipopeptides as self-adjuvanting immunocontraceptive vaccines*. J Immunol, 2002. **169**(9): p. 4905-12.
432. Fields, G.B. and R.L. Noble, *Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids*. International Journal of Peptide and Protein Research, 1990. **35**(3): p. 161-214.
433. Li, W., et al., *Multimerization of a proline-rich antimicrobial peptide, Chex-Arg20, alters its mechanism of interaction with the Escherichia coli membrane*. Chemistry & Biology, 2015. **22**(9): p. 1250-1258.
434. Li, W., et al., *The effect of selective D- or Na-methyl arginine substitution on the activity of the proline-rich antimicrobial peptide, Chex1-Arg20*. Frontiers in Chemistry, 2017. **5**(1).
435. Li, W., et al., *C-terminal modification and multimerization increase the efficacy of a proline-rich antimicrobial peptide*. Chemistry – A European Journal, 2017. **23**: p. 390-396.
436. BenMohamed, L., S.L. Wechsler, and A.B. Nesburn, *Lipopeptide vaccines--yesterday, today, and tomorrow*. Lancet Infect Dis, 2002. **2**(7): p. 425-31.
437. Moyle, P.M. and I. Toth, *Self-adjuvanting lipopeptide vaccines*. Curr Med Chem, 2008. **15**(5): p. 506-16.
438. Zaman, M. and I. Toth, *Immunostimulation by synthetic lipopeptide-based vaccine candidates: structure-activity relationships*. Front Immunol, 2013. **4**: p. 318.
439. Ennion, S., S. Hagan, and R.J. Evans, *The role of positively charged amino acids in ATP recognition by human P2X1 receptors*. J Biol Chem, 2000. **275**(45): p. 35656.
440. Miura, K., et al., *Toll-like receptor 2 and palmitic acid cooperatively contribute to the development of nonalcoholic steatohepatitis through inflammasome activation in mice*. Hepatology, 2013. **57**(2): p. 577-89.
441. Huang, S., et al., *Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways*. J Lipid Res, 2012. **53**(9): p. 2002-13.
442. Erridge, C. and N.J. Samani, *Saturated fatty acids do not directly stimulate Toll-like receptor signaling*. Arterioscler Thromb Vasc Biol, 2009. **29**(11): p. 1944-9.
443. Takeuchi, O., et al., *Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins*. J Immunol, 2002. **169**(1): p. 10-4.
444. Okusawa, T., et al., *Relationship between structures and biological activities of mycoplasmal diacylated lipopeptides and their recognition by toll-like receptors 2 and 6*. Infect Immun, 2004. **72**(3): p. 1657-65.
445. Jin, M.S. and J.O. Lee, *Structures of the toll-like receptor family and its ligand complexes*. Immunity, 2008. **29**(2): p. 182-91.
446. Botos, I., D.M. Segal, and D.R. Davies, *The structural biology of Toll-like receptors*. Structure, 2011. **19**(4): p. 447-59.
447. Jin, M.S., et al., *Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide*. Cell, 2007. **130**(6): p. 1071-82.
448. Nüsse, O., *Biochemistry of the phagosome: the challenge to study a transient organelle*. ScientificWorldJournal, 2011. **11**: p. 2364-81.
449. Gordon, S., *Phagocytosis: An Immunobiologic Process*. Immunity, 2016. **44**(3): p. 463-475.
450. Shen, Y., et al., *Toll-like receptor 2- and MyD88-dependent phosphatidylinositol 3-kinase and Rac1 activation facilitates the phagocytosis of Listeria monocytogenes by murine macrophages*. Infect Immun, 2010. **78**(6): p. 2857-67.

451. Fang, L., et al., *TLR2 mediates phagocytosis and autophagy through JNK signaling pathway in Staphylococcus aureus-stimulated RAW264.7 cells*. Cell Signal, 2014. **26**(4): p. 806-14.
452. Fatehchand, K., et al., *TLR2 ligands modulate Fcγ receptor expression and function in monocytes (P4397)*. The Journal of Immunology, 2013. **190**(1 Supplement): p. 205.8-205.8.
453. Daigneault, M., et al., *The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages*. PLoS One, 2010. **5**(1): p. e8668.
454. Mogensen, T.H., *Pathogen recognition and inflammatory signaling in innate immune defenses*. Clin Microbiol Rev, 2009. **22**(2): p. 240-73, Table of Contents.
455. Yamazaki, S., et al., *TLR2-dependent induction of IL-10 and Foxp3⁺ CD25⁺ CD4⁺ regulatory T cells prevents effective anti-tumor immunity induced by Pam2 lipopeptides in vivo*. PLoS One, 2011. **6**(4): p. e18833.
456. Yanagawa, Y. and K. Onoe, *Enhanced IL-10 production by TLR4- and TLR2-primed dendritic cells upon TLR restimulation*. J Immunol, 2007. **178**(10): p. 6173-80.
457. Heuking, S., et al., *Fate of TLR-1/TLR-2 agonist functionalised pDNA nanoparticles upon deposition at the human bronchial epithelium in vitro*. J Nanobiotechnology, 2013. **11**: p. 29.
458. Hotze, E.M., T. Phenrat, and G.V. Lowry, *Nanoparticle aggregation: challenges to understanding transport and reactivity in the environment*. J Environ Qual, 2010. **39**(6): p. 1909-24.
459. Garcia-Alvarez, R., et al., *In vivo formation of protein corona on gold nanoparticles. The effect of their size and shape*. Nanoscale, 2018. **10**(3): p. 1256-1264.
460. Carrigan, S.D. and M. Tabrizian, *Reducing Nonspecific Adhesion on Cross-Linked Hydrogel Platforms for Real-Time Immunoassay in Serum*. Langmuir, 2005. **21**(26): p. 12320-12326.
461. Park, E.-J., et al., *Repeated-dose toxicity and inflammatory responses in mice by oral administration of silver nanoparticles*. Environmental Toxicology and Pharmacology, 2010. **30**(2): p. 162-168.
462. Park, Y.-M., et al., *Nanoparticle-Based Vaccine Delivery for Cancer Immunotherapy*. Immune Netw, 2013. **13**(5): p. 177-183.
463. Zhao, H., et al., *The Application of Nanoparticle-Based Drug Delivery Systems in Checkpoint Blockade Cancer Immunotherapy*. J Immunol Res, 2018. **2018**: p. 3673295.
464. Liu, X., et al., *Characterization of a human antibody fragment Fab and its calcium phosphate nanoparticles that inhibit rabies virus infection with vaccine*. PLoS One, 2011. **6**(5): p. e19848.
465. Uskoković, V. and V.M. Wu, *Calcium Phosphate as a Key Material for Socially Responsible Tissue Engineering*. Materials (1996-1944), 2016. **9**(6): p. 434.
466. Li, W., et al., *The Effect of Selective D- or N(alpha)-Methyl Arginine Substitution on the Activity of the Proline-Rich Antimicrobial Peptide, Chex1-Arg20*. Front Chem, 2017. **5**: p. 1.