Development of high performance Fluoridated Hydroxyapatite coatings for biological applications

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2007

Wang, Y. S. (2007). Development of high performance Fluoridated Hydroxyapatite coatings for biological applications. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/5281>

<https://doi.org/10.32657/10356/5281>

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**DEVELOPMENT OF HIGH PERFORMANCE
FLUORIDATED HYDROXYAPATITE COATINGS
FOR BIOLOGICAL APPLICATIONS**

**WANG YONGSHENG
SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING
2007**

Development of High Performance Fluoridated Hydroxyapatite Coatings for Biological Applications

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School of Mechanical and Aerospace Engineering

A thesis submitted to the Nanyang Technological University
in fulfillment of the requirement for the degree of
Doctor of Philosophy

2007

Abstract

Hydroxyapatite (HA) coated titanium alloy (generally, Ti6Al4V) implant is the most preferred bioimplant due to bioactivity of HA and the excellent mechanical properties of titanium alloy substrate. However, long-term stability and integrity are seriously hindered due to the rapid dissolution rate of HA coating. Recently, fluorine-containing hydroxyapatite has attracted a great deal of attention due to its lower bioresorption rate and comparable biocompatibility to that of pure HA. As such, long-term stability and integrity of the fluoridated hydroxyapatite (FHA) coated implant can be expected. This project aims at developing novel bioactive fluoridated hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_{2-x}\text{F}_x$ ($0 \leq x \leq 2$)) coating on titanium alloy (Ti6Al4V) substrate with high adhesion strength by sol-gel dip-coating method. Influences of incorporated fluorine ions on mechanical properties and biological properties are studied in detail.

With the selected $10\text{Ca}(\text{NO}_3)_2-(3-x/12)\text{P}_2\text{O}_5-(x/6\text{HPF}_6)$ ($0 \leq x \leq 2$) dip-coating system, dense and uniform FHA coatings ($\sim 1.5 \mu\text{m}$) have been successfully deposited on Ti6Al4V substrates by sol-gel dip-coating method. The X-ray photoelectron spectroscopy (XPS), X-ray diffraction (XRD) analysis and Fourier transform infra-red spectroscopy (FTIR) results confirm that fluorine ions have been successfully incorporated into the HA lattice structure, and the resulting FHA coating is homogeneous and of high purity (with Ca/P ratio approximately at the stoichiometric value of 1.67).

The incorporation of fluorine ion shows significant beneficial effect on the mechanical properties of coated samples. The pull-out tensile test reveals that with increase of the degree of fluoridation, the adhesion strength increases up to about 40%; and the dissolution behavior has no adverse influences on the adhesion properties. The scanning scratch test reveals that with the increase of fluorine concentration, the critical load increases, and the coating-substrate interfacial failure mode changes from brittle to ductile, indicating a much stronger adhesion for higher fluoridation degree. The interfacial shear strength measured with a shear strain lag method increases from $\sim 393 \text{ MPa}$ for pure HA coating to $\sim 572 \text{ MPa}$ for FHA coatings with the highest

fluoridation degree, indicating that more incorporated fluorine ions lead to the higher interfacial shear strength. In addition, with incorporation of fluorine ions, fracture toughness increases about 200 to 300%. With the increase of fluoridation degree, the decrease in residual stress and the formation of more complex chemical bonds in transitional region (at coating-substrate interface) are the main reasons for those enhancements in mechanical properties.

Incorporation of fluorine ions in HA lattice structure lowers the solubility of HA in Tris-buffered physiological saline (TPS) solution. The lowest solubility can be achieved at a fluoridation degree of 0.8-1.1. In comparison with the dissolution behavior in TPS, the presence of glucose (1g/L) in TPS (G-TPS) has negligible influence on the dissolution behavior of FHA coatings. As for the tests in bovine serum albumin (BSA, 40g/L) modified TPS (A-TPS), although an increase in the total amount of dissolved coating is observed, the order of dissolution rate of FHA coatings is not influenced by the addition of BSA.

In vitro bioactivity test in conventional simulated body fluid (c-SBF) indicates that the nucleation and growth rate of bone-like apatite deposited from the solution onto coating surface is significantly dependant on the degree of fluoridation: the bioactivity is increase firstly with the increasing fluoridation degree to 0.8-1.1, and then decreases remarkably. As for the test in glucose-modified SBF (1g/L, G-SBF), in comparison with the test in c-SBF, negligible influence is observed on the nucleation and growth of bone-like apatite on FHA coating surfaces. In contrast, the addition of BSA in SBF (40g/L, A-SBF) has significant retardation effect on the precipitation (nucleation and growth) of bone-like apatite from A-SBF onto FHA coatings.

In vitro cell responses are evaluated with osteoblastic cell (MG63) in terms of initial cell attachment and spreading, cell morphology, cell proliferation and differentiation. No significant difference is observed for the amount of initially attached cells on all FHA coating surface during the first 4 hours of culturing. Similar cell morphologies and good cell viability are observed. Coatings fluoridated to 0.8-1.1 have stronger stimulating effect on cell proliferation and differentiation activities.

Abstract

In summary, incorporation of fluorine ions into HA lattice structure can benefit both the mechanical properties and *in vitro* biological properties of the coatings: with the incorporation of fluorine ions, the residual stress is significant reduced, while the adhesion properties and toughness are remarkably enhanced; the lowest solubility and the most positive stimulating effect (testing in acellular solutions and osteoblastic cell responses) were observed at a fluoridation degree of ~ 0.8 - 1.1 , at which the coating takes the chemical formulation of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_{1.2-0.9}\text{F}_{0.8-1.1}$.

Acknowledgement

Firstly, I would like to express my sincere and deepest appreciation to my supervisor Professor Sam Zhang and co-supervisor Dr Xianting Zeng of Singapore Institute of Manufacturing Technology for their constant support, encouragement and invaluable guidance through this work. I am deeply impressed with their erudition and working spirit and benefit from them throughout the whole research progress. My appreciation also goes to Dr. Kui Cheng, for his help and suggestions in this study. My sincere thanks go to following students, Dr. Deen Sun, Dr. Bui Xuan Lam, Mr. Soon Eng Ong, Mr. Yibin Li, Mr. Xuejun Zhu, Mr. Yunxiang Tong, Mr. Dongjie Xu and Ms. Jinling Xu for their continuous warm help from the beginning of this work.

Deep gratitude goes to Mr. Thomas Lew, Ms. Yong Mei Yok, Mr. Koh Soon Hong, Ms. Chow Shiau Kee of Materials Lab A and B, Mr. Nelson Ng and Ms. Kok Su Ling in Biomaterials Lab (MSE, NTU), Ms. Phang-Tay Peng Choo in Environmental Lab (CEE, NTU), Ms. Jinye Shen and Ms Ruoshan Tan in BCPE lab (MAE, NTU) and Ms. Min Qian of Singapore Institute of Manufacturing Technology, for their experimental assistance, helpful discussion and their kind assistance in my research.

I take this opportunity to thank all the thin film group members who have been giving me so much help and encouragement in my study.

I would also like to express my gratitude to Singapore Institute of Manufacturing Technology (SIMTech-NTU collaboration project U03-S-389B) and the Agency for Science Technology and Research, Singapore (A*Star project 032101 0005) for providing me with facilities and materials to carry out the experiments.

Last but not least, I am sincerely indebted to my parents, my sister and my brother, for their encouragement and great love. Also my loving appreciation goes to my wife, Ms. Yan Xie, for her love and support. This work is dedicated to them.

List of Publications

1. Sam Zhang, Xianting Zeng, Yongsheng Wang, Kui Cheng, Wenjian Weng. Adhesion strength of sol-gel derived fluoridated hydroxyapatite coatings. *Surface and Coatings Technology*, 200, 2006:6350-6354
2. Yongsheng Wang, Sam Zhang, Xianting Zeng, Kui Cheng, Min Qian, Wenjian Weng. In vitro behavior of fluoridated hydroxyapatite coatings in organic-containing simulated body fluid. *Materials Science and Engineering C*, 27, 2007: 244-250.
3. Sam Zhang, Yongsheng Wang, Xianting Zeng, Kui Cheng, Min Qian, Deen Sun, Wenjian Weng, Weiyi Chia. Evaluation of interfacial shear strength and residual stress of sol-gel derived fluoridated hydroxyapatite coatings on Ti6Al4V substrates. *Engineering Fracture Mechanics*, 74, 2007:1884-1893.
4. Yongsheng Wang, Sam Zhang, Xianting Zeng, Lwin Lwin Ma et al. Osteoblastic cell response on fluoridated hydroxyapatite coatings. *Acta Biomaterialia*, 3, 2007:191-197.
5. Yongsheng Wang, Sam Zhang, Xianting Zeng, Lwin Lwin Ma, Khiam Aik Khor, Min Qian. Initial attachment of osteoblastic cells onto sol-gel derived fluoridated hydroxyapatite coatings. *Journal of Biomedical Materials Research A* (accepted).
6. Sam Zhang, Yongsheng Wang, Xianting Zeng, Khiam Aik Khor, Wenjian Weng, Deen Sun. Evaluation of adhesion strength and toughness of fluoridated hydroxyapatite coatings. *Thin Solid Films* (accepted).
7. Soon-Eng Ong, Sam Zhang, Hejun Du, Yongsheng Wang, Lwin-Lwin Ma, In-vitro cellular behavior on amorphous carbon containing silicon, *Thin Solid Films* (accepted).
8. Kui Cheng, Sam Zhang, Wenjian Weng, Khor Khiam Aik, Shundong Miao, Yongsheng Wang, The adhesion strength and residual stress of colloidal-Sol-gel prepared biphasic beta-TCP/FHA coatings, *Thin Solid Films* (accepted).

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List of Symbols and Abbreviations**Symbols**

γ_{NL}	Interfacial energy between nucleus-liquid
γ_{SL}	Interfacial energy between substrate-liquid
γ_{SN}	Interfacial energy between substrate-nucleus
ε_f	Maximum elastic strain of coating
θ	Contact angle
λ	Average steady-state crack spacing
ν_f	Poisson's ration of coating
ν_s	Poisson's ratio of substrate
σ_e	Experimentally determined tensile stress of coating
σ_f	Tensile strength of the coating
σ_R	Residual stress
$\tau_{\max}$	Interfacial shear strength
$\phi(\theta)$	Function of contact angle
Ω	Unit volume of the species under consideration
Δk	Curvature difference before and after removal of coating
ΔG	Unit free energy change
ΔG^0	Unit free energy change of an equilibrium status
ΔU	Strain energy difference before and after cracking
f	shape factor
h_T	Total thickness of the substrate plus coating
k	Boltzmann constant
n	The number of ion unit
t	Coating thickness
x	Designed fluoridation degree in the dipping sol
E_f	Elastic modulus (Young's modulus) of coating

List of Symbols and Abbreviations

E_s	Elastic modulus (Young's modulus) of substrate
J	Nucleation rate
K_{IC}	Fracture toughness
K_f	Kinetic factor
K_{sp}	Solubility product of stress free coating
K_{sp}^σ	Solubility product under stress of σ
R	Gas constant
R_a	Average roughness
R_c	Coating region
R_q	Root mean square roughness
R_s	Substrate region
R_t	Transitional region
S	Supersaturation
T	Temperature
X	Measured fluoridation degree of FHA coating

Abbreviations

α -TCP	α -Tricalcium phosphate
β -TCP	β -Tricalcium phosphate
c-SBF	Simulated body fluid
ACP	Amorphous calcium phosphate
ALP	Alkaline phosphatase
A-SBF	Bovine serum albumin modified SBF
ATCC	American Type Culture Collection
A-TPS	Bovine serum albumin modified TPS
BE	Binding energy
BSA	Bovine serum albumin
CSM	Continuous stiffness mode
CTE	Coefficient of thermal expansion
DCPD	Dicalcium phosphate dihydrate

List of Symbols and Abbreviations

EDX/EDS	Energy Dispersive X-ray/Spectroscopy
EMEM	Eagle's Minimum Essential Medium
FA	Fluorapatite
FCS	Fetal calf serum
FHA	Fluoridated hydroxyapatite
FTIR	Fourier Transform Infra-Red Spectroscopy
G-SBF	Glucose-modified simulated body fluid
G-TPS	Glucose-modified TPS
HA	Hydroxyapatite
IAP	Ionic activity product
OC	Osteocalcin
OCP	Octacalcium phosphate
OP	Optical profiler
PBS	Phosphate buffered saline solution
SEM	Scanning Electron Microscopy
SST	Scanning scratch test
TEM	Transmission electron microscopy
ToF-SIMS	Time-of-flight secondary ion mass spectrometry
TPS	Tris-buffered physiological saline solution
TTCP	Tetracalcium phosphate
XPS	X-ray Photoelectron Spectroscopy
XRD	X-ray Diffraction Analysis

Chapter 1 Introduction

1.1 Background

The basic purpose of a biomaterial is to replace a part or a function of the body in a safe, reliable, economic, and physiological acceptable manner. During the last 90 years, man-made biomaterials, including metals, polymers, ceramics and composites, were widely used in clinical applications [1]. Of the biomaterials, bioceramics are extensively investigated because of their lack of toxicity on human body. Among these bioceramics, hydroxyapatite has been long recognized as substitute materials for bones and teeth in orthopedics and dentistry due to their chemical and biological similarity to human hard tissues and also the direct bonding capability to the surrounding tissues. It has been the most preferred materials for the application in orthopaedics and has received intense attentions over the past 25 years [2, 3]. Hydroxyapatite not only improves the rate of osseointegration, but can also establish a high bone-implant interfacial strength by forming a strong chemical bonding with natural bone. However, its intrinsic poor mechanical properties, such as brittleness, low fracture toughness, low impact strength etc, have restricted further applications as load-bearing implants for hard tissue repairing/replacement in spite of its excellent biocompatibility and bioactivity [4].

On the other hand, titanium and its alloy show good biocompatibility, whereas in the case of other metals used as biomaterials, the accumulation of toxic metal ions due to dissolution and corrosion in physiological environment in the body often lead to severe problems [5]. Furthermore, the mechanical properties of titanium and its alloys

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are superior to those bioceramics such as hydroxyapatite and bioglass. However, the ability of inducing bone in-growth and implant fixation need to be improved in order to shorten the time of implant-bone osteointegration [6]. A great deal of approaches has been proposed and among the different solutions, hydroxyapatite coatings on their surface attracted the highest interest in the last few years. The hydroxyapatite coated titanium alloy implants can integrate the advantages of both materials, the bioactivity of hydroxyapatite and the excellent mechanical properties of titanium alloy substrates. Furthermore, hydroxyapatite coating is believed to protect the titanium alloy substrates from corrosion and dissolution, and can also serve as a barrier for the release of toxic metal ions from the metallic substrates into the living body [7].

Various fabrication methods have been developed and frequently employed for the preparation of hydroxyapatite coatings on metallic substrates, which include pulsed laser deposition (PLD), thermal spraying, electrophoretic deposition, biomimetic processes and sputtering etc [8]. All of these methods have achieved certain degree of success in depositing hydroxyapatite coatings on metallic substrates, but one or more of the following disadvantages were observed: complicated preparation process, inhomogeneous hydroxyapatite coating and formation of phases other than hydroxyapatite in the coating, high processing temperature, utilization of sophisticated equipment etc. In order to avoid these shortcomings, an alternative coating method, namely, sol-gel technique has been developed. Compared with other fabrication methods, the sol-gel technique demonstrates more advantages, such as the ability for tailoring chemical compositions, improved homogeneity at molecular level, ability to produce uniform fine grained structure, and low cost and easy operation [9].

Chapter 1 Introduction

Therefore, sol-gel technique can be one of the most promising methods for the fabrication of metallic implants coated with hydroxyapatite thin film.

Previous studies on hydroxyapatite-coated implants have shown good fixation to the host bones and increased bone ingrowth into the implants [7, 10]. However, there are still many concerns about the application of hydroxyapatite coatings, particularly with regard to the long-term stability. One of the most serious concerns raised is the resorption or degradation of hydroxyapatite coatings in a biological environment. Faster dissolution or resorption is likely to result in faster and stronger fixation in the initial period of implantation, but could also lead to disintegration of the coating, with rapid loss of the bonding strength and mechanical fixation, delamination, and the production of particles, which has been cited as a potential complication [11, 12]. By contrast, slow controlled resorption may allow the surrounding bone the opportunity to replace resorbed coating and maintain long-term stability [13]. Hence, the stability and integrity of the coating layer should be carefully considered prior to actual clinical applications.

It is widely known that fluorine is an essential trace element in human hard tissue and the presence of fluorine ions not only improves the quantity and quality of bone formation but can also effectively protect the dental enamel from caries by forming less soluble fluoridated hydroxyapatite [14]. Thereby, it has been suggested to incorporate fluorine ion into HA structure by replacing OH group to control the degradation rate. As reported, fluorapatite showed markedly less degradation in comparison with HA while maintaining the comparable bioactivity [15]. Recently, investigation on fluorapatite and fluoridated hydroxyapatite coatings as the substitutes

for pure HA coating has attracted a great deal of attention in areas requiring long-term chemical and mechanical stability, and is becoming the most promising approach to fulfill the requirements of hard tissue replacement and/or repairing [16, 17]. However, because the development of FHA coatings is still in the initial stage, the understanding about their properties is far from adequate, especially the relationship between the coating properties, e.g. the mechanical properties and biological performances etc, and the incorporated fluorine ions (with different concentration) is still not well understood and/or even not established. Considering the great potential applications of FHA coatings, further studies are absolutely required to achieve comprehensive understanding upon the performances of FHA coatings.

1.2 Objectives

The objective of this project is to systematically investigate the influence of incorporated fluorine ions on the mechanical and biological properties of FHA coatings. The novel bioactive fluoridated hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_{2-x}\text{F}_x$, (x is the degree of fluoridation: $x=0$, pure HA; $x=2$, pure FA; $0 < x < 2$), coatings on titanium alloy (Ti6Al4V) substrate will be deposited by using the sol-gel dip-coating method. The coating properties will be optimized with respect to the degree of fluoridation to achieve high bioactivity, reduced solubility, and excellent adhesion strength. Consequently, a long-term stability and integrity of the fluoridated hydroxyapatite coated implants can be expected.

1.3 Scopes

(1) Sol-gel preparation of FHA coatings

With the selected precursors, fluoridated hydroxyapatite coatings with different degrees of fluoridation ($0 \leq x \leq 2$) will be prepared by using dip-coating method on Ti6Al4V substrates. Phase composition, surface morphology, chemical groups and chemical compositions of the coatings will be investigated with X-ray diffraction analysis (XRD), scanning electron microscopy (SEM), Fourier transform Infra-red spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS) respectively.

(2) Mechanical properties of FHA coatings

The tensile adhesion strength will be investigated by using pull-out based tensile testing methods. In addition, the coating-substrate interfacial shear strength will be evaluated with shear strain lag method. Furthermore, a scanning scratch testing method and secondary ion mass spectrometry analysis will also be conducted to get a clear understanding regarding the properties of coating-substrate interface. Other mechanical properties including coating toughness, elastic modulus etc, will be estimated with nanoindentation. As one of the main influencing factors on coating performance, residual stress will also be evaluated using “wafer” curvature method. Based on the above evaluations, the influences as well as mechanisms of incorporated fluorine ions on those mechanical properties will be thoroughly considered and discussed.

On the other hand, in order to study the influence of dissolution behavior on coating adhesion strength, pull-out based tensile testing method is employed to evaluate the

adhesion properties after the dissolution test in Tris-buffered physiological saline solution (TPS) and modified TPS solution.

(3) *In vitro* behavior of FHA coatings

Firstly, the dissolution behavior of FHA coatings will be studied in three kinds of solutions, i.e. Tris-buffered physiological saline solution (TPS), glucose-containing Tris-buffered physiological saline solution (G-TPS), and bovine serum albumin-containing Tris-buffered physiological saline solution (A-TPS). The coatings are soaked in those solutions separately at a stable temperature of 37°C for up to 3 weeks. Calcium ion concentration in the solutions at different time slots will be analyzed with inductively coupled plasma (ICP) atomic emission spectrometer to monitor the dissolution behavior.

Secondly, the bioactivity of FHA coatings will be investigated by immersing the coatings in acellular conventional simulated body fluid (C-SBF) and organic-modified (glucose and albumin) SBF at 37°C. Influences of incorporated fluorine ions as well as the presence of organic components on coatings' bioactivity will be discussed.

Thirdly, osteoblastic cell (MG63) will be employed to investigate the cell responses to FHA coatings. Protein adsorption, initial cell attachment and spreading, cell proliferation, and cell differentiation (alkaline phosphatase and osteocalcin activities) will be well investigated to understand the influences incorporated fluorine ions as well as different fluoridation degree on cell responses.

1.4 Organization

This report is organized as follows: a literature review on HA/FA and their coatings is presented in Chapter 2; Chapter 3 describes the experimental setup and methodology in details used in this project; Chapter 4 presents the experiment results followed by detailed discussion; and the last part, Chapter 5 gives the conclusions and future work.

Chapter 2 Literature Review

Throughout the history of humankind, attempts have always been made for the development of biomaterials to repair or replace any deficient tissue caused by illness or injury to relieve pain, improve function and thus benefit the welfare of human beings. For example, the Romans, Chinese and Aztec used gold in dentistry more than 2000 years ago [1]. Till now, a huge amount of materials has been developed to be used as biomaterials and all of them could be classified into the following four categories as indicated in Table 2-1: metals, ceramics, polymers, and composites [4]. Each biomaterial has its own advantages and disadvantages. For instance, advantages of polymeric materials over the others are their excellent flexibility in physical properties varying from viscous fluid to tough solid; but a serious drawback of polymers is their insufficient mechanical strength when used as medical devices in orthopaedic and oral surgeries. That is to say, the selection of a biomaterial for a specific application will depend on both the particular repairing or replacement situation and the properties of biomaterials. Therefore, this project is focused on the investigation of new implants for load bearing applications in hard tissue replacement like hip joint, teeth roots and other artificial bones etc. The demands for those load bearing implants have increased enormously in current industrial era due to the increasing injuries caused by accidents and the improvement of social awareness about the right to existence. According to the American Academy of Orthopaedic Surgeons, for example, over 120,000 total hip replacement operations are performed each year [18]. As such, it is meaningful and valuable for the development of high performance and reliable implants.

Table 2-1 Classification of biomaterials and their use in the body [4, 19]

Biomaterials	Common examples	Advantages	Disadvantages	Clinical Applications
Metals	Stainless steels, Ti and its alloys, Co-Cr alloys,	Strong, tough, ductile	Difficult to make, may corrode, bioinert	Joint replacements, bone plates and screws, dental root, implants, pacer and suture wires
Ceramics	Aluminum oxide, calcium phosphates including hydroxyapatite, carbon	Very biocompatible, inert, strong in compression	Brittle, not resilient, and difficult to make	Dental, femoral head of hip replacement, coating of dental and orthopedic implants
Polymers	Nylon, silicone rubber, polyester, polytetrafluoroethylene, etc.	Resilient, easy to fabricate	Not strong, deforms with time, may degrade	Sutures, blood vessels, hip socket, ear, nose, other soft tissues, sutures
Composites	Carbon-carbon, wire or fiber reinforced bone cement	Strong, tailor-made	Difficult to make	Joint implants, heart valves

2.1 Hard tissue replacement

2.1.1 Basic requirements

Many kinds of materials currently have been developed, including metals and alloys, polymers, ceramics and composites etc, and widely used in biomedical fields (as shown in Table 2-1). However, no matter what the source, biomaterials must meet several criteria to perform successfully as load bearing implants in orthopedic and dental applications [20, 21]. First of all, they must be biocompatible, or able to function *in vivo* without eliciting any intolerable response in the body, either locally or systemically. On the other hand, those appropriate biomaterials must be able to withstand the often hostile environment of the body, and show better properties such as resistance to corrosion and degradation, such that the body environment does not adversely affect material performance over the intended performance lifetime of the implant. Furthermore, adequate mechanical properties, e.g. tensile/compressive

strength, elastic modulus, fatigue endurance etc, are also the critical criteria for the selection of biomaterials to be used as devices intended to replace or reinforce load-bearing skeletal structures. In addition, they must be capable of reproducible fabrication to the highest standards of quality control and, of course, at a reasonable cost. Biomaterials that meet these criteria are fundamental to the practice of orthopedic surgery and to ensure the success of implantation.

2.1.2 Selections of materials for hard tissue replacement

From the viewpoint of biocompatibility and bioactivity, hydroxyapatite (HA), as one of the bioactive ceramics, is the most suitable candidate due to its chemical and biological similarity to human hard tissue [22], and the direct bonding capability to surrounding tissues [23, 24]. However, the lack of reliability caused by its intrinsic brittleness (Table 2-1 and Table 2-2) restrict its successful application as load bearing implants. On the other hand, metallic materials, e.g. Co-Cr alloys, stainless steel, titanium and titanium alloys, are the most preferred selection by considering their excellent mechanical properties (Table 2-2). However, all of these metallic biomaterials are bioinert, and would release some metal ions caused by corrosion in such biological environment into human body.

In view of the advantages and disadvantages of metallic biomaterials and bioactive ceramics, an advisable and practicable solution is to develop HA coatings on the surface of metallic implants. It is believed that such kind implants can well combine the expected biological properties of HA and the excellent mechanical properties of metallic substrates. On the other hand, the HA coating also protects the metallic

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substrates from corrosion and serves as barrier layer for the release of toxic metal ions into human body.

Among all metallic materials used as biomaterials, titanium and titanium alloys are the most preferred choice due to their good biocompatibility, superior corrosion resistance in comparison with others. Moreover, the relatively lower modulus of titanium and titanium alloys (Table 2-2) is another strongpoint over other metallic biomaterials, because it could alleviate the stress shielding effect resulted from the modulus mismatch between the bone and the metallic implants. Therefore, more and more attentions have been focused on the development of HA coated titanium or titanium alloys implants to fulfill the requirements of load bearing implants.

Table 2-2 Mechanical properties of bone and some biomaterials [5, 19]

Types of materials	Young's modulus (GPa)	Ultimate tensile strength (MPa)	Tensile elongation rate (%)	Fracture toughness (Mpa ^{1/2})
Alumina	350	\	<1	\
Hydroxyapatite	85	40-100	\	~1
Stainless steel	200	1000	10	100
Co-Cr alloys	230	450-1300	10-30	100
Ti-Al-V and c.p. Ti	100-140	500-1150	15-25	80
PMMA	2.8	55	8	\
Bone	17	130	2	2-12

2.2 Hydroxyapatite and hydroxyapatite coatings

2.2.1 Calcium phosphates and hydroxyapatite

Calcium phosphate (CaP) compounds, as one kind of the most important bioceramics, are increasingly important in the field of biomaterials and, in particular, as bone

substitutes. The advantages of calcium phosphate biomaterials for hard tissue repair and augmentation are well known in orthopedic and dental surgery because of their biocompatibility and osteoconductivity [25]. Many kinds of calcium orthophosphate salts are listed in Table 2-3. The most important property of CaPs is probably the solubility in water because the *in vivo* behavior of CaPs can be predicted to a large extent by their solubility. If the solubility of a CaP, e.g., HA, is less than the mineral part of bone, it degrades extremely slowly if at all. If the solubility of a CaP is greater than that of the mineral part of bone, it degrades. Therefore, using different solubilities of CaPs, the *in vivo* degradation rates of CaPs can be predicated and follow the order of [26-28]:

(ACP—Amorphous calcium phosphate; TTCP—Tetracalcium phosphate; α -TCP— α -Tricalcium phosphate; DCPD—Dicalcium phosphate dehydrate; DCP—Dicalcium phosphate; OCP—Octocalcium phosphate; β -TCP— β -Tricalcium phosphate)

It can be said that MCPM and MCP are most soluble and HA is the most insoluble in neutral solution. However, even though all parameters except for the composition were kept constant, this above order was not always observed experimentally; the surface of a highly soluble CaP is reactive and may become covered with a poorly soluble CaP, hence reducing its degradation rate. Therefore, all of the calcium orthophosphate compounds in Table 2-3 can be converted into Hydroxyapatite at a pH higher than 5 [29].

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Table 2-3 calcium phosphate compounds with their respective Ca/P molar ratio [22, 26]

Name	Formula	Ca/P molar ratio	Mineral	Abbreviation
Monocalcium phosphate monohydrate	$\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$	0.50	\	MCPM
Dicalcium phosphate	CaHPO_4	1.00	Monetite	DCP
Dicalcium phosphate dihydrate	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	1.00	Brushite	DCPD
Octocalcium phosphate	$\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$	1.33	\	OCP
Precipitated hydroxyapatite	$\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x}$	1.50-1.67	\	PHA
Amorphous calcium phosphate	$\text{Ca}_{10-x}\text{H}_{2x}(\text{PO}_4)_6(\text{OH})_2$	\	\	ACP
Monocalcium phosphate	$\text{Ca}(\text{H}_2\text{PO}_4)_2$	0.50	\	MCP
α -Tricalcium phosphate	$\alpha\text{-Ca}_3(\text{PO}_4)_2$	1.50	\	α -TCP
β -Tricalcium phosphate	$\beta\text{-Ca}_3(\text{PO}_4)_2$	1.50	\	β -TCP
Sintered hydroxyapatite	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	1.67	Hydroxyapatite	HA
Oxyapatite	$\text{Ca}_{10}(\text{PO}_4)_6\text{O}$	1.67	\	OXA
Tetracalcium phosphate	$\text{Ca}_4(\text{PO}_4)_2\text{O}$	2.00	Hilgenstockite	TeTCP/TTCP

HA (hydroxyapatite), $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, a basic calcium phosphate (CaP), is one of the main components that exists approximately 5% in human body weight as bone mineral that provides storage and control of calcium uptake and release. The word hydroxyapatite consists of “hydroxy” as hydroxyl ion and apatite which is the mineral name. The ideal Ca/P molar ratio is 1.67 and the calculated density is about 3.219 g/cm^3 . The apatite structure is shared by a large group of relatively common minerals, which may be generally defined by the formula:

where A may be Ca, Sr, Ba, Cd, Pb, Mg, Zn etc., B may be P, V, As, B, CO_3 etc., and Z may be OH, OD, CO_3 , F, Cl, O, vacancy etc. The commonest varieties are fluorapatite ($\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$, FA), chlorapatite ($\text{Ca}_{10}(\text{PO}_4)_6\text{Cl}_2$, ClA), hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, HA), francolite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{CO}_3)(\text{F},\text{OH})_2$) etc [30]. The apatite family of minerals belongs to the hexagonal system, with a space group, $P6_3/m$. This space group is characterized by a six-fold c-axis perpendicular to three equivalent a-axes (a_1 , a_2 , a_3) at angles 120° to each other. The smallest building unit, known as the

unit cell, contains a complete representation of the hydroxyapatite crystal, consisting of Ca, PO₄, and OH groups closely packed together in an arrangement shown in Figure 2-1 [31, 32]. This crystal structure of hydroxyapatite is projected along the c-axis onto the basal plane. The unit cell dimensions of HA are: $a = 0.942$ nm and $c = 0.688$ nm.

Figure 2-1 Crystal structure of hydroxyapatite, viewed along the c-axis [31, 32]

The ten calcium atoms belong to either Ca (I) or Ca (II) subsets depending on their environment. Four calcium atoms occupy the Ca (I) positions: two at level $Z = 0$ and the other two at $Z = 0.5$. Six calcium atoms occupy the Ca (II) positions: one group of three calcium atoms describing a triangle located at $Z = 0.25$, the other group of three at $Z = 0.75$, surrounding the OH groups located at the corner of the unit cell at $Z = 0.25$ and $Z = 0.75$, respectively Figure 2-1. The six phosphate (PO₄) tetrahedral are in a helical arrangement from levels $Z = 0.25$ to $Z = 0.75$. The network of PO₄ groups

provides the skeletal framework that gives the apatite structure its stability. The oxygen atoms of the phosphate groups are described as one O_I, one O_{II} and two O_{III}. The OH groups lie on the corners of the projected basal plane, meaning that the hydroxyl ions order along c-axis (Z orientation) [31, 32].

2.2.2 Hydroxyapatite coatings

2.2.2.1 Preparation methods of hydroxyapatite coatings

Since a strong metallic implant coated with a bioactive layer (HA coating) has proved to be promising and practicable, many techniques have been developed for the fabrication of HA coatings onto metallic implant surface. The methods that are commonly used include pulsed laser deposition (PLD) or laser ablation [33-35], hot isostatic pressing method (HIP) [36, 37], thermal spraying technique including plasma spraying [38-40], flame spraying [41, 42], and high velocity oxy-fuel combustion spraying (HVOF) [43-45], electrophoretic deposition method [46-48], biomimetic coating method [49-51], sputtering coating technique [52-54], and sol-gel method [55-59]. Each method has its own advantages and disadvantages upon the coating properties, such as coating chemistry, phase composition, crystallinity, mechanical properties as well as biological properties etc. A comparison of these techniques is summarized in Table 2-4.

Hydroxyapatite coatings deposited with these methods possess different properties, which can affect the biological responses as well as the ultimate coating performances. Generally, phase composition, mechanical properties and biological properties are the critical aspects to evaluate the performances of HA coated metallic implants.

Chapter 2 Literature Review

Table 2-4 Comparison of different methods for HA coating preparation [8, 60]

	Method	Coating thickness	Advantages	Disadvantages	References
High temperature deposition techniques	Pulsed laser deposition	< 5 μm	Coating crystalline and amorphous; dense or porous; active atmosphere conditions	Expensive, time consuming, multiphase coating including α -TCP, β -TCP, TTCP besides HA and amorphous HA	[33-35]
	Hot isostatic pressing	0.2-2.0 mm	Produces dense coatings	Can not coat complex substrates; high temperature required; thermal expansion mismatch; elastic property differences; expensive	[36, 37]
	Thermal spraying	30-200 μm	High deposition rates; can obtain various coating thickness and can be used for complex substrate shapes; low cost	High temperature induces decomposition; rapid cooling produces amorphous coatings;	[38-45, 61, 62]
	Electrophoretic deposition	0.1-2.0 mm	Rapid deposition rates; can coat complex substrates	Non-uniform thickness; impurity; poor biological fixation to the metal substrates	[46-48, 63]
Low temperature deposition techniques	Biomimetic coating	< 30 μm	Low processing temperatures; can form bonelike apatite; can coat complex shapes; can incorporate bone growth stimulating factors	Time consuming; requires replenishment and a constant pH of simulated body fluid	[49-51]
	Sputter coating	< 3 μm	Uniform coating thickness on flat substrates; dense coating	Ca/P ratio of the coating is higher than that of synthetic HA if RF magnetron sputtering is used; expensive; time consuming; producing amorphous coatings	[52-54, 64]
	Sol-gel method	< 1 μm	Low processing temperature; can coat complex shapes; can obtain very thin coatings; higher purity and homogeneity; low cost	Some processes require controlled atmosphere and high sintering temperature	[55-59, 65]

2.2.2.2 Phase composition

According to the processing temperature, all coating methods can be classified into two groups, low temperature and high temperature deposition techniques [66, 67] as shown in Table 2-4. Deposition of HA coatings with high temperature techniques have to suffer a high temperature process. Generally, the deposition temperature is much higher than the melting point of HA (about 1450°C [68]) and the transformation temperature of HA to other phases (around 1000°C). Therefore, at elevated temperature during the deposition process, phase decomposition of HA to other phases such as TCP, TTCP, CaO etc. is inevitable (c.f. Table 2-3). The following decomposition reactions commonly represent the decompositions during deposition of HA coatings by using such high temperature deposition techniques [44, 69, 70]:

Besides the decomposition, another critical issue related to high temperature deposition of HA coating is the formation of amorphous phase due to the rapid solidification occurred on the metallic substrate surface [71-73]. Consequently, the final coatings prepared with those high temperature deposition methods are multiphase coatings containing HA and a certain amount of other impure phases, such as amorphous hydroxyapatite (ACP), oxyapatite, Tricalcium phosphate (TCP), tetracalcium phosphate (TTCP), calcium oxide (CaO) etc.

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As for the HA coatings prepared with those low temperature deposition techniques (except biomimetic method), although an annealing process (generally, the temperature is lower than 1000°C) is usually required to remove the unwanted chemical components (to obtain the final HA coating) or to increase the coating crystallinity, the phase composition is rather simple than those coatings obtained by using high temperature deposition methods. Generally, the final coating is a pure HA coating or with a small amount of CaO, and/or TCP [52, 74], OCP [75], carbonated hydroxyapatite [58, 76, 77], CaCO₃ [78] etc. For the biomimetic method, by considering that the process is usually carried out in SBF or concentrated/modified SBF solutions, the main coating phase composition is carbonated hydroxyapatite and/or OCP [51, 79] rather than a single phase coating of HAs [80].

No matter what kind of impure phases, all of them have crucial effects on the performance of coated implants, especially on the dissolution behavior of the coatings [8, 28, 81]. As mentioned in §2.2.1, all of the others phases in the coating have larger solubility than that of HA. Although the faster dissolution produces a supersaturated environment, which allows physiologically produced HA to precipitate on the coating and enhance the bone ingrowth, it also leads to the serious resorption or degradation of the coatings, and even the failure of the implants [28, 82]. On the other hand, the impure phase, CaO, has no biocompatibility and dissolves significantly faster than TCP, thus it is a detrimental phase that should be avoided [8]. As such, both the purity and the crystallinity of the coating should be strictly controlled in order to obtain the expected effective HA coating layers. Thereby, based on the above discussion, the phase composition of HA coatings depend highly on the selection of deposition

method. From this viewpoint, the lower temperature deposition methods are much preferred to deposit the expected HA coatings on metallic substrates.

2.2.2.3 Mechanical properties

In view of the successful implantation as well as long-term stable performance, mechanical properties are important for those HA coated metallic implants. Among all the mechanical properties required, adhesion strength (or bonding strength) between the coating and metallic substrate is the most concerned property for those load-bearing implants. Two kinds of pull-out based methods are widely used to evaluate the adhesion strength: uniaxial pull-out tensile test method (as shown in Figure 2-2a) for the determination of tensile adhesion strength and shear pull-out test (Figure 2-2b) for the measurement of shear adhesion strength [80, 83, 84]. Both of these two methods are tested using epoxy or super glues to fix the coating onto the counterpart, accordingly, the bonding strength will be highly influenced by many factors, e.g. uniformity of the epoxy layer, penetration depth of the glues, porosity and thickness of the coating etc. Therefore, it could be imagined that a wide range of adhesion strength would be obtained, even though for the coatings deposited with the same deposition method. According to the documented results, the adhesion strengths of HA coatings on metallic substrates prepared with different deposition techniques were summarized in Table 2-5. On the other hand, cohesion failure (failure within the coating layer) was always observed in those pull-out based tests, indicating that the obtained results are not the actual adhesion strengths between the coating and substrate. In other words, those pull-out based testing methods are highly influenced by the coating characteristics and can only provide limited information about the adhesion properties at the coating-substrate interface. Moreover, as reported, in *in*

vivo studies [8, 85] suggested that the failure of HA coated implants mainly occurs at the coating-substrate interface, and the failure probability at this interface increased with the period of implantation due to the strength of the coating-bone interface tends to increase with healing time. Therefore, more other evaluation techniques/methods, e.g. scratch test etc, are indeed important and necessary to get a sound evaluation of adhesion properties [86].

Figure 2-2 Schematic diagrams of pull-out based testing methods for assessing of adhesion strength [80, 83, 84]: a) tensile adhesion strength test, b) shear adhesion strength test

Along with the adhesion strength, other properties including toughness and residual stress are becoming more and more important in order to get a rounded understanding on the performance of HA coated implants. Here, it must be pointed out that those related investigations are just focused on thermal sprayed HA coatings, hence, the following cited results totally come from this field.

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Table 2-5 Adhesion strength of HA coatings deposited with different deposition methods

	<i>Pulsed laser deposition</i>	<i>Hot isostatic pressing</i>	<i>Thermal spraying</i>	<i>Electrophoretic deposition</i>	<i>Biomimetic coating</i>	<i>Sputter coating</i>	<i>Sol-gel method</i>
Tensile adhesion strength (MPa)	30-58	/	10-60	<14	<15	>60	15-60
Shear adhesion strength (MPa)	/	4.0-5.5	11-26.8	8-23	<9.5	/	<17
References	[35, 87]	[36]	[62, 88-92]	[63, 93, 94]	[80, 95]	[64, 96, 97]	[98-100]

Fracture toughness, serves as a decisive factor in evaluating the functionality of coated implants, determines the level to which the material can be stressed in the presence of cracks, or equivalently, the magnitude of cracking which can be tolerated at a specific stress level. Regarding the interfacial fracture toughness of HA coated Ti6Al4V implants, Filliaggi et al [101] used a short bar chevron notch test and obtained the K_{IC} values of 0.6-1.41 MPam^{1/2}. By using a single-edge notch-bend test, Tsui et al [102] reported some similar values of K_{IC} of about 0.28-1.1 MPam^{1/2}. In addition, an indentation based method was also employed by Li et al [103], and the corresponding value was reported as 0.48 MPam^{1/2} for K_{IC} .

On the other hand, residual stresses definitely exist in the thermal sprayed HA coatings due to the rapid solidification process and the mismatch of thermal expansion coefficients of HA coating and metallic substrate. Residual stress in the coating might vary with coating thickness, deposition parameters etc., therefore, both tensile and compressive residual stresses have been reported with different values. For instance, tensile residual stresses of 200-450 MPa and 20-40 MPa were reported by Brown et al [104] and Tsui [102] et al respectively. In contrast, a compressive residual stress of

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about 35-78 MPa was reported by Yang et al [90, 105]. The presence of residual stress in the coating serves as an important influencing factor in determining the durability of the coated implants. Previous work has demonstrated that the existence of residual stress in HA coatings can alter the concentration of supernatant species in solution: tensile residual stresses enhancing dissolution and compressive residual stress impeding dissolution [106, 107]. In addition, tensile residual stress also will promote multiple cracking of the coating, and the compressive residual stress can weaken the bonding and bonding strength at the coating-substrate interface. Therefore, since both the tensile and compressive residual stresses exert detrimental influences on HA coated implants, it is desirable to produce coatings on metallic implants without any unexpected residual stress.

In view of the fact that most of the coating deposition method require an annealing or firing process to get the final expected coatings, e.g. to increase the crystallinity and/or enhance the adhesion strength, it is hardly to say that the adhesion strengths of HA coatings prepared with low temperature deposition methods are weaker than those coatings obtained with high temperature deposition techniques. Also, other testing techniques/methods should be introduced in order to get a rounded understanding upon the adhesion properties. In addition, some other important mechanical properties, e.g. toughness, residual stress etc, should be well investigated before the actual clinical applications, and also those investigations can provide the strict comparison between different deposition methods to determine which of them is the most suitable technique to prepared such HA coating.

2.2.2.4 Biological performance of hydroxyapatite coatings

It is well known that any biomaterial must be thoroughly evaluated to determine its biocompatibility/bioactivity and whether it functions appropriately in the actual biomedical applications. Generally, two kinds of evaluation techniques are employed for such purposes: *in vitro* (in glass tube) and *in vivo* (in living organism) tests. Although, *in vivo* tests are the most direct and reliable evaluation methods for biomaterials, their results are normally difficult to obtain and interpret due to lack of animal sources and the complexity of different cellular responses (during *in vivo* tests, the cells that migrate to the implant surface contain different cell lineage, and the final results are demonstrated by the fact that the progeny of these cells may form a variety of tissue types adjacent to the implant) [108]. However, *In vitro* testing can provide more rapid and relatively inexpensive data compared with *in vivo* testing. Moreover, *in vitro* testing can provide useful initial screening of materials and can aid in understanding the performance of a material *in vivo*. Those valuable insights also could help to determine whether an implant/device needs further evaluation in expensive *in vivo* experimental models and minimize the amount of animals required in *in vivo* testing [1]. *In vitro* tests of biomaterials can be carried out in any cell-free or cell-containing environment to study their biocompatibility and bioactivity. In particular, cell-free solutions allow the study of chemical and mineralogical changes of the material under conditions that simulate the physiological interactions between the material surface and the surrounding tissues.

2.2.2.4.1 *In vitro* test in acellular simulated body fluid

In 1991, Kokubo et al proposed that the essential requirement for an artificial material to bond to living bone is the formation of bonelike apatite on its surface when

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implanted into the living body [109]. In the same year, Kokubo et al firstly developed an acellular conventional simulated body fluid (C-SBF) with similar inorganic ion concentrations to those of human blood plasma (as shown in Table 2-6), in order to reproduce the formation process of bone-like apatite on biomaterials *in vitro*. Since then, C-SBF has been routinely used as an effective *in vitro* testing method to predict the *in vivo* bone bioactivity of various biomaterials. Furthermore, this test can be used for the determination of the number of animals used during *in vivo* testing, as well as the duration of animal experiments. In addition, such test can also assist the efficient development of new types of bioactive materials.

Table 2-6 Ion concentrations of Kokubo's SBF (C-SBF) and human blood plasma [110, 111]

	Ion concentration (mM)							
	Na ⁺	Cl ⁻	HCO ₃ ⁻	K ⁺	Mg ²⁺	Ca ²⁺	HPO ₄ ²⁻	SO ₄ ²⁻
Kokubo et al (C-SBF)	142	147.8	4.2	5	1.5	2.5	1	0.5
Human blood plasma	142	103	27	5	1.5	2.5	1	0.5

For a material to be bioactive *in vivo*, it must possess the ability to induce bone-like apatite formation on its surface in SBF. Many studies have been done on HA coated metallic implants in SBF, which verified the intrinsic bioactivity of HA to be used as coatings [45, 97, 112-114]. On the other hand, the response of HA coatings in SBF was observed to be highly affected by coating phase composition, crystallinity, morphology etc [28, 115, 116], and the precipitation rate of bone-like apatite was directly dependent on the Ca²⁺ ion concentration in the SBF at the vicinity of the coating surface [58, 117]. As indicated in §2.2.2.2, besides HA phase, there also exists some other phases including TCP, TTCP, CaO etc, which possess higher solubilities than that of HA phase. Therefore, in SBF testing, the dissolution of such impure

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phases will accelerate the precipitation rate of bone-like apatite through significantly enhancing the Ca^{2+} ion concentration at a localized area near to the implant surface [89, 118]. To some extent, this indicates that the existence of impure phases in HA coating could improve the bioactivity of HA coated metallic implants.

2.2.2.4.2 Cell response to HA coating

Cell culture methods have been used to evaluate the biological compatibility of material for more than two decades. Investigation on cell responses to materials can provide more details of understanding cell-materials interactions and can aid in establishing actual biological responses to artificial materials [119]. Because those HA-coated metallic implants are used for hard tissue replacement/repairing, *in vitro* models using osteoblastic cells are essential and valuable tools for the initial assessment of those candidate implants. Osteoblastic cells, which arise from pluripotential mesenchymal stem cells, have a set of distinguishing characteristics that include the ability to synthesize osteoid or bone matrix and to mineralize the osteoid to get the calcified bone [120].

2.2.2.4.2.1 Cell-implant interactions

The fixation of implants to bone is based on the process of osteointegration, which leads to direct apposition of mature living bone onto the implant surface [121, 122]. Since that osteointegration process is strictly mediated by osteoblastic cells, the fate of such implants is thus determined by the response of cells to the material's surface. Therefore the implant should create favorable conditions for osteoblast attachment, spreading, growth, differentiation and functionalization. Virtually, under

physiological environment, all implant surfaces become immediately coated with a 1-10 nm thick adsorbed protein layer before cells can adhere to the material [123, 124], as shown in Figure 2-3. The rapid adsorption of proteins effectively translates the structure and composition of the foreign surface into a biological language, which are also response for the following host responses [125]. As such, when the cells arrive at the implant surface, they can only “see” a protein-covered surface. Those adsorbed proteins offer necessary binding sites to those anchorage-dependent cells, leading to the initial cell attachment onto the surface of biomaterials (Figure 2-3). Thus, when we talk about cell-surface interactions, it is ultimately an interaction between cells and surface adsorbed proteins (or other biomolecules). If the surface of the implant is bioactive to the cells, they will then gradually spread well throughout the implant surface. Only after a cell have attached onto the implant surface and spread towards a certain situation, can the subsequent activity such as cell proliferation and differentiation take place. Also, it is reported that a well-spread cell is favorable to DNA synthesis and cell growth [126-128]. Therefore, it can be claimed that the initial cell attachment and spreading onto the implant surface are the crucial prerequisites for those anchorage-dependent osteoblastic cells to determine their intracellular signal transduction, gene expression, long-term viability as well as the successful osteointegration.

Figure 2-3 Schematic illustration of the successive events for cell-implant interactions [124, 129] Roughly, three continuous stages could be described for cell-implant interaction: the immediate adsorption of proteins onto implant surface followed by cell attachment and cell spreading.

2.2.2.4.2.2 Cell proliferation and differentiation

Roughly, the progress from an initially attached osteoblastic cell to well-differentiated mature osteoblast (osteocyte) can be divided into three stages, i.e. proliferation, differentiation or extracellular matrix synthesis and maturation, and mineralization stages, as shown in Figure 2-4 [130].

Figure 2-4 Temporal expression of cell growth and osteoblast phenotypes[130]. The three periods of osteoblastic cells are illustrated with expression of representative genes: Proliferation, H4 histone reflects DNA synthesis, c-fos and c-jun (AP-1), and type I collagen (COL-I); Differentiation, alkaline phosphatase (ALP) is expressed as an early differentiation marker, osteocalcin (OC) and osteopontin (OP) are recognized as late markers of differentiation; Mineralization, OC and OP are also the early markers of mineralization indicating the onset mineralization of ECM, accumulation of calcium ion (Ca+2) is another indicator of mineralization.

During the first several days following cell attachment and spreading, cells try to reproduce themselves; and an active proliferation is directly reflected by the exponential increase of cell number as well as the deposition of type I collagen. On the other hand, several genes related to cell cycle (e.g. histone) and cell growth (e.g. c-myc, c-fos, and c-jun) are actively expressed [130], and can be used to monitor those mitotic activities. Immediately after the down-regulation of proliferation, cell phenotypes associated with osteoblastic cell differentiation are detected, indicating that the cells step into differentiation stage. The most frequently used markers of

osteoblast differentiation are alkaline phosphatase (ALP), and osteocalcin (OC). As an early differentiation marker, ALP is an enzyme that is associated with calcification. This is an early marker of osteoblastic cell differentiation because activity is greatest just before mineralization actually begins. It is reported that an enhanced expression of this enzyme is apparently needed just before the onset of matrix mineralization, providing localized enrichment of inorganic phosphate—one of the components of apatite (the mineral phase of bone) [108, 131]. High levels of bone ALP are usually detected in both preosteoblasts (osteoblastic cells) and osteoblasts *in vivo* and in differentiating osteoblastic cells *in vitro*. Osteocalcin (OC), known as a late marker of cell differentiation, is a small molecular weight protein (5.7 kDa) produced by osteoblasts and has the ability to chelate Ca^{2+} to form bone minerals [130, 132]. For this reason, it is a particularly effective marker of a well-differentiated osteoblastic cell. Generally, once mineralization is initiated, alkaline phosphatase will decrease while osteocalcin accumulation continues, reflecting an expression related to deposition of mineral for the latter.

2.2.2.4.2.3 In vitro test using cell culture

As one of calcium phosphate ceramics, sintered HA has well established as bioactive material that enhances osteoblastic cell viability and osteointegration with host bone [133]. Those valuable biological properties are well maintained when HA are used as coatings on metallic implants. In comparison with those bioinert metallic implants, many studies reported that the fabricated HA coatings on their surface not only can help to stimulate cell growth and DNA synthesis, but also can enhance the ALP and OC activities, and the mineralization of extracellular matrix [134-138]. However, those desired biological properties of HA coatings are highly dependent on coating

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surface roughness. Lee et al investigated the cell responses to HA coatings with different roughness values (Ra), i.e. 0.67 μ m and 10.37 μ m, and concluded that smoother surface was more favorable for cell attachment than the rougher one [139]. Moreover, Kim et al [65] reported that the higher surface roughness (Ra = 0.84 μ m) could significantly enhance the number of attached cells compared with the lower surface roughness (Ra = 0.22 μ m), but no significant difference regarding cell proliferation and differentiation. While an enhanced effect on cell differentiation activities and the formation of bone nodules was observed in other researchers' studies [138, 140, 141].

On the other hand, several other studies also indicated that the behavior of cultured osteoblastic cells are affected by the crystallinity and phase composition of the prepared HA coatings [136]. Kim et al claimed that on the coatings with higher crystallinity (>76%), the cells attached and proliferated well and expressed ALP and OC to a higher degree as compared to the poorly crystallized coatings (~43%crystallinity) [65]. Whereas, Chou et al reported that the lower crystalline coatings with some other impure phases (CaO, TCP etc) were favorable to cell attachment but exerted inhibiting effects on cell proliferation due to the elevated medium pH value caused by the dissolution of impure phase [142]. On the contrary, other studies illustrated insignificant influences on cell attachment, proliferation and differentiation or mineralization with respect to different crystallinity and/or phase composition [143].

Although it is commonly accepted that the HA coatings could improve the *in vitro* biological properties for those metallic implants, there still exists some inconsistent or

even conflicting viewpoints regarding the influences of coating properties on cell responses. Considering those differences in coating preparation, experimental setup, testing methods etc, it is hard to judge which one is more acceptable.

2.2.2.4.3 In vivo evaluation of HA-coated implants

Practically speaking, the goal *in vivo* assessment of implants or devices is to determine and predict whether such implants/devices present potential harm to the human body by evaluations under conditions simulating clinical end-uses applications [144]. *In vivo* models allow the evaluation of toxicity of a biomaterial and the efficacy of a biomaterial within a therapeutic application. Therefore, those osteointegration (the close apposition of bone to implant surface) examinations during *in vivo* studies are essential for clinical application and for further analysis about the effect of the implant on the expression of osteoblast phenotype.

Histological analysis of HA-coated implant has shown that bone ingrowth with osseous integration occurs as early as 10 days following implantation [145]. Thereby, HA coating could provide the crucial initial fixation requirement for the success of an implant. Furthermore, a histomorphometric study by Moroni et al [146], comparing the percentage of bone bonding to HA-coated and uncoated implants in dogs, revealed a significant increase in bone amount in the HA-coated implants. They also showed enhancement of bone-to-pin osseointegration and interfacial strength in HA-coated pins as compared to uncoated pins in a sheep study [147]. Figure 2-5 shows the typical histological micrograph upon enhancing effect of HA coating on bone-implant fixation. Similar results are also reported in other researchers' studies [148-151]. Along with this, extensive investigations have been conducted on the bonding

strength of HA-coated implants during *in vivo* tests. For instance, according to the studies of Oonishi et al [152], the adhesion strength between the implant and bone was ~ 0.53 and ~ 1.35 MPa for uncoated and HA-coated implants respectively at two weeks after implantation, while those bonding strengths increased to ~ 7.5 and ~ 14.15 MPa at six weeks correspondingly. Other short-term *in vivo* tests also indicated the similar trend regarding this issue [153-155]. Essentially, it is revealed that those quicker and stronger responses of HA coatings *in vivo* significantly benefit from the fast dissolution of other impure phases, which results in an increase of Ca^{2+} and PO_4^{3-} ion concentrations in local area around the implant-bone interface. Nevertheless, from the viewpoint of long-term stability, whether the loss of HA coating could maintain such desired bonding strength between the implant and surrounding tissue is questioned.

Figure 2-5 Histological sections after 8 weeks implantation (x40). Non-coated Ti6Al4V implant (NC, left one) showed that there only partial contact between the implant and surrounding bone (B); HA-coated implant (HC) indicated a well bonded implant-bone (B) interface [156].

2.2.3 Problems involved in hydroxyapatite coatings

A prerequisite for any implant used in orthopedic or dental is permanent fixation to the surrounding tissues with no intervening gaps or fibrous tissues [23]. According to

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the *in vivo* and *in vitro* studies as well as more than a decade's clinical practice with HA-coated prostheses, there is general agreement that the originally pursued benefits of HA coatings, that is, earlier fixation and stability with more bone ingrowth or outgrowth, can be achieved. However, doubts still exist concerning the durability of the fixation [10]. One of the most important events occurring at the bone-implant interface is the resorption of the HA coatings, also called degradation or coating loss [11, 155]. Although, some resorption or dissolution is, of course, essential to trigger bone-implant bonding, the fast resorption could lead to disintegration of the coating, with rapid loss of the bonding strength between it and substrates, resulting in delamination, the production of particles, and loss of mechanical fixation. It is reported that a decrease as high as 31.6% was observed for plasma-sprayed HA coatings after only 2 weeks immersion in SBF [89]. Some other studies have shown resorption of HA coatings up to 2 years after implantation, and a complete loss of a 60 μm -thick HA coating after 4 years [8]. While, Aebli et al carried out a histological study of a proximally HA-coated femoral component, and found that the HA coating had completely degraded after 9.5 years implantation [157]. Therefore, all of the expected functions of HA coatings, as discussed in §2.1.2, will definitely disappear along with the fast loss of HA coatings.

What is worse, particles or particulates, as the products of HA degradation, may cause severe damage or complications to the bone-implant system. It is reported that the HA particles can be resorbed by macrophages if their size is sufficient small compared to the macrophages (approximately 30 μm) [158]. When a macrophage phagocytizes the particles, the cells release cytokines, prostaglandins, and collagenases almost immediately. If the particles do not dissolve within the life span of the macrophage,

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more macrophages will accumulate at the site in response to the release of cytokines to digest the dead macrophages and undissolved HA particulates. As well, particles larger than a macrophage ($>30\text{ }\mu\text{m}$), will not be digested by macrophages and will probably become engulfed by a giant cell. The excessive cellular reaction to HA particulates and the stimulation of a foreign-body response could lead to a decrease in local pH value, which disrupts the bone remodeling process, causing the resorption of both HA coating and bone [159, 160]. Additional problems could arise if particulate debris travels to the implant-bone interface, producing third-body wear and component loosening/failure. Morscher et al investigated 6 revisions of HA-coated implants follow-up over ten years after successful primary implantation [161]. They found that HA granules embedded in all of the examined implants, what's more, as higher as 66.7% of them had loosened and 50% of them showed severe osteolysis of the proximal femur.

Considering the above problems regarding HA-coated implants, from the standpoint of long-term stability, the well-recognized short-term advantages of HA coatings on improving bone ingrowth and osteointegration may ultimately be overtaken by detrimental sequelae. Understanding that the essential issue of the above problems is the high rate of degradation or resorption, accordingly, two solutions can be suggested: one is to increase the coating thickness to ensure that the coating could endure certain long time degradation; another one is to control and reduce the coating solubility. As for the first solution, actually, it is very limited and infeasible, because the increase of coating thickness could not only exert some detrimental influences on mechanical properties, but also could aggravate the produce of coating debris [162, 163]. Therefore, to reduce the coating degradation rate is the most possible choice. And

fortunately, it has been proved that the substitution of hydroxyl ions in HA lattice structure by fluorine ions is an effective way to lower its solubility, hence, a long-term stable performance can be expected for the fluoridated hydroxyapatite coating.

2.3 Fluoridated hydroxyapatite and fluoridated hydroxyapatite coating

2.3.1 Fluoridated hydroxyapatite and its properties

2.3.1.1 Introduction to fluoridated hydroxyapatite

The principal inorganic matrix mineral phase of bone and teeth is based on hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, but additionally, contains different quantities of cations like Na^+ , K^+ and Mg^{2+} which occupy Ca^{2+} sites and anions such as CO_3^{2-} , F^- , and Cl^- substituting OH^- groups [30, 164]. These substitutions, especially F^- , induce complex structures at the unit-cell level and play a significant role in influencing the physical and biological properties of the resulting material. It is well known that fluorine is an essential trace element required for normal dental and skeletal development. Over the past decades, it was widely investigated and accepted that the incorporation of fluorine ions can not only strengthen the tooth enamel, but also provides the essential protection to maintain the healthy status of teeth. It is proved that tooth surface enriched with fluorine ion was more resistant to caries, suggesting that F^- serves as a prophylactic in the occurrence dental caries [164]. It is commonly known that the presence of fluorine, which was introduced in toothpaste or drinking water, is significantly effective for reducing tooth decay and preventing caries formation [14, 165]. On the other hand, it has been shown that the presence of fluorine has certain beneficial effects in increasing the quantity and quality of bone

formation and can be effective for the prevention and treatment of osteoporosis [166]. Therefore, although fluorine is only a trace element in human body, it is really indispensable for the healthy maintenance of human hard tissues. The notable function of fluorine is suggested to be due to the replacement of hydroxyl groups by fluorine ions in hydroxyapatite, resulting in the formation of less soluble fluoridated hydroxyapatite, which can enable the hard tissues to suffer from less dissolution or possible resorption.

The formation of fluoridated hydroxyapatite is easily achieved by replacing OH groups with fluorine ions in hydroxyapatite structure. The crystallographic feature of HA pertains to the P63/m space group; its most striking feature is the calcium triangle channel formed by calcium atoms that align along the (001) axis (101). The substitution of a hydroxyl ion by a fluorine ion will produce solid solution of hydroxyapatite and fluorapatite according to the degree of substitution, with a composition of $\text{Ca}_{10}(\text{PO}_4)_6\text{F}_x(\text{OH})_{2-x}$, (x is the degree of fluoridation: $x = 0$, pure HA; $x = 2$, pure FA; $0 < x < 2$, fluoridated hydroxyapatite, FHA). The arrangement of OH groups and F^- ions in HA, FA and FHA are shown in Figure 2-6. The respective positions for the OH groups are, O at $z = 0.2$ or 0.3 , and at 0.7 or 0.8 ; H, at 0.06 or 0.44 and at $z = 0.56$ or 0.94 . For F^- ions, their positions are at $z = 0.25$ and at 0.75 [31]. Also, experiments have indicated that the OH groups are deposited slightly away from the center of the calcium triangle plane and polarized in the c -axis, while F^- ion is deposited in the center of calcium triangle center. Although the fluorine ion is substituted for the hydroxyl ion in OH lattice position and the complete chemically homogeneous solid solution of HA/FA can be formed, there are many distinct differences between HA, FHA and FA.

Figure 2-6 Arrangement of OH^- groups and F^- ions in HA, FA and FHA crystal structures (Arrangement along c -axis) [31, 167]. $\text{Ca}_{10}(\text{PO}_4)_6\text{F}_x(\text{OH})_{2-x}$, x is the degree of fluoridation: $x=0$, pure HA; $x=2$, pure FA; $0 < x < 2$, fluoridated hydroxyapatite, FHA.

Due to the difference in the size of the fluorine ion (0.132 nm) and the hydroxide ion (0.168 nm), there are some changes of the lattice parameters between the HA, FHA and FA. Generally for pure HA, the lattice constants are reported as: $a = b = 0.9418$ nm, and $c = 0.6882$ nm, while for pure FA these parameters are: $a = b = 0.9368$ nm, and $c = 0.68841$ nm. As for the FHA, the lattice parameters have a regular change corresponding to the different degree of substitution. The a -axis and c -axis cell parameters of the FHA with different fluorine ion contents are represented in Figure 2-7. A change in the a -axis lattice parameter can obviously be noted, whereas the c -axis lattice parameter does not show any discernable difference [3, 168]. The a -axis parameter decreases markedly with increasing fluorine content.

Accordingly, the changes of the lattice parameters can be distinguished from the XRD results. The short-range XRD patterns with a slower step size were collected for the HA, FHA and FA as shown in Figure 2-8 [169]. It was found that the (300) reflection peak of FA was shifted toward a higher 2θ relative to that from HA, while the (300)

peak of FHA was intermediate between the above two patterns. Also, there are the same shift tendencies of the (211) peaks for the three kinds of apatites. These results are in good agreement with other reports [168, 170]. The shifts of these characteristic peaks indicate the change of a -axis of the HA lattice.

Figure 2-7 Changes in a) a -axis and b) c -axis cell parameters, respectively as a function of the degree of fluoridation [3]. The a cell dimension contracts linearly with increasing fluorine content, while no clear trend is displayed for c dimension.

Figure 2-8 Short-range XRD patterns of HA, FHA and FA [169]. It was found that the peaks, e.g. (211) and (300), of FA were shifted toward a higher 2θ relative to that of HA, while those peaks belonging to FHA were intermediate between FA and HA.

The fluorine-hydroxyl substitution in the channel along the c-axis in the HA structure can explain why there is no change in cell parameter dimensions in the c-axis direction, while the contraction observed for the a-axis lattice parameter of the FHA can be attributed to the smaller size of the fluorine ion, relative to the hydroxide ion. Thus all the fluorine ions enter the FHA apatite structures. They are not incorporated into other apatite forms [171].

2.3.1.2 Properties of fluoridated hydroxyapatite

2.3.1.2.1 Thermal stability

It has reported that the incorporation of fluorine ions can improve the thermal stability of hydroxyapatite and can help to retain its phase purity up to a temperature of 1400°C [172, 173]. TGA investigations showed that at about 800°C, a weight loss was observed for HA due to the release of OH groups from HA (dehydration of HA), further increase of temperature caused the decomposition of HA into other CaP phases (refer to the reactions 2-(1-5) listed in §2.2.2.2). In contrast, fluoridated hydroxyapatite ($x > 0.4$), maintained a stable weight gain (or the weight loss was too weak to be detected) even calcined up to the temperature of 1400°C [70, 167]. The weight loss observed in TGA investigations at higher temperature indicates the formation of new phases, which can be well characterized with X-ray diffraction analysis (XRD). Kim et al [172] reported that, after sintering at 1400°C for 3 hours, the fluorinated HA had a degree of decomposition lower than 10%, while that of HA and HA composite could be ~40-70%. Rodriguez-Lorenzo et al [3] reported that after being calcined at 900°C for 12 hours for HA, FHA and FA, TCP peaks could be observed only in the XRD patterns of HA and FHA, that's to say, FA, with the

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highest fluorine concentration, has the strongest thermal resistance. Similarly, some other results can be found in other researchers' reports [170]. On the other hand, the grain growth rate, which is treated as another indicator of thermal stability, reveals that incorporation of fluorine ions can retard the grain growth, indicating that the incorporated fluorine ion makes the HA to be more stable [170, 174].

Those above observations suggest that when certain amount of fluorine ions could be successfully incorporated into HA lattice structure, the thermal stability of HA matrix would be greatly increased or the decomposition would be effectively retarded. However, it does not mean that the more the fluorine ions incorporated into HA lattice structure, the better the enhancement effect on the thermal stability. Chen et al [167] reported that the when the degree of fluoridation higher than 0.8 (in their study, $x \geq 1.2$), the thermal stability was drastically improved, while, no significant improvement could be observed when the x value increased from 1.2 to 2. On the other hand, Gross et al [174] calculated the activation energy for grain growth of fluoridated hydroxyapatite, as shown in Figure 2-9, and revealed that the activation energy was a function of incorporated fluorine ion and the highest barrier was achieved in the x range of 0.8-1.2, implying that a medium degree of fluoridation could be more effective to immobilize the fluorine and hydroxyl ions within the lattice. What is more, Senamaud et al [70] directly pointed out that the stabilizing effect induced by the incorporated fluorine ions achieved the maximum outcome at $x=1$.

Figure 2-9 Activation energy for grain growth of fluoridated hydroxyapatite [174]. The highest barrier for grain growth occurs for the FHA with a degree of fluoridation between 0.8-1.2.

This stabilizing effect can be explained by considering the crystal structure of hydroxyapatite [167, 173]. In hydroxyapatite structure, the most probable arrangement of OH groups should be randomly organized along c-axis with about 50% of the OH groups direct in one way and the other 50% in the opposite way (in other words, not all the OH groups are not in the highest structural arrangement of –OHOH...OH–, or –HOHO...HO–). This arrangement confers a certain degree of disorder on the crystal structure of hydroxyapatite (as shown in Figure 2-6, the left one). Once the OH groups are partially substituted by F[–], the hydrogen atoms of neighboring OH groups will be attracted to the nearby F[–] because of the higher affinity between the existing hydrogen ions of the hydroxyl ions and the fluorine ions with respect to the oxygen ions. Thus a quite well-ordered apatite structure is produced (Figure 2-6, the middle one) [32]. The partial substitution of hydroxide ions induces a stabilizing effect which reduces the mobility of the remaining hydroxide ions and consequently affects the resistance to decomposition. Theoretically, the F[–] concentration of 50% in the FHA should be enough to remove the disorder in HA lattice structure and hence to stabilize the structure due to the alternating arrangement

of F^- between each pair of OH groups. However, by considering the random replacement of OH by F^- in the OH positions ($z = 0.25$ or 0.75), the fluorine ion concentration required to stabilize the structure should be in a range near to 50%, e.g. 0.8-1.2 etc [174]. As for the higher degree of fluoridation, e.g. $x > 1.6$, the repulsive interaction of neighboring fluorine ions will cause certain negative influences on the stability of the fluoridated hydroxyapatite [70, 175].

2.3.1.2.2 Mechanical properties

Fluorine ion incorporates into hydroxyapatite to form chemically homogeneous distribution within bone and teeth, leading to a modification of the chemical and mechanical properties of these tissues. The change in mechanical properties could be related to the modified apatite composition, a change in crystallite dimensions and orientation, a higher mineral content within the bone, or a modification to the bone architecture [176]. Each of these factors can contribute to a modification to the mechanical properties. In some treatments, such as the treatment for osteoporosis, high concentrations of fluoride salts are administered and this results in elevated fluoride levels within bone. Studies have not been performed to reveal how an increased level of fluoride influences the mechanical properties of the inorganic phase within bone. Since bone consists of a chemically enriched hydroxyapatite, it is important to initially assess the influence of each chemical species at increasing concentration on the mechanical properties of hydroxyapatite. The logical progression will then involve the assessment of chemical species in parallel to ascertain the chemical species that exerts the highest influence on properties. Here, the influences of incorporated fluorine ions on the mechanical properties of HA will be reviewed. Gross et al [177] systematically investigated the influences of fluorine ion

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incorporation on mechanical properties of the sintered fluoridated hydroxyapatite, including hardness, fracture toughness, elastic modulus and brittleness, by microindentation method. They reported that hardness remained constant (~ 490 Hv) until 80% fluoridation was reached ($x = 1.6$), and the final Vickers hardness is about 550 for FA ($x = 2$). The elastic modulus increased linearly with fluorine content from ~ 110 GPa for HA to ~ 130 GPa for FA. The fracture toughness was affected the most by fluorine incorporation into the lattice, as shown in Figure 2-10, toughness increased gradually and reached a peak at about $x = 1.2$, then decreased rapidly with further increase of degree of fluoridation. In contrast, the brittleness index decreased gradually to a minimum at about $x = 1.2$ followed by a sharp rise. Some similar results can be found in other published papers [170]. Kim et al [172] also reported that FHA composite exhibited improved mechanical properties, such as strength, toughness, and harness, having values of more than ~ 2 -4 times higher than those of pure hydroxyapatite. On the contrary, Barinov et al [178] found that both the fracture toughness and the hardness decreased from $0.77 \text{ MPam}^{1/2}$ and 1.87 GPa for HA to $0.69 \text{ MPm}^{1/2}$ and 1.63 GPa for (10%AF+90%HA) respectively. This discrepancy maybe caused by the difference in initial preparation of FHA powders.

On the other hand, sintering temperature as well porosity has significant influences on the mechanical properties of fluoridated hydroxyapatite. For instance, an increase in hardness as well as elastic modulus was usually observed with the decrease of porosity. Therefore, in view of this aspect, it is reasonable that large discrepancy could be found in different researchers' work [170, 177-179].

Figure 2-10 Fracture toughness of fluoridated hydroxyapatite. Toughness increased gradually and reached a peak value at about $x=1.2$, then rapidly decreased with further increase of degree of fluoridation. On the other hand, an inverse relationship was observed between toughness and porosity.

2.3.1.2.3 Solubility of fluoridated hydroxyapatite

Fluoride has been found effective for the prevention of dental caries, and the cariostatic property of fluorine is believed to be related to the formation of a less soluble fluorine-containing mineral (fluoridated hydroxyapatite) [165, 180]. With a view to the application as a biomaterial, the introduction of fluorine ions into hydroxyapatite is based on the desired property: low degree of dissolution in body fluid. Regarding the dissolution process of apatites (including HA, FHA, and FA), Dorozhkin [181] proposed a surface mechanism for the dissolution process of apatite. This mechanism was based on chemical properties and crystal structure of apatite and seemed to take into consideration all current main experimental and theoretical results. The mechanism consists of five successive dynamic chemical reactions (e.g., FA):

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Based on the above successive chemical reactions, two general chemical reactions can be drawn for the description of FA, FHA and HA dissolution:

Reaction (2-11) describes chemical transformation of apatite into acidic calcium phosphates, while reaction (2-12) describes the dissolution process as a reversible chemical reaction. That is to say, there exist two competitive processes: demineralization and remineralization of the FHA phase. If the demineralization process is predominant in the testing solution, then the coating will be dissolved until a dynamic balance is established for these two competitive processes (2-12). Because FA as well FHA has the smaller solubility than that of HA, the presence of fluorine ions in the solution, e.g. supplemented in daily drinking water, can effectively promotes the remineralization process (reaction (2-12) moves to left) to reduce the .loss of mineral phases (formation of caries) [182].

Besides the dissolution behaviors described above, the inhibiting effect of incorporated fluorine ions on the dissolution behaviors should be further addressed. Firstly, the incorporated fluorine ion can effectively inhibit the dissolve of hydroxyl groups [183, 184]. It is reported that dissolution behavior is a diffusion-related process, and the diffusion of hydroxyl ions within the apatite structure is a critical factor to control the rate of dissolution. A quantitative measure of the difference in dissolution of the OH^- and F^- can be obtained from a comparison of the diffusion

constants for both species. Based on the calculations of de Leeuw et al [183], a diffusion constant of about $5.74 \times 10^{-9} \text{ cm}^2/\text{s}$ was calculated for OH^- , while almost a zero value was obtained for that of F^- . Therefore, when water is present at HA surface, the hydrated HA reveals the onset of dissolution through the escape of hydroxyl groups from HA surface into the solution. However, if there are some incorporated fluorine ions, the mobility for diffusion of hydroxyl ions will be reduced due to the additional strong hydrogen bonding between the “inert” F^- and OH^- (c.f. §2.3.1.2.1). On the other hand, the incorporated fluorine ions can cause a decrease in a lattice index (c.f. §2.3.1.1), resulting in a more compact structure [185]. Therefore, in the vicinity of the fluorine ions, the calcium ions remain more closely and stronger resistance toward dissolution.

However, it does not mean that the more the incorporated fluorine ions, the stronger the resistance to dissolution. Although a calculated result reported by Driessens [186] suggested a continuous decrease of solubility with increasing degree of fluoridation (pH4-8), the experiment-based results done by Moreno et al revealed a different trend in response to the degree of fluoridation [180]. As shown in Figure 2-11, the experimentally determined minimum solubility was located at a degree of substitution of ~ 0.56 (pH7, and here, the chemical formula was defined as $\text{Ca}_5(\text{PO}_4)_3\text{F}_x(\text{OH})_{1-x}$). The reason also was attributed to the stabilizing effect of fluorine ions on the apatite structure as discussed in §2.3.1.2.1.

Figure 2-11 Solubility of fluoridated hydroxyapatite as a function of the degree of fluoridation, a-pH5, b-pH6, c-pH7 [180]. Here the chemical formula was defined as $\text{Ca}_5(\text{PO}_4)_3\text{F}_x(\text{OH})_{1-x}$.

2.3.2 Current status of fluoridated hydroxyapatite coating

2.3.2.1 Methods for coating preparation

The replacement of hydroxyl groups by fluorine ions in HA structure will lead to the formation of fluoridated hydroxyapatite, which has a more compact crystal structure, lower solubility, and comparable biocompatibility compared to pure HA. Therefore, in view of long-term stability, fluoridated hydroxyapatite has been considered as the most promising substitute for HA to be used as coatings on metallic implants. Recently, more and more attentions have been attracted for the investigations of FHA coatings,

Thermal spray technique, as the most employed method to preparation HA coating, has also been used for the deposition of FHA coatings. According to the documented literatures, it seems that the first trial of depositing FHA coatings could be retrospectively to the year of 1988, when Lugschneider et al [187] reported the successful preparation

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of FA coatings on titanium alloy substrate. However, the deposition of FHA coatings was firstly achieved indeed by Gineste et al [188] in 1999 by using plasma spraying. After that, a few other related studies have been reported [189, 190]. Considering the using of high temperature during coating preparation, as discussed in §2.2.2.1, decomposition is really considered as the inevitable restriction for coating preparation.

In order to avoid using high temperature, Shikhanzadeh et al [191] developed a low temperature method, i.e. electrochemical deposition method, to produce fluoridated hydroxyapatite. Only based on FTIR result and, they concluded that the fluorine ions have been successfully incorporated into HA (they were failed to provide the concentration of F^-). What is more, till now, no more other related publications regarding this method (to prepare FHA coating) could be found, neither by Shikhanzadeh's group nor other researchers.

Recently, two other deposition methods, i.e. pulsed laser deposition (PLD) [192] and electron beam deposition [193], were also introduced to produce FHA coatings. Besides the difficulty to control the coating composition (chemical composition and purity), the requirements of high vacuum and sophisticated equipment, time-consuming etc inevitably restrict their wide and further applications.

Along with these, sol-gel method also has been developed and now is becoming the most preferred method to deposit FHA coatings [17, 194, 195]. In comparison with other methods, sol-gel method possesses some unique advantages [9]:

- Increased homogeneity due to mixing on the molecular scale
- Ability to produce uniform fine-grained structures

- Use of different chemical routes (alkoxides or aqueous based)
- Control of the coating composition, thickness etc.
- Easy doping of other expected ions
- Low cost and easy operation

In view of those advantages, it is reasonable that sol-gel method is considered as the most promising method to produce FHA coatings. Based on the documented results, an overview of different calcium, phosphate and fluoride precursors used for preparation of FHA coatings is listed in Table 2-7.

Table 2-7 Overview of different Ca-, P- and F-precursors for preparation of FHA coatings

Ca-precursor	P-precursor	F-precursor	References
Ca(NO ₃) ₂ ·4H ₂ O	(NH ₄) ₂ HPO ₄	NH ₄ F	[194]
	P(C ₂ H ₅ O) ₃	NH ₄ F	[16, 195, 196]
	P ₂ O ₅	CF ₃ COOH HPF ₆ NH ₄ PF ₆	[17, 197-199]

2.3.2.2 Influences of incorporated fluoridated ions on coating properties

Although the development of FHA coatings as substitute for HA is still in its initial stage, there are still some valuable research findings in this field, which could provide useful information for further studies. With the incorporation of fluorine ion, some influences on coating properties have been observed and reported in some studies. Firstly, as expected initially, the incorporation of fluorine ions could significantly reduce the solubility of the obtained FHA coatings, no matter in the physiological saline solutions [16, 196], phosphate buffered saline solution (PBS) [195], TRIS buffered solution [197] or citric acid modified phosphate buffer solution (CPBS, pH4.7-6.95) [198]. However, only two related studies reported the response of solubility to different degree of fluoridation, but they provided the conflicting results regarding this point: Kim et al [16] reported that the solubility was roughly reduced

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with the increase of fluoridation degree; while Lee et al [193] showed a different dissolution curve, which was similar to Figure 2-11 (pH7). It is hardly to judge which one is incorrect by considering the differences in experimental setup as the lack of some critical data (e.g. the actual fluorine concentration in the coating etc.).

On the other hand, as an unexpected effect, Lee et al [193] and Weng et al [198] reported that certain degree of fluoridation could enhance the bonding strength between the coatings and substrates. The reason was roughly attributed to the decrease of coefficient of thermal expansion (CTE) with the incorporation of fluorine ions, resulting in a similar CTE of FHA coating to that of Ti substrate. In contrast, Kim et al [195] reported an insignificant effect of incorporated fluorine ions ($x = 1$) on adhesion strength. Therefore, further studies should be performed to ascertain whether/why the incorporated fluorine ions could improve the adhesion strength.

As for the influences on biological properties, previous *in vivo* histological evaluations demonstrated that, both HA and FA coatings possessed the comparable ability to improve bone contact and ingrowth (apposition) after an implantation period of 6 weeks to 6 months, but HA coating showed a severe reduction in thickness in comparison with that of FA coating [15, 200, 201]. Fluoridated HA coating, as demonstrated by Martini et al [202], could not only offer positive effect of providing a suitable substrate to enhance new bone formation, but also serve as an effective protective coating to limit detachment of titanium particles (caused by the wear and friction occurring at the implant surface-bone interface). Moreover, based on *in vivo* histological and histomorphometrical evaluation, Gineste et al [188] conclude that a stronger degradation resistance was observed for FHA ($x \approx 0.8$) coating compared

with that of FA or HA coating, while maintained good interactions with surrounding tissues. Besides the above *in vivo* evaluations, some *in vitro* tests were also carried out and indicated that certain incorporated fluorine ions ($\text{HPF6}/\text{Ca} = 1/45$ and $1/30$) could drastically enhance the deposition of bone-like apatite in SBF [197]. However, with respect to the *in vitro* cell responses, there are some inconsistent or even conflicting conclusions between different researchers, for instance, Kim et al [16, 195] reported an insignificant effect of incorporated fluorine ions ($x = 1$) on cell proliferation, ALP and OC activities; while Lee et al [193] demonstrated an adverse influence on cell proliferation and ALP levels for those partially substituted HA coatings; on the contrary, Cheng et al [203] suggested that the coating with a designed degree of fluoridation of $\text{HPF6}/\text{Ca} = 4/90$ showed stimulating effect on cell proliferation. Therefore, as the basic and crucial *in vitro* test, further studies upon cell response should be carried out to get better understanding about the functions of incorporated fluorine ions.

2.4 Summary

Hydroxyapatite, with a similar inorganic composition to that of human bone and teeth, is well known as a bioactive ceramic and has been used as a coating on metallic implants to fulfill the requirements of hard tissue replacement/repairing. Those HA-coated metallic implants can combine both the excellent biological performance of HA and the excellent mechanical properties of the metallic substrate. Although short-term *in vivo* evaluations as well as some clinical applications demonstrate the satisfactory performance of HA coating in inducing bone growth and osteointegration, the long-term stability is still the most questioned issue in view of the high

degradation/resorption rate of HA coating in such a biological environment as well as the complications caused by coating dissolution.

Recently, fluorapatite has attracted a great deal of attention for its lower resorption rate and comparable biocompatibility to HA. Fluorapatite can form a full range solid solution with HA by replacing OH with F. Hence, it is possible to improve the stability of HA by incorporation of fluorine ions while without any adverse influence on its biocompatibility. Some previous studies have demonstrated the feasibility of preparing FHA coatings on metallic implants by employing some coating deposition methods, and sol-gel technique is regarded as the most promising method to prepare FHA coatings due to its advantages, especially the easy doping of fluorine ions and coating composition control. Based on the current status of the development of FHA coatings, to date, however, the relationship between the coating properties, e.g. the mechanical properties and biological performances etc, and the incorporated fluorine ions (with different concentration) is still not well understood and even not established. Furthermore, as the crucial index, the adhesion properties between the coating and substrate should be well analyzed by using other techniques in addition to the commonly used pulled-out based testing methods. Therefore, in this project, by using sol-gel coating deposition method, a systematic investigation on the influences of incorporated fluorine ions on the properties of FHA coatings will be conducted. The coating properties will be optimized with respect to the degree of fluoridation to achieve high bioactivity, reduced solubility, and excellent adhesion strength. This study on sol-gel derived FHA coatings could contribute to their further biomedical applications.

Chapter 3 Experimental

3.1 Overview of experiment flow

In this project, fluoridated hydroxyapatite coatings with different fluoridation degrees will be prepared with sol-gel dip-coating method and thereafter characterized. Issues include chemical and physical properties, mechanical and *in vitro* biological properties. An overview of the whole process is illustrated in Figure 3-1.

Figure 3-1 Overview for the study of sol-gel derived fluoridated hydroxyapatite coatings

3.2 Preparation of HA and FHA coatings

3.2.1 Preparation of Sols

3.2.1.1 Sol for HA coating

Generally, metal alkoxides are most preferred for the sol-gel process. In the case of a few metals (for example, metals of Group I & II), it might not be convenient to use alkoxides due to their unavailability and/or difficulties in synthesis. Thus alternative precursors may have to be employed, but care has to be taken to choose such precursors as: i) they can be converted into oxides by thermal or oxidative decomposition; and ii) their solubility in organic solvents preferable from the initial stage or during the sol-gel processing would be a further advantage. Thus out of the inorganic salts, metal nitrates are probably the best candidates as other salts like sulphate or chloride are thermally more stable and in their case, it may be difficult to remove the anionic portion effectively from the final products. As for the selection of Ca-precursor for this project, calcium nitrate would be the most preferred choice. It has a better solubility in absolute ethanol, lower decomposition temperature, and a better oxidizability from nitrate ion, which can help the decomposition of the residual organic substance.

In view of the chemical properties of P-precursor candidatures and the advantages of sol-gel technology, phosphorous pentoxide (P_2O_5) was selected as P-precursor, and absolute ethanol as the organic solvent. After P_2O_5 was dissolved in the absolute ethanol, the solution was further refluxed for 24 hours, then the resulting species were $PO(OEt)_v(OH)_{3-v}$ ($0 \leq v \leq 2$), in which the fraction of different phosphor species were 0.043 for H_3PO_4 , 0.58 for $PO(OEt)(OH)_2$ and 0.377 for $PO(OEt)_2(OH)$, respectively.

This P-containing solution has been proven to be a stable one and can form a stable sol with Ca-precursor for the fabrication of HA coating.

Following the selection of precursors, a certain amount of calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, Sigma-Aldrich, AR) was dissolved in absolute ethanol giving rise to a 2M Ca-containing solution. Also, a certain amount of phosphorous pentoxide (P_2O_5 , Merck, GR) was dissolved in absolute ethanol to form a 2M P_2O_5 ethanol solution. The P_2O_5 ethanol solution was refluxed for 24 hours to form a clear P-containing solution. Then the two solutions were mixed in the Ca/P molar ratio of 1.67 as the sol for the fabrication of HA coating. Figure 3-2 shows the preparation process of the sol for the fabrication of HA coating.

Figure 3-2 Preparation of the sol for deposition of HA coating

3.2.1.2 Sol for FHA coatings

Many F-containing chemicals can be selected as F-precursor to introduce fluorine ions into hydroxyapatite. According to previous work reported by Cheng et al[17], hexafluorophosphoric acid (HPF_6) could be the better one as F-precursor for the preparation of sol for FHA coating.

Figure 3-3 shows the preparation of sols for FHA coatings. The amount of HPF_6 (Sigma-Aldrich, GR) was determined by the designed degree of substitution (OH^- was substituted by F^-). Then the F precursor was mixed with a certain amount of P-containing ethanol solution. After that, the Ca-containing ethanol solution was added drop-wise into the P-F mixture, while maintaining vigorous stirring, to obtain a Ca/P ratio of 1.67, and the reaction mixture was refluxed for 24 hours to obtain the sol for the fabrication of FHA coating.

Figure 3-3 Preparation of the sols for deposition of FHA coatings

3.2.1.3 Grouping of the sols

In order to study the changes of the properties of FHA ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_{2-x}\text{F}_x$) coatings, sols with different contents of fluorine should be designed for the preparation of FHA coatings containing different fluorine contents, $x=0, 2/6, 4/6, 1, 8/6, 10/6$ and 2.0 . According to the designed theoretical degree of fluoridation (x value), seven different groups of sols were prepared, and the samples were coded as F0, F1, F2, F3, F4, F5, and F6 for further discussion (Table 3-1). One group was prepared for HA coating, and the other 6 groups were prepared for the FHA coatings. The Ca/F molar ratio was used to determine the volume of HPF_6 before the preparation of sol and the concentration of fluorine ions in the prepared sol.

Table 3-1 Designed sols with different fluorine contents for $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_{2-x}\text{F}_x$ coatings

Name	Ca:F:OH	Chemical formula	x value	Remark
F0	30:0:6	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	0	HA
F1	30:1:5	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_{5/3}\text{F}_{1/3}$	$2/6=1/3$	
F2	30:2:4	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_{4/3}\text{F}_{2/3}$	$4/6=2/3$	
F3	30:3:3	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})\text{F}$	$6/6=3/3=1.0$	
F4	30:4:2	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_{2/3}\text{F}_{4/3}$	$8/6=4/3$	
F5	30:5:1	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_{1/3}\text{F}_{5/3}$	$10/6=5/3$	
F6	30:6:0	$\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$	$12/6=6/3=2.0$	FA

3.2.2 Preparation of substrates

In this project, commercial titanium alloy (Ti6Al4V) was selected as substrates for the HA and FHA coatings. Ti6Al4V pieces, with the size of $20 \times 30 \times 1.2\text{mm}$ were cut from a larger piece of Ti6Al4V plate. Before coating deposition, degreasing and sand blasting were carried out to create a clean surface with certain roughness. These substrates were firstly abraded with silica carbide sandpapers (grit range of #180-#1000), and then ultrasonically washed in acetone for 15 minutes, followed by

washing in deionized water for 3 times. Then the substrates were dried and prepared for the dip-coating.

3.2.3 Coating deposition process

A typical process flowchart prepared for the HA and FHA coatings was given in Figure 3-4. Dip-coating method was adopted for the fabrication of HA and FHA coatings on the titanium alloy substrates. The prepared substrates were immersed vertically into the HA/FHA sol and withdrawn at a speed of 3cm/min, and then the as-dipped coatings were dried at 150°C for 15 minutes followed by the further firing at a selected higher temperature—600°C. The deposition run was repeated 4 times in order to achieve the desired coatings thickness.

Figure 3-4 Process flowchart for the deposition of HA/FHA coatings

3.2.4 Coating composition, morphology and structure

3.2.4.1 Chemical composition

X-ray Photoelectron Spectroscopy (XPS), also called ESCA (Electron Spectroscopy for Chemical Analysis), can be used to identify different elements and different chemical states of an element, especially for the surface analysis. It is highly surface specific due to the short range of the photoelectrons that are excited from the samples. The energy of the photoelectrons leaving the samples is determined and this gives a spectrum with a series of photoelectron peaks. The binding energy indicated by the peaks is characteristic of each element. The peak areas can be used to determine the composition of the samples. The shape of each peak and the binding energy can be slightly altered by the chemical state of the emitting atom. The XPS technique can analyze the surfaces of both organic and inorganic materials that are insulating or conductive. In this project, the surface chemical composition and the state of each element, was analyzed by the Kratos-Axis Ultra System. The spectrophotometer employed monochromatic Al K α X-ray source (1486.7eV) operating at 15kV and an ambient pressure of 10^{-9} Torr. The measured binding energy scale of all XPS spectra was corrected by using C1s (BE = 284.8eV).

3.2.4.2 Chemical groups

Fourier Transform Infra-Red Spectroscopy (FTIR) provides the information on structural information and determines what chemical groups are in a specific compound. It identifies different types of chemical bonds in a molecule by producing an infrared absorption spectrum. Chemical bonds in different environments will absorb varying intensities at varying frequencies. IR spectroscopy involves collecting

absorption information and analyzing it in the form of a spectrum. The IR spectrum shows the characteristic O-H, P-O and C-O transmittance bands representing OH, PO₄ and CO₃ groups respectively in the HA and FHA coatings. In this project, the FTIR studies were carried out on a BioRad FTS-3000 spectrometer in the range of 600-4000 cm⁻¹ using transmission mode with a resolution of 4 cm⁻¹.

3.2.4.3 Phase composition

X-ray Diffraction Analysis (XRD) is a non-destructive analysis tool that provides quantitative information on the phases in the coatings. Based on the Bragg equation, the diffraction pattern for monochromatic X-ray as a function of the diffraction angle contains two primary pieces of information: i) the overall pattern of the diffracted peak positions represents the lattice plane spacing, which relates to the specific crystalline phases present; ii) individual peak profiles contain the distribution of d-spacing at that crystallographic (h k l) direction, which in turn is related to compositional or structural distortions of the assemblage of atoms in that plane. Thus the diffraction peaks, line broadening and intensity effects of the patterns were used to characterize the degree of crystallinity, the presence of any other phases, and the changes of the crystal structures.

The system of Philips X'pert 1830 (using monochromatic CuK α radiation) was set at step size 0.02° with scan rate of 1.00°/min. Count rate was taken at 2 θ ranging from 20°-50°. Voltage and current were maintained at 40 KV and 30 mA respectively.

3.2.4.4 Coating morphology, surface roughness and contact angle

LEICA S360 Scanning Electron Microscopy (SEM) was employed to observe the surface morphology and cross-sectional morphology (coating thickness) of HA/FHA coatings. The working current of the filament is ~110 mA with an acceleration voltage of 20 kV. The ImagePro image analysis software was used for the determination of the coating thickness. Along with the surface morphology, surface roughness (Rq) was measured with a non-contact optical profiler (OP, WYKO NT2000, Veeco Instruments Ins. USA). Contact angle was measured using the sessile drop technique with First Ten Angstroms 200 Goniometer.

3.2.5 Interfacial analysis

The chemical composition depth profile near the interface was analyzed by using time-of-flight secondary ion mass spectrometry (ToF-SIMS). An Ar⁺ ion gun with acceleration voltage of 4 kV and filament current of 15 mA was used to etch the samples at a gas pressure of 6.65×10^{-6} Pa. Positive ion mass depth profiling was conducted using time of flight secondary ion mass spectrometry (ToF-SIMS IV, manufactured by CAMECA/IONTOF, Germany) for elemental distribution. The bombarding Ar ion was operated at 3 keV; Ga⁺ impingement beam was powered at 25 keV; Ar sputtering area was set as $300 \times 300 \mu\text{m}^2$ while the Ga⁺ impingement area for analyses was $150 \times 150 \mu\text{m}^2$.

3.3 Mechanical properties

3.3.1 Young's modulus

Young's modulus of the coatings and substrate were determined using nano-indentation equipment (NANO indenter XP, MTS Nano Instruments, USA) with a Berkovich indenter (a three-side pyramid with the same area-to-depth ratio as that of Vicker's indenter). Indentation experiments were performed using a continuous stiffness measurement (CSM) technique. In order to avoid the substrate effect, the coating was indented so that the indentation depth is less than 1/10 of the coating thickness.

3.3.2 Toughness

In present study, a nanoindentation-based energy analysis method, which was developed by Li et al[204, 205], was employed to evaluate the coating fracture toughness (K_{IC}). Theoretically, the energy approach examines the energy difference before and after the cracking, which is responsible for the fracture of the coating. The energy difference would then be referred as the energy release in the through-thickness cracking in the coating. The energy release can be obtained from a "step" that would be observed in the load-displacement curve for the indentation. Therefore, based on the energy difference before and after the crack generation, the fracture toughness of the coating can be determined as [205]:

$$K_{IC} = \left[\frac{\Delta U}{t} \cdot \frac{E_f}{2\pi C_R \cdot (1 - \nu_f^2)} \right] \quad (3-1)$$

where ν_f is the poisson's ration of the coating, $2\pi C_R$ is the crack length in the coating plane, t and E_f are the coating thickness and elastic modulus respectively, ΔU is the strain energy difference before and after cracking.

Nanoindentations were carried out directly using a NANO Indenter XP system (MTS Nano Instruments, USA) with a Berkovich indenter. This instrument monitors and records the dynamic load and displacement of the indenter during indentation process. Indentations and associated cracks were observed using SEM, and the length of cracks of the indentation was analyzed using Image-Pro Plus image measurement software.

3.3.3 Residual stress

A “wafer curvature method” was used to evaluate the residual stress of FHA coatings [206, 207]. On a relatively thin substrate, the residual stress distributed in the coating layer will cause the substrate to bend. The induced curvature for the coated substrate depends on the force, the elastic properties as well as the thickness for both substrate and coating. In the current study, specimens (about $4 \times 4 \text{ mm}^2$) were cut from coated larger samples using a diamond saw. Then the thickness of FHA coated substrate was gently reduced to 0.2-0.3 mm from one side by grinder-polisher (ECOMET 6). More details for the thinning procedures can be found elsewhere [207]. The radius of curvature of the FHA coated substrate was measured by an optical profiler imaging system (WYKO, NT2000, Veeco Instruments Inc, USA) and the thickness of the specimens were determined by micrometer. Then the retained FHA coating was removed carefully. After that, the radius of curvature was measured again as before.

The measured curvature difference before and after the removal of the coating, Δk , is related to the residual stress, σ_R , in the coating, though:

$$\sigma_R = \frac{\Delta k \cdot E_f \cdot [t^2 + (E_s \cdot h_T^3)/(E_f \cdot t) + (E_f \cdot t^3)/(E_s \cdot h_T) + h_T^2 + 3 \cdot (t + h_T)^2]}{6 \cdot (t + h_T) \cdot (1 - \nu_s)} \quad (3-2)$$

where, h_T is the total thickness of the substrate plus coating, t is the coating thickness, ν_s is the Poisson's ratio of the substrate (0.34 for Ti6Al4V), E_s and E_f are the Yong's modulus of the substrate and the coating, respectively.

3.3.4 Adhesion strength

3.3.4.1 Pull-out tensile test

The adhesion strength (note: the term “adhesion strength” or “bonding strength” was used simply to represent the present pull-out test results shortly) of FHA coatings was measured using a Universal Instron mechanical testing system (Instron 5569). In order to avoid the misalignment during uniaxial tensile test, a new set of clamping fixture was designed, as illustrated in Figure 3-5, eight-millimeter diameter Al rods were glued onto the coating surface with an epoxy resin (Epoxy Adhesives DP460, 3M, Scotch-WeldTM, USA) and cured at room temperature for 24 hours. The glued samples were set in a jig (Figure 3-5), and the rods were pulled at a cross-head speed of 1mm/min until the coating layer failed.

On the other hand, in order to investigate the influence of dissolution behavior on the adhesion strength, *in vitro* dissolution tests were firstly carried out by soaking FHA coatings in a Tris-buffered physiological saline solution (0.9%NaCl, pH7.4) at a constant temperature of 37°C for 3 weeks (details, please refer to §3.3.2). After that,

the samples were taken out followed by washing with DI water for 3times, and then were prepared for tensile tests as described above.

After the tensile test, evaluation of the failure mode was done by looking at the fracture surface with SEM analyzing the chemical composition of its surface by EDS. A one-way ANOVA method was used to evaluate the statistical significance of the pull-out adhesion strength of different FHA coatings. In all case, the results were considered statistically different with $p < 0.05$.

Figure 3-5 Schematic illustration of the pull-out tensile test for the evaluation of adhesion strength

3.3.4.2 Scanning scratch test

Scratch tester was used to evaluate the bonding strength between the coating and the substrate as well as the mechanical durability of the coating surface by pressing a spherical indentator to the coating using a diamond cone as the stylus. Scratch tester (SHIMADZU, SST-101) was used in this project mainly for the determination of bonding strength at the interface between the HA/FHA and the titanium alloy

substrate. To measure adhesive strength of the coatings, some specific setup parameters were selected, as shown in Table 3-2.

Table 3-2 Parameters used in scanning scratch testing

Tip radius of stylus	15 μ m
Full scale load	1000mN
Down speed	2 μ m/s
Scanning amplitude	50 μ m
Scratch speed	2 μ m/s

3.3.4.3 Evaluation of interfacial shear strength

Given the testing constraints imposed by the thickness of sol-gel derived coating, the well developed shear lag strain method by Agrawal and Raj [208, 209] was used to evaluate the interfacial shear strength between the coating and the substrate. Basically, this method relies on the development of transverse crack patterns in a brittle coating when the relatively ductile supporting substrate is plastically deformed under an applied uniaxial load (as shown in Figure 3-6). This crack behavior can be adequately described by a shear-lag analysis that directly relates crack density to the load transfer capabilities of the interface, and also this shear lag theory predicts the establishment of a steady state of constant crack density observed at relatively high strain levels [210]. For a coating of thickness t , the interfacial shear strength $\tau_{\max}$ can be determined by a simplified expression [208]:

$$\tau_{\max} = \sigma_f \cdot \frac{\pi \cdot t}{1.5\lambda} \quad (3-3)$$

where, λ is the average steady-state crack spacing and σ_f is the tensile strength of the film, i.e., the coating. σ_f can be determined experimentally by measuring the

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maximum elastic strain, ε_f , of the coating (at which the initial formation of cracks is detected):

$$\sigma_f = \varepsilon_f \cdot E_f \quad (3-4)$$

where, E_f is the Young's modulus of the coating. In current study, FHA coatings were deposited on I-shape Ti6Al4V substrates with a gage length (G) of 18 mm and 4mm in width (W) (as shown in Figure 3-7). FHA coated specimens were pulled uniaxially using a Universal Instron mechanical testing system (Instron 5569) operated under the selected stain control model with a strain gauge attached to monitor the precise strain levels.

Figure 3-6 Crack density, or the inverse of transverse crack spacing λ , as a function of strain; an immediate increase in crack density is observed above the failure strain of the coating, ε_f , which can be experimentally defined as shown. By shear lag method, a steady-state or constant crack density is expected at higher strain levels.

To obtain the maximum elastic strain ε_f , specimens were pulled sequentially to an increment of 0.2% strain, followed by SEM (LEICA S360) examination of random areas within the central gauge region to detect cracks that may have formed. ε_f was defined as the strain level at which a transverse crack could be routinely detected by

SEM in a random area at a fixed magnification. Larger strain increments were employed to obtain steady state of crack density and to evaluate the crack spacing. Based on the SEM micrographs taken at random areas within the gauge region with a fixed magnification (e.g. 3000 $\times$), a systematic collection of crack spacing data for each specimen and each strain level was conducted using Image-Pro Plus image measurement software. Here, crack spacing was defined as the distance between two successive transverse cracks [210]. Average steady state crack spacing is obtained by routine statistical analysis and then used to calculate the interfacial shear strength.

Figure 3-7 Schematic diagram of the geometry of the prepared Ti6Al4V substrate for shear strength evaluation. G and w are the gage length and width of the substrate, respectively.

3.4 *In vitro* investigations

3.4.1 Bioactivity in acellular solutions

As mentioned in §2.2.2.4.1, conventional simulated body fluid (C-SBF) with ion concentrations nearly to those of human blood plasma is widely used to evaluate bioactivity of various implants *in vitro*. Here, Kokubo's SBF (C-SBF) solution was also used to test bioactivity of the prepared FHA coatings on Ti6Al4V substrates.

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The C-SBF solution was prepared in house according to Kokubo's protocol by dissolving appropriate quantities of the related reagent-grade chemicals in deionized water [110]. Sodium chloride, NaCl; sodium hydrogen carbonate, NaHCO₃; potassium chloride, KCl; di-potassium hydrogen phosphate trihydrate, K₂HPO₄•3H₂O; magnesium chloride hexahydrate, MgCl₂•6H₂O; calcium chloride, CaCl₂; hydrochloric acid, HCl (1M); and tri-hydroxymethyl aminomethane, NH₂C(CH₂OH)₃. Reagents were added, one by one after each reagent was completely dissolved in the deionized water according to the order given in Table 3-3. After all the reagents were added and dissolved, the solution was then raised to 37°C and maintained at this temperature while titrating the solution to a pH of 7.4 with 1M HCl or NH₂C(CH₂OH)₃. During the titration process, the solution was also continuously diluted with consecutive additions of deionized water to make the final designed volume. Finally, the obtained C-SBF solution was kept in a polyethylene or polystyrene bottle and stored at 4-8°C (if precipitations are found in the solution, it can not be used anymore).

Table 3-3 Reagents for preparing simulated body fluid (37°C, pH 7.40, 1L)

Reagent	Amount of Reagent	Order of Addition
NaCl	7.996g	1
NaHCO ₃	0.350g	2
KCl	0.224g	3
K ₂ HPO ₄ •3H ₂ O	0.228g	4
MgCl ₂ •6H ₂ O	0.305g	5
1M-HCl	40ml	6
CaCl ₂	0.278g	7
Na ₂ SO ₄	0.071g	8
NH ₂ C(CH ₂ OH) ₃	6.057g	9

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However, as shown in Table 3-4, actual body fluid contains not only the inorganic components but also various kinds of organic components (such as carbohydrates and proteins) and these organic components would exert noticeable influence on the implants. Therefore, in order to study the effect of organic components on the bioactivity of HA and FHA coatings, another two kinds of organic-containing SBF were prepared. In this project, bovine serum albumin (BSA, Merck) and glucose (D(+)-Glucose anhydrous, Merck) were selected respectively for the preparation of protein and carbohydrate-containing SBF solutions. The glucose modified SBF solution (G-SBF) and BSA modified SBF solution (A-SBF) were obtained by dissolving 1 g/L for glucose and 40 g/L for BSA respectively in the prepared SBF solution. These organic-modified SBF solutions were adjusted to a pH value of 7.4 at 37°C, and were stored at 4-8°C.

Table 3-4 Chemical composition of human blood plasma compared to the ion concentration of Kokubo's SBF (C-SBF) [110, 211]

	Inorganic ion concentration (mM)								Organic composition (mg/dL)			
	Ca ²⁺	HPO ₄ ²⁻	Na ⁺	Cl ⁻	Mg ²⁺	K ⁺	HCO ₃ ⁻	SO ₄ ²⁻	Albumin	Globulin	Fibrinogen	Glucose
Blood plasma	2.5	1	142	103	1.5	5	27	0.5	3300-4000	880-3530	340-430	100
Kokubo's C-SBF	2.5	1	142	148	1.5	5	4.2	0.5	/	/	/	/

The coatings were placed in sterilized bottle containing solution with a liquid/area ratio of 50 ml/cm². Before soaking in the solution, the samples were washed ultrasonically in acetone for 10 min, and then sterilized in ethanol. The soaking took place in a temperature-controlled shaking water bath for various periods of 2, 4, 7, 14, 21 and 28 days at 37°C±0.1°C. After the desired immersion, the samples were taken out, gently washed with deionized water and dried at room temperature before the

effect characterization. After the testing, both solutions and samples were kept for posterior analysis.

Scanning electron microscopy (SEM, LEICA S360) was used to examine the surface morphology changes that occurred after the immersion in these three kinds of simulated solutions. The Ca^{2+} ion concentration of the each solution was measured with inductively coupled plasma atomic emission spectrometer (ICP-AES, PerkinElmer Optima 2000). An average of three measurements was taken for each sample.

3.4.2 Dissolution behavior

The dissolution behavior of the FHA coatings was firstly investigated by soaking in a Tris-buffered physiological saline solution (TPS, 0.9%NaCl, pH7.4) at a constant temperature of 37°C for designed periods of time (up to 21 days). As mentioned in Table 3-4, besides the inorganic components, there are a lot of organic components in blood plasma. Therefore, in order to study the influences of such organic components on dissolution behavior of FHA coatings, bovine serum albumin (BSA, Merck) and glucose (D(+)-Glucose anhydrous, Merck) were selected respectively for the preparation of protein and carbohydrate modified TPS solutions (G-TPS and B-TPS). The amount of glucose and BSA added in TPS were the same as in SBF. The immersion processes for all FHA coatings were carried out in the same way for these three testing solutions.

At end of the period, the sample was taken out and the concentration of Ca^{2+} in the solution was analyzed with inductively coupled plasma atomic emission spectrometer

(ICP-AES, PerkinElmer Optima 2000) to monitor the dissolution behavior of these FHA coatings.

3.4.3 Cell responses

3.4.3.1 Cell and culture conditions

Human osteosarcoma MG63 cells obtained from American Type Culture Collection (ATCC, Rockville, USA) were used to assay the osteoblastic cell response on coating surface. The MG63 cell line was originally derived from a human osteosarcoma, and this cell line consistently and reproducibly exhibits a number of fundamental phenotypic characteristics of osteoblasts, enabling comparisons to be made between different experiments without the inevitable wide variation of primary bone cells obtained from different cell sources and of different passage in tissue culture [212, 213].

In general, most systems currently in use share routine basic culture environments and methods of maintenance. In this project, osteoblastic cells (MG63) are routinely grown in monolayer culture in standard culture flasks (subculture) or on the samples 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). Incubation conditions are maintained at 37°C in a humidified atmosphere with 5% CO₂ in an incubator (Hera Cell, Heraeus). For cell assay, the FHA coated samples were cut into small size (10×10×1 mm) and sterilized in an autoclave at 121°C for 20 minutes and cultured in 24-well tissue-culture test plates to observe cell morphology, proliferation and differentiation.

3.4.3.2 Protein adsorption onto FHA coatings

To evaluate protein adsorption onto FHA coatings, smaller samples (10×20 mm) were cut from the prepared coatings, and ultrasonically washed in acetone for 20min followed by sterilizing in an autoclave at 121°C for 20 minutes. Then the samples were soaked in culture medium at a constant temperature of 37°C for 20 minutes. After that, the samples were gently rinsed in deionized water for 3 times and then immersed separately in 4 ml 0.1M EDTA-disodium salt solution for 5hours at 37°C. Each sample received two more washes in the same EDTA solution (4 ml) for 1hour at 37°C. The solution was saved separately. The quantitative measurement of adsorbed proteins was conducted using a commercial Micro BCA protein assay kit (PIERCE, USA). All the experimental processes were carried out strictly according to the protocol. By using the microplate assay mode, the measurement was read at 570 nm in a microplate reader and the amount of adsorbed proteins was calculated from a standard curve. Each set of tests was performed in triplets, Statistical analysis was carried out using one-way analysis of variance (ANOVA) and statistically significant was considered at $p<0.05$.

3.4.3.3 Initial cell attachment and spreading

For cell attachment assay, the FHA coated samples were cut into small size (10×10×1 mm) and sterilized in an autoclave at 121°C for 20 minutes and cultured in 24-well tissue-culture test plates. Cells were seeded on the sterilized coating surface at a density of 4×10^5 cells/ml and cultured for up to 4 hours. At each culture period (0.5, 1, 2 and 4 hours), the samples were taken out and removed to new 24-well tissue-culture plates. After being washed 2 times with phosphate buffered saline solution (PBS), cells were detached with trypsin/EDTA and stained with trypan blue followed by

counting the living cells using a haemocytometer (Becton Dickinson, Germany). Statistical analysis was carried out on cell counting using one-way analysis of variance (ANOVA) at an average of 3-5 replicates and significance was considered at $p < 0.05$.

Scanning electron microscopy (SEM) was employed to examine the morphology of initially attached and spread MG63 cells. For this purpose, cells were seeded on the prepared FHA samples (10×10 mm) at cell density of 3×10^4 cells/ml and incubated for 0.5, 1, 2 and 4 hours. At each culture period, cells were washed with PBS for two times, then fixed with 1.5 ml 2.5% glutaraldehyde for 1 hour at room temperature followed by dehydrating with a series of graded ethanol/water solutions (50%, 70%, 80%, 95% and 100% respectively). Then hexamethyldisilazane was added in each well to preserve the original morphology of the cells. The test plates were kept in a fume hood to dry at room temperature. The samples were coated with gold (30s) before observation under SEM (JEOL, JSM-5600LV) operating at 10 kV.

3.4.3.4 Cell morphology

A cell density of 5.3×10^3 cells/ml was used to observe cell morphologies on FHA coatings (10×10×1 mm) after an incubation of 3 days. The attached cells on the samples were rinsed twice with PBS, and then were fixed with 1.5 ml 2.5% glutaraldehyde for 1 hour at room temperature. After washing again with PBS, dehydration was performed by immersing the samples in a series of graded ethanol/water solutions, starting with 50%, followed by 70%, 80%, and 95% respectively [214]. The final dehydration was done in absolute ethanol for half an hour. Then a mount of 0.5ml hexamethyldisilazane was added in each well to

preserve the original morphology of the cells, the test plates were kept in fume hood to dry at room temperature. After gold coating (30s), cell morphology was observed under scanning electron microscope (SEM, LEICA S360) at an accelerating voltage of 10 kV.

3.4.3.5 Cell proliferation

Cells were seeded on the sterilized coating surface (10×10×1 mm) at a density of 4.8×10^4 cells/ml and cultured for up to 7 days. At each culture period (1, 2, 3, 5 and 7 days), the samples were taken out and removed to new 24-well tissue-culture plates. After being washed 2 times with phosphate buffered saline solution (PBS), cells were detached with trypsin/EDTA and stained with trypan blue followed by counting the living cells using a haemocytometer (Becton Dickinson, Germany). Total of 5-7 counts were carried out for each sample and the average value was taken as the number of cells grown on the surface of FHA coatings. Statistical analysis was carried out using one-way analysis of variance (ANOVA) and significance was considered statistically at $p < 0.05$.

3.4.3.6 Differentiation assay

To evaluate the functionality of the cultured cells on FHA coatings, intracellular alkaline phosphatase (ALP) activity and osteocalcin (OC) expression were analyzed. Cells were seeded on each sample at a density of 6×10^4 cells/ml and cultured for 7, 14 and 21 days with weekly change of culture medium. At harvest, all cell layers were washed 2 times with PBS and then detached from the coating surface with trypsin/EDTA. After centrifugation, the cell pellets were washed with DI-water for 2

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times and finally re-suspended by vortexing in 0.6 ml lysis buffer solution (1% Triton X-100 in Tris HCl buffer, pH7.4). Following one cycle of freezing/thawing process, cells were further lysed by sonicating them for 5 minutes at 4°C. Finally, after being centrifuged at 10000 rpm for 5 minutes at 4°C, the supernatants were collected as cell lysates for the assay of alkaline phosphatase level, osteocalcin concentration as well as total protein synthesis.

The ALP activity was measured using the phosphate detection kit (BIOMOL GREEN, USA). All the experimental processes were strictly conducted under the guidance of the provided protocol. By using the microplate assay mode, the measurement was read at 620 nm in a microplate reader and the ALP activity was calculated from a standard curve.

The quantitative measurement of osteocalcin in the cell lysates was conducted using an immunoenzymatic assay (EASIA ELISA kit, BioSource, USA). The experimental process was carried out thoroughly and strictly on the directions of the manufacture. The absorbance was measured using micropalater reader at 405 nm against a reference filter set at 620 nm and the OC expression level was evaluated based on a standard curve.

The measured alkaline phosphatase activity and osteocalcin concentration were finally normalized to the total protein content, which was determined using a commercial Micro BCA protein assay kit (PIERCE, USA).

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Statistical analysis was carried out on ALP and OC levels using one-way analysis of variance (ANOVA) at an average of 3-5 replicates. Differences were considered statistically significant at $p < 0.05$.

Chapter 4 Results and Discussion

FHA coatings on titanium alloy substrates were fabricated by a dip-coating method using different sols with designed compositions in Chapter 3. In this chapter, the experiment results will be presented in three parts followed by corresponding discussions. The first part, §4.1, will be focused on the chemical and physical properties of the FHA coatings, including coating chemical compositions, chemical groups in the coatings, phases and purity, surface and interface analysis etc.; the second part, §4.2, will be concentrated on the analysis of mechanical properties, which contains the aspects of residual stress, adhesion strength, and coating toughness etc; in the last part, §4.3, the *in vitro* behaviors of FHA coatings will be presented, including dissolution testing, bioactivity testing in acellular solutions, and osteoblastic cell responses to the FHA coatings.

4.1 Chemical and physical properties

4.1.1 Chemical composition

First of all, the status of fluorine ions in prepared FHA coatings is the most concerned issue. Generally, only two possibilities can account for the presence of fluorine ions in the coatings: a) fluorine ions could be offset with calcium cations to form CaF_2 as a separate phase in the coatings. That is to say, the resulting coating should be composed of two different mineral compounds such as CaP and CaF_2 . b) Fluorine ions could be incorporated into the HA lattice structure by replacing OH groups to form fluoridated HA coatings. These two possibilities for the chemical status of

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fluorine ions could be well discerned by XPS. The XPS spectra of fluorine (F1s) in FHA coatings are analyzed and the results are shown in Figure 4-1. The F1s peak is evident in the wide scan (Figure 4-1 a), and with increasing fluorine concentration in the sols, an increasing intensity of F1s peak is observed, indicating that more fluorine ions are incorporated into the coating. XPS narrow scan (Figure 4-1 b) reveals only one peak located at 684.3 eV belonging to F1s [215, 216], which is the fingerprint of fluorine in FHA or FA structure, indicating that fluorine ions have been successfully incorporated into HA lattice structure. Furthermore, by considering that the binding energy of F1s in CaF_2 is about 685.5 eV [217, 218] and the F1s peaks in Figure 4-1b are strictly symmetric, it is reasonable to conclude that there has no CaF_2 in all prepared FHA coatings, or its amount is too small to be detected by XPS (the resolution of XPS is about 0.1 at%).

Figure 4-1 XPS patterns of FHA coatings: a) Survey scan; b) F1s narrow scan. There is only one peak located at 684.3eV belong to F1s, which is the fingerprint peak of fluorine in FHA or FA structure. With increase of fluorine concentration in the prepared dipping sols, the F1s peak intensity also increasing, indicating more fluorine ions incorporated into HA lattice structure.

On the other hand, in the recorded XPS narrow scan spectra of FHA coatings (as shown in Figure 4-2), all the HA and FHA coatings have the same peaks of Ca2p and P2p respectively (the peak positions). The binding energy values for those peaks of $\text{Ca}2p_{1/2}$, $\text{Ca}2p_{3/2}$ and P2p are located at 350.7, 347.2 and 133.4 eV respectively, which

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correspond well to those typical values of Ca2p and P2p in literature for HA and FHA [100, 219]. In our present coating preparation process, besides the existence of Ca cations in HA or FHA lattice structure, another most possible state is in the form of CaF_2 . As reported, the $\text{Ca}2\text{p}_{3/2}$ has a binding energy of 348.3 eV in CaF_2 , which is about 1 eV higher than that of HA (or FHA) [218]. According to the XPS results (Figure 4-2a), there are only two distinguished peaks belonging to $\text{Ca}2\text{p}_{1/2}$ (350.7 eV) and $\text{Ca}2\text{p}_{3/2}$ (347.2 eV) respectively, indicating that there is only one kind of chemical bonding status for Ca in the coatings. This result is in accordance with the F1s narrow results (no CaF_2 in FHA coatings). As for the P2p (Figure 4-2b), there is only one distinguished peak located at 133.4 eV, indicating that P is in the form of PO_4^{3-} in the coatings (typical value in HA and FHA).

Figure 4-2 XPS narrow scan spectra for a) Ca2p and b) P2p of different FHA coatings. For Ca2p, there are two peaks located at 350.7 eV and 347.2 eV respectively, which are the typical binding energy values of $\text{Ca}2\text{p}_{1/2}$ and $\text{Ca}2\text{p}_{3/2}$ peaks in HA and/or FHA. As for P2p, only one distinguished peak located at 133.4 eV, which is in good agreement with those reported values in HA and FHA.

The coating compositions, i.e. the concentration of Ca, P and F in FHA coating, are determined by ratio of the area under the respective elemental peak in XPS narrow scan spectrum. As such, the measured Ca/P ratio is in the range of 1.63~1.70 (as shown in Table 4-1), which is close to the stoichiometric value of 1.67. The Ca/F

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molar ratios as well as the atomic percentage of fluorine (at%F) in FHA coatings are also calculated and listed in Table 4-1. On the other hand, Figure 4-3 also shows the measured fluorine ion concentration in the coating (indicated by capital X) in comparison with the designed value (indicated by small x , c.f. Table 3-1). The dash line shows the ideal situation where all fluorine designed in the dipping sols completely incorporates into HA lattice. The data points are the measured fluorine concentrations in the deposited FHA coatings. Although fluorine ion concentration measured in the coating (X value) increases with the increase of designed Ca/F value (x value) in the dipping sols (Table 3-1, Table 4-1 and Figure 4-3), a discrepancy is observed between the designed x value and the measured fluorine incorporation, especially at high concentration, which is attributed to the loss of fluorine ions in the form of HF during sol-gel dip-coating process [220].

Table 4-1 Ca/P, Ca/F molar ratio and at%F of FHA coatings

	F0	F1	F2	F3	F4	F5	F6
Ca/P	1.65	1.64	1.66	1.63	1.63	1.70	1.67
Ca/F	\	30.84	15.33	10.91	8.04	6.64	6.00
at%F	0	0.64	1.36	1.96	2.44	3.07	3.29

Figure 4-3 Fluorine concentration in FHA coatings: there is a discrepancy between the designed x value and the measured fluorine incorporation (X value), especially at high concentration, which is attributed to the loss of fluorine in the form of HF during sol-gel dip-coating process.

4.1.2 Chemical groups

Figure 4-4 reports the FTIR results of different FHA coatings. In the range of 1000-1150 cm^{-1} , there are 3 peaks at 1010 cm^{-1} , 1082 cm^{-1} , and 1120 cm^{-1} related to the triply asymmetric ν_3 stretching mode of phosphate group (PO_4); another one peak located at about 961 cm^{-1} represents the ν_1 symmetric stretching vibration of PO_4 (Figure 4-4a). These PO_4 peaks existing in the coatings indicate the formation of the apatite structure [194, 221]. Those ν_1 and ν_3 bands of phosphate vibrations in fluoridated HA coatings are very close to those of pure HA (F0) coating (the resolution of FTIR equipment is 2-4 cm^{-1}), indicating that the phosphate environment is very similar for HA and fluoridated HA coatings. On the other hand, in the spectra (Figure 4-4a), two bands recorded between 1400 and 1500 cm^{-1} are assigned to the ν_3 stretching vibration mode of carbonate (CO_3), and another weak vibration situated at around 872 cm^{-1} is due to the ν_2 mode of carbonate groups (CO_3) [57, 169]. The presence of carbonate groups in the coatings is reasonable considering the coating preparation processes.

Since the incorporation of fluorine ions is achieved by replacing OH groups in HA lattice structure, the vibration of O-H must be influenced by the incorporated fluorine ions. Figure 4-4b illustrates the changes occurring in the O-H stretching region at around 3500-3600 cm^{-1} . There is only one vibration band located at about 3573 cm^{-1} for pure HA coating (F0) due to the stretching of O-H in an infinite OH-chain (c.f. Figure 2-6) [169], while passing to coating F1, the introduction of fluorine ions (F1 in Figure 4-4) causes a new vibration band to appear at about 3545 cm^{-1} , and the peak at 3573 cm^{-1} for HA on the other hand, slightly shifts to 3570 cm^{-1} with the incorporation of fluorine ions (Figure 4-4b). It is believed that the new-formed peak at

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3545 cm^{-1} is caused by the direct influences of incorporated fluorine ions on the stretching vibration of OH groups through the hydrogen bonds along OH channel (c-axis, c.f. §2.2.1 and §2.3.1.1), i.e. -OH-F- [218, 222]. The shift of the peak at 3573 cm^{-1} to lower wavenumber (3570 cm^{-1}) is attributed to the influences of incorporated fluorine ions on those OH groups which are not directly adjacent to F^- (c.f. §2.3.1.1) [222].

Figure 4-4 FTIR spectra of FHA coatings: a) there are 3 peaks at 1010cm^{-1} , 1082cm^{-1} , and 1120cm^{-1} are attributed to the triply asymmetric ν_3 stretching mode of phosphate group (PO_4); another peak located at about 961cm^{-1} represents the ν_1 symmetric stretching vibration of PO_4 . b) The incorporation of fluorine ions causes significant influences on the stretching vibration band OH groups (located at 3573cm^{-1} for HA).

The intensity and position of those two vibration bands depend strongly on the degree of fluoridation. As shown in Figure 4-4b, with the increase of fluoridation degree, the peak intensity of 3570 cm^{-1} is reduced drastically (F2), and even can not be detected (disappeared) for those coatings with higher fluoridation degree (F3, F4, F5 & F6), thus indicating that all of the OH groups are linked to the fluorine ions through hydrogen bonds. As for the vibration band at 3545 cm^{-1} (F1 in Figure 4-4), its position shifts to a lower wavenumber when more fluorine ions are incorporated (about 3542

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cm^{-1} for F2 and 3540 cm^{-1} for other FHA coatings). On the other hand, this band at 3545 cm^{-1} firstly increases in intensity with increasing fluorine concentration (from F1 to F2), then decreases gradually with further increase of fluoridation degree (for F3, F4, F5 & F6). This indicates that: at lower fluoridation degree (F1 & F2), more incorporated fluorine ions (from F1 to F2) will form more hydrogen bonds with adjacent OH groups, resulting in the initial increase of peak intensity; while at higher degree of fluoridation (F3-F6), the content of OH will be reduced with more incorporated fluorine ions, leading to the gradual decrease of peak intensity. Theoretically, a fluoridation degree of 0.67 (x value) is the critical value to ensure that all of the OH groups could form hydrogen bonds with the adjacent fluorine ions (-OH-F-HO-), and hence the peak at 3570 cm^{-1} will disappear [222]. In this present study, this result shows that the fluoridation degree (X value) should be between 0.6 (F2) and 0.8 (F3), which is in consistent with the theoretical value. In addition, it is reported that the vibration band at 3540 cm^{-1} will eventually vanish when all OH groups are substituted by F^- [194]. While, in our study, there still exists a small peak in F6 coating, indicating an incomplete replacement of OH groups due to the loss of fluorine ion during coating preparation process (c.f. §4.1.1).

4.1.3 Phases and purity

Phase composition of FHA coatings deposited on titanium alloy substrates were investigated using XRD analysis. As shown in Figure 4-5, all of the prepared FHA coatings have the similar diffraction patterns, and the characteristic peaks, i.e. (002), (211), (112), (300) etc, are roughly identical to those of the reference HA structure (JCPDS file card #9-432), indicating the formation of HA (F0) and FHA phases in the prepared coatings. Besides the peaks of HA/FHA and the substrates, there are no

other phases, such as CaF_2 , TCP, CaO etc, that could be observed in these patterns, suggesting a high purity of HA or FHA phases obtained by the current sol-gel dip-coating method. This also can be proved by the XPS analysis (c.f. §4.1.1). Although some of the resulting Ca/P ratios (Table 4-1) show a slight deviation from the stoichiometric values of the pure HA or FHA, the amounts of those phases maybe too small to be detected by XRD scanning analysis.

Figure 4-5 XRD patterns of prepared FHA coatings. The characteristic peaks of FHA structures, i.e. (002), (211), (300), could be observed in all diffraction patterns, indicating the formation of typical FHA structure. In addition, no other impurity phase can be observed in all the XRD patterns. The dot line indicates the standard peak position of HA in JCPDS file card #9-432.

There are some differences between pure HA (F0) and fluoridated HA coatings observed from these XRD diffraction patterns due to the incorporation of fluorine ion into HA lattice structure (c.f. §2.3.1.1). As shown in Figure 4-5, all the reflection peaks of fluoridated HA coatings ($X > 0$) are much clearly (indicated by peak intensity) shown in the XRD patterns in comparison with that of pure HA coating, especially for the peaks of (002) and (202) in the coatings with higher fluoridation degrees (F4-F6). Such change (peak intensity) of those reflection peaks is mainly attributed to the increase of crystallinity of FHA coatings. The calculated result indicates that with the

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increase of fluoridation degree, the crystallinity of the coating increases gradually from ~51.2% for pure HA coatings to ~55.5% for F4 and ~60.8% for F6. Similar results regarding the enhancing effect of incorporated fluorine ions on crystallinity are also reported in documents. On the other hand, according to the XRD patterns (Figure 4-5), the (211) and (300) peaks of F6 coating are shifted toward a high 2θ relative to that from F0 (HA) coating (the peak located at 31.8° and 32.9° , respectively), while the (211) and (300) peaks of other FHA coatings are intermediate between the two, but closer to that of F6. The (002) peak is not significantly changed.

The incorporation of fluorine ions into HA lattice is also analyzed from the shift of the characteristic diffraction peaks. The shifts in (211) and (300) peaks reflect the change in lattice parameters of HA lattice structure, i.e. the value of *a*-axis and/or *c*-axis [168-170]. With the obtained XRD diffraction patterns (Figure 4-5), the peaks were indexed based on the hexagonal unit and the cell parameters were calculated from refining the diffraction patterns. The calculated cell parameters (*a*-axis and *c*-axis) of all the prepared coatings are presented in Figure 4-6 respectively. The *a*-axis parameter decreases gradually from 0.9418 nm for HA down to about 0.9375 nm for F6 (Figure 4-6a), while a similar value (~0.6882 nm) in *c*-axis parameter is calculated for all prepared FHA coatings (Figure 4-6b). Similar results were also reported in other previous researchers' studies (c.f. §2.3.1.1) [3, 169]. The decrease in *a*-axis parameter for those fluoridated HA coatings with increasing fluoridation degree is attributed to the substitution of the smaller fluorine ion (0.132 nm) for the larger hydroxyl ion (0.168 nm) in the channel along *c*-axis in the apatite structure. On the other hand, the shift of those related characteristic diffraction peaks towards higher diffraction angle and the calculated contraction in *a*-axis parameter also imply that

fluorine ions have been successfully incorporated into the apatite structure. In comparison with the standard *a*-axis lattice parameter of pure FA (0.9368 nm), F6 possesses a little larger calculated *a*-axis value (0.9375 nm), indicating that not all the fluorine ions in the designed sols have been incorporated into the coating, which is in accordance with the chemical composition analysis in §4.1.1.

Figure 4-6 Changes in a) *a*-axis and b) *c*-axis cell parameters, respectively, as a function of degree of fluorination for the FHA coatings. With increasing fluorine content incorporated into the HA structure, there is a decrease for *a*-axis value while *c*-axis maintains a constant value.

4.1.4 Surface and interface

The SEM micrographs of different FHA coatings on titanium alloy substrates are shown in Figure 4-7. All the coatings are homogeneous with a few micropores, and even some micro cracks. The presence of such micropores and micro cracks is resulted from the evaporation and/or decomposition of the solvent as well as the change of surface tension during drying and firing processes. The fluorine concentration in the dipping sols plays an important role on the surface properties of the prepared FHA coatings. As can be seen in Figure 4-7a-d, the coatings tend to gain rougher surface with increasing fluorine ion content. Coating surface roughness (*R_q*) measured with optical profiler indicates a slight increase with increasing fluorine ion

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concentration in the dipping sols (as shown in Figure 4-8), attributing to the formation of nc-CaF₂ in the dipping sols during coating preparation process.

Figure 4-7 Surface morphology of FHA coatings: a) F0, b) F2, c) F4, d) F6. The surfaces of all FHA coatings are dense and uniform. With the incorporation of more fluorine ions, the coating tends to get a rougher surface.

On the other hand, the incorporation of fluorine ions can also change the surface energy of FHA coatings. As indicated by measured contact angle, with increase in fluorine ion concentration, the contact angles on FHA coatings gradually increase from 50.5° on F0 (HA) to 55.5° on F6 (as shown in Figure 4-8). Though the surface roughness may have influence on the coating wettability, the increase in contact angle should be attributed mainly to the incorporation of fluorine ions, as fully fluoridated HA ($x=2$, FA) has a much higher contact angle (62°) than that of HA (49°) [223]. Some other researchers also found the same variation trend regarding the contact

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angle of fluoridated HA and explained it from the standpoint of influences caused by incorporation of fluorine ions [224]. On the other hand, according to Vogler's definition of a hydrophobic surface (contact angle $>65^\circ$) [225], all of the fabricated FHA coatings in this study are in the range of hydrophilic.

Figure 4-8 Measured surface roughness (Rq) and contact angle of different FHA coatings. The coating roughness (Rq) shows a slight increase with the incorporation of fluorine ion; similarly, with increasing in fluorine ion concentration, the contact angle on FHA coatings gradually increased from 50.5° on F0 to 55.5° on F6.

For all prepared FHA coatings, approximately the same coating-substrate interface morphologies are observed. Typically, the cross-section morphologies are shown in Figure 4-9 (for F0 and F6 coatings). As indicated in Figure 4-9, these coatings are dense and uniform with a thickness of about $1.5\ \mu\text{m}$. Moreover, it seems that those coatings bond well with the substrates, and there is no delamination or mismatch at the interface, suggesting an improved chemical and mechanical affinity between the coating and substrate.

Figure 4-9 Cross-section morphology of HA and FHA coatings: a) F0, b) F6. The cross-section micrographs show that the coatings are dense with a thickness of about 1.5 μm.

In order to get the information about the distribution of elements at/near the interface between the coating and substrate, SIMS composition depth profiles analysis was carried out and the typical results were shown in Figure 4-10. As for F0 (HA) coating (Figure 4-10a), according to the distribution of the concerned elements along the cross section, Ca, O, P and Ti, the cross section can be divided into three regions at/near the interface: the substrate (Rs) region, transitional region (Rt), and the coating region (Rc). The transitional region (Rt) has a thickness of about 85 nm and within the transitional region, the Ca and P concentration decrease drastically towards the substrate, while O element decrease gradually from the coating region (Rc) to the substrate region (Rs). While the concentration of Ti element increases gradually from the transitional region (Rt) to the substrate region (Rs). It can be speculated from the existence of transitional region and the distribution of the elements along the cross section that certain Ti-P-Ca-O compounds have formed at/near the interface [58, 100]. Similar to that of F0 (HA) coating, as expected, also three regions can be observed for the cross section analysis of F6 coating with respect to the element (Ca, P, F, O and Ti) distribution along the coating cross section (Figure 4-10b). Ca and P have a

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significant decrement in the Rt region near to the coating region while the O and F concentrations decrease gradually from Rc region to the substrate (Rs), and F has a similar changing rate to that of O. Similar to that of F0, the concentration of Ti also increases from the transitional region (Rt) to the substrate region (Rs). Comparing the depth profiles of F0 (HA) and F6, it can be observed that with the incorporation of fluorine ions, the thickness of transitional region (Rt) increases from about 85 nm for HA coating to about 175 nm for F6 coating. That is to say, the presence of fluorine ions can attract more O into the transitional region to form some kind of Ca-P-F-O-Ti complex chemical compounds [220].

Figure 4-10 SIMS depth profile analysis at the interface: a) F0 and b) F6. Three regions can be divided for the cross sections of these coatings: the coating region (Rc), a transitional region (Rt), and the substrate region (Rs). Within the transitional region, the Ca and P concentration decrease drastically from the coating towards the substrate, while the O and/or F concentration decrease gradually towards the substrate; and Ti concentration increase gradually from transitional region to the substrate. In comparison with HA coating (a), the incorporation of fluorine ions results in a larger transitional region thickness (b).

4.1.5 Discussion

The prepared $\text{Ca}(\text{NO}_3)_2\text{-PO}(\text{OEt})_{3-v}(\text{OH})_v\text{-OH}(\text{OEt})$ sol was used for the deposition of HA coating. Because the decomposition temperature of NO_3 is about 561°C [226], it is reasonable that all NO_3 groups have been decomposed at the selected firing temperature of 600°C in the present study, and a pure HA phase could be expected. As shown in Figure 4-4, no peaks belong to NO_3 groups can be observed indicating all of them have been removed during the firing process.

For the deposition of fluoridated hydroxyapatite coatings, HPF_6 was selected as the fluorine containing reagent and was added into the above sol ($\text{Ca}(\text{NO}_3)_2\text{-PO}(\text{OEt})_{3-v}(\text{OH})_v\text{-OH}(\text{OEt})$) according to the designed degree of fluoridation. During the refluxing process (c.f. §3.1.1.2), HPF_6 is hydrolyzed and alcoholized, and the fluorine ions gradually released through hydrolysis/alcoholysis immediately react with Ca ions in the solution to form nano-crystalline CaF_2 particles (nc- CaF_2) [17]. The violent boiling in the solution during the fluxing effectively prevents the growth of such nc- CaF_2 particles, and thus the stable dipping solutions will be obtained. Such nc- CaF_2 (about 12 nm) in the dipping solutions will agglomerate into clusters with a size of about 100 nm [199]. It has been reported that after refluxing, no F signal could be detected with liquid NMR, indicating that all the fluorine ions have transferred from the liquid phase into solid phase, i.e. from HPF_6 to nc- CaF_2 [17, 199]. Accordingly, the subsequent reactions with F involved for the preparation of fluoridated hydroxyapatite coatings are reactions with nc- CaF_2 . Therefore, it is quite important to determine whether the fluorine ions have been successfully incorporated into the HA lattice structure. The XRD patterns, as shown in Figure 4-5, reveal that no CaF_2 peaks can be detected (only the peaks belong to FHA structures), indicating that fluorine

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ions do not exist in the form of CaF_2 in the coatings. On the other hand, the changes of lattice parameters (Figure 4-6) imply that the fluorine ions have been successfully incorporated into the HA lattice structure. Further confirmation about the status of fluorine ions comes from the XPS results. The wide scan spectra (Figure 4-1a) provide a global concept on all the concerned elements, F1s, Ca2p, P2p, O1s etc, suggesting the existence of all these elements in the coatings. Furthermore, the narrow scan analysis of Ca2p (Figure 4-2a) and F1s (Figure 4-1b) reveal that no extra peaks could be detected except those fingerprint peaks belonging to FHA structures. In addition, the change of peak position as well as peak intensity of OH groups in Figure 4-4b (FTIR patterns) also serves as an indicator regarding the presence of fluorine ions in the HA lattice structure.

Confirmed that the fluorine ions have been successfully incorporated into HA crystal structure, the chemical composition of the FHA coatings were also analyzed. The measured fluorine ion concentration (Figure 4-3) as well as Ca/F molar ratio (Table 4-1) in the coatings increases with the increase of the designed Ca/F ratios of the dipping sols (Table 3-1), indicating the incorporation of more fluorine ions into FHA coatings. The discrepancy between the designed x value and the measured fluorine ion concentration in the coating (X value), especially at high concentration, is attributed to the loss of fluorine in the form of HF during deposition of FHA coatings [220]. Obviously, on the other hand, all of coatings show typical HA or FHA phases and no other impure phases, e.g. TCP, CaO etc, can be observed in those corresponding XRD patterns (Figure 4-5). The XPS composition analysis shows that the Ca/P molar ratios of those coatings are in the range of 1.63~1.7 (Table 4-1), which are similar to that of the stoichiometric value ($\text{Ca/P} = 1.67$) of pure HA or FHA. Comparing with other

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coating deposition methods, such as plasma spraying, PLD, HVOF etc [33, 40, 45], the sol-gel dipping coating method is indeed a most preferred technique to produce the expected HA and FHA coatings.

Fluorine ion concentration in the dipping sols has considerable influences on coating surface properties. As mentioned above, during the preparation of FHA dipping sols, the released fluorine ions will react with calcium cations to form nc-CaF₂ particles. Because of the strong electronegativity of fluorine ion, it is believed that hydrogen bonding easily forms between F in nc-CaF₂ and H in P containing groups (i.e. PO(OEt)_{3-v}(OH)_v) in those dipping sols [199]. The more the fluorine ion in the sols, the more nc-CaF₂ particles form. Accordingly, with a higher fluorine ion concentration in the dipping sol, the nc-CaF₂- PO(OEt)_{3-v}(OH)_v network constructed through the hydrogen bonding has poor resistivity to the shrinkage during the drying and firing process, resulting in a relatively rougher surface (c.f. Figure 4-7 and Figure 4-8) [17]. On the other hand, it has been reported that the incorporation of fluorine ions into HA lattice structure will gradually reduce the surface energy (a larger surface contact angle), and a lowest value is obtained for FA (all OH groups have been substituted by fluorine ions) [223]. While, in this present study, by confirming that fluorine ions have been successfully incorporated into the crystal structure, the increase in contact angle (Figure 4-8) is attributed to the incorporation of fluorine ions.

In addition, the incorporation of fluorine ions also accounts for the development of transitional region (Rt) as well as the chemical bonds in Rt. Actually, the formation of Rt and chemical bonds at/near the interface is finished at the same time during coating preparation. The formation process may be explained as follows: First, chemi- and

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physisorption process during dipping and drying. Since native oxide (TiO_y , $y \leq 2$) forms spontaneously on titanium and titanium alloy surface upon exposure to air, hydroxide ions and water molecules are adsorbed by Ti cations that leads to formation of Ti-OH bonds on the outmost surface [227]. XPS depth profiling confirmed a 5-8nm titanium oxide layer as soon as the Ti6Al4V substrates were prepared. As mentioned above, the fluorine ions released from HPF_6 can react with Ca cations to form nc- CaF_2 , and the F- in nc- CaF_2 easily forms hydrogen bonding with H in OH groups (mainly in $\text{PO}(\text{OEt})_{3-v}(\text{OH})_v$). On the other hand, it is also reported that nc- CaF_2 has good affiliation with titanium alloy substrate [220]. Therefore, when the Ti6Al4V substrate is immersed into the dipping sol, nc- CaF_2 could be easily adsorbed onto the substrate surface through formation of hydrogen bond. The higher the fluorine concentration in the dipping sol, the more nc- CaF_2 forms and adsorbs on the substrate surface. It has been reported that the adsorbed nc- CaF_2 on the titanium alloy substrate surface attract more O near to the interface through the formation of hydrogen bonding, such that, extra OH groups will be attracted to/near to the substrate surface, resulting in the widening of the transitional region [228]. On the other hand, due to the amphoteric property of Ti-OH, the substrate surface will be positively charged in current dipping sol ($\text{pH} < 1$). Thus surface adsorption of electronegative groups, such as NO_3^- , P-containing groups etc. will follow. Previous studies by Filiaggi et al [101] have indicated that the interactions at the interface of the substrates and the dipping sol have great influence on the ultimate adhesion property.

Following the adsorption, an important step is the diffusion during firing when the surface-adsorbed nc- CaF_2 , P-containing groups etc. react with titanium to form certain chemical bonds if the temperature is high enough to activate the formation process.

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Diffusion of fluorine and oxygen from the coating into the transition region encourages this reaction and results in accumulation of O and F in the region (Figure 4-10). Since higher fluorine content in the dipping sol attracts more O near to the interface, firing in open atmosphere provides inexhaustible oxygen for the process. As a result, complex Ti-P-O-F-Ca bonds may be formed in the transient region. In comparison with pure HA coating (Figure 4-10), not only the O and F, but also the Ca and P have longer diffusion distances in F6. The formation of transitional region (whether in HA or FHA coatings) as well as the influences of fluorine ions on the development of transitional regions (formation of chemical bonds in Rt region) would finally affect the coating-substrate interface properties.

4.1.6 Summary

In this part, the chemical and physical properties of sol-gel derived fluoridated hydroxyapatite coatings on Ti6Al4V substrate have been extensively investigated. Studies in this part can be summarized as follows:

- The binding energy of F1s revealed by the single fingerprint peak at ~684.3eV in XPS narrow scan patterns indicates that fluorine ions have been successfully incorporated into HA lattice structure. With the increase of fluoridation degree, the peak intensity of F1s also increase, suggesting more fluorine ions have been incorporated into HA lattice structure.
- Since the incorporated fluorine ion will take the place of OH group in HA lattice structure, the variation of peaks related to OH groups in FTIR spectra (peaks between 3500-3600 cm^{-1}) is sensitive to the degree of fluoridation. Reversely, these changes in OH group related peaks also can be employed to identify whether the fluorine ions has been incorporated into HA lattice structure.

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- Due to the size difference between fluorine ion (0.132 nm) and hydroxyl ion (0.168 nm), the incorporated fluorine ions cause a decrease in a-axis lattice parameter, leading to a more compact structure. The shift of the typical peaks (e.g. (211) etc.) in XRD patterns is the direct outcome of such influence. Similarly, such shift in XRD patterns also can be used to identify the successful incorporation of fluorine ions.
- Surface and interface micrographs reveal that the coatings are dense and uniform with a coating thickness of $\sim 1.5 \mu\text{m}$.
- SIMS composition depth profile reveals that there is a transitional region (Rt) at the interface of coating-substrate, and this region increases with the increasing fluoridation degree. It is believed that some certain complex compounds containing Ti-P-Ca-O-(F) have formed in this region.
- No other impure phases could be observed in XRD patterns and the Ca/P molar ratio is nearly equal to that of stoichiometric value of 1.67.

4.2 Mechanical properties

4.2.1 Residual stress

Residual stress is inherently induced in any coating deposited by a method with a high temperature process due to the differences in the thermal properties between the coating and the substrate. Considerable efforts have been made in recent years to understand and measure the residual stresses that were developed during the preparation of HA coatings [102, 105]. Popular among the various methods is the X-ray diffraction (XRD) method which is a nondestructive and simple technique for residual stress measurement. However, since this method is based on the shift of the peaks in XRD pattern, the use of this method is limited by some factors of the coating, e.g. surface roughness, phase composition and especially the doping of some expected ions etc. Therefore, in this study, the residual stress in the fluoridated hydroxyapatite coatings is evaluated by the curvature method (c.f. §3.3.3). Figure 4-11 illustrates a typical example of the measured curvatures for FHA coatings (F3). A larger curvature radius was obtained after removing of the coating, indicating that a tensile residual stress existed in FHA coatings.

In order to calculate the residual stress, the moduli of the coatings were determined with nanoindentation by using continuous stiffness measurement (CSM) mode. The moduli calculated from nanoindentation test were determined as 47, 54 and 74 GPa for HA (F0), F3 and F6 coatings, respectively. The reported elastic moduli for HA and fluoridated hydroxyapatite was between 20-140 GPa [62, 90], which were highly influenced by the porosity, crystallinity, phase composition as well as the degree of fluoridation, etc. In the present study, the same fabrication process was used for all

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the coatings, thus the increased elastic modulus with increasing of fluoridation was believed to come from the incorporation of fluorine ions into HA lattice structure, in agreement with Gross et al [177]. The average modulus of the Ti6Al4V substrate was determined by the same nanoindentation method to be 115 ± 5 GPa. This value agrees well with the reported value of about 114 GPa for this kind of material.

According to equation (3-2), the calculated values of the residual stresses are shown in Figure 4-12. With increasing of fluorine content in the coating, the residual stress continuously reduces from ~ 273 MPa for F0 (HA) to 190 MPa for F3 and to ~ 137 MPa for F6. In comparison with the F0 (HA) coating, the reduction in residual stress is significant for F3 ($p < 0.05$) coating and F6 ($p < 0.01$) coating. Although the mean level of residual stress for F6 coating is slightly lower than that of F3 coating, the difference is not significant (considered at $p < 0.05$). In other words, the incorporation of fluorine ions into HA lattice structure significantly reduces the residual stress up to about 50%.

Figure 4-11 Typical profilometry results of measured curvatures for F3: a) before removing the coating, b) after removing the coating. The reduction of curvature caused by the removing of FHA coatings indicates a tensile residual stress existing in the coatings.

Figure 4-12 Calculated residual stresses for the prepared FHA coatings based on the curvature testing. The incorporation of fluorine ions results in a decrease of residual stress—the higher the fluoridation degree, the lower the residual stress in FHA coatings.

Residual stress is the internal stress existing in a body that is under no external load condition [229]. The residual stress in a coating is generated during the deposition of the coatings. It is reported that the presence of residual stress in the coating has significant influence on coating properties and durability of the coating *in vivo* [107]: it not only affects the dissolution properties of HA coating, but also weakens the bonding strength between the coating and substrate (c.f. §2.2.2.3). Therefore, the investigation on residual stress in the FHA coating is quite important in order to get a comprehensive evaluation about the performance of FHA-coated implants. A great number of possible parameters can cause residual stresses depending on deposition technique. Structure-related residual stress results from the following three aspects: thermal stress, growth-induced stress and structural mismatch (including lattice distortion) induced stress [230]. In a sol-gel deposition process, the thermal stress comes from the drying and firing process coupled with the difference in the coefficient of thermal expansion (CTE) between the coating and the substrate, the growth-induced stress comes from the coating shrinkage driven by capillary stresses during drying and firing process, and the structural mismatch-induced stress comes

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from the difference in crystal structure between the coating and the substrate as well as lattice distortion within the coating as a result of defects or incorporation of foreign molecules or molecule groups. The thermal stress, σ_{Rt} , can be calculated by [206]:

$$\sigma_{Rt} = \frac{\Delta\alpha \cdot \Delta T \cdot E_f}{1 - \nu_s} \quad (4-1)$$

Where, $\Delta\alpha$ is the difference of CTE between the FHA coating and the Ti6Al4V substrate, ΔT is the temperature variation during drying or firing. CTE of HA is $15 \times 10^{-6}/K$ while that of fluorapatite (or FA) is $9.1 \times 10^{-6}/K$, which is much closer to that of Ti6Al4V substrate ($8.9 \times 10^{-6}/K$) [56]. Thereafter, the residual stress caused by thermal mismatch should be tensile and will decrease with increasing fluorine content in the coatings. Using these values together with room temperature $25^\circ C$, firing temperature $600^\circ C$, Poisson Ratio for Ti6Al4V 0.34, Young's Modulus of the coating as determined by nanoindentation, equation (4-1) gives rise to a residual stress of ~ 227 MPa for HA coating and ~ 77 MPa for F6. This reduction comes from the reduction in difference in CTE. Obviously these values are smaller than the curvature-measured residual stress: 273 MPa for HA (F0), 190 MPa for F3 and 137 MPa for F6. The difference is due to other mechanisms of residual stress generation: Capillary shrinkage during the drying and firing process is one of them (growth-induced residual stress). Lattice distortion due to lattice defect (existence of CO_3 or other ions/molecular groups) is another (structure-mismatch induced residual stress). In our FHA coatings carbonate groups did appear as observed in Figure 4-4.

According to the documented literature, different results were obtained regarding the residual stress in HA coatings, not only the difference in the value, but also the stress state existing in the coatings. Brown et al [104] reported tensile residual stresses (200-

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450 MPa) in HA coatings deposited by APS and HVOF/CDS processes, which is consistent with our present result of HA coating (F0, c.f. Figure 4-12). On the other hand, Tsui et al [102] and Han et al [106] reported a tensile residual stress of about 20-88 MPa in plasma-sprayed HA coating, which were opposed by Yang et al [105] and Sergo et al [231], who reported a compressive residual stress in plasma sprayed HA coatings. These contradictory results are still in dispute. The differences may result from the coating preparation method, process, as well as the method employed for the measurement of residual stress. In our present study, a tensile residual stress is measured in all FHA coatings, and the incorporation of fluorine ion can effectively reduce the residual stress in the coating.

4.2.2 Adhesion strength

4.2.2.1 Pull-out tensile adhesion strength

4.2.2.1.1 Adhesion strength of as-prepared FHA coatings

The adhesion strength between the coatings (as-prepared coatings) and the Ti6Al4V substrates is shown in Figure 4-13. As for pure HA coating (F0), the evaluated bonding strength is about 19 MPa. With the incorporation of fluorine ions into the coating (F2), the bonding strength increases significantly ($p < 0.05$) to about 27 MPa. However, further increase in fluoridation degree has no significant improvement ($p > 0.05$) in bonding strength. Taking into account the average value and standard deviation (as indicated by the error bars) of the testing results, the bonding strength appears to be relatively the same value ($p > 0.68$) at about 27 MPa for those as-prepared fluoridated HA coatings (F2, F4 and F6). In other words, the current pull-out

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tensile test indicates that the difference in fluoridation degree has no significant ($p > 0.05$) influences on the measured bonding strength.

Figure 4-13 Adhesion strength (pull-out tensile test) of as-prepared FHA coatings: with the incorporation of fluorine ions, the adhesion strength increases from ~19MPa for pure HA coating (F0) to ~27MPa for all fluoridated hydroxyapatite coatings (F2, F4 and F6). * indicates a significant different with respect to F0 coating ($p < 0.05$).

It is believed that the bonding strength between the coating and substrate comes from two aspects, one is the mechanical interlocking and the other one is the chemical bonding. In current work, since the substrates had the same finish, the mechanical interlocking can be considered identical. Therefore, the increase in bonding strength is attributed to the stronger chemical bonds, which were developed at the coating-substrate interface during coating deposition process, especially at the firing stage. SIMS depth profile analysis (Figure 4-10) can provide such evidence for the formation of chemical bonds at/near the coating/substrate interface. As discussed in §4.1.4, the addition of F^- ions will widen the transitional region (R_t) and cause the formation of more complex chemical bonds in this region, leading to a higher coating-substrate bonding strength for FHA coatings in comparison with that of pure HA coating.

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A typical analysis of the fracture surface of the coatings with SEM and EDS after the pull-out tensile tests is shown in Figure 4-14. A mixed failure mode is commonly observed, which consists of adhesion failure occurred at the coating-substrate interface, cohesion failure occurred within the coating and gluing failure occurred at the epoxy-coating interface. By evaluating from the fracture surface (Figure 4-14), the area fraction of adhesion and cohesion failure is only about 20%-30%, suggesting that the gluing fracture is the dominated failure model in current study. Such that, limited information is reflected regarding the adhesion properties of coating-substrate interface during this current pull-out tensile test. Therefore, although the increase in adhesion strength is insignificant when the fluoridation degree increases from F2 to F6, it is hardly to say that the incorporation of more fluorine ions has no significant influence on the coating-substrate adhesion properties. Further investigation would be carried out to evaluate the influence of fluoridation degree on the interfacial adhesion properties by using the scratch testing method (c.f. §4.2.4.2).

On the other hand, by considering the failure mode of pull-out tensile test, it is safe to say that the minimal bonding strength is about 19 MPa and 27 MPa for HA and FHA coatings respectively. According to the ISO standards (c.f. ISO 13779) [232], the recommended bonding strength (pull-out tensile test) should be not less than 15 MPa. Therefore, from the standpoint of bonding strength, our present coatings prepared by sol-gel dip-coating method could be well-accepted for biomedical applications. Moreover, with respect to the documented results (Refer to Table 2-5), the values of bonding strength reported for plasma sprayed HA coatings were usually in the range of ~20-30 MPa, and the bonding strengths of sol-gel derived HA coatings were generally lower than 30 MPa. As such, the FHA coatings in this study have

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comparable bonding strengths to the reported values for HA coatings deposited with any technique. In addition, regarding the bonding strength of FHA coatings, Weng et

Figure 4-14 Typical fracture surface of FHA coatings after pull-out tensile test: A mixed failure mode is commonly observed, which consists of adhesion failure occurred at the coating-substrate interface, cohesion failure occurred within the coating and gluing failure occurred at the epoxy-coating interface (the area fraction is about 70-80%).

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al reported that FHA coating (Ca/F = 10:6 in their dipping sol) has a higher bonding strength (20.6 MPa) than pure HA coating (15 MPa), indicating that the incorporation of fluorine ion can improve the bonding strength [198]. Similarly, by using electron beam deposition method, Lee et al concluded that the bonding strength was improved from ~20 MPa for HA coating to ~40 MPa for FHA ($x < 1.5$) coatings [193]. In contrast, a much higher value of ~70 MPa was reported by Kim et al for their sol-gel derived FHA coatings ($x = 1$) after annealing at 600°C, but it should be noticed that their substrate was zirconia [196]. Thereby, based on the above discussion, it is reasonable to conclude that the FHA coatings in present study have comparable bonding strength to the reported values and could well satisfy the basic requirement of bioimplant regarding the bonding strength.

4.2.2.1.2 Adhesion strength of FHA coatings after dissolution testing

In order to investigate the influences of dissolution behavior on the adhesion strength, pull-out tensile tests were also carried out to evaluate the bonding strength between the coating and substrates after 21days immersion testing in TPS and A-TPS. As shown in Figure 4-15 and, after soaking in TPS for 3weeks, all the coatings show a significantly higher ($p < 0.05$) adhesion strength in comparison with that of as-prepared coatings correspondingly (in Figure 4-13). The increase in the measured adhesion strength is about 54.6%, 59.7%, 63.8% and 61.5% for pure HA (F0), F2, F4, and F6 coatings respectively (after soaking in TPS for 3weeks). On the other hand, as illustrated in Figure 4-15, comparing the bonding strength of pure HA coating (F0) with that of fluoridated HA coatings (F2, F4 and F6), a significant improvement ($p < 0.05$) can be observed. Whereas, by considering the average value and the standard deviation, there has no significant difference ($p > 0.05$) between different

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fluoridated HA coatings (F2-F6) even though the average value of bonding strength increases gradually from ~39 MPa for F2 to ~46 MPa for F6. Similarly, the bonding strengths of FHA coatings were also measured after soaking in A-TPS for 3 weeks, and the evaluated results are quite similar (insignificant, $p < 0.05$) to those of TPS (Figure 4-15), indicating that the presence of albumin in the testing solutions has no significant influences (whether negative or positive) on coating adhesion strength.

Figure 4-15 Adhesion strength of FHA coatings after dissolution tests in TPS and A-TPS: the adhesion strength increases from ~29 MPa for pure HA coating (F0), to ~39 MPa for F2 and ~46 MPa for F4 and F6 respectively. * $p < 0.028$ with respect to F0 after soaking in A-TPS; ** $p < 0.0468$ with respect to F0 after soaking in TPS.

Typical tensile fracture surface micrographs and EDS analysis of FHA coatings after dissolution tests in TPS and/or A-TPS is shown in Figure 4-16. Similar to that without soaking (c.f. Figure 4-14), a mixed failure mode containing adhesion failure, cohesion failure and gluing failure is also observed for FHA coating after soaking in TPS and A-TPS. However, the area fraction of gluing failure drastically drops to ~30-40% (from ~70-80% before soaking, Figure 4-14). In other words, the total area fraction of adhesion and cohesion failure increase from around 25% before soaking to around 65% after soaking. Therefore, it is believed that the increase in area fraction of total

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adhesion and cohesion failures after soaking in TPS and modified TPS contributes much more to the increase in pull-out adhesion strength of FHA coatings. In other words, it also implies that the adhesive bonding strength of coating-substrate interface in present study is quite strong.

The increase in area fraction of adhesive failure (as well as the increase in bonding strength) of the sol-gel derived FHA coating after soaking in TPS and/or A-TPS in this study is mainly attributed to two aspects: the dense, uniform FHA coating and the increase of surface roughness after soaking in those solutions. As discussed in §4.1.4, the coatings are dense and uniform, such that, when they were soaked in TPS or A-TPS, the solution had not any possible opportunity to interact with the coating-substrate interface, thus no direct adverse influence (especially the corrosion behavior) on the properties of coating-substrate interface. In other words, such dense and uniform coating can serve well as a protective layer to protect the interface as well as the metallic substrate from corrosion attack in a biological environment. On the other hand, in comparison with the as-prepared coatings, there is a significant increase in coating surface roughness after soaking in TPS or A-TPS for 3 weeks due to the dissolution behavior (please refer to Figure 4-8 and Table 4-3). For instance, the as-prepared F2 coating has a roughness of ~290 nm (Rq), while a value of ~360 nm (Rq) is achieved after 21 days immersion in TPS. Accordingly, as illustrated in Figure 4-17, it is believed that the rougher surface would provide more effective contact area and “catching points” for the epoxy-coating interaction interface during pull-out tensile test. Therefore, the fracture surface mainly occurred within the coating and at the coating-substrate interface, leading to a higher adhesion strength. That is to say, in comparison with the pull-out tensile test of as-prepared coatings, it is much believable

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that the interfacial bonding strength is at least 29 MPa for HA coating and 39 MPa for fluoridated HA coatings respectively.

Figure 4-16 Typical fracture surface of FHA coatings after dissolution tests: A mixed failure mode is commonly observed, which consists of adhesion failure occurred at the coating-substrate interface, cohesion failure occurred within the coating and gluing failure occurred at the epoxy-coating interface (the area fraction is about 30-40%).

Figure 4-17 Schematic illustration of the contact between the coating and the epoxy: the rougher surface after dissolution tests could provide more effective contact area as well as the catching points for the epoxy-coating interaction.

It has been reported that the adhesion strength of plasma sprayed HA coatings inevitably suffer a significant reduction (up to 75% decrease) after the *in vitro* tests in SBF. For instance, Gu et al reported that the bonding strength was reduced from 27.38 MPa before soaking to about 18.7 MPa after soaking in SBF for 2 weeks, resulting a total reduction of 31.6% in original bonding strength [89]. Similarly, a significant reduction of about 27% was observed by Yang et al after only one week immersion in SBF [153]. The main reason was attributed to the intrinsic drawbacks of thermal spray techniques, which cause quite impure phases (e.g. CaO, TCP etc) [45] as well as the transversal cracks across the coating thickness [92]. Such impure phases and transversal cracks result in continuation of chemical dissolution of the coatings, which weakens the bonding of lamella in the coating and the bonding of the interface between the coating and the substrate. In contrast, as discussed above, the coatings prepared with sol-gel method in present study do not show any detectable adverse influence caused by dissolution behavior on the interfacial adhesion strength. Therefore, the coatings, especially the FHA coatings seem to be more promising to be used in biomedical applications.

4.2.2.2 Scratch testing

A typical scratch track of the coating is shown in Figure 4-18. Figure 4-19 is the corresponding friction response (in terms of relative voltage output) as a function of load, for F0 (HA) and F6 coatings. At the beginning of the scratch, coefficient of friction increases as load increases due to cohesive failure because of the “soft” nature of the coating. Before point 1 (c.f., Figure 4-18 and Figure 4-19), there are fluctuations as a result of the surface roughness. After point 1, the indenter starts to plough into the coating, resulting in a steeper increase in coefficient of friction. As the load increases to point 2, or 370 mN for pure HA (curve *a*), the indenter completely peels off the coating and scratches onto the substrate causing an abrupt increase in friction. This load is taken as the adhesion strength for the coating represented by curve *a* and is entered in Figure 4-19 as the first data point. Curve *b* records that for F6. After the indenter digs into the coating (note the change of the slope in the curve), there is no abrupt increase in friction till the indenter reaches the substrate at about 470 mN. Comparing Curves *a* and *b* in Fig. 5, *b* appears smoother (less fluctuation in friction before the indenter digs in and the coating adheres to the substrate better (slower slope after the indenter digs in, and lack of abrupt increase). The abrupt increase of friction signals sudden brittle peeling off of the coating. Lack of this abrupt change demonstrates that *b* has a more ductile interface or better coating-substrate bonding [233].

Figure 4-18 Typical scratch track of FHA coatings: at point 1, the indenter starts to plough into the coating, and at point 2, the indenter completely peels off the coating and scratches onto the substrate causing an abrupt increase in friction.

Figure 4-19 Coefficient of friction in terms of relative output voltage as a function of normal load in the scratch test of FHA coating: a) F0 (HA) coating, b) F6 coating. at point 1, the indenter starts to plough into the coating, resulting in a steeper increase in coefficient of friction, At point 2, the indenter completely peels off the coating and scratches onto the substrate causing an abrupt increase in friction.

Figure 4-20 summarizes the adhesion strength (critical load L_c) of all the FHA coatings as a function of fluoridation degree. With increasing fluorine ion concentration, the critical load gradually increases from ~ 379 mN for F0 (HA) to

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~476 mN for F6. With lower degree of fluoridation (F1, $x \leq 0.33$), the critical load increases slightly (insignificant, $p > 0.05$) in comparison with that of F0. Further increase of fluoridation degree ($x \geq 0.33$) results in significant improvement ($p < 0.05$) on the critical load of FHA coatings (Figure 4-20), however, no significant difference ($p > 0.05$) could be observed between those FHA coatings with higher fluoridation degrees ($x \geq 0.33$, i.e. F2, F3, F4, F5, and F6).

Figure 4-20 Adhesion strength of FHA coatings on Ti6Al4V. Incorporation of fluorine ions into HA structure can enhance the critical load, but the significant improvement is only achieved when the degree of fluoridation is equal to or greater than 0.67 (x value). * indicates a significant difference ($p < 0.05$) with respect to F0.

Coatings adhere to substrates through either mechanical interlocking or chemical bonding or both. In current study, since all substrates have the same finishing, mechanical interlocking is deemed identical, thus the increase in adhesion strength is attributed to stronger chemical bonds developed at the coating-substrate interface during the firing process. As discussed in §4.1.4, within the transitional region, the Ca and P concentration decrease significantly towards the substrate, while the F and O decreases very gradually from the coating to the substrate. In other words, there exists relatively large amount of F and O in the transition region. It has been shown that

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along the cross section, certain Ti-P-Ca-O-F chemical bonds form at the interface that could contribute to the increase in adhesion strength. Formation of chemical bonds at the interface may be also responsible for the change in failure mode (from brittle to more ductile). The formation of the chemical bonding between the coating and the substrate (transitional region) has been discussed in §4.1.5.

Residual stress at the interface resulted from the deposition process may exert adverse effect on adhesion strength. The difference in thermal expansion coefficient for the coating and substrate results in thermal mismatch that in turn contributes negatively to adhesion strength via increased residual stress. Incorporation of fluorine ion into apatite structure decreases the coefficient of thermal expansion (CTE) from $15 \times 10^{-6}/\text{K}$ to $9.1 \times 10^{-6}/\text{K}$ when HA is changed to fluorapatite (FA), which is much closer to that of the Ti6Al4V substrate ($8.9 \times 10^{-6}/\text{K}$): the difference in CTE between HA and Ti-alloy is 68.5% while between FA and Ti-alloy it is only 2.2%! As a result, incorporation of fluorine in HA reduces the thermal mismatch and thus residual stress and consequently improves the adhesion strength. The reduction in residual stress also contributes to better ductility.

4.2.2.3 Evaluation of interfacial shear strength

4.2.2.3.1 Calculation of interfacial shear strength

The interfacial shear strength between FHA coating and substrate was evaluated by using the shear lag strain method (c.f. §3.3.4.3). Despite some differences in surface morphology, the initial formation of transverse cracks as well as the steady state crack density is observed for all the FHA coatings. Figure 4-21 shows a typical sequence of

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crack development towards a steady state in FHA coatings (typically here is F0). The initial occurrence of cracks could not be detected in all areas examined until the strain level reached 1.4% (Figure 4-21 a). With the increase of strain, more regular transverse crack patterns developed in the coatings and maintained a steady state at a strain level of $\sim 3\%$ (Figure 4-21 b). Further increase of strain level did little contribution to the crack density and delamination of the coating occurred at higher strain level ($\sim 5\%$). The average steady state crack spacing was measured as $4.91 \pm 0.15 \mu\text{m}$. Typical crack density (number of cracks per unit length) for F0 (HA) coating was illustrated in Figure 4-22 as a function of strain. It should be noted that this steady state of crack density was achieved rather quickly after the initiation of cracks in the coating. Similarly, for F3 coating, the average steady state crack spacing was measured as $5.19 \pm 0.08 \mu\text{m}$, a little larger than that of F0 (HA); for F6 coating, the maximum elastic strain of the coating occurred at 1.2%; the steady state crack spacing of about $4.82 \pm 0.12 \mu\text{m}$ was achieved rapidly at a strain value of 3%.

Figure 4-21 SEM micrographs of the typical transverse crack patterns for FHA coatings: a) at $\varepsilon_f \approx \varepsilon = 1.4\%$, b) in the steady-state crack density region, $\varepsilon = 3\%$. The initial occurrence of cracks could not be detected in all areas examined until the strain level reached 1.4% (a), and a steady state of the crack density is obtained at a strain level of $\sim 3\%$ (b).

Substituting the related parameters into equation (3-3), the maximum interfacial shear strength of the coatings was calculated as 393, 459 and 572 MPa for F0 (HA), F3 and

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F6 respectively. The critical coating parameters as well as the calculated interfacial shear strength are summarized in Table 4-2. The calculated interfacial shear strength for F6 coating is significantly greater than that of F0 (HA) coating ($p<0.01$) and F3 coating ($p<0.05$). There has no significant difference between the interfacial shear strength of HA and F3 coatings ($p<0.062$). That is to say, a larger amount of incorporated fluorine ions would contribute more benefit to the coating-substrate interfacial shear strength, and a maximum improvement of ~45.5% in the interfacial shear strength as a result of the incorporation of fluorine ions could be observed for F6 coatings compared with that of HA coatings.

Figure 4-22 Transverse crack density as a function of strain for FHA coatings (for F0): The initial occurrence of cracks could not be detected in all areas examined until the strain level reached 1.4%, and a steady state of the crack density is obtained at a strain level of ~3%.

Table 4-2 Calculated interfacial shear strength and related coating parameters

	F0 (HA)	F3	F6
λ (μm)	4.91 $\pm$ 0.03	5.19 $\pm$ 0.04	4.82 $\pm$ 0.04
E (GPa)	42.73 $\pm$ 4.4	53.83 $\pm$ 6.58	73.62 $\pm$ 6.14
ε_f (%)	1.4	1.4	1.2
σ_f (MPa)	598.22 $\pm$ 61.6	753.62 $\pm$ 92.12	883.44 $\pm$ 73.68
τ_{max} (MPa)	392.98 $\pm$ 23.53	459.23 $\pm$ 37.91 [#]	571.99 $\pm$ 44.31 ^{*,**}

([#] $p<0.062$ with respect to F0; ^{*} $p<0.01$ with respect to F0; ^{**} $p<0.05$ with respect to F3)

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4.2.2.3.2 Relevance of interfacial shear strength values

The shear lag method employed in this study is derived by Agrawal and Raj based on the assumption of a limited coating thickness, according to thermodynamic criterion for coating delamination. With substantially larger coating thickness, delamination will become prevalent due to excessive transverse stresses distributed in the coating. What's more, this effect will be aggravated by the defects in the coating, where a greater contribution from local tensile stress can be expected. In the present study, no delamination was observed in all the coatings even at large substrate strains (>3%). Coating buckling and delamination become more remarkable only at very large strain (>5%), mainly induced by the Poisson's effect of substrate contraction normal to the loading direction.

In evaluation of the shear strength $\tau_{\max}$ using the shear lag strain method, errors could be introduced in the determination of σ_f which relies on the accurate measurement of ε_f and E_f through equation (3-4). The incremental straining technique was used to obtain the critical strain ε_f for crack initiation. Thus the obtained value could be influenced by the inherent variability of the coating itself, e.g. distribution of defects, size of the flaw etc, as well as the difficulty in experimentally detecting crack initiation, which may occur in a very small scale before the formation of detectable cracks. The sol-gel derived FHA coatings contained a certain amount of pores as well as micro cracks in it, which may promote the occurrence of transverse cracks. Therefore, considering the difficulty in detecting small-sized transverse cracks, it is hard to say whether the obtained results are overestimated or underestimated. However, as a first level approximation, the current method in determination

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of ε_f serves the purpose. Although the determination of E_f is another source of error, the obtained values for FHA coatings by nanoindentation method are reasonable as compared with other reported results [177, 234]. Consequently, the calculated shear strength values for all FHA coatings, with comparable values to published results [234], suggest the employed shear lag strain method is usable.

The evaluated interfacial shear strength for all the fluoridated hydroxyapatite coatings on Ti6Al4V substrates increases with the increasing fluorine concentration in the coatings. The shear strength results in current study (393, 459 and 572 MPa for F0 (HA), F3 and F6 respectively) are at least an order of magnitude greater than the shear adhesion strengths reported for plasma-sprayed and/or high-velocity oxy-fuel sprayed hydroxyapatite coatings in spite of the different coating thicknesses [62, 235]. As reported in the *in vivo* tests, the shear strength between bone and HA-coated implant was about 16.65 MPa after 24-week implantation in dogs [153], while others reported that the shear strength between the interface of bone and HA-coated implants was about 19.66 MPa after 5 months implantation in dogs [154].

4.2.2.3 Influences of residual stress on interfacial shear strength

The interfacial shear strength $\tau_{\max}$ in equation (3-3) is derived on the assumption of zero residual stress. As residual stress exists (in tensile) in the coating, the tensile stress needed to break the coating will be less. Should there is no residual stress, more stress will be needed to break the coating. Let σ_e be the experimentally determined tensile stress, then stress required to break the coating or σ_f in equation (3-3) should be modified to:

$$\sigma_f = \sigma_e + \sigma_R \quad (4-2)$$

Plugging back to equation (3-3), thus the interfacial shear strength would be 573 MPa for F0 (HA), 575 MPa for F3 and 661 MPa for F6. Compare to the shear strength values calculated before residual stress consideration, it can be seen that the existence of residual stress can reduce the interfacial shear strength as large as 31.4% (for HA).

4.2.3 Toughness

Fracture toughness plays a decisive role in implant functionality by determining the level to which the material can be stressed in the presence of cracks, or equivalently, the magnitude of cracking which can be tolerated at a specific stress level. Fracture toughness determination in coatings and thin films is usually based on an indentation technique. In the present study, the indentation was carried out, and the typical load-displacement curves of indentations together with the corresponding SEM micrograph are shown in Figure 4-23. The coating around the indenter has bulged upwards (Figure 4-23a), indicating delamination and buckling of the coating. In the load-displacement curve, there is one step observed (Figure 4-23b), which is caused by the energy release corresponding to the coating delamination and buckling during the nanoindentation process. The strain energy release (ΔU) can be evaluated from the load-displacement (the step), and the crack length ($2\pi C_R$) in the coating plane is evaluated from the SEM micrographs, therefore, based on equation (3-1), the fracture toughness of FHA coatings can be calculated and are summarized in Figure 4-24. The incorporation of fluorine ions has a significant effect on the coating fracture toughness: with the increase of fluoridation degree, the fracture toughness continuous increases from $\sim 0.123 \text{ MPam}^{1/2}$ for F0 (HA) to $0.26 \text{ MPam}^{1/2}$ for F3 and to $0.31 \text{ MPam}^{1/2}$ for F6

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(with respect to F0, $p < 0.01$ for F3 and F6), but the difference between F3 and F6 is insignificant ($p > 0.1$). In other words, the incorporation of fluorine ions into HA lattice structure can enhance the fracture toughness up to about 1.5 times.

Figure 4-23 SEM micrographs of nanoindentation FHA coatings a) and their corresponding load-displacement curves b)

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The reasons for the increase in fracture toughness are mainly attributed to the following aspects caused by the incorporation of fluorine ions. Firstly, the incorporation of fluorine ions causes a higher elastic modulus (E_f), which contributes more to the fracture toughness (c.f. equation (3-1)). The elastic moduli were measured as about 47, 54 and 74 GPa for F0 (HA), F3 and F6, respectively (c.f. §4.2.1). Other researchers also reported the similar trend regarding the enhancing effect of incorporated fluorine ions on Young's modulus of FHA. Secondly, high adhesion strength also benefits the fracture toughness. As discussed above, the more the fluorine ions incorporated into HA lattice structure, the higher the adhesion strength. What is more, according to the scratch test, it is found that the coating-substrate interface becomes more ductile with higher fluoridation degree. As such, the crack propagation will be well restricted near to the interface, resulting in a smaller crack length ($2\pi C_R$). Finally, residual stress also plays a significant role during the measurement of fracture toughness. As reported, the presence of tensile residual stress is favorable for the crack opening in the coating during indentation process [236]. Therefore, the reduction of residual stress will reduce the crack-sensitivity. In our present study, a decrease in the tensile residual stress was observed with the increase of fluoridation degree (c.f. Figure 4-12), which will reduce the driving force for cracking opening, resulting in a smaller crack length (higher fracture toughness). On the other hand, as discussed above, the reduction of residual stress also benefits the adhesion strength, which also indirectly improves the coating toughness. Therefore, considering the above positive influencing effects, higher fracture toughness is expected for the fluoridated hydroxyapatite coatings (Figure 4-24, F3 and F6).

Figure 4-24 Fracture toughness of the prepared coatings: It shows that the fracture toughness is a function of fluorine concentration, the higher the fluoridation degree, the higher the fracture toughness. *indicates a significant increase of fracture toughness with respect to F0.

4.2.4 Discussion

4.2.4.1 Integration of different adhesion testing methods

Apart from the biological performances that render the usefulness of bioceramic coatings, other features, especially the adhesion properties between the coating and substrate, also serve as the fundamental requirement for the long-term osteointegration of orthopaedic and dental implants [154, 202]. Therefore, as the prerequisite to any prepared bioceramic-coated implant, it is essential and crucial to thoroughly investigate the adhesion properties of coating-substrate interface to ensure a long-term fixation. Many methods have been developed for the preparation of HA and FHA coatings, but in comparison, the sol-gel method has the advantages of composition homogeneity, low cost, ease in operation and doping of ions, which is used widely. It is also the choice of method in this study. In common with other coating deposition techniques, the adhesion properties of sol-gel derived FHA coatings are well investigated.

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The most frequently used method to measure coating-substrate adhesion strength is the pull-out tensile test, according to which the minimum adhesion strength of 15 MPa is stipulated by ISO standards (ISO 13779) [232]. The measured values (Figure 4-13 and Figure 4-15) of our sol-gel derived FHA coatings with pull-out test can totally satisfy such requirement. However, the fracture surface always contains three failure modes simultaneously (as shown in Figure 4-14 and Figure 4-16), i.e. adhesion failure, cohesion failure and gluing failure, and the area fraction of adhesion failure is quite small. Such that, only limited information could be provided about the adhesion properties of coating-substrate interface (actually, due to the mixed failure modes of fracture surfaces, the measured value should be named as bonding strength instead of adhesion strength). What's more, this method is also very sensitive to a number of influencing factors, including alignment of the applied force, epoxy properties, coating surface roughness, infiltration of epoxy etc [153, 207]. Therefore, it is far from satisfactory to evaluate the adhesion properties of coating-substrate interface by using pull-out test singly. In other words, other techniques need further investigation upon the coating-substrate adhesion.

Generally, for a bioceramic-coated load-bearing implants, the adhesion behaviors of coating-substrate interface can be roughly classified into tensile and shear adhesion. Therefore, besides the tensile strength, the interfacial shear strength also serves as a crucial factor for those implants used as tooth root and hip joint replacement. Although, certain quantitative evaluations have been done with the commonly used pull-out shear test (c.f. §2.2.2.3), in viewing the intrinsic drawbacks of “pull-out test”, those obtained shear strength should be “cohesive shear strength” rather than the “interfacial shear strength” [91]. However, the shear lag strain approach described by

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Agrawal and Raj, which utilizes the regular crack patterns obtained through the designed tests, appears useful and relatively straightforward for the determination of interfacial shear strength. The interfacial shear strength evaluated with the shear lag strain method in current study (as shown in Table 4-2) are at least an order of magnitude greater than the pull-out shear strengths reported for plasma-sprayed and high-velocity oxy-fuel sprayed hydroxyapatite. On the other hand, since the reported interfacial shear strength between the coating surface and surrounding tissues *in vivo* was about 16.65 MPa after 24-week implantation. Therefore, from the standpoint of interfacial shear strength, our current sol-gel derived FHA coatings appear quite promising to be used as long-term load-bearing implants, especially for the coatings with higher fluorine content.

The scratch test is generally accepted as one of simple and popular methods in assessing the adhesion properties of coating-substrate interface. Basically, it is carried out by drawing a diamond tip over the coating surface to produce a scratch. The applied normal load is increased linearly until a critical load is reached at which the adhesion failure is induced at the coating-substrate interface. Such that, the critical load can be taken as a semi-quantitative measurement of the coating-substrate adhesion strength, and the failure mode can provide further qualitative information upon the coating-substrate interface. As shown in Figure 4-20, the increase in critical load suggests a good adhesion between the coating and substrate, and the recorded load-cartridge output curve (Figure 4-19) reveals that the coating-substrate interface becomes more ductile with the incorporation of fluorine ions. That is to say, the scratch test can provide a lot of remarkable information about the coating-substrate interface, even though some of them are just qualitative. Therefore, by considering the

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features of pull-out tensile test, shear lag strain method and scratch test, the combination of these three testing methods could be more helpful to get a comprehensive understanding upon the coating-substrate adhesion properties.

4.2.4.2 Influences of fluorine ions on coating mechanical properties

Incorporation of fluorine ions into biomaterials plays an important role in the physicochemical properties of implants. The most important aspects include solubility and mechanical properties. As for the influences of fluorine ions on the coating mechanical properties, mainly, the functionalization is achieved through the following two approaches: the incorporation of fluorine ions into the HA lattice structure and the combination of fluorine ions at the coating-substrate interface.

It is commonly accepted that the substitutions within the c-axis channel of hydroxyapatite have considerable influence on its intrinsic physical properties, e.g. hardness, elastic modulus toughness, as well as the thermal behaviors etc [177, 237] (c.f. §2.3.1.2). The incorporation of fluorine ions by replacing OH groups in HA lattice structure results in a more compact structure (Figure 4-6), which is also reflected by a greater true density of 3.21 g/cm^3 for fluorapatite in comparison with pure hydroxyapatite with a density value close to 3.16 g/cm^3 . On the other hand, the incorporation of fluorine ions can also improve the crystallinity and the thermal stability of HA. It is believed that the higher density, higher crystallinity, and higher thermal resistance contribute to the increase of hardness, elastic modulus as well as the fracture toughness [177]. As shown in our present study, there is an increase in coating elastic modulus, and the fracture toughness (Figure 4-24), resulting from the combination of all the above effects caused by incorporation of fluorine ions. A

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situation of relevance regarding the influence of fluorine ions on mechanical properties of HA is the dental enamel. It is well established that the outer fluorine rich layer (fluoridated hydroxyapatite) of the enamel can provide the higher mechanical properties, which is quite crucial to protect the inner dentin. In addition, the incorporation of fluorine ions into HA lattice structure reduces the coefficient of thermal expansion from $15 \times 10^{-6}/\text{K}$ for pure HA to $9.1 \times 10^{-6}/\text{K}$ for pure fluorapatite, which is much closer to that of the Ti6Al4V substrate ($8.9 \times 10^{-6}/\text{K}$). Therefore, incorporation of fluorine ions in HA reduces the thermal mismatch between the coating and the substrate and thus reduces the residual stress in the coating, which will be more beneficial to the coating-substrate adhesion properties.

The combination of fluorine ions at the coating-substrate interface makes great contribution to the adhesion properties. As discussed in §4.1.4 and §4.1.5, during the coating preparation process, the presence of fluorine ions helps to the development of transitional region between the coating and the substrate, which contains strong complex Ti-P-Ca-F-O chemical bonds (Figure 4-10), resulting in direct enhancement on tensile adhesion strength (Figure 4-13) and interfacial shear strength (Table 4-2). The increase in the critical load (Figure 4-20) of scratch test also indicates the formation chemical bonds at coating-substrate interface due to the incorporation of fluorine ions. Moreover, the change of failure mode (Figure 4-19) of scratch test from a brittle mode for F0 to a ductile mode for F6 suggests that with the incorporation of fluorine ions, the interface between the coating and substrate becomes more ductile. On the other hand, the incorporation of fluorine ions can drastically reduce the residual stress in the coatings (Figure 4-12), which serves as an indirect enhancing effect of fluorine ions on the adhesion properties. It is reported that in a different

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situation when the residual stresses are varied by processing, the HA revealing high bonding strength fractures mainly inside the HA under lower residual stress, as residual stress increases, the bonding strength decrease and fracture tends to occur more easily along the coating-substrate interface. Therefore, it is believed that the decrease in residual stress in the FHA coatings with the incorporation of fluorine ions improves the adhesion properties between the coating and the substrate.

In addition, it has been reported that current plasma sprayed HA coating as a bioactive layer on metallic implant has been questioned primarily because of the decay of the coating and the weakness of the coating-substrate interface [90], especially after the *in vitro* immersion test. Such degradation of adhesion strength is in general attributed to the dissolution behavior related to the coating structures, e.g. impurity phases, crystallinity, cracks etc, which would impair the mechanical properties of the coating after *in vivo* or *in vitro* tests. However, in our current study, for instance, after soaking in TPS for 21 days, the pull-out adhesion strength is about 29 MPa for pure HA and over 39 MPa for fluoridated HA coatings (Figure 4-15), which is much higher than the recommended adhesion strength of 15 MPa by ISO [232]. What is more important, the soaking tests in TPS has no detectable detrimental influences on the interfacial adhesion properties (a significant increase is observed for the pull-out adhesion strength before and after soaking in TPS). Therefore, from the standpoint of adhesion, since the documented results (including clinic use, *in vivo* and *in vitro* tests) reveal that implantation failures always occur at the coating-substrate interface due to the insufficient adhesion between the coating and the substrate, it is believed that the fluoridated hydroxyapatite coatings prepared with the sol-gel dip-coating method could be well satisfied with requirements of the biomedical applications.

4.2.5 Summary

From studies in this part, some key points can be drawn:

- A tensile residual stress was measured in FHA coating, and the incorporation of fluorine ion can effectively reduce the residual stress in the coating (the residual stress continuously reduces from ~273 MPa for F0 to ~190 MPa for F3 and to ~137 MPa for F6).
- The pull-out tensile test shows that the adhesion strength is ~19 MPa for pure HA coating, and ~27 MPa for fluoridated HA coatings. After soaking in tris-buffered physiological saline solution for 3 weeks, the adhesion strength increases to ~29 MPa for pure HA coating and to over ~39 MPa for fluoridated HA coatings. The presence of albumin has no significant influence on adhesion strength.
- Fracture surface analysis (after pull-out tensile test) reveals a mixed failure mode for all coatings: adhesion failure occurred at the coating-substrate interface, cohesion failure within the coating, and gluing failure occurred at the epoxy-coating interface. The total area fraction of adhesion and cohesion failures is ~20-30% and ~60-70% for the coatings before and after immersion tests respectively.
- Scanning scratch test reveals that the coating adheres to the substrates up to 35% better as the fluorine concentration increase in the coating. Moreover, with the increase of fluoridation degree, the coating-substrate interfacial failure mode changes from brittle to ductile, indicating a much stronger adhesion for coatings with higher fluoridation degree.
- The interfacial shear strength of FHA coatings is measured by the shear lag strain method to range from ~393 MPa for pure HA coating to ~495 MPa for F3 and to ~572MPa for F6, suggesting that more incorporated fluorine ions lead to a higher interfacial shear strength.

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- The incorporation of fluorine ion has significant effect on the coating fracture toughness: $\sim 0.12 \text{ MPam}^{1/2}$ of pure HA coating increases to $\sim 0.26 \text{ MPam}^{1/2}$ for F3 and to $\sim 0.31 \text{ MPam}^{1/2}$ for F6. The increase from “without” to “with” fluorine ion incorporation is more than doubled and statistically significant.

4.3 *In vitro* behaviors

4.3.1 Dissolution resistance of FHA coatings

4.3.1.1 Influences of fluoridation degree on coating dissolution behaviors

The dissolution behaviors of FHA coatings in Tris-buffered physiological saline solution (TPS) are shown in Figure 4-25. The concentration of Ca^{2+} released from the coatings into the solution increase with the soaking time and the dissolution rates decrease gradually, especially after a soaking duration of 7 days (Figure 4-25). Evidently, for all coatings, the dissolution curve contains two distinct stages: the initial sharp rise in the coating dissolution curve during the first 7 days, followed by a “constant rate” dissolution of the remaining coating. As discussed in §4.1.3 and §2.2.1, besides the HA or FHA phases in the prepared coatings, other phase, e.g. the amorphous phase, must be existing in the coatings, and their solubilities follow the order of [26-28]:

Therefore, those extremely soluble phases (e.g. ACP) on or near the coating surface are dissolved into the solution very quickly, leading to the sharp increase of Ca^{2+} concentration in the solution during the first 7 days. Since the removal of those more soluble phases near the surface of the coating, a layer of highly crystalline, porous HA or FHA is left behind. Subsequent dissolution of this remaining layer continues at essentially the rate of pure HA or FHA. Because of the high resistance to dissolution, the remaining coating partially shields the underlying more soluble phases from surrounding TPS solution. Dissolution of the underlying coating thus becomes diffusion limited, resulting in the effective dissolution rate to approach that of the pure crystalline HA or FHA phases (almost, a linear increment in Ca^{2+} concentration).

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On the other hand, the dissolution behaviors (see inset of Figure 4-26 for all coatings after 21 days) indicates that the variation of solubility is a function of fluoridation degree: initially, the solubility gradually decreases with the incorporation of fluorine ions and reaches a minimum with F3 and F4, and then an increase can be observed for further increase of fluoridation degree. In other words, all the fluoridated HA coatings have a lower Ca^{2+} concentration compared with that of pure HA (F0), but the dissolution rate follow the order of $\text{F0} > \text{F2} > \text{F6} > \text{F3} \approx \text{F4}$ (c.f. Figure 4-25). As discussed in §2.3.1.2.3, this can be explained on the basis of disordered structure of HA and the hydrogen bonding in the hydroxyl group to fluorine ions occurring in the structure [180, 183-185]: in HA crystal structure, H^+ ion in a hydroxyl group could bond with O^{2-} from different directions along c-axis; once an OH group is substituted by a fluorine ion (F^-), a stronger hydrogen bond forms between the fluorine ion and the nearby OH group, forming a more ordered structure with enhanced chemical and thermal stability. As such, the greatest contributions to the stability of crystal structure will be located at a percentage of 50% substitution of OH by fluorine ion (or at $x = 1$). In our present study, from Figure 1, F3 ($x = 3/3$) and F4 ($x = 4/3$) corresponds to measured X of 0.84 and 1.04 respectively. These coatings have the minimum solubility than others (c.f., inset of Figure 4-25). Taking into consideration of the random nature of the substitution of F^- for OH^- , it is reasonable to assume that our current study indicates a probably optimum range of 0.8-1.1 as the degree of fluoridation for a minimum solubility, which is also in accordance with other reported results [180, 193]. On the other hand, the incorporated fluorine ions can cause a decrease in a lattice index (c.f. Figure 4-6), resulting in a more compact structure. Therefore, in the vicinity of the fluorine ions, the calcium ions remain more close and

stronger resistance toward dissolution, leading to a lower solubility in comparison with the pure HA coating (F0).

Figure 4-25 Dissolution behavior of FHA coatings in Tris-buffered physiological saline solution (TPS): concentration of Ca^{2+} is function of both soaking time and fluoridation degree. Inset: total Ca^{2+} concentration after 21 days. It indicates that dissolution decreases as fluorine ions are incorporated, with F3 and F4 reaching the minimum.

4.3.1.2 Dissolution behaviors in organic-modified TPS solutions

Glucose and albumin, as the most common organic components of human blood plasma, were employed to investigate the influences carbohydrate and proteins on the dissolution behaviors of FHA coatings. Figure 4-26a shows the dissolution behavior of FHA coatings during the incubation in glucose-modified (1 g/L) Tris-buffered physiological saline solution (G-TPS) for up to 21 days. In comparison with the dissolution behaviors in TPS (Figure 4-25), actually, the presence of glucose has no significant influences on the dissolution behaviors of these coatings. For instance, for F4 coating as shown in Figure 4-27 (comparing Figure 4-26a with Figure 4-25), one does not find too much difference (insignificant) regarding the amount of released Ca^{2+} ions in the testing solutions during each incubation period (e.g. at 14 days, the Ca^{2+} concentration is about 1.64 ppm/cm² in G-TPS and about 1.55 ppm/cm² in TPS).

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respectively). On the other hand, the addition of glucose does not change the order of dissolution rate of these coatings, i.e. all the fluoridated HA coatings have a lower Ca^{2+} concentration compared with that of pure HA, and the dissolution rate follow the order of $\text{F0} > \text{F2} > \text{F6} > \text{F3} \approx \text{F4}$ (c.f. Figure 4-25 and Figure 4-26a).

Since the proteins in blood plasma, e.g. albumin, fibrinogen and globulin etc (c.f. Table 3-4), have been shown to inevitable interact with the HA coatings (in an ionic manner) [125, 211, 238], it is then necessary to determine the role of proteins in the dissolution behaviors of FHA coatings. Albumin, as one of the most common component of proteins in blood plasma, was selected in current work to investigate its influence on dissolution behaviors of FHA coatings. As shown in Figure 4-26b, during the 21days period, a continuous increase in Ca^{2+} release was observed for all coatings. However, in comparison with the testing in TPS (Figure 4-25) or G-TPS (Figure 4-26a), the presence of albumin results in significantly higher Ca^{2+} concentration released in the solution during the whole testing period. As an example, Figure 4-27 clearly depicts the dissolution behaviors of F4 coatings in different testing conditions. The higher Ca^{2+} release for coatings in A-TPS suggests that the labile ions such as Ca^{2+} can be easily removed by anionic side chains found on proteins (BSA in the investigation).

It has been reported that the isoelectric point of BSA is 4.7, therefore, BSA will be negatively charged in the Tris-buffered physiological saline solution with a pH value of 7.4 [239]. Therefore, the labile ions, such as calcium, can be easily removed from the coating surface by anionic side chains found on proteins. These anionic groups drive calcium ions into solution. From the dissolution study, calcium released in

Figure 4-26 Dissolution behavior of FHA coatings in organic-buffered TPS solutions: a) Glucose-modified TPS (G-TPS); b) BSA-modified TPS (A-TPS). The concentration of Ca^{2+} is function of both soaking time and fluoridation degree. The dissolution decreases as fluorine ions are incorporated, but the minimum solubility is obtained for F3 and F4.

Figure 4-27 Dissolution behavior of F4 coating in different testing conditions: The addition of glucose has no significant influence on dissolution behaviors of FHA coatings in comparison with the tests in TPS. However, in comparison with the testing in TPS or G-TPS, the presence of albumin results in significantly higher Ca^{2+} concentration released in the solution during the whole testing period.

albumin-containing TPS is significantly greater when compared to the tests in TPS or G-TPS. On the other hand, two competitive processes are commonly used to describe the surface reactions during the dissolution tests, i.e. dissolution and reprecipitation. For the FHA coatings in the present study, the dissolution/reprecipitation process can be described as:

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If the dissolution process is predominant, the coating will be dissolved continuously until a thermodynamic equilibrium is established for these two concurrent processes and vice versa. Therefore, after incubating the coatings in the testing solutions, they will be inevitably dissolved at first in such an unsaturated environment and a thin Nernst layer will be formed in the solution near to the coating surface, which results in certain reduction of coating dissolution rate. When albumin is added into the TPS solution at the pH value of 7.4, the released calcium ion will be immediately grasped by the negatively charged albumin in the solution, resulting in an extra consumption of Ca^{2+} in the Nernst layer (in other words, the Ca^{2+} concentration on the right hand side of equation (4-3) is reduced in comparison with albumin-free TPS solutions). Such that, more Ca^{2+} will be released from the coating into the protein-containing solution in order to compensate the consumption of Ca^{2+} by the albumin. Consequently, a higher calcium ion concentration is produced in albumin-modified TPS in comparison with TPS and G-TPS.

4.3.1.3 Influences of residual stress on dissolution behavior

Residual stress may affect the thermodynamics of dissolution behaviors by altering the chemical potential of the coatings. For those stress-free coatings, the unit free energy change of an equilibrium status, ΔG^0 , can be described as [106, 231]:

$$\Delta G^0 = -RT \ln K_{sp} \quad (4-4)$$

where R is the gas constant, T is the temperature and K_{sp} is the solubility product of the corresponding coating. If the constraint of a stress-free solid is removed and a stress, σ_{ss} , is applied, such as a residual, the system is no longer in equilibrium

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because the chemical potential of the coatings is altered by an amount proportional to the stress, namely, $\sigma_{ss} \cdot \Omega/3$ per unit volume (here Ω is the unit volume of the species under consideration). As a result, according to the thermodynamic analysis, with the presence of residual stress, additional free energy for the dissolution equilibrium becomes available in the form of elastic strain energy stored in the coatings, and the new equilibrium condition is represented by an altered value of the solubility product, K_{sp}^{σ} :

$$\Delta G^0 - \frac{\sigma_{ss} \cdot \Omega}{3} = -RT \ln K_{sp}^{\sigma} \quad (4-5)$$

The negative sign implies that a compressive residual stress will hinder whereas a tensile residual stress will enhance the dissolution. Therefore, the ratio of the solubility product under different stresses (e.g. σ_1 and σ_2) is:

$$\frac{K_{sp}^{\sigma_1}}{K_{sp}^{\sigma_2}} = \exp \left[(\sigma_1 - \sigma_2) \cdot \frac{\Omega}{3RT} \right] \quad (4-6)$$

This expression can be used to estimate the change of solubility product owing to the presence of different residual stresses.

In the present study, as discussed in §4.2.2, since the residual stresses in those prepared coatings are tensile stresses, it can be concluded that all the coatings will exhibit a higher solubility than the stress-free coatings correspondingly. Based on equation (4-6), the ratio of the solubility product under certain residual stress and stress-free conditions ($\sigma_2=0$) can be expressed as:

$$\frac{K_{sp}^{\sigma_1}}{K_{sp}} = \exp \left[\sigma_1 \cdot \frac{\Omega}{3RT} \right] \quad (4-7)$$

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By substituting the evaluated residual stress into equation (4-7), the ratio of $K_{sp}^{\sigma_1} / K_{sp}$ was calculated as 274, 49.7 and 16.7 for F0, F3 and F6 respectively. It can be seen that the residual stress has remarkable influence on the dissolution behavior of those coatings on implant surfaces. Although the residual stress may be overestimated in our present study, which will result in a much higher $K_{sp}^{\sigma_1} / K_{sp}$ ratio (because it follows the function of $f(x) = \exp(x)$), the calculated results here just show how the influence of residual stresses on the dissolution behaviors of the deposited coatings.

On the other hand, as discussed above, the tensile residual stress will result in a larger solubility product and the higher the fluoridation degree of the coating, the lower the residual tensile stress. For the coatings of F3 and F6, the calculated $K_{sp-F3}^{\sigma_{R-F3}} / K_{sp-F6}^{\sigma_{R-F6}}$ ratio is about 2.97, that is to say, the solubility product of F3 is about 2.97 times greater than that of F6. But it does not mean that F6 will possess the smallest solubility. According to the study done by Moreno et al, the theoretical ratio (intrinsic value) of K_{sp-F6} / K_{sp-F3} is calculated to be about 3.63 for stress-free samples of F3 and F6. By considering the influences of residual stress, the final solubility displayed should be the combined behaviors of intrinsic dissolution and residual stress induced dissolution, therefore, the actual solubility product of F6 is still about 1.22 times higher than that of F3. In other words, the dissolution behavior of the prepared FHA coatings still follows the trend reported by Moreno, and the minimal solubility is achieved at around F3 and F4 (c.f. Figure 4-25 and Figure 4-26).

4.3.1.4 Surface morphology after dissolution tests

The dissolved surface morphologies of FHA coatings after soaking in TPS, G-TPS and A-TPS were observed with SEM. As an example, Figure 4-28 shows the SEM

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micrographs of F6 after soaking in different solutions for 21 days. The morphologies exhibit a microstructurally rough surface instead of the original smooth surface (as-prepared coating, c.f. Figure 4-7d) after the dissolution tests in TPS or modified TPS solutions. As the quantitative description of surface roughness, the R_q values of FHA coatings after dissolution tests are listed in Table 4-3. In comparison with that of the original surface (Figure 4-8), the surface roughness (R_q) has a significant increase for each corresponding coating after soaking in TPS and modified TPS solutions. The formation of those rough surfaces is due to the difference in the solubility of different phases in the coatings. As discussed in §4.3.1.1, the quickly preferential dissolution of those more soluble impure phases (especially the amorphous phase) from the coating surface leaves only the high dissolution-resistance phases (HA or FHA) on the coating surface with a porous morphology, which finally results in the increase of surface roughness after soaking in the testing solutions.

On the other hand, for each coating (e.g. F6), there is no significant difference between the surface morphologies after soaking in TPS (Figure 4-28a) and G-TPS (Figure 4-28b), but its surface looks more “porous” (Figure 4-28c) after soaking in A-TPS. According to the measurement of Ca^{2+} concentration released into the solutions (Figure 4-25 and Figure 4-26), the higher Ca^{2+} concentration in the A-TPS will be responsible for the more porous surface in comparison with the other two tests in TPS and G-TPS.

Figure 4-28 Surface morphologies of FHA coatings after 28-day soaking in a) TPS, b) G-TPS, c) A-TPS. The morphologies exhibit a rougher surface in comparison with the original surface. The presence of glucose and BSA has no significant influence on the change of surface morphology after dissolution testing.

Table 4-3 Surface roughness (R_q , nm) of FHA coatings after soaking in TPS, G-TPS and A-TPS

	F0	F2	F3	F4	F6
TPS	357.65±10.31	358.85±16.88	357.44±12.39	364±11.4	369.44±10.27
G-TPS	347.61±11.03	352.04±12.44	368.43±33.01	358.36±14.87	361.35±6.87
A-TPS	367.38±21.94	366.59±11.33	373.78±13.21	362.63±8.46	375.22±12.1

It is believed that the initial dissolution behaviors are quite beneficial to the healing process as well the long-term successful osteointegration [202]. Firstly, the initial fast dissolution of the coating will result in a local super saturated environment (e.g. Ca^{2+}), which is more favorable for the nucleation and growth of bone-like apatite, speeding up the formation of chemical bonds between the implant and surrounding tissues. On the other hand, a rough implant surface appears to be favorable for cell attachment,

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and, particularly suitable for primary implant stability as compared to a smooth implant surface. Moreover, since it was found that higher shear strength between the implant and surround tissues was obtained with coarser surface morphology *in vivo* [240], the roughening effect of initial dissolution on the coating surface (c.f. Table 4-3) is thought to be beneficial for the fixation of implants.

4.3.2 Bioactivity testing in acellular solutions

4.3.2.1 Testing in c-SBF

Characteristic surface morphologies of FHA coatings after *in vitro* tests in c-SBF with different soaking time are shown in Figure 4-29. For pure HA (F0), after 2 days immersion in c-SBF, some precipitated particles could be observed on its surface (Figure 4-29a), indicating the induction of apatite deposition (nucleation). After 14 days, there was a continuous precipitated apatite layer (Figure 4-29b), which is completely different with the original coating surface (Figure 4-7a). Further increase of soaking time has no influence on the morphology of the newly grown apatite layer except increase in thickness. The bioactivity of the coatings is significantly influenced by the incorporation of fluorine ions. At lower fluoridation degree ($x \leq 1.33$), i.e. coatings of F2, F3 and F4, after 2 days immersion in c-SBF, a drastic increase in nucleation rate is observed in comparison with that of pure HA coating (Figure 4-29a, c, d, and e). To have a continuous apatite layer formed, F2, F3 and F4 only required 7 days (typically, as shown in Figure 4-29f) in contrast to the required 14 days for pure F0 (HA). That is to say, F2, F3 and F4 exhibit higher ability than HA to induce the deposition of apatite layer (nucleation and growth) in c-SBF. Further increase of fluoridation degree will exert certain retarding influences on the induction of apatite

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formation. As shown in Figure 4-29g and h, no obvious nucleation of apatite can be observed on F6 after 2 days immersion, but a continuous apatite layer could be obtained only after 28 days, indicating that F6 has a slower ability to induce apatite deposition (nucleation and growth) but the comparable bioactivity to other coatings. Therefore, at this stage, it is reasonable to say that certain degree of fluoridation (x value between 0.67-1.33) has positive bio-activation effect on the coating bioactivities, which is in accordance with some previously reported results in other researchers' work [197]. In addition, at low magnification, the surface of the new precipitated bone-like apatite layer appears dense and sometimes contains spherical apatite particles (Figure 4-29b, f and h). At higher magnification (Figure 4-29i and j), however, porosity can still be seen in the new formed apatite layers. No significant difference could be observed between the high magnification surface morphologies of the new formed apatite layer on pure HA coating (F0, Figure 4-29i) and on other fluoridated hydroxyapatite coatings (typically, Figure 4-29j).

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Figure 4-29 SEM micrographs of FHA coatings after testing in c-SBF: a) F0-2days, b) F0-14days, c) F2-2days, d) F3-2days, e) F4-2days, f) F2, F3 and F4 after 7days in c-SBF, g) F6-2days, h) F6-28days, i) high magnification surface morphology of F0 after 14days in c-SBF, and j) typical high magnification surface morphology of the new precipitated bone-like apatite layer on other fluoridated hydroxyapatite coating surfaces.

The variation of Ca^{2+} concentration in c-SBF is shown in Figure 4-30 as a function of soaking time. The ups and downs of the Ca^{2+} concentration indicate dissolution and precipitation of apatite from the solution to the surfaces of the coatings. In Figure 4-30, during the first 4 days, the pure HA (F0) coating experienced more dissolution than reprecipitation that resulted in the increase of Ca^{2+} concentration in the solution. The Ca^{2+} released from the coating may result in a supersaturation of Ca^{2+} , a situation

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more favorable for nucleation and growth [241, 242]. Therefore, spontaneous growth of apatite layer takes place which consumes calcium and phosphate ions in c-SBF causing the gradually decrease of Ca^{2+} concentration. With the incorporation of fluorine ions into the HA lattice structure, the dissolution process only maintained for 2 days for F2 and F3, and no obvious dissolution process could be observed for F4 coating. What is more, the F2, F3 and F4 possessed much stronger ability to induce the apatite nucleation and deposition process (Figure 4-29c-f). Further increase of fluorine ion content (F6) would cause some adverse effects on the nucleation and growth process of apatite reprecipitation. No obvious nucleation can be observed after 2 days immersion in c-SBF for F6 coating (Figure 4-29g) though partial coating was dissolved in these days. Moreover, only after 28 days immersion in c-SBF, a continuous deposited apatite layer was obtained for the F6 samples (Figure 4-29h). Also, partially similar phenomena have been reported by K. Cheng et al [197] regarding the influences of incorporated fluorine ions on the *in vitro* bioactivity of FHA coating in acellular SBF solution.

Figure 4-30 Concentration of Ca^{2+} after soaking tests in c-SBF for up to 28 days: The ups and downs of the Ca^{2+} concentration indicate dissolution and precipitation of apatite from the solution onto the surfaces of the coatings.

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For the precipitation of bone-like apatite from c-SBF, the following criteria must be satisfied [243]: i) Precipitation must be thermodynamically feasible and favorable. This can be true only when the solution is supersaturated with respect to certain specific phase, i.e.

$$S = IAP/K_{sp} > 1 \quad (4-8)$$

where S is the supersaturation (supersaturated when $\beta > 1$), IAP is the ionic activity product, and K_{sp} is the solubility. ii) Nucleation must be energetically and probabilistically possible. Appropriate ions and radicals need to collide and the bond energies thus dissipated have to be able to overcome the increase in surface energy associated with a nucleus of critical size. iii) Stable nuclei have to be able to grow.

Basically, the thermodynamic driving force for Ca-P precipitation from SBF onto FHA coating surface can be calculated based on the classical equation of free energy change in supersaturated solutions [243]:

$$\Delta G = -\frac{RT}{n} \cdot \ln S = -\frac{RT}{n} \cdot \ln(IAP/K_{sp}) \quad (4-9)$$

where ΔG is the Gibbs energy per mole of ionic units that compose Ca-P in solution, R is the gas constant ($8.314 \text{ JK}^{-1}\text{mol}^{-1}$), T is the absolute temperature, and n is the number of ion units in a Ca-P molecule. And the nucleation rate, J , can be estimated as (heterogeneous nucleation):

$$J = K_f \exp\left(-\frac{\Delta G}{kT}\right) = K_f \exp\left(-\frac{f \cdot v^2 \cdot \gamma_{NL} \cdot \phi(\theta)}{k^3 \cdot T^3 \cdot (\ln S)^2}\right) \quad (4-10)$$

with

$$\phi(\theta) = (2 + \cos \theta) \cdot (1 - \cos \theta)^2 / 4 \quad (4-11)$$

and

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$$\cos \theta = (\gamma_{SL} - \gamma_{SN}) / \gamma_{NL} \quad (4-12)$$

In which, K_f is the kinetic factor, f is the shape factor, v is the volume occupied by one molecule within the unit cell, k is the Boltzmann constant, $\phi(\theta)$ is the function of contact angle (θ) for a nucleus on the coating surface, γ_{NL} , γ_{SL} , and γ_{SN} are the interfacial energy between nucleus-liquid, substrate-liquid, and substrate-nucleus respectively.

In order to ascertain the influences of fluorine ion on the precipitation of bone-like apatite from c-SBF, firstly, we'd like to discuss the precipitation process of Ca-P from c-SBF onto pure HA coating surface. A schematic representation on the deposition of bone-like apatite on HA coating surface is depicted in Figure 4-31. Briefly, upon immersion, coating species are released from the surface due to the presence of some phases with higher solubility and enter the diffusive layer (diffusive layer—a very thin layer in the solution near to the coating surface, where the surface potential of the coating (ψ_0) falls off to ψ_0/e). The precipitation of CaP from c-SBF onto HA coating surface mainly depends on the ion concentrations (IAP) in the diffusive layer rather than the bulk ion concentrations of the solution. If the release rate exceeds the diffusion rate, the solution is locally supersaturated, and the IAP is increased. This oversaturation results in the stronger driving force (equation (4-9)) for the precipitation (nucleation and growth) of CaP on HA coating surface [244]. The ICP results (c.f. Figure 4-30) confirm that the dissolution and reprecipitation of calcium occurred concurrently during the first few days of immersion, which led to the precipitation of a bone-like apatite layer on HA coating surface [245].

Figure 4-31 A schematic illustration of bone-like apatite precipitation process from c-SBF onto HA coatings: a) coating is introduced in SBF solution; b) coatings species are dissolved, increasing the IAP in the diffusive layer; c) deposition of bone-like apatite phases on HA coating surfaces.

With the incorporation of fluorine ions into HA lattice structure, the physical and chemical properties of the coatings are drastically modified. Although the incorporation of fluorine ions can reduce the solubility of the coatings, which may lead to the reduction in IAP values, it is hardly to say that such reduction in IAP will exert significant influence on the driving force ΔG (equation (4-9)) by considering the related ion concentrations of SBF (for instance, Ca^{2+} concentration in c-SBF is 100 ppm, while the dissolved Ca^{2+} concentration for pure HA coating is less than 2.8 ppm after immersion in TPS for 21 days). On the other hand, the precipitation of CaP from c-SBF onto coating surface strongly relies on the ions and ion concentrations in the diffusive layer (as shown in Figure 4-31). For FHA coatings ($x > 0$), when they are immersed into c-SBF solution, some fluorine ions will be released into the diffusive layer due to the dissolution behavior. Since the fluorine ion is intrinsically strongly electronegative, such ions in the diffusive layer will attract more cations, especially Ca^{2+} , into this layer, leading to the increase in zeta potential in this layer, which in

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turn will attract more other anions (e.g. PO_4^{3-}) into this layer. Therefore, in the local region (diffusive layer), it is believed that the IAP value of FHA coatings ($x > 0$) is much higher than that of pure HA, which enhances the driving force for the precipitation (nucleation and growth) of CaP from solution onto FHA coating surfaces (c.f. Figure 4-29).

However, with more fluorine ion ($x > 4/3$) incorporated into HA lattice structure, an adverse influence is observed upon the precipitation of CaP from c-SBF onto coating surface (c.f. Figure 4-29). The reason is still not clear, but it may be caused by the fact that more fluorine ions in the coating will lead to more dissolved fluorine ions in the diffusive layer, which may form some CaF_2 , resulting in the extra consumption of Ca^{2+} . Such that, not only the Ca^{2+} concentration is reduced in diffusive layer, but also the inducing effect of fluorine ions is restrained, which finally reduces the driving force for the precipitation of CaP. Therefore, it is believed that certain degree of fluoridation will benefit the precipitation of bone-like apatite (CaP) from c-SBF onto coating surface, indicating much better bioactivity of such coatings [197].

4.3.2.2 Testing in G-SBF

Glucose-modified SBF (G-SBF) solution was employed in order to investigate the influences of glucose (1 g/L) on coating bioactivity. Figure 4-32 shows the typical surface morphologies FHA coatings after *in vitro* tests in G-SBF. Since no significant differences can be observed between F2, F3 and F4 samples, the typical surface morphologies of these three kind of samples after testing in G-SBF are shown in Figure 4-32c and d. Comparing with the bioactivities in c-SBF (Figure 4-29), actually, for the same coating, glucose has no significant influences on process of apatite

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nucleation and growth. For instance, for F4 coating, comparing Figure 4-29e with Figure 4-32c and Figure 4-29f with Figure 4-32d, one does not find too much difference except some pin-holes are observed after 2days in G-SBF, and also after 7days a new continuous apatite layer is formed on its surface (Figure 4-32d). Therefore, these results indicate that glucose does not seem to have significant influence on the nucleation and growth of bone-like apatite on the prepared pure HA

Figure 4-32 SEM micrographs of FHA coatings after soaking in G-SBF: a) F0-2days, b) F0-14days, c) F2, F3 and F4 after 2days in G-SBF, d) F2, F3 and F4 after 7days in G-SBF, e) F6-2days, f) F6-28days.

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(F0) and other fluoridated hydroxyapatite coatings (F2-F6), which is also observed on pure HA coatings by other researchers [246, 247]. The precipitate apatite layer on all FHA coating surfaces in G-SBF shows porous morphology at high magnification, which is quite similar to those layers obtained in c-SBF (c.f. Figure 4-29i and j).

Figure 4-33 describes the Ca^{2+} concentration in G-SBF for each sample as a function of soaking time. As glucose has no obvious influences on the precipitation process of either HA (F0) or fluoridated hydroxyapatite coatings (Figure 4-32), it is not surprising that the Ca^{2+} concentration profiles does look very much the same as that of in Figure 4-30 (for testing in c-SBF).

Figure 4-33 Concentration of Ca^{2+} after soaking tests in G-SBF for up to 28days: The ups and downs of the Ca^{2+} concentration indicate dissolution and precipitation of apatite from the solution onto the surfaces of the coatings. The Ca^{2+} concentration profile looks the same as that of c-SBF.

4.3.2.3 Testing in A-SBF

4.3.2.3.1 Morphological study

To date, the biological behavior of CaP materials has been studied through *in vitro* tests in SBF solution, with inorganic ion composition and pH value similar to

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biological fluid. But it is well known that the hard tissue environment is more complex especially because proteins such as serum albumin and other blood proteins are present. The initial event that occurs upon exposure of a biomaterial to a physiological environment is the rapid adsorption of proteins onto the material from the surrounding fluid phase. Bovine serum albumin (BSA) is a well-characterized protein that is often chosen to study the interaction of proteins with solid surfaces [248].

As discussed in §4.3.2.1, after 2 days in c-SBF, obvious apatite nucleation is observed on F0 surface, and after 14 days, a continuous layer is formed. But soaking in the protein-containing SBF or A-SBF for 2 days results in much less new apatite nucleation on F0 (Figure 4-34a) than that in SBF (Figure 4-29a). Even after 28 days, the apatite formation is still sporadic (Figure 4-34b). While for F2, F3 and F4, after soaking in A-SBF for 2 days (Figure 4-34c, e and g), there is only sporadic nucleation of new apatite on their surface. Comparing with Figure 4-29c-e and Figure 4-29c, e and g, one can easily see that albumin addition drastically slowed down nucleation of apatite from A-SBF. This retardation effect is likely carried out to the growth stage of the newly nucleated apatite particles. No continuous apatite layer can be observed on F2 coating (only covered by large amount of wormlike apatite nucleation and some particles) even after 28 days immersion in A-SBF (Figure 4-34d) and only after 28 days can the new apatite layer becomes continuous for F3 and F4 (Figure 4-34f and Figure 4-34h, respectively), which only needs only 7 days in standard c-SBF (Figure 4-29f) or glucose modified SBF (Figure 4-32d). The cracks observed in Figure 4-34 f) and h) were caused by drying shrinkage of the newly formed gel-like apatite structure during specimen preparation. While, for F6 coating, the presence of BSA in SBF can

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not see significant influence on the nucleation rate of apatite at the initial stage (the first 2 days, Figure 4-34i) compared with those of c-SBF and G-SBF results in the first 2 days (Figure 4-29h and Figure 4-32f), but there did not form any continuous apatite layer after 28 days in A-SBF (only the sporadic particles could be observed in Figure 4-34j). Therefore, protein addition in SBF adversely affects apatite nucleation and growth on all FHA coatings. According to Combes et al [249], the slow deposition and growth comes from the fact that the adsorption of albumin reduces the interfacial energy of apatite nuclei with the solution, which deprives the driving force for the growth of the new bone-like apatite layers. The SEM micrograph with high magnification indicates that the new formed apatite layer on F3 and F4 after 28 days in A-SBF also possesses a porous microstructure (Figure 4-34k), which is quite similar to those layers obtained in c-SBF and G-SBF (c.f. Figure 4-29i-j).

(Figure 4-34)

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Figure 4-34 SEM micrographs of HA and FHA coatings after soaking in A-SBF: a) F0-2 days, b) F0-28 days, c) F2-2 days, d) F2-28 days, e) F3-2 days, f) F3-28 days, g) F4-2 days, h) F4-28 days, i) F6-2 days, j) F6-28 days, k) high magnification of F3 and F4 after 28days in A-SBF. After soaking in A-SBF for 28 days, the continuous bone-like apatite layer is only observed on F3 and F4 samples.

4.3.2.3.2 Variation of Ca^{2+} concentration

Figure 4-35 shows the Ca^{2+} concentration in A-SBF after the *in vitro* test as a function of soaking time. It is interesting to note that the curves show a sudden drop in the first 2 days for both pure HA (F0) and other fluoridated hydroxyapatite coatings. After that, calcium concentration increases and then slowly decreases. The increase in Ca^{2+} is accompanied with the dissolution of the coatings into A-SBF, while the decrease in Ca^{2+} indicates the precipitation of apatite from A-SBF onto FHA coating surface. The sudden drop of Ca^{2+} concentration is resulted from the adsorption of BSA on the coating surface, which captures large amount of Ca^{2+} from the solution. The interactions between BSA and the coatings are quite complex. Generally, after the coating is immersed into the A-SBF solution, BSA will be immediately absorbed onto the surface and thus influence both the dissolution of the coating into the solution and the precipitation of apatite from the solution onto the coating surface [124, 250]. It has been suggested that the proteins compete with ions, e.g. Ca^{2+} and PO_4^{3-} etc, in the solution for the same surface binding sites [245]. Therefore the adsorption of BSA to the surface of the coating reduces the number of nucleation and growth sites for apatite. On the other hand, the isoelectric point of BSA is 4.7, therefore, BSA will be negatively charged in the physiological solution with a pH value of 7.4, thus tends to bind positive ions like Ca^{2+} in the solution [251]. This is strongly affects the available Ca^{2+} ions for nucleation and growth of apatite. Thus, in the present study, there is a very slow nucleation and growth rate for all coatings in A-SBF (Figure 4-34) compared with the conditions of BSA free SBF solutions (Figure 4-29 and Figure 4-32). The growth rate has been drastically reduced in A-SBF, because F3 and F4 coating only induce a continuous precipitation layer after 28 days (Figure 4-34f and h)

while for the other coatings, no continuous layers could be observed even after 28 days immersion (only larger amount of nuclei could be observed).

Figure 4-35 Concentration of Ca^{2+} after soaking test in A-SBF solution for up to 28 days: The ups and downs of the Ca^{2+} concentration indicate dissolution and precipitation of apatite from the solution onto the surfaces of the coatings.

4.3.2.3.3 Incorporation of BSA into the precipitated apatite layer

Since the precipitation of apatite from A-SBF onto FHA coating surface is modified by the presence of BSA, it is believed that BSA was incorporated into the new formed apatite layer along with the precipitation process. The FTIR spectra of F4 after immersion in c-SBF and A-SBF for 28 days are shown in Figure 4-36. In comparison with the coating before immersing in c-SBF and A-SBF (c.f. Figure 4-4), the peak located at 962 cm^{-1} (ν_1 symmetric stretching vibration mode of PO_4) is observed for all the deposited apatite layers from c-SBF and A-SBF. While, the ν_3 stretching mode of phosphate group (PO_4) in the range of $1000\text{--}1150\text{ cm}^{-1}$ is a broad band for the new formed apatite layer instead of well split sharp peaks in the coating before immersion test (c.f. Figure 4-4), indicating that the precipitated apatite layer is amorphous and poorly crystallized. The IR peaks of carbonate are observed clearly around $1400\text{--}1500\text{ cm}^{-1}$ and near 872 cm^{-1} (Figure 4-36), suggesting that the obtained new apatite layer is

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carbonated apatite. The broad peaks in Figure 4-36b) at about 1634 cm^{-1} and $3000\text{--}3700\text{ cm}^{-1}$ are relevant to strong H-O-H vibration (water bands) [252]. With the addition of BSA into the SBF solution, important differences in the FTIR band pattern are detected mainly in the range of $1500\text{--}1700\text{ cm}^{-1}$. As shown in Figure 4-36a), the absorption band located at around 1650 cm^{-1} is assigned to amide I band, which arises from C=O stretching (80% of the band intensity), C=N stretching (10%), and N=H bending (10%) in BSA. Another peak at about 1540 cm^{-1} , labeled as amide II, is composed of N-H bending (60%) and C-N stretching (40%) vibration [239, 250]. In addition, it should be noted that the broad band at about $3000\text{--}3700\text{ cm}^{-1}$ in Figure 4-36a) should include both contribution of water band and the vibration of N-H in BSA; and amide I band of BSA is also overlapped with the water band (at around 1634 cm^{-1} in Figure 4-36b). The presence of amide bands should be attributed to two aspects: the adsorbed BSA on the new formed apatite surface and BSA incorporated in the precipitated apatite layer.

Figure 4-36 FTIR spectra of F4 coating after immersion in a) A-SBF and b) c-SBF for 28 days: peak located at around 962 cm^{-1} and the broad band in the range of $1000\text{--}1150\text{ cm}^{-1}$ indicates that the new layer is an poorly crystallized apatite layer. Absorption bands of carbonate at about 872 cm^{-1} and $1400\text{--}1500\text{ cm}^{-1}$ indicate a carbonated apatite layer. The components located at around 1650 cm^{-1} and 1540 cm^{-1} are assigned to amides I and II in BSA.

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As discussed above, the precipitation of apatite from A-SBF onto FHA coating surface is modified by BSA. It has been reported that BSA would be incorporated into the new formed apatite layer [253]. In the current investigation, XPS was employed ascertain whether BSA has been incorporated into the precipitated apatite layer. Figure 4-37 a) shows the N1s narrow scan spectra of the new formed apatite layer on F4 after immersing in A-SBF for 28 days. The N1s peak with a binding energy of ~ 400 eV confirms the presence of BSA [248]. After etching for 15min, the N1s peak can be still observed clearly despite some decrease in peak intensity in contrast to the spectrum without etching process (Figure 4-37a). In order to confirm that BSA has been incorporated into the precipitated apatite layer, additional XPS study on the adsorption of BSA on F4 coating surface was carried out. After immersing the F4 sample in A-SBF for 20min, the sample was taken out and washed gently with DI-water for 3 times, then used for XPS analysis. As shown in Figure 4-37b), after etching for 15min, the N1s peak is too weak to be identified, indicating that the adsorbed BSA on the coating surface has been roughly removed through the etching process. Therefore, it is believed that after etching for 15min, the adsorbed protein on the new formed apatite layer (Figure 4-37a) has been roughly removed. The decrease in N1s peaking intensity after etching 15min (Figure 4-37a) is caused by the removal of adsorbed BSA on the apatite layer surface, and the N1s peak there (Figure 4-37a) suggests the incorporation of BSA into the precipitated apatite layer (to some extent, the precipitated apatite layer can be considered as some kind of composite containing BSA and apatite).

Figure 4-37 XPS narrow scan spectra of N1s for a) the precipitated apatite layer on F4 after immersion in A-SBF for 28 days, and b) the adsorbed BSA on F4 after immersion in A-SBF for 20min. After an etching process of 15min, it is believed that the adsorbed BSA on coating surface could be roughly removed.

4.3.2.3.4 Retarding effect of BSA on apatite precipitation

As discussed above, the influence of BSA on the precipitation of apatite layer from A-SBF onto coating surface is quite complex due to its own “adsorption” property. It is well known that proteins are long chains of amino acids and are highly surface-active molecules due to their amphiphilic properties [254]. The carboxyl or amino groups in proteins can dissociate in aqueous solution at a suitable pH value, resulting in COO^- and NH_3^+ ions covalently attached to the protein macromolecule. Generally, in an acidic solution, a typical protein becomes positively charged because of the presence of NH_3^+ and COOH groups, and in a basic solution it is charged negatively because of NH_2 and COO^- groups. When pH value equals the isoelectric point (pI) of the protein, the net charge is zero. While, for BSA, its isoelectric point is 4.7 [254], which means that at the physiological pH value (7.4), this kind of protein is negatively charged and tend to bind positive ions like Ca^{2+} ions present in the environment solution (but it can also bind a certain amount of negatively charged ions, e.g. PO_4^{3-}).

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According to the current results (Figure 4-29 and Figure 4-34), the presence of BSA seems to have an inhibitory effect on the precipitation (nucleation and growth) rate of newly formed bone-like apatite layer. Based on the above discussion, the cause of this retardation could be twofold: firstly is the adsorption of BSA on the coating surface. It has been reported that the proteins compete with calcium and phosphate ions in solution for the same surface binding sites [250, 255]. Therefore, the adsorbed BSA on coating surfaces occupy the sites where precipitation would occur, thus blocking the number of active nucleation/growth sites. On the other hand, as the ability of a surface to nucleate mineral phases is closely to the magnitude of interfacial energies (equation (4-12)), a coating which exhibits a good nucleatory ability in a protein-free solution may be less efficient in the presence of proteins. The adsorption of protein on coating surface can cause a decrease of γ_{SL} (equation (4-12)) and thus lower the nucleation ability of the coatings (equation (4-9)) [249]. Secondly, BSA can also bind the calcium and phosphate ions in SBF solution, thus those calcium and phosphate ions, which ought to participate in the precipitation of bone-like apatite layer, are masked by the macromolecules of BSA. Such that, the effective amount of calcium and phosphate ions used for the formation of new CaP from SBF onto coating surface is drastically reduced, leading to the decrease in driving force ΔG (equation (4-9)) for the precipitation process. Consequently, those inhibition effects of BSA in SBF solution finally result in the slowing down of nucleation rate and growth rate of new bone-like apatite. These current results are in accordance with the investigation of other researchers' work [246, 249] regarding the influences of proteins on the in vitro tests in acellular SBF solutions.

In addition, since all FHA coatings (F0-F6) have comparable ability for the adsorption of proteins onto their surfaces (c.f. §4.3.3.1), it is believed that the intensity of the inhibition effect of BSA on the precipitation of bone-like apatite from SBF onto FHA coating surfaces is roughly the same. Therefore, by considering the results in SBF and A-SBF, we can say that the coating with a fluoridation degree of about 0.8-1.1 (for F3 and F4 coatings) has the most promising bioactivity in comparison with other coatings.

4.3.3 Cell responses to FHA coatings

4.3.3.1 Protein adsorption

The interaction between osteoblastic cells and implants depends on the surface aspects of implants, which may be described according to their chemical and physical properties. These surface characteristics determine how biological molecules will be adsorbed onto implant surface and will influence the following cell-implant interactions. It is known that proteins exist in blood plasma or serum in great numbers; once in touch with an implant, they quickly adhere to the implant surface because they are intrinsically highly surface active. It is through this adsorbed protein layer that cells sense foreign surfaces [125, 256]. The adsorption of proteins plays a pivotal role for the subsequent cell attachment and the eventual cell morphology (c.f. §2.2.2.4.2.1). Figure 4-38 summarizes the amount of adsorbed proteins on FHA coatings measured by the Micro-BCA method. No significant difference ($p>0.05$) is observed in the total amount of adsorbed proteins between different FHA coatings after immersing in the culture medium for 20 minutes (all at the level of $\sim 1.6 \mu\text{g}/\text{cm}^2$). But this does not mean that fluorine incorporation has no influence on protein

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adsorption. It has been demonstrated that greater contact angle results in reduction of protein adsorption [257, 258]. At the same time, however, larger surface area results in more adsorption. As a result of the competition between increase of contact angle and increase of surface roughness (thus surface area), the net change in protein adsorption seems negligible.

Figure 4-38 Protein adsorption on FHA coatings after 20 minutes immersion in cell culture medium. No significant difference ($p>0.05$) is observed for the amount of adsorbed proteins ($\sim 1.6 \mu\text{g}/\text{cm}^2$) on different FHA coating surfaces.

4.3.3.2 Initial cell attachment and spreading

4.3.3.2.1 Quantitative assay of cell attachment

The initial cell attachment onto FHA coatings was directly monitored by quantifying cell numbers at each culture period (0.5, 1, 2 and 4h). The results were shown in Figure 4-39. Similar amounts of cells were attached onto different FHA coatings at each time slot. That indicates the incorporated fluorine ions have no significant influence (whether positive or negative) on initial cell attachment. In other words, all FHA coatings show comparable cell attachment, and there is no significant difference from that of the pure HA (see sample F0). That is understandable since cells attach to

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the coating surface through attachment on a layer of protein which in turn attach to the coating [129, 256]. As the number of protein attached to the surface is the same on FHA and on pure HA, it follows that there is no difference in number of cells attached on these coatings. Since FHA coatings are hydrophilic and a similar amount of adsorbed proteins is observed, naturally, about the same amount of anchorages would be provided for cell attachment. Besides protein adsorption, surface roughness could also exert some influence on initial cell attachment. Deligianni et al reported that the number of cells attached onto HA surface experienced insignificant increase when the surface roughness (R_a) increased from 0.73 μm to 2.85 μm [259]; however, a significant difference was observed when the surface roughness reached 4.68 μm . In our study, the roughness variation (max ~ 33 nm in R_a) is too small to cause any significant increase in cell attachment.

Figure 4-39 Cell attachment assay onto FHA coatings culture for up to 4 hours: Similar amounts of cells were attached onto different FHA coatings at each time slot, suggesting that the incorporated fluorine ions have no significant influence on initial cell attachment.* indicates a significant increase of attached cell numbers at 4h with respect to that at 0.5h.

With increase of incubation time, a slight increase in the cell attachments is observed.

The increase becomes significant ($p < 0.05$) after 4h incubation compared with that of 0.5h. Most cells are believed to arrive at the coating surface in 0.5h due to the

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exertion of gravity and the physiochemical interactions between the cells and the adsorbed molecules on coating surface [260], but a large amount of weakly adhered cells are removed during the washing. After longer incubation, most cells attach well on the coating surface thus an increase in cell attachment is seen.

4.3.3.2.2 Morphological change of cells during the first 4hours

The individual cell spreading process and the corresponding morphologies were studied with SEM. Since the cells do not reach the coating surface at the same time or spread at the same rate, the morphology may not be the same at a culture interval (Figure 4-40). Therefore, the morphological sequences reported and the micrographs shown represent the most typical individual cell morphology at that time interval.

Figure 4-40 A typical cell morphology at lower magnification after incubation for 4hours. Because the cells do not reach the coating surface at the same time or spread at the same rate, the morphology may not be the same at each culture interval. Therefore, the morphological sequences reported and the micrographs shown represent the most typical cell morphology at that time interval.

The cell spreading process as monitored by the change of cell morphologies was the same on all FHA coatings. Typically, the morphological change sequences of

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osteoblastic cell spreading on HA (F0, without incorporation of fluorine ions) and fluoridated HA (F4 as a representative) are shown in Figure 4-41 and Figure 4-42 respectively. After cultured for 0.5h, the osteoblastic cells still appeared spherical with a rough surface texture containing numerous blebs (Figure 4-41a and Figure 4-42 a). Those blebs are believed to increase the total surface area of the cells to accommodate the excess membrane as the cells round up from the flattened state in response to trypsin [260]. At the base of cells, filopodia were developed and extended on coating surface. The filopodia enabled the cells to contact and attach on the surface. That was the first step. With increase of incubation time, the filopodia gradually extended outward for spreading/webbing of cytoplasm. After 1h incubation (Figure 4-41b and Figure 4-42 b), though the main body of cells was still almost spherical, the cells was well attached to the surface with extended filopodia as well as lamellipodia formed by the spreading of cytoplasm. As time passed, cytoplasm continued to spread outwards, leading to flattening of the cells. After 2h (Figure 4-41c and Figure 4-42 c), the main body of the cells became cone-shaped and surrounded by cytoplasm. At this stage, high adhesion strength is expected due to the increase of contact area between the cells and the coating surfaces [261]. The progression of cytoplasm webbing finally resulted in a fully flattened cell, as shown in Figure 4-41d and Figure 4-42 d, where even the nucleus could be seen. The cells attached to the surface tightly and their shape changed from spherical to polygonal, similar to that after 72h (as shown in Figure 4-43). As such, the cell attachment is completed within 4h incubation.

Figure 4-41 Morphological changes of osteoblastic cells after incubating on F0 for up to 4 hours: a) 0.5h, b) 1h, c) 2h, d) 4h. The process of initial cell attachment and spreading can be described as the following continuous steps: contact and attachment of cells a), centrifugal growth of filopodia b), cytoplasm spreading c) and cell flattening d).

Based on the above observations, the process of cell attachment and spreading happens in the following steps, similar to that observed with osteoblast or other cells on HA, alumina, bone and tissue culture polystyrene samples [256, 260]: (1) adsorption of proteins on coating surface (surface roughness plays a positive role); (2) contact and attachment of cells onto the protein layer (surface roughness does not have effect unless very rough—more than a few microns difference); (3) centrifugal growth of filopodia; (4) cytoplasm spreading and finally (5) cell flattening. Fluorine incorporation in HA causes an insignificant increase of surface roughness ($\sim 33\text{nm}$ in R_a) and decrease in wettability (increased contact angle), it does not affect protein adsorption or initial cell attachment. In other words, all FHA coatings are the same for

Figure 4-42 Morphological changes of osteoblastic cells after incubating on F4 for up to 4 hours: a) 0.5h, b) 1h, c) 2h, d) 4h. The process of initial cell attachment and spreading can be described as the following continuous steps: contact and attachment of cells a), centrifugal growth of filopodia b), cytoplasm spreading c) and cell flattening d).

cell attachment and spreading as pure HA coating (Figure 4-41 and Figure 4-42). According to Folkman et al [126], well-spread cells after 4hours incubation are more favorable for cell proliferation and differentiation that follows. Consequently, incorporation of fluorine ions in HA has no adverse effect on initial cell activity. It should be emphasized that these different stages are not discretely separable but are different phases of a contiguous progress. Recognition and studies on of these different morphological events during initial cell adhesion and spreading on FHA-coated implants would be of great value in understanding the behavior of osteoblastic cells in vitro and in vivo.

4.3.3.3 Cell morphology

Initial cell attachment and spreading indicate that the prepared FHA coatings are favorable for initial cell-coating contact. Further cell spreading and morphology also should be investigated in order to evaluate cell viability on those coating surfaces. Figure 4-43 shows typical SEM micrographs of MG63 cells after culture duration of 3 days on FHA coatings. No significant difference is observed in cell morphology on different FHA coatings. All cells spread well and grew favorably throughout the coating surface. Under higher magnification (Figure 4-43 b, c and d), the cells are seen flattened and attach tightly on coating surfaces with their filopodium and lamellipodium, suggesting good cell viability on all FHA coatings. As discussed in §2.2.2.4.2.1, the well-spread cells on all FHA coating surface are expected to be favorable for DNA synthesis and cell growth.

Figure 4-43 Morphology of MG63 cell after 3 days on: a) F0 (at lower magnification), b) F0, c) F4 and d) F6. All cells spread well and grow favorably throughout the coating surface, suggesting good cell viability of all FHA coatings.

4.3.3.4 Cell proliferation

Following the initial cell attachment and spreading, cells will step into the stage of proliferation. In this project, cell proliferation was directly monitored by quantifying cell numbers at each culture period and the results are shown in Figure 4-44. Within the first 2 days, cell number increased slightly and no significant difference (considered at $p < 0.05$) observed among the coatings. A greater proliferation rate occurs between 2 to 5 days followed by a slow-down till 7 days. The cell numbers on F3 and F4 were significantly ($p < 0.05$) higher than that on F0, F2 and F6 after 5 days. No significant difference is observed among F0, F2 and F6 over the entire evaluation period. Comparing with the initial cell density, cells were drastically proliferated on all FHA coating surfaces after 7 days (at least 4 times as many as the seeding density). That means that all FHA coatings, regardless of the degree of fluoridation, have favorable viability with MG63 cells, thus have comparable bioactivities *in vitro*. On the other hand, by considering that the amount of initially attached cells was almost the same for all FHA coatings (c.f. Figure 4-39), the cell numbers on F3 and F4 coatings were significantly higher than on other coatings after 7 days (Figure 4-44), indicating that F3 ($X = 0.84$) and F4 ($X = 1.04$) coatings have stronger stimulations on cell proliferation. Similarly, Harrison et al [262] reported that a higher number of mouse embryonic stem cells were obtained with a fluoridation level of 0.3-1.1 in FHA discs. Qu et al [257] reported that after 7 days, osteosarcoma cells (SAOS-2) on FHA discs with fluoride content of 0.3-0.567 (mol F⁻/mol apatite) had a significantly higher total amount of protein than that on pure HA, indicating a higher proliferation rate on those discs. As such, certain amount of fluorine in the coatings is believed to benefit cell proliferation considerably.

Figure 4-44 Cell proliferation of MG63 on FHA coatings: all coatings have favorable viability with MG63 cells regardless of the degree of fluoridation. The significantly higher cell numbers on F3 and F4 after 7 days indicates that F3 and F4 coatings have more stimulation on cell proliferation.

4.3.3.5 Cell Differentiation

As an early differentiation marker, it is reported that ALP is associated with calcification and an enhanced expression of this enzyme is apparently needed just before the onset of matrix mineralization, providing localized enrichment of inorganic phosphate, one of the components of apatite, the mineral phase of bone [108, 131]. In our present study, Figure 4-45 reveals the intracellular alkaline phosphatase activity of MG63 cells on FHA coatings over the culture period. There was a very significant increase ($p < 0.01$) in ALP level for F0 and F6 in the first two weeks followed by an insignificant (since $p > 0.05$) increase in the third week. In contrast, although a significant increase ($p < 0.01$) was achieved for F2 and F3, and an insignificant change for F4 within the first two weeks, they all presented a significant decrease ($p < 0.01$) in ALP activity in the following week (down-regulation), indicating that more cells cultured on F2, F3 and F4 have stepped into next differentiation stage (mineralization) [108]. F4 displayed the highest ALP level and no significant difference in others

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samples within the first week. At week two, the ALP levels in F2, F3 and F4 were significantly higher than others ($p<0.05$), suggesting that the cells shifting to a more differentiated stage. Though no significant difference could be observed for all FHA coatings after three weeks, we could confirm that F2, F3 and F4 were in the stage of down-regulation of ALP activity. Even though some researchers reported that the incorporation of fluorine ions has insignificant effect on ALP activities [16, 196], in our present study, significantly higher value was obtained on F4 after one week, and significantly higher levels on F2, F3 and F4 at week 2 followed by significant decrease at week 3 indicated that certain amount of fluorine in the coating can stimulate early cell differentiation. This confirms that certain amount of fluorine ions stimulate early cell differentiation [257, 263].

Figure 4-45 Intracellular alkaline phosphatase activities of MG63 cells on FHA coatings. * At week 1, F4 had a significantly higher ALP activity than that of other coatings ($p<0.01$). ** At week 2, F2, F3 and F4 had a significantly higher ALP activity than that of F0 ($p<0.05$).

Along with ALP activity, osteocalcin (OC) is regarded as a late marker of osteoblastic cell differentiation [130]. It is reported that OC is specifically synthesized by osteoblasts at the late stage of differentiation and has the ability to chelate Ca^{2+} to form bone minerals, thus it also serves as a marker of mineralization [130, 132]. In the

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present study, the level of intracellular osteocalcin secreted by MG63 cells on different coatings, as illustrated in Figure 4-46, were similar to each other at week 1, then a significant rise ($p < 0.05$) was observed for F3 and F4 compared with other samples between week 1 and week 2. Finally, all of the samples reached a similar peak value at week 3. On the late differentiation marker, although all the coatings presented comparable levels of osteocalcin after 3 weeks (Figure 4-46), significantly higher amount of osteocalcin were obtained on F3 ($X = 0.84$) and F4 ($X = 1.04$) at week 2, suggesting that certain amount of fluorine incorporation could stimulate more cells to step into the late differentiation stage. Therefore, by considering the ALP activities and OC levels, coatings with actual fluorine content in the range of 0.8-1.1 have stronger stimulation on cell differentiation as well as cell mineralization process, which is consistent with the reference [264].

Figure 4-46 Intracellular osteocalcin assay results for MG63 cells cultured on FHA coatings. * indicates that at week 2, the Osteocalcin levels of F3 and F4 were significantly higher than that of F0 ($p < 0.05$).

Based on our *in vitro* cell culture studies, the difference observed in cell responses (including cell proliferation, ALP activity and osteocalcin level) for different FHA coatings suggests that the incorporated fluorine ions play a critical role in influencing

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the cell behaviors. Even though chemical or physical properties of the fluoridated HA coatings exert influences on cell response, the main player should be the combined ionic effects along with the dissolution behavior. The fluorine concentration in the range of $\sim 10^{-7}$ - 10^{-5} M (released from NaF) stimulates proliferation and ALP level of osteoblastic cells [265, 266]. The *in vivo* tests also indicate that the presence of low doses of NaF could stimulate the osteoblastic activity, resulting in a higher osteocalcin concentration [267]. Ca is found to enhance osteoblast differentiation, whereas the presence of inorganic phosphate (Pi) slows down osteoblast differentiation and mineralization [268]. Meleti et al [269] also concludes that cells display a profound loss of mitochondrial membrane potential in the presence of Pi, suggesting that Pi activates cell apoptosis through induction of a mitochondrial membrane transition. Understandably the results of the present study are the net result of ion effects of dissolved Ca, F and P (c.f. §4.3.1). Assuming a stoichiometric dissolution behavior in FHA coatings, F⁻ released can be estimated as in the range of $\sim 10^{-7}$ - 10^{-5} M in all of the fluoridated HA coatings. This range falls, perfectly in the above mentioned effective range. The stimulating effects of Ca and F were compromised by the adverse effects of P. As a result, the most positive outcome after the comprises were observed on coatings with fluorine concentration (*X* value) between 0.8-1.1 (F3 and F4).

4.3.3.6 Discussion

It is essential for the efficacy of orthopaedic or dental implants to establish a strong, durable, and stable solid interface with complete fusion between the material's surface and the bone tissue with no fibrous tissue interface. The interface between bone and an implant is determined by many factors, including cellular responses to and

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interactions with the implants. An ideal implant imitates natural biological tissue, and the interface exhibits intimate apposition of bone to the implant or even chemical bonding between bone and implant. On the other hand, the biocompatibility of an implant is very closely related to cell behavior on contact with them and particularly to cell responses to their surfaces. Therefore, a complete understanding of the responses of the bone-forming osteoblastic cells to a solid implant could help in assessing and optimizing the implant performance, and especially to optimize the surface properties of the implants. Generally, the responses of a single type of cell, such as osteoblastic cell, are difficult to assess with animal models (*in vivo* test) because of the numerous and complex events that occur when a biomaterial is placed in a bloody wound site. In contrast, *in vitro* models are useful for the examination of one cell line at a time.

In this project, MG63 osteosarcoma cell is employed to evaluate the cell responses to FHA coatings. This cell line consistently and reproducibly exhibits a number of fundamental phenotypic characteristics of osteoblasts, enabling comparisons to be made between different experiments without the inevitable wide variation of primary bone cells obtained from different cell sources and of different passage in tissue cultures [213]. As an anchorage-dependent cell line (osteoblastic cell, MG63), initial cell attachment and spreading are crucial prerequisite in determination of long-term viability of cells on the implant surface, involving cell proliferation, differentiation, mineralization and successful osteointegration [108, 126, 213], while cells that cannot adhere and spread have been reported to undergo programmed cell death via apoptosis [270]. In this work, it is shown that all fluoridated HA coatings show similar capability for cell attachment as that of pure HA coating during the first 4hours (c.f.

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Figure 4-39). Once the cells attach onto FHA coating surface, they will progressively spread through the coating surface (typically shown in Figure 4-41 and Figure 4-42). Surface reactivity is believed to be a key factor in determining the morphological and functional responses observed during the cell-implant interactions. According to previous work reported by Folkman et al [126], it is believed that the well-spread cells after 4h incubation (c.f. Figure 4-41 and Figure 4-42) are more favorable for the following cell proliferation and differentiation on those FHA coatings. Moreover, after 4h incubation, the cells attached onto the coating surface tightly and the shape changed from spherical to polygonal, which looks much like the morphology after cultured for 72h (c.f. Figure 4-43). Therefore, the incorporated fluorine ions have no adverse effect on cell attachment and spreading, and osteoblastic cells could form intimate interfaces with the various FHA coatings, which suggests a high degree of biological acceptance of the materials.

The interaction between osteoblastic cells and implants depends on the surface aspects of implants, which may be described according to their chemical and physical properties. These surface characteristics determine how biological molecules will be adsorbed onto implant surface and will influence the following cell-implant interactions. It is known that proteins will immediately adsorb on implant surface after implantation of an implant in human body, and cells do not interact with a naked material either in vitro or in vivo. Thereafter, cells in contact with a surface depend on the protein adsorption. In the present study, although there are some differences in surface roughness and surface energy etc (c.f. Figure 4-8) between different FHA coatings, the amount of totally adsorbed proteins appears to be the same in view of the average value and the standard derivation (c.f. Figure 4-38). It is believed that the incorporated fluorine ions mediate the adsorption of proteins through their influence

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on surface roughness and surface wettability, therefore, a similar amount of adsorbed proteins is observed for all FHA coatings. On the other hand, the attachment and spreading of osteoblastic cells also imply insignificant difference in coating surface properties (especially in the ability of protein adsorption) between different FHA coatings.

Following the attachment and spreading, those osteoblastic cells will step into the stage of proliferation and differentiation (c.f. **2.2.2.4.2**) [130], which are evaluated by cell counting, ALP activity and OC production at each time period respectively. As shown in Figure 4-44, all coatings have favorable viability with MG63 cells thus have comparable bioactivities in vitro. But at the same time, according to the proliferation rate, the cell numbers for F3 and F4 are significantly higher than other coatings after 7 days (c.f. Figure 4-44), indicating that certain degree of fluoridation could stimulate the proliferation rate of MG63 cells in vitro. Because of the same amount of initially attached cells (c.f. Figure 4-39) as well as the same ability for cell spreading (c.f. Figure 4-42 and Figure 4-43), the enhanced cell proliferation rate on F3 and F4 is attributed to the effect of fluorine ions incorporation. Along with the cell proliferation, the cell functionality was assessed by measuring the osteoblast differentiation markers, i.e. ALP and OC. The enzyme ALP exists in intracellular membranes and its catalytic activity enhances the secretion of other extracellular matrix proteins, thus it is considered as a marker for osteoblastic cells at a relatively early stage [195]. As a later marker of cell differentiation, OC is a bone-specific differentiation marker, and is known to stimulate bone mineralization at the later stage. Generally, once mineralization is initiated, alkaline phosphatase will decrease while osteocalcin accumulation continues, reflecting an expression related to deposition of mineral for

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the latter. In our present study, the significant increase in ALP levels during the second week suggested that the cells shifting to a more differentiated stage (c.f. Figure 4-45). On the other hand, the significant higher value was obtained for F4 after one week, and the significant higher levels for F2, F3 and F4 at week 2 indicated that certain fluorine content in the coating could really stimulate the early cell differentiation behavior [263]. Regarding the late differentiation marker, although all the coatings presented the comparable levels of osteocalcin after 3 weeks (c.f. Figure 4-46), significant up-regulations were observed for F3 and F4 at week 2 compared with other coatings. That is to say, coatings with a fluorine content in the range of 0.8-1.1 (X value) have stronger capability of stimulating cell differentiation behaviors as well as cell mineralization process [264].

Such differences (enhancing effects) observed in cell proliferation and differentiation (ALP and OC activities) suggest that the incorporated fluorine ions play certain crucial role in changing the activities of osteoblastic cell functions. Even though some other chemical or physical properties of the fluoridated HA coatings will exert certain influences on cell response, the main reason is attributed to the combined ionic effects along with the dissolution behavior (the released Ca^{2+} , PO_4^{3-} and F^-). Practically, the fluorine ion itself is known to effectively enhance the proliferation and differentiation (ALP activity, and synthesis of bone-specific proteins) of osteoblastic cells. The calculated fluorine concentration for FHA coatings released in this study ($\sim 10^{-7}$ - 10^{-5} M) is quite in accordance with the range ($\sim 10^{-7}$ - 10^{-5} M) suggested for the enhancement of osteoblastic functions. On the other hand, other ions, i.e. Ca^{2+} and PO_4^{3-} , would also play an important role in influencing the cell responses. It is reported that Ca ions could enhance the osteoblastic functions, whereas the presence

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of inorganic phosphate (Pi) has adverse influences on osteoblastic functionalization. For the fluoridated HA coating, the incorporated fluorine ions can control the solubility of the coating itself, thus control the released ion concentration (F^- , Ca^{2+} , and PO_4^{3-}) in the medium (c.f. §2.3.1.2.3 and §4.3.1). Therefore, by considering the variation of coating solubility, the reduction in enhancing effect of F^- and Ca^{2+} caused by the decrease of solubility will be compensated, at the same time, by the weakening in the adverse effect of phosphate ions. Consequently, the cell responses are the outcome of the combined ion effects of F^- , Ca^{2+} and PO_4^{3-} . Still, the elucidation of the relationship between ionic dissolution and cell responses requires further tests, such as ion-mediated gene expression etc. However, from this study, by considering all the *in vitro* biocompatible and bioactivity tests (dissolution tests, bioactivity tests in acellular solutions and cell responses), it is recommended that the best or optimal fluoridated hydroxyapatite takes the form of $Ca_{10}(PO_4)_6(OH)_{1.2-0.9}F_{0.8-1.1}$ (X value between 0.8-1.1).

4.3.4 Summary

To summarize, the *in vitro* behaviors (dissolution behavior, bioactivity in acellular solutions, and cell responses) of FHA coatings have been studied, we found that:

- Incorporation of fluorine ions into HA lattice structure lowers the solubility of HA in tris-buffered physiological saline solution (TPS), and the lowest solubility can be achieved at a fluoridation degree of 0.8-1.1 (X value).
- The presence of glucose (1 g/L) in TPS (G-TPS) has negligible influence on the dissolution behavior of FHA coatings. The introduction of albumin (BSA, 40 g/L) into TPS (A-TPS) has a stimulating effect on the release of Ca^{2+} from FHA coatings into the solution, but the order of dissolution rate of FHA coatings is not

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influenced by the addition of BSA ($F_0 > F_2 > F_6 > F_3 \approx F_4$).

- In vitro bioactivity test in conventional simulated body fluid (c-SBF) indicates that the nucleation and growth rate of bone-like apatite is dependent on the degree of fluoridation: the bioactivity increases firstly with increasing F^- content till F_4 , and then decreases remarkably.
- The presence of glucose in SBF (1 g/L, G-SBF) has negligible influence on the bioactivity. While the presence of BSA at a concentration of 40 g/L (A-SBF) has significant retardation effect on apatite precipitation from SBF onto FHA coating surfaces.
- Incorporation of fluorine ion in HA does not affect the amount of proteins adsorbed on the coating surface ($\sim 1.6 \mu\text{g}/\text{cm}^2$). This, in turn, results in no effect on initial cell attachment and spreading (during the first 4 hours). The process of cell attachment and spreading happens in the following steps: adsorption of proteins on coating surface, contact and attachment of cells, centrifugal growth of filopodia, cytoplasm spreading and flattening.
- Cells adhere and spread well across the entire coatings surface regardless of F^- content after 3 days of incubation, suggesting good cell viability on all FHA coatings.
- Incorporated fluorine ion has positive stimulating effects on cell proliferation, alkaline phosphate activities and, osteocalcin levels, especially at fluoridation degrees in the range of 0.8-1.1. The influence on cell phenotypes comes from the combined ion effects of Ca, P and F released from the coating. For the best resistance to dissolution and the highest cell activities, it is recommended that the fluoridated hydroxyapatite take the form of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_{1.2-0.9}\text{F}_{0.8-1.1}$.

Chapter 5 Conclusions and Recommendations

5.1 Conclusions

In this project, fluoridated hydroxyapatite (FHA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_{2-x}\text{F}_x$) coatings have been deposited on Ti6Al4V substrates by sol-gel dip-coating method. The mechanical properties, including residual stress, elastic modulus, adhesion properties between coating and substrate, and coating fracture toughness, have been systematically investigated. The dissolution behavior, *in vitro* bioactivity as well as osteoblastic cell responses to the FHA coatings, have been extensively characterized. Based on the results shown in Chapter 4, the following conclusions can be drawn:

1. Dense and uniform FHA coatings ($\sim 1.5 \mu\text{m}$ in thickness) on Ti6Al4V substrates have been successfully deposited by sol-gel dip-coating method. XPS, XRD and FTIR results confirm that the fluorine ions have been successfully incorporated into the HA lattice structure, and the resulting FHA coatings were homogeneous and of high purity. Besides the amorphous phase, no other impure phases existed in FHA coatings. The Ca/P ratio is nearly equal to that of stoichiometric value of 1.67.
2. The incorporation of fluorine ions has significant influences on the mechanical properties of FHA-coated implants. The pull-out tensile test shows that the adhesion strength ranges from $\sim 19 \text{ MPa}$ for pure hydroxyapatite (F0) to $\sim 27 \text{ MPa}$ for fluoridated HA coatings. After soaking in Tris-buffered physiological saline solution for 21 days, the adhesion strength increases to $\sim 29 \text{ MPa}$ in the case of

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pure HA and to over 39MPa in the case of fluoridated HA coatings. The scanning scratch test reveals that with the increase of fluorine concentration, the critical load increases, and the coating-substrate interfacial failure mode changes from brittle to ductile, indicating a much stronger adhesion for a higher fluoridation degree. As for the interfacial shear strength evaluated with the shear lag strain method, with increasing fluorine content in the coating, the interfacial shear strength between the coating and substrate increases (range from ~393 MPa for F0 to ~572 MPa for F6). In addition, with incorporation of fluorine ions, the fracture toughness of the coating doubles as compared with pure HA coating (F0). The enhancement in mechanical properties is attributed to two aspects: the reduction of residual stress with increasing fluorine ion content in the coating and the formation of complex chemical bonds in the transitional region between the coating and the substrate.

3. Incorporation of fluorine ions in HA lattice structure lowers the solubility of HA in Tris-buffered physiological saline (TPS) solution. The lowest solubility can be achieved at a fluoridation degree of 0.8-1.1 (F3 and F4). In comparison with the dissolution behavior in TPS, the presence of glucose (1 g/L) in TPS (G-TPS) has negligible influence on the dissolution behavior of FHA coatings. As for the tests in bovine serum albumin (BSA, 40 g/L) modified TPS (A-TPS), although an increase in the total amount of released Ca^{2+} is observed for each coating, the order of dissolution rate of FHA coatings is not influenced by the addition of BSA ($\text{F0} > \text{F2} > \text{F6} > \text{F3} \approx \text{F4}$).

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4. *In vitro* bioactivity test in conventional simulated body fluid (c-SBF) indicates that the nucleation and growth rate of bone-like apatite deposited from the solution onto the coating surface is significantly dependent on the degree of fluoridation: the bioactivity increases firstly with increasing F^- content till F4 coating and then decreases remarkably. As for the test in glucose-modified SBF (1 g/L, G-SBF), in comparison with the test in c-SBF, the negligible influence is observed on the nucleation and growth of bone-like apatite on FHA coating surfaces. In contrast, the addition of BSA in SBF (40 g/L, A-SBF) has significant retardation effect on the precipitation (nucleation and growth) of bone-like apatite from A-SBF onto FHA coatings.

5. The initial osteoblastic cell (MG63) attachment and spreading on FHA coatings (during the first 4 hours) happen in the following steps: adsorption of proteins on coating surface; contact and attachment of cells; centrifugal growth of filopodia; cytoplasm spreading and flattening. The protein adsorption test shows that all FHA coatings have a similar amount of adsorbed proteins ($\sim 1.6 \mu\text{g}/\text{cm}^2$) after soaking in the complete cell culture medium, which results in an insignificant difference for the amount of initially attached cells among different FHA coatings. The well-spread cells on all prepared FHA coating surfaces indicate that the incorporation of fluorine ions have no adverse effect on the initial cell attachment and spreading process, suggesting that the fluoridated hydroxyapatite coatings have comparable bioactivity to that of pure hydroxyapatite in terms of the initial cell attachment and spreading.

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6. The incorporated fluorine ions have positive stimulating effect on osteoblastic cell proliferation, and all cells adhere and spread well throughout the entire coating surface regardless of different fluoridation degrees. Although all FHA coatings show a positive stimulating effect on cell differentiation (ALP activity and osteocalcin level) behaviors, the coatings fluoridated to 0.8-1.1 have stronger stimulating effect compared with other coatings. The influences on cell phenotypes come from the combined ion effects of Ca, P and F released from the coating. For the best dissolution resistance and cell activities, it is recommended that the fluoridated hydroxyapatite should take the general form of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_{1.2-0.9}\text{F}_{0.8-1.1}$.

5.2 Recommendations

Although a great deal of research has been conducted in developing and investigating the sol-gel derived fluoridated hydroxyapatite coatings for biomedical applications, a number of other interesting areas are still remaining to be explored and improved. Some of these are described below:

1. Fatigue properties of FHA-coated implants. For those load bearing implants, it is believed that the durability and reliability basically depend on its resistance to fatigue and biodegradation due to routine activities, e.g. walking, running and stair climbing etc. The fatigue behavior can cause cracks in the coating and this crack (if normal to the loading direction) may continue to grow toward the coating/substrate interface. If this kind growth can be along the interface, failure caused by delamination will be observed for the implants. Therefore, fatigue behaviors should be carried out before the practical biomedical applications of

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FHA-coated implants. For instance: fatigue behavior of FHA-coated implants in a corrosive environment (e.g. SBF); dissolution behaviors of FHA coatings under fatigue testing etc.

2. *In vivo* tests of FHA-coated implants. Although *in vitro* testing can provide a rapid and relatively inexpensive insight about the possible implants, *in vivo* testing is the most direct, essential and reliable method to evaluate the interaction/performance of any implant with living body, e.g. cytotoxicity, degradation, osteoinductivity etc. Therefore, it is crucial to evaluate the *in vivo* bioactivity of FHA-coated implants by implanting them into bones of living animals, which could provide more reliable guidance for further improvement and/or application of FHA-coated implants.
3. Functional graded multilayered FHA coatings. According to the current study, the higher the fluoridation degree, the stronger the adhesion properties. However, the most promising fluoridation degree is about 0.8-1.1 for the expected bioactivities. Therefore, fabrication of functional graded multilayered FHA coatings should be a favorable choice: the outer layer is F3/F4 to satisfy and ensure the biological requirements, while the inner layer is the F6 to achieve and maintain the strong adhesion strength.

References

- [1] B. D. Ratner, A. S. Hoffman, F. J. Schoen, J. E. Lemons, *Biomaterials Science-An Introduction to Materials in Medicine*, Academic Press, San Diego, California, 1996.
- [2] H. Oonishi, Orthopedic Applications of Hydroxyapatite, *Biomaterials* 12 (1991) 171-178.
- [3] L. M. Rodriguez-Lorenzo, J. N. Hart, K. A. Gross, Influence of fluorine in the synthesis of apatites. Synthesis of solid solutions of hydroxy-fluorapatite, *Biomaterials* 24 (2003) 3777-3785.
- [4] J. D. Bronzino, *The Biomedical Engineering Handbook* (2nd Edition), CRC Press, Boca Raton, 2000.
- [5] C. Leyens, M. Peters, *Titanium and titanium alloys: fundamentals and applications*, Weinheim, Wiley-VCH, 2003.
- [6] J. Gallardo, P. Galliano, A. Duran, Bioactive and protective sol-gel coatings on metals for orthopaedic prostheses, *Journal of Sol-Gel Science and Technology* 21 (2001) 65-74.
- [7] J. L. Ong, D. C. N. Chan, Hydroxyapatite and their use as coatings in dental implants: A review, *Critical Reviews in Biomedical Engineering* 28 (2000) 667A-707A.
- [8] L. M. Sun, C. C. Berndt, K. A. Gross, A. Kucuk, Material fundamentals and clinical performance of plasma-sprayed hydroxyapatite coatings: A review, *Journal of Biomedical Materials Research* 58 (2001) 570-592.
- [9] D. M. Liu, T. Troczynski, W. J. Tseng, Water-based sol-gel synthesis of hydroxyapatite: process development, *Biomaterials* 22 (2001) 1721-1730.
- [10] D. C. Greenspan, Bioactive ceramic implant materials, *Current Opinion in Solid State & Materials Science* 4 (1999) 389-393.
- [11] R. D. Bloebaum, D. Beeks, L. D. Dorr, C. G. Savory, J. A. Dupont, A. A. Hofmann, Complications with Hydroxyapatite Particulate Separation in Total Hip-Arthroplasty, *Clinical Orthopaedics and Related Research* (1994) 19-26.
- [12] S. Overgaard, M. Lind, K. Josephsen, A. B. Maunsbach, C. Bunger, K. Soballe, Resorption of hydroxyapatite and fluorapatite ceramic coatings on weight-bearing implants: A quantitative and morphological study in dogs, *Journal of Biomedical Materials Research* 39 (1998) 141-152.
- [13] D. A. Puleo, A. Nanci, Understanding and controlling the bone-implant interface, *Biomaterials* 20 (1999) 2311-2321.
- [14] D. H. Leverett, Fluorides and the Changing Prevalence of Dental-Caries, *Science* 217 (1982) 26-30.
- [15] H. Caulier, J. Vanderwaerden, Y. Paquay, J. G. C. Wolke, W. Kalk, I. Naert, J. A. Jansen, Effect of Calcium-Phosphate (Ca-P) Coatings on Trabecular Bone Response - a Histological Study, *Journal of Biomedical Materials Research* 29 (1995) 1061-1069.
- [16] H. W. Kim, H. E. Kim, J. C. Knowles, Fluor-hydroxyapatite sol-gel coating on titanium substrate for hard tissue implants, *Biomaterials* 25 (2004) 3351-3358.
- [17] K. Cheng, G. R. Han, W. J. Weng, H. B. Qu, P. Y. Du, G. Shen, J. Yang, J. M. F. Feireira, Sol-gel derived fluoridated hydroxyapatite films, *Materials Research Bulletin* 38 (2003) 89-97.
- [18] N. Marsha, T. Leonard, A. Randall, Special Feature: Consistency in Postoperative Education Programs Following Total Hip Replacement, *Topics in Geriatric Rehabilitation* 15 (2000) 68-76.

References

-
- [19] R. E. Smallman, R. J. Bishop, Modern Physical metallurgy and Materials Engineering-Science, Process, Application Elsevier, 1998.
 - [20] L. L. Hench, Biomaterials: a forecast for the future, Biomaterials 19 (1998) 1419-1423.
 - [21] D. G. Poitout, Biomechanics and Biomaterials in Orthopedics. , Springer, London, 2004.
 - [22] J. W. Boretoes, M. Eden, Contemporary Biomaterials - Material and Host Response, Clinical Applications, New Technology and Legal Aspects, William Andrew Publishing, New Jersey, USA, 1984.
 - [23] R. Vedantam, C. Ruddlesdin, The fully hydroxyapatite-coated total hip implant - Clinical and roentgenographic results, Journal of Arthroplasty 11 (1996) 534-542.
 - [24] R. Roop Kumar, M. Wang, Functionally graded bioactive coatings of hydroxyapatite/titanium oxide composite system, Materials Letters 55 (2002) 133-137.
 - [25] H. Yuan, Y. Li, J. D. de Bruijn, K. de Groot, X. Zhang, Tissue responses of calcium phosphate cement: a study in dogs, Biomaterials 21 (2000) 1283-1290.
 - [26] M. Böhner, Calcium orthophosphates in medicine: from ceramics to calcium phosphate cements, Injury-International Journal of the Care of the Injured 31 (2000) S37-S47.
 - [27] G. Vereecke, J. Lemaitre, Calculation of the solubility diagrams in the system $\text{Ca}(\text{OH})_2\text{-H}_3\text{PO}_4\text{-KOH-HNO}_3\text{-CO}_2\text{-H}_2\text{O}$, Journal of Crystal Growth 104 (1990) 820-832.
 - [28] P. Ducheyne, S. Radin, L. King, The Effect of Calcium-Phosphate Ceramic Composition and Structure on Invitro Behavior .1. Dissolution, Journal of Biomedical Materials Research 27 (1993) 25-34.
 - [29] K. Byrappa, T. Ohachi, Crystal Growth Technology, Willam Andrew, Springer, New York, 2003.
 - [30] H. Aoki, Medical Applications of Hydroxyapatite, Ishiyaku Euroamerica, Toyoko, 1994.
 - [31] L. L. Hench, J. Wilson, An Introduction to Bioceramics, World Scientific, Singapore, 1993.
 - [32] M. I. Kay, R. A. Young, Crystal structure of hydroxyapatite, Nature 204 (1964) 1050-1052.
 - [33] Q. H. Bao, C. Z. Chen, D. G. Wang, Q. M. Ji, T. Q. Lei, Pulsed laser deposition and its current research status in preparing hydroxyapatite thin films, Applied Surface Science 252 (2005) 1538-1544.
 - [34] M. Katto, M. Nakamura, T. Tanaka, T. Nakayama, Hydroxyapatite coatings deposited by laser-assisted laser ablation method, Applied Surface Science 197 (2002) 768-771.
 - [35] C. K. Wang, J. H. C. Lin, C. P. Ju, H. C. Ong, R. P. H. Chang, Structural characterization of pulsed laser-deposited hydroxyapatite film on titanium substrate, Biomaterials 18 (1997) 1331-1338.
 - [36] T. Onoki, T. Hashida, New method for hydroxyapatite coating of titanium by the hydrothermal hot isostatic pressing technique, Surface & Coatings Technology 200 (2006) 6801-6807.
 - [37] Y. Q. Fu, A. W. Batchelor, K. A. Khor, Hot isostatic pressing of hydroxyapatite coating for improved fretting wear resistance, Journal of Materials Science Letters 17 (1998) 1695-1696.
 - [38] Y. P. Lu, G. Y. Xiao, S. T. Li, R. X. Sun, M. S. Li, Microstructural inhomogeneity in plasma-sprayed hydroxyapatite coatings and effect of post-heat treatment, Applied Surface Science 252 (2006) 2412-2421.
-

References

- [39] L. S. Ozyegin, F. N. Oktar, G. Goller, E. S. Kayali, T. Yazici, Plasma-sprayed bovine hydroxyapatite coatings, *Materials Letters* 58 (2004) 2605-2609.
- [40] K. A. Khor, P. Cheang, Plasma sprayed hydroxyapatite(HA) coatings produced with flame spheroidised powders, *Journal of Materials Processing Technology* 63 (1997) 271-276.
- [41] P. Cheang, K. A. Khor, Thermal spraying of hydroxyapatite (HA) coatings: Effects of powder feedstock, *Journal of Materials Processing Technology* 48 (1995) 429-436.
- [42] H. Oguchi, K. Ishikawa, S. Ojima, Y. Hirayama, K. Seto, G. Eguchi, Evaluation of a high-velocity flame-spraying technique for hydroxyapatite, *Biomaterials* 13 (1992) 471-477.
- [43] J. D. Haman, K. K. Chittur, D. E. Crawmer, L. C. Lucas, Analytical and mechanical testing of high velocity oxy-fuel thermal sprayed and plasma sprayed calcium phosphate coatings, *Journal of Biomedical Materials Research* 48 (1999) 856-860.
- [44] H. Li, K. A. Khor, P. Cheang, Effect of the powders' melting state on the properties of HVOF sprayed hydroxyapatite coatings, *Materials Science and Engineering a-Structural Materials Properties Microstructure and Processing* 293 (2000) 71-80.
- [45] R. S. Lima, K. A. Khor, H. Li, P. Cheang, B. R. Marple, HVOF spraying of nanostructured hydroxyapatite for biomedical applications, *Materials Science and Engineering a-Structural Materials Properties Microstructure and Processing* 396 (2005) 181-187.
- [46] X. W. Meng, T. Y. Kwon, Y. Z. Yang, J. L. Ong, K. H. Kim, Effects of applied voltages on hydroxyapatite coating of titanium by electrophoretic deposition, *Journal of Biomedical Materials Research Part B-Applied Biomaterials* 78B (2006) 373-377.
- [47] J. Ma, C. H. Liang, L. B. Kong, C. Wang, Colloidal characterization and electrophoretic deposition of hydroxyapatite on titanium substrate, *Journal of Materials Science-Materials in Medicine* 14 (2003) 797-801.
- [48] A. Stoch, A. Brozek, G. Kmita, J. Stoch, W. Jastrzebski, A. Rakowska, Electrophoretic coating of hydroxyapatite on titanium implants, *Journal of Molecular Structure* 596 (2001) 191-200.
- [49] S. Bharati, M. K. Sinha, D. Basu, Hydroxyapatite coating by biomimetic method on titanium alloy using concentrated SBF, *Bulletin of Materials Science* 28 (2005) 617-621.
- [50] E. C. S. Rigo, A. O. Boschi, M. Yoshimoto, S. Allegrini, B. Konig, M. J. Carbonari, Evaluation in vitro and in vivo of biomimetic hydroxyapatite coated on titanium dental implants, *Materials Science & Engineering C-Biomimetic and Supramolecular Systems* 24 (2004) 647-651.
- [51] F. Barrere, P. Layrolle, C. A. van Blitterswijk, K. de Groot, Biomimetic coatings on titanium: a crystal growth study of octacalcium phosphate, *Journal of Materials Science-Materials in Medicine* 12 (2001) 529-534.
- [52] V. Nelea, C. Morosanu, M. Iliescu, I. N. Mihailescu, Microstructure and mechanical properties of hydroxyapatite thin films grown by RF magnetron sputtering, *Surface & Coatings Technology* 173 (2003) 315-322.
- [53] T. G. Nieh, A. F. Jankowski, J. Koike, Processing and characterization of hydroxyapatite coatings on titanium produced by magnetron sputtering, *Journal of Materials Research* 16 (2001) 3238-3245.

References

- [54] D. R. Cooley, A. F. Vandellen, J. O. Burgess, A. S. Windeler, The Advantages of Coated Titanium Implants Prepared by Radiofrequency Sputtering from Hydroxyapatite, *Journal of Prosthetic Dentistry* 67 (1992) 93-100.
- [55] A. Duran, A. Conde, A. G. Coedo, T. Dorado, C. Garcia, S. Cere, Sol-gel coatings for protection and bioactivation of metals used in orthopaedic devices, *Journal of Materials Chemistry* 14 (2004) 2282-2290.
- [56] Z. Sam, X. T. Zeng, Y. S. Wang, K. L. Cheng, W. J. Weng, Adhesion strength of sol-gel derived fluoridated hydroxyapatite coatings, *Surface & Coatings Technology* 200 (2006) 6350-6354.
- [57] E. Tkalec, M. Sauer, R. Nonninger, H. Schmidt, Sol-gel-derived hydroxyapatite powders and coatings, *Journal of Materials Science* 36 (2001) 5253-5263.
- [58] A. Montenero, G. Gnappi, F. Ferrari, M. Cesari, E. Salvioli, L. Mattogno, S. Kaciulis, M. Fini, Sol-gel derived hydroxyapatite coatings on titanium substrate, *Journal of Materials Science* 35 (2000) 2791-2797.
- [59] K. A. Gross, C. S. Chai, G. S. K. Kannangara, B. Ben-Nissan, L. Hanley, Thin hydroxyapatite coatings via sol-gel synthesis, *Journal of Materials Science-Materials in Medicine* 9 (1998) 839-843.
- [60] W. L. Jaffe, D. F. Scott, Total hip arthroplasty with hydroxyapatite-coated prostheses, *Journal of Bone and Joint Surgery-American Volume* 78A (1996) 1918-1934.
- [61] K. A. Gross, C. C. Berndt, Thermal processing of hydroxyapatite for coating production, *Journal of Biomedical Materials Research* 39 (1998) 580-587.
- [62] H. Li, K. A. Khor, P. Cheang, Properties of heat-treated calcium phosphate coatings deposited by high-velocity oxy-fuel (HVOF) spray, *Biomaterials* 23 (2002) 2105-2112.
- [63] Y. Han, T. Fu, J. Lu, K. Xu, Characterization and stability of hydroxyapatite coatings prepared by an electrodeposition and alkaline-treatment process, *Journal of Biomedical Materials Research* 54 (2001) 96-101.
- [64] S. J. Ding, C. P. Ju, J. H. C. Lin, Characterization of hydroxyapatite and titanium coatings sputtered on Ti-6Al-4V substrate, *Journal of Biomedical Materials Research* 44 (1999) 266-279.
- [65] H. W. Kim, H. E. Kim, V. Salih, J. C. Knowles, Sol-gel-modified titanium with hydroxyapatite thin films and effect on osteoblast-like cell responses, *Journal of Biomedical Materials Research Part A* 74A (2005) 294-305.
- [66] E. Montserrat, Developing Hydroxyapatite CAPS coatings on Metallic Implants for Tissue Replacement, in: Nanyang Technological University, 2003.
- [67] S. J. Yankee, B. J. Pletka, H. A. Luckey, W. A. Johnson, Thermal Spray Research and Applications, in: T. F. Bernecki (Ed.) 3rd National Thermal Spray Conference, ASM International, Long Beach, California, 1990, pp. 433-438.
- [68] Y. Chen, C. Gan, T. Zhang, G. Yu, P. Bai, A. Kaplan, Laser-surface-alloyed Carbon Nanotubes Reinforced Hydroxyapatite Composite Coatings, *Applied Physics Letters* 86 (2005) 251905.
- [69] M. Ogiso, Y. Yamashita, T. Matsumoto, Differences in microstructural characteristics of dense HA and HA coating, *Journal of Biomedical Materials Research* 41 (1998) 296-303.
- [70] N. Senamaud, D. Bernache-Assollant, E. Champion, M. Heughebaert, C. Rey, Calcination and sintering of hydroxyfluorapatite powders, *Solid State Ionics* 101 (1997) 1357-1362.

References

- [71] Y. Cao, J. Weng, J. Y. Chen, J. M. Feng, Z. J. Yang, X. D. Zhang, Water vapour-treated hydroxyapatite coatings after plasma spraying and their characteristics, *Biomaterials* 17 (1996) 419-424.
- [72] K. A. Gross, C. C. Berndt, H. Herman, Amorphous phase formation in plasma-sprayed hydroxyapatite coatings, *Journal of Biomedical Materials Research* 39 (1998) 407-414.
- [73] K. A. Gross, V. Gross, C. C. Berndt, Thermal analysis of amorphous phases in hydroxyapatite coatings, *Journal of the American Ceramic Society* 81 (1998) 106-112.
- [74] Y. Yan, J. G. C. Wolke, A. De Ruijter, L. Yubao, J. A. Jansen, Growth behavior of rat bone marrow cells on RF magnetron sputtered hydroxyapatite and dicalcium pyrophosphate coatings, *Journal of Biomedical Materials Research Part A* 78A (2006) 42-49.
- [75] S. Rossler, A. Sewing, M. Stolzel, R. Born, D. Scharnweber, M. Dard, H. Worch, Electrochemically assisted deposition of thin calcium phosphate coatings at near-physiological pH and temperature, *Journal of Biomedical Materials Research Part A* 64A (2003) 655-663.
- [76] M. Metikos-Hukovic, E. Tkalcec, A. Kwokal, J. Piljac, An in vitro study of Ti and Ti-alloys coated with sol-gel derived hydroxyapatite coatings, *Surface & Coatings Technology* 165 (2003) 40-50.
- [77] C. Wang, J. Ma, W. Cheng, R. Zhang, Thick hydroxyapatite coatings by electrophoretic deposition, *Materials Letters* 57 (2002) 99-105.
- [78] L. A. de Sena, M. C. de Andrade, A. M. Rossi, G. D. Soares, Hydroxyapatite deposition by electrophoresis on titanium sheets with different surface finishing, *Journal of Biomedical Materials Research* 60 (2002) 1-7.
- [79] A. Bigi, E. Boanini, B. Bracci, A. Facchini, S. Panzavolta, F. Segatti, L. Sturba, Nanocrystalline hydroxyapatite coatings on titanium: a new fast biomimetic method, *Biomaterials* 26 (2005) 4085-4089.
- [80] J. Ma, H. F. Wong, L. B. Kong, K. W. Peng, Biomimetic processing of nanocrystallite bioactive apatite coating on titanium, *Nanotechnology* 14 (2003) 619-623.
- [81] C. K. You, S. Oh, S. Kim, Influences of heating condition and substrate-surface roughness on the characteristics of sol-gel-derived hydroxyapatite coatings, *Journal of Sol-Gel Science and Technology* 21 (2001) 49-54.
- [82] J. Weng, Q. Liu, J. G. C. Wolke, X. Zhang, K. de Groot, Formation and characteristics of the apatite layer on plasma-sprayed hydroxyapatite coatings in simulated body fluid, *Biomaterials* 18 (1997) 1027-1035.
- [83] Implants for Surgery-Hydroxyapatite-Part 4: Determination of Coating adhesion strength in: British Standard, BS ISO 13779-4, 2002.
- [84] L. D. Piveteau, B. Gasser, L. Schlappbach, Evaluating mechanical adhesion of sol-gel titanium dioxide coatings containing calcium phosphate for metal implant application, *Biomaterials* 21 (2000) 2193-2201.
- [85] T. Albrektsson, Hydroxyapatite-coated implants: A case against their use, *Journal of Oral and Maxillofacial Surgery* 56 (1998) 1312-1326.
- [86] J. Wang, P. Layrolle, M. Stigter, K. de Groot, Biomimetic and electrolytic calcium phosphate coatings on titanium alloy: physicochemical characteristics and cell attachment, *Biomaterials* 25 (2004) 583-592.
- [87] F. J. Garcia-Sanz, M. B. Mayor, J. L. Arias, J. Pou, B. Leon, M. Perez-Amor, Hydroxyapatite coatings: A comparative study between plasma-spray and pulsed laser deposition techniques, *Journal of Materials Science: Materials in Medicine* 8 (1997) 861-865.

References

-
- [88] S. J. Ding, Y. M. Su, C. P. Ju, J. H. Chern Lin, Structure and immersion behavior of plasma-sprayed apatite-matrix coatings, *Biomaterials* 22 (2001) 833-845.
 - [89] Y. W. Gu, K. A. Khor, P. Cheang, In vitro studies of plasma-sprayed hydroxyapatite/Ti-6Al-4V composite coatings in simulated body fluid (SBF), *Biomaterials* 24 (2003) 1603-1611.
 - [90] Y. C. Yang, E. Chang, S. Y. Lee, Mechanical properties and Young's modulus of plasmasprayed hydroxyapatite coating on Ti substrate in simulated body fluid, *Journal of Biomedical Materials Research Part A* 67A (2003) 886-899.
 - [91] H. Li, K. A. Khor, P. Cheang, Titanium dioxide reinforced hydroxyapatite coatings deposited by high velocity oxy-fuel (HVOF) spray, *Biomaterials* 23 (2002) 85-91.
 - [92] S. W. K. Kweh, K. A. Khor, P. Cheang, Plasma-sprayed hydroxyapatite (HA) coatings with flame-spheroidized feedstock: microstructure and mechanical properties, *Biomaterials* 21 (2000) 1223-1234.
 - [93] X. F. Xiao, R. F. Liu, Effect of suspension stability on electrophoretic deposition of hydroxyapatite coatings, *Materials Letters* 60 (2006) 2627-2632.
 - [94] M. Wei, A. J. Ruys, B. K. Milthorpe, C. C. Sorrell, J. H. Evans, Electrophoretic deposition of hydroxyapatite coatings on metal substrates: A nanoparticulate dual-coating approach, *Journal of Sol-Gel Science and Technology* 21 (2001) 39-48.
 - [95] J. Pena, I. Izquierdo-Barba, A. Martinez, M. Vallet-Regi, New method to obtain chitosan/apatite materials at room temperature, *Solid State Sciences* 8 (2006) 513-519.
 - [96] S. J. Ding, Properties and immersion behavior of magnetron-sputtered multi-layered hydroxyapatite/titanium composite coatings, *Biomaterials* 24 (2003) 4233-4238.
 - [97] S. J. Ding, C. P. Ju, J. H. C. Lin, Immersion behavior of RF magnetron-assisted sputtered hydroxyapatite/titanium coatings in simulated body fluid, *Journal of Biomedical Materials Research* 47 (1999) 551-563.
 - [98] A. Balamurugan, G. Balossier, S. Kannan, S. Rajeswari, Elaboration of sol-gel derived apatite films on surgical grade stainless steel for biomedical applications, *Materials Letters* 60 (2006) 2288-2293.
 - [99] H. W. Kim, Y. H. Koh, L. H. Li, S. Lee, H. E. Kim, Hydroxyapatite coating on titanium substrate with titania buffer layer processed by sol-gel method, *Biomaterials* 25 (2004) 2533-2538.
 - [100] M. F. Hsieh, L. H. Perng, T. S. Chin, Hydroxyapatite coating on Ti6Al4V alloy using a sol-gel derived precursor, *Materials Chemistry and Physics* 74 (2002) 245-250.
 - [101] M. J. Filiaggi, N. A. Coombs, R. M. Pilliar, Characterization of the Interface in the Plasma-Sprayed Ha Coating/Ti-6al-4v Implant System, *Journal of Biomedical Materials Research* 25 (1991) 1211-1229.
 - [102] Y. C. Tsui, C. Doyle, T. W. Clyne, Plasma sprayed hydroxyapatite coatings on titanium substrates Part 1: Mechanical properties and residual stress levels, *Biomaterials* 19 (1998) 2015-2029.
 - [103] H. Li, K. A. Khor, P. Cheang, Young's modulus and fracture toughness determination of high velocity oxy-fuel-sprayed bioceramic coatings, *Surface and Coatings Technology* 155 (2002) 21-32.
 - [104] S. R. Brown, I. G. Turner, H. Reiter, Residual stress measurement in thermal sprayed hydroxyapatite coatings, *Journal of Materials Science: Materials in Medicine* 5 (1994) 756-759.
 - [105] Y. C. Yang, E. Chang, Measurements of residual stresses in plasma-sprayed hydroxyapatite coatings on titanium alloy, *Surface and Coatings Technology* 190 (2005) 122-131.
-

References

- [106] Y. Han, K. W. Xu, J. Lu, Dissolution response of hydroxyapatite coatings to residual stresses, *Journal of Biomedical Materials Research* 55 (2001) 596-602.
- [107] Y. C. Yang, E. Chang, Influence of residual stress on bonding strength and fracture of plasma-sprayed hydroxyapatite coatings on Ti-6Al-4V substrate, *Biomaterials* 22 (2001) 1827-1836.
- [108] B. D. Boyan, C. H. Lohmann, D. D. Dean, V. L. Sylvia, D. L. Cochran, Z. Schwartz, Mechanisms involved in osteoblast response to implant surface morphology, *Annual Review of Materials Research* 31 (2001) 357-371.
- [109] T. Kokubo, H. Takadama, How useful is SBF in predicting in vivo bone bioactivity?, *Biomaterials* 27 (2006) 2907-2915.
- [110] T. Kokubo, Bioactive Glass-Ceramics - Properties and Applications, *Biomaterials* 12 (1991) 155-163.
- [111] C. Ohtsuki, H. Kushitani, T. Kokubo, S. Kotani, T. Yamamuro, Apatite Formation on the Surface of Ceravital-Type Glass-Ceramic in the Body, *Journal of Biomedical Materials Research* 25 (1991) 1363-1370.
- [112] L. Cleries, J. M. Fernandez-Pradas, J. L. Morenza, Behavior in simulated body fluid of calcium phosphate coatings obtained by laser ablation, *Biomaterials* 21 (2000) 1861-1865.
- [113] A. Stoch, W. Jastrzebski, E. Dlugon, W. Lejda, B. Trybalska, G. J. Stoch, A. Adamczyk, Sol-gel derived hydroxyapatite coatings on titanium and its alloy Ti6Al4V, *Journal of Molecular Structure* 744-747 (2005) 633-640.
- [114] J.-i. Hamagami, Y. Ato, K. Kanamura, Fabrication of highly ordered macroporous apatite coating onto titanium by electrophoretic deposition method, *Solid State Ionics* 172 (2004) 331-334.
- [115] K. A. Khor, H. Li, P. Cheang, S. Y. Boey, In vitro behavior of HVOF sprayed calcium phosphate splats and coatings, *Biomaterials* 24 (2003) 723-735.
- [116] S. R. Radin, P. Ducheyne, The effect of calcium phosphate ceramic composition and structure on in vitro behavior. II. Precipitation, *Journal Of Biomedical Materials Research* 27 (1993) 35-45.
- [117] Y. P. Lee, C. K. Wang, T. H. Huang, C. C. Chen, C. T. Kao, S. J. Ding, In vitro characterization of postheat-treated plasma-sprayed hydroxyapatite coatings, *Surface and Coatings Technology* 197 (2005) 367-374.
- [118] K. A. Khor, H. Li, P. Cheang, Characterization of the bone-like apatite precipitated on high velocity oxy-fuel (HVOF) sprayed calcium phosphate deposits, *Biomaterials* 24 (2003) 769-775.
- [119] C. Knabe, F. C. M. Driessens, J. A. Planell, R. Gildenhaar, G. Berger, D. Reif, R. Fitzner, R. J. Radlanski, U. Gross, Evaluation of calcium phosphates and experimental calcium phosphate bone cements using osteogenic cultures, *Journal of Biomedical Materials Research* 52 (2000) 498-508.
- [120] J. E. Aubin, F. Liu, L. Malaval, A. K. Gupta, Osteoblast and Chondroblast Differentiation, *Bone* 17 (1995) S77-S83.
- [121] G. C. Menezes, C. N. Elias, M. Attias, F. C. Silva-Filho, Osteoblast adhesion onto titanium dental implants, *Acta Microscopica* 12 (2003) 13-20.
- [122] K. M. Dorota, L. S. Malgorzata, M. Mazur, J. Komender, Osteogenic cell contact with biomaterials influences phenotype expression, *Cell and Tissue Banking* 6 (2005) 55-64.
- [123] P. Tengvall, *How Surfaces Interact with the Biological Environment*, CRC Press, New York, USA, 2003.
- [124] B. Kasemo, Biological surface science, *Current Opinion in Solid State & Materials Science* 3 (1998) 451-459.

References

- [125] C. J. Wilson, R. E. Clegg, D. I. Leavesley, M. J. Percy, Mediation of biomaterial-cell interactions by adsorbed proteins: A review, *Tissue Engineering* 11 (2005) 1-18.
- [126] J. Folkman, A. Moscona, Role of cell shape in growth control, *Nature* 273 (1978) 345-349.
- [127] L. C. Baxter, V. Frauchiger, M. Textor, I. Gwynn, R. G. Richards, Fibroblast and osteoblast adhesion and morphology on calcium phosphate surfaces, *European Cells and Materials* 4 (2002) 1-17.
- [128] A. Okumura, M. Goto, T. Goto, M. Yoshinari, S. Masuko, T. Katsuki, T. Tanaka, Substrate affects the initial attachment and subsequent behavior of human osteoblastic cells (Saos-2), *Biomaterials* 22 (2001) 2263-2271.
- [129] J. M. Schakenraad, H. J. Busscher, Cell Polymer Interactions - the Influence of Protein Adsorption, *Colloids and Surfaces* 42 (1989) 331-343.
- [130] G. S. Stein, J. B. Lian, Molecular Mechanisms Mediating Proliferation Differentiation Interrelationships During Progressive Development of the Osteoblast Phenotype, *Endocrine Reviews* 14 (1993) 424-442.
- [131] V. Kartsogiannis, K. W. Ng, Cell lines and primary cell cultures in the study of bone cell biology, *Molecular and Cellular Endocrinology* 228 (2004) 79-102.
- [132] L. C. Gerstenfeld, S. D. Chipman, J. Glowacki, J. B. Lian, Expression of Differentiated Function by Mineralizing Cultures of Chicken Osteoblasts, *Developmental Biology* 122 (1987) 49-60.
- [133] L. L. Hench, Bioceramics - from Concept to Clinic, *Journal of the American Ceramic Society* 74 (1991) 1487-1510.
- [134] C. Massaro, M. A. Baker, F. Cosentino, P. A. Ramires, S. Klose, E. Milella, Surface and biological evaluation of hydroxyapatite-based coatings on titanium deposited by different techniques, *Journal of Biomedical Materials Research* 58 (2001) 651-657.
- [135] A. Bigi, B. Bracci, F. Cuisinier, R. Elkaim, M. Fini, I. Mayer, I. N. Mihailescu, G. Socol, L. Sturba, P. Torricelli, Human osteoblast response to pulsed laser deposited calcium phosphate coatings, *Biomaterials* 26 (2005) 2381-2389.
- [136] Y. Yang, K.-H. Kim, J. L. Ong, A review on calcium phosphate coatings produced using a sputtering process--an alternative to plasma spraying, *Biomaterials* 26 (2005) 327-337.
- [137] J. W. Xie, M. J. Baumann, L. R. McCabe, Osteoblasts respond to hydroxyapatite surfaces with immediate changes in gene expression, *Journal of Biomedical Materials Research Part A* 71A (2004) 108-117.
- [138] L. H. Li, H. W. Kim, S. H. Lee, Y. M. Kong, H. E. Kim, Biocompatibility of titanium implants modified by microarc oxidation and hydroxyapatite coating, *Journal of Biomedical Materials Research Part A* 73A (2005) 48-54.
- [139] T. M. Lee, R. S. Tsai, E. Chang, C. Y. Yang, M. R. Yang, The cell attachment and morphology of neonatal rat calvarial osteoblasts on the surface of Ti-6Al-4V and plasma-sprayed HA coating: Effect of surface roughness and serum contents, *Journal of Materials Science-Materials in Medicine* 13 (2002) 341-350.
- [140] D. Perizzolo, W. R. Lacefield, D. M. Brunette, Interaction between topography and coating in the formation of bone nodules in culture for hydroxyapatite- and titanium-coated micromachined surfaces, *Journal of Biomedical Materials Research* 56 (2001) 494-503.
- [141] V. Borsari, G. Giavaresi, M. Fini, P. Torricelli, A. Salito, R. Chiesa, L. Chiusoli, A. Volpert, L. Rimondini, R. Giardino, Physical characterization of different-roughness titanium surfaces, with and without hydroxyapatite coating, and their effect

References

- on human osteoblast-like cells, *Journal of Biomedical Materials Research Part B-Applied Biomaterials* 75B (2005) 359-368.
- [142] L. Chou, B. Marek, W. R. Wagner, Effects of hydroxylapatite coating crystallinity on biosolubility, cell attachment efficiency and proliferation in vitro, *Biomaterials* 20 (1999) 977-985.
- [143] M. C. Siebers, X. F. Walboomers, S. C. G. Leeuwenburgh, J. G. C. Wolke, J. A. Jansen, The influence of the crystallinity of electrostatic spray deposition-derived coatings on osteoblast-like cell behavior, in vitro, *Journal of Biomedical Materials Research Part A* 78A (2006) 258-267.
- [144] J. M. Anderson, Biological responses to materials, *Annual Review of Materials Research* 31 (2001) 81-110.
- [145] R. J. Furlong, J. F. Osborn, Fixation of Hip Prostheses by Hydroxyapatite Ceramic Coatings, *Journal of Bone and Joint Surgery-British Volume* 73 (1991) 741-745.
- [146] A. Moroni, V. L. Caja, C. Sabato, E. L. Egger, F. Gottsauner-Wolf, E. Y. S. Chao, Bone ingrowth analysis and interface evaluation of hydroxyapatite coated versus uncoated titanium porous bone implants, *Journal of Materials Science: Materials in Medicine* 5 (1994) 411-416.
- [147] A. Moroni, S. Toksvig-Larsen, M. C. Maltarello, L. Orienti, S. Stea, S. Giannini, A comparison of hydroxyapatite-coated, titanium-coated, and uncoated tapered external-fixation pins: An in vivo study in sheep, *Journal of Bone and Joint Surgery - Series A* 80 (1998) 547-554.
- [148] P. A. Ramires, A. Wennerberg, C. B. Johansson, F. Cosentino, S. Tundo, E. Milella, Biological behavior of sol-gel coated dental implants, *Journal of Materials Science-Materials in Medicine* 14 (2003) 539-545.
- [149] T. Gerber, T. Traykova, K. O. Henkel, V. Bienengraeber, Development and in vivo test of sol-gel derived bone grafting materials, *Journal of Sol-Gel Science and Technology* 26 (2003) 1173-1178.
- [150] C. You, I. S. Yeo, M. D. Kim, T. K. Eom, J. Y. Lee, S. Kim, Characterization and in vivo evaluation of calcium phosphate coated cp-titanium by dip-spin method, *Current Applied Physics* 5 (2005) 501-506.
- [151] N. Aebli, J. Krebs, H. Stich, P. Schawalder, M. Walton, D. Schwenke, H. Gruner, B. Gasser, J. C. Theis, In vivo comparison of the osseointegration of vacuum plasma sprayed titanium- and hydroxyapatite-coated implants, *Journal of Biomedical Materials Research Part A* 66A (2003) 356-363.
- [152] H. Oonishi, M. Yamamoto, H. Ishimaru, E. Tsuji, S. Kushitani, M. Aono, Y. Ukon, The Effect of Hydroxyapatite Coating on Bone-Growth into Porous Titanium-Alloy Implants, *Journal of Bone and Joint Surgery-British Volume* 71 (1989) 213-216.
- [153] C. Y. Yang, R. M. Lin, B. C. Wang, T. M. Lee, E. Chang, Y. S. Hang, P. Q. Chen, In vitro and in vivo mechanical evaluations of plasma-sprayed hydroxyapatite coatings on titanium implants: The effect of coating characteristics, *Journal of Biomedical Materials Research* 37 (1997) 335-345.
- [154] C. K. Chang, J. S. Wu, D. L. Mao, C. X. Ding, Mechanical and histological evaluations of hydroxyapatite-coated and noncoated Ti6Al4V implants in tibia bone, *Journal of Biomedical Materials Research* 56 (2001) 17-23.
- [155] J. E. Dalton, S. D. Cook, In vivo mechanical and histological characteristics of HA-coated implants vary with coating vendor, *Journal Of Biomedical Materials Research* 29 (1995) 239-245.

References

-
- [156] T. Seno, Y. Izumisawa, I. Nishimura, S. Maehara, T. Yokoyama, K. Yamashita, T. Kotani, The interfacial strength in sputtering-hydroxyapatite-coating implants with Arc-deposited surface, *Journal of Veterinary Medical Science* 65 (2003) 419-422.
 - [157] N. Aebli, J. Krebs, D. Schwenke, H. Stich, P. Schawalder, J. C. Theis, Degradation of hydroxyapatite coating on a well-functioning femoral component, *Journal of Bone and Joint Surgery-British Volume* 85B (2003) 499-503.
 - [158] C. M. Mullermai, C. Voigt, U. Gross, Incorporation and Degradation of Hydroxyapatite Implants of Different Surface-Roughness and Surface-Structure in Bone, *Scanning Microscopy* 4 (1990) 613-624.
 - [159] K. A. Gross, W. Walsh, E. Swarts, Analysis of retrieved hydroxyapatite-coated hip prostheses, *Journal of Thermal Spray Technology* 13 (2004) 190-199.
 - [160] I. A. Silver, R. J. Murrills, D. J. Etherington, Microelectrode Studies on the Acid Microenvironment beneath Adherent Macrophages and Osteoclasts, *Experimental Cell Research* 175 (1988) 266-276.
 - [161] E. W. Morscher, A. Hefti, U. Aebi, Severe osteolysis after third-body wear due to hydroxyapatite particles from acetabular cup coating, *Journal of Bone and Joint Surgery-British Volume* 80B (1998) 267-272.
 - [162] K. Ozeki, A. Mishima, T. Yuhta, Y. Fukui, H. Aoki, Bone bonding strength of sputtered hydroxyapatite films subjected to a low temperature hydrothermal treatment, *Bio-Medical Materials and Engineering* 13 (2003) 451-463.
 - [163] A. K. Lynn, D. L. DuQuesnay, Hydroxyapatite-coated Ti-6Al-4V : Part 1: the effect of coating thickness on mechanical fatigue behaviour, *Biomaterials* 23 (2002) 1937-1946.
 - [164] T. S. B. Narasaraju, D. E. Phebe, Some physico-chemical aspects of hydroxylapatite, *Journal of Materials Science* 31 (1996) 1-21.
 - [165] G. N. Jenkins, Review of fluoride research since 1959, *Archives of Oral Biology* 44 (1999) 985-992.
 - [166] R. F. Sognnaes, Fluoride protection of bones and teeth, *Science* 150 (1965) 989-993.
 - [167] Y. M. Chen, X. G. Miao, Thermal and chemical stability of fluorohydroxyapatite ceramics with different fluorine contents, *Biomaterials* 26 (2005) 1205-1210.
 - [168] R. Murugan, T. S. S. Kumar, K. P. Rao, Fluorinated bovine hydroxyapatite: preparation and characterization, *Materials Letters* 57 (2002) 429-433.
 - [169] M. Wei, J. H. Evans, T. Bostrom, L. Grondahl, Synthesis and characterization of hydroxyapatite, fluoride-substituted hydroxyapatite and fluorapatite, *Journal of Materials Science-Materials in Medicine* 14 (2003) 311-320.
 - [170] I. Manjubala, M. Sivakumar, S. N. Nikkath, Synthesis and characterisation of hydroxy/fluoroapatite solid solution, *Journal of Materials Science* 36 (2001) 5481-5486.
 - [171] S. Matsuya, Y. Matsuya, S. Takagi, L. C. Chow, Effect of fluoride on apatite formation from $\text{Ca-4(PO}_4\text{)(2)O}$ in 0.1 mol L⁻¹ KH_2PO_4 , *Journal of Materials Science-Materials in Medicine* 9 (1998) 325-331.
 - [172] H. W. Kim, Y. J. Noh, Y. H. Koh, H. E. Kim, Enhanced performance of fluorine substituted hydroxyapatite composites for hard tissue engineering, *Journal of Materials Science-Materials in Medicine* 14 (2003) 899-904.
 - [173] A. Krajewski, A. Ravaglioli, N. Roveri, A. Bigi, E. Foresti, Effect of Fluoride, Chloride and Carbonate Ions Introduced by Cyclic Ph Fluctuation on the Physicochemical Properties of Apatite-Based Ceramics, *Journal of Materials Science* 25 (1990) 3203-3207.
-

References

-
- [174] K. A. Gross, L. M. Rodriguez-Lorenzo, Sintered hydroxyfluorapatites. Part 1: Sintering ability of precipitated solid solution powders, *Biomaterials* 25 (2004) 1375-1384.
- [175] N. H. de Leeuw, Density functional theory calculations of local ordering of hydroxy groups and fluoride ions in hydroxyapatite, *Physical Chemistry Chemical Physics* 4 (2002) 3865-3871.
- [176] A. H. M. Ng, G. Hercz, R. Kandel, M. D. Gryn timer, Association between fluoride, magnesium, aluminum and bone quality in renal osteodystrophy, *Bone* 34 (2004) 216-224.
- [177] K. A. Gross, L. M. Rodriguez-Lorenzo, Sintered hydroxyfluorapatites. Part II: Mechanical properties of solid solutions determined by microindentation, *Biomaterials* 25 (2004) 1385-1394.
- [178] S. M. Barinov, F. Rustichelli, V. P. Orlovskii, A. Lodini, S. Oscarsson, S. A. Firstov, S. V. Tumanov, P. Millet, A. Rosengren, Influence of fluorapatite minor additions on behavior of hydroxyapatite ceramics, *Journal of Materials Science-Materials in Medicine* 15 (2004) 291-296.
- [179] K. A. Gross, K. A. Bhadang, Sintered hydroxyfluorapatites. Part III: Sintering and resultant mechanical properties of sintered blends of hydroxyapatite and fluorapatite, *Biomaterials* 25 (2004) 1395-1405.
- [180] E. C. Moreno, M. Kresak, R. T. Zahradnik, Fluoridated hydroxyapatite solubility and caries formation, *Nature* 247 (1974) 64-65.
- [181] S. V. Dorozhkin, Surface reactions of apatite dissolution, *Journal of Colloid and Interface Science* 191 (1997) 489-497.
- [182] E. Z. Kurmaev, S. Matsuya, S. Shin, M. Watanabe, R. Eguchi, Y. Ishiwata, T. Takeuchi, M. Iwami, Observation of fluorapatite formation under hydrolysis of tetracalcium phosphate in the presence of KF by means of soft X-ray emission and absorption spectroscopy, *Journal of Materials Science-Materials in Medicine* 13 (2002) 33-36.
- [183] N. H. de Leeuw, Resisting the onset of hydroxyapatite dissolution through the incorporation of fluoride, *Journal of Physical Chemistry B* 108 (2004) 1809-1811.
- [184] R. A. Young, W. Van der Lugt, J. C. Elliott, Mechanism for fluorine inhibition of diffusion in hydroxyapatite, *Nature* 223 (1969) 729-730.
- [185] A. S. Posner, *The Mineral of Bone*, Clinical Orthopaedics and Related Research (1985) 87-99.
- [186] F. C. M. Driessens, Relation between apatite solubility and anti-cariogenic effect of fluoride, *Nature* 243 (1973).
- [187] E. Lugschneider, T. Weber, M. Knepper, Production of biocompatible coatings of hydroxyapatite and fluorapatite, *National Thermal Spray Conference* (1988) 337-343.
- [188] L. Gineste, M. Gineste, X. Ranz, A. Ellefterion, A. Guilhem, N. Rouquet, P. Frayssinet, Degradation of hydroxylapatite, fluorapatite, and fluorhydroxyapatite coatings of dental implants in dogs, *Journal of Biomedical Materials Research* 48 (1999) 224-234.
- [189] K. A. Bhadang, K. A. K. A. Gross, Influence of fluorapatite on the properties of thermally sprayed hydroxyapatite coatings, *Biomaterials* 25 (2004) 4935-4945.
- [190] L. Savarino, M. Fini, G. Ciapetti, E. Cenni, D. Granchi, N. Baldini, M. Greco, G. Rizzi, R. Giardino, A. Giunti, Biologic effects of surface roughness and fluorhydroxyapatite coating on osteointegration in external fixation systems: An in vivo experimental study, *Journal of Biomedical Materials Research Part A* 66A (2003) 652-661.
-

References

-
- [191] M. Shirkhanzadeh, M. Azadegan, V. Stack, S. Schreyer, Fabrication of Pure Hydroxyapatite and Fluoridated-Hydroxyapatite Coatings by Electrocrystallization, *Materials Letters* 18 (1994) 211-214.
- [192] D. Ferro, S. M. Barinov, J. V. Rau, R. Teghil, A. Latini, Calcium phosphate and fluorinated calcium phosphate coatings on titanium deposited by Nd:YAG laser at a high fluence, *Biomaterials* 26 (2005) 805-812.
- [193] E. J. Lee, S. H. Lee, H. W. Kim, Y. M. Kong, H. E. Kim, Fluoridated apatite coatings on titanium obtained by electron-beam deposition, *Biomaterials* 26 (2005) 3843-3851.
- [194] M. Cavalli, G. Gnappi, A. Montenero, D. Bersani, P. P. Lottici, S. Kaciulis, G. Mattogno, M. Fini, Hydroxy- and fluorapatite films on Ti alloy substrates: Sol-gel preparation and characterization, *Journal of Materials Science* 36 (2001) 3253-3260.
- [195] H. W. Kim, J. C. Knowles, V. Salih, H. E. Kim, Hydroxyapatite and fluor-hydroxyapatite layered film on titanium processed by a sol-gel route for hard-tissue implants, *Journal of Biomedical Materials Research Part B-Applied Biomaterials* 71B (2004) 66-76.
- [196] H. W. Kim, Y. M. Kong, C. J. Bae, Y. J. Noh, H. E. Kim, Sol-gel derived fluor-hydroxyapatite biocoatings on zirconia substrate, *Biomaterials* 25 (2004) 2919-2926.
- [197] K. Cheng, W. J. Weng, H. B. Qu, P. Y. Du, G. Shen, G. R. Han, J. Yang, J. M. F. Ferreira, Sol-gel preparation and in vitro test of fluorapatite/hydroxyapatite films, *Journal of Biomedical Materials Research Part B-Applied Biomaterials* 69B (2004) 33-37.
- [198] W. J. Weng, S. Zhang, K. Cheng, H. B. Qu, P. Y. Du, G. Shen, J. Yuan, G. R. Han, Sol-gel preparation of bioactive apatite films, *Surface & Coatings Technology* 167 (2003) 292-296.
- [199] K. Cheng, S. Zhang, W. J. Weng, Sol-gel preparation of fluoridated hydroxyapatite in $\text{Ca}(\text{NO}_3)_2\text{-PO}(\text{OH})(3-x)(\text{OEt})(x)\text{-HPF}_6$ system, *Journal of Sol-Gel Science and Technology* 38 (2006) 13-17.
- [200] E. J. Blom, J. G. C. Verheij, J. M. A. de Blieck-Hogervorst, L. Di Silvio, C. Klein, Cortical bone ingrowth in growth hormone-loaded grooved implants with calcium phosphate coatings in goat femurs, *Biomaterials* 19 (1998) 263-270.
- [201] H. Caulier, S. Vercaigne, I. Naert, J. vanderWaerden, J. G. C. Wolke, W. Kalk, J. A. Jansen, The effect of Ca-P plasma-sprayed coatings on the initial bone healing of oral implants: An experimental study in the goat, *Journal of Biomedical Materials Research* 34 (1997) 121-128.
- [202] D. Martini, M. Fini, M. Franchi, V. D. Pasquale, B. Bacchelli, M. Gamberini, A. Tinti, P. Taddei, G. Giavaresi, V. Ottani, Detachment of titanium and fluorohydroxyapatite particles in unloaded endosseous implants, *Biomaterials* 24 (2003) 1309-1316.
- [203] K. Cheng, W. J. Weng, H. M. Wang, S. Zhang, In vitro behavior of osteoblast-like cells on fluoridated hydroxyapatite coatings, *Biomaterials* 26 (2005) 6288-6295.
- [204] X. D. Li, B. Bhushan, Measurement of fracture toughness of ultra-thin amorphous carbon films, *Thin Solid Films* 315 (1998) 214-221.
- [205] X. D. Li, D. F. Diao, B. Bhushan, Fracture mechanisms of thin amorphous carbon films in nanoindentation, *Acta Materialia* 45 (1997) 4453-4461.
- [206] M. Watanabe, D. R. Mumm, S. Chiras, A. G. Evans, Measurement of the residual stress in a Pt-aluminide bond coat, *Scripta Materialia* 46 (2002) 67-70.
- [207] Y. Xie, H. M. Hawthorne, Measuring the adhesion of sol-gel derived coatings to a ductile substrate by an indentation-based method, *Surface & Coatings Technology* 172 (2003) 42-50.
-

References

-
- [208] D. C. Agrawal, R. Raj, Measurement of the Ultimate Shear-Strength of a Metal Ceramic Interface, *Acta Metallurgica* 37 (1989) 1265-1270.
- [209] D. C. Agrawal, R. Raj, Ultimate Shear Strengths of Copper Silica and Nickel Silica Interfaces, *Materials Science and Engineering a-Structural Materials Properties Microstructure and Processing* 126 (1990) 125-131.
- [210] M. J. Filiaggi, R. M. Pilliar, D. Abdulla, Evaluating sol-gel ceramic thin films for metal implant applications .2. Adhesion and fatigue properties of zirconia films on Ti-6Al-4V, *Journal of Biomedical Materials Research* 33 (1996) 239-256.
- [211] K. Ohta, H. Monma, J. Tanaka, H. Eda, Interaction between hydroxyapatite and proteins by liquid chromatography using simulated body fluids as eluents, *Journal of Materials Science-Materials in Medicine* 13 (2002) 633-637.
- [212] J. Clover, M. Gowen, Are Mg-63 and Hos Te85 Human Osteosarcoma Cell-Lines Representative Models of the Osteoblastic Phenotype, *Bone* 15 (1994) 585-591.
- [213] S. N. Nayab, F. H. Jones, I. Olsen, Effects of calcium ion implantation on human bone cell interaction with titanium, *Biomaterials* 26 (2005) 4717-4727.
- [214] M. A. Harrison, *General techniques of cell culture*, Cambridge University Press, 1997.
- [215] Y. Tanizawa, H. Tsuchikane, K. Sawamura, T. Suzuki, Reaction Characteristics of Hydroxyapatite with F- and Po3f2- Ions - Chemical-States of Fluorine in Hydroxyapatite, *Journal of the Chemical Society-Faraday Transactions* 87 (1991) 2235-2240.
- [216] H. Tanaka, A. Yasukawa, K. Kandori, T. Ishikawa, Surface structure and properties of fluoridated calcium hydroxyapatite, *Colloids and Surfaces a-Physicochemical and Engineering Aspects* 204 (2002) 251-259.
- [217] M. A. Stranick, M. J. Root, Influence of Strontium on Monofluorophosphate Uptake by Hydroxyapatite - Xps Characterization of the Hydroxyapatite Surface, *Colloids and Surfaces* 55 (1991) 137-147.
- [218] P. Layrolle, A. Lebugle, Synthesis in pure ethanol and characterization of nanosized calcium phosphate fluoroapatite, *Chemistry of Materials* 8 (1996) 134-144.
- [219] S. Kaciulis, G. Mattogno, L. Pandolfi, M. Cavalli, G. Gnappi, A. Montenero, XPS study of apatite-based coatings prepared by sol-gel technique, *Applied Surface Science* 151 (1999) 1-5.
- [220] K. Cheng, S. Zhang, W. J. Weng, The F content in sol-gel derived FHA coatings: an XPS study, *Surface & Coatings Technology* 198 (2005) 237-241.
- [221] A. Stoch, W. Jastrzebski, A. Brozek, J. Stoch, J. Szaraniec, B. Trybalska, G. Kmita, FTIR absorption-reflection study of biomimetic growth of phosphates on titanium implants, *Journal of Molecular Structure* 555 (2000) 375-382.
- [222] F. Freund, R. M. Knobel, Distribution of fluorine in hydroxyapatite studied by infrared spectroscopy, *Journal of Chemical Society-Dalton Trans* (1977) 1136-1140.
- [223] H. J. Busscher, A. W. J. Vanpelt, H. P. Dejong, J. Arends, Effect of Spreading Pressure on Surface Free-Energy Determinations by Means of Contact-Angle Measurements, *Journal of Colloid and Interface Science* 95 (1983) 23-27.
- [224] H. Qu, M. Wei, Effect of fluorine content on mechanical properties of sintered fluoridated hydroxyapatite, *Materials Science & Engineering C-Biomimetic and Supramolecular Systems* 26 (2006) 46-53.
- [225] E. A. Vogler, Structure and reactivity of water at biomaterial surfaces, *Advances in Colloid and Interface Science* 74 (1998) 69-117.
- [226] D.R.Lide, *Handbook of Chemistry and Physics*, CRC Press, Boca Raton, 2004.
- [227] D. M. Brunette, P. Tengvall, M. Textor, P. Thomsen, *Titanium in Medicine*, Springer, Berlin, 2001.
-

References

- [228] K. Cheng, S. Zhang, W. J. Weng, X. T. Zeng, The interfacial study of sol-gel-derived fluoridated hydroxyapatite coatings, *Surface & Coatings Technology* 198 (2005) 242-246.
- [229] J. W. Dally, *Experimental Stress Analysis*, McGraw-Hill Press, New York, 1978.
- [230] S. Zhang, D. Sun, Y. Q. Fu, H. J. Du, Q. Zhang, Effect of sputtering target power density on topography and residual stress during growth of nanocomposite nc-TiN/a-SiN_x thin films, *Diamond and Related Materials* 13 (2004) 1777-1784.
- [231] V. Sergo, O. Sbaizero, D. R. Clarke, Mechanical and chemical consequences of the residual stresses in plasma sprayed hydroxyapatite coatings, *Biomaterials* 18 (1997) 477-482.
- [232] *Implants for Surgery-Hydroxyapatite-Part 2: Coatings of Hydroxyapatite*, in: British Standard, BS ISO 13779-2, 2000.
- [233] S. Zhang, X. L. Bui, *Adhesion Aspects of Thin Films*, VSP, Utrecht, 2005.
- [234] L. Gan, H. Wang, R. M. Pillar, Evaluating interface strength of calcium phosphate sol-gel-derived thin films to Ti6Al4V substrate, *Biomaterials* 26 (2005) 189-196.
- [235] F. Brossa, A. Cigada, R. Chiesa, L. Paracchini, C. Consonni, Postdeposition Treatment Effects on Hydroxyapatite Vacuum Plasma Spray Coatings, *Journal of Materials Science-Materials in Medicine* 5 (1994) 855-857.
- [236] T. Fett, A. B. K. Njiwa, J. Rodel, Crack opening displacements of Vickers indentation cracks, *Engineering Fracture Mechanics* 72 (2005) 647-659.
- [237] H. W. Kim, J. C. Knowles, L. H. Li, H. E. Kim, Mechanical performance and osteoblast-like cell responses of fluorine-substituted hydroxyapatite and zirconia dense composite, *Journal of Biomedical Materials Research Part A* 72A (2005) 258-268.
- [238] Y. Liu, E. B. Hunziker, N. X. Randall, K. de Groot, P. Layrolle, Proteins incorporated into biomimetically prepared calcium phosphate coatings modulate their mechanical strength and dissolution rate, *Biomaterials* 24 (2003) 65-70.
- [239] B. Feng, J. Chen, X. Zhang, Interaction of calcium and phosphate in apatite coating on titanium with serum albumin, *Biomaterials* 23 (2002) 2499-2507.
- [240] Y. Nakashima, K. Hayashi, T. Inadome, K. Uenoyama, T. Hara, T. Kanemaru, Y. Sugioka, I. Noda, Hydroxyapatite-coating on titanium arc sprayed titanium implants, *Journal of Biomedical Materials Research* 35 (1997) 287-298.
- [241] Q. Zhang, J. Chen, J. Feng, Y. Cao, C. Deng, X. Zhang, Dissolution and mineralization behaviors of HA coatings, *Biomaterials* 24 (2003) 4741-4748.
- [242] J. Xie, C. Riley, K. Chittur, Effect of albumin on brushite transformation to hydroxyapatite, *Journal of Biomedical Materials Research* 57 (2001) 357-365.
- [243] A. T. C. Wong, J. T. Czernuszka, Transformation Behavior of Calcium-Phosphate .1. Theory and Modeling, *Colloids and Surfaces a-Physicochemical and Engineering Aspects* 78 (1993) 245-253.
- [244] S. Lazic, Microcrystalline Hydroxyapatite Formation from Alkaline-Solutions, *Journal of Crystal Growth* 147 (1995) 147-154.
- [245] S. W. Ha, R. Reber, K. L. Eckert, M. Petitmermet, J. Mayer, E. Wintermantel, C. Baerlocher, H. Gruner, Chemical and morphological changes of vacuum-plasma-sprayed hydroxyapatite coatings during immersion in simulated physiological solutions, *Journal of the American Ceramic Society* 81 (1998) 81-88.
- [246] S. V. Dorozhkin, E. I. Dorozhkina, S. Agathopoulos, J. M. F. Ferreira, The influence of glucose and bovine serum albumin on the crystallization of a bone-like

References

- apatite from revised simulated body fluid, in: *Bioceramics* 16, vol 254-2, Trans Tech Publications Ltd, Zurich-Uetikon, 2004, pp. 327-330.
- [247] S. V. Dorozhkin, E. I. Dorozhkina, M. Epple, Precipitation of carbonate apatite from a revised simulated body fluid in the presence of glucose, *Journal of Applied Biomaterials and Biomechanics* 1 (2003) 200-207.
- [248] C. M. Pradier, D. Costa, C. Rubio, C. Compere, P. Marcus, Role of salts on BSA adsorption on stainless steel in aqueous solutions. I. FT-IRRAS and XPS characterization, *Surface and Interface Analysis* 34 (2002) 50-54.
- [249] C. Combes, C. Rey, M. Freche, In vitro crystallization of octacalcium phosphate on type I collagen: influence of serum albumin, *Journal of Materials Science-Materials in Medicine* 10 (1999) 153-160.
- [250] H. Zeng, K. K. Chittur, W. R. Lacefield, Dissolution/reprecipitation of calcium phosphate thin films produced by ion beam sputter deposition technique, *Biomaterials* 20 (1999) 443-451.
- [251] T. Asaoka, T. Ando, T. Meguro, I. Yanato, Development of a structure based protein function prediction method: calcium binding protein, *Chem-Bio Informatics Journal* 3 (2003) 96-113.
- [252] S. Takemoto, Y. Kusudo, K. Tsuru, S. Hayakawa, A. Osaka, S. Takashima, Selective protein adsorption and blood compatibility of hydroxy-carbonate apatites, *Journal of Biomedical Materials Research Part A* 69A (2004) 544-551.
- [253] H. B. Wen, J. R. de Wijn, C. A. van Blitterswijk, K. de Groot, Incorporation of bovine serum albumin in calcium phosphate coating on titanium, *Journal of Biomedical Materials Research* 46 (1999) 245-252.
- [254] H. S. Azevedo, I. B. Leonor, C. M. Alves, R. L. Reis, Incorporation of proteins and enzymes at different stages of the preparation of calcium phosphate coatings on a degradable substrate by a biomimetic methodology, *Materials Science & Engineering C-Biomimetic and Supramolecular Systems* 25 (2005) 169-179.
- [255] A. Krajewski, R. Malavolti, A. Piancastelli, Albumin adhesion on some biological and non-biological glasses and connection with their Z-potentials, *Biomaterials* 17 (1996) 53-60.
- [256] M. A. Malik, D. A. Puleo, R. Bizios, R. H. Doremus, Osteoblasts on Hydroxyapatite, Alumina and Bone Surfaces In vitro - Morphology During the 1st 2-H of Attachment, *Biomaterials* 13 (1992) 123-128.
- [257] H. B. Qu, M. Wei, The effect of fluoride contents in fluoridated hydroxyapatite on osteoblast behavior, *Acta Biomaterialia* 2 (2006) 113-119.
- [258] H. H. Liao, A. S. Andersson, D. Sutherland, S. Petronis, B. Kasemo, P. Thomsen, Response of rat osteoblast-like cells to microstructured model surfaces in vitro, *Biomaterials* 24 (2003) 649-654.
- [259] D. D. Deligianni, N. D. Katsala, P. G. Koutsoukos, Y. F. Missirlis, Effect of surface roughness of hydroxyapatite on human bone marrow cell adhesion, proliferation, differentiation and detachment strength, *Biomaterials* 22 (2001) 87-96.
- [260] Rajarama.R, D. E. Rounds, S. P. S. Yen, A. Rembaum, Scanning Electron-Microscope Study of Cell Adhesion and Spreading In vitro, *Experimental Cell Research* 88 (1974) 327-339.
- [261] F. Grinnell, M. Milam, P. A. Sreere, Studies on Cell Adhesion .3. Adhesion of Baby Hamster Kidney-Cells, *Journal of Cell Biology* 56 (1973) 659-665.
- [262] J. Harrison, A. J. Melville, J. S. Forsythe, B. C. Muddle, A. O. Trounson, K. A. Gross, R. Mollard, Sintered hydroxyfluorapatites--IV: the effect of fluoride substitutions upon colonisation of hydroxyapatites by mouse embryonic stem cells, *Biomaterials* 25 (2004) 4977-4986.

References

- [263] B. H. Yoon, H. W. Kim, S. H. Lee, C. J. Bae, Y. H. Koh, Y. M. Kong, H. E. Kim, Stability and cellular responses to fluorapatite-collagen composites, *Biomaterials* 26 (2005) 2957-2963.
- [264] H. W. Kim, E. J. Lee, H. E. Kim, V. Salih, J. C. Knowles, Effect of fluoridation of hydroxyapatite in hydroxyapatite-polycaprolactone composites on osteoblast activity, *Biomaterials* 26 (2005) 4395-4404.
- [265] J. R. Farley, J. E. Wergedal, D. J. Baylink, Fluoride Directly Stimulates Proliferation and Alkaline-Phosphatase Activity of Bone-Forming Cells, *Science* 222 (1983) 330-332.
- [266] J. P. Rodriguez, G. Rosselot, Sodium fluoride induces changes on proteoglycans synthesized by avian osteoblasts in culture, *Journal of Cellular Biochemistry* 83 (2001) 607-616.
- [267] P. Dandona, A. Coumar, D. S. Gill, J. Bell, M. Thomas, Sodium-Fluoride Stimulates Osteocalcin in Normal Subjects, *Clinical Endocrinology* 29 (1988) 437-441.
- [268] S. Ma, Y. Yang, D. L. Carnes, K. Kim, S. Park, S. H. Oh, J. L. Ong, Effects of dissolved calcium and phosphorous on osteoblast responses, *Journal of Oral Implantology* 31 (2005) 61-67.
- [269] Z. Meleti, I. M. Shapiro, C. S. Adams, Inorganic phosphate induces apoptosis of osteoblast-like cells in culture, *Bone* 27 (2000) 359-366.
- [270] K. Anselme, Osteoblast adhesion on biomaterials, *Biomaterials* 21 (2000) 667-681.