2012

Multi-functions of carbonated calcium deficient hydroxyapatite (CDHA)

Huan Zhou
The University of Toledo

Follow this and additional works at: http://utdr.utoledo.edu/theses-dissertations

Recommended Citation
http://utdr.utoledo.edu/theses-dissertations/497

This Dissertation is brought to you for free and open access by The University of Toledo Digital Repository. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of The University of Toledo Digital Repository. For more information, please see the repository’s About page.
A Dissertation

entitled

Multi-Functions of Carbonated Calcium Deficient Hydroxyapatite (CDHA)

by

Huan Zhou

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the
Doctor of Philosophy Degree in Biomedical Engineering

Dr. Sarit B. Bhaduri, Committee Chair

Dr. Arunan Nadarajah, Committee Member

Dr. A. Champa Jayasuriya, Committee Member

Dr. Patricia A. Relue, Committee Member

Dr. Yong X. Gan, Committee Member

Dr. Stephen Callaway, Committee Member

Dr. Patricia R. Komuniecki, Dean
College of Graduate Studies

The University of Toledo

May 2012
An Abstract of

Multi-Functions of Carbonated Calcium Deficient Hydroxyapatite (CDHA)

by

Huan Zhou

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the
Doctor of Philosophy Degree in Biomedical Engineering

The University of Toledo
May 2012

Natural bone is a complex composite mainly constituted of inorganic minerals and organic collagen molecules. Calcium phosphate (CaP) based materials have been proposed as the predominant bone substitute for bone tissue engineering applications due to their chemical similarity to bone mineral. Amorphous carbonated calcium deficient hydroxyapatite (CDHA) is an important compound among CaP materials because of the amorphous crystallite structure. The presence of extra ions in its lattice structure not only influences cell attachment and proliferation of osteoblasts, but also helps in bone metabolism.

Biomimetic coating approach is the most widely used approach to produce CDHA coatings to implant. It is a process using simulated body fluid (SBF) to deposit bone-like CDHA coating to various material surfaces. The CDHA formation mechanism, SBF compositions and reacting conditions of biomimetic coating have already been sufficiently studied and compared in the past 20 years.

It is an attempt in this thesis to explore new applications of SBF in biomedical research, focusing on different biomaterial applications: 1) based on the low temperature reaction condition of SBF, bisphosphonate incorporated CDHA coatings were deposited
onto Ti6Al4V surface for the treatment of osteoporosis; 2) amorphous calcium phosphate nanospheres with extra elements in the lattice structure were prepared by a novel microwave assisted approach, providing a new potential of CaP materials production; 3) CDHA particles formed in SBF can be used as great fillers with biopolymers for preparing biocomposites for biomedical applications; 4) based on the high activity of CDHA amorphous structure and the stabilization ability of ethanol, yttrium and europium doped calcium phosphates were prepared using CDHA as a sacrificing template.

In the end, future work based on these observations in the thesis is addressed, including areas of drug delivery, biocomposite fabrication and preparation of functionalized calcium phosphate materials.
Acknowledgments

I would like to thank my advisor, Dr. Sarit B Bhaduri for his continual support and guidance throughout my Ph.D program. Without the mentoring of him, I could not have completed this work. I would like to thank Dr. Joseph Lawrence and Dr. Ahmed Touny for their initial guidance into the materials research. I would also like to acknowledge the significant contributions made by my committee members Dr. Arunan Nadarajah, Dr. A. Champa Jayasuriya, Dr. Patricia A. Relue, Dr. Yong X. Gan, and Dr. Stephen Callaway during my research journey. Additionally, I would like to thank Tamara Phares in the Bioengineering Department for her help in biological aspects. I am grateful for my fellow friends in UT for their support and cooperation during my studies. I would also like to thank my family for all of their love and support.
# Table of Contents

Abstract .................................................................................................................................................. iii

Acknowledgments ................................................................................................................................... v

Table of Contents ................................................................................................................................... vi

List of Tables .......................................................................................................................................... xi

List of Figures ....................................................................................................................................... xii

List of Abbreviations ............................................................................................................................ xvi

1 Introduction .......................................................................................................................................... 1

   1.1 Bone Substitute Materials ............................................................................................................. 1

   1.2 Calcium Phosphate Materials ........................................................................................................ 2

   1.3 Biomimetic Coating ...................................................................................................................... 5

   1.4 Modified Applications of CDHA .................................................................................................. 7

   1.5 Summary ..................................................................................................................................... 10

2 Biomimetic Coating of Bisphosphonate Incorporated CDHA on Ti6Al4V ....................................... 11

   2.1 Abstract ....................................................................................................................................... 11

   2.2 Introduction .................................................................................................................................... 11

   2.3 Experiments................................................................................................................................. 14

      2.3.1 Sample pre-treatment ........................................................................................................... 14

      2.3.2 SBF preparation .................................................................................................................... 15

      2.3.3 Drug loading ......................................................................................................................... 16

      2.3.4 Analysis ................................................................................................................................ 16

      2.3.5 Preliminary evaluation of CDHA and cell proliferation ....................................................... 18
2.4 Results......................................................................................................................................... 19
  2.4.1 Morphology of CDHA-AS coatings using different methods.............................................. 19
  2.4.2 AS release prolife ................................................................................................................. 20
  2.4.3 Preliminary evaluation of CDHA and cell proliferation....................................................... 21
  2.5 Discussion................................................................................................................................... 26
  2.6 Conclusion................................................................................................................................... 31
3 Novel Microwave Synthesis of ACP nanospheres ............................................................................. 33
  3.1 Abstract ....................................................................................................................................... 33
  3.2 Introduction ................................................................................................................................. 33
  3.3 Materials and Methods ................................................................................................................ 36
  3.4 Results ......................................................................................................................................... 39
  3.5 Discussion ................................................................................................................................... 46
  3.6 Conclusion................................................................................................................................... 52
4 Fabrication Aspects of PLA-CaP/PLGA-CaP Composites for Orthopedic Applications: A Review .54
  4.1 Abstract ....................................................................................................................................... 54
  4.2 Introduction ................................................................................................................................. 54
  4.3 Material aspects of PLA-CaP/PLGA-CaP composites ........................................................... 57
  4.4 Fabrication aspects of PLA-CaP/PLGA-CaP composites ........................................................... 63
    4.4.1 Microencapsulation Process ................................................................................................. 64
    4.4.2 Solvent Casting .................................................................................................................... 67
    4.4.3 Phase Separation ................................................................................................................ 68
    4.4.4 Electrospinning .................................................................................................................... 70
    4.4.5 Coating Technique .............................................................................................................. 72
    4.4.6 Supercritical Gas Foaming ................................................................................................... 74
    4.4.7 Melting ................................................................................................................................. 76
    4.4.8 Solid Free Fabrication/Rapid Prototyping .......................................................................... 79
4.5 Performance of PLA-CaP/PLGA-CaP composites ................................................................. 85
  4.5.1 Mechanical Performance ................................................................................................. 85
  4.5.2 Biological Performance ................................................................................................. 89
4.6 Challenges ............................................................................................................................ 92
  4.6.1 Mechanical Integrity of the Composite .......................................................................... 92
  4.6.2 Incorporation of Proteins/Drugs/Genes and Stem Cells ................................................ 95
  4.6.3 Long-term degradation ................................................................................................. 98
4.7 Summary ............................................................................................................................. 100

5 Fabrication of Novel PLA/CDHA Bionanocomposite Fibers for Tissue Engineering Applications via
Electrospinning .......................................................................................................................... 102
  5.1 Abstract ............................................................................................................................ 102
  5.2 Introduction ....................................................................................................................... 103
  5.3 Materials and Methods ..................................................................................................... 106
    5.3.1 CDHA powders synthesis ............................................................................................ 106
    5.3.2 PLA/CDHA solution preparation ............................................................................... 107
  5.3.3 Electrospinning approach ............................................................................................. 108
    5.3.4 Physical Evaluation ....................................................................................................... 108
    5.3.5 In vitro degradation tests ............................................................................................. 109
    5.3.6 Bioactivity test .............................................................................................................. 110
    5.3.7 Osteoblast cell culture ................................................................................................ 111
5.4 Results ................................................................................................................................. 112
    5.4.1 Physical Evaluation ....................................................................................................... 112
    5.4.2 In vitro degradation tests ............................................................................................. 115
    5.4.3 Bioactivity test .............................................................................................................. 116
    5.4.4 Osteoblast cell culture ................................................................................................ 117
5.5 Discussion ............................................................................................................................ 119
5.6 Conclusion ........................................................................................................................................ 124

6 Deposition of Bisphosphonate Incorporated PLA/CDHA Composite Coating via Electrospraying 125
   6.1 Abstract ........................................................................................................................................ 125
   6.2 Introduction .................................................................................................................................. 125
   6.3 Materials and Methods .................................................................................................................. 128
      6.3.1 Substrates ............................................................................................................................. 128
      6.3.3 PLA/CDHA solution preparation ......................................................................................... 129
      6.3.4 Electrospraying ..................................................................................................................... 130
      6.3.5 Characterization ..................................................................................................................... 130
      6.3.6 Bioactivity testing ................................................................................................................... 131
      6.3.7 In vitro testing ......................................................................................................................... 132
      6.3.8 AS incorporation and release ............................................................................................... 133
   6.4 Results .......................................................................................................................................... 134
   6.5 Discussion .................................................................................................................................... 141
   6.6 Conclusion ................................................................................................................................... 146

7 Production of Yttrium Phosphate Using CDHA as Precursors .......................................................... 148
   7.1 Abstract ........................................................................................................................................ 148
   7.2 Introduction .................................................................................................................................. 148
   7.3 Materials and Methods .................................................................................................................. 149
   7.4 Results .......................................................................................................................................... 151
   7.5 Discussion .................................................................................................................................... 154
   7.6 Conclusion ................................................................................................................................... 155

8 Synthesis of Eu$^{3+}$ Doped Calcium Phosphate Nanospheres .......................................................... 156
   8.1 Abstract ........................................................................................................................................ 156
   8.2 Introduction .................................................................................................................................. 156
   8.3 Materials and Methods .................................................................................................................. 158
List of Tables

1.1 Properties of reported CaP compounds [6, 7] .................................................................................... 4
1.2 Ion concentrations of human plasma and SBF, mM [29-31] ............................................................. 7
2.1 Compositions of 1L 1.5x t-SBF ....................................................................................................... 15
2.2 Compositions of 1L PBS ................................................................................................................. 18
3.1 Sample preparation for microwave ACP analysis with different study purposes: 1) influence of total ionic concentration; 2) influence of Mg$^{2+}$ concentration; 3) comparison of the impacts of Mg$^{2+}$ concentration and total ionic concentration; 4) effect of HCO$_3^-$; 5) influence of microwave heating time; 6) comparison of microwave assisted precipitation and conventional precipitation .................... 38
4.1 Search results in Science citation index expanded when using different key words ....................... 59
4.2 Information on various CaP materials used to manufacture PLA-CaP/PLGA-CaP composites [18, 37, 127] ................................................................................................................................................. 62
4.3 Physical properties of PLA-CaP/PLGA-CaP composites fabricated using different techniques .... 86
4.4 Results of in vitro and in vivo experiments using different PLA-CaP/PLGA-CaP composites ..... 91
5.1 Modified 1.5x t-SBF solution composition for a total volume of 1L ............................................ 107
5.2 Mixture compositions and working conditions ............................................................................. 108
5.3 1.5x t-SBF solution composition for a total volume of 1L ............................................................ 110
6.1. Modified 1.5x t-SBF solution compositions for a total volume of 1L ........................................... 129
6.2. 1.5x t-SBF solution compositions for a total volume of 1L .......................................................... 131
7.1 Recipe of SBF1, for a total volume of 1 L .................................................................................... 150
7.2 Recipe of SBF2, for a total volume of 1 L .................................................................................... 151
8.1 Different solution composition ...................................................................................................... 158
List of Figures

2-1 SEM images of samples .................................................................................................................. 20
2-2 The average AS content/area of each groups .................................................................................. 21
2-3 AS release prolif of different groups prepared using method I or II ........................................... 21
2-4 XRD patterns of samples soaked in 1.5× t-SBF solution with different concentrations of AS (0, 10^{-6}, 10^{-5} and 10^{-4}M) ........................................................................................................... 22
2-5(a) SEM images of the surfaces of CDHA coatings deposited from 1.5x t-SBF solutions with different concentrations of AS, magnification is 1 K ................................................................. 23
2-5(b) SEM images of the surfaces of CDHA coatings deposited from 1.5x t-SBF solutions with different concentrations of AS, magnification is 5 K ........................................................................... 24
2-5(c) SEM images of the surfaces of CDHA coatings deposited from 1.5x t-SBF solutions with different concentrations of AS, magnification is 100 K .................................................................................. 24
2-6(a) Osteoblast cell numbers on CDHA coatings deposited from 1.5x t-SBF solutions with different concentrations of AS after 24 hours and 6 days ....................................................................... 25
2-6(b) Osteoblast cell numbers surrounding strips with CDHA coatings deposited from 1.5x t-SBF solutions with different concentrations of AS after 24 hours and 6 days .............................................. 26
2-7 SEM images of osteoblast cells on the CDHA coatings deposited from 1.5× t-SBF solutions with different concentrations of AS after 6 days: (a) 10^{-4}, and (b) 10^{-5} mol/L .............................................................. 26
3-1 XRD data of precipitates from different SBF compositions after 5 min microwave heating and related product after CaCl_2 aqueous solution incubation .................................................................................. 40
3-2 FTIR data of precipitates from different SBF compositions after 5 min microwave heating and related product after CaCl_2 aqueous solution incubation ........................................................................ 41
3-3 SEM images of different samples................................................................................................... 43
3-4 Ca/P and Mg/P molar ratio comparisons of different sample groups........................................... 44
3-5 TEM images of (a) & (b) nanospheres and (c) related clusters after transition ......................... 44
3-6 Results of osteoblast cell culture.................................................................................................. 45
3-7 SEM of pressed nanospheres after culture medium incubation .................................................. 46
4-1 Structure of (a) PLA; (b) PGA; and (c) PLGA ........................................................................... 59
4-2 Fabrication techniques can be separated into two groups for cortical or cancellous bone applications based on related product porosity ................................................................. 64
4-3 SEM and TEM image of the HA nanoparticle-coated PLLA microspheres prepared after evaporation of CH$_2$Cl$_2$ from the CH$_2$Cl$_2$ solution of PLA-in-water emulsion...................................................... 66
4-4 Schematic of solvent casting ...................................................................................................... 67
4-5 Schematic of phase separation .................................................................................................. 69
4-6 SEM micrographs of pure PLA and PLA/nano-HA scaffold fabricated via phase separation technique: (a,b) Pure PLLA and cross-section; (c,d) nHAP/PLLA and cross-section ....................... 69
4-7 A typical electrospinning set-up ................................................................................................. 71
4-8 SEM photographs of the surfaces of (a, b) non-treated and (c, d) alkali-treated PLA fabrics immersed in HA ethanol suspension ................................................................................................. 74
4-9 Schematic of supercritical gas foaming ..................................................................................... 75
4-10 Similarity of cancellous bone structures and composite foam macrostructures ....................... 76
4-11 Schematic of melting extrusion ................................................................................................. 79
4-12 Schematic of low-temperature deposition manufacturing ........................................................ 81
4-13 SEM images of the cross-section of the PLLA/TCP composite scaffold.................................... 82
4-14 Schematic of 3D printing ......................................................................................................... 83
4-15 Schematic of selective laser sintering ....................................................................................... 84
4-16 PLA-HA scaffold fabricated via indirect SFF ......................................................................... 85
4-17 SEM surface and cross-section images of the samples after immersion in SBF for 7 days........ 90
5-1 XRD patterns of (a) CDHA nanocrystals, (b) PLA pellets, (c) PLA/CDHA fibers .................. 112
5-2 FTIR data of (a) CDHA nanocrystals, (b) PLA pellets, (c) PLA/CDHA fibers ............. 113
5-3 Results of SEM and EDS characterization ................................................................. 114
5-4 TEM images of (a) as-produced CDHA precipitates, (b) PLA/CDHA fibers, chloroform:DMF ratio is 9:1 .............................................................. 115
5-5 Results of in vitro degradation test ............................................................................ 116
5-6 SEM images of (a) PLA fibers after 1 week soaking in 1.5x t-SBF, and (b) PLA/CDHA fibers after 1 week soaking in 1.5x t-SBF ....................................................... 117
5-7 XRD patterns of (a) PLA fibers after 1 week soaking in 1.5x t-SBF, and (b) PLA/CDHA fibers after 1 week soaking in 1.5x t-SBF ............................................................................................ 117
5-8 Results of in vitro cell culture on PLA and PLA/CDHA fibers ................................ 118
5-9 SEM image of nano-CaP particles expressed by osteoblast cells after 7 days proliferation on PLA/CDHA fibers .............................................................................. 118
6-1 Electrospraying setup for PLA/CDHA coatings deposition ........................................... 130
6-2 SEM images of products performed at different flowing rate (working distance was 20 cm, working voltage was 15 kV) ...................................................................................... 135
6-3 SEM images of beads formed at different working distance (flowing rate was 30 ml/h, working voltage was 25 kV) ...................................................................................... 135
6-4 SEM images of beads formed at different working voltage (flowing rate was 30 ml/h, working distance was 10 cm) ...................................................................................... 135
6-5 CDHA nanocrystals characterization: (a) SEM image of CDHA nanocrystals; (b) EDS result of CDHA nanocrystals ........................................................................................................ 136
6-6 XRD patterns of oxidized Ti6Al4V substrate, CDHA, PLA, and composite coatings ........ 137
6-7 FTIR data of PLA, CDHA, and PLA/CDHA coatings ...................................................... 137
6-8 Results of SEM characterization ............................................................................... 138
6-9 EDS mapping analysis: (a) focused PLA/CDHA coatings area, (b) Ca element distribution, (c) C
element distribution ................................................................................................................................................. 138

6-10 SEM images of samples after 7 days biomimetic coating: (a) oxidized Ti6Al4V substrates; (b) oxidized Ti6Al4V substrates with PLA/CDHA coatings ......................................................................................................................... 139

6-11 Weight change of oxidized Ti6Al4V substrates with/without PLA/CDHA coatings after 7 days SBF incubation ................................................................................................................................................................. 139

6-12 Results of in vitro testing ....................................................................................................................................... 140

6-13 SEM images of osteoblast cells on the surfaces of oxidized Ti6Al4V substrates, PLA coatings and PLA/CDHA coatings ........................................................................................................................................................................ 140

6-14 AS release study ......................................................................................................................................................... 141

7-1 (a) SEM image of CDHA coatings and (b) SEM image of YPO₄ coating ................................................................. 151

7-2 EDS result of coating after incubation in YCl₃ aqueous solution ......................................................................................... 152

7-3 EDS results of different time period prepared CDHA coated Ti6Al4V after incubation in YCl₃ aqueous solution ........................................................................................................................................................................ 152

7-4 CDHA microspheres precipitated from SBF2 .................................................................................................................. 153

7-5 SEM image of YPO₄ microspheres related EDS mapping of Y, Ca, and P elements ..................................................... 153

7-6 SEM image of reacted microspheres without EtOH pre-treatment ............................................................................. 154

8-1 SEM images of ACP nanospheres after 24 hrs reaction in different medium ............................................................. 159

8-2 Eu/Ca molar ratio plotted with EtOH volume ratio in solution ................................................................................. 160
List of Abbreviations

ACP ......................... Amorphous Calcium Phosphate
AS .............................. Alendronate Sodium

BPs .............................. Bisphosphonates

CaP .......................... Calcium Phosphate Materials
CDHA ......................... Carbonated Calcium Deficient Hydroxyapatite

DCPA ......................... Dicalcium Phosphate Anhydrous
DCPD .......................... Dicalcium Phosphate Dihydrate
DMF .............................. Dimethyformamide

EDS .......................... Energy Dispersive X-ray Spectroscopy

FBS .............................. Fetal Bovine Serum
FMOC .......................... 9-Fluorenylmethyl Chloroformate

FTIR .......................... Fourier Transform Infrared Spectroscopy

HA .............................. Hydroxyapatite
HEPES ....................... 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HPLC .......................... High Performance Liquid Chromatography

MEM .......................... Minimum Essential Medium

OCP .......................... Octacalcium Phosphate

PBS .......................... Phosphate Buffered Saline
PLA ........................... Polylactic Acid
PLGA .......................... Poly(lactide-co-Glycolide)
PGA ........................... Polyglycolic acid

SBF .......................... Simulated Body Fluids
SEM .......................... Scanning Electron Microscope
SiC ........................... Silicon Carbide

TCP .......................... Tricalcium Phosphate
TEM .......................... Transmission Electron Microscope
**Tris** .................................. Tris-hydroxymethylaminomethane

**XRD** ........................... X-Ray Diffraction
Chapter 1

Introduction

1.1 Bone Substitute Materials

Bone defects are a serious health problem world-wide with over 500000 bone repair procedures performed annually [1]. Bone tissue usually has the ability to repair itself, but when complicated pathological fractures or large bone defects occur, the healing process fails.

Bone grafting is a surgical procedure in which a defect is repaired by the implantation of a bone substitute. The objective of a bone graft is the full or partial restoration of a bone’s function. Bones ought to provide mechanical support for the body, protect vital organs, produce hematopoetic cells and serve as reservoir for calcium phosphates to preserve mineral homeostasis. Nowadays, autografts, allografts, and xenografts are commonly used for the treatment of bone fractures, showing good clinical results under certain conditions. However their applications are limited due to infection, disease transfer, rejection, lack of graft supplies, and high harvesting cost [2-4].

Bone substitute materials can be roughly divided into three main types governed by the bone tissue response. In broad terms, inert (more strictly, nearly inert) materials illicit no or minimal tissue response (biocompatibility). Active materials encourage bone
bonding to surrounding tissue (bioactivity). Degradable, or resorbable materials are incorporated into surrounding tissues or even dissolve after a period of time (biodegradability). Usually, metallic materials are typically biocompatible (Ti alloys etc.) and partially biodegradable (such as Mg incorporated alloys). Ceramics may be biocompatible, bioactive or partially biodegradable. Polymers may be biocompatible or biodegradable.

In their orthopedic applications, based different mechanical and biological properties, bone substitute materials play different roles. Metallic materials are normally used for load bearing situations. Ceramics such as alumina and zirconia are used for wear applications in joint replacements, while calcium phosphate (CaP) is used for bone bonding applications to assist implant integration. Biopolymers such as polyethylene with high mechanical strength are used as articulating surfaces against ceramic components in joint replacements, but polyesters, chitosan, collagen etc. are used as matrix for bone cells proliferation.

1.2 Calcium Phosphate Materials

In the past 40 years, CaP materials have attracted much attention as a bone substitute materials due to their chemical similarity to the mineral phase of bone. Therefore, they are the predominant bioactive bioceramics widely used and investigated [5]. The most important advantage of CaP being a bioactive material is that bone can form a direct chemical bonding to CaP, without forming a fibrous interface layer with poor mechanical stability, which usually occurs in many biocompatible materials after implantation. The reactions at the CaP-bone interfaces include the release of calcium phosphate ions from the implant to induce supersaturation of calcium and phosphate ions in the local body
fluid. This results in deposition of bone mineral to the surface of implants. Furthermore, this modified surface can rapidly accommodate protein adsorption, cell adhesion, cellular bone matrix formation trigger and mineralization. The applications of CaP include, coating of orthopedic and dental implants, alveolar ridge augmentation, scaffolds for bone growth and as powders for total hip or knee surgery.

On the basis of chemical composition, reported applicable CaP biomaterials are classified as hydroxyapatite (HA), octacalcium phosphate (OCP), amorphous calcium phosphate (ACP), tricalcium phosphate (TCP), calcium deficient hydroxyapatite (CDHA), their chemical formula and relevant properties are listed in Table 1.1 [6, 7]. Similarly, the synthesis techniques of these CaP materials have been sufficiently reviewed in literature [6-8]. In general, they are prepared based on the precursors containing Ca\(^{2+}\) and PO\(_4^{3-}\) separately in varied reaction conditions.

The most stable CaP phase in an aqueous environment with neutral pH values is HA. DCPA, DCPD and OCP can only be synthesized under aqueous conditions and at low temperatures since they already contain water molecules in their chemical formula. TCP can only be synthesized at high temperatures (in excess of 800°C) [9,10]. Generally, these CaPs are used either as single phase or as a multiphasic combination of two or more phases in the biomedical field [11]. Other interesting possibilities have been explored with CaP. The usage of polymeric or metal-oxide fillers embedded within a CaP matrix and vice-versa [12] has been investigated by various researchers. Coating metallic [13-16] and polymeric [17] fillers with CaP has been documented to increase their biocompatibility.
Table 1.1 Properties of reported CaP compounds [6, 7]

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Ca/P molar ratio</th>
<th>Solubility at 25 °C, -log(Kₜ)</th>
<th>pH stability range in aqueous solution at 25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dicalcium phosphate dihydrate (DCPD)</td>
<td>CaHPO₄·2H₂O</td>
<td>1.0</td>
<td>6.59</td>
<td>2.0-6.0</td>
</tr>
<tr>
<td>Dicalcium phosphate anhydrous (DCPA)</td>
<td>CaHPO₄</td>
<td>1.0</td>
<td>6.90</td>
<td>Stable above 100 °C</td>
</tr>
<tr>
<td>Octacalcium phosphate (OCP)</td>
<td>Ca₈(HPO₄)₂(PO₄)₄·5H₂O</td>
<td>1.33</td>
<td>96.6</td>
<td>5.5-7.0</td>
</tr>
<tr>
<td>α-Tricalcium phosphate (α-TCP)</td>
<td>α -Ca₃(PO₄)₂</td>
<td>1.5</td>
<td>25.5</td>
<td>Cannot be synthesized from aqueous solution</td>
</tr>
<tr>
<td>β-Tricalcium phosphate (β-TCP)</td>
<td>β -Ca₃(PO₄)₂</td>
<td>1.5</td>
<td>28.9</td>
<td>Cannot be synthesized from aqueous solution</td>
</tr>
<tr>
<td>Amorphous calcium phosphate (ACP)</td>
<td>CaₓHᵧ(PO₄)ₓ·nH₂O, n= 3–4.5; 15–20% H₂O</td>
<td>1.2-2.2</td>
<td>Varies</td>
<td>5-12</td>
</tr>
<tr>
<td>Calcium-deficient hydroxyapatite (CDHA)</td>
<td>Ca₁₀₋ₓ(HPO₄)ₓ(PO₄)₆₋ₓ(OH)₂₋ₓ f (0 &lt;ₓ &lt; 1)</td>
<td>1.5-1.6</td>
<td>85.1</td>
<td>6.5-9.5</td>
</tr>
<tr>
<td>Hydroxyapatite (HA)</td>
<td>Ca₁₀(PO₄)₆(OH)₂</td>
<td>1.67</td>
<td>116.8</td>
<td>9.5-12</td>
</tr>
</tbody>
</table>

Again, natural bone is a complex composite mainly constituting of inorganic minerals and organic collagen molecules. In this system collagen serves as the matrix for cell growth and tissue repair while the inorganic mineral phase improves bone mechanical strength and regeneration of bone [18]. The higher tensile strength and fracture toughness of bone are attributed to the tough and flexible collagen fibers reinforced by apatite crystals. These two components of bone are assembled into a highly
organized complex structure that gives stability to the body. In general, the first level of organization consists of HA particles that are entrenched between the ends of adjacent collagen molecules. The HA/collagen composites are then joined to form large collagen fibrils or lamellar sheets. Finally, both collagen fibrils and lamellar sheets are organized to form bone structure at varied load conditions.

It should be noted that minerals in bone are poorly crystalline, calcium-deficient hydroxyapatite (CDHA), with small amounts of ions such as sodium (Na\(^+\)), potassium (K\(^+\)), magnesium (Mg\(^{2+}\)), and carbonate (CO\(_3^{2-}\)) present in the lattice structure, as compared to synthesized CaP materials with pure chemical composition [18-20]. The amorphous crystallite and extra ions influence cell attachment, proliferation of osteoblasts and help in bone metabolism.

1.3 Biomimetic Coating

There are many ways to synthesize CDHA including simultaneous addition of calcium and orthophosphate containing solutions into boiling water followed by boiling the suspension for several hours, hydrolysis of precursors, and biomimetic coating [6]. Among these techniques, biomimetic coating is the most widely applied in biomaterial research. For instance, it not only can produce active CDHA materials but can also combine CDHA with different biocompatible materials for improved mechanical and biological performances [21-28].

Biomimetic coating is a process using simulated body fluid (SBF) to deposit bone-like CDHA coating to various material surfaces. SBF, with ion concentrations, temperature, pH, which are almost equal to those of human blood plasma, was first prepared by Kokubo et al. They used this technique to prove the formation of apatite on
glass-ceramics *in vivo* and *in vitro* based on the components CaO and SiO$_2$ [21]. It was later reported once these CaO and SiO$_2$ based glass-ceramics were soaked in SBF solutions, a poorly crystallized calcium-deficient and carbonate-containing bone-like apatite (CDHA) formed on the surface [22]. It was reported that glass-ceramics provided favorable sites for the apatite nucleation on their surfaces. Hydrated silica layer was formed by the exchange of calcium ions from the glass-ceramics and H$_3$O$^+$ ions in SBF [23]. In addition, titanium pre-treated with NaOH solution was reported to induce spontaneous formation of bone-like apatite layer when soaked in SBF [24]. The sodium titanate layer formed in the pre-treatment reacted with H$_3$O$^+$ ions in SBF to produce Ti-OH groups, which induced the nucleation of apatite as layered hydrated silica. De Andrade et al. demonstrated the conversion of sodium titanate to calcium titanate was based on the better stability of later titanate in a larger activity-pH domain. Thereby, a univalent cation is replaced by a divalent one, and the formed calcium titanate cation can attach itself to one bond of phosphate anion from the surroundings as a nucleation site [25]. Moreover, based on this nucleation kinetic, SBF has also been applied to deposit bone-like CDHA coating layer to a series of substrates including ceramics, metals and biopolymers once suitable surface treatments are applied [21-28].

Several SBF recipes were developed since its first introduction (Table 1.2). The SBF solution first introduced by Kokubo et al. is referred as c-SBF [21]. However, the hydrogen carbonate (HCO$_3^-$) ion concentration in the c-SBF solution is only at the level of 4.2 mM, which is “6.4 times” lower than the 27 mM level of human blood plasma. Because an appropriate level of carbonate ions is required in the process of biomineralization of carbonated, poorly crystalline calcium phosphate, c-SBF was
reported to be quite sluggish in forming a bone-like coating layer after 1 week at 37°C environment [29]. As an alternative, a new tris-hydroxymethylaminomethane (tris, 
C₄H₁₁NO₆) – buffered SBF solution (t-SBF) has been reported [30]. The composition of this SBF solution has an increase of HCO₃⁻ ion concentration from 4.2 mM to 27 mM and decrease of Cl⁻ ion concentration from 147.8 mM to 125.0 mM as compared to c-SBF [29, 30]. Except t-SBF, several other SBF compositions were also reported such as revised simulated body fluid (r-SBF), ionized simulated body fluid (i-SBF) and modified simulated body fluid (m-SBF), all showing ability to deposit bone-like CDHA to substrates [31].

Table 1.2 Ion concentrations of human plasma and SBF, mM [29-31]

<table>
<thead>
<tr>
<th></th>
<th>Blood plasma</th>
<th>c-SBF</th>
<th>t-SBF</th>
<th>r-SBF</th>
<th>i-SBF</th>
<th>m-SBF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>142.0</td>
<td>142.0</td>
<td>142.0</td>
<td>142.0</td>
<td>142.0</td>
<td>142.0</td>
</tr>
<tr>
<td>K⁺</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>1.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>103.0</td>
<td>147.8</td>
<td>125.0</td>
<td>103.0</td>
<td>103.0</td>
<td>103.0</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>27.0</td>
<td>4.2</td>
<td>27.0</td>
<td>27.0</td>
<td>27.0</td>
<td>10.0</td>
</tr>
<tr>
<td>HPO₄⁻</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Ca/P</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>1.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Buffer</td>
<td>Tris</td>
<td>Tris</td>
<td>HEPES</td>
<td>HEPES</td>
<td>HEPES</td>
<td>HEPES</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
<td>7.25-7.4</td>
<td>7.4</td>
<td>7.4</td>
<td>7.4</td>
<td>7.4</td>
</tr>
</tbody>
</table>

1.4 Modified Applications of CDHA

It has been addressed above that CDHA modified implant surface can rapidly accommodate protein adsorption. This affinity makes biomimetic coating a possible approach to deposit drug/protein loaded CDHA coatings onto implant surfaces to promote bone regeneration. For example, Labberzadeh et al. soaked poly(lactide-co-glycolide) (PLGA) scaffold into SBF to deposit a CDHA layer. The
protein adsorption and release studies showed that while the protein release pattern was similar for PLGA and mineralized PLGA scaffolds, precipitation of the CDHA layer on PLGA led to enhanced protein adsorption and slower protein release [32]. Yao et al. loaded penicillin-based antibiotics to anodized nanotubular titanium via a co-precipitation drug with CDHA method, in which drug molecules were mixed in SBF to collectively precipitate with CDHA crystals [33]. Results showed that such co-precipitated coatings on anodized nanotubular titanium could release drug molecules for up to 3 weeks. Whereas, previous studies have demonstrated only a 150-minute release of antibiotics through simple physical adsorption. In Chapter 2, this SBF based drug co-precipitation method was applied to load a bisphosphonate drug alendronate sodium (AS) to Ti6Al4V alloy surface. The most important objective of promoting osseointegration at the alloy surface will be achieved by CDHA and the treatment of osteoporosis will be aided by alendronate sodium. The work compared different loading methods of AS with CDHA formation and the resulted crystallite change of CDHA caused by the binding of AS to CDHA precursors. Also, the biocompatibility of prepared AS-CDHA coatings was evaluated.

One the other hand, one disadvantage of biomimetic coating is its long deposition time, taking approximately 21 days to form uniform CDHA coatings on implant surfaces. It was reported that increased ionic strength of SBF can accelerate CDHA coating formation rate. Many groups use 1.5x SBF to produce uniform CDHA coatings as an alternative to 1x SBF [29]. In some situations, the ionic concentrations can further be intensified to 5x or 10x for specific rapid coating deposition [34, 35]. For example, our lab has reported a SBF with 10 times the calcium and phosphate ionic strength of body
fluid with the ability to deposit uniform CDHA coating in less than 6 hours [34]. In contrast, such intensified SBF solution can be used to prepare CDHA microspheres at suitable environmental conditions [36]. In Chapter 3, a novel environment was applied to such concentrated SBF solutions through microwave irradiation. In this environment, highly active amorphous calcium phosphate (ACP) nanospheres were prepared. This is a precursor to CDHA and a powerful tool in the mechanism study of CDHA/bone apatite formation. To sum up, the mechanism and impacting factors of ACP nanospheres formation were studied.

In recent years, fabrication of inorganic-organic composites mimicking the composite nature of real bone has attracted research interest in the field of bone regeneration. Materials used for such bone-mimicking composites are usually biopolymers and inorganic ceramics. CaP is the most investigated inorganic ceramic in such composites combined with various biopolymers. In Chapter 4, we reviewed the incorporation of CaP ceramics and polylactic acid (PLA) and its co-polymer PLGA, which are the only synthetic and biodegradable polymers with an extensive FDA approval history [37]. During review, it was observed the incorporation of CDHA particles to PLA is rare. Therefore, in Chapter 5 and Chapter 6, CDHA particles prepared from SBF were incorporated with PLA via electrospinning and electrospraying.

Freshly prepared CDHA materials from SBF are highly active material for reaction because they are usually amorphous. Therefore, they are favorable precursors for specific elements, doping to reveal distinct properties. In Chapter 7, Y doped CDHA microspheres and YPO₄ microspheres were prepared using a CDHA template with the preservation of the microsphere structure. Y doped CDHA microspheres can be potential materials for
bone regeneration because literature reports show promoted cytocompatibility for osteoblasts adhesion as compared to undoped CaP materials [38, 39]. YPO₄ microspheres are a potential material for internal radiation therapy, in which ⁸⁹Y can be activated to Yttrium-90 (⁹⁰Y) by neutron bombardment for irradiation before implantation [40]. In Chapter 8, Eu doped ACP materials were also prepared for biological fluorescent labeling.

1.5 Summary

Chapters 2-8 are written such that they each may be viewed as an independent work with a different focusing area. Different applications of CDHA prepared from SBF were studied, ranging from a drug carrier, mechanism study, composite filler, and sacrificing template. A variety of studies including CDHA formation, material characterization, degradation testing, \textit{in vitro} cell culture etc. were applied for the evaluation of prepared materials for biomedical applications. All these interesting properties all contribute to excellent activity of CDHA.
Chapter 2

Biomimetic Coating of Bisphosphonate Incorporated CDHA on Ti6Al4V

2.1 Abstract

Bi-functional coatings of carbonated calcium deficient hydroxyapatite (CDHA) on Ti alloys were developed by using a biomimetic coating process. The bi-functionality was achieved by loading alendronate sodium (AS), an approved bisphosphonate (Bp) drug used for the treatment of osteoporosis, into the inner layers of CDHA coatings. Three possible methods of loading AS into CDHA coatings were systematically studied and compared. The results indicated that the co-precipitation method had greater benefits and can modify the release profile of AS by incorporating AS in the inner layers of the coatings. As a preliminary study, the influences of applied AS dosage to CDHA coatings were evaluated using XRD and SEM. In vitro tests indicated that the AS content on CDHA coatings played a significant role, and optimum AS content in local area is beneficial for osteoblast cells proliferation. It is expected that the CDHA-AS coatings via the co-precipitation approach have potential for bone tissue engineering applications.

2.2 Introduction

The prime motivation behind this study is to develop bi-functional coatings of carbonated calcium deficient hydroxyapatite (CDHA) on titanium alloys. The goal is to
combine the CDHA with a drug belonging to the bisphosphonate (Bp) family and achieve the bi-functionality. The most important objective of promoting osseointegration at the metal surface will be achieved by CDHA and the treatment of osteoporosis will be aided by alendronate sodium (AS) an approved drug in the Bp family. The idea is to deliver the drug locally, at an optimal level of dosage using a benign low-temperature biomimetic process.

Calcium phosphate (CaP) coatings play an important role in the success of osteointegration of metallic implants for bone tissue regeneration [41, 42]. In addition, CaP coatings can potentially work as drug-carrier system to further promote bone healing [43, 44]. However, the conventional high-temperature coating processes such as plasma spraying [41] not only lower the bioactivity of CaP materials, but also limit the possibility to incorporate bioactive agents into these coatings. Furthermore, a high temperature process removes the carbonates from the HA structure. Hence, the biomimetic coating process is a viable alternative.

Biomimetic coating technique is based on the use of simulated body fluid (SBF), with ion concentrations, temperature, and pH similar to physiological conditions. SBF has the ability to induce deposition of bone-like CDHA coatings on the surface of Ti and its alloys when suitably treated [21, 24, 45]. The chemical composition of as-deposited CDHA is relatively close to the mineral component of bone, which is poorly crystalline hydroxyapatite (HA) with small amounts of sodium (Na⁺), potassium (K⁺), magnesium (Mg²⁺), chloride (Cl⁻), and carbonate (CO₃²⁻) present in the lattice structure [5, 18, 46]. It is biocompatible, and bioactive, as well as biodegradable. The extra elements in small quantities were reported to influence cell adhesion, proliferation of osteoblasts and help
in bone metabolism [46].

For more than three decades, bisphosphonates (Bps) have been used as primary agents for the treatment of bone related diseases such as osteoporosis [47]. All Bp drugs share the same main backbone (P-C-P) structure. This structure provides the high affinity of Bp to CaP crystals, in which the phosphoryl groups are bound simultaneously to the same calcium atom with the formation of a bidentate or possibly tridentate chelate [48]. Such high affinity, combined with its inhibitory effects on osteoclasts, makes Bp an important bioactive agent for the treatment of osteoporosis. In addition, unique side chains of Bp in R1 and R2 positions determine different chemical properties: the hydroxyl group at R1 position increases the affinity of Bp to CaP crystals, while the ability to fight against osteoclast cells is related to chemical moiety in R2 position [49]. Various Bps with different side chains are available in the market including olpadronate, alendronate, zoledronate and pamidronate [47, 49].

The systemic delivery of Bps, by methods such as oral administration, was reported to have limitations such as low absorption in target tissue, and side effects such as gastric injury and oral ulcers [49-51]. In recent years, local delivery of Bp to the target area of bone has become popular. In the early stage of implantation during bone replacement when significant bone loss can occur, Bp can provide a stable and integrated interface between the implant and the bone [52]. For example, Tengvall et al. reported a 28% increase in the pull-out force and 90% increase in pull-out energy of Bp-loaded implants in rats as opposed to those without Bp [53]. Peter et al. examined zoledronate-loaded HA-coated Ti implants, reporting excellent results in terms of bone growth around the implants, especially when the zoledronate content is optimum (between 0.2 and 2.0 µg
Because of its reported effects on bone healing, it may be beneficial to incorporate Bp into CDHA-coated metallic implants for bone replacement applications.

In this work, we evaluate three possible methods of loading Bp into CDHA coatings and compare them systematically. These methods include: I) classical Bp aqueous solution contact with as-deposited CDHA coatings [54-56]; II) co-precipitation of Bp-CDHA coatings; III) alternative layer by layer coatings of CDHA and Bp by several cycles. The effects of coating method on the morphology of CDHA coatings were investigated and the loading and release profile of Bp were also assessed. The best loading method was then selected and different Bp concentrations were used to evaluate the effects of Bp content on CDHA coatings formation. As a preliminary study, the optimum Bp dosage to be incorporated into CDHA coatings was determined using *in vitro* tests on osteoblast cells.

### 2.3 Experiments

#### 2.3.1 Sample pre-treatment

A Ti6Al4V plate (McMaster-Carr, CA) was cut into small strips with dimensions of 5mm ×5mm ×1mm, which were used as substrates for CDHA coating. The strips were polished by using #1000 silicon carbide (SiC) paper (Struers, Denmark) and cleaned using distilled water. A set of four strips were immersed in 50mL of 5M sodium hydroxide (NaOH) solution at 60°C for 24 hours for surface etching, and then transferred in 50mL of distilled water for 24 hours at 37°C in order to remove extra NaOH on the sample surface. NaOH was purchased from Fisher Scientific. The NaOH solution was prepared by dissolving NaOH into DI water with stirring. The purity of NaOH is listed as
99.8%. Subsequently, these strips were dried at 37°C for 24 hours.

### 2.3.2 SBF preparation

The SBF used in this work was 1.5x t-SBF, which is a Tris (C₄H₁₁NO₆) buffered SBF solution developed by our group [29, 30]. The composition of t-SBF closely mimics the composition of human blood plasma. The ionic concentrations of t-SBF solution were intensified 1.5 times to accelerate CDHA coating formation. The composition is shown in Table 2.1. The reagents sodium bicarbonate (NaHCO₃) and sodium sulfate (Na₂SO₄) were purchased from Acros Organics (Pittsburgh, PA). Sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na₂HPO₄), magnesium chloride (MgCl₂.6H₂O), hydrochloric acid (HCl), calcium chloride (CaCl₂.2H₂O), and Tris-Base buffer were purchased from Fisher Scientific (Pittsburgh, PA). The purity of all chemicals is listed as over 99.5%. The reagents were measured with 0.002g of error. All reagents were dissolved in 700 ml of DI water one by one in the order listed in Table 2.1 and extra DI water was added to make the final solution volume 1L using a 1L cylinder with 5ml error. The SBF solution was stored at 4°C, when not in use.

**Table 2.1. Compositions of 1L 1.5x t-SBF**

<table>
<thead>
<tr>
<th>Order</th>
<th>Reagent</th>
<th>1.5x t-SBF (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaCl</td>
<td>9.8184</td>
</tr>
<tr>
<td>2</td>
<td>NaHCO₃</td>
<td>3.4023</td>
</tr>
<tr>
<td>3</td>
<td>KCl</td>
<td>0.5591</td>
</tr>
<tr>
<td>4</td>
<td>Na₂HPO₄</td>
<td>0.2129</td>
</tr>
<tr>
<td>5</td>
<td>MgCl₂.6H₂O</td>
<td>0.4574</td>
</tr>
<tr>
<td>6</td>
<td>1M HCL</td>
<td>15 mL</td>
</tr>
<tr>
<td>7</td>
<td>CaCl₂.2H₂O</td>
<td>0.5822</td>
</tr>
<tr>
<td>8</td>
<td>Na₂SO₄</td>
<td>0.1080</td>
</tr>
<tr>
<td>9</td>
<td>Tris-Base</td>
<td>9.0945</td>
</tr>
<tr>
<td>10</td>
<td>1M HCL</td>
<td>50 mL</td>
</tr>
</tbody>
</table>
2.3.3 Drug loading

The FDA-approved aldenaronate sodium (AS) was purchased from Fisher Scientific (NJ). Three different methods were used to load AS into CDHA coatings on Ti6Al4V via incubating strips into solution with different components (4 strips in each 50ml solution). In method I, the pretreated Ti6Al4V substrates were soaked vertically at 37˚C in 50 mL of 1.5× t-SBF solution in tightly sealed 100ml Pyrex glass bottles (Corning Life Sciences, MA) for 15 days to obtain the CDHA coatings. The 1.5x t-SBF solution was changed every 48 hours. The CDHA coated strips were then separated into two groups (I-1 and I-2). In group I-1, the strips were soaked in 50mL of AS solution with a concentration of $1 \times 10^{-4}$ mol/L for 6 days, with solution changed every 48 hours. In group I-2, the strips were soaked for another 5 days in 1.5x t-SBF and then immersed in AS solution with a concentration of $3 \times 10^{-4}$ mol/L for 24 hours. In method II, same protocol as method I was used with AS solution replaced by AS-1.5x t-SBF. Again, the coated strips were separated into two groups (II-1 and II-2). In group II-1, the strips were soaked in AS-1.5x t-SBF with $1 \times 10^{-4}$ mol/L AS for 6 days. In group II-2, the strips were soaked in AS-1.5x t-SBF with $1 \times 10^{-4}$ mol/L AS for 24 hours. In method III, the pretreated Ti6Al4V strips were coated with 1.5 x t-SBF for 24 hours followed by AS solution for the next 24 hours. The layer by layer coating process was continued by alternating between 1.5x t-SBF and AS solution for a total of 21 days. The total amount of AS used for each method was the same. After 21 days of CDHA coating and AS incorporation using different methods, all strips were dried in an oven at 37˚C for further analysis.

2.3.4 Analysis

The morphology of CDHA-AS coatings formed using different methods was
examined using a Scanning Electron Microscope (SEM, S4800, Hitachi). A reverse phase high performance liquid chromatography (HPLC) protocol utilizing pre-column derivatization of the primary amine group of AS with 9-fluorenylmethyl chloroformate (FMOC, C_{15}H_{11}ClO_{2}) was used to obtain the AS release profile [57]. Chemicals for HPLC analysis: sodium citrate (Na_3C_6H_5O_7), sodium phosphate dibasic anhydrous (Na_2HPO_4), acetonitrile (CH_3CN), methanol (CH_3OH), dichloromethane (CH_2Cl_2), orthophosphoric acid (H_3PO_4), sodium tetraborate decahydrate (Na_2B_4O_7·10H_2O), and FMOC were purchased from Fisher Scientific (Pittsburgh, PA). Na_3C_6H_5O_7, Na_2HPO_4, and Na_2B_4O_7·10H_2O have purity above 99.0% and all liquids used are HPLC grade. 0.1 M sodium citrate solution, 0.1 M sodium borate solution and FMOC solution (0.1 %, w/v) was prepared (FMOC was dissolved in acetonitrile). Phosphate buffer solution (PBS) was prepared for AS release, the composition of PBS is outlined in Table 2.2. NaCl, KCl, Na_2HPO_4 and potassium phosphate monobasic (KH_2PO_4) were purchased from Fisher Scientific with purity over 99.5%. The AS-treated strips were soaked in well plates with 100 μL phosphate buffer solution (PBS), which was collected and changed every 8 hours. The collected solution was stored in a refrigerator at 5˚C for further analysis. Some of the strips were treated with HCl in order to dissolve CDHA for further assessment of total AS loading. The chromatographic analyses were performed using the HPLC system (Agilent Technologies HPLC 1100) with a reverse phase (PRP-1) column (10 μm particle size, 250 x 4.1 mm, Hamilton, NV). The mobile phase was prepared by mixing methanol, acetonitrile and a buffer containing 0.05 M sodium citrate and 0.05 M sodium phosphate dibasic anhydrous (pH 8, adjusted using orthophosphoric acid). The volume ratio of methanol, acetonitrile, and buffer was 5:20:75. A flow rate of 0.5 ml/min was used in the
HPLC at room temperature. FMOC derivative was detected using a UV detector operated at a wavelength of 266 nm.

Table 2.2. Compositions of 1L PBS

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>1.44</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.24</td>
</tr>
</tbody>
</table>

2.3.5 Preliminary evaluation of CDHA and cell proliferation

Ti6Al4V strips were coated with CDHA-AS using the optimum drug loading method based on the results obtained from prior experiments. Different AS concentrations were used to investigate the effects of resulting AS contents on CDHA coatings. AS concentrations of $0, n \times 10^{-6}$, $n \times 10^{-5}$ and $n \times 10^{-4}$ mol/L were used, and $n$ was 1 or 3 depending on the application procedure. The CDHA coatings on Ti6Al4V strips, formed at different AS concentrations were characterized by X-Ray Diffraction (XRD, Ultima III, Rigaku). For all samples, data was collected at 2θ angles ranging from 10˚ to 45˚ at a scan speed of 1˚ per minute. The XRD was operated at 40 kV and 44 mA with a monochromated Cu-Kα radiation source. The morphology of CDHA coatings formed at different AS concentrations was examined using the SEM. The optimum AS dosage was assessed by *in vitro* tests using 7F2 mouse osteoblast cells (CRL-12557, American Type Culture Collection, Manassas, VA). First the cells were cultured at 37°C and 5% CO$_2$ in alpha minimum essential medium ($\alpha$-MEM, Thermo Scientific HyClone), augmented by 10% Fetal Bovine Serum (FBS, Thermo Scientific HyClone). The culture medium was changed every other day until the cells reached a confluence of ~90%. A 48 well plate (Corning® cell culture plates with CellBIND® surface) was used for *in vitro* testing.
Approximately 10,000 cells in 500 μL culture medium were seeded to each well containing the sterilized coated strip. The numbers of cells on the strips and surrounding strips in well were counted after 24 hours using cytotox 96® non-radioactive cytotoxicity assay kit (Promega, WI) and after 6 days using a hemocytometer. For statistics, all cell culture experiments were performed in triplicates. The morphology of the osteoblast cells on the surface of the strips was examined using SEM after sample preparation by alcohol dehydration, critical point drying and sputter coating using gold.

2.4 Results

2.4.1 Morphology of CDHA-AS coatings using different methods

The NaOH-etched Ti6Al4V showed a porous surface morphology as seen in Fig. 2-1a. Both method I (Fig. 2-1b) and method II (Fig. 2-1c) produced uniform CDHA-AS coatings on the surface of Ti6Al4V. These coatings were in the form of a dense layer with growth of CDHA globules on top, similar to previous work [29]. However, method III produced very thin and loose CDHA layers which were more likely the debris rather than coatings (Fig. 2-1d). Hence, it was evident that method III cannot produce uniform CDHA-AS coatings on Ti6Al4V.
Fig. 2. SEM images of a) NaOH etched Ti6Al4V surface; b) AS-CDHA coatings produced using method I; c) AS-CDHA coatings produced using method II; d) AS-CDHA coatings produced using method III

2.4.2 AS release profile

The average AS content/area of each group are shown in Fig. 2-2. The AS release profiles of group I and group II are shown in Fig. 2-3. A quick release of AS in group I-2 in PBS was observed in the first 24 hours, while the release of AS in group I-1 was more stable and continuous. 80% of AS in group II-2 was released in the first 16 hours as compared to a more sustained AS release profile in group II-1. On the other hand, group II-1 also had a more stable and continuous AS release profile as compared group I, even after 96 hours.
2.4.3 Preliminary evaluation of CDHA and cell proliferation

Based on AS release profiles, method II-1 was selected as the preferred method and used for the preparation of CDHA-AS coatings with different AS contents. The samples prepared using method II-1 with different AS contents will then be used to evaluate the influence of AS content on CDHA microstructure and cell proliferation.

XRD patterns of CDHA coatings formed in 1.5x t-SBF solutions with different
amounts of AS (AS concentrations of 0, 10^{-6}, 10^{-5} and 10^{-4} mol/L) are shown in Fig. 2-4. There are two main features observed in the XRD patterns. First, the prominent XRD peaks at 20 angle 26° and 32° belonging to CDHA were observed on all strips (JCPDS-ICDD card no 18-03030). The deposition of CDHA on Ti6Al4V strips in presence of AS in 1.5x t-SBF is therefore confirmed. It was observed that the intensity of Ti peaks is much higher in strips formed in 1.5x t-SBF solution with 10^{-4} mol/L AS as compared to strips in conventional 1.5x t-SBF. Second, there is a broadening of CDHA peak as a function of AS content, as shown in Fig. 2-4.

Fig. 2-4 XRD patterns of samples soaked in 1.5× t-SBF solution with different concentrations of AS (0, 10^{-6}, 10^{-5} and 10^{-4}M); “A” represents CDHA peaks, and “□” represents Ti6Al4V peaks

Fig. 2-5(a) through Fig. 2-5(c) show the SEM images from the surfaces of CDHA coatings deposited using 1.5x t-SBF solutions with different amounts of dissolved AS (AS concentrations of 0, 10^{-6}, 10^{-5} and 10^{-4} mol/L) at different magnifications. Three main features were observed in the SEM micrographs. First, in Fig. 2-5(a) all strips were found to form an under-coat layer first, followed by growth of CDHA globules on it. With the
decrease of AS concentrations in 1.5x t-SBF, CDHA globules number per area decreased but their size increased. Second, micro-cracks were observed on CDHA coatings precipitated from 1.5x t-SBF solutions with AS concentration of $10^{-5}$ and $10^{-4}$ mol/L. At higher magnification, the size of the micro-cracks on CDHA coatings formed in 1.5x t-SBF solution with AS concentration of $10^{-4}$ mol/L was observed to be much larger than ones from 1.5x t-SBF solution with AS concentration of $10^{-5}$ mol/L (Fig. 2-5(b)). Third, CDHA nano-crystals as shown in Fig. 2-5(c), transformed from worm-like feature to needle-like feature as a result of increase in AS concentration.

Fig. 2-5(a) SEM images of the surfaces of CDHA coatings deposited from 1.5x t-SBF solutions with different concentrations of AS: (a) 0, (b) $10^{-6}$, (c) $10^{-5}$, and (d) $10^{-4}$ mol/L, magnification is 1 K
Fig. 2-5(b). SEM images of the surfaces of CDHA coatings deposited from 1.5x t-SBF solutions with different concentrations of AS: (a) 0, (b) $10^{-6}$, (c) $10^{-5}$, and (d) $10^{-4}$ mol/L, magnification is 5 K.

Fig. 2-5(c). SEM images of the surfaces of CDHA coatings deposited from 1.5x t-SBF solutions with different concentrations of AS: (a) 0, (b) $10^{-6}$, (c) $10^{-5}$, and (d) $10^{-4}$ mol/L, magnification is 100 K.

The *in vitro* tests in Fig. 2-6(a) indicate that the AS content on CDHA coatings influenced osteoblast cells proliferation. After 6 days of cell culture, it was observed that
the strips with coatings formed in $10^{-6}$ mol/L AS-1.5x t-SBF had the highest number of osteoblast cells ($p<0.05$), and cell number decreased when AS content on CDHA coating increased. In addition, the numbers of osteoblasts surrounding strips in wells after 24 hrs and 6 days were shown in Fig. 2-6(b), which also indicted the adverse effect of high content released AS to osteoblasts in the environment ($p<0.05$). Osteoblast cell morphologies after 6 days on strips with different AS content were similar as shown in Fig. 2-7. The only difference is the number of filipodia (as an indicator for osteoblast cells proliferation) on coatings formed in 1.5x t-SBF with $10^{-4}$ mol/L AS was much lower than other strips.

![Fig. 2-6(a). Osteoblast cell numbers on CDHA coatings deposited from 1.5x t-SBF solutions with different concentrations of AS: (1) 0, (2) $10^{-6}$, (3) $10^{-5}$, and (4) $10^{-4}$ mol/L after 24 hours and 6 days](image-url)
Fig. 2-6(b). Osteoblast cell numbers surrounding strips with CDHA coatings deposited from 1.5× t-SBF solutions with different concentrations of AS: (1) 0, (2) $10^{-6}$, (3) $10^{-5}$, and (4) $10^{-4}$ mol/L after 24 hours and 6 days.

Fig. 2-7. SEM images of osteoblast cells on the CDHA coatings deposited from 1.5× t-SBF solutions with different concentrations of AS after 6 days: (a) $10^{-4}$, and (b) $10^{-5}$ mol/L.

2.5 Discussion

It is well known that a hydrated layer of sodium titanate and titanium oxide, and features of nano-scale roughness can form on the exposed surfaces of Ti6Al4V substrates.
after 24 hours of NaOH etching at 60°C [58, 59]. In this work, such phenomenon was also observed as shown in Fig. 2-1a. The chemically formed sodium titanate can be transformed into Ti-OH via ion exchange of its $\text{Na}^+$ ions with $\text{H}_3\text{O}^+$ ions in the surrounding aqueous environment, and these formed Ti-OH groups can in turn react with $\text{Ca}^{2+}$ ions present in solution to form amorphous calcium titanate, which were the sites for CDHA deposition [60]. In method I and method II, a dense layer of CDHA was successfully deposited before AS incorporation. However, in method III, the situation was totally different: the CDHA deposition was obstructed when AS was incorporated in the initial step of CDHA formation. It was reported that Bp can form strong bonding to potential CaP crystal growth sites in order to block further accumulation of reactant ions from solution [61, 62]. In method III, amorphous calcium titanate was formed in the first 24 hours of 1.5x t-SBF immersion. The following AS-aqueous solution treatment introduced AS to these amorphous calcium titanate as a protective layer to avoid further CDHA growth on Ti6Al4V surface. Such protective layer is not 100% inhibitory to CDHA growth because AS were randomly adsorbed on these amorphous calcium titanate sites and AS can be released back to aqueous environment. However, in method III in turns soaking Ti6Al4V in 1.5x t-SBF and AS aqueous solution intensified such inhibitory effect of AS, resulting in poor CDHA coating formation on Ti6Al4V surface. On the other hand, adsorbed AS cannot destroy formed CDHA coatings, and thus both method I and method II can produce uniform CDHA-AS coatings.

CDHA is a potential carrier for AS administration. Jesse et al. studied the delivery of zoledronate from Biphasic Calcium Phosphate (BCP, consisting of different ratio of HA and beta-tricalcium phosphate) and Calcium Deficient Hydroxyapatite (CDA,
Zoledronate reportedly formed a calcium complex in the crystalline form on BCP materials while getting absorbed on CDA surface via polar covalent interactions with calcium atoms. It was indicated that CDA caused sustained release of zoledronate over a period of time as compared to fast release of zoledronate from BCP in biological evaluation [64]. The chemical composition of CDHA \( \text{Ca}_{10-x-y}[\text{(HPO}_4)_x\text{(CO}_3)_y\text{(PO}_4)_{6-x-y}]\text{(OH)}_{2-x} \) is similar to CDA with extra carbonate elements presented in the lattice structure [29, 65], making CDHA more reactive in physiological environment. Oliveira et al. produced CDHA coatings with Bp on starch-based degradable biomaterials via immersing CDHA coated material into Bp containing solution, and reported in a buffered solution with pH at 7.4 around 30% of the drug released in the first 24 hours [56]. In addition, the inhibitory effect of AS to CDHA dissolution possibly prolonged the release of AS from coatings [66, 67]. For the stabilization of the implant, it is preferred that sustained and stable release of Bp takes place from the implant. Local high Bp concentration caused by a quick release mechanism can result in cytotoxic effects to bone cells [68, 69].

In the AS release profile study, method I and method II were compared. The conceptual base of method I and method II are different: method I is based on the adsorption of AS to CDHA coatings surface and method II is focusing on incorporating AS into inner layers of CDHA coatings together with CDHA precursors suspended in solution. The results demonstrated that once AS successfully incorporated into inner layers of CDHA coatings via co-precipitation (group II-1), a sustained and stable release profile of AS can be obtained (Fig. 2-3). In method I, the AS release profile difference between group I-1 and group I-2 was possibly caused by the prolonged incorporation
time in group I-1, enabling AS to slowly diffuse into the inner-layers of CDHA. On the other hand, the loading content difference between group I-1 and group I-2 (Fig. 2-2) was possibly attributed to two factors: 1) higher concentration of AS intensified adsorption of AS to formed CDHA coatings; 2) bonded AS released back to aqueous environment, proportional to the time of contact with aqueous environment. In method II, AS was initially bonded to CaP precursors suspended in 1.5x t-SBF. This caused two phenomena: 1) AS inhibited the CaP precursors to bond to formed CDHA coatings, and thus decreasing loading content of AS, especially when AS concentration in the solution was high; 2) when AS inhibitory effects was lower (low AS concentration), layers of CHDA-AS can be slowly built on pre-formed CDHA coatings, which can thus produce sustained and stable release of AS. Therefore, method II-1 was considered as the best AS incorporation process among these tested methods.

The intensity of peaks of Ti6Al4V in XRD analysis was inversely proportional to the thickness of deposited CDHA coatings. The increase of Ti peaks intensity as a function of AS concentration in 1.5x t-SBF following method II-1 indicated the presence of AS in SBF delayed the growth of CDHA coatings on Ti6Al4V strips. This phenomenon matched the inhibitory effect of AS to CDHA crystals growth. The micro-cracks formation related to AS content shown in Fig. 2-5(a) and Fig. 2-5(b) can also be attributed to the inhibition of CDHA bulk growth caused by AS. In CDHA deposition, CDHA precursors were deposited to random regions on the surface of formed CDHA coatings, which further worked as nucleation sites and expand CDHA coatings to overlap the gaps among different regions and finally to cover the whole surface area to form uniform coating layers. The loading of AS to these CDHA precursors or formed CDHA
coatings, isolated local area into separated islands and inhibited growth of new CDHA to overlap gaps. In addition, AS suspended in aqueous solution can accumulate in the gaps to further avoid CDHA formation. The increase in CDHA globule numbers per area and the decrease of CDHA globules size as a function of AS content indicated the fact that AS adsorption inhibited nucleation of CDHA crystals.

The phenomenon that CDHA nano-crystals transformed from worm-like feature to needle-like feature as a result of AS concentration increase shown in Fig. 2-5(c) was caused by the incorporation of AS to CaP crystal structure making rearrangements to further crystal growth. Octacalcium phosphate (OCP) is considered as an important intermediate material in the formation of CDHA in SBF. It was argued by some groups that instead of the role of calcium titanate in CDHA nucleation, CDHA deposition can also take place via OCP and then transforms into CDHA via hydrolysis and partial dissolution of OCP [70, 71]. Once AS incorporated into the OCP structure via bonding to Ca\(^{2+}\) ions, it will interrupt the partial dissolution of OCP and the in situ reorganization of the lattice ions in the conversion of OCP to CDHA. Such interruptions were directly reflected in the particle features. In addition, the phenomenon of CDHA peak broadening as a function of AS content observed in XRD data can also be attributed to the incorporation of AS to OCP/CDHA lattice in biomimetic coating process. The adsorption of AS modified the crystal lattice growth and resulted in the transformation of crystals from worm-like to needle-like features, making the crystal structure more amorphous. Such XRD peak broadening was reported before by Boanini et al. in HA synthesis together with alendronate by physical absorption [72, 73]. Also, based on our observations in the poor coatings formed via method III and this CDHA nano-crystals
feature transformation caused by AS incorporation, it is suggested both calcium titanate and OCP play important roles in CDHA coating formation.

Kashii et al. reported the administration of alendronate to rats caused microstructure of bone matrix to become less anisotropic, leading to deterioration of bone material properties [74]. Because bone matrix anisotropy and its material property are strongly influenced by mineral orientation, it was supposed that alendronate obstructed the right mineral orientation formation. The AS incorporation process via method II-1 can be considered as a \textit{in vitro} model to mimic the process of bone matrix formation incorporated with AS \textit{in vivo}. The results also indicated that the newly formed CDHA structure was obstructed by the adsorption of high content of AS.

The \textit{in vitro} testing results (Fig. 2-6) indicated that low AS content in local area is beneficial to osteoblast cells proliferation, while high AS content can induce adverse effects. The SEM images (Fig. 2-7) also support this comment. It has been reported that micromole level concentration of AS in medium is toxic to osteoblast proliferation [32, 33]. This corroborates with the cell numbers variation related to AS concentration observed in our experiments. The round morphology of cells on strips using 10$^{-4}$ mol/L AS indicated this incorporation concentration can trigger toxic effect to osteoblast cells.

2.6 Conclusion

Three possible methods of loading AS to CDHA coatings was systematically studied and compared. Co-precipitation of CDHA and AS can modify the release prolife of AS by incorporating AS in the inner layers of CDHA coatings. The effects of AS on the CDHA coatings formation were strongly influenced by the applied dosage. \textit{In vitro} testing indicated low AS content in local area is beneficial to osteoblast cells proliferation. This
preliminary study showed the possibility and benefits to produce CDHA-AS coatings via co-precipitation process for bone tissue engineering applications.
Chapter 3

Novel Microwave Synthesis of ACP nanospheres

3.1 Abstract

Amorphous calcium phosphate (ACP) is an important precursor phase in tissue mineralization. It shows high solubility and excellent remineralization ability. Commercially viable techniques for producing ACP are high cost/low efficiency processes. This chapter describes a novel microwave assisted ACP synthesis route as an alternative to current ACP synthesis methods. An important feature of the process is the use of supersaturated biomimetic fluids (SBFs), which are based on Kokubo-like simulated body fluids. However, our present compositions are substantially different in that they no longer simulate the body fluid compositions. The effects of solution composition and processing parameters were studied. The mechanism of ACP synthesis under microwave irradiation process is also discussed. The as-synthesized ACP nanospheres were characterized and showed good reactivity and biocompatibility. These as-synthesized nanoparticles can be potential candidates for biomedical applications and remineralization mechanism study.

3.2 Introduction

Calcium phosphate (CaP) materials are known to be bone substitute materials for
bone tissue engineering applications [5, 18]. Of the CaP family members, amorphous calcium phosphate (ACP) has attracted increasing interests due to its high solubility and excellent remineralization ability, as compared to other CaP materials [75, 76]. It has been widely regarded as the metastable precursor phase for the subsequent formation of CaP in biological organisms, playing a critical role in the process of tissue mineralization [75-78]. It has been incorporated into various biomaterials in the forms of coatings, cements, ceramics or composites, showing good performances both in vitro and in vivo [75].

ACP can be generally synthesized via two routes: either using a low temperature or a high energy processing/high temperature route [75]. The low temperature route of ACP synthesis is based on the double decomposition of a calcium and phosphate salts in aqueous medium, water-alcohol or alcoholic medium [75, 79-82]. The high energy processing/high temperature route involves rapid quenching of molten CaPs or physical deposition techniques [75, 83-85]. However, the high energy processing/high temperature route has the inability to control the composition of synthesized ACP materials and impurities such as contaminated elements or other crystalline phases may also be introduced [75]. Therefore, the low temperature route is commonly preferred for the control of composition and phase characteristics of the as-synthesized ACP. The main difficulty encountered in the wet route preparation of ACP is related to its instability and reactivity in solution. Usually, alkaline pH environment [75], presence of inorganic species such as Mg$^{2+}$, P$_2$O$_7^{4-}$, CO$_3^{2-}$, Zr$^{4+}$, Si$^{4+}$ [86-88], organic molecules such as poly(ethylene glycol), poly(propylene fumarate) [76, 79, 82], or alcohol [80, 81] are used to stabilize ACP.
In this chapter we present the results of a novel microwave assisted “spontaneous precipitation” technique to synthesize spherical-ACP nanoparticles. There are several interesting aspects of this chapter. First, this technique uses supersaturated biomimetic fluids (SBF) as the precursor solution for ACP precipitation. SBF is usually referred to as simulated body fluids, as developed by Kokubo et al., with ion concentrations, temperature, and pH almost similar to physiological conditions [21]. SBF is commonly applied to induce deposition of bone-like apatite coatings on surface of material substrates with bioactivity [21, 24, 27, 29]. It is important to note here that we have carefully avoided the use of the word “simulated”. The rationale here is that most conventional SBF compositions are, in fact, supersaturated. By avoiding the restriction of “simulated composition”, the SBF composition can be further modified in terms of enhancing the ionic strength/adding other ions. Our definition broadens the utility of using SBFs to a great extent. Indeed, the ionic concentration strength of SBF can be increased to accelerate the coating deposition kinetics [34, 36, 89]. In the literature, these supersaturated SBFs with increased ionic concentration of Ca$^{2+}$ and PO$_4^{3-}$/HPO$_4^{2-}$ proportional to human body fluids, usually are described as “n x SBF” or “SBF x n”. However, the concentrations of other ions such as Mg$^{2+}$ and HCO$_3^{2-}$ were also intentionally adjusted. Therefore, it is not factual to simply describe all these supersaturated solution as “n x SBF” or “SBF x n”. Second, the application of microwave irradiation along with the assistance of Mg$^{2+}$ ions to SBFs resulted in mass precipitation of spherical ACP nanoparticles. To the best of our knowledge, such result was not reported before. It is expected that such ACP nanoparticles can have important applications in tissue engineering applications. Third, as-synthesized ACP nanospheres
can be a potential tool for the mechanism study of mineralization. Finally, this is a simple, fast, cheap, and scalable process for the synthesis of highly reactive ACP nanoparticles.

3.3 Materials and Methods

Modified SBF compositions were prepared based on the SBF composition, as described by Hofmann et al. [21]. Mg\(^{2+}\) and HCO\(_3^\text{-}\) concentrations in SBF were reduced to intensify CaP nucleation and growth. Aqueous solutions containing dissolved KCl, NaCl, NaHCO\(_3\), MgCl\(_2\).2H\(_2\)O, Na\(_2\)SO\(_4\), CaCl\(_2\).2H\(_2\)O, KH\(_2\)PO\(_4\) (Fisher Scientific, NJ, USA) were prepared in 1 L DI water in 3 L-capacity Pyrex beakers, as shown in Table 3.1. The reagents were dissolved in solution one by one, and added based on the order listed in Table 3.1. For each composition case, transparent solutions were obtained, stable at room temperature. The standard SBF was named as SBF1 with Ca\(^{2+}\) and PO\(_4^{3-}\)/HPO\(_4^{2-}\) concentrations similar to body fluids. If the total ionic concentration of SBF was intensified n times, the solution was referred to SBFn. If in SBFn single ion such as Mg\(^{2+}\) and HCO\(_3^\text{-}\) was additionally adjusted to m times of its concentration in SBF1 for a specific purpose, the solution was defined as SBFn-Xm, X representing the adjusted ion. The pH value of each solution was measured using pH meter (Mettler Toledo). Effects of total concentration and specific ions (Mg\(^{2+}\) and HCO\(_3^\text{-}\)), influence of microwave heating time, and comparisons to conventional SBF precipitation kinetics (incubated in 37 °C water bath or placed on 100 °C hot plate) were all studied.

150 ml sample beakers with 100 ml as-prepared solution were then placed onto 10 × 10 × 1 cm alumina insulating fiberboards and covered with an upside down 250 ml-capacity glass beaker. To proceed with microwave-assisted process, the sample assembles were placed into a household microwave (MW) oven (Emerson, max. power
100 W, 2450 MHZ, NJ, USA) for 5 min. Sample SBF2 was additionally heated for 1 min and 9 min separately for the study of the influence of heating time. The MW oven was operated at its maximum power. At the end of microwave heating, the samples were moved to a cold water bath to cool down. Finally, the precipitates were collected after centrifuge at 3000 rpm for 5 min. All precipitates were completely dried in 37 °C oven overnight. In order to examine the instability of as-synthesized ACP nanospheres, 0.05 g powders were soaked to 50 ml 2.5 mM CaCl₂ aqueous solution for 48 hours at 37 °C, followed by characterization.

All the precipitates were characterized by x-ray diffraction (XRD, X'Pert Pro MPD, PANalytical) with monochromated Cu Kα radiation operated at voltage 40 kV and current 44 mA setting. Fourier transform infrared spectroscopy (FTIR, UMA-600 Microscope, Varian Excalibur Series) was applied for chemical bands analysis, recorded with 256 scans with resolution of 1 cm⁻¹ between 4000 and 700 cm⁻¹. The morphological features of precipitates were visualized using scanning electron microscope (SEM, S4800, Hitachi). Energy dispersive x-ray spectroscopy (EDS) in SEM was used to analyze the elements molar ratio in precipitates. Inner structure and conversion of ACP precipitates was investigated via transmission electron microscopy (TEM, HD-2300, Hitachi) with a voltage 200 kV. The surface charges of nanopspheres were measured using zeta potential/particle sizer (380 ZLS, Nicomp).

7F2 mouse osteoblast cells (CRL-12557, American Type Culture Collection) were used for cell culture studies on precipitates from SBF2 and SBF2-Mg24, hydroxyapatite (HA) and dicalcium phosphate dihydrate (DCPD) were used as control groups. Osteoblast cells were first grown at 37°C and 5% CO₂ in alpha minimum essential
medium (α-MEM, Thermo Scientific HyClone), augmented by 10% Fetal Bovine Serum (FBS, Thermo Scientific HyClone). Harvested Osteoblasts were seeded to wells (BD Flacon™ 12 wells cell culture plates) of sterilized pellets (SBF2, SBF2 24x Mg, HA and DCPD) separately, approximately 5000 osteoblast cells were seeded to each well. Cell numbers on pellets were counted after 24 hours, 7 days using CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega). All experiments were performed triplicate for one-way ANOVA statistical analysis purpose. Osteoblast morphology on samples was examined using SEM.

Table 3.1 Sample preparation for microwave ACP analysis with different study purposes: 1) influence of total ionic concentration; 2) influence of Mg$^{2+}$ concentration; 3) comparison of the impacts of Mg$^{2+}$ concentration and total ionic concentration; 4) effect of HCO$_3$; 5) influence of microwave heating time; 6) comparison of microwave assisted precipitation and conventional precipitation

<table>
<thead>
<tr>
<th>g/L</th>
<th>KCl</th>
<th>NaCl</th>
<th>NaHCO$_3$</th>
<th>MgCl$_2$2H$_2$O</th>
<th>Na$_2$SO$_4$</th>
<th>CaCl$_2$2H$_2$O</th>
<th>KH$_2$PO$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1SBF1</td>
<td>0.28384</td>
<td>6.9966</td>
<td>0.2268</td>
<td>0.04063</td>
<td>0.0284</td>
<td>0.36755</td>
<td>0.13602</td>
</tr>
<tr>
<td>1,2,5,6 SBF2</td>
<td>0.56768</td>
<td>13.9932</td>
<td>0.4536</td>
<td>0.08125</td>
<td>0.0568</td>
<td>0.7351</td>
<td>0.27218</td>
</tr>
<tr>
<td>1SBF4</td>
<td>1.13536</td>
<td>27.9864</td>
<td>0.9072</td>
<td>0.1625</td>
<td>0.1136</td>
<td>1.4702</td>
<td>0.54436</td>
</tr>
<tr>
<td>1SBF8</td>
<td>2.2707</td>
<td>55.9728</td>
<td>1.8144</td>
<td>0.325</td>
<td>0.2272</td>
<td>2.9404</td>
<td>1.08872</td>
</tr>
<tr>
<td>1SBF1-Mg4</td>
<td>0.28384</td>
<td>6.9966</td>
<td>0.2268</td>
<td>0.16063</td>
<td>0.0284</td>
<td>0.36755</td>
<td>0.13602</td>
</tr>
<tr>
<td>2,3 SBF2-Mg8</td>
<td>0.56768</td>
<td>13.9932</td>
<td>0.4536</td>
<td>0.325</td>
<td>0.0568</td>
<td>0.7351</td>
<td>0.27218</td>
</tr>
<tr>
<td>2SBF2-Mg16</td>
<td>0.56768</td>
<td>13.9932</td>
<td>0.4536</td>
<td>0.65</td>
<td>0.0568</td>
<td>0.7351</td>
<td>0.27218</td>
</tr>
<tr>
<td>2SBF2-Mg24</td>
<td>0.56768</td>
<td>13.9932</td>
<td>0.4536</td>
<td>0.975</td>
<td>0.0568</td>
<td>0.7351</td>
<td>0.27218</td>
</tr>
<tr>
<td>4SBF2-HCO$_3$</td>
<td>0.56768</td>
<td>13.9932</td>
<td>0</td>
<td>0.08125</td>
<td>0.0568</td>
<td>0.7351</td>
<td>0.27218</td>
</tr>
<tr>
<td>3,5,6 SBF4-Mg16</td>
<td>1.13536</td>
<td>27.9864</td>
<td>0.9072</td>
<td>0.65</td>
<td>0.1136</td>
<td>1.4702</td>
<td>0.54436</td>
</tr>
</tbody>
</table>
3.4 Results

In composition SBF2-HCO$_3$O, no precipitations took place after 5 min microwave heating because the solution was too acidic (pH=4.8). Other solutions with the same weight ratio between NaHCO$_3$ and KH$_2$PO$_4$ all showed a similar pH value (pH=6.76±0.04) and resulted in precipitates after microwave heating. In composition SBF2, longer microwave heating time resulted in more evaporation of the solution, approximately 95 ml after 1 min, 60 ml left after 5 min heating and 15 ml left after 9 min heating. No precipitation took place even after 48 hours sample SBF2 incubated in 37 °C water bath. Sample SBF4-Mg16 however could produce precipitates after 48 hours water bath incubation. Heating samples (SBF2 and SBF4-Mg16) using 100 °C hot plate took more than 10 min to make the solution cloudy, indicating the occurrence of precipitation. Above results indicated that pH, ionic concentrations and temperature all served as the driving forces for precipitation. Additionally, zeta potentials of all precipitates were close to zero.

The XRD results of prepared particles are shown in Fig. 3-1. It was observed precipitates from conventional concentrated SBF (SBF2, SBF4 & SBF8) all have similar XRD patterns, similar to bone-like apatites formed in SBF solutions as reported before [21, 36]. However, increasing the Mg$^{2+}$ concentration caused the crystals to be more amorphous (SBF2-Mg8 and SBF2-Mg24). It is interesting to notice a sharp and intense peak near 32 degree was observed in SBF2-Mg24. This peak becomes weak but is still detectable in SBF2-Mg8. Crystallization of these amorphous materials after incubating in CaCl$_2$ aqueous solution was also observed (SBF2-Mg24-CaCl$_2$), indicating the as-synthesized materials are precursors for apatite with high reactivity.
Fig. 3-1 XRD data of precipitates from different SBF compositions (SBF2, SBF4, SBF8, SBF2-Mg8, and SBF2-Mg24) after 5 min microwave heating and related product after CaCl₂ aqueous solution incubation (SBF2-Mg24-CaCl₂).

The FTIR data of precipitates are shown in Fig. 3-2. Precipitates from conventional SBFs (SBF2, SBF4 & SBF8) all showed carbonate bands, while increasing the Mg²⁺ prevented the incorporation of carbonate ions to the precipitate lattice structures. On the other hand, the absorbance spectra of the phosphate region (900-1200 cm⁻¹) of precipitates formed in SBF2-Mg24 solution exhibited a broad, relatively featureless contour. Decreasing the Mg²⁺ concentration and increasing of total ionic concentration, the spectra of phosphate region exhibited the presence of several visible peaks and shoulders (SBF2-Mg8, SBF2, SBF4 & SBF8). This phenomenon indicated the incorporation of Mg²⁺ can make synthesized materials more amorphous [90]. Incubation
of precipitates from SBF2-Mg24 in CaCl2 aqueous solution sharpened the spectra in the phosphate region (900-1200 cm⁻¹), indicating a conversion from ACP to apatite.

![FTIR spectra](image)

Fig. 3-2 FTIR data of precipitates from different SBF compositions (SBF2, SBF4, SBF8, SBF2-Mg8, and SBF2-Mg24) after 5 min microwave heating and related product after CaCl2 aqueous solution incubation (SBF2-Mg24-CaCl2)

Fig. 3-3 shows the results of SEM characterization. ACP nanospheres can be produced from SBF1 and SBF2 using 5 min microwave heating (Fig. 3-3a & 3b). But precipitates from SBF4 and SBF8 were clusters composed of needle-like nanocrystals (Fig. 3-3c & 3d). The clusters shown in Fig. 3-3d are organized microspheres as compared to the clusters with random shape shown in Fig. 3-3c. However, once the Mg²⁺ concentration of SBF was intensified, nanospheres were produced instead of clusters (Fig. 3-3e). Fig. 3-3f and Fig. 3-3g show the increase of Mg²⁺ concentration can decrease
precipitated nanospheres diameters. Precipitates from SBF4-Mg16 using 48 h water bath are shown in Fig. 3-3h. They are agglomerated microspheres composed of plate-like crystals instead of clusters composed of needle-like nanocrystals found in 5 min microwave heating. The precipitates after 100 °C hot plate heating are shown in Fig. 3-3i & 3j. The precipitates from SBF2 were randomly distributed nanocrystals but the precipitates from SBF4-Mg16 were microspheres with plate-like substructure. Fig. 3-3k & 3l show the conversion of nanospheres to clusters after incubation in CaCl$_2$ aqueous solution, indicating incorporation of Mg$^{2+}$ to ACP can delay its conversion to apatite.

The EDS analysis results of these prepared precipitates are shown in Fig. 3-4. In Fig. 3-4a, the Ca/P and Mg/P molar ratio of precipitates from different SBF concentrations were compared. With the increase strength of SBF, the Ca/P ratio increased from values close to 1 to values close to 1.67, but the Mg/P ratio decreased. In Fig. 3-4b the effects of increased Mg$^{2+}$ concentration in SBF2 based solution are shown, indicating the decrease of Ca/P ratio to 1 and the increase of Mg/P ratio. The comparisons of the impacts of Mg$^{2+}$ concentration and total ionic concentration are shown in Fig. 3-4c, describing the fact that with increasing ionic concentrations, Ca/P increased and Mg/P decreased.

In the TEM data in Fig. 3-5a, the structure of the as-prepared ACP nanosphere from SBF2 was observed to be composed of an inner dense core with a shell. The shell has the ability to further induce needle-like CaP crystals deposition to the inner core. In addition, the conversion from ACP nanospheres to bonded CaP clusters composed of needled-like crystals with the shell moving outwards if the microwaved solution was not immediately cooled down (Fig. 3-5b & 5c).
Fig. 3-3 SEM images of (a) precipitates from SBF1; (b) precipitates from SBF2; (c) precipitates from SBF4; (d) precipitates from SBF8; (e) precipitates from SBF4-Mg16; (f) precipitates from SBF2-Mg8; (g) precipitates from SBF2-Mg24; (h) precipitates from SBF4-Mg16 after 48 hours 37 °C water bath; (i) precipitates from SBF2 after 10 min heating on 100 °C hot plate; (j) precipitates from SBF4-Mg16 after 10 min heating on 100 °C hot plate; (k) precipitates from SBF2 after incubation in CaCl$_2$ aqueous solution; (l) precipitates from SBF2-Mg24 after incubation in CaCl$_2$ aqueous solution.
Fig. 3-4 Ca/P and Mg/P molar ratio comparisons of (a) precipitates from different SBF concentrations (SBF1, SBF2, SBF4, and SBF8); (b) precipitates from SBF2 with different Mg$^{2+}$ concentration intensification (0x, 8x, 16x, 24x Mg$^{2+}$); (c) precipitates from SBF1-Mg4, SBF2-Mg8, SBF4-Mg16

Fig. 3-5 TEM images of (a) & (b) nanospheres and (c) related clusters after transition

The osteoblast cell numbers after 7 days on samples HA, SBF2 and SBF2-Mg24 showed no significant difference (one-way ANOVA analysis, P>0.05), indicating obtained ACP precursors are biocompatible (Fig. 3-6). On the other hand, the features of nanospheres from SBF2 and SBF2-Mg24 after 1d and 7d incubation in culture medium
showed significant differences. For example, ACP nanospheres from SBF2 started conversion after 1d and converted to apatite coating layer with plate-like substructure in some area after 7 days incubation (Fig. 3-7a & 7b); ACP nanospheres from SBF2-Mg24 were stable after 7 days, but random deposition of apatite precursors to material surface formed a new apatite layer with high porosity in some surface area (Fig. 3-7c & 7d).

Fig. 3-6 Results of osteoblast cell culture: (a) cell numbers after 1 and 7 days; (b) osteoblast cells on sample SBF2 after 7 days; (c) osteoblast cells on sample SBF2-Mg24 after 7 days
Fig. 3-7 SEM of pressed nanospheres after culture medium incubation: (a) sample SBF2 after 1d; (b) sample SBF2 after 7d; (c) sample SBF2-Mg24 after 1d; (d) sample SBF2-Mg24 after 7d

3.5 Discussion

The presence of ACP precursors in concentrated SBF in the initial precipitation stage has been reported by many groups [91-93]. The ACP precursors in the solution form apatite nuclei either on the surface of immersed bioactive substrate or in the SBF solution. Those nucleated on the substrate can further grow into a bone-like apatite coating layer, while those nucleated in the solution form precipitates in the form of agglomerated microspheres or irregular clusters with plate-like substructure varied with temperature and SBF compositions [34, 36, 94]. In our case, the precipitates from 37 °C water bath or 100 °C heating matched the results of previous reports, indicating without any specific
assistance, it is hard to obtain ACP materials from SBF due to the high instability of ACP.

In contrast, ACP nanospheres were successfully produced via microwave heating from solution SBF2 and SBF4-Mg16. It was a clear evidence that the microwave heating induced specific mechanism to nucleation (nanospheres structure instead of clusters) and conversion (stabilized structure) of ACP crystals in aqueous solution. The peak near 32 degree in XRD characterization observed in SBF2-Mg24 and SBF2-Mg8 is considered as a symbol of the interphase transition between highly active amorphous CaP and maturated CaP, reflecting crystallite level of obtained CaP nanospheres. This peak was also observed in our previous experiment in depositing apatite to PLA and PLA/calcium deficient hydroxyapatite (CDHA) electrospun fibers using SBF [95].

From the TEM image (Fig. 3-5a), a specific light layer was observed as the shell of the ACP nanospheres. There are two hypothesis of the fact of this layer. First, it is known that binding of free Ca\(^{2+}\) and PO\(_4^{3-}\)/HPO\(_4^{2-}\) largely depends on the affinity of their positive and negative charges. In SBF this layer easily attracts more free ions for nucleation after the end of microwave schedule as shown in Fig. 3-5c. Therefore, it is hypothesized that during microwave heating, some specific positive charge barrier formed on the shell to inhibit the continuous binding between PO\(_4^{3-}\)/HPO\(_4^{2-}\) and Ca\(^{2+}\), making the layer a highly active sites full of PO\(_4^{3-}\)/HPO\(_4^{2-}\) or Ca\(^{2+}\) for CaP growth. But such barrier should disappear once microwave was turned off to continue CaP growth. If this assumption is true, the zeta potential of ACP nanospheres should not be neutral because this active shell was exposed to the environment. However, the zeta potential measurements showed the precipitates produced were almost neutral instead of highly charged. Another hypothesis is this specific light layer is highly active ACP with low
crystallinity, while stabilized CaP is usually dark in TEM. The application of microwave energy to SBF cannot interrupt the initial nucleation of CaP, but drive Ca$^{2+}$ and PO$_4^{3-}$/HPO$_4^{2-}$ to form the most favored structure under microwave irradiation. Once the applied microwave energy is turned off, as-synthesized ACP nanospheres especially the parts of light shell perform like other ACP precursors in SBF solution to convert into apatite clusters. One proof of this hypothesis is the precipitates formed in SBF4 and SBF8 were observed to be clusters instead of ACP nanospheres. On the other hand, precipitates from SBF1 and SBF2 were ACP nanospheres because CaP nucleation activity is related to the total ionic concentration [34, 94]. Additionally, increased Mg$^{2+}$ concentration also stabilized the formed ACP nanospheres (SBF4-Mg16). It is well known that the presence of Mg$^{2+}$ ions in ACP structure and solution can inhibit further apatite crystal growth to formed spherical-ACP [86, 87]. This phenomenon indicated nanospherical form is the most favored structure under microwave irradiation. The question is why this precipitation process with microwave irradiation favored the nanospherical structure of ACP and inhibited the conversion of ACP to matured CaP in heating stage when the strength of SBF kept on increasing due to water evaporation. First, we believe spherical structure is favored in CaP nucleation and growth, especially in a stable environment (low pH, with stabilizer etc.), shown both in our results (Fig. 3-3d, h &j) and previous reports [36, 93]. As a result, the ACP in nanospherical (and not other irregular forms) nucleated initially during CaP precipitation in SBF with low pH. Once these nanospheres appeared, they boosted the kinetics of CaP precipitation. Second, the conversion of ACP to apatite or the growth of needle-like crystals to nanospheres was interrupted in this microwave heating process, with excessive Ca$^{2+}$ and PO$_4^{3-}$/HPO$_4^{2-}$ kept
on forming ACP nanospheres instead. Lerner et al. [96] argued from a thermodynamic stability perspective that in basic aqueous solution containing Ca$^{2+}$ and PO$_4^{3-}$ with microwave irradiation, ACP is the first phase to precipitate as compared to octacalcium phosphate (OCP) and HA. The explanation is that ions in water need activation energy to overcome energy barrier to release their hydration water molecules in order to get close to the ions already integrated on the formed crystal surface (formed ACP precursors), while the formation of a solid (ACP precursors) containing the hydration sphere, like the ions have in solution, requires much less energy barrier than the anhydrous products OCP and HA [96, 97]. In our system, energy applied via microwave irradiation and appearance of ACP precursors triggered an irreversible CaP precipitation process. Excessive ions in solution have two possible routes to precipitate: 1) to attach to the as-synthesized ACP nanospheres surface to convert ACP to other CaP materials; 2) to continue to form ACP nanospheres. On considering the energy barrier, formation of more ACP nanospheres is energy favored. These are the reasons why the final products in our microwave technique are all ACP precursor nanospheres.

The role of HCO$_3^-$ in SBF is to control the pH and to be incorporated to formed CaP lattice structure, as discussed elsewhere [29, 89]. In our results, removing HCO$_3^-$ from SBF lowered pH, resulting in no precipitation even the solution was boiling with microwave heating. This indicated the CaP nanospheres were not stable at low pH environment similar to other CaP materials. The presence of carbonate in FTIR data of precipitates (Fig. 3-2) showed the incorporation of HCO$_3^-$ to the lattice structure. However, it is surprising to find the absence of carbonate bands in the FTIR spectra of ACP nanospheres with intensified Mg$^{2+}$ ions, contrary to previous precipitation studies
One possible reason is in the high temperature environment and in the highly amorphous crystal structure caused by incorporation of Mg\(^{2+}\), carbonate is not stable in lattice structure.

Further, it was observed that added Mg\(^{2+}\) ions also showed the tendency to make Ca/P ratio close to 1 in ACP nanospheres (Fig. 3-4) and decrease the obtained nanospheres size (Fig. 3-3b, f & g). It has been reported that though Mg\(^{2+}\) can disturb the crystal growth process of ACP to HA or OCP, it has no detectable effects on the formation of DCPD with Ca/P ratio 1 [98, 99]. However, in highly supersaturated solution, such inhibitory effect caused by increased Mg\(^{2+}\) concentration can be overcome with the preservation of the nanospherical structure (SBF4-Mg16). The phenomenon that Mg\(^{2+}\) concentration in solution can decrease as-synthesized nanospheres size can be ascribed to the fact that though the spherical entities of ACP can be formed very fast, and Mg\(^{2+}\) appears not to stop this growth process, its CaP formation inhibitory effect still limited the size of formed nanospheres to some extent.

The mechanism of ACP-HA conversion is still hotly debated [100, 101]. One theory is the dissolution and recrystallization mechanism [100], assuming that the transformation from ACP to HA involves three basic steps: (1) the dissolution and hydration of ions from ACP; (2) the transfer of these hydrated ions; and (3) the nucleation and subsequent growth of HA. However, in our TEM results single nanoneedles nucleated to spherical-ACP shell and ACP core were stable during conversion, opposite to this assumption. Tao et al. evaluated the conversion of ACP to HA using gold nanoparticles as probes [101]. In their report they revealed ACP – HA conversion is an aggregation process and Kirkendall effect coupled with an interfacial reaction: 1)
spontaneous aggregation of nanospheres; 2) the HA nanoneedles nucleated at the ACP/solution interface and extended outward radially, while the internal ACP phase provided material flow flux for the phase conversion to HA. Finally the ACP core is eliminated as the HA ball grew, leaving a hollow core with a size similar to that of the former ACP core. Our TEM results matched this mechanism description. In addition, the SEM image of fractured nanospheres caused by applying some stress showed a hollow core (Fig. 3-7c). Therefore, our results partially support this ACP-HA conversion theory and our microwave-assisted ACP products can be a potential powerful tool for the study of mineralization.

In the in vitro osteoblast culture testing, sample 2x SBF and 2x SBF 24x Mg showed the similar biocompatibility as HA, indicating they are potential materials for tissue engineering applications. However, there were differences in features after 1d and 7d incubation in culture medium. For composition SBF2, without the increased Mg\(^{2+}\) incorporation, it acted as a conventional ACP to convert to HA, and finally dense maturated apatite coatings with plate-like substructures were obtained in some areas. It was also indicated in that ionic concentration of culture medium, as the calcium in the solution was consumed by ACP precursors in pellets, new spherical-ACP precursors cannot be formed and deposited to pellet surface. However, for sample SBF2-Mg24 spherical-ACP precursors were observed to randomly deposit to pellet surface after 24 hours. In addition, these precursors agglomerated and kept stable even after 7 days in culture medium to form a coating layer with porosity, functioned like biomimetic coating process via SBF. The only possible reason for this phenomenon is the presence of Mg\(^{2+}\) in the ACP nanospheres. Barrere et al. reported the Mg\(^{2+}\) is essential for CaP coating
formation on Ti6Al4V substrate surface. In the experiment, the high attached Mg$^{2+}$ in the vicinity of Ti6Al4V initiated CaP coating formation and inhibited CaP growth favoring tiny CaP globules on Ti6Al4V substrates [35]. Therefore, it is assumed that during incubation the incorporated Mg$^{2+}$ inhibited the ACP-HA conversion, but promoted deposition of spherical ACP precursors. In highly concentrated SBF solution like the one used in report of Barrere et al., these ACP precursors can be converted into HA coating composed of tiny globules after a short time [35]. In culture medium without such high ionic concentration, these freshly deposited spherical ACP precursors preserved the spherical-structure instead. Agglomeration and conversion to some extent can still be observed after 7 days, because these newly formed ACP precursors are highly unstable and reactive. In this 7 day cell culture, the continuing conversion of synthesized ACP possibly influenced osteoblast cells proliferation. Therefore long-term in vitro and in vivo experiments will be necessary in future better understanding the biological performance of microwave-synthesized ACP.

3.6 Conclusion

In conclusion, this study shows a novel microwave-assisted “spontaneous precipitation from supersaturated biomimetic fluids (SBFs)” process to synthesize ACP in the form of nanospheres. We demonstrated the influences of different factors and the mechanism of ACP formation in this microwave route: 1) pH and ionic concentrations served as important factors in ACP precipitation; 2) Mg$^{2+}$ stabilized the formed ACP nanospheres by inhibiting apatite crystal growth; 3) formation of new ACP nanospheres instead of conversion of formed ACP to OCP and HA is energy favored under microwave irradiation. The reactivity and biocompatibility of synthesized ACP were also evaluated.
The results show the potential of this technique in the mass production of ACP with efficiency and low cost. The as-synthesized ACP precursors can be used alone or combined with various biomaterials for tissue engineering applications.
Chapter 4

Fabrication Aspects of PLA-CaP/PLGA-CaP Composites for Orthopedic Applications: A Review

4.1 Abstract

For several decades, composites made of polylactic acid-calcium phosphates (PLA-CaP) and polylactic acid-co-glycolic acid-calcium phosphates (PLGA-CaP) have seen widespread uses in orthopedic applications. This chapter reviews the fabrication aspects of these composites, following the ubiquitous materials science issue of “processing-structure-property” correlations. Various fabrication processes such as microencapsulation, phase separation, electrospinning, supercritical gas foaming etc. are reviewed with specific examples of their applications in fabricating these composites. The effect of the incorporation of CaP materials on the mechanical and biological performance of PLA/PLGA is addressed. Also, this chapter describes the state of art on challenges and innovations concerning CaP dispersion, incorporation of bio-molecules/stem cells and long-term degradation of the composites.

4.2 Introduction

Bone, a critical component of the skeletal system in human body works as the site for load bearing and mineral metabolism. It is a dynamic system with physiological remodeling, removal, and replacement capabilities throughout life [102]. Due to this
self-healing property, small defects can be easily treated by bone itself. However, in the case of extensive bone defects such as bone fracture, this self-healing mechanism for the reconstruction of large bone segments is not feasible. Nowadays, autografts, allografts, and xenografts are commonly used for the treatment of bone fracture. Even though these bone grafts are reported to achieve good clinical results under certain conditions, their applications are limited due to infection, disease transfer, non-unions, rejection, lack of dependable graft supplies, and high harvesting cost [2-4]. In recent years, synthetic grafts have been developed as substitutions of bone grafts, which are primarily used for treatment of bone fracture [5, 6]. They are readily available and can be processed into different shapes for specific orthopedic applications with excellent mechanical and biological performance with no risk of infection and disease transfer [4, 37, 103-107].

Natural bone is a complex composite mainly constituting of biological apatite and molecules of collagen. In this system, collagen serves as the matrix for cell growth and tissue repair while apatite serves as the inorganic phase to improve mechanical strength and regeneration of bone [18]. The higher tensile strength and fracture toughness of bone are attributed to the tough and flexible collagen fibers reinforced by apatite crystals. In recent years, fabrication of inorganic-organic composites mimicking the composite nature of real bone has attracted research interest in the field of bone regeneration. In such composites, the inorganic additives with good compressive strength reinforce the elastic polymer phase to generate bioactive materials with improved mechanical performance and degradation profiles [37, 108].

Materials used for such bone-mimicking composites are usually biopolymers and inorganic ceramics. Biopolymers can be natural (collagen, hyaluronic acid etc.) or
synthetic (polyesters etc.). Natural biopolymers occur in nature and can be extracted; and synthetic biopolymers are industrially synthesized for biomedical applications. Even though natural biopolymers favor bone cell attachment and biochemical response in specific situations, there are concerns regarding antigenicity and immunogenicity, potential disease transmission, sourcing, poor mechanical properties, and lack of controlled biodegradability. As compared to natural biopolymers, synthetic biopolymers can be tailored to control their properties such as degradation rate and mechanical strength. They can be easily processed into composite scaffolds with desirable morphological features conducive to tissue in-growth [105, 109, 110]. Inorganic ceramics mainly include calcium phosphate (CaP) ceramics and bioactive glasses [111]. Most of them are bioactive and can induce strong bone bonding between implanted materials and bone tissues in order to withstand shear and distraction loads [111]. The family of CaP ceramics is the predominant bone substitute materials. The main driving force behind the use of CaP ceramics is their chemical similarity to the mineral component of hard tissue in the body [5, 18]. The complete list of important CaP materials has been well summarized by Dorozhkin et al. [6, 18]. Unfortunately, as for any ceramic material, CaP ceramics lack the necessary mechanical and elastic properties of the calcified tissues and exhibit high brittleness, and thus their mechanical performance after implantation is limited [37, 109]. Incorporation of CaP phase into biopolymer matrices such as polylactic acid (PLA) and its co-polymer poly (lactic acid-co-glycolic acid) (PLGA) can improve mechanical performance as compared to CaP only [37, 112, 113].

Investigations on composites composed of CaP phases and synthetic biopolymers for orthopedic applications appear to be a popular topic in biomaterials research. The efforts
include variation in composition, fabrication methods and clinical evaluation [37, 109, 111]. In this chapter, we focus and emphasize on the fabrication aspects of PLA-CaP and PLGA-CaP composites from a materials engineering perspective. From now on these two individual composites will be abbreviated as PLA-CaP/PLGA-CaP composites.

The motivation for this chapter comes from the fact that PLA-CaP/PLGA-CaP composites are a rather narrow but important focus area in biomedical engineering. It is noted that research on PLA-CaP/PLGA-CaP composites has been vigorous. A diverse range of fabrication techniques resulted in a wide range of desirable properties and microstructures. Yet, to the best of our knowledge, a compressive review on the fabrication aspects is truly limited. In preparing this chapter, the authors are guided by the ubiquitous materials engineering issue: “processing-structure-property” correlations. An emphasis is placed on how a fabrication process dictates the development of the microstructure, which in turn, results in enhancement in properties leading to potential applications. The chapter, therefore, includes all of the above topics: beginning with materials issues and ending with biomedical applications.

### 4.3 Material aspects of PLA-CaP/PLGA-CaP composites

Among the important synthetic biopolymers, polyesters play the most important role. Fully biodegradable synthetic polyesters such as PLA, polyglycolic acid (PGA), polycaprolactone (PCL), and polyhydroxybutyrate (PHB) are commercially available. Among these polyesters, studies on PLA and PGA are predominant (Table 4.1).

PLA (Fig. 4-1a) is a thermoplastic, biodegradable, biocompatible, synthetic polymer with high strength and modulus that can be made from annually renewable resources for use in either industrial packaging or medical device market [114]. It can be easily
processed into shapes such as screws, pins, and plates for orthopedic applications and fabricated into scaffolds for replacement and regeneration of tissues, or devices for controlled delivery of bio-molecules [1, 110, 115, 116]. Its stereochemical structure can easily be modified by polymerizing a controlled mixture of the L- or D-isomers (lactate) to yield high molecular-weight amorphous or crystalline polymers [114]. PLA has a slow degradation rate between 10 months and 4 years due to the hydrophobic methyl group in the backbone. It is degraded by simple hydrolysis of the ester bond and the rate depends on the molecular weight, isomer ratio, and crystallinity.

PGA (Fig. 4-1b) is a rigid thermoplastic material, with high crystallinity, modulus and strength. PGA can be synthesized via polymerization of glycolic acid. It has a faster degradation rate (6-12 weeks) as compared to PLA. Due to its high degree of crystallinity, it is insoluble in many common organic solvents, making the solution processing of PGA non trivial [117]. Therefore, many known solvent based composite fabrication techniques are not widely used to prepare PGA-CaP composite. As an alternative, PLGA (Fig. 4-1c), the co-polymer of PLA and PGA is used in order to combine the advantages of both polymers. It is synthesized by means of random ring-opening co-polymerization of monomers glycolic acid and lactic acid. The degradation rate of PLGA is much faster than PLA due to the component glycolic acid in the backbone and also the degradation rate can be adjusted by varying the amounts of the glycolic acid and lactic acid [118]. PLGA can be easily fabricated into different structures for bone implant applications [110, 117]. It is important to mention that PLA and PLGA are the only synthetic and biodegradable polymers with an extensive FDA approval history [37]. More detailed information on PLA, PLGA and PGA are available in the literature [114, 118-121].
Table 4.1. Search results in Science citation index expanded when using different key words

<table>
<thead>
<tr>
<th>Key words</th>
<th>Literature Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polylactic acid</td>
<td>13916</td>
</tr>
<tr>
<td>Polyglycolic acid</td>
<td>9500</td>
</tr>
<tr>
<td>Polycaprolactone</td>
<td>7977</td>
</tr>
<tr>
<td>Polyhydroxybutyrate</td>
<td>1360</td>
</tr>
<tr>
<td>Polylactic acid + bone</td>
<td>1652</td>
</tr>
<tr>
<td>Polyglycolic acid + bone</td>
<td>1469</td>
</tr>
<tr>
<td>Polycaprolactone + bone</td>
<td>717</td>
</tr>
<tr>
<td>Polyhydroxybutyrate + bone</td>
<td>95</td>
</tr>
</tbody>
</table>

(a) **Polylactic acid (PLA)**  
(b) **Polyglycolic acid (PGA)**

(c) **Poly (lactic acid-co-glycolic acid) (PLGA)**

Fig. 4-1. Structure of (a) PLA; (b) PGA; and (c) PLGA

An important drawback with these biopolymers is that degradation of PLA and PLGA generate acidic products that can lower of the local solution pH. The resultant
acidic medium accelerates further degradation in an autocatalytic manner, which triggers inflammatory and foreign body reactions \textit{in vivo} [122-124]. In addition, their hydrophobic surface property lowers cell adhesion and eventually their poor mechanical strength limits their applications in load bearing situations [125, 126]. These limitations can be overcome by incorporating additional inorganic fillers or coatings to their matrix.

The CaP materials on the contrary are bioactive and osteoinductive; some are even quite biodegradable. Even so, the major limitation to use CaP materials as bone implant is their brittleness. Their poor mechanical behavior is even more pronounced when they are fabricated into scaffolds with high porosity to mimic bone structure. Therefore, CaP materials are commonly used as fillers or coatings with biopolymers instead of their stand-alone use. The important data on various phases of CaP family used for making PLA-CaP/PLGA-CaP composites are outlined in Table 4.2. Among these members, hydroxyapatite (HA, Ca\textsubscript{10}(PO\textsubscript{4})\textsubscript{6}(OH)\textsubscript{2}) and tricalcium phosphate (TCP, Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{2}) are the most commonly used CaP ceramics in fabricating PLA-CaP/PLGA-CaP composites [37, 104, 105, 108].

HA is the principal inorganic constituent of hard tissues and is one of the most stable and least soluble of all CaP materials [6]. HA is usually synthesized via wet-chemical methods or semi solid-state reactions [6, 7]. Wet-chemical methods use aqueous solutions of compounds containing Ca\textsuperscript{2+} and PO\textsubscript{4}\textsuperscript{3-} ions with pH > 7, influenced by many factors including the concentrations of the starting salts, mixing sequence and rate, solution pH, reaction temperature, and holding time. Semi solid-state reactions refer to hydrothermal and hydrolysis of other calcium orthophosphates to synthesize HA. In addition, other techniques such as water-free synthesis, electro-chemical synthesis, and hydrothermal
treatment of coral were also developed for HA synthesis [6, 7, 128-130]. There are variations to HA synthesis process. One such process is the reduction of particle size from micron to nano-scale in order to provide high surface area to volume ratio to improve osteoconductivity and solubility. This reduction of particle size improves the dispersion of HA particles in the PLA or PLGA matrix, which eventually enhances the mechanical performance [131, 132]. Another variation calls for the preparation of calcium deficient hydroxyapatite (CDHA, \( \text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x} \) \((0 < x < 1)) instead of HA [6, 18]. The vacancy of ions in the lattice structure of CDHA makes the presence of other ions in the crystal structure. The extent depends on the counter-ions from the chemicals used for the preparation (such as \( \text{Na}^+, \text{Cl}^- \) etc.). Such type of ion-substituted CDHA (\( \text{Na}^+, \text{K}^+, \text{Mg}^{2+}, \text{Sr}^{2+} \) for \( \text{Ca}^{2+} \); \( \text{CO}_3^{2-} \) for \( \text{PO}_4^{3-} \) or \( \text{HPO}_4^{2-} \); \( \text{F}^-, \text{Cl}^-, \text{CO}_3^{2-} \) for \( \text{OH}^- \)) when combined with water forms biological apatite which is the main inorganic part of natural bone [46, 133]. This makes CDHA a very promising compound which can be used as bone substitutes. Simultaneous addition of calcium and orthophosphate containing solutions into boiling water followed by boiling the suspension for several hours, hydrolysis of precursors and biomimetic coating can be used to synthesize CDHA materials [6, 21, 27, 30, 134-136].

Instead of HA, another CaP material TCP is widely used in the medical field with higher dissolution property [18, 37]. TCP can be further classified as \( \alpha \)-TCP and \( \beta \)-TCP. \( \beta \)-TCP can be prepared via calcination of precursors (CDHA with Ca/P ratio =1.5) at temperatures above 800°C [6, 18]. In combination with HA, \( \beta \)-TCP forms biphasic calcium phosphate (BCP) that can also be used as bone substitution material and filler for composite fabrication [137, 138]. At temperatures above 1125 °C, BCP transforms into
α-TCP, which is more reactive in aqueous systems than β-TCP and can be hydrolyzed to HA [6, 18]. The solubility of TCP materials is much higher than HA. However, from a crystallographic point of view, HA is more similar to natural bone tissue apatite than TCP [139].

Table 4.2. Information on various CaP materials used to manufacture PLA-CaP/PLGA-CaP composites [18, 37, 127]

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ca/P ratio</th>
<th>Chemical formula</th>
<th>Solubility at 25°C, -log(Ks)</th>
<th>pH stability</th>
<th>Space group</th>
<th>Unit cell parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>1.67</td>
<td>Ca_{10}(PO_{4})<em>{6}(OH)</em>{2}</td>
<td>~116.8</td>
<td>9.5-12</td>
<td>Monoclinic</td>
<td>a = 9.84214(8), b = 2a, c = 6.8814(7)Å, γ = 120° (monoclinic)</td>
</tr>
<tr>
<td>CDHA</td>
<td>1.5-1.67</td>
<td>Ca_{10-x}(HPO_{4})<em>{x}(P</em>{2}O_{5})<em>{6-x}(OH)</em>{2-x} (0 &lt; x &lt; 1)</td>
<td>~85.1</td>
<td>6.5-9.5</td>
<td>Monoclinic</td>
<td>a = b = 9.4302(5), c = 6.8911(2)Å, γ = 120° (hexagonal)</td>
</tr>
<tr>
<td>α-TCP</td>
<td>1.5</td>
<td>α-Ca_{9}(PO_{4})_{2}</td>
<td>25.5</td>
<td>N/A</td>
<td>Monoclinic</td>
<td>a = 12.887(2), b = 27.280(4), c = 15.219(2)Å, β = 126.20(1)°</td>
</tr>
<tr>
<td>β-TCP</td>
<td>1.5</td>
<td>β-Ca_{9}(PO_{4})_{2}</td>
<td>28.9</td>
<td>N/A</td>
<td>Rhombohedral</td>
<td>a = b = 10.4183(5), c = 37.3464(23)Å, γ = 120°</td>
</tr>
<tr>
<td>DCPD</td>
<td>1.0</td>
<td>CaHPO_{4}·2H_{2}O</td>
<td>6.59</td>
<td>2.0</td>
<td>Monoclinic</td>
<td>a = 5.812(2), b = 15.180(3), c = 6.239(2)Å, β = 116.42(3)°</td>
</tr>
<tr>
<td>DCPA</td>
<td>1.0</td>
<td>CaHPO_{4}</td>
<td>6.9</td>
<td>N/A</td>
<td>Triclinic</td>
<td>a = 6.910(1), b = 6.627(2), c = 6.998(2)Å, α = 96.34(2)°, β = 103.82(2)°, γ = 88.33(2)°</td>
</tr>
<tr>
<td>ACP</td>
<td>1.0-2.2</td>
<td>Ca_{x}H_{y}(PO_{4})<em>{z}, H</em>{2}O, n = 3–4.5; 15–20% H</td>
<td>N/A</td>
<td>5-12</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Other CaP materials such as brushite (DCPD, CaHPO_{4}·2H_{2}O) and monetite (DCPA, CaHPO_{4}) also have the potential of being used to fabricate PLA-CaP/PLGA-CaP composites. However, relevant reports in this research area are rare [37, 140, 141]. One reason is the acidic property of HPO_{4}^{2-} in these materials, which in turn increases the local pH during the degradation process thus limiting their use in the PLA/PLGA matrix.

Amorphous calcium phosphate (ACP) is another potential CaP candidate for PLA-CaP/PLGA-CaP composites. ACP has attracted increasing interests due to its high solubility and excellent re-mineralization ability, as compared to other CaP materials. It
has been widely regarded as the metastable precursor phase for the subsequent formation of CaP in biological organisms, playing a critical role in the process of tissue mineralization. Generally, ACP can be synthesized via two routes: one using a low temperature method based on the double decomposition of a calcium and phosphate salts in aqueous medium, water-alcohol or alcoholic medium [75, 79-82] and the other using a high temperature method involving rapid quenching of molten CaPs [75, 83-85]. However, the high temperature route has the inability to control the composition of synthesized ACP materials and impurities such as contaminated elements or other crystalline phases [75]. Therefore, the low temperature route is commonly preferred for the control of composition and phase characteristics of the as-synthesized ACP.

4.4 Fabrication aspects of PLA-CaP/PLGA-CaP composites

Bone can be divided into cortical bone (5-13% porosity) and cancellous bone (30-90%) based on porosity of the structure [142]. Human cortical bone exhibits mechanical and physical properties such as compressive strength (130-180 MPa); flexural strength (135-190 MPa); tensile strength (50-151 MPa); and elastic modulus (12-18 GPa) with the ranges provided in the parenthesis. Cancellous bone, in comparison exhibits compressive strength (4-12 MPa), tensile strength (1-6 MPa) and elastic modulus (0.1-0.5 GPa) with the ranges provided in the paranthesis [142-150]. Careful consideration of the bone type and mechanical properties are needed for synthetic bone implants. For cortical bone applications, the right stiffness of the implant is needed in order to provide stability for load bearing but not resulting in strain shielding. On the other hand, for cancellous bone applications, the 3D structure is more important for aesthetic reasons [37].

There are a number of techniques available for the fabrication of composites
composed of biopolymers and CaP materials. The composites can be in the form of microspheres, film, disk, coatings or porous scaffold for cortical or cancellous bone applications. The details have been illustrated in different published reviews [37, 105, 108]. In this section, we only focus on the fabrication of PLA-CaP/PLGA-CaP composites and related structure porosity correlation (Fig 4-2). Fabrication techniques can be separated into two distinct groups for cortical or cancellous bone applications based on related product porosity. The various fabrication techniques are presented and discussed in the subsequent paragraphs.

Fig. 4-2. Fabrication techniques can be separated into two groups for cortical or cancellous bone applications based on related product porosity

4.4.1 Microencapsulation Process

The microencapsulation process in which the removal of the hydrophobic polymer solvent is achieved by evaporation, has been widely reported in recent years for the preparation of microspheres and microcapsules based on biodegradable polymers and copolymers of hydroxy acids [151]. It is widely applied to entrap agents such as drugs into polymer structure for long-term release. As an alternative to common drugs, CaP materials can also be incorporated into PLA/PLGA microspheres as cores or fillers to
form PLA-CaP/PLGA-CaP composites. Use of emulsion is the most widely used technique. The protocol calls for adding phase(s) containing CaP and PLA/PLGA to stabilize aqueous medium to form oil (o)/water (w) or w/o/w emulsion system with stirring to from microspheres [152-154]. Polyvinyl alcohol (PVA) is the common stabilizer use to control the size distribution of fabricated microspheres. As an alternative to conventional emulsion, new approaches were developed to produce PLA-CaP/PLGA-CaP microspheres. Nagata et al. developed surfactant-free emulsion solvent evaporation method by adding (NH$_4$)$_2$HPO$_4$ solutions to a mixture of Ca(CH$_3$COO)$_2$ and PLA [153, 155, 156]. In this study, the end groups COOH of PLA play an important role as nucleation sites for calcium phosphate precipitate at the oil/water interface and the resultant calcium phosphate plays a role to act as a stabilizer for microsphere fabrication [153, 155, 156]. In contrast, fabrication of HA coated PLA microspheres using nano-HA particles instead of precursors ((NH$_4$)$_2$HPO$_4$ and Ca(CH$_3$COO)$_2$) as particulate emulsifier was reported by Fujii et al. [157]. In their process, nano-HA was initially dispersed in water with pH 6.5 and then the HA dispersed aqueous solution was hand-shaken with PLA-CH$_2$Cl$_2$ solution. The HA-coated PLA microspheres were prepared by the evaporation of CH$_2$Cl$_2$ from the emulsion (Fig. 4-3). Xu et al. fabricated negatively charged PLGA microspheres using anionic surfactant sodium dodecyl sulfate (SDS), followed by depositing HA coating to microspheres via dual constant composition method [158]. The coating process is controllable and efficient enough (1~6 hours) to avoid potential PLGA degradation. As an alternative to conventional emulsion to produce microspheres, PLGA-coated BCP particles using solvent-non-solvent system were produced by Uskoković group [137, 138, 159-161].
their experiment, chemically synthesized granules or nanoparticles of BCP were added into completely dissolved PLGA. After mixing, methanol was added to the solution. After solvent evaporation with stirring, BCP particles were fully covered by PLGA.

These fabricated PLA-CaP/PLGA-CaP microparticles show necessary properties to work as fillers or delivery system for bone regeneration [48, 49, 71, 78, 79, 81, 82]. However, they cannot be directly used for load-bearing applications since it comprises of loose particles. This limitation can be overcome by incorporating these particles into a structure which has more integrity [83-87].

![Image](image.png)

Fig. 4-3. (a) SEM image of the HA nanoparticle-coated PLLA microspheres prepared after evaporation of CH$_2$Cl$_2$ from the CH$_2$Cl$_2$ solution of PLA-in-water emulsion. White arrows indicate free HA nanoparticles, (b) Magnified SEM image that shows the single microsphere. Inset shows the surface morphology of the microsphere, (c) TEM image of an ultrathin cross section of the microspheres, and (d) Magnified TEM image of the ultrathin cross section showing HA nanoparticles in coated PLLA microspheres that form the shell [157]
4.4.2 Solvent Casting

Solvent casting is a conventional technique to fabricate polymer film based on organic solvent evaporation, which can also be applied to prepare polymer based composite structure. In this process PLA/PLGA and CaP are mixed or self-assembled in solvent(s), and then cast into molds such as dishes, glass slides or plates after solvent evaporation with CaP randomly dispersed in the polymer matrix (Fig. 4-4) [168-172]. Furthermore, salt leaching can be combined with solvent casting to tailor pores on the surface. Kothapalli et al. reported the fabrication of PLA/HA composites with 90% porosity and interconnectivity using solvent casting/salt leaching method [173]. However, salt leaching assisted solvent casting can only be used to fabricate 2D or very thin 3D structures. Otherwise, salt particles may remain trapped in the polymer matrix without exposure to aqueous environment to create porosity. There are two concerns as regards to using solvent casting method to produce PLA-CaP/PLGA-CaP composites: one is the fact that this process uses toxic organic solvents; the second is during the solvent evaporation, CaP particles can spontaneously precipitate from the polymer solution due to poor affinity and can cause non-uniform dispersion of CaP in PLA/PLGA matrix.

Fig. 4-4. Schematic of solvent casting: PLA-CaP/PLGA-CaP is mixed and casted in molds, after solvent(s) evaporation, CaP particles are randomly dispersed in the polymer matrix
4.4.3 Phase Separation

Phase separation technique can applied to produce 3D porous scaffolds. This process, first reported in 1995, is based on the fact that once a thermal treatment is induced, a homogenous polymer solution becomes thermodynamically unstable and tends to separate into a multi-phase system including a polymer-rich phase and a polymer-lean phase to lower the free energy [169]. After removal of the solvent, the polymer-rich phase solidifies to form the structure while polymer-lean phase becomes pores [174]. CaP particles can be added to the polymer system, and dispersed in the matrix after solvent processing (Fig. 4-5). Nejati et al. fabricated PLA/HA matrix via phase separation using needle-like nano-sized HA particles as filler, the porosity of the scaffold was up to 85% with macropore diameter in the range of 64-175 μm (Fig. 4-6) [134]. Fabrication of PLGA/nano-HA composite scaffold via freeze-drying phase separation using different PLGA solution concentrations and HA contents was reported by Huang et al. [175]. In the report, high PLGA solution concentration decreased porosity of the fabricated scaffold because viscosity increase caused by high concentration of PLGA solution prevented phase separation; additionally, the pore structure became more and more irregular and some pores also closed with increasing HA content. Yang et al. fabricated porous PLGA/β-TCP scaffold using phase separation and porogen leaching [176]. It was observed that in the scaffold, the process of porogen leaching generated pores with 75-400 μm diameters. Also, pores with diameters less than 20 μm were produced by phase separation. Additionally, the toxic solvent chloroform was extracted by ethanol to reduce scaffold toxicity. A work done by Wang et al. illustrated the fabrication of nano-fibrous PLA/HA composites (100-750 nm) with interconnected microporous
structure (1-10 μm), high porosity up to 90% and homogeneous HA dispersion via phase separation in order to mimic the architecture of natural extracellular matrix (ECM) for tissue engineering application [177]. Similar to solvent casting method, residual toxic organic solvent is still a concern in this solvent-based technique.

![Diagram of phase separation](image)

**Solution preparation**  
**Solvent removal**

Fig. 4-5. Schematic of phase separation, the system is composed of thermal treatment for phase separation and solvent removal for phase solidification

![SEM micrographs](image)

Fig. 4-6. SEM micrographs of pure PLA and PLA/nano-HA scaffold fabricated via phase separation technique: (a,b) Pure PLLA and cross-section; (c,d) nHAP/PLLA and cross-section [134]
4.4.4 Electrospinning

Electrospinning is a simple and versatile technique capable of generating non-woven fibrous mats directly from a variety of polymers and composite materials [178]. The applications of electrospun fibers include filtration, textile manufacturing, catalysts and medical fields. The use of electrospun fibers in biomedical applications has been dramatically increased of late because electrospun fibers offer a range of attractive features such as large surface areas, high porosities, and ease of incorporation of functional components (drug, gene, enzyme etc.), making them ideal candidates for tissue engineering applications [178-180]. In the process of electrospinning, an electric force is applied to overcome surface tension forces of biopolymer solution loaded in capillary tube to generate a jet ejected from the tip of capillary tube and moves in the direction of the external electric field, elongates according to external and internal electrical forces, and finally gets deposited on a conductive substrate in the form of fibers (Fig. 4-7) [174, 175]. A number of processing parameters (applied voltage, flow rate, working distance, solution conductivity, solution viscosity, solvent(s) property etc.) can greatly influence the features of electrospun fibers such as surface porosity, fiber dimension etc. [179, 181]. The ability to co-spin PLA/PLGA with CaP additives offers the possibility to fabricate fibrous PLA/CaP or PLGA/CaP composite scaffolds via electrospinning with high surface area and interconnected channels suitable for tissue regeneration applications. For example, Jeong et al. fabricated PLA/HA nanofibrous scaffold via electrospinning, and reported the incorporation of HA can shrink the fiber size from 365±83 nm to 135±13 nm due to the interaction between HA and PLA and the impact of HA on the mixture viscosity [182]. PLA/CDHA electrospun fibers were prepared by Zhou et al. and also
reported the fiber size shrinkage related to CDHA incorporation [95]. Additionally, they also showed that the surface feature of the fibers can be adjusted from smooth to porous via controlling the solvent composition. In these composites fabricated via co-spinning, CaP particles are usually embedded along the polymer fiber matrix. As an alternative, Cui et al. fabricated PLA fibers with calcium nitrate entrapment and incubated fibers in phosphate solution to form CaP on the PLA matrix instead of co-spinning PLA with CaP to improve CaP dispersion uniformity in the PLA matrix [183]. In addition, Touny et al. recently introduced a novel reactive electrospinning process to fabricate PLA/monetite fibers [140]. In their process, PLA was initially mixed with H$_3$PO$_4$ in organic phase, Ca(OH)$_2$ powders were well dispersed in another organic phase and then slowly poured into PLA-H$_3$PO$_4$ mixture with vigorous stirring. This mixture was finally used to fabricate fibers via electrospinning. Monetite was produced via the reaction of precursors during electrospinning, which uniformly dispersed in the PLA matrix. In addition, the heat and water produced during the reaction induced pore formation on PLA surface to further improve surface area of the fibers.

Fig. 4-7. A typical electrospinning set-up, composed of syringe, needle, power supply, collector and a pump
The limitations of electrospinning process are the usage of toxic solvent(s) that may cause adverse effects to cells if not totally removed and difficulty in controlling pore size and pore shape [105]. Also, there are several processing parameters involved in the electrospinning process that needs to be optimized in order to obtain the desired composite. This optimization process is cumbersome and time consuming when compared to the solvent casting or other fabrication techniques.

4.4.5 Coating Technique

Coating technique can be categorized into two types: depositing CaP coating on PLA/PLGA matrix or depositing PLA/PLGA on the CaP scaffold. The well-known method of CaP deposition is the biomimetic coating process via soaking the matrix in simulated body fluid (SBF) to deposit bone-like apatite (CDHA) on the targeted materials surface (Ti, biopolymer, bioceramics etc.) since 1990s [21, 22]. Zhang et al. soaked PLA scaffold into SBF to deposit CDHA coatings on PLA surface to enhance osteoinductive properties [27]. Labberzadeh et al. incubated PLGA scaffold in SBF to deposit a CDHA layer, as site for protein adsorption and release studies. They observed the protein release pattern was similar for PLGA and mineralized PLGA scaffolds, but precipitation of the CDHA layer on PLGA led to enhanced protein adsorption and slower protein release [163]. A drawback of this process is that the biomimetic coating is time consuming (about 21 days). As an alternative, production of nano-HA coated PLA composite by soaking alkali treated PLA matrix into nano-HA/ethanol suspension for only 1 hour was developed by Yanagida et al. [184]. In their report, carboxyl groups were introduced to PLA surface by alkali treatment and their interactions with calcium ions on HA facilitated the HA coating deposition on PLA surface (Fig. 4-8). Aerosol deposition method (ADM)
is also a novel method to deposit HA film on PLA substrates [185, 186]. In this process, PLA substrates were coated with HA particles, which were deposited by means of a high velocity helium carrier gas. Subsequently, HA coated PLA substrates were heat treated to prevent HA coating from being peeled through washing with water. The process of depositing PLA/PLGA to CaP scaffold involves simply soaking scaffolds into PLA/PLGA containing organic phases followed by drying to remove the solvent. Miao et al. deposited PLGA coating to HA/β-TCP scaffold, and observed macroporosity of the scaffolds after infiltration and coating with the PLGA was slightly decreased due to the observed thin polymer coating present on the strut surfaces [112]. Zhao et al. deposited PLA/HA composite coatings on HA macroporous scaffold using an alternative method [113]. They injected PLA/HA solution into the porous HA scaffolds by a vacuum pump and eventually removed extra solutions by centrifugation method to ensure the interconnectivity of the scaffolds. Porosity of the scaffold slightly decreased from 85.0 ± 3.6% to 82.0 ± 2.7% after coating while both the mechanical and biological properties increased. The results demonstrated that the centrifugation method showed a great potential to prepare polymer-lined scaffolds with improved 3D interconnectivity and reinforced adhesion force between the polymer coating and the substrate.
This technique does not involve too many parameter optimization when compared to the solvent casting and electrospinning techniques respectively. However, fabrication of different sizes and shapes may still be limited.

4.4.6 Supercritical Gas Foaming

Supercritical gas foaming is a solvent-free fabrication technique to produce scaffolds with controlled architecture and properties. The process utilizes the nucleation and growth of gas bubbles (internal phase) dispersed throughout a polymer (continuous phase) by sudden depressurization. The porous structure of polymer is formed when the dispersed gas phase is removed from the continuous phase of polymer [187-189]. In the process of fabrication of PLA-CaP/PLGA-CaP scaffold, a homogenous mixture of CaP particles and biopolymers (PLA/PLGA) is initially prepared with thermal treatment (usually melt-extrusion) in a pressure chamber followed by CO\textsubscript{2} diffusion to form a melt polymer-gas solution with a saturation pressure and temperature above the melting
temperature of the polymer (Fig. 4-9). Foam expansion is then carried out by sudden gas release with additional cooling, which increases polymer viscosity and creates permanent foam architecture by solidification and re-crystallization of the polymer. The rate of cooling was reported to have a significant effect on the porous structure. Cooling too rapidly fixed small closed pores, whereas cooling too slowly did not allow freezing of the structure, resulting in the structure to collapse [188, 189]. Mathieu et al. fabricated PLA/β-TCP and PLA/HA scaffolds via supercritical gas foaming in order to mimic natural bone with porosity over 80% [189]. The characterization results demonstrated that the as-fabricated 3-D scaffold had anisotropy in morphology with pores oriented in the foaming direction, which made the axial modulus of the scaffolds up to 1.5 times greater than the transverse modulus. This composite also showed viscoelastic behavior with increased modulus for higher strain rates similar to natural bone.

Fig. 4-9. Schematic of supercritical gas foaming: melted polymer-CaP-gas solution with a saturation pressure and temperature above the melting temperature of the polymer undergoes sudden gas release and cooling to produce porous polymer matrix with CaP dispersion.
These results indicated that the supercritical gas foamed composite scaffolds have suitable architecture and properties for bone tissue regeneration applications (Fig. 4-10). Comparison of supercritical gas foamed PLGA/HA composite and solvent cast PLGA/HA composite was conducted by Kim et al. [190, 191]. First, supercritical gas foaming eliminated the application of toxic organic solvent. Second, the foamed PLGA/HA composite showed interconnected porous structures without a skin layer and exhibited superior enhanced mechanical properties when compared to the scaffolds that were fabricated via solvent casting. Third, foamed PLGA/HA composite had abundant exposed HA particles on scaffold surface, which were expected to promote bone regeneration.

![Similarity of cancellous bone structures and composite foam macrostructures](image)

Fig. 4-10. Similarity of cancellous bone structures and composite foam macrostructures [189]

### 4.4.7 Melting

Melt extrusion is based on the low melting point of PLA/PLGA and high melting
point of CaP materials (Fig. 4-11). For example, Damadzadeh et al. used different HA content and particle size to produce PLA/HA and PLGA/HA composites via extrusion [192]. As alternatives to melt extrusion, processes such as forging, pressing, sintering, and microwave irradiation based heating were also applied to produce dense composites. Shikinami et al. extruded PLA/HA granules into thick billet, which was forged at 103 °C into thin billets and cut into devices with different shapes and size such as screws, pins and plates [193]. The composites satisfied initial mechanical strengths while maintaining them for as long as necessary (up to 24 weeks) for internal bone fixation devices. Mohn et al. prepared composites of PLGA and spherical amorphous TCP nanoparticles via blending directly or through a two-step approach, including solvent casting, extrusion followed by hot pressing. The latter route yielded good particle dispersion while blending alone led to inhomogeneous mixtures [194]. Ignjatovic et al. developed cold/hot pressing approach to manufacture PLA/HA composites with different density, porosity, compressive strength, and elastic modulus by controlling pressing parameters such as temperature, pressure, and time [195-199]. Sintering method to produce PLA/calcium metaphosphate (CMP) composite for bone regeneration was reported by Jung et al. [200]. In their process, mixture of PLA, CMP, and salt particles were pressed into discs followed by heat treatment at 210 °C for 30 min to generate a scaffold with a homogeneously interconnected porous structure without a skin layer. This resulted in a narrower pore size distribution, higher mechanical strength and higher exposure of CMP to surrounding environment in comparison with scaffolds made by a solvent casting method. Sintered microsphere scaffolds were also reported since 2001. It was a multifunctional scaffold with a connected pore system and mechanical properties in the
mid-range of cancellous bone [201]. In this process, pre-prepared PLGA microspheres were poured into a mold and heated to above glass transition temperature of PLGA for 24 hours. Upon heating, the polymer chains were activated to intertwine with neighboring polymer chains and thus formed contacts between neighboring microspheres. Following heating, the matrix was allowed to cool down to room temperature, which eliminated the mobility of the polymer chains. As a result, those contacts formed with adjacent spheres during high-temperature polymer fusion became fixed to provide mechanical support.

PLGA/CaP sintered microsphere scaffold was also fabricated using PLGA/CaP microspheres [164-166]. As the sintering temperature or time increased, the compressive mechanical properties significantly increased while the porosity of the scaffolds decreased. Consequently, the spherical morphologies of the microspheres were lost [164]. Jin et al. introduced a novel method to generate PLGA/β-TCP composites through polymerization using microwave energy [202]. Initially, l-lactide and glycolide were melted completely at 140 °C and β-TCP particles were dispersed into polymer solution with stannous octoate as an initiator. The mixture was then polymerized using microwave for 2 h with an irradiation power of 100 W (around 150 °C) followed by subsequent cooling. In these composites, the β-TCP powders were well dispersed in the PLGA matrix up to 30-wt% content. This behavior was attributed to the in-situ heating induced by the microwave energy.
4.4.8 Solid Free Fabrication/Rapid Prototyping

Solid free fabrication (SFF), or rapid prototyping (RP) is a series of digital information controlled 3D scaffold fabrication techniques, which converts computer aided design (CAD) into a specific cross-sectional format, expressing 3D model as layers. Such digital layered-3D model is then manufactured layer by layer using SFF machine and with each layer representing the cross-section of the digital model at specific levels. The applications of SFF include design visualization, metal casting, architecture, geospatial, healthcare and entertainment/retail. SFF has been applied to fabricate complex 3D scaffold with delicate architecture for tissue engineering applications [105, 203, 204]. In general, SFF can be grouped into 3 categories: melt-dissolution deposition method, particle bonding method, and indirect SFF fabrication method [205]. Melt-dissolution deposition method is a process wherein each layer is created by extrusion of a strand of material through an orifice while it moves across the plane of the layer cross-section. Once the deposited material cools, it solidifies itself and attaches to the previous layer [206]. In particle-bonding method, particles are selectively bonded to a thin layer of
powder material, which are then bonded in multilayers to form a complex 3D scaffold. During fabrication, surrounding unprocessed powders supports the object of interest. After completion of all layers, the object then is removed from the bed of unbonded powder [206]. Indirect SFF fabrication method involves fabrication of a negative mold based on the scaffold design. The scaffold is then cast using desired materials. Finally, the mold is sacrificed after final the scaffold is obtained [204, 207]. Such techniques enable the user to control both the external and the internal morphology of the final construct. In addition, it also requires less raw scaffold material while increasing the range of materials that can be used. The original material properties are conserved since it does not require further heating [205]. Several review articles and books have summarized and compared SFF scaffold fabrication approaches in detail [203-206, 208]. Therefore, this section will focus mainly on recently reported PLA-CaP/PLGA-CaP composite scaffolds fabricated via SFF/RP.

Low-temperature deposition manufacturing (LDM) is a process belonging to melt-dissolution deposition technique (Fig. 4-12). Its scaffold-building cycle is performed in a low temperature environment under 0 °C, so that it can preserve bioactivity of scaffold materials because of its benign processing conditions [209]. PLA/TCP scaffold has been successfully fabricated via LDM. The LDM system uses a pump nozzle to deposit PLA/TCP dioxane slurry to build scaffolds layer by layer based on 3D digital models [209]. The as-fabricated scaffolds had high porosity up to 89.6% and mechanical property values were close to human spongy bone (Fig. 4-13). Multi-nozzle Deposition Manufacturing (MDM) is an improved version of LDM. Scaffolds with different properties were fabricated in a low temperature environment under 0 °C by not only a
single-nozzle deposition process, but also by a bi-nozzle and tri-nozzle deposition processes [210]. Therefore, it can easily create functionally graded scaffolds with gradient materials and gradient morphologies, where bioactivity are customized and preserved. Yan et al. prepared a BMP (bone morphogenetic proteins) loaded PLGA/TCP scaffold using a tri-nozzle deposition manufacturing [210]. It was composed of two regions: spongy parts with BMP and compact parts. The compact part of the scaffold comprised of small pore size without the BMP which was expected to degrade slower and produce limited bone regeneration than the spongy part with larger pore size. The compact part had superior mechanical properties and provided better support during the process of bone repair. It took a relatively long time of about 1 year for the compact part to be resorbed by the body before the regenerated bone resumed its full function by the tissue regeneration triggered by BMP.

Fig. 4-12. Schematic of low-temperature deposition manufacturing, the system builds scaffold layer by layer directly computer driven by digital model in low temperature environment under 0 °C

3D printing (3DP) is a particle bonding technique, which selectively binds powder
particles with a liquid binder to form solid 3D objects one layer in each cycle (Fig. 4-14). After the binder is dried, the completed scaffold is retrieved and unattached particles are removed [204]. Sherwood et al. used PLGA/PLA/TCP/NaCl mixture to fabricate 3D scaffolds via 3D printing [211]. In this process, liquid CO$_2$ was used to remove the residual binder. Finally, the salt leaching created the micro-pores. The resulting scaffold composed of three regions with different functions: The upper cartilage region had 90% porosity and composed of PLGA/PLA, with macroscopic staggered channels to facilitate homogenous cell seeding. The lower, cloverleaf-shaped bone portion had 55% porosity and consisted of PLGA/TCP composite, designed to maximize bone ingrowth while maintaining critical mechanical properties. The transition region between these two sections contained a gradient of materials and porosity to prevent delamination. Roy et al. implanted 3D printed PLGA/β-TCP scaffold in rabbit calvarial defect for 8 weeks to promote bone healing and observed scaffold with macroscopic channels [212]. The scaffold had a controlled gradient porosity and pore size. The results showed that it had higher percentages of new bone area as compared to scaffold without porosity [212].

Fig. 4-13. SEM images of the cross-section of the PLLA/TCP composite scaffold: (a) low magnified and (b) high magnified [211]
Fig. 4-14. Schematic of 3D printing, powder particles are delivered to powder bed and assembled with a liquid binder to form solid 3D objects one layer in each cycle.

Selective laser sintering (SLS) is another particle bonding technique, which uses a deflected laser beam selectively to scan over the powder surface following the cross-sectional profiles carried by the slice data (Fig. 4-15) [208]. The interaction of the laser beam with the powder elevates the powder temperature to reach the glass-transition temperature, causing surfaces in contact to deform and fuse together. PLGA/carbonated HA (CHA) scaffold was prepared by Zhou et al. using SLS [197]. It was observed that the degree of fusion of the PLA/CHA composite powder was lower than that of the pure PLA powder, making it easier to remove excessive powder from pores. The increased viscosity of the composite material could explain this. Also, the CHA nanoparticles on the powder surface might act as a barrier against fusion.
There are limited reports on indirect SFF fabrication of PLA-CaP/PLGA-CaP scaffolds [213]. A sintered HA ceramic scaffold base was fabricated containing a primary and secondary pore network. The primary network was designed for tissue growth into the ceramic body while the secondary network served as the polymer mold. The secondary network was contiguous with the primary pore network in the manufactured scaffolds. PLA was melt cast into half of the base as outlined in the global pore melt casting techniques. Ceramic-free regions within the scaffold were created by selective application of a ceramic solvent, yielding a non-blend, discrete composite with mechanical interdigitation (interlocking) of the ceramic and polymer phases. Finally, porous discrete composites that include regions of pure sintered HA, PLA, and combinations of the two in the same scaffold were produced (Fig. 4-16). The polymer wall surrounding the periphery of the polymer half serves to entrap seeded chondroblasts and prevent migration of osteoblasts into the polymer region. Thus, it prevents bone formation in the region of cartilage growth. Conversely, osteoblast migration and bone
production are occurring in the HA region.

Fig. 4-16. PLA-HA scaffold fabricated via indirect SFF [213]

4.5 Performance of PLA-CaP/PLGA-CaP composites

In PLA-CaP/PLGA-CaP composites, PLA/PLGA is usually referred to as the continuous phase or matrix, while CaP materials are referred to as the dispersed phase or fillers. The continuous phase is responsible for supporting the dispersed materials by maintaining their relative positions. The dispersed phase is usually responsible for enhancing physical and biological properties of the matrix [37]. For enhancement in mechanical properties of the PLA-CaP/PLGA-CaP composites, CaP works as a reinforcing material to meet performance criteria for a particular application or implant [173, 214]. For biocompatibility and bioactivity of the PLA-CaP/PLGA-CaP composites, incorporation of CaP materials into PLA/PLGA matrix greatly promotes cell proliferation and tissue regeneration [190, 215].

4.5.1 Mechanical Performance

As seen in Table 4.3, the influence of one type of composite manufacturing compared to another on the implant’s mechanical performance is difficult to access. This is because different papers focus on selected mechanical properties (compressive
modulus, tensile strength, Young’s modulus, compressive strength etc.) and many variables (porosity, ratio of CaP, CaP type etc.). The strengthening effect of CaP in PLA/PLGA matrix can be explained by the fact that the PLA/PLGA matrix is a load transfer medium and thus transfers the load to the intrinsically rigid CaP crystals. However, the incorporation of CaP can result in decrease of tensile strength of PLA/PLGA matrix due to the brittleness of CaP materials [172]. Another issue is the content of incorporated CaP in the composite. When the CaP content is low, the PLA/PLGA matrix is continuous, which ensures good interfacial bonding with CaP particles and thus yielding improved compressive strength. In composites with greater CaP content, the amount of PLA/PLGA matrix surrounding the CaP particles is lesser. This results in poor integrity of the composite, causing low compressive properties [176, 216].

Table 4.3. Physical properties of PLA-CaP/PLGA-CaP composites fabricated using different techniques

<table>
<thead>
<tr>
<th>Techniques</th>
<th>Materials</th>
<th>Architecture</th>
<th>Mechanical Properties</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent casting</td>
<td>PLA, amorphous-TCP (0-30 wt%)</td>
<td>film</td>
<td>Young’s modulus: up to 2800 MPa (30 wt% amorphous-TCP)</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>PLA, HA (0-50 wt%)</td>
<td>scaffold with porosity 86%-92%</td>
<td>Compressive modulus: up to 9.87 ± 1.8 MPa (50 wt% HA) Yield strength: up to 0.44±0.01MPa (50 wt% HA)</td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>PLGA, HA (0-15 wt%)</td>
<td>film</td>
<td>Tensile strength: up to 3.9 ± 0.37 MPa (10 wt% HA) Young’s modulus: up to 16.56 ± 5.96 MPa (10 wt% HA)</td>
<td>170</td>
</tr>
<tr>
<td>Method</td>
<td>Component(s)</td>
<td>Morphology</td>
<td>Properties</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------------</td>
<td>---------------------------------------</td>
<td>------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Phase separation</td>
<td>PLGA, β-TCP (0-60 wt%)</td>
<td>scaffold with porosity 83.5%-91.4%</td>
<td>Compressive modulus: up to 6.64 ± 1.03 MPa (30 wt% β-TCP)</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>PLA, HA (0-30% wt%)</td>
<td>fibrous scaffold with porosity up to 90%</td>
<td>Compressive modulus: up to 0.63 MPa (20 wt% HA)</td>
<td>177</td>
</tr>
<tr>
<td>Electrospinning</td>
<td>PLA, HA (0 to 16.7 wt%)</td>
<td>fibers</td>
<td>Young's modulus: up to 4.711 ± 0.019 MPa (16.7 wt% HA)</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tensile strength: up to 0.262 ± 0.007 MPa (16.7 wt% HA)</td>
<td></td>
</tr>
<tr>
<td>CaP coating</td>
<td>PLA, CDHA</td>
<td>scaffold with porosity up to 95%</td>
<td>Compressive modulus: approximately 10 MPa (with CDHA)</td>
<td>27</td>
</tr>
<tr>
<td>PLA/PLGA coating</td>
<td>PLGA, HA, β-TCP</td>
<td>scaffold with porosity up to 87%</td>
<td>Compressive modulus: up to 665 ± 50 kPa (with PLGA)</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Compressive strength: up to 66 ± 7 kPa (with PLGA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLA, HA</td>
<td>Scaffold with porosity 82.0 ± 2.7 %</td>
<td>Compressive strength: approximately 1.7 MPa (with PLA)</td>
<td>113</td>
</tr>
<tr>
<td>Supercritical gas foaming</td>
<td>PLGA, HA (50 wt%)</td>
<td>scaffold with porosity 91 ± 3%</td>
<td>Compressive modulus: 4.5 ± 0.3 MPa</td>
<td>190, 191</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Young's modulus: 26.9 ± 0.2 MPa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLA, HA/β-TCP (0-10 wt%)</td>
<td>scaffold with porosity 74% to 81%</td>
<td>Young's modulus: up to approximately 180 MPa (10% β-TCP)</td>
<td>189</td>
</tr>
<tr>
<td>Melt extrusion</td>
<td>PLA/PLGA, HA/β-TCP (0-30 wt%)</td>
<td>rod-like profiles with a diameter of 2–2.5 mm</td>
<td>Young's modulus: up to 4.5 ± 0.2 GPa (30 wt% HA and 70 wt% PLA)</td>
<td>192</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Flexural strength: up to 136 ± 5 MPa (10 wt% HA and 90 wt% PLA)</td>
<td></td>
</tr>
<tr>
<td>Process</td>
<td>Composition</td>
<td>Porosity</td>
<td>Mechanical Properties</td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------------------------------</td>
<td>----------------------------------</td>
<td>---------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Forging</td>
<td>PLA, HA (0-50 wt%)</td>
<td>billet</td>
<td>Flexural strength: up to 270 ± 4.1 MPa (40 wt% HA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Flexural modulus: up to 12.3 ± 0.2 GPa (50 wt% HA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tensile strength: 154.1±0.8 MPa (PLA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Young’s modulus: up to 2.4 ± 0.9 GPa (50 wt% HA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Compressive strength: up to 115.3 ± 3.9 MPa (PLA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Compressive modulus: up to 6.5 ± 0.2 GPa (50 wt% HA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold/Hot Pressing</td>
<td>PLA, HA (N/A)</td>
<td>3D block</td>
<td>Compressive strength: up to 140 MPa</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Compressive modulus: up to 10 GPa</td>
<td></td>
</tr>
<tr>
<td>Sintering</td>
<td>PLGA, HA (20 wt%)</td>
<td>scaffold with porosity 30%</td>
<td>Compressive strength: up to approximate 4 MPa</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Compressive modulus: up to 200 MPa</td>
<td></td>
</tr>
<tr>
<td>SFF</td>
<td>PLA, TCP (50 wt%)</td>
<td>scaffold with porosity up to 89.6%</td>
<td>Compressive modulus: 60.11 MPa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLGA, TCP (23-33.3 wt %)</td>
<td>Scaffold with porosity from 50%</td>
<td>Tensile strength: up to 5.7 ± 1 MPa (33.3 wt% TCP)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>to 90% in different regions</td>
<td>Young's modulus: up to 233 ± 27 MPa (23 wt% TCP)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Compressive strength: up to 13.7 ± 0.8 MPa (23 wt% TCP)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Compressive modulus: up to 450 ± 79 MPa (23 wt% TCP)</td>
<td></td>
</tr>
</tbody>
</table>

From the bone mimicking perspective, it is very hard to produce PLA-CaP/PLGA-CaP composite or any biocomposites perfectly matching the mechanical properties and highly organized structure of cancellous or cortical bone. Hence, further
in-depth research is required on the manufacturing aspect of PLA-CaP/PLGA-CaP composites in order to make them mimic the skeletal bone structure.

4.5.2 Biological Performance

Bioactivity is referred to the ability of materials to possess direct bone bonding capabilities once implanted. SBF has been widely accepted as a powerful test to evaluate the bioactivity of the target material [218]. PLA and PLGA have the ability to form apatite once incubated in SBF, because the hydrolysis of them during biodegradation results in -COOH which can bind to Ca\(^{2+}\) for further apatite nucleation and growth [27, 32]. On considering the degradation rate difference between PLA and PLGA, PLGA is more favored for apatite deposition. However, it is possible to modify the surface of PLA/PLGA to increase the functional group –COOH [182]. The incorporation of highly bioactive CaP can intensify the deposition of bone-like apatite from SBF. Kim et al. bonded HA particles to PLA surface, and reported considerable level of apatite formation on treated PLA surface after 7 days in SBF solution as compared to the flat surface of untreated PLA (Fig. 4-17) [219]. Zhou et al. also reported such phenomenon when incubating PLA/CDHA and PLA electrospun fibers in SBF for 7 days [95].
The results of using PLA-CaP/PLGA-CaP composites in vitro and in vivo are presented in Table 4.4. The table illustrates the increased biocompatibility and efficient bone forming capacity of PLA-CaP/PLGA-CaP composites as compared to controlled PLA/PLGA groups. All results confirm that PLA-CaP/PLGA-CaP composites have greater potential for clinical applications.
Table 4.4. Results of in vitro and in vivo experiments using different PLA-CaP/PLGA-CaP composites

<table>
<thead>
<tr>
<th>Composites</th>
<th>Method</th>
<th>Cell culturing or implantation</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>in vitro experiment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLGA/HA [170]</td>
<td>Solvent casting</td>
<td>Chondrocytes isolated from joint of porcine rear leg</td>
<td>6 days (cell number is 1.3 times higher than control group)</td>
</tr>
<tr>
<td>PLGA/HA [175]</td>
<td>Phase separation</td>
<td>Mesenchymal stem cells from rabbit tibia</td>
<td>2 weeks (significantly higher cell growth and alkaline phosphatase (ALP) activity)</td>
</tr>
<tr>
<td>PLA/β-TCP [220]</td>
<td>Electrospinning</td>
<td>Human adipose stem cells</td>
<td>2 weeks (high DNA content and ALP activity)</td>
</tr>
<tr>
<td>PLGA-HA [165]</td>
<td>Sintering</td>
<td>Human mesenchymal stem cells</td>
<td>3 weeks (enhance cell proliferation at early point, but differentiation and mineralization were promoted at later point)</td>
</tr>
<tr>
<td></td>
<td><strong>in vivo experiment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLGA/BCP [137]</td>
<td>Emulsion</td>
<td>Implantation in alveolar bones</td>
<td>24 weeks (significantly higher bone density as compared to control group)</td>
</tr>
<tr>
<td>PLA/β-TCP [152]</td>
<td>Emulsion</td>
<td>Implantation in rabbit femur</td>
<td>4 weeks (mineralized bone with repairing of soft tissue)</td>
</tr>
<tr>
<td>PLGA/TCP [215]</td>
<td>Electrospinning</td>
<td>Implantation in rabbit calvarial bone</td>
<td>4 weeks (mineralized bone)</td>
</tr>
<tr>
<td>PLGA/HA [190]</td>
<td>Supercritical gas foaming with salt leaching</td>
<td>Implantation in rat skulls</td>
<td>8 weeks (mineralized bone with lamellar structures)</td>
</tr>
</tbody>
</table>
PLA/β-TCP [221] | Supercritical gas foaming | Implantation in sheep femur and tibia | 12 months (mineralized bone)

PLA/HA [222] | Cold/hot pressing | Implantation in mice femur | 12 weeks (good growth in surrounding connective tissue)

PLGA/β-TCP [212] | SFF | Implantation in rabbit calvarial bone | 8 weeks (mineralized bone)

4.6 Challenges

4.6.1 Mechanical Integrity of the Composite

As mentioned before, uniform dispersion of CaP particles in the composite structure is always a concern during fabrication especially when high ratio CaP materials are used as fillers. CaP particles tend to combine together and form aggregates via interlocking, electrostatic attraction, van der Waals forces, liquid bridges and solid bridging [219]. Also, CaP shows low affinity towards PLA/PLGA. Therefore, CaP particles are poorly dispersed or are easily agglomerated, resulting in uneven distribution within the polymeric matrix. In addition, in solvent-based fabrication process, the phase separation between CaP and polymer solution occurs when high content of CaP are used. This is due to lack of affinity of CaP to organic solvents, which results in the difficulties during processing and produces uneven dispersion of CaP in the composite structure. The agglomeration of CaP in the polymer matrix can cause decrease in mechanical strength, since these agglomerations start to act as stress concentration points giving rise to brittle failure with a lower ultimate tensile strength [216]. Application of strong ultrasonication to destroy CaP agglomerates is the most common approach to preparing PLA/CaP or
PLGA/CaP mixture in solvent(s) [214, 224, 225]. As an alternative, Takamatsu et al. used ball milling to decrease CaP aggregates [226]. In their process, PLA/β-TCP solution was ball-milled at a velocity of 80 rpm at ambient temperature for periods up to 48 hours. Drying and then hot pressing followed this. Young’s modulus and bending strength of composites increased with increase in ball-milling time. This is a result of absence of large β-TCP aggregates in ball-milled composites. In addition, it was reported that the choice of dispersant also influences CaP dispersion in polymer solution. The ability of various conventional organic dispersants (chloroform, dimethylformamide, tetrahydrofuran, acetone, ethanol) to form homogeneous and stable PLA/HA solution was compared by Deng et al. [227]. It was observed that dimethylformamide is an effective dispersant to de-aggregate HA particles in solvent-based PLA/HA composites fabrication. The application of surfactant is another approach to improve CaP dispersion. Kim et al. introduced a surfactant 12-hydroxystearic acid (HSA) to address the HA dispersion and agglomerate problem [228]. TEM analysis of the as-fabricated PLA/HA electrospun fibers revealed uniform distribution of HA powders throughout the PLA polymeric matrix; while products using commercial HA powders without HSA showed severe agglomeration of HA in PLA matrix. It resulted in separation of the PLA-rich part from HA agglomerates.

The size and morphology of CaP fillers are also important to the composite mechanical strength. Nejati et al. used both needle-like nano HA and micro-sized HA to fabricate HA filled PLA composite scaffolds via phase separation. The mechanical testing results showed the compressive strengths of nanocomposite scaffolds were increased to 8.46 MPa. Compared to that, compressive strengths of pure PLA and HA microcomposite
scaffolds were 1.79 and 4.61 MPa, respectively [134]. This is attributed not only to the larger interfacial area between nano HA and PLA matrix, but also to uniform dispersion of nano HA. On the other hand, it may also be argued that irregular CaP particles are preferred because PLA/PLGA liquid can penetrate into CaP particle surface and can form a mechanical interlock with the particle when transited back to solid form during composite fabrication [216].

To further promote the adhesion between CaP and PLA/PLGA, formation of strong chemical bonding between PLA/PLGA and CaP has been studied [156, 229-239]. The most commonly applied technique is to modify CaP particle surface. In some polymer/HA composites reports, coupling agent molecules such as silane, zirconyl salts, hexamethylene etc. were grafted on HA particles surface through covalent bonds by chemical reaction with hydroxyl groups on the surface of HA [229-231]. Rakovsky et al. modified the surface of CDHA by reaction with hexamethylene diisocyanate (HDI) in a non-aqueous suspension [232]. Composites of CDHA–HDI with PLA were high pressure consolidated at room temperature at 2.5 GPa; the composite had a compressive strength of approximately 300 MPa. Hong et al. made HA particles surface grafted with PLA via ring-opening polymerization of L-lactide in the presence of stannous octanoate (Sn(Oct)2) as catalyst. The results showed that the PLA molecules grafted greatly improved the adhesive strength between HA and PLA matrix as inter-connecting molecules [233, 234]. To further enhance bonding, the surface morphology of CaP can also be roughened. Hong et al. produced lenticular carbonated HA particles with 30–40 nm in diameter and 100–200 nm in length via precipitation, which had a coarse surface with many global protuberances, which could be in favor of the interaction with the polymer matrix [235].
However, the grafting rate is limited due to a reduced amount of hydroxyl groups on HA particles. In order to increase the hydroxyl group numbers, HA particles were first grafted with L-lactic acid and then with PLA. Chemical bonding between Ca atoms on HA surface and carboxyl groups of L-lactic acid or PLA were formed with grafting ratios up to 40% [236]. These grafted CaP particles were also reported to show homogeneous dispersion at nanoscale in PLA/PLGA matrix such as electrospun fibers as compared to conventional CaP particles [236, 237]. In another approach, the surface of HA was initially coated using phosphonic acid based agent to introduce more number of reactive hydroxyl groups on the HA particles for PLA bonding [238]. The diametric tensile strength of PLA/HA composite prepared from PLA-grafted HA was found to be over twice that of the composite prepared from the non-modified HA. Moreover, the tensile strength of the improved composite was 23 % higher than that of PLA alone. On the other hand, PLA/PLGA can also be modified to improve its adhesion to CaP particles. Alkali treatment of PLA can induce carboxyl groups formation on PLA surface, which can then interact with calcium ions on HA to drive the HA coating deposition on PLA surface [156]. Yet another approach is to use poly(α-methacrylic acid) (PMMA) to introduce carboxyl groups to PLA surface via photo-oxidization and UV induced polymerization and the modified PLA could then act as a template to manipulate the nucleation and growth of HA crystals [239].

4.6.2 Incorporation of Proteins/Drugs/Genes and Stem Cells

There is significant scope in the loading of proteins/drugs/genes to further promote tissue regeneration. However, this does not allow for extreme temperature ranges or extremely aggressive chemical conditions during processing. The incorporation of
proteins, drugs, genes and stem cells in the composites will be discussed in detail in the subsequent paragraphs.

In PLA/PLGA matrix, CaP materials can also work as loading sites for proteins for further enhancing bone regeneration. Woo et al. examined the adsorption of serum protein and adhesion of molecules on PLA and PLA/HA scaffolds prepared by thermal induced phase separation process [240]. PLA/HA scaffolds adsorbed much higher amount of serum proteins, adhesion proteins, and peptides (fibronectin, vitronectin, RGD, and KRSR). Furthermore, β1 and β3 integrins and phosphorylation of Fak and Akt proteins in the cells on the PLA/HA scaffolds were significantly more abundant than those on PLA scaffolds, which suggested that enhanced adsorption of serum adhesion proteins to PLLA/HAP scaffolds protect the cells from apoptosis possibly through the integrin-Fak-Akt pathway. Therefore, proteins (such as growth factor BMPs) can be directly loaded to composite surface via soaking composites scaffold into bio-molecules containing aqueous solution [241-243]. Instead of direct absorption of proteins to composite surface, they can be also be combined into the inner matrix of the composite. Kwon et al. prepared rhGDF-5 (recombinant human growth and differentiation factor-5)/β-TCP/PLGA composites via blending rhGDF-5 coated β-TCP with PLGA solution, rhGDF-5 is a growth/differentiation factor for bone growth and in vivo study indicated this composite greatly promotes bone healing as compared to control group (PLGA/β-TCP) [244].

For drug delivery applications, emulsion is the most common approach to produce microspheres for drug delivery. Shi et al. used both oil/water (o/w) and water/oil/water (w/o/w) emulsion methods to fabricate PLGA/HA microspheres loaded with alendronate
to study the release kinetics [162]. Alendronate belongs to bisphosphonates, which is one of the primary agents against osteoclast-mediated bone loss. They also have high affinity towards HA crystals [49]. It was reported that in w/o/w emulsion system, the affinity between HA (in organic phase) and alendronate (in water phase) was inhibited due to limited HA exposure to alendronate caused by PLGA-containing organic phase, resulting in encapsulation efficiency of alendronate below 40%. On the other hand, PLGA/HAlendronate microspheres fabricated via o/w emulsion showed approximately 90% encapsulation efficiency. All PLGA/Ha-alendronate systems exhibited controlled release without remarkable initial burst release. AS alternative to loose microparticles, drugs are also loaded to 3D implant composite. Chu et al. fabricated a PLGA/5-fluorouracil/HA scaffold via SFF as an implantable drug delivery system; 5-fluorouracil is an anti-cancer drug [245]. The relationship between drug release rate and the amount of HA showed a bi-linear kinetics and an HA content of about 5-20 percent seemed to be a reasonable amount of additive to control the release rate of 5-fluorouracil. Xie et al. added icaritin to PLGA/TCP slurry to fabricate PLGA/TCP/icaritin composite scaffold material with slow release of icaritin during scaffold degradation for enhancing bone repair [246].

DNA can also be loaded to composites in order to induce bio-molecules generation in vivo and enhance bone regeneration. PLGA/HA electrospun composite scaffold with DNA loaded was constructed by Nie et al. via three different approaches including naked DNA coated outside, DNA-loaded chitosan nanoparticles coated outside, and DNA-loaded chitosan nanoparticles encapsulated inside the fibers [247]. Human marrow stem cells culture experiment showed that the scaffolds with encapsulated DNA/chitosan...
nanoparticles had higher cell attachment, higher cell viability, and desirable transfection efficiency of DNA.

PLA-CaP/PLGA-CaP composite scaffolds have been shown to be good candidates for stem cells loading in order to induce cooperative cell-cell and cell-matrix interactions, thus promoting bone regeneration. Li et al. transplanted PLGA/HA electrospun composite cultured with/without MSCs into cartilage defects in rats and both scaffolds had better tissue morphology, structure integrity, matrix staining, and much thicker newly formed cartilage than the control group. The histological score for PLGA/HA-MSC is better than that for PLGA/HA, showing that the MSC play an important role in tissue repair [248]. Montjovent et al. seeded human fetal bone cells on to supercritical gas foamed PLA/β-TCP scaffold and implanted the composite in rat. Composite scaffold resorption as well as cortical and trabecular bone repair were assessed. The composite scaffold degradation was coupled to the rate of tissue regeneration, leading to a structural integrity of the constructs. A complete bone ingrowth induced by PLA/β-TCP composite scaffold with bone cells was observed as compared to blank PLA/β-TCP composite scaffold [249]. Bone marrow stromal cells were also seeded to PLA/β-TCP composite scaffold and implanted it to bone defects [250]. It was observed that the mineralization in the defects sites occurred after 30 days and a callus was formed around the periphery of the implants and along the adjacent host bone after 90 days; while the empty bone defects had not fully healed after 90 days and the bone regeneration did not progress after 60 days.

4.6.3 Long-term degradation

The degradation behavior of PLA/PLGA matrix is influenced by the degradation properties of combined CaP fillers. It has been reported that the incorporation of HA can
greatly inhibit the degradation and water uptake of PLA matrix, while acting as a physical barrier to block off the entry of water, and thus slowing down PLA degradation kinetics [225]. As an alternative, TCP promotes the degradation of PLA/PLGA matrix by intensifying water uptake. In an experiment reported by Loher et al., pure PLGA matrix only had 0.5% mass loss and 2% water uptake after six weeks, while 30% amorphous TCP incorporation resulted in 7% mass loss and 14% water uptake after six weeks. It is possible that the high degradation rate of amorphous TCP generated pores in the composite, thus resulting in a larger polymer surface area being exposed to the surrounding liquid to promote PLGA degradation [168]. The inflammation caused by degradation of PLA/PLGA is one of the reasons to incorporate CaP materials into PLA/PLGA matrix [161, 177, 182]. However, in long-term investigations, the inflammation problem is still a concern in the application of PLA-CaP/PLGA-CaP composite due to the difference in degradation rates between CaP fillers and PLA/PLGA matrix. Ignataus et al. implanted PLA or PLA/TCP scaffolds into female merino sheep [251]. A mild inflammatory response was observed in the first 12 months but a strong inflammatory reaction was seen after 24 months. The incorporation of α-TCP can improve the biocompatibility of the scaffold to avoid inflammation reaction in vivo. After a total degradation of α-TCP after 6 months, PLA degradation can cause potential inflammation reaction in vivo. After 2 years, the strengths were significantly decreased. A better tailoring of the contents and compositions of PLA/PLGA matrix and CaP fillers is necessary for successful long-term clinical applications. On the other hand, biodegradation of the composite and deposition of apatite are two competing process in vivo. Ideally, it is desired that there is a smooth transition between a primarily polymer
based composite to a mineralized bone material in the bone regeneration process. By carefully tailoring the CaP material content and composition, the composite structure, and the degradation rate of PLA/PLGA can possibly provide the smooth transition from composite to mineralized bone.

Designing and implementing an *in vitro* system to mimic long-term *in vivo* degradation of PLA-CaP/PLGA-CaP composite scaffold is a new study as an alternative to the more expensive *in vivo* animal experiments. However, such long-term studies focusing on PLA-CaP/PLGA-CaP composite are rare. Yang et al. developed an *in vitro* dynamic system for the long-term evaluation of composite degradation, which showed potential in investigation of long-term mechanical and chemical transition of composite scaffolds [252]. In contrast to simple incubation in phosphate buffered saline (PBS) at 37 °C, PLGA/ β-TCP composite scaffolds were incubated in weekly refreshed PBS at 37 °C with dynamic loading to mimic the *in vivo* environment. The results indicated that the cyclic loading under dynamic conditions accelerated the degradation of the PLGA component in the composite. This system could provide fundamental experimental results for the degradation behavior of biodegradable polymeric scaffolds used in bone tissue engineering *in vivo*. More work is still required to develop a long-term study.

### 4.7 Summary

In order to substitute bone grafts, the development of composites made of PLA/PLGA and CaP materials for bone tissue engineering applications has gained greater interest. The biological response, mechanical properties, composite architecture, degradability of the PLA/PLGA-CaP composites can be influenced by fabrication techniques, properties and contents of raw materials, and the interaction between matrix
and fillers. PLA/PLGA-CaP composites can be tailored to meet different bone tissue engineering application requirements. However, there are still a number of issues that still remain to be improved (e.g. inflammation, long-term degradation, scale-up, mechanical strength improvement etc.) for even wider application of PLA/PLGA-CaP composites in clinical applications.
Chapter 5

Fabrication of Novel PLA/CDHA Bionanocomposite Fibers for Tissue Engineering Applications via Electrospinning

5.1 Abstract

The main theme here is to fabricate PLA (poly lactic-acid)/ CDHA (carbonated calcium deficient hydroxyapatite) bionanocomposites, where both the constituents are biocompatible and biodegradable with one dimension in nanometer scale. Such materials are important in tissue engineering applications. The bionanocomposite fibers were fabricated via electrospinning. There are two important signatures of this chapter. First, CDHA, rather than HA, is added to PLA as the second phase. As opposed to HA, CDHA mimics the bone mineral composition better and is biodegradable. Therefore, PLA/CDHA fibers should have better biodegradability while maintaining a physiological pH during degradation. To the best of our knowledge, this is the first attempt of electrospinning of such a composite. Second, the CDHA nanoparticles were synthesized using the benign low temperature biomimetic precipitation technique, the only route available for the retention of carbonate ions in the HA lattice. The structural properties, degradation behavior, bioactivity, cell adhesion, and growth capability of as-fabricated PLA/CDHA bionanocomposites were investigated. The results show that the
incorporation of CDHA decreased PLA fiber diameters, accelerated PLA degradation, buffered pH decrease caused by PLA degradation, improved the bioactivity and biocompatibility of the scaffold. These results prove that PLA/CDHA bionanocomposites have the potential in tissue regeneration applications.

5.2 Introduction

The major theme of this work is to fabricate PLA (poly-lactic acid)/CDHA (carbonated calcium deficient hydroxyapatite) bionanocomposites for tissue engineering applications. To the best of our knowledge, this is the first example of an electrospun PLA/CDHA bionanocomposite scaffold for tissue engineering. As explained in the following, the presence of CDHA is critical to proper functioning of PLA based scaffold, yet has not been attempted before. For such applications, electrospinning has emerged as a potential process for the fabrication of temporary fibrous scaffolds to serve as a substrate for implanted cells and newly formed tissues [179]. The term “bionanocomposites” can be defined by referring to the field of nanocomposites. The latter term has been coined by adding the prefix ‘Bio” to “nanocomposites”. Roy and Roy originally used “nanocomposites” in describing the resulting structure of sol-gel processed ceramics and glasses [253, 254]. Komarneni presented a review of such developments [255]. By following the definition of nanocomposites, bionanocomposites can be defined by their constituents and their size. As opposed to single-phase materials, nanocomposites are heterogeneous materials consisting of two or more phases with the restriction that at least one dimension of the constituents should of the order of 1 to 50 nm range [255]. However, such size restrictions are not rigorously applied as along as one of the constituents meets the criterion. The constituents can be organic or inorganic in
any combination. A seminal paper laying out the concepts of bionanocomposites has been published by Dardar et al. [256]. In our specific case, an organic material (PLA) is the matrix and an inorganic material (CDHA) is the second phase. Both the matrix and the second phase are in nanometer scale.

The reason for the choice of PLA as the matrix is simple: it is a FDA approved biomaterial used for applications as sutures, pins, screws and drug delivery systems [257, 258]. It can also be used to fabricate fibrous scaffolds via electrospinning [179]. However, when it comes to tissue engineering, PLA has disadvantages such as low cell adhesion due to its hydrophobic surface property, and inflammatory reactions in vivo caused by its degradation product, lactic acid [122, 125]. The incorporation of Calcium Phosphate (CaP) particles into PLA matrix can buffer the localized pH decrease, and improve cell adhesion, osteoconductivity, and mechanical properties [182, 259].

There are two important signatures of this work. First is the use of CDHA as the second phase. Of CaP materials used in composite fabrication, hydroxyapatite (HA, Ca$_{10}$(PO$_4$)$_6$(OH)$_2$) is a very common second phase [108]. While HA is structurally similar to the main inorganic component of nature bone, it is still compositionally different. The mineral component of bone is carbonated calcium phosphate (CDHA) with small amounts of sodium (Na$^+$), potassium (K$^+$), magnesium (Mg$^{2+}$), carbonate (CO$_3^{2-}$) and water (H$_2$O) along with the major mineral content of calcium (Ca$^{2+}$) and phosphate (PO$_4^{3-}$) [46]. As a result of this subtle difference, CDHA is a bioactive material, while HA is not, in spite of its biocompatibility. Unfortunately, most of the papers reported in the literature use HA as the second phase in fabricating composites with a whole range of biopolymers as matrices including PLA [108, 177, 182].
Second, nanocrystalline CDHA powders are synthesized using a biomimetic process. Being a benign low temperature process, it can retain the carbonate ions in CDHA lattice. No high temperature technique can achieve this. Kokubo has developed simulated body fluid (SBF) to biomimetically synthesize CDHA coatings [21]. The process involves immersing metallic, ceramic, or polymeric substrates into SBF solution held at 37°C. Such a process must meet three criteria: 1) the SBF composition should closely mimic the ionic species and concentrations of human plasma; 2) the pH of the SBF should be fixed at that of the human blood (7.40); 3) continuous process temperature should always be kept at the physiologic temperature of 37°C. A composition of SBF (c-SBF), pioneered and popularized by Kokubo, meets the above criteria [21]. The problem associated with c-SBF is that concentration of \( \text{HCO}_3^- \) is 4.2 mM, which is much less than that of human plasma. Tas first realized this problem and revised this original c-SBF composition, which we refer to as (t-SBF) [30]. There are several other compositions also available in the literature [260-262]. Our group has provided ample proof of the efficiency of t-SBF in fabricating monolithic CDHA coatings [29, 263]. During the CDHA deposition process, an amorphous calcium phosphate precursor is always present [91, 92]. Such precursors are considered as transient solution precursors for the formation of CDHA precipitates with a size range of 0.7-1.0 nm. The CaP clusters in the solution form apatite nuclei either on the surface of a substrate or in the SBF solution. Those nucleated on the substrate can further grow into a CDHA coating layer, while those nucleated in the solution form CDHA precipitates [94]. In this work, these nucleated particles are collected for further use. In addition, during the nucleation and precipitation processes, trace elements can be combined into lattice structure. These CDHA precipitates have the potential to be ideal
second phase materials for biopolymer matrix because they are biocompatible, as well as bioactive.

Formation of monolithic PLA/CDHA composites has been reported in the literature before. Durucan et al. reported fabrication of CDHA-PLA/PLGA composites via the hydrolysis of TCP-PLA/PLGA composites [136]. Zhang et al. soaked PLA scaffold into simulated body fluid (SBF) to deposit CDHA coatings on PLA surface [27]. Rakovsky et al. fabricated CDHA/PLA nanocomposite via high pressure consolidation, CDHA was synthesized via wet chemical synthesis [232]. But this is the first time that electrospun PLA/CDHA bionanocomposites have been fabricated. This chapter describes: 1) the synthesis of CDHA using the t-SBF, 2) incorporate the CDHA nanoparticles into the nanofibers of PLA via the process of electrospinning, and 3) to evaluate the physical properties, degradation behavior, bioactivity, cell attachment, and growth capability of as-fabricated PLA/CDHA bionanocomposites.

5.3 Materials and Methods

5.3.1 CDHA powders synthesis

CDHA was synthesized using modified 1.5x t-SBF, with reduced content of Tris-Base compared to conventional 1.5x t-SBF reported before [29]. The compositions are shown in Table 5.1. The reagents were dissolved in solution one by one, and added based on the order listed in Table 5.1. NaHCO$_3$ and Na$_2$SO$_4$ were purchased from Acros Organics. NaCl, KCl, NaH$_2$PO$_4$, MgCl$_2$.6H$_2$O, CaCl$_2$.2H$_2$O, Tris-base buffer (C$_4$H$_{11}$NO$_6$), and HCl were purchased from Fisher Scientific. The solution was later stored at 37°C environment for 24 hours. Subsequently, CDHA precipitates were collected after filtration and dried in oven overnight. Further characterization was applied
to prove such precipitates were exactly CDHA.

Table 5.1 Modified 1.5x t-SBF solution composition for a total volume of 1L

<table>
<thead>
<tr>
<th>Order</th>
<th>Reagent</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaCl</td>
<td>9.8184</td>
</tr>
<tr>
<td>2</td>
<td>NaHCO₃</td>
<td>3.4023</td>
</tr>
<tr>
<td>3</td>
<td>KCl</td>
<td>0.5591</td>
</tr>
<tr>
<td>4</td>
<td>Na₂HPO₄</td>
<td>0.2129</td>
</tr>
<tr>
<td>5</td>
<td>MgCl₂.6H₂O</td>
<td>0.4574</td>
</tr>
<tr>
<td>6</td>
<td>1M HCL</td>
<td>15 mL</td>
</tr>
<tr>
<td>7</td>
<td>CaCl₂.2H₂O</td>
<td>0.5822</td>
</tr>
<tr>
<td>8</td>
<td>Na₂SO₄</td>
<td>0.1080</td>
</tr>
<tr>
<td>9</td>
<td>Tris-Base</td>
<td>6.063</td>
</tr>
<tr>
<td>10</td>
<td>1M HCl</td>
<td>29 mL</td>
</tr>
</tbody>
</table>

5.3.2 PLA/CDHA solution preparation

PLA pellets were supplied by Jamplast (Ellisville, MO). The weight average molecular weight (Mw) and poly dispersity of PLA are 114,000 and 1.435 respectively. These values were determined by gel permeation chromatography relative to polystyrene standards using a Shimadzu LC–10ADVP liquid chromatography equipped with a Shimadzu ELSD-LT ultraviolet (UV) detector (Columbia, MD). Chloroform (CHCl₃) and Dimethylformamide (DMF) were used as medium for the presentation of electrospinnable mixtures and were purchased from Fisher Scientific. PLA pellets were initially dissolved in chloroform. Following that, CDHA precipitates were added to PLA solution to make a mixture. To uniformly disperse CDHA crystals in PLA solution, mixture was treated by 1 hour ultrasonication to destroy CDHA agglomerates. After further addition of DMF to the mixture and 4 hours of stirring, the solution was ready for electrospinning. The volume ratio of chloroform and DMF was adjusted to tailor the fiber surface morphology (Table 5.2).
Table 5.2 Mixture compositions and working conditions

<table>
<thead>
<tr>
<th>No.</th>
<th>CDHA ratio (%)</th>
<th>Chloroform:DMF (ml/ml)</th>
<th>Working Distance (cm)</th>
<th>Applied Voltage (kV)</th>
<th>Feeding Rate (ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0%</td>
<td>9:1</td>
<td>25</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0%</td>
<td>7:3</td>
<td>25</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>25%</td>
<td>9:1</td>
<td>25</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>25%</td>
<td>7:3</td>
<td>25</td>
<td>20</td>
<td>1</td>
</tr>
</tbody>
</table>

5.3.3 Electrospinning approach

A horizontal electrospinning setup was used in this study to fabricate fibrous composite scaffold. It was composed of a high voltage power supply, a pump, a syringe, a flat tip needle and a conducting collector plate. In general, the morphology of fibers can be influenced by various processing parameters such as applied voltage, feeding rate, and working distance [179]. The operating parameters applied in the experiment were based on our previous research (unpublished data). A certain volume of PLA/CDHA solution (constant total mass/volume ratio at 1:10 (g/ml)) was loaded into the syringe and injected through a needle (gauge 22) at constant operating parameters to fabricate PLA/CDHA fibers containing 25% CDHA mass ratio; neat PLA fibers were also electrospun with the same operating parameters as control (Table 5.2). Fibers were collected and left to dry in a fume hood to remove residual solvents. Once no weight loss of as-produced fibers was observed by using XP105 DeltaRange analysis balance (Mettler Toledo), residual solvents were considered to be totally removed.

5.3.4 Physical Evaluation

CDHA nanocrystals, PLA pellets, and as-fabricated PLA/CDHA fibers all were characterized by x-ray diffraction (XRD, Ultima III, Rigaku) with monochromated Cu
Kα radiation, operated at voltage 40 kV and 44 mA setting. All samples were examined at 20 angles from 10° to 45° at a scanning speed of 1° per minute. Fourier transform infrared spectroscopy (FTIR, UMA-600 Microscope, Varian Excalibur Series) was also applied for chemical analysis of nanocrystals and fibers. The transmittance of each sample was recorded with 256 scans with resolution of 4 cm\(^{-1}\) between 4000 and 700 cm\(^{-1}\). The morphological features of precipitates and fibers were visualized by scanning electron microscope (SEM, S4800, Hitachi). Energy dispersive x-ray spectroscopy (EDS) analysis was applied to examine the distribution of precipitates in fibers. Nano-structures of precipitates and PLA/CDHA composites were investigated using transmission electron microscopy (TEM, HD-2300, Hitachi) with a voltage 200 kV and the zeta contrast mode.

5.3.5 In vitro degradation tests

To clearly prove the degradation efficiency of CDHA against HA, a comparison of degradation between CDHA and synthesized HA was carried out. HA was synthesized via chemical method using Ca(OH)\(_2\) and H\(_3\)PO\(_4\). Both the chemicals were purchased from Fisher Scientific. A measured weight of 0.05g powders was added into tubes containing 15 ml phosphate buffer solution (PBS) (pH: 7.4), and all tubes are incubated in vitro at 37°C for different times. Three samples of each type were taken out at the end of each degradation period, and pH change of the PBS with time was measured. Centrifugation was applied to pipette out PBS, and powders were washed using DI water and dried in air to remove residential water. The weights of dried powders were measured.

PLA/CDHA and PLA fibers (chloromform:DMF ratio is 7:3) were electrospun on to microscope cover glasses (18 mm diameter, purchased from Fisher Scientific) for in vitro
degradation test. The weight of each glass substrate and sample were recorded and placed in closed tubes containing 25 mL PBS and incubated in vitro at 37°C for different times. Three samples of each type were taken out from the incubator at the end of each degradation period, pH values of the PBS and the weights of the dried samples were measured.

5.3.6 Bioactivity test

For a material to be bioactive in vivo, it must have the ability to induce apatite formation on its surface in vitro. In order to show that the bioactivity of CDHA and PLA was maintained in the process of mixture preparation and electrospinning, PLA/CDHA and PLA fibers (chloroform:DMF ratio is 7:3) were electrospun to microscope cover glass (18 mm diameter, purchased from Fisher Scientific) for bioactivity test. Samples were placed in closed tubes containing 25 mL 1.5x t-SBF and incubated in vitro at 37°C for one week with solution replenished every 48 hours. The chemical compositions of 1.5x t-SBF are shown in Table 5.3. The apatite formation on fibers surface were characterized using XRD and SEM.

Table 5.3 1.5x t-SBF solution composition for a total volume of 1L

<table>
<thead>
<tr>
<th>Order</th>
<th>Reagent</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaCl</td>
<td>9.8184</td>
</tr>
<tr>
<td>2</td>
<td>NaHCO₃</td>
<td>3.4023</td>
</tr>
<tr>
<td>3</td>
<td>KCl</td>
<td>0.5591</td>
</tr>
<tr>
<td>4</td>
<td>Na₂HPO₄</td>
<td>0.2129</td>
</tr>
<tr>
<td>5</td>
<td>MgCl₂.6H₂O</td>
<td>0.4574</td>
</tr>
<tr>
<td>6</td>
<td>1M HCL</td>
<td>15 mL</td>
</tr>
<tr>
<td>7</td>
<td>CaCl₂.2H₂O</td>
<td>0.5822</td>
</tr>
<tr>
<td>8</td>
<td>Na₂SO₄</td>
<td>0.1080</td>
</tr>
<tr>
<td>9</td>
<td>Tris-Base</td>
<td>9.0855</td>
</tr>
<tr>
<td>10</td>
<td>1M HCl</td>
<td>50 mL</td>
</tr>
</tbody>
</table>
5.3.7 Osteoblast cell culture

7F2 mouse osteoblast cells (CRL-12557, American Type Culture Collection) were used for cell culture studies on PLA/CDHA (3:1) fibers (chloromform:DMF ratio is 7:3). PLA/CDHA fibers were fixed onto microscope cover glass strips (Fisher Scientific) to ensure they can settle in the wells with medium. PLA fibers were also fixed as controls, and microscope cover glass strips were used as blank. Osteoblast cells are mononucleate cells that are responsible for bone formation. Cells were first grown at 37°C and 5% CO₂ in alpha minimum essential medium (α-MEM, Thermo Scientific HyClone), augmented by 10% Fetal Bovine Serum (FBS, Thermo Scientific HyClone). The culture medium was replenished every other day until the cell reached a confluence of 90%. Osteoblasts were seeded to wells (BD Flacon™ 12 wells cell culture plates) of sterilized PLA and PLA/CDHA fibers (chloromform:DMF ratio is 7:3), approximately 20,000 osteoblast cells were seeded to each well. Cell numbers on samples were counted after 24 hours, 4 days and 7 days using CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega). For statistical analysis, all experiments were performed at least triplicate. Osteoblast morphology after attachment was examined using SEM. Glutaraldehyde and HMDS were purchased from Fisher Scientific, ethanol was purchased from Pharmco-Aaper. These chemicals were used for SEM samples preparation. Prior to SEM characterization, samples were soaked in primary fixative of 3.5% glutaraldehyde. Subsequently samples were washed by PBS buffer and dehydrated with increasing concentrations of ethanol (30%, 50%, 70%, 90%, 95%, 100%) for 15 minutes each. After the dehydration of ethanol, samples were kept in solution of HMDS (hexamethyldisilazane)-ethanol (1:2, v/v) for 15 minutes, HMDS-ethanol (2:1, v/v) for 15 minutes, and 2 changes for 15
minutes each with 100% HMDS. Finally, HMDS was removed and samples were air dried in a hood overnight. The samples were then ready to be sputter coated with gold for SEM characterization.

5.4 Results

5.4.1 Physical Evaluation

The XRD patterns of precipitated nanocrystals, PLA pellets, PLA fibers and PLA/CDHA fibers are shown in Fig. 5-1. For precipitates, the main XRD peaks at 20 angle 25.9° and 31.8° confirmed the formation of HA lattice (JCPDS-ICDD card no 18-03030). The crystal domain sizes of precipitates along the c direction were calculated by JADE software based on Scherrer’s equation using (002) diffraction peak, and were equal to 210 Å. PLA pellets examined by XRD showed high crystallite with a strong peak observed at 16.5°. Both PLA and CDHA XRD peaks were observed on the XRD patterns of PLA/CDHA fibers, which confirmed the presence of CDHA and PLA in the composite fibers.

![XRD patterns](image)

Fig. 5-1 XRD patterns of (a) CDHA nanocrystals, (b) PLA pellets, (c) PLA/CDHA fibers

The FTIR data of nanocrystalline CDHA, PLA, and PLA/CDHA fibers are shown in
Fig. 5-2. From FTIR data of CDHA, the presence of carbonate in nanocrystalline CDHA was confirmed by the absorption bands observed at 1470-1420 and 875 cm$^{-1}$. Absorption band of PO$_4^{3-}$/HPO$_4^{2-}$ belonged to CDHA at 1040-1020 cm$^{-1}$ was also observed on FTIR data of PLA/CDHA fibers, which confirmed the presence of CDHA nanocrystals in PLA/CDHA fibers. PLA/CDHA fibers also showed a new absorption peak appeared at 1600 cm$^{-1}$ as compared to PLA and CDHA nanocrystals.

[Figure 5-2: FTIR data of (a) CDHA nanocrystals, (b) PLA pellets, (c) PLA/CDHA fibers]

Images of Fig. 5-3a through Fig. 5-3g were the results of SEM characterization. As shown in Fig. 5-3a, CDHA precipitates were consisted of needle-like nano-crystals. In Fig. 5-3b, the presence of elements Mg and Na in precipitates were observed by EDS analysis. XRD and FTIR data demonstrated above combined with the EDS analysis proved the precipitates were exactly CDHA. PLA fibers electrospun with different organic solvents composition are shown in Fig. 5-3c and Fig. 5-3d, higher DMF ratio in solvent not only reduced fibers diameter but also eliminated porous surface of PLA fibers. Such differences were also observed in Fig. 5-3e and Fig. 5-3f, which were fibers from PLA/CDHA solution with different organic solvents ratios. The SEM images show that the size of the fibers was decreased by the incorporation of CDHA filler to PLA matrix.
PLA/CDHA fibers shown in Fig. 5-3f are fibers in nano-size. The EDS elements mapping shown in Fig. 5-3g and Fig. 5-3h demonstrated the uniform distribution of precipitates in fibers.

Fig. 5-3 Results of SEM characterization: (a) CDHA precipitates, (b) EDS analysis of CDHA precipitates, (c) PLA fibers, chloroform:DMF ratio is 9:1, (d) PLA fibers, chloroform:DMF ratio is 7:3, (e) PLA/CDHA fibers, chloroform:DMF ratio is 9:1, (f) PLA fibers, chloroform:DMF ratio is 7:3, (g) EDS mapping of Ca, (h) EDS mapping of C

TEM images of CDHA precipitates are shown in Fig. 5-4a. Precipitates were needle
like nano-crystals with dimensions of 20-40 nm in c-axis. In Fig. 5-4b is the TEM images of PLA/CDHA fibers, where CDHA particles were dispersed in the internal channel of PLA matrix as fillers. Some CDHA agglomerates were also observed.

![Fig. 5-4 TEM images of (a) as-produced CDHA precipitates, (b) PLA/CDHA fibers, chloroform:DMF ratio is 9:1](image)

5.4.2 In vitro degradation tests

The comparison of degradation rate between CDHA and HA is shown in Fig. 5-5a. CDHA is also slightly more basic to cause pH of the solution to slightly increase as shown in Fig. 5-5b. Fig. 5-5c is the mass loss behavior of PLA/CDHA fibers and PLA fibers in PBS, PLA/CDHA fibers showed a faster degradation rate as compared to PLA fibers. Fig. 5-5d is the pH change of PBS caused by degradation of PLA/CDHA and PLA fibers. Both fibers led to pH decrease in the first weeks, but the pH of PBS incubated with PLA/CDHA fibers was buffered back to almost 7.4 in the following weeks as compared to decreasing pH of PBS incubated with PLA fibers.
Results of in vitro degradation test: (a) weight loss of CDHA and HA particles in PBS, (b) pH change of PBS caused by CDHA and HA degradation, (c) weight loss of PLA/CDHA and PLA fibers in PBS, (d) pH change of PBS caused by PLA/CDHA and PLA fibers degradation.

5.4.3 Bioactivity test

Results of bioactivity test were shown in Fig. 5-6. Both PLA and PLA/CDHA fibers surfaces were deposited with apatite (Fig. 5-6a&6b). However, the apatite coating on PLA/CDHA fibers were more uniform. The XRD results also indicated the same results with stronger apatite peaks on PLA/CDHA fibers (Fig.7).
5.4.4 Osteoblast cell culture

Results of *in vitro* cell culture are shown in Fig. 5-8. The results were compared by
One-way ANOVA analysis: PLA/CDHA electrospun fibers can promote osteoblast cells growth after 7 days as compared to PLA electrospun fibers and blank (p<0.05). Osteoblast cells exhibited a flat appearance and were spread out over the surfaces of all samples. Formation of nano-CaP particles expressed by osteoblasts on PLA/CDHA fibers after 7 days was observed as shown in Fig. 5-9.

![Graph showing cell numbers over time](image)

**Fig. 5-8 Results of *in vitro* cell culture on PLA and PLA/CDHA fibers**

![SEM image of nano-CaP particles](image)

**Fig. 5-9 SEM image of nano-CaP particles expressed by osteoblast cells after 7 days proliferation on PLA/CDHA fibers**
5.5 Discussion

Bone-like CDHA is an important compound among CaP materials because of its bioactivity [18]. CDHA composition is relatively close to the mineral component of bone, which is poorly crystalline hydroxyapatite (HA) with small amounts of elements such as sodium (Na$^+$), potassium (K$^+$), magnesium (Mg$^{2+}$), and carbonate (CO$_3^{2-}$) present in the lattice structure, as compared to crystallized CaP materials. These extra elements were reported to influence cell attachment, proliferation of osteoblasts and help in bone metabolism [29, 262]. Based on the results of XRD, SEM, TEM, FTIR and EDS characterization of precipitates, bone-like CDHA nanocrystals were successfully synthesized from the spontaneous precipitation occurred in modified 1.5x t-SBF. The crystal domain size of CDHA precipitates was similar to that recorded deproteinated bone apatite, quantitatively confirmed by the similarity of the two crystal domain sizes (210 and 213 Å, respectively) [264].

One function of addition of tris-base buffer in SBF is to make pH of SBF solution stable in a long period of time as described above. The purpose of reducing the tris-base amount is to intensify the precipitation of CDHA, and the result after 24 hour precipitation fit the expectation. How to harvest CDHA precipitates is still a concern of CDHA application in industry in future. One possible approach is to use highly supersaturated SBF solution to produce precipitates with similar compositions as bone minerals [16, 36].

In degradation test, CDHA was observed to show a faster degradation as compared to stoichiometric HA, which can be attributed to the presence of trace elements in lattice structure. A pH increase caused by CDHA degradation in PBS was also observed. This
behavior can be possibly attributed to a series of chemical reactions occurred in 1.5x
t-SBF during CDHA precipitation. A phenomenon that pH of SBF solution increased
spontaneously and finally reached a stable value caused by decomposition of bicarbonate
ions has been reported before [31, 265]. In the CDHA precipitation process, OH\(^{-}\) ions are
incorporated into the lattice structure of CDHA, which can be released into PBS to
increase pH when degradation of CDHA occurred. Therefore, when CDHA nanocrystals
were used as second phase in PLA matrix, they can buffer the pH decrease caused by the
degradation of PLA as shown in Fig. 5-5d.

The incorporation of CaP materials with PLA has been a popular topic in biomaterial
research. Many approaches were studied such as electrospinning, casting, and
supercritical gas foaming [37]. However, no studies were reported on electrospun of
CDHA and PLA bionanocomposite. This is the main highlight of this study.
Electrospinning is a process to fabricate fibrous scaffold with high surface area and
interconnected channels suitable for tissue regeneration. This process can be operated at
ambient temperature, which is beneficial to the preservation of carbonate in CDHA lattice.
Additionally, such electrospun PLA/CDHA scaffold shows possibility to be used as
bio-molecules delivery system. PLA is a conventional biodegradable material used for
drug carrier, and act as barrier to avoid robust release of carried molecules; CDHA can
work as drug loading sites for bio-molecules.

The XRD patterns and FTIR data of PLA/CDHA fibers (Fig. 5-1 and Fig. 5-2)
confirmed the presence of CDHA and PLA in the composite fibers. The results were
similar to PLA/HA fibers reported in the literature before [217]. The new absorption band
of PLA/CDHA fibers observed on FTIR was assigned to COO\(^{-}\) resulting from the
interaction of COOH in PLA with Ca$^{2+}$ in CDHA lattice. Therefore, CDHA precipitates were not only dispersed in the PLA matrix, but also reacted with PLA, resulting in chemical bonding between each other.

As shown in Fig. 5-3, the compositions of solvents were observed to influence fibers morphology. Higher DMF content not only decreased fibers diameter but also eliminated porous structure on fiber surface. Its effect on fiber diameter is attributed to its higher conductivity as compared to chloroform, which consequently increased electromagnetic force during electrospinning which stretched jet into thinner fibers [258]. The difference in pore structure can be explained by the volatility difference between DMF and chloroform. The low volatility of DMF led to a decrease in the evaporation rate of the mixed solvents to form stable jet being injected from the needle during electrospinning. Otherwise, quick evaporation caused by chloroform produced pores on PLA fibers surface. Such phenomenon caused by quick solvent evaporation was also described previously in electrospinning of biopolymers [266, 267]. The incorporation of CDHA to PLA matrix can also decrease fibers diameter, which is related to the change in viscosity of PLA solution caused by CDHA additives and the chemical bonding formed between CDHA and PLA in PLA/CDHA fibers. Due to dual effects of DMF and interaction between PLA/CDHA, fibers diameter was further reduced.

The TEM images of electrospun PLA/CDHA fibers showed dispersion of CDHA in PLA matrix (Fig. 5-4b). Only PLA/CDHA fibers (chloroform:DMF ratio is 9:1) were characterized using TEM because small diameter fibers get easily damaged by electron beam in TEM. The use of zeta contrast mode provided some benefits for fibers characterization as compared to normal TEM. First, the intra-fiber structure formed on
the fibers surface was analyzed. Internal channels were observed with connections to outer pores. Therefore, PLA works as a shield with other co-electrospun materials in the inner cores, and such nano-pores can potential provide channels for bio-molecules diffusion. Second, the existence of CDHA and its dispersion level can also be characterized. As shown in Fig. 5-4b, there are some agglomerates of CDHA particles in PLA/CDHA electrospun fibers. As described above, CDHA particles were formed via nucleation during precipitation process, which usually promotes some weak bonding between the CDHA nanocrystals. Therefore, physical force applied via ultrasonication in this experiment was not strong enough to destroy such bonding; stronger ultrasonication or external physical force is necessary in future to finely disperse CDHA particles.

The *in vitro* degradation test showed incorporation of CDHA to PLA matrix promotes faster degradation rate. This mechanism of degradation of PLA matrix with added CDHA is similar to degradation of PLGA with added TCP (tricalcium phosphate.) In an experiment reported by Loher et al., pure PLGA matrix only had 0.5% mass loss and 2% water uptake after six weeks, while 30% α-TCP incorporation resulted in 7% mass loss and 14% water uptake after six weeks [168]. The high degradation rate of α-TCP particles generated pores in the composite to result in a larger polymer surface being exposed to the surrounding media to promote PLGA degradation. It is believed that the same mechanism is applicable in this case as well. A larger section of PLA surface is exposed to the surrounding media due to the degradation of CDHA. The accumulation of hydrophilic degradation products of CDHA inside the PLA/CDHA fibers, further draws media into PLA matrix during the whole degradation process. In comparison, in previously published papers about PLA/HA electrospun fibers, HA acted as a physical
barrier and blocked off the entry of water to slow down PLA degradation [225, 266]. The most important result of pH monitoring is that the adverse effects caused by PLA degradation can be overcome by the buffering effect of CDHA.

Formation of apatite on the surface of samples depends on the materials’ intrinsic ability to form apatite in SBF. The ability of biomaterials to develop bone-like apatite upon soaking in SBF has been correlated to bone bonding formation ability in vivo [218]. Both CDHA nanocrystals and PLA can play roles in apatite deposition. CDHA nanocrystals were similar to apatite formed in 1.5x t-SBF, which can work as nucleation sites for apatite deposition. The apatite formation mechanism PLA is related to the hydrolysis of PLA, which results in a negatively charged PLA surface. The positively charged calcium ions in the solution are attracted to the hydrolyzed PLA surface and apatite forms through the attraction of phosphate groups from the solution [27]. The difference of coating thickness and uniformity observed between PLA and PLA/CDHA fibers can be attributed the incorporation of CDHA into the PLA matrix to intensify the apatite coating formation. Therefore, the apatite formation on PLA/CDHA coatings demonstrates their potential bone bonding ability.

Incorporation of CDHA into PLA matrix promotes osteoblasts cells proliferation as shown in Fig. 5-8, which further corroborates the results of bioactivity test. The results confirmed the important role of the CDHA in the stimulation of osteoblast cell response and thus its significance in the bone regeneration.

Based on these results, PLA/CDHA electrospun fibers can be applied for potential bone tissue regeneration. More studies are still required to assess the practical usefulness of this composite fiber, such as the assessment of bone tissue engineering potential with
stem cells.

5.6 Conclusion

In this study, bone-like carbonated calcium deficient nanocrystalline CDHA particles were synthesized using a biomimetic technique using 1.5X t-SBF. Ultrasonication of CDHA nanocrystals and choice of chloroform and DMF in certain ratios (7:3) led to the uniform distribution of CDHA in PLA resulting in bionanocomposites with each of the constituents with one dimension in nanometer scale. The results show that the incorporation of CDHA decreased PLA fiber diameters, accelerated PLA degradation, buffered pH decrease caused by PLA degradation, improved the bioactivity and biocompatibility of the scaffold. Such PLA/CDHA bionanocomposite has the potential to be used in tissue regeneration applications.
Chapter 6

Deposition of Bisphosphonate Incorporated PLA/CDHA Composite Coating via Electrospraying

6.1 Abstract

Composite coatings composed of carbonated calcium deficient hydroxyapatite (CDHA) and poly lactic acid (PLA) were deposited to Ti6Al4V via electrospraying. The operation parameters, structural properties, bioactivity, cell adhesion, and growth capability of as-fabricated PLA/CDHA coatings were investigated. The as-deposited composite coating show good biocompatibility and bioactivity. The as-deposited coating is applied as carrier for alendronate sodium (AS) local release. AS, an approved bisphosphonate (Bp) drug used for the treatment of osteoporosis, was incorporated into composite coatings matrix via co-electrospraying. Its release behavior was studied, showing a long-term sustained release. This approach can be a potential coating technique in biomedical field.

6.2 Introduction

There are two main themes in this chapter: one is to develop composite coatings composed of carbonated calcium deficient hydroxyapatite (CDHA) and poly lactic acid (PLA) on titanium (Ti) alloys Ti6Al4V by electrospraying; second is to incorporate alendronate sodium (AS), an approved bisphosphonate (Bp) drug used for the treatment
of osteoporosis, to the composite coatings for long-term local release. There are many interesting concepts combined in this chapter. First, the composite coatings deposition process is performed at low-temperatures, enabling the retention of important phases such as carbonate and bio-molecules into coatings. Second, both CDHA and PLA are biocompatible and biodegradable [95]. Therefore, the surrounding tissues can replace them and form interfacial bonds to Ti6Al4V substrates. Additionally, osseointegration at Ti6Al4V surface can be promoted by the presence of biologically active CDHA. Third, the coating setup is very simple and the whole coating process is controllable and can be finished in a few minutes. Fourth, as opposed to the simple dip coating [268], the electrospraying process has the ability to produce a surface topography, useful for cell attachment and proliferation. Fifth, CDHA acts as a buffer towards pH decrease caused by the degradation of PLA [95]. Finally, AS was incorporated into this composite coatings and showed long-term sustained release behavior.

Calcium phosphate (CaP) materials are often used as biologically active coatings on implant surfaces due to their similarity with the mineral phase present in bone and teeth [269]. Coating procedures that load bio-molecules into CaP coatings for enhanced osseointegration or disease treatment are of high interests of lately [270]. The most popular CaP coating process now is plasma-spraying technique, due to its high deposition rate and the ability to coat large areas [270]. However, important phases such as carbonated apatite (which is important in bone composition and metabolism) and biological agents such as growth factors cannot be deposited using plasma-spraying due to the high temperature condition [46, 270]. Biomimetic coating process based on the application of simulated body fluids (SBF) is one promising technique for generating
such bio-molecules loaded apatite coatings on implant materials [271]. The limitation is time consuming in an industry operation [272]. Electrospraying is considered as a promising technique to deposit CaP coatings onto implant surfaces [273, 274]. It has a simple set-up, with high deposition efficiency. In general, an organic solvent containing CaP particles or CaP precursors is pumped through a nozzle; and a high voltage is applied between the nozzle and heated substrate. Consequently, droplets coming out the nozzle disperse into a spray, and this spray is deposited upon the substrate. When the solvent has evaporated, a coating is formed on the substrate. However, the application of high temperature in this electrospraying process has the same disadvantage as plasma-spraying.

In drug delivery field, electrospraying has been employed to generate biodegradable polymer micro-particles loaded with drug [275, 276]. These biodegradable polymers can assist sustained bio-molecules release prolife as barrier system. As an alternative to microparticles, biodegradable polymers have been fabricated as coatings with/without bio-molecules on metallic-implant surface via electrospraying under normal temperate [277, 278].

Biopolymers have been combined with CaP particles to construct biopolymers/CaP composite, in which biopolymers work as continuous phase responsible for filling the volume, as well as it surrounds, and supports the dispersed materials by maintaining their relative positions; and CaP acts as the dispersed phase responsible for enhancing one or more properties of the matrix [264]. Therefore, it is possible to apply CaP as filler into biopolymer coatings on metallic-implant surface to improve coatings chemical and physical properties. In addition, with the physical support of biopolymers, no extra
thermal treatment is required to maintain CaP particles in relative positions. Furthermore, bio-molecules can be co-sprayed with biopolymer/CaP solution onto implant surface for promoting tissue regeneration.

In this chapter, we report the deposition of AS incorporated PLA/CDHA composite coatings onto the surface of Ti6Al4V substrate via electrospraying. This chapter describes: 1) incorporate the CDHA nanoparticles into the PLA coating via the process of electrospraying, 2) influences of working parameters to coatings topology, 3) evaluations of the physical properties, bioactivity, cell adhesion, and growth capability of as-fabricated PLA/CDHA electrosprayed coatings, and 4) release behavior of incorporated AS from composite coatings.

6.3 Materials and Methods

6.3.1 Substrates

Ti6Al4V plate (McMaster-Carr) was cut into small strips with dimensions of 10×10×1 mm. The strips were polished by #1000 silicon carbide (SiC) paper (Struers). They were pretreated by 20% H₂O₂ at 37°C environment for 24 hours to create titanium oxide layer on surface. Finally they were cleaned using ethanol and acetone prior to coating deposition.

6.3.2 CDHA nanocrystals synthesis

CDHA was produced using modified 1.5x t-SBF, with reduced content of Tris-base (C₄H₁₁NO₆) compared to conventional 1.5x t-SBF reported before [95]. The compositions are shown in Table 6.1. NaHCO₃ and Na₂SO₄ were purchased from Acros Organics. NaCl, KCl, NaH₂PO₄, MgCl₂.6H₂O, CaCl₂.2H₂O, Tris-base buffer (C₄H₁₁NO₆), and HCl were purchased from Fisher Scientific. The reagents were dissolved in solution
one by one, and added based on the order listed in Table 6.1. The solution was later stored at 37˚C environment for 24 hours. Later on, precipitates were collected after filtration and dried in 60˚C oven overnight.

Table 6.1. Modified 1.5x t-SBF solution compositions for a total volume of 1L

<table>
<thead>
<tr>
<th>Order</th>
<th>Reagent</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaCl</td>
<td>9.8184</td>
</tr>
<tr>
<td>2</td>
<td>NaHCO₃</td>
<td>3.4023</td>
</tr>
<tr>
<td>3</td>
<td>KCl</td>
<td>0.5591</td>
</tr>
<tr>
<td>4</td>
<td>Na₂HPO₄</td>
<td>0.2129</td>
</tr>
<tr>
<td>5</td>
<td>MgCl₂.6H₂O</td>
<td>0.4574</td>
</tr>
<tr>
<td>6</td>
<td>1M HCL</td>
<td>15 mL</td>
</tr>
<tr>
<td>7</td>
<td>CaCl₂.2H₂O</td>
<td>0.5822</td>
</tr>
<tr>
<td>8</td>
<td>Na₂SO₄</td>
<td>0.1080</td>
</tr>
<tr>
<td>9</td>
<td>Tris-Base</td>
<td>6.063</td>
</tr>
<tr>
<td>10</td>
<td>1M HCl</td>
<td>29 mL</td>
</tr>
</tbody>
</table>

6.3.3 PLA/CDHA solution preparation

PLA pellets were provided by Jamplast (Ellisville, Mo). The weight average molecular weight (Mw) and poly dispersity of PLA are 114,000 and 1.435 respectively. These values were determined by gel permeation chromatography relative to polystyrene standards using a Shimadzu LC–10ADVP liquid chromatography equipped with a Shimadzu ELSD-LT ultraviolet (UV) detector (Columbia, MD). PLA pellets were initially dissolved in chloroform (CHCl₃) in a 1.5:20 (w/v) ratio. After that, CDHA nanocrystals were added to PLA solution to make a mixture (1:3, w/w). To uniformly disperse CDHA nanocrystals in solution, the mixture was treated by 10 min ultrasonication to destroy any CDHA agglomerates. Followed by 4 hours stirring, solution was ready for electrospraying.
6.3.4 Electrospraying

A horizontal electrospraying setup was used in this study to deposit composite coatings to Ti6Al4V substrates as shown in Fig. 6-1. It was composed of a high voltage power supply, a pump, a syringe, a flat tip needle and a conducting collector plate. Ti6Al4V substrates were fixed on the collecting plate. Solution loaded in syringe was injected through a needle (gauge 20) at different injection rates (2 ml/h, 20 ml/h, 30 ml/h, 40 ml/h) under controlled voltages (5 kV, 10 kV, 15 kV, 20 kV, 25 kV), with varied working distances (5 cm, 10 cm, 15 cm, 20 cm, 25 cm) to evaluate the influences of electrospraying parameters on PLA/CDHA coatings. Finally, fabrication of hybrid coatings via electrospraying was performed for 30 s based on optimized operation parameters (30 ml/h, 10 kV, 10 cm), and coated samples were dried in air for 48 hours to remove residual solvents.

Fig. 6-1. Electrospraying setup for PLA/CDHA coatings deposition

6.3.5 Characterization

CDHA, PLA and composite coatings all were characterized by X-ray diffraction (XRD, Ultima III, Rigaku) with monochromated Cu Kα radiation, operated at a voltage 40 kV and a current 44 mA setting. All samples were examined at 2θ angles from 10° to
45° at a scanning speed of 1° per minute. Fourier transform infrared spectroscopy (FTIR, UMA-600 Microscope, Varian Excalibur Series) was also applied for chemical analysis of CDHA, PLA and composite coatings. The transmittance of each sample was recorded with 256 scans with resolution of 4 cm⁻¹ between 4000 and 700 cm⁻¹. The morphological features of CDHA particles, and composite coatings were visualized by scanning electron microscope (SEM, S4800, Hitachi). Energy dispersive X-ray spectroscopy (EDS) analysis was applied to examine the chemical composition of CDHA nanocrystals and the distribution of CDHA nanocrystals in coatings.

6.3.6 Bioactivity testing

For a material to be bioactive in vivo, it must have the ability to induce apatite formation on its surface in vitro. Oxidized Ti6Al4V substrates with/without PLA/CDHA coatings were soaked into 1.5x t-SBF solution (chemical compositions is shown in Table. 6.2) for 7 days with solution replenished every 48 hours to evaluate the bioactivity improvement on substrate surface caused by PLA/CDHA coatings.

Table 6.2. 1.5x t-SBF solution compositions for a total volume of 1L

<table>
<thead>
<tr>
<th>Order</th>
<th>Reagent</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaCl</td>
<td>9.8184</td>
</tr>
<tr>
<td>2</td>
<td>NaHCO₃</td>
<td>3.4023</td>
</tr>
<tr>
<td>3</td>
<td>KCl</td>
<td>0.5591</td>
</tr>
<tr>
<td>4</td>
<td>Na₂HPO₄</td>
<td>0.2129</td>
</tr>
<tr>
<td>5</td>
<td>MgCl₂.6H₂O</td>
<td>0.4574</td>
</tr>
<tr>
<td>6</td>
<td>1M HCL</td>
<td>15 mL</td>
</tr>
<tr>
<td>7</td>
<td>CaCl₂.2H₂O</td>
<td>0.5822</td>
</tr>
<tr>
<td>8</td>
<td>Na₂SO₄</td>
<td>0.1080</td>
</tr>
<tr>
<td>9</td>
<td>Tris-Base</td>
<td>9.0855</td>
</tr>
<tr>
<td>10</td>
<td>1M HCl</td>
<td>50 mL</td>
</tr>
</tbody>
</table>
6.3.7 *In vitro testing*

7F2 mouse osteoblast cells (CRL-12557, American Type Culture Collection) were used for cell attachment studies on the composite coatings. Oxidized Ti6Al4V substrates and PLA-Ti6Al4V substrates were used as comparison. PLA-Ti6Al4V substrates were produced by depositing melting PLA to the surface of Ti6Al4V substrates followed by quenching. Cells were first grown at 37°C and 5% CO2 in alpha minimum essential medium (α-MEM, Thermo Scientific HyClone), augmented by 10% Fetal Bovine Serum (FBS, Thermo Scientific HyClone). The culture medium was replenished every other day until the cell reached a confluence of 90%. Osteoblasts were seeded to wells (BD Flacon™ 12 wells cell culture plates) of sterilized strips, approximately 20,000 osteoblast cells were seeded to each well. Cell numbers on strips were counted after 24 hours and 4 days using CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega). For statistically meaningful results, all experiments were performed triplicate. Osteoblast morphology after 4 days was examined using SEM. Glutaraldehyde and HMDS were purchased from Fisher Scientific, ethanol was purchased from Pharmco-Aaper. These chemicals were used for SEM samples preparation. Prior to SEM characterization, samples were soaked in primary fixative of 3.5% glutaraldehyde. Subsequently samples were washed by PBS buffer and dehydrated with increasing concentrations of ethanol (30%, 50%, 70%, 90%, 95%, 100%) for 15 minutes each. After the dehydration of ethanol, samples were kept in solution of HMDS (hexamethyldisilazane)-ethanol (1:2, v/v) for 15 minutes, HMDS-ethanol (2:1, v/v) for 15 minutes, and 2 changes for 15 minutes each with 100% HMDS. Finally, HMDS was removed and samples were air dried in a hood overnight. The samples were then ready to be sputter coated with gold for
SEM characterization.

6.3.8 AS incorporation and release

AS was added to PLA/CDHA solution (0.3% w/v), with ultrasonication and magnetic stirring. After 2 hours stirring, the solution was loaded to syringe and electrosprayed (30 ml/h, 10 kV, 10 cm) to Ti6Al4V plates as coatings. The coatings were scratched off and incubated in 2 ml micro centrifuge tube with 600 ml PBS for AS release study. AS loaded PLA coating was also fabricated as control group using same operation parameters. In different time period, 200 ml PBS was removed for analysis and 200 ml PBS fresh solution was refilled to the micro-centrifuge tube to keep constant solution volume. A reverse phase High Performance Liquid Chromatography (HPLC) protocol utilizing pre-column derivatization of the primary amine group of AS with 9-fluorenlymethyliclorafoformate (FMOC, C₁₅H₁₁ClO₂) was used to obtain the AS release profile. Chemicals for HPLC analysis; sodium citrate (Na₃C₆H₅O₇), sodium phosphate dibasic anhydrous (Na₂HPO₄), acetonitrile (CH₃CN), methanol (CH₃OH), dichloromethane (CH₂Cl₂), orthophosphoric acid (H₃PO₄), sodium tetraborate decahydrate (Na₂B₄O₇·10H₂O), and FMOC were purchased from Fisher Scientific (NJ). Solution containing 0.1 M sodium citrate and 0.1 M sodium borate and FMOC solution (0.1%, w/v) was prepared (FMOC was dissolved in acetonitrile). The chromatographic analyses were performed using the HPLC system (Agilent Technologies HPLC 1100) with a reverse phase (PRP-1) column (10 μm particle size, 250 x 4.1 mm, Hamilton, NV). The mobile phase was prepared by mixing methanol, acetonitrile and a buffer containing 0.05 M sodium citrate and 0.05 M sodium phosphate dibasic anhydrous (pH 8, adjusted using orthophosphoric acid). The volume ratio of methanol, acetonitrile, and buffer was 5:20:75. 200μL collected solution
was removed to 2mL centrifuge tube. And then 200μL solution containing 0.1M sodium citrate and 0.1M sodium borate, and 160μL FMOC solution were added. The mixed solution was then kept at room temperature for 30 minutes for the derivatization to proceed. After 30 minutes, 1mL dichloromethane was added and the tube was vortexed for another 30 seconds, then centrifuged at 1500 rpm for 15 minutes to remove the excessive reagent from the aqueous layer. Finally, about 200μL aqueous layer was transferred to an HPLC vial. A flow rate of 0.5 ml/min was used in the HPLC at room temperature. FMOC derivative was detected using a spectrophotometric detector operated at a wavelength of 266 nm.

6.4 Results

The effect of flowing rate of coating materials on the morphology of the coated surface was examined. Fig. 6-2 shows the morphology of coated surface while varying the flowing rate of PLA/CDHA mixture. It was observed maintaining the applied voltage, working distance while increasing flowing rate resulted in transition of deposited products from fibers with beads to beads with high porosity surface. These micro-beads can overlap each other to form coatings with high roughness. The effect of working distance on the beads size and morphology are shown in Fig. 6-3. Low working distance induced the presence of CDHA nanocrystals on the surface of beads instead of dispersed in PLA matrix. On contrast, high working distance increased the surface porosity of deposited beads. The coatings deposited using various applied voltages are shown in Fig. 6-4. High applied voltage also induced the presence of CDHA nanocrystals on the surface of beads, and favored individual beads deposition.
Fig. 6-2. SEM images of products performed at different flowing rate (working distance was 20 cm, working voltage was 15 kV): (a) 2 ml/h (x50), (b) 2 ml/h (x2.5 k), (c) 30 ml/h (x50), (d) 30 ml/h (x2.5 k)

Fig. 6-3. SEM images of beads formed at different working distance (flowing rate was 30 ml/h, working voltage was 25 kV): (a) 10 cm, (b) 15 cm, (c) 20 cm

Fig. 6-4. SEM images of beads formed at different working voltage (flowing rate was 30 ml/h, working distance was 10 cm): (a) 5 kV, (b) 10 kV, (c) 25 kV

The SEM and EDS results of CDHA are shown in Fig. 6-5. Precipitates were
consisted of nano-crystals with dimensions of 20-40 nm in c-axis. These CDHA nanocrystals were aggregated as complex. The presence of elements Ca, P, Mg and Na were observed in precipitates via EDS analysis.

Fig. 6-5. CDHA nanocrystals characterization: (a) SEM image of CDHA nanocrystals; (b) EDS result of CDHA nanocrystals

The XRD patterns of Ti6Al4V substrates, CDHA, PLA pellets and composite coatings are shown in Fig. 6-6. Ti6Al4V substrates oxidized using H2O2 for 24 hours had an oxide layer too thin to give any oxide diffractions. For CDHA, the main XRD peaks at 20 angle 26° and 32° confirmed the formation of hydroxyapatite (HA) lattice (JCPDS-ICDD card no 18-03030). The crystal domain sizes of precipitates along the c direction were calculated by JADE software based on Scherrer’s equation using (002) diffraction peak, and were observed to be 210 Å. PLA examined by XRD showed high crystallite with a strong peak observed at 17°. The XRD pattern of composite coatings showed peaks belonging to Ti6Al4V, CDHA and PLA separately, which confirmed the composition of the coating, a composite of PLA and CDHA.
The FTIR data of CDHA, PLA pellets and composite coatings are shown in Fig. 6-7. From FTIR data of CDHA, the presence of carbonate in CDHA was confirmed by the absorption bands observed at 1470-1420 and 875 cm\(^{-1}\). Absorption band of \(\text{PO}_4^{3-}/\text{HPO}_4^{2-}\) belonged to CDHA at 1040-1020 cm\(^{-1}\) was also observed on FTIR data of hybrid coatings, proving the presence of CDHA in coatings. A new absorption peak of coatings appeared at 1600 cm\(^{-1}\) as compared to PLA and CDHA, which was assigned to COO\(^-\) resulting from the interaction of COOH in PLA with Ca\(^{2+}\) in CDHA lattice.
Images of Fig. 6-8a through Fig. 6-8c are the results of SEM characterization of Ti6Al4V substrate and composite coatings. After H$_2$O$_2$ oxidation treatment, micro-cracks were found on the surface of Ti6Al4V substrates as shown in Fig. 6-8a. After coating deposition by electrospraying, the whole area of substrate was coated with high roughness (Fig. 6-8b&8c). These coatings were composed with randomly deposited beads with certain porosity. The EDS elements mapping shown in Fig. 6-9 demonstrated the uniform distribution of CDHA in composite coatings.

Fig. 6-8. Results of SEM characterization: (a) Ti6Al4V after oxidization, (b) PLA/CDHA coatings electrosprayed under optimized condition (x30 K), (c) PLA/CDHA coatings surface morphology (x8 K)

Fig. 6-9. EDS mapping analysis: (a) focused PLA/CDHA coatings area, (b) Ca element distribution, (c) C element distribution

After 7 days bioactive testing, apatite particles were observed to be randomly deposited on the surface oxidized Ti6Al4V substrate (Fig. 6-10a) instead of uniform
coating found on the surface of PLA/CDHA coating (Fig. 6-10b). As shown in Fig. 6-10a, micro-cracks on Ti6Al4V substrate were partially covered by deposited apatite. While on the PLA/CDHA coatings surface, bulk agglomerates of apatite were observed (Fig. 6-10b). The weight changes were also recorded (Fig. 6-11): after 7 days SBF incubation, Ti6Al4V substrate showed a weight increase after apatite deposition, but the one with PLA/CDHA coatings showed a weight decrease on contrast, even higher amount of apatite coatings were deposited on the surface.

**Fig. 6-10.** SEM images of samples after 7 days biomimetic coating: (a) oxidized Ti6Al4V substrates; (b) oxidized Ti6Al4V substrates with PLA/CDHA coatings

**Fig. 6-11.** Weight change of oxidized Ti6Al4V substrates with/without PLA/CDHA coatings after 7 days SBF incubation
The in vitro testing results are shown in Fig. 6-12. The results were compared by one-way ANOVA analysis. At 24 hours, PLA/CDHA coatings had the similar attached osteoblasts as compared to oxidized Ti6Al4V substrates, and Ti6Al4V substrates with PLA coating. After 4 days, the number of osteoblasts on Ti6Al4V-composite coating was the highest (p<0.05). The SEM images of osteoblast cells on different substrates were shown in Fig. 6-13. No significant difference of cells morphology was observed. Osteoblast cells exhibited a flat appearance and were spread out over the surfaces of all samples. All of the surfaces of the experimental groups were covered with a confluent layer of osteoblast cells.

Fig. 6-12. Results of in vitro testing

Fig. 6-13. SEM images of osteoblast cells on the surfaces of oxidized Ti6Al4V substrates, PLA coatings and PLA/CDHA coatings
The AS release study results are shown in Fig. 6-14. It was observed that AS in PLA coatings released in a very short time. But the release of AS from PLA/CDHA coatings lasted months.

![Graph showing AS release study](image)

Fig. 6-14. AS release study

### 6.5 Discussion

Bone-like CDHA is an important compound among CaP materials because of its activity [95, 269]. CDHA composition is relatively close to the mineral component of bone, which is poorly crystalline hydroxyapatite (HA) with small amounts of elements such as sodium (Na\(^+\)), potassium (K\(^+\)), magnesium (Mg\(^{2+}\)), and carbonate (CO\(_3^{2-}\)) present in the lattice structure, as compared to crystallized CaP materials [46, 269]. As opposed to pure HA, CDHA is biodegradable because of the presence of trace elements. The crystal domain size of CDHA precipitates was similar to that recorded deproteinated bone apatite, quantitatively confirmed by the similarity of the two crystal domain sizes (210 and 213 Å, respectively) [264]. One function of addition of tris-base buffer in SBF is to make pH of
SBF solution stable in a long period of time via the chelating the aqueous Ca ions present in the SBF. The purpose of reducing the tris-base amount is to intensify the precipitation of CDHA, and the result after 24 hour precipitation fit the expectation.

The incorporation of CaP materials with PLA has been a popular topic in biomaterial research. Many approaches were studied such as electrospinning, solvent casting, and supercritical gas foaming to fabricate PLA/CaP composites [95, 173, 177, 182]. Deposition of PLA/CaP composite coatings onto implant surface as potential drug carriers to further improve tissue regeneration is still a new topic in these years. This is one of the highlights of this study. In addition, incorporation of CDHA with PLA can improve the encapsulation efficiency and release prolife of special bio-molecules compared to alone PLA composites. The room temperature requirement in electrospraying approach is beneficial to the drug loading in coatings and the preservation of carbonate in CDHA lattice to ensure the high bioactivity of CDHA.

In the field of biopolymer fabrication, the main function of electrospraying is to produce biopolymer micro-particles. A stable electrospraying system can be established only with low electric conductivity, a low dielectric constant, and a high vapor pressure of a solvent such as chloroform. The addition of a solvent with the opposite solvent properties can destroy electrospraying stability [275]. That’s the reason in our study we only applied chloroform as the solvent. Flowing rate is an important factor in controlling coatings morphology. It was observed that decreasing flowing rate induced the transition of products from loose fibers with beads to beads with porosity. At low flowing rate, high molecular weight PLA is favored to produce fine fibers with certain electrical driven force applied [182, 275]. The number of charge carriers per volume in a jet segment is
higher when the flowing rate is lower, which resulted in an increase in the electrical force to produce individual particles from jets into fine fibers to decrease surface tension. Therefore, in our case formation of fibers with beads instead of fine fibers at low flowing rate was caused by the low conductivity of chloroform, which limits the electrical force on jet. In high flowing rate, formation of micro-beads is more favored than fibers due to the low applied force per particles from jet. In addition, more chloroform rich-areas are presented in beads than fibers to occupy space due to the decrease of surface/volume ratio as compared to fine fibers. After the evaporation of chloroform, those solvent rich-areas were converted into micro-pores on beads surface. The other function of chloroform presented in beads is to assist the bridge formation between separate beads via PLA diffusion in chloroform, and after solvent evaporation beads were bonded to each other via newly formed PLA bridges in local areas. Another factor influences applied electrical force is voltage gradient, which is net result of applied voltage and working distance. The increasing working distance decreased voltage gradient, consequently inducing the pores formation on beads surface; while the increasing applied voltage increased voltage gradient to produce smaller beads. The presence of CDHA nanocrystals can be considered as a phase separation between CDHA crystals and PLA layer. It was possibly related to the voltage gradient, which caused the charge difference between CDHA and PLA solution, thus caused difference in velocities from the jet to the collector. The velocity difference was intensified at higher voltage gradient. As a result, some CDHA crystals appeared on the beads surface instead buried in the PLA layer when applied voltage was high and working distance was low. This phenomenon can potentially improve bioactivity of coatings surface due to the high exposure of CDHA to the
surrounding environment. A combination of parameters (flowing rate of 30 ml/h, voltage of 10 kV, and working distance of 10 cm) was tried to produce coatings on Ti6Al4V substrates for further characterization. In this working condition, fibers with beads were all removed from the hybrid coatings and a PLA/CDHA composite coating can be deposited on Ti6Al4V substrate with high surface roughness.

In the XRD patterns of PLA/CDHA coatings, the diffraction peaks detected were located at the same 2θ angle of Ti6Al4V, PLA and CDHA. No extra diffraction peaks were observed. It is interesting to notice the preservation of PLA crystallite after electrospraying, which was different from the absence of PLA crystallite peaks in electrospinning [279]. PLA with high crystalline can delay the degradation of PLA, which can consequently delay lowering of pH change. The new absorption band found on FTIR data of PLA/CDHA coatings was assigned to COO\(^{-}\) resulting from the interaction of COOH in PLA with Ca\(^{2+}\) in CDHA lattice. Therefore, CDHA precipitates were not only uniformly dispersed in the PLA matrix, but also reacted with PLA, resulting in strong bonding between each other.

As shown in Fig. 6-8a, micro-cracks were found on the surface of Ti6Al4V substrates, which was caused by the released oxygen formed in the oxidation reaction between \(\text{H}_2\text{O}_2\) and Ti6Al4V. The oxidized layer of Ti6Al4V was bioactive, able to induce the formation of strong bone bonding as reported by other groups [280]. Uniform composite coatings without the appearance of fibers were successfully deposited to the surface of oxidized Ti6Al4V substrates. The whole area can effectively covered by the PLA/CDHA coatings after 1 min electrospraying (Fig. 6-8b). Hence, this new approach is a very rapid coating process. As shown in Fig. 6-8c, pores were observed on the
surface of agglomerate-like PLA/CDHA composites, which were possibly caused by random deposition of beads and evaporation of residual chloroform during drying. The porosity slightly increased the surface area of the coatings, which may be beneficial to bio-molecules release in future.

Formation of apatite on the surface of samples depends on the materials’ intrinsic ability to form apatite in SBF. The ability of biomaterials to develop bone-like apatite upon soaking in SBF has been correlated to bone bonding formation ability in vivo [218]. For PLA/CDHA coatings, both CDHA precipitates and PLA play roles for apatite deposition. CDHA precipitates were similar to apatite in 1.5x t-SBF, which can work as epitaxial nucleation sites for apatite deposition. The apatite formation mechanism on PLA is related to the hydrolysis of PLA, which results in a negatively charged PLA surface. The positively charged calcium ions in the solution are attracted to the hydrolyzed PLA surface and apatite forms through the attraction of phosphate groups from the solution [27]. For oxidized Ti6Al4V substrates, accumulation of OH− ions on the surface, caused by titanium oxide hydrolysis and its interaction with SBF solution, led to a more negatively charged surface that works similar to the negatively charged PLA surface [280]. The difference of coating uniformity observed between PLA/CDHA coatings and oxidized Ti6Al4V substrates can be contributed to two factors: 1) the hydrolysis level of PLA was stronger than oxidized Ti6Al4V layer; 2) the incorporation of CDHA into the coatings to intensify the apatite coating formation. The ability of biomaterials to develop bone-like apatite upon soaking in SBF has been correlated to bone bonding formation ability in vivo. Therefore, the apatite formation on PLA/CDHA coatings demonstrates their potential bone bonding ability.
The highest cell numbers in the in vitro osteoblast cells studies on PLA/CDHA coatings showed its great biocompatibility. Incorporation of CDHA into PLA improved osteoblasts cells response after 4 days as compared to PLA coatings and Ti6Al4V substrates. The results demonstrate PLA/CDHA coatings produced by electrospraying are non-toxic and can support osteoblast cells proliferation. Based on these results, electrosprayed PLA/CDHA coatings can be applied as alternative coating to currently biomedical coating techniques.

All Bp drugs share the same main backbone (P-C-P) structure. This structure provides the high affinity of Bp to CaP crystals, in which the phosphoryl groups can bind simultaneously to the calcium atom with the formation of a bidentate or possibly tridentate chelate [48, 49]. Therefore, the huge difference of AS release behavior between PLA and PLA/CDHA coatings can be attributed to the fact that CDHA in PLA matrix works as loading site for AS for long term release. Once CDHA slowly degraded in medium, AS was also released to the aqueous environment. As an alternative, AS itself cannot be entrapped in PLA matrix alone with CDHA assist, it quickly diffused to aqueous environment due to its high affinity to water.

One concern in this method of AS loading is the possible toxic effect of organic solvent to the function of AS drug. In vitro study using osteoclast to evaluate the activity of AS would be necessary in future.

6.6 Conclusion

The aim of this study was to investigate the possibility of fabricating PLA/CDHA composite coatings on the surface of Ti6Al4V substrates as bi-functional implants for promoting bone formation, and working as drug carrier. The results demonstrated
coatings composed of CDHA and PLA with great biocompatibility and bioactivity were successfully deposited on to the surface of Ti6Al4V substrates by electrospraying under certain conditions. Fabricated PLA/CDHA coatings were favored by osteoblast cells. This approach is rapid, simple, and can be operated at normal room temperature. Long-term release of AS was achieved via incorporating AS to PLA/CDHA coating. Based on our observations, this approach can be a promising coating technique in biomedical field.
Chapter 7

Production of Yttrium Phosphate Using CDHA as Precursors

7.1 Abstract

In this chapter, CDHA is used as the precursor to produce YPO₄ as an alternative to known YPO₄ manufacturing techniques. The as-prepared YPO₄ was characterized using SEM and EDS. Freshly prepared CDHA showed the highest reaction activity to react with YCl₃ aqueous solution for the sacrifice of Ca²⁺ ions in the structure, with the cost of losing its initial structure. However, once CDHA materials were pre-treated using ethanol to stabilize their structure. The formed YPO₄ can preserved the structure of CDHA materials such as coatings and microspheres.

7.2 Introduction

Yttrium (Y) is a rare earth element. In recent years many researchers have explored the use of yttrium phosphate (YPO₄) in the field of ceramic materials. YPO₄ appears to be valuable for use as a coating to protect ceramic materials from a high temperature, moisture-containing environment. This is because of its great chemical compatibility, resistance to thermal expansion, and water vapor corrosion, when exposed to a high temperature environment [281, 282]. YPO₄ can also be used as phosphorus once doped with other ions for optical, luminescence and electronic applications [283-285]. In
addition, Y(90)PO4 can be used for in situ radiotherapy [286, 287]. Several amalgamations of YPO₄ by various researchers have been reported, such as high-temperature solid state reactions, hydrothermal precipitation, and micro-emulsion [282, 284, 288, 289]. A new transient template method using CDHA was developed in our lab, as an alternative to these techniques demonstrated above. It has the capability to deposit uniform YPO₄ coating, to produce YPO₄ microspheres at room temperature. It is hoped this new system can bring some innovative concepts to YPO₄ manufacture.

7.3 Materials and Methods

Two SBF solutions were prepared for different purposes, coating deposition (SBF1) and microspheres preparation (SBF2). SBF1 has been reported by our group for rapid coating deposition of Ti6Al4V substrates [34]. The composition of SBF1 is listed in Table 7.1. NaHCO₃ was added 10 minutes before coating deposition to trigger CaP nucleation and precipitation. SBF2 was developed by the SBF composition introduced by Hoffman et al. [36]. The amount of MgCl₂ was intensified to produce separated CDHA microspheres, because Mg²⁺ can delay the crystals growth of HA [98, 99]. The composition of SBF2 is listed in Table 7.2.

Ti6Al4V substrates were initially treated with 5M NaOH solution as described in chapter 3 to produce nucleation sites for CaP on its surface. The pretreated Ti6Al4V substrates were incubated into SBF1 for 6 hours at a 37°C environment, with the solution changed after 3 hours. One coated Ti6Al4V substrate was dried in an oven to be used as a blank control and other Ti6Al4V substrates were incubated in 70% EtOH for stabilization and moved to a YCl₃ aqueous solution for the reaction. The reaction was carried out in a 37°C environment for 24 hours. After that all samples were dried in an oven. Considering
the fact that CDHA can slowly mature with the loss of water content in its structure, an additional experiment was conducted to prove the concept that CDHA activity is related to the YPO$_4$ formation. One CDHA coated Ti6Al4V substrate prepared 1 month ago and one CDHA coated Ti6Al4V substrate prepared 12 months ago were also soaked into 0.05 mol/L YCl$_3$ aqueous solution for 48 hours. After drying, all samples were characterized using SEM and EDS.

Table 7.1 Recipe of SBF1, for a total volume of 1 L

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>58.443</td>
</tr>
<tr>
<td>KCl</td>
<td>0.3728</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>3.6754</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>1.0165</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>0.24</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>0.84</td>
</tr>
</tbody>
</table>

SBF2 solution was packaged into several 50 ml centrifuge tubes and incubated in a 37°C water bath. The precipitates were collected after 48 hours via centrifugation and dried in an oven. Some precipitates were preserved for further SEM characterization and EDS analysis, others were pretreated using 90% EtOH and further incubated into 0.05 mol/L YCl$_3$ aqueous solution in a 37°C environment for 24 hours. Materials without EtOH pre-treatment were used as a blank control. The final products were collected and dried for characterization.
Table 7.2 Recipe of SBF2, for a total volume of 1 L

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>1.4192</td>
</tr>
<tr>
<td>NaCl</td>
<td>34.983</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1.134</td>
</tr>
<tr>
<td>MgCl₂.6H₂O</td>
<td>0.2233</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>0.142</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>1.83775</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.68045</td>
</tr>
</tbody>
</table>

7.4 Results

CDHA coatings can be successfully deposited to the surface of pretreated Ti6Al4V substrate with a substructure of plate-like crystals (Fig. 7-1a). After incubation in YCl₃ aqueous solution, the coating on Ti6Al4V showed a totally different morphology with a porous characteristic (Fig. 7-1b). The EDS results showed that the new coating layer is composed of YPO₄ instead of CDHA (Fig. 7-2). On the other hand, the CDHA coated Ti6Al4V substrate prepared 1 month ago showed the presences of Ca²⁺ and Y³⁺ combined with PO₄³⁻ in the structure after incubation in YCl₃ aqueous solution (Fig. 7-3a). However, the CDHA coated Ti6Al4V substrate prepared 12 months ago preserved its crystal composition without any penetration of Y³⁺ (Fig. 7-3b).

![Fig. 7-1 (a) SEM image of CDHA coatings and (b) SEM image of YPO₄ coating](image-url)
CDHA microspheres were successfully precipitated from SBF2, showing a substructure of plate-like crystals (Fig. 7-4). After YCl₃ aqueous solution incubation, CDHA microspheres were observed to become YPO₄ microspheres with trace amount of Ca ions (Fig. 7-5). The surface morphology of microspheres became less porous with a coarse feature after incubation. However, samples without EtOH pre-treatment were observed to become a mess with spherical structure loss and particles agglomeration (Fig. 7-6).
Fig. 7-4 CDHA microspheres precipitated from SBF2

Fig. 7-5 SEM image of YPO$_4$ microspheres related EDS mapping of Y, Ca, and P elements
7.5 Discussion

It is known that CDHA can become stable once it loses its water content in its amorphous structure region. Therefore, the freshly synthesized CDHA materials exhibit the highest activity. Additionally, no reaction occurred to the CDHA coatings with a 1 year history. Freshly synthesized CDHA works as a precursor for YPO₄. This phenomenon is controlled by the fact that YPO₄ is more stable than CDHA in aqueous environment. It is also interesting to note that after EtOH pre-treatment, the structure features of CDHA, such as coating and microspheres, were preserved, and all Ca²⁺ were replaced by Y³⁺. No significant damages were observed. The cracks of YPO₄ coatings were thought to be caused by the release of Ca²⁺ ions during reaction.

On the other hand, the phenomenon that replaced Ca²⁺ with Y³⁺ depends on the activity of CDHA, and can make the production of Y³⁺ doped CDHA particles possible
(for example, using 100% EtOH and prolong the incubation time). In addition, as Y belongs to the rare earth elements, and they showed similar chemical properties and applications, it is possible to prepare various rare earth elements doped CaP materials or phosphors using CDHA.

7.6 Conclusion

In conclusion, YPO$_4$ can be produced from CDHA as a precursor material. The reaction level depends on the activity of CDHA, which decreases with water content loss. The formed YPO$_4$ can preserve the structure of CDHA materials such as coating and microspheres. It is a new approach to prepare YPO$_4$ coatings and microspheres.
Chapter 8

Synthesis of Eu$^{3+}$ Doped Calcium Phosphate Nanospheres

8.1 Abstract

The goal is to develop a new method to prepare lanthanide-doped calcium phosphate materials via replacement reaction between calcium and lanthanide. Amorphous calcium phosphate nanopsheres doped with Eu$^{3+}$ were prepared using this method. Moreover, it was observed that the lanthanide content can be controlled by adjusting EtOH content in solution.

8.2 Introduction

The most commonly used fluorescent labels for biological applications are fluorescent proteins (such as red fluorescent protein), and organic dyes (such as fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate) [290]. These label materials are excited by a specific amount of energy associated with a specific excitation wavelength, $\lambda_{\text{excitation}}$. Electrons from the ground state can excite to an elevated energy state caused by the amount of energy absorbed. The electron then undergoes non-radiative energy decay and this energy loss results in the emission of a photon with a specific emission wavelength, $\lambda_{\text{emission}}$. The difference between the excitation wavelength and the emission wavelength is known as the Stokes shift. When the shift is large enough,
two wavelengths can be distinguished by detector. However, they have several limitations such as broad spectrum profiles, low photobleaching thresholds, and poor photochemical stability [290]. As an alternative to these fluorescent labels new fluorescent materials such as semiconductor quantum dots, lanthanide-doped inorganic nanoparticles and fluorescent dyes coated silica beads were developed [291-293]. However, semiconductor quantum dots can provide good fluorescent results, but are limited to optical blinking and toxicity [294, 295]. Fluorescent dye coated silica beads are limited to the properties of the applied fluorescent dyes.

Lanthanide-doped inorganic nanoparticles show very interesting luminescent properties which make them useful for biological fluorescent labeling [293]. Their fluorescence is characterized by narrow emission bandwidths determined by the lanthanide ions, high photochemical stability and long fluorescence lifetime (up to several milliseconds) [293]. However, the synthesis and biological applications of lanthanide-doped inorganic nanoparticles is still in the early stages with a lot of open area for investigation.

Calcium Phosphate materials have been considered as matrix materials for Lanthanide doping in recent years [296]. It is known calcium phosphates, such as CDHA, allow substitution ions into their lattice structure with relative ease. When lanthanide series elements are substituted into the crystal, the doped material has shown fluorescence capabilities. However, to the best of our knowledge, the lanthanide-doped calcium phosphate materials are mainly synthesized via co-precipitation [297]. Here a new approach for preparation of lanthanide-doped calcium phosphate nano materials is introduced. It is an attempt to bring new concept to lanthanide-doped calcium phosphate
Amorphous calcium phosphate nanospheres, SBF2-Mg16, were prepared following the procedures described in chapter 3. The achieved nanospheres were dried for further applications. Europium nitrate (Eu(NO$_3$)$_3$) was purchased from Sigma-Aldrich (MO, USA). As listed in Table 8.1, 0.008 g Eu(NO$_3$)$_3$ was used to react with 0.05 g SBF2-Mg16 nanospheres in 20 ml solution with different composition. Eu(NO$_3$)$_3$ was initially dissolved in solution followed by pouring in SBF2-Mg16 nanospheres. The tubes with powders and solution were kept in a 37 °C environment for 24 hours. After that, all powders were collected via centrifuging. They were dried and characterized using SEM and EDS.

Table 8-1 Different solution composition

<table>
<thead>
<tr>
<th>Solution</th>
<th>composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100% H$_2$O</td>
</tr>
<tr>
<td>2</td>
<td>95% H$_2$O+5%EtOH</td>
</tr>
<tr>
<td>3</td>
<td>90% H$_2$O+10%EtOH</td>
</tr>
<tr>
<td>4</td>
<td>70% H$_2$O+30%EtOH</td>
</tr>
<tr>
<td>5</td>
<td>50% H$_2$O+50%EtOH</td>
</tr>
<tr>
<td>6</td>
<td>5% H$_2$O+95%EtOH</td>
</tr>
</tbody>
</table>

8.4 Results

It was observed Eu$^{3+}$ ions are highly active when they contacted with PO$_4^{3-}$ ions in an aqueous environment. In solution 1, the suspended white powders rapidly agglomerated and form a cluster suspended in water. With increasing content of EtOH in solution, such nucleation phenomenon was retarded especially when EtOH content reached 30% or more. In SEM characterization (Fig 8-1), ACP nanospheres were found to be damaged.
and Ca\(^{2+}\) was replaced by Eu\(^{3+}\) when no EtOH presented in the solution. However, the nanosphere structure can be preserved when high content EtOH was applied. The surface feature of blank ACP nanospheres should be smooth. As shown in SEM, the surface of ACP nanospheres became rough when no EtOH presented in the solution. The EDS results showed Eu\(^{3+}\) was successfully incorporated into the lattice structure of ACP nanospheres via reaction with PO\(_4\)\(^{3-}\). In addition, the content ratio of Eu\(^{3+}\) in ACP nanospheres can be controlled via adjusting the content of EtOH in solution (Fig. 8-2).

Fig. 8-1 SEM images of ACP nanospheres after 24 hrs reaction in (a) water; (b) 5\% EtOH solution; (c) 10\% EtOH solution; (d) 30\% EtOH solution; (e) 50\% EtOH solution; (f) 95\% EtOH solution
8.5 Discussion

To verify the potential to produce Eu$^{3+}$ doped ACP nanospheres based on the high activity of ACP and the high affinity between PO$_4^{3-}$ and Eu$^{3+}$, an experiment was done. In a saline solution containing Ca$^{2+}$ and PO$_4^{3-}$, the addition of Eu$^{3+}$ directly resulted in white precipitates formation. Therefore, it is impossible to achieve Eu$^{3+}$ doped ACP nanospheres by adding Eu(NO$_3$)$_3$ to SBF solution before microwave heating. EtOH is usually used as a medium to preserve ACP during storing to avoid the conversion of ACP to other CaP materials. As a result, using EtOH as a solution component is supposed to limit the activity of ACP thus resulting in the inhibition of rapid reactions between Eu$^{3+}$, Ca$^{2+}$ and PO$_4^{3-}$. The SEM data proved this concept and Eu$^{3+}$ can be incorporated into ACP nanospheres with the right molar ratio of Ca element. In literature, it is recommended that the molar ratio between Eu and Ca is 5% [298]. In this method, this ratio can be obtained when the EtOH content is approximately 37%.

8.6 Conclusion

A new method to prepare Eu doped CaP material is illustrated and the content of doped Eu can be controlled via adjusting EtOH volume ratio in the solution.
Chapter 9

Conclusion and Future Directions

9.1 Conclusion

The primary goal of this thesis is to investigate different functions of CDHA in biomedical applications. CDHA has been successfully applied as drug carrier, composite filler, and template, and its synthesis was also developed using microwave irradiation.

The possible bisphosphonate drug loading method discussed in Chapter 2 was an attempt to develop a CDHA-bisphosphonate coating for local drug delivery and bone regeneration. The results indicated that the release of bisphosphonate can be sustained once it is loaded in the inner layers of the CDHA coatings. It was also observed the dosage of loaded bisphosphonate controls the formation of CDHA and biological performance of the coating. This preliminary study showed the possibility and benefits to produce CDHA-coatings via a co-precipitation process for bone tissue engineering applications. This preliminary study showed the ability to produce CDHA-AS coatings via a co-precipitation process for bone tissue engineering applications.

The microwave assisted synthesis of amorphous calcium phosphate (ACP) illustrated in Chapter 3 provides a novel ACP synthesis route which further develops and enriches the current ACP synthesis methods. This technique relies on the use of microwave
irradiation and the assistance of Mg$^{2+}$ ions. Synthesis conditions were studied and the mechanism of forming ACP nanospheres was discussed. It was found both pH and ionic concentrations play critical roles in microwave-assisted synthesis of ACP nanospheres. The formation of ACP nanospheres by this technique is energy favored. More importantly, the synthesized ACP nanoparticles showed good reactivity and biocompatibility, potential to be used for biomedical applications, and the study of the remineralization mechanism. It is a great innovation of calcium phosphate on timely topic.

The art of fabrication of PLA-CaP/PLGA-CaP composites was demonstrated in Chapter 4, which reviews recent reported fabrication techniques and related mechanical/biological performance of prepared composites. Based on this review, CDHA was fabricated with PLA using elect rspinning and electrospraying into composites for biomedical applications as addressed in Chapter 5 and Chapter 6. They both showed great biocompatibility and bioactivity as compared to blank PLA matrix.

The application of CDHA to produce a Y/Eu-CaP variant was also demonstrated through incorporation of Y/Eu into CDHA crystal lattice via reaction in an aqueous environment. To emphasize, it was also observed with the assistance of EtOH, the reaction can be controlled and the structure of CDHA template can be preserved. This pathway can be a new method for preparation of CaP variant with multi-functional ions in its crystal structure.

9.2 Future Directions

The concept of doping different ions to CDHA has been verified in the thesis. Additionally, it can introduce different functions to CaP materials. For example, Y can promote cell growth, improve the mechanical and thermal performance of CaP;
fluorescence ability can be introduced to CaP particles with Eu. These methods can be expanded to many ion types for specific biomedical applications. All these concepts can be a future direction. In addition, the kinetic study of this conversion process will be investigated for further doping optimization studies.

In the thesis, the manufacture of ACP using microwave irradiation was introduced. However, the applications of these ACP nanospheres are not fully studied. There are possibilities that these ACP nanospheres can be incorporated into various biopolymer matrices to prepare composites, or applied as drug/protein/gene carriers. In addition, their compositions can be adjusted by controlling the reacting solution compositions and using the doping method described above.

ACP nanospheres, doped ACP nanospheres, doped CDHA particles can all be potential fillers for fabricating biocomposites for biomedical applications. They can introduce new properties to biopolymer matrix after incorporation. In addition, these doped materials can also be incorporated into bone cement structure to further improve the cement mechanical strength and dissolution stability.
Reference


10. Tas AC, Jalota S, Bhaduri SB. Synthesis of HA-seeded TTCP (Ca(PO₄)₂O) powders at 1230 °C from Ca(CH₃COO)₂.H₂O and NH₄H₂PO₄. J Am Ceram Soc


18. Dorozhkin SV, Epple M. Biological and medical significance of calcium phosphates.


45. Ma J, Wong H, Kong LB, Peng KW. Biomimetic processings of nanocrystallite


62. Åberg J, Brohede U, Mihranyan A, Strømme M, Engqvist H. Bisphosphonate incorporation in surgical implant coatings by fast loading and co-precipitation at low


88. Skrtic D, Antonucci JM, Eanes ED, Brunworth RT. Silica- and zirconia-hybridized


97. Christoffersen J, Christoffersen MR. Kinetics of spiral growth of calcite crystals and
determination of the absolute rate constant. J Cryst Growth 1990;100:203-211.


106. Porter JR, Ruckh TT, Popat KC. Bone tissue engineering: a review in bone


143. Keaveny T, Hayes W. Mechanical properties of cortical and trabecular bone.


153. Nagata F, Miyajima T, Teraoka K, Yokogawa Y. Preparation of porous poly(lactic acid)/hydroxyapatite microspheres intended for injectable bone. Key Eng


161. Ignjatović N, Ajduković Z, Savic VP, Uskoković DP. Size effect of calcium


186. Kitajima A, Tsukamoto M, Akedo J. Hydroxyapatite film coated poly-l-lactic


194. Mohn D, Ege D, Feldman K, Schneider OD, Imfeld T, Boccaccini AR. Stark WJ.


202. Jin HH, Min SH, Song YK, Park HC, Yoon SY. Degradation behavior of
poly(lactide-co-glycolide)/ β-TCP composites prepared using microwave energy.


211. Sherwood JK, Riley SL, Palazzolo R, Brown SC, Monkhouse DC, Coates M,


219. Bae JY, Won JE, Park JS, Lee HH, Kim HW. Improvement of surface bioactivity of poly(lactic acid) biopolymer by sandblasting with hydroxyapatite


249. Montjovent MO, Mark S, Mathieu L, Scaletta C, Scherberich A, Delabarde C, Zambelli PY, Bourban PE, Applegate LA, Pioletti DP. Human fetal bone cells


258. Lassalle V, Ferreira ML. PLA nano- and microparticles for drug delivery: an


1998;41:227-236.


293. Mondejar SP, Kevtun A, Epple M. Lanthanide-doped calcium phosphate


