PEGylated calcium phosphate nano-carriers

Han, Jamie YuLing

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PEGylated Calcium Phosphate Nano-Carriers

Han YuLing Jamie

School of Material Science and Engineering

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Han YuLing Jamie

School of Material Science and Engineering

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the requirement for the degree of Doctor of Philosophy

Abstract

Calcium phosphate (CaP) nanoparticles (NPs) grafted with methoxy poly(ethylene) glycol (mPEG) were studied as carriers in this thesis. The objectives of this thesis are to (1) develop a superior technique in synthesizing CaP NPs to study and compare the effect of protein amount and type on loading efficiency with conventional co-precipitation (co-ppt) method and (2) develop two procedures to chemically graft mPEG onto CaP NPs and determine which is the better technique. Also, the effect of mPEG chain length, in the prevention of protein adsorption was studied to establish the optimal chain length. It was shown that protein-loaded CaP synthesized by the IM technique is more homogenous in morphology and more importantly, has considerably higher loading efficiency as compared to co-ppt. In addition, protein were found to be released continuously for IM samples while for co-ppt samples, a burst release with little or no subsequent release was observed, thus concluding that IM was a superior technique in loading proteins to CaP NPs. Subsequently, CaP NPs were PEGylated via Direct and Linker techniques. The former was found to be better in terms of higher surface density and has relatively lower cumulative percentage mass of protein adsorbed to it in the protein binding tests. It was also found that surface density of mPEG of chain length 2000 grafted onto CaP is 67 % higher as compared to mPEG of chain length 5000, which resulted in lower adsorption of specific and non-specific protein. Cellular uptake studies with mouse leukemic monocyte macrophage cell line RAW 264.7 showed that CaP-isc-mPEG₂₀₀₀ was able to delay protein from adsorbing for up to 12 h while CaP-isc-mPEG₅₀₀₀ was able to do so for only up to 3 h.

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List of Abbreviations

A _v	avogadro number
BET	brunauer– Emmett–Teller
BSA	bovine serum albumin
CaP	calcium phosphate
co-ppt	co-precipitation technique
D _{ave}	average distance between two mPEG chains
DCPD	dicalcium phosphate dehydrate
EPR	enhanced permeation and retention
HA	hydroxyapatite
HMDI	hexamethylene diisocyanate
FCM	flow cytometry
FBS	fetal bovine serum
FESEM	field emission scanning electron microscope
FTIR	fourier-transformed infrared spectroscopy
IgG	human immunoglobulin G
IM	inverse micelles
isc-mPEG2000	isocyanate derived mPEG of molecular weight 2000
isc-mPEG5000	isocyanate derived mPEG of molecular weight 5000
LE	loading efficiency
LIM	liposomes-in-microsphere
LSZ	lysozyme
LUVs	large unilamellar vesicles
MGB	myoglobin

MPS	mononuclear phagocyte system
MLV	large multilamellar vesicles
NP	nanoparticles
OCP	octacalcium phosphate
PCL	poly(caprolactone)
PBS	phosphate buffer solution
PMMA	poly (methylmethacrylate)
PEEK	polyether ether ketone
PEG	poly(ethylene) glycol
mPEG	monomethoxy-capped PEG
PLGA	poly D,L-lactide-co-glycolide
PLA	poly D,L-lactide
PGA	poly(glycolic) acid
PVA	poly(vinyl alcohol)
pHEMA	poly(hydroxyethyl methacrylate)
PPA	pyrophosphoric acid
Rhd	rhodamine 6G
SLN	solid lipid nanoparticles
SUV	small unilamellar vesicles
TCP	tricalcium phosphate
TGA	thermogravimetric analysis
TEM	transmission electron microscopy
VPA	vinyl phosphonic acid
XPS	x-ray photoelectron spectroscopy
XRD	x-ray diffraction
X _c	fraction of crystalline phase

Chapter 1 Introduction

1.1 Background

Cancer is an abnormal condition where a cluster of cells grow uncontrollably and in the process damages healthy cells around it. Cancer can occur in many areas of the body including the breasts, lungs, liver, ovaries and stomach. Conventional cancer treatments and diagnostics involve the use of surgery, catheters, chemotherapy and radiation. Chemotherapy and radiation therapies are generally not localized and in the process of treatment, often harm normal cells thus resulting in side effects like nausea, fatigue, hair loss and pain. Therefore, it is essential to treat cancer locally to increase the efficiency of treatment and eliminate all possible side effects.

Targeted delivery of therapeutic proteins for cancer therapy involving the use of carriers has an enhanced efficacy compared to treatment with naked protein drugs [1]. During transport, the carrier shields the protein from premature degradation or activation due to enzymes activities and physiological conditions, which may otherwise affect its efficiency and even induce side effects. In addition, the use of protein-loaded nano-carriers confer several other advantages including the capability for tissue-specific targeting (active targeting) [2]. With the ability to confine treatment to tumour sites and reducing damage to surrounding healthy cells, it is potentially an improved treatment over conventional ones like chemotherapy and radiotherapy.

Tumour sites have blood vessels that are often dilated and fenestrated with an average pore size of less than a micron [3, 4]. As such, circulating carriers will be able to infiltrate through this leaky tumour vasculature [5]. The poor lymphatic drainage [6] further

enhanced permeation and retention (EPR) effect of nanoparticles (NPs) within the tumour region thus achieving passive targeting [7, 8] and hence improve delivery efficiency. Therefore, the longer the circulation time *in vivo*, the more efficient passive targeting will be. Longer circulation of carriers can be attained when carriers are able to evade detection by opsonin proteins and internalization by the mononuclear phagocyte system (MPS). As such, carriers ought to satisfy three requirements: (1) small in size, in the range of nanometer, to extravagate through blood vessels, tissues and infiltrate tumour structure. (2) Good loading efficiency in encapsulating a variety of drug and protein and dyes for tracking and imaging and (3) encompass properties that prevent adsorption of opsonin proteins and uptake by cells for effective passive targeting.

The choice of material as carrier in a delivery system is essential as the interaction of the carrier with the cargo directly affects its loading efficiency. Furthermore, the feasibility to carry out surface modification on the carrier, for enhanced delivery efficiency has to be taken into consideration. To date, there have been studies on a variety of material systems such as organic materials; lipids [9] and polymers [2], and inorganic materials, such as iron oxide [10] silica [11, 12] and calcium phosphate (CaP) [13, 14] as carriers. Over the years, CaP particles have been studied increasingly for its use as carriers in a delivery system. CaP comes in a variety of forms like hydroxyapatite (HA) and tricalcium phosphate (TCP) and was previously used for protein purification, scaffold and coatings over metallic implants for enhanced biocompatibility. The advantages of using CaP particles for delivery are its biocompatibility, bioresorbability, stability in varying conditions and relative ease of fabrication.

1.2 Problem statement

Conventional methods of loading proteins into CaP or HA using co-precipitation (co-ppt) yield low encapsulation efficiency that is ineffective for delivery. Low encapsulation efficiency is often contributed to by the ease of desorption of protein that has been adsorbed on the surface [15, 16]. The risk of desorption contradicts the function of a carrier – to deliver and to protect loaded agents. One way to resolve this problem of desorption is to encapsulate the protein within CaP NP carriers. The carriers then serve to protect the protein from premature degradation of the therapeutic agents. Loading protein in CaP using co-ppt yields low loading efficiency with a portion of protein adsorbed on the surface. To date, at least to the authors' knowledge, there have been no methodical studies on loading and releasing model proteins using inverse-micelles (IM) techniques. Thus, in this study, the development of IM technique for the synthesis and loading of therapeutic agents in CaP will be carried out for comparison with conventional co-ppt.

CaP's high affinity for absorbing biomolecules works against it as a delivery system. The ability to attract serum proteins [17] such as opsonins C3, C4 and C5 from the complement system [18] and immunoglobulins, would result in detection and internalization by the MPS, hence reducing delivery efficiency [19]. Ideally, the surface of CaP carriers should be modified to prevent opsonin proteins from attaching to it which will result it to be internalized by cells. Therefore, to prevent protein adsorption, the surface of the NPs has to be hydrophilic and able to provide steric hindrance. Numerous studies found that the chain length of poly(ethylene) glycol (PEG) is a vital factor in preventing protein adsorption and the optimum chain length is dependent on the nature of the core material [20-22]. The use of PEG for preventing protein adsorption and cellular uptake has been extensively studied for surface modification on polymeric materials [20, 22], gold [23, 24], titanium [25], silica particles [26, 27]. Several studies have carried out

surface modifications of CaP and HA with inorganic molecules such as hexyl and decyl phosphates, pyrophosphoric acid (PPA) [28], hexamethyldisilazane [29], phenyl phosphonate moieties [30], often with the intention to manipulate surface properties and increase colloidal stability. Surface modification of HA with organic polymers has been investigated using PEG of MW of 1500 [31] and vinyl phosphonic acid (VPA) [32], usually to improve adhesion between the ceramic and polymer composites as well as to enhance protein and cell attachment. Based on the author's knowledge, there have been no systematic studies on surface modification of CaP nano-sized particles by grafting organic PEG to specifically delay protein adsorption and cell internalisation. In addition, the relationship between PEG chain length, amount of protein adsorbed and cell uptake efficiency has not yet been established. The lack of study in this area highlights two essential points, which are to develop and find the best method to graft PEG to CaP and subsequently determine the optimum PEG chain length that is most efficient in preventing and delaying protein adsorption and cell uptake.

1.3 Hypothesis and novelty

This thesis was designed to test two hypotheses:

Primary hypothesis: IM technique is able to attain a higher loading efficiency as compared to loading by conventional co-ppt technique, as protein is loaded in CaP NPs by when it is entrapped in CaP's matrix during precipitation, instead of loading it on their surface.

Secondary hypothesis: Different grafting techniques of CaP NP with PEG would have an impact on cellular uptake, and would expect to be lower than non-PEGylated CaP NP.

As there are no methodical studies on loading and releasing model proteins that use inverse-micelles (IM) technique, the novelty of the first part of this thesis is the development of this method and subsequent loading and release studies. The affinity of CaP with biomolecules such as proteins is evident through many studies. However, the loading efficiency of proteins has been reported to be low [15, 16, 33]. Hence the new technique and the results it yields will provide valuable knowledge to researchers who intend to encapsulate protein in CaP NPs or similar cargo.

The novelty of second part of this thesis is the development of grafting techniques, as well as the study of the effect of grafted PEG chain length of CaP NPs. As no other studies have surface modified PEG onto CaP using these techniques and studied the effect of chain length of PEG grafted on CaP on protein adsorption and cellular uptake. The new knowledge provided in this thesis will help researchers in this field understand the effect of PEG on CaP NPs and possibly open new ideas and application. In this thesis, two PEG grafting techniques are developed for CaP NP and evaluated for their ability to reduce non-specific protein adsorption and subsequently cellular uptake.

1.4 Objectives

In line with the problem statements and hypothesis, it is therefore imperative to develop a technique to synthesize and load therapeutic agents in CaP NPs while leaving their surface available for further modification. It is also essential to develop novel methods for the surface grafting of inorganic CaP NPs with organic mPEG and to optimize this surface modification process.

There are two aspects to this work.

- (1) Developing a superior technique in synthesizing CaP NPs.

- a. Study and compare the effect of protein amount and type on loading efficiency with co-ppt method.
- (2) Developing two procedures to chemically graft mPEG onto CaP NPs.
- a. Study and compare the two grafting techniques (linker method and direct method) to determine which yields greater surface density and protein repellent properties.
 - b. Investigate and compare the effects of mPEG chain length for optimum properties to prevent protein adsorption.
 - c. Study the uptake of dye-loaded CaP NPs and dye-loaded PEGylated CaP NPs by mouse leukaemic monocyte macrophage cell line (RAW 264.7 cells)

1.5 Scope

In order to achieve the objectives, the research work was done within the following scope. In this work, only two synthesis routes were explored to produce protein loaded CaP NPs. The conventional co-ppt method has been used as a reference for comparison with the inverse micelle technique developed. The proteins to be encapsulated are limited to bovine serum albumin (BSA) and lysozyme (LSZ), and the rhodamine 6G (Rhd) dye was used for subsequent uptake studies. BSA and LSZ were chosen for their difference in structure and isoelectric point (IEP) values. The release of the cargo and dissolution studies was carried out in conditions simulating physiological conditions at varying pH.

PEG was chosen over other polymers for the surface modification of CaP due to its hydrophilicity and the thin water film that would resultantly form, preventing the adherence of protein [34-36]. In addition, the chain would provide a steric barrier that prevents protein from attaching to the NPs. With decreased or delayed protein attachment,

there will be delayed recognition by the MPS thus resulting in longer circulation *in vivo* [37]. Monomethoxy capped PEG (mPEG) was chosen to limit the chemical reaction to one end of the chain. The mPEG used in the surface modification study is limited to molecular weights of 2000 Da and 5000 Da. These molecular weights were studied and reported to be optimal for preventing protein adsorption and hence cellular uptake.

With respect to the study of interaction of NPs with proteins, human immunoglobulin G (IgG) and BSA were selected for specific and non-specific protein binding test to study the protein repellent properties of mPEG grafted onto CaP. IgG was chosen as it is one of the opsonin protein in the body and would be a good model. BSA was selected as albumin serum is one of the most abundant plasma proteins in the body. For cellular uptake studies, mouse leukaemic monocyte macrophage cell line (RAW 264.7) was selected as it is a common cell line for *in vitro* studies on MPS.

Chapter 2 Literature review

2.1 Carrier system

2.1.1 Organic material as carriers

Organic materials explored as carriers have been widely studied. Lipid, natural or synthetic polymer particles, such as liposomes, solid lipid nanoparticles (SLN), poly (methyl methacrylate) (PMMA) or poly D,L-lactide-co-glycolide (PLGA) NPs, has been studied as carriers of drugs and DNA. Lipid based and polymeric materials are suitable for such use due to their biocompatibility and biodegradability into non-toxic by-products. Their properties are easily manipulated by varying polymer composition and/or molecular weight. This allows a greater control over the rate of cargo release.

Liposomes are spherical concentric phospholipid bilayers encapsulating aqueous compartments, and were first proposed for drug delivery by Gregoriadis [38-41]. They can be classified based on their size and numbers of lipid bilayers, such as large multilamellar vesicles (LMVs), small unilamellar vesicles (SUVs) or large unilamellar vesicles (LUVs). There are several advantages of using liposomes; they can be formulated into small structures (80-100 nm in size), are able to encapsulate hydrophilic and/or hydrophobic therapeutic agents in the aqueous interior and/or within the bilayer [42] and its surface can be modified to alter its properties [43]. Liposomes have been studied to load and deliver anti-microbials [44], chemotherapeutics [45] as well as DNA and siRNAs [46, 47], insulin and BSA [48-50].

Despite the many advantages, there have been major drawbacks to the use of liposome for targeted delivery due to poor control over its release mechanism, low encapsulation

efficiency and loading precision due to the fact that it have no definite shape and size [51]. In addition, liposomes have poor large-scale manufacturing ability and poor stability during storage [52, 53]. They are also susceptible to disintegration through biochemical attack by high density lipoproteins.

There have been progressions in the development of stabilized liposomes using PEG coating to create long-circulating liposomal carriers [54, 55]. Currently, PEGylated liposomal formulations of doxorubin (known as Doxil ® or Caelyx ®) is approved for clinical use in AIDs-related Kaposi's sarcoma, recurrent and metastatic breast cancer [56]. Studies on ligand-targeted liposome were also carried out using antibodies and growth receptors such as anti-CD20, folic acid, transferrin [43, 57]. Some studies have found that drug loaded liposomes that have been grafted with ligands experienced increased drug delivery efficiency with decreased systemic drug exposure [58, 59]. However, challenges for ligand-targeted liposomes include the retention of the ligand's functionality and the ability of the attached ligands to maintain long circulation that is critical for a carrier during *in vivo* circulation.

Polymer-based systems are considered to be biologically more stable in terms of storage and *in vivo* circulation as compared to lipid-based systems [60]. They possess good targeting ability while the latter is generally more biocompatible. Polymeric NPs may consist of a polymeric matrix as the core or the polymer may be a wall surrounding an organic or aqueous core [61]. Generally, hydrophobic drugs have better solubility and are more readily incorporated in the organic compartments like the polymer matrix or in the organic core as compared to hydrophilic compounds. Some of the more common polymers explored as carriers of protein include gelatins [62], chitosan [63], poly D,L-lactide (PLA) [64], poly(glycolic) acid (PGA), PMMA, polyesters such as PLGA [65]

and poly(caprolactone) (PCL). Synthesis of polymeric NPs can start from either a monomer or from a pre-formed polymer [60].

The advantages of polymer-based systems are that they have excellent pharmacokinetic control, biocompatibility and are able to load and deliver a wide range of therapeutic agents. Moreover, most polymers have good potential for surface modification and can be functionalized onto other types of NPs to alter their surface properties and improve their biodistribution properties. PEG is one polymer that is commonly used to graft onto the surface of NPs via covalent bonds [66] to reduce immunogenicity and phagocytosis of carriers, with the aim to increase delivery efficiency [67, 68]. In fact, there are many polymers that are approved by the Food and Drug Administration (FDA) such as polyether ether ketone (PEEK-OPTIMA®), PLGA and PCL. Several micro-sized polymers loaded with proteins have been approved by FDA, for instance, Leuprolide acetate may be used in treating hormone-responsive cancer like breast and prostate cancer or uterine fibroids using PLGA (Lupron Depot ®) and recombinant human growth hormone encapsulated in PLG microspheres for treatment of growth failure (Nutropin Depot ®).

Both liposomes and polymeric-based systems have excellent properties and drawbacks. Feng and Chien [69] proposed to combine both systems to enhance their advantages and eliminate disadvantages. For instance, the liposomes-in-microspheres (LIM) system is one where proteins are loaded into liposomes prior to encapsulation by polymer. The bioactivity of proteins would be preserved in the liposomes which is in turn stabilised by the polymeric matrix [69]. Another advantage of LIM is its ability to load both hydrophobic and hydrophilic protein for delivery in one particle. However, these systems

are unable to carry radioactive elements for radiotherapy due to their stability problems and sensitivity to changes in pH.

2.1.2 Inorganic carriers

Inorganic particles have also been explored as carriers of proteins, drugs and other biomolecules. Ceramic-based and metallic-based NPs were studied as carriers for instance, silica [11, 70] and gold [71]. Silica is a common inorganic material studied as a potential carrier as it is biocompatible, has low toxicity, is hydrophilic and has good chemical and mechanical stability. Furthermore, its porous structure can be altered to control the diffusion rate of the loaded cargo. In addition, silica will not be affected by varying pH values in terms of swelling or changes in porosity or microbiological attack [72]. Silica particles have been explored as carriers of drugs like ibuprofen and naproxen [70]. Another inorganic material studied as potential carrier is magnesium phosphate. Amorphous magnesium phosphate was loaded with pDNA and achieved an entrapment efficiency of 99 % [73]. However, these NPs are 100-130 nm in size and have a high tendency to aggregate. Gold nano-rods were also studied as carriers for the controlled release of DNA into cells [71]. Thiol-modified EGFP-encoded DNA fragments were modified onto nano-rods. The DNA was released when irradiation caused the transformation of nano-rods to spherical form.

2.2 Calcium phosphate as a carrier

2.2.1 Properties of calcium phosphate

CaP NPs have been explored as a carrier in a delivery system [15, 74, 75] and its excellent biocompatibility, biodegradability and bioactivity [76] makes it a popular choice amongst inorganic material. CaP's has high affinity with biological substances

allows it to be utilized as adsorbents in column chromatography for purification and separation of nucleic acids and proteins [77]. CaP has also been studied as a material for potential drug carriers for sustained release [74, 75, 78, 79] of antibiotics [80], growth factors [75], and even bioimaging agents [81, 82].

There are two vital factors contributing to the efficiency of a carrier. They are the encapsulation efficiency of therapeutic agents and delivery by passive targeting. The high affinity of biomolecules for CaP facilitates the loading of proteins onto CaP NPs through adsorption [83]. However, this works against it for passive targeting of tumours. The ability to attract proteins especially opsonin proteins would result in detection and internalization by the MPS, hence reducing delivery efficiency [19]. The excellent biological reactivity of apatites is contributed to by the ease with which surface HPO_4^{2-} groups can be exchanged with CO_3^{2-} ions or organic species such as RPO_4^{2-} , RSO_4^{2-} and RCOO^- [84], the presence of several forms of P-OH [85] as well as negative and positive sites [86, 87] on the surface that are responsible for the adsorption of mineral and organic species.

2.2.2 Mechanism of formation and degradation of CaP

The exact chemical pathway for the formation of CaP is unknown, however, it is generally acknowledged as having the model of crystal growth via an initial amorphous state [88, 89]. The phases of CaP change from an initial amorphous agglomerate units followed by solid state transformation to dicalcium phosphate dehydrate (DCPD) then to octacalcium phosphate (OCP) and finally to HA at $\text{pH} > 6.7$ [88-90]. Hence, the general mechanism for the formation of HA NPs occurs in several stages [91]. The stages are, (1) homogenous nucleation, (2) aggregation of amorphous CaP particles forming spherical units of 20 nm – 30 nm in size [92], (3) aggregation of spherical particles into chain-like

structures, (4) growth of these structures and (5) secondary precipitation and phase transformation. There are several factors influencing the exact pathway of precipitation including synthesis conditions like ionic concentration, synthesis temperature, aging duration, Ca/P ratio, pH and the presence of additives. The pH affects precipitation by shifting the phosphate ions. As the pH lowers, the phosphate species change from PO_4^{3-} to HPO_4^{2-} to H_2PO_4^- . The more protonated they become, the less favorable precipitation will be.

Similar to the formation mechanism of CaP, there is no exact mechanism on its dissolution. A few dissolution models have been proposed and most are on the basis of slightly acidic or near neutral pH values ($4 < \text{pH} < 8$) [93]. The degradation of CaP materials is pH dependent such that the greater extend of degradation increases pH decreases [94]. Cell mediated degradation of CaP does occur as shown by Frayssinet et al. [95], where internalized CaP NPs located in the cells' acidic lysosomes undergo degradation. The by-products of degradation are PO_4^{3-} and Ca^{2+} ions and this will lead to an increase in concentration of Ca^{2+} ions in the cytoplasm and thus in the mitochondria. Generally, the mitochondria have high capacity for Ca^{2+} uptake and function as a buffer compartment for Ca^{2+} ions coming from the cytoplasmic matrix [96, 97] or as essential precursors for calcification in cells [98]. In certain cases, CaP crystals will be formed inside the mitochondria [99, 100] and eventually lead to mitochondrial malfunction [101], depriving the cell of energy and stopping all metabolic processes resulting in immediate and unconditional cell death.

2.2.3 Loading techniques for CaP NPs

There are a variety of methods to load protein onto CaP NPs; surface adsorption, co-precipitation and using inverse micelles.. BSA [16, 102], myoglobin (MGB) [103] LSZ

[104] and pDNA [14] were loaded in CaP NPs based on the techniques mentioned. These techniques work on a common basis; the interaction of functional groups on proteins and the P-sites (negatively charged) and C-sites (positively charged) on CaP [86, 87].

a. Loading via surface adsorption

Loading of proteins via surface adsorption is a straightforward method which involves physical adsorption of proteins onto pre-synthesized CaP NPs [105]. It works on the basis of interaction between the groups on protein with CaP's surface groups (P-sites and C-sites) [86, 87]. This technique has four main drawbacks; (1) the amount of protein loaded is limited by the CaP NPs' surface area resulting in low loading efficiency. Kandori et al. adsorbed BSA [16] and MGB [103] onto CaP NPs, and achieved low loading efficiency of approximately 5.96 wt% and 0.126 wt% respectively. (2) As protein is loaded onto the surface, it allows desorption due to a lack of strong interactions between the particles and protein, thus resulting in burst release [15]. This contributes to the low loading efficiency which is vital for a carrier. (3) The protein on the surface is susceptible to premature deactivation by enzymes, pH and temperature during delivery *in vivo*, (4) resulting in a reduction of sites available for surface modification when proteins adsorbed onto CaP NPs.

Adsorption of proteins onto CaP NPs is a convenient technique and is one of the earlier methods explored [102, 104, 106] for protein loading. In one study, Boonsongrit et al. studied BSA-loaded HA microspheres via adsorption method encapsulated with PLGA to investigate the effect of this polymer protective layer. The loading efficiency was found to be low at 8.0 wt%. Release studies show that samples without PLGA coating had a burst release of 70 wt% within 0.5 h with no observed release after 24 h. In contrast, the release of BSA loaded HA encapsulated with PLGA polymer was significantly reduced to

approximately 10 wt% with sustained release up to 216 h [15]. In another study, the feasibility of loading pDNA onto CaP was investigated. CaP shell was coated over the adsorbed DNA to protect it from premature degradation within the cytoplasm of the cells or during storage. The formation of the shell was done by co-precipitating CaP over DNA-CaP NPs. Transfection efficiency performed on T-HUVEC (transformed human umbilical vein endothelial cells) in serum-free RPMI1640 was found to increase with the presence of shell [107]. These studies demonstrated that loading by adsorption often result in low loading efficiencies and the ease of desorption of this method and that it is vital to protect the protein loaded onto NPs to increase delivery efficiency.

b. Loading via co-precipitation

Co-precipitation is a another common technique to produce CaP NPs as its size and shape can be accurately controlled [108]. Variations of this technique have been employed to synthesize and load therapeutic agents in CaP NPs, by co-precipitating CaP NPs with proteins. This method works on the basis of interaction between proteins and ions. As protein molecules has negative and positive charged groups, it attracts positive calcium ions and negative phosphate ions [16, 109]. The proteins then acts as templates where nucleation and growth occurs and in the process entrapping the proteins in the CaP matrix [110]. The nuclei that formed on the protein would be stabilized due to lowered interfacial energy [111]. The protein loaded will be located within and on the surface of the CaP NP. The drawbacks of this co-ppt technique are its low loading efficiency, the tendency of protein to be loaded on the surface which in turn causes ease of desorption leading to a burst release. These are unfavorable characteristics for carriers. Protein on the surface reduces the available surface for surface modification too.

Liu et al. [16] precipitated calcium-deficient HA with BSA at varying pH (7.5, 8.5 and 9.5). BSA was added into solution containing phosphate ions prior to adding the solution containing calcium ions. Another sample was made by adding BSA into solution with calcium ions prior to the addition of the solution containing phosphate ions. The BSA loaded via either sequence was found to have a loading efficiency of 4.5 wt% to 17.5 wt% and they experienced a burst release of 1 wt% within 8 h, after which no BSA release was observed. Similarly, in another study [112], BSA was loaded into HA with an efficiency of 18 wt% and its release in PBS of pH 7.2 shows a burst release of 40 wt% and a total of 90 wt% release over 4 days. It was concluded that the burst release was due to the BSA bound to the surface. This shows that loading via co-ppt method is not favourable due to some proteins being bound to the surface of the carriers which then leads to burst release. Thus, this drives the need to develop a better loading technique.

c. Loading via inverse micelles

Another less explored route to load cargo in CaP NPs is by using micelles [113]. Inverse micelle is an aqueous core in an organic phase, stabilized by surfactants. This technique has the ability to produce NPs of uniform shape with controlled size, narrow size distribution [114] with low degree of agglomeration and high chemical purity [115, 116]. It works on a similar basis to co-ppt except that the precipitation process occurs in a smaller environment - in the aqueous core of inverse micelles, in the presence of proteins. The surfactants stabilizing the aqueous core confine the growth of CaP, therefore entrapping proteins in the matrix of CaP NPs. The schematic diagram of the formation of protein-loaded CaP NPs via IM technique is shown in **Fig. 2.1**.

Fig. 2.1: Schematic diagram of the formation mechanism of protein-loaded CaP NPs through IM technique

Bisht et al. [14] and Roy et al. [13] used inverse micro-emulsion technique to load pDNA into CaP NPs. The inverse micelle was bounded by a single surfactant (AOT) in hexane. Bisht et al. achieved 0.588 wt% loading efficiency in CaP NPs, 100-120 nm in size. DNase treatment was done to detect the degradation of DNA and it showed that pDNA that was encapsulated in the matrix of CaP NPs was protected from degradation while the free pDNA degraded. The benefit of this technique is its ability to encapsulate protein within the carrier and not load it on the surface, thus protecting it from degradation and desorption. However, current studies on this loading technique are limited to loading of pDNA only. Moreover, the loading efficiency is still relatively low at 0.588 wt% which is similar to the adsorption and co-ppt loading methods, and more importantly, no release studies has been carried out. Hence, it is essential to further optimize this emulsion technique to improve on the loading efficiency that is vital for a carrier. In addition, it is important to explore the loading of model proteins such as BSA and LSZ for comparison with co-ppt technique.

2.2.4 CaP as carriers of dyes

In addition to loading proteins and DNA, dyes were loaded onto CaP NPs for delivery and imaging purposes. As CaP undergoes dissolution in acidic pH [117], the fluorescence of the released dye would indicate the location of tumours and this allow tracking of the state of CaP carriers and thus giving an indication of cargo delivery. Morgan et al. loaded a variety of organic fluorophores in CaP via reverse emulsion using a single surfactant aided by a nucleating agent. Dissolution studies in varying pH have demonstrated that release of fluorophores is pH-dependent [118]. These results suggest that the CaP/HA NPs is capable of loading proteins, DNAs and even dyes, and were shown to be a promising material that can be used as carriers.

2.3 Release mechanism of proteins from carriers

2.3.1 Protein release from polymer-based carriers

The release mechanism of organic carriers can be broadly classified as: (1) diffusion, (2) degradation, (3) swelling followed by diffusion and (4) active efflux. Polymer-based carriers were explored by many other studies to load and release drugs [61, 119] and proteins [120, 121]. However, keeping within the scope of this thesis, only protein release will be discussed in this thesis. The release mechanism of protein from biodegradable polymers is Primarily based on two routes: protein diffusion through water-filled channels and by matrix degradation [122, 123]. In general, proteins at the surface of the carrier will result in a burst release and in the process forms pores on the surface. More proteins within the matrix are able to diffuse through these pores – diffusion control stage. At the same time, the release medium enters through these pores, filling the carrier's matrix. With time, the polymer chains undergo hydrolytic degradation into shorter chains and

eventually leach out as oligomers and releasing proteins simultaneously, the, protein release is degradation-controlled [124].

2.3.2 Protein release from CaP carriers

The locations of the loaded protein; on the surface or within the matrix, and the amount of protein loaded may influence the release mechanism due to a change in physiochemistry of the material. Proteins can be loaded onto carriers by surface adsorption or entrapment in the CaP carriers. Surface adsorption that involves the attraction of proteins to P and/or C sites on CaP surface, often, experiences burst release [15, 16]. This is due to the competition between the positively and negatively charged ions in the release medium with the adsorbed proteins [87].

On the other hand, proteins loaded within the matrix of the carrier undergo a different release mechanism, depending on the interaction between protein and carrier. Proteins may be loaded in the matrix via two methods; (1) entrapment within the matrix during nucleation and growth [114, 125], and (2) entrapment in the pores of the carrier [126]. For protein loaded in the pores of the carrier, a large burst release is often observed due to proteins on the surface, followed by fast release of proteins [127, 128]. This is a diffusion controlled release as the proteins released were triggered by the release medium that entered the pores. For proteins loaded in the carrier during nucleation and growth, the presence of protein in the carrier's matrix may alter its properties to a certain degree. The presence of impurities such as proteins and, ions in the matrix may cause a change in structure and crystallinity. Leonor et al. [125], among other studies [129-131] explored the effects of proteins incorporated in CaP matrix. It was found that the presence of protein in CaP decreases its crystallinity. It was also reported that the addition of a dopant generates defects and disorders in the HA crystal lattice, thus decreasing the crystallinity

of HA nanocarriers, and increasing dissolution of HA NPs in a physiological environment [112, 132]. The dissolution of HA with reference to Dorozhkin [93] is shown in **equation 1**:

As the pH of the buffer solution decreases, the H^+ concentration in the buffer solution increased, causing the dissolution equilibrium to be shifted towards the right, making dissolution of HA or CaP is faster [105, 112], thus increasing protein release rate.

2.4 Cellular uptake of nano-carriers

Intravenous delivery of cargo in carriers by injecting them into the bloodstream would evoke the immune system to remove the foreign bodies from the body rapidly by MPS [133] or by the complement system. This is unfavorable for a delivery system as it decreases delivery efficiency. The internalization of carriers by cells is known as endocytosis, with one form of endocytosis. Phagocytosis is a specific form of endocytosis and is based on an opsonization process motivated by the film tension theory [134]. In this process, when a foreign material such as a carrier is detected, opsonin proteins such as the complement proteins, IgG, IgM and fibronectin are adsorbed onto the carriers. The opsonin proteins are recognized by specific or non-specific receptors present at the surface of the macrophage plasma membrane, triggering the internalisation of the particle by the cell into an intracellular compartment known as the phagosome. The phagosome is then transported from regions near the cell membrane, subsequently fusing with the lysosome which is ultimately degraded by enzymes and slightly acidic condition ($\sim \text{pH } 4.5$) [135, 136].

2.5 Passive targeting and protein repellent carriers

2.5.1 Mechanism of passive targeting

Passive targeting is the targeting of carriers to a site of interest (tumour) by extravagation through blood vessels, and tissues, and infiltration into the leaky, tumour vasculature. Once within the vasculature, the carriers are retained due to EPR effect. With a higher concentration of carriers in the tumour region, the possibility of interaction and uptake by tumour cells increases so that carriers are able to release their cargo in the cells. In addition, the acidic environment of the tumours can then be exploited to break down the carriers and release their cargo. Kubo et al. [137] utilised a magnetic field to concentrate magnetic liposomes containing drug to tumour sites in mice. It was observed that there was greater anti-tumour activity when the magnetic field was applied to bring the carriers to the sites as compared to without applying magnetic field. In order to carry out passive targeting, the carrier's time in the blood should be increased so as to enhance the chance to reach the tumours. One way to prolong circulation in blood is to prevent detection and internalisation by macrophages which means that the carriers have to avoid opsonin proteins from being adsorbed onto their surface. For this reason, the efficiency of a carrier is largely dependent on its ability to prevent proteins from adsorbing onto its surface.

2.5.2 Mechanism of surface modification for protein repellent properties

Surface modification with polymers can prevent proteins from adsorbing via a combination of two main mechanisms [138, 139]; steric repulsion and the presence of a water film. With a layer of polymer and a water film present at the surface, the mode of adsorption of the protein would change and it can occur by (1) primary adsorption due to the diffusion of proteins through the water film and polymer layer to the underlying surface, and (2) secondary adsorption at the polymer surface due to protein-polymer

interactions [140, 141]. These two mechanisms are inter-related as the layer of hydration and effect of steric repulsion are dependent on the polymer coverage. The polymer coverage that is measured as the surface density is in turn dependent and interlinked to chain length of the grafted polymer. The schematic of the mechanism and relations is shown in **Fig. 2.2**.

Hydration coverage is based on the hydrophilicity of the polymer. A hydrophilic surface would attract water molecules to form a thin layer of water film over the surface. This layer of water molecule minimizes the interfacial free energy thus resulting in a low interaction with environmental proteins [142]. Consequently, it prevents the two mechanisms from occurring as its low interfacial free energy prevents primary adsorption and diffusion of proteins, and also protein-polymer interaction [35, 36]. Therefore, with a reduction of protein adsorption, opsonization and internalisation by cells will be lowered too. Carriers coated with hydrophilic polymers have prolonged half-lives, which is likely due to the decreased opsonization and subsequent clearance by macrophages [143]. Attaching PEG to carriers not only greatly increase the hydration on the surface of the NPs, it protects any loaded protein from enzymatic degradation when they are used for *in vivo* applications [144, 145]. Similarly, Zahr et al. found that grafting hydrophilic PEG decreases cellular uptake by two thirds as compared to unPEGylated or bare NPs [146]. Jeon et al. studied the effect of interaction of varying protein size with polyethylene oxide and concluded that the layer of hydrophilic chains on the NP's surface repels plasma proteins [147] and potentially leads to longer circulation time. It is important to note that the adsorption of proteins onto NPs is also influenced by properties such as the size and the surface charge of nano-sized carriers and varying parameters.

Size of carriers is one of the parameter that influences uptake of NPs. The exact size that is the most efficient in avoiding uptake has not yet been determined. This is probably because there are several factors that influence the degree of protein adsorption. It is essential to note that the parameters apply differently in different material systems of which the effect of NPs' size and its uptake by cells is a good illustration. Silica NPs of 200 nm were found to have the largest uptake dosage to COS-7 cells thus attaining the greatest efficiency for gene transfection as compared to smaller NPs [148]. Similarly polystyrene of 200 nm was observed to have the highest uptake by Caco-2 cells [149]. On the other hand, gold particles of 50 nm was found to have the highest internalization by HeLa cells [24]. While there is no conclusive study with respect to co-relating size and uptake, the general notion is that larger NPs will be cleared at a higher rate by the MPS. Thus, to prevent this problem, agglomeration of NPs must be avoided. As nano-sized particles have high surface area to volume ratio and high surface energy, they tend to aggregate to reduce its surface energy. This a common problem for particles that do not have a stable system, for instance HA, magnesium phosphate [73] and gold NPs.

Fig. 2.2: Schematic of relation between the mechanisms that prevent protein adsorption

The surface charge of the NPs largely influence the *in vivo* clearance rate and cellular uptake by electrostatic interactions since the cell surface is slightly negatively charged. Harush-Frenkel et al. found that positively charged PEGylated PLA NPs were internalized rapidly via the clathrin-mediated pathway [150]. The positively charged surface would have a stronger interaction with the negatively charged cells' surfaces, resulting in a faster cellular uptake compared to neutral and negative surfaces [146, 151]. One solution to prevent aggregation is to alter the surface with polymers. Modifying the surface can lead to a three pronged approach to decreasing protein adsorption and cellular uptake. (1) It can prevent aggregation of the NPs so as to retain its original size, (2) increase its hydrophilicity of the NPs and (3) alter its surface charge. Some common techniques of surface modification include functionalizing the surface of the NPs with hydrophilic polymers. These polymers tend to mask surface charge and provide a steric barrier that prevents adherence of proteins. In addition, it provides stability and prevents aggregation. Some of the more common polymers used to modify surfaces for longer circulation time of NPs are PEG, polaxamers and polyamines [151, 152] with PEG being the more commonly used one.

2.5.3 Polymers studied for protein repellent properties

There are many biocompatible hydrophilic polymers such as PEG, poly(hydroxyethyl methacrylate) (pHEMA), poly(vinyl alcohol) (PVA) and poly(acrylamide) that have been used as coatings to reduce protein adsorption. PEG is the most commonly used polymers and besides having excellent properties such as biocompatibility, amphiphilicity, it is non-toxic and thus can be eliminated by a combination of renal and hepatic pathways. In addition, it has the lowest level of protein adsorption and cellular internalization as compared to other polymers [153]. More importantly, it is approved by FDA for human intravenous, oral and dermal applications.

PEG is a hydrophilic polymer with a polyether backbone and two hydroxyl groups on each end of the chain. The hydroxyl groups are able to chemically react with other functional groups such as isocyanate, amine to form covalent bonds. Polymerisation of ethylene oxide is usually carried out by anionic polymerization. Polymerisation reactions can be altered to obtain a variety of chain lengths with low polydispersities. PEG has the ability to be functionalized with other groups such as bromo, amino, aminoethyl, carboxymethyl, succinimidodisuccinate, tosylate, mesylate, aldehyde, octadecylamine, monopalmitate, stearyloxy derivative of PEG [154, 155]. These PEG derivatives increases the variety of chemical reactions including those that requires mild conditions. An important derivative of PEG is the monomethoxy-capped PEG where the OH group at one end is replaced by a methyl group, thus limiting its reaction with other OH group at the other end of the chain as shown in **Fig. 2.3**. This simplifies the reaction of the PEG and ensures a one to one reaction with the other functional group and is suitable for forming covalent bonds with proteins and lipids [156]. The grafting of PEG onto a surface via covalent bond is more stable as it does not allow the PEG to be displaced by plasma proteins easily [157]. The interactions of complement proteins and cells receptors with PEG is based on the two mechanisms and depends greatly on the surface density which is in turn affected by chain length as seen in **Fig. 2.2**.

2.5.4 Effects of mPEG surface density

The surface density of PEG chains grafted on NPs' surface is a vital parameter that influences the interaction of complement proteins and interactions with cells. In general, the greater the density of PEG chains per area nm^2 , the more efficient it is in preventing protein from being adsorbed and hence uptake by cells [22].

Fig. 2.3: Chemical structure of PEG and mPEG.

Many studies investigate the optimum surface density of PEG, however, similar to the relationship between cellular uptake and size, optimal surface density varies with core material. For instance PEG coated PEG-PLA was studied by Gref et al. [158] and Bazile et al. [159]. The optimum surface density was found to be in the range of 1 to 0.44 chains per nm² and 0.5 to 0.67 chains per nm² respectively. In another study, Shi et al. reported that the optimum PEG surface density for niosomes ranges from 0.53 to 0.45 chains per nm², which is within a range similar to that found by Bazile et al.

2.5.5 Effects of mPEG chain length

The length and conformation of the PEG chains were reportedly able to modify steric effects around the particles consequently changing protein adsorption significantly [20, 160, 161]. Chain length of PEG is an important parameter as it affects the surface density when grafted onto a surface [162]. There has been many reports investigating the optimum chain length of PEG that when covalently grafted can reduce protein adsorption and cell internalisation. An optimal molecular weight of the PEG chain between 2000 Da to 5000 Da has been described in the literature [163] for different material systems. The work of Xu et al. [27] gives evidence that increasing PEG molecular weight from PEG 650 Da to PEG 6000 Da decreased the amount of protein adsorbed; however, the trend exhibited asymptotic behaviour. In a study on grafting silica particles with PEG, it was

observed that PEG of molecular weight above 3000 Da did not change the amount of BSA adsorbed significantly [164]. On the other hand, Szleifer's theory proposed that the attraction of proteins to PEG is insignificant and protein's adsorption is independent once the PEG chain length exceeds ethylene oxide units corresponding to a PEG molecular weight larger than 2200 [165]. Ma et al. investigated the interaction of stealth block copolymer NPs with blood and found that there was no significant change in uptake and protein adsorption for PEG with molecular weight above 5000 Da [166]. Similarly, Gref et al. concluded that the maximum protein-repellent effect corresponded to PEG of 5000 Da [20]. Hence, the lack of consensus for the optimal chain length is mainly due to a variety of factors including size of NPs and the type of core material.

2.6 PEGylation of inorganic NPs

Surface functionalisations of inorganic NPs are often used to achieve colloidal stability and to increase hydrophilicity or hydrophobicity often with the intention to reduce interaction between proteins and cells. Gold NPs [23], silica NPs [12, 167] and HA NPs have been previously studied for such purposes.

Gold NPs were surface modified with thiol-modified PEG of three different chain lengths (900 Da, 1500 Da and 5000 Da) to enhance stability and resistance to protein. Liu et al. [168] found that stability of the gold NPs increased with increasing chain length while there were no difference in peptide adsorption between gold modified with combination of peptide /PEG of 900 Da and peptide/PEG 5000 Da. In another study, gold NPs were grafted with thiol-PEG-TNF (TNF is the tumor necrosis factor to be loaded) for enhanced delivery efficiency. *In vivo* tests with mice showed that the surface modified particles accumulated in MC-38 colon carcinoma tumours with almost no accumulation in the RES

or other organs. The presence of the particle was indicated by the change in colour of the tumour [169].

The OH groups on the surface of silica are able to react very well with isocyanate functional groups and thus, using diisocyanates as a linker, PEG was able to form covalent bonded with it [12]. Bagwe et al. [167] modified the surface of silica NPs with various functional groups like carboxylate, amine, amine/phosphonate, PEG, octadecyl, and carboxylate/octadecyl groups to prevent aggregation as well as to reduce protein adsorption. It was found that silica NPs modified with both carboxylate and octadecyl groups had the least amount of non-specific protein bound to it. Similarly, in another study, silica nano-sized and micro-sized particles were PEGylated by adding PEG of different chain length (3000 Da, 6000 Da and 35000 Da) during the condensation process of TMOS. Results showed that the amount of BSA adsorbed by PEGylated silica NPs was significantly lower as compared to bare silica NPs. Comparing across PEG chain lengths, there was not much difference in terms of BSA adsorption on PEGylated silica NPs grafted with PEG of chain length above 3000 Da. These studies give evidence that PEG can be grafted onto inorganic particles and this would significantly reduce the amount of protein adsorbed onto its surface.

In the case of CaP or HA NPs, the surface Ca^{2+} and P-OH groups can be exploited for reactions to alter surface properties groups. Kandori et al. succeeded in grafting oleylphosphate molecules onto the surface of HA by precipitating calcium hydroxide and phosphoric acid in the presence of disodium oleylphosphate to make it more hydrophobic [170]. HA NPs were coated with silica to modify their surface properties so as to enhance their colloidal stability [171]. Similar to silica systems, diisocyanate linkers were explored for the alteration of HA NPs' surface using their surface OH groups [31]. Liu et

al. grafted PEG of MW 1500 Da to HA NPs' surface using a diisocyanate linker - hexamethylene diisocyanate (HMDI) to prove that grafting can be done. No further tests were carried out to investigate its surface density or its ability to prevent or delay protein from being adsorbed [31]. Thus, surface modification of CaP has not been extensively studied with emphasis to the prevention of and/or its delay of protein adsorption and hence cellular uptake.

Chapter 3 Material and Methods

3.1 Materials

The synthesis of CaP NPs requires calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, Sigma-Aldrich, Germany), ammonium dihydrogenphosphate ($\text{NH}_4\text{H}_2\text{PO}_4$, Sigma-Aldrich, UK), ammonium hydroxide 25% (NH_4OH , J.T. Baker, Inc., USA), cyclohexane 99% (Fisher Chemicals, USA), poly(oxyethylene)₅ nonyl phenol ether (NP5, Sigma-Aldrich, USA), poly(oxyethylene)₉ nonyl phenol ether (NP9, Sigma-Aldrich, USA) were used as received. Ethanol 99% (Merck), phosphate buffer solution (PBS, Sigma-Aldrich, Singapore) and de-ionized (DI) water were used in the washing of synthesized CaP NPs.

Surface modification of NPs requires pyrophosphoric acid (PPA, Riedel-de Haën), hexamethylene diisocyanate (Alfa Aesar), mono methoxy poly(ethylene glycol) 5000 (mPEG₅₀₀₀, Sigma-Aldrich, Singapore), isocyanate derived mono-methoxy poly(ethylene) glycol MW 2000 (isc-mPEG₂₀₀₀), isocyanate derived mono-methoxy poly(ethylene) glycol MW 5000 (isc-mPEG₅₀₀₀) Sigma-Aldrich, Singapore), dibutyltin dilaurate (DBDL, Fluka), anhydrous tetrahydrofuran (THF, Sigma-Aldrich, Singapore), hydrated tetrahydrofuran (THF, Tedia, U.S.A.). Retrieval of samples requires ethanol 99% (Merck), diethyl ether (Sigma-Aldrich, Singapore), 99.98% anhydrous N-N-dimethyl formamide (DMF, Sigma-Aldrich, Singapore). All chemicals were used as received.

Protein used for loading as well for non-specific and specific protein binding test uses bovine serum albumin (BSA, Sigma-Aldrich), lysozyme (LSZ, Sigma-Aldrich, Singapore) and technical grade $\geq 80\%$ human immunoglobulin G (IgG, Sigma-Aldrich,

Singapore) respectively. The proteins were used without further purification. Dye loading uses Rhodamine 6G dye (1 μ l/ml) (Rhd, Fluka, Switzerland).

Cell culture studies involved mouse leukaemic monocyte macrophage cell line RAW 264.7 (ATCC # TIB-71), Dulbecco's modified essential medium (DMEM, Invitrogen Cat. # 11995-065) as media with 10 % fetal bovine serum (FBS, Gemini Cat.# 100-106) (BioWhittaker, U.S.A.), 1 % penicillin/streptomycin (Invitrogen Cat.# 10378-016), 2 mM L-glutamine (Invitrogen Cat.# 10378-016), DAPI nucleic acid stain (DAPI, Invitrogen), 4 % paraformaldehyde (PFA, Sigma-Aldrich, Singapore) and Phosphate Buffer Solution (PBS, Sigma-Aldrich, Singapore).

3.2 Synthesis of CaP NPs

3.2.1 Synthesis and loading of protein and dye in CaP NPS via inverse micelles

An organic solution consisting of cyclohexane (oil phase), surfactants NP5 and NP9 with weight ratio of oil to surfactant at 7:3, and NP5 to NP9 at 1:1, was first prepared and mixed using a magnetic stirrer. Following which, 1 M aqueous $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ solution and 1 M aqueous $\text{NH}_4\text{H}_2\text{PO}_4$ solution were prepared and their pH were adjusted to 11 using ammonium hydroxide. For the loading of proteins or dyes, proteins (20 mg, 30 mg and 60 mg) were added to and dissolved in aqueous $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$. The solutions were each added individually into a separate oil-surfactant mixture to form an inverse micro-emulsion. They were subjected to continuous agitation until homogenous before adding one emulsion to another to react for an hour. Retrieval and purification of CaP NPs were done by re-suspending the emulsion in ethanol (dissolves cyclohexane and surfactants), and mixed using a vortex followed by centrifugation. This cycle was repeated twice to

remove any traces of the oil phase. Following which, this wash cycle was performed again three times, but this time with DI water. The final wash cycle was done twice with PBS (pH 7.4) and once more with DI water to effectively remove any by-products and unloaded protein. The resulting precipitate was freeze-dried (Alpha 1–4 LSC, Christ, Germany) before further characterization.

3.2.2 Synthesis and loading of protein in CaP NPs via co-ppt route

Aqueous solutions of 1 M $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 1 M $\text{NH}_4\text{H}_2\text{PO}_4$ were similarly prepared and their pH were adjusted to 11 using ammonium hydroxide. For the loading of proteins, 20, 30 and 60 mg of proteins were added to and dissolved in aqueous $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$. $\text{NH}_4\text{H}_2\text{PO}_4$ was then added drop-wise to the calcium solution and stirred continuously for 3 h at room temperature. Retrieval and purification of CaP NPs was done by centrifugation and re-suspending the precipitate in DI water and mixed using a vortex followed by centrifugation once again. This cycle was repeated thrice to remove any trace ammonia solution. The resulting precipitate was freeze-dried (Alpha 1–4 LSC, Christ, Germany) before further characterization. The samples were labeled accordingly, but with an asterix at the end to denote co-ppt technique, e.g.: CaP-BSA-20*.

3.2.3 Surface modification of CaP NPs

a. Linker method: Grafting of mPEG onto CaP NPs via PPA and HMDI

PPA was dissolved in acetone to form concentration of 40 mM. CaP powder was oven-dried at 120°C for 48 h. 3 g of CaP powder was mixed with PPA solution under continuous agitation for 5 h at 60°C in a reflux set up, similar to Tanaka et al.[28]. Retrieval of CaP NPs was done by centrifuging and washing twice in acetone, followed by thrice in DI water. The precipitate was then freeze-dried (Alpha 1–4 LSC, Christ,

Germany) before further characterization. CaP-PPA is oven-dried at 120°C for 48 h to remove traces of moisture.

CaP-PPA was added to 9 ml of anhydrous DMF under agitation similar to what has been prepared previously [172]. HMDI followed by DBDL was added to the suspension under continuous agitation at 60°C for 4 h. mPEG was added next under continuous agitation for 12 h. Surface modification reactions were carried out inside a glove-box with an inert N₂ environment.

Retrieval of CaP NPs was done by centrifuging and washing twice in hydrated DMF, followed by twice and thrice in ethanol and DI water respectively. The precipitate was then freeze-dried (Alpha 1–4 LSC, Christ, Germany) before further characterization. CaP-PPA was oven-dried at 120°C for 48 h to remove traces of moisture.

Preliminary studies were carried out with three varying parameters; PPA concentration, mass ratios of HMDI to CaP and mass ratio of mPEG₅₀₀₀ to CaP, to determine the optimum condition for linker technique. PPA concentration of 40 mM, mass ratio of HMDI to CaP of 1:1 and mass ratio of mPEG₅₀₀₀ to CaP of 6:1 was used in this study as it resulted in the highest mPEG₅₀₀₀ surface density grafted.

b. Direct method: Grafting of mPEG onto CaP NPs via isocyanate derived mPEG

CaP is oven-dried at 120°C for 48 h to remove traces of moisture and re-suspend in anhydrous DMF. isc-mPEG₂₀₀₀ was dissolved in anhydrous DMF and added to CaP suspension. DBDL was added to the suspension and was left under constant agitation for 24 h. The isc-mPEG₂₀₀₀ to CaP mass ratio is fixed at 3:1. Steps taken are done inside a

glove-box with an inert N₂ environment. The preparation of CaP grafted with isc-mPEG₅₀₀₀ was carried out similarly as described above. Retrieval, purification and drying of grafted CaP NPs was carried out as described previously.

3.3 Characterisation

3.3.1 Fourier-transformed Infrared Spectroscopy (FTIR)

The samples were analyzed using FTIR (FTIR Spectrum GX, Perkin–Elmer, USA), by first mixing them with oven-dried KBr powder (0.6 mg of sample to 140 mg KBr) before compressing into a pellet. Sample spectra were collected over the range of 4000 cm⁻¹ to 400 cm⁻¹ at a resolution of 4 cm⁻¹. The reported data represents the average of 64 scans for improved signal to noise ratio.

3.3.2 X-ray diffraction (XRD)

The crystallinity of CaP NPs was identified using XRD (XRD-6000, Shimadzu, Tokyo, Japan) with CuK α radiation, operating at 40 kV and 40 mA. Data were collected over θ range from 20° to 55° with a step size of 0.05° and dwell time of 10 s.

3.3.3 Transmission electron microscopy (TEM)

Particle shape and size was characterized using TEM (JEM 2010, JOEL, Tokyo, Japan) at an accelerating voltage of 200 kV. Powder samples were prepared by resuspending a small quantity with ethanol using ultra-sonication in a water bath. A carbon coated copper grid was used to collect the samples from the solution and the images were captured via an in-built camera. Selected-area electron diffraction (SAED) patterns were obtained for 3 samples; CaP and CaP loaded with protein via co-ppt and IM to determine its crystallinity.

3.3.4 Field Emission Scanning Electron Microscope (FESEM)

Morphology of powder samples was analyzed with FESEM (JSM 6340F, JOEL, Tokyo, Japan). Samples were prepared by scattering powder onto carbon tape adhered to a platform, prior to coating with platinum coater (JFC-1600 Auto fine coater JEOL, Japan) for 75 seconds. Micrographs were captured at vacuum condition with working distance of 8 mm, accelerating voltage of 5 kV and emission current of 12 μ A.

3.3.5 Nanosizer

Hydrodynamic sizes of CaP NPs were measured using a NanoSeries Zetasizer (Malvern Instruments, UK). Powder samples were added to 0.1 wt% sodium hexametaphosphate (SHMP) solution to form a colloid via ultra-sonication. A 0.2 μ m cellulose membrane filter was used to remove any contamination before measurement. Results were obtained from data groups of n = 5 and were expressed as mean $\pm$ standard deviation (SD).

3.3.6 Thermogravimetric Analysis (TGA)

TGA (TA-Q500 Thermogravimetric analyzer) was used to heat samples from room temperature to 800°C at a rate of 10°C/min. The relative change in weight with temperature provides qualitative and quantitative information of proteins loaded in the sample or mPEG grafted onto the sample.

Loading efficiency (LE %) was calculated as shown in **equation 2** below [173]:

$$\text{LE (\%)} = (\text{Mass}_{\text{protein in sample}} / \text{Total Mass}_{\text{loaded sample}}) \times 100 \% \quad (2)$$

CaP-PPA-HMDI samples were tested to determine the amount of weight loss prior to grafting mPEG and these values were used to subtract off from the mass of PEGylated samples as in **equation 3** in order to derive the mass of PEG grafted.

$$\% \text{ Mass}_{\text{mPEG grafted}} = \% \text{ mass loss}_{\text{PEGylated sample}} - \% \text{ mass loss}_{\text{sample prior to PEGylation}} \quad (3)$$

The number of mPEG chains grafted on per unit mass of NPs can be derived from corresponding mass of mPEG (found in **equation 3**), molecular weight of mPEG to find the number of moles and dividing it with avogadro number (A_v). With the known number of chain of mPEG grafted per unit mass of PEGylated sample and surface area (m/g) found by nitrogen adsorption analysis (section 3.3.6), the surface density of mPEG grafted and this average distance between two mPEG chains (D_{ave}) can be calculated. The equations are as shown below [172]:

$$\# \text{ chains of mPEG} = [(\% \text{ Mass}_{\text{mPEG grafted}} / 100\%) / \text{Mr mPEG}] \times A_v \quad (4)$$

$$\text{Surface density} = \# \text{ chains of mPEG} / \text{Surface area of CaP} \quad (5)$$

$$D_{\text{ave}} = (1/\text{Surface density})^{0.5} \quad (6)$$

3.3.7 Surface area measurement by Nitrogen adsorption analysis

The specific surface area of CaP NPs was determined by the Brunauer–Emmett–Teller (BET) method using the Micromeritics Surface area analyzer (ASAP 2000, USA). Nitrogen (N_2) adsorption–desorption isotherms were measured after degassing the samples at 200°C for 4 h.

3.3.8 X-ray photoelectron spectroscopy (XPS)

The surface elemental analysis of protein-loaded CaP NPs was analysed by XPS (VG ESCALab 220i-XL). Samples were scanned using Al K Alpha source with spot size of 400 μm and energy step size of 0.1 eV. The information depth is 5-10 nm and the take-off angle used is 50° with respect to the surface normal. A survey scan is done with binding energy (BE) 0 eV to 1300 eV. The corresponding BE of the peaks produced are then identified by matching it with the table of known BEs of elements. Advantage software is used to carry out curve fitting and thus determine the atomic concentration percentage. The element of interest for CaP and protein-loaded CaP NPs is nitrogen which is present in proteins but not in pure CaP NPs.

3.3.9 Protein release studies

Protein-loaded CaP NPs were suspended in PBS at different pH values of 5, 6 and 7.4. Samples were kept at 37 °C with mechanical stirring. At specific time points, the suspension was centrifuged at 6500 rpm for 5 min and the supernatant was drawn. BCA protein assay kit (Thermo Scientific Pierce) was used along with UV spectrophotometer (UV-2501, Shimadzu) to obtain the absorbance peak at 562 nm for protein released. Two sets of samples each were prepared and each measurement was performed in triplicates. The amount of protein release was calculated in reference to a pre-determined standard concentration-intensity calibration curve.

3.3.10 Dissolution studies

Inductively coupled plasma emission spectrometer (ICP, PerkinElmer Optima 2000) was employed to determine the concentration of Ca^{2+} ions for dissolution studies of CaP NPs during protein release. The protein-loaded CaP NPs were suspended in PBS pH 5, 6 and

7.4 at 37°C for 1, 3 and 7 days. The suspensions were centrifuged and the supernatant was drawn and filtered using 0.2 µm size filter followed by measurement. Two sets of samples each were prepared and each measurement was performed in triplicates.

3.4 In vitro studies on stealth properties

3.4.1 Protein binding test

BSA and IgG were the protein used in non-specific and specific binding test. Protein was dissolved in DI water to form a solution of 0.5 mg/ml. 5 mg of NPs was added to 5 ml of the solution and were incubated at 37°C for 1, 3, 6, 24 and 48 h. The NPs were centrifuged and the supernatant was drawn and tested using Bicinchoninic acid protein assay kit (BCA, Thermo Scientific Pierce) and subjected to multi-plate reader. The intensity was translated into concentration and amount of protein using a calibration curve of BSA and IgG done prior to conducting this test.

3.4.2 Cellular uptake studies

a. Cell culture

RAW 264.7 cells were used to study the phagocytic uptake of the NPs. Cells were cultivated in DMEM supplemented with 10 % FBS and 1 % penicillin–streptomycin at 37°C in a humidified environment of 5 % CO₂. The medium was replenished every other day and cells were sub-cultured after reaching confluences. For quantification and imaging of particle uptaken by cells, Rhd dye was loaded in Cap NPs via the IM route as described in 3.2.1. The same Rhd-loaded CaP NPs undergo PEGylation as described in 3.2.3b.

b. Quantification of particle cellular uptake studies using flow cytometry (FCM)

Cells were seeded in 24 well plate, upon reaching confluence, the culture medium was replaced with 40 μ l of each of the following suspension CaP-Rhd, CaP-Rhd-mPEG₂₀₀₀ and CaP-Rhd-mPEG₅₀₀₀ and fresh media were added to reach a final volume of 1.5 ml per well and incubated for 1, 3, 6, 12 and 24 h. At each time point, media was removed and the well washed 3 times with PBS. Cells were trypinised, diluted with media and centrifuged. Cells were re-suspended in PBS and subjected to FCM Calibur (Beckton Dickinson) with Argon-ion laser. 488 nm excitation wavelength and collected emission through 560 nm to 640 nm band pass filter were used.

c. Cellular uptake studies using confocal microscopy

Cells were seeded on confocal glass slips in a 6 well plate at a density of 300 000 cells/well. 40 μ l of NP suspension were added to the well, media were added to reach a final volume of 3 ml per well and incubated for 1, 3, 6, 12 and 24 h. At each time point, media was removed and the well washed 3 times with PBS. Cells were fixed with 4 % PFA and washed with PBS three times and mounted onto glass slides using anti-fade mounting reagent. Confocal microscopy (Carl Zeiss LSM 510 META upright) was used to capture images.

3.5 Statistical analysis

Statistical analyses were carried out on nanosizer results, specific and non-specific protein binding tests as well as FCM. F-test (two sample variance) was carried out first to determine to test with equal or unequal variances for student's t-test. The two-sample student's t-test was used to determine statistical significance between pairs of results with $p < 0.05$ considered to be significant.

Chapter 4 Utilizing inverse micelles to synthesize Calcium phosphate nanoparticles as protein carriers

The use of CaP NPs as carriers for targeted delivery of therapeutic proteins is highly feasible due to its many advantages. As CaP is chemically similar to bone mineral, it has excellent properties such as biocompatibility and bioresorbability. In addition, its high affinity for biomolecules can be exploited for the loading of proteins. As there have been no methodical studies on the loading and release of model proteins using IM techniques, this chapter explores the use of inverse micelles for the synthesis and loading of two model proteins in CaP for comparison with the conventional co-ppt method. The loading efficiency and release profiles of CaP loaded with different protein amounts (BSA and LSZ) via both methods were evaluated to determine which is the more superior technique.

4.1 Results

4.1.1 Characterisation of CaP NPs

FTIR spectra of CaP NPs, with and without proteins, synthesized via IM and co-ppt routes are shown in **Fig. 4.1**. Samples produced from both techniques exhibited similar characteristic phosphate peaks; triply degenerated asymmetric stretching mode of the P–O bond at 1100–1000 cm^{-1} , non-degenerated symmetric stretching mode of the P–O bond at 990–920 cm^{-1} , triply degenerated bending mode of the P–O bond at 603 cm^{-1} and 566 cm^{-1} and double degenerated bending mode of the O–P–O bond at 472 cm^{-1} . These peaks are in agreement with the FTIR of HA as reported by Koutsopoulos [174]. The peaks at 1421 cm^{-1} , 1457 cm^{-1} and 873 cm^{-1} indicate B-type carbonate (CO_3^{2-}) substitution, where CO_3^{2-} substitutes the phosphate positions in the HA lattice [175]. Additional peaks observed for protein-loaded CaP NPs were contributed by the presence of the proteins. For both BSA

and LSZ loaded samples, peaks at 1655 cm^{-1} , 1550 cm^{-1} and 1400 cm^{-1} correspond to amide I (C=O stretching vibration of the amide group coupled to the bending of the N-H bond and the stretching of the C-N bond), amide II band (N-H deformation and C-N stretching vibrations) and amide III (C-N stretching mode and N-H bending mode) respectively [16]. The peaks at 2922 cm^{-1} and 2958 cm^{-1} represent C-H stretching vibrations of $-\text{CH}_2-$ bonds. These peaks therefore show that both BSA and LSZ are loaded regardless of the synthesis method used. However, protein-loaded CaP NPs via co-ppt yielded much weaker amide peaks relative to its corresponding P-O peak at $1100\text{--}1000\text{ cm}^{-1}$ (**Fig. 4.1**). The difference in relative peak intensities suggests that there are more amide bonds present in protein-loaded CaP NPs when loaded via the IM method than when loaded by the co-ppt method (given that the same mass of each samples was subjected to FTIR). As FTIR is a qualitative analysis, the quantification of protein loaded in CaP NPs is to be confirmed using TGA.

TGA was carried out next to ascertain the amount of proteins that were loaded in the CaP NPs. The percentage of BSA and LSZ proteins loaded in CaP NPs through IM ranged from 24.6 wt% to 27.3 wt%, and 12.7 wt% to 15.5 wt% respectively, as shown in **Table 4.1**. Comparatively, LE% was found to be much lower when loaded using co-ppt method. The LE% ranged from 3.4 wt% to 8.0 wt% and 0.5 wt% to 0.7 wt% for CaP NPs loaded with BSA and LSZ respectively. This low LE% of loading protein via co-ppt method was also observed by Liu et al. who precipitated calcium-deficient HA with BSA [16]. These results quantitatively confirmed that the IM method is superior in loading proteins, giving much higher LE %, than the co-ppt method.

Diffraction patterns of CaP NPs synthesized via both methods are shown in **Fig. 4.2**. Regardless of synthesis methods, both yielded identical XRD patterns and exhibited

characteristic XRD patterns of HA, with the peak at 31.8° corresponding to the (211) diffraction plane of HA [174]. The fraction of crystalline phase (X_c) can be evaluated from **equation 7** according to Landi et al. [176]:

$$X_c = 1 - V_{112/300}/I_{300} \quad (7)$$

where I_{300} is the intensity of (300) diffraction peak and $V_{112/300}$ is the intensity of the hollow between (112) and (300) diffraction peaks. The calculated crystallinity of pure CaP NPs synthesized in IM and by co-ppt are 0.21 and 0.50 respectively, showing that CaP NPs synthesized by IM technique had lower crystallinity.

Fig. 4.1: FTIR spectra of CaP loaded with protein (a) BSA and (b) LSZ using IM method and (c) BSA and (d) LSZ using co-ppt method (denoted by *) from 4000 cm^{-1} to 400 cm^{-1}

Fig. 4.2: XRD spectra of CaP loaded with BSA and LSZ proteins synthesized by IM and co-ppt (denoted by *) methods from 20° to 55°

Table 4.1: Size and loading efficiency of CaP NPs loaded with BSA and LSZ via IM and co-ppt methods. Sizer results were obtained from data groups of $n = 5$ and were expressed as mean $\pm$ standard deviation (SD).

Sample	Size (nm) $\pm$ S.D		Loading efficiency (%)	
	IM	co-ppt	IM	co-ppt
CaP	47 $\pm$ 3	53 $\pm$ 8	-	-
CaP-BSA-20	49 $\pm$ 5	59 $\pm$ 4	24.6	3.4
CaP-BSA-30	49 $\pm$ 4	55 $\pm$ 2	25.4	4.5
CaP-BSA-60	48 $\pm$ 2	57 $\pm$ 6	27.3	8.1
CaP-LSZ-20	50 $\pm$ 5	55 $\pm$ 7	12.7	0.7
CaP-LSZ-30	49 $\pm$ 2	58 $\pm$ 3	14.6	0.7
CaP-LSZ-60	48 $\pm$ 4	56 $\pm$ 4	15.5	0.9

Another observation that can be drawn from the XRD spectra is that the crystallinity of CaP NPs generally decreases with protein loading. This is shown from the less defined peaks of the loaded samples as compared to pure CaP NPs. The intensities of peaks (310) (marked with triangles) and (002) (marked with circles) for samples produced by IM were observed to decrease. The presence of proteins during particle formation probably interfered with the crystalline structure, resulting in Ca NPs that were less crystalline, this observation was also supported by Wen et al. [130]. The SAED pattern shown in **Fig. 4.3** indicates that unloaded CaP is more crystalline than protein-loaded CaP. While, protein-loaded CaP produced via co-ppt method with lower loading efficiency is more crystalline than when loaded via IM route. This is analogous with the XRD pattern shown in **Fig. 4.2**.

TEM micrographs of CaP NPs samples are shown in **Fig. 4.4**. CaP NPs synthesized from IM or co-ppt techniques were generally 50 nm in size. CaP NPs produced by IM method were found to have short aspect ratio and were ellipsoidal in shape, while CaP NPs from co-ppt were found to have poor surface morphology and were irregular in shape. As inverse micelles confined particle nucleation and growth, they were able to produce particles that were more regular and homogeneous in shape. Conversely, due to the quick precipitation of CaP NPs through the co-ppt technique, there was less control over particle nucleation and growth, which gave rise to irregular particles, this was also reported previously by Loo et al. [108]. Also, it was observed that the type of protein and amount loaded do not affect particle size and shape. In summary, the IM technique produces ellipsoidal protein-loaded CaP NPs that were more regular and homogeneous in size and shape. The hydrodynamic sizes were also measured and these are summarized in **Table 4.1** for protein loaded CaP NPs produced by IM and co-ppt techniques. Hydrodynamic sizes were approximately 50 nm, which is generally in good agreement

with the TEM micrographs. Statistical analysis showed no significant difference in hydrodynamic sizes for CaP NPs produced by IM or co-ppt methods. Also, there was no significant difference in the hydrodynamic sizes of CaP NPs loaded with and without proteins.

It was of interest to know whether protein was adsorbed onto the particle surfaces for both synthesis techniques. Particles with adsorbed proteins may not be suitable for further surface modification for either passive or active targeting. In addition, protein adsorbed

Fig. 4.3: SAED patterns of unloaded CaP produced by (a) co-ppt (b) IM route and protein loaded CaP (c) CaP-BSA-30* synthesized by co-ppt and (d) CaP-BSA-30 synthesized by IM route

Fig. 4.4: TEM images of (a) CaP, (b) CaP*, and protein-loaded samples fabricated by IM route (c) CaP-BSA-20 and by co-ppt technique (d) CaP-BSA-20*

onto particle surfaces will often lead to desorption and a large initial burst release. In order to affirm the presence of protein on the CaP's surface, XPS analysis was carried out to measure N % on the surface of protein-loaded CaP NPs. **Fig. 4.5** shows the XPS spectra for protein-loaded CaP NPs synthesized by both techniques at N1s peak at binding energy of 402 eV. The corresponding N % for protein loaded CaP is shown in **Table 4.2**. BSA loaded CaP was found to have N % of 1.9 % and 16.9 % when produced by IM and co-ppt respectively. Similarly, for LSZ loaded CaP, the values are 1.0 % and

5.1 % for samples produced by IM and co-ppt respectively. Thus, samples fabricated by IM were found to have considerably lower N % with respect those synthesized by the co-ppt method. These values indicate that an insignificant amount of protein was adsorbed onto the surface of CaP NPs via the IM technique, while the contrary is true for co-ppt samples. This result would therefore suggest that most of the loaded proteins in co-ppt would be on the surface of CaP NPs, while proteins were encapsulated within the CaP matrix for particles synthesized through the IM technique.

Fig. 4.5: XPS spectra of nitrogen scan at 402.2 eV for samples synthesized by IM route (a) CaP, (b) CaP-BSA-20, (c) CaP-LSZ-20 and by co-ppt method (d) CaP-BSA-20* and (e) CaP-LSZ-20*

Table 4.2: N atomic percentage for protein loaded CaP synthesized via two methods.

Sample	N atomic percentage (%)	
	IM	co-ppt
CaP	0	0
CaP-BSA-20	1.9	16.9
CaP-LSZ-20	1.0	5.1

4.1.2 Protein release studies

The protein release profiles for CaP NPs loaded with 20 mg of BSA and LSZ synthesized via both methods are shown in **Fig. 4.6** and **4.7**. Only results of CaP NPs synthesized with 20 mg of proteins are shown, as release profiles are similar regardless of protein amount added to CaP during the synthesis process. From the results, the release profiles are shown to be influenced by the synthesis route (IM vs. co-ppt), the type of protein loaded (BSA vs. LSZ) and the pH of the medium (pH 5 – pH 7.4).

The effect of the synthesis route on release profiles can be observed by comparing **Fig. 4.6** and **4.7**. Samples produced via IM show a smaller initial burst release than those produced via co-ppt for both BSA and LSZ proteins. The initial burst release of the proteins could be due to desorption of surface adsorbed proteins. From **Fig. 4.6a** and **4.6b**, a steady release of proteins was observed for IM samples over up to 18 days, while co-ppt samples had little subsequent release after the initial burst. The release profile of proteins from CaP NPs was also observed to be pH dependent. This can be explained by the stability of CaP NPs at lower pH and from their degree of crystallinity. CaP-BSA-20 (**Fig. 4.6a**) shows a faster release of proteins at pH 5, followed by pH 6 and pH 7.4. It has been reported that amorphous CaP undergoes dissolution in acidic environment [94, 117], which explains the greater extent of dissolution of CaP NPs in PBS at lower pH conditions (pH 5 and pH 6) than at the physiological pH 7.4.

Fig. 4.6: Release profiles in cumulative percentage mass of protein-loaded CaP NPs in PBS pH 5, 6 and 7.4, synthesized by IM technique (a) CaP-BSA-20 and by co-ppt method (b) CaP-BSA-20*

Fig. 4.7: Release profiles in cumulative mass of protein released for protein loaded CaP NPs in PBS 5, 6 and 7.4 synthesized by IM technique (a) CaP-LSZ-20 and co-ppt method (b) CaP-LSZ-20*

As such, the less crystalline nature of CaP NPs synthesized through IM (**Fig. 4.2**) also aids in the release of proteins over time. **Fig. 4.7a** and **4.7b** plots the cumulative amount of LSZ released from IM and co-ppt technique respectively. The amount released was observed to be substantially larger for IM samples than co-ppt samples, which is

attributed to the higher LE% of proteins for IM CaP NPs (**Table 4.1**). The IM technique therefore allows for higher protein loading, thus producing protein carriers that contain higher payloads.

Degradation or dissolution of CaP NPs was characterized by measuring the concentration of Ca^{2+} ions released from the particles into the medium through ICP-MS. The concentration of Ca^{2+} released from CaP-BSA-20 and CaP-BSA-20* in PBS at pH 5, 6 and 7.4 is shown in **Fig. 4.8**. For CaP-BSA-20, the concentration of Ca^{2+} in pH 5 is 9 times and 7 times higher than that in pH 7.4 in days 1 and 7 respectively. The concentration of Ca^{2+} ions released from CaP-BSA-20* when compared to CaP-BSA-20 was considerably lower at each pH and time point. It is therefore evident that CaP NPs were degrading and samples that are less crystalline would degrade faster in an acidic environment as compared to a neutral condition [130, 177]. These results are consistent with the release profiles, and explain for the release of proteins from CaP NPs at different pH conditions.

Fig. 4.8: Concentration of Ca^{2+} present in PBS pH 5, 6 and 7.4 after suspending CaP-BSA-20 and CaP-BSA-20* for 1, 3 and 7 days at 37°C

4.2 Discussion

Proteins can be loaded onto CaP NPs via surface adsorption, co-precipitation and inverse emulsion. As it is important to evaluate synthesis techniques based on material properties and synthesis condition, the summary of current techniques used to produce protein-loaded CaP NPs, their advantages and disadvantages is listed in **Table 4.3**. Surface adsorption requires pre-synthesized nanoparticles to be incubated in protein solution for 48 h to 60 h at controlled temperatures. Surface adsorption is a simple technique to load proteins, however, it has several drawbacks; the proteins on the surface desorbs easily and are susceptible to prematured deactivation by external conditions such as pH and enzyme interaction *in vivo*. Encapsulation of proteins in CaP protects them from external conditions and this can be achieved by using co-ppt and inverse emulsions methods.

Therefore, in this study, a new method of loading protein by encapsulating them within CaP NPs was developed. Its performance was subsequently compared against to the conventional method of loading using co-ppt route. Comparison was made in terms of the synthesis process of loading proteins. Co-ppt technique involves adding protein to calcium or phosphate precursors prior to precipitation. The loading of protein using co-ppt techniques explored by other studies such as Liu et al. [16] involved precipitating HA in the presence of BSA at different pH. This method results in NPs having needle-like morphology (40 -70 nm x 4-12 nm). In another study, Dasgupta et al. [112] loaded BSA into HA by aging BSA in calcium and phosphate precursors for 24 h at room temperature. The resultant NPs were rod-like, of size 37 ± 13 nm x 11 ± 3 nm (measured using TEM). While, Co-ppt method of loading in the current study involved stirring the solution for 3 h at room temperature, yielded NPs with size of approximately 58 nm (measured using nanosizer). In general, proteins loaded onto CaP NPs via co-ppt techniques often result in

needle-like morphology with protein loaded in the particle and on its surface and is consistent with the co-ppt particles synthesized in this study.

For the synthesis of CaP NPs involving inverse micelles, precipitation of calcium, and phosphate ions in the presence of protein occur in a nano-environment as shown in **Fig. 2.1**. The one study that loaded protein in CaP using inverse micelles was conducted by Bisht et al. who loaded of pDNA using inverse micelles. It involves a preparation duration of 12 h and a subsequent synthesis duration of another 12 hours at 4°C [14]. In comparison, the current IM method had the shortest reaction time under room temperature yet it produces protein-loaded NPs of uniform morphology of size 50 nm (measured using nanosizer). The IM technique has shown to be better than the co-ppt technique in the current study as well as other studies in terms of a shorter reaction time, uniform morphology of 50 nm in size, loading of protein within the CaP NPs and thus reduces burst release.

Table 4.3: Comparison of various techniques for loading protein onto CaP particles.

Method	Cargo type	Reaction condition	Advantage	Disadvantages
Surface adsorption	BSA [102] and LSZ [33]	Pre-synthesized HA NPs were added to protein solution at pH 6 and incubated at 15°C for 48 h (LSZ) and 60 h (BSA).	• Ease of loading	• Relatively longer preparation under controlled temperature.
	BSA [15]	HA microsphere were added to BSA solution and incubated at 37 °C under agitation (100 rpm) for 24 h		• Ease of desorption leads to poor release efficiency
	BSA [105]	Firstly, inverse-micelles method that requires more than 24 h to obtain dry CaP NPs was carried out. Size of CaPs was found to be 30 to 42 nm. CaP was then mixed with various		• Protein susceptible to premature deactivation by enzymes, pH and

		concentrations of BSA in PBS of pH 7.5 on a shaker for 6 h at 37 °C.		temperature
Co-ppt	BSA and LSZ (current work)	Different amount of BSA was added to calcium solution prior to precipitating with phosphate precursor at pH 11 and reacted for 3 h at room temperature. Resultant particles were needle-like with length of about 50 nm.		
	BSA [16]	Precipitation occurs at pH 7.5, 8.5 and 9.5) BSA was added to calcium solution prior to precipitating and repeated, this time, adding BSA to phosphate solution. No reaction time was stated. Resultant particles were needle-like with dimension of 4–12 nm in diameter and 40–70 nm measured using TEM.	<ul style="list-style-type: none"> • Ease of loading • Encapsulation protects protein from external premature deactivation by external conditions 	<ul style="list-style-type: none"> • Poor release efficiency
	BSA [112]	BSA solution added to calcium precursor followed by adjustment of pH to 9. Phosphate precursor was then added and left to age for 24 h at room temperature. Protein-loaded NPs had length and width of approximately 37 nm and 11 nm.		
	pDNA [178]	pDNA was added to calcium precursor solution. It was added to phosphate precursor by a pump at a flow rate of 13.4 µl/s while bring mixed by a vortexer at a speed of 500 rpm. Reaction time not stated.		
Inverse micelles	pDNA [14]	In 25 ml of 1M AOT in hexane, 70 µl aqueous solution of 1.36M Calcium chloride and aqueous solution of 2.94 µg of pDNA, were added and stirred for 12 h to form microemulsion A. In	<ul style="list-style-type: none"> • Long synthesis time of at least 24 h. • Synthesis carried out at 	<ul style="list-style-type: none"> • Relative low yield based on µl scale used.

	another 25 ml of AOT solution, 50 μ l of 0.2 M Tris–HCl buffer (pH 7.4), 70 μ l aqueous solution of 0.35 M Na_2HPO_4 and aqueous solution of 2.94 μ g of pDNA were added and stirred for 12 h to form microemulsion B. Both were added at slow rate of 20 drops per min at 4°C and stirred for another 12 h [14].	4°C	
BSA LSZ (current work)	Two surfactants (NP5 and NP9) were first mixed in cyclohexane. Protein was added to Calcium precursor and it was then added to one of the oil-surfactant mixture. Phosphate precursor solution was added to the other oil-surfactant mixture. They were stirred for 30 min before adding them together and stir for 1 h.	<ul style="list-style-type: none"> • Short synthesis time. • Synthesis procedure is carried out at room temperature. 	• Low yield

This study's focus is essentially the development of a technique to enhance protein-loading on CaP NPs and the subsequent release of the protein. Therefore, a comparison of protein-loading capability and release profile between common loading techniques done by different studies is vital. **Table 4.4** shows the LE% and release profiles of respective protein-loading techniques from the current work as well as the studies described in **Table 4.3**. LE% for BSA and LSZ loaded by IM technique was 24.6 wt% - 27.3 wt % and 12.7 wt% - 15.5 wt% respectively with a small initial burst release of 10 wt% followed by slow release for the next 18 days of up to 100 wt%, 60 wt% and 30 wt% in pH 5, 6 and 7.4 respectively. On the other hand, low LE% and burst release of protein were observed for protein loaded by surface adsorption method. Proteins MGB and BSA were adsorbed on micro-sized particles and NPs with LE% of 5.96 wt% and 8.0 wt% respectively [15, 103]. The low LE% is due to the relatively lesser binding sites due to

limited surface area as compared to encapsulation of proteins in the NPs' matrix. There has been no release studies on MGB-loaded HA NPs while for BSA-loaded HA NPs, approximately 10 wt% of BSA was released after 24 h in water, while in physiological buffer solution and 10mM PBS, 70 wt% of loaded BSA within 0.5 h and 80 wt% after 24 h was observed respectively. The ease of desorption is due to the lack of strong interactions between surface binding sites and the protein which allows it to be easily displaced by competitive ions in the medium [15, 87]. In addition, protein on the surface of the NPs is susceptible to premature deactivation due to changes in the environment; temperature, pH and enzymes.

Loading of proteins via co-ppt technique was explored in this chapter for direct comparison with the IM technique. However, for a more thorough examination, co-ppt techniques investigated by other studies to load protein into HA or CaP NPs were included in **Table 4.4**. In this current study of protein-loaded CaP using co-ppt method, it was observed that the maximum LE% was 8.1 wt% and a burst release of 28 wt%, 30 wt% and 40 wt% in pH 5, 6 and 7.4 within 6 h, followed by little subsequent release. The difference in ability to release after the initial burst is due to the slow dissolution rate due to increased crystallinity of the CaP which is in turn due to the amount of protein in the CaP matrix [112, 130]. In other studies that loaded BSA onto CaP using the co-ppt method, Liu et al. [16] had a maximum LE% of 17.5 wt% while Dasgupta et al. [112] had an LE% of 18 wt%. Correspondingly, their release in buffer solution of pH 6 was 1 wt% within the first 8 hours after which no release was observed and in PBS pH 7.2, a 40 wt% burst release within first 4 h and subsequent 90 wt% release in the next 4 days. In general, burst releases of protein followed by little or no release was observed for protein loaded onto CaP NPs by co-ppt method.

Comparing the performance of all techniques in the current work as well as past studies, the IM technique was found to have the highest LE% and it had the smallest burst release regardless of pH with continuous release over the next 18 days for PBS pH 5 and 6, while in pH 7.4, only a small burst was observed. This shows that protein loaded in CaP via the IM technique had the potential to be a carrier for intravenous delivery as it does not release during circulation in blood until it reaches the slightly acidic tumour site. On the other hand, from other studies, low LE% was observed in addition to unfavourable release profiles of relatively large burst release followed by 90 wt% release over 4 days at pH 7.2 [112] or little release of 1 wt% in buffer solution at pH 6 [16]. Thus IE is an enhanced technique of loading protein into CaP NPs.

The uniformity in size and the relatively higher LE% for the IM method could be attributed to the particle formation mechanisms involved. The IM synthesis route is based on reactions between proteins, Ca^{2+} and PO_4^{3-} ions in a nano-environment within the micelles, which were found to be about 50 nm from nanosizer measurements. The micelle therefore acts as a cage that constricts particle growth.

The isoelectric points (IEP) of BSA and LSZ are 4.7 and 11 respectively [179] and at synthesis pH they are negatively and neutrally charged respectively [87, 104]. Therefore, within the micelles, electrostatic attraction will occur between the Ca^{2+} ions and the negatively charged BSA while the LSZ attraction to the ions will be insignificant if any. The micelles containing the Ca^{2+} ions and protein would thus collide, interact and react with those micelles containing PO_4^{3-} ions. With sufficient energy, fusion of the micelles occurs, allowing the ionic species to react in the core and/or at the interface [180]. PO_4^{3-} ions will interact with the Ca^{2+} ions forming CaP NPs through nucleation and growth [114] and the proteins are resultantly entrapped in the CaP NPs matrix. As growth is

confined within the surfactant-bound aqueous core, the particles formed were ellipsoidal in shape, and approximately 50 nm. Due to the short reaction time and presence of proteins, the resultant CaP NPs had low crystallinity (**Fig. 4.2** and **4.3**). This was also similarly observed in previous studies reported by others [117, 181]. **Fig. 2.1** shows the schematic diagram of the formation mechanism of CaP NPs through inverse micelles. On the other hand for co-ppt, proteins are free to move in the large volume of aqueous solution. Oppositely charged ions react to form CaP NPs. Subsequently, protein is loaded through surface adsorption instead of being loaded within the particle matrix. This accounts for the significantly lower LE% for co-ppt as compared to using IM.

LE% was also observed to be dependent on protein type. BSA was observed to have a higher LE% as compared to LSZ and a possible reason for this may be due to the difference in their IEP and structures. Negatively charged BSA has a flexible structure and can alter its conformation to a certain extent [182]. When it is electrostatically attracted to Ca^{2+} sites, its flexibility allows it to conform and accommodate itself onto/into the CaP matrix. On the other hand, neutrally charged LSZ has no attraction to the ions and together with its relatively rigid structure [183] it is not able to change its conformation as easily, thus resulting in a lower LE%.

Table 4.4: Comparison of performance of various techniques to load proteins.

Method	Protein type	Loading efficiency (wt%)	Release profile	Advantage	Disadvantages
Surface adsorption	MGB [103]	5.96	No release study carried out.		• Ease of desorption
	BSA [15]	8.0	Release was carried out in distilled water, psychological buffer solution and PBS at pH	• Ease of loading	leads to poor release efficiency • Protein

			7.4. In water, approximately 10 % of BSA was released after 24 h. 70 % within 0.5 h and 80 % after 24 h in physiological buffer solution was observed. For 10mM PBS, similar release trend was observed.		susceptible to prematured deactivation by enzymes, pH and temperature.
Co-precipitation	BSA [16]	4.5 to 17.5	Release study was carried out in buffer solution (NaOH and C ₆ H ₈ O ₇) at pH 6 stirred at 37°C. 1 wt% within 8 h after which no release was observed	<ul style="list-style-type: none"> • Ease of loading • Encapsulation protects protein from external premature deactivation by external conditions 	<ul style="list-style-type: none"> • Poor release efficiency • Poor loading efficiency.
	BSA [112]	18	Release study was carried out in PBS at pH 7.2. 40 % burst release within first 4 hours. It achieved about 90 % release in the next 4 days.		
	pDNA [178]	Not applicable *	No release study of pDNA carried out.		
Inverse micelles	pDNA [14]	5.88µg per mg of CaP. (Equivalent to 0.588 wt%)	No release study of pDNA carried out.		<ul style="list-style-type: none"> • Low loading efficiency.
	BSA LSZ (current work)	24.6 - 27.3 12.7-15.5	Small burst release of 10 % and slow release for the next 18 days up to 100 %, 60 % and 30 % in pH 5, 6 and 7.4	<ul style="list-style-type: none"> • Higher loading efficiency, • Low burst release • Release occurs at acidic pH and not at physiological pH of 7.4. 	<ul style="list-style-type: none"> • Low yield

*Binding efficiency was calculated instead of loading efficiency, hence, unsuitable for comparison.

Release kinetics was different across different synthesis routes and pH conditions. For samples in pH 7.4, some protein release was observed within the first 6 h, possibly due to surface protein desorption. However, no further release was subsequently observed. It was observed that release kinetics were higher at lower pH than at neutral conditions as reported by Tung [117], Morgan et al. [118] and Wen et al. [130]. The release at lower pH 5 and pH 6 was studied to simulate the release of proteins in the slightly acidic pH conditions that may be found in certain tumours [184]. The system synthesized through IM technique allows a greater release of its payload at lower pH, and can therefore potentially be used for therapeutic exploitation.

CaP NPs synthesized through IM were also observed to have continuous sustained release of proteins over weeks (low pH conditions), while almost no further release was observed for co-ppt samples after their initial burst release. This again confirms that the dissolution of CaP NPs (**Fig. 4.8**), which was aided by the low crystallinity of IM samples, releases the entrapped proteins within the particle matrix. The release results is further supported by a study carried out by Wen et al. [130], it was claimed that BSA cannot be detected without the occurrence of dissolution of CaP crystals in PBS of a neutral pH while a continuous release over 14 days was observed in an acidic PBS. Therefore, from the comparison of results, CaP NPs synthesized through IM were therefore better able to provide a sustained release than co-ppt samples.

4.3 Summary

In this study, protein-loaded CaP NPs were synthesized through IM and co-ppt synthesis techniques. The IM technique allows for the synthesis of CaP NPs of 50 nm of homogenous morphology, whereas irregular CaP NPs were produced by co-ppt. Results also show that the IM technique is more efficient in loading proteins as compared to co-

ppt, due to the entrapment of proteins within the inverse micelles and subsequently in the particle matrix. Protein-loaded CaP NPs have a lower degree of crystallinity, which influences the dissolution rates and ultimately the protein release kinetics. An initial burst release was observed for co-ppt samples, whereas a continuous release of proteins was observed for IM-produced CaP NPs. This study therefore shows the superiority of the IM technique in preparing pH responsive CaP NPs as protein carriers.

Chapter 5 Surface modification of calcium phosphate nanoparticles for enhanced protein repellent properties

Delivery systems based on passive targeting works by delaying opsonin proteins from adsorbing onto the carriers and hence uptake by macrophages. This would then allow the NPs to circulate longer and eventually by EPR effect, accumulate in the tumour's leaky vasculature. CaP's high affinity for absorbing biomolecules makes it attract proteins which work against it as a carrier by attracting opsonin proteins, and thus, it is necessary to modify its surface to prevent or delay proteins from adsorbing onto it. PEG is commonly used for the surface modification of other polymeric materials [20, 22], gold [23, 24], titanium [25], silica particles [26, 27] for this purpose. There have been no systematic studies on surface modification of CaP NPs by covalently attaching organic PEG to specifically delay protein adsorption and cell internalisation. In addition, the relation between PEG chain length grafted onto CaP and its efficiency to prevent protein adsorption and cell uptake has not been established for CaP NPs. Therefore, this chapter strives to develop two methods to graft PEG to CaP NPs and thus determine the best method. In addition, it also aims to find the optimum PEG chain length that yields the most efficiency in preventing and delaying protein adsorption and cell uptake with mouse leukaemic monocyte macrophage cell line. With regards to the linker technique, PEGylated CaP-PPA40 are presented, as preliminary studies show that this PEGylated sample works best.

5.1 Results

5.1.1 Comparing grafting techniques

In **Fig. 5.1**, the FTIR spectra of CaP PEGylated by both methods, shows characteristic CaP peaks. In addition, the presence of mPEG was evident from twin peaks at 2935 cm^{-1} and 2857 cm^{-1} which represented the asymmetrical and symmetrical stretching mode of $-\text{CH}_2-$ respectively. The peaks at 3340 cm^{-1} and 1620 cm^{-1} correspond to primary urethane N-H stretching vibrations and primary urethane NH_2 group deformation vibrations respectively. Peaks at 1580 cm^{-1} , 1471 cm^{-1} , 1331 cm^{-1} and 1254 cm^{-1} are associated with symmetric stretching vibrations of the $-\text{N}=\text{C}=\text{O}$ group, amide II band (N-H deformation and C-N stretching vibrations), amide I band (C=O stretching vibrations) and urethane amide IV band (coupled C-N and C-O stretching vibrations) respectively [172, 185]. These peaks strongly suggested that mPEG was covalently grafted to CaP via urethane bond. The presence of grafted PEG can be affirmed by the presence of translucent layer seen in the TEM micrographs in **Fig. 5.2 a-c**.

The hydrodynamic size and surface density for the PEGylated CaP grafted by the two techniques is shown in **Table 5.1**. The size increased after PEGylation, from 60 nm to 134 and 157 nm for direct and linker technique respectively and is coherent with FESEM micrographs in **Fig. 5.2 d-f**. The surface density of PEG grafted, as determined by **equations 4-6** was found to be 0.8 chains/nm^2 and 0.6 chains/nm^2 for direct and linker technique respectively.

The effectiveness of grafted PEG to delay adsorption of protein was determined by the amount of protein adsorbed in specific and non-specific protein binding tests. The cumulative IgG mass adsorbed on the samples over 48 h (shown in **Fig. 5.3**) ranges from 50 wt% to 70 wt% for CaP NPs. After PEGylation the cumulative protein mass adsorbed

on the NP decreased significantly to the range of 5 wt% - 10.5wt % and 2.5 wt% - 10.3 wt% corresponding to samples with surface density of 0.6 chains/nm² and 0.8 chains/nm² respectively. There is no statistical difference for the amount of protein adsorbed between the two surface densities after 0.5 h. Two trends can therefore be established; (1) the presence of grafted PEG onto NPs reduces the amount of protein adsorbed significantly and (2) PEGylated CaP with higher mPEG surface density results in lower amount of protein adsorbed. Similar trends were observed for non-specific protein binding test using BSA as shown in **Fig. 5.4**. The cumulative protein mass dropped from the range of 19 wt% - 28 wt% for CaP NPs to the range of 0.5 wt% - 3 wt% and 0.6 wt% - 2.5 wt% for PEGylated CaP with the corresponding surface density of 0.6 chains/nm² and 0.8 chains/nm². These tests once again provide evidence that grafted PEG significantly reduces protein being adsorbed onto NPs' surface. Between the two different surface densities, there is no statistical difference in terms of the amount of protein adsorbed. These tests indicate that mPEG grafted on CaP NPs reduces IgG and BSA. This is vital for a carrier as the reduction of serum and opsonin protein adsorbed is an essential criteria for prolonged circulation time for passive targeting [186].

Fig. 5.1: FTIR spectra of CaP, and CaP PEGylated by linker (CaP-PPA40-mPEG₅₀₀₀) and direct (CaP-isc-mPEG₅₀₀₀) techniques from 4000 cm⁻¹ to 400 cm⁻¹

Table 5.1: Size and surface density of PEGylated CaP grafted via the two techniques. Hydrodynamic size results were obtained from data groups of $n = 5$ and were expressed as mean $\pm$ standard deviation (SD).

PEGylation techniques	Samples	Size (nm)	Surface density (chains/nm ²)
-	CaP	60 ± 4	-
Direct	CaP-isc-mPEG ₅₀₀₀	134 ± 4	0.80
Linker	CaP-PPA40-mPEG ₅₀₀₀	157 ± 5	0.60

Fig. 5.2: TEM and FESEM micrographs of (a,d) CaP, (b,e) PEGylated CaP-isc-mPEG₅₀₀₀ by direct method and (c,f) CaP-PPA40-mPEG₅₀₀₀ by linker method.

(a)

(b)

Fig. 5.3: Cumulative mass of IgG adsorbed during the specific protein binding tests over 48 h for CaP and PEGylated CaP of surface density 0.6 chains/nm^2 and 0.8 chains/nm^2 for linker and direct techniques respectively. (a) Comparison between CaP and PEGylated CaP and (b) comparison between surface densities due to different PEGylation techniques. Bars with a blue dot indicate statistical difference.

(a)

(b)

Fig. 5.4: Cumulative mass of BSA adsorbed during the non-specific protein binding tests over 48 h for CaP and PEGylated CaP of surface density 0.6 chains/nm² and 0.8 chains/nm² for linker and direct techniques respectively. (a) Comparison between CaP and PEGylated CaP and (b) comparison between surface densities due to different PEGylation techniques.

5.1.2 Optimising chain length

The optimisation of PEG chain length (of molecular weight 2000 Da and 5000 Da) uses direct techniques to graft due to the comparatively lesser protein adsorbed as shown in the previous section. The FTIR for CaP grafted with mPEG₂₀₀₀ shown in **Fig. 5.5** has similar peaks to that of mPEG₅₀₀₀. As stated in section 5.1.1, the additional peaks at 2935 cm⁻¹, 2857 cm⁻¹, 1254 cm⁻¹, 1620 cm⁻¹ and 1580 cm⁻¹ strongly suggest that mPEG was covalently grafted to CaP via urethane bond. The presence of mPEG layer over CaP is confirmed by the translucent layer seen in the TEM micrographs (**Fig. 5.6**). This layer is measured to be 12 ± 3 nm and 26 ± 6 nm for CaP-isc-mPEG₅₀₀₀ and CaP-isc-mPEG₂₀₀₀ respectively. The hydrodynamic size and surface density of CaP grafted with mPEG₂₀₀₀ via direct method is shown in **Table 5.2**. Sample grafted with PEG of shorter chain length, CaP-isc-mPEG₂₀₀₀ was found to be slightly smaller in size of approximately 122 nm and had a higher surface density of 1.34 chains/nm², while the sample grafted with longer chain length (CaP-isc-mPEG₅₀₀₀) is larger at 134 nm in size with surface density of 0.8 chains/nm² (determined by **equations 4 to 6**). Hence shorter chain resulted in higher surface density.

Table 5.2: Size and surface density of PEGylated CaP grafted via two techniques. Sizer results were obtained from data groups of n = 5 and were expressed as mean ± standard deviation (SD).

PEGylation method	Samples	Size (nm)	Surface density (chains/nm ²)
	CaP	50 ± 2	-
Direct	CaP-isc-mPEG ₅₀₀₀	134 ± 5	0.80
Direct	CaP-isc-mPEG ₂₀₀₀	122 ± 4	1.34

Fig. 5.5: FTIR spectra of CaP, and CaP PEGylated by direct method using mPEG of chain lengths 5000 Da and 2000 Da technique from wavelength 4000 cm^{-1} to 400 cm^{-1}

Fig. 5.6: TEM micrographs of CaP PEGylated by direct method with varying chain length (a) CaP-isc-mPEG₅₀₀₀ and (b) CaP-isc-mPEG₂₀₀₀

(a)

(b)

Fig. 5.7: Cumulative mass of IgG adsorbed during the specific protein binding tests over 48 h for CaP and PEGylated CaP of surface density 1.34 chains/nm^2 and 0.8 chains/nm^2 for direct techniques respectively. (a) Comparison between CaP and PEGylated CaP and (b) comparison between surface densities due to differences in chain length. Bars with a blue dot indicate statistical difference.

(a)

(b)

Fig. 5.8: Cumulative mass of BSA adsorbed during the non-specific protein binding tests over 48 h for CaP and PEGylated CaP of surface density 1.34 chains/nm² and 0.8 chains/nm² for direct techniques respectively. (a) Comparison between CaP and PEGylated CaP and (b) comparison between surface densities due to differences in chain length.

In order to investigate the co-relation of chain surface density and protein adsorption, specific and non-specific protein binding test were carried out on PEGylated CaP shown in Fig. 5.7 and 5.8. For specific protein binding test, (Fig. 5.7), CaP grafted with PEG showed a significant reduction of IgG adsorbed as compared to bare CaP. The percentage mass of IgG adsorbed for bare CaP was found to be 48 wt% at 0.5 h and 70 wt% at 48 h. After PEGylation, this value dropped to 2.5 wt% - 10.3 wt% for CaP-isc-mPEG₂₀₀₀ and 3 wt% - 8.9 wt% for CaP-isc-mPEG₅₀₀₀. Across chain length, CaP-isc-mPEG₂₀₀₀ generally has lower protein adsorption as compared to CaP-isc-mPEG₅₀₀₀, and was found to be statistically lower at the 6 h and 24 h time points.

For non-specific protein binding test (**Fig. 5.8**), over 0.5 h to 48 h, 19 wt% - 28.7 wt% of cumulative BSA mass was adsorbed on bare CaP NPs. The amount adsorbed decreased significantly after PEGylation to 0.5 wt% - 2.2 wt% for CaP-isc-mPEG₂₀₀₀ and 0.6 wt% - 2.5 wt% for CaP-isc-mPEG₅₀₀₀ with no statistical difference between them. The protein binding tests give evidence that grafting PEG onto CaP was able to considerably reduce the amount of protein from being adsorbed onto the surface. Across chain length, CaP grafted with shorter chain length mPEG₂₀₀₀, had higher surface density and was found to have a lower cumulative percentage mass of protein adsorbed for both types of protein.

In order to affirm that PEGylated CaP is able to prevent protein adsorption and hence internalization by cells, cellular uptake studies was carried out. CaP was loaded with Rhod dye prior to PEGylation with mPEG₂₀₀₀ and mPEG₅₀₀₀. The NPs internalized in RAW cells are then able to be quantified and analyzed by FCM and confocal microscopy. The presence of Rhod loaded in CaP NPs was confirmed by the FTIR spectra shown in **Fig. 5.9**. It was observed that the characteristic peaks of CaP are present, in addition to new peaks at 2941 cm⁻¹, 2912 cm⁻¹, 1272 cm⁻¹, 3380 cm⁻¹, 1645 cm⁻¹ and 1533 cm⁻¹. These

peaks correspond to Rhd dye C-H stretching vibrations of $-\text{CH}_3$ bonds, amide I ($\text{C}=\text{O}$ stretching vibration of the amide group coupled to the bending of the N-H bond and the stretching of the C-N bond), amide II (N-H deformation and C-N stretching vibrations), C-O stretching vibration and overlap with C-N stretching aromatic amines respectively.

PEGylated Rhd-loaded CaP NPs were incubated with mouse macrophage cell line, RAW 264.7 over a 24 h period to study the effect of PEGylation and PEG chain length on cell uptake. Uptake of Rhd-loaded NPs by cells would increase the fluorescence of the cells such that the greater the amount of NPs internalized by cells, the higher the fluorescence intensity. FBS in media used during incubations with NPs account for the opsonisation events. FCM measures the fluorescence of a cell population of 10,000, this would be normalized to the intensity of the negative control (auto fluorescence of cells without NPs). **Fig. 5.10** shows fluorescence intensity of the cells after incubating PEGylated CaP NPs with cells, thus indicating internalization of NPs. In general, PEGylated CaP was observed to have a lower cellular uptake as compared to CaP.

Fig. 5.9: FTIR spectra of CaP loaded with Rhd dye using IM method from wavelength 4000 cm^{-1} to 400 cm^{-1}

Fig. 5.10: Flow cytometer analysis on the uptake of CaP and CaP PEGylated with mPEG of different chain length by RAW 264.7 cells. Each measurement is an average of three specimens. Bars with a red dot indicate statistical difference.

Across chain length, CaP-isc-mPEG₂₀₀₀ with higher surface density of 1.34 chains/nm² was observed to have a significantly lower uptake as compared to CaP for up to 12 h, while CaP-isc-mPEG₅₀₀₀ with surface density of 0.8 chains/nm² was found to decrease uptake at up to 3 h. At 24 h, there is no statistical difference between CaP and PEGylated CaP. This quantitative analysis on uptake clearly shows that PEGylated CaP with higher surface density is more efficient in delaying uptake for 12 h as compared to 3 h for the one with lower surface density. This FAC supports results that were obtained from protein binding tests.

Fig. 5.11 shows the confocal microscopic images of RAW 264.7 cells after incubating with CaP and CaP-isc-mPEG₂₀₀₀ NPs at varying time points to study cellular uptake. The images show cells at x-y plane with fixed z section. The nucleus is stained with DAPI and

thus emits blue fluorescence while Rhd dye in the NPs gives red fluorescence. Initially, there was little red fluorescence observed, indicating little uptake. At the third hour, the intensity of red fluorescence is stronger for CaP as compared to the PEGylated CaP-isc-mPEG₂₀₀₀. Beyond third hour, it is hard to detect any differences in fluorescence visually. These images are coherent with the results from FCM (**Fig. 5.10**). The location of the internalized NPs were found to be in the cytoplasm of the cell as observed in the confocal images taken on a fixed x-y plane with varying z-section shown in **Fig. 5.12**.

Fig. 5.11: Confocal microscopic images that show the uptake of (a) CaP and (b) Rhod loaded CaP-isc-mPEG₂₀₀₀ by RAW 264.7 cells over 24 h. Overlay images shows the red fluorescing CaP NPs in the cytoplasm of the cells and blue nucleus of the cells stained by DAPI. The scale bar represents 20µm.

Fig. 5.12: Confocal microscopic images of RAW cell after 6 h of incubating with CaP-isc-mPEG₅₀₀₀ taken along the x-y plane with varying Z-section. The image labeled (1) corresponds to the top of the cell and subsequent label corresponds to each depth into the cell. The scale bar represents 20 μ m.

5.2 Discussion

5.2.1 Comparing grafting techniques

Two techniques were developed to graft mPEG onto the surface of CaP NPs. Both techniques utilize the reaction of the isocyanate group with OH groups on the surface of CaP to form a covalent urethane bond. Surface modification via formation of covalent bonds is stable as it does not allow the PEG to be displaced by plasma proteins [157]. P-OH is believed to be the surface functional group responsible for this reaction [28, 31]. The direct method of grafting uses isocyanate derived mPEG chains to react with surface OH groups of CaP. The linker technique involves the use of PPA to react with P-OH group on CaP to form additional O-PO(OH)₂ groups on the surface with phosphoric acids as the by-products. This was to increase the number of surface OH groups prior to grafting mPEG onto it using HMDI as a linker. The diisocyanate linker formed urethane bonds with both OH groups on the surface and OH groups of the mPEG chain. According to Nelson and Toy et al. [187], CaP has three types of P-OH groups and Tanaka et al. proposed that CaP undergoes reactions as seen in **equations 8 and 9** with PPA as follows [188]:

The FTIR spectrum confirmed that urethane bonds were formed after PEGylation for both techniques. CaP PEGylated by the linker technique has a lower surface density of 0.6 chains/nm² and with a size of 157 nm as compared to the direct technique resultants of 0.8 chains/nm² and a size of 137 nm. The values for surface density suggest that the

Liu et al. who grafted isocyanate onto HA to improve the interface of the HA/polymer composite [31]. The higher surface density of PEGylated CaP via the direct technique would have a stronger steric barrier as well as greater hydration coverage over the surface.

The surface densities of CaP-PPA40-mPEG₅₀₀₀ and CaP-isc-mPEG₅₀₀₀ are 0.6 chains/nm² and 0.8 chains/nm² respectively and these values correspond to an average spacing between two mPEG chains (D_{ave}) of 1.12 nm and 1.29 nm respectively (using **equation 6**). The protein binding tests carried out showed that PEG grafted onto CaP NPs does indeed reduce to a large extent, the cumulative percentage mass of BSA and IgG adsorbed. The protein binding tests show that the layer of PEG can only delay adsorption and uptake but it does not completely prevent protein from being adsorbed onto the NPs. This is probably due to the fact that the particle surface was not entirely covered by PEG chains [191]. This observation can be supported by Osterberg et al. [192] that showed that protein adsorption was strongly dependent on the total surface area that was not covered by a polymer. Furthermore, interactions between proteins and CaP surface that are not protected by PEG are very likely to occur especially since CaP has such a high affinity for biomolecules [83]. The attraction of proteins to CaP NPs is due to the myriad of bonding abilities it has with proteins. CaP has both positive and negative (Ca^{2+} and PO_4^{3-}) sites [85] and its surface HPO_4^{2-} groups can be exchanged with CO_3^{2-} ions or organic species such as RPO_4^{2-} , RSO_4^{2-} and $RCOO^-$ [84]. Proteins express both positive and negative charge groups [193] hence their interactions with CaP NPs can vary as follows [147]; repulsive and attractive ionic interactions, hydrogen bonding; van der Waals (VDW) interactions; hydrophobic interactions; hydration forces; acid–base interactions; and steric repulsion resulting from polymer constriction [147].

The chain length and surface density of the grafted PEG on the surface are two vital parameters for delaying protein adsorption and cellular uptake. These two parameters are inter-related; chain length influences the surface density as depicted in the schematic in **Fig. 2.2**. Protein adsorption is restricted by two mechanisms; hydration coverage and steric hindrance. Upon grafting, water molecules bind loosely to its ether group of the PEG chains and as it folds, it forms coils and in the process, traps water molecules thus increasing the hydration of the CaP's surface. Ultimately, the surface of PEGylated CaP NPs is covered with a layer of water molecules and PEG chains [142, 191]. The layer of water molecule minimizes the interfacial free energy thus resulting in low attraction with the surrounding proteins [34-36]. While steric hindrance refers to the mechanical barrier created by PEG chains that prevent proteins from direct contact with the surfaces of CaP NPs. Therefore for greater efficiency in preventing protein adsorption, PEG has to cover as much CaP surface as possible [141, 192, 194].

PEGylated CaP surface modified with the direct method yields higher surface density and a lower percentage mass of protein being adsorbed. Furthermore, this technique is a one step procedure and thus, it is decided that this method is suitable to carry out the studies on varying PEG chain length.

5.2.2 Comparing chain length

The results in section 5.1.2 give strong evidence that with its higher surface density, PEGylated CaP, when grafted with a shorter chain length of mPEG₂₀₀₀, is more efficient in preventing protein adsorption and cellular uptake. Grafting mPEG₂₀₀₀ increases the surface density by approximately 67 % as compared to mPEG₅₀₀₀. Correspondingly, CaP-isc-mPEG₂₀₀₀ with higher surface density was found to have lower cumulative percentage

mass of protein adsorbed and delayed uptake by RAW cells for 12 h as compared to 3 h for the higher surface density, CaP-isc-mPEG₅₀₀₀.

With a greater surface density, the distance between two mPEG chain is smaller (see **equation 6**) and thus there exists a larger extent of steric barrier and hydration coverage. Previous studies on PEGylation with PEG of 2000 Da found that a distance of 1–1.5 nm [158] and a more narrow range of 1.2–1.4 nm found by Bazile et al. [159] is necessary to prevent protein adsorption. The surface densities of CaP-isc-PEG₂₀₀₀ and CaP-isc-PEG₅₀₀₀ are 1.34 chains/nm² and 0.8 chains/nm², corresponding to 0.86 nm and 1.12 nm (converted using **equation 6**) respectively, exceeding and falling within the range respectively. Although both PEGylated samples have values that are sufficient to provide steric coverage and obstruct proteins from adsorbing, it is apparent from the protein binding tests as well as the cellular uptake studies that higher surface density is more efficient in preventing protein adsorption and thus cellular uptake. Vittaz et al. [195], amongst many others [147, 192], has showed that grafting long PEG chains at a low surface density did not provide good protection against protein adsorption. The results are supported by Szleifer's theory that postulated that the attraction of proteins to PEG is insignificant and that protein adsorption is independent of PEG chain length once it exceeds ethylene oxide units corresponding to a PEG MW larger than 2200 [165].

5.3 Summary

In this study, two methods to graft were developed using P-OH groups on the surface. The linker method uses PPA to increase P-OH group and HMDI as a linker to form urethane bonds with CaP and mPEG. The direct method of grafting uses isocyanate derived mPEG to react with CaP's surface OH. FTIR proved that urethane bonds formed for both methods and TGA shows that the surface density of the PEGylated CaP grafted

via direct method was found to be relatively higher than that for linker method. The possible side reactions of HMDI that may occur during PEGylation probably lowered the efficiency of grafting. Protein binding tests showed that PEGylated samples reduced the amount of proteins adsorbed significantly especially for the sample CaP-isc-mPEG₅₀₀₀ that was produced by direct method as it has higher surface density of PEG. As the direct method is shown to be considerably better in addition to its straightforward procedure, this method is used to graft mPEG₂₀₀₀ to compare and determine the effect of chain length on protein binding tests and hence cellular uptake studies. Both specific and non-specific protein binding tests shows that the shorter mPEG₂₀₀₀ chain having a higher surface density of 1.34 chains/nm², prevents protein from being adsorbed at a larger extent as compared to the longer mPEG₅₀₀₀ chain with surface density of 0.8 chains/nm². Cellular uptake studies with mouse macrophage 264.7 RAW cells were analysed with FCM. It was observed that, with respect to uptake of CaP, CaP-isc-mPEG₂₀₀₀ was able to delay uptake up to 12 h while with the longer chain length, CaP-isc-mPEG₅₀₀₀, uptake can be delayed up to 3 h. This is because shorter chain length has a higher surface density and thus, higher efficiency in preventing protein adsorption and cellular uptake. Therefore, based on the results from this study, it can be concluded that direct method with mPEG chain length of 2000 Da works best for CaP NPs.

Chapter 6 Conclusion and future work

6.1 Conclusion

This project is on the study of PEGylated CaP as nano-carriers. CaP has been explored as carriers previously, but the conventional co-ppt method was found to have low loading efficiency, with some protein being adsorbed on the surface. Furthermore, there is a lack of systematic studies on the loading and release of model proteins. Therefore, this study is motivated to improve the loading efficiency of CaP NPs by developing a better technique to load and synthesize them. The first objective is to develop a technique so as to study and compare the effect of protein amount and type on loading efficiency against the co-ppt method. The results give strong evidence that the current IM technique produced homogenous NPs 50 nm in size, with lower crystallinity and significantly higher loading efficiency as compared to co-ppt. The IM method was able to load proteins within the CaP matrix while proteins were found to be on the surface of CaP when synthesized via co-ppt. The release of proteins occurs when CaP undergoes dissolution at acidic pH with CaP of lower degree of crystallinity having a higher dissolution rate. CaP loaded with proteins via IM was found to be released continuously while burst release was observed for co-ppt samples.

In addition to protein loading, another vital aspect for a carrier is to be efficient in passive targeting, and hence it has to avoid detection and subsequently, clearance by the MPS. The ability to prevent proteins from adsorbing onto CaP is especially important since proteins have a high affinity to CaP. Hence the second objective of this project is to develop and compare two new techniques of grafting mPEG onto CaP in terms of surface density and preventing protein from being adsorbed onto the CaP surface. The sub-objective is to determine the optimum PEG chain length that works best for CaP NPs. The

results show that direct method is relatively better than linker method. In terms of the surface density of mPEG grafted onto CaP, the direct method has a higher value that results in a lower cumulative percentage mass of protein adsorbed. After establishing the better method, the mPEG chain length was varied, and it was found that the surface density of mPEG₂₀₀₀ is 67 % higher as compared to mPEG₅₀₀₀. The higher surface density resulted in less protein adsorption for specific and non-specific protein binding tests. Cellular uptake studies showed that compared to CaP, CaP-isc-mPEG₂₀₀₀ was able to delay protein from adsorption for up to 12 h while CaP-isc-mPEG₅₀₀₀ was able to do so for up to 3 h.

6.2 Future work

The results from the current study on loading proteins into CaP nano-sized carriers and the grafting of mPEG of different chain length can pave the way for more in-depth studies in the future. The areas that could be looked into for future investigation to refine and substantiate the current work will be listed below.

6.2.1 Loading of drug into CaP using inverse micelles

Model proteins BSA and LSZ were used in this study for loading into CaP NPs so as to investigate the efficiency of the current IM method as compared to the conventional co-ppt method. However, this study did not explore the feasibility of loading drugs. It would be ideal to establish CaP NPs as a carrier of proteins as well as drugs and other small molecules. The motivation for this is that a wide range of therapeutic agents currently used for cancer therapy are drugs. Therefore, the study of loading and releasing drugs of different amounts and drugs with varying structures will result in a better understanding of CaP as a carrier.

6.2.2 Modeling of proteins and/or drugs in CaP NPs carriers

Another aspect that is worth looking into is the modeling of CaP as potential carriers. The current work investigated the release profiles and dissolution of protein loaded CaP. The results obtained showed that future work involving the correlation of material properties, such as crystallinity and size, to the release profile and rate of dissolution at varying temperature and pH is feasible. Crystallinity and size of the NPs are relatively easy to measure as compared to the rate of dissolution and release profile especially *in vivo* conditions. Therefore, the knowledge to predict the dissolution rate and hence, release profile of cargo by simply knowing the crystallinity, size and test conditions such as the temperature and pH will be valuable.

6.2.3 Study of bioactivity of proteins loaded and released using inverse micelles

The work presented in this thesis shows that the IM technique is able to load proteins into CaP NPs, and also depicted their release in varying pH was shown. Protein's stability and bioactivity are important as its function depends on its structure. The extent of denaturation (the loss of its native structure) might be affected by various parameters such as the synthesis condition, release condition and duration and method of storing the samples. Therefore, it is essential to investigate the bioactivity of proteins loaded by the IM technique and subsequently, their release in PBS of varying pH values. In this study, BCA protein assay kit determined the amount of protein released and the measurements are not unaffected by the protein's structure. Hence, the use of an enzyme assay kit and/or reverse-phase HPLC (high-performance liquid chromatography) is necessary to measure the protein's level of activity or percentage of the protein's native structure retained respectively.

6.2.4 Grafting mPEG of varying chain lengths

The current study developed, explored and compared two techniques to chemically graft PEG to CaP's surface. In addition, two chain lengths were studied to determine which works best for CaP. The purpose of grafting PEG is to study its ability in preventing protein adsorption so as to reduce cellular uptake. As the scope of grafting CaP with PEG was limited, there were parameters that could not be investigated. Results showed that both techniques worked and hence, is feasible to utilize these methods to explore grafting with PEG of varying length and structure. The examination on PEG chain structure (dendrimers) and a wider range of PEG chain length (below 2000 Da) can be further varied. Specific and non-specific protein binding tests as well as cellular uptake studies can be carried out to optimize the type of PEG that works best for CaP NPs. In addition, the release of proteins from CaP NPs PEGylated with different PEG chain lengths can be explored to investigate the effects of PEG and its chain length on release profiles of the proteins.

6.2.5 Biodistribution of PEGylated CaP NPs

Dye-loaded CaP NPs in the current study were shown to be successfully PEGylated. Protein binding tests as well as cellular uptake confirmed that PEG does indeed reduce and delay protein adsorption. Cellular uptake studies with mouse macrophage cells were analysed with FCM and confocal microscopy and they showed that PEGylation helped reduce cell internalization. The study of bio-distribution of these PEGylated CaP NPs would be a more precise study on the effectiveness of PEG *in vivo* to avoid and delay internalization by MPs. This knowledge would be valuable and can be used to validate the results of this current study.

Chapter 7 References

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Appendix: List of Publications and Conferences

Publications

1. Han, J. Y., Tan, T. T. Y., and Loo, J. S. C., Utilizing inverse micelles to synthesize calcium phosphate nanoparticles as nano-carriers. *J. Nanoparticle Res.*, 2011. 13: p 3441-3454.
2. Han, Y. J., Loo, S. C. J., Phang, N. T., Boey, F., Ma, J., Controlled size and morphology of EDTMP-doped hydroxyapatite nanoparticles as model for 153 Samarium-EDTMP doping. *J. Mater. Sci. – Mater. Med.*, 2008. 19: p 2993-3003.
3. Han, Y. J., Loo S. C. J., Lee, J. and Ma, J., Investigation of the bioactivity and biocompatibility of different glass interface with hydroxyapatite, fluorohydroxyapatite and 58S bioactive glass. *Biofactors*, 2007. 30: p 205-216.

Conferences

1. Joint Symposium on the MSE for 21st Century 2009 (Kyoto): Surface modification of hydroxyapatite nano-particles for protein repellent properties. Jamie Han Y. L and Asst/P Joachim Loo.
2. AsiaNano Conference 2008: Synthesis of Mono-dispersed Hydroxyapatite Nanoparticles using Double Inverse Microemulsion Method. Y. L. J., Han and S. C. J., Loo
3. International Conference on Cellular & Molecular Bioengineering (ICCMB) 2007: Investigation of the bioactivity and biocompatibility of different glass interface with

hydroxyapatite, fluorohydroxyapatite and 58S bioactive glass. Han, Y. L., Loo, S. C. J., and Ma, J.