Simultaneous incorporation of magnesium and fluorine ions in hydroxyapatite coatings on Ti6Al4V implant.

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**SIMULTANEOUS INCORPORATION OF MAGNESIUM
AND FLUORINE IONS IN HYDROXYAPATITE
COATINGS ON Ti6Al4V IMPLANT**

**CAI YANLI
SCHOOL OF MECHANICAL AND AEROSPACE
ENGINEERING
2011**

**SIMULTANEOUS INCORPORATION OF MAGNESIUM
AND FLUORINE IONS IN HYDROXYAPATITE
COATINGS ON Ti6Al4V IMPLANT**

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List of Abbreviations

α -TCP	α -Tricalcium phosphate
β -TCP	β -Tricalcium phosphate
β -TCMP	Magnesium substituted β -tricalcium phosphate
AAS	Atomic absorption spectroscopy
ACP	Amorphous calcium phosphate
ALP	Alkaline phosphatase
BE	Binding energy
BHA	Bi-layer structured HA
DCPD	Dicalcium phosphate dihydrate
EDS	Energy dispersive spectroscopy
EMEM	Eagle's minimum essential medium
FA	Fluorapatite
FHA	Fluoridated hydroxyapatite
FMA	Mg and F co-substituted hydroxyapatite with fixed F and varied Mg concentration
FTIR	Fourier transform infra-red spectroscopy

GDOES	Glow discharge optical emission spectroscopy
GIXRD	Grazing incidence X-ray diffraction
HA	Hydroxyapatite
ICP-OES	Inductively coupled plasma emission spectrometer
MFA	Mg and F co-substituted hydroxyapatite with fixed Mg and varied F concentration
Mg _x FHA	$\text{Ca}_{(10-x)}\text{Mg}_x(\text{PO}_4)_6\text{F}_1(\text{OH})_1, 0 \leq x \leq 2$
MgF _y HA	$\text{Ca}_9\text{Mg}_1(\text{PO}_4)_6\text{F}_y(\text{OH})_{(2-y)}, 0 \leq y \leq 2$
OC	Osteocalcin
OCP	Octacalcium phosphate
PBS	Phosphate buffered saline
SBF	Simulated body fluid
SEM	Scanning electron microscopy
TBS	Tris-buffered saline
XPS	X-ray photoelectron spectroscopy

Publication List

1. Yanli Cai, Sam Zhang, Xianting Zeng, Yongsheng Wang, Min Qian, Wenjian Weng. Improvement of bioactivity with magnesium and fluorine ions incorporated hydroxyapatite coatings via sol-gel deposition on Ti6Al4V alloys. THIN SOLID FILMS, 2009, 517:5347-5351.
2. Yanli Cai, Jingjing Zhang, Sam Zhang, Subbu S Venkatraman, Xianting Zeng, Hejun Du, Debasish Mondal. Osteoblastic cell response on fluoridated hydroxyapatite coatings: the effect of magnesium incorporation, BIOMEDICAL MATERIALS, 2010, 5:054114.
3. Yanli Cai, Sam Zhang, Xianting Zeng, Min Qian, Deen Sun, Wenjian Weng. Interfacial study of magnesium-containing fluoridated hydroxyapatite coatings, THIN SOLID FILMS, 2011, 519:4629-4633.
4. Yanli Cai, Sam Zhang, Xianting Zeng, Deen Sun. Effect of fluorine incorporation on long-term stability of magnesium-containing hydroxyapatite coatings, JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE, 2011, 22:1633-1638.

Summary

Hydroxyapatite (HA) coated titanium alloys are available as hard tissue implant material. However, the performance is not satisfactory due to insufficient short-term osseointegration and long-term stability of the implant. Studies show that cation incorporation (Mg) into HA resulted in better biological performances, i.e. short-term osseointegration, and anion incorporation (F) into HA decreased the dissolution rate and improved the adhesion strength, i.e. long-term stability. As such, a combination of good short-term osseointegration and long-term stability of the implant is achieved by properly incorporating both cation and anion into HA.

This project aims at developing a bi-layer structured Mg and F ions co-substituted HA coating on Ti6Al4V substrate to achieve an integration of short-term osseointegration and long-term stability. $\text{Ca}_{10-x}\text{Mg}_x(\text{PO}_4)_6\text{F}_1(\text{OH})_1$ or Mg_xFHA in short, $\text{Ca}_9\text{Mg}_1(\text{PO}_4)_6\text{F}_y(\text{OH})_{(2-y)}$ or MgF_yHA in short, and the bi-layer structured HA (BHA in brief) coatings are deposited by a sol-gel dip-coating method. The compositional dependence of co-substitution of Mg and F ions is systematically studied on material structure, in vitro bioactivity, cell response, dissolution rate, and adhesion strength.

The results show that as-deposited Mg_xFHA coatings have a single FHA phase when $x \leq 1.0$. A small amount of Mg-substituted β -tricalcium phosphate (β -TCMP)

appears together with FHA phase when $1.0 < x \leq 2.0$. The phases of as-deposited MgF_yHA coatings depend on F concentration. Without F incorporation, β -TCMP is the main phase, and HA as the secondary phase. When y is 0.5, HA becomes the main phase and only a little β -TCMP presents. Single FHA phase forms when $y \geq 1.0$. Grazing incidence X-ray diffraction, X-ray photoelectron spectroscopy, fourier transform infra-red spectroscopy results indicate that Ca ions and OH groups in HA crystal structure are substituted with Mg and F ions, respectively.

High Mg concentrations ($1.0 \leq x \leq 2.0$) in the Mg_xFHA coatings improve the in vitro bioactivity a lot. However, F concentration does not influence the bioactivity of the MgF_yHA coatings. MG63 cells attach and spread well on all the Mg_xFHA and MgF_yHA coatings. In the Mg_xFHA coatings, Mg ions show a significant stimulating effect on cell proliferation and late cell differentiation when x is 1.5. F concentration studied does not affect cell numbers grown on the MgF_yHA coatings. However, it maintains a positive stimulating on cell differentiation.

The incorporation of Mg increases the dissolution rate of Mg_xFHA coating in the tris-buffered saline (TBS) solution, and the maximum is achieved at $x = 1.5$. F incorporation decreases the dissolution rate of MgF_yHA coating in the TBS solution, especially at the measured fluoridation degree of 0.72 – 1.16. Mg substitution enhances the adhesion strength, but comparable among different Mg concentrations. Greatly enhanced adhesion strength is achieved by F incorporation at the measured

F concentration around 1.0.

Finally, $\text{Mg}_{1.5}\text{FHA}/\text{MgF}_{1.5}\text{HA}$ bi-layer structured HA coating is developed. Single FHA phase forms in the coating with the substitution of Mg and F ions in HA lattice. It shows comparable in vitro bioactivity with pure HA coating, but more significant cell proliferation. The long-term stability of the bi-layer structured HA coating is much better than pure HA coating, exhibiting lower dissolution rate and higher adhesion strength.

Chapter 1 Introduction

1.1 Background

In orthopaedic medical engineering, there are two main concerns regarding the application of implants: short-term osseointegration and long-term stability. Osseointegration is the stable anchorage of an implant achieved by direct bone-to-implant contact [1]. Stability refers to a long-term service life of the implant with the ability to carry and sustain the static and dynamic loads [2]. It is obviously very important to achieve both of them within the shortest possible healing time and with a very small failure rate.

Metallic, ceramic and polymeric biomaterials have been widely used as bone replacements. Metallic alloys like titanium and titanium alloys are often employed as basic materials for load bearing implants because of their excellent mechanical properties. Even though these materials are mostly bio-inert, they are not bioactive, i.e., they do not form a mechanically stable link between the implant and bone tissue. Thus it is difficult to integrate into the body. As such, the metal surface has to be properly engineered. Many ways have been sought to improve the mechanical contact at the interface. The most effective way is to coat the metal with calcium phosphate ceramics that can promote bone-bonding between the metal and the bone. Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, HA) has similar composition and molecular

structure as that of the natural bone thus has been widely used as coating on metallic implant to promote cellular activities and stimulate formation of new bone. It has been suggested that the more similar to bone mineral of HA in composition, crystallinity, crystal structure, crystal size and morphology, the better osteoconductivity would be achieved [3].

However, bulk HA or HA coatings vary extensively from natural bone mineral in composition. Natural bone is a non-stoichiometric apatite containing many trace elements, such as Na^+ (*ca.* 0.7 wt%), K^+ (*ca.* 0.03 wt%), Mg^{2+} (*ca.* 0.55 wt%), Cl^- (*ca.* 0.10 wt%), and F^- (*ca.* 0.02 wt%), which play important roles in the physiological systems [4]. Pure HA does not have these composition, therefore its bioactivity is inferior to the bone mineral [5], which adversely affects the short-term osseointegration. Pure HA coating does not have strong adhesion strength on metallic substrate either. Moreover, it dissolves rapidly in body fluid, which in turn creates a long-term stability problem.

To resemble bone mineral in composition, various cations and anions are incorporated into HA or HA coatings, which are reviewed detailedly in Chapter 2. In brief, incorporation of cations like Mg^{2+} into HA decreases its crystallinity and improves the biological performances, i.e. good bioactivity, better cell behaviors [6]. Incorporation of anions (F^-) into HA crystal structure decreases the solubility of the HA coating [3, 7] and enhances the adhesion strength between the coating and the

substrate [8]. Therefore, it is interesting and desirable to incorporate both Mg and F ions into HA coating on Ti6Al4V substrates to have a combination of their individual functions in one coating/substrate system to achieve both short-term osseointegration and long-term stability.

1.2 Hypothesis and Objectives

- **Hypothesis**

This project is to develop a surface modification system for hard tissue implant using a sol-gel dip-coating method to fabricate an ion-substituted hydroxyapatite coated metallic substrate. We hypothesized that the simultaneous incorporation of magnesium and fluorine ions into hydroxyapatite coatings on titanium substrate promotes in vitro bioactivity and cellular response, as well as decreases dissolution rate and enhances adhesion strength.

- **Objectives**

The objectives of this project aim at investigating the simultaneous incorporation of Mg and F ions into the molecular structure of HA and their effects on in vitro bioactivity, cellular response, dissolution rate, and adhesion strength. Consequently, a bi-layer structured Mg and F ions co-substituted HA coating is developed to achieve an integration of short-term osseointegration

and long-term stability in one coating/substrate system.

1.3 Scope

The work needed to achieve above objective roughly consists of the following three domains:

1. The realization of simultaneous substitution of Mg at Ca location and F at OH position: grazing incidence X-ray diffraction (GIXRD), X-ray photoelectron spectroscopy (XPS) and fourier transform infra-red spectroscopy (FTIR) were used to examine the substitution.
2. The effect of Mg concentration on phase composition, microstructure, bioactivity, cell response, dissolution rate and adhesion strength of coatings with fixed F content (Mg_xFHA), where $0 \leq x \leq 2$. The effect of F concentration on phase composition, microstructure, bioactivity, cell response, dissolution rate and adhesion strength of coatings with fixed Mg content (MgF_yHA), where $0 \leq y \leq 2$.

For both the effects of Mg and F ions, the phase composition was determined by GIXRD and microstructure by scanning electron microscopy (SEM). In vitro bioactivity was evaluated in simulated body fluid (SBF). Cell response was assessed by human osteosarcoma MG63 cells. Dissolution rate was

studied in tris-buffered saline (TBS) solution. Adhesion strength was measured by pull-off test.

3. The development of a bi-layer structured Mg and F ions co-substituted HA coating for good short-term osseointegration and long-term stability at the same time: Mg_xFHA as the upper layer and MgF_yHA as the bottom layer. Both short-term osseointegration and long-term stability were compared with that of pure HA coating. Short-term osseointegration was assessed through in vitro bioactivity and cell response, dissolution rate and adhesion strength for long-term stability.

1.4 Organization

This report contains following chapters: a general introduction of the project is briefed in Chapter 1; Chapter 2 presents a literature review on hard tissue implants, HA and HA coatings, incorporation of cations and anions into HA and HA coatings; Chapter 3 describes the problems, strategy and methodology; the experimental results are shown and discussed in Chapter 4; Chapter 5 gives the conclusions and suggested recommendations.

Chapter 2 Literature Review

This chapter reviews the recent progress of hydroxyapatite (HA) coating for implant replacement application. It covers the hard tissue implants, HA crystal structure, ion substitution, fabrication methods, mechanical and biological properties, with emphasis on advances of cation/anion substitution in HA and its effects on mechanical and biological properties.

2.1 Hard tissue implants

Hard tissue implants are inserted into a bone fracture to facilitate healing or compensate for a lack or loss of bone tissue. The materials chosen for implants should be non-toxic and non-antigenic, as well as allowing the growth of immune and phagocytic cells and native tissues. The materials used in orthopaedic surgery include ceramics, polymers, metals (such as stainless steel, cobalt-chromium, pure titanium, Ti6Al4V alloy, NiTi shape memory alloy), and resorbable materials (such as bioglass, hydroxyapatite and bone grafts). The mechanical properties of some implant materials with bone are compared in Table 2-1.

Table 2-1 Comparison of mechanical properties between some implant materials
and bone [9, 10]

Materials	Ultimate tensile strength (MPa)	Elastic modulus (GPa)	Yield strength (MPa)
Bone	90-140	10-40	—
Hydroxyapatite	100	10	—
cpTi	785	105	692
Ti6Al4V	960-970	110	850-900
NiTi	800	70-110	100
Stainless steel	465-950	200	170-750
CoCrMo	600-1795	200-230	275-1585

In orthopaedic medical engineering, titanium and titanium alloys are the most preferred candidates because of their good biocompatibility, superior corrosion resistance and low modulus. However, the development of orthopaedic operation requires new demands for the man-made hard tissue implants. For example, regulation of biological processes, real integration with surrounding tissues, degradation of materials in biological system, infection resistance after implantation and shortened healing times [11].

One feasible strategy to cater to some of these demands is to deposit coatings onto

metals. The most commonly applied ones are calcium phosphate coatings, especially HA coatings. HA coatings have several advantages, such as similar composition with natural bone, the ability to form carbonated HA on their surfaces, the ability to promote cellular functions and expressions, provision of an appropriate scaffold for bone formation, the ability to bind and concentrate bone morphogenetic proteins [12]. Therefore, extensive attention has turned to develop titanium and titanium alloys coated with HA.

2.2 Hydroxyapatite and hydroxyapatite coatings

2.2.1 Calcium phosphates and hydroxyapatite

The application of calcium phosphates as bone substitute materials originates from their chemical similarity to the inorganic constituents of bone and teeth. They are non-toxic and bioactive, not only leading to an intimate physicochemical bond between the implant and bone, but also providing a scaffold or template for formation of new bone [13]. However, they are brittle with a poor fatigue resistance, restricting its application as load-bearing materials. Thus calcium phosphates are usually used as fillers or coatings for biomedical applications [14]. A brief description of biologically relevant calcium phosphates is presented in Table 2-2.

Table 2-2 Properties of biologically relevant calcium phosphates [13, 15, 16]

Compound	Formula	Ca/P ratio	Solubility at 37 °C, -log(Ksp)	Density (g/cm ³)
Monocalcium phosphate monohydrate (MCPM)	Ca(H ₂ PO ₄) ₂ •H ₂ O	0.5	No data	2.23
Monocalcium phosphate anhydrate (MCPA)	Ca(H ₂ PO ₄) ₂	0.5	No data	2.58
Dicalcium phosphate dehydrate (DCPD)	CaHPO ₄ •2H ₂ O	1.0	6.63	2.32
Dicalcium phosphate anhydrate (DCPA)	CaHPO ₄	1.0	7.02	2.89
Octacalcium phosphate (OCP)	Ca ₈ (HPO ₄) ₂ (PO ₄) ₄ •5H ₂ O	1.33	95.9	2.61
α-tricalcium phosphate (α-TCP)	α-Ca ₃ (PO ₄) ₂	1.5	25.5	2.86
β-tricalcium phosphate (β-TCP)	β-Ca ₃ (PO ₄) ₂	1.5	29.5	3.07
Hydroxyapatite (HA)	Ca ₁₀ (PO ₄) ₆ (OH) ₂	1.67	117.2	3.16
Tetracalcium phosphate (TTCP)	Ca ₄ (PO ₄) ₂ O	2.0	37-42	3.05

In general, the lower the Ca/P ratio, the more acidic and water soluble the calcium phosphate is. Among all the calcium phosphates, HA is the most stable and the least

soluble. The reason lies in the lattice structure. HA possesses a hexagonal structure with a $P6_3/m$ space group and cell dimensions $a = b = 9.42 \text{ \AA}$, and $c = 6.88 \text{ \AA}$, where $P6_3/m$ refers to a space group with a six-fold symmetry axis, a three-fold helix and microplane. It has an exact stoichiometric Ca/P ratio of 1.67 and is chemically very similar to the mineralized human bone [5]. The hexagonal HA structure comprises of Ca^{2+} sites surrounded by the tetrahedral PO_4^{3-} , OH^- or F^- ions occupying columns parallel to the hexagonal axis, as illustrated in Figure 2-1. The crystal structure presents two nonequivalent Ca^{2+} sites: one set of Ca^{2+} ions is aligned in columns (site I: Ca(I)); the other one forms equilateral triangles centered on the screw axes (site II, Ca(II)) [17, 18].

Figure 2-1 Crystal structure of hydroxyapatite [17, 19]

2.2.2 Hydroxyapatite coatings

HA coating has chemical similarity to bone and teeth mineral, therefore it is used on orthopaedic (e.g. hip-joint prosthesis) and dental implants. It enhances the osseointegration without forming fibrous tissues, but instead, an extremely thin and epitaxial bonding layer surrounds bone tissues [20]. So far, many deposition methods have been explored, including plasma spray methods [21-23], chemical treatment methods [24, 25], pulsed laser deposition or ablation [26, 27], sol-gel methods [28-31], electrochemical deposition [32-34], surface induced mineralization [35, 36], etc. Among these methods, the sol-gel method has its own advantages, such as low processing temperature, no requirements on shapes, high purity and homogeneity, low cost, and so on [37].

No matter which method is used to deposit HA on metal substrate, HA coatings behave the similar way after the implantation. HA coatings achieve a strong bonding with living bone in a relatively short period. The formation of the bone-implant bonding is schematically shown in Figure 2-2. Briefly, it can be described as follows: (1) initial dissolution of HA coating causes a rise of calcium and phosphate ion concentration in a local environment around the coating; (2) crystals precipitation on HA coating and ion exchange with surrounding tissues; (3) formation of a carbonated calcium phosphate layer with the incorporation of a collagenous matrix and bone growth towards the implant; (4) bone remodeling in

area of stress transfer by fast resorption of both carbonated HA in bone mineral and HA coating; (5) further bone ingrowth and remodeling at the bone-implant interface and biological fixation through the bidirectional growth of a bonding layer [38].

Figure 2-2 Schematic diagram of the formation of bone-implant bonding [38]

Although HA coated titanium implants combine both good biocompatibility of HA and excellent mechanical properties of metal substrate, significant differences still exist in between synthetic HA and biological apatite. For example, the bioactivity, in vivo resorption and degradation of synthetic HA and HA coatings were inferior to the bone mineral, studies have also shown poor adhesion strength between the

coating and substrate would lead to the loosening of the implant and ultimately cause implant failure.

The quality of the HA coating is the most important issue affecting the implant fixation, resorption, degradation, stability, etc. The factors influencing its quality include phase composition, crystallinity, microstructure, surface roughness, etc., all of which can lead to different outcomes with regard to bioactivity and stability. To improve the quality issues, numerous designs and methods have been proposed, one of which is to mimic the nano- and micro-scale features and/or chemistry of living tissues. In biological systems, calcium phosphates occur mainly in the form of poorly crystallized non-stoichiometric sodium-, magnesium-, and carbonate-containing HA (often called “biological apatite”) as the principle inorganic constituent of hard tissues. Two distinct features of biological apatite are small dimensions and low crystallinity, and the major properties are summarized in Figure 2-3. Since the apatite structure has an extraordinary ability to accept substitutions and vacancies as well as its adaptability to the biological functions of different tissues [39], it is possible to incorporate different trace elements into the apatite structure to tailor properties both physical, chemical and biological.

Figure 2-3 Crystal structure of biological apatite, powder X-ray diffraction patterns and infrared spectra of enamel, dentine and bone [40-42]

2.3 Incorporation of trace elements into HA

Bone has an apatite structure, and the general chemical formula is expressed as $\text{M}_{10}(\text{XO}_4)_6\text{Y}_2$, where M is a bivalent ion, XO_4 a trivalent ion and Y a monovalent ion. In general, it is represented as $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, and many trace elements are contained in the bone mineral. Table 2-3 gives the detailed information of the chemical composition of the most important human calcified tissues.

Table 2-3 Comparative composition and structural parameters of inorganic phases of adult-human calcified tissues [40, 41]

Composition	Enamel	Dentin	Bone	HA
Calcium [wt%]	36.5	35.1	34.8	39.6
Phosphorus (as P) [wt%]	17.7	16.9	15.2	18.5
Ca/P (molar ratio)	1.63	1.61	1.71	1.67
Sodium [wt%]	0.5	0.6	0.9	—
Magnesium [wt%]	0.44	1.23	0.72	—
Potassium [wt%]	0.08	0.05	0.03	—
Carbonate (as CO_3^{2-}) [wt%]	3.5	5.6	7.4	—
Fluoride [wt%]	0.01	0.06	0.03	—
Chloride [wt%]	0.30	0.01	0.13	—
Pyrophosphate (as $\text{P}_2\text{O}_7^{4-}$) [wt%]	0.022	0.10	0.07	—
Total inorganic [wt%]	97	70	65	100
Total organic [wt%]	1.5	20	25	—
Water [wt%]	1.5	10	10	—
α -axis [$\text{\AA}$]	9.441	9.421	9.41	9.430
c -axis [$\text{\AA}$]	6.880	6.887	6.89	6.891
Crystallinity index (HA=100)	70-75	33-37	33-37	100
Typical crystal sized [nm]	100 μm $\times 50 \times 50$	35 $\times$ 25 $\times$ 4	50 $\times$ 25 $\times$ 4	200-600

As shown in Table 2-3, many trace elements are present in human calcified tissues. They do not only play pivotal roles in the behavior of biological apatite, but also contribute to various biochemical reactions in the human body. For example, sodium (Na^+) is a monovalent ion available in abundance next to calcium and phosphorus, playing a significant role in bone metabolism and osteoporosis [43, 44]. Magnesium (Mg^{2+}) has its own significance in cell proliferation, calcification process and bone fragility. Moreover, it indirectly influences mineral metabolism. Mg deficiency may directly result in a decrease in osteoblastic bone formation, and delay the onset of mineralization of newly formed cartilage matrix and bones [4, 45, 46]. Potassium (K^+) is active in the mineralization and biochemical processes [47]. Fluorine (F^-) is well recognized for its potential behavior of decreasing the solubility of the apatite and its prevention role in dental caries in a bacteria containing, acidic environment. Also, F^- promotes the mineralization and crystallization of calcium phosphate in the bone forming process [7, 48, 49]. The significance of chloride (Cl^-) ions, resides in their ability to develop an acidic environment on the surface of bone that activates osteoblasts in the bone resorption process [50, 51].

With the presence of trace elements, partial/total substitutions may occur without significant changes in crystal structure. This is due to the apatite structure readily accepting a wide variety of anionic and cationic dopants [39]. Numerous attempts of substituted apatite have been reported. The substitution of Ca^{2+} with Mg^{2+} [30,

41, 52], Zn^{2+} [41, 53-55], Sr^{2+} [25, 56], Pb^{2+} [57], etc., the replacement of PO_4^{3-} by HPO_4^{2-} [58], CO_3^{2-} [40, 41, 59-62] or SiO_4^{4-} [63-65], and OH^- by F^- [29, 41, 66-68] or Cl^- [69]. In this review, the substitution of different kinds of cations and anions into HA will be extensively studied, with emphasis on the substitution effect on the phase evolution, mechanical and biological properties.

2.3.1 Incorporation of cation into HA

The chemical composition of hexagonal HA (within the ideal $\text{P6}_3/\text{m}$ space group) is $\text{Ca(I)}_4\text{Ca(II)}_6(\text{PO}_4)_6(\text{OH})_2$ (c.f. Figure 2-1). The local atomic configurations are displayed in Figure 2-4. The Ca(I) site is surrounded by six PO_4^{3-} tetrahedral and coordinated by nine oxygen ions. The Ca(II) site is seven coordinated with six oxygen ions from PO_4^{3-} and one oxygen ion from OH^- [70, 71]. It has been reported that the larger cations usually occupy the Ca(II) sites and the smaller ones prefer the Ca(I) sites, because the nearest distance among Ca(I) sites is smaller than that among Ca(II) sites [72]. The substitution of cations into HA tends to cause the HA structure disordering, and influence the phase, mechanical and biological properties.

Figure 2-4 Crystal structure of HA viewed along the *c*-axis (a) and the local atomic structures of Ca(I) (b) and Ca(II) (c) [19, 71]

Among the incorporated cations, Sr^{2+} and Pb^{2+} ions could replace Ca^{2+} ions in the whole range of composition [73, 74], while the incorporation of Mg^{2+} ions into HA crystal structure is still under debate. Generally, it seems that cations having larger atomic numbers tend to be more easily exchanged with Ca^{2+} in HA. However, ionic size and/or electronegativity of cations also affect the substitution for Ca^{2+} in HA. The ionic size difference between Ca^{2+} and M^{2+} causes the variations in the interatomic distances between substitutional divalent cations and the surrounding oxygens, thus the formation energy varies. The relevance of the formation energies to the ionic sizes was investigated and plotted in Figure 2-5. It was found that the formation energies increased more with increasing the ionic size difference between Ca^{2+} and M^{2+} , because larger lattice relaxations of the surrounding oxygen by substitution of the smaller or larger sized cations expense more significant elastic energy [70].

Figure 2-5 Formation energies of substitutional divalent cations against experimental ionic radii (Ionic substitution in HA under an aqueous solution at pH 7) [70]

2.3.1.1 Phase evolution and substitution of cation into HA

The substitution of Ca^{2+} with M^{2+} leads to the changes of as-formed phases. Figure 2-6 shows the phase evolution of Mg-substituted HA powders (MgHA) ($\text{Ca}_{10-x}\text{Mg}_x(\text{PO}_4)_6(\text{OH})_2$, x varies from 0 to 10) prepared by the mechanochemical-hydrothermal method. With increasing x (lower than 6.0), the HA diffraction peaks broaden and weaken, indicating the gradual decrease in crystallinity of the apatite phase. At $x = 6.0$, only an amorphous phase (Mg-containing amorphous calcium phosphate, Mg-ACP) is detected; at $x = 10.0$, only the $\text{NH}_4\text{MgPO}_4 \cdot \text{H}_2\text{O}$ phase is produced instead of HA or ACP. These results reveal that Mg has limited solubility in HA and it is difficult to obtain a pure HA phase with a complete Mg substitution ($x = 10.0$) [52]. The decrease of crystallinity with increasing Mg content was also

proved by other researchers [72, 75]. The gradual decrease of crystallinity is one of the indications that the incorporation of Mg into HA crystal structure leads to the increased lattice disorder [52]. Heat treatment of as-prepared MgHA powders at 900 °C for 1 hour in air shows that MgHA phase becomes thermally unstable with the increasing Mg content. Besides HA peaks at low Mg contents ($x = 0.5 - 1.0$), weak peaks of whitlockite $[(Ca, Mg)_3(PO_4)_2]$ are also observed; at $x = 2.0 - 4.0$, HA-derived peaks disappear, and other phases such as $(Ca, Mg)_3(PO_4)_2$, $Ca_4Mg_5(PO_4)_6$, β - $Ca_2P_2O_7$ form [52].

Figure 2-6 XRD patterns of as-prepared Mg-HA powders [52]

When the synthesis method is different, the substitution amount of Mg ions in HA

lattice changes. Ren F. et al. [18] synthesized Mg-substituted apatite ($\text{Ca}_{10-x}\text{Mg}_x(\text{PO}_4)_6(\text{OH})_2$, x varies from 0 to 10) by the wet-chemical precipitation method. Phase identification revealed that the apatite phase could be preserved only in the Mg concentration range of 0 – 1.5. However, the Mg/(Ca + Mg) molar ratio in the final apatite was significantly lower than that in the initial solutions. In their research, the upper limit of Mg substitution in HA lattice was between 5 and 7 mol%. The thermal stability of Mg-substituted apatite was studied by thermogravimetric analyzer (TGA) (Figure 2-7). The decomposition temperature of HA to β -TCP in the region of 690 – 800 °C decreases with Mg content as indicated in the TGA curves. Since Mg substitution in HA lattice could destabilize the crystal structure, the decrease of the decomposition temperature confirms that Mg enters the HA lattice because the surface adsorbed Mg on the apatite surface would not affect the decomposition temperature of HA to β -TCP.

Figure 2-7 TGA curves for Mg-substituted apatite [18]

Mg incorporated apatite coating ($\text{Ca}_{10-x}\text{Mg}_x(\text{PO}_4)_6(\text{OH})_2$, x varies from 0 to 2) has been deposited on Ti6Al4V substrates by the sol-gel method [76]. With the incorporation of Mg, Mg-substituted β -TCP (whitlockite, β -TCMP) forms in the coating and dominates at $x = 1.0 - 2.0$ (Figure 2-8). These results are consistent with Mg-substituted HA powders as discussed above: Mg-substituted HA is thermally unstable and Mg stabilizes whitlockite with the increasing Mg contents.

Figure 2-8 XRD patterns of the coatings with different Mg contents [76]

The substitution of cation into HA lattice can be reflected by tracing the change of the lattice parameters. The incorporation of larger or smaller cations into the HA lattice will cause the expansion or contraction of the unit cell. In MgHA powder, the unit cell dimensions a and c decrease almost linearly with increasing x_{Mg} ($x_{\text{Mg}} = \text{Mg}/(\text{Mg}+\text{Ca})$) (shown in Figure 2-9), induced by the substitution of Ca^{2+} ions (0.99 Å) by smaller Mg^{2+} (0.69 Å) ions [72]. The same results were also found by other

researchers [77].

Figure 2-9 Unit cell dimensions vs. x_{Mg} : a (○); c (□) [72]

When the larger cations such as Sr^{2+} ions (1.13 Å) are incorporated into the apatite, the lattice parameters of both a and c linearly increase with the addition of Sr (Figure 2-10) [74], indicating structural incorporation of Sr^{2+} into HA lattice. Similar results were also reported by Li Z.Y. et al. [78].

Figure 2-10 Variation in lattice parameters, a and c for $(\text{Sr}_x\text{Ca}_{1-x})_5(\text{PO}_4)_3\text{OH}$ [74]

2.3.1.2 Mechanical properties

Currently, most researchers are focusing on the preparation of cation incorporated

HA bulk, and the mechanical properties of bulk materials depend on the structures processed. For example, when Mg ions were added into the apatite reactant solution to be introduced into the cement system, it resulted in a reduction in compressive strength of cement [30]. When nanocrystalline Mg and Zn incorporated HA was synthesized, the addition of 1.0 wt% of MgO enhanced the compressive strength of hydroxyapatite by 33.8%, and the addition of 1.0 wt% of ZnO increased it by 6.2% [79].

Adhesion strength between the coating and the substrate is a critical factor for the successful implantation and long-term stability of the coated implant. Normally, scratch and pull-off tests are widely used. Scratch testing is a simple method to determine the coating adhesion and failure modes [80-82]. While a sample is displaced at a constant speed, elastic and/or plastic deformation takes place. At a certain load, damage occurs in the scratching region: the adhesive strength of the coating-substrate system is characterized by the critical load L_c . For the increasing load scratch test, it can be defined as the smallest load at which some recognizable failure occurs and the load bearing capacity is lost. As the scratch test is basically a comparison test, the critical load L_c determined by this method is the representative of coating adhesion behavior [82].

The pull-off test is useful in providing information on the adhesion properties at the interface of the coating and the substrate, and is a relatively quantitative method for

adhesion strength assessment. The adhesion strength is determined by applying a uniaxial tensile load to a cylindrical test assembly composed of one coated specimen bonded to an uncoated component. The coated and uncoated components are bonded together by the adhesive. The materials and dimensions of the uncoated component shall be the same as those of the coated specimen. After the preparation, the test assembly is placed in the grips of the tensile testing machine, and the tensile load is applied at a constant velocity until complete separation of the components is achieved [83]. Together with other testing methods of adhesion strength, the detailed bonding characteristics of the coating and the substrate can be obtained.

ASTM C633 standard governs the determination of the degree of adhesion of a coating to a substrate or the cohesion strength of the coating in a tension normal to the surface. According to the standard, two kinds of failure modes are defined: 1) adhesive failure happens at the coating-substrate interface, and is referred to as “the adhesion strength of the coating”; 2) cohesive failure occurs within the coating, and is referred to as “the cohesion strength”. The failure between the epoxy and the coating is also regarded as the cohesive failure [84]. However, a combination of these failures is often observed in one specimen. Figure 2-11 gives a schematic illustration of the failure modes for clear identification. Figure 2-11(a) shows the typical adhesive failure, Figure 2-11(b) for the typical cohesive failure, and a combination of both adhesive and cohesive failure in Figure 2-11(c).

Figure 2-11 Schematic illustration of the failure modes ((a): adhesive failure; (b) cohesive failure; (c) combination of all the failures)

The adhesion strength between Mg incorporated apatite coating and Ti6Al4V substrate was evaluated by the scratch test [85]. The adhesion strength increases almost linearly as more Mg ions are incorporated in the apatite coatings. The chemical bonds Ca-P-H-O-Ti-Mg at the interface of the coating and substrate contribute to the enhancement of adhesion strength. More Mg-Ti-O chemical bonds are formed as more Mg ions are incorporated in the coating, giving rise to higher adhesion strength in Mg-containing apatite coatings.

2.3.1.3 Biological performances

Understanding the performance of both natural and synthetic biomaterials is important for the development of new and more biocompatible implant materials. The evaluation involves both in vitro and in vivo assessments. In vitro assessment uses cell and/or tissue derived from both animals and humans and maintained in tissue culturing conditions. In vivo test uses animal models to evaluate the biological responses to biomaterials in living animals. By obtaining the clinical data

during the trial period and histological data from the examination of post-mortem tissues, we are able to assess the tissue responses to the implanted materials.

The prerequisite for the biomaterials to bond with living bone is the formation of a layer of biologically active hydroxylcarbonate apatite (HCA) on the biomaterial surface when it is exposed to physiological fluids. The behavior of HCA formation on biomaterials in a simulated body fluid (SBF) is known as the *in vitro* bioactivity, which is used to evaluate the *in vivo* bioactivity of bone-forming biomaterials [86].

Zn incorporated HA with Zn concentrations (Zn/Zn+Ca) at 0.5, 1, 2 and 5 at% was immersed into biological fluids to study the physico-chemical reactions at the materials periphery [87]. A Ca-P-Mg layer formed at the periphery of the HA incorporated with 5 at% Zn, representing the bioactive properties of Zn-incorporated HA. Biomimetic Mg-substituted HA (~1.0 wt% of Mg) prepared via wet-chemical synthesis made the apatite more bone-like, enhanced the solubility of the material at the physiological pH value, and improved the cell behaviors of MSCs and MG63 in terms of adhesion, proliferation and metabolic activation compared with stoichiometric HA [88]. Sr-containing HA (Sr-HA) cement prepared for primary hip replacement showed good bioactivity and bone-bonding ability after the implantation using a rabbit model, and direct contact was confirmed between the Sr-HA cement and cancellous and cortical bone under load-bearing conditions [89, 90].

Cation-incorporated HA coatings were also evaluated by in vitro and in vivo tests. Sr-HA coatings on Ti6Al4V substrates were prepared by plasma spraying aiming at the applications of load bearing implants such as artificial hip replacement. The coating exhibited strong bonding with substrate, its good bioactivity was demonstrated by forming a bone-like apatite layer on the surface of the coating when immersed in the SBF solution, and its excellent biocompatibility was indicated by cell culture tests with human osteoblasts [91]. Also, Sr-substituted apatite coating could be grown on Ti6Al4V substrate through biomimetic synthesis. The presence of Sr ions in the solution inhibited the coating formation and resulted in the decrease of coating thickness. The as-formed Sr-substituted biomimetic coatings presented a bone-like structure similar to human bone, which might have a positive effect in enhancing bone formation and contributing to better osseointegration [56]. Mg-containing apatite coatings prepared by the sol-gel method promoted the formation of apatite layer in the SBF solution, and showed good bio-responses with MG63 cells just like the HA coatings [92, 93].

2.3.2 Incorporation of anion into HA

There are two kinds of anions in HA crystal structure, phosphate group (PO_4^{3-}) and hydroxyl group (OH^-). PO_4^{3-} can be replaced by HPO_4^{2-} , CO_3^{2-} or SiO_4^{4-} , and OH^- by F^- or Cl^- . In this review, the emphasis is on the substitution of OH^- groups. In turn, the effect of anion incorporation into HA will be discussed through phase

evolution, solubility, mechanical and biological properties.

2.3.2.1 Phase evolution and substitution of anion into HA

When OH^- groups in HA are partially substituted by F^- ions, fluoride-substituted or fluoridated hydroxyapatite (FHA) forms with a composition of $\text{Ca}_{10}(\text{PO}_4)_6\text{F}_x(\text{OH})_{2-x}$ ($0 < x < 2$). If all the OH^- groups are substituted by F^- ions, it is called fluorapatite (FA, $\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$). FA has a very similar crystal structure with HA and belongs to the same space group as HA. The arrangement of OH^- groups and F^- ions in HA, FHA and FA are shown in Figure 2-12. Theoretically, F^- ions easily replace OH^- groups in HA on thermodynamic ground ($\Delta E_x = -0.4 - 0.6 \text{ kJ mol}^{-1}$). The lowest energy of the crystal structure is reached at $x = 1.0$ with a configuration that HA crystal consists of alternating planes of F^- ions and OH^- groups, where they are alternating in the c -axis, but co-located within the a/b -plane [94].

Figure 2-12 Arrangement of OH^- groups and F^- ions in HA, FHA and FA crystal structures [95, 96]

The incorporation of F ions in HA improved the thermal and chemical stability of HA. Figure 2-13 is the TGA data of HA, FHA and FA powders heated from 25 °C to 1400 °C in dry air, which shows different degrees of weight loss of samples with different F contents [96]. For pure HA powder, the weight loss from 25 °C to 200 °C is caused by the evaporation of absorbed water in the powder, that from 800 °C to 850 °C due to the release of OH⁻ groups from the HA crystals, and the decomposition of HA and further loss of OH⁻ groups are happened above 1000 °C. However, the decomposition stage is not observed with F incorporation, suggesting the retarded decomposition and improved thermal stability of HA. Moreover, the dilatometer analysis showed that the onset sintering temperature of FHA powder was increased to 1150 °C compared with that of HA powder around 1000 °C.

Figure 2-13 TGA data of HA, FHA and FA powders heated to 1400 °C in dry air [96]

When F⁻ ions are incorporated into HA lattice, the lattice parameters are changed accordingly. Since F⁻ (1.32 Å) is smaller than OH⁻ (1.68 Å), the substitution results

in a contraction in the a -axis, but no significant change along the c -axis [97-99]. The substitution is reflected from XRD patterns (Figure 2-14): the (300) peak of FA shifts towards a higher 2θ relative to that of calc-HA (calcined from HA at 1000 °C for 1 hour in air) [98, 100].

Figure 2-14 Short-range XRD patterns of calc-HA, calc-fHA and FA [98]

The incorporation of F^- ions also influences the vibration behavior of OH^- groups [29, 97]. As shown in Figure 2-15, the bands at 3570 and 630 cm^{-1} are assigned to the stretching and wagging of free OH^- respectively. With increasing F content, the intensities of OH^- peaks decrease, and an additional $OH-F$ stretching band appears at a lower frequency due to the partial substitution of OH^- groups with F^- ions. When OH^- groups in HA were completely replaced by F^- ions, the OH^- peak at 3570 cm^{-1} disappears. The split and shift of OH stretching peak at 3570 cm^{-1} can be an indication of the substitution of OH^- groups with F^- ions.

Figure 2-15 FTIR spectra of the coatings: (a) HA, (b) 25FHA, (c) 50FHA, (d) 75FHA, (e) FA [29]

XPS is another way to identify the F incorporation into HA structure. In Figure 2-16, F 1s is not detected in the original sample (a). As F concentration increases, the peak at 684.3 eV intensifies. While for CaF_2 , F 1s peak locates at 685.0 eV. Moreover, the binding energy of Ca 2p shifts to 347.4 eV, which is different from that in the original sample (at 347.1 eV) and that for CaF_2 (at 348.3 eV). This is due to the chemical shift relative to the incorporation of F^- ions in the apatite [101]. The fingerprint of F 1s in FHA or FA at 684.3 eV was also reported by other researchers [102, 103].

Figure 2-16 XPS spectra of F 1s (A) and Ca 2p (B) of CaHA treated with various F concentration (mol/dm^3): (a) 0, (b) 0.01, (c) 0.03, (d) 0.05, (e) 0.10, (f) CaF_2 [101]

When OH^- groups in HA are partially replaced by Cl^- ions, chlorine-substituted HA (chlorapatite, Clap) is obtained [69]. The hexagonal apatite structure retains for all the chlorine substituted samples with no major structural distortion. The calculated lattice parameters of the samples provide the evidence for the incorporation of Cl^- ions in the apatite structure. A significant expansion of all the parameters of a -axis and c -axis was observed for Clap with respect to that of HA due to the substitution of bigger Cl^- ions ($1.81 \text{ \AA}$) for OH^- groups ($1.68 \text{ \AA}$).

2.3.2.2 Solubility

Solubility is an important factor influencing both the short-term osseointegration and the long-term stability. Immediately after the implantation, a certain extent of

dissolution of the coating is necessary to increase the local ion concentrations of calcium and phosphate to stimulate the formation of new bone. From the viewpoint of the long-term stability of the implant, the dissolution resistance of the coating should be strong enough to avoid the detachment of the coating from the substrate. Therefore, it is vital to investigate the solubility of the coating. Factors affecting the solubility of a material include the preparation method, the resultant crystallinity and density, and the extent of ion substitutions into the apatite lattice [104].

It is well known that fluoride can prevent the caries. The mechanism of reduced solubility of HA by addition of fluoride has been investigated intensively [105-108]. Moreno E.C. et al. [108] discussed the solubility of FHA ($\text{Ca}_5(\text{PO}_4)_3(\text{OH})_{1-x}\text{F}_x$) theoretically and experimentally. A series of solid solutions with different fluoridation degrees x , from 0 to 1, were prepared. The solubility of these compounds was obtained by equilibrating them for more than one month with a series of dilute phosphoric acid solutions at 37 °C. The solubility product constant K_x at various substitution concentrations was calculated according to the experimental data as shown in Figure 2-17. From Figure 2-17, a minimum in K_x is seen at a substitution degree of 0.56. The decreased solubility can be explained on the basis of the disordered structure of HA and the hydrogen bonding between OH^- groups and F^- ions. Also, the solubility of heterogeneous FHA was studied by Okazaki M. et al. [109, 110]. The apparent solubility of FHA decreased with the increased fluoridation degree no matter the fluoride was supplied in the initial or

final half of the experimental period.

Figure 2-17 Solubility of the solid solutions as a function of the degree of fluoride substitution $(\text{Ca}_5(\text{PO}_4)_3(\text{OH})_{1-x}\text{F}_x)$, a: pH 5; b: pH 6; c: pH 7 [108]

Regardless of the methods of preparing FHA coatings (such as thermal spraying [111], electron-beam deposition [29], sol-gel deposition) [7, 49], similar dissolution behaviors were found, i.e. the incorporation of F^- ions decreased the solubility of HA coatings as shown in Figure 2-18.

Figure 2-18 Dissolution behavior of FHA coatings in physiological saline solution:

Ca^{2+} concentration as a function of soaking time [49]

Besides the experimental studies of the effect of F incorporation on the solubility of HA, a computer modeling study was also done using molecular dynamic simulations [94, 112]. In pure HA material, the onset of dissolution is induced through the leaching of surface hydroxyl groups from the material into solution in the presence of water. When F^- ions partially replace OH^- groups, OH^- group may dissolve, but the F^- ion at the surface does not dissolve as readily but remains in the crystal. Moreover, the surface Ca^{2+} ions persist more closely coordinated to the material surface in the vicinity of the substituted F^- ions. Thus the incorporation of F^- ions into the lattice makes the materials more dissolution resistant through two aspects: the resistance towards dissolution into the solvent of F^- ions themselves and strong anchoring of the surface Ca^{2+} ions.

2.3.2.3 Mechanical properties

Fluorine incorporation into HA also changes the mechanical properties of HA. Bulk FHA has been widely synthesized, and their mechanical properties were measured using indentation, including hardness, elastic modulus, fracture toughness and brittleness [113, 114]. Both the fluoridation degree and the sintering temperature have an effect on the mechanical properties of bulk HA, which are not reviewed here. However, the dependence of F incorporation on the mechanical properties of FHA coatings is reviewed below in detail.

Wang Y.S. et al. [8, 115, 116] have systematically investigated the mechanical properties of sol-gel derived FHA ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_{2-x}\text{F}_x$, x varies from 0 to 2) coatings on Ti6Al4V substrates. The adhesion strength between the coating and the substrate was evaluated through both scratch and pull-out tensile tests. Scratch test revealed that the adhesion strength was up to 35% better as the F concentration increased in the coating, and this adhesion enhancement was much more obvious at higher firing temperatures. The pull-out tensile test showed that the adhesion strength increased up to about 40% from ~19 MPa for HA to ~26 MPa for FHA ($x = 1$) as shown in Figure 2-19. After soaking in tris-buffered physiological saline (TPS) solutions for 21 days, the enhancement became more significant because the rougher surface after the dissolution resulted in stronger bonding between the epoxy and the coating, and in turn reduced the area fraction of the “adhesive

failure”.

Figure 2-19 Adhesion strength of FHA coating before and after soaking in TPS solutions [116]

The toughness, interfacial shear strength, and residual stress of FHA coatings have also been evaluated [8, 115, 116]. At the fluoridation degree of $x = 1$, the toughness of the coating doubled as compared with HA coating. The interfacial shear strength was evaluated by the shear strain lag method, and the “wafer curvature method” was employed to measure the residual stresses. The resultant interfacial shear strength increased from ~393 MPa (pure HA) to ~459 MPa (1.96 at% fluorine) and further to ~572 MPa (3.29 at% fluorine). The residual stress in the coating also decreased from ~273 MPa to ~190 MPa and further to ~137 MPa as F concentration in the coating increased.

Other researchers also studied the mechanical properties of FHA coatings on metal

substrates. For instance, Kim H.W. et al. [97] deposited a HA/FHA double-layered film on titanium by a sol-gel method, which was highly dense and uniform, and adhered to the substrate strongly with an adhesion strength of about 40 MPa.

2.3.2.4 Biological performances

Fluoride has an affinity for skeletal tissue, and also has a specific mitogenic activity and attraction for cells of skeletal origin. Fluoride seems to be an enhancer of bone cell growth factors, and requires the presence of appropriate growth factors to induce calcification. It may act primarily on the osteoprogenitor cells and/or undifferentiated osteoblasts cells rather than stimulating the proliferation of highly differentiated osteoblasts. It is important in bone healing and bone regeneration because it will induce the differentiation of osteoprogenitor cells and undifferentiated precursor cells into osteoblasts (Figure 2-20) [12].

Figure 2-20 Effect of fluoride in cell differentiation and new bone formation [12]

In vitro cell culture tests are widely used to evaluate the effect of fluorine content in FHA bulks and coatings on the cell activities in terms of cell attachment, morphology, proliferation and differentiation [49, 117-119]. For FHA bulks, more cell attachment and proliferation were observed on FHA bulks than on pure HA, and cells on FHA bulks demonstrated a higher alkaline phosphatase (ALP) activity than those on pure HA after 3 weeks of culturing [119]. Wang Y.S. et al. [49] found that F incorporation had positive stimulating effect on cell proliferation, ALP activities and osteocalcin levels, especially at the fluoridation degree of 0.8 – 1.1. However, Kim H.W. et al. [7] found that the osteoblast-like MG63 and HOS cells grew and proliferated favorably on all the HA and FHA coatings as well as on pure Ti. The osteoblast cells proliferated on FHA coatings to a lower extent than on pure HA coating obtained by electron-beam deposition, and the ALP activity of the cells on the FHA coatings was not significantly different from that on the HA coating [29]. The positive or negative effect of fluorine ions in FHA coatings on the cell behaviors are still under argument.

In addition, several phenomena were observed during in vivo studies of FHA coatings. The degradation of plasma sprayed HA, FA and FHA coatings on dental implants evaluated in dog jaws till 12 months indicated good integration between FHA coating and bone tissue. HA and FA coatings (even at 100 μm thickness) were almost completely degraded within the implantation period. In contrast, FHA coatings did not show significant degradation during the same period [120].

Savarino L. et al. [121] have investigated the in vivo compatibility and degradation of plasma sprayed FHA, HA coatings and titanium oxide (TiO_x). The bone at the interface was not mineralized in all samples after two weeks of implantation. After 12 weeks, the newly formed bone tissue at the interface with both FHA and HA coating was shown to be fully mineralized. Also, the in vivo tests done by Caulier H. et al. [122] proved that no significant differences were found in bone reaction among uncoated, plasma-sprayed HA and FA coatings on titanium on the initial bone healing of oral implants.

2.3.3 Problems involved in cation or anion incorporated HA

To make the implant more acceptable to bone cells and stimulate rapid integration with bone rather than fibrous encapsulation, the surface coating should have suitable biochemical and structural characteristics to elicit wanted cell activity and finally result in full osseointegration. Moreover, the resultant bone-implant interface must have the internal strength and bonding capacity to the underlying metal substrate so as to endure the forces produced by the implant loading. Cation or anion substitution into HA improves the mechanical and biological properties in comparison with pure HA. For example, the cations like Mg^{2+} incorporation is beneficial to the biological performance of HA, while the anions like F^- incorporation improves the adhesion strength between the coating and the substrate. However, it is found that the short-term osseointegration and the long-term stability

are difficult to be addressed at the same time if only cation or anion is incorporated. As such, simultaneous incorporation of cation and anion into HA is proposed to exert their special advantages in biological and mechanical performances and achieve the short-term osseointegration and the long-term stability of the implant, which will be reviewed in the next section.

2.3.4 Simultaneous incorporation of cations and anions into HA

The apatite structure has the ability to accept a wide number of possible substitutions in its lattice, and trace elements are important to bone mineral, so the simultaneous incorporation of several elements into apatite structure is expected to improve the performances of apatite. So far, many attempts have been made to synthesize the apatite substituted with different cations and anions. The simultaneous incorporation of cations and anions into HA is summarized in Table 2-4. As shown in Table 2-4, aqueous precipitation is the most commonly used method for the synthesis of cations and anions incorporated bulk HA, while sol-gel method is preferred to deposit HA and derivative coatings, which can control the precise composition of the coating.

Table 2-4 Summary of simultaneous incorporation of cations and anions into HA

	Cation	Anion	Method	References
Bulk	K ⁺	Cl ⁻	Melt salts method	[123]
	Al ³⁺	F ⁻	Aqueous precipitation method	[124]
	Sr ²⁺	F ⁻	Aqueous precipitation method	[124]
	Zn ²⁺	F ⁻	Aqueous precipitation method	[124]
	Mg ²⁺	F ⁻	Aqueous precipitation method	[124-127]
	Mg ²⁺	F ⁻	Heterogeneous synthesis with two-step supply system	[128]
	Mg ²⁺	CO ₃ ²⁻	Aqueous precipitation method	[129, 130]
	Mg ²⁺	CO ₃ ²⁻	Precipitation method with gradient Mg supply system	[131]
	Mg ²⁺	CO ₃ ²⁻	Neutralization method	[88]
	Mg ²⁺	CO ₃ ²⁻ , F ⁻	Aqueous precipitation method	[132]
Coating	Na ⁺ , Mg ²⁺	F ⁻	Aqueous precipitation method	[48]
	Na ⁺ , K ⁺ , Mg ²⁺	F ⁻ , Cl ⁻	Aqueous precipitation method	[133]
	Zn ²⁺	F ⁻	Sol-gel method	[54, 55, 134, 135]

When cations and anions are incorporated into HA simultaneously, crystallographic properties, thermal behaviors, mechanical and biological properties, etc. differ from

individual substitution of cations or anions into HA. For example, cations themselves (Al^{3+} , Mg^{2+} , Zn^{2+} or Sr^{2+}) at low levels in the solution had negative effects on the properties of apatite, e.g., lower crystallinity, lower Ca/P ratios, higher HPO_4/PO_4 , and higher dissolution in an acid buffer. When only F^- ions were present in the precipitation solution, positive effects on the apatite were observed, e.g., greater crystallinity, higher Ca/P, lower HPO_4/PO_4 , and lower dissolution. However, the simultaneous presence of F^- ions with the cations (Al^{3+} , Mg^{2+} , Zn^{2+} or Sr^{2+}) minimized the negative effects caused by the cations, suppressed the formation of Mg-substituted β -TCP (β -TCMP), and allowed a greater incorporation of cations in the apatite [124].

When Mg^{2+} and F^- ions were co-existent in the apatite, the decrease of crystallinity observed at lower F content with the increasing of Mg concentration was restored at higher F content, peaking at half of the maximum F content [126]. When CO_3^{2-} ions were added into Mg-containing fluoridated apatite, this specific crystallinity behavior disappeared. The overall crystallinity was lower than that of Mg-free and CO_3 -free FHA, and the crystallinity increased with the increasing F content. The negative effect of Mg^{2+} ions on the crystallinity was inhibited by the presence of CO_3^{2-} ions [132]. As discussed above, the substitution of F^- ions into apatite resulted in a contraction in the a -axis, but no significant change along the c -axis [97-99]. When Mg^{2+} and CO_3^{2-} ions were introduced into FHA, there was a little difference. As F content increased, the a -axis decreased greatly, while the c -axis

scarcely changed; comparatively, it was still a little lower than that of pure HA [132]. In aqueous solution, the substitution of PO_4^{3-} with CO_3^{2-} ions resulted in a decrease of *a*-axis dimension, thus a greater reduction of *a*-axis was caused together with the substitution of F^- ions. Since Mg concentration was a constant, the *c*-axis scarcely changed with a small decrease compared with that of pure HA.

Besides crystallographic properties, the thermal stability was improved by the incorporation of multiple elements. The thermal stability of HA phase was enhanced up to 1400 °C compared with the stoichiometric HA decomposing into α -TCP beyond 1200 °C. Also, significant improvements in the mechanical properties (flexural strength, hardness, etc.) were obtained for the sintered co-substituted HA, especially at higher concentrations of substituted elements [48, 133].

Zinc-containing FHA (ZnFHA) films were deposited on Ti6Al4V substrates by the sol-gel dip-coating method [54, 55]. The results showed that the doping of Zn lowered down the film crystallinity and promoted the precipitation of apatite particles from the SBF solution, and the cell proliferation on the ZnFHA films was significantly increased compared with FHA film. The reduced bacterial attachment on ZnFHA films was also observed by other researchers [135]. The presence of Zn might accelerate the release of F ions from the film, which was found to act as enzyme inhibitors affecting the bacterial metabolism, and thereby leading to the

minimum bacterial attachment compared with Ti, TiN and FHA films.

2.3.4.1 Current status of Mg and F ions incorporated HA

Among different cations and anions, Mg and F ions are selected in this project. As the fourth abundant element in human body, Mg could reduce the risks of cardiovascular disease, promote the catalytic reactions, and control the biological functions. Mg content in the natural bone is affected by a great variability depending on aging: it is contained in high concentrations in cartilage and natural bone tissue during the initial stages of osteogenesis, while it tends to disappear when the bone is mature. For example, 1.0 wt% Mg was found in the new-born rats, and this level decreased to 0.5 wt% after one year [130]. So, it is vital to introduce Mg ions during the stage of new bone formation. Furthermore, F ions as well are recognized for its potential behavior on the long-term stability of the apatite coating, such as the improvement of mechanical properties and dissolution resistance.

Okazaki M. [126, 128] has systematically investigated the crystallographic properties of Mg-free and Mg-containing fluoridated apatite (Mg-FHAp_I and Mg-FHAp_{II} with different Mg concentration) synthesized by aqueous precipitation and two-step supply system. The inverse of the half-value breadth calculated from (300) and (002) reflections is represented as the crystallinity along the *a*-axis and *c*-axis, as shown in Figure 2-21. The crystallinity varies in each series with increasing F content. A minimum crystallinity of FHAp is observed at the low F

content, and then the crystallinity increases with the increasing F content (Figure 2-21A). At lower Mg content, Mg-FHAp_I, this minimum crystallinity is invisible, and an overall increase of crystallinity is found (Figure 2-21B). At higher Mg content, Mg-FHAp_{II}, the crystallinity steadily reaches a maximum at about half of the maximum F content (Figure 2-21C), and the whole crystallinity of Mg-FHAp_{II} is lower than that of Mg-FHAp_I. The minimum crystallinity seen at lower F content in FHAp might be due to the imbalance between F⁻ ions and OH⁻ groups, which is relieved by the increase of F content. When Mg²⁺ ions are added, especially at higher concentration, the positive effect of F⁻ ions on the crystallinity is weakened by the addition of Mg²⁺ ions.

Figure 2-21 Crystallinity of Mg-free (FHAp, A) and Mg-containing fluoridated hydroxyapatite (Mg-FHAp_I, B and Mg-FHAp_{II}, C) [126]

The influence of Mg and F ions on the crystallinity of Mg-containing fluoridated apatite was also reported by Hidouri M. et al. [125]. As shown in Figure 2-22, a slight broadening of the reflections is observed as the increased Mg concentration (Figure 2-22b-d), indicating the lower crystallinity of Mg-containing fluoridated apatite compared with pure fluoridated apatite (Figure 2-22a).

Figure 2-22 XRD patterns of as-prepared powders with different Mg concentrations:

(a) Fap; (b) 0.25MgFap; (c) 0.50MgFap; (d) 1.00MgFap [125]

The lattice dimensions of three series of FHAp were calculated from (300) and (002) reflections using silicon as standard, as shown in Figure 2-23. The decrease of a -axis of Mg-free and Mg-containing FHAp with the increased fluoridation degree suggests the substitution of F^- ions into the apatite crystal structures. The c -axis also decreases with the increased Mg content over the whole range of F content due to the substitution of Ca^{2+} with Mg^{2+} ions. This substitution can also be reflected

from the shifting of (002) peaks (Figure 2-24). With the increasing Mg content, (002) peak shifts towards higher degree. In addition, the decreased a - and c -axis with the increasing of Mg content further confirmed the substitution [125].

Figure 2-23 Dimensions of a - and c -axis for Mg-free (FHAp) and Mg-containing fluoridated hydroxyapatite (Mg-FHAp_I and Mg-FHAp_{II}) [126]

Figure 2-24 (002) peak reflections of as-prepared powders: (a) Fap; (b) 0.25MgFap; (c) 0.50MgFap; (d) 1.00MgFap [125]

The thermal behavior of Mg-containing FHA was investigated by Hidouri M. [125]. As-prepared powders were calcined at different temperatures to determine possible changes of sintered phases. Take 1.00MgFap as an example (Figure 2-25). A secondary phase $\text{Mg}_2\text{F}(\text{PO}_4)$ is detected in the range of 650 °C to 1120 °C, and the amount of this phase increases with increasing Mg concentration. $\text{Mg}_2\text{F}(\text{PO}_4)$ phase disappears at 1300 °C due to its dissolution in the liquid phase or incorporation into the apatite structure. Since no decomposition of the material is observed at 1300 °C, it is concluded that the incorporation of Mg into apatite does not affect its thermal stability.

Figure 2-25 XRD patterns of as-prepared 1.00MgFap powder sintered at different temperatures [125]

Figure 2-26 shows the solubility of Mg-free and Mg-containing FHAp as the

function of dissolved Ca^{2+} concentration in 0.5 mol/L acetate buffer solutions (pH 4.0, 37 °C). The solubility of Mg-free FHAp is lower than that of Mg-containing FHAp at lower F content, but the difference between them is negligible at higher F content [126]. Even if the inclusion of Mg^{2+} ions increases the solubility of FHA, the strong bonding of F^- ions with neighbouring ions may be maintained to stand against the acid attack, so that the solubility of different series at higher F content does not vary extensively.

Figure 2-26 Solubility of Mg-free (FHAp) and Mg-containing fluoridated hydroxyapatite (Mg-FHAp_I and Mg-FHAp_{II}) as dissolved Ca^{2+} concentration [126]

Most recently, Sun Z.P. et al. [127] synthesized $\text{Mg}^{2+}/\text{F}^-$ -doped nanophase HA powder by a precipitation method followed by sintering at 1100 °C for 1 hour. The highest microhardness was achieved in 1% Mg- and 2.5% F-doped HA. Cytocompatibility testing shown in Figure 2-27 demonstrates no statistical

difference in osteoblast cell densities after 4 hours between HA, HA1Mg, HA2.5Mg, HA7.5Mg, HA1Mg2.5F, and HA2.5Mg2.5F. Significant increases of osteoblast cell densities are found on HA7.5Mg2.5F and HA2.5Mg7.5F (where 7.5Mg and 2.5F refers to the mole percent of Mg and F in HA, respectively). However, the reason for the greater osteoblast adhesion on these two samples is still unknown.

Figure 2-27 Four-hour osteoblast adhesion test (* $p < 0.05$ with respect to HA7.5Mg2.5F, # $p < 0.05$ with respect to HA2.5Mg7.5F) [127]

2.4 Summary

HA coated titanium and titanium alloys are widely used for hard tissue implants, especially for artificial hip joints and dental implants. However, pure HA coating

has drawbacks such as inferior bioactivity to bone mineral (influence the short-term osseointegration), and poor adhesion strength between the coating and the substrate (affect the long-term stability). To tackle these drawbacks, a number of ions have been incorporated into HA to replace Ca^{2+} , OH^- or PO_4^{3-} . Incorporation of cations (Mg^{2+}) into HA not only changes the lattice parameters and decreases the crystallinity, but also improves the biological performances, i.e. good bioactivity, better cell behaviors. The incorporation of anions (F^-) into HA lowers the dissolution rates of HA coatings. Moreover, the incorporation of F^- ions into HA coating improves the mechanical properties of HA coating, especially the adhesion strength. However, the effect of anion on the biological performances is still under debate. Since the performance of cations or anions incorporated HA is not satisfactory due to their insufficient short-term osseointegration and long-term stability, simultaneous incorporation of cations and anions into HA is proposed. The preliminary investigation shows that the simultaneous incorporation of cations and anions results in different crystallinity, thermal stability, solubility, mechanical and biological performances from those of cations or anions incorporated HA. However, up to date, the exploration on simultaneous incorporation of cations and anions (especially Mg and F ions) into HA, is not systematical and needs further investigation, especially when they are employed as coatings on metal substrates.

Chapter 3 Problem Statement and Methodology

3.1 Problem statement and strategy

For the real application of HA coated titanium alloys in hard tissue implants, both the short-term osseointegration and the long-term stability must be fulfilled at the same time. However, pure or cation/anion incorporated HA coating on titanium could not meet these two demands. Improved biological performances of cation incorporated HA are beneficial for the short-term osseointegration, while anion incorporated HA is advantageous to the long-term stability of the implant. In this project, simultaneous incorporation of cation (Mg) and anion (F) into HA is proposed, aiming to achieve both the short-term osseointegration and the long-term stability in one coating/substrate system. A sol-gel dip-coating method is selected to control the precise composition of the coating and easily realize a multi-layered structure.

3.2 Project overview

In this project, a sol-gel dip-coating method is used to deposit Mg and F ions incorporated HA coatings, including Mg_xFHA , MgF_yHA and bi-layer structured HA coatings. The coatings are investigated in terms of the co-substitution mechanism, in vitro bioactivity, cell response, dissolution rate, and adhesion strength. An overview of the whole project is illustrated in Figure 3-1.

Figure 3-1 Overview of the whole project

3.3 Coating preparation

3.3.1 Substrate preparation

Titanium alloy (Ti6Al4V) was selected as the substrate. Ti6Al4V substrates ($20 \times 30 \times 1.2 \text{ mm}^3$ or $20 \times 30 \times 2 \text{ mm}^3$) were firstly abraded with silica carbide (SiC) sandpapers (grit range of #180-#1200), then ultrasonically cleaned in acetone for 15

minutes and then in deionized water for 15 minutes. After cleaning, the substrates were dried in compressed air and prepared for dip-coating.

3.3.2 Sols' preparation

Calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, Merck, AR) and magnesium nitrate hexahydrate ($\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, Merck, AR) were dissolved in ethanol to prepare 2 M of Ca-precursor and Mg-precursor. They were mixed together to form a Ca-Mg mixture according to the designed amount. 2 M of P-precursor was prepared by dissolving phosphorous pentoxide (P_2O_5 , Merck, GR) in ethanol followed by a refluxing process for 24 hours. Then F-precursor (diluting hexafluorophosphoric acid (HPF_6 , Sigma-Aldrich, GR) in ethanol) was added to P-precursor to obtain a P-F mixture. The Ca-Mg mixture was added drop-wise into the P-F mixture to form a solution with a (Ca, Mg)/P ratio of 1.67. This mixture was further refluxed for 24 hours to obtain the sol. The sketch map of the whole process is shown in Figure 3-2.

Figure 3-2 Sketch map of the sols preparation

For Mg_xFHA coatings, the designed substitution degree of Ca by Mg is indicated by the x value in the general formula of $\text{Ca}_{(10-x)}\text{Mg}_x(\text{PO}_4)_6\text{F}_1(\text{OH})_1$, where $x = 0, 0.5, 1.0, 1.5$ and 2.0 . The corresponding samples were labeled as FMA0, FMA1, FMA2, FMA3 and FMA4, or the corresponding coatings as Mg_0FHA , $\text{Mg}_{0.5}\text{FHA}$, Mg_1FHA , $\text{Mg}_{1.5}\text{FHA}$ and Mg_2FHA for further discussion (Table 3-1).

For MgF_yHA coatings, the designed fluoridation degree is indicated by the y value in the general formula of $\text{Ca}_9\text{Mg}_1(\text{PO}_4)_6\text{F}_y(\text{OH})_{(2-y)}$, where $y = 0, 0.5, 1.0, 1.5$ and 2.0 . The corresponding samples were labeled as MFA0, MFA1, MFA2, MFA3 and MFA4, or the corresponding coatings as MgF_0HA , $\text{MgF}_{0.5}\text{HA}$, MgF_1HA , $\text{MgF}_{1.5}\text{HA}$ and MgF_2HA for further discussion (Table 3-2).

Table 3-1 Designed sols with different Mg contents for Mg_xFHA coatings

Name	Formula	<i>x</i> value
FMA0	Ca ₁₀ (PO ₄) ₆ F ₁ (OH) ₁	0
FMA1	Ca _{9.5} Mg _{0.5} (PO ₄) ₆ F ₁ (OH) ₁	0.5
FMA2	Ca ₉ Mg ₁ (PO ₄) ₆ F ₁ (OH) ₁	1.0
FMA3	Ca _{8.5} Mg _{1.5} (PO ₄) ₆ F ₁ (OH) ₁	1.5
FMA4	Ca ₈ Mg ₂ (PO ₄) ₆ F ₁ (OH) ₁	2.0

Table 3-2 Designed sols with different F concentrations for MgF_yHA coatings

Name	Formula	<i>y</i> value
MFA0	Ca ₉ Mg ₁ (PO ₄) ₆ (OH) ₂	0
MFA1	Ca ₉ Mg ₁ (PO ₄) ₆ F _{0.5} (OH) _{1.5}	0.5
MFA2	Ca ₉ Mg ₁ (PO ₄) ₆ F ₁ (OH) ₁	1.0
MFA3	Ca ₉ Mg ₁ (PO ₄) ₆ F _{1.5} (OH) _{0.5}	1.5
MFA4	Ca ₉ Mg ₁ (PO ₄) ₆ F ₂	2.0

3.3.3 Coating deposition

Sol-gel dip-coating method was adopted to deposit coatings on Ti6Al4V substrates, and the flow chart of the process is given in Figure 3-3. The optimized procedures

were employed as follows [136]. As-prepared substrate was dipped vertically into the sol and withdrawn at a speed of 3 cm min^{-1} for the first layer. The as-dipped coating was then dried at $150 \text{ }^{\circ}\text{C}$ for 15 minutes followed by firing at $600 \text{ }^{\circ}\text{C}$ for 15 minutes. This dipping-drawing-drying-firing process was further repeated 4 times at a speed of 4.5 cm min^{-1} for a thicker coating with a thickness of $1.5 - 2 \text{ }\mu\text{m}$.

Figure 3-3 Flow chart of the coating deposition

3.3.4 Fabrication of bi-layer structured HA coating

Finally, a bi-layer structured HA (briefed as BHA) coating was deposited on Ti6Al4V substrate. The upper layer was the Mg_xFHA coating reflecting the best

bioactivity and cellular response, while the MgF_yHA coating as the bottom layer showing the lowest dissolution rate and the highest adhesion strength. According to the above criteria and the results in Chapter 4, $\text{Mg}_{1.5}\text{FHA}$ was selected as the upper layer, and $\text{MgF}_{1.5}\text{HA}$ as the bottom layer, illustrated in Figure 3-4. The dipping-drawing-drying-firing process was repeated three times for the bottom layer, and two times for the upper layer in accordance with total five times for the whole coating. Pure HA coating ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) was also deposited as control.

Figure 3-4 Design of bi-layer structured HA coating on Ti6Al4V substrate

3.4 Coating characterization

3.4.1 Phase evolution

Grazing incidence X-ray diffraction (GIXRD) is a powerful tool to determine the diffraction pattern from a very thin film or coating. Rigaku Ultima 2000 was employed to analyze the phases of the samples. The incidence angle was fixed at 1° , and the scanning range was from 20° to 50° with a step size of 0.02° . X-ray was $\text{CuK}\alpha$ radiation ($\lambda = 1.5406 \text{ \AA}$) working at 40 kV and 40 mA.

3.4.2 Chemical composition

X-ray photoelectron spectroscopy (XPS, Kratos-Axis Ultra System) was used to determine the chemical composition of the coatings and the chemical states of each element. Monochromatic Al K α X-ray source (1486.7 eV) was employed and the vacuum of the chamber was 2×10^{-9} torr. The survey scan was recorded in the range of 0-1200 eV in 1 eV step for each sample, followed by narrow scan for each elemental peak in 0.1 eV step. Selectively, narrow scan for different elements was performed after etching by Ar $^{+}$ for 10 minutes. Then, the coating composition was calculated according to the narrow scans before and after the etching. The measured binding energy of all XPS profiles was calibrated using C 1s (BE = 284.8 eV).

3.4.3 Chemical groups

Fourier transform infra-red spectroscopy (FTIR, BioRad FTS-3000) can identify the chemical groups and show the characteristic bands of PO $_4^{3-}$, CO $_3^{2-}$, and OH $^{-}$ groups. In this project, FTIR studies were carried out in the range of 4000-700 cm $^{-1}$ with a resolution of 4 cm $^{-1}$.

3.4.4 Surface and interface

Surface morphology was observed by scanning electron microscopy (SEM, JEOL JSM-5600LV). The working current of the filament was ~60 mA with an acceleration voltage of 15 or 20 kV. The coatings were gold coated before the

observation. As-deposited sample was cut into small pieces ($10 \times 10 \times 2 \text{ mm}^3$) followed by cold mounting. The mounted sample was polished for cross-sectional view by SEM.

The elements distribution along the depth direction was obtained by glow discharge optical emission spectroscopy (GDOES, GD Profiler 2, HORIBA JOBIN YVON).

The corresponding parameters were set as follows: pressure 650 Pa, power 50 W, module 7.5 V, and phase 3.5 V.

To examine the chemical bonding near the interface of the coating and the substrate, the coatings were firstly polished with SiC sandpapers until the substrate could be seen, then measured by X-ray photoelectron spectroscopy (XPS, Kratos-Axis Ultra System) using monochromatic Al K α X-ray source (1486.7 eV). The measured binding energy was calibrated using C 1s (BE = 284.8 eV).

3.5 Short-term osseointegration

3.5.1 Bioactivity test

Kokubo's simulated body fluid (SBF) [137] was prepared for the in vitro bioactivity test by dissolving following reagent grade chemicals in deionized water: NaCl, NaHCO₃, KCl, K₂HPO₄•3H₂O, MgCl₂•6H₂O, CaCl₂, Na₂SO₄, then 1 M HCl and NH₂C(CH₂OH)₃ were used to adjust the pH value of the solution to 7.4 at $37 \pm 0.1 \text{ }^\circ\text{C}$. Table 3-3 gives the order and the reagent for the preparation of the SBF

solution. The inorganic ion concentrations in the SBF solution are almost the same as that in human blood plasma, which can be used to simulate the human body environment for the in vitro bioactivity test.

Table 3-3 Reagents for preparing Kokubo's SBF [137]

Order	Reagent	Amount
1	NaCl	8.035 g
2	NaHCO ₃	0.355 g
3	KCl	0.225 g
4	K ₂ HPO ₄ •3H ₂ O	0.231 g
5	MgCl ₂ •6H ₂ O	0.311 g
6	1.0 M HCl	39 ml
7	CaCl ₂	0.292 g
8	Na ₂ SO ₄	0.072 g
9	NH ₂ C(CH ₂ OH) ₃	6.118 g
10	1.0 M HCl	0-5 ml

Before soaking in the SBF solution, the samples were ultrasonically washed in acetone for 10 minutes and then sterilized in ethanol. Then, the samples were immersed in the SBF solution in the sterilized bottles for various periods till 28 days at 37±0.1 °C in a water bath. After immersion, the samples were taken out,

gently washed with deionized water and dried at room temperature. Then, GIXRD (Rigaku Ultima 2000) was employed to analyze the phases of the samples using CuK α radiation ($\lambda = 1.5406 \text{ \AA}$) at 40 kV and 40 mA with a step size of 0.02° , SEM (JEOL JSM-5600LV) for the observations of the surface morphology. FTIR (BioRad FTS-3000) was carried out in the range of $4000\text{--}400 \text{ cm}^{-1}$ with a resolution of 4 cm^{-1} to identify the chemical groups by scratching the coating from the substrate and then mixed with KBr powder. XPS (Kratos-Axis Ultra System) was used to determine the chemical composition of the coatings after the in vitro bioactivity test.

3.5.2 Cell culture test

3.5.2.1 Cell and culture conditions

Human osteosarcoma MG63 cells purchased from American Type Culture Collection (ATCC, Rockville, MD) were used to evaluate the osteoblastic cell response on the coating surface. Cell culture was conducted at 37°C in a humidified 5% CO_2 atmosphere in a standard culture medium containing Eagle's Minimum Essential Medium (EMEM, ATCC) supplemented with 10% fetal calf serum (FCS, ATCC) and 1% penicillin/streptomycin (ATCC). For the cell assay, the coated samples were cut into small pieces ($10 \times 10 \times 2 \text{ mm}^3$) and sterilized in an autoclave at 121°C for 20 minutes before the cell culture tests.

3.5.2.2 Cell morphology

Cells at a density of 6.0×10^3 cells ml^{-1} were seeded on the coating surface to observe the cell morphology. After incubation for 3 days, cells were fixed with 2.5% glutaraldehyde for 1 hour at room temperature followed by dehydration with a series of graded ethanol/water solutions (50%, 70%, 80%, 95% and 100%, respectively). Then 0.5 ml of hexamethyldisilazane was added to each well to preserve the original morphology of the cells. Finally, the test plates were dried in the fume hood at room temperature. The samples were gold coated before the observation by SEM (JEOL JSM-5600LV).

3.5.2.3 Cell proliferation

CellTiter 96[®] non-radioactive cell proliferation assay (Promega) was employed to measure cell proliferation on the coatings. Cells were seeded on the sterilized coating surface at a density of 5.0×10^4 cells ml^{-1} and cultured for 1, 2, 3 and 5 days. After each culture period, the samples were taken out and transferred to new 24-well cell culture plates. After being washed twice with phosphate buffered saline (PBS) solution, cells were detached with trypsin/EDTA and then centrifuged to obtain cell pellets. After being washed twice with deionized water, the cell pellets were diluted in EMEM. 100 μl of cell solution was taken out into a 96-well plate, and 15 μl of dye solution was added to each well. After incubation at 37 °C for 4 hours, 100 μl of solubilization/stop solution was added to each well, and then the

plate was incubated at 37 °C for 1 hour. The absorbance at 570 nm wavelength was then recorded using a microplate reader after mixing the contents of the wells into a homogeneous colored solution. Recorded absorbance was converted to cell numbers using a standard curve.

3.5.2.4 Cell differentiation

The intracellular alkaline phosphatase (ALP) activity and osteocalcin (OC) expression were analyzed to evaluate the functionality of cultured cells on the coatings. Cells were seeded on each sample at a density of 6.0×10^4 cells ml⁻¹. After the cells were cultured for 7, 14 and 21 days, the samples were washed twice with PBS, and then cells were detached from the coating surface with trypsin/EDTA. After centrifugation, cell pellets were washed twice with deionized water and finally resuspended in 0.6 ml of lysis buffer solution (1% Triton X-100 in Tris-HCl buffer, pH 7.4). Following one cycle of freeze/thawing process, cells were further lysed by sonicating them for 10 minutes. Finally, after being centrifuged at 10000 rpm for 5 minutes, the supernatants were collected for the assay of ALP activity, OC concentration as well as total protein concentration.

The ALP activity and OC expression were measured using a phosphate detection kit (BIOMOL GREEN, USA), and an immunoenzymatic assay (EASIA ELISA kit, BioSource, USA), respectively. The experimental processes were conducted strictly according to the provided protocols. The measurement was read at 620 nm in a

microplate reader for the ALP activity and at 405 nm against a reference filter at 650 nm for the OC expression. The ALP activity and OC expression were then calculated based on standards curves. Finally, the measured ALP activity and OC expression were normalized to the total protein content, which was then determined using a commercial Micro BCA protein assay kit (Pierce, USA).

Statistical analysis was carried out on cellular tests using one-way analysis of variance (ANOVA) at an average of 3-5 replicates. The significance level (α) is set as 0.05 (i.e., $p < 0.05$).

3.6 Long-term stability

3.6.1 Dissolution test

A tris-buffered saline (TBS) solution was used to investigate the dissolution behavior of the coatings. The TBS solution contains 150 mM NaCl and 20 mM Tris ($\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3$), and the pH value of the solution was adjusted to 7.4 – 7.6 by 1 M HCl.

Before the dissolution test, samples were ultrasonically washed in acetone for 10 minutes and then sterilized in ethanol. Then, the samples were immersed in the TBS solution in the sterilized bottles for various periods up to 28 days at 37 ± 0.1 °C in a water bath. After immersion, the samples were taken out, gently washed using deionized water and dried at room temperature.

The concentrations of Ca^{2+} and Mg^{2+} ions after each period were measured by an inductively coupled plasma emission spectrometer (ICP-OES, PerkinElmer Optima DV2000), or an atomic absorption spectroscopy (AAS, SHIMADZU AA-6800). SEM (JEOL JSM-5600LV) was utilized to observe the surface morphology after the dissolution test, and surface roughness (R_q) was measured by a confocal imaging profiler (SENSOFAR).

3.6.2 Pull-off test

Pull-off test measures adhesion strength of the coating on the substrate. Good adhesion strength indirectly indicates long-term stability. The adhesion strength of the coatings on Ti6Al4V substrates was measured by a universal instron mechanical testing system (Instron 5569). A clamping fixture was designed to avoid the misalignment during the uniaxial tensile test. Al rod (8 mm diameter) was glued onto the coating surface with epoxy resin (Epoxy Adhesives DP460, 3M, Scotch-Weld™, USA) and cured at room temperature for 24 hours. This rod-sample system was then put into a steel holder and the rod was ready for clamping by the Instron fixture (Figure 3-5). In testing, the rod was pulled at a cross-head speed of 1 mm min^{-1} until the coating failure.

Figure 3-5 Schematic illustration of pull-off test

“Pull-off adhesion strength” was used without distinguishing adhesive and cohesive failure, calculated using: $X = 4F/(\pi d^2)$, where X is the “pull-off adhesion strength”, F is the actual force applied to the test surface at rupture, and d is the equivalent diameter of the original surface area stressed [138]. After the test, SEM (JEOL JSM-5600LV) was used to evaluate the failure mode at the fracture surface. The optical images of the whole coating were captured using a normal camera and then the area of adhesive failure on the fracture surface was measured by Image-Pro Plus 6.0. Statistical analysis was carried out using one-way analysis of variance (ANOVA) at an average of ten replicates. Differences were considered statistically significant at $p < 0.05$.

Chapter 4 Results and Discussion

Mg_xFHA, MgF_yHA, and BHA coatings were fabricated on Ti6Al4V substrates by a sol-gel dip-coating method using different sols with designed compositions as described in Chapter 3. In this chapter, the experimental results will be present in three parts followed by corresponding discussions. The first part, section 4.1, will focus on the effect of magnesium incorporation in fluoridated hydroxyapatite (Mg_xFHA) coatings. The second part, section 4.2, will concentrate on the effect of fluorine substitution in magnesium-containing hydroxyapatite (MgF_yHA) coatings. Finally, a bi-layer structured magnesium and fluorine ions co-substituted HA coating (Mg_{1.5}FHA/MgF_{1.5}HA) will be evaluated in both the short-term osseointegration and the long-term stability in section 4.3.

4.1 Effect of magnesium incorporation in fluoridated hydroxyapatite (Mg_xFHA) coatings

Magnesium ions are incorporated into fluoridated hydroxyapatite coatings to improve the biological performances. The as-deposited coatings are denoted by Mg_xFHA (Ca_(10-x)Mg_x(PO₄)₆F₁(OH)₁, x varies from 0 to 2). The co-substitution of Mg and F ions in the Mg_xFHA coatings, the effect of Mg incorporation on short-term osseointegration and long-term stability of the Mg_xFHA coatings are discussed. The emphasis is on the in vitro bioactivity and cell response.

4.1.1 Co-substitution of Mg and F ions in the Mg_xFHA coatings

When Mg and F ions are incorporated into Mg_xFHA coatings simultaneously, the corresponding physical, chemical, biological and mechanical properties are influenced. In this part, the co-substitution of Mg and F ions in the Mg_xFHA coatings are discussed from phase evolution, co-substitution mechanism, surface and interface properties.

4.1.1.1 Phase evolution

GIXRD patterns of Mg_xFHA coatings on Ti6Al4V substrates are shown in Figure 4-1. The diffraction peaks of all the samples are assigned to apatite phase and Ti6Al4V substrate. With reference to pure HA (PDF#09-0432), no any other calcium phosphate is found in the coating. All the main diffraction peaks in FMA0 sample have a slight shift towards higher degree, especially the (300) peak shifting from 32.902° of pure HA to 33.040°. That is because some of the hydroxyl groups (OH⁻) are substituted by the fluorine ions (F⁻) to form fluoridated hydroxyapatite (FHA) [118]. There is not much difference in peak positions in low concentration of Mg_xFHA (see FMA1 and FMA2) except slight peak shift towards higher degree. It is expected that the substitution takes place of smaller Mg²⁺ (0.69 Å) for larger Ca²⁺ (0.99 Å) ion. As Mg concentration increases further, i.e. FMA3 and FMA4, a new phase of Mg-substituted β-tricalcium phosphate (β-TCMP) with weak intensity is detected, signaling it is beyond the maximum “solubility” of Mg in HA crystal

structure.

Figure 4-1 GIXRD patterns of as-deposited Mg_xFHA coatings on Ti6Al4V substrates

In our previous study [92], β -TCMP was formed as the main phase when Mg was incorporated into HA coatings with the same concentration as FMA2 in the sol, because the substitution of Mg^{2+} into Ca^{2+} positions made the apatite structure resemble β -TCP. Moreover, Mg favored the conversion of HA into β -TCMP during the heat treatment [75, 139]. However, when Mg and F ions are incorporated into HA coatings at the same time, only a small amount of β -TCMP is found in FMA3 and FMA4, meaning F-incorporation in HA increases the “solubility” of Mg in the HA structure. Similar results were also reported by other researchers who prepared apatite powders in the presence of Mg and F ions [124]. The shrinkage along c -direction caused by the substitution of OH^- groups by F^- ions would lead to larger “rattling” space thus the lattice structure becomes less stable. As a result, Mg ions

might have more chances to enter into the HA crystal structure to occupy the Ca position.

4.1.1.2 Co-substitution of Mg and F ions in the Mg_xFHA coatings

Theoretically, Mg²⁺ (0.69 Å) is substituted for Ca²⁺ (0.99 Å), and F⁻ (1.32 Å) replaces OH⁻ (1.68 Å) in HA crystal structure when Mg or F ions are incorporated into HA powders or coatings. The incorporation of foreign ions into HA crystal structure influences the lattice parameters, chemical states, mechanical and biological properties of HA correspondingly. Therefore, it is vital to confirm the substitution of Mg and F ions into HA crystal structure, which is studied from lattice parameters, chemical states and chemical groups.

4.1.1.2.1 Lattice parameters

When fluorine ions are incorporated into HA crystal structure, the lattice parameters are changed. There is a significant decrease in the *a*-axis from 9.418 Å for pure HA down to 9.3710 Å for pure FA, but the *c*-axis shows no obvious change [97-99]. In this case, the shift of (002) peak and the change of *c*-axis can reflect the substitution of Ca²⁺ with Mg²⁺ when Mg and F ions are simultaneously incorporated into HA coating. Table 4-1 lists the two-theta of (002) peak and calculated *c*-axis of Mg_xFHA coatings. As Mg concentration increases from 0 to 1.0, the (002) peak shifts towards higher degree due to the substitution of larger Ca²⁺

(0.99 Å) with smaller Mg^{2+} (0.69 Å). However, when Mg concentration increases further to 1.5 and 2.0, (002) peak shifts to lower degree compared with $x = 1.0$, but still higher than that when $x = 0.5$. The same trend is also observed from the change of c -axis. The change of c -axis is related to the phase analysis stated above. At higher Mg concentrations (FMA3 and FMA4), β -TCMP is detected as well as FHA, implying Mg^{2+} can't completely replace Ca^{2+} . The formation of β -TCMP competes with the Ca^{2+} substitution on the assumption of Mg^{2+} in the sol. Therefore, it is reasonable that there is a little increase of c -axis at higher Mg concentrations. Although the change of c -axis is not linear with the increasing of Mg concentration, the decrease of c -axis indicates the replacement of Ca^{2+} by Mg^{2+} in the HA crystal structure.

Table 4-1 Two-theta of (002) peak and calculated c -axis of Mg_xFHA coatings

Sample	FMA0	FMA1	FMA2	FMA3	FMA4
Two-theta (°)	25.84	25.86	25.94	25.90	25.90
c -axis (Å)	6.89	6.88	6.86	6.87	6.87

4.1.1.2.2 Chemical composition

The chemical compositions of Mg_xFHA coatings are confirmed by XPS, as shown in Figure 4-2. The survey scans (Figure 4-2(a)) demonstrate that all the elements in

Mg_xFHA coatings are detected (the presence of C 1s in all the samples is due to inevitable surface contamination even from air).

Figure 4-2 XPS profiles of Mg_xFHA coatings on Ti6Al4V substrates ((a): survey scan; (b): F 1s narrow scan; (c): Mg 2p narrow scan)

The narrow scans of F 1s are shown in Figure 4-2(b), the binding energy of F 1s at 684.3 ± 0.1 eV reveals that F ions have been incorporated into the HA crystal structure (F 1s at 684.3 ± 0.1 eV is the fingerprint of F in FHA or FA, while the peak of F 1s in CaF₂ is at 686.7 eV [8, 103]). This is also supported by the GIXRD

analysis. Figure 4-2(c) is the narrow scans of Mg 2p. With increasing Mg in the sols, Mg 2p peak gradually intensifies, indicating more Mg ions are included in the HA coating. Moreover, only one symmetrical peak is located at 50.3 eV belonging to Mg 2p, that means one chemical state of Mg existing in the coating, which is the fingerprint of Mg^{2+} in apatite structure [92]. Therefore, this result confirms that Mg ions have been incorporated into the HA lattice. In both Mg-containing HA and Mg-substituted β -TCP, Mg bonds with phosphate groups, thus the binding energy of Mg 2p between them does not have difference. As a result, only one symmetrical peak of Mg 2p is detected for FMA3 and FMA4 samples, though a second phase of β -TCMP is discovered by GIXRD.

Element concentration in the coating can be determined by the ratio of area under the respective elemental peak in an XPS narrow scan. Figure 4-3 shows the measured Mg concentration in the coating (indicated by capital X) in comparison with the designed value (indicated by small x , c.f. Table 3-1) before and after Ar^+ etching. The squares show the ideal situation when all the designed Mg are completely incorporated into the HA crystal structure. The circles represent the measured Mg concentrations on the surface of the coatings before the Ar^+ etching, and triangles for that after the etching. Although the measured Mg concentration (X value) increases with the increase of designed x value, a discrepancy is observed between the designed and measured concentration, especially after $x > 0.5$. After the etching, the measured Mg concentration has shown to decrease significantly

with contents much lower than that on the surface of the coating. The difference in the Mg concentration before and after the etching indicates that only a portion of Mg is incorporated into the HA crystal structure ($x > 0.5$), and the remaining ions are abundant on the surface of the coating. After the removal of the Mg on the surface of the coating, the measured Mg determines the concentration of Mg incorporated into HA crystal structure, and the corresponding formula is $\text{Ca}_{9.2-9.5}\text{Mg}_{0.8-0.5}(\text{PO}_4)_6\text{F}_1(\text{OH})_1$, i.e. about 1.2 – 1.9 wt% of Mg replaces Ca in the HA crystal structure.

Figure 4-3 Comparison of the measured Mg concentration in the Mg_xFHA coatings before and after the etching with the designed Mg concentration in the sols

4.1.1.2.3 Chemical groups

FTIR identifies chemical groups in different coatings, as shown in Figure 4-4.

There are three peaks at 1002-1015 cm^{-1} , 1086-1088 cm^{-1} and 1120-1122 cm^{-1} for each coating, due to the triply asymmetric ν_3 stretching vibration of the phosphate group (PO_4^{3-}). A weak peak at 964-965 cm^{-1} represents the ν_1 symmetric stretching vibration of PO_4^{3-} . The presence of typical peaks of PO_4^{3-} indicates the formation of apatite on the substrate [140]. Also, there are two weak peaks between 1400 and 1500 cm^{-1} , ascribing to the ν_3 stretching vibration of carbonate (CO_3^{2-}) [141]. The existing of carbonate groups in the coating is from the coating preparation process.

Figure 4-4 FTIR spectra of Mg_xFHA coatings on Ti6Al4V substrates

Furthermore, there is a weak peak around 3540 cm^{-1} relating to the F incorporation into the apatite crystal structure. For pure HA coating, a peak at about 3570 cm^{-1} is detected due to the stretching of O-H in an infinite OH-chain, while for FHA coatings, the new peak at about 3540 cm^{-1} is caused by the direct influences of incorporated F on the stretching vibration of OH groups through the hydrogen

bonds along OH channel, i.e. -OH-F- [97]. The presence of the peak at 3540 cm^{-1} is another evidence of the replacement of OH^- groups by F^- ions.

4.1.1.3 Surface and interface

Figure 4-5 shows the SEM surface morphologies of the Mg_xFHA coatings on Ti6Al4V substrates. All the coatings are homogeneous with a few micropores. The presence of these micropores is caused by the evaporation and/or decomposition of the solvent as well as the change of surface tension during the drying and firing process. The Mg concentration in the dipping sols plays an important role on the surface properties of the prepared Mg_xFHA coatings. As can be seen in Figure 4-5, the coatings tend to be smoother with increasing Mg concentration.

Figure 4-5 SEM surface morphology of Mg_xFHA coatings on Ti6Al4V substrates

All the Mg_xFHA coatings have similar cross-sectional views after mounting and polishing, as shown in Figure 4-6 (taking FMA0 and FMA4 as examples). The coatings are dense and uniform with a thickness of about 1.5 – 2.0 μm . No delamination is seen at the interface even after repeated abrading and polishing, suggesting good bonding between the coating and the substrate.

Figure 4-6 Cross-sectional view of Mg_xFHA coatings on Ti6Al4V substrates

((a): FMA0; (b): FMA4)

GDOES provides elemental information and is used to acquire elemental distribution from coating to substrate through depth profiling. GDOES depth profile of FMA4 is plotted in Figure 4-7. It shows the distribution of main elements of the

coating and the substrate along the depth direction, including Ca, P, O, Mg, F, Ti and Al.

Figure 4-7 GDOES depth profile of FMA4 (Mg20: 20 times of Mg concentration;
F100: 100 times of F concentration)

Along the depth direction, the elemental distribution at cross-section can be divided into three regions: the coating region (R_c), the transitional region (R_t), and the substrate region (R_s). The masses of Ca, P, O, and F in the coating are very stable in the coating region. The mass of Mg decreases from the coating to the substrate, in agreement with our previous study that Mg ions can only be partially incorporated into HA lattice, whereas extraneous ions are abundant on the surface of the coating (section 4.1.1.1). At the boundary between the R_c and the R_t , the concentrations of Ca, P, Mg and F begin to decrease, whereas there is a slight increase in O concentration followed by a sharp decrease. The increase of O might owe to the

presence of F ions, because nc-CaF₂ formed in the dipping sol could be easily absorbed on the substrate surface through hydrogen bond, which could attract more O near the interface [8]. As can be seen in Figure 4-6, the transitional region R_t is around 700 nm. As all the elements of the coating and substrate are seen in the R_t , diffusion of coating elements into the substrate and substrate elements into the coating occur at the interface. At the boundary between the R_t and the R_s , all the elements in the coating disappear and the elements of the substrate, Ti and Al, begin to stabilize.

The chemical bonding near the interface of the coating and the substrate is analyzed through XPS. Figure 4-8 presents the results of XPS narrow scan near the interface of the Mg₂FHA coating and the substrate. Ti 2p spectrum is shown in Figure 4-8(a). Only Ti⁴⁺ is detected, Ti 2p_{3/2} at 458.3 eV and Ti 2p_{1/2} at 464.0 eV, which can be attributed to TiO₂/TiO₃²⁻ [142, 143]. Ca 2p (Figure 4-8(b)) contains two different states: Ca 2p_{3/2} at 347.2 eV is the typical binding energy of Ca²⁺ in HA or FHA, and Ca 2p_{3/2} at 346.4 eV corresponds to that in CaTiO₃ [142, 144]. In consideration of the elements diffusion during the firing stage, it is reasonable that CaTiO₃ forms at the coating/substrate interface. Figure 4-8(c) shows O 1s spectrum. The main component at the binding energy of 529.7 eV is attributable to O²⁻ species in TiO₂/TiO₃²⁻, while that at 531.3 eV can be assigned to O²⁻ in HA or FHA [142, 144]. Correspondingly, the binding energy of P 2p of PO₄³⁻ in HA or FHA is located at 133.2 eV (not shown here) [144]. Mg 2p spectrum in Figure 4-8(d)

presents only one peak at 50.4 eV, which is the typical binding energy of Mg^{2+} . As reported in the literature [145], MgTiO_3 could be formed around 400 °C. Also, Mg is detected in the transitional region (R_t) (Figure 4-7). Therefore, it is speculated that MgTiO_3 also forms during the firing at 600 °C. From above GDOES and XPS analysis, it can be concluded that chemical bonds especially Ca-O-Ti and Mg-O-Ti bonds form at the coating/substrate interface.

Figure 4-8 XPS spectra of Ti 2p (a), Ca 2p (b), O 1s (c) and Mg 2p (d) near the interface of the Mg_2FHA coating and the substrate

4.1.1.4 Discussion

For the deposition of FHA coatings, HPF_6 was selected as the F-precursor and mixed with P-precursor to form a P-F mixture. Ca-precursor was added dropwise to the P-F mixture, and then the solution was refluxed for 24 hours to obtain the sol [8, 49, 115, 116, 146]. During the refluxing process, HPF_6 is hydrolyzed and alcoholized. Through hydrolysis and alcoholysis, F ions are gradually released and immediately react with Ca ions in the solution to form nano-crystalline CaF_2 particles (nc- CaF_2). In the subsequent drying and firing process, these particles react with the rest of Ca and P groups to form FHA phase. The formation of FHA can be described from the reaction of the nc- CaF_2 during the drying and firing process. Writing FHA as $3(\text{Ca}_3(\text{PO}_4)_2)\text{CaF}_x(\text{OH})_{2-x}$, the overall chemical process can be simplified into a two-step reaction [103]:

where M_{Ca} and M_{P} stand for the ligands coordinated to Ca and P, respectively, i- $\text{Ca}_3(\text{PO}_4)_2$ stands for the intermediate compound between Ca and P with Ca/P of 1.5, and $x \leq 2$. For the deposition of FMA0 ($\text{Ca}_{10}(\text{PO}_4)_6\text{F}_1(\text{OH})_1$), the chemical process happens according to above reactions.

When Mg-precursor is mixed with Ca-precursor and added dropwise to P-F mixture, the chemical process is similar with that of FHA coating. Write Mg_xFHA

$(\text{Ca}_{10-x}\text{Mg}_x(\text{PO}_4)_6\text{F}_1(\text{OH})_1$ as $3(\text{Ca}_{3-x/3}\text{Mg}_{x/3}(\text{PO}_4)_2)\text{CaF}_1(\text{OH})_1$, the overall chemical process can be simplified into a two-step reaction according to (1) and (2):

In the case of $x > 1.0$, Mg is too much to be completely incorporated into the apatite structure, some $\text{i-Ca}_{3-x/3}\text{Mg}_{x/3}(\text{PO}_4)_2$ will be left to form $\beta\text{-TCMP}$ ($\text{Ca}_{3-x/3}\text{Mg}_{x/3}(\text{PO}_4)_2$).

For the ion incorporated HA coatings, it is important to confirm whether the ions have been successfully incorporated into the HA crystal structure. The incorporation of Mg and F ions in the HA crystal structure is reflected from the lattice parameters, chemical states and chemical groups. Since c -axis is almost unchanged with the F incorporation, Mg substitution in the HA lattice is indicated by the shift of (002) peak towards a higher degree and the decreased c -axis compared with that of FMA0. The binding energy of Mg 2p at ~ 50.3 eV further confirms the Mg^{2+} substitution in the HA structure. The binding energy of F 1s at ~ 684.3 eV reveals the incorporation of F in the HA crystal structure. Furthermore, the peak at 3540 cm^{-1} in FTIR spectra relates to the -OH-F- bond in OH channel, indicating the presence of F in the HA lattice.

The chemical compositions of Mg_xFHA coatings are also analyzed. An increased intensity of Mg 2p is observed with increased Mg concentration in the sols (Figure

4-2(c)), indicating more Mg ions are included into the coatings. However, the measured Mg composition after the etching is lower than that on the surface of the coating. It suggests that excessive Mg is abundant on the surface of the coating besides those incorporated into the apatite crystal structure, which is also proved by GIXRD. A small amount of β -TCMP forms at high Mg concentration (FMA3 and FMA4), suggesting the limit substitution of Ca ions with Mg ions. The distribution of Mg along the depth direction is also reflected by the GDOES profile (Figure 4-7): the mass of Mg decreases from the coating to the substrate. The detection of Mg 2p near the coating/substrate interface verifies the diffusion of Mg from the coating into the substrate. Furthermore, the formation of Mg-O-Ti as well as Ca-O-Ti bonds at the coating/substrate interface is speculated according to the chemical states analysis of Ti 2p, O 1s, Ca 2p and Mg 2p (Figure 4-8), which would have a positive effect on interfacial properties.

According to the XPS analysis, about 1.2 – 1.9 wt% of Mg replaces Ca in the HA crystal structure. Since the optimum Mg amount in artificial HA ceramics can vary with different applications, it is crucial to control the Mg substitution in the HA lattice over the widest possible range by controlling the synthesis procedure. In magnesium/carbonate co-substituted HA, the maximum Mg substitution is about 0.35 wt%, while carbonate ions at 0.9 wt% into the HA lattice [130]. Up to 1.5 wt% of Mg was incorporated into the apatite structure with or without added carbonate when apatite was directly precipitated in the solution under controlled conditions of

pH and temperature [129]. Under hydrothermal conditions at 120 °C, MgHA powders were synthesized with up to 5 wt% of Mg in HA [75]. Also, Mg-substituted HA powders containing 0.24 – 28.4 wt% of Mg were prepared by the mechanical-hydrothermal method [52]. It can be seen that the substitution levels of Mg into apatite structure mainly depend on the preparation methods. Mg-substituted HA ceramics with different Mg concentration have been proposed for the applications in the orthopaedic and dental fields. Bonfield et al. suggested to synthesize Mg (up to 0.5 wt%) and CO₃ (up to 1 wt%) co-substituted HA by precipitation from aqueous solutions as a raw material for the fabrication of bone implants [147]. Dolci et al. claimed HA powder (crystal size ranging between 0.5 and 200 nm) containing up to 25.4 wt% of Mg for the preparation of odontostomatologic applications [148]. Kakei et al. [149] analyzed the concentrations of calcium, magnesium, phosphate and carbonate ions in the mineral phase of rat calvaria specimens at different development stages. The results showed that Mg ions were present at a high concentration (approx. 1.0 wt%) in the bone mineral of new-born rats, and this level decreased to 0.5 wt% after one year. The chemistry of the bone mineral was clearly related to the formation and growth stages of new bone. 1.2 – 1.9 wt% of Mg incorporated into the HA crystal structure in the prepared coatings are very close to the amount of Mg in the new bone, very promising for the applications as bone repair or replacement biomaterials.

4.1.2 Short-term osseointegration

It is a great concern that a stable anchorage achieves by direct bone-to-implant contact within the shortest time, so it is worthy of investigating the short-term osseointegration in vitro. Moreover, Mg incorporation has shown to result in better biological performances. Thus, it is expected to have positive effects on the in vitro bioactivity and cell response of the Mg_xFHA coatings, which are studied below.

4.1.2.1 In vitro bioactivity

The formation of a layer of biologically active hydroxylcarbonate apatite on the biomaterial surface in the SBF solution is a criterion of the in vitro bioactivity. In this part, the phase evolution, surface morphology, chemical groups and chemical composition of the coatings during the bioactivity test are studied to examine the bioactivity of Mg_xFHA coatings.

4.1.2.1.1 Phase evolution during the in vitro bioactivity test

Figure 4-9 exhibits the GIXRD profiles of Mg_xFHA coatings after soaking in the SBF solutions for 7 and 28 days. After 7 days, as shown in Figure 4-9(a), an obvious difference is observed around 32° for FMA3 and FMA4, while not obvious for FMA0, FMA1 and FMA2 as compared with that in Figure 4-1. For FMA3 and FMA4, the peaks of (211), (112) and (300) are combined into one broad peak, which implies the poorly crystallized apatite grown in the solution – evidence of

precipitation of a new apatite layer on the primary coating. The diffraction peaks of substrate and calcium phosphate are found in all the samples. Figure 4-9(b) shows the GIXRD profiles after 28 days. By now, broad peaks around 32° are also obvious for FMA2, which are assigned to the newly formed apatite layer. Some other intermediate products like $\text{CaH}_2\text{P}_2\text{O}_7$ and $\text{CaP}_4\text{O}_{11}$ are also detected.

Figure 4-9 GIXRD profiles of Mg_xFHA coatings on Ti6Al4V substrates after soaking in the SBF solutions for 7 days (a) and 28 days (b)

4.1.2.1.2 Surface morphology during the in vitro bioactivity test

Figure 4-10 shows SEM images of Mg_xFHA coatings before and after soaking in the SBF solutions for 7 and 28 days. After soaking in the SBF solution for 7 days, the surface morphology of FMA3 shows a newly formed porous layer (Figure 4-10 FMA3a). Similar observations are found on FMA4 (Figure 4-10 FMA4a). Other surfaces exhibit some grooves due to the dissolution of the coatings. After 28 days (the b-series images), FMA2b, FMA3b and FMA4b exhibit similar porous layer as FMA3a and FMA4a (precipitation of apatite), but FMA0b and FMA1b still show the dissolution characteristics.

Figure 4-10 Surface morphology of Mg_xFHA coatings on Ti6Al4V substrates after soaking in the SBF solutions for 7 days (a) and 28 days (b)

The dissolution and precipitation behavior of HA coating are two of the main factors governing the bioactivity of HA coating [150]. When samples are immersed in the SBF solution, both the dissolution of the coating and the precipitation of the

new apatite layer occur at the same time. When the precipitation is dominant, the new apatite layer forms. At the beginning of the process, the dissolution dominates. The kinetics of this process can be described by the empirical equation [150]:

$$\frac{dc}{dt} = k_1 s_1 (c_\infty - c)^n \quad (5)$$

where dc/dt is the dissolution rate, k_1 is the rate constant for dissolution, s_1 represents the specific area, c_∞ stands for the equilibrium concentration, c for the concentration of solution at different times and n is the effective order of reaction.

As soon as the coatings are immersed in the SBF solution, the difference of coatings in terms of $(c_\infty - c)$ can be ignored. Thus, the specific area of the coatings, s , has main influence on the dissolution rate. The dissolution increases with the increase of the contact surface area with the solution. The specific area of the coatings depends on the crystallite size of the coating: the larger the crystallite size is, the less the specific area of the coating is. Scherrer formula ($D_{hkl} = K\lambda(\beta_{1/2}\cos\theta)^{-1}$) is used to calculate the crystallite size in different coatings. In the formula, D_{hkl} is the crystallite size measured in the direction perpendicular to the diffraction planes hkl ; K is the Scherrer constant (0.91 is used here); λ is the wavelength of X-ray (1.5406 Å); $\beta_{1/2}$ is the broadening of the diffraction peak at half-maximum intensity; θ is the half value of the diffraction angle 2θ for hkl . The crystallite size of Mg_xFHA coatings calculated at (002) is 187.8, 182.0, 173.9, 155.6 and 169.7 Å, respectively. Therefore, $Mg_{1.5}FHA$ coating has the highest dissolution rate,

followed by Mg₂FHA, Mg₁FHA, Mg_{0.5}FHA and Mg₀FHA coating. Namely, the higher the Mg concentration in the coatings (i.e. Mg_{1.5}FHA and Mg₂FHA), the faster the dissolution is. Another reason attributing to the high dissolution rate of Mg_{1.5}FHA and Mg₂FHA is the existence of small amount of β -TCMP, which has higher solubility than Mg-containing HA [151]. As a result, the concentrations of calcium ions and phosphate groups increase sharply, leading to the increase of local supersaturation, which is beneficial to nucleation and growth of the new apatite crystals. The crystal growth (precipitation) can be described by a similar empirical equation [150]:

$$-\frac{dc}{dt} = k_2 s_2 (c - c_\infty)^n \quad (6)$$

where $-dc/dt$ is the crystal growth rate (precipitation), k_2 is the rate constant for crystal growth (precipitation), s_2 is a function of the total number of available nucleation sites, c_∞ stands for the equilibrium concentration, c for the concentration of solution at different times and n is the effective order of reaction. The dissolution and precipitation are reversible, of which the direction is determined by the difference between the concentration of the solution (c) and the equilibrium concentration (c_∞). When $c_\infty > c$, the precipitation process predominates. Since the dissolution of Mg_{1.5}FHA and Mg₂FHA proceeds much faster than other coatings, Mg_{1.5}FHA and Mg₂FHA firstly reverse to the precipitation. This is evident in Figure 4-19(a) where new apatite layer is observed only after 7 days. The new apatite layer is also observed on FMA2 after 28 days, while not found on FMA0 and FMA1

even after 28 days. The rate of precipitation is governed by the concentration difference ($c - c_{\infty}$) and the number of available nucleation sites (s_2). This again confirms that high Mg concentration favors the formation of new apatite layer. Both surface morphology observation and phase analysis show that the existence of Mg in the coating significantly affects the bone growth (formation of new apatite layer) in the SBF solution, i.e. the in vitro bioactivity of the coating. Higher concentration of Mg has better in vitro bioactivity.

4.1.2.1.3 Chemical groups after in vitro bioactivity test

The chemical groups are identified by FTIR after the in vitro bioactivity test, as shown in Figure 4-11. Compared with the FTIR spectra of the as-deposited coatings (Figure 4-4), some changes are seen. For all the coatings, the weak peak between 1120 cm^{-1} and 1122 cm^{-1} vanishes. The two peaks located at around 603 cm^{-1} and 565 cm^{-1} correspond to the phosphate group (PO_4^{3-}), ascribing to the deformation vibration of PO_4^{3-} [22]. In addition, in Mg_0FHA and $\text{Mg}_{0.5}\text{FHA}$ coatings, two peaks around 1041 cm^{-1} and 1095 cm^{-1} belonging to (PO_4^{3-}) are visible. In Mg_1FHA , $\text{Mg}_{1.5}\text{FHA}$ and Mg_2FHA coatings, PO_4^{3-} peak around 1031 cm^{-1} broadens, indicating the poor crystallinity of apatite. Besides PO_4^{3-} peaks, carbonate group (CO_3^{2-}) peaks are also detected in Mg_1FHA , $\text{Mg}_{1.5}\text{FHA}$ and Mg_2FHA coatings between $1400\text{--}1500\text{ cm}^{-1}$ due to the ν_3 stretching vibration of carbonate, and another peak at about 872 cm^{-1} ascribes to the ν_2 mode of CO_3^{2-} [141]. Since the

CO_3^{2-} peaks do not show in the Mg_0FHA and $\text{Mg}_{0.5}\text{FHA}$ coatings, it is reasonable that CO_3^{2-} forms during the growth of new apatite layer. The changes in chemical groups before and after soaking in the SBF solutions support the phase analysis and surface morphology observation that a new apatite layer forms on the surface of FMA2, FMA3 and FMA4.

Figure 4-11 FTIR spectra of Mg_xFHA coatings on Ti6Al4V substrates after soaking in the SBF solutions for 28 days

4.1.2.1.4 Chemical composition after the in vitro bioactivity test

The chemical compositions of Mg_xFHA coatings after the bioactivity test are studied using the XPS technique. The survey scan profiles are shown in Figure 4-12. The main elements of the apatite coatings, Ca, P and O, are detected in all the samples. F 1s is visible in FMA0 and FMA1 profiles, while not in FMA2, FMA3 and FMA4. It suggests a new apatite layer on the primary coating of FMA2, FMA3

and FMA4, while not on that of FMA0 and FMA1. A small amount of Mg is also detected after the in vitro bioactivity test. For FMA2, FMA3 and FMA4, Mg may be contained in the newly formed apatite layer during the biomimetic growth in the SBF solutions [152]. While for FMA0 and FMA1, the detected Mg might be due to the immersion in Mg-containing SBF solutions.

Figure 4-12 XPS survey scan profiles of Mg_xFHA coatings on Ti6Al4V substrates after soaking in the SBF solutions for 28 days

In summary, after soaking in the SBF solutions for 28 days, phase analysis shows the formation of poorly crystallized apatite layer on the surface of FMA2, FMA3 and FMA4. FTIR spectra further confirm the existence of CO_3^{2-} as well as PO_4^{3-} in the new layer. This newly formed layer is also observed from SEM images, and all the elements in the apatite coatings are also detected by XPS. Whereas, no obvious change is found in the phases, chemical groups and chemical compositions in

FMA0 and FMA1, and only the dissolution features are observed. Therefore, it is concluded that hydroxylcarbonate apatite layer forms on the surfaces of FMA2, FMA3 and FMA4, i.e. Mg in the coating results in better in vitro bioactivity as revealed by faster formation of a new apatite layer on the surface of the coating as submerged in the SBF solution.

4.1.2.2 Cell response

Osteoblastic cells are responsible for the synthesis and mineralization of bone during both initial bone formation and later bone remodeling. Human osteosarcoma MG63 cells exhibit fundamental phenotypic characteristics of osteoblastic cells, thus they are cultured for the evaluation of cell response on the coatings. Cell response on the Mg_xFHA coatings is studied in terms of cell morphology, cell proliferation and differentiation.

4.1.2.2.1 Cell morphology

Figure 4-13 shows typical SEM micrographs of MG63 cells after 3 days of culture on Mg_xFHA coatings. The dark areas signify the spreading of cell while the rough base is due to the coating dissolution in the culture medium. No significant difference is observed in cell morphology on different coatings. All cells spread well and grow favorably on the coating surface. Under higher magnification (Figure 4-13(c)), the cell's filopodium and lamellipodium are seen to be flat and

adhered tightly on the coating surface, suggesting good viability on the coating.

The well spread cells on all Mg_xFHA coatings are expected to be favorable for cell growth.

Figure 4-13 Cell morphology after incubation on Mg_xFHA coatings for 3 days: (a) FMA0, (b) FMA3 and (c) FMA2 (higher magnification of a single cell)

4.1.2.2.2 Cell proliferation

Figure 4-14 shows the quantification of cell numbers at each cell culture period to monitor the cell proliferation process. On the first day, there is no significant difference between the coatings. After incubation for 3 days, FMA1 and FMA3 have significantly higher cell numbers than that on FMA0. After 5 days, the cell

numbers on FMA3 are significantly higher than those on all the other coatings. Compared with the initial seeded cell density, all the coatings show a significant increase in cell numbers after incubation for 5 days (four to six times as many as the seeded cell density). It indicates that all the coatings, regardless of the degree of Mg incorporation, have favorable viability with MG63 cells. Meanwhile, the most cell numbers on FMA3 after 5 days predict that FMA3 coating (a relatively higher Mg concentration) stimulates cell proliferation more.

Figure 4-14 Cell proliferation on Mg_xFHA coatings in terms of cell density: *At day 3, FMA1 and FMA3 have a significantly higher cell numbers than that on FMA0 ($p < 0.05$). ** At day 5, cell numbers on FMA3 are significantly higher than all the other coatings ($p < 0.05$).

4.1.2.2.3 Cell differentiation

To evaluate the cell differentiation, ALP activity, an early differentiation marker,

was firstly measured. Figure 4-15 reveals the intracellular ALP activity of MG63 cells cultured on Mg_xFHA coatings for different periods. A significant increase ($p < 0.05$) in ALP activity is observed for all the coatings except for FMA4 in the first 2 weeks. From day 14 to day 21, a down-regulation is seen for all the coatings: significant decrease for FMA0, FMA1 and FMA3 and insignificant change for FMA2 and FMA4. The significant decrease indicates that more cells step into the next differentiation stage. However, there is no significant difference between Mg-incorporated coatings (from FMA1 to FMA4) and Mg-free one (FMA0).

Figure 4-15 Intracellular ALP activities of MG63 cells cultured on Mg_xFHA coatings over the culture period: no significant difference between Mg-incorporated coatings (from FMA1 to FMA4) and Mg-free one (FMA0).

As a late marker of cell differentiation, OC expression is also measured as shown in Figure 4-16. At day 7, FMA3 exhibits a significantly higher OC expression than all the other coatings ($p < 0.05$), showing the fastest entry to the late differentiation

stage. Then, a significant decrease in OC expression follows at day 14 and 21, which implies that more cells finish the differentiation stage and shift to other stages. Also, FMA2 at day 7 has a significant higher OC expression than FMA0 and FMA4 ($p < 0.05$). However, FMA0 reaches its maximum OC expression at day 14 over the whole culture period, meaning most cells undergo the late cell differentiation at day 14. Above comparison reveals that Mg incorporation can accelerate late cell differentiation. However, considering Mg concentration on the accelerating effect, it is not the higher the better, since it is not observed on FMA4.

Figure 4-16 Intracellular OC expression of MG63 cells cultured on MgFHA coatings over the culture period: * FMA3 has a significantly higher OC expression than all the other coatings ($p < 0.05$).

4.1.2.2.4 Effect of Mg incorporation on cell response

Significantly larger cell numbers on FMA3 at day 5 (Figure 4-14) and the much

higher OC expression on FMA3 at day 7 (Figure 4-16) suggest the stimulating effect of Mg ions on the osteoblastic cell responses. Similar results have been reported by Yamasaki Y, et al. [131]. Mg ions at the surface of FGMgCO₃ apatite (Mg-rich surface and functionally graded CO₃ apatite) induced more osteoblast cells to be attracted to the FGMgCO₃Ap-collagen composite than to Mg-free CO₃Ap-collagen composite.

The healing of damaged bone should originate from the binding or adhesion of cells to biomaterials, which is thought to be mediated primarily by membrane-associated adhesion receptors belonging to the integrin family [153]. The expression of specific integrin will determine cell proliferation, differentiation, or even death. Moreover, divalent cations have been suggested to play a regulatory role in integrin-dependent cell adhesion. The binding of integrins to specific ligands depends on the presence of divalent cations, with most but not all cells preferring Mg²⁺ to Ca²⁺ ions. It was also reported that Mg²⁺ enhanced cell adhesion to collagen type I and to laminin in a concentration-dependent manner [154]. Zreiqat H. et al. also proved that the adhesion of human bone-derived cell to Mg²⁺ ions modified bioceramic (Al₂O₃-Mg²⁺) was increased compared to that on Mg²⁺-free Al₂O₃. This cation-promoted cell adhesion depends on integrin-associated signal transduction pathways involving signaling protein [155].

Since the F concentration is fixed for all the coatings, the stimulating effect on cell

responses should come from incorporation of Mg^{2+} ions. Mg^{2+} ions seem to contribute to cell adhesion on the surface of the coating through the action of the integrin family existing on the cell membrane. Mg^{2+} ions bind cells to the coating surface to accelerate cell adhesion, proliferation, differentiation and finally bone formation. Considering that FMA3 shows a significant difference in cell proliferation (the most cell numbers) and late cell differentiation (the highest OC expression), it is probable that Mg^{2+} ions both in the apatite lattice and the surface of apatite crystals act directly or indirectly to promote cell responses. However, regarding the Mg^{2+} concentration in the coating, it is not the higher the better. In the present study, Mg_xFHA coating exhibits optimum cell responses at $x = 1.5$. Furthermore, the dissolved Mg^{2+} ions from the coating can contribute to becoming a source for the remodeling of new bone.

4.1.3 Long-term stability

The long-term stability study of surface coated medical implants is to relieve mainly two concerns: dissolution rate of coating and adhesion strength between coating and substrate. Therefore, in this part, the long-term stability of the Mg_xFHA coatings is investigated on both the dissolution rate of coating and the adhesion strength between the coating and the substrate.

4.1.3.1 Dissolution rate

The dissolution behaviors of Mg_xFHA coatings in the tris-buffered saline (TBS) solution are shown in Figure 4-17. The concentration of Ca^{2+} released from the coating into the solution increases (Figure 4-17), while the dissolution rate of the coating decreases (Figure 4-18) with the soaking time. The dissolution curves divide into two distinct stages: the initial sharp increase of the Ca^{2+} concentration and the sharp decrease of the dissolution rate during the first 7 days, followed by a slow increase in Ca^{2+} concentration and finally a nearly constant dissolution rate. The different dissolution behaviors in the two stages are due to the different driving force to the dissolution. In the first stage, the dissolution should be surface dissolution limited. In the prepared coatings, other phases, such as amorphous phase must be existent in the coatings. The solubilities of some calcium phosphates are as follows [10]:

Therefore, more soluble phases on or near the coating surface are dissolved into the solution very quickly, leading to the sharp increase of Ca^{2+} concentration during the first 7 days. As the driving forces for all the coatings are same, there is no significant difference on the dissolution behavior among different coatings. After the removal of these more soluble phases near the surface of the coating, the dissolution of the coating becomes diffusion limited, and the effective dissolution rate is approaching to that of the phases in the coating. As discussed in section

4.1.1.1, FHA phases are the main phases in all the coatings, and a small amount of β -TCMP exists in FMA3 and FMA4. Because β -TCMP is more soluble than FHA, FMA3 and FMA4 releases more Ca^{2+} into the solution after the first 7 days, and FMA3 shows the fastest dissolution.

Figure 4-17 Dissolution behaviors of Mg_xFHA coatings in the TBS solution: Ca^{2+} concentration as a function of both soaking time and Mg concentration

Figure 4-18 Dissolution rates of Mg_xFHA coatings in the TBS solution as a function of both soaking time and Mg concentration

On the other hand, the dissolution behavior shows that Mg incorporation into the coating has a negative effect on the dissolution resistance of the coating, which was also reported by other researchers [126]. The possible reason is that the substitution of Ca atom with Mg atom in the apatite structure destabilizes the crystal structure. When the central Ca atom is replaced by Mg atom, the structure becomes asymmetrical. Hydroxyl groups locate on one side of the central atom, and the six external calcium atoms displace contralaterally. Also, the replacement of the central Ca atom by Mg atom shortens the bond lengths between the central atom and surrounding oxygens and shrinks the structure [156]. When Mg ions are simultaneously incorporated into the HA coating with F ions, this deformation may not only destabilize the apatite structure, but also influence the ordered structure formed by the F incorporation in the apatite structure. It is well known that the solubility of HA coating can be decreased by the substitution of OH^- with F^- in the HA lattice [7, 49, 111]. In the HA crystal structure, H^+ in a OH^- can bond with O^{2-} from different directions along the c -axis; once an OH^- is replaced by a F^- , a stronger hydrogen bond forms between the F^- and the nearby OH^- , forming a more ordered structure, especially at a 50% substitution [49]. Thus, the replacement of Ca^{2+} by Mg^{2+} results in the increased solubility of Mg_xFHA coating ($x > 0$) in comparison with FHA coating.

The release of Mg^{2+} ions from the coating into the solutions is also measured. Taking FMA3 as an example, the Mg^{2+} concentration increases with the soaking

time, shown in its typical dissolution behavior in Figure 4-19. As shown in the inset of Figure 4-19, the Mg^{2+} concentration of different coatings exhibits the same trend as that of the Ca^{2+} concentration after soaking in the TBS solution for 28 days. The dissolution behavior of Mg^{2+} can be explained in terms of coating composition, which is measured by XPS (Figure 4-3). Excessive Mg distributes on the surface of the coatings, and the measured Mg concentration increases with the increase of the designed Mg in the sol. Therefore, the coating with higher Mg concentration releases more Mg^{2+} ions into the solution ($\text{FMA1} < \text{FMA2} < \text{FMA3}$), however, FMA4 (the highest Mg concentration) releases less Mg^{2+} than FMA3. This might be due to the extra Mg ions on the surface of the coating, which protect the coatings from dissolution because the vacancies or tangling bonds which are active in dissolution are filled with Mg ions, thus leading to slower dissolution.

Figure 4-19 Dissolution behavior of FMA3 in the TBS solution: Mg^{2+} concentration as a function of soaking time (inset: total Mg^{2+} concentration after 28 days)

After the dissolution test, the surface morphologies of the dissolved Mg_xFHA coatings have no significant difference, as shown in Figure 4-20. Compared with those before the dissolution test (Figure 4-5), a rough and porous surface is observed on both FMA3 and FMA4, and the pore diameter is 2 – 3 μm . As a result of different solubility of different phases in the coating, the rapid dissolution of the more soluble phases leaves behind a porous morphology with the dissolution resistant phases (HA or FHA) within the coating.

Figure 4-20 Surface morphology of Mg_xFHA coatings on Ti6Al4V substrates after soaking in the TBS solutions for 28 days ((a): FMA3; (b): FMA4)

Since the initial dissolution of the coating is beneficial to the healing process as well as the osseointegration [157], the current dissolution behaviors of different coatings are promising. When a synthetic HA ceramic was exposed in vivo, a small amount of the ceramic was phagocytized but the major remaining part acted as a secondary nucleator as evidenced by the appearance of a newly formed mineral [158]. The initial sharp dissolution of the coating increases the concentrations of

calcium and phosphate ions to create a locally supersaturated environment, causing the precipitation and growth of biological apatite crystals from the surface of the initial synthetic crystals with simultaneous incorporation of various ions presented in the biologic fluid. This is favorable to speed up the formation of links between the implant and bone. On the other hand, it is easier for osteoblast cells to attach and grow on a rough and porous surface and then form new bone.

4.1.3.2 Adhesion strength

The adhesion strength between the coating and the substrate is measured by the pull-off test. The typical morphology of rupture surfaces of the Mg_2FHA coating after the pull-off test is shown in Figure 4-21. It can be seen that both the adhesive and cohesive failure are present. Figure 4-21(a) shows the typical adhesive failure: a piece of the coating is detached from the substrate and others still adhere to the substrate. Cohesive failure can be observed in Figure 4-21(b). It happens within the coating, and is spot flaking around the surface of the coating.

Figure 4-21 Typical morphology of rupture surfaces of the Mg_2FHA coating

Since both adhesive and cohesive failures are present, the “pull-off adhesion strength” is used not to distinguish adhesive and cohesive failure, shown in Figure 4-22. Without Mg incorporation (FMA0), the pull-off adhesion strength is about 12 MPa. When Mg is incorporated, it increases significantly ($p < 0.05$) to about 18 MPa. However, there is no significant difference between different Mg concentrations.

Figure 4-22 Pull-off adhesion strength between Mg_xFHA coatings and Ti6Al4V substrates: Mg incorporation enhances the pull-off adhesion strength significantly ($p < 0.05$), but no significant difference between different Mg concentrations.

Both the mechanical interlocking and the chemical bonding contribute to the adhesion between the coating and substrate. In this work, all the substrates were polished to grade #1200 of SiC sandpapers to achieve the same finish. Moreover, Blackwood D.J. et al. proved that the adhesion became independent of surface

finish at levels smoother than #600 [159]. Therefore, the mechanical interlocking is considered as identical among all the samples, thus the main contribution to the adhesion strength is the chemical bonds at the interface of the coating and substrate formed during the coating deposition process, especially at the firing stage. During the firing process, the elements of the coating and substrate diffuse towards each other. As can be seen in Figure 4-7, all the elements are present in the transitional region (R_t). Without Mg incorporation, chemical bonds between Ti-O-Ca-P-F formed at the interface, such as Ca-O-Ti bonds, which could contribute to higher adhesion strength [8]. When Mg ions are incorporated, Mg-O-Ti bonds form at the interface as well as Ca-O-Ti bonds, which would make the chemical bonds even stronger and result in much higher adhesion strength. Since more Mg ions are abundant on the surface the coating, Mg concentration near the interface might be not significantly different among Mg_xFHA coatings, thus there is no significant difference between different Mg concentrations.

4.1.4 Summary

In summary, $Ca_{10-x}Mg_x(PO_4)_6F_1(OH)_1$ (Mg_xFHA , $x = 0, 0.5, 1, 1.5, 2$) coatings were deposited on Ti6Al4V substrates by the sol-gel dip-coating method. The substitution of Mg and F ions in HA crystal structure was discussed. The dependence of Mg concentration on in vitro bioactivity, cell response, dissolution behavior, and adhesion strength of Mg_xFHA coatings were systematically studied.

The following conclusions are drawn.

1. The resultant phase composition of the coating highly depends on Mg content.
When $x \leq 1.0$, a single FHA phase is formed; when $1.0 < x \leq 2.0$, a small amount of β -TCMP phase appears together with dominant FHA phase.
2. GIXRD, XPS and FTIR results indicate that Ca ions and OH groups in HA are substituted by Mg and F ions, respectively.
3. The simultaneous incorporation of Mg and F ions in HA coating improves the in vitro bioactivity of the coating. This improvement becomes more significant at high Mg concentrations ($1.0 \leq x \leq 2.0$).
4. Cells spread well on all the coatings. When the designed Mg substitution degree reaches 1.5, Mg ions show a significant stimulating effect on cell response exhibiting the most cell proliferation and the fastest late cell differentiation.
5. The incorporation of Mg increases the dissolution rate of Mg_xFHA coating in the TBS solution, and the maximum is achieved at $x = 1.5$.
6. Mg substitution enhances the adhesion strength between the coating and the substrate, but comparable among different Mg concentrations.
7. The optimal Mg content is at $x = 1.5$ to achieve the best in vitro bioactivity and cellular response, i.e. the best short-term osseointegration.

4.2 Effect of fluorine substitution in magnesium-containing hydroxyapatite (MgF_yHA) coatings

Fluorine ions are incorporated into magnesium-containing hydroxyapatite coatings to enhance the long-term stability. The as-deposited coatings are denoted by MgF_yHA ($\text{Ca}_9\text{Mg}_1(\text{PO}_4)_6\text{F}_y(\text{OH})_{(2-y)}$, y varies from 0 to 2). The co-substitution of Mg and F ions in the MgF_yHA coatings, the effect of F substitution on short-term osseointegration and long-term stability of the MgF_yHA coatings are discussed. The emphasis is on the dissolution rate and adhesion strength.

4.2.1 Co-substitution of Mg and F ions in the MgF_yHA coatings

As discussed in section 4.1, when Mg and F ions are incorporated into MgF_yHA coatings simultaneously, the corresponding physical, chemical, biological and mechanical properties are influenced. In this part, the co-substitution of Mg and F ions in the MgF_yHA coatings is also discussed from phase evolution, co-substitution mechanism, surface and interface properties.

4.2.1.1 Phase evolution

GIXRD patterns of as-deposited MgF_yHA coatings are shown in Figure 4-23. Without F incorporation (MFA0), β -TCMP is detected as the main phase, and HA phase exists as the secondary phase. It is due to the presence of Mg ions, because the transformation temperature from apatite to β -TCMP could be lowered about

100 °C than that to pure β -TCP (700 – 800 °C) when Mg ions were added. Furthermore, Ca ion vacancies in the ideal β -TCP structure are too small to accommodate Ca ions, but allow for the inclusion of Mg ions, which thereby stabilizes the structure resulting from the difference in size of Ca^{2+} ions (0.99 Å) and Mg^{2+} ions (0.69 Å) [37, 139]. Since the designed (Ca, Mg)/P ratio in MgF_0HA is 1.67, exceeding the stoichiometric molar ratio of 1.5 for β -TCP, it is understandable that Mg ions preferentially incorporate into β -TCP lattice and the excess of Ca ions form HA phase.

Figure 4-23 GIXRD patterns of as-deposited MgF_yHA coatings on Ti6Al4V substrates

When F is incorporated, FHA becomes the main phase in all the MgF_yHA coatings ($y > 0$), confirmed by the slight shifting of the main diffraction peaks towards higher degrees compared with that of HA [118]. Furthermore, it is worthy of noting

that when F concentration increases from 0 to 0.5, i.e. $\text{MgF}_{0.5}\text{HA}$ (MFA1), β -TCMP is only detected with weak intensity; as F concentration further increases, no more β -TCMP forms. This is a clear indication of F ions' suppression ability of β -TCMP formation. In other words, F promotes the substitution of Ca^{2+} with Mg^{2+} in the HA lattice, in agreement of our previous findings (section 4.1.1.1) as well as other researches [124].

4.2.1.2 Co-substitution of Mg and F ions in the MgF_yHA coatings

As stated in section 4.1.1.2, the substitution of Mg and F ions in the HA crystal structure needs to be confirmed so as to investigate the effects caused by the incorporation of Mg and F ions. The same as that carried out on Mg_xFHA coatings, the corresponding lattice parameters, chemical states and chemical groups of MgF_yHA coatings are studied.

4.2.1.2.1 Lattice parameters

The shift of (211) and (300) peaks and the shrinkage of a -axis take place when fluorine ions are incorporated into the HA crystal structure [97-99]. In the MgF_yHA coatings, the two-theta of (300) peak and calculated a -axis are listed in Table 4-2. As F concentration increases from 0 to 1.0, the (300) peak shifts towards higher degree due to the substitution of larger OH^- (1.68 Å) with smaller F^- (1.32 Å). However, at F concentration of 1.5, the two-theta of (300) peak remains the same

with that of 1.0. When F concentration increases further to 2.0, (300) peak shifts a little to higher degree. The same trend is also observed from the change of a -axis. The gradual shrinkage of a -axis along with the increasing of F concentration indicates the replacement of OH^- groups by F^- ions in the HA crystal structure. As mentioned in section 4.1.1.2, the shift of (002) peak and the decrease of c -axis are indications of Mg substitution in the HA lattice. In pure HA, the two-theta of (002) is 25.88° and c -axis is $6.88 \text{ \AA}$ as shown in PDF#09-0432. In the MgF_yHA coatings, the (002) peak shifts to a higher degree of 26.02° or 26.04° , and calculated c -axis is $\sim 6.84 \text{ \AA}$. Since the designed Mg concentration is all the same in the MgF_yHA coatings, there is no obvious difference of c -axis between different coatings. Anyway, the substitution of Mg ions in the HA lattice is confirmed by the shrinkage of c -axis.

Table 4-2 Two-theta of (300) peak and calculated a -axis of MgF_yHA coatings

Sample	MFA0	MFA1	MFA2	MFA3	MFA4
Two-theta ($^\circ$)	33.00	33.16	33.22	33.22	33.24
a -axis ($\text{\AA}$)	9.39	9.35	9.33	9.33	9.33

4.2.1.2.2 Chemical composition

XPS analysis of MgF_yHA coatings (chemical states and F concentration) are shown

in Figure 4-24. The survey scans (Figure 4-24(a)) present the existence of all the elements in MgF_yHA coatings including the contaminated C 1s. Since F concentration varies in MgF_yHA coatings, F incorporation and concentration in the coatings are analyzed. In the narrow scans of F 1s (Figure 4-24(b)), only one peak is detected, and the binding energy is at 684.3 ± 0.1 eV in different coatings, which is the fingerprint of F in FHA or FA structure [8, 103], revealing the incorporation of F ions in the HA crystal structure. Moreover, the intensity of F 1s peak increases when F concentration increases, indicating more F ions are incorporated. Figure 4-24(c) exhibits the comparison of the designed and the measured F concentration in the coatings. The straight line shows the ideal situation when all the designed F in the sols are completely incorporated into the HA crystal structure. The data points are F concentrations determined by XPS in the as-deposited coatings. There is a discrepancy between the designed and measured F concentrations, especially at higher levels. The loss of fluorine might be due to the formation of HF during the sol-gel dip-coating process [103]. Fluorine concentration in the coating is measured in the range of 0.39 – 1.16 mol F^- /mol apatite. Except for F incorporation, Mg 2p at 50.3 eV is also detected (not shown here), confirming the substitution of Mg in the HA lattice.

Figure 4-24 XPS analysis of MgF_yHA coatings: (a) XPS survey scans of MgF_yHA coatings; (b) XPS narrow scans of F 1s; (c) fluorine concentration in MgF_yHA coatings (straight line: designed; solid square: measured)

4.2.1.2.3 Chemical groups

Figure 4-25 shows FTIR spectra of MgF_yHA coatings. The typical peaks of phosphate group (PO_4^{3-}) are in the range of $1000 - 1200 \text{ cm}^{-1}$ due to the triply asymmetric ν_3 stretching vibration and between $960 - 970 \text{ cm}^{-1}$ caused by ν_1 symmetric stretching vibration [140]. Two weak peaks between 1400 and 1500 cm^{-1}

ascribe to the ν_3 stretching vibration of carbonate (CO_3^{2-}) [141]. Without F incorporation (MFA0), there is a peak at 3570 cm^{-1} due to the stretching vibration of free OH groups. When F ions are incorporated, the stretching peak of OH shifts to a lower frequency at 3540 cm^{-1} due to the stretching of OH-F band when the OH groups are partially replaced by the F ions [29, 97]. Moreover, the intensity of the peak at 3540 cm^{-1} decreases as F concentration increases, implying more F ions are incorporated into HA lattice. It's worthy of noting that this peak still presents in MFA4 with weak intensity, meaning the existence of OH-F band in the coating. In other words, F^- ions don't completely replace OH^- groups when the designed fluoridation degree is 2.0, because the OH stretching band disappears in the case of the pure FA [29]. This is in agreement with the measurement of F concentration in the coatings (Figure 4-24(c)).

Figure 4-25 FTIR spectra of MgF_yHA coatings on Ti6Al4V substrates

4.2.1.3 Surface and interface

Surface morphologies of Mg_yFHA coatings observed by SEM are seen in Figure 4-26. Basically, the coatings are uniform. However, there are a few micropores on the coatings due to the evaporation and/or decomposition of the solvent as well as the change of surface tension during the drying and firing process.

Figure 4-26 SEM surface morphology of MgF_yHA coatings on Ti6Al4V substrates

Similar cross-sectional view is observed in different MgF_xHA coatings after mounting and polishing, as shown in Figure 4-27. The coating thickness is measured as $1.5 - 2.0 \mu\text{m}$. After repeated abrading and polishing, the coatings are still dense and uniform and bond well on the substrates.

Figure 4-27 Cross-sectional view of Mg_xFHA coatings on Ti6Al4V substrates ((a): MFA0; (b): MFA2)

The elemental distribution from the coating to the substrate is acquired by GDOES. A typical GDOES depth profile is plotted in Figure 4-28, showing the distribution of Ca, P, O, Mg, F, Ti and Al along the depth direction. Three regions are identified at the cross-section along the depth direction: the coating region (R_c), the transitional region (R_t), and the substrate region (R_s). Tangent method is used to determine the boundaries between two adjacent regions. In the coating region R_c , most of the elements in the coating are stable. At the boundary of R_c and R_t , the concentration of Ca, P, Mg and F begins to decrease, whereas there is an abrupt increase of O concentration followed by a sharp decrease. The increase of O might

be due to the presence of F ions, because of nc-CaF₂ formed in the dipping sol could be easily absorbed on the substrate surface through hydrogen bonds, which could attract more O near the interface [8]. In the region of R_t , all the elements of the coating as well as the substrate are present. Also, the width of the R_t is about 500 nm. Thus it is speculated that the mutual diffusion of the elements in the coating and the substrate take place during the coating deposition process. At the boundary between the R_t and the R_s , Ti and Al stabilize, and Ca, P, O, Mg and F disappear.

Figure 4-28 GDOES depth profile of MFA3 (Mg20: 20 times of Mg concentration;
F100: 100 times of F concentration)

XPS narrow scans near the interface of the MgF_{1.5}HA coating and the substrate are shown in Figure 4-29. The chemical states of Ti, Ca, O and Mg are analyzed. Figure 4-29(a) shows Ti 2p spectrum. Only Ti⁴⁺ is detected, Ti 2p_{3/2} at 458.5 eV and

Ti 2p_{1/2} at 464.2 eV, the typical binding energy in titanium dioxide (TiO₂) or titanate group (TiO₃²⁻) [142, 143]. Ca 2p spectrum in Figure 4-29(b) contains two different states: Ca 2p_{3/2} at 347.4 eV is the typical binding energy of Ca²⁺ in HA or FHA; Ca 2p_{3/2} at 346.7 eV can be attributed to CaTiO₃ [142, 144]. Since the elemental interdiffusion takes place during the firing stage, it is reasonable that CaTiO₃ forms near the interface of the coating and the substrate, i.e. Ca-O-Ti bonds form at the coating/substrate interface. O 1s spectrum is shown in Figure 4-29(c). The main component at the binding energy of 529.9 eV can be assigned to O²⁻ species in TiO₂/TiO₃²⁻, and that at 531.3 eV corresponds to O²⁻ in HA or FHA [142, 144]. P 2p locating at 133.4 eV (not shown here) is attributable to PO₄³⁻ in HA or FHA [144], further proving the apatite formation. Figure 4-29(d) presents Mg 2p spectrum. Only one peak at 50.6 eV is detected, which is the typical binding energy of Mg²⁺. While Mg2p of pure Mg metal is around 49.8 eV, so Mg bonds with O and forms Mg-O bonds or Mg-O-Ti bonds.

Figure 4-29 XPS spectra of Ti 2p (a), Ca 2p (b), O 1s (c) and Mg 2p (d) near the interface of the $\text{MgF}_{1.5}\text{HA}$ coating and the substrate

4.2.1.4 Discussion

Without F incorporation, MgF_0HA coating is represented as $\text{Ca}_9\text{Mg}_1(\text{PO}_4)_6(\text{OH})_2$.

The Ca/P ratio is 1.5, lower than that of pure HA (1.67), thus the formation of β -TCP is preferred as its Ca/P ratio is 1.5, especially with the presence of Mg ions [75, 139]. In this case, Mg ions are preferentially incorporated into β -TCP to form β -TCMP. Supposing Mg ions are incorporated into β -TCP at its structurally possible maximum substitution of 14.25 mol% [160], then the assumption of Ca

ions and P-containing groups is 66.86% and 77.97% of total Ca and P in the sol, respectively. The excess of Ca ions and P-containing groups in the sol further forms HA.

When F-precursor is added, F ions released through the hydrolysis and alcoholysis of HPF_6 react with Ca ions in the solution to form nc- CaF_2 particles. Because of the strong electronegativity of F ion, it is believed that hydrogen bonding easily forms between F in nc- CaF_2 particles and H in P-containing groups. The more F ions in the sol, the more nc- CaF_2 particles form. The formation of nc- CaF_2 particles competes with the assumption of Ca ions in the sol with the assembly of β -TCMP. Moreover, the conversion of β -TCP to the apatite can be promoted by the presence of F ions with the increasing F amount and reaction temperature [161], thus it is reasonable that the formation of FHA phase is preferred. As seen in Figure 4-23, FHA becomes the main phase as soon as F ions are incorporated. However, when the F/Ca ratio is over 0.2 (that in pure fluorapatite), the formation of excessive nc- CaF_2 particles results in a reduction of Ca/P ratio in the sol. As a result, the reaction between the extra P-containing groups with nc- CaF_2 particles produces volatile HF, which escapes from the coating during the heating process [103]. This is the reason that the measured F concentration in MFA4 ($\text{Ca}_9\text{Mg}_1(\text{PO}_4)_6\text{F}_2$) is lower than that in F6 ($\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$) [49].

The incorporation of Mg and F ions in the HA crystal structure is confirmed

through the lattice parameters, chemical states and chemical groups. The shift of (211) and (300) peaks and the shrinkage of a -axis indicate the incorporation of F ions in the HA crystal structure. The binding energy of F 1s at $\sim 684.3 \pm 0.1$ eV is another evidence of F incorporation. Furthermore, the shifting of the stretching peak of OH to a lower frequency at 3540 cm^{-1} is due to the stretching of OH-F band when the OH^- groups are partially replaced by the F^- ions. Mg substitution in the HA lattice is also confirmed by the shrinkage of c -axis and the binding energy of Mg 2p at ~ 50.3 eV.

The depth profiling illustrates the distribution of the elements along the depth direction. The mutual diffusion takes place at the coating/substrate interface. Mg concentration decreases from the surface to the substrate, while F concentration keeps constant. Ca-O-Ti and Mg-O-Ti bonds contribute to the interfacial properties, and the existence of F ions in the transitional region may also have an effect though it is not detected by XPS near the interface of the coating and substrate due to the detection limit. The effects of Mg and F substitution in the HA lattices as well as the inclusion in the coatings on the properties of MgF_yHA coatings are discussed below.

4.2.2 Short-term osseointegration

Although the incorporation of fluorine ions into magnesium-containing hydroxyapatite coatings is expected to improve the long-term stability of the

implant, it is still important to examine the short-term osseointegration. In this part, the in vitro bioactivity and cell response of the MgF_yHA coatings are studied by simulated body fluid and human osteosarcoma MG63 cells.

4.2.2.1 In vitro bioactivity

After soaking in the SBF solutions for 28 days, the phases are indentified by GIXRD, shown in Figure 4-30. The GIXRD profiles are different from that of as-deposited MgF_yHA coatings (Figure 4-23). A broad peak is seen around 32° on all the samples, indicating a poorly crystallized apatite. Also, main diffraction peaks of Ti6Al4V substrate are not observed, which should be at 40.5° , 38.5° and 35.4° . The absence of the diffraction peaks of the substrate also suggests the formation of a new layer on as-deposited coatings because the thickness of the detected layer is over the detection limit of GIXRD (usually several micrometers). Besides the apatite phase, β -TCMP is present in MFA0. Since β -TCMP is the main component of as-deposited MgF_0HA , it is understandable that it still exists after soaking in the SBF solution. Since the poorly crystallized apatite forms on all the MgF_yHA coatings, it suggests comparable bioactivity of MgF_yHA coatings regardless of the fluoridation degree.

Figure 4-30 GIXRD profiles of MgF_yHA coatings on Ti6Al4V substrates after soaking in the SBF for 28 days

Formation of new apatite phase is also supported by the surface morphology observation. Figure 4-31 shows typical surface morphologies of MgF_1HA coating after soaking in the SBF solution for 28 days. After the sol-gel deposition (Figure 4-26 MFA2), some slight grooves are observed on the coating surface caused by the evaporation and/or decomposition of the solvent as well as the change of surface tension during the drying and firing process. After soaking in the SBF solution for 28 days (Figure 4-31(a)), the surface is completely different, quite flat and homogeneous. From the high magnification (Figure 4-31(b)), this newly formed layer has submicro-scale network structure, which is the typical microstructure of bone-like apatite grown in the SBF solution.

Figure 4-31 SEM images of MgF_1HA coating on Ti6Al4V substrates after soaking in the SBF for 28 days ((b) is the high magnification of (a))

4.2.2.2 Cell response

The study of cell response on the MgF_yHA coatings is carried out the same as that on the Mg_xFHA coatings, including cell morphology, cell proliferation and differentiation.

4.2.2.2.1 Cell morphology

Typical SEM images of MG63 cells after cultured on MgF_yHA coatings for 3 days are shown in Figure 4-32. Taking MFA1 and MFA3 as examples, no significant difference is observed in cell morphology on different coatings (Figure 4-32(a, b)). All cells spread well and grow favorably on the coating surface. Cells attach tightly on the coating surface with their filopodium and lamellipodium (Figure 4-32(c, d)), suggesting good viability on all the coatings regardless of F concentration.

Figure 4-32 Cell morphology after cultured on MgF_yHA coatings for 3 days: MFA1

(a, c), MFA3 (b, d)

4.2.2.2.2 Cell proliferation

Cell proliferation is quantified through cell numbers at each cell culture period.

Figure 4-33 shows cell proliferation on MgF_yHA coatings in terms of cell density.

During the whole cell culture period, no significant difference is observed among different coatings ($p < 0.05$). However, cell numbers increase sharply after incubation for 5 days, over five times compared with the initial seeded cell density.

It indicates that all the coatings, regardless of the fluoridation degree, have favorable viability with MG63 cells.

Figure 4-33 Cell proliferation on MgF_yHA coatings in terms of cell density: no significant difference is observed on different coatings ($p < 0.05$)

4.2.2.2.3 Cell differentiation

Alkaline phosphatase (ALP) is an important intracellular enzyme, and considered as a prerequisite for normal skeletal mineralization. ALP activity is the most widely recognized early marker of osteoblastic cell differentiation [162]. In this work, ALP activity was measured to evaluate the early cell differentiation, as revealed in Figure 4-34. Significant increase ($p < 0.05$) in ALP activity is observed for all the coatings except for MFA0 in the first 2 weeks. Both MFA2 and MFA3 have a significantly higher ALP activity than MFA0 after 14 days. Then, a down regulation is seen on most of the coatings. This trend in ALP activity indicates that F ions stimulate early cell differentiation most at the second week with a designed fluoridation degree of 1.0 – 1.5 (the measured one of 0.72 – 0.97).

Figure 4-34 Intracellular ALP activities of MG63 cells cultured on MgF_yHA coatings over the culture period: * MFA2 and MFA3 have a significantly higher ALP activity than MFA0 ($p < 0.05$).

Osteocalcin (OC) is the predominant noncollagenous protein of bone and dentin, and plays roles in bone formation and remodeling [163]. Late cell differentiation is assessed by OC expression, as shown in Figure 4-35. At day 7, MFA2 exhibits a significantly higher OC expression than all the other coatings ($p < 0.05$). However, no significant difference is observed between different coatings after 14 and 21 days. Although comparable levels of OC expression are found on different coatings after 14 and 21 days, the fastest entry of MFA2 to the late differentiation stage suggests that F incorporation in the coating can accelerate cells to late differentiation stage when the designed fluoridation degree is around 1.0 (the measured F concentration is 0.72).

Figure 4-35 Intracellular OC expression of MG63 cells cultured on MgF_yHA coatings over the culture period: * MFA2 has a significantly higher OC expression than all the other coatings ($p < 0.05$)

4.2.2.2.4 Effect of F incorporation on cell response

It is well known that fluorine can directly stimulate cell proliferation and alkaline phosphate activity [164]. In our previous studies, when only F ions were incorporated into HA coatings, a significantly higher ($p < 0.05$) cell proliferation was observed on FHA coatings at the fluoridation degree of 0.8 to 1.1 [49]. However, this stimulation effect of F incorporation is not shown when Mg ions are co-substituted into HA coating. It might be due to the presence of Mg ions. Mg ions were proved to accelerate osteoblast cell adhesion on FGMgCO₃Ap-collagen composite [6]. Also in section 4.1.2.2, Mg ions were shown to have more profound stimulation effect on cell proliferation than on early cell differentiation. Therefore, when F ions were co-substituted into HA with Mg ions, the effect of Mg ions on

cell proliferation might cover that of F ions. Consequently, no significant difference is observed on cell proliferation with varied F concentrations.

On the other side, the stimulating effect of fluorine is dose-dependent, because higher dose might cause toxic effect [164, 165]. Generally, a moderate level of F content ($\text{Ca}_{10}(\text{PO}_4)_6\text{F}_{\sim 1.0}(\text{OH})_{\sim 1.0}$) is recommended in clinical application. For example, in Cheng et al.'s work, when x in the range of 0.67 – 1.68 ($\text{Ca}_{10}(\text{PO}_4)_6\text{F}_x(\text{OH})_{2-x}$), sol-gel derived FHA coating is the most suitable for implantation [118]. Wang et al. further optimized the fluoridation degree, and they found that FHA coating exhibited the best dissolution resistance and cell activities at the molar level of fluorine ions from 0.8 to 1.1 [49]. Also in the case of electrochemical deposition, FHA coating with fluoridation degree from 1.0 to 1.25 had the most positive stimulating effects on cell proliferation and ALP activities [93]. In our study, the coatings with a measured fluoridation degree of 0.72 – 0.97 exhibit the most predominant stimulating effect on cell differentiation (ALP activity and OC expression), which is in agreement with above mentioned.

Moreover, it seems that the effect of F ions on cell behaviors varies at different stages. Inoue M. et al. investigated in vitro response of osteoblast-like and odontoblast-like cells to unsubstituted and substituted apatites, and they found that F ions contributed more to differentiation rather than to proliferation in the early period [166]. Similar results were also reported by Kim H.W. et al. They studied the

effect of fluoridation of hydroxyapatite in hydroxyapatite-polycaprolactone (HA-PCL) composites on osteoblast activity, and found that the proliferation of MG63 cells on FHA-PCL composite was at a similar level to those on HA-PCL composite. However, the ALP activity and OC production were increased significantly by the increase of fluoridation of HA [167]. Our study presents the similar results: there is no significant difference on cell proliferation, but the stimulating effect is observed on both early and late stage of cell differentiation (significantly higher ALP activity and OC expression at the measured fluoridation degree of 0.72).

4.2.3 Long-term stability

Fluorine substitution in magnesium-containing hydroxyapatite coatings is expected to improve the long-term stability of the implant. In this part, the long-term stability of the MgF_yHA coatings is investigated in detail. The dissolution behaviour is monitored by the change of Ca^{2+} concentration in the TBS solution, and the adhesion strength between the coating and the substrate is measured by the pull-off test.

4.2.3.1 Dissolution rate

The substitution of foreign ions into HA lattice disrupts the crystal structure, and in turn, alters the dissolution properties of the materials in solution. In other words,

the dissolution rate can be traced by the appropriate substitution of foreign ions into HA lattice. The dissolution behavior of MgF_yHA coatings with the fixed Mg content and the varied fluoridation degree is monitored by the change of Ca^{2+} concentration in the TBS solution, as shown in Figure 4-36. All the coatings release more Ca^{2+} ions with the increasing soaking time. However, MgF_0HA coating without F incorporation dissolves the fastest, exhibiting the highest Ca^{2+} concentration and the fastest dissolution rate (Figure 4-37). When more F ions are incorporated, less Ca^{2+} ions are released from $\text{MgF}_{0.5}\text{HA}$, and the least Ca^{2+} ions from MgF_1HA , $\text{MgF}_{1.5}\text{HA}$ and MgF_2HA coating. The dissolution behavior of MgF_yHA coatings reveals that MgF_0HA coating has the highest dissolution rate, MgF_1HA , $\text{MgF}_{1.5}\text{HA}$, and MgF_2HA coating has the lowest but comparable dissolution rate. The dissolution rate is dependent of the solubility of the phases in the coating. From Figure 4-23, the phases of MgF_yHA coatings are detected as follows: most β -TCMP and little HA in MgF_0HA , most FHA and little β -TCMP in $\text{MgF}_{0.5}\text{HA}$, single FHA in MgF_1HA , $\text{MgF}_{1.5}\text{HA}$ and MgF_2HA . Although β -TCMP is more stable and less soluble than β -TCP, even that with 10.1 mol% Mg has a lower solubility than that of HA below pH 6.0, it is still more soluble than HA at pH 7.4 [168, 169]. Thus it is more soluble than FHA because the incorporation of F ions in the HA lattice lowers the solubility of HA [7, 49]. Also, it is reported that the resorption of calcium phosphates as implants takes place in this order: $\text{FHA} < \text{Mg-TCP} (\beta\text{-TCMP}) < \text{HA} < \beta\text{-TCP} < \alpha\text{-TCP}$ [170]. Therefore, it is reasonable that

MgF₀HA coating releases the most Ca²⁺ ions, less in MgF_{0.5}HA coating, and the least in MgF₁HA, MgF_{1.5}HA and MgF₂HA coatings.

Figure 4-36 Dissolution behaviors of MgF_yHA coatings in the TBS solution: Ca²⁺ concentration as a function of both soaking time and F concentration

Figure 4-37 Dissolution rates of MgF_yHA coatings in the TBS solution as a function of both soaking time and F concentration

Surface morphology of MgF_yHA coatings after the dissolution test is observed (Figure 4-20). After soaking in the TBS solution for 28 days, all the coatings exhibit a rough surface caused by the rapid dissolution of more soluble phases leaving less soluble phases in the coating. However, different dissolution characteristics are found when F concentration varies. For example, large pores or grooves are seen in MgF_0HA (without F incorporation), but only small pores in MgF_2HA (with F incorporation). The difference between the surface morphology is also reflected by the surface roughness (R_q). The R_q of MgF_0HA and MgF_2HA after the dissolution test is $0.845 \pm 0.013 \mu\text{m}$ and $0.429 \pm 0.032 \mu\text{m}$, respectively. These kinds of the dissolution characteristics correspond to above dissolution behaviors of MgF_yHA coatings. The prior dissolution of $\beta\text{-TCMP}$ in MgF_0HA leaves large grooves and pores on the coating, and slow dissolution of FHA in MgF_2HA only induces small pores.

Figure 4-38 Surface morphology of MgF_yHA coatings on Ti6Al4V substrates after soaking in the TBS solutions for 28 days ((a): MFA0, (b): MFA4)

The dissolution kinetics and mechanism of fluoridated hydroxyapatite (FHA) have been widely studied at different temperature and pH values [171-175]. It was found that the dissolution rates of FHA are pH independent at neutral and basic conditions ($7 \leq \text{pH} \leq 10$). The dissolution seems to be initiated by the relatively rapid removal of F and/or OH and Ca near the surface. After the initial preferential release of F, OH and Ca, stoichiometric release of Ca, P and F takes place [173]. For pure HA, the presence of water induces the onset of dissolution through the escape of hydroxyl groups from the surface of apatite. The surface calcium ions also show the onset of dissolution at a lower extent: the bonds between calcium and lattice oxygen ions are lengthened by the surface water molecules. However, when fluorine ions replace hydroxyl groups partially in HA lattice, hydroxyl groups may dissolve, but fluorine ions at the surface remain in the crystal. Moreover, the surface calcium ions are anchored on the surface by the strong interaction with fluorine ions [94]. Therefore, the incorporation of fluorine ions into HA lattice makes the material more dissolution resistant. It can be explained from the change of HA crystal structure. In pure HA lattice, there is a certain degree of disorder with randomly oriented OH groups because OH groups are diatomic and located off-center from the symmetry position in the *c*-direction. When OH groups are replaced by F ions, there will be no elastic strain caused by the substitution of OH groups by F ions because F^- (1.32Å) ion is smaller than OH^- group (1.68Å). Also, F ions can locate on the symmetry positions in HA lattice [94]. The substitution of

OH group by F ions stabilizes randomization of OH groups in the columns by reversion the OH alignment within the column [176]. Moreover, fluorine is very electronegative and has an even greater capacity than oxygen in forming hydrogen bonds. Hydrogen bonds formed between F ions and adjacent OH groups further stabilize the crystal structure. Therefore, the crystal structure of FHA is more stable than HA, resulting lower dissolution rate than that of HA. This is also supported by the calculated excess heats of solid solution that the replacement of OH groups in HA by F ions is an exothermic process [176].

4.2.3.2 Adhesion strength

After the pull-off test, the surface morphology is observed by SEM as shown in Figure 4-39. Typical adhesive failure is seen on MFA0 (without F incorporation) (Figure 4-39(a)): big pieces of the coating are torn from the substrate. While on MFA3 (with F incorporation) (Figure 4-39(b)), the coating still adheres well on the substrate without or with only a little detached coating. The fraction of the adhesive failure on the whole surface of the coating is estimated by the Image-Pro Plus 6.0 after capturing the images of the whole coating, listed in Table 4-3. The percentage of the adhesive failure in MFA0, MFA1 and MFA2 is more than 20%, but that in MFA3 and MFA4 is less than 10%. Thus it can be speculated that the adherence of the coating on the substrate of MFA3 and MFA4 is stronger than that on others.

Figure 4-39 Surface morphology of MgF_yHA coatings after the pull-off test ((a): MFA0; (b): MFA3)

Table 4-3 Fraction of adhesive failure on MgF_yHA coatings after the pull-off test

Sample	MFA0	MFA1	MFA2	MFA3	MFA4
Adhesive failure (%)	23.4±4.9	24.1±8.6	30.1±7.8	2.7±4.3	9.0±7.5

Correspondingly, the pull-off adhesion strength is compared in Figure 4-40. Without F incorporation (MFA0), the pull-off adhesion strength is about 16 MPa. When F is incorporated at the fluoridation degree of 0.5 – 1.0, no significant difference is observed. However, in the range of 1.5 – 2.0, the pull-off adhesion strength increases significantly ($p < 0.05$) to 29 and 24 MPa. It suggests that F incorporation into Mg-containing HA coating enhances the adhesion strength at higher F concentration. The trend of the calculated pull-off adhesion strength quite

matches that of the fraction of the adhesive failure. Moreover, the adhesion strength between the coating and the substrate should be much higher if the area of the adhesive failure is considered.

Figure 4-40 Pull-off adhesion strength between MgF_yHA coatings and Ti6Al4V substrates: significantly higher pull-off adhesion strength on MFA3 and MFA4 ($p < 0.05$).

The influence of F incorporation on the adhesion strength between the coating and the substrate owes to the changes of HA crystal structure, the incorporated F concentration, the reduction of the coefficient of thermal expansion, and the chemical bonding. As discussed in section 4.2.3.1, the incorporation of F ions into HA lattice stabilizes the crystal structure by reversing the OH alignment within the column and forming hydrogen bonds between OH groups and nearby F ions. Theoretically, the structure is the most stable at around 50% substitution due to the

alternating arrangement of the F ions between each pair of OH groups [96]. The fluorine concentration measured by XPS is shown in Figure 4-24(c). There is a discrepancy between the measured and designed F concentration. In $\text{MgF}_{1.5}\text{HA}$ and MgF_2HA , the measured F concentration is around 1.0 or nearly 50% substitution, thus they have the most stable crystal structure. Correspondingly, significantly higher pull-off adhesion strength is observed in MFA3 and MFA4. Another effect of F ions is the reduction of the coefficient of thermal expansion. The coefficient of thermal expansion of pure HA, pure FA and Ti6Al4V is $15 \times 10^{-6}/\text{K}$, $9.1 \times 10^{-6}/\text{K}$, and $8.9 \times 10^{-6}/\text{K}$, respectively. The substitution of F ions in HA lattice reduces the coefficient of thermal expansion, so does the thermal mismatch between the coating and the substrate, thus the residual stress in the coating is reduced [8]. Furthermore, the chemical bonds among Ti-O-Ca-Mg-P-F at the interface formed during the drying and firing process, such as Ca-O-Ti and Mg-O-Ti bonds, also contribute to the enhancement of the adhesion strength between the coating and the substrate.

4.2.4 Summary

In summary, $\text{Ca}_9\text{Mg}_1(\text{PO}_4)_6\text{F}_y(\text{OH})_{(2-y)}$ (MgF_yHA , $y = 0, 0.5, 1, 1.5, 2$) coatings were deposited on Ti6Al4V substrates by the sol-gel dip-coating method. The substitution of Mg and F ions in HA crystal structure was studied. The effect of F concentration on in vitro bioactivity, cell response, dissolution behavior, and adhesion strength of MgF_yHA coatings were systematically discussed. The

following conclusions are drawn.

1. F content has significant effect on resultant phases of the coating. β -TCMP is the main phase without F incorporation, and HA as the secondary phase. When F ions are incorporated at $y = 0.5$, HA becomes the main phase and only a little β -TCMP presents. When $y \geq 1.0$, single FHA phase forms.
2. GIXRD, XPS and FTIR results confirms the substitution of Mg and F ions in the HA crystal structure.
3. All the coatings exhibit comparable in vitro bioactivity regardless of F concentration.
4. Cells attach tightly and spread well on all the coating surfaces. F concentration studied does not affect cell numbers grown on the coatings. However, it maintains a positive stimulating on cell differentiation.
5. F incorporation decreases the dissolution rate of MgF_yHA coating in the TBS solution, especially at the measured fluoridation degree of 0.72 – 1.16.
6. Adhesion strength is enhanced by F incorporation at the measured F concentration around 1.0.
7. The optimal F concentration is at $y = 1.5$ (measured F concentration around 1.0) to achieve the lowest dissolution rate and the highest adhesion strength, i.e. the best long-term stability.

4.3 $\text{Mg}_{1.5}\text{FHA}/\text{MgF}_{1.5}\text{HA}$ bi-layer structured HA coating

As discussed in section 4.1 and 4.2, Mg_xFHA and MgF_yHA coatings exhibited distinct properties in the short-term osseointegration and the long-term stability. Mg incorporation in Mg_xFHA coatings took a great effect on the short-term osseointegration, but not significant on the long-term stability. $\text{Ca}_{8.5}\text{Mg}_{1.5}(\text{PO}_4)_6\text{F}_1(\text{OH})_1$ ($\text{Mg}_{1.5}\text{FHA}$) showed the best in vitro bioactivity and cellular response, i.e. the best short-term osseointegration. However, F incorporation in MgF_yHA coatings enhanced the long-term stability much, but the effects on the short-term osseointegration were comparable. $\text{Ca}_9\text{Mg}_1(\text{PO}_4)_6\text{F}_{1.5}(\text{OH})_{0.5}$ ($\text{MgF}_{1.5}\text{HA}$) presented the lowest dissolution rate and the highest adhesion strength, i.e. the long-term stability.

Therefore, a bi-layer structured HA coating was developed on the basis of systematical investigation of both Mg_xFHA and MgF_yHA coatings, i.e. $\text{Mg}_{1.5}\text{FHA}$ was selected as the upper layer, and $\text{MgF}_{1.5}\text{HA}$ as the bottom layer. This bi-layer structured HA coating is denoted by BHA coating. The physical and chemical properties as well as the short-term osseointegration and the long-term stability were evaluated compared with pure HA coating.

4.3.1 Physical and chemical properties

Although the physical and chemical properties of both $\text{Ca}_{8.5}\text{Mg}_{1.5}(\text{PO}_4)_6\text{F}_1(\text{OH})_1$

and $\text{Ca}_9\text{Mg}_1(\text{PO}_4)_6\text{F}_{1.5}(\text{OH})_{0.5}$ were studied in section 4.1 and 4.2, they may behave differently when deposited into a bi-layer structured coating. In this part, the phase formation, chemical states, chemical groups, surface and interface properties of BHA coating are investigated.

4.3.1.1 Phase formation

Phase comparison between pure HA and BHA are done by GIXRD and shown in Figure 4-41. Typical diffraction peaks of HA are detected on pure HA deposited sample: main peaks of (211), (112), (300) and (002) locate at 31.7° , 32.2° , 32.8° and 25.9° . While for BHA, only FHA phase is detected. Compared with HA, all the main diffraction peaks of BHA shift a little to higher degree: (211) at 32.0° , (300) at 33.2° , and (002) at 25.94° . The shifting of diffraction peaks to higher degree corresponds to the shrinkage of *a*- and *c*-axis as calculated in Table 4-4, indicating the incorporation of Mg and F ions in the HA lattice as discussed in section 4.1.1 and 4.1.2.

Figure 4-41 Phase comparison between HA and BHA by GIXRD

Table 4-4 Calculated a -axis and c -axis of HA and BHA coating

	a -axis (Å)	c -axis (Å)
HA	9.44	6.87
BHA	9.35	6.85

The phase formation of BHA coating is illustrated by comparing the phases of bottom layer with MFA3 ($\text{MgF}_{1.5}\text{HA}$) (Figure 4-42(a)), and BHA with FMA3 ($\text{Mg}_{1.5}\text{FHA}$) (Figure 4-42(b)). Since the designed composition of bottom layer is the same as that of MFA3 ($\text{MgF}_{1.5}\text{HA}$) and the only difference is the deposition times, the phases of the bottom layer and MFA3 are identical as shown in Figure 4-42(a). In other words, the phase formed on the substrate is independent of the thickness of the coating. When the upper layer is deposited on the bottom layer to

form BHA coating, a little difference in phases is observed in Figure 4-42(b): a weak peak of β -TCMP detected in FMA3 is not present in BHA. This is because the gradient of Mg concentration in the upper and bottom layer from Mg1.5 to Mg1.0. Mg diffusion during the firing process results in a lack of Mg to form β -TCMP, thus only FHA phase forms after the upper layer is deposited.

Figure 4-42 Illustration of phase formation of BHA coating ((a): Comparison of MFA3 and bottom layer of BHA; (b): Comparison of FMA3 and BHA)

4.3.1.2 Chemical states

The chemical states of the main elements in the coatings are measured by XPS, as shown in Figure 4-43. HA and BHA coatings have the same peaks of Ca 2p and P 2p respectively. The binding energy of Ca 2p_{1/2}, Ca 2p_{3/2} and P 2p locates at 350.7±0.1, 347.2±0.1 and 133.3 eV, which are the typical binding energy of Ca 2p and P 2p in HA and FHA [142, 144]. In BHA coating, Mg 2p and F 1s are detected at the binding energy of 50.6 and 684.3 eV, indicating the substitution of Mg and F ions in the HA lattice as discussed in Mg_xFHA and MgF_yHA coatings.

Figure 4-43 XPS narrow scan spectra of Ca 2p (a) and P 2p (b) in HA and BHA coating, Mg 2p (c) and F 1s (d) in BHA coating

4.3.1.3 Chemical groups

FTIR spectra identify the chemical groups in HA and BHA coatings, shown in Figure 4-44. In pure HA coating, there are three peaks belonging to phosphate group (PO_4^{3-}) at 959, 1005 and 1086 cm^{-1} . The peaks of PO_4^{3-} in BHA coating are very close to those of pure HA coating, locating at 966, 1012 and 1093 cm^{-1} , indicating the formation of apatite structure [140]. The weak peaks in the range of

1400 – 1500 cm^{-1} ascribe to carbonate (CO_3^{2-}) [141]. In pure HA coating, the stretching vibration of OH group is at 3570 cm^{-1} , however, this peak shifts to 3540 cm^{-1} in BHA coating due to the vibration of OH-F, suggesting the partial substitution of OH groups with F ions in the HA crystal structure [29, 97].

Figure 4-44 FTIR spectra of HA and BHA coatings

4.3.1.4 Surface and interface

Figure 4-45 shows surface morphology and cross-sectional view of BHA coating. The surface of the coating is very homogeneous without any cracks or pores (Figure 4-45(a)). The cross-sectional view (Figure 4-45(b)) exhibits a well bonding between the coating and the substrate without any delamination. Also, it is observed that the coating thickness is about 1.5 μm .

Figure 4-45 Surface (a) and cross-sectional (b) view of BHA coating on Ti6Al4V substrate

The depth profiling of BHA is carried out by GDOES, as shown in Figure 4-46. As analyzed in Mg_xFHA and MgF_yHA , the coating region (R_c), the transitional region (R_t) and the substrate region (R_s) are divided. In the coating region R_c , it is roughly set to the upper layer ($\text{Ca}_{8.5}\text{Mg}_{1.5}(\text{PO}_4)_6\text{F}_1(\text{OH})_1$) and the bottom layer ($\text{Ca}_9\text{Mg}_1(\text{PO}_4)_6\text{F}_{1.5}(\text{OH})_{0.5}$) according to the deposition times though there is no obvious boundary between them. Mg concentration slightly decreases along the depth direction, but F concentration exhibits the contrary trend, which are in accordance with the designed Mg and F concentration in the upper and bottom layer. In the region of R_t , all the elements of the coating as well as the substrate are present, demonstrating the mutual diffusion of the elements in the coating and the substrate. At the boundary between R_t and R_s , Ti and Al stabilize, and Ca, P, O, Mg and F disappear.

Figure 4-46 GDOES depth profile of BHA (Mg20: 20 times of Mg concentration;
F200: 200 times of F concentration)

4.3.2 Short-term osseointegration

The upper layer of BHA coating is designed with the best bioactivity and cellular response. However, whether the best bioactivity and cellular response can be maintained needs further verification, which is also done through the bioactivity and cell culture tests.

4.3.2.1 In vitro bioactivity

In vitro bioactivity of HA and BHA coating is evaluated by immersing the samples in the SBF solution for different days. After 7 days, the phase evolution is examined by GIXRD (Figure 4-47). A broad peak is observed around 32° on both HA and BHA due to the formation of poorly crystallized apatite. Moreover, the

absence of the diffraction peaks of Ti6Al4V substrate suggests that the total thickness of the newly formed layer and the primary coating is more than the detection depth of GIXRD (usually several micrometers). Since similar phase evolution happens on both HA and BHA, it suggests comparable *in vitro* bioactivity.

Figure 4-47 GIXRD profiles of HA and BHA coatings on Ti6Al4V substrates after soaking in the SBF for 7 days

Surface morphology observation is another proof to demonstrate the formation of new apatite layer. Figure 4-48 shows surface morphologies of BHA and HA coatings after soaking in the SBF solution for 7 days. Many cracks are seen in Figure 4-48(a) and (b), and some pieces of the layer are peeled off from the primary coating as shown in Figure 4-48(c) and (d), revealing that the thickness of the peeled layer is around 10 μm , much thicker than primary coating ($\sim 1.5 \mu\text{m}$).

Formation of a much thicker layer is an evidence of newly formed apatite layer as detected in Figure 4-47. The cracks and peeling off are due to the shrinkage upon drying as well as the stress relief during dehydration. They will not appear in the moist body environment [177]. Moreover, the coating is further dehydrated during pumping process of gold sputtering for SEM observation, and the stress released leads to further cracking of the coating.

Figure 4-48 SEM images of BHA (a) and HA (b) coatings on Ti6Al4V substrates after soaking in the SBF for 7 days ((c, d): the cross-sectional views of (a, b))

4.3.2.2 Cell response

Figure 4-49 shows SEM images of MG63 cells after cultured on BHA and HA coatings for 3 days. No significant difference is observed in cell morphology on different coatings (Figure 4-49). All cells spread well and grow favorably on the coating surface. Cells attach tightly on the coating surface with their filopodium and lamellipodium, suggesting good viability on both BHA and HA coatings.

Figure 4-49 Cell morphology after cultured on BHA (a, c) and HA (b, d) coatings for 3 days

Cell numbers on HA and BHA coatings at each cell culture period are compared in Figure 4-50. On the first and second day, there is no significant difference between the coatings. After incubation for 3 and 5 days, the cell numbers on BHA coating are significantly higher than those on HA coating. It suggests that BHA coating stimulates cell proliferation more.

Figure 4-50 Cell proliferation on HA and BHA coatings in terms of cell density:
significantly higher cell density on BHA coating at day 3 and 5 ($p < 0.05$)

4.3.3 Long-term stability

The bottom layer of BHA coating is designed with the lowest dissolution rate and the highest adhesion strength. However, it is worthy of examining whether the BHA coating exhibits the same properties. The long-term stability of BHA coating is measured through the dissolution and pull-off tests.

4.3.3.1 Dissolution rate

Dissolution behaviors of HA and BHA coatings are compared by the change of Ca^{2+} concentrations in the TBS solution, as shown in Figure 4-51. Ca^{2+} concentrations increase with the soaking time, but HA coating releases more Ca^{2+} ions during the whole period, indicating a higher dissolution rate. The average

reduction of the coating thickness after soaking in the TBS solution for 28 days is estimated to be about 0.5 and 0.4 μm in HA and BHA coatings, respectively, according to the dissolution amount, coating area and coating density (using density of pure HA, 3.16 g/cm^3 [13]). Thus the dissolution is limited in the upper layer of BHA (about 0.6 μm thick theoretically), and the comparison of dissolution rate between HA and BHA becomes that between HA and the upper layer. Although the designed Mg concentration in the upper layer is higher than that in the bottom layer, β -TCMP is not detected (Figure 4-41), and only FHA phase forms. Since FHA has lower dissolution rate than HA as discussed in section 4.1.3.1 and 4.2.3.1, it is reasonable that BHA coating releases less Ca^{2+} ions.

Figure 4-51 Comparison of dissolution behaviors of HA and BHA coatings in the

TBS solution: Ca^{2+} concentration as a function of soaking time

4.3.3.2 Adhesion strength

Figure 4-52 compares the pull-off adhesion strength between HA and BHA coating on Ti6Al4V substrates. Without distinguishing the adhesive and cohesive failure, the pull-off adhesion strength between HA coating and Ti6Al4V substrate is about 10 MPa, and about 20 MPa for BHA coating, nearly two times higher. Compared with MFA3 (the designed composition of the bottom layer, of which the pull-off adhesion strength is 29 MPa), the pull-off adhesion strength of BHA is much lower. It is caused by the elements interdiffusion during the drying and firing process, thus the elements concentrations in BHA coating are not identical to the designed ones. Although it is much lower than that of MFA3, it is still significantly higher than that of pure HA, a lot more than the minimum 15 MPa stipulated by ISO standards (ISO 13779) for biomedical applications [116].

Figure 4-52 Pull-off adhesion strength between HA and BHA coating on Ti6Al4V substrates: the pull-off adhesion strength of BHA is two times higher than that of HA.

4.3.4 Discussion

Functionally graded (gradient), bi-layered or multi-layered materials have been developed to optimize systems for required properties [6, 97, 100, 178, 179]. This idea originates from the biological systems. For example, in biological system, bone has a functionally graded structure from cortical compact on surface towards inner cancellous; or in teeth, there is a negative gradient of fluorine concentration from the surface of tooth enamel toward the dentine-enamel junction [100]. Through simulating their compositions or functions, the solubility, the ion-specific cellular responses, or other properties can be regulated.

In this project, $\text{Mg}_{1.5}\text{FHA}/\text{MgF}_{1.5}\text{HA}$ bi-layer structured HA coating is developed aiming at the combination of both the short-term osseointegration and the long-term stability in one coating/substrate system. However, the as-deposited BHA coating is not a real “bi-layer” structure due to the elements diffusion in the drying and firing process. After the final heat treatment, it becomes a whole coating without any distinct boundary. However, the distribution of Mg and F ions in the coating along the depth direction is still in agreement with the designed trend.

Compared with pure HA coating, BHA coating contains both Mg and F ions in the HA crystal structure: the shrinkage of both a - and c -axis, typical binding energy of Mg 2p and F 1s at 50.6 and 684.3 eV, a new band at 3540 cm^{-1} . The substitution of Mg and F ions tailors both biological and mechanical properties of BHA coating.

The upper layer ($\text{Mg}_{1.5}\text{FHA}$) with higher Mg concentration induces the formation of bone-like apatite in a short time and facilitates cell spreading and proliferation. The bottom layer ($\text{MgF}_{1.5}\text{HA}$) with higher F concentration bonds well with Ti6Al4V substrate and can remain longer in the physiological environment due to its lower dissolution rate. Although some individual properties of BHA coating are not the most superior compared with Mg_xFHA and MgF_yHA coatings, its integrated properties fulfill the requirements of the short-term osseointegration and the long-term stability. Therefore, $\text{Mg}_{1.5}\text{FHA}/\text{MgF}_{1.5}\text{HA}$ bi-layer structured HA coating has large potentials being employed as hard tissue implants in the orthopaedic fields.

4.3.5 Summary

In summary, $\text{Mg}_{1.5}\text{FHA}/\text{MgF}_{1.5}\text{HA}$ bi-layer structured HA coating was deposited on Ti6Al4V substrate by the sol-gel dip-coating method. The phase formation, chemical states, chemical groups and interfacial properties were studied. The in vitro bioactivity, cell response, dissolution behavior and adhesion strength of BHA coating were evaluated and compared with those of pure HA coating. The following conclusions are drawn.

1. Single FHA phase forms in $\text{Mg}_{1.5}\text{FHA}/\text{MgF}_{1.5}\text{HA}$ bi-layer structured HA coating with the substitution of Mg and F ions in the HA lattice.
2. $\text{Mg}_{1.5}\text{FHA}/\text{MgF}_{1.5}\text{HA}$ bi-layer structured HA coating shows comparable in

vitro bioactivity with pure HA coating.

3. Cells spread well on both HA and BHA coatings. However, $\text{Mg}_{1.5}\text{FHA}/\text{MgF}_{1.5}\text{HA}$ bi-layer structured HA coating stimulates cell proliferation more.
4. $\text{Mg}_{1.5}\text{FHA}/\text{MgF}_{1.5}\text{HA}$ bi-layer structured HA coating is more dissolution resistant than pure HA coating.
5. Adhesion strength between $\text{Mg}_{1.5}\text{FHA}/\text{MgF}_{1.5}\text{HA}$ bi-layer structured HA coating and the substrate is two times more than that of pure HA coating.

Chapter 5 Summary, Conclusions and Recommendations

5.1 Summary and Conclusions

In this project, magnesium (Mg) and fluorine (F) ions were incorporated into hydroxyapatite (HA) coatings simultaneously on Ti6Al4V substrates by the sol-gel dip-coating method, including $\text{Ca}_{10-x}\text{Mg}_x(\text{PO}_4)_6\text{F}_1(\text{OH})_1$ (Mg_xFHA) and $\text{Ca}_9\text{Mg}_1(\text{PO}_4)_6\text{F}_y(\text{OH})_{(2-y)}$ (MgF_yHA), $x, y = 0, 0.5, 1.0, 1.5$ and 2.0 . The substitution of Mg and F ions in the HA crystal structure was discussed. The dependence of Mg and F concentrations on in vitro bioactivity, cell response, dissolution behavior and adhesion strength of Mg_xFHA and MgF_yHA coatings were systematically studied. Finally, $\text{Mg}_{1.5}\text{FHA}/\text{MgF}_{1.5}\text{HA}$ bi-layer structured HA (BHA) coating was developed and evaluated by both short-term osseointegration and long-term stability compared with pure HA coating. Based on the results shown in Chapter 4, the following conclusions are drawn.

1. In the Mg_xFHA coatings, the resultant phase composition of the coating highly depends on Mg content. When $x \leq 1.0$, a single fluoridated hydroxyapatite (FHA) phase is formed; when $1.0 < x \leq 2.0$, a small amount of Mg-substituted β -tricalcium phosphate (β -TCMP) phase appears together with dominant FHA phase. In the MgF_yHA coatings, F concentration has significant effect on

resultant phases of the coating. β -TCMP is the main phase without F incorporation, and HA as the secondary phase. When F ions are incorporated at $y = 0.5$, HA becomes the main phase and only a little β -TCMP presents. When $y \geq 1.0$, single FHA phase forms.

2. The results of grazing incidence X-ray diffraction, X-ray photoelectron spectroscopy and fourier transform infra-red spectroscopy indicate that calcium ions and hydroxyl groups in the HA crystal structure are substituted with Mg and F ions in both Mg_xFHA and MgF_yHA coatings, respectively.
3. High Mg concentrations ($1.0 \leq x \leq 2.0$) in the Mg_xFHA coatings improve the in vitro bioactivity a lot. However, all the MgF_yHA coatings exhibit comparable bioactivity regardless of F concentration.
4. Cells spread well on all the Mg_xFHA and MgF_yHA coatings. In the Mg_xFHA coatings, when Mg substitution degree reaches 1.5, Mg ions show a significant stimulating effect on osteoblastic cell responses exhibiting the most cell proliferation and the fastest late cell differentiation. F concentration studied does not affect cell numbers grown on the MgF_yHA coatings. However, it maintains a positive stimulating on cell differentiation.
5. The incorporation of Mg increases the dissolution rate of Mg_xFHA coating in the tris-buffered saline (TBS) solution, and the maximum is achieved at $x = 1.5$.

F incorporation decreases the dissolution rate of MgF_yHA coating in the TBS solution, especially at the measured fluoridation degree of 0.72 – 1.16.

6. Mg substitution enhances the adhesion strength, but comparable among different Mg concentrations. Adhesion strength is enhanced by F incorporation at the measured F concentration around 1.0.
7. Single FHA phase forms in $\text{Mg}_{1.5}\text{FHA}/\text{MgF}_{1.5}\text{HA}$ bi-layer structured HA coating with the incorporation of Mg and F ions in the HA lattice. It shows comparable in vitro bioactivity with pure HA coating, but stimulates cell proliferation more. The long-term stability of $\text{Mg}_{1.5}\text{FHA}/\text{MgF}_{1.5}\text{HA}$ bi-layer structured HA coating is much better than pure HA coating, exhibiting lower dissolution rate and higher adhesion strength.

5.2 Recommendations

Although a great deal of work has been done in developing and investigating the Mg and F ions co-substituted HA coatings via the sol-gel dip-coating method, a number of other interesting researches are still worthy of exploring. Some of these are described below:

1. Molecular dynamic simulation of simultaneously incorporated Mg and F ions in HA crystal structure. Computer simulation is a powerful tool providing a deeper understanding of the physical and chemical origin of the ion substitution

in HA crystal structure, the stability and some surface properties. Comparing with the experimental data, it can further reveal the co-substitution mechanism as well as the dependence of Mg and F concentrations on biological and mechanical properties.

2. In vitro studies under loaded conditions. Cyclic loading of coated implants might cause fatigue failure or accelerated dissolution, especially at the coating/substrate interface. Thus it is another important method to evaluate the stability of the coating. Combining with the studies under static conditions, more information can be provided on the evaluation of the long-term stability of the coating.
3. In vivo animal test of Mg and F co-substituted HA coated implants. It provides the most direct and reliable interaction and performance of the implant with living tissues. Then the further improvement and/or application of Mg and F co-substituted HA coated implants can be greatly promoted.

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