Hydroxyapatite coated polymer microspheres for drug delivery

Zhang, Zhe

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

HYDROXYAPATITE COATED POLYMER
MICROSPHERES FOR DRUG DELIVERY

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MICROSPHERES FOR DRUG DELIVERY**

ZHANG ZHE

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**SCHOOL OF MECHANICAL AND AEROSPACE
ENGINEERING**

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HYDROXYAPATITE COATED POLYMER MICROSPHERES FOR DRUG DELIVERY

ZHANG ZHE

School of Mechanical and Aerospace Engineering

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List of Abbreviations

BSA	bovine serum albumin
DCM	dichloromethane
DCPD	dicalcium phosphate dehydrate
DDS	drug delivery system
FESEM	field emission scanning electron microscopy
FTIR	fourier transform infrared
HA	hydroxyapatite
OCP	octacalcium phosphate
PAH	polyanhydride
PCL	poly(ϵ -caprolactone)
PGA	poly(glycolic acid)
PLA	poly(lactic acid)
PLGA	poly(lactic-co-glycolic acid)
POE	poly(ortho ester)
PVA	poly(vinyl alcohol)
SBF	simulated body fluid
SDS	sodium dodecyl sulfate
TGA	thermo gravimetric analysis
TTP	pentasodium tripolyphosphate
XRD	X-ray diffraction

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Summary

Hydroxyapatite (HA) can bond with protein via ion interaction and it is dissolvable in body fluid therefore it is of great interest to use as possible drug carrier for controlled release. Processing of HA-coated microspheres to incorporate protein (as drug) has been studied. However, long soaking time, poor drug coverage of the spheres and high pH conditions hinder practical applications.

In this project, a new two-step process is used to prepare HA on polymer microspheres. As step 1, the polymer microspheres are soaked into calcium ion solution for 2 hours, then as step 2, phosphate ions are added to the solution. The concentration of supersaturated solution and soaking time of the microspheres are studied as two essential factors for the HA growth. 1.25mM calcium ion and 0.75mM phosphate ion are identified as the preferred concentration for HA growth on poly(lactic-co-glycolic acid) microspheres. In this process, HA is formed in the first hour of incubation as compared to several days in other processes. A full coverage of plate-like structured HA coating with sharp edge is achieved after only 24 hours.

Two approaches of incorporation of drug into the HA coating are tested. One is to soak the HA-coated microspheres into a drug-containing solution (thus

“drug-soaked HA”) and the other is to add the drug into the HA-forming solution and let the drug co-deposit on the microsphere at the time of HA formation (thus “drug-co-deposited HA”).

As a model drug, bovine serum albumin is used in the test. For “drug-soaked HA”, HA-coated microspheres are soaked in 100, 500 and 1000 μ g/ml protein solution for 12 hours. For “drug-co-deposited HA”, 10, 100, 500 and 1000 μ g/ml protein solutions are mixed respectively into 0.75mM phosphate ion solution and then poured into the calcium solution containing the polymer microspheres and stirred for 24 hours. It is found that the protein in the supersaturated solution can strongly inhibit the nucleation and growth of HA formation. At 1000 μ g/ml, there is no formation of HA. At 10 μ g/ml, very little organic linked structure is observed. At 100 and 500 μ g/ml, clear net-linked structure of HA suggests protein is incorporated in crystal lattice of HA coating.

The drug release profiles show that the “drug-soaked HA” has burst release and a fast second linear stage; the “drug-co-deposited HA” results in a slow linear release right from the beginning (no burst release). The results show that the co-deposition of drug with HA in the coating process provides a possible route for long-term controlled release.

Chapter 1 Introduction

1.1 Background

This section will offer a simple introduction of drug delivery system and the CaP mineral hydroxyapatite.

1.1.1 Drug delivery system

Beginning in the 1960s, the modern view of drug delivery began to touch different functions of biomedical applications. The release rate of the drug could be constant over a long period; in other cases, release could be cyclic during a long period; or it could be triggered by the temperature, pH or other external environmental events. The important purpose for controlling drug delivery is to make drug more effective in clinical treatment while eliminating the potential adverse effects of both under- and overdosing. Typical examples of controlling drug release are taken as slow release of water-soluble drugs, fast release of water-resisting or low solubility drugs, delivery to specific target sites, and simultaneous delivery of two or more drugs.

In these conditions, drug delivery system (DDS) arises not only as protection of the drug, but also as a device to control the drug release for varied requirements of biomedical application. An ideal drug delivery system should be satisfied following conditions: easy to fabricate, strong mechanical property, biocompatible,

convenient to administer and remove, capable of achieving high drug loading efficiency, controllable of release rate and target delivery of drug, and safe regarding non-inflammatory reactions *in vivo*.

Protein pharmaceuticals have advantages in high specificity and activity at relatively low concentrations, compared with small molecular chemical drugs[1]. These features make protein pharmaceuticals necessary for curing human diseases, and especially for tissue repair. However, for protein pharmaceutical delivery device, there are some challenging tasks on loading protein drugs with high efficiency, maintaining bioactivity of the drug in the loading process, and controlling drug release rate at a constant .

1.1.2 Hydroxyapatite

According to the Ca/P ratio and crystal phase, CaPs can be classified to amounts of species. Among all the CaPs, hydroxyapatite (HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is the most stable, the most dense and has the lowest solubility[2]. CaP deposition attained from aqueous solutions by means of a biomimetic method is usually identified as amorphous or less crystallized HA. Octacalcium phosphate (OCP) and dicalcium phosphate dehydrate (DCPD, brushite) are also reported as CaP deposition[2], however, they would quickly transform to stable HA due to their metastable phases in aqueous solution.

Nowadays, HA as a commercial product has been widely used in tissue engineering,

especially in application of new bone formation[3] since it is similar in composition to natural bone. The HA coating applied on different templates such as glass-ceramics, polymer scaffold and metal for implant is well developed. Another point about HA shows it has a high affinity to bind with protein molecules and DNA because of the electrostatic force of positive (Ca^{2+}) and negative (PO_4^{3-}) ions on its surface. This makes HA unfold to new application in chromatographic purification[4] and drug delivery. Bioactive protein molecules such as albumin, growth factor TGF- β 1 and IGF-1, BMP-2 are reported to bond on the surface of HA coating for protein drug release in new bone regeneration treatment[5-8]. Recently, the HA coating has also been applied to a polymer micro/nanosphere template for protein drug delivery. The solutions containing calcium and phosphate ions with high pH is designed to accelerate HA deposition, however, this method is not suitable for most biodegradable polymer microspheres.

1.2 Objective

This project is focused on the preparation of HA coated microspheres under normal physiological temperature and pH by means of the biomimetic method; and release profile of “drug-soaked HA” and “drug-co-deposited HA” coating on polymer microspheres.

1.3 Scope

This project will aim to fulfill the following sections:

1. Preparation of polymer microspheres by the emulsion-evaporation method
2. The effect of the concentration of solution containing Ca and P on HA growth on the surface of polymer microspheres
3. The effect of the incubation time of microspheres on HA growth
4. The effect of drug in the Ca and P supersaturated solution on HA formation
5. The loading efficiency study on “drug-soaked HA” and “drug-co-deposited HA” coating on polymer microspheres
6. The drug release profile of “drug-soaked HA” and “drug-co-deposited HA” coating

Chapter 2 Literature Review

This chapter will provide some useful information on biodegradable microspheres which are very popular used in drug delivery system; and hydroxyapatite as a coating biomaterial on scaffold, film or micro/nanospheres by means of the biomimetic method.

2.1 Microspheres as drug delivery system

Recently, microspheres have become commonly used in drug delivery system because they can be easily administered to some specific target sites through oral supply and injection. There are some advantages of microspheres as a drug delivery device: they can enhance the half-life of the drug, protect the bioactivity of the drug from biodegradation by varied enzymes in the body, improve the absorptivity and solubility of drugs, realize pulse or sustained release, and target the needed tissue.

Biodegradable polymers, either synthetic or natural, are capable of being hydrolyzed into biocompatible small molecular byproducts through chemical or enzyme catalysts and do not induce an inflammatory reaction in the body, therefore they are of great interest in fabrication of microspheres. Table 2.1 shows the list of commercial protein-based biodegradable microspheres and a few

examples of biodegradable polymers used in microsphere preparation are listed in Table 2.2.

Table 2.1 List of proteins-based biodegradable microspheres[1]

Protein/Peptide	Product Name	Company	Application
Leuprolide acetate	Lupron Depot®	Takeda-Abott	Prostate cancer
Recombinant human growth hormone	Nitropin Depot®	Genentech-Alkermes	Growth hormone deficiency
Goserelin acetate	Zoladex®	I.C.I.	Prostate cancer
Octreotide acetate	Sandostatin LAR® depot	Novartis	GH suppression anticancer
Triptorelin	Decapeptyl®	Debiopharm	LHRH agonist
Recombinant bovine somatotropin	Polisac®	Monsanto	To increase milk production in cattle
Buserelin acetate	Suprecur® MP	Aventis	Prostate cancer
Buserelin acetate	Profect® Depot	Aventis	Prostate cancer
Leuprolide acetate	Eligard®	Sanofi-Synthelabo	Prostate cancer
Goserelin acetate	Zoladex®	Astrazeneca	Prostate cancer
Triporelin	Trelstar™ Depot	Pfizer	Prostate cancer
Lanreotide	Somatuline® LA	Ipsen	Acromegaly

Table 2.2 List of biodegradable polymers used in microsphere drug delivery

system

	classification	polymer
natural polymer	protein-based polymers	collagen, albumin, gelatin
	polysaccharides	agarose, alginate, carrageenan, hyaluronic acid, dextran, chitosan, cyclodextrins
synthetic polymer	polyester	poly(lactic acid), poly(glycolic acid), poly(hydroxy butyrate), poly(ϵ -caprolactone), poly(α -malic acid), poly(dioxanones)
	polyanhydrides	poly(sebacic acid), poly(adipic acid), poly(terphthalic acid) and various copolymers
	polyamides	poly(imino carbonates), polyamino acids
	phosphorous-based polymers	polyphosphates, polyphosphonates, polyphosphazenes
	others	poly(cyano acrylates), polyurethanes, polyortho esters, polydihdropyrans, polyacetals

2.1.1 Natural polymeric microspheres

Some natural hydrogel such as alginate and chitosan are widely used to prepare microspheres by means of the ionic cross-linking method due to their good biodegradability, biocompatibility and high mucoadhesive properties[9-25].

2.1.1.1 Alginate

Commercially used alginate is extracted from three kinds of brown algae including *Laminaria hyperborean*, *Ascophyllum nodosum*, and *Macrocystis pyrifera*[26].

Sodium alginate is a water-soluble linear polysaccharide comprised of α -L-guluronic (G-block) and β -D-mannuronic (M-block) acid in alternate bond.

Because of the particular structure of monomer units, the G-block and M-block regions exhibit completely different geometries. Two neighboring G-blocks create a diamond-shaped hole that is ready for conjugating with ions. Gelation of alginate is realized by G-block cross-linked with divalent metal ions such as Ca^{2+} , Sr^{2+} , and Ba^{2+} except Mg^{2+} [27]. The gelation process is mainly achieved by chelation with divalent ions. The Na ions from the G-block exchange with divalent Ca ions, and these G-blocks aggregate to form the egg-box structure shown in Fig 2.1[28].

Fig 2.1 Egg structured alginate gel formed by chelation with Ca^{2+} and chemical structure of polymer chain of two consecutive G-blocks and M-blocks[29]

Some studies demonstrate that the alginate microsphere has large sized pore and shows the physical and chemical instability under high pH environment[30]. These limitations may lead to low drug encapsulation and quick release in drug delivery application.

2.1.1.2 Chitosan

Chitosan is the only cationic biodegradable polymer because of the amide groups in its chemical structure (Fig 2.2). It shows high drug encapsulation and excellently sustained drug release, as well as good bioadhesion properties. The most widely used chitosan is α -chitosan from the chitin of crab and shrimp shell[31]. It forms a gel with the popular ionic cross-linker pentasodium tripolyphosphate (TPP) in a mild aqueous environment. Glutaraldehyde is another agent chosen as a chemical cross-linker reacting with amide groups for gel formation[32]. In contrast to ionic gelation, chitosan microspheres prepared by

chemical cross-linking have high density and low porosity.

Fig 2.2 Chemical structure of chitosan[33]

2.1.2 Synthesized polymeric microspheres

Most synthesized polymers used to prepare microspheres are hydrophobic. Thus, emulsion-evaporation method is commonly chosen for microsphere fabrication. It also shows other advantages in drug encapsulated microsphere process: it can maintain the chemical stability and bioactivity of the drugs, provide high encapsulation efficiency, and control the ideal size distribution of the microspheres.

Among the biodegradable synthesized microspheres, the most commonly investigated microspheres in drug delivery system are the following polymers: poly(ortho ester), polyanhydride, poly(ε-caprolactone), poly(lactic acid), poly(glycolic acid) and poly(lactic-co-glycolic acid).

2.1.2.1 Poly(ortho ester)

Since the 1970s, poly(ortho ester) (POE) as a biodegradable polymer has evolved into four species: POE I, POE II, POE III, and POE IV[34]. The chemical structures of the species are shown in Fig 2.3. Compared to other polyesters that

degrade homogeneously in aqueous solution throughout the polymer matrix, POE with high hydrophobicity and water impermeability performs degradation by surface erosion. The morphology of POE microspheres is shown in Fig 2.4, as reported by David N.N.[35]. Depending on the rate of surface erosion, the drug loaded POE microspheres maintain constant drug release rate without a significant burst release[34]. However, low pH environment could accelerate erosion rate due to the pH-sensitivity of POE, which limits its application in drug delivery system.

Fig 2.3 Chemical structures of four POE species[36]

Fig 2.4 Micrograph of POE microspheres[35]

2.1.2.2 Polyanhydride

Polyanhydride (PAH) is a hydrophobic polymer which linked with hydrolytically anhydride (Fig 2.5). The degradation of PAH is administrated by the polymer monomer composition. Using the emulsion-evaporation method, PAH microspheres are harvested as shown in Fig 2.6[37]. The degradation of PAH depends on the water uptake rate and undergoes surface erosion in aqueous solution, which is the same mechanism as POE, and performs the zero-order drug release profile as the microsphere drug carriers. Since degraded monomer acids are non-mutagenic and non-cytotoxic byproducts, PAH shows the minimal inflammatory reaction *in vivo*. One drawback of PAH is that it is difficult to make storage in anhydrous condition because of the hydrolytic instability of the anhydride bond.

Fig 2.5 Chemical structure of PAH[37]

Fig 2.6 Micrograph of PAH microspheres by phase-contrast light microscopy[38]

2.1.2.3 Poly(ϵ -caprolactone)

Poly(ϵ -caprolactone) (PCL) is a semi-crystalline hydrophobic polymer and its chemical structure is shown in Fig 2.7. Stephen D.K. et al used PCL microspheres for taxol release, and the morphology of drug-load PCL microspheres is shown in Fig 2.8[39]. Due to its high crystalline and hydrophobicity, the degradation of PCL is quite slow, which provides the drug for long-term delivery. A new design developed in blending PCL with other biodegradable polymers to provide opportunities to change drug release rate[40]. There are two steps for biodegradation of PCL-blended microspheres *in vitro* or *in vivo*. At the first beginning, the PCL-blended microspheres maintain their morphology and mass. Degradation of PCL-blended microspheres starts with weight loss due to the continuous chain cleavage and small fragments diffusion out of the polymer matrix. Its byproducts from biodegradation are non-acidic which is good for biomedical application since non-acidic fragments do not induce local pH change.

Fig 2.7 Chemical structure of PCL[41]

Fig 2.8 Micrograph of PCL microspheres[39]

2.1.2.4 Poly(lactic-co-glycolic acid)

Poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) are all in hydrophobic polyester family which biodegraded by hydrolysis of the ester linkages. PLGA is a copolymer which is synthesized by means of random ring-opening polymerization of glycolic acid and lactic acid. The chemical structures of PGA, PLA and PLGA are shown in Fig 2.9.

Fig 2.9 Chemical structure of PGA, PLA and co-polymer PLGA[36]

PLGAs have been used as Food and Drug Administration (FDA) approved therapeutic devices due to its biodegradability and biocompatibility. Depending on the ratio of lactide to glycolide chosen, various PLGAs are harvested. PLGAs are identified in regard to the monomers' ratio: taking PLGA (80:20) as an example, it

is a copolymer comprised with 80% lactic acid and 20% glycolic acid. All PLGAs are amorphous, and show a very low glass-transition temperature in the range of 40~60°C. In comparison with PGA and PLA which show low solubility in some oil solvents, PLGA can be dissolved in most of common solvents such as chlorinated solvents, acetone or ethyl acetate. PLGA as a member of polyester family also degrades as a result of the hydrolysis of the ester linkages in the presence of water which is the same as PLA and PGA. In general, the duration of the PLGA degradation is correlated to the ratio of glycolic acid and lactic acid. The PLGA with higher amount of glycolic acid performs the shorter duration needed for degradation. Therefore, widely used PLGA (50:50) exhibits faster degradation duration. Aside from the ratio of monomers, PLGA end-capped with carboxyl groups illustrates shorter degradation duration than that end-capped with ester groups.

Since the body can effectively deal with monomers of PLGA biodegradation, very minimal systemic toxicity was found to affect the application of PLGA in protein and DNA delivery[42]. One commercially marked drug delivery device by PLGA is Lupron Depot[®], commonly used for the treatment of advanced prostate cancer.

2.2 HA coating on polymer, metal or ceramic by biomimetic method

2.2.1 Simulated body fluid

In 1991, Kububo proposed that the necessary requirement for artificial implants to

adhere with bone cells is the formation of bone-like apatite or HA on their surface[43]. Like apatite formed *in vivo*, it also can be produced in simulated body fluid (SBF), a solution with ion concentrations nearly equal to those of human blood plasma. Table 2.3 shows the ion concentrations in SBF solution and human blood plasma as a reference. Since SBF or concentrated SBF can supply with abundant calcium and phosphate ions, apatite formed on the surface of the implant in SBF solution becomes a classical method for the apatite coating study.

Table 2.3 Ion concentrations of SBF in comparison with those in human blood plasma[44]

Ion	Ion concentrations (mm)	
	Blood plasma	SBF
Na ⁺	142.0	142.0
K ⁺	5.0	5.0
Mg ²⁺	1.5	1.5
Ca ²⁺	2.5	2.5
Cl ⁻	103.0	147.8
HCO ₃ ⁻	27.0	4.2
HPO ₄ ²⁻	1.0	1.0
SO ₄ ²⁻	0.5	0.5
pH	7.2–7.4	7.40

2.2.2 HA deposition by biomimetic method

HA coating deposition by biomimetic method has been studied for quite a few years. It is known as similarity to the body's physiological environments, and it can trigger the deposition of apatite coating on different substrates, including polymer scaffolds, cements and ceramics, metal surfaces and fixation rods. In

order to better understand the coating deposition procedure in aqueous solution, Xiong Lu et al summarized a theoretical analysis of HA coating precipitation[45].

2.2.2.1 Driving force of apatite deposition

The thermodynamic driving force of apatite deposition is obtained based on the free energy change in supersaturated solutions[46-47]:

$$\Delta G = -\frac{RT}{n} \ln(S) = -\frac{RT}{n} \ln\left(\frac{A_p}{K_{sp}}\right), \quad (2.1)$$

where ΔG is the Gibbs energy of apatite (CaP) ionic units in solution; R , the universal gas constant ($8.314 \text{ JK}^{-1} \text{ mol}^{-1}$); T , the absolute temperature; n , the number of ion units in one mole of apatite; S is the ratio of the activity product of ion units of precipitates (A_p) to the corresponding solubility product (K_{sp}), defined as supersaturation.

Among all CaP products formed in the solution, HA is the most stable; other CaPs such as OCP or DCPD formed at the beginning tend to transform into HA. Therefore, only the equation of HA precipitation in aqueous solution is studied here.

The corresponding supersaturation (S) is calculated as follows:

$$S(\text{HA}) = \frac{a^5(\text{Ca}^{2+})a^3(\text{PO}_4^{3-})a(\text{OH}^-)}{K_{sp}(\text{HA})} \quad (2.3)$$

where $K_{sp}(\text{HA})$ equals to 2.35×10^{-59} [48].

The activity coefficient γ_i , of each ion unit can be calculated from the modified

Debye-Hückel equation:

$$\log \gamma_i = -Az_i^2 \left[\frac{I^{1/2}}{1+I^{1/2}} - 0.3I \right] \quad (2.4)$$

where A is the Debye-Hückel constant varying with temperature, 0.5211 at 37°C;

z_i , the charges of ions; I, the total strength of ions in the solution and calculated as

$$I = \frac{1}{2} \sum_i c_i z_i^2 \quad (2.5)$$

where c_i is the mole number of each ion unit.

SBF was often chosen as a source of Ca and P for apatite formation by biomimetic method; the equation for the ionic activity calculations would take in account magnesium and other ions in SBF that could affect HA precipitation [49-51]. In that case, classic theories of thermodynamics and kinetics would be not easy to apply since all chemical reactions in the solution must be considered.

2.3.2.2 Nucleation rates

The CaP nanocrystal nucleation rate (J) can be calculated based on the classical equation of heterogeneous nucleation:

$$J = K \exp \left(-\frac{\Delta G}{kT} \right) = K \exp \left(-\frac{16\pi\theta^2\gamma^3 f(\theta)}{3k^3 T^3 (\ln S)^2} \right) \quad (2.6)$$

where k, the Boltzmann constant; T, the absolute temperature. Depending on this equation, the nucleation rate (J) of apatite formation is proportional to the kinetic factor (K), and determined by the activation energy of nucleation (ΔG). Here, ΔG is primarily affected by interfacial energy γ , the supersaturation S and the contact angle function $f(\theta)$. v is defined as the CaP molecular volume, and determined by the crystal structure. The kinetic factor (K) is proportional to the probability (P)

that ion units of CaP form a nucleus in the solution, i.e., $K=K'P$. Boistelle et al proposed the equation to calculate the P value of HA precipitation as follows[52]:

$$P = \frac{9! [Ca^{2+}]^5 [PO_4^{3-}]^3 [OH^-]}{5! 3! ([Ca^{2+}] + [PO_4^{3-}] + [OH^-])^9} \quad (2.7)$$

Attained from their experiment, K' value could be calculated as $13.64 \times 10^{-24} \text{cm}^{-3} \text{s}^{-1}$.

Based on the studies by Wu and Nancollas, the interfacial energy (γ) is also experimentally calculated as 10.4mJm^{-2} for HA [53-54].

2.2.3 HA coating on different substrates

Since the SBF solution was first proposed by Kokubo et al.[55-56], many efforts have been made to prepare adherent and homogeneous HA layers on different substrates for bone tissue application.

2.2.3.1 Glass-Ceramic

Kokubo et al found that a layer of amorphous apatite formation on the CaO-SiO₂-based glass-ceramics when they studied *in vitro* bioactivity of glass-ceramics in SBF solution [55, 57]. He demonstrated that this layer was about 7 μm in thickness, and comprised of plate-structure CaPs after 10 days immersion in SBF solution, as shown in Fig 2.10. The mechanism of apatite formation on CaO-SiO₂-based glass-ceramic is illustrated in Fig 2.11[58-59]. The calcium ions released from degradation of glass-ceramic increases ion activity in SBF solution, which lead to induce apatite deposition. In addition, the hydrated silica on the

surface of glass-ceramic supplies a preferable substrate for apatite nucleation. Consequently, an apatite layer is formed and adhered on the surface of glass-ceramic. After apatite nuclei formation, they continue to the growth stage by consuming the calcium and phosphate ions in SBF solution due to high supersaturation with respect to apatite under normal physiological conditions. This finding is not only significant for an *in vitro* bioactivity study of artificial implant, but also for apatite or HA coating preparation under physiological temperature and pH in the application of tissue engineering. Simultaneously, biocompatibility and bioadhesion between tissue cell and substrate are obviously increased by introducing the new apatite layer.

Fig 2.10 Micrographs of surface and cross-section of apatite-coated glass-ceramic soaked in SBF for 10 days[57]

Fig 2.11 Mechanism of apatite formation on the surfaces of CaO-SiO₂ based glasses and glass-ceramics[59]

2.2.3.2 Metal

Titanium and its alloys are popularly for use in reconstruction surgery as dental and orthopedic implants due to their excellent biocompatibility with bone[60]. The biocompatibility of bone tissue results from the unique characteristics of the titanium and bone interface. Hydrated titanium oxide on the surface of titanium implants was formed slowly in physiological aqueous environment and contributed to incorporate with calcium and phosphate ions[61]. Based on this theory, in order to improve the bioactivity of titanium implant, a TiOH gel coating was modified on the surface of titanium as a template for bone-like apatite growth as P.J. Li reported[62]. A thick and dense apatite layer comprised of small HA particles was observed after two weeks immersion in SBF solution (Fig 2.12). This is the first report to introduce HA coating on metal substrate.

Fig 2.12 Micrographs of HA deposited on the gel-derived titania coating after being soaked in SBF for 2 weeks[62]

Kokubo et al continue to create a novel alkali and heat treatment method to modify the chemical group of Ti and its alloy surface to induce HA nucleation and growth[63]. First, Ti template was soaked in NaOH or KOH solution for alkali treatment. After being rinsed several times by DI water, Ti template was pre-heated at 600°C for heat treatment, and then cooled down to room temperature. In general, the surface of Ti or its alloys is usually covered with a thin layer of TiO₂. The TiO₂ layer could be dissolved in alkali solution, following the chemical reaction:

While Ti metal is hydrated by the following chemical process[59]:

The hydrated titania with negative charge can be interacted with positively charged alkali ions to form a thin gel layer on the surface of Ti metal or its alloys. However, the gel layer is not stable and shows poor adherence with metal. Heat treatment was the other necessary process to stabilize this thin layer. HA particles were successfully bonded on the gel layer of Ti alloy, as shown in Fig 2.13. The mechanism of HA growth on alkali and heat treated titanium metal or its alloy is also performed in Fig 2.14. Nowadays, this method has been applied as a standard for all modifications of metal surface.

Fig 2.13 Micrographs of the surface (b) and the cross-section (c) of Ti metal treated with NaOH and at 600°C after soaking in SBF solution for 4 weeks[63]

Fig 2.14 Structure change of titanium metal by (a) alkali (b) heat treatment and (c) the process of HA formation in SBF[63]

2.2.3.3 Polymer scaffold

Recently, porous and 3-dimensional polymer scaffolds are developed as a substrate for cell growth and proliferation in tissue engineering and cells can secrete an extracellular matrix to induce new tissue formation[64]. Polymer scaffold is chosen as artificial implant due to biocompatibility, biodegradability, high mechanical strength, and osteoconductivity. Biocompatibility and biodegradability are correlated with polymer nature and can be administrated by an appropriate choice of polymer. In order to improve mechanical strength and osteoconductivity, some researchers designed a layer of apatite coating on the surface of polymer scaffold.

The polysaccharide chitosan with amino groups, as a natural biodegradable polymer, is widely applied as a porous 3-D scaffold [65-66]. I.Manjubala et al used the freeze-dried method to fabricate macro-porous chitosan scaffold (Fig 2.15 (a)

and (b)) [67-68]. They designed a modified double diffusion chambers containing separate calcium and phosphate ions to induce apatite deposition on the chitosan scaffold. The CaP particles can be clearly observed on the top and cross-section of the scaffold as Fig 2.15 (c) and (d) shows. Apatite layer formed on the surface of the chitosan scaffold results from the amino groups of chitosan. Positively charged amino groups can bind phosphate ions in the solution to supply the site for apatite nucleation. After the nuclei formation, apatite starts to grow by consuming calcium and phosphate ions in the solution.

Fig 2.15 Micrographs of (a) and (c) Top view of chitosan scaffold and mineralized scaffold, (b) and (d) cross-section of chitosan scaffold and mineralized

scaffold[67]

Aside from the amino group, the carboxyl group is another effectively negative-charged chemical group which can induce apatite formation by means of absorption of calcium ions[69]. Alginate hydrogel by ionic or chemical cross-linking is used as scaffold material for tissue engineering due to its biocompatibility and biodegradability [29, 70]. S.G. Darilis et al investigated nucleation of HA on alginate scaffold for stem-cell based bone tissue application[71] and HA nanoparticles fully covering the surface of the alginate gel were observed in Fig 2.16. Since alginate gel was prepared by using CaCl_2 as a cross-linker, phosphate ions in the solution were immediately absorbed on the surface of the gel; in addition, there are abundant carboxyl groups in the alginate structure, which could absorb calcium ions in the solution simultaneously. Thus, HA particles exhibit rapid precipitation on alginate gel scaffold.

Fig 2.16 Micrographs for control alginate gel (left) and HA coated on the surface of alginate gel after 4 weeks immersion in SBF (right)[71]

Recently, many researchers have shown interest in synthesized co-polymer PLGA as a 3-D macroporous scaffold in tissue engineering since it is capable of

controlling biodegradability by composition, in comparison to natural polymers. In order to increase cell bioadhesion and osteoconductivity, many efforts are made to deposit a layer of apatite on PLGA scaffold[72-80]. W.L. Murphy first reported HA coating on the PLGA scaffold by biomimetic method in SBF solution, as shown in Fig 2.17[74]. Later, they modified an NaOH treatment for PLGA scaffold before soaking to increase the surface hydroxyl and carboxyl groups, then immersed scaffold into SBF solution for HA crystal growth[75]. The presence of these increasing groups accelerates calcium ion binding to the polymer surface and improves mineral growth. The morphology of mineral coated on modified PLGA is shown in Fig 2.18. Chou et al carried out a two-step soaking method with supersaturated solution as 5×SBF (SBF1) and Mg^{2+} and HCO_3^- free 5×SBF (SBF2) for HA growth[78]. Unlike apatite precipitated from original SBF, the coating obtained by two-step soaking method is comprised of plate-like structured HA nanocrystals (Fig 2.19).

Fig 2.17 Micrograph of HA crystals deposition on PLGA scaffold after 16 days incubation[74]

Fig 2.18 Micrographs of HA deposited on NaOH treated PLGA scaffold after 16 days incubation in SBF (left: magnification $\times 80$; right: magnification $\times 3000$)[75]

Fig 2.19 Micrographs of apatite coated PLGA scaffold by sequentially immersing in SBF1 and SBF2 for 12h[78]

2.3 Co-precipitation of drug and HA on the metal or scaffold

Using biomimetic method HA can be deposited on the templates from the solution at normal physiological temperature. This provides probability for co-precipitation of protein drug and HA because protein can maintain its bioactivity at normal physiological temperature. Many efforts were made on study of dissolution rate and crystal phase of drug/HA co-precipitation [81-86].

H.B. Wen et al investigated the effect of protein (bovine serum albumin, BSA) on

crystalline phase and morphology of co-precipitation coating on titanium alloy[84].

HA coating with the absence of protein shows straight plate-like units with sharp edges; while co-precipitation coating shows link-net structured crystal plates with some organic-like substance (Fig 2.20).

Fig 2.20 Micrographs of HA (A,B) and HA/BSA (bathing concentration 2mg/ml) (C,D) coatings prepared on Ti alloy[84]

Y. Liu et al focus on the dissolution rate of CaP/BSA co-precipitation in different bathing concentrations of BSA (Fig 2.21), which is directly correlated with the drug release rate[86]. They demonstrated that co-precipitation coating showed lower dissolution rate than CaP coating without BSA. However, there is no obvious difference on dissolution from co-precipitation with various bathing concentrations of BSA.

Fig 2.21 Release of Ca²⁺ ions (a) from CaP coatings prepared biomimetically in the absence or presence of BSA (bathing concentration of 1 mg/ml), (b) from CaP/BSA co-precipitated coatings with different bathing concentrations of BSA (0.1–100 μg/ml)[86]

2.4 HA coating on micro/nanospheres

Depending on the nucleation and growth theory of HA formation on scaffold, it can be applied to microparticle or nanoparticle as a substrate. The difference between their structures determines their biomedical application: HA coating on scaffold or alloy is applied to improving the bioactivity and bioadhesion; while HA coated

microparticles/nanoparticles are carriers for bioactive molecular drug delivery because HA can provide strongly chemical bonding with protein and DNA.

2.4.1 Supersaturated solution

2.4.1.1 SBF or concentration modified SBF

SBF or concentrated modified SBF solution, as a calcium and phosphate source, is usually chosen as supersaturated solution for apatite formation. Murphy et al prepared HA coated PLGA microspheres using SBF solution which is similar with classic method of biomimetic HA coating on scaffolds[87]. After immersing PLGA microspheres in SBF solution for 7 days at 37°C, HA coating on the surface of microspheres was successfully produced. The morphology of uncoated and coated microspheres was showed in Fig 2.22. Mineral HA with plate-like structure was clearly observed after microsphere soaking. It showed that the biomimetic method for HA coating formation is also suitable for particle as a substrate. The protein release rate from binding with HA and encapsulation in PLGA microspheres was also investigated(Fig 2.23). Both approaches showed sustained release, however, the total amount of bound protein release significantly higher than the encapsulated protein after 30 days. Even more importantly, the bound protein release rate is nearly constant, as a linear kinetics showed in the release curve without a burst release stage. It demonstrated that the release of protein drug binding with HA coating did not result from surface diffusion and started with dissolution of mineral HA. The dissolution rate of HA coating determined the bound protein release

kinetics. This study opens a new view to control protein drug release in drug delivery system.

Fig 2.22 Schematic of mineral-coated PLGA microspheres and micrographs for uncoated and coated PLGA microsphere[87]

Fig 2.23 A) Comparison of cumulative release of BSA bound to HA coated PLGA microspheres and BSA encapsulated in PLGA microspheres. Micrographs of B) HA coated microspheres and C) coated PLGA microsphere after the 30 day release period.[87]

They also study the effect of the amount of the nucleate agent and surfactant in supersaturate solution on the growth of HA coating[88]. The morphology of HA coated microspheres with different concentrations addition of microspheres as nucleate agent was showed in Fig 2.24. 0.25% (w/v) of PLGA microspheres is preferable amount addition for HA coating growth without aggregation. During the growth of the mineral HA coating, the presence of surfactant (Tween 20) significantly inhibited the formation of the mineral and changed the surface morphology of the coating, as shown in Fig 2.25.

Fig 2.24 Micrographs of HA coated microspheres after 7 day soaking in SBF solution with different amount addition of microspheres: (A) 0.25% (w/v); (B) 0.50% (w/v); (C) 0.75% (w/v); (D) 1.00% (w/v) [88]

Fig 2.25 The morphology on the surface of microspheres formed (A) in the presence of 0.1% (v/v) Tween20 and (B) in the absence of Tween20[88]

Kang et al also chose PLGA microspheres, 150-300µm in size, as substrate for HA growth[89]. While, SBF with inorganic ion concentrations three times those of human blood plasma was modified as a source of Ca and P. After different immersion times, the surface morphology of uncoated PLGA and HA coated PLGA microspheres was shown in Fig 2.26. HA coating is successfully formed on the surface after an immersion of 5 days, however, some obvious cracks are observed on the surface of coating. Poor coverage of the coating is mainly caused by Mg^{2+} , HCO_3^- and other ions in the SBF which is bonded with Ca^{2+} and PO_4^{3-} to affect the nucleation and growth of HA on the microspheres.

Fig 2.26 Micrographs of (A, B) PLGA microsphere and (C, D, E, and F) apatite coated PLGA microsphere incubated in SBF for (C, D) 3 days and (E, F) 5 days.

The scale bars indicate 10 μm in A, C, and E and 1 μm in B, D, and F[89]

Apart from negatively charged PLGA microspheres, positively charged chitosan microspheres are also a good choice for HA growth because the $-\text{NH}_3$ group of chitosan can bond with phosphate ions to induce apatite nucleation in the solution. Jayasuriya, A.C. et al used chitosan as a template to prepare apatite coated chitosan microspheres for the release of growth factor IGF-1[90]. The important point in their work is that they used 5 times concentration of SBF for rapid

biomineralization. Some researchers use an incubation period of 5-7 days in order to provide a complete HA coating precipitation in 1.5×SBF or 3×SBF solution. It is quite long immersion duration for preparation of drug delivery devices. Jayasuriya et al increased the concentration of SBF to five times to obtain complete coverage of HA coating in 24h incubation. A thin layer comprised of HA nanoparticles on the surface of chitosan is shown in Fig 2.27. However, the morphology of the HA coating is quite different from Murphy's work which shows plate-like structure in SBF solution. Morphology difference is may caused by high concentration effect of Mg^{2+} , HCO_3^- and other ions in 5×SBF on nucleation and growth of HA.

Fig 2.27 Morphology of chitosan microspheres before (a) and after (b)
biomineralization[90]

Min Lee et al modified Jayasuriya's work and designed a two-step immersion process to prepare apatite coated alginate/chitosan microspheres for osteogenic protein delivery[91]. They first added alginate/chitosan microspheres into 5×concentrated SBF (SBF1) for a 6h nucleation process; apatite nuclei coated

microspheres were then poured into Mg^{2+} and HCO_3^- free SBF (SBF2) for 12h of continuing growth. By a two-step method, an apatite coating with plate-like structure is completely formed in less than one day, as shown in Fig 2.28 (b). The problem in his work is high extent of aggregation occurs (Fig 2.28 (a)).

Fig 2.28 Micrographs of (a) alginate/chitosan microparticles after soaking in SBF1, (b) after soking in SBF2[91]

2.4.1.2 Solution containing only Ca and P

A classic biomimetic method of HA coating formation is by immersing the substrate in SBF, but this usually takes up to several days to produce a complete coating. The other problems need to solve contains big aggregation and poor coverage. The constant composition precipitation in the solution is a rapid method for HA particle formation, as first reported by Tomson, M.B. and Nancollas, G.H[92]. Q. G., Xu et al successfully applied this method to prepare an HA coating fully covered on the surface of negative liposomes[93]. In his work, a stable supersaturated solution with respect to HA was prepared by mixing CaCl_2 and

KH_2PO_4 (Ca/P ratio 1.67), 37 °C and pH 7.8 as neutral and basic pH tends to induce formation of HA[94]. Then the liposomes as nucleation agent were added to induce the nucleation and growth of HA nanocrystals. In order to monitor the whole process for HA precipitation, a computer-control system was designed as shown in Fig 2.29. Ion meter was used to measure calcium ion activity and pH meter was to monitor pH changes in the solution and maintain a pH of 7.8 throughout the whole process by adding KOH. The titrants comprised of 25mM CaCl_2 (Titrant 1), 15mM KH_2PO_4 and 50mM KOH (Titrant 2) supplies Ca^{2+} and PO_4^{3-} to maintain the concentration of Ca and P in the solution as a constant. By using this system, an HA coating is successfully grown on liposomes in 6 h (Fig 2.30). However, regarding commercial development, this system is too complicated and is not available for mass production.

Fig 2.29 Diagram of computer-controlled constant composition method[93]

Fig 2.30 Bright field image and corresponding electron diffraction pattern of HA coated liposome nanospheres [93]

Anitha Ethirajan et al developed a novel two-step process for HA coating formation, as shown schematically in Fig 2.31[95]. The addition of calcium ions for bonding with nanoparticles, then followed by a dropwise addition of phosphate ions, corresponds to the stoichiometric Ca/P ratio of 1.67 for HA formation. The concentration of Ca and P in the solution is very low, 0.5mM Ca^{2+} and 0.3mM PO_4^{3-} . The pH value in his work, used at a constant of 10, leads to the generation of HA coated nanoparticles. As a result, HA coating is formed after 24h but is not fully covered, as shown in Fig 2.32. A pH of 10 throughout the whole experiment is not suitable for most biodegradable microspheres/nanospheres due to quick hydrolysis under alkaline condition.

Fig 2.31 Schematic illustration of HA deposition on carboxyl-functionalized nanoparticle by consecutive addition of Ca^{2+} and PO_4^{3-} [95]

Fig 2.32 Micrographs of HA coated nanoparticles, in bright field (left) and dark field (right) image modes[95]

2.4.2 Modified chemical groups of microspheres

Anitha Ethirajan et al also investigated the effect of the amount and category of surface chemical groups on HA growth[95]. They demonstrated that in the same incubation time, the amount of HA nanocrystals increased along with increases in

the surface carboxyl groups of nanoparticles. Negatively charged surfactant (sodium dodecyl sulfate, SDS) present on the surface of nanoparticles can affect the bonding of calcium ions with carboxyl groups and inhibit HA growth. However, Q.G., Xu et al provided a different conclusion with respect of SDS modified microspheres template[96]. They prepared negatively charged SDS-modified PLGA microspheres with zeta-potential of -79.1mV in comparison to the commonly used poly(vinyl alcohol) (PVA) surfactant for PLGA preparation of only -10mV. They illustrated that a complete HA coating could be formed on SDS modified PLGA microspheres in 6h immersion (shown in Fig 2.33), but there was almost no HA crystal observed on the PLGA microspheres with PVA surfactant. Thus, they concluded that SDS, as the polar and ionic head of $-\text{OSO}_3$, was a strong chelating group for calcium ions.

Fig 2.33 Micrographs of negatively charged PLGA microspheres with SDS surfactant (a, b) and HA coated PLGA microspheres under different soaking time: (c, d) 1 h, (e, f) 3 h and (g, h) 6 h[96]

Since chitosan itself has low capability to induce HA formation in normal SBF solution, Leonor, I.B. et al prepared silica-treated chitosan microspheres as a template for HA growth[97]. From the image in Fig 2.34, HA particles formation was observed within 1 day; after 7 days of immersion, the HA coating fully covered on two kinds of silica-treated chitosan microspheres.

Fig 2.34 Micrographs of chitosan microparticles pre-treated with a calcium silicate solution under condition I (Row (a)) or II (Row (b)) and subsequently soaked in SBF for 1, 3 and 7 days[97]

Chapter 3 Experiment Design

3.1 Problem statement and strategies

Nowadays, drug release, especially protein drug release from polymer microspheres such as PLGA, is often met with a primary burst release of molecules over short timescales and decreased activity of biological molecules due to microsphere manufacture processing. Some researchers have high interest in biomineralized HA coated polymer microspheres for protein drug delivery. It is a novel idea that the drug release rate is a constant, determined by the dissolution rate of HA as a result of the drug loading on the surface of HA coating. However, the main problems with the preparation of HA coated microspheres are long incubation duration, high pH to induce HA deposition which is not suitable for most biodegradable microspheres and poor coverage of HA coating on the surface of microspheres.

This project develops a novel two-step method under physiological temperature and pH, aiming to harvest a PLGA microsphere completely covered by an HA layer in a short incubation time. Two kinds of drug loading are designed for controlled release: One is to soak the HA-coated microspheres into a drug-containing solution (“drug-soaked HA”) and the other is to add the drug into the HA-forming solution and let the drug co-deposit on the microsphere at the time of HA formation (“drug-co-deposited HA”).

3.2 Experiment procedure

3.2.1 PLGA microsphere preparation

Microspheres were prepared by an emulsion diffusion-evaporation technique, based on the work of M.N.V. Ravi Kumar et al [98] and Qingguo Xu et al [99]. The methodology was taken as follows: 100 mg of PLGA is dissolved in 5 ml dichloromethane (DCM) at room temperature. The organic phase was then dropped into an aqueous surfactant containing 50 mg of poly(vinyl alcohol)(PVA) in 5 ml distilled water while stirring. The emulsion is stirred at 1400rpm at room temperature using a magnetic stirrer. After 1h stirring, the emulsion was moved into a 90ml 0.1% wt PVA solution resulting in PLGA hardness and micro-precipitation. Stirring is continued at 500rpm overnight to completely remove organic solvent DCM.

The resultant PLGA microspheres were collected by centrifugation at 6000 rpm (B4i multifunction centrifuge, France) and rinsed three times with distilled water to remove any residual surfactant. The PLGA microspheres were then lyophilized overnight, and kept in a sealed tube until use.

3.2.2 Deposition of HA coating on PLGA microspheres

In this project, coating of the PLGA microspheres was carried out by a two-step biomimetic method, first calcium ions, and followed by phosphate ions. 20mg of microspheres was added into 20ml of CaCl_2 solution. The hybrid solution was then stirred at room temperature for 2 hours to allow the bonding of calcium ions with the carboxyl group of PLGA. 20ml of K_2HPO_4 solution was added dropwise under stirring.

The molar ratio of calcium to phosphate was maintained at 1.67 with respect to HA.

The mixture was then moved to 37°C oven under stirring. The pH was neutral throughout the experiment. When the experiment proceeded for a certain time (1h, 4h, 8h, 16h and 24h), the HA coated microspheres were collected by centrifuge at 5000rpm for 10min, and rinsed three times with distilled water. The resultant microspheres were freeze dried for future measurement.

3.2.3 HA coating Characterization

This part will introduce the characterization methods for HA coating in detail.

3.2.3.1 Particle size and surface charge

The particle size and surface charge of microspheres were investigated by laser light scattering on a Malvern Particle Sizer (Malvern Instruments, Malvern, UK). The freeze dried microspheres were re-suspended in 0.1% wt Tween20 solution by ultrasonic in an ultrasonic bath (DC200, USA) for 2 min. The mean particle size is obtained from three batches of microspheres. The surface charges of microspheres were measured by their zeta-potentials in distilled water. Each result was the average of 12 measurements, and all measurements were carried out under 20°C and at an angle of 90°.

3.2.3.2 Field emission scanning electron microscopy

The surface morphology and the extent of aggregation of HA coated PLGA microspheres were performed by field emission scanning electron microscopy (FESEM, JSM-6340F, Japan) at an accelerating voltage of 3kV. The emulsion containing HA coated PLGA microspheres was dropped on the silica wafer and dried in the air for test. Since the conduction of samples is very weak, gold coater (JFC-1600,

Japan) was used to give a thin gold film on samples to improve the surface conduction.

3.2.3.3 Fourier Transform Infra-red

For powder samples, 95% potassium bromide (KBr) and 5% dried microspheres was mixed to compress a disc for test. Infrared spectra were obtained by Fourier Transform Infrared (FTIR) spectrometer (SpectrumGX, PerkinElmer, US) in the transmission mode. 16 scans over the range of $400\text{--}4000\text{cm}^{-1}$ were performed at a resolution of 2cm^{-1} .

3.2.3.4 X-ray diffraction

The emulsion containing HA coated PLGA microspheres was drop on a silica wafer and dried in the air for examination. Data was obtained by X-ray diffraction (XRD, Bruker AXS D8 Advance, Germany) at 35kV and 50mA with Cu K α radiation. Scan range is between 10° and 60° 2θ and scan step is 0.02° . Scan rate is 1° per minute.

3.2.3.5 Thermo gravimetric analysis

The thermo gravimetric analysis (TGA) of samples was carried out by TGA thermal analyser (Shimadzu DTG60H, Japan). 15mg dried PLGA or HA coated PLGA microspheres were heated in a platinum crucible in a range of $30\text{--}800^\circ\text{C}$ at rate of $10^\circ\text{C}/\text{min}$ under air flow.

3.2.4 Drug loading

In this study, we chose bovine serum albumin (BSA) as a model drug. Two kinds of drug loading are designed as follows.

3.2.4.1 Drug loading via soaking of HA coated microspheres

Drug loading on HA coated microspheres was proceeded to soak coated microspheres into BSA solution with different initial concentration, 100 μ g/ml, 500 μ g/ml and 1000 μ g/ml. After 12h incubation, the solution was centrifuged to separate microspheres. The amount of BSA in the supernatant was measured by BCA protein assay using microplate reader. The loading amount of drug is calculated by following equation:

The loading amount of drug = the total amount of drug – the amount of drug in the solution after incubation (3.1)

3.2.4.2 Drug loading via co-precipitation of HA and drug during HA formation

Phosphate solution containing BSA as a precursive solution was used for co-precipitation of HA and drug. The whole process is similar to the preparation of HA coating on microspheres. 20mg PLGA microspheres were added into CaCl₂ solution first. After 2h of rigorous stirring, phosphate solution containing different concentrations of BSA (10 μ g/ml, 100 μ g/ml, 500 μ g/ml, and 1000 μ g /ml) was added drop by drop. The molar ratio of calcium to phosphate for initial use was still maintained at 1.67. Then, the mixtures were left in an oven at 37°C under stirring for co-precipitation. After 24h of incubation, microspheres with a layer of BSA

co-deposited HA were collected by centrifuge at 5000rpm for 10min. The resultant microspheres were frozen and then lyophilized overnight. Characterization of drug co-deposited HA coating is similar to HA coating.

For co-precipitation of HA and BSA on PLGA microspheres, drug has already been loaded into coating in the preparation process. The solution separated after centrifuge was kept for the measurement of BSA concentration by BCA protein assay. The loading amount of drug is calculated by equation(3.1).

3.2.5 Drug release

Drug release profiles of “drug-soaked HA” and “drug-co-deposited HA” coating were carried out in phosphate buffer solution (PBS). 10mg of BSA loaded, freeze-dried microspheres with two kinds of loading method were respectively immersed in 1ml PBS (pH 7.4) at 37°C under 50rpm shaking. The release medium was changed daily. The total release duration in this work is 30 days. The release amount of BSA was measured by BCA protein assay using microplate reader.

Chapter 4 Results and Discussion

4.1 PLGA microspheres

PLGA microspheres have been prepared by means of the oil in water (O/W) emulsion-evaporation method. The SEM micrographs show uniform distribution and smooth surface of the microspheres (Fig 4.1 (a) and (b)).

Fig 4.1 SEM micrographs of negatively charged PLGA microspheres (a) 600× (b)

6000×magnification

The average particle size of PLGA microspheres is measured as $6.67 \pm 0.82 \mu\text{m}$. The zeta-potential of these microspheres is $-27.0 \pm 1.87 \text{mV}$, which indicated the negatively charged surface caused by the carboxyl groups in chemical structure of PLGA. It is an important point for choosing PLGA as a template for HA growth, since the rapid formation of HA crystals is well-pronounced for particles with high amount of carboxyl groups[95]. Generally, calcium ion could be bonded with carboxyl group on the surface of PLGA microspheres through electrostatic force. After addition of phosphate ion, HA crystals start to nucleate and grow on the microspheres due to the bonding of Ca and P.

4.2 Study on the formation of HA coating

In this project, we proposed a two-step biomimetic method for HA coated microspheres preparation. The mechanism and characterization of HA formation on microsphere by this method will be discussed in this part.

4.2.1 The mechanism of formation of HA coating on PLGA microsphere

A novel two-step process under physiology temperature and pH for HA growth is created and illustrated in Fig 4.2. At first, after the addition of microspheres into calcium solution, calcium ions in the solution are partly absorbed on the surface of microspheres due to bonding with carboxyl groups. This process would supply a Ca-rich surface for apatite nucleation. Residual calcium ions in the solution provide

a supply for HA continuing growth. Secondly, when phosphate ions are added to the suspension, they would interact with the calcium ions at the microspheres surface to form the very first layer of HA, and thereafter, HA growth would take place. The whole growth is carried out at 37°C as biomimetic process.

Fig 4.2 Schematics of HA coating formation on PLGA microspheres

4.2.2 The factors on formation of HA coating

The factors that affect HA formation are proposed as the amount of $-\text{COOH}$ group, the concentration of the Ca and P solution and immersion time of the substrate. In this project, we fixed the amount of the $-\text{COOH}$ group on PLGA as a constant index. The study of HA growth was focus on the ions concentration of solution and immersion time of microspheres.

4.2.2.1 The effect of concentration of Ca and P solution on the formation of HA coating

Solutions containing Ca and P with different concentrations are showed in Table 4.1. We set the ratio of Ca and P as a constant of 1.67 with respect to HA for the two solutions. After directly mixing the Ca and P solution, Solution2 shows suspension with white particles precipitation, and Solution1 is still clear, as shown in Fig 4.3. It indicates that HA particles are formed without a nucleation agent from Solution2 of high concentration once Ca and P ions touch each other in aqueous solution.

Table 4.1 Ca and P solution with different concentration (Ca/P=1.67)

Solution	Ca^{2+} (mM)	PO_4^{3-} (mM)	Precipitation
1	1.25	0.75	No
2	2.5	1.5	Yes

Fig 4.3 Photographs for Ca and P mixed solution with different concentration: (a)

Solution1 (b) Solution2

The formation of HA coating was investigated after the addition of PLGA microspheres in the two solutions for 16h incubation. For soaking in Solution1, a complete apatite coating is fully covered on the surface of microspheres (Fig 4.4 (a)). From magnification images (Fig 4.4 (b) and (c)), the surface of coating on the microspheres shows a plate-like structure and the coated microspheres are still uniform, without aggregation. In contrast to Solution1, after soaking in Solution2, the bare smooth surface of microspheres and big particles with irregular shape can be observed in Fig 4.4 (d) and (e).

Fig 4.4 SEM micrographs of HA formation after PLGA microspheres immersed in
Solution1 (a, b, c) and Solution2 (d, e)

Samples were also scanned by FTIR analysis, as shown in Fig 4.5. FTIR spectra show phosphate absorption peaks of HA in both solutions at 1041cm^{-1} (stretching vibration of PO_4^{3-}) and $601, 563\text{cm}^{-1}$ (deformation vibration of PO_4^{3-}) in comparison to PLGA microspheres before immersion. It demonstrates that the coating with plate-like structure in Solution1 and big particles with irregular shapes in Solution2 are both correlated with HA.

Fig 4.5 FTIR spectra for PLGA microspheres (a), HA coated PLGA microspheres immersed in Solution1 (b) and Solution2 (c) (* represents phosphate groups)

Depending on SEM images and FTIR spectra, HA particles formed in Solution2 could be quickly self-precipitated in the solution rather than on the surface of the microspheres due to high concentration of Ca and P. Solution 1 with 1.25mM of Ca^{2+} and 0.75mM of PO_4^{3-} , is preferable concentration for HA nanocrystals nucleation and growth on the microsphere substrate.

4.2.2.2 The effect of immersion time on the formation of HA coating

The effect of immersion time on HA coating growth was examined by FTIR based

on samples at 1h, 4h, 8h, 16h and 24h, as shown in Fig 4.6. After the first hour of incubation, the peaks for stretching and deformation vibration of the phosphate groups are obtained, but very weak. With lengthened immersion time, the phosphate groups show stronger peaks at 1041, 601, and 563 cm^{-1} . The peaks around 3500 cm^{-1} derived from the hydroxyl group are very weak for all the samples, hinting the HA coating is poorly crystallized[100].

Fig 4.6 FTIR spectra for HA coated microspheres with different immersion time: (a) 1h; (b) 4h; (c) 8h; (d) 16h; (e) 24h (* represents phosphate groups)

The morphology of PLGA microspheres before immersion as a control is shown in Fig 4.7 (a). Some HA crystals can be observed only after 1h incubation (Fig 4.7 (b)). Combined with the FTIR spectra, it is not difficult to see that a rapid nucleation and growth of HA takes place in the first hour. However, resulting from the short

duration, HA crystals only grow on some parts of the microsphere. After 4h and 8h of immersion, HA crystals are formed on more areas of microsphere surface (Fig 4.7 (c) and Fig 4.7 (d)), but still do not cover completely. Until 16h, complete coverage is seen as shown in Fig 4.7 (e). The coating is composed of amounts of HA crystals with plate-like structure on the surface of the microspheres. After soaking for 24h, plate-like structure with sharp edge is clearly observed, as shown in Fig 4.7 (f).

Fig 4.7 SEM micrographs for uncoated microspheres (a) and HA coated microspheres with different incubated time: (b) 1h, (c) 4h, (d) 8h, (e) 16h, (f) 24h

Simultaneously, the size distribution of microspheres before and after coating is studied as Table 4.2 shows. After 16h immersion, the thickness of HA coating nearly reached $1.5\mu\text{m}$. However, there is slight increasing in 24h incubation duration in comparison to 16h. The reason is probably attributed to the consumption of Ca and P, and decrease of ions concentration.

Table 4.2 Average size of HA coated microspheres with different soaking time

Soaking Time(h)	0	16	24
Size(μm)	6.67 ± 0.82	8.16 ± 1.34	8.41 ± 1.73

The phase analysis of the coating was carried out by XRD as shown in Fig 4.8. The strong characteristic peaks at 26° and 32° corresponding to HA are obtained for coated PLGA microspheres throughout the different soaking time. The broadening and overlapping of peaks demonstrates that the coating is comprised of poorly crystallized HA, consistent with the FTIR result. Poor crystallization of HA coating contributing to higher dissolution rate may be exploited in controlling drug delivery.

Fig 4.8 XRD spectra for uncoated PLGA microspheres (a) and HA coated microspheres with different incubated time: (b) 1h, (c) 4h, (d) 8h, (e) 24h

(● represents HA characteristic peak)

Since HA is fully covered on surface of PLGA microspheres after 16h incubation, TGA is used to analyze the weight of HA coating after 16h and 24h, as shown in

Fig 4.9. In the temperature range of 300-400°C, a significant weight loss is obtained, which corresponds to the thermal decomposition of PLGA. Heating at 500 °C, there is no weight at all for PLGA microspheres, indicating that all microspheres were burned out. 9.12% and 10.73% of HA remains for HA coated microspheres after 16h and 24h incubation, respectively. Depending on the data and spectra, there is a slight weight increasing with incubation time from 16h to 24h. However, the increase is very small because most of the calcium and phosphate ions in the solution are consumed and the kinetic balance is found between HA and Ca/P in the solution.

Fig 4.9 TGA spectra for PLGA microspheres (a) and HA coated PLGA microspheres after 16h (b) and 24h incubation (c)

4.3 Study on drug-co-deposited HA coating

In this project, BSA which is 66kDa in molecular weight and 3.5 ± 0.3 nm in size is chosen as a model protein drug. The size distribution of BSA is shown in Fig 4.10.

Fig 4.10 Size distribution of BSA

The nanosized BSA for loading on HA coating usually takes place on the surface of coating, and the loading efficiency has its limitation. (Some data regarding to the loading efficiency are shown in the next part.) Due to protein bonding with HA, drug-co-deposited HA on the microspheres can be formed at the time of the HA nanocrystal deposition. The advantages of co-precipitation are that there is no need to re-load the drug after HA formation, and the drug release duration will may be lengthened.

The two-step biomimetic method is still suitable for drug-co-deposited HA preparation, due to its mild condition and maintenance of drug's bioactivity. This provides the possibility of studying drug-co-deposited HA coating on the polymer microspheres.

We propose the hypothesis that the drug has little or no effect on the nucleation and growth of HA, and loading efficiency is improved with increasing the initial drug concentration for drug-co-deposited HA. The different concentrations of the drug were designed to study the drug loading and HA growth. After 24h of incubation, the amount of drug incorporated in co-precipitation coating was calculated as data shown in Table 4.3. The results are completely different from our hypothesis. The concentration of initial drug increases from 100 to 500 $\mu\text{g/ml}$, the amount of precipitated drug increases quite a little (from 43.5 to 55.0 $\mu\text{g/ml}$). When the initial drug increases up to 1000 $\mu\text{g/ml}$, there is no drug on the microspheres (0 $\mu\text{g/ml}$). This strange condition hints that BSA may strongly affect the growth of HA crystal, since the amount of the deposited drug is related to HA formation.

Table 4.3 Concentration of BSA before and after co-precipitation

Sample	1	2	3	4
The concentration of drug in phosphate solution	10 $\mu\text{g/ml}$	100 $\mu\text{g/ml}$	500 $\mu\text{g/ml}$	1000 $\mu\text{g/ml}$
The concentration of drug precipitated in the coating	N.A.	43.5 $\pm$ 2.5 $\mu\text{g/ml}$	55.0 $\pm$ 6.2 $\mu\text{g/ml}$	0 $\mu\text{g/ml}$

In order to confirm the drug effect on HA growth, FTIR measurement was used to analyze the coated microspheres without or with BSA drug co-precipitation as Fig 4.11 displays. For Fig 4.11 (a) spectra as a control of HA coated microspheres without BSA, the peaks at 1041, 601 and 563 cm^{-1} are associated with the phosphate groups. When 10, 100 and 500 $\mu\text{g/ml}$ BSA were added for co-precipitation, additional peaks at 1648 and 1541 cm^{-1} in FTIR spectra were observed, which can be ascribed to the amide I and amide II band of protein respectively (Fig 4.11 (b), (c) and (d)). The amide I band represents the stretching vibrations of C=O in the backbone of the protein, while amide II results from the combination of C-N stretching and N-H bending vibrations. These peaks demonstrate that the incorporation of BSA drug into the HA coating. However, the condition differs after a 1000 $\mu\text{g/ml}$ addition for co-precipitation, as shown in Fig 4.11 (e). In the spectra, no characteristic peak that corresponded with either phosphate groups or amide groups is observed. It illustrates that drug-co-deposited HA, even only HA is not formed after 24h incubation, as the spectra is similar to uncoated bare PLGA microspheres.

Fig 4.11 FTIR spectra for HA coated microspheres (a) and co-precipitation of HA and BSA coated microspheres with initial BSA concentration (b) 10µg/ml (c) 100µg/ml (d) 500µg/ml (e) 1000µg/ml (* represents phosphate groups; ● represents amide groups)

The morphology of drug-co-deposited HA coating on the surface of PLGA microspheres was observed by FESEM. The fully covered HA coating without BSA, as a control, is shown in Fig 4.12 (a) and (b). The coating is comprised of many straight plate-like structured HA nanocrystals with sharp edges. However, some significant differences were found between the distinct concentrations of BSA drug for co-precipitation. At a concentration of 10µg/ml of BSA, a completely uniform HA layer on the microspheres is observed, but the partial areas show the organic net-linked structure lacking of a sharp edge (Fig 4.12 (c) - (e)). In Fig 4.12(d), there are three red circles marked to clearly show the organic

net-linked structure. When higher concentration of 100 μ g/ml BSA addition, more net-link structured co-precipitations appear, as shown are in Fig 4.12 (f) and (g); and the coating is not as dense as the one obtained in the control. A marked difference appears when the concentration of initial BSA is increased to 500 μ g/ml (Fig 4.12 (h) and (i)). The surface of the microsphere is partially covered with apatite after 24h of incubation. Moreover, all formed apatite shows organic net-linked structure which is completely different from plate-like structured HA coating without the addition of BSA. When up to 1000 μ g/ml BSA is added, the smooth surface observed in Fig 4.12 (j) proves that there is no apatite formation on the surface of PLGA microspheres after 24h of immersion, which is consistent with the FTIR spectra. These demonstrate that BSA can strongly influence the duration and structure of HA formation as incorporation in the crystal lattice of HA.

Fig 4.12 Micrographs of HA coated PLGA microspheres (a) [(b) 20000× magnification] and co-precipitation of HA and drug on the microspheres with BSA

concentration (c)10 μ g/ml [(d) 20000 $\times$ and (e) 40000 $\times$ magnification], (f)100 μ g/ml [(g) 20000 $\times$ magnification], (h)500 μ g/ml [(i) 20000 $\times$ magnification], (j)1000 μ g/ml

4.4 Loading efficiency of drug-soaked HA and drug-co-deposited HA coating

The amount of the drug absorbed on the HA coating and incorporated in the co-precipitation of HA and BSA were measured and calculated in Table 4.4 and Fig 4.13.

Table 4.4 The amount of BSA from “drug-soaked HA” and “drug-co-deposited HA”

Initial loading (μ g/ml)	100	500	1000
Absorbed on the surface of HA coating (μ g/ml)	52.4 $\pm$ 7.8	325.1 $\pm$ 34.3	356.5 $\pm$ 25.2
deposited in co-precipitation of BSA and HA coating (μ g/ml)	43.5 $\pm$ 2.5	55.0 $\pm$ 16.2	0

Fig 4.13 The amount of BSA from “drug-soaked HA” and “drug-co-deposited HA”

Depending on the positive calcium ion and negative phosphate ion of HA, protein drug can strongly bond with HA by electrostatic force between calcium ion and carboxyl group, and phosphate ion and amino group, respectively. Loading efficiency of “drug-soaked HA” is studied by soaking coated microsphere in BSA solution with varying BSA concentrations of 100, 500, and 1000µg/ml. From Table 4.4 and Fig 4.13, the amount of bound BSA increases along with an increase in initial BSA amount, that is very clearly obtained for the initial amount increasing from 100 to 500µg/ml. However, condition differs from 500 to 1000µg/ml, since the HA coating has nearly reached its absorption limit at this point.

The “drug-co-deposited HA” on the surface of PLGA microspheres provides one-step method for HA growth and drug loading without an additional absorption process. However, the protein drug BSA, as a foreign polymer, can strongly influence the nucleation and growth of HA coating. Thus, the loading profile of “drug-co-deposited HA” is different from that of “drug-soaked HA” because the amount of precipitated drug is related with HA formation. The amount of the precipitated drug in “drug-co-deposited HA” is also shown in Table 4.4 and Fig 4.13. Compared to the loading profile of “drug-soaked HA”, there is little increase for the precipitated drug with the initial drug increasing from 100 to 500 μ g/ml, and non-precipitated drug is measured against 1000 μ g/ml of the initial drug. These results illustrate that HA growth is inhibited by the presence of protein and the amount of drug bound with HA decreases simultaneously.

4.5 In vitro drug release

In this work, BSA release profile from “drug-soaked HA” and “drug-co-deposited HA” with the same initial concentration (500 μ g/ml) of BSA was investigated, as shown in Fig 4.14. The release kinetics of “drug-soaked HA” display two stages: a burst release after first day incubation and following a linear release until 30 days. After 1day immersion, 20% of BSA releases from HA coating. The rapid release rate is caused by diffusion of BSA out of HA coating. Since BSA is absorbed on the surface of HA, in the beginning of release, partial instable BSA diffuses by

exchanging with ions in the medium. While, bound BSA in the HA coating releases at a constant rate which is correlated with the dissolution of HA. After 30 days incubation, cumulative BSA release achieves 76%. Compared with “drug-soaked HA”, BSA release from “drug-co-deposited HA” shows a linear kinetic without burst release stage. In addition, “drug-co-deposited HA” provides quite lower release rate than “drug-soaked HA”. After 30 days incubation, cumulative BSA release from “drug-co-deposited HA” is obtained as 16%. It suggests that BSA is incorporated in HA crystal lattice which is consistent with the morphology showed in Fig 4.12. This novel drug delivery device offers the probability for long-term release profile.

Fig 4.14 Comparison of cumulative release of BSA from “drug-soaked HA” and “drug-co-deposited HA”

We also studied the BSA release from “drug-co-deposited HA” with different initial concentration of BSA (100 and 500 μ g/ml). As shown in Fig 4.15, no difference of BSA release rate was observed as a function of initial BSA concentration. It demonstrates that BSA release profile of “drug-co-deposited HA” is independent with initial BSA concentration.

Fig 4.15 Cumulative release of BSA from “drug-co-deposited HA” with initial BSA concentration 100 and 500 μ g/ml

Chapter 5 Conclusions and Recommendations

5.1 Conclusions

The current work primarily focused on preparation of HA coated microspheres, BSA loading on “drug-soaked HA” and “drug-co-deposited HA”, and release profile of these two kinds at pH 7.4. Based on the results shown in Chapter 4, the following conclusions are drawn.

1. HA can precipitate on the surface of PLGA microspheres and quickly form a uniform plate-like structured coating by a two-step method: adding in Ca-containing solution followed by addition of P-containing solution under physiological temperature and pH.
2. The concentration of Ca and P directly influence the HA formation on the microsphere or HA self-precipitation from the solution. 1.25mM calcium ion and 0.75mM phosphate ion are identified as the preferred concentration for HA nucleation and growth on the surface of PLGA microspheres.
3. Full coverage of the coating on the PLGA microspheres is obtained after 16h soaking. With 24h of incubation, the coating morphology is plate-like structure with the sharp edge. Since CaP coating was formed in the solution without high temperature process, low crystalline HA coating is obtained and confirmed by XRD.

4. BSA has strong influence on HA growth due to its incorporation into crystal lattice of HA. Organic net-linked structure was observed as initial 100 μ g/ml BSA drug added, and the whole coating was not as dense as HA coating with the absence of drug. Increasing initial concentration to 500 μ g/ml, an incompletely covered coating is observed. There is no co-precipitation as 1000 μ g/ml BSA addition.
5. Loading efficiency of “drug-soaked HA” is enhanced as increasing initial BSA amount until loading capability tend to saturation.
6. BSA release profile from “drug-soaked HA” shows burst release and fast second linear stage; while “drug-co-deposited HA” performs a slower linear release without first burst release. It demonstrates that the BSA release of “drug-soaked HA” is related with BSA diffusion process, and “drug-co-deposited HA” is related with the degradation of HA coating. In addition, release profile of “drug-co-deposited HA” is independent of initial BSA concentration.

5.2 Recommendations

BSA release profile is carried out under pH 7.4 in this work. Considering the *in vivo* environment, local inflammatory response may fall down the pH of the medium. Thus, at an early stage, protein drug release from coated microspheres will be subjected to low pH environment. The study of BSA release at low pH is significant for HA or HA/BSA coated microspheres.

BSA as a model protein strongly influences the formation of HA on microspheres.

Unlike negatively charged BSA at pH of 7, whether other neutral or positively charged protein shows the similar effect on deposition of HA is another problem available to study.

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