

DESIGN, FABRICATION AND EVALUATION OF 2D TO 3D NANOSTRUCTURED
CERAMIC/POLYMER COMPOSITES FOR ORTHOPEDIC REGENERATION AND
CONTROLLED DRUG DELIVERY

BY

HUINAN LIU

B.S., UNIVERSITY OF SCIENCE AND TECHNOLOGY BEIJING, 1997

M.S., UNIVERSITY OF SCIENCE AND TECHNOLOGY BEIJING, 2000

M.S., PURDUE UNIVERSITY, 2005

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This dissertation by Huinan Liu is accepted in its present form
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as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

Date _____

Thomas J. Webster, Advisor

Recommended to the Graduate Council

Date _____

Jeffrey R. Morgan, Reader

Date _____

Edith Mathiowitz, Reader

Date _____

G. Tayhas R. Palmore, Reader

Date _____

Jeffrey M. Karp, Reader

Approved by the Graduate Council

Date _____

Sheila Bonde, Dean of the Graduate School

CURRICULUM VITAE

HUINAN LIU

EDUCATION

Brown University, Providence, Rhode Island
Ph.D., Biomedical Engineering, May 2008

Purdue University, West Lafayette, Indiana
M.S., Materials Science and Engineering, December 2005

University of Science and Technology Beijing (USTB), Beijing, China
M.S., Materials Science and Engineering, March 2000
B.S., Diploma with First-class Honors, Major in Materials Science and Engineering
and Minor in Scientific English Literature, July 1997

RESEARCH EXPERIENCE

Brown University, Biomedical Engineering, Research Assistant, 2006-Present

Ph.D. Dissertation Project: Design, Fabrication and Evaluation of 2D to 3D
Nanostructured Ceramic/Polymer Composites for Orthopedic Regeneration and
Controlled Drug Delivery.

Advisor: Dr. Thomas J. Webster

Advisory Committee: Dr. Jeffrey R. Morgan, Dr. Edith Mathiowitz, Dr. G. Tayhas
Palmore, and Dr. Jeffrey M. Karp (Harvard-MIT)

The main goal of my research is to create novel biomaterials and drug delivery systems with highly controlled nano-to-macro hierarchical structures that repair or replace damaged bone tissue and restore its normal biological functions. My research develops a multidisciplinary approach to assemble orthopedic tissue substitutes that can deliver structural, biological and mechanical signals to bone cells at the nano-scale and eventually heal damaged bone tissue at the macro-scale in a more effective way. This is accomplished by applying the concepts of nanotechnology, tissue engineering and controlled drug delivery into orthopedic systems to closely mimic natural bone in terms of its chemistry, nanostructure, biological organization and distinctive mechanical properties. Specific studies are listed as the following.

- Designed CAD models to build 3D nanophase ceramic/polymer composite scaffolds by a novel aerosol-based 3D printing technique.
- Characterized such nanocomposites and 3D scaffolds by Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), transmission electron microscopy (TEM), energy dispersive X-ray analysis (EDX), X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM) and etc.
- Investigated mechanical properties (such as elastic modulus, fracture behavior, compressive and tensile strength) of such nanocomposites in comparison to natural bone.
- Developed physical and chemical methods to load bone morphogenetic proteins (BMPs) and associated peptides into scaffolds for regulating cellular behavior and consequently treating various bone diseases.
- Conducted *in vitro* analysis to determine differentiation of human mesenchymal stem cells and functions of osteoblasts (bone forming cell) on the nanocomposite scaffolds. Fluorescence microscopy and confocal laser scanning microscopy were used to characterize cell adhesion and infiltration. Biochemical assays were used to characterize long-term cell functions.
- Studied drug loading efficiency and drug release rate related to degradation kinetics of materials.

Other Projects:

- Investigated the relationship between biological properties and material characteristics of nano-to-micron particulate calcium phosphates.
- Studied *in vitro* cytocompatibility of novel machinable calcium phosphate/lanthanum phosphate (LaPO_4) composites for orthopedic applications.
- Characterized osteoblast interactions with calcium phosphate/barium titanate (BaTiO_3) composites.
- Investigated osteoblast adhesion on calcium phosphates with various Ca/P ratios.
- Investigated osteoblast adhesion on nanograined hydroxyapatite/calcium titanate composites and tricalcium phosphate/calcium titanate composites.

Purdue University, Materials Science and Engineering, Research Assistant, 2003-2005

M.S. Thesis Project: Nanophase Titania/PLGA (Poly-Lactide-Co-Glycolide) Composites for Bone Tissue Engineering Applications.

General Research Institute for Nonferrous Metals at Beijing, China, Research Engineer, 2000-2003

- Studied microstructure and properties of orthopedic implant materials, mainly focused on titanium alloys.
- Simulated the processing of titanium alloys by the Finite Element Method (FEM).

University of Science and Technology Beijing (USTB), Research Assistant, 1997-2000

- Developed the Expert System for the designing of extrusion dies.
- Predicted the properties of microalloyed steel plates based on the artificial neural network model.
- Investigated functionally gradient materials, such as synthesis, process and performance of stainless steel and zirconia composites.

RESEARCH INTERESTS

Biomaterials, Ceramics, Polymers, Nanocomposites, Nano-to-micron 3D Fabrication, Rapid prototyping, Orthopedic prostheses, Vascular grafts, Neural implants, Health impacts of nanomaterials, Biomimetic tissue engineering, and Drug delivery systems for controlled tissue regeneration and disease treatment.

PUBLICATIONS

Patent Application

- Liu H, Ergun C and Webster TJ. Novel Machinable Calcium Phosphate/Lanthanum Phosphate Composites for Orthopedic Application. Disclosed to Brown University, August 2007.

Book Chapters

- Liu H and Webster TJ. “Bioinspired Nanocomposites for Orthopedic Applications”, in Nanotechnology for the Regeneration of Hard and Soft Tissues, Webster TJ (ed), World Scientific, pp. 1-52, 2007.
- Liu H, Park G and Webster TJ. “Biocomposites”, in Encyclopedia of Biomaterials and Biomedical Engineering, Wnek G and Bowlin G (eds.), Marcel Dekker, Inc., pp. 1-17, 2006.

Journal Articles (Peer-reviewed)

- Liu H and Webster TJ. Nanomedicine for Implants: A Review of Studies and Necessary Experimental Tools. *Biomaterials*. 28(2): 354-369, 2007.
* **Rated as ScienceDirect Top 25 Hottest Articles**
- Liu H, Yazici H, Ergun C and Webster TJ. An In Vitro Evaluation of the Ca/P Ratio Factor in Cytocompatibility of Nano-to-Micron Particulate Calcium Phosphates for Bone Regeneration. *Acta Biomaterialia*. Accepted, 2007.
- Ergun C, Liu H and Webster TJ. Osteoblast Adhesion on Novel Machinable Calcium Phosphate/Lanthanum Phosphate Composites for Orthopedic Applications. *Journal of Biomedical Materials Research*. Accepted, 2007.
- Liu H and Webster TJ. The Promise of Aerosol Printed 3D Nanostructured Ceramic/Polymer Composites as Next Generation Orthopedic Tissue Engineering Scaffolds. *Materials and Processes for Medical Devices*. In press, 2007.
- Ergun C, Liu H, Webster TJ, Olcay E, Yilmaz S and Sahin FC. Increased Osteoblast Adhesion on Nanoparticulate Calcium Phosphates with Higher

Ca/P Ratios. *Journal of Biomedical Materials Research A*. 85(1): 236-241, 2008.

- Ergun C, Liu H, Halloran JW and Webster TJ. Increased Osteoblast Adhesion on Nanograined Hydroxyapatite and Tricalcium Phosphate Containing Calcium Titanate”, *Journal of Biomedical Materials Research*. 80A(4): 990-997, 2007.
- Liu H, Slamovich EB and Webster TJ. Increased Osteoblast Functions among Nanophase Titania/Poly(lactide-co-glycolide) Composites of the Highest Nanometer Surface Roughness. *Journal of Biomedical Materials Research*. 78A(4): 798-807, 2006.
- Liu H, Slamovich EB and Webster TJ. Less Harmful Acidic Degradation of Poly(lactic-co-glycolic acid) Bone Tissue Engineering Scaffolds Through Titania Nanoparticle Addition. *International Journal of Nanomedicine*. 1(4): 541-545, 2006.
- Liu H, Slamovich EB and Webster TJ. Increased Osteoblast Functions on Nanophase Titania Dispersed in Poly-lactic-co-glycolic Acid Composites. *Nanotechnology*. 16(7): S601-608, 2005.
- Liu H, Slamovich EB and Webster TJ. Increased Osteoblast Functions on Poly-lactic-co-glycolic-acid with Highly Dispersed Nanophase Titania. *Journal of Biomedical Nanotechnology*. 1(1): 83-89, 2005.
- Palin E, Liu H, and Webster TJ. Mimicking the Nanofeatures of Bone Increases Bone-forming Cell Adhesion and Proliferation. *Nanotechnology*. 16(9): 1828-1835, 2005.
- Liu H and Xie J. Database System for Design of Extrusion Dies. *Beijing Keji Daxue Xuebao/Journal of University of Science and Technology Beijing*. 23(1): 63-72, 2001.
- Chen J, Xie J and Liu H. Extrusion Characteristics of Composite Powders of Stainless Steel and Zirconia. *Beijing Keji Daxue Xuebao/Journal of University of Science and Technology Beijing*. 19(6): 590-598, 1997.

Conference Proceedings

- Liu H and Webster TJ. Nano-Dispersed Particulate Ceramics in Poly-Lactide-Co-Glycolide Composites Improve Implantable Bone Substitute Properties. *2007 Materials Research Society Symposium Proceedings*, Nanophase and Nanocomposite Materials. Boston, MA, November 2007.
- Liu H and Webster TJ. Nanostructured Titania/PLGA Composite Scaffolds Improve Cytocompatibility and Mechanical Strength for Better Bone Regeneration. *2007 AIChE Annual Meeting Proceeding*. Salt Lake City, UT, November 2007.
- Liu H and Webster TJ. Favored Osteoblast Interactions with Aerosol Printed 3D Nano-to-Macro Hierarchical Architectures: The Promise of Nanocomposites as Orthopedic Prostheses. *2007 NSTI Nanotechnology Proceeding*. Santa Clara, CA, May 2007.
- Liu H and Webster TJ. Ceramic/Polymer Nanocomposite Tissue Engineering Scaffolds for More Effective Orthopedic Applications: From 2D Surfaces to Novel 3D Architectures. *2006 Materials Research Society*

Symposium Proceedings, Biosurfaces and Biointerfaces. Boston, MA, November 2006.

- Liu H and Webster TJ. From Nano to Micro: Nanostructured Titania/PLGA Orthopedic Tissue Engineering Scaffolds Assembled by Three-dimensional Printing. *2006 AIChE Annual Meeting Proceeding*. San Francisco, CA, November 2006.
- Liu H, Slamovich EB and Webster TJ. Less Harmful Acidic Degradation of Poly(lactic-co-glycolic acid) with Well-dispersed Titania Nanoparticle. *2006 NSTI Nanotechnology Proceeding*. Boston, MA, May 2006.
- Liu H, Slamovich EB and Webster TJ. Surface Roughness Values Closer to Bone for Titania Nanoparticle/Poly-lactic-co-glycolic Acid (PLGA) Composites Increases Bone Cell Adhesion. *2005 Materials Research Society Symposium Proceedings*, Vol. 873E, Biological and Bio-inspired Materials and Devices. San Francisco, CA, March 2005.
- Liu H, Slamovich EB and Webster TJ. Enhanced Osteoblast Functions on Nanophase Titania in Poly-lactic-co-glycolic Acid (PLGA) Composites. *2004 Materials Research Society Symposium Proceedings*, Vol. 845, Nanoscale Materials Science in Biology and Medicine, pp. 315-320. Boston, MA, November 2004.
- Liu H, Slamovich EB and Webster TJ. Osteoblast Functions on Nanophase Titania in Poly-lactic-co-glycolic Acid (PLGA) Composites. *2004 AIChE Annual Meeting Proceeding*, pp. 1271-1273. Austin, TX, November 2004.
- Liu H, Slamovich EB and Webster TJ. Improved Dispersion of Nanophase Titania in PLGA Enhances Osteoblast Adhesion. *Ceramic Transactions, Ceramic Nanomaterials and Nanotechnology III - Proceedings of the 106th Annual Meeting of the American Ceramic Society*, Vol. 159, pp. 247-255. Indianapolis, IN, April 2004.

PRESENTATIONS

Podium Presentations

- Improved Mechanical Properties of Nanophase Titania/PLGA (Poly-Lactide-Co-Glycolide) Composites for Orthopedic Applications. *2008 34th Annual Northeast Bioengineering Conference*, Providence, RI, April 2008.
- Nanostructured Titania/PLGA Composite Scaffolds Improve Cytocompatibility and Mechanical Strength for Better Bone Regeneration. *2007 AIChE Annual Meeting*, Salt Lake City, UT, November 2007.
- Reduced Macrophage Functions On Nanomaterials. Presented on Dr. Thomas J Webster's Behalf. *2007 BMES Annual Meeting*, Los Angeles, CA, September 2007.
- Favored Osteoblast Interactions with Aerosol Printed 3D Nano-to-Macro Hierarchical Architectures: The Promise of Nanocomposites as Orthopedic Prostheses. *2007 NSTI Nanotechnology Conference*, Santa Clara, CA, May 2007.

- Three Dimensional Nanophase Ceramic/Polymer Composites for Bone Tissue Engineering. *Northeast BMES 2007 Meeting*, Stony Brook, NY, March 2007.
- Bio-inspired 2D to 3D Nanocomposite Scaffolds. *Division of Biology and Medicine Seminar*, Brown University, Providence, RI, March 2007.
- Novel Bio-nanocomposites for Orthopedic Applications. **Invited**, *Graduate Materials Links (GML) Symposium on Interdisciplinary Graduate Research*, Northeastern University, Boston, MA, February 2007.
- Nanophase Ceramic/Polymer Composites for More Effective Bone Regeneration: From 2D to 3D. **Invited**, *Regional Bioengineering and Biotechnology Conference 2007*, UMass Dartmouth, MA, February 2007.
- Polymer/Ceramic Nanocomposite Tissue Engineering Scaffolds for More Effective Orthopedic Applications. *2006 MRS Fall Meeting*, Boston, MA, November 2006.
- From Nano to Micro: Nanostructured Titania/PLGA Orthopedic Tissue Engineering Scaffolds Assembled by Three-dimensional Printing. *2006 AIChE Annual Meeting*, San Francisco, CA, November 2006.
- Ceramic/Polymer Nanocomposites for Orthopedic Applications. **Invited**, *2006 MS&T Meeting*, Cincinnati, OH, October 2006.
- Nanomedicine for Increasing Tissue Growth. **Invited**. Presented on Dr. Thomas J Webster's Behalf. *2006 MS&T Meeting*, Cincinnati, OH, October 2006.
- Decreased Degradation of Poly(lactic-co-glycolic acid) Bone Tissue Engineering Scaffolds Through Titania Nanoparticle Addition. *2006 NSTI Nanotechnology Conference*, Boston, MA, May 2006.
- Osteoblast Long-term Functions on Nanophase Ceramic/polymer Composites. *School of Materials Engineering Seminar*, Purdue University, West Lafayette, IN, June 2005.
- Nanophase Titania/Poly-lactic-co-glycolic acid (PLGA) Scaffolds for Bone Tissue Engineering Applications: Titania Dispersion and Osteoblast Response. *30th Society for Biomaterials Annual Meeting*, Memphis, TN, April 2005.
- Mimicking the Surface Roughness of Bone in Titania Nanoparticle/Poly-lactic-co-glycolic acid (PLGA) Composites Increases Bone Cell Adhesion. *2005 MRS Spring Meeting*, San Francisco, CA, March 2005.
- Osteoblast Adhesion on Nanophase Ceramic/Polymer Composites. *School of Materials Engineering Seminar*, Purdue University, West Lafayette, IN, February 2004.

Poster Presentations

- Nano-dispersed Particulate Ceramics in Poly-Lactide-Co-Glycolide Composites Improve Implantable Bone Substitute Properties. *2007 MRS Fall Meeting*, Boston, MA, November 2007.
- Well Dispersed Nano-Titania in PLGA Composites Promote Bone Cell Functions and Mechanical Strength. *2007 BMES Annual Meeting*, Los Angeles, CA, September 2007.

- Enhanced Osteoblast Function and Infiltration into Nanostructured Titania/Poly(lactide-co-glycolide) Aerosol 3D Printed Orthopedic Tissue Engineering Scaffolds. *32th Society for Biomaterials Annual Meeting*, Chicago, IL, April 2007.
- Increased Osteoblast Adhesion on Nanograined Hydroxyapatite/Calcium Titanate and Tricalcium Phosphate/Calcium Titanate Composites. *2006 MRS Fall Meeting*, Boston, MA, November 2006.
- PLGA/Titania Nanoparticle Composites for More Effective Orthopedic Applications. *2006 BMES Annual Meeting*, Chicago, IL, October 2006.
- Nanophase Titania/PLGA (Poly-Lactide-co-Glycolide) Composites for Bone Tissue Engineering Applications. *Methods in Bioengineering*, Massachusetts Institute of Technology, Cambridge, MA, July 2006.
- Nanophase Titania/Poly(lactic-co-glycolic acid) Composites for Drug Delivery Applications. *2006 AAPS Annual Meeting*, Boston, MA, June 2006.
- Degradation Kinetics of Poly(lactide-co-glycolide) Mediated by Titania Nanoparticles. *31th Society for Biomaterials Annual Meeting*, Pittsburgh, PA, April 2006.
- Nanophase Titania/Poly-lactic-co-glycolic Acid (PLGA) Composites for Orthopedic Applications. *2005 Composites at Lake Louise*, Lake Louise, Canada, October 2005.
- Nanophase Titania/PLGA (poly-lactide-co-glycolide) Composites for Bone Tissue Engineering Applications. *2005 Graduate Student Poster Competition*, School of Materials Engineering, Purdue University, West Lafayette, IN, November 2005.
- * **Received First-prize Research Award.**
- Improved Osteoblast Functions on Nanophase Titania in PLGA Composites. *2004 BMES Annual Meeting*, Philadelphia, PA, October 2004.
- Improved Dispersion of Nanophase Titania in Polymer Composites Enhance Osteoblast Adhesion. *106th ACerS Annual Meeting*, Indianapolis, IN, April 2004.
- Improved Dispersion of Nanophase Titania in Polymer Composites Enhance Osteoblast Adhesion. *2004 Sigma Xi Graduate Student Poster Competition*, Purdue University, West Lafayette, IN, February 2004.
- * **Received Second-prize Poster Award.**

TEACHING EXPERIENCE

Teaching Assistant Positions

- Transforming Society-Technology and Choices for the Future, Brown University, Providence, RI, Spring 2007.
Taught a guest lecture and led discussions on how to start research projects; developed the sample solutions for some homework problems; graded homework and exam problems.

- Structure and Properties of Materials, Purdue University, West Lafayette, IN, Spring 2005.
Served as an instructor for 2 review sessions twice per week; held weekly office hours; designed and graded biweekly quizzes; graded weekly homework and exams.
- Materials Processing Laboratory, Purdue University, West Lafayette, IN, Spring 2004.
Prepared labs; gave lab introductions and demonstrations; helped students with their lab projects.
- Fundamentals of Database, USTB, Beijing, China, Spring 1999.
Led a programming lab in Visual Foxpro; helped students debug code; graded programming assignments; helped students with the final project.

Pedagogical Training and Teaching Certificates

- Certificate III, Professional Development, The Harriet W. Sheridan Center for Teaching and Learning in Higher Education, Brown University, Providence, RI, 2007-2008.
- Certificate II, Classroom Tools, The Harriet W. Sheridan Center for Teaching and Learning in Higher Education, Brown University, Providence, RI, 2007-2008.
- Certificate I, Teaching Effectiveness, The Harriet W. Sheridan Center for Teaching and Learning in Higher Education, Brown University, Providence, RI, 2006-2007.

Teaching Consultation/Services

- Served as a teaching consultant for The Harriet W. Sheridan Center for Teaching and Learning in Higher Education, Brown University, Providence, RI, 2007-2008.
- Served as the Sheridan Center graduate liaison for the Division of Engineering, Brown University, Providence, RI, 2007-2008.
- Served on the graduate student panel for the WiSE (Women in Science and Engineering) program, Brown University, Providence, RI, 2007.

Advising

- Served as a research mentor for 3 undergraduate students, Purdue University, West Lafayette, IN, 2004-2005.

TEACHING INTERESTS

Undergraduate-Level Courses: Structure and properties of materials, Thermodynamics, Tissue engineering, Cell biology and Physiology.

Graduate-Level Courses: Advanced composite materials, Material characterization techniques, Biomaterials and Nanomedicine.

OTHER ACADEMIC ACTIVITIES/SERVICES

Professional Societies

- Member of MRS (Materials Research Society)
- Member of ACerS (The American Ceramic Society)
- Member of ASM (ASM International - Materials Information Society)
- Member of TMS (The Minerals, Metals & Materials Society)
- Member of AIST (Association for Iron & Steel Technology)
- Member of BMES (Biomedical Engineering Society)
- Member of AAPS (American Association of Pharmaceutical Scientists)
- Member of AIChE (American Institute of Chemical Engineers)

Reviewer

- Reviewed manuscripts for Biomaterials, International Journal of Nanomedicine, and Journal of Biomedical Materials Research.
- Reviewed manuscripts for Proceedings of MRS Annual Spring and Fall Meeting.

Conferences

- Chaired Bioinstrumentation II Track Session. *2008 34th Annual Northeast Bioengineering Conference*, Providence, RI, April 2008.
- Symposium Assistant. *2007 MRS Fall Meeting*, Boston, MA, November 2007.
- Chaired Undergraduate Platform Session IV: Tissue Engineering on Dr. Thomas J Webster's Behalf. *2007 BMES Annual Meeting*, Los Angeles, CA, September 2007.
- Chaired Nanostructured Scaffolds for Tissue Engineering Session on Dr. Thomas J Webster's Behalf. *2006 AIChE Annual Meeting*, San Francisco, CA, November 2006.

AWARDS AND HONORS

- Nominated as a Full Member of Sigma Xi (The Scientific Research Society), 2008
- First Place for Materials Engineering Graduate Student Association Research Competition, Purdue University, West Lafayette, IN, 2005.
- Member of Alpha Sigma Mu Honor Society (International Professional Honor Society For Materials Science and Engineering), Purdue University, West Lafayette, IN, 2003-2005.
- Second Place Poster Award for Sigma Xi Graduate Student Research Competition, Purdue University, West Lafayette, IN, 2004.
- Outstanding Graduate Student Scholarship, USTB, Beijing, 1998.
- Excellent Graduate Award, First-class Honor issued by the Government, Beijing Municipal Commission of Education, Beijing, 1997.
- Excellent Bachelor Thesis Award, USTB, Beijing, 1997.
- IET Outstanding Undergraduate Student Fellowship, USTB/IET Fund, Beijing,

1996.

- Outstanding Undergraduate Student Scholarship, USTB, Beijing, 1993-1997.

LANGUAGES

English, Chinese (native).

GRADUATE-LEVEL COURSES TAKEN AT PURDUE UNIVERSITY

- Microstructural Characterization Techniques
- Powder Processing (Colloid Science and Ceramics)
- Quantitative Analysis of Microstructure (Stereology)
- Deposition Processing of Thin Films and Coatings
- Phase Equilibria in Multicomponent Systems (Advanced Thermodynamics)
- Scanning Electron Microscopy (SEM) Skills
- Transmission Electron Microscopy (TEM) Skills
- Energy Dispersive X-ray Micro Analysis (EDX) Skills
- Steel: Classification and Properties for Application in Automobiles
- Polymer Synthesis
- Polymers in Pharmaceutical and Biomedical Systems
- Statistics Methods for Biology
- Atomic Force Microscopy (AFM) Skills

GRADUATE-LEVEL COURSES TAKEN AT BROWN UNIVERSITY

- Biomaterials
- Small Wonders: The Science, Technology, and Health Impacts of Nanomaterials
- Drug and Gene Delivery
- Techniques in Molecular and Cell Science
- Cell Physiology and Biophysics
- Principles in Experimental Surgery

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First and foremost, I would like to sincerely thank my advisor, Dr. Thomas J. Webster, for his extensive support, encouragement, and enthusiastic guidance. Professionally, his strong insight in science and technology has inspired me into this most exciting interdisciplinary field, the development of novel biomaterials for treating diseases. I have learned so much from him not only intellectually but also spiritually. I really appreciate the time, training and caring that he invested in me throughout my graduate studies, all of which made this dissertation possible.

I would also like to thank Dr. Jeffrey R. Morgan, Dr. Edith Mathiowitz, Dr. G. Tayhas Palmore, and Dr. Jeffrey M. Karp (Harvard-MIT) for serving as valuable members of my graduate committee and providing me helpful input and constructive suggestions for this project. I highly appreciate their time, inspiring comments and encouragements.

I am also very grateful to many people who assisted me to use various instruments and/or share their experiences, including Senior Research Engineer Mr. Anthony W. McCormick for his technical supports for using instruments in the Center for Advanced Materials Research, Dr. Robbert Creton and Mr. Geoffrey Williams for their assistance for using microscopes in the Leduc Bioimaging Facility, Dr. Michael Renn from

Optomec, Inc. for his assistance with M³D[®] systems, and Dr. Christopher Bull, Mr. Brian R. Corkum and Mr. Charlie Vickers for their assistance and supports for working in the Joint Engineering and Physics Instrument Shop.

I would like to thank all my group members in Dr. Webster's nanomedicine laboratory for their supports and suggestions. I also appreciate great supports from professors, graduate students and staff in the Division of Engineering and the Division of Biology and Medicine during my past two years of graduate studies at Brown University. I thank all my friends at Brown. They made my life at Brown more memorable.

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I dedicate my greatest thankfulness to my beloved family, my parents, my husband and my brother. My dearest husband, Dr. Dmytro V. Zagrebelnyy, as my best friend in life has provided the most important mental support for my graduate studies. Without his love, support and motivation, I can not overcome all the difficulties I encountered at Brown. My family encourages me to move forward and makes my life meaningful and colorful. Without my family, I could not have pursued my dream and fulfilled my passion for discovery and innovation in science and technology.

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CHAPTER 1. INTRODUCTION

1.1. Increasing Demand for More Effective Orthopedic Prostheses

Annually, an estimated 1.5 million individuals in the United States suffer from a bone fracture caused by some form of bone disease [1]. It is projected that the prevalence of bone diseases will increase significantly as the United States population ages, as shown in Figure 1.1 [1,2]. The most adverse effects of bone diseases (such as osteopenia, osteoporosis, bone cancer, etc.) relate to fractures. Osteoporosis is a leading underlying cause of bone fracture which affects both males and females at all ages, although to varying degrees. Other bone disorders, such as Paget's disease, osteogenesis imperfecta, rickets, and osteomalacia also have adverse influences on bone structure, strength, and density, and subsequently lead to bone fractures.

Orthopedic prostheses are often required to repair or replace damaged bone tissue due to various diseases, injuries and genetic malformations. In 2001, about 165,000 hip joints and 326,000 knees were replaced in hospitals in the United States according to the National Center for Health Statistics [3,4]. Health statistics also highlight that the number of new implantation and revision surgeries have gradually increased over the past decade, as shown in Figure 1.2 [3,4]. A majority of the patients who receive an orthopedic implant may have to undergo several revision surgeries in their lifetime since the average longevity of current orthopedic implants is only 10 to 15 years [5]. Direct care

expenditures for fractures, such as surgery and therapy, cost approximately 18 billion dollars per year in the United States. Indirect costs, such as lost productivity for patients, may add billions of dollars to this figure [1]. In the coming decades, these costs could double or triple if surgical removal and revision surgery become necessary after implantation when an orthopedic implant fails under physiological loading conditions.

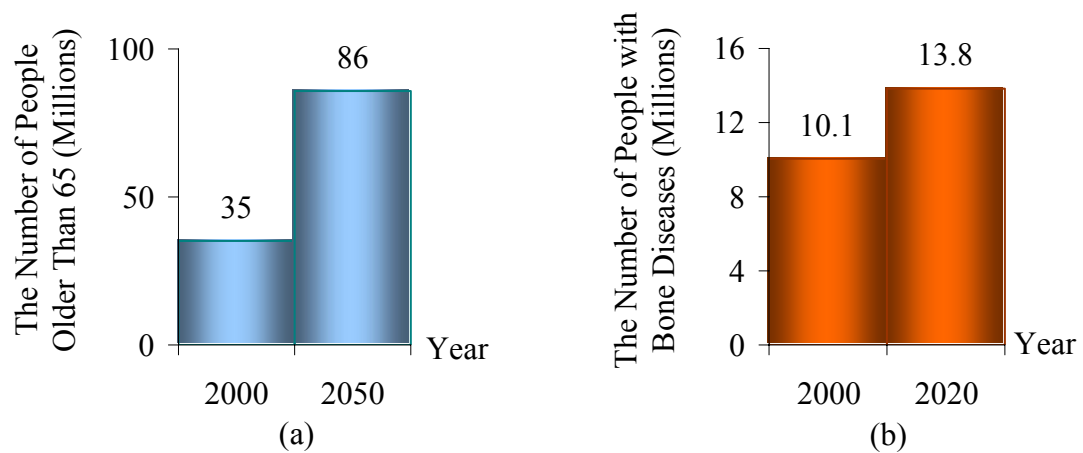


Figure 1. 1: The number of people with bone diseases will increase as the population ages. (a) The number of people older than 65 in 2000 and 2050 in the United States. (b) The number of people with bone diseases in 2000 and 2020 in the United States. (Data obtained from [1,2]).

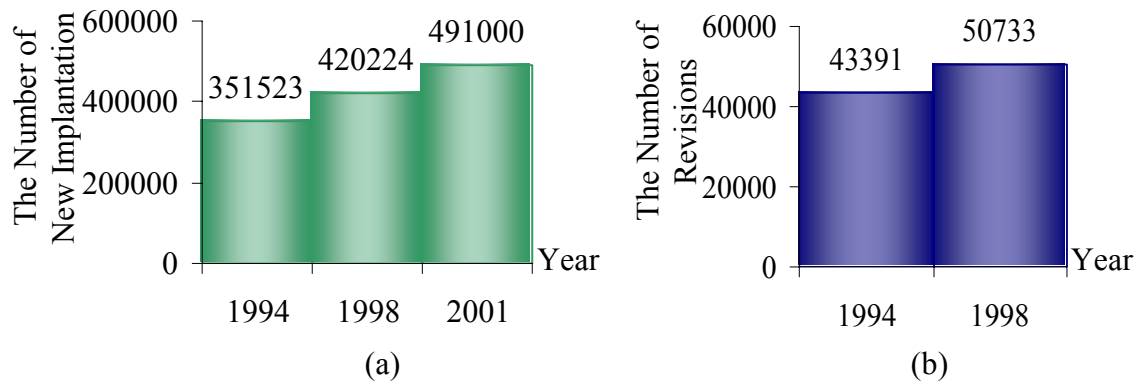


Figure 1. 2: The number of new implantation surgeries and the number of revision surgeries have both gradually increased over the past decade. (a) The number of newly implanted total joints in 1994, 1998 and 2001. (b) The number of revision surgeries in 1994 and 1998. (Data obtained from [3,4]).

Therefore, in order to ease the discomfort of patients and lower medical costs, it is of great importance to design, fabricate and evaluate novel orthopedic prostheses that can provide improved clinical efficacy. This dissertation presents a series of studies that have been conducted on bio-inspired nanocomposites for the purpose of developing more effective orthopedic prostheses. Specifically, chapter 1 introduces key considerations when developing orthopedic prostheses and the rationale for investigating nanostructured ceramic/polymer composites. Chapter 2 covers the fabrication, characterization and cytocompatibility of two-dimensional (2D) nanophase titania in polymer composites as well as degradation kinetics of the polymer mediated by the dispersion of nano-titania. Chapter 3 discusses design and fabrication of three-dimensional (3D) nanophase ceramic/polymer composites using a novel aerosol-based 3D printing technique as well as cell interactions with such 3D printed structures. Chapter 4 reports on the mechanical properties of nanophase ceramic/polymer composites and their significance for orthopedic applications. Chapter 5 reports on the use of nanophase ceramic/polymer

composites for controlled drug delivery applications. Lastly, chapter 6 summarizes the major conclusions drawn from these studies highlighted key design criteria for improving orthopedic prostheses through nanotechnology, and proposals for future research.

1.2. Problems with Current Bone Substitutes

Traditionally, autografts, allografts, xenografts and metal implants have been used to repair fractures and other bone defects. However, these substitutes are far from ideal as each has its own specific problems and limitations [6].

1.2.1. Autografts

An autograft is the tissue removed from one portion of the skeleton and transferred to another location in the same individual. It is commonly taken in the form of cancellous bone from the patient's iliac crest, but compact bone can be used as well [7]. Historically, autografts have been the gold standard for bone replacements for many years because they provide osteogenic cells as well as essential osteoinductive factors needed for bone healing and regeneration [8]. However, autografts are always associated with donor shortage and donor site morbidity, which severely limit their applications. The number of patients requiring a transplant far exceeds the available supply of donor tissue [9]. Clearly, other bone substitutes are needed to reduce this deficit.

1.2.2. Allografts and Xenografts

An allograft is the tissue transplanted between genetically non-identical members of the same species while a xenograft is the tissue transplanted between members of different species. Clearly, allografts and xenografts have the risk of disease transmission and, thus, may involve a severe immune response [10,11].

1.2.3. Metals and Metal Alloys

Due to the above stated issues with natural grafts, synthetic materials have been the material of choice for the majority of orthopedic applications. Metals and metal alloys, such as stainless steel, CoCrMo alloy and Ti6Al4V alloy, have been the dominant materials used in orthopedics. However, the average longevity of current metal-based orthopedic implants is only 10 to 15 years [5].

Implant loosening over time is the leading cause of clinical failure in the short term, as a result of insufficient osteoblast (bone forming cell) functions and excessive fibroblast (fibrous tissue forming cell) activities. Moreover, mismatches in the mechanical properties of metallic implants and physiological bone result in “stress shielding” problems in the long term according to Wolff’s law [12-14]. That is, the implanted material shields healing bone from mechanical loading, resulting in necrosis of the surrounding bone and subsequent implant loosening. Table 1.1 highlights some physical and mechanical properties of metals which are currently used for bone replacements. Obviously, metals have much higher density and mechanical properties than actual bone. All these conditions generate clinical complications and necessitate

additional revision surgery. In addition, metallic implants are permanent and, thus, can not be remodeled or replaced with time with healthy bone; this results in chronic clinical problems (such as possible consistent inflammation and malnutrition of surrounding bone tissue).

Table 1. 1: Selected physical and mechanical properties of metal alloys that are currently used as bone replacements. (Data obtained from [15]).

Metal alloys	Density (g/cm ³)	Elastic Modulus (GPa)	Yield Strength (MPa)	Ultimate Tensile Strength (MPa)	Elongation (%)
Stainless Steel (316L Annealed)	8	193	172	485	40
CoCrMo (F75 Cast)	8.3	220	450	655	8
Ti6Al4V	4.42	100	795	860	10

All of these clinical problems that are associated with natural grafts and metallic implants emphasize a critical need for novel synthetic orthopedic prostheses that possess similar structure, properties, and functions to physiological bone. In this manner, it is important to first understand the composition, structure, and resulting properties of bone.

1.3. Basic Science of Bone

The skeleton is a remarkable organ that serves both a structural function (providing mobility, support and protection for other internal organs) and a reservoir function (e.g., as the storehouse for essential minerals). This section introduces the

chemistry, architecture, mechanical properties and physiological functions of natural bone so as to closely mimic or match its composition, microstructure and properties using synthetic materials.

1.3.1. Bone as a Nano-Composite Material

Natural bone is a composite material composed of organic compounds (mainly collagen) reinforced with inorganic compounds (minerals). Apparently, the single mineral phase of bone is too brittle and easy to break while the single collagen phase is too soft and does not have mechanical stability (such as compression strength). The composite chemistry of bone provides both strength and resilience so that the skeleton can absorb energy when stressed without breaking. The detailed composition of bone differs depending on species, age, dietary history, health status and anatomical location. In general, however, the inorganic phase accounts for about 70% of the dry weight of bone and the organic matrix makes up the remainder [16].

1.3.1.1. Inorganic Phase

The inorganic or mineral component of bone is primarily rod-like (20 to 80 nm long and 2 to 5 nm in diameter) crystalline hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ or HA. Small amounts of impurities which affect cellular functions may be present in the mineralized HA matrix; for example, magnesium, strontium, sodium, or potassium ions may replace calcium ions, carbonate may replace phosphate groups, whereas chloride and fluoride may replace hydroxyl groups. Because the release of ions from the mineral phase

of the bone matrix controls cell-mediated functions, the presence of impurities may alter certain physical properties of bone (such as solubility) and consequently important biological aspects which are critical to normal bone function. For example, magnesium present in the mineralized matrix may enhance cellular activity and promote the growth of HA crystals and subsequent new bone formation [1].

1.3.1.2. Organic Phase

Approximately 90% of the organic phase of bone is Type I collagen; the remaining 10% consists of noncollagenous proteins and ground substances. Type I Collagen found in bone is synthesized by osteoblasts and is secreted as a triple helical procollagen into the extracellular matrix, where collagen molecules are stabilized by cross-linking of reactive aldehydes among the collagen chains. Generally, each of the 12 types of collagen found in body consists of 3 polypeptide chains composed of approximately 1,000 amino acids each. Specifically, Type I collagen (molecular weight 139,000 Daltons) possesses 2 identical $\alpha 1(I)$ chains and 1 unique $\alpha 2$ chain; this configuration produces a fairly rigid linear molecule that is 300 nm long [17]. The linear molecules (or fibers) of Type I collagen are grouped into triple helix bundles having a periodicity of 67 nm, with gaps (called hole-zones) between the ends of the molecules and pores between the sides of parallel molecules. The collagen fibers provide the framework and architecture of bone, with the HA particles located between the fibers.

Noncollagenous proteins, for example, growth factors and cytokines (such as insulin-like growth factors and osteogenic proteins), bone inductive proteins (such as osteonectin, osteopontin, and osteocalcin), and extracellular matrix compounds (such as

bone sialoprotein, bone proteoglycans, and other phosphoproteins as well as proteolipids) provide minor contributions to the overall weight of bone but have major contributions to its biological functions. During new bone formation, noncollagenous proteins are synthesized by osteoblasts, and mineral ions (such as calcium and phosphate) are deposited into the hole-zones and pores of the collagen matrix to promote HA crystal growth. The ground substance is formed from proteins, polysaccharides and mucopolysaccharides which acts as a cement, filling the spaces between collagen fibers and HA crystals.

In conclusion, bone itself is a nanostructured composite composed of nanometer-sized HA well-dispersed in a mostly collagen matrix (Figure 1.3). Although the inorganic and organic components of bone have structural and some regulatory functions, the principal regulators of bone metabolism are bone cells which will be discussed in section 1.3.3.

1.3.2. Architecture, Microstructure and Mechanical Properties of Bone

Cancellous bone and compact bone are two of the most important naturally occurring forms of bone, as shown in Figure 1.3. Cancellous bone (also called trabecular or spongy bone) is characterized by a three-dimensional sponge-like branching lattice structure with 50 to 90% porosity and large pores which are up to several millimeters in diameter. Cancellous bone, primarily found at the epiphyses and metaphyses of both long and cuboidal bones, approximates an isotropic material and is mainly subjected to compression under physiological loading conditions. In contrast, compact bone (also

called cortical bone) is characterized by less than 30% porosity and is composed of small pores up to 1 mm in diameter. Compact bone, primarily found at the diaphysis of long bones (such as the femur and the tibia), is highly anisotropic with reinforcing structures along its loading axis.

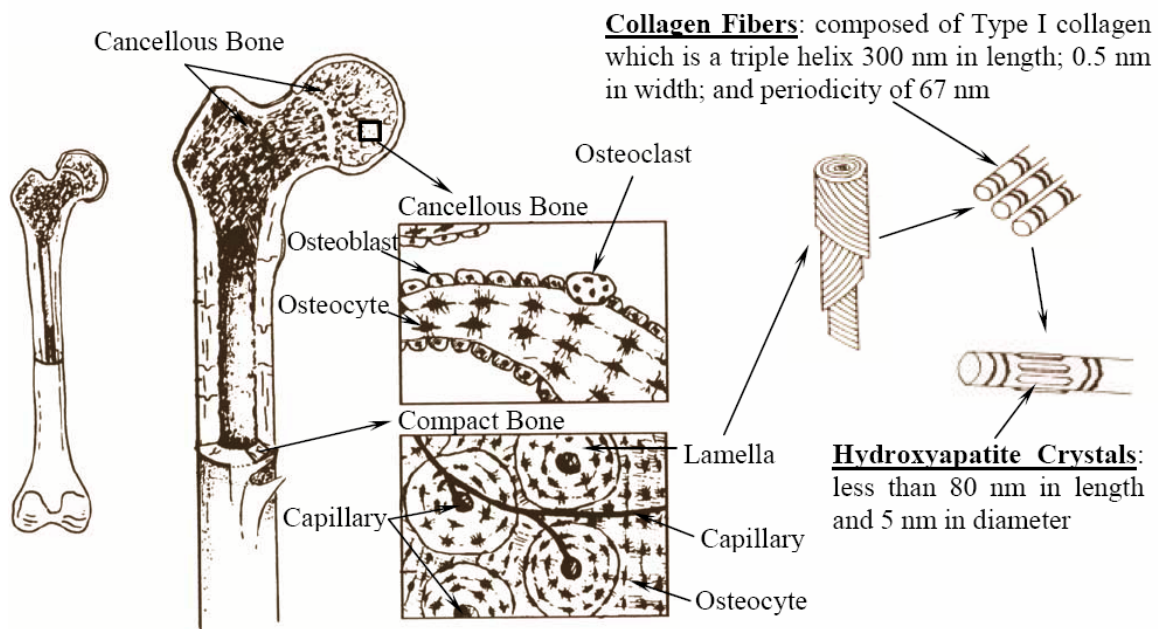


Figure 1. 3: Schematic structure of a human femur. (Adapted and redrawn from [18]).

Compact bone is usually more dense and, thus, stronger than cancellous bone. The relative density and some mechanical properties of bone are shown in Table 1.2. These properties also change with sex, age, dietary history, health status, and anatomical locations. Diseased bone usually has lower density and weaker mechanical properties than respective healthy bone.

Table 1. 2: Relative density and mechanical properties of healthy human bone. (Data obtained from [19-22]).

Property	Cancellous Bone	Compact Bone (Longitude)	Compact Bone (Transverse)
Relative Density	0.05-0.7	0.7-1.8	
Elongation (%)	5-7	1-3	
Elastic Modulus (GPa)	0.05-0.5	17-30	7-13
Ultimate Tensile Strength (MPa)	2-20	130-150	50-60
Compressive Strength (MPa)	2-12	100-230	
Bending Strength (in Ringer's Solution) (MPa)	N/A	50-150	
Fracture Toughness, K_{IC} ($\text{MPa}\sqrt{\text{m}}$)	N/A	2-12	

At the microstructural level, bone consists of two structures: woven and lamellae. Woven bone is immature or a primitive form of bone and is normally found in the metaphyseal region of growing bone as well as in fracture callus and diseased (such as Pagetic) bone. Woven bone is composed of relatively disoriented coarse collagen fibers and, thus, has isotropic characteristics. In contrast, lamellae bone is a more mature bone that results from the remodeling of woven or previously existing bone. Lamellae bone is highly organized and contains stress-oriented collagen fibers which results in anisotropic properties with greatest strength parallel to the longitudinal axis of the collagen fibers. Lamellae bone is formed into concentric rings (approximately 4-20 rings) called osteons with a central blood supply called a Haversian system.

It is not only the complex architecture of natural bone that makes it difficult to replace, but also its dynamic ability. Bone has the ability to regenerate when damaged and also to remodel when the loading conditions change, for example, the mass of bone mineral can be increased with exercise, making bones less likely to fracture [23]. Therefore, it is important to understand how bone cells coordinate functions during this bone remodeling process.

1.3.3. Bone Remodeling and Bone Cells

Bone as a living organ can change in size, shape, position, and properties by its remodeling process throughout its lifetime to respond to different kinds of stress produced by physical activity or mechanical loads. The remodeling process involves the removal of old bone and regeneration of new bone at the same site. Therefore, bone has the capability of self-repairing under excessive mechanical stresses by activating the remodeling process through the formation of a bone-modeling unit (BMU). This process involves three major types of bone cells: osteoblasts (bone-forming cells), osteocytes (bone-maintaining cells), and osteoclasts (bone-resorbing cells).

Bone remodeling continues throughout human life so that most of the adult skeleton is replaced about every 10 years. Figure 1.4 depicts how bone cells cooperate in the bone remodeling process. Osteoclasts are activated by growth factors, cytokines, and proteins present in the bone matrix to resorb old bone. Osteoblasts are then activated by growth factors (such as insulin-like growth factors I and II) secreted by osteoclasts and/or osteocytes to deposit calcium-containing minerals. Osteocytes regulate new bone formation by modulating osteoblast differentiation from non-calcium depositing to

calcium depositing cells through the secretion of growth factors (such as insulin-like growth factor I and the tissue growth factor β) [24].

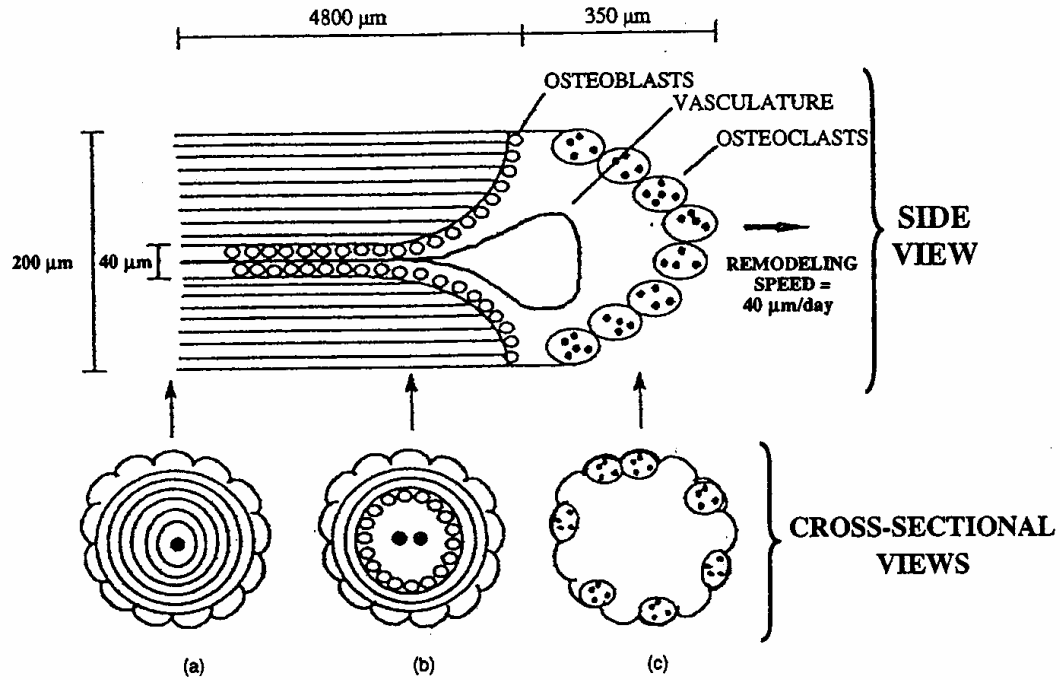


Figure 1. 4: Schematic diagram of the coordinated bone cell functions that maintain homeostasis during bone remodeling. (Adapted and redrawn from [25]).

1.3.3.1. Osteoblasts

Osteoblasts are located on the periosteal and endosteal surfaces of bone with an average diameter of 10 to 50 μm and contribute to new bone synthesis. Figure 1.5 schematically describes the time course of osteoblast proliferation and differentiation on a newly implanted biomaterial. After initial adhesion to the surface of an implant, osteoblasts actively proliferate and express genes for Type I collagen, vitronectin, and fibronectin [26]. At the end of proliferation, the extracellular matrix development and

maturation begin and osteoblasts start to differentiate from non-calcium to calcium depositing cells. Alkaline phosphatase activity and mRNA expression for proteins (such as osteopontin, and collagenase) increase tenfold [26]. As the mineralization process begins and mineral nodules form, osteoblasts synthesize and deposit bone sialoprotein, osteocalcin (a calcium-binding protein), and other matrix proteins. Osteocalcin interacts with HA and is thought to mediate the coupling to bone resorption by osteoclasts and bone formation by osteoblasts and/or osteocytes.

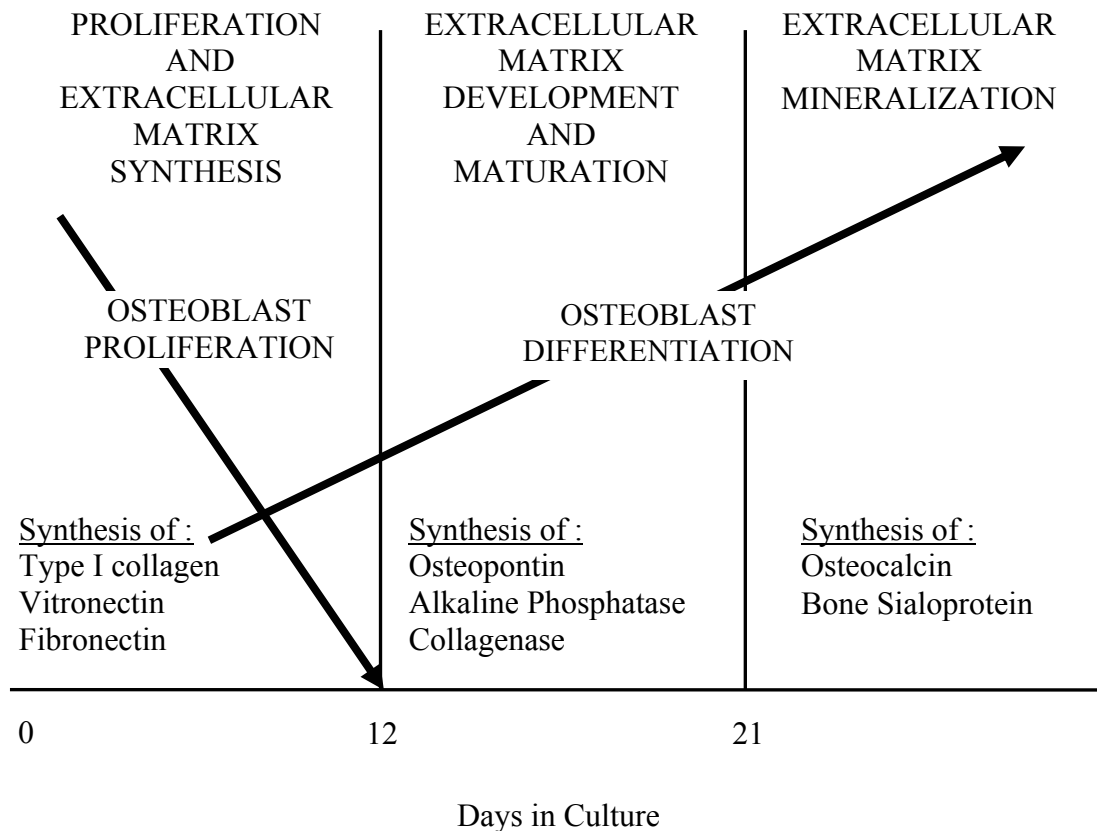


Figure 1. 5: Time course of osteoblast functions on a newly implanted biomaterial. (Adapted and redrawn from [26]).

1.3.3.2. Osteocytes

Osteocytes are mature osteoblasts embedded in the mineralized bone matrix and also contribute to new bone synthesis but to a lesser extent than osteoblasts. The principal difference between osteocytes and osteoblasts is their relative location in bone. Osteocytes are arranged concentrically around the central lumen of an osteon and in between lamellae (Figure 1.3). Osteocytes possess extensive long branches with which they establish contacts and communications with adjacent osteocytes through small channels called canaliculi. Due to their three-dimensional distribution and interconnecting structure, osteocytes are believed to be sensitive to physiological stress and strain signals in bone tissue and help to mediate or balance (i) osteoblastic activity to deposit new bone and (ii) osteoclastic activity to dissolve old bone.

1.3.3.3. Osteoclasts

Osteoclasts are derived from pluripotent cells of bone marrow and lie in the regions of bone resorption in pits called Howship's lacunae. Osteoclasts, primarily responsible for bone resorption, are distinguished by their large size which is up to 100 μm in diameter and their multiple nuclei which could be up to 100 per cell. When osteoclasts sweep across disrupted bone surfaces to dissolve bone, they first form ruffled cell membrane edges to increase their total surface area of attachment onto the resorptive surfaces. Then, osteoclasts produce tartrate-resistant acid phosphatase (also known as TRAP) which results in the release of hydrogen ions through the carbonic anhydrase system and subsequently decreases the pH of the local environment. The

lowered pH increases the solubility of HA crystals and the organic components of the bone matrix are removed by acidic proteolytic digestion.

The remodeling process is vital for bone health, for a variety of reasons. First, the remodeling process helps bone repair or replace small cracks or deformities in the areas of cell damage resulted from repeated stresses. Second, remodeling maintains the resilience of bone by replacing old, brittle bone with new regenerated bone. Third, the remodeling is important for functions of the skeleton, providing storage space for calcium and phosphorus. Specifically, the formation phase of remodeling can take up calcium and phosphorus and replenish this storage space when mineral supplies are ample while the resorption phase can supply these minerals to the other parts of body when needed.

Importantly, the extent of bone remodeling that occurs at an implant surface will determine the fate of the prosthetic device. For example, loosening and failure of the implant may result from either: (1) little or no remodeling in the bone surrounding an implant, which may lead to malnourished juxtaposed bone, or (2) too much remodeling in the bone surrounding an implant, which may lead to excessive bone resorption, or osteolysis and eventual implant loosening.

1.4. Essential Requirements for Orthopedic Prostheses

Orthopedic prostheses must have a series of suitable properties for the purpose of bone regeneration. The successful design of orthopedic prostheses involves comprehensive considerations of macro-, micron- and nano-structural properties of the prostheses and their interactions with natural tissue. Such properties affect not only cell

survival, proliferation, differentiation, signaling, and growth, but also their gene expression and the preservation of their phenotype, which eventually determines the success or failure of the implant by mediating healing.

1.4.1. Considerations of Synthetic Material-Tissue Interfaces

Cellular and molecular events that occur at the tissue-material interface will clearly control the extent of bone remodeling around the prostheses and determine the eventual clinical success or failure of the implant. Implantation surgeries inevitably introduce “foreign body” substances into living tissue and subsequently cause a series of host tissue responses, including inflammation and wound healing, which involve the recruitment of a variety of cell types and proteins to the tissue-material interface [27].

There are four types of tissue response to materials, as shown below in Table 1.3. The relative level of reactivity of a material influences the thickness of the interfacial zone or layer between the material and tissue. Analyses of implant failures during the past 20 years generally show failure originating at the biomaterial-tissue interface [28]. When biomaterials are nearly inert and the interface is not chemically or biologically bonded, there is relative movement and the progressive detrimental development of a fibrous capsule in hard tissues [28].

Table 1. 3: Types of tissue response to implanted materials. (Adapted and redrawn from [28]).

Implanted Materials	Tissue Response
Toxic	The surrounding tissue dies
Nontoxic and biologically inactive (nearly inert)	A fibrous tissue of variable thickness forms
Nontoxic and biologically active (bioactive)	An interfacial bond forms
Nontoxic and resorbable	The surrounding tissue replaces it

However, such detrimental fibrous tissue formation can be avoided if certain optimal chemistries are chosen. For example, for bone regeneration, initial protein-mediated osteoblast adhesion to the surface of orthopedic materials is imperative for subsequent new bone formation, leading to successful osseointegration. The initial adsorption of proteins to material surfaces is important for cell adhesion.

1.4.1.1. Protein-Material Interactions

Before cells (such as osteoblasts) adhere to an implant surface, proteins will adsorb onto the surface within milliseconds to potentially interact with select cell membrane receptors, as shown in Figure 1.6 [17].

Accessibility of adhesive domains (such as specific amino acid sequences) of adsorbed proteins may either enhance or inhibit subsequent cellular attachment. Adsorption of particular proteins (such as vitronectin, fibronectin, and laminin) from body fluids determines the subsequent adhesion and growth of specific desirable or undesirable cells on the surface [17]. The type, concentration, conformation, and bioactivity of plasma proteins adsorbed onto materials depend on surface chemistry,

hydrophilicity or hydrophobicity, charge, topography, roughness, and energy. For example, maximum vitronectin adsorption was noted on hydrophilic surfaces with high surface roughness [29,30]. Compared to rough surfaces, very smooth surfaces favor fibroblast functions over osteoblast functions to subsequently result in fibrous tissue formation called fibrosis, which should be avoided for a successful implant [31]. It has also been reported that the adsorption of calcium on titanium surfaces enhanced binding of select proteins since many proteins have calcium binding sites, but the adsorption of other ions (such as magnesium) does not affect select protein adsorption [17].

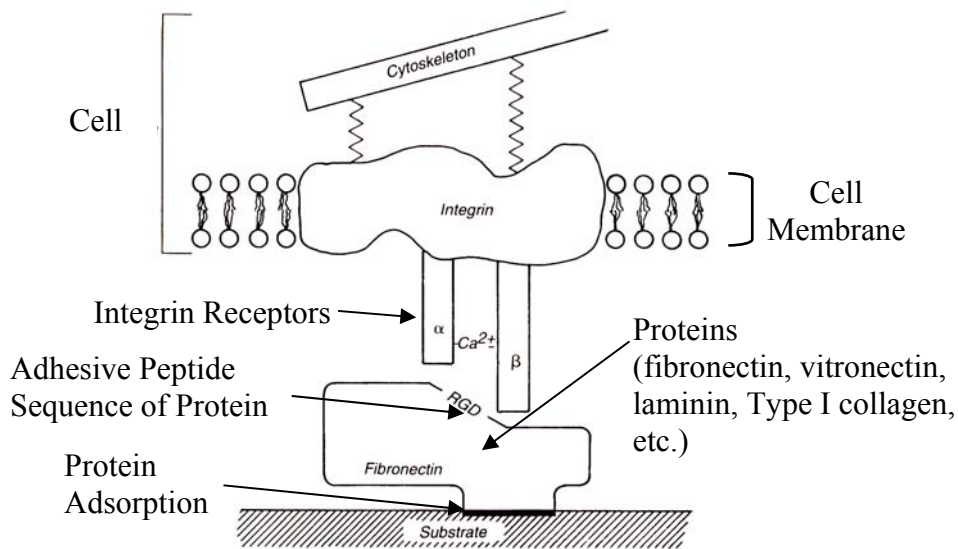


Figure 1. 6: Schematic representation of protein-mediated cell adhesion on biomaterial surfaces. (Adapted and redrawn from [17]).

The strength of adhesion between the material surface and the adsorbed proteins will determine if the proteins will remain adherent or be replaced by other proteins with a higher affinity to the surface [32-34]. The adsorbed protein layer composition and configuration then dictates what adhesive protein ligands will be exposed, thus,

determining the cell adhesive nature of the surface [35,36]. Specifically, the Arginine-Glycine-Aspartic Acid (RGD) peptide sequence present in vitronectin, fibronectin, collagen, and laminin are known to promote the adhesion of several types of cells to biomaterial surfaces [28].

1.4.1.2. Protein-Mediated Cell Interactions with Surfaces

Cells interact with their external environment through signals (specifically, chemical, electrical, and mechanical) transmitted through the cell membrane. For this reason, understanding cellular interactions with a biomaterial surface requires elucidation of molecular processes that occur at the cell membrane-biomaterial interface. For example, cellular adhesion, a prerequisite step for anchorage-dependent cell functions has been well examined at the molecular level. Cell-binding regions of extracellular matrix proteins (such as the RGD peptide sequence) and respective cell-membrane-intercalated receptors have been identified as being among the most important mechanisms for cell adhesion to substrates (Figure 1.6).

Initial bone cell interactions with a surface indicate material toxicity, cytocompatibility, and eventually its potential to support new bone formation. Therefore, nowadays, how the surface characteristics influence initial cellular activity (such as cell adhesion, morphology, proliferation, and differentiation) *in vitro* attracts more and more attention because it is relatively easy for *in vitro* cytocompatibility studies to eliminate a wide range of extraneous factors to examine a specific cellular response as compared with *in vivo* studies [37].

1.4.2. Desirable Properties of Synthetic Materials for Orthopedic Applications

When developing synthetic materials for orthopedic applications, the properties highlighted in the following sections must be considered thoroughly because these properties control, either directly or indirectly, the efficacy and destiny of bone substitutes, critical for clinical success.

1.4.2.1. Biocompatibility

Orthopedic prostheses should be compatible to cells and be well integrated into the host tissue without eliciting a severe immune response, cytotoxicity, or formation of scar tissue [7]. Factors that determine cytocompatibility can be affected not only by intrinsic chemistry of materials but also by techniques used for material synthesis and fabrication. For example, residual chemicals involved in polymer processes (such as organic solvents, initiators, stabilizers, cross-linking agents, catalysts, or unreacted monomers) may leach out of implanted materials under physiological conditions. Therefore, not only the intact biomaterial, but also any leachable components and degradation products, must be biocompatible. Specifically, the release of acidic by-products from some degradable materials may cause tissue necrosis or inflammation due to a quick drop in local pH [38].

1.4.2.2. Biodegradability

The ideal orthopedic prostheses should be biodegradable and bioresorbable with a controllable degradation and resorption rate to match cell/tissue growth *in vitro* and *in*

vivo. The degradation rate of the materials and the rate of new tissue formation must be appropriately coupled to each other in such a way that by the time the injury site is totally regenerated, the implant is totally degraded. The degradation rate of an implant can be altered by many factors (such as its structure and the molecular weight of the component materials). The structures in prostheses (such as surface-to-volume ratio, porosity, pore size and shape) may also play important roles in degradation kinetics, as do dimensions and geometries. The choice of implantation site, the amount of mechanical loading, and the rate of metabolism of degradation products *in vivo* also influence the degradation time of the implanted prostheses.

1.4.2.3. Mechanical Properties

Orthopedic prostheses should also have adequate mechanical properties to match the intended site of implantation. *In vitro*, the scaffolds should have sufficient mechanical strength to withstand hydrostatic pressures and to maintain spacing required for cell in-growth and matrix production [39]. *In vivo*, because bone is always under physiological stresses (such as compression, tension, torsion, and bending), the mechanical properties of the implanted materials should closely match those of living bone so that early healing of the injured site can be possible. If the mechanical strength of an implant is much higher than bone, resulting stress-shielding effects will slow down bone healing. If the mechanical strength of an implant is much lower than bone, obviously, it will break down under load-bearing conditions.

1.4.2.4. Surface Properties

Orthopedic prostheses should have appropriate surfaces to favor cell attachment, proliferation and differentiation. Surface properties, both chemical and topographical, can control and affect bioactivity and osteoconductivity. Chemical properties are related to the ability of proteins to initially adsorb and, subsequently, for cells to adhere to the material surface. Topographical properties are of particular interest when osteoconductivity is concerned. Osteoconduction is the process by which osteogenic cells migrate to the surface of the scaffold through a fibrin clot, which is established immediately after the material is implanted. This migration of osteogenic cells through the clot will cause retraction of the temporary fibrin matrix. Hence, it is of the utmost importance that the fibrin matrix is well secured to the implant, otherwise, when osteogenic cells start to migrate, the fibrin will detach from the implant due to wound contraction. As opposed to a smooth surface, it has been previously shown that a rough surface will be able to imprison the fibrin matrix and hence facilitate the migration of osteogenic cells to the implant surface [40,41].

1.4.2.5. Osteoinductivity

Osteoinduction is the process by which mesenchymal stem cells and pluripotent osteoprogenitor cells are recruited to a bone healing site. These cells are then stimulated to the osteogenic differentiation pathway. However, when the portion of bone that requires regeneration is large, natural osteoinduction is not enough for accelerating bone healing. Therefore, the orthopedic implant itself should be osteoinductive to promote bone formation. Recombinant human bone morphogenetic proteins (rhBMPs), such as

rhBMP-2 and rhBMP-7, were found to be osteoinductive and capable of inducing new bone formation. Recent research has demonstrated that combining rhBMPs with bone scaffolds could significantly increase osteoinductivity of the scaffolds and hence promote new bone growth and accelerate healing [42].

1.4.2.6. Interconnected 3D Structures

The ideal orthopedic prostheses should have 3D bone-like interconnected porous structures with appropriate organization, porosity and scale to favor tissue integration and vascularization, as well as support flow transportation of nutrients and metabolic waste. Pore size is a very important factor because bone scaffolds with large void volume and large surface-area-to-volume ratio maximize space to help cells, tissues, and blood vessels penetrate. To attain a high surface area per unit volume, however, smaller pores are preferable as long as the pore size is greater than the diameter of osteoblasts (typically, 10 μm). If the pores employed are too small, pore occlusion by the cells may happen. This will prevent cellular penetration and neovascularization of the inner areas of bone scaffolds. It is reported that interconnected larger pores facilitate diffusion and cell migration within the scaffolds, improving nutrient supply and waste removal, and, thus, increasing the viability of cells at the center of the scaffolds [38]. Currently, researchers are still searching for the optimal pore size and shape for various bone tissue engineering applications. It is also crucial to control the suitable porosity of scaffolds by adjusting available fabrication techniques to match the porosity of true bone. Importantly, the porosity, pore structures, and pore size affect the mechanical and biological properties of scaffolds.

1.4.2.7. Feasible Fabrication Techniques and Sterilizability

Orthopedic prostheses should be fabricated reproducibly on a large scale using versatile processing techniques for a variety of shapes and sizes to match bone defects in patients. As with all implanted materials, bone substitutes must be easily sterilizable to prevent infection. The method of sterilization, however, must not interfere with bioactivity of biomaterials or alter their chemical composition, which could influence their cytocompatibility or degradation properties.

Keeping these requirements in mind, several orthopedic materials for bone regeneration will be further reviewed in section 1.5.

1.5. Suitable Orthopedic Materials

The selection of the most appropriate material to produce an orthopedic prosthesis is a very important step towards the construction of a successful product. As mentioned, the properties of constituent materials will determine, to a great extent, the properties of the final implant. So far, a wide variety of natural and synthetic biomaterials, such as polymers, ceramics, and a combination of them, have been studied for orthopedic and dental applications. Table 1.4 highlights some physical and mechanical properties of materials of particular interest for bone repair.

Table 1. 4: Selected properties of materials used for bone repair. (Adapted and redrawn from [43-46]).

Materials	Density (g/cm ³)	Elastic Modulus (GPa)	Ultimate Strength (MPa)
Polymers			
Polyethylene (PE)	0.91-0.96	0.88-1	30-35 (Tensile)
Poly(methyl methacrylate) (PMMA)	1.15-1.2	2.1-3.4	22-48 (Tensile) 64-103 (Compressive)
Tyrosine-derived Polycarbonate	1.2	1.2-1.5	51-67 (Tensile)
Ceramics			
Alumina	3.8-4.0	365-380	6-55 (Tensile) 1000-2700 (Compressive)
Zirconia	5.7-5.95	190-210	>300 (Tensile) 1500-2000 (Compressive)
HA	3.15-3.22	40-117	8-50 (Tensile) 100-294 (Compressive)
Composites			
Epoxy/carbon fiber	1.55-1.63	46-215	579-1240 (Tensile)
Polypropylene fumarate /Tricalcium phosphate (PPF/TCP)	N/A	0.4-1.2	16.7-17.9 (Compressive)
Bioglass [®]	2.2-3.7	35	42-84 (Tensile)

1.5.1. Biodegradable Polymers

Bioresorbable natural and synthetic polymers have attracted increasing attention for their use as scaffold materials in the last ten years [47]. Many practical advantages arise because these polymers such as PLGA (poly-lactide-co-glycolide) allow for precise control of chemical composition (e.g., the lactide/glycolide ratio in the PLGA

copolymers), crystallinity, molecular weight, molecular weight distribution, as well as microstructure and macrostructure (including porosity) [48-50]. This allows adequate control of bone scaffold properties (such as degradation rate and mechanical strength), thus, creating optimal conditions for cell survival, proliferation, and subsequent tissue formation. The degradation products of these polymers can be removed by natural metabolic pathways.

1.5.1.1. PLGA as a Biodegradable Polymer

The most commonly used synthetic polymers are biodegradable aliphatic polyesters. Poly(glycolic acid) (PGA, also called as polyglycolide), poly(lactic acid) (PLA, also called as polylactide), and their copolymers poly(lactic-co-glycolic acid) (PLGA, also called as poly-lactide-co-glycolide), as a family of aliphatic polyesters, are some of the most popular scaffold polymers [51-53].

PLGA was originally developed for use in resorbable surgical sutures and biodegradable drug delivery systems. These polymers (PLA, PGA, and PLGAs) are approved by the U.S. Food and Drug Administration (FDA) for certain human clinical applications. The first commercial suture, Dexon[®] (composed of poly-lactide-co-glycolide), was available in 1970 and the first FDA-cleared drug product was the Lupron Depot[®] drug-delivery system (TAP Pharmaceutical Products Inc.; Lake Forest, IL) which was a controlled release device for the treatment of advanced prostate cancer that used biodegradable microspheres of 75/25 weight ratio of lactide/glycolide to administer leuprolide acetate over periods of time up to 4 months (replacing daily injections). Since then there has been intensive development of medical devices composed of PGA, PLA,

and their copolymers [54]. The use of biodegradable polymers in orthopedic devices for fixation of fractures of long bones was first clinically implemented in Finland in 1984 [55,56]. Since the 1990s, the applications of PLA, PGA, and PLGA in tissue engineering have been extensively investigated [57].

DL-lactides and glycolides are polymerized via a cationic ring-opening reaction in the presence of stannous octoate as a catalyst to form a random copolymer called poly(DL-lactide-co-glycolide) or PLGA. A representative polymerization reaction is shown in Figure 1.7. PLGA gradually degrades into the endogenous natural metabolites lactic acid and glycolic acid by non-enzymatic hydrolysis of ester bonds in its backbone [58,59]. The polymers that undergo hydrolytic cleavage tend to have more predictable degradation rates *in vivo* than polymers whose degradation is mediated predominantly by enzymes because the levels of enzymatic activity may vary widely not only among different patients but also among different tissue sites in the same patient. But, the availability of water is virtually constant in all soft/hard tissues and varies little from patient to patient. The degradation products of PGA, PLA and PLGA are nontoxic, natural metabolites, and are eventually eliminated from the body in the form of carbon dioxide and water.

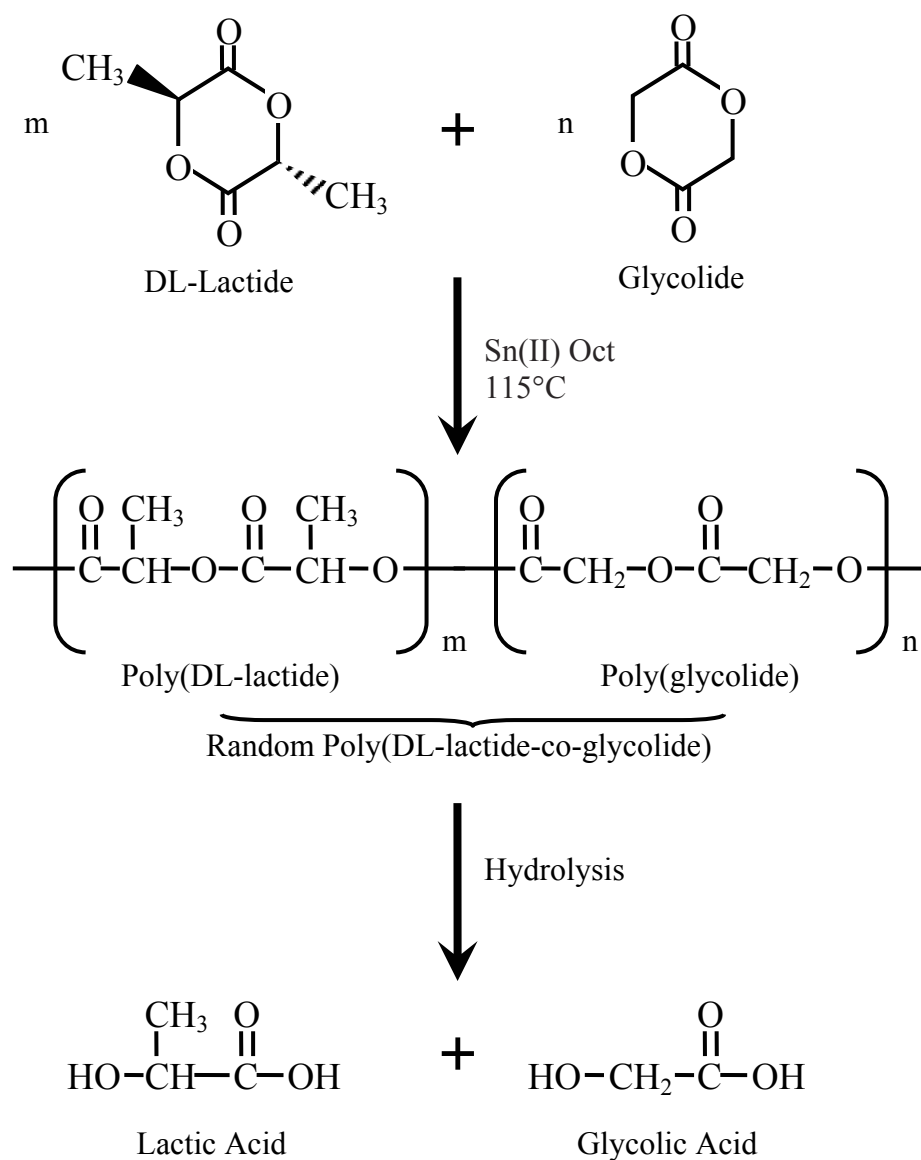


Figure 1. 7: Synthesis of poly(DL-lactide-co-glycolide) (PLGA) and decomposition into respective acids by hydrolysis.

The PLGA degradation process has been divided into three steps that begin at the outer perimeter of the device and move gradually into the interior, followed by catastrophic disintegration [60]. In step 1, water diffuses into the polymer and hydrolytic random chain scission of ester bonds begins. In step 2, the molecular weight decreases and low-molecular-weight oligomers in the inner part of the matrix begin to diffuse out of

the thinning outer layer. At this stage, an acidic environment is formed. When the molecular weight of these oligomers is low enough to allow solubilization in the medium, weight loss begins. In the final step 3, a polymer shell remains after the oligomers solubilize and slow degradation of the shell takes place. PLGA usually degrades through random scission mode under normal conditions (i.e. in water or phosphate buffer medium of pH 7.4 at 37 °C). However, PLGA degrades through unzipping mode (chain-end scission) under harsh conditions (such as high acidity, high temperature, or high energy radiation) [61]. Clearly, this complex degradation process indicates the difficulties in controlling the release rate.

The degradation rate of these polymers, such as PGA, PLA, and PLGA, can even be tailored to satisfy the requirements from several weeks to several years by altering the ratio of polylactic to polyglycolic acid, molecular weight, molecular weight distribution, crystallinity, hydrophilicity, pH of the surrounding fluids, as well as specimen size, geometry, porosity, surface properties and sterilization methods [62]. The degradation rate becomes slower as the molecular weight becomes higher. The lower the crystallinity is, the higher the chance of penetration of water molecules to initiate hydrolysis of the chains. Gamma irradiation used for sterilization at doses of 2-3 Mrad can result in significant backbone degradation since aliphatic polymers are sensitive to radiation damage. These materials are usually sterilized by exposure to ethylene oxide. Unfortunately, the use of ethylene oxide gas represents a serious safety hazard as well as potentially leaving residual traces in the polymeric devices. They must be degassed for extended periods of time.

Polymer crystallinity is a measure of the alignment of polymeric chains along each other. The presence of bulky side groups, branches and freely mobile atoms (like oxygen in the backbone bonds) adversely influences the alignment of neighboring chains and, thus, crystallinity. Because lactic acid has a chiral center, PLA can exist in four stereoisomeric forms, poly(L-lactic acid), poly(D-lactic acid), meso-poly(DL-lactic acid), and the racemic mixture of poly(L-lactic acid) and poly(D-lactic acid). Stereoregular poly(L-lactic acid) is semicrystalline, while the racemic poly(DL-lactic acid) is amorphous.

In the same conditions, hydrophilic PGA degrades faster in aqueous solutions (or *in vivo*) than the hydrophobic PLA because the adsorption of water molecules is higher into the chain of the former polymer, although the ester bonds in each have about the same chemical reactivity towards water. The extra methyl group in the PLA repeating unit (compared with PGA) makes it more hydrophobic, reduces the molecular affinity to water, and, thus, leads to a slower hydrolysis rate. Therefore, it seems that the higher the glycolic acid content, the faster the degradation rate. However, the lifetime of PLGA is shorter at a PLA/PGA ratio of 50/50 [63], because the more crystalline domains of PGA form as the amount of glycolic acid in the copolymer increases. In the crystalline state, the polymer chains are densely packed and organized to resist the penetration of water. Consequently, polymer backbone hydrolysis tends to only occur at the surface of the crystalline regions, which takes a much longer time than hydrolysis in an amorphous polymer or in an amorphous region of a semicrystalline polymer.

The mechanical properties of biodegradable polymers depend on their chemical structure, crystallinity, molecular weight, or molecular orientation. Table 1.5 highlights the mechanical properties of selected biodegradable polymers [18,21,64,65].

Table 1. 5: Mechanical properties of selected biodegradable polymers. (Data obtained from [18,21,64,65]).

Polymers	Elastic Modulus (GPa)	Tensile Strength (MPa)	Ultimate Elongation (%)
PGA (polyglycolide)	>6.9	>68.9	15-20
PLLA (semicrystalline)	2.4-4.2	55.2-82.7	5-10
PDLLA (amorphous)	1.4-2.8	27.6-41.4	3-10
PLGA	1.4-2.08	41.4-55.2	3-10
PCL (poly(ϵ -caprolactone))	0.21-0.34	20.7-34.5	300-500

Clearly, degradation leads to a loss of mechanical properties and an increase in crystallinity as a result of content loss. PGA loses mechanical integrity between two and four weeks while PLA takes many months or even years to lose mechanical integrity *in vitro* or *in vivo* [66,67]. The amorphous regions of semicrystalline polymers are subjected to degradation earlier than the crystalline regions, leading to an increase in crystallinity. The heterogeneity index (HI, M_w/M_n), an indicator of molecular weight distribution, increases upon PLGA degradation, indicating a faster decrease in M_n (number average molecular weight) in comparison to a decrease in M_w (weight average molecular weight).

1.5.1.2. Other Biodegradable Polymers

There are other aliphatic polyesters, such as poly(ϵ -caprolactone) (PCL), which has been studied for bone tissue engineering applications [68]. PCL degrades at a significantly slower rate than PLA, PGA, and PLGA [69-71]. A slow degradation rate makes PCL less attractive for general tissue engineering applications, but more attractive for long-term implants and controlled drug release applications. PCL-based copolymers have recently been synthesized to improve degradation properties [72]. Poly(propylene fumarate) (PPF) is also an important synthetic biodegradable polymer and can degrade through hydrolysis of the ester bonds similar to glycolide and lactide polymers [73]. The mechanical properties of PPF can vary greatly depending on the synthesis method and the cross-linking agents used [74].

Naturally derived polymers, such as collagen, have also been used for bone regeneration [75-77]. Collagen is a fibrous protein and a major natural extracellular matrix component. On the one hand, collagen (as the most popular natural polymer for tissue regeneration by far) has very attractive biological properties (such as biocompatibility) desirable for bone regeneration; on the other hand, there are concerns over collagen because of poor handling and poor mechanical properties to support bone loading requirements. Denatured collagen (gelatin) has also been processed into porous materials for bone tissue repair [78-80]. To increase the strength of these natural materials, they are often combined with ceramics [81].

1.5.2. Bioceramics

The main advantage of using ceramics lies in their high cytocompatibility with bone cells. For orthopedic applications, alumina, zirconia, titania, and calcium phosphates (such as calcium tetrphosphate ($\text{Ca}_4\text{P}_2\text{O}_9$), tricalcium phosphate (TCP, $\text{Ca}_3(\text{PO}_4)_2$), hydroxyapatite (HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) and its derivatives, as well as their combinations) are the most common types of bioceramics that have been used to facilitate bone tissue regeneration [82,83]. These ceramics are widely considered to be osteoconductive because their surface properties support osteoblast adhesion, growth, and differentiation and are also reported to be osteoinductive as a result of their capacity to bind and concentrate bone morphogenetic proteins (BMPs) *in vivo* [84]. Moreover, selected ceramics, such as HA and TCP, can react with physiological fluids and form tenacious bonds to hard and soft tissues through cellular activity, thus, classifying them as “bioactive” [85, 86]. In this dissertation, titania and calcium phosphate-based bioceramics were chosen as model ceramics. Therefore, their structure, properties, and medical applications will be discussed in the following sections.

1.5.2.1. Titania

1.5.2.1.1. Crystal Structure of Titania

Titania, also called titanium dioxide, has four possible phases: amorphous, the metastable crystalline forms of brookite and anatase, and the high temperature stable phase rutile. The control of titania crystal structure is important because each of the four possible phases possesses vastly different properties. Both anatase and rutile crystallize in the tetragonal system and are produced commercially, while brookite in the rhombic

system is rare, difficult to produce, and has no technological importance identified so far. Transformation from amorphous to anatase requires sintering temperatures near 300 °C. Above 700 °C, the monotropic conversion of anatase to rutile takes place rapidly. Therefore, rutile is the most thermally stable although anatase is also stable over long time periods below its phase transformation temperature.

However, when manufacturing temperatures are high (such as above 1000 °C), the oxygen partial pressure increases continuously as oxygen is liberated and consequently lower oxides of titanium (such as TiO) can be formed. This is accompanied by changes in color and electrical conductivity. Above 400 °C, a significant yellow color develops, caused by thermal expansion of the lattice; this is reversible.

In all three titania crystal structures, one titanium atom in the lattice is surrounded octahedrally by six oxygen atoms, while each oxygen atom is surrounded by three titanium atoms in a trigonal arrangement. The three structures correspond to different manners of linking the octahedra at their corners and edges, as shown in Figure 1.8 [87,88]. In rutile, the structure is based on octahedra of titanium oxide which share two edges of the octahedron with other octahedra and forms chains. It is the chains themselves which are arranged into a four-fold symmetry. In anatase, the octahedra share four edges hence the four fold axis. Rutile has the most compact atomic structure and, thus, has the highest density and hardness.

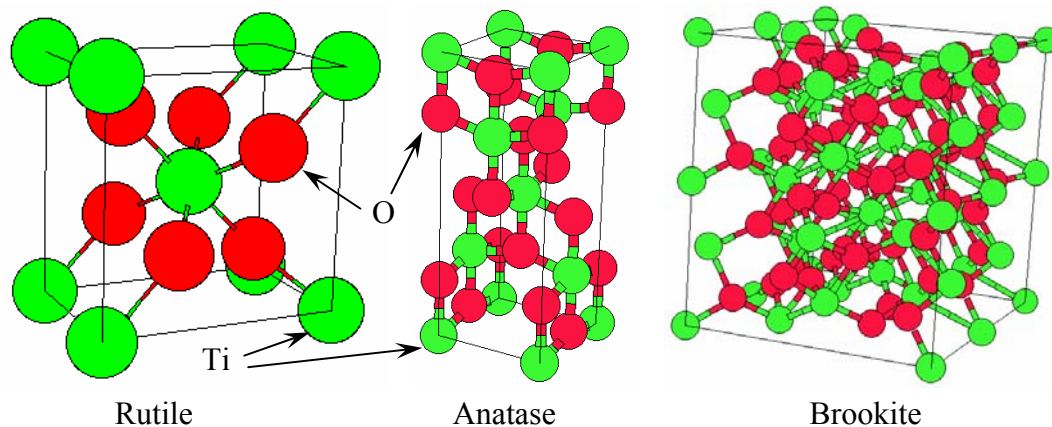


Figure 1. 8: Crystallographic unit cell of the three phases of titania. Green (light) balls represent titanium cations while red (dark) balls represent oxygen anions. (Adapted and redrawn from [87,88]).

1.5.2.1.2. Chemical, Physical, Mechanical and Thermal Properties of Titania

Both the anatase and rutile phase of titania are chemically very stable and resist various atmospheric contaminants (such as sulfur dioxide, carbon dioxide, and hydrogen sulfide). Under normal conditions, they are not readily reduced, oxidized, or attacked by most inorganic and organic reagents. Titania dissolves slightly in bases, hydrofluoric acid, and hot concentrated sulfuric acid. Therefore, titania is stable and nontoxic, making it medically preferred due to its chemical inertness [89].

Physical and mechanical properties of anatase and rutile are summarized in Table 1.6 [90,91]. The temperature for which these values are valid is room temperature.

Table 1. 6: Typical physical and mechanical properties of titania. (Data obtained from [90,91])

Properties	Anatase	Rutile
Crystal System	Tetragonal	Tetragonal
Lattice Constants		
a (nm)	0.3785	0.4593
c (nm)	0.9514	0.2959
Theoretical Density (kg/m ³)	3.89×10^3	4.26×10^3
Melting Point (°C)	Convert to rutile	1830-1850
Boiling Point (°C)	Convert to rutile	2500
Hardness, Mohs Scale	5.5-6	7-7.5
Vickers Hardness (GPa)	N/A	7-11
Poisson's Ratio	N/A	0.27
Young's Modulus (GPa)	N/A	283
Shear Modulus (GPa)	N/A	90
Modulus of Rupture (MPa)	N/A	140
Transverse Rupture Strength (MPa)	N/A	69-103
Compressive Strength (MPa)	N/A	680
Fracture Toughness, K_{IC} (MPa·m ^{0.5})	N/A	2.5
Thermal Expansion (K ⁻¹)	N/A	9.4×10^{-6}
Thermal Conductivity (W·m ⁻¹ ·K ⁻¹)	N/A	11.7

1.5.2.1.3. Surface Properties of Titania

Usually, the surface of titania is saturated by coordinatively bonded water, which then forms hydroxyl ions. Depending on the type of bonding of the hydroxyl groups to titanium, these groups possess acidic or basic character. The surface of titania is, thus, always polar. The surface covering of hydroxyl groups has a decisive influence on dispersibility of titania particles because adsorbed water vapor promotes the sticking of

powders to surfaces and agglomeration of powders. Heating above the boiling point of water is required to remove the adsorbed water completely.

1.5.2.1.4. Medical Applications of Titania

Oxidized layers (mainly titania) spontaneously form on traditional titanium orthopedic implant surfaces when exposed to air, water or other media (except under certain artificial conditions like ultra-high vacuum). Titania on the surface improves the stability (corrosion resistance) and biocompatibility of implants and is often used as coatings on implants. For example, the bioactivity of a titania coating can be easily improved by inducing deposition of apatite in Kokubo's simulated body fluid (SBF) [92]. The enhanced bioactivity of both titania and titania with grown apatite is attributed to both the epitaxial effect and the abundant Ti-OH group on their surfaces [92]. Selective adsorption of vitronectin (a protein known to mediate osteoblast adhesion) to titania surfaces was observed to be more than on an unoxidized titanium surfaces [93]. Anodization of titanium implants to create titania also improved osteoblast adhesion leading to more new bone formation around the implant [94].

Moreover, it has been reported that (i) titania coated implants enhance osteoblast-like cell proliferation and alkaline phosphatase activity *in vitro* as compared to uncoated pure titanium implants and (ii) a sol-gel derived titania coating stimulated the immediate contact with connective tissue *in vivo* whereas the titanium controls formed a gap and an extensive fibrous capsule at the implant-tissue interface [95,96]. It has also been reported that osteoblast adhesion increased with increasing crystallinity, while differentiation was

stimulated more on anatase than on rutile [97]. Very few studies, however, have been conducted on titania as a component of biocomposites for orthopedic applications.

1.5.2.2. Calcium Phosphates

Calcium phosphate-based bioceramics have received great attention as bone substitutes due to their chemical similarity to natural bone, their bioactivity and promising applications for less invasive orthopedic surgeries [98]. Importantly, the stability, reactivity, degradability, mechanical properties and biological properties of calcium phosphates depend to a great extent on their ratios of calcium (Ca) to phosphorous (P) [99-102]. The Ca/P ratios of calcium phosphates in bulk or in coatings vary according to which of the following phases are present: alpha and beta-tricalcium phosphate (TCP, β -Ca₃(PO₄)₂), tetracalcium phosphate, octacalcium phosphate, and hydroxyapatite (HA or Ca₁₀(PO₄)₆(OH)₂). Among these phases, pure crystalline HA is known to be the most stable and strongest phase [103]. HA is one of the most used calcium phosphates in the fabrication of orthopedic implants.

1.5.2.2.1. Crystal Structure of Hydroxyapatite

The crystal structure of HA is hexagonal rhombic with lattice constants of $a=0.942$ nm and $c=0.688$ nm, as shown in Figure 1.9 [104-107]. This unit cell can be arranged along a preferred orientation due to its hexagonal symmetry, which contributes to the anisotropic properties of natural bone. The ideal Ca/P molar ratio in stoichiometric HA is $1/0.6 \approx 1.67$. However, no biological HA shows a stoichiometric Ca/P ratio. For example, in bone and dental enamel, the crystallinity of HA is low and natural HA is

often doped with other ions, such as K^+ , Na^+ , Mg^{2+} , and Zn^{2+} , substituting for the Ca^{2+} ions [108]. HA has the ability to accept compositional variations through exchange of ions in its three sub-lattices, as shown in Figure 1.10. Therefore, HA can be modified and developed in response to the requirements of specific applications.

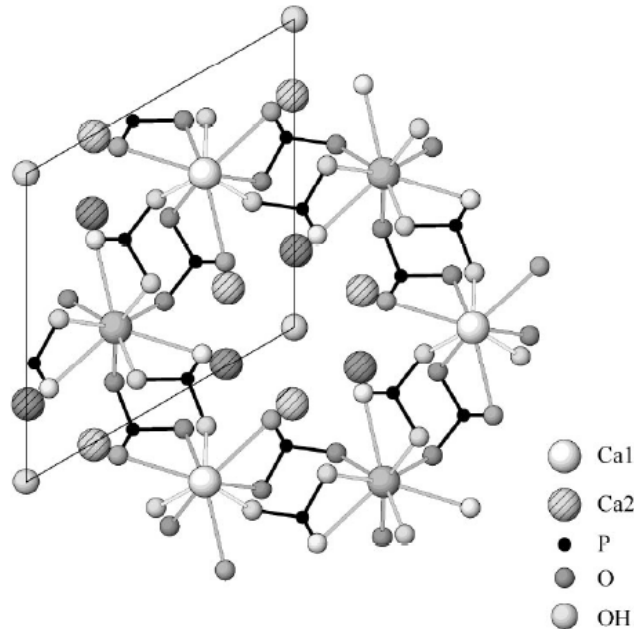


Figure 1. 9: Crystal structure of hydroxyapatite (HA) projected onto the (0001) plane (Hexagonal, $a=0.942$ nm and $c=0.688$ nm). (Adapted and redrawn from [107]).

The crystallinity of HA has a remarkable physiological meaning for skeletal systems. The more crystalline the HA becomes, the more difficult ions interchange and bone grows [109]. The less crystalline HA allows faster bone growth because its non-stoichiometric structure can store necessary elements through substitution. These elements facilitate bone regeneration carried out by osteoblasts. It has been reported that HA doped with zinc and magnesium promoted responses of osteoblasts and, in consequence, new bone formation [110].



Figure 1. 10: Generic formulation of apatite minerals, and potential substitutions in the three sub-lattices.

1.5.2.2.2. Chemical, Physical, Mechanical and Biological Properties of HA

Hydroxyapatite (HA), as a member of calcium phosphate-based bioceramics, has been widely used as bulk implants or as coatings on orthopedic and dental implants in order to achieve fast chemical bonding between bone and an implant [111,112]. Specifically, it has been documented that bone apposition is significantly improved at the surface of a HA-coated compared to uncoated metallic implant (thus, providing a stronger bone-implant interface) [113]. Physical and mechanical properties of HA are summarized in Table 1.7 in comparison with TCP.

Table 1. 7: Physical and mechanical properties of HA in comparison with TCP. (Data obtained from [22,114,115]).

Property	Hydroxyapatite (Sintered, Crystalline, Purity>99.2%)	β -Tricalcium Phosphate (Sintered, Purity>99.7%)
Crystal System	hexagonal rhombic	N/A
Lattice Constants		
a (nm)	0.94125 nm	N/A
c (nm)	0.68765 nm	N/A
Density (g/cm ³)	3.16	3.07
Vickers Hardness (HV)	600	N/A
Compressive Strength (MPa)	500-1000	460-687
Bending Strength (MPa)	115-200	140-154
Young's Modulus (GPa)	80-110	33-90
Fracture Toughness, K_{IC} (MPa $\sqrt{m}$)	0.5-1.5	N/A
Slow Crack Growth, Susceptibility Coefficient, n (unitless)*	12-27	N/A

*Note: The dynamic fatigue resistance of HA is related to the testing environment (such as pH, etc.) [116]. For example, Raynaud et al. found that the n decreased from 22.5 ± 2 in air to 10 ± 4 in Ringer's solution for dense materials [117]. Wakamatsu et al. gave a constant subcritical crack growth parameter $n=19$ for sintered HA in 37 °C distilled water [118].

Calcium phosphate materials may degrade in extracellular fluids due to an acidic wound healing response and/or by cellular activity within compartments of low pH [119].

As mentioned, the long term stability of calcium phosphate derived materials depends to a great extent on their Ca/P ratios. The lower the Ca/P ratio is, the larger are the acidity and solubility of calcium phosphates. For $\text{Ca/P} < 1$, both acidity and solubility are extremely high; both parameters decrease substantially for Ca/P ratios close to 1.67 (stoichiometric HA) [120]. Moreover, less crystalline phases of calcium phosphates (such as TCP) degrade much faster than the crystalline phase HA [121]. In addition, the dopants found naturally in HA dramatically change its properties. It is known that the bone regeneration rate depends on presence of certain elements that are released during the resorption of calcium phosphates, besides several other factors such as porosity, composition, and solubility of materials. For instance, small amounts of strontium, zinc or silicates stimulate the action of osteoblasts [109].

In addition to intentionally designing calcium phosphate materials to be more biodegradable or more stable, there are several unintentional cases that may lead to a lack of purity in the produced HA phase. For example, many coating processes lead to bulk or localized Ca/P ratios that can deviate from the standard HA stoichiometric value of 1.67. Calcium oxide (CaO) can be induced from either thermal decomposition [122] or from intentional additions for improving thermal stability [123]. Tricalcium phosphate is another common product of thermal decomposition that may occur during HA coating processes.

The main advantages of using bioceramics in orthopedic applications include high cytocompatibility with bone cells and possibly biodegradability leading to bone ingrowth. However, when used alone as a single phase material, they are inherently brittle, difficult to process into complex shapes and can not match the mechanical properties of true bone.

For example, the fracture toughness of HA ($0.5\text{-}1.5 \text{ MPa}\sqrt{\text{m}}$) is much lower than that of the cortical bone ($2\text{-}12 \text{ MPa}\sqrt{\text{m}}$) [124 - 126]. Therefore, bioceramics should be considered as major components of biocomposites for bone regeneration.

1.5.3. Bio-inspired Ceramic/Polymer Composites

Ceramic/polymer composites have been considered as the third-generation orthopedic biomaterials due to their closer-matched properties with natural bone compared to first (metals or metal alloys) and second generation (ceramics) bone substitute materials [98]. The design of ceramic/polymer composites offers an exceptional approach to combine the advantages of bioactive, strong ceramics and biodegradable, flexible polymers to optimize physical, mechanical, and biological properties of scaffolds for bone regeneration. In the past few years, the development of ceramic/polymer composites as orthopedic materials has attracted more and more attention [49,127,128].

First, in ceramic/polymer composites, osteoblast functions can be enhanced from better cell seeding and growth environments due to improved osteoconductivity properties provided by the bioactive ceramic phase [129-133]. For example, Ma et al. prepared highly porous PLA/HA composite scaffolds with a thermal-induced phase separation technique and demonstrated that osteoblast survival percentages and proliferation rates in the PLA/HA scaffolds were higher than in the pure PLA scaffolds [133].

Second, ceramic particles (such as Bioglass[®], HA and TCP) used as inclusions in biodegradable polyesters can provide a pH buffering effect at the polymer surface and tailor the desired degradation and resorption kinetics of the polymer matrix; thus, preventing acceleration of polymer degradation, avoiding the formation of an unfavorable environment for cells, and reducing side-effects (such as inflammation) from acidic degradation by-products [49].

Third, the stiffer particulate ceramic phase in polymer composites is important for improving mechanical properties of implants [134-137]. Specifically, Thomson et al. demonstrated that the compressive yield strength increased from 0.95 ± 0.11 MPa for PLGA foams to 2.82 ± 0.63 MPa for foams with PLGA/HA fiber weight ratios of 7/6 [128]. Moreover, Marra et al. reported that the Young's modulus increased from 2.5 ± 0.7 MPa to 12.5 ± 3.2 MPa when 10 wt. % HA was incorporated into a PCL/PLGA blend with a weight ratio of 10/90 [130]. Wei et al. have also demonstrated that the compressive modulus of HA/PLA scaffolds increased with HA content [6]. Specifically, the modulus increased from 4.3 MPa for the plain PLA scaffolds to 8.3 MPa when the weight ratio of HA to PLA was 50/50 [6].

Most importantly, ceramic/polymer composites can be formulated to mimic many aspects of natural bone. As mentioned, natural bone is a nanostructured composite composed of a polymer matrix (mainly collagen) reinforced with nanometer-sized ceramic particles (mainly carbonated HA). Recent research in this field suggests that better osteoconductivity would be achieved if synthetic materials were fabricated to resemble bone in terms of its microstructure [138,139]. For example, Du et al. synthesized HA/collagen composites with a porous microstructure similar to bone and

these materials promoted the deposition of a new bone matrix. Furthermore, they showed that osteoblasts within this biologically-inspired composite eventually acquired a three-dimensional polygonal shape that integrated with juxtaposed bone fragments [138,139]. Therefore, it is clear that one approach for the design of next generation orthopedic prostheses is to further closely mimic bone from the structural perspective.

1.6. Nanostructured Biocomposites as Next-Generation Orthopedic Materials

It has been reported that the response of host organisms (including at the protein and cellular level) to nanomaterials is different than that to conventional (micron-scale) materials and the remarkable recognition capabilities of cells and biomolecules when combined with the unique properties of nanomaterials can lead to novel tissue substitutes and controlled drug delivery systems with significantly improved performances [140]. Nanomaterials typically have basic structural units less than 100 nm in at least one dimension and for that reason, have significantly improved mechanical, electrical magnetic, catalytic, optical and biological properties compared to conventional formulations of the same materials [141 - 144]. Although nanomaterials have revolutionized numerous other fields, the question has been raised concerning how the nanomaterials can benefit orthopedic medicine. Therefore, it is important to elucidate the promise that nanostructured biocomposites can bring into the field of bone regeneration.

1.6.1. Desirable Cell Interactions with Nanocomposites

The rationale for the growing impact of nanotechnology in medicine is that biological systems are inherently composed of nanoscale building blocks and pathophysiological processes always involve interactions at the molecular or cellular level.

In orthopedics, particularly, the dependence of osteoblast adhesion on nanomaterials was first reported in 1999 [145]. Specifically, alumina with grain sizes between 49 and 67 nm and titania with grain sizes between 32 and 56 nm promoted osteoblast adhesion compared to their respective micron-grained materials. Further investigations of these nanoceramics (alumina, titania, hydroxyapatite) demonstrated that *in vitro* osteoblast proliferation and long term functions (as measured by intracellular and extracellular matrix protein synthesis such as collagen and alkaline phosphatase, and calcium-containing mineral deposition) were enhanced on ceramics with grain sizes less than 100 nm [146]. In addition to osteoblast functions, enhanced osteoclast (bone-resorbing cell) functions were also observed on nanophase ceramics compared to conventional ceramics. For example, osteoclast synthesis of tartrate-resistant acid phosphatase (TRAP) and subsequent formation of resorption pits were up to two times greater on nanophase HA compared to conventional HA [147]. Coordinated functions of osteoblasts and osteoclasts are critical for the formation and maintenance of healthy new bone [148]. Therefore, the results of promoted functions of osteoblasts coupled with greater functions of osteoclasts could ensure healthy remodeling of juxtaposed bone formed at the surfaces of nanophase ceramics. Moreover, decreased functions of competitive cells, such as fibroblasts (cells that contribute to fibrous encapsulation and

callus formation events that may lead to implant loosening and subsequent failure), were observed on nanophase ceramics compared to conventional ceramics [149]. Specifically, the ratio of osteoblast to fibroblast adhesion increased from 1:1 on conventional alumina to 3:1 on nanophase alumina [149]. In fact, decreasing alumina grain size from 167 to 24 nm increased osteoblast adhesion 51% and at the same time decreased fibroblast adhesion 235% after 4 hours [145].

Nanophase ceramics have demonstrated desirable interactions with the select cells. As mentioned, however, ceramics are inherently brittle, difficult to process into complex shapes required for orthopedic applications and can not match the mechanical properties of true bone for load-bearing when they are used alone, because natural bone is composed of both malleable organic components (mainly type I collagen) and stiff inorganic components (HA). Nanophase ceramic/polymer composites combine the advantages of strong, bioactive ceramics and flexible, biodegradable polymers to optimize their physicochemical, biological and mechanical properties for bone regeneration. Moreover, nanophase ceramics in polymer composites can mimic the nanostructure and associated properties of bone and can potentially be combined with bone morphogenetic proteins (BMPs) to further control new bone growth.

Previous studies have also determined bone cell functions on nanophase ceramic/polymer composites with various ceramic/polymer ratios [150,151]. Specifically, composites of PLGA combined separately with 30 wt.% nanophase alumina, titania and HA showed the greatest osteoblast responses [150]. Moreover, up to three times more osteoblasts adhered to PLGA composites when 30 wt.% nanophase titania was incorporated compared to 30 wt.% conventional titania. Fibroblasts, as competitive cells

to osteoblasts, also deserve some attention. As mentioned, fibroblast functions decreased on nanophase compared to conventional ceramics (alumina, titania and HA), as well as on PLGA with nanoscale surface features compared to conventional PLGA [149,152].

1.6.2. Rationale for Cell Interactions with Nanomaterials

From these studies concerning the degree to which the select cells interact with nanomaterials, it is important to further understand the mechanisms as to why nanomaterials demonstrate unique biological properties.

1.6.2.1. Natural Tissue is Nanostructured

One straightforward explanation lies in the fact that natural tissues and associated extracellular matrices are composed of nanostructured materials. Natural bone is a good example of a nanostructured composite material. There are three scale levels of hierarchical structures in bone: (i) the nanostructure (a few nanometers to a few hundred nanometers), including non-collagenous organic proteins, fibrillar collagen and embedded mineral (HA) crystals; (ii) the microstructure (from 1 to 500 micrometers), including lamellae, osteons and Haversian systems; and (iii) the macrostructure, including cancellous and cortical bone [137]. These three levels of oriented structures assemble into heterogeneous and anisotropic bone, as shown in Figure 1.11.

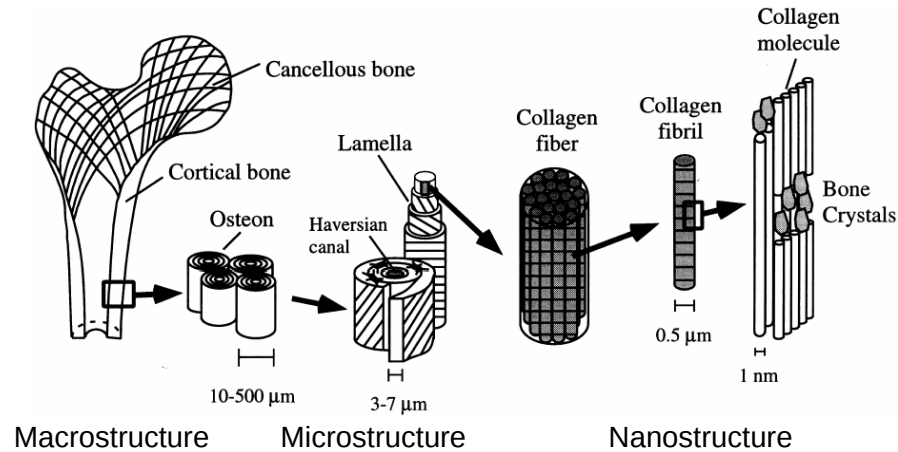


Figure 1. 11: Diagram illustrating three scale levels of hierarchical structures of bone. (Adapted and redrawn from [137]).

1.6.2.2. Unique Surface Properties of Nanomaterials

Although the aforementioned reason to study nanomaterials (to mimic dimensions of components of tissues) has been stressed by many researchers, another more scientific reason relies on altered protein adsorption on nanomaterials due to their unique surface properties and energetics. As mentioned, proteins adsorb onto the surface within milliseconds to potentially mediate cell attachment. The availability of specific cell-adhesive epitopes (such as the RGD sequence) of adsorbed proteins mediates subsequent bone cell adhesion [153]. Investigations into the underlying mechanisms revealed that the concentration, conformation, and bioactivity of vitronectin (a protein contained in serum that is known to mediate osteoblast adhesion) was responsible for the select, enhanced adhesion of osteoblasts (a crucial prerequisite for subsequent, anchorage-dependent-cell functions) on nanomaterials [154].

The type, concentration, conformation and bioactivity of proteins adsorbed onto the materials depend on their surface chemistry, hydrophilicity or hydrophobicity, charge,

topography, roughness, and energetics [155-159]. Nanomaterials have higher surface areas, higher surface roughness, higher portions of surface defects (edge/corner sites and grain boundaries) resulting from both decreased grain size and decreased diameter of surface pores. Moreover, nanophase ceramics possess enhanced surface wettability due to greater surface roughness and greater numbers of grain boundaries on their surfaces. All these unusual properties affect their interactions with proteins since all proteins are nanoscale entities. For example, vitronectin has a linear structure 15 nm in length and is preferentially adsorbed to the small defects (pores) on the nanomaterials, such as 0.98 nm pores present on nanophase titania compacts [154].

Moreover, increased surface areas and nanoscale surface features on nanomaterials can expose more available sites for proteins to interact with and, thus, alter the amount of protein adhesion as well as protein conformation that are crucial for subsequent cellular interactions. Miller et al. examined fibronectin interactions with nanomaterials with various nanoscale surface features under an atomic force microscope (AFM) and visualized for the first time how proteins respond differently to surface feature scales [140,160]. Specifically, fibronectin (5 $\mu\text{g/mL}$) adsorbed to PLGA surface with 500 nm spherical bumps showed little to no interconnectivity between fibronectin molecules; fibronectin (5 $\mu\text{g/mL}$) adsorbed to PLGA surface with 100 nm spherical bumps showed well-spread fibronectin molecules with a highest degree of interconnectivity leading to a network masking of the underlying PLGA nanometer surface features [160].

It has been reported that nanophase ceramics (alumina, titania, HA) significantly promoted specific protein adsorption (vitronectin and fibronectin) compared to the

respective conventional ceramics [161]. Specifically, adsorption of vitronectin was 10% greater on nanophase compared to conventional alumina [154]. Furthermore, protein conformation plays a critical role in mediating subsequent cell interactions. Increased unfolding of vitronectin adsorbed on nanophase ceramics compared to conventional ceramics was also observed [154]. Vitronectin unfolding further promoted the availability of specific cell-adhesive epitopes (RGD sequence) for subsequent enhanced osteoblast adhesion [154]. Moreover, increased protein adsorption was also observed on nanocomposites, specifically, when nano-HA rather than micron-HA was introduced to poly(L-lactic acid) (PLLA) [6].

1.6.3. Advantageous Mechanical Properties of Nanocomposites and Rationale

Ceramic/polymer nanocomposites may be synthesized to possess hardness, bending, compressive and tensile strengths that are higher than conventional composites but are more similar to physiological bone. Indeed, greater mechanical properties have been reported for polymer composites with a reduction in ceramic grain size into the nanometer range [162]. For example, McManus et al. reported that the bending moduli of composites of PLA with 40 and 50 wt.% nanophase (<100 nm) alumina, titania and HA were significantly greater than respective composite formulations with conventional coarser grained ceramics [162]. Specifically, compared to a bending modulus of 60 ± 3 MPa for plain PLA and 870 ± 30 MPa for conventional titania/PLA composites with a weight ratio of 50/50, the bending modulus of nanophase titania/PLA composites with a weight ratio of 50/50 was 1960 ± 250 MPa [162].

Mechanical deformation theory indicates that as grain size is reduced, high-volume fraction of interfacial regions compared to bulk materials leads to increased deformation by grain-boundary sliding and short-range diffusion-healing events, thus, increased ductility in nanocrystalline ceramics may be observed.

1.7. Hypothesis and Objectives

It has been mentioned previously that traditional bone substitutes (autografts, allografts, xenografts and metallic implants) do not meet increasing clinical demands as a result of either limited sources of natural grafts or short implantable lifetimes (10-15 years) of current synthetic implants [163]. Therefore, the long term objective of this study is to develop a new approach to design and fabricate orthopedic implant systems that heal damaged bone tissue in a natural and more effective way. This will be accomplished by combining nanotechnology, tissue engineering and controlled drug delivery into orthopedic prostheses to closely mimic natural bone in terms of its chemistry, highly ordered nano-to-macro hierarchical structures and associated physicochemical, mechanical and nanoscale surface properties (Figure 1.12).

The specific hypothesis behind this proposed research is that the chemistry and special surface properties (topography, surface area and surface roughness) of ceramic/polymer nanocomposites as well as bone-like three dimensional (3D) structures built from the nanocomposites will enhance the initial adhesion, long-term functions and infiltration of bone cells. The success of such prostheses for bone regeneration can be further ensured by incorporating bone morphogenetic proteins (BMPs) and controlling

their release, which also provide versatility of this proposed approach in treating various bone diseases.

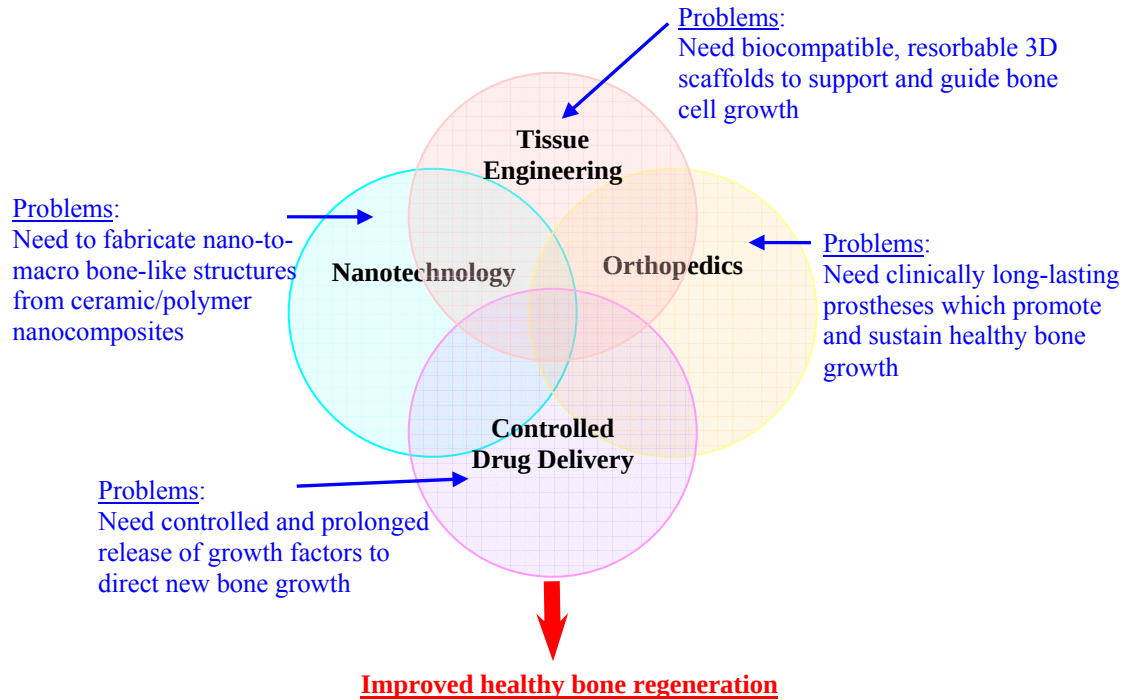


Figure 1. 12: Diagram illustrating the multidisciplinary approach of this dissertation which will combine nanotechnology, tissue engineering and controlled drug delivery into orthopedic prosthetic design to promote healthy bone regeneration.

The hypothesis is established on the following considerations. First, from the chemistry and material points of view, natural bone is a nanocomposite composed of nanometer HA crystals well dispersed in a mostly collagen matrix [137]. The ceramic/polymer nanocomposites optimally mimic the bone chemistry, which is beneficial for regenerating bone in a more natural way. Second, the nanocomposites can be fabricated to sufficiently mimic the nanoscale surface topography and roughness that bone cells naturally are accustomed to in the body, thus, favoring bone cell functions.

Third, from the clinical perspective, the main reason for failure of current metallic bone substitutes lies in a lack of osseointegration (that is, insufficient juxtaposed bone growth at the bone-implant interface) which leads to early and intermediate loosening of implants [164-166]. Thus, the interactions of implants with cells, especially osteoblasts and mesenchymal stem cells, are key considerations for orthopedic implant systems. Fourth, combining BMPs into the nanocomposites provides an extra control over cell functions and orientation on the nanocomposites because BMP-2 and BMP-7 (also called osteogenic protein-1), for example, are growth factors that induce the differentiation of mesenchymal stem cells and osteoprogenitor cells into osteoblasts [167-168,169,170]; induce bone tissue formation; influence the bone pattern formation [167-170]; and are chemotactic for osteoblasts [171] (that is, the characteristic movement or orientation of osteoblasts in response to a BMP-2 or BMP-7 concentration gradient) [172,173].

Lastly, natural bone assembles its 3D hierarchical structure from nanoscale building blocks. Currently, however, it is a scientific challenge to manipulate nanoscale structures and integrate them into macro architectures and systems while preserving their nanoscale structures and components within the fabricated macro-scale assemblies. Novel 3D fabrication techniques are highly needed to assemble such nanomaterial building blocks to create macro bone-like structures that can be used clinically. For these reasons, it is believed that the proposed novel aerosol-based 3D printing will accomplish an important role towards treating damaged bone.

The specific aims of this project are designed to assess cell interactions with 2D and 3D nanocomposites that have attractive bone-like surface properties and hierarchical architectures and also to control the delivery of BMPs using such nanocomposites for

more effective bone regeneration. These specific aims will be addressed in Chapter 2 (2D nanocomposites), Chapter 3 (3D nanocomposites), and Chapter 5 (nanocomposites as controlled drug delivery carriers). In addition to these perspectives, Chapter 4 will address the nanocomposites in terms of their mechanical properties necessary for orthopedic applications. Accomplishing these specific aims will provide the foundation to assess the ability of nanostructured ceramic/polymer composites to promote and direct bone cell functions to heal and reestablish normal physiological bone function.

CHAPTER 2. NANOSTRUCTURED 2D CERAMIC/POLYMER COMPOSITES: FROM MATERIAL CHARACTERISTICS TO OSTEOBLAST RESPONSES

2.1. Specific Problems and Aims

One of the key factors that influence properties of polymer composite materials is the dispersion status of ceramics. Even when the chemistry, phase composition, and crystal structures of the ceramic component are kept the same in a composite material, its final properties may vary significantly depending on ceramic dispersion (agglomeration). This is important since considering bone at the nanostructural level, HA crystals are located specifically in discrete spaces within collagen fibrils and this unique architectural dispersion and arrangement grant natural bone distinctive biological and mechanical properties such that no synthetic materials have yet ever fully mimicked [98]. The dispersion of ceramics in polymers is definitely one of the most important issues for all of composite engineering. No literature studies, however, have addressed the relationship between ceramic dispersion in polymer composites and their resulting biological properties (particularly for orthopedic applications).

Agglomeration significantly increases as ceramic particle sizes decrease into the nanometer regime and as the percentage of a ceramic phase increases to more than 2 wt% in a polymer matrix. Therefore, the objective of the studies to be presented in this chapter was to disperse nanophase ceramics in polymers and compare biological properties of

well-dispersed to agglomerated nanocomposites. A 30/70 ceramic/polymer weight ratio was chosen since previous studies have demonstrated that this ratio is optimal for osteoblast adhesion [150]. Both nanophase ceramics and single phase polymers were used as controls.

In this study, PLGA (poly-lactide-co-glycolide) was chosen as the model polymer since it is biodegradable, widely utilized in tissue engineering applications, and has been approved by the FDA for certain human clinical applications. Nanophase titania was utilized as the model ceramic since it is readily formed at the surfaces of the current widely-used titanium orthopedic implants and has excellent cytocompatibility properties as shown in previous studies [146]. Sonication was controlled and used at low to high powers to achieve various dispersion states of nanophase titania in PLGA composites and to imitate the nano-sized surface features of bone. The resulting surface characteristics (such as topography, surface area and surface roughness) of the composites were studied by scanning electron microscopy (SEM) and atomic force microscopy (AFM), and were compared to natural bone. The objective of the *in vitro* study was to investigate osteoblast adhesion and subsequent long-term functions (such as total protein synthesis, total collagen synthesis, alkaline phosphatase activity and calcium deposition) on nanophase titania/PLGA composites with nano-dispersion or micron-agglomeration. The relationship between osteoblast functions and the surface properties of the titania/PLGA composites were also addressed to aid in understanding cell-material interactions. Moreover, the degradation behavior of nanophase titania/PLGA composites was investigated to provide a fundamental guideline for tailoring PLGA degradation kinetics in the composites, which is necessary for optimizing bone regeneration and drug delivery.

2.2. Materials and Methods

2.2.1. Materials Preparation

2.2.1.1. Nanophase Titania/PLGA Composites

PLGA (poly-lactide-co-glycolide) pellets (50/50 wt.% poly(DL-lactide/glycolide); molecular weight: 100,000-120,000 g/mol; intrinsic viscosity: 66-80 cm³/g; polydispersity: 1.8; density: 1.34 g/cm³; glass transition temperature T_g: 45-50 °C) were purchased from Polysciences, Inc. (Warrington, PA).

Nanophase titania powder (Nanotek[®]) was purchased from Nanophase Technologies Corporation (Romeoville, IL). The purity of the titania powder was 99.5+%, the particle size was 32 nm which was calculated from BET adsorption measurements, the particle morphology was nearly spherical as shown in the TEM image (Figure 2.1), and the crystalline phase was 80% anatase/20% rutile [174]. Bulk and true density of this titania powder were 0.25 g/cm³ and 3.96 g/cm³, respectively.

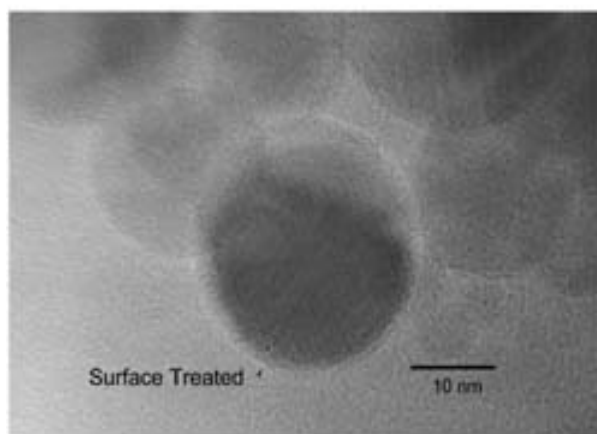


Figure 2. 1: TEM image of nanophase titania powder. Magnification bar is 10 nm.

PLGA pellets were dissolved in chloroform (Mallinckrodt Technical) at 40 °C in a water bath for 40 minutes. Nanophase titania powder was then added to the PLGA solution to give a 30/70 ceramic/polymer weight ratio.

The composite mixture was sonicated using a W-380 sonicator (Heat System – Ultrasonics, Inc.) with its tip immersed in the mixture. The output power settings of sonicator were from 118.75 W to 332.5 W, as shown in Table 2.1. The W-380 sonicator permits the application of ultrasonic energy to the suspensions on a pulsed basis. In this study, the pulse width was set at 60% of the duty cycle out of 1 second cycle time. This intermittent operation permits high intensity sonication while avoiding heat build-up in the processed suspensions. The temperature of composite mixture was monitored before and after sonication using a thermometer (VWR) placed 10 mm below the tip of the ultrasonic horn where the temperature was the highest in the composite mixture, as shown in Table 2.2.

After sonication, the suspension was cast into a Teflon petri dish (Chemware[®], 50 mm diameter ×15 mm height, Cole-Parmer Instrument Company, Vernon Hills, IL) evaporated in air at room temperature for 24 hours and dried in an air vacuum chamber at room temperature for 48 hours. The schematic procedure is shown in Figure 2.2. Finally, the composite films (0.5 mm in thickness) were cut into 1 cm × 1 cm squares for material characterization and cell functional studies [175].

Table 2. 1: Nanophase titania/PLGA composites, controls and references that were studied in this chapter.

Materials	Parameters
PLGA	Pure PLGA, control
PTC25	PLGA/titania composites sonicated at 118.75 W for 10 minutes
PTC35	PLGA/titania composites sonicated at 166.25 W for 10 minutes
PTC45	PLGA/titania composites sonicated at 213.75 W for 10 minutes
PTC70	PLGA/titania composites sonicated at 332.5 W for 10 minutes
TCG	Green titania compacts, control
TCS	Sintered titania compacts, control
Glass	Etched in 1 N NaOH, reference

Table 2. 2: The temperature of composite suspensions before and after sonication.

Samples	Temperature (°C)	
	Before sonication	After 10-minnute sonication
PTC25	25	31
PTC35	25	36
PTC45	25	40
PTC70	25	47

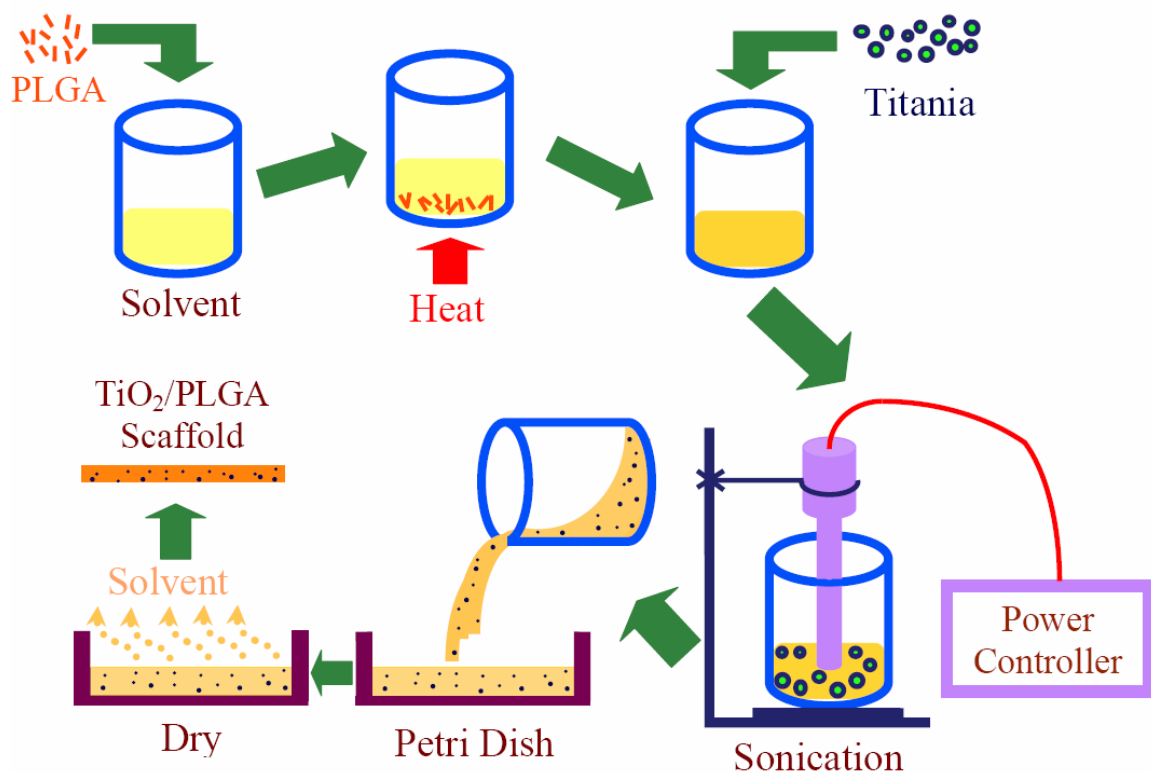


Figure 2. 2: The schematic procedures for preparing nanophase titania/PLGA composites using a solvent-casting technique.

2.2.1.2. Control Materials

PLGA films as well as green (non-sintered) and sintered titania compacts were prepared as described below and were used as control materials.

2.2.1.2.1. PLGA

For polymer films, PLGA pellets were dissolved in chloroform at 40 °C in a water bath for 40 minutes, cast into a Teflon petri dish, evaporated in air at room temperature for 24 hours and dried in an air vacuum chamber at room temperature for 48 hours. The

films (0.3 mm in thickness) were then cut into 1 cm × 1 cm squares for use in material characterization and cell experiments.

2.2.1.2.2. Nanophase Titania Compacts

Green titania disks were prepared by dry pressing nanophase titania powders (obtained as described above) in a tool-steel die via a uniaxial pressing cycle from 0.6 to 3 GPa over a 10 minute period into pellets of 0.8 mm in thickness. The green compacts were then heated in air at a rate of 10 °C/minute from room temperature to a final temperature of 600 °C, sintered at 600 °C for 2 hours and were cooled down at the same rate as the heating rate. These compacts were termed as sintered titania. Previous studies have demonstrated no grain growth and phase transformation when titania was sintered under these conditions [146].

2.2.1.3. Reference Materials

Borosilicate glass coverslips (Fisher Scientific; 1 cm in diameter) were used as reference materials for all of the *in vitro* experiments according to standard protocols [175]. The glass coverslips were degreased by soaking in acetone (Mallinckrodt) for 10 minutes, sonicating in acetone (Mallinckrodt) for 10 minutes, soaking in 70% ethanol (AAPER) for 10 minutes, and sonicating in ethanol for 10 minutes. Lastly, the coverslips were etched in 1 N NaOH for 1 hour at room temperature. For use in *in vitro* cell experiments, glass coverslips were then rinsed thoroughly with deionized (DI) water and dried in an oven at about 65 °C for 1 hour.

All the substrates used in the cell experiments were previously summarized in Table 2.1.

2.2.1.4. Sterilization of Materials

Composite samples and PLGA controls were sterilized by soaking in 70% ethanol for 30 minutes and were dried completely before performing experiments with cells. Titania compacts were sterilized by exposing them to UV light for 1 hour on each side. Glass references were sterilized in a steam autoclave at 120 °C for 30 minutes.

2.2.1.5. Preparation of Bone Slices

Devitalized porcine femurs were dissected in an effort to compare surface properties of the nanophase titania/PLGA composites sonicated at different powers to natural bone. For this purpose, the diaphyses of the porcine femurs were purchased from a supermarket (Wal-Mart) and were cut into slices (1 cm × 1 cm × 1 mm) using a handsaw. Bone slices were then degreased and ultrasonically cleaned of adhering tissue and marrow in acetone according to established lab protocols [176]. The outer uncut surface of bone slices were characterized using a scanning electron microscope (SEM) and an atomic force microscope (AFM) as described in the next section.

2.2.2. Characterization Methods

2.2.2.1. Scanning Electron Microscopy (SEM) and Quantitative Image Analysis

Surface topography of the nanophase titania/PLGA composites (prepared as described in section 2.2.1) were characterized according to standard scanning electron microscopy techniques using a JEOL JSM-840 Scanning Electron Microscope at a 5 kV accelerating voltage and 3×10^{-11} Amp probe current. Substrates were sputter-coated with a thin layer of gold-palladium using a Hummer I Sputter Coater (Technics) in a 100 millitorr vacuum argon environment for 3 minutes with 0.01 Amp of current. SEM images taken at 15 kX magnifications were used to determine differences in the topography. Quantitative image analysis methods were used to determine titania coverage on the surface of the nanocomposites [102].

2.2.2.2. Atomic Force Microscopy (AFM) and Characteristic Data Analysis

Atomic force microscopy (AFM) can be used as a powerful tool to gain a better understanding of the surface of materials on scales from μm to nm or even down to atomic resolution. AFM produces topographical data by scanning a sharp tip, situated at the end of a microscopic cantilever, over a surface. The advantage of using AFM for surface characterization is that the measurements could cover many orders of magnitude of length scales and acquire three-dimensional data in a digital format which allows extensive mathematical analyses of the data.

In this study, AFM was used to characterize the three-dimensional surface features as well as surface roughness and surface area of the materials of interest to the

present study. Specifically, height images of each sample were collected according to established tapping mode techniques using a MultimodeTM SPM (Digital Instruments Inc., Santa Barbara, CA). The typical tip (NSC15, Mikromasch) curvature radius of the probe used in the present study was less than 10 nm. The measurements were conducted in ambient air using a scan rate of 1 Hz and 256 scanning lines. The scan field of view was $5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$. The resulting height images were analyzed using Nanoscope imaging software.

Z values (or heights) were used in calculating the substrate surface area in a $25\text{ }\mu\text{m}^2$ scanned area. The surface area was found using the following equation:

$$A = l \times b \quad (2.1)$$

where l was the length traveled by the AFM tip over 256 scanning lines and b was the width of 256 scanned lines.

Root mean square (RMS) surface roughness values (nm) were calculated using height information from AFM scans captured on an area of $25\text{ }\mu\text{m}^2$. The imaging software uses the following equation 2.2 to compute surface roughness for a three-dimensional $N \times N$ pixel image:

$$R_q = \sqrt{\frac{\sum_{i=1}^N (Z_i - Z_{ave})^2}{N}} \quad (2.2)$$

where R_q was the rms surface roughness (standard deviation of height), Z_{ave} was the average of the Z values (or heights) within the given scanning area, Z_i was the Z value of current pixel, and N was the number of pixel points within the given area of samples.

All surface roughness values and surface area data were collected from five different $5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$ AFM scan spots. The average RMS surface roughness and surface area value were calculated.

In addition, height images of each sample were also collected from five different $1\text{ }\mu\text{m} \times 1\text{ }\mu\text{m}$ AFM scan spots using the same procedures described above. The average RMS surface roughness and surface area values were calculated based on $1\text{ }\mu\text{m} \times 1\text{ }\mu\text{m}$ AFM scans.

2.2.3. *In vitro* Cytocompatibility Studies

2.2.3.1. Cell Culture

Human osteoblasts (bone-forming cells; CRL-11372 American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin/streptomycin (P/S; Hyclone) under standard cell culture conditions, that is, a sterile, $37\text{ }^{\circ}\text{C}$, humidified, 5% CO_2 /95% air environment. Cells at population numbers 6-9 were used in the experiments without further characterization.

2.2.3.2. Osteoblast Adhesion

Figure 2.3 illustrates the experimental procedures used to determine osteoblast adhesion on the substrates. All sterilized substrates listed in Table 2.1 were placed in 12-well tissue culture plates (Corning, New York) and were rinsed three times with sterilized phosphate buffered saline (PBS; a solution containing 8 g NaCl, 0.2 g KCl, 1.5 g

Na_2HPO_4 , and 0.2 g KH_2PO_4 in a 1000 ml of DI water adjusted to a pH of 7.4; all chemicals from Sigma).

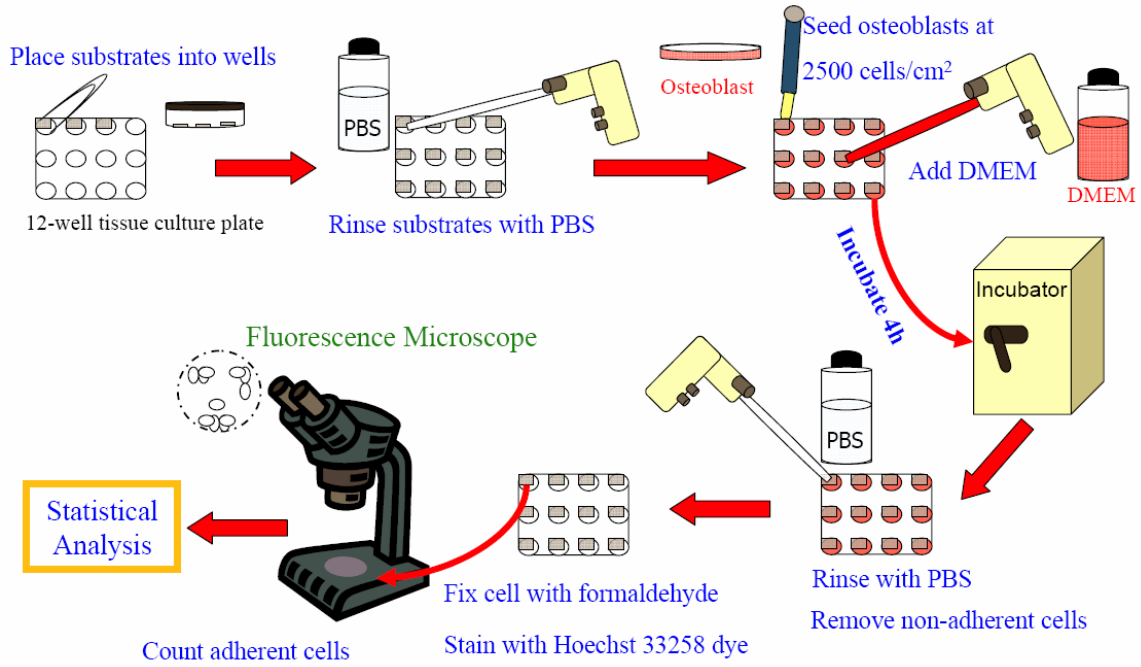


Figure 2. 3: The schematic diagram of the experimental procedures followed for determining osteoblast adhesion.

Osteoblasts were seeded at a concentration of 2500 cells/cm² onto the substrates of interest in 2 ml of DMEM supplemented with 10% FBS and 1% P/S and were then incubated under standard cell culture conditions for 4 hours. After that time period, non-adherent cells were removed by rinsing with PBS and adherent cells were then fixed with formaldehyde (Fisher Scientific, Pittsburgh, PA) and stained with Hoechst 33258 dye (Sigma); the cell nuclei were, thus, visualized and counted under a fluorescence microscope (Leica, excitation wavelength 365 nm and emission wavelength 400 nm).

Cell counts were expressed as the average number of cells on eight random fields per substrate.

All experiments were run in triplicate and repeated at three separate times. Cell adhesion was evaluated based on the mean number of adherent cells.

2.2.3.3. Osteoblast Morphologies

Osteoblast morphologies on the composites and the controls were observed using a JEOL JSM-840 Scanning Electron Microscope. For this purpose, after the 4 hour adhesion test, adherent osteoblasts on the substrates were fixed with 2 % glutaraldehyde (Electron Microscopy Sciences) in 0.1 M cacodylate (pH 7.4; Electron Microscopy Sciences) for 30 minutes at 4 °C. After washing with cacodylate buffer, the cells were secondarily fixed with 1 % osmium tetroxide (Electron Microscopy Sciences) in 0.1 M cacodylate (pH 7.4) for 30 minutes at 4 °C. The cells were dehydrated through a series of ethanol solutions (from 30, 50, 70, 90, to 100 %; AAPER) and were finally dried by critical point drying (CPD; LADD Research Industries). Specifically, the specimens were immersed in liquid CO₂ until there was a complete exchange of liquid CO₂ for the ethanol in the specimens. The specimens were then heated above 34 °C under 7.6 MPa where all liquid CO₂ was converted to gaseous CO₂ and the specimens were dry. Before imaging, all the specimens were sputter-coated with a thin layer of gold-palladium using a Hummer I Sputter Coater (Technics) in a 100 millitorr vacuum argon environment for 3 minutes with 0.01 Amp of current. Cell morphologies were imaged using a 5 kV accelerating voltage, and a 3×10^{-11} Amp probe current. Magnifications varied according to the distribution of cells on the substrates.

2.2.3.4. Osteoblast Long-term Functions

Osteoblasts were seeded at a density of 100,000 cells/cm² onto the substrates of interest and were cultured in DMEM supplemented with 10% FBS, 1% P/S, 50 µg/ml L-ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma) under standard cell culture conditions for 7, 14, and 21 days. Cell culture media was changed every other day during the osteoblast long-term function experiments. At the end of the prescribed time periods, the substrates were first rinsed three times with 50 mM Tris-buffered saline (TBS; a solution consisting of 8.77 g NaCl, 6.61g Tris-HCl, and 0.97 g Tris Base in a 1000 ml of DI water adjusted to a pH of 7.4; all chemicals from Sigma). Then, the osteoblasts were lysed using distilled water and three freeze-thaw cycles to determine total protein content, total collagen content and alkaline phosphatase activity in the supernatant according to standard protocols as described below.

2.2.3.4.1. Total Protein Content

Total protein content in the cell lysates was determined using a commercial Coomassie PlusTM --- The Better Bradford Assay Kit (Pierce Biotechnology, Inc.) following manufacturer's instructions. For this purpose, aliquots of each protein-containing, distilled water supernatant of cell lysates were mixed with a Coomassie PlusTM Reagent in a 96-well microplate and incubated for 10 minutes at room temperature. Light absorbance of these samples was measured at 595 nm on a spectrophotometer (Spectra MAX 190; Molecular Devices). Total intracellular protein synthesized by osteoblasts cultured on the substrates of interest to the present study was determined from a standard curve of absorbance versus known concentrations of albumin

run in parallel with experimental samples. The total intracellular proteins synthesized by osteoblasts were normalized by substrate surface area and expressed as $\mu\text{g}/\text{cm}^2$. All experiments were run in triplicate and repeated at three separate times.

2.2.3.4.2. Total Collagen Content

Cell lysates were prepared as described above. Collagen is main organic component of bone. To test this, aliquots of the distilled water supernatant were dried onto a 96-well microplate through incubating at 37 °C for i) 16 hours and for ii) 24 hours in the presence of a desiccant (W.A. Hamond Drierite Company). Thereafter, the microplate was rinsed three times with distilled water, 1 minute per wash and 200 μL per well. Then 100 μL of a 0.1% Sirius Red stain (Sirius Red powder in picric acid; Sigma) was dispensed into each well and incubated for 1 hour at room temperature. After that, the microplate was washed five times with 200 μL of 0.01 M HCl (Mallinckrodt Technical) for 10 seconds per wash to remove the unbound stain. The collagen bound stain was then washed with 200 μL of 0.1 M NaOH for 5 minutes for desorption. The eluted stain was then mixed several times into a multichannel pipette and was placed into a second microplate. Finally, absorbance was read at 540 nm in a spectrophotometer (Spectra MAX 190; Molecular Devices). A standard curve was plotted as known concentrations of collagen run parallel with experimental samples versus absorbance at 540 nm and the collagen content of the samples were calculated from this curve. Total collagen content was normalized by substrate area and expressed as mg/cm^2 .

2.2.3.4.3. Alkaline Phosphatase Activity

Alkaline phosphatase is an enzyme whose production signifies increased osteoblast differentiation to calcium depositing cells [177]. An Alkaline Phosphatase Assay Kit, a commercial kit from Upstate Cell Signaling Solutions, was used to assay alkaline phosphatase activity in the cell lysates prepared as described above. For this purpose, aliquots of the distilled water supernatants of cell lysates were mixed with 5 μ L NiCl_2 , 5 μ L BSA, 5 μ L phosphopeptide solution in the wells of a microplate. Then, the reaction was incubated for 15 minutes at 37 °C. Alkaline phosphatase activity was detected by the addition of 100 μ L Malachite Green solution. The assay was read with blank and standards by a spectrophotometer (Spectra MAX 190; Molecular Devices) at 650 nm. Alkaline phosphatase activity was calculated by comparing absorbance values to a standard curve of absorbance versus known concentrations of potassium phosphate monobasic run in parallel with experimental samples. One unit of activity was equivalent to 1 nmol p-Nitrophenyl phosphate (pNPP) hydrolyzed per minute. The activity was normalized by substrate area and expressed as nanomoles of converted pNPP/min/cm².

2.2.3.4.4. Quantification of Calcium Deposition

Lastly, the ultimate indicator of osteoblast differentiation (calcium deposition) was determined in this study. For this purpose, after the cells were lysed and removed, the substrates of interest (and remaining calcium-containing mineral deposited on them) were treated with 0.6 N HCl (Mallinckrodt Technical) at 37 °C overnight. After the prescribed time period, the amount of calcium present in the acidic supernatant was quantified using a commercially available kit (Sigma) and following the manufacturer's

instructions. Light absorbance of the samples was measured at 575 nm using a spectrophotometer (Spectra MAX 190; Molecular Devices). Total calcium was calculated from standard curves of absorbance versus known concentrations of calcium standards (Sigma) run in parallel with the experimental samples. Calcium concentration values were normalized by substrate area and expressed as $\mu\text{g}/\text{cm}^2$. All experiments were run in triplicate and repeated at three separate times.

2.2.3.5. Acellular Calcium Deposition Studies

Not only can calcium be deposited by osteoblasts, but it may also precipitate from surrounding cell culture media. To determine this, all the substrates of interest to the present study were incubated in DMEM supplemented with 10% FBS, 1% P/S, 50 $\mu\text{g}/\text{ml}$ L-ascorbic acid (Sigma) and 10 mM β -glycerophosphate (Sigma) under standard cell culture conditions for 7, 14, and 21 days. Cell culture media was changed every other day during these acellular calcium deposition experiments. At the end of the prescribed time periods, the substrates were rinsed three times with 50 mM Tris-buffered saline (TBS; a solution consisting of 8.77 g NaCl, 6.61g Tris-HCl, and 0.97 g Tris Base in a 1000 ml of DI water adjusted to a pH of 7.4; all chemicals from Sigma). The substrates of interest and remaining calcium-containing mineral deposited on them from DMEM were then treated with 0.6 N HCl (Mallinckrodt Technical) at 37 °C overnight. After the prescribed time period, the amount of calcium present in the acidic supernatant was quantified using a commercially available kit (Sigma) and following the manufacturer's instructions. Light absorbance of the samples was measured at 575 nm using a spectrophotometer (Spectra MAX 190; Molecular Devices). Total calcium was calculated from standard

curves of absorbance versus known concentrations of calcium standards (Sigma) run in parallel with the experimental samples. Calcium concentration values were normalized by substrate area and expressed as $\mu\text{g}/\text{cm}^2$. All experiments were run in triplicate and repeated at three separate times.

2.2.4. *In vitro* Degradation Studies

For ceramic/polymer composite degradation experiments, initial dry substrates of interest were weighed (W_0) and sterilized. Then, all the substrates were immersed into 3 mL of PBS (along with blank PBS as a reference) and were incubated under standard cell culture conditions. After 21, 28, and 35 days, specimens were removed from PBS, abundantly rinsed with DI water to remove the soluble inorganic salt, and dried in an air vacuum chamber at room temperature for 48 hours to reach constant mass. At each time point, samples were weighed (W_t) and the percentage of weight loss (%WL) with respect to incubation time was calculated according to equation (2.3).

$$\%WL = \frac{(W_0 - W_t)}{W_0} \times 100\% \quad (2.3)$$

At least three samples of each kind were measured and the results averaged. The pH of the supernatant buffer was monitored three times a week during the experiment.

2.2.5. Statistical Analysis

Numerical data were analyzed using standard analysis of variance (ANOVA) techniques; statistical significance was considered at $p < 0.05$. All data analyzed by

ANOVA were from experiments run in triplicate and repeated at least three separate times.

2.3. Results

2.3.1. Materials Characterization

2.3.1.1. Surface Topography Determined by SEM

2.3.1.1.1. Nanophase Titania/PLGA Composites

Titania particles of different agglomeration sizes were visible on the surface of the composites, as shown in Figure 2.4. Scanning electron micrographs suggest that the distribution of ceramic particles was different on the surface of the composite scaffolds depending on the sonication power utilized; specifically, there were more titania particles on the surface of each scaffold after sonication with higher power. Finer titania particles were also observed on the surface with increasing sonication powers. That is, larger ceramic agglomerations tended to break into smaller particles in the polymer solution after higher powers of sonication. Because of this, the amount of surface area occupied by titania increased on the surface of the composite scaffolds with higher sonication powers. Specifically, 10.6%, 10.2%, and 10.1% compared to 5.7% of the surface area occupied was titania on PTC70, PTC45, PTC30 and PTC25 composites, respectively. At higher sonication powers, titania particles became smaller and were more evenly dispersed in the PLGA matrix. However, there were no significant differences in terms of titania surface coverage for PTC45 and PTC70 compared to PTC35.

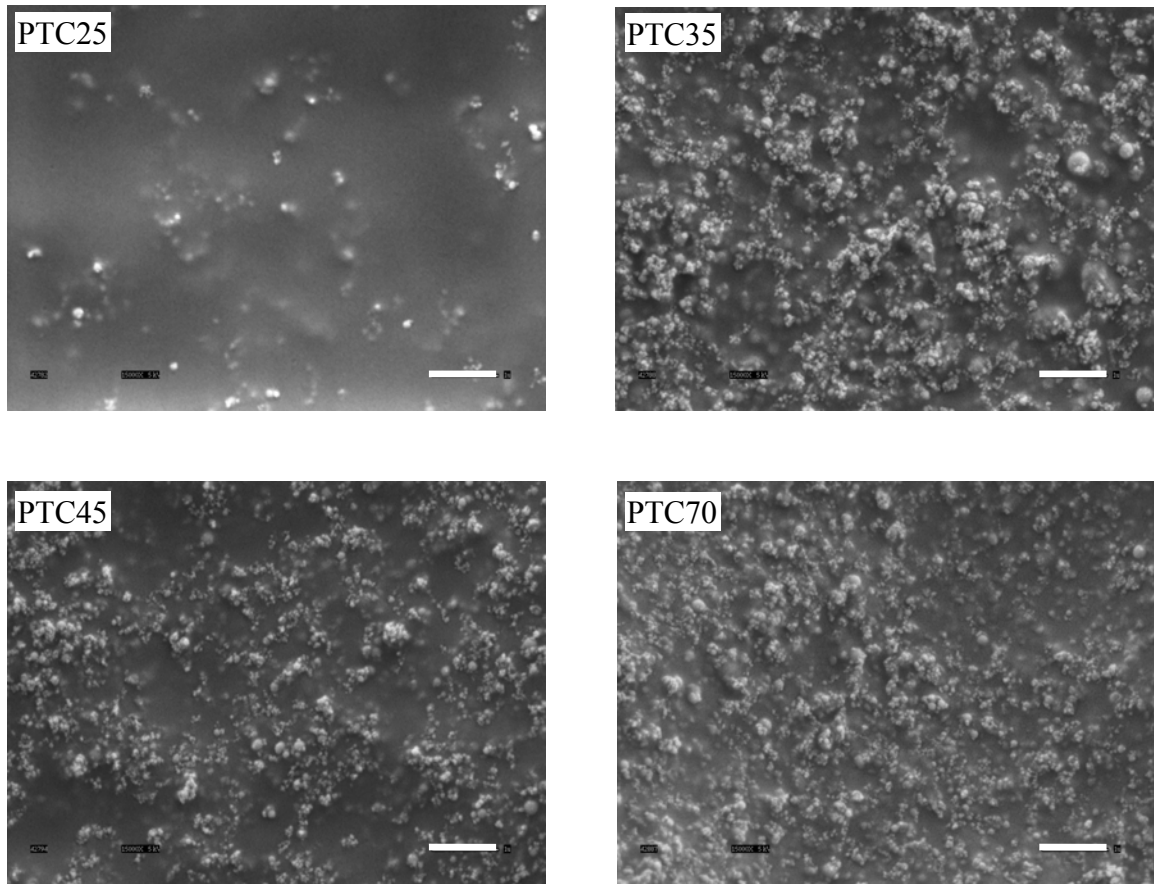


Figure 2. 4: SEM micrographs of nanophase titania/PLGA composites: PTC25, PTC35, PTC45, and PTC70. Original magnification: 15 kX; magnification bars: 1 μ m.

2.3.1.1.2. Control Materials

Pure PLGA and titania compacts (both green and sintered) are shown in Figure 2.5. It can be seen that the surface of PLGA was rather smooth while the surface of titania compacts were more rough and, thus, more similar to the surface structure of natural bone. Figure 2.5 shows outer surface of natural bone. Figure 2.6 shows inner surface of natural bone. Clearly, the inner surface of natural bone has more porous structures for nutrient and waste transportation.

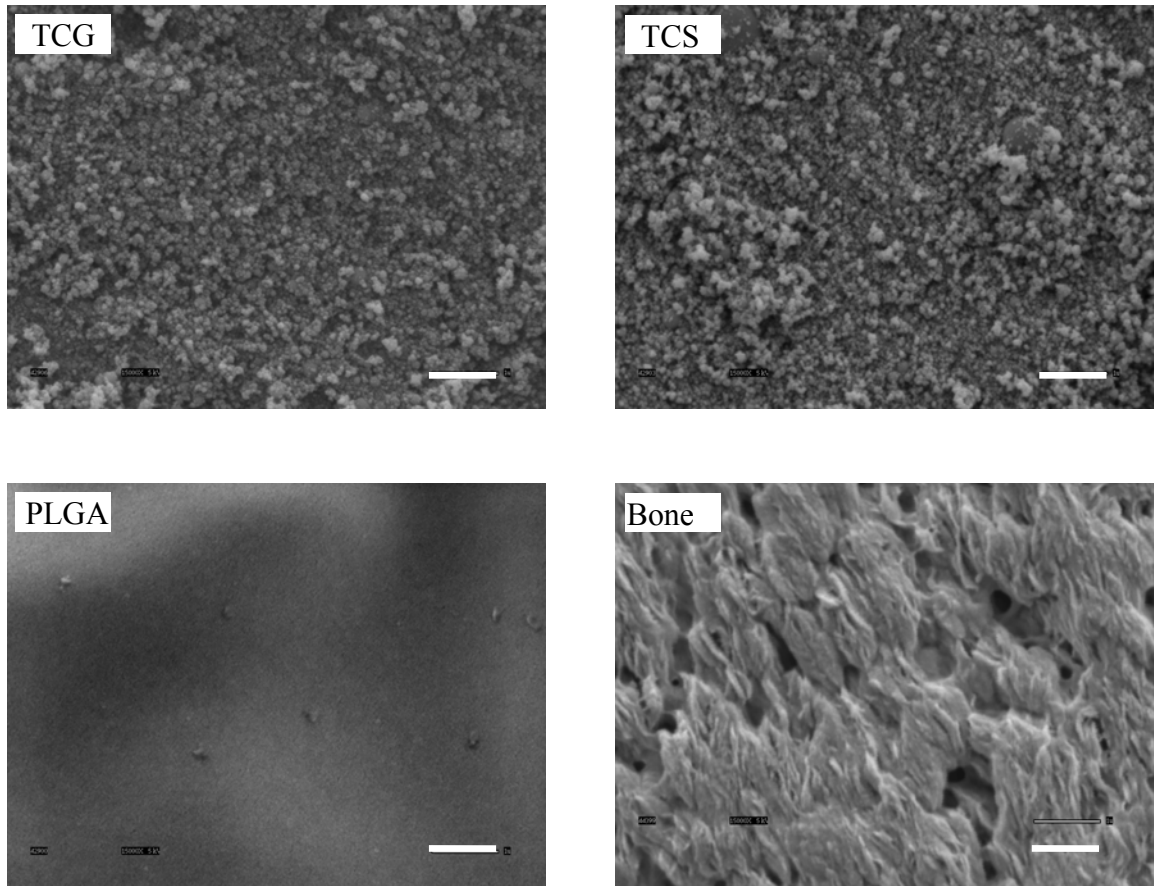


Figure 2. 5: SEM micrographs of control materials and natural bone: PLGA, TCG (green titania compacts), TCS (sintered titania compacts) and outer surface of bone. Original magnification: 15 kX; magnification bars: 1 μ m.

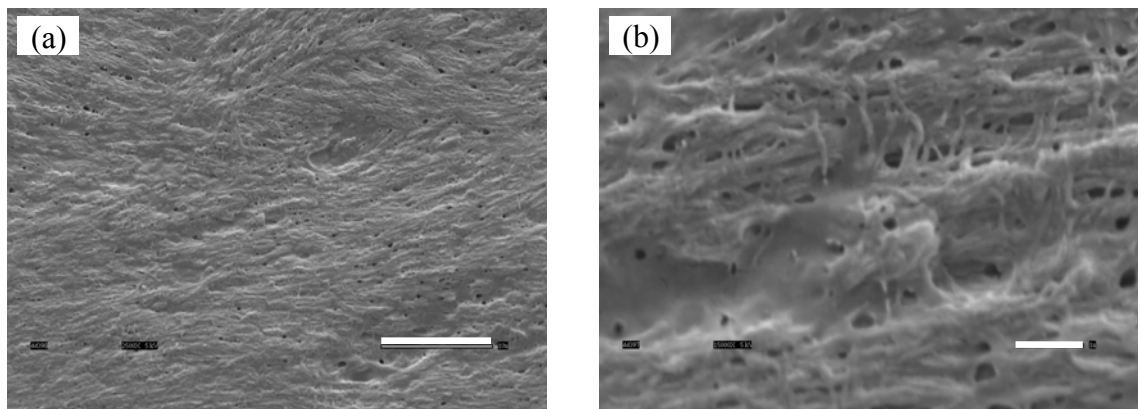


Figure 2. 6: SEM micrographs of inner surface of bone. (a) Original magnification: 2500 X, magnification bar: 10 μ m. (b) Original magnification: 15 kX, magnification bar: 1 μ m.

2.3.1.2. Nanometer Surface Features Determined by AFM

Atomic force microscopy results demonstrated that all the titania/PLGA composites fabricated at different sonication powers had nanometer scale surface roughness from 20 nm to 120 nm according to $5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$ AFM scans. AFM images confirmed SEM results that the dispersion of ceramic particles improved on the surface of the composites when higher sonication powers were utilized (Figure 2.7) and that the surface of PLGA was rather smooth while the surface of titania compacts were more rough and, thus, more similar to the structure of natural bone (Figure 2.8).

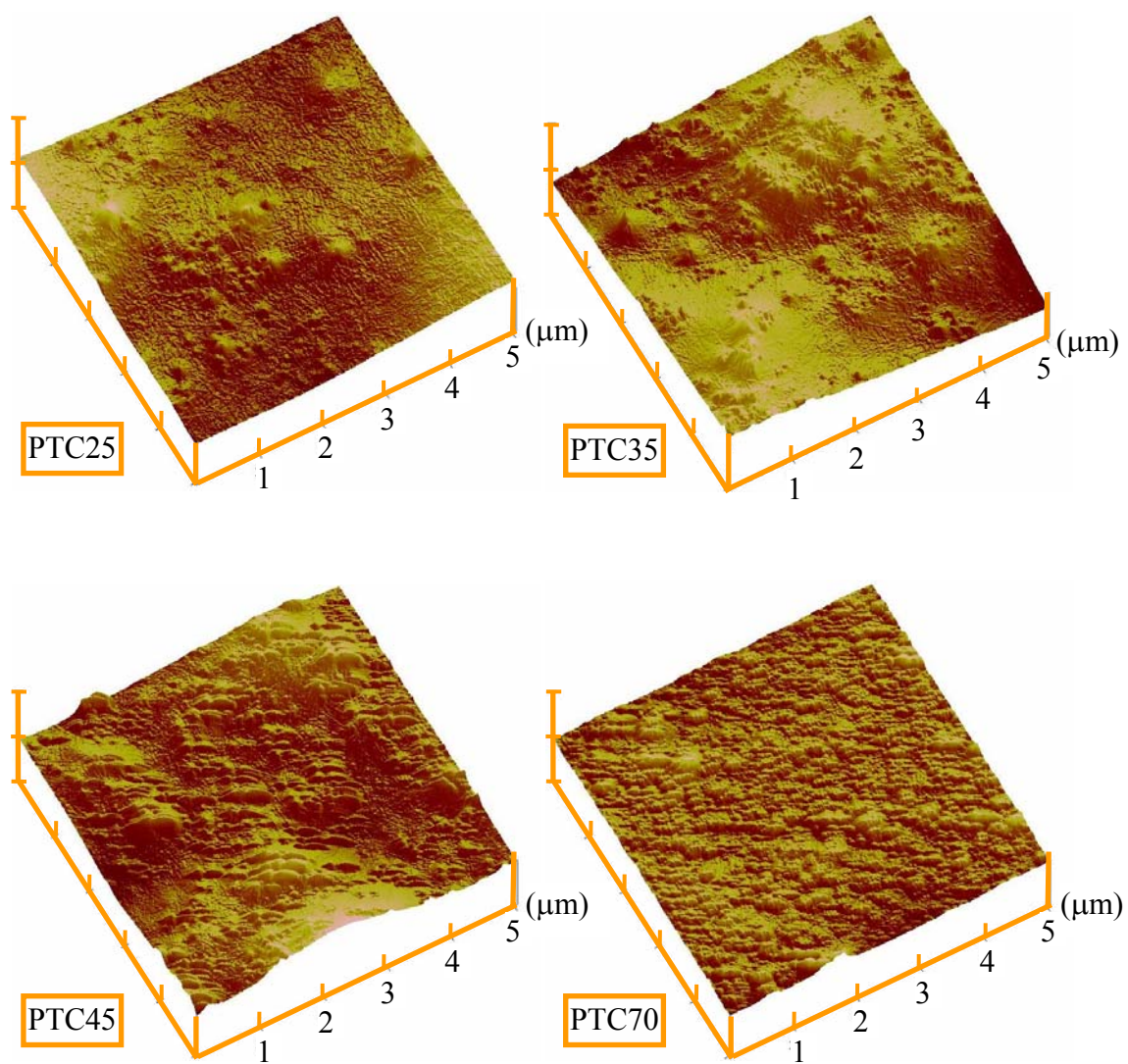


Figure 2. 7: AFM micrographs of materials of interest: PTC25, PTC35, PTC45, and PTC70. Original scan size is $5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$. Data Z-scale is 300 nm.

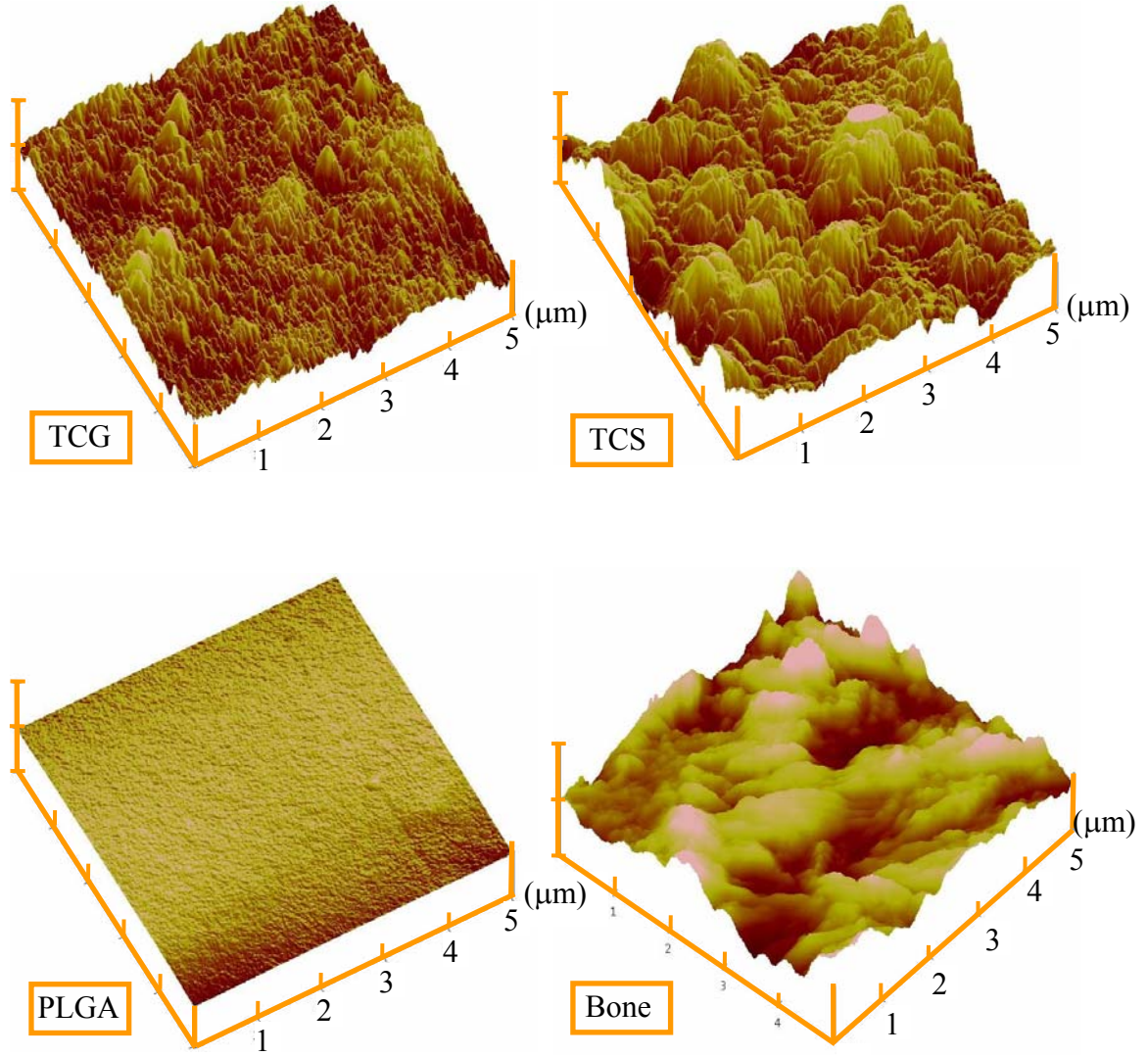


Figure 2. 8: AFM micrographs of materials of interest: PLGA, TCG, TCS, and bone. Original scan size is 5 $\mu\text{m} \times 5 \mu\text{m}$. Data Z-scale is 300 nm.

Moreover, the surface roughness of all the substrates compared to natural bone was plotted in Figure 2.9. Results showed that: (i) the surface roughness of all the composites was significantly greater than PLGA, (ii) the surface roughness of the titania compacts were significantly greater than all the composites, and (iii) the surface roughness of PTC35 was significantly greater than PTC25, PTC45 and PTC70. However,

there was no significant difference in surface roughness between titania compacts and natural bone. Importantly, the composite with the surface roughness values closest to bone was PTC35.

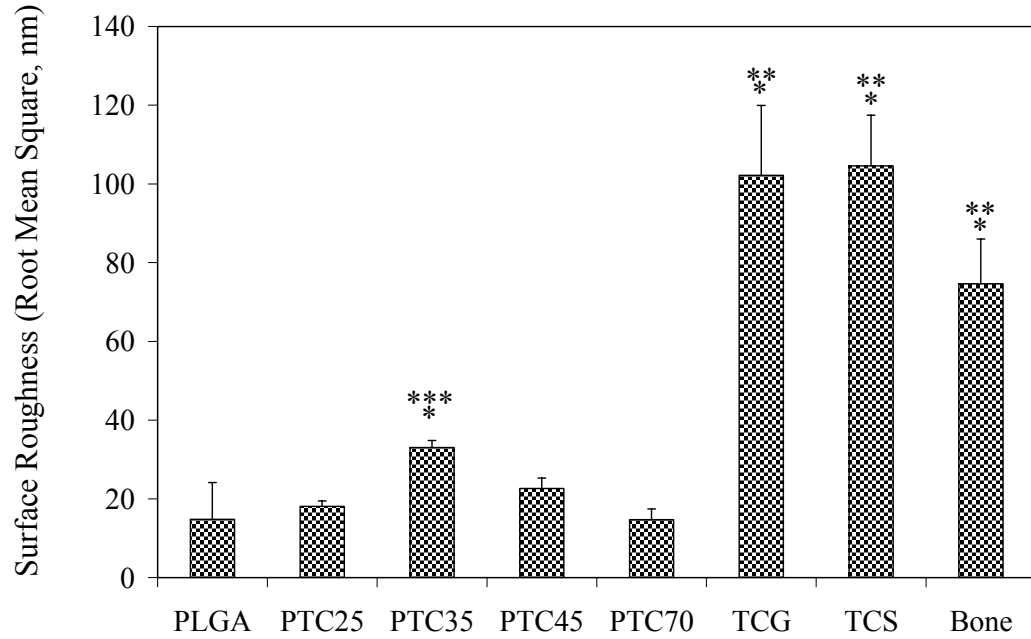


Figure 2. 9: Surface roughness (root-mean-square) of PLGA, PTC25, PTC35, PTC45, PTC70, TCG, TCS, and natural bone. Values are mean $\pm$ SEM; $n=5$; * $p < 0.05$ compared to PLGA; ** $p < 0.05$ compared to all the composites; *** $p < 0.05$ compared to PTC25, PTC45 and PTC70. AFM scan size is $5 \mu\text{m} \times 5 \mu\text{m}$.

Results from AFM surface analysis also provided quantitative evidence of the surface area of substrates of interest compared to natural bone, as shown in Table 2.3. The surface area of all the composites was significantly greater than PLGA, and the surface area of the titania compacts were significantly greater than all the composites.

Table 2. 3: Surface area values of the substrates of interest compared to bone. Values are mean $\pm$ SEM; n = 3; * p < 0.05 compared to PLGA; # p < 0.05 compared to all the composites. AFM scan size is 5 $\mu\text{m} \times 5 \mu\text{m}$.

Samples	Average Surface Area (μm^2 per unit of scanned area [25 μm^2])
PLGA	25.0401 $\pm$ 0.0250
PTC25	25.1135 $\pm$ 0.0005*
PTC35	25.3437 $\pm$ 0.0208*
PTC45	25.5063 $\pm$ 0.0301*
PTC70	25.3384 $\pm$ 0.0231*
TCG	51.1387 $\pm$ 7.0515* #
TCS	41.1670 $\pm$ 7.9951* #
Bone	29.2762 $\pm$ 1.6143* #

In addition, AFM results from 1 $\mu\text{m} \times 1 \mu\text{m}$ scans demonstrated very similar trends as AFM results from 5 $\mu\text{m} \times 5 \mu\text{m}$ scans. AFM images from 1 $\mu\text{m} \times 1 \mu\text{m}$ scans were shown in Figure 2.10 and Figure 2.11. The average RMS surface roughness and surface area were calculated from 1 $\mu\text{m} \times 1 \mu\text{m}$ AFM scans, as shown in Figure 2.12 and Table 2.4.

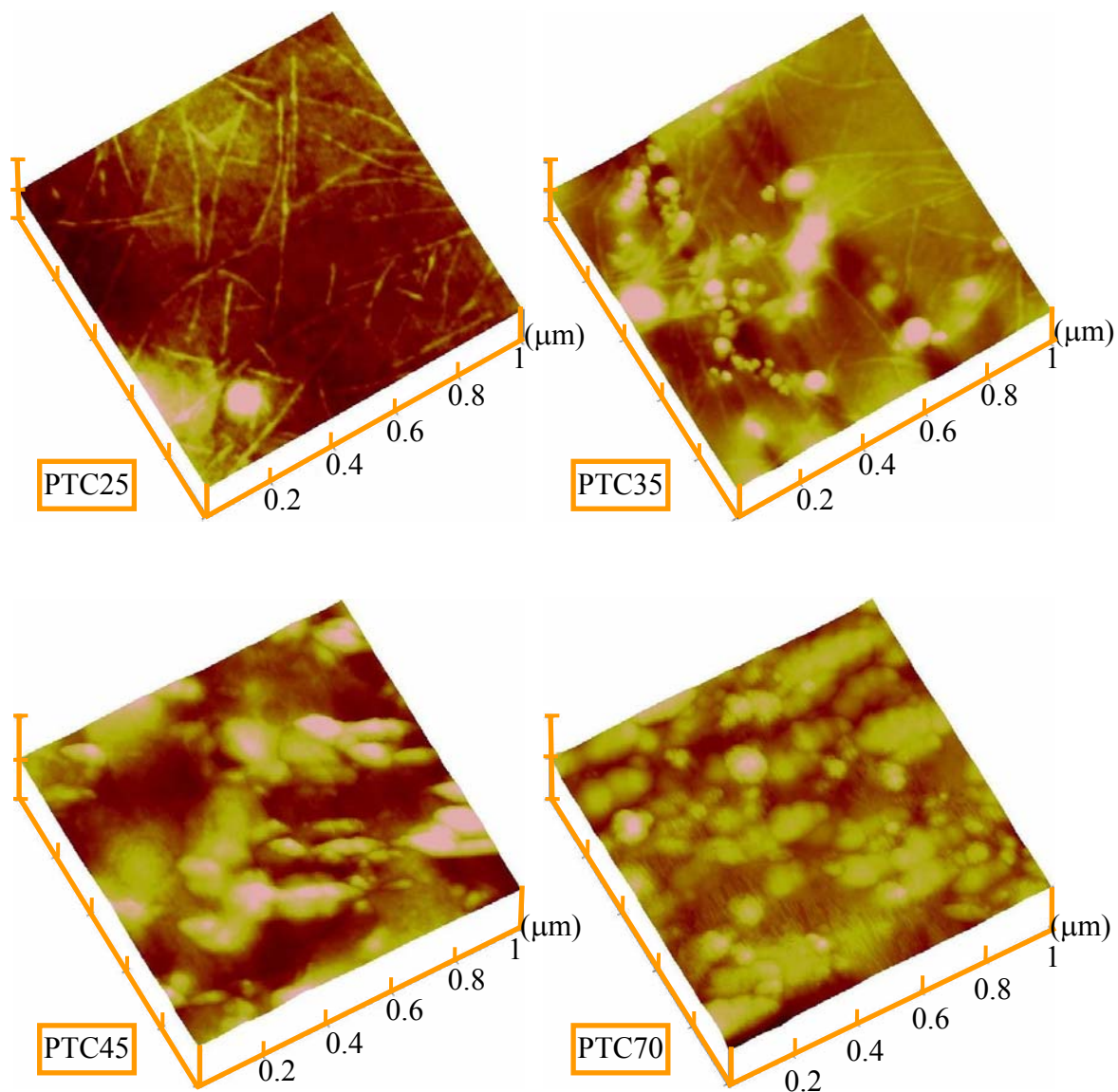


Figure 2. 10: AFM micrographs of materials of interest: PTC25, PTC35, PTC45, and PTC70. Original scan size is $1\text{ }\mu\text{m} \times 1\text{ }\mu\text{m}$. Data Z-scale is 200 nm.

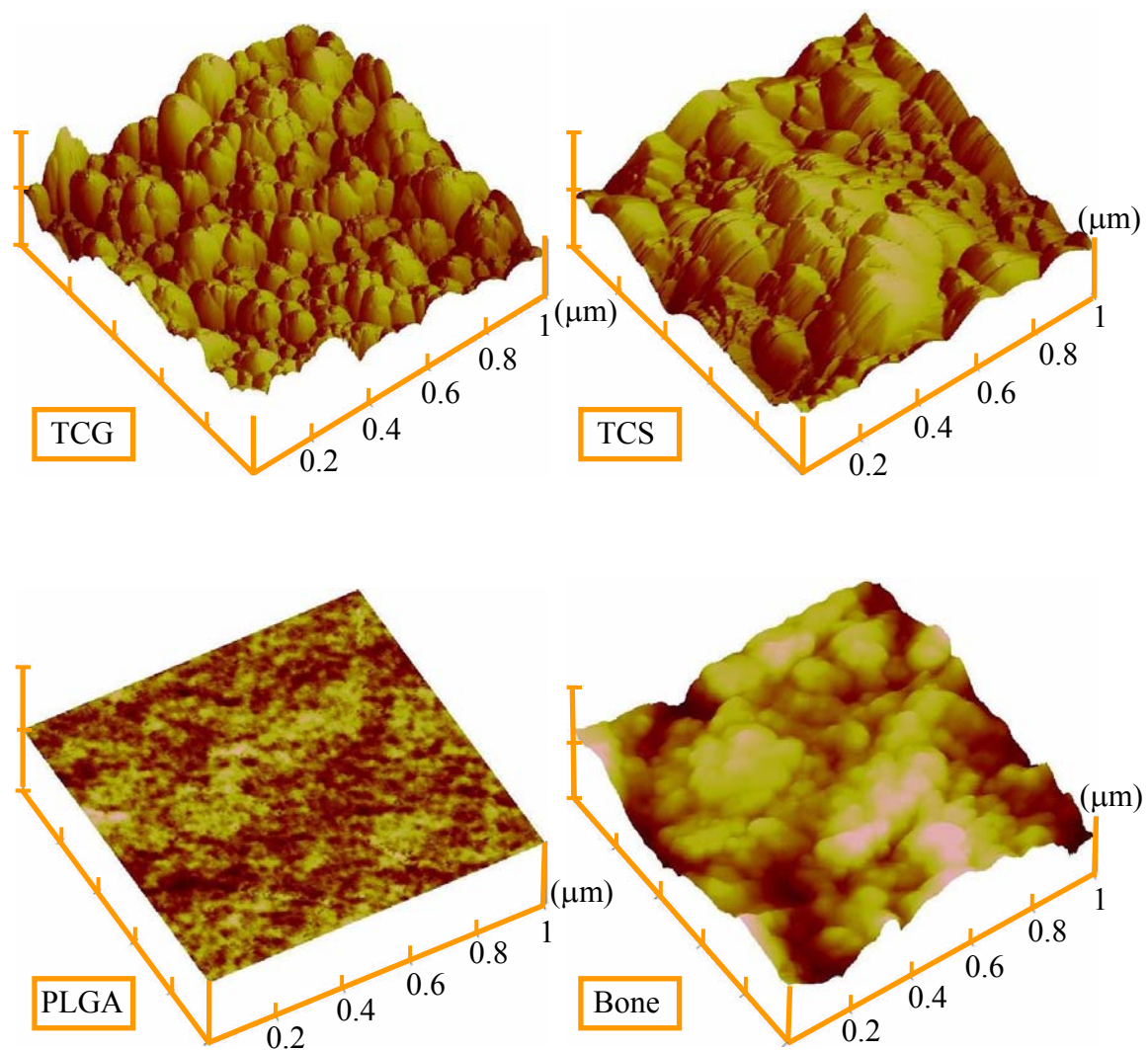


Figure 2. 11: AFM micrographs of materials of interest: PLGA, TCG, TCS, and bone. Original scan size is $1\ \mu\text{m} \times 1\ \mu\text{m}$. Data Z-scale is 200 nm.

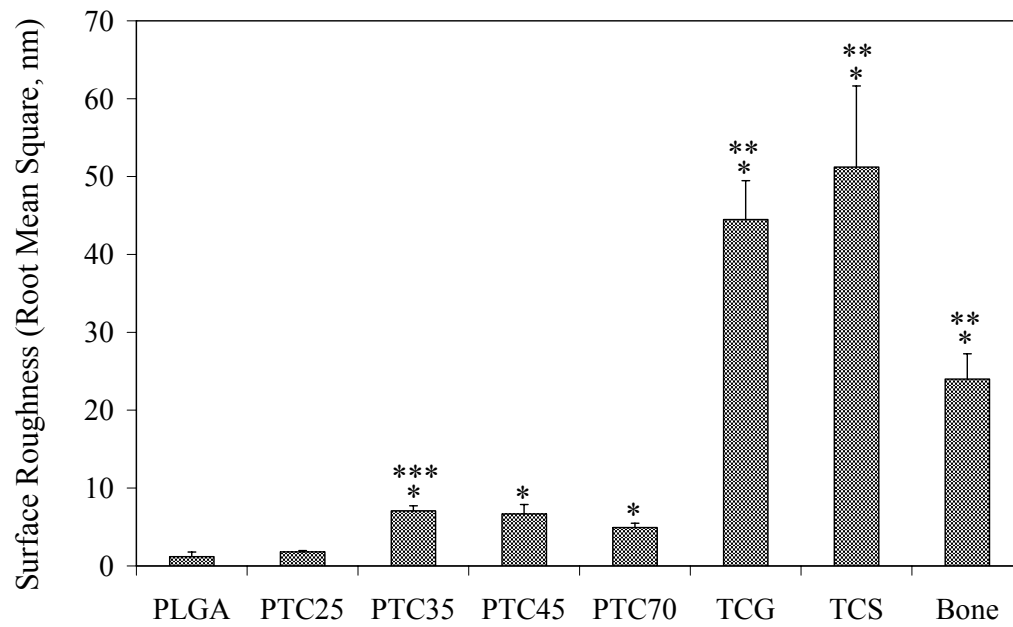


Figure 2. 12: Surface roughness (root-mean-square) of PLGA, PTC25, PTC35, PTC45, PTC70, TCG, TCS, and natural bone. Values are mean $\pm$ SEM; $n=5$; * $p < 0.05$ compared to PLGA; ** $p < 0.05$ compared to all the composites; *** $p < 0.05$ compared to PTC25. AFM scan size is $1 \mu\text{m} \times 1 \mu\text{m}$.

Table 2. 4: Surface area values of the substrates of interest compared to bone. Values are mean $\pm$ SEM; n = 3; * $p < 0.05$ compared to PLGA; # $p < 0.05$ compared to all the composites. AFM scan size is $1 \mu\text{m} \times 1 \mu\text{m}$.

Samples	Average Surface Area (μm^2 per unit of scanned area [$1 \mu\text{m}^2$])
PLGA	1.0047 ± 0.0020
PTC25	1.0040 ± 0.0004
PTC35	$1.0333 \pm 0.0023^*$
PTC45	$1.0467 \pm 0.0072^*$
PTC70	$1.0288 \pm 0.0071^*$
TCG	$1.7813 \pm 0.1827^{* \#}$
TCS	$1.6900 \pm 0.0468^{* \#}$
Bone	$1.3078 \pm 0.0542^{* \#}$

2.3.2. *In Vitro* Cytocompatibility

2.3.2.1. Osteoblast Adhesion

Adhesion is a critical initial step for the interaction between osteoblasts and materials. Results showed that osteoblast adhesion was significantly greater on the TCG and TCS than all the composites, as shown in Figure 2.13. Moreover, osteoblast adhesion was significantly greater on all the composites than on PLGA. Most importantly, osteoblast adhesion was significantly greater on the PTC35 than on the PTC25, PTC45 and PTC70 composites. Osteoblast adhesion was not significantly different between the TCG and TCS.

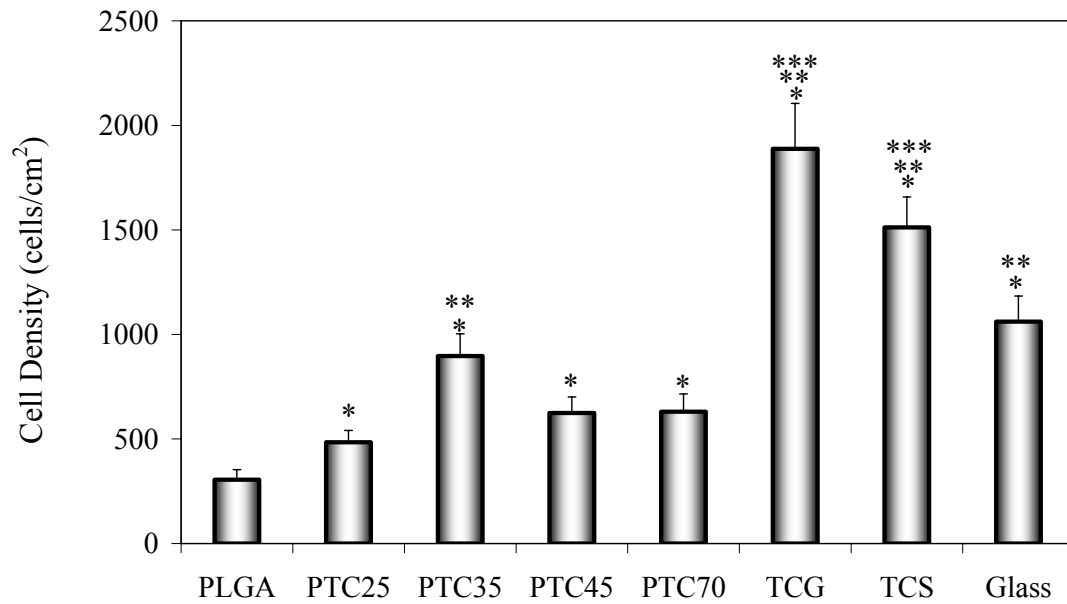
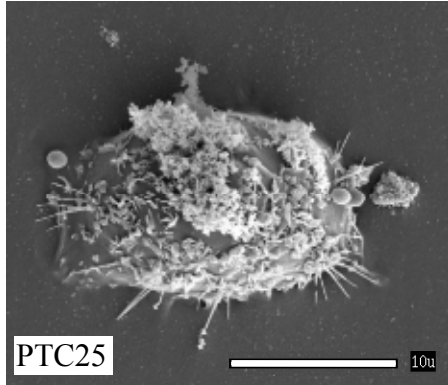


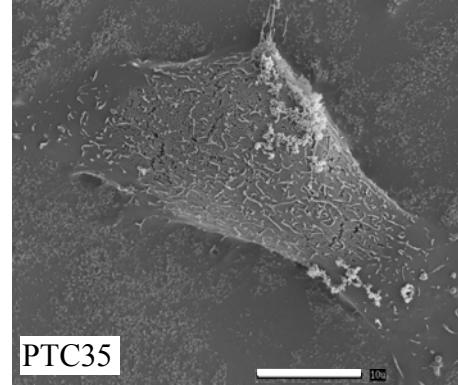
Figure 2. 13: Osteoblast adhesion on PLGA, PTC25, PTC35, PTC45, PTC70, TCG, TCS, and reference: Glass. Values are mean $\pm$ SEM; N = 3; * p < 0.05 compared to PLGA; ** p < 0.05 compared to PTC25; *** p < 0.05 compared to all the composites.

2.3.2.2. Osteoblast Morphologies

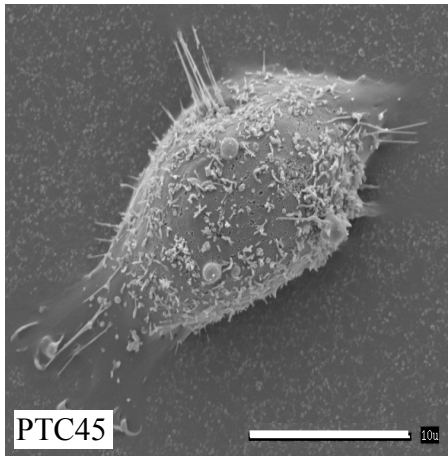
The typical morphologies of adherent osteoblasts on the substrates of interest after a 4-hour incubation time are presented in Figures 2.14 and 2.15. The average length of the major axis of typical adherent osteoblasts on the substrates of interest was measured, as shown in Figure 2.14 and 2.15.



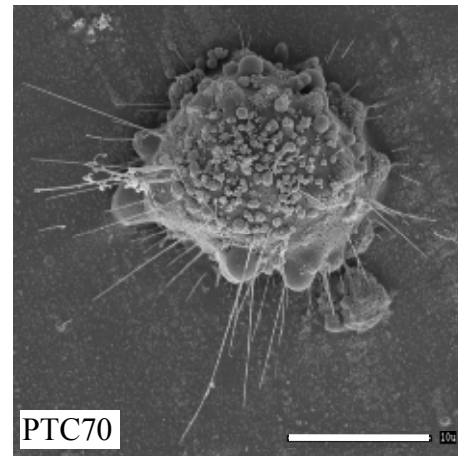
The average length of
the major axis: 19 μm .



The average length of
the major axis: 36 μm .

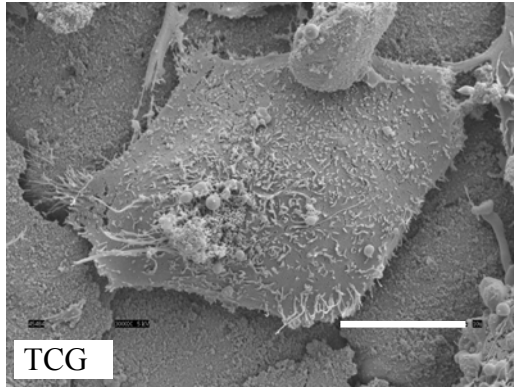


The average length of
the major axis: 28 μm .

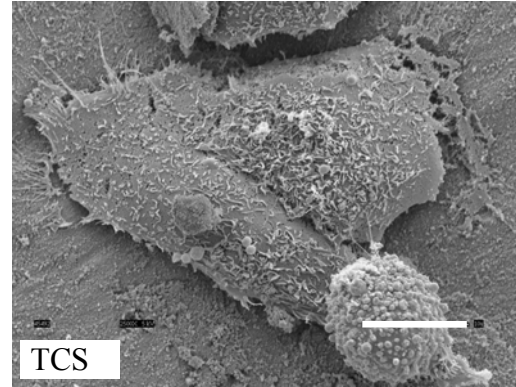


The average length of
major axis: 17 μm .

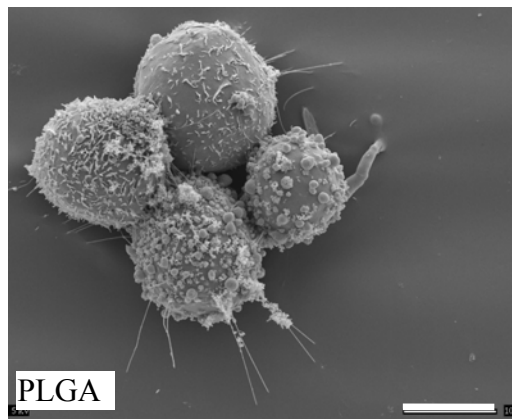
Figure 2. 14: SEM micrographs of osteoblasts adhering on the materials of interest: PTC25, PTC35, PTC45, and PTC70. Incubation time is 4 hours. The average length of the major axis of typical adherent osteoblasts on the substrates of interest was shown below each micrograph. Magnification bars: 10 μm .



The average length of the major axis: 33 μm .



The average length of the major axis: 34 μm .



The average length of the major axis: 14 μm .

Figure 2. 15: SEM micrographs of osteoblasts adhering on the materials of interest: PLGA, TCG, and TCS. Incubation time is 4 hours. The average length of the major axis of typical adherent osteoblasts on the substrates of interest was shown below each micrograph. Magnification bars: 10 μm .

Osteoblasts on TCG and TCS possessed their typical very flat polygonal shape. In contrast, less spread osteoblasts were observed on PLGA and PTC70 substrates. Generally, osteoblasts were more spread on TCG and TCS than on any composite and PLGA. Osteoblasts on the composites were better spread than that on PLGA.

2.3.2.3. Osteoblast Long-term Functions

2.3.2.3.1. Synthesis of Total Protein

There were detectable amounts of total proteins secreted by osteoblasts on all substrates after 7, 14 and 21 days of culture (Figure 2.16).

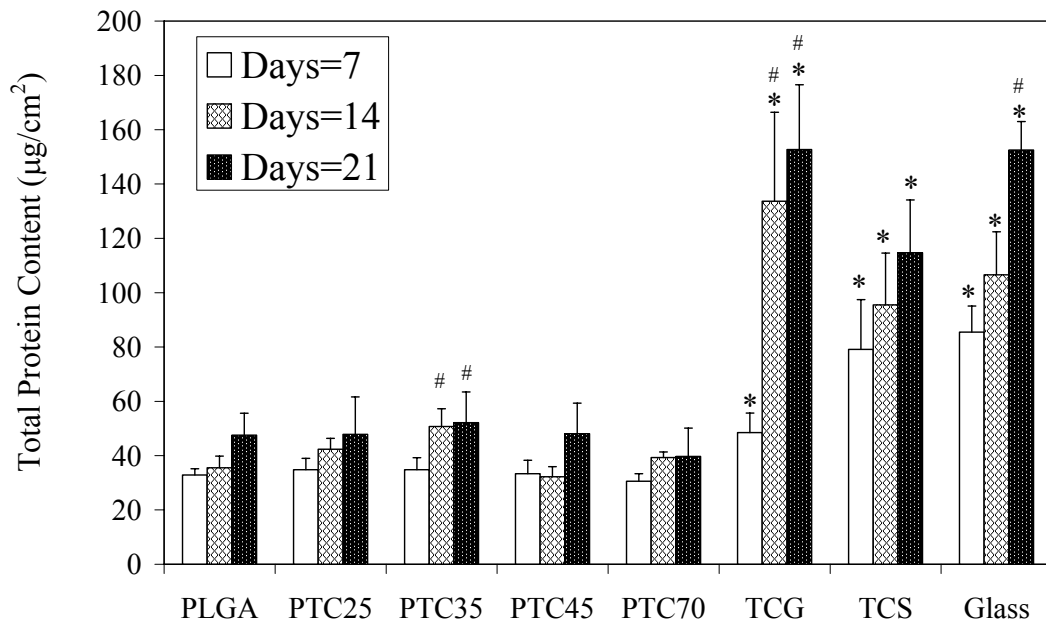


Figure 2. 16: Total protein content in osteoblasts cultured on PLGA, PTC25, PTC35, PTC45, PTC70, TCG, TCS; and reference: Glass. Values are mean $\pm$ SEM; N = 3; * p < 0.05 compared to PTC25, PTC35, PTC45, PTC70, and PLGA at respective days. # p < 0.05 compared to the respective substrates at 7 days.

Generally, total protein content increased with longer time periods of culture. Specifically, total protein content increased significantly on the PTC 35 and TCG after 14 and 21 days of culture compared to 7 days of culture while total protein content on all the other composites (PTC25, PTC45, and PTC70) and PLGA did not increase significantly after 14 and 21 days of culture compared to 7 days of culture.

Importantly, total protein content was significantly greater on TCG and TCS than that on all the composites and PLGA after respective 7, 14, and 21 days of culture. In contrast, the total protein content was not significantly different between TCG and TCS substrates after respective 7, 14, and 21 days of culture. After 7 days of culture, total protein content was not significantly different among all the composites and PLGA. However, total protein content was significantly greater on PTC35 than all the other composites and PLGA after 14 days of culture. There was no statistical difference of total protein content detected among all the composites and PLGA after 21 days of culture.

2.3.2.3.2. Total Collagen Content

There were detectable amounts of total collagen synthesized by osteoblasts on all substrates after 7, 14 and 21 days of culture (Figure 2.17). Generally, total collagen synthesis by osteoblasts increased with longer time periods of culture. For example, total collagen content increased significantly on all the substrates after 21 days of culture compared to 7 days of culture.

After 7 days of culture, total collagen synthesis was significantly greater on TCG and TCS than on all the composites and PLGA; significantly greater on PTC35 than on the other composites and PLGA. After 14 days of culture, total collagen synthesis was significantly greater on TCG and TCS than on all the composites and PLGA; significantly greater on PTC35 than on PTC45 and PLGA. After 21 days of culture, total collagen synthesis was significantly greater on TCG and TCS than on all the composites and PLGA; significantly greater on PTC35, PTC45 and PTC70 than on PLGA and PTC25; not significantly different between PTC35, PTC45 and PTC70; not significantly

different between the TCG and TCS; and not significantly different between PTC25 and PLGA.

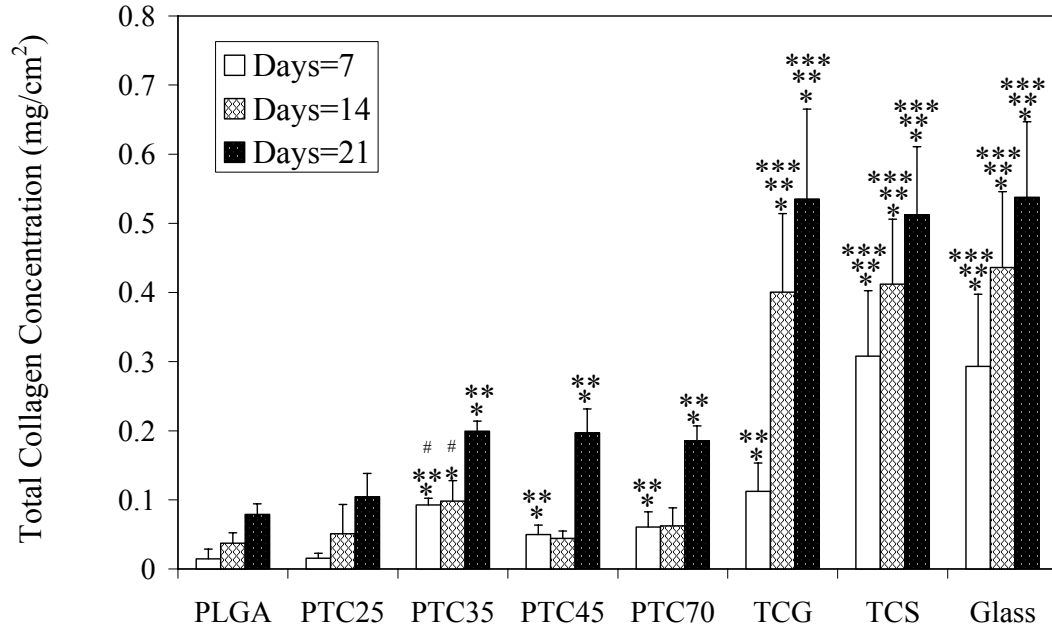


Figure 2. 17: Total collagen content in osteoblasts cultured on PLGA, PTC25, PTC35, PTC45, PTC70, TCG, TCS; and reference: Glass. Values are mean $\pm$ SEM; N = 3; * p < 0.05 compared to PLGA at respective days; ** p < 0.05 compared to PTC25 at respective days; *** p < 0.05 compared to all the composites at respective days; # p < 0.05 compared to PTC45 at respective days.

2.3.2.3.3. Alkaline Phosphatase Activity

There were detectable amounts of alkaline phosphatase synthesized by osteoblasts on all the substrates after 7, 14 and 21 days of culture (Figure 2.18). Generally, alkaline phosphatase activity increased with longer time periods of osteoblast culture. Specifically, alkaline phosphatase activity increased significantly on all the substrates after 21 days of culture compared to 7 days of culture.

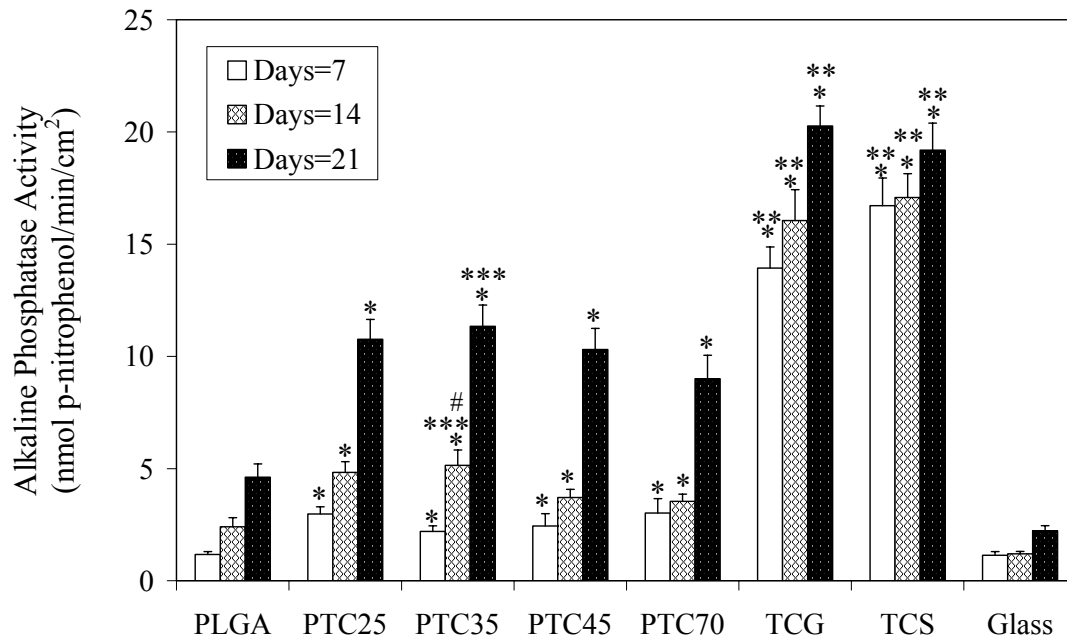


Figure 2. 18: Alkaline phosphatase activity in osteoblasts cultured on PLGA, PTC25, PTC35, PTC45, PTC70, TCG, TCS; and reference: Glass. Values are mean $\pm$ SEM; N = 3; * p < 0.05 compared to PLGA at respective days; ** p < 0.05 compared to all the composites at respective days; *** p < 0.05 compared to PTC70 at respective days; # p < 0.05 compared to PTC45 at respective days.

After 7 days of culture, alkaline phosphatase activity was significantly greater on TCG and TCS than on all the composites; significantly greater on all the composites than on PLGA; and not significantly different among all the composites. After 14 days of culture, alkaline phosphatase activity was significantly greater on TCG and TCS than on all the composites; significantly greater on all the composites than on PLGA; and significantly greater on PTC35 than on PTC45 and PTC70. After 21 days of culture, alkaline phosphatase activity was significantly greater on TCG and TCS than on all the composites; significantly greater on all the composites (twice more) than on PLGA; and significantly greater on PTC35 than on PTC70. Alkaline phosphatase was not significantly different between TCG and TCS at respective 7, 14 and 21 days of culture.

2.3.2.3.4. Extracellular Calcium Deposition

There were detectable amounts of calcium deposited by osteoblasts on all the composites, PLGA, TCG, TCS and glass after 7, 14 and 21 days of culture (Figure 2.19). There were significantly greater amounts of calcium deposited by osteoblasts on all the composites and titania compacts at 21 than at 7 and 14 days of culture. However, calcium deposited on PLGA and glass did not increase at 21 days of culture compared to at 7 and 14 days of culture.

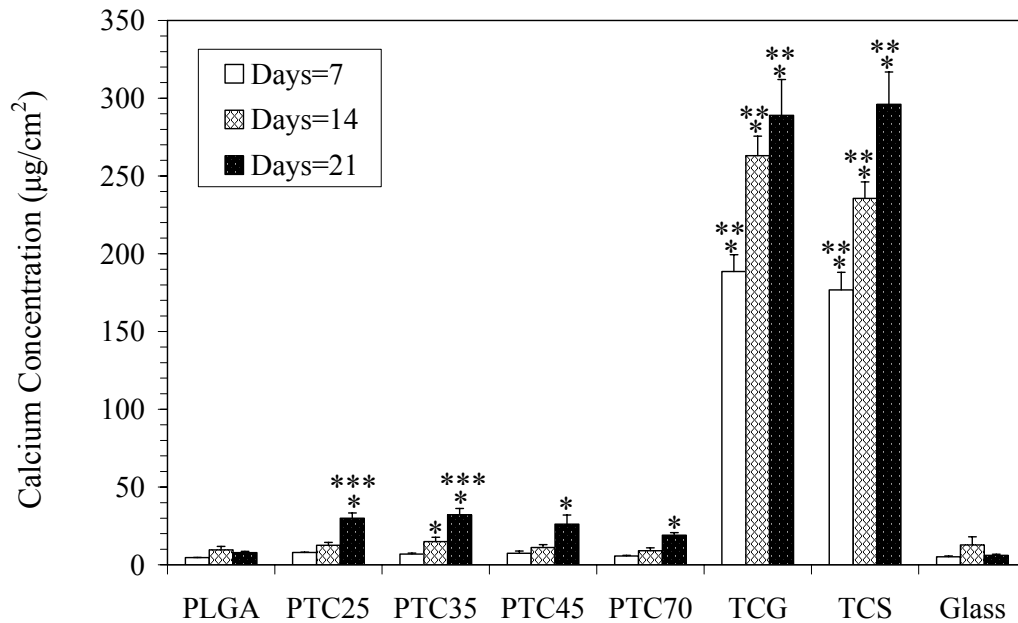


Figure 2. 19: Calcium deposited by osteoblasts cultured on PLGA, PTC25, PTC35, PTC45, PTC70, TCG, TCS; and reference: Glass. Values are mean $\pm$ SEM; $n = 3$; * $p < 0.05$ compared to PLGA at respective days; ** $p < 0.05$ compared to all the composites at respective days; *** $p < 0.05$ compared to PTC70 at respective days.

After respective 7, 14, and 21 days of culture, calcium deposition was significantly greater on TCG and TCS than on all the composites and PLGA. There was no significantly different calcium deposition by osteoblasts between TCG and TCS at any

time period. After 7 days of culture, only very small amounts of calcium deposited on the composites and PLGA and, thus, statistical difference was not detected. After 14 days of culture, calcium concentration was significantly greater on PTC35 than on the other composites and PLGA. After 21 days of culture, the amount of calcium was significantly greater on all the composites than on PLGA. Importantly, the amount of calcium deposited by osteoblasts was significantly greater on PTC35 than on PTC70 after 21 days of culture.

2.3.2.4. Acellular Calcium Deposition

Acellular calcium precipitated on all the composites, PLGA, TCG, TCS and glass after 7, 14 and 21 days of culture (Figure 2.20). There were significantly greater amounts of calcium precipitated on all the composites and titania compacts at 21 than at 7 days of culture. However, calcium precipitated on PLGA and glass did not increase at 21 days of culture compared to at 7 and 14 days of culture.

After respective 7, 14, and 21 days of culture, acellular calcium precipitation was significantly greater on TCG and TCS than on all the composites and PLGA. There was no significantly different calcium precipitation between TCG and TCS at any time period. After 7 days of culture, the amount of acellular calcium precipitated was significantly greater on PTC35 and PTC45 than that on PTC25, PTC70 and PLGA; not significantly different between PTC35 and PTC45; and not significantly different between PTC25 and PLGA. After 14 days of culture, the amount of acellular calcium precipitated was significantly greater on all the composites than that on PLGA and not significantly different among the composites. After 21 days of culture, the amount of acellular calcium

precipitated was significantly greater on all the composites than on PLGA; significantly greater on PTC35 than on PTC25 and PTC70; but not significantly different between PTC35 and PTC45.

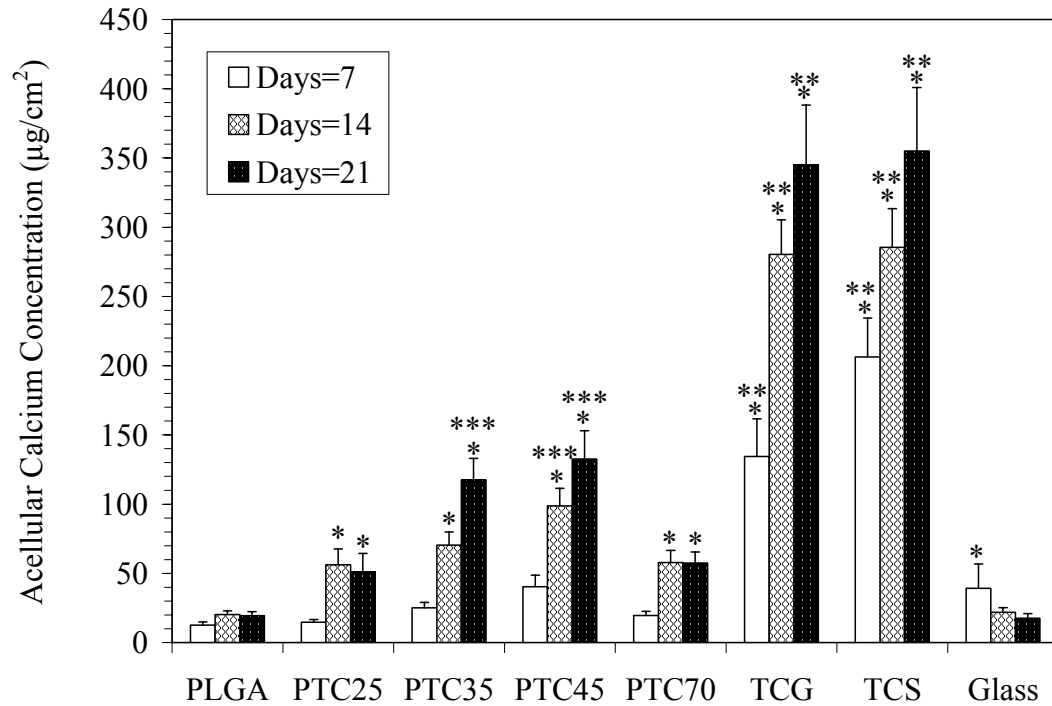


Figure 2. 20: Acellular calcium precipitated on PLGA, PTC25, PTC35, PTC45, PTC70, TCG, TCS; and reference: Glass. Values are mean $\pm$ SEM; N = 3; * p < 0.05 compared to PLGA at respective days; ** p < 0.05 compared to all the composites at respective days; *** p < 0.05 compared to PTC25 and PTC70 at respective days.

2.3.3. Evaluation of *In Vitro* Degradation

There were detectable amounts of weight loss of PLGA and all the composites after 21, 28 and 35 days of incubation in PBS under standard incubation conditions (Figure 2.21).

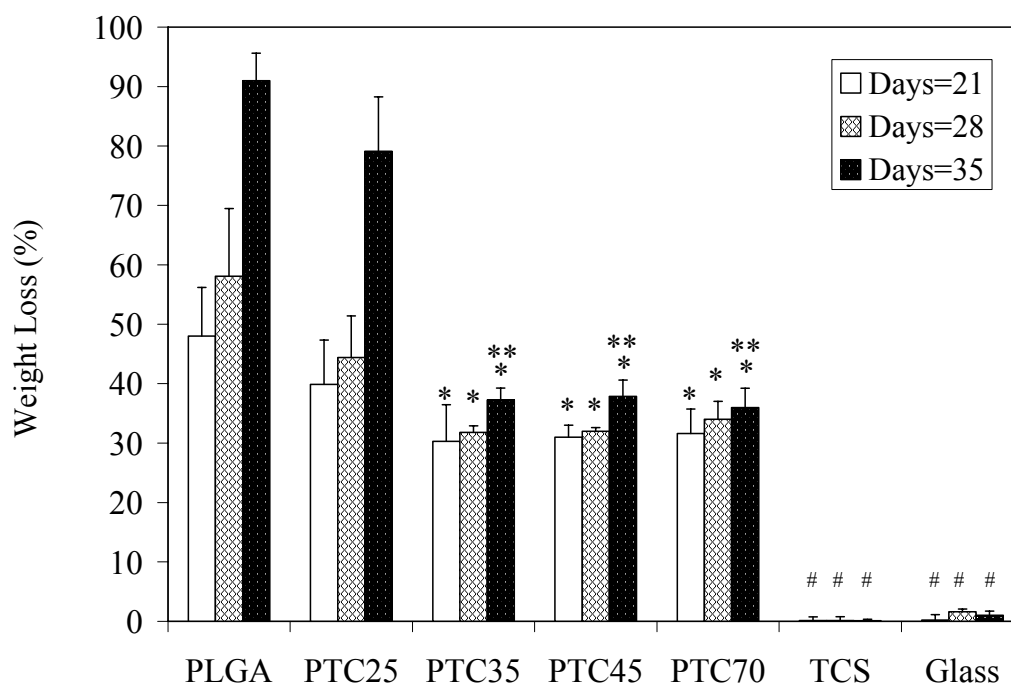


Figure 2. 21: Percent weight loss for PLGA, PTC25, PTC35, PTC45, PTC70, TCS, and Glass incubated in PBS under standard incubation conditions. Values are mean $\pm$ SEM; N = 3; * p < 0.05 compared to PLGA at respective days; # p < 0.05 compared to all the composites at all days; ** p < 0.05 compared to PTC25 at respective days.

The percentage of weight loss of pure PLGA was the greatest among all the substrates incubated at respective days. As expected, no weight loss was observed on titania compacts and glass. Among all the composites, the weight loss of PTC25 was greater than the others at 35 days of incubation. This indicated that the dispersion status of nanophase titania in PLGA played an important role in decreasing the degradation rate of these nano-composite.

Moreover, the buffering effect of titania particles towards PLGA weight loss was more significant after longer time periods of incubation, which correlated to the pH buffering effect of titania particles, as shown in Figure 2.22. Specifically, the pH drop

was less than 16% for all the composites during the first 21 days of incubation while it was 19% for pure PLGA. During 22 to 35 days of incubation, the pH drop was faster than the first 3 weeks of incubation. Specifically, the pH drop was 70% for pure PLGA after 35 days of incubation while it was 68% for PTC25, 56% for PTC35, and only 43% for PTC70.

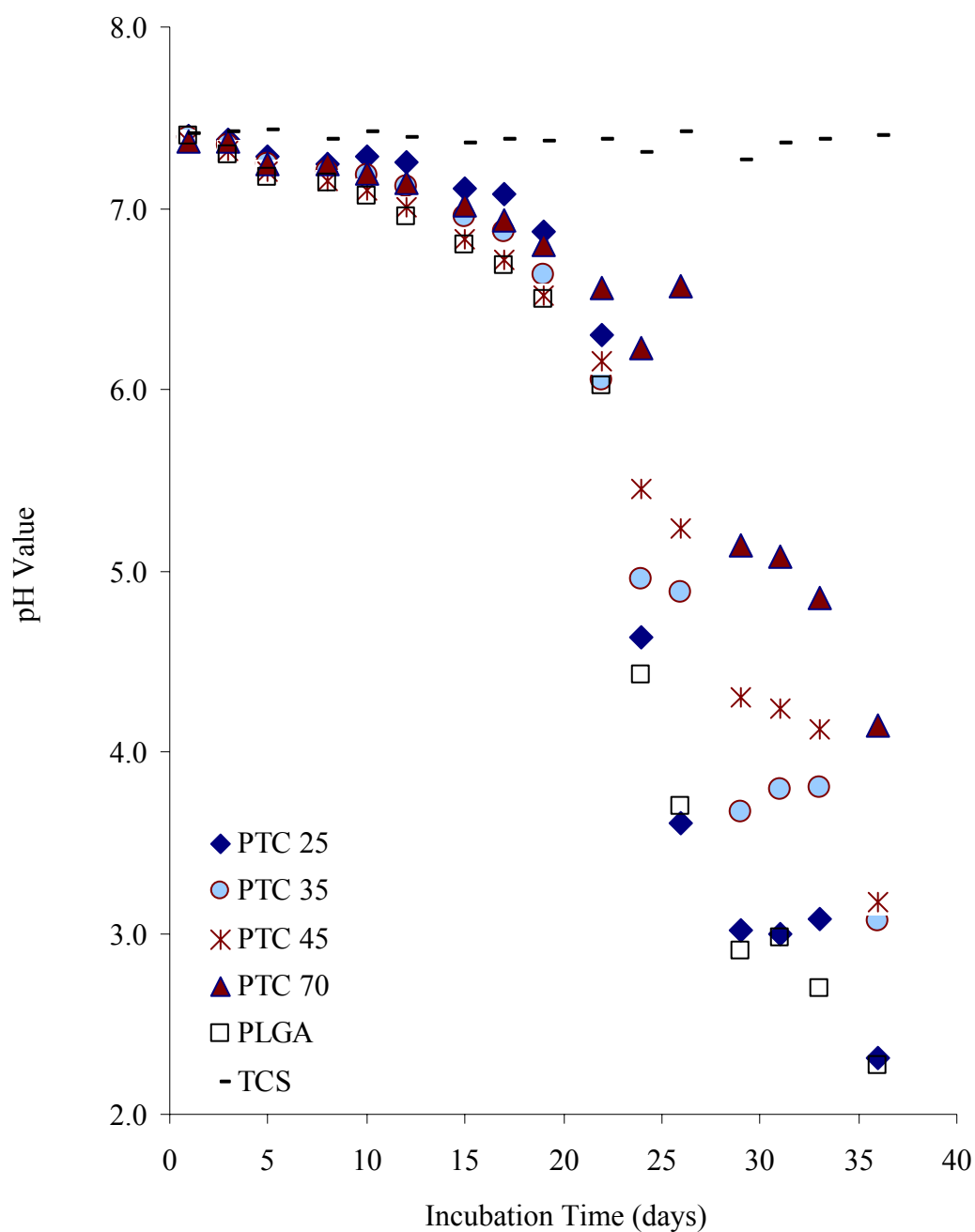


Figure 2. 22: pH variation with incubation time for PLGA, PTC25, PTC35, PTC45, PTC70, TCS, and Glass incubated in PBS under standard incubation conditions. Values are mean $\pm$ SEM; N = 3. The SEM bars were not shown in this figure for the purpose of clarity.

2.4. Discussion

2.4.1. Bio-inspired Nanophase Titania/PLGA Composites as Bone Substitutes

Compared to metals, metal alloys (such as titanium and titanium alloys) and conventional ceramics (grain sizes greater than 100 nm), nanophase ceramics (such as titania) have improved cytocompatibility properties [145,146]. In the present study, the improved cytocompatibility of nanophase titania was documented by greater osteoblast adhesion, synthesis of alkaline phosphatase, and calcium mineral deposition over PLGA [175]. However, practically, ceramics are inherently brittle and difficult to deform into complex shapes with acceptable mechanical properties for load-bearing orthopedic applications when they are used alone.

Therefore, nanophase titania/PLGA composites (as formulated in the present study) offer an opportunity to take advantage of the great cytocompatibility properties of nanophase titania with improved malleability properties due to the addition of a polymer. In addition, through the use of PLGA, the composite may degrade as new bone grows, thus allowing for increased interlocking strength and a potentially higher degree of implant success. Moreover, nano-sized titania particles used as inclusions in biodegradable PLGA can provide a pH buffering effect to the polymer and to tailor the degradation kinetics of the PLGA matrix.

As mentioned, natural bone is composed of nanostructured constituent such as Type I collagen, HA crystals, and proteins. Thus, it stands to reason that cells are naturally exposed to nanostructured surface features in the body. Previous studies conducted by our research group provided evidence that greater weight percentages of

nanophase titania in PLGA scaffolds increased functions of osteoblasts (such as adhesion, alkaline phosphatase activity and calcium deposition) when many material properties (such as chemistry, crystallinity, and crystal phase) were kept constant in comparison scaffolds [178]. Specifically, for example, nanophase titania/PLGA composites with a 30/70 weight percent ratio demonstrated greater osteoblast functions than nanophase titania/PLGA composites with a 20/80 weight percent ratio and conventional titania/PLGA composites with a 30/70 weight percent ratio [178]. Therefore, in this study, nanophase titania/PLGA composites with a 30/70 weight percent were chosen since the 30/70 weight percent ratio was proven to be optimal for osteoblast functions.

However, when titania particles decrease into the nanometer regime and the amount of titania particles in PLGA increases, the tendency for particle agglomeration is dramatically higher and may consequently counteract the advantages of adding nanophase titania to PLGA in the first place. The strong tendency for nanoparticles to agglomerate could result in unevenly distributed nanoparticles and subsequently inhomogeneous modification of the properties of polymer matrix; clearly, the mechanical and biological properties of nano-composites will depend irregularly on the amount of titania loaded. Therefore, it is important to study and discuss the dispersion behavior of nanophase titania in PLGA and its influence on subsequent cell functions.

2.4.2. Dispersion of Nanophase Titania in PLGA Composites

2.4.2.1. Why Dispersion Is Necessary for Nanocomposites

Nanocomposites represent a new prospective branch in the field of conventional ceramic/polymer composites for orthopedic applications. It has been shown that an overall enhancement of composite properties can be achieved under certain conditions by the addition of nanoparticles instead of conventional micron-sized particles. For example, Zhang et al. reported that tensile strength, percent elongation, and tear strength of EPDM (ethylene-propylene-diene monomer) rubber composites reinforced with 40 wt.% magnesium hydroxide ($\text{Mg}(\text{OH})_2$) particles increased significantly as the particle size decreased. Specifically, when the average particle size of $\text{Mg}(\text{OH})_2$ decreased from 2 μm to 50 nm, the tensile strength increased from 3 MPa to 10 MPa; the percent elongation increased from 280% to 430%; the tear strength increased from 15 KPa to 35 KPa [179]. This is likely to be the combined result of the stress concentration effect becoming negligible as the size of particles approach that of the molecules and a synergistic effect yet unknown becoming dominant at the nanometer scale.

Furthermore, the presence of nanoparticles provides improvements in other properties as well (such as scratch resistance, erosion resistance, wear resistance, and fire resistance) [180]. For example, it was reported that the scratch indentation of titania-filled epoxy composites decreased from 60 μm to 30 μm when the filled titania particle size decreased from 0.24 μm to 32 nm [181]. However, these positive effects of adding nanoparticles into a polymer do not appear simultaneously, but rather depend on the dispersion state and microstructure of nanoparticles in polymer matrix. Most importantly, when the microstructural homogeneity of the nanocomposites improves, their mechanical

properties (such as strength and hardness), which are crucial for bone tissue engineering applications, increase even more significantly [182].

Polymer-based nanocomposites have attracted considerable attention owing to their unique properties resulting from nanoscale microstructures which have been characterized by the larger fraction of filler atoms that reside at the surface of the nanoparticles leading to stronger interfacial interactions with the surrounding polymer matrix compared to larger particle-filled composites. Properties of polymer-based nanocomposites are a function of the dispersion state of the nano-sized reinforcing ceramic particles. If the ultra-fine phase dimensions of the nanoparticles are maintained after compounding with the polymer matrix, such nanocomposites will need a far less filler content to achieve a more significant improvement in elastic modulus and strength than conventional composites. For example, polypropylene (PP) reinforced with 1 μm $\text{Al}(\text{OH})_3$ particles achieved elastic modulus values of 1700 MPa at 15 vol.% $\text{Al}(\text{OH})_3$ particle and 2520 MPa at 36 vol.% $\text{Al}(\text{OH})_3$, while the same composites reinforced with 55 μm $\text{Al}(\text{OH})_3$ particles only had elastic modulus values of 1660 MPa at 15 vol.% $\text{Al}(\text{OH})_3$ and 1747 MPa at 36% $\text{Al}(\text{OH})_3$ [183]. Therefore, nanocomposites are much lighter in weight and easier to process than respective conventional particulate filled polymers.

The present work demonstrated the importance of the dispersion status of titania nanoparticles in a PLGA matrix, specifically, on the cytocompatibility properties of such nanocomposites. As expected, the dispersion of nanophase titania in PLGA was enhanced by increasing the intensity of sonication. That is, higher ultrasonic energy broke larger titania agglomerates into smaller titania particles, which were more easily dispersed in

PLGA suspensions, and subsequently remained on the surface of the nanocomposites after the solvent (chloroform) evaporated. The composites (such as PTC35 or the composite sonicated at 166.25 W) with greater amounts of nanophase titania on the surface promoted greater osteoblast adhesion and long-term functions (such as alkaline phosphatase activity and calcium-containing mineral deposition).

As previously mentioned, nanophase titania/PLGA composites with a 30/70 of weight percent ratio were chosen as model composites in this study. The volume percentage of nanophase titania in PLGA was calculated according to the equation 4.1.

$$V_{\text{titania}} = \frac{\frac{W_{\text{titania}}}{\rho_{\text{titania}}}}{\frac{W_{\text{titania}}}{\rho_{\text{titania}}} + \frac{W_{\text{PLGA}}}{\rho_{\text{PLGA}}}} \quad (4.1)$$

V_{titania} is the volume fraction of titania in PLGA, which was calculated as 12.7 vol.%. W_{titania} is the weight fraction of titania, which was 0.30; W_{PLGA} is the weight fraction of PLGA, which was 0.70; ρ_{titania} is the theoretical density of titania with an 80/20 anatase/rutile phase content, which was 3.96 g/cm³; ρ_{PLGA} is the density of PLGA, which was 1.34 g/cm³.

Since the predominant feature of nanoparticles lies in their ultra-fine dimension, a large fraction of the filler atoms can reside at the interface and can lead to a strong interfacial interaction [184], but only if the nanoparticles are well dispersed on the nanometer level into the surrounding polymer matrix. As the interfacial structure plays a critical role in determining the properties of composites, nanocomposites coupled with a great number of interfaces could be expected to provide unusual properties, and the

shortcomings induced by the heterogeneity of conventional (or micron) particle filled composites would also be avoided [182].

The microstructural appearance of 12.7 vol. % particles of different sizes dispersed in a polymer matrix was sketched in Figure 2.23. Consequently, the so-called nanoparticle filled polymers sometimes contain a number of loose clusters of particles and exhibit properties even worse than conventional particle/polymer systems.

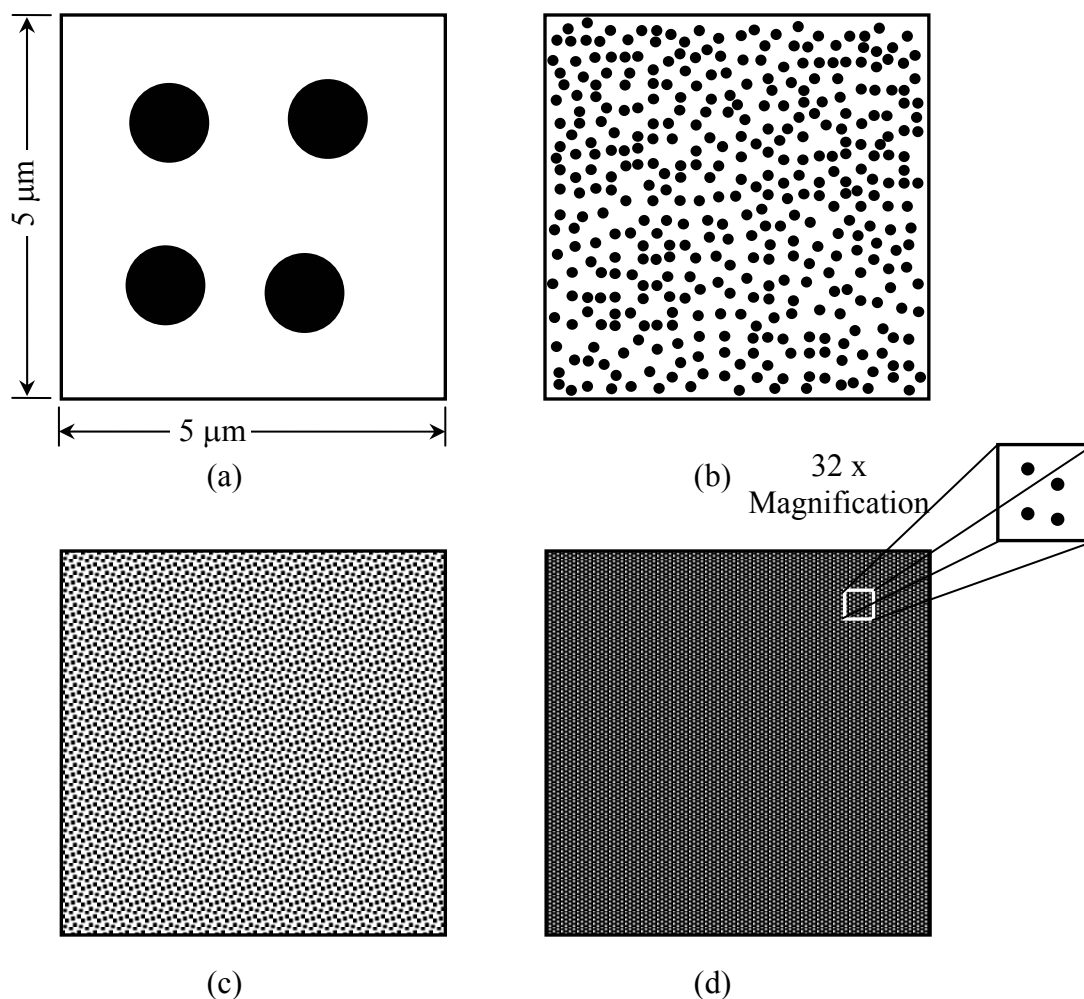


Figure 2. 23: Schematic of theoretical microstructure of ceramic/polymer composites. (a) 12.7 vol. % of particles with 1000 nm diameters (4 particles within an area of $25 \mu\text{m}^2$); (b) 12.7 vol. % of particles with 100 nm diameters (404 particles within an area of $25 \mu\text{m}^2$); (c) 12.7 vol. % of particles with 50 nm diameters (1617 particles within an area of $25 \mu\text{m}^2$); and (d) 12.7 vol. % of particles with 30 nm diameters (4492 particles within an area of $25 \mu\text{m}^2$).

When nanophase titania particles are incorporated into PLGA, the mechanical properties (such as bending modulus) of the composites increased from 500 ± 60 MPa to 1470 ± 90 MPa, especially if a rather uniform dispersion of the nanoparticles exists [162]. This implies, on the one hand, that titania nanoparticles can effectively improve polymer properties more than conventional micron titania particles. On the other hand, when the

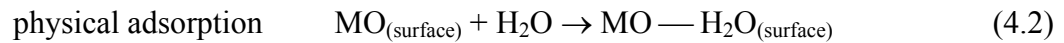
nanoparticles are unevenly dispersed in the matrix, the nanocomposites serve as composites filled with micrometer-sized agglomerate fillers, in which crack-initiation and coalescence occur more easily in the particulate-rich phases. In such studies, the presence of powder agglomeration caused a remarkable change in the powder-packing structure [185]. In addition, the agglomerated particles in a suspension caused an increase in viscosity at a given shear rate. Irregular powder packing reduces the volume fraction of free flowing solvents because the solvent is entrapped within the agglomerates and, thus, reduces the evaporation rate of solvent, and even results in the remainder of the organic solvent in the final product [186].

However, very few studies have been presented to date concerning the relationship between the microstructural details determined by the dispersion status of nanoparticles in a polymer matrix and subsequent biological properties of such bulk nanocomposites. This is very surprising, since this is a topic which has both a fundamental and applied significance for the development of nanocomposites for tissue engineering applications. Therefore, the objective of the present *in vitro* work focused on the performance of composites with identical species and the same amount of the reinforcing components, but with a different dispersion status. In particular, the effects of the dispersion status on the biological behavior of nanophase titania/PLGA composites were studied, so as to provide knowledge for an optimum material preparation for orthopedic applications. Dispersion status of nanophase titania/PLGA biocomposites can manipulate surface features of such nanocomposites, such as surface roughness and surface area, which consequently influence *in vitro* cell responses.

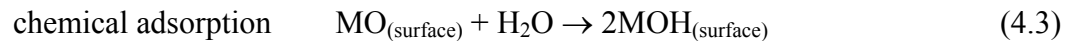
2.4.2.2. Mechanism of Agglomeration of Nanophase Titania Particles

Groups of particles that are relatively weakly bonded together by physical bonds may behave as fragile, larger pseudo-particles called soft agglomerates. If particles are strongly bonded together by chemical bonds, the larger particle is not as easily dispersed and is referred to as aggregate or hard agglomerate. In soft agglomerates of non-magnetic powder, the weak physical bonds may be Van der Waals forces, electrostatic attraction, or capillary adhesion forces. Electrostatic forces occur because of surface-adsorbed ions or because of the transfer of electrons between particles in regions of contact. Electrostatic and Van der Waals forces produce relatively fragile agglomerates. However, even these fragile agglomerates can impede the flow of nanocomposite suspensions and cause an uneven distribution of properties of nanocomposites.

Usually, the surface oxide of particles (such as titania) tend to adsorb water molecules from the atmosphere physically or chemically. Evidence of surface adsorption of water is provided by infrared adsorption studies, heats of immersion, and the thermal behavior of the adsorption-desorption kinetics [187]. Reactions of the type:



and



have been suggested (see Figure 2.24) [187]. M represents a metal atom. The polar hydroxyl (–OH) groups may cause the surface to attract and physically adsorb other molecules, thus, having a crucial effect on the agglomeration of titania particles.

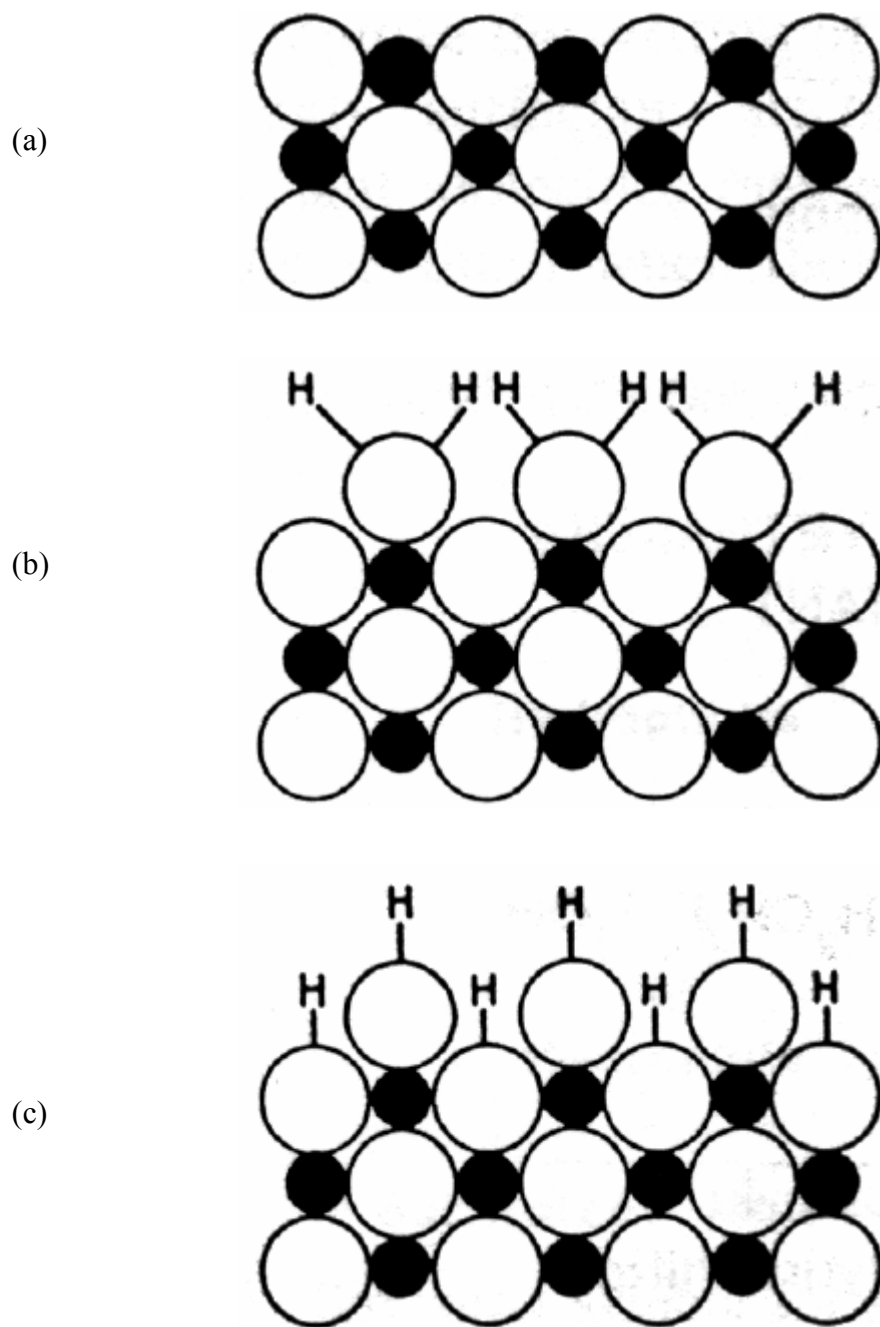


Figure 2. 24: Schematic of the cross section of the atomic structure of an oxide showing (a) a dry surface, (b) a surface with physically adsorbed water and (c) a surface with chemically adsorbed water. Dark solid balls represent metal atoms and light hollow balls represent oxygen. (Adapted and redrawn from [187]).

Nanophase titania may form physically-bonded or chemically-bonded agglomerates in a PLGA chloroform solution. Physically-bonded agglomerates are fragile

and easier to break while chemically-bonded agglomerates are more difficult to break. Therefore, an absolutely homogeneous dispersion of nanophase titania particles in a PLGA matrix is a very difficult task due to the existence of chemical bonding. Sonication at different powers was utilized in this study to break down soft agglomerates and to produce nanostructured composites. The significant improvement in dispersion status of nanophase titania in PLGA composites is beneficial for the miscibility of particle/matrix, even though the particles could not be completely dispersed in the form of primary nanoparticles in the polymer matrix.

2.4.2.3. Dispersion of Nanophase Titania Particles in PLGA by Sonication

In order to take full advantages of the benefits mentioned above, a technique to uniformly disperse the nanophase titania particles in the PLGA was required. For non-medical applications, such as electronic systems, surfactants have been usually used to stabilize the dispersion of ceramic particles in polymer-based composites and optimize the electrical properties of the composites. For example, Rao et al. reported that block copolymer surfactants (i.e. polystyrene-b-epoxy modified polybutadiene) could improve the BaTiO₃ particle (average diameter 65 nm) dispersion in epoxy composites and, thus, increased the dielectric constant of the composites from 10 to 42 with 40 vol.% BaTiO₃ particle loading, which allowed the composites to achieve a higher dielectric constant at relatively lower ceramic loading level for embedded capacitor applications [188]. However, for biomedical applications, cytocompatibility of surfactants has to be considered and tested. Unfortunately, so far, common surfactants widely used in structural, mechanical and electrical systems are either cytotoxic or have adverse

influences on cytocompatibility properties. Furthermore, even if biocompatible surfactants were found, they are still not a good choice for dispersion of very fine nanoparticles for bone tissue engineering applications because bone substitutes are exposed to mechanical loading conditions. Adding the surfactants decreases packing density of the particles and, thus, impairs mechanical properties of ceramic/polymer composites.

Sonication has been found to be effective for submicron and nano-powders which are hard to disperse by other methods [189-191]. It is generally believed that sonication helps to improve the dispersion status of nanoparticles and consequently the homogeneity of composites microstructure. For example, it was reported that 1.5 to 3.0 wt.% spherical silicon carbide (SiC) nanoparticles 29 nm in diameter were dispersed into a SC-15 resin using a sonicator (Sonics Vibra Cell Ultrasonic Liquid Processor) at 55% of the amplitude for about 30 minutes and the dispersion of the nanoparticles was visually observed to be uniform [192]. It was also demonstrated that uniform incorporation of SnO₂ nanoparticles into polyethylene oxide (PEO)-LiClO₄ composites was achieved by sonication and conductivity of such nanocomposites improved due to better dispersion [193]. Nano nickel particles that were dispersed into polycarbosilane (PC) by sonication, and then mixed with SiC fibers demonstrated better mechanical properties and continuous controllable resistivity [194]. However, in all these studies, single-power sonicators were used and, that is, the sonication powers were not controlled.

Sonication involves the formation and collapse of cavities that produce local high velocity jets and pressure gradients. The resulting mechanical forces on the aggregated particles are strong enough to break up the weakly bonded particles, such as those joined

by the Van der Waals forces. In the present study, the dispersion quality looked acceptable immediately after sonication (whether at high or low powers) according to visual observations and there were no visibly clear phase separations. During solvent evaporation, however, particle sedimentation was observed on samples sonicated at low powers.

It was observed that the best dispersion stability was achieved at a sonication period of 10 minutes. Most aggregates were broken in the early stage of sonication and the dispersion quality did not improve that much after 10 minutes. If sonication was set as longer than 10 minutes, the temperature would rise up so that chloroform would have evaporated during sonication. Subsequently, the composite mixture would become too thick to flow and could not be cast into the mold. Similar events were reported by Park et al. that 4 vol.% iron oxide (Fe_3O_4) particles with an average diameter of approximately 26 nm were sonicated in resin solutions at a power of 400 W for 5, 11, and 20 minutes and the best particle dispersion quality was obtained at a sonication time of 11 minutes [195].

In the present study, initially, nanoparticles formed agglomerates because of the Van der Waals forces between them. During sonication, agglomerates broke up and titania particles were surrounded by the polymer molecules. Although these separated particles tried to re-agglomerate after sonication because of the Van der Waals forces, they could not form direct contact with one another because individual titania particles were still separated by the intervening polymer molecules. That is, once the particles were separated by sonication, steric equilibrium was achieved. It is, thus, easy to maintain a dispersed state until all the solvent was removed by evaporation. As expected,

it was clearly shown that the improved dispersion was achieved under higher sonication power in Figures 2.4 and 2.7. That is, higher ultrasonic energy broke large titania agglomerates into smaller titania particles, which were more easily dispersed in PLGA suspensions and took longer time for sedimentation (as will be discussed in next section 2.4.2.4), and consequently increased the coverage of titania on the surface of the composites. Although sonication was effective, it was also determined to be rather difficult to achieve a completely uniform dispersion of nanoscale particles due to the presence of hard agglomerates.

2.4.2.4. Sedimentation of Nanophase Titania Particles

After sonication, the composite mixture was cast into a Teflon petri dish and chloroform was allowed to evaporate in air at room temperature. It was observed that chloroform evaporated and the composites solidified within 6 hours after casting. The time (t) for titania particles to settle to a height (H) could be estimated using Stokes equation (4.4) if the composite mixture was assumed as a Newtonian fluid with laminar flow.

$$t = \frac{18H \cdot \eta_L}{D^2 \cdot (\rho_p - \rho_L) \cdot g} \quad (4.4)$$

η_L is the viscosity of the suspension, approximated by the viscosity of chloroform at room temperature, which was $5.63 \times 10^{-4} \text{ kg} \cdot \text{m}^{-1} \cdot \text{s}^{-1}$. D is the titania particle size. ρ_p is density of titania particle. ρ_L is the density of suspension, approximated by the density of chloroform, which was $1.48 \times 10^3 \text{ kg} \cdot \text{m}^{-3}$. g is the acceleration due to gravity, which was 9.8 m/s^2 . t is the time that it would take titania particles with a diameter of D to settle

down a height H in suspension L . H is the height of the composite suspension after casting into the petri dish, estimated by the equation

$$H = \frac{V}{A} = \frac{8000}{\pi(\frac{50}{2})^2} \approx 4mm \quad (4.5)$$

V is the volume of the composite suspension; A is surface area of the petri dish used in this study.

According to the Stokes equation, if the particle size was 200 nm, it would take 12 hours for titania particles to settle down. If the particle size was 100 nm, it would take 47 hours for titania particles to settle down. If the particle size was 50 nm, it would take 188 hours (7.8 days) for titania particles to settle down. Smaller particles will take even longer time to settle down, which is much longer than the evaporation time of chloroform. Therefore, theoretically, the evaporation process of chloroform was much faster than the sedimentation process of titania particles which were smaller than 300 nm.

2.4.3. Quantification of Essential Surface Properties

Of particular importance to the present study is how the nanometer surface features of nanophase titania/PLGA composites controlled by sonication at different powers influenced osteoblast adhesion and their long-term functions. As in all of the present studies, it was an important objective of the present study to elucidate various properties of nanophase composites that promoted osteoblast functions. In all materials, there are several possibilities: surface roughness, surface area, chemistry, degree of crystallinity, crystal phase, and so on. The present study was carefully designed to control as many of these properties as possible and evaluate only the consequences of changing

the degree of titania dispersion status and subsequent topographical structure which influence surface properties (such as surface roughness and surface area). Moreover, for the first time, surface roughness and surface area of natural bone were measured to compare with the nanocomposites of interest to the present study.

Not surprisingly, AFM results showed that sonication significantly increased the RMS (root mean square) roughness values of the surfaces of the nanocomposites. Actually, from the SEM pictures in Figure 2.4, it can be seen that when sonication power increased and subsequently improved the dispersion of nanophase titania particles, the surface microstructure became more uniform which was closer to the microstructure of natural bone since nano-HA crystals are uniformly dispersed in a collagen matrix in natural bone, as shown in Figure 2.5 and 2.6.

Besides directly imaging surfaces, surface structures can also be quantified by several fundamental parameters, for example, RMS roughness defined previously in equation 2.2, a measure of the deviation in height above or below some reference point; R_a , a measure of the arithmetic average of the absolute height of all pixels; or R_{p-v} , a measure of the maximum peak-to-valley height. All of these parameters may be used to characterize the surface roughness, but the RMS value is the most commonly used for the analysis of AFM data. The advantage of RMS is not only its simplicity, but also in terms of statistical significance. Since it is the standard deviation of the height, it describes the spread of the height distribution about the mean value. Because the surfaces in this study were produced by a method with some degree of spatial randomness, they were expected to exhibit Gaussian or near-Gaussian height distributions, suggesting that RMS is an appropriate description of roughness.

Obviously, RMS roughness values alone are not sufficient to describe 3-D surface features. The most apparent limitation of RMS (also R_a and R_{p-v}) for describing surfaces is a lack of spatial information. A single roughness value provides no insight into the width or spacing of surface features, and roughness for surfaces with different spatial variations of features that may be identical [196-198]. Fortunately, this could be compensated by measuring surface area at the same time, which provides spatial information of surface features. Generally, the parameters used for quantifying surface features such as surface area and surface roughness are dependent on the scan size, the size of the image in relation to the largest feature size, and the type of post-processing performed on the image data. It should be noted that the scan area was $25\ \mu\text{m}^2$ and $1\ \mu\text{m}^2$ in this study and the measurements of surface area and roughness were based on five randomly selected areas.

It is also important to be aware that roughness analysis can be biased by AFM imaging artifacts. For example, it is unlikely that the sample is exactly perpendicular to the tip; therefore, AFM images usually have some planar artifact (sample tilt) that is not representative of the surface. Roughness measured with this artifact intact is an overestimate, while improper removal of the artifact will also result in misrepresentation. Therefore, in this study only linear plane fitting and flattening (no higher-order fits) were used for all the surfaces so as to achieve comparable results among samples. The issue of the effect of probe tip radius and geometry on limited spatial resolution and image artifacts has also been considered extensively by researchers [199,200].

These parameters (such as surface area and roughness) discussed above influence protein adsorption, osteoblast adhesion and long-term functions which will be discussed in the following section.

2.4.4. Osteoblast Functions on Nanophase Titania/PLGA Composites

The development of bone-implant interfaces depends on the direct interactions of osteoblasts with the biomaterial. Osteoblast adhesion and long-term functions are therefore essential for bone-biomaterial interactions. Early *in vitro* studies of osteoblast-biomaterial interaction were more concentrated with the effect of diverse materials rather than any surface properties on cell adhesion, proliferation and differentiation [201]. However, it is now understood that the surface properties of biomaterials play a critical role in the establishment of cell-biomaterial interfaces. *In vitro* cytocompatibility studies are increasingly concerned with the influence of surface topography and consequent adsorption of proteins on cell attachment and proliferation [202-204].

As mentioned, the dispersion of nanophase titania in PLGA was enhanced by increasing the intensity of sonication. A key objective of this study was to determine the influence of nanophase titania dispersed in PLGA by sonication at various powers on osteoblast functions. The composites (such as PTC35) with greater amounts of nanophase titania dispersed on their surface promoted greater osteoblast adhesion and long-term functions (such as alkaline phosphatase activity and calcium-containing mineral deposition). Thus, as demonstrated in SEM pictures, osteoblast functions may have been enhanced simply because more titania was present on the surfaces of composites under high power sonication and osteoblasts preferred titania over PLGA. However, when the

sonication power increased to higher than 166.25 W (35% maximum power), although the titania coverage on the composites was about the same, osteoblast functions (such as adhesion) decreased.

It is intriguing to consider why osteoblast adhesion and long-term functions were different on the nanophase titania/PLGA composites which had the same percentage of nanophase titania but were prepared using different sonication powers and, thus, had various surface properties (such as various nanometer scale roughness).

2.4.4.1. Surface Roughness Influences Osteoblast Functions

In this light, it is important to mention that previous studies have shown that protein interactions are much different on surfaces with nanometer compared to conventional roughness. Specifically, the adsorption of vitronectin has been reported to be much greater on nanophase compared to conventional titania although both titania in comparison had the same chemistry [161]. Moreover, exposure of select epitopes that mediate osteoblast adhesion (such as RGD) was greater when vitronectin was adsorbed on nanometer compared to conventional ceramics [161]. The same events may be happening here. That is, since PTC35 possessed the highest nanometer scale surface roughness among all the composites, the present results suggest that osteoblast adhesion and long-term functions may be closely related to surface roughness of titania/PLGA composites.

Nanophase titania compacts, nanocomposites, and PLGA used in this study had nanometer scale surface roughness from 20 nm to 120 nm according to $5\ \mu\text{m} \times 5\ \mu\text{m}$ AFM scans. Interestingly, PTC35 had nanometer surface roughness values closer to

natural bone compared to any other composite formulated here. The composite PTC35 with nanometer surface roughness values almost double that of PTC70, allowed for better cell adhesion, alkaline phosphatase activity and greater calcium mineral deposition. That is, when sonication power increased, titania agglomerates decreased to finer particles, which promoted titania coverage on the surface of composites and subsequently enhanced nanometer surface roughness; greater osteoblast adhesion and long-term functions on the PTC35 composite, thus, resulted. However, when the sonication power was greater than 166.25 W (PTC35), titania coverage on the composite surface did not significantly further increase. But the surface roughness measured at $5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$ AFM scans actually decreased because very fine titania particles tended to smoothen the surface of the composites; decreased osteoblast adhesion and long-term functions for the PTC70 (compared to PTC35), thus, resulted.

Cell morphology is an essential regulator for cell adhesion and proliferation. Studies have demonstrated that well spread cells divide at a higher rate than those cells with a rounded shape [205]. In the present study, more well spread osteoblasts were observed on the titania compacts than PLGA and the composites; more well spread osteoblasts were observed on PTC35 than the other composites and PLGA.

Therefore, roughness definitely had a significant positive effect on the greater osteoblast adhesion and longer-term functions since some well-dispersed titania/PLGA composites had the same surface composition in the present study.

Clearly, initial events during cell-biomaterials interactions, such as cell adhesion, affect longer-term functions (such as proliferation, synthesis of proteins and calcium mineral deposition). In the present study, enhanced synthesis of alkaline phosphatase and

deposition of calcium-containing mineral was observed on the titania/PLGA composites which increased osteoblast adhesion the most (PTC35). Thus, it is unclear at this time whether enhanced osteoblast long-term functions resulted simply from more cells adhering in the first place or whether those adherent osteoblasts differentiated at a faster rate. A much higher seeding density ($100,000 \text{ cells/cm}^2$) was used for longer-term experiments than that for adhesion (2500 cells/cm^2) which may have compensated the influence of initial osteoblast adhesion on longer-term functions; that is, the number of initial adherent cells on the surface may not make a considerable difference as time goes. Further studies will be needed to determine the exact mechanism by which the nanophase titania/PLGA composites with the highest nanometer surface roughness (PTC35) promoted osteoblast functions.

2.4.4.2. Surface Area Influences Osteoblast Functions

Another explanation for promoted osteoblast functions may be greater surface area. Previous studies have shown that, compared with larger grain size titania compacts, nanophase titania had about 35% more surface area for cell adhesion [206]. The results from this study also demonstrated that osteoblast adhesion, collagen synthesis, alkaline phosphatase synthesis, and calcium deposition were greater on the composites with higher surface area. However, when normalized to this increased surface area, osteoblast adhesion, proliferation, and deposition of calcium-containing mineral were still enhanced on nanometer compared to conventional bulk titania [206]. This indicates that increased surface area was not the only contributing factor to greater osteoblast functions on nanophase titania compacts.

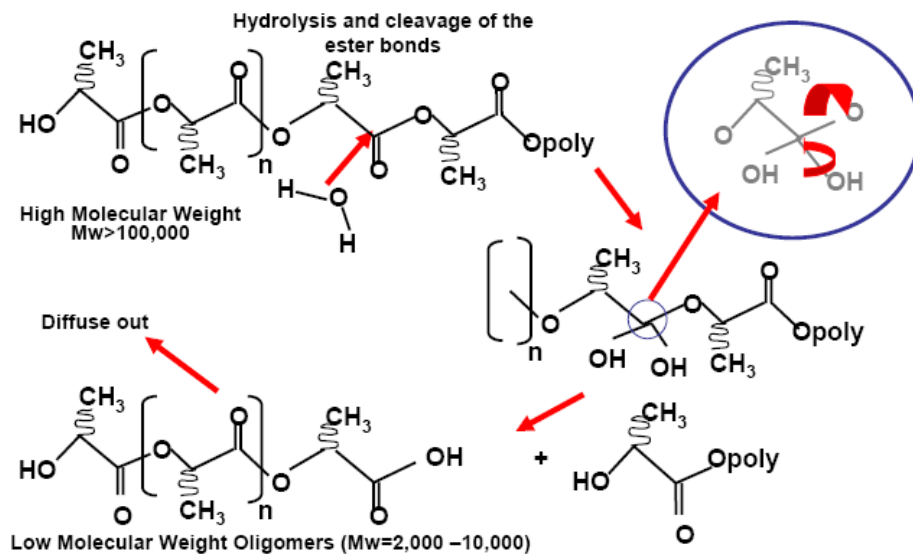
In this study, acellular calcium deposition on nanophase titania/PLGA composites and PLGA and titania controls depended on the surface area of the materials since they followed very similar trend if comparing the data shown in Table 2.3 and Figure 2.20.

2.4.5. Degradation Behavior of Nanophase Titania/PLGA Composites

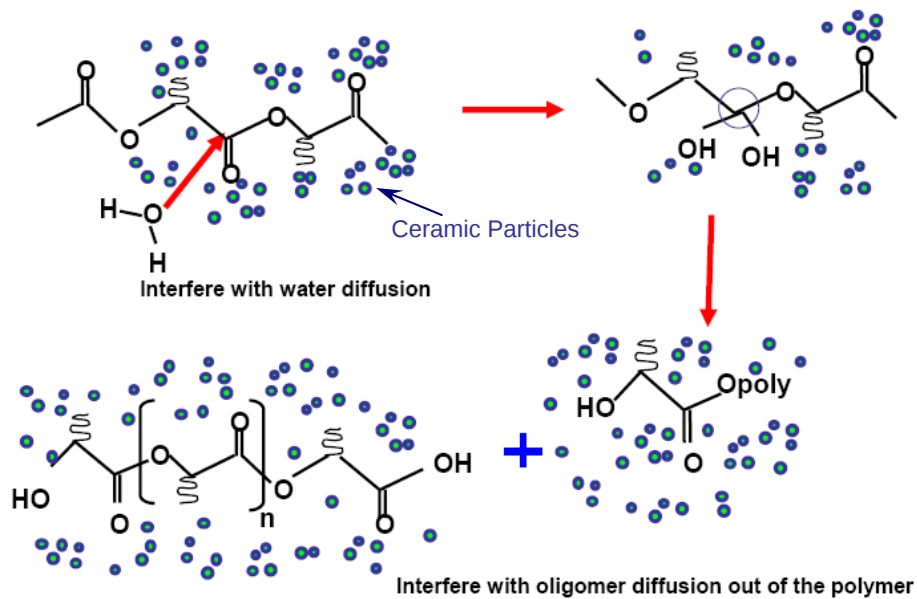
As mentioned, PLGA can degrade with random chain scission by ester hydrolysis in a process auto-catalyzed by the generation of carboxylic acid end groups, and ceramic particles used as inclusions in PLGA can tailor the degradation kinetics of the polymer matrix and prevent acceleration of polymer degradation. Zhang et al. reported that adding HA particles into PLGA (50/50 PLA/PGA) could decrease the degradation rate of PLGA and buffering pH drop during the degradation process [207]. Maquet et al. demonstrated that the degradation kinetics of PLGA (75/25 PLA/PGA) was delayed by the presence of Bioglass[®] and the molecular weight of PLGA decreased in a slower rate in the presence of Bioglass[®] [208]. However, the influence of nanophase titania and its dispersion status on the degradation kinetics of PLGA have not been completed so far. This study provided the first evidence that nanophase titania present in PLGA can improve the structural stability and mediate the degradation behavior of PLGA.

Moreover, it is important to understand the mechanisms of PLGA degradation mediated by ceramic particles. Surprisingly, this issue has not been thoroughly addressed in the literature [207,208]. In this study, therefore, how the presence of ceramic particles influences PLGA degradation in the composites was speculated and illustrated in Figure 2.25. Initially, ceramic particles interfere with the diffusion of water molecules into the polymer chains, which decrease the probability of hydrolysis and, thus, decrease the

PLGA degradation rate. At a later stage of PLGA degradation, ceramic particles interfere with the diffusion of the intermediate degradation products (oligomers) out into the surrounding media, which slow down the pH drop in the media and, thus, further decrease the PLGA degradation rate.



(a) Diagram illustrating the mechanisms of PLGA degradation.



(b) Diagram illustrating the mechanisms of PLGA degradation mediated by ceramic particles.

Figure 2. 25: Diagrams illustrating (a) the mechanisms of PLGA degradation and (b) the mechanisms how ceramic particles influence PLGA degradation.

More importantly, nanophase titania/PLGA composites demonstrated much less weight loss than pure PLGA at 21, 28, and 35 days of incubation. Specifically, the weight loss of PTC35, PTC45 and PTC70 was approximately 20% less than pure PLGA at 21 days of incubation; 30% less than pure PLGA at 28 days of incubation; and 50% less than pure PLGA at 35 days of incubation. This buffering effect of titania particles in weight loss is more significant after longer time periods of incubation, which is correlated to the pH buffering effect of titania particles. The buffering effect of titania particles on pH is significant at the later stage of the degradation, that is, after 21 days of incubation. Less acidic degradation of PLGA is less harmful to surrounding bone cells.

It is also important to note that the weight loss of PTC25 was very close to PLGA, and even no statistical significance between them was observed. This indicated that the dispersion status of nanophase titania in PLGA certainly played an important role in the degradation behavior of the nanocomposites.

2.4.6. Toxicity of Nanophase Titania/PLGA Composites

2.4.6.1 Toxicity of PLGA and Its Degradation Products

PLGA has been proven to be a successful biodegradable polymer for biomedical applications because there is very minimal systemic toxicity associated with PLGA [209,210]. For example, Basarkar et al. evaluated *in vitro* toxicity of PLGA nanoparticles (mean particles size: 740-1000 nm) in human embryonic kidney (HEK293) cells (ATCC, CRL-1573) using a commercial MTT assay. It was reported that PLGA nanoparticles were non-toxic at concentrations of 2.5-50 μg per well in 100 μL media after 24 hours of

culture and average cell viabilities were more than 90% of the control (not treated with PLGA nanoparticles). Furthermore, PLGA undergoes hydrolysis in the body to produce lactic acid and glycolic acid, which are normal by-products of various physiological metabolic pathways and are removed from the body through citric acid cycle (also known as tricarboxylic acid cycle or the Krebs cycle) [209]. In aerobic organisms, the citric acid cycle is a metabolic pathway that is involved in the chemical conversion of carbohydrates, fats and proteins into carbon dioxide and water to generate usable energy. The degradation products of PLGA are neutralized and eventually eliminated from the body with the urine. Therefore, once PLGA completely degrades, nothing foreign will be left in the body.

2.4.6.2 Toxicity of Nano-Titania Particles

Well-dispersed titania nanoparticles in the PLGA composites decreased the PLGA degradation rate, reduced the rapid pH drop induced by lactic acid and glycolic acid (intermediate degradation products of PLGA), and provided the composites with longer periods of mechanical integrity for bone regeneration. As mentioned, PLGA degradation rate could be tailored to match new bone growth rate. When new bone grows faster than PLGA degrades, titania nanoparticles could be incorporated into new bone matrix through bone mineralization process. Even if small amounts of nano-titania particles were detached from the composites due to PLGA degradation, these small amounts of nano-titania particles should not affect osteoblast viability [211,212]. Specifically, it was reported that the number of viable osteoblasts when cultured with 1000 µg/mL of nano-titania particles for 2 or 6 hours was similar to the cell cultures

without particles. Brunner et al. further confirmed that nano-titania particles were not toxic to rodent 3T3 fibroblast cells by measuring the MTT-conversion and DNA content in the cell cultures, after these cells were exposed to low concentrations (less than 30 $\mu\text{g/mL}$) of titania nanoparticles in the media for 3 days [213]. Human dermal fibroblasts and human lung epithelial cells were also used to investigate cytotoxicity of titania nanoparticles and cell inflammatory response to them [214]. Cytotoxic and inflammatory effects were not observed in the presence of relatively low concentrations (less than 100 $\mu\text{g/mL}$) of titania nanoparticles. It was reported that these cellular responses exhibited classic dose-response behavior and the effects increased with time of exposure [214]. Moreover, it was suggested that cytotoxicity of nano-titania particles could be further reduced by decreasing their ability to generate reactive oxygen species (ROS) [214]. Titania nanoparticle surfaces are prone to dissociative adsorption of water and transformation of chemisorbed water into $\text{OH}^\bullet$ radicals under illumination. Such radicals are reactive and are capable of generating ROS and oxidizing biological species [214]. In the absence of light, however, titania nanoparticles did not provoke an appreciable ROS level [215]. Therefore, toxicity of titania nanoparticles is minimal in the body due to the dark environment.

2.5. Conclusions

The results from this *in vitro* study demonstrated that nanophase titania has exceptional cytocompatibility with osteoblasts. Specifically, osteoblast adhesion and long-term functions on nanophase titania compacts were greater than on any nanophase

titania/PLGA composite. Moreover, PLGA when combined with nanophase titania allowed for better osteoblast adhesion, collagen synthesis, alkaline phosphatase activity, and calcium mineral deposition than what occurred for pure PLGA. It demonstrated for the first time that the nanophase titania/PLGA composites with the closest surface roughness to natural bone at the nanoscale (provided by well-dispersed titania nanoparticles in PLGA) promoted osteoblast adhesion and calcium deposition the most. Among the composites, when considering all data together, PTC35, enhanced osteoblast functions the most.

Nanophase titania in PLGA composites also provided better degradation kinetics which favors cell survival and enhanced functions.

In conclusion, this study suggests that nanophase titania/PLGA composites with proper dispersion status have excellent cytocompatibility properties crucial in designing better orthopedic materials for bone regeneration. In order to take full advantage of the nanophase titania/PLGA composites, however, adjusting dispersion of titania and mimicking the surface properties of natural bone (such as surface roughness) are key considerations.

CHAPTER 3. OSTEOBLAST INTERACTIONS WITH NANOSTRUCTURED 3D CERAMIC/POLYMER COMPOSITES

3.1. Scientific Challenges and Specific Aims

Chapter 2 demonstrated that nanoscale surface features provided by well-dispersed nanophase titania in PLGA composites promoted osteoblast adhesion and long-term functions (such as alkaline phosphatase activity and calcium-containing mineral deposition). However, to date, relatively few advantages of the macro assembly of nanocomposites composed of nanophase ceramics and degradable polymers have been incorporated into the orthopedic clinical arena due to the limited availability and flexibility of traditional 3D fabrication techniques for nanocomposites. The challenge lies in how to integrate nanoscale structures or components in a cost-effective, scalable and repeatable way into macro architectures while preserving their nano-features.

A successful synthetic orthopedic prosthesis requires a hierarchical internal structure with interconnecting pores for nutrition transportation, cell infiltration and vascularization as well as nanoscale surface features favorable for cell attachment and long-term functions. This Chapter, therefore, focuses on further mimicking bone by building 3D structures from titania/PLGA nanocomposites because, similarly, natural bone assembles its 3D hierarchical architecture from nanostructured building blocks. The objective of this study was to test the effectiveness of a novel aerosol-based 3D printing

technique for nanocomposite fabrication and *in vitro* cytocompatibility (such as osteoblast adhesion and infiltration into these 3D printed nanocomposite scaffolds).

3.1.1. Problems of Current 3D Fabrication Techniques

Solvent-casting/porogen-leaching (SC/PL) techniques have been widely used to fabricate 3D porous polymer scaffolds for tissue engineering applications. Salt is the most commonly used porogen because it is easily available and very easy to handle. Briefly, this technique involves producing a suspension of polymers or ceramic/polymer composites in a solvent. Salt particles are ground and sieved into small particles and those of the desired size (most researchers use 100-200 μm range particles) are transferred into a mold. A polymer or composite suspension is then cast into the salt-filled mold. The solvent is then removed by evaporation in air and/or in vacuum. After the evaporation of the solvent, the salt crystals are leached away by immersion in water to form a porous structure. In this technique, the pore size can be controlled by the size of the porogen particles and the porosity can be controlled by the amount of porogen added into the polymer or composite suspension. However, solvent-casting/porogen-leaching techniques have two main disadvantages. First, certain critical variables such as pore shape and inter-pore openings are still not well controlled in this technique. Second, if nanophase ceramic particles were used to make nanocomposite scaffolds in this technique, nanoparticles may interfere with the porogen leaching process, which will result in residual porogen particles in the final tissue engineering products, and, thus, have adverse effects on their cytocompatibility.

Another technique, called phase separation and emulsion freeze drying, has been developed based on the thermodynamic principle for the fabrication of 3D porous polymer scaffolds [216-217]. This technique involves liquid-liquid phase separation and solid-liquid phase separation. Liquid-liquid phase separation was mainly used for preparing polymer scaffolds. Solid-liquid phase separation, also called emulsion freeze drying, could be applied to both polymers and composites. Briefly, this technique could be achieved by lowering the temperature to induce solvent crystallization from a polymer or composite suspension (solid phase formation in a liquid phase). After the removal of the solvent crystals (sublimation or solvent exchange), the space originally taken by the solvent crystals becomes pores. For example, Liu et al. used this technique to prepare collagen/hydroxyapatite composite scaffolds [218]. Specifically, hydroxyapatite powder was added into a collagen solution, and homogenized by a speed stirrer. The mixture was then poured onto petri dishes, and rapidly transferred into a refrigerator at -30 °C to solidify the mixture and induce solid-liquid phase separation. The solidified mixture was maintained at that temperature for 2 hours, and then lyophilized for 2 days. The final collagen/hydroxyapatite scaffolds were porous with three-dimensional interconnected fiber microstructure and demonstrated an uneven pore size from 50 to 150 μm . Although this technique is advantageous as it does not require a porogen and an extra washing/leaching step, the phase diagrams of the polymer-solvent or composite-solvent systems must be fully characterized which would significantly increase the difficulties in controlling the process especially when composites are involved. Moreover, the pores formed using phase separation techniques usually have irregular shapes, have diameters

on the order of a few to tens or hundreds of microns and are often not uniformly distributed.

One of the common shortcomings of these traditional fabrication technologies (such as solvent-casting/porogen-leaching and phase separation) is the lack of precise control of the 3D internal and external nano-architecture of the final products especially when a second phase (ceramic nanoparticles) is involved [219,220]. This is not desirable since osteoblasts prefer well-ordered structures rather than random structures [221-224]. Moreover, these traditional techniques can not produce scalable consistent results for clinical applications.

3.1.2. Nanofabrication: A Novel Aerosol-Based 3D Printing

A novel aerosol-based 3D printing technique (Maskless Mesoscale Materials DepositionTM or M³DTM system) developed by OPTOMECH[®] provides a great promise in breaking through these difficulties associated with traditional fabrication techniques. Here it is proposed to use the M³DTM system to build desired 3D bone-like macro structures to take full advantage of nanocomposites for orthopedic applications (Figure 3.1). One direct and simple reason is that natural bone, similarly, assembles its 3D macro hierarchical structures from nanostructured building blocks (nano-HA and collagen) (Figure 1.11.).

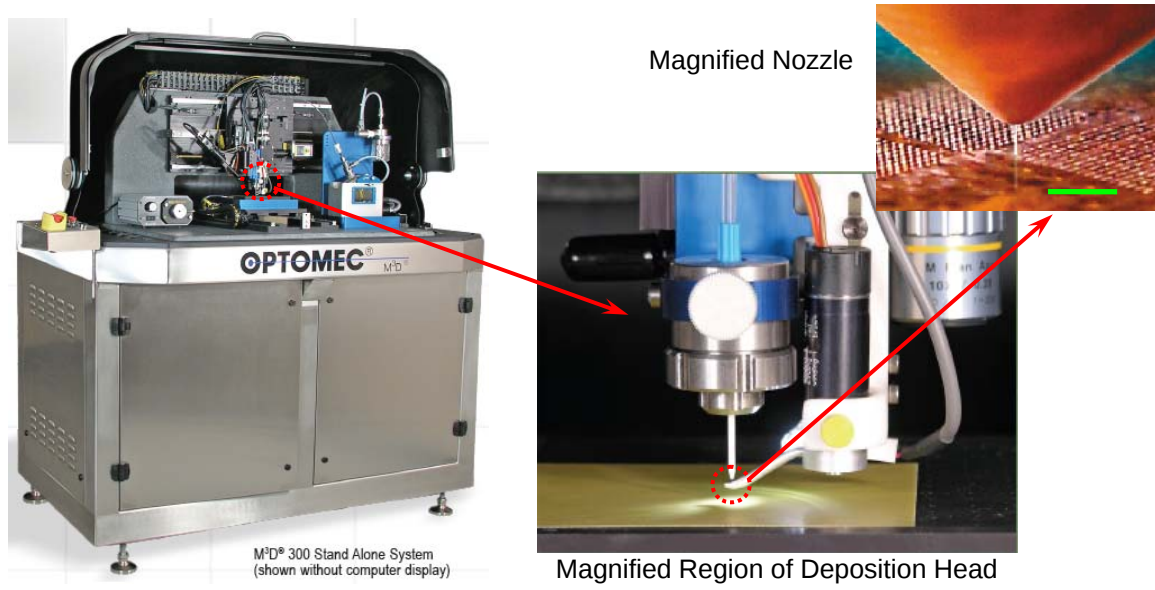


Figure 3. 1: Illustration of the M³DTM system developed by OPTOMECH[®]. Left is the M³DTM system. Right is a close up of the deposition head and nozzle used to deposit nanophase ceramic/polymer composites in a controlled manner. Bar=100 μm. (Adapted and redrawn from [225]).

The M³DTM system offers great promise towards its applications in the next generation of nanocomposite orthopedic implant systems due to many more scientific reasons, especially when considering the following benefits offered by its special features. First, the M³DTM system is capable of producing complex-shaped scaffolds with well-controlled 3D architectures and internal pore structures layer-by-layer from pre-designed CAD models. This technique makes it possible to directly assemble bone substitutes with a variety of shapes and sizes to match bone defects in the patients based on their medical information from computer-aided tomography (CT) and/or magnetic resonance imaging (MRI) (that can be translated into CAD models). Second, the M³DTM system can deposit a wide variety of materials, including any materials that can be suspended in liquid (metals, ceramics, polymers, composites and biological materials), on virtually any 2D planar surfaces or 3D non-planar substrates. The ability to deposit materials on a 3D non-

planar substrate is made possible by the relatively high (more than 5 mm) standoff point of the deposition head and long focal length of the material beam exiting the nozzle. There is no physical contact between the nozzle and the substrate and therefore conformal writing can be achieved. Third, the M³DTM system follows an additive (bottom-up) manufacture that does not need tooling, masks or any porogens, thus, offering a cost-effective high resolution deposition in contrast to traditional subtractive (top-down) methods. Fourth, the M³DTM system provides a scalable and repeatable 3D nanofabrication technique that has the ability to deposit materials at speeds up to 1 mm³/s with a feature resolution down to 10 µm in line width and 50-100 nm in thickness (single layer deposition) [226]. The M³DTM system is also capable of depositing a single layer as high as 5 microns for a larger scale of applications [226]. Fifth, the M³DTM system offers low temperature processing [227], which is particularly beneficial for fabricating nanomaterials because it helps prevent grain growth typically induced by high temperature sintering.

The major challenges of using this 3D printing technique lie on designing CAD (computer-aided design) models for bone-like structures, dispersion of nanophase ceramics in the polymer solutions and controlling rheological properties of nanocomposite suspensions for optimal aerosolization. Sonication at controlled powers is necessary to disperse nanophase ceramics in polymer composites. Various ceramic/polymer/solvent ratios have to be manipulated to obtain stable suspensions, critical for maximizing 3D printing efficiency. Many factors (size and shape of nanoparticles, dispersion/agglomeration of nanoparticles, volume fraction, steric repulsion, Brownian motion, electrostatic forces, hydrodynamic forces) affect stability of

suspensions. Measuring the rheology of a suspension offers an indication of the colloidal state, which is important for determining its processing behavior for 3D printing. A rheometer can be used to measure viscosity as a function of shear rate to determine non-Newtonian flow behavior, thus, determining dispersion/agglomeration as well as shear thinning or thickening of the nanocomposite suspensions of interest.

3.2. Materials and Methods

3.2.1. Preparation of 3D Nanophase Titania/PLGA Scaffolds

PLGA (poly-lactide-co-glycolide) pellets (50/50 wt.% poly(DL-lactide/glycolide); molecular weight: 100,000-120,000 g/mol) were purchased from Polysciences, Inc. (Warrington, PA). Nanophase titania powder (Nanotek[®]) was purchased from Nanophase Technologies Corporation (Romeoville, IL). The purity of the titania powder was 99.5+%, the particle size was 32 nm which was calculated from BET adsorption measurements, the particle morphology was nearly spherical as shown in the TEM image (Figure 2.1), and the crystalline phase was 80% anatase/20% rutile [175].

PLGA pellets were dissolved in chloroform (Sigma-Aldrich) at 40 °C in a water bath for 40 minutes. Nanophase titania was then well dispersed in PLGA solutions by controlled sonication using a S-250D Branson[®] Digital Sonifier (Branson, Inc., Danbury, CT) with its tip immersed in the mixture. This sonifier permits the application of ultrasonic energy to the suspensions on a pulsed basis. In this study, the intensity was set at 400 W and the pulse width was set as 60% of the duty cycle out of 1 second cycle time. This intermittent operation permits high intensity sonication while avoiding heat build-up

in the processed suspensions. The weight ratio of nano-titania/PLGA in the composites was 30/70.

As a first attempt to further mimick bone in its 3D architecture, a novel aerosol-based 3D printing technique was used to build 3D structures from titania/PLGA nanocomposites. The M³DTM system was developed by OPTOMECH[®]. This technique uses aerodynamic focusing of aerosol streams for the high-resolution deposition of chemical precursor solutions or colloidal suspensions. The M³DTM system consists of 3 basic modules, as shown in Figure 3.2. (i) An aerosol (mist) generation module for atomizing material suspensions. A dense aerosol of tiny droplets is generated using an ultrasonic transducer (for suspensions with a viscosity of less than 10 cP) or a pneumatic atomizer (for suspensions with a viscosity of 10-1000 cP) [228]. (ii) A flow guidance module for carrying and focusing the aerosol. An annular and co-axial flow of the aerosol stream is carried by a gas flow to the deposition head and focused by a second gas sheath in the deposition head through a nozzle towards the deposition platform. The M³DTM flow guidance head is capable of focusing an aerosol stream to as small as a tenth of the size of the nozzle orifice for higher resolution structures. (iii) A CAD module (in-flight processing) for controlling the pattern of the aerosol droplets. The deposition is driven by a CAD model that is pre-written into a standard .DFX file. Patterning is accomplished by a computer-driven deposition platform or by translating the flow guidance head while the deposition platform remains fixed.

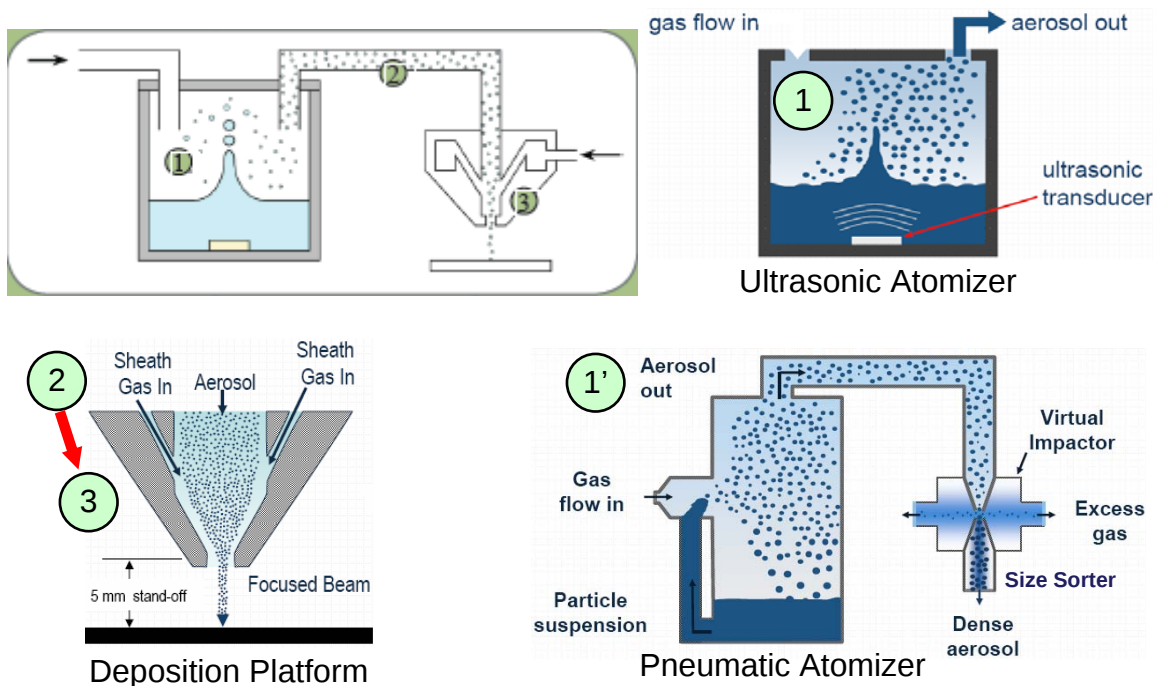


Figure 3. 2: Diagram illustrating the basic principles of the aerosol-based 3D printing. (1) The well-dispersed nanocomposite suspensions are aerosolized in an atomizer (ultrasonic or pneumatic) to create a dense aerosol of tiny droplets. (2) The aerosol is carried by a gas to the deposition head. (3) The aerosol is focused by a second gas sheath in the deposition head and “sprayed” onto the deposition platform layer by layer. (Adapted and redrawn from [225]).

The suspension of well dispersed nano-titania in PLGA composites was aerosolized in the M³D™ ultrasonic atomizer to create a dense aerosol of tiny droplets; the aerosol was carried by a gas to the deposition head and focused by a second gas flow within the deposition head; and finally the resulting high velocity stream was “sprayed” onto the substrate layer by layer according to pre-designed CAD (computer-aided design) models (Figure 3.2). The final 3D nanocomposite scaffolds were 1 cm × 1 cm squares with a thickness of 0.5 mm.

The 3D printed nanocomposite scaffolds were dried in air at room temperature for 24 hours and dried in an air vacuum chamber at room temperature for 48 hours. These 3D

composites were sterilized by soaking in 70% ethanol for 30 minutes and were dried completely before performing experiments with cells.

3.2.2. Characterization of 3D Nanophase Titania/PLGA Scaffolds

Field emission scanning electron microscopy (FESEM) was used to characterize the nano-to-micron structure and surface features of these 3D scaffolds. Surface topographies and 3D structures of the 3D printed nanophase titania/PLGA composites were characterized according to standard scanning electron microscopy techniques using a LEO 1530 Field Emission Scanning Electron Microscope (FESEM) at a 5 kV accelerating voltage and 3×10^{-11} Amp probe current.. Substrates were sputter-coated with a thin layer of gold-palladium using a Hummer I Sputter Coater (Technics) in a 100 millitorr vacuum argon environment for 3 minutes with 0.01 Amp of current. The areal analysis technique was used to quantitatively measure the pore size and porosity. SEM images taken at 60 kX magnifications were used to determine nanophase titania dispersion in PLGA.

3.2.3. *In Vitro* Osteoblast Interactions with 3D Nanophase Titania/PLGA Scaffolds

Human osteoblasts (bone-forming cells; CRL-11372 American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin/streptomycin (P/S; Hyclone) under standard cell culture conditions, that is, a

sterile, 37 °C, humidified, 5% CO₂/95% air environment. Cells at passage numbers 5-6 were used in the experiments.

All sterilized scaffolds were placed in tissue culture plates (Corning, New York) and were rinsed three times with sterilized phosphate buffered saline (PBS). Osteoblasts were seeded at a concentration of 3500 cells/cm² onto the substrates of interest in 2 ml DMEM supplemented with 10% FBS and 1% P/S and were then incubated under standard cell culture conditions for 4 hours. After that time period, non-adherent cells were removed by rinsing with PBS and adherent cells were then stained with DAPI nucleic acid stain (Invitrogen).

Confocal laser scanning microscopy was used to evaluate osteoblast attachment on the surface and infiltration into the porous structures. The cell nuclei were visualized and counted under a Leica TCS SP2 AOBS spectral confocal microscope (excitation wavelength 358 nm and emission wavelength 461 nm). Two detection channels were used in this study: one was for imaging fluorescence from stained cells and another one was for collecting bright field images of scaffolds. Leica's confocal software (LCS version 2.5) was used for 3D-scanning image acquisition and 3D reconstruction. Cell counts were expressed as the number of cells adherent around the pores and adherent on the surfaces away from the pores by averaging twenty fields of view. All experiments were run in triplicate. Numerical data were analyzed using Student *t* test; statistical significance was considered at $p < 0.05$.

Osteoblasts morphologies on the composite scaffolds were observed using a LEO 1530 FESEM. For this purpose, after the 4 hour adhesion test, adherent osteoblasts on the substrates were fixed with 2 % glutaraldehyde (Electron Microscopy Sciences) in 0.1 M

cacodylate (pH 7.4; Electron Microscopy Sciences) for 30 minutes at 4 °C. After washing with cacodylate buffer, the cells were secondarily fixed with 1 % osmium tetroxide (Electron Microscopy Sciences) in 0.1 M cacodylate (pH 7.4) for 30 minutes at 4 °C. The cells were dehydrated through a series of ethanol solutions (from 30, 50, 70, 90, to 100 %; AAPER) and were then critical point dried (CPD; LADD Research Industries). All the specimens were sputter-coated with a thin layer of gold-palladium using a Hummer I Sputter Coater (Technics) in a 100 millitorr vacuum argon environment for 3 minutes with 0.01 Amp of current.

3.3. Results and Discussions

3.3.1. Well-Ordered 3D Nanophase Titania/PLGA Scaffolds

The 3D printed nanophase titania/PLGA composite scaffolds had well-ordered 3D structures as designed (Figure 3.3a). The SEM results demonstrated that the printed nano 3D scaffolds have a well-controlled, repeatable inner structure and, moreover, possessed uniformly dispersed titania nanoparticles which provided for nanoscale surface features throughout the PLGA matrix. The pores had a cubic shape and pore sizes were controlled at 100 μm . The porosity was 32% according to the areal analysis. As mentioned, the pore size, shape and percentage can be precisely controlled by pre-designed CAD models using this aerosol based 3D printing technique.

The architecture of these 3D scaffolds was significantly different from that formed from porogen leaching or phase separation techniques which produce randomly packed pores. Clearly, the uncontrolled, random pore structures cause a lack of

predictable biological properties and mechanical properties. The advantage of this interconnected, ordered pore network created in this study is that it provides a regulated pathway of suitable dimensions for nutrient and waste transportation as well as vascularization throughout the scaffolds. Moreover, the surfaces of such nanocomposite scaffolds demonstrated uniform dispersion of titania nanoparticles after 3D printing (Figure 3.3b). It was previously reported in Chapter 2 that well dispersed titania nanoparticles in PLGA promoted initial osteoblast adhesion and long-term functions such as calcium deposition.

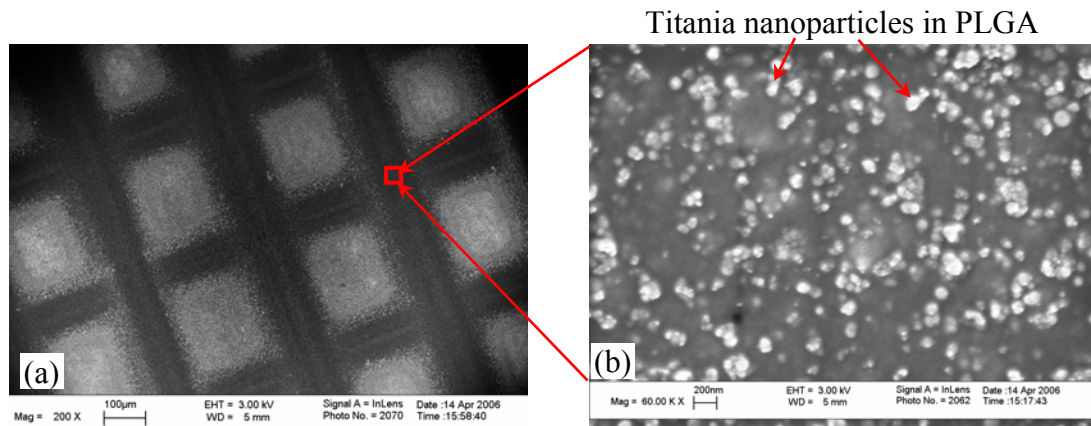


Figure 3. 3: SEM micrograph of (a) 3D nanocomposite scaffolds, Bar=100 µm; (b) a magnified region of the 3D nanocomposite surface. Bar=200 nm.

3.3.2. Increased Osteoblast Interactions with 3D Printed Nanocomposites

The *in vitro* osteoblast adhesion results demonstrated that these 3D scaffolds further promoted osteoblast infiltration into porous structures compared to previous nanostructured surfaces. The SEM image in Figure 3.4a shows a well-spread osteoblast attached on the nanocomposite surface. The confocal image in Figure 3.4b shows

enhanced osteoblast adhesion on pore structures of such 3D printed nanocomposite scaffolds. The results demonstrated that osteoblasts preferred the nanoscale roughness of the pore walls over that of the outer surface of the 3D nano-scaffolds. Thus, this study suggests that these novel 3D nano-scaffolds consisting of nanophase titania/PLGA composites created via the 3D printing technique are very promising for more effective orthopedic tissue engineering applications.

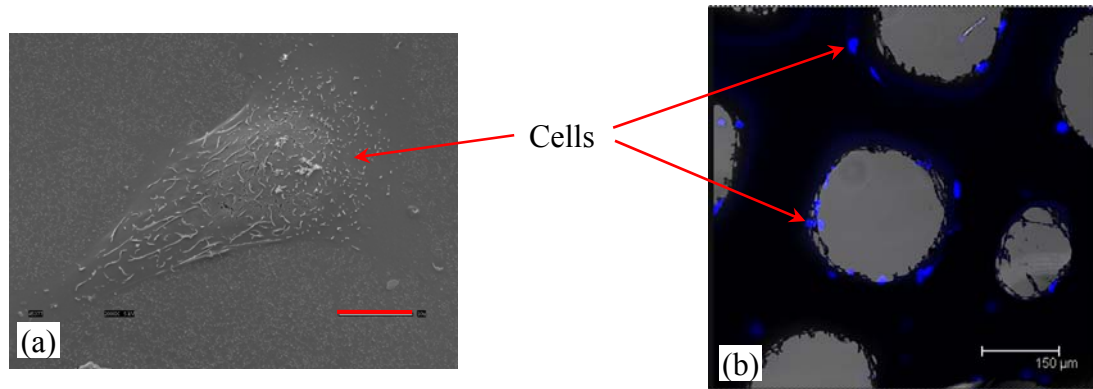


Figure 3. 4: (a) SEM micrograph of an osteoblast adhering on the nanocomposite surface, Bar=10 μm . (b) Confocal micrograph of osteoblasts adhering around pore structures of 3D printed nanocomposite scaffolds. Bar=150 μm .

Quantitative results demonstrated that osteoblast infiltration onto the pore structures was 4.2 times greater than osteoblast adhesion onto the rest of scaffold surfaces, as shown in Figure 3.5. Increased osteoblast infiltration into 3D porous structures is a crucial prerequisite for enhancing subsequent new bone ingrowth.

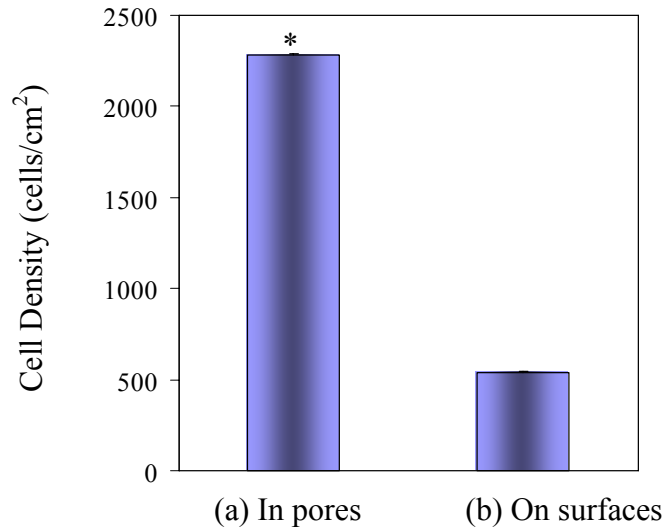


Figure 3. 5: (a) The average number of osteoblasts adherent to pore structures. (b) The average number of osteoblasts adherent to the surfaces away from pores. Values are mean $\pm$ SD; $n = 3$; * $p < 0.05$ compared to (b).

3.4. Conclusions

Results of this study have evaluated a means of fabricating a hierarchical macro-structure from ceramic/polymer nanocomposites that can mimic properties of natural bone, thus, providing a new material and approach for more effective orthopedic applications. The aerosol-based 3D printing technique produced well-ordered bone-like structures and preserved nano-dispersion of ceramic in polymer composites. The 3D printed nanocomposites promoted bone cell infiltrations and subsequent bone ingrowth.

Considering these exciting results, future work is needed to focus on understanding cell interactions with various nanostructured 3D patterns and determining the mechanisms for improved osteoblast functions and infiltration on these 3D nanocomposite scaffolds. The mechanisms of cell-material interactions can be

determined by selective and competitive protein adsorption. This will provide a fundamental mechanism explaining protein mediated cellular behavior on the 3D printed nanocomposites.

CHAPTER 4. MECHANICAL PROPERTIES OF NANOPHASE CERAMIC/POLYMER COMPOSITES

4.1. Problems and Specific Aims

Previous chapters demonstrated that well-dispersed nano-particulate titania in poly-lactide-co-glycolide (PLGA) composites promoted osteoblast (bone-forming cell) adhesion and long-term functions (such as collagen synthesis and calcium-containing mineral deposition) compared to pure PLGA and more agglomerated titania in PLGA composites. The controlled dispersion of titania nanoparticles in PLGA also furthered decreased the weight loss of bone scaffolds, reduced harmful acidic pH changes during PLGA degradation, and prolonged the mechanical integrity of the scaffolds. It is intriguing and necessary to examine mechanical properties of such nanocomposites for orthopedic applications.

Mismatches in the mechanical properties of metallic implants and physiological bone result in stress-shielding problems [162]. Metallic materials widely used in orthopedic applications have much stronger mechanical properties (such as elastic modulus) than natural bone, which can weaken the newly formed bone interface due to stress-shielding. Because natural bone is under continuous physiological stresses (such as compression, tension, torsion, and/or bending), the mechanical properties of orthopedic implant materials should closely match those of living bone. This is necessary to

minimize stress and strain imbalances during physiological loading conditions which will lead to implant failure.

The objective of the present study, therefore, was to characterize the mechanical properties of PLGA with well-dispersed nanophase titania. The dispersion of titania in PLGA was controlled by sonication and was characterized by field emission scanning electron microscopy and image analysis techniques. For this purpose, two major stresses (compression and tension) that natural bone experiences under physiological loading conditions were characterized using an Instron Material Testing System.

4.2. Materials and Methods

4.2.1. Material Preparation for Mechanical Tests

4.2.1.1. Specimens for Tensile and Compressive Tests

4.2.1.1.1. Nanophase Titania/PLGA Composites for Mechanical Tests

PLGA (50/50 wt.% poly(dl-lactide/glycolide); Polysciences) was dissolved in an organic solvent and titania nanoparticles (Nanophase Technologies) were added into the PLGA solution to provide a 70/30 polymer/ceramic weight ratio. The composite mixture was then processed using a Misonix 3000 sonicator (Misonix, Inc.) with its microtip immersed in the mixture. After sonication, the composite suspension was cast into a Teflon mold that was specially designed for dog-bone shaped tensile specimens, evaporated in air at room temperature for 24 hours and dried in an air vacuum chamber at room temperature for 48 hours. The design of the casting mold for tensile specimens will be described in the later section 4.2.1.2. The dispersion status of final composite scaffolds

was controlled by sonication settings. These PLGA/titania composites (PTC) were termed as PTCa (a=agglomerated) and PTCd (d=dispersed) according to their titania dispersion states. PLGA was used as a control and was prepared by the solvent-casting technique described above except that no ceramics were added. These mold-cast tensile specimens had the same dimension (Figure 4.1). The gage length was 25 mm; the gage width was 10 mm; and the thickness was 0.5 mm. Similar procedures were used to prepare the specimens for compressive tests except that the casting mold was for compressive specimens with a circular shape. The gage diameter of compressive specimens was 10 mm and the thickness was 0.5 mm.

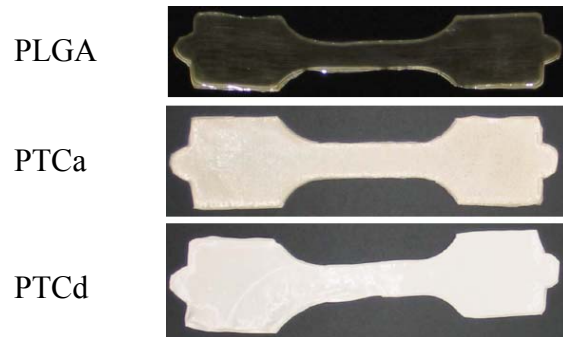
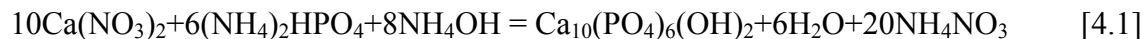


Figure 4. 1: The tensile specimens of PLGA, PTCa and PTCd. The gage length x width x thickness = 25 x 10 x 0.5 mm.

4.2.1.1.2. Nanophase HA/PLGA Composites for Mechanical Tests

Nanophase HA was synthesized using a wet chemistry precipitation method by mixing solutions of calcium nitrate and ammonium phosphate in an alkaline pH region [229]. Specifically, a 1 M calcium nitrate solution and a 0.6 M ammonium phosphate solution were prepared by dissolving their respective solid state powders in deionized (DI) water separately. The produced ammonium phosphate solution was mixed with DI water

which was adjusted to pH 10 by ammonium hydride. The pre-made 1 M calcium nitrate solution was then added into the mixture of ammonium phosphate and ammonium hydride at a rate of 3.6 ml/min. Precipitation occurred as soon as the calcium nitrate was added. Chemically, the HA precipitation occurred through the reaction [4.1]:



Precipitation continued for 10 minutes at room temperature with constant stirring. The supernatant was collected, centrifuged (Eppendorf centrifuge, Model 5810 R) to reduce 75% of the solution volume and placed into a 125 ml Teflon liner (Parr Instrument). The Teflon liner was sealed tightly in a Parr acid digestion bomb (Parr Instrument) and treated hydrothermally at 200 °C for 20 hours to obtain nanocrystalline HA. The hydrothermal treatment demonstrated a great advantage to prepare a stoichiometric, ultrafine HA powder with a homogeneous shape and size distribution due to higher applied pressures than atmospheric [230,231]. After the hydrothermal treatment, nano-HA particles were rinsed with DI water and dried in an oven at 80 °C for 12 hours.

PLGA (50/50 wt.% poly(dl-lactide/glycolide); Polysciences) was dissolved in an organic solvent and synthesized HA nanoparticles were added into the PLGA solution to provide a 30/70 ceramic/polymer weight ratio. The composite mixture was then processed using a Misonix 3000 sonicator (Misonix, Inc.) with its microtip immersed in the mixture. After sonication, the composite suspension was cast into a Teflon mold that was specially designed for dog-bone shaped tensile specimens, evaporated in air at room temperature for 24 hours and dried in an air vacuum chamber at room temperature for 48 hours. The design of the casting mold for tensile specimens will be described in the later

section 4.2.1.2. The dispersion status of final composite scaffolds was controlled by sonication settings. These HA/PLGA composites (PHA) were termed as PHAa (a=agglomerated) and PHAd (d=dispersed) according to their HA dispersion states. PLGA was used as a control and was prepared by the solvent-casting technique described above except that no ceramics were added. These mold-cast tensile specimens had the same dimension (Figure 4.2). The gage length was 25 mm; the gage width was 10 mm; and the thickness was 0.5 mm. Similar procedures were used to prepare the specimens for compressive tests except that the casting mold was for compressive specimens with a circular shape. The gage diameter of compressive specimens was 10 mm and the thickness was 0.5 mm.

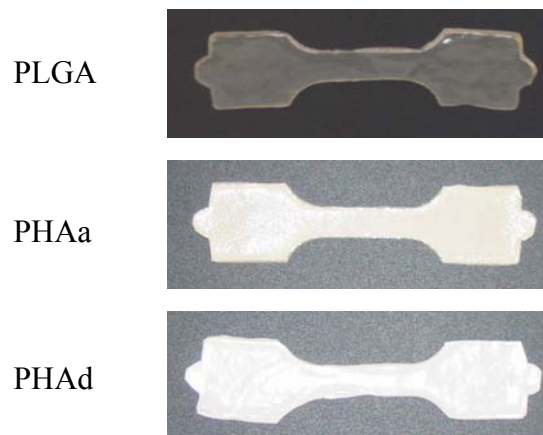


Figure 4. 2: The tensile specimens of PLGA, PHAa and PHAd. The gage length x width x thickness = 25 x 10 x 0.5 mm.

4.2.1.2. Design of Casting Molds for Tensile Specimens

The casting molds for tensile specimens were designed based on ASTM (American Society for Testing and Materials) standards D638, D882, D3039, and ISO

(International Organization for Standardization) standard 37 [232-237]. Figure 4.3 shows an example of casting molds for preparing tensile specimens.

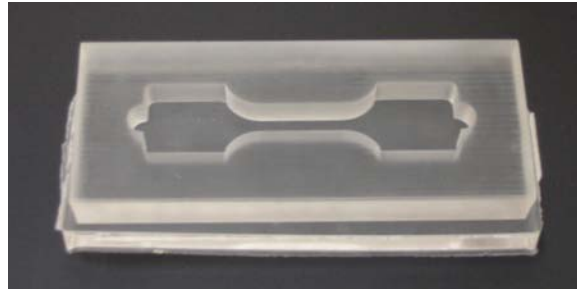


Figure 4. 3: The casting mold for tensile specimens. The gage length was designed as 25 mm; the gage width was designed as 10 mm; and the depth was designed as 10 mm.

4.2.3. Characterization of Materials Before Mechanical Tests

Surface properties of the titania/PLGA nanocomposites and HA/PLGA nanocomposites were characterized before mechanical tests using a Field Emission Scanning Electron Microscope (FESEM, LEO 1530) at a 3 kV accelerating voltage. The nanocomposites and PLGA were sputter-coated with a thin layer of gold-palladium, using a Hummer I Sputter Coater (Technics) in a 100 mTorr vacuum argon environment for 3 min at 10 mA of current.

4.2.4. Mechanical Tests: Tensile and Compressive Tests

All composites and PLGA were subjected to tensile and compressive tests using an Instron 5882 mechanical testing system (Figure 4.4). A 50 kN load cell was used to

measure the load. All samples were pulled at a constant crosshead speed until failure. The extension and compression rate were set at 10mm/min. Load/displacement curves were obtained using the LabTech software program. Data points and specimen dimensions were transferred to a spreadsheet for conversion to stress versus strain points. Figure 4.4 provides an example of such a tensile test. The load and displacement data were collected and translated to stress and strain. The tensile and compressive moduli were calculated from the stress-strain curves.



PTCd

Figure 4. 4: The experimental setup for tensile tests. The specimen was PTCd (well-dispersed titania in PLGA composites).

4.2.5. Fracture Analysis After Tensile Tests

Fracture surfaces and cross-sections of the titania/PLGA nanocomposites, HA/PLGA nanocomposites and PLGA were characterized after tensile tests using a Field Emission Scanning Electron Microscope (FESEM, LEO 1530) at a 3 kV accelerating voltage. For observing fracture cross-sections, specimens were mounted on specially

designed 45°/90° holders. The nanocomposites and PLGA were sputter-coated with a thin layer of gold-palladium, using a Hummer I Sputter Coater (Technics) in a 100 mTorr vacuum argon environment for 3 min with 10 mA of current.

4.2.6. Statistical Analysis

All mechanical tests were repeated three times (3 specimens each time) for each type of specimens. Numerical data were analyzed using standard analysis of variance (ANOVA) techniques and standard pair-wised comparison tests; statistical significance was considered at $p < 0.05$.

4.3. Results

4.3.1. Material Characterization Before Mechanical Tests

4.3.1.1. Nanophase Titania/PLGA Composites Before Mechanical Tests

Scanning electron micrographs suggest that the distribution of nano-titania particles was much different in the PTCa and PTCd samples although both of them had the same weight percentage of titania (that is, 30 wt.%) in PLGA, as shown in Figure 4.5. Specifically, there were less titania particles on the top surface of PTCa than PTCd because the agglomerates larger than 100 nm descended faster than the solvent evaporation rate according to the established Stoke's Equation. The amount of surface area occupied by titania increased on the top surface of PTCd (10.1%, Figure 4.5c) compared to PTCa (5.7%, Figure 4.5a) because the solvent evaporation was much faster

than the sedimentation of well-dispersed titania particles less than 100 nm. Moreover, for the PTCa, the top surface was much different from the bottom surface, which indicates the difference in the distribution of nano-titania agglomerates. More agglomerates concentrated on the bottom side of PTCa. For the PTCd, however, there was no significant difference between its top and bottom surfaces.

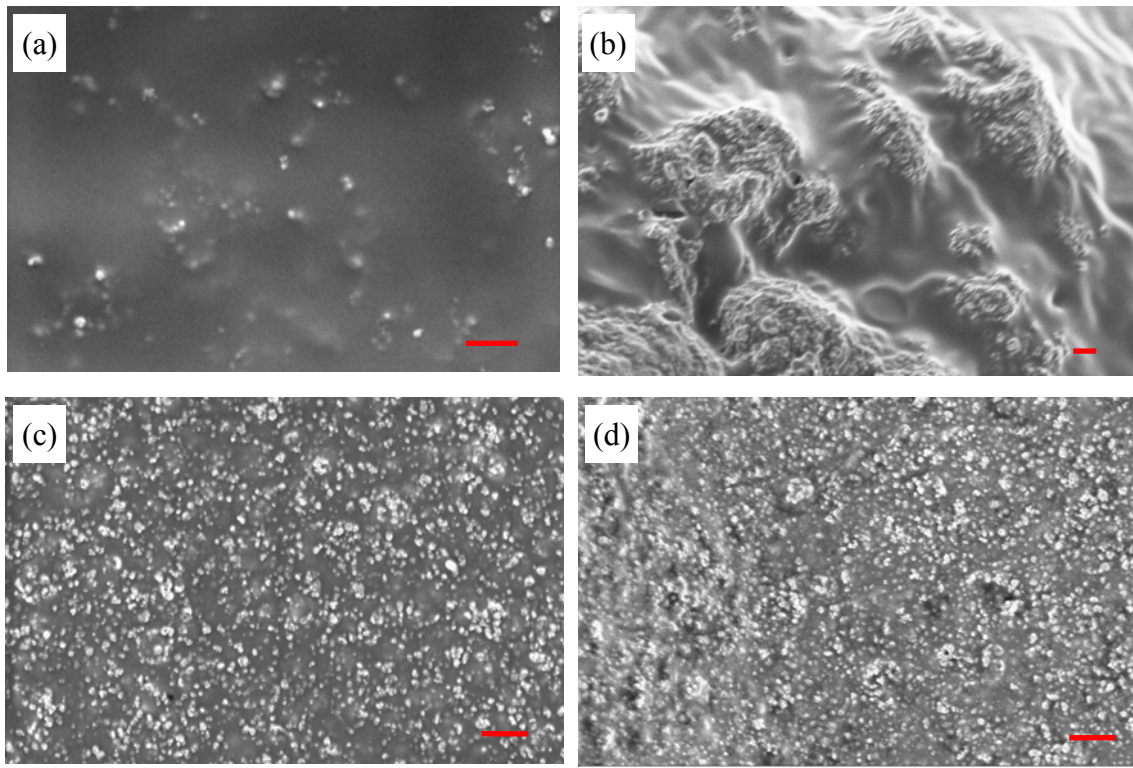


Figure 4. 5: SEM micrographs of nanophase titania/PLGA composites: (a) the top surface of PTCa, (b) the bottom surface of PTCa, (c) the top surface of PTCd, and (d) the bottom surface of PTCd. Magnification bars: 1 μm .

4.3.1.2. Nanophase HA/PLGA Composites Before Mechanical Tests

Nanophase HA synthesized by the wet chemistry method demonstrated a relative uniform particle size, as shown in Figure 4.6. The linear image analysis results demonstrated that the nano-HA had an average particle size of 36 nm.

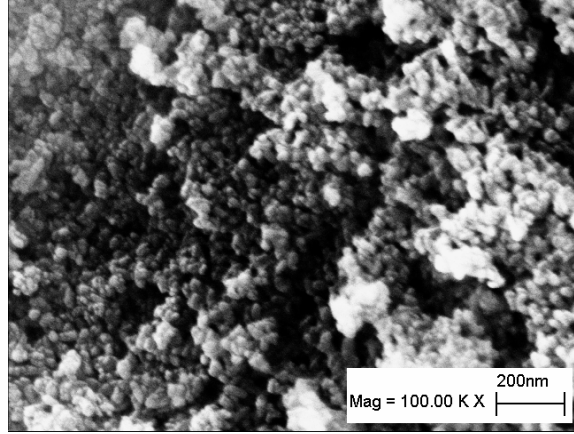


Figure 4. 6: SEM micrographs of particulate HA synthesized by the wet chemistry method. Original magnification is 100 kX, scale bar is 200 nm.

Scanning electron micrographs suggest that the distribution of nano-HA particles was much different in the PHAa and PHAd samples although both of them had the same weight percentage of HA (that is, 30 wt.%) in PLGA, as shown in Figure 4.7. Specifically, there were less HA particles on the top surface of PHAa than PHAd because the agglomerates larger than 100 nm descended faster than the solvent evaporation rate according to the established Stoke's Equation. The amount of surface area occupied by HA increased on the top surface of PHAd (11.2%, Figure 4.7c) compared to PHAa (7.1%, Figure 4.7a) because the solvent evaporation was much faster than the sedimentation of well-dispersed HA particles less than 100 nm. Moreover, for the PHAa, the top surface was much different from the bottom surface, which provided evidence of the difference

in the distribution of nano-HA agglomerates. More agglomerates concentrated on the bottom side of PHAa. For the PHAd, however, there was no significant difference between its top and bottom surfaces.

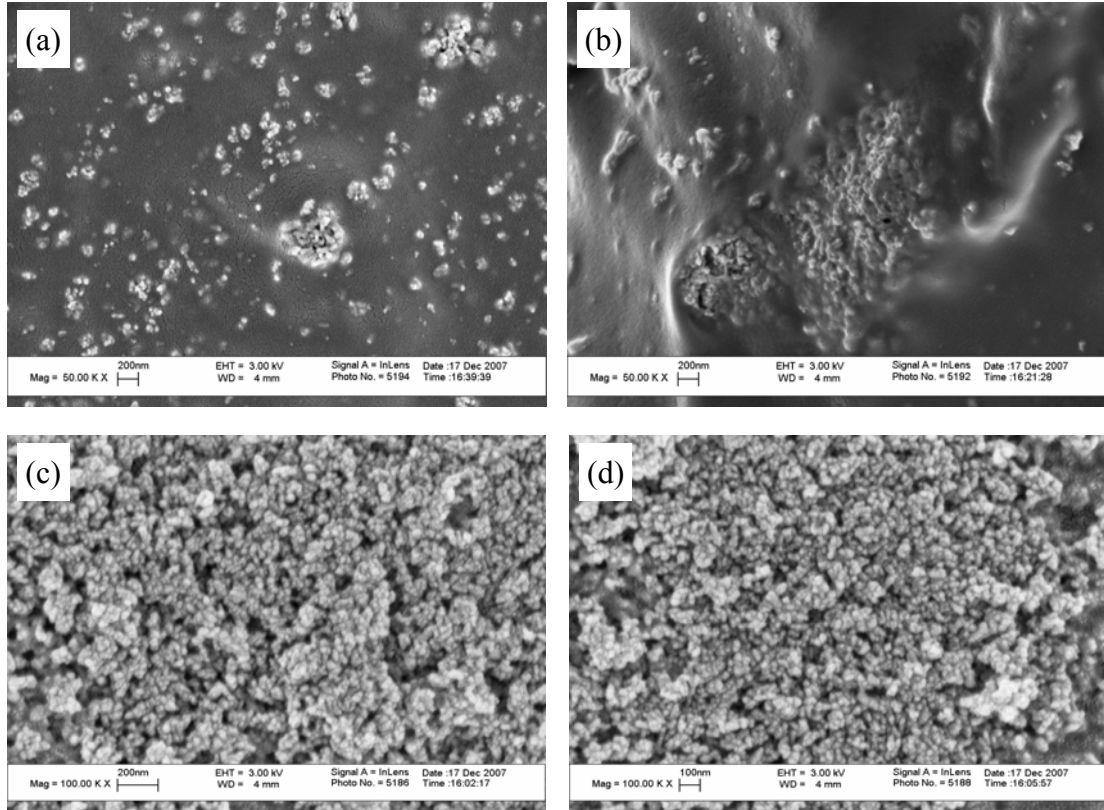


Figure 4. 7: SEM micrographs of nanophase HA/PLGA composites: (a) the top surface of PHAa, (b) the bottom surface of PHAa, (c) the top surface of PHAd, and (d) the bottom surface of PHAd. Original magnification is 50 kX for (a,b) and 100 kX for (c,d). Magnification bars are 200 nm for (a,b,c) and 100 nm for (d).

4.3.2. Mechanical Properties

4.3.2.1. Mechanical Properties of Nanophase Titania/PLGA Composites

These nanophase titania/PLGA composites enhanced mechanical properties of scaffolds compared to the polymer control according to the results of tensile and compressive tests.

The tensile stress-strain curves were calculated from load-extension data of tensile tests (Figure 4.8). The stress was the load divided by cross-section area of tensile specimens. The strain was the extension divided by the gage length of tensile specimens. The tensile modulus, tensile strength at yield, ultimate tensile strength (UTS), elongation at yield and elongation at break were calculated according to the established equations [238]. The tensile moduli of the materials of interest were calculated from the stress-strain curves and are illustrated in Figure 4.9. The tensile modulus of the PTCd was about 2 times higher than the PTCa and the tensile modulus of the PTCa was about 3 times higher than the PLGA.

Tensile strength at yield, UTS, elongation at yield and elongation at break were calculated from the stress-strain curves and are illustrated in Figure 4.10 and Figure 4.11. As shown in Figure 4.9, Figure 4.10 and Figure 4.11, PTCd had greater elastic modulus, tensile strength at yield and UTS than PTCa and PLGA, while PTCd had less elongation at yield and elongation at break than PTCa and PLGA.

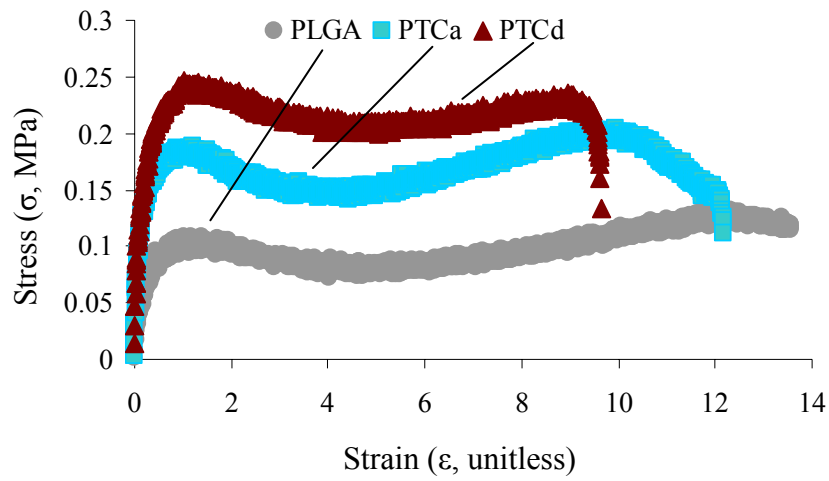


Figure 4. 8: The typical stress-strain curves of PLGA, PTCa and PTCd calculated from the load-extension data from tensile tests.

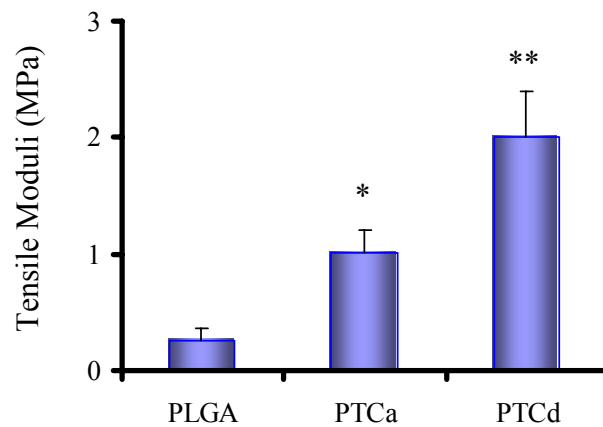


Figure 4. 9: The tensile moduli of the materials of interest. Values are mean $\pm$ SEM; $n = 3$; * $p < 0.05$ compared to PLGA; and ** $p < 0.05$ compared to PTCa.

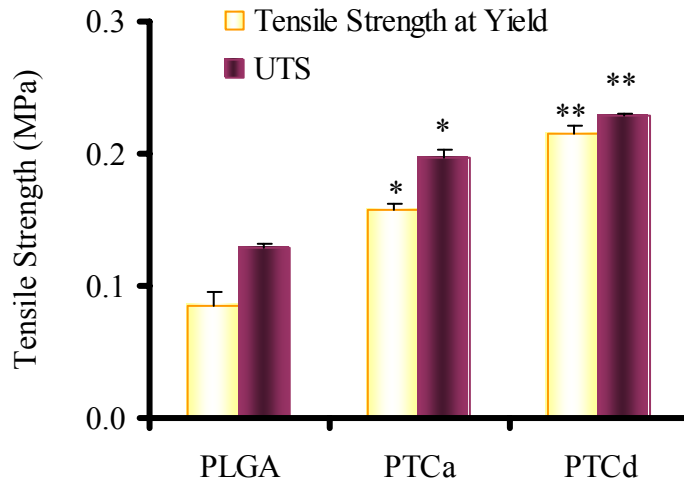


Figure 4. 10: The tensile strength at yield and the ultimate tensile strength (UTS) of the materials of interest. Values are mean $\pm$ SEM; $n = 3$; * $p < 0.05$ compared to PLGA; and ** $p < 0.05$ compared to PTCa.

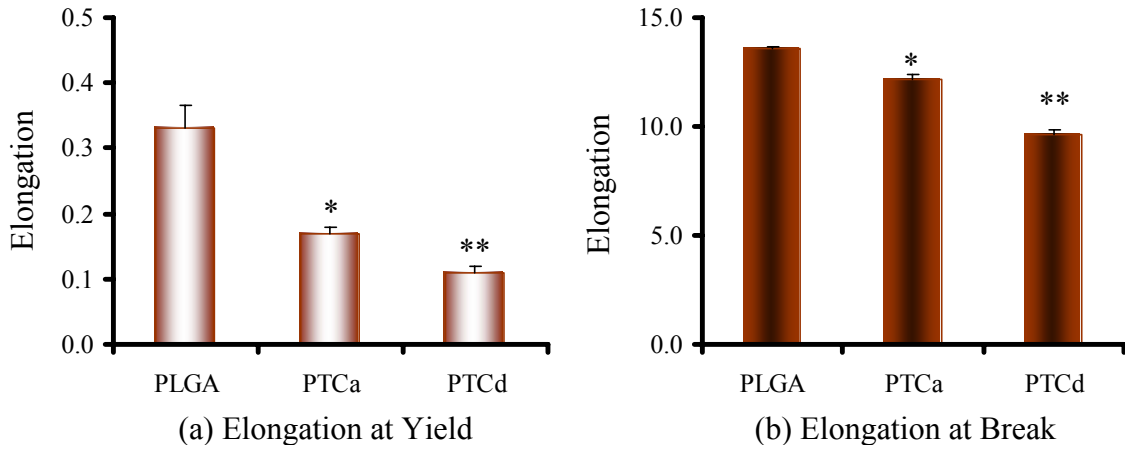


Figure 4. 11: The elongation at yield and the elongation at break for the materials of interest. Values are mean $\pm$ SEM; $n = 3$; * $p < 0.05$ compared to PLGA; and ** $p < 0.05$ compared to PTCa.

The compressive moduli were calculated from the data of compressive tests and are shown in Figure 4.12. The compressive modulus of the PTCd was about 2 times higher than the PTCa and the compressive modulus of the PTCa was about 2 times higher

than the PLGA. In comparison to natural bone, the compressive modulus of the PTCd was closer to low-density cancellous bone (~ 10 MPa) than PTCa and PLGA, although it was still lower than high-density compact bone (~ 10 GPa) [239,240].

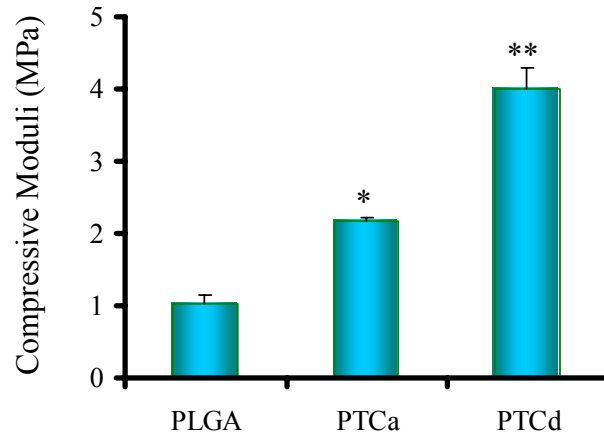


Figure 4. 12: The compressive moduli of the materials of interest. Values are mean $\pm$ SEM; $n = 3$; * $p < 0.05$ compared to PLGA; and ** $p < 0.05$ compared to PTCa.

4.3.2.2. Mechanical Properties of Nanophase HA/PLGA Composites

These nanophase HA/PLGA composites enhanced mechanical properties of scaffolds compared to the polymer control according to the results of tensile and compressive tests.

The tensile stress-strain curves were calculated from load-extension data of tensile tests (Figure 4.13). The stress was the load divided by cross-section area of tensile specimens. The strain was the extension divided by the gage length of tensile specimens. The tensile modulus, tensile strength at yield, ultimate tensile strength (UTS), elongation at yield and elongation at break were calculated according to the established equations [238]. The tensile moduli of the materials of interest were calculated from the stress-

strain curves and are illustrated in Figure 4.14. The tensile moduli of the PHAd and PHAa were greater than the PLGA.

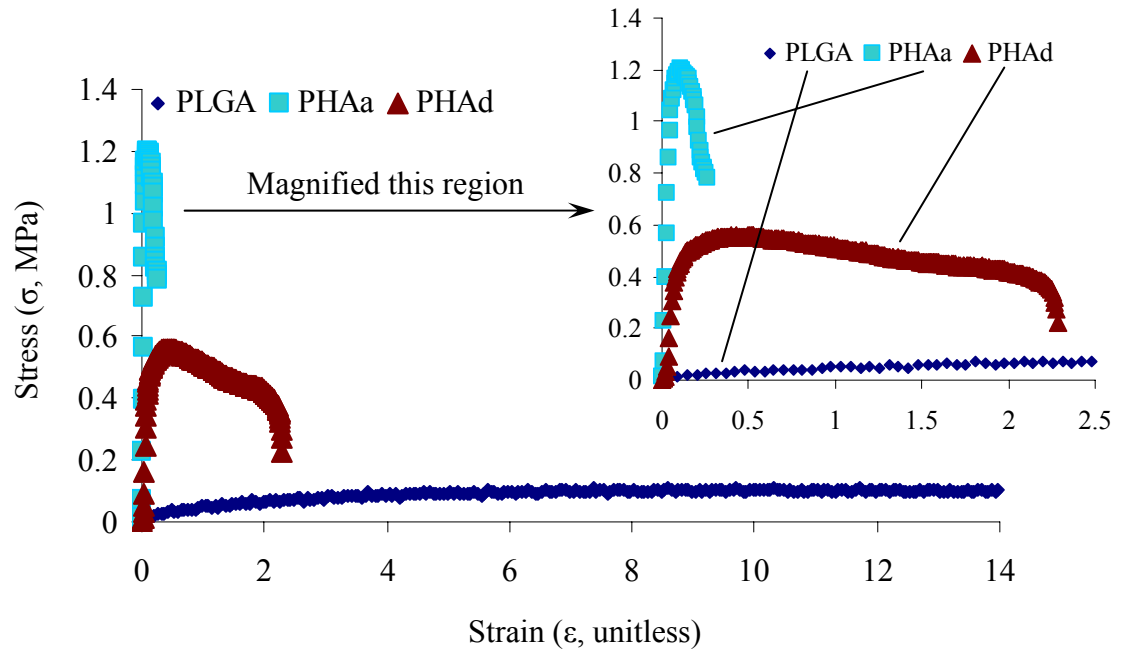


Figure 4. 13: The typical stress-strain curves of PLGA, PHAa and PHAd calculated from the load-extension data from tensile tests.

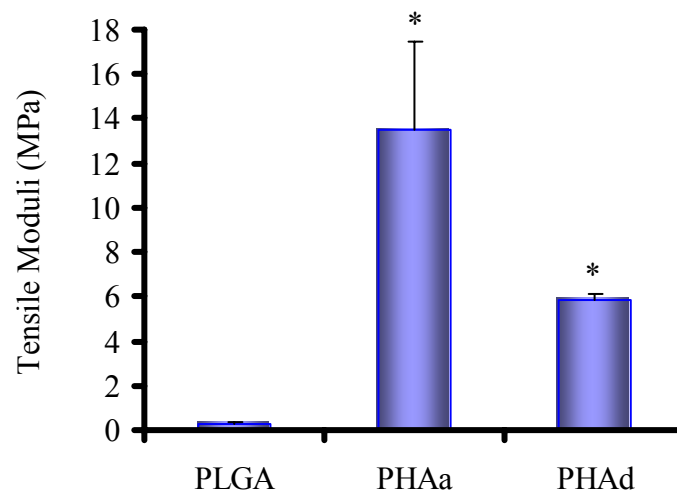


Figure 4. 14: The tensile moduli of the materials of interest. Values are mean $\pm$ SEM; n = 3; * p < 0.05 compared to PLGA.

Tensile strength at yield, UTS, elongation at yield and elongation at break were calculated from the stress-strain curves and are illustrated in Figure 4.15 and Figure 4.16. As shown in Figure 4.14, Figure 4.15 and Figure 4.16, PHAa and PHAd had greater elastic modulus, tensile strength at yield and UTS than the PLGA, while PHAa and PHAd had less elongation at yield and elongation at break than the PLGA.

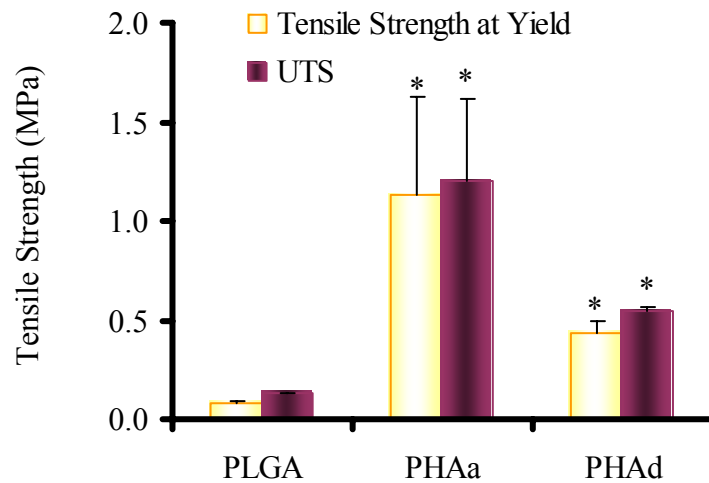


Figure 4. 15: The tensile strength at yield and the ultimate tensile strength (UTS) of the materials of interest. Values are mean $\pm$ SEM; n = 3; * p < 0.05 compared to PLGA.

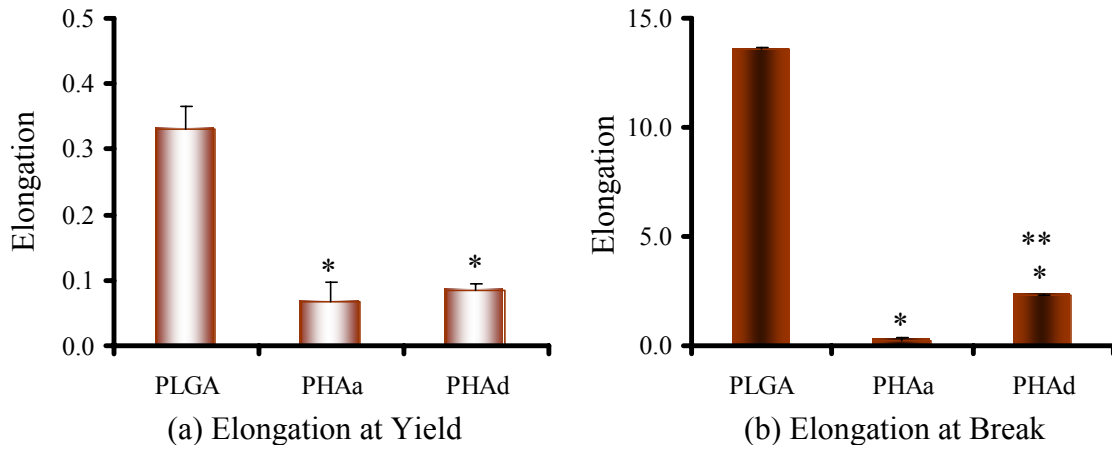


Figure 4.16: The elongation at yield and the elongation at break for the materials of interest. Values are mean $\pm$ SEM; $n = 3$; * $p < 0.05$ compared to PLGA; and ** $p < 0.05$ compared to PHAa.

The compressive moduli were calculated from the data of compressive tests and are shown in Figure 4.17. The compressive moduli of the PHAa and PHAd were greater than the PLGA. In comparison to natural bone, the compressive moduli of the PHAa and PHAd were closer to low-density cancellous bone (~ 10 MPa) than the PLGA, although it was still lower than high-density compact bone (~ 10 GPa) [240].

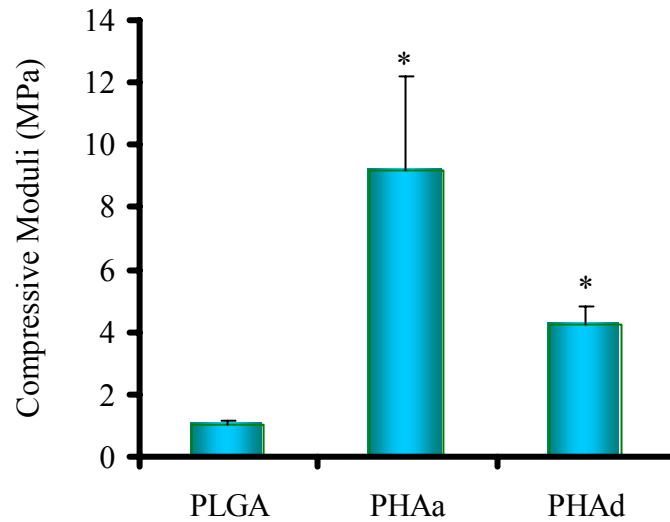


Figure 4. 17: The compressive moduli of the materials of interest. Values are mean $\pm$ SEM; $n = 3$; * $p < 0.05$ compared to PLGA.

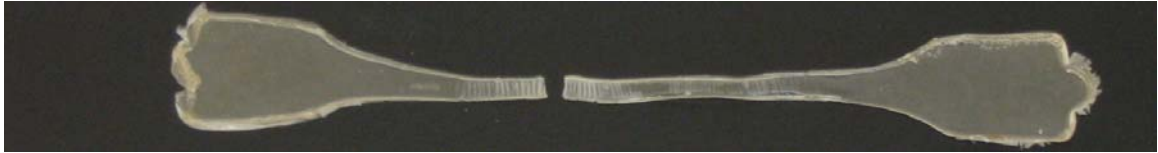
4.3.3. Fracture Analysis

4.3.3.1. Macroscopic View of Fractures

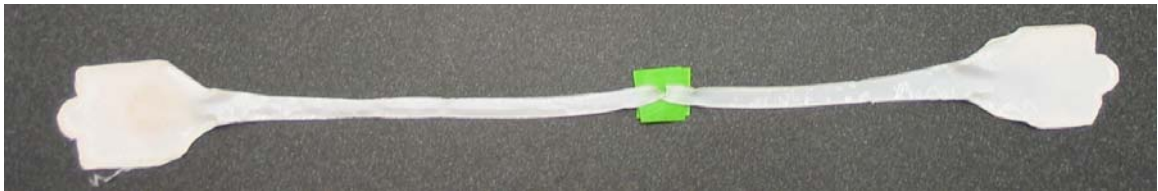
The stress-strain behaviors were different with nanophase titania/PLGA composites, nanophase HA/PLGA composites and single-phase PLGA, as shown in previous Figures 4.8 and 4.13. The stress-strain relations of nanophase ceramic/PLGA composites demonstrated an initial linear and following nonlinear deformation until fracture. The differences in tensile behaviors clearly influenced the fracture appearances of the nanocomposites. Macroscopic fracture appearances of the nanocomposites and PLGA control are shown in Figure 4.18. The fracture surfaces of the nano-HA/PLGA composites appeared more brittle compared to nano-titania/PLGA composites and PLGA. This difference in fracture surfaces clearly demonstrated that variations in the ceramic

phases (titania or HA) and their dispersion states in the polymer matrix caused different fracture behaviors and effectively altered the mechanical performance of the composites.

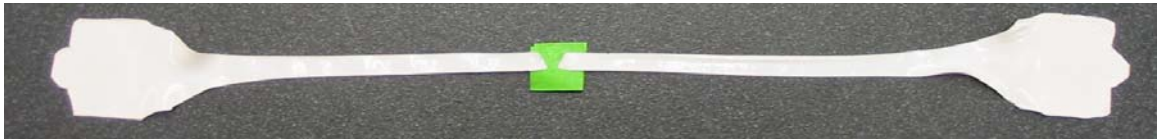
(a) PLGA



(b) PTCa



(c) PTCd



(d) PHAa



(e) PHAd



Figure 4. 18: Macroscopic fracture appearances of nanophase titania/PLGA composites, nanophase HA/PLGA composites and PLGA.

4.3.3.2. Microscopic View of Fractures

The fracture sites of the tensile specimens were visualized using a FESEM. Figure 4.19 shows representative microscopic appearances of the PLGA fracture surfaces after tensile tests. The river-like bands appeared in Figure 4.19 (a,c,d) were termed as “splay” or “silver streak”. These silver streaks were very different from microcracks that appeared in Figure 4.19 (b,d) and nanopores that appeared in Figure 4.19 (e,f) due to their unique characteristics. First, the silver streaks still contained 30-50 vol. % of polymers while there were no polymers inside the microcracks or the nanopores. Second, these silver streaks maintained certain strength compared to the microcracks and the nanopores. Third, the silver streaks were reversible while the microcracks and nanopores were irreversible. The silver streaks could be reduced or even removed under the compressive stress or heat (Temperature above T_g). Moreover, the density and refractive index of the splay region decreased compared to the original non-deformed polymers due to the void formation. The formation of silver streaks and the derived branches was associated with the tensile stress concentration and the dislocations. When the volume gain induced by the extension along the direction of the load could not compensate the volume loss due to the contraction along the direction perpendicular to the load, the silver streaks and voids would begin to form. The orientation of the silver streaks was perpendicular to the direction of load, as shown in Figure 4.19.

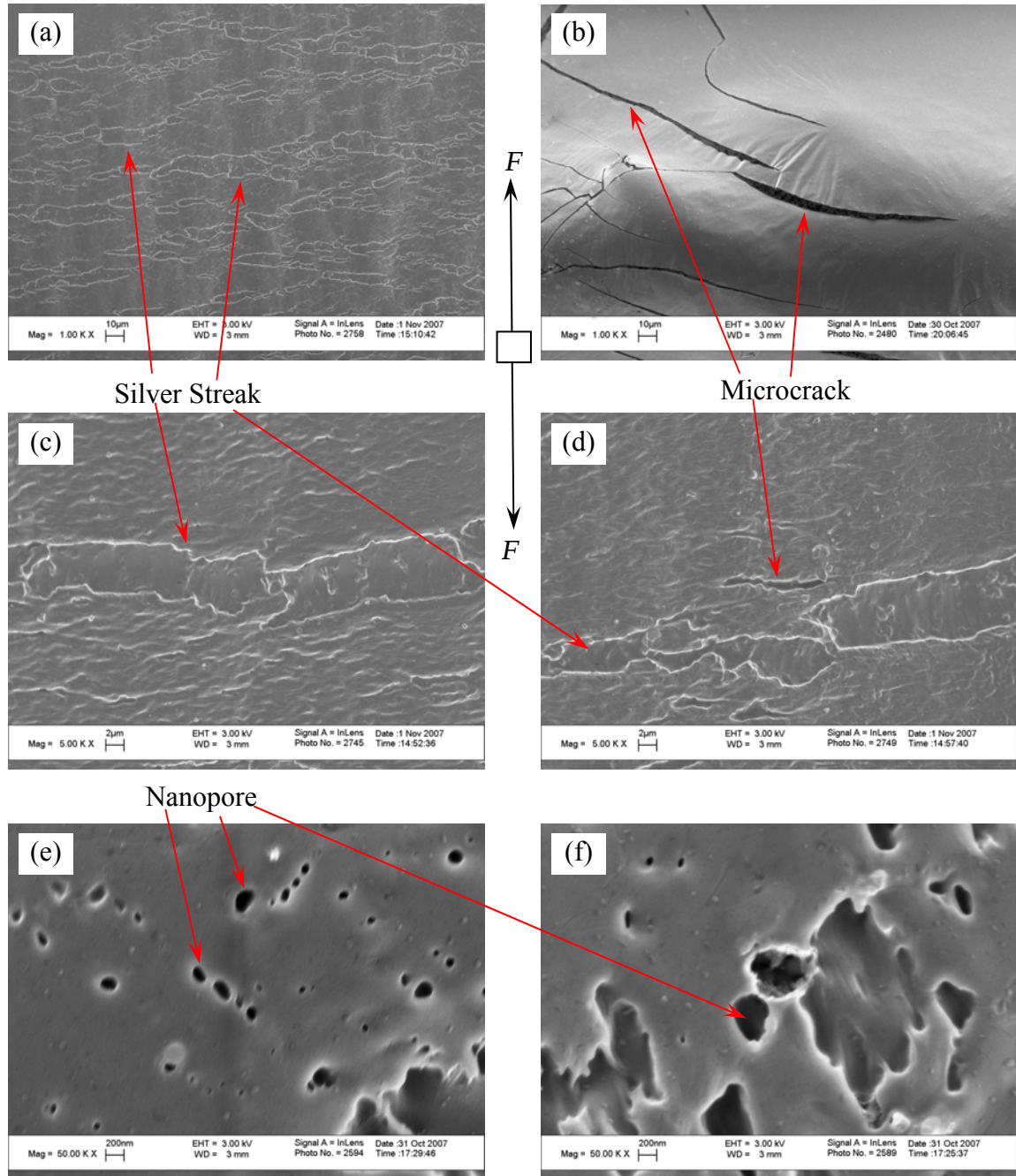


Figure 4. 19: Microscopic fracture appearances of PLGA after tensile tests. Original magnifications are 1 kX for (a,b), 5 kX for (c,d) and 50 kX for (e,f). Magnification bars are 10 μm for (a,b), 2 μm for (c,d) and 200 nm for (e,f). F shows the direction of the load.

Figure 4.20 and Figure 4.21 show representative microscopic fracture appearances of the PTCa (agglomerated nano-titania/PLGA composites) after tensile tests.

Figure 4.20(a) shows the fracture cross-section of the PTCa. Figure 4.20(b,c,d) shows the top surfaces of PTCa near the fracture cross-section. Figure 4.21 shows the bottom surfaces of the PTCa near the fracture cross-sections. The debonding of ceramic phases from the polymer matrix was evident for PTCa, as shown in Figure 4.21 (b,c,d). The silver streaks were observed on the top surface in Figure 4.20(b). The microcracks and nanopores were observed on the top surfaces in Figure 4.20(d). The crack tip initiation and propagation were observed, as shown in Figure 4.20(c).

Figure 4.22 and Figure 4.23 show representative microscopic fracture appearances of the PTCd (well dispersed nano-titania/PLGA composites) after tensile tests. Figure 4.22(a) shows the fracture cross-section of the PTCd. Figure 4.22(b,c,d) shows the top surfaces of PTCd near the fracture cross-section. Figure 4.23 shows the bottom surfaces of the PTCd near the fracture cross-sections. The debonding of ceramic phases from the polymer matrix was also observed for PTCd, as shown in Figure 4.22(b,c). The silver streaks, however, were not observed on the top surface of PTCd. The microcracks and nanopores were present on the top surfaces in Figure 4.22(b,d). The crack tip initiation, propagation and branching were observed, as shown in Figure 4.22(d) and 4.23(a).

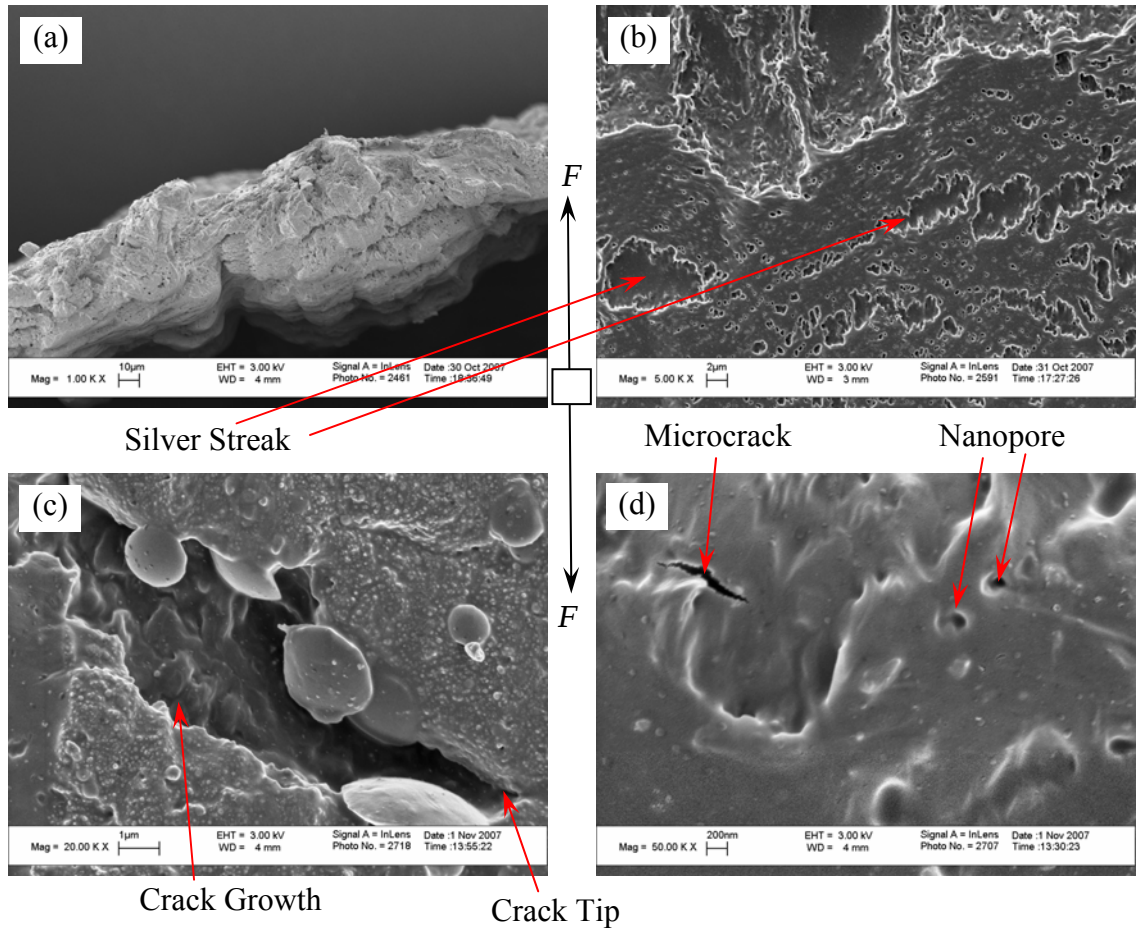


Figure 4. 20: Microscopic fracture appearances of PTCa (agglomerated nanotitania/PLGA composites) after tensile tests. The fracture cross-section is shown in (a). The top surfaces of PTCa near the fracture cross-section are shown in (b,c,d). Original magnifications are 1 kX for (a), 5 kX for (b), 20 kX for (c) and 50 kX for (d). Magnification bars are 10 μm for (a), 2 μm for (b), 1 μm for (c) and 200 nm for (d). F shows the direction of the load.

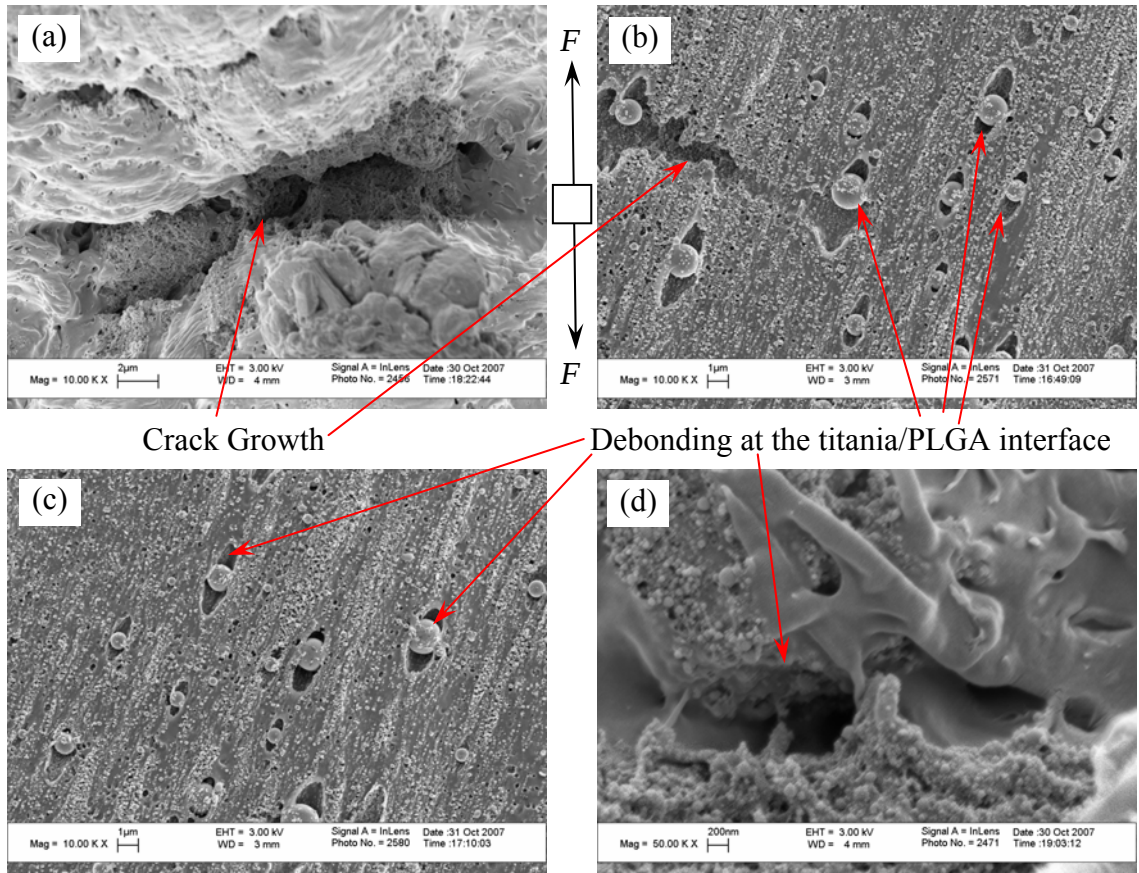


Figure 4. 21: Microscopic fracture appearances of PTCa (agglomerated nano-titania/PLGA composites) after tensile tests. The bottom surfaces of the PTCa near the fracture cross-sections. Original magnifications are 10 kX for (a,b,c) and 50 kX for (d). Magnification bars are 2 μm for (a), 1 μm for (b,c) and 200 nm for (d). F shows the direction of the load.

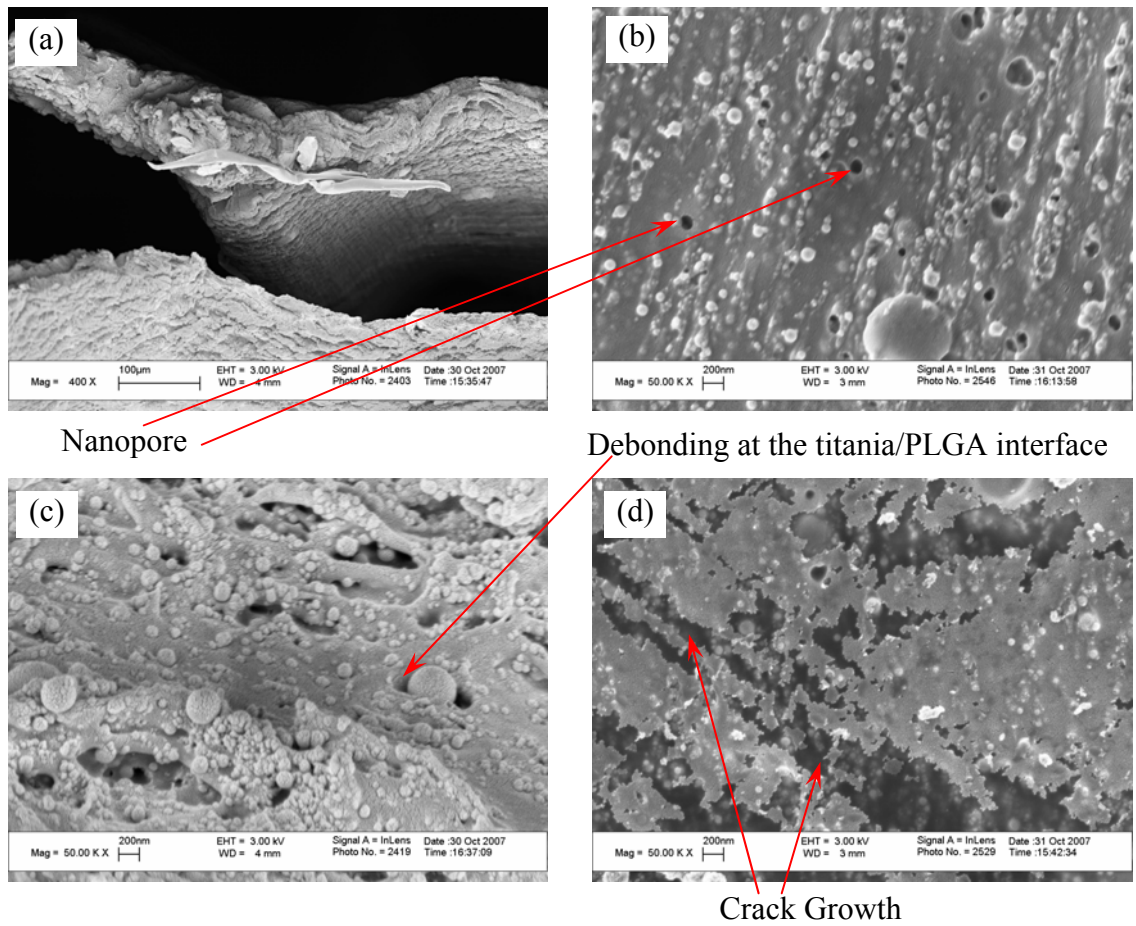


Figure 4. 22: Microscopic fracture appearances of PTCd (well-dispersed nanotitania/PLGA composites) after tensile tests. The fracture cross-section is shown in (a). The top surfaces of PTCd near the fracture cross-section are shown in (b,c,d). Original magnifications are 400 X for (a) and 50 kX for (b,c,d). Magnification bars are 100 μm for (a) and 200 nm for (b,c,d).

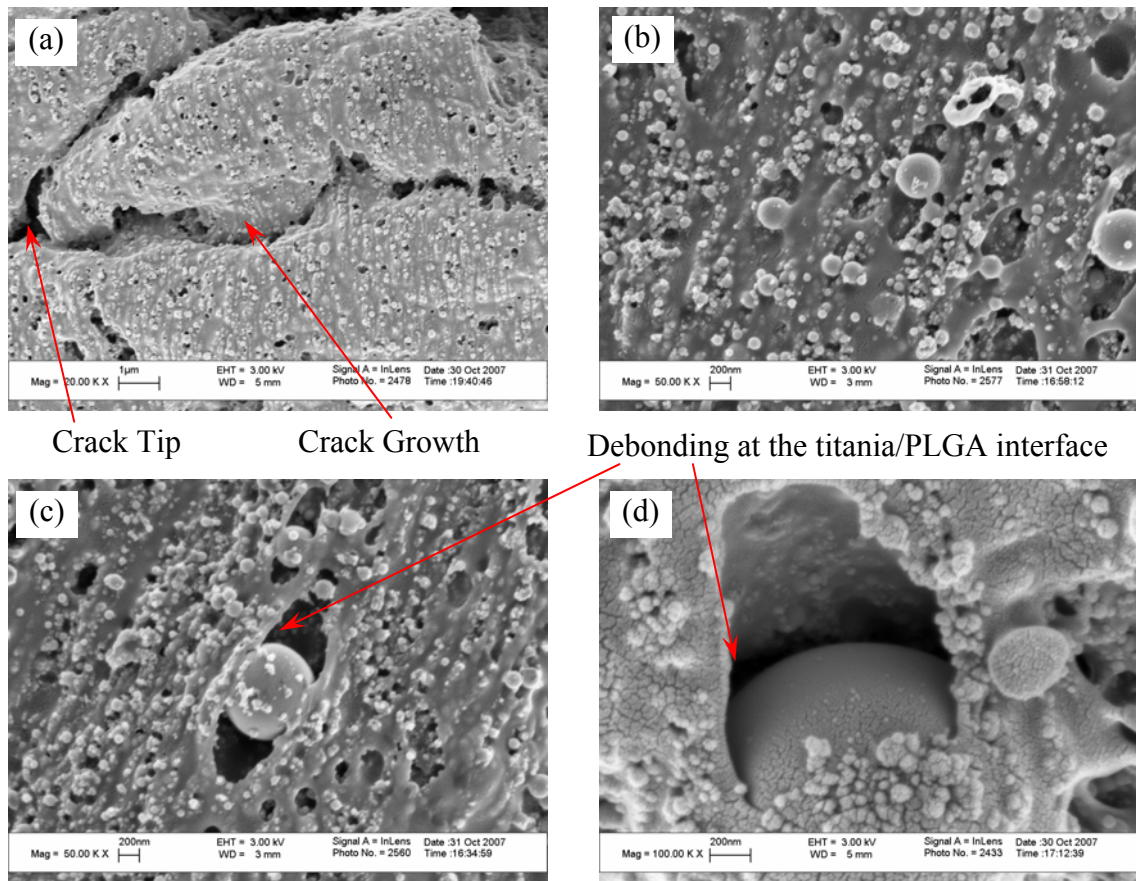


Figure 4. 23: Microscopic fracture appearances of PTCd (well-dispersed nano-titania/PLGA composites) after tensile tests. The bottom surfaces of the PTCd near the fracture cross-sections. Original magnifications are 20 kX for (a), 50 kX for (b,c) and 100 kX for (d). Magnification bars are 1 μm for (a) and 200 nm for (b,c,d).

Microcracks and nanopores were observed on both well-dispersed and agglomerated nanophase titania in PLGA composites. However, the amount of microcracks and the size of microcracks were different on PTCa and PTCd. The cracks propagated along the interfaces of the ceramic and the polymer matrix. There were also instances when the crack did not initiate at the interface, but at the polymer phase (Figure 4.20d). In these rare cases, it is speculated that the high local stress concentrations were created due to poor distribution of ceramic particles.

4.4. Discussion

It is intriguing to speculate why nanophase ceramic/polymer composites developed here have improved mechanical properties and tailored their fracture behaviors. Since the predominant feature of nanoparticles lies in their ultra-fine dimension, a large fraction of filler atoms can reside at the PLGA-ceramic interface which can lead to a stronger interfacial interaction, but only if the nanoparticles are well dispersed at the nanometer level in the surrounding polymer matrix. As the interfacial PLGA-ceramic structure plays a critical role in determining the mechanical properties of composites, nano-composites with a great number of smaller interfaces could be expected to provide unusual properties, and the shortcomings induced by the heterogeneity of conventional (or micron) particle filled composites would also be decreased or even avoided. For example, it was reported that a better bonding between the polymer matrix and the reinforcing phase resulted in a higher elastic modulus and a higher strength [241,242]. McManus et al. also reported that the bending moduli of composites of PLA with 40 and 50 wt.% nanophase (<100 nm) alumina, titania and HA were significantly greater than respective composite formulations with conventional coarser grained ceramics [162]. Specifically, compared to a bending modulus of 60 ± 3 MPa for plain PLA and 870 ± 30 MPa for conventional titania/PLA composites with a weight ratio of 50/50, the bending modulus of nanophase titania/PLA composites with a weight ratio of 50/50 was 1960 ± 250 MPa [162].

Scientifically, it is a great challenge to completely transfer desirable mechanical properties (such as Young's modulus E , compressive strength and hardness) of nanoscale ceramics into macroscale ceramic/polymer nanocomposites, although single-phase nano-

ceramics possess exceptional compressive strength, stiffness and hardness. Mechanical properties of nanoparticle-filled polymer composites have been significantly improved compared to conventional larger particle-filled polymer composites, but they are still far below the expected theoretical and experimental values determined by the individual nanoscale building blocks, except at a very low volume fraction of the reinforcing phase [243-246]. Non-ideal mechanical properties of ceramic/polymer composites are largely related to the difficulties in achieving well-dispersed large volume fractions of the reinforcing nano-ceramics in polymer composites and a lack of nanostructural control in the composites. As mentioned, nanoparticles have a strong tendency to agglomerate in the composites, especially when they took up more than 2 wt.% of the composites. Nano-ceramics are, thus, very difficult to be incorporated homogeneously, individually into the polymer, as completely dispersed ceramics rather than intercalated structures. Moreover, it is also important to control an effective load transfer from the polymeric matrix to the nanoscale components and understand mechanical interactions of the two constituents at the nanoscale.

For loading-bearing orthopedic applications, it is important to produce a nanocomposite with mechanical properties closer to the theoretical values. The approaches include controlling spatial distribution and orientation of nanoparticles in a polymer matrix at the nanoscale, and retaining this order at the macroscale. For example, Podsiadlo et al. assembled a homogeneous, optically transparent clay (montmorillonite, MTM)/polymer (poly(vinyl alcohol), PVA) nanocomposite with planar orientation of the aluminosilicate nanosheets using a bottom-up layer-by-layer (LBL) assembly process. The tensile strength (UTS) of these multilayer MTM/PVA composites reached 400 ± 40 MPa

and the Young's modulus reached 106 ± 11 GPa, one order of magnitude greater than that of PVA. This was contributed to the nanoscale dimension of the inorganic MTM phase, and the nearly perfect orientation and fine dispersion of the MTM nanoplatelets. A highly effective load transfer between nanosheets and the polymer was, thus, achieved by combining highly ordered nanoscale building blocks with dense covalent and hydrogen bonding that stiffened the polymer chains. The greater mechanical properties of PVA/MTM nanocomposites were resulted from several mechanisms at the nanoscale. The degree of structural organization (afforded by the LBL process) of the clay platelets in the composite maximizes the number of polymer/MTM interactions and constrains the polymer-chain motion, which resulted in a highly efficient load transfer between the polymer phase and the stiff MTM platelets.

All these theories and postulations discussed above can be applied to nanophase ceramic/polymer composites to promote their mechanical properties for load-bearing orthopedic applications.

4.5. Conclusions

The dispersion of ceramic nanoparticles (titania or HA) in PLGA promoted mechanical properties of orthopedic materials as compared to the PLGA and the agglomerated ceramic/PLGA composites. For example, well-dispersed nano-titania/PLGA composites promoted the tensile modulus, tensile strength at yield, ultimate tensile strength and compressive modulus as compared to PLGA and the more agglomerated nano-titania/PLGA composites. As expected, nano-HA/PLGA

nanocomposites also demonstrated greater tensile modulus, tensile strength and compressive modulus than the PLGA. Although the well-dispersed nano-HA/PLGA composites (PHAd) had a slightly lower tensile modulus, tensile strength and compressive modulus compared to PHAa, PHAd presented a much better ductility (greater elongation at yield and greater elongation at break) than PHAa.

In conclusion, when collectively considering these results, the combination of the ductile PLGA with a strong and biocompatible well-dispersed nano-ceramic phase can be very promising for customizing mechanical properties of next generation orthopedic prostheses. Therefore, coupled with prior studies demonstrating greater osteoblast functions, the combination of PLGA with nano-ceramics may provide better candidate materials for more effective orthopedic applications, from both biological and mechanical perspectives.

CHAPTER 5. NANOPHASE CERAMIC/POLYMER COMPOSITES AS CONTROLLED DRUG DELIVERY CARRIERS FOR TREATING BONE DISEASES

5.1. Problems and Specific Aims

Pharmaceutical agents are often required to stimulate new bone formation for the treatment of bone injuries or diseases (such as osteoporosis). However, there are several problems associated with current drug delivery methods. First, conventional systemic administration of these agents can not effectively reach targeted sites and, thus, they can cause non-specific bone formation in areas not affected by injury or disease. Second, even if intentionally delivered or implanted locally to the damaged bone tissue, these agents tend to rapidly diffuse into adjacent tissues due to weak physical bonding to their drug carriers, which limits their potential to promote prolonged bone formation in targeted areas of bone. Therefore, this study explored chemical bonding methods for immobilizing bone morphogenetic proteins (BMPs) to nanophase hydroxyapatite (nano-HA) to improve local bone growth and interfacial bonding strength to juxtaposed bone. Moreover, the use of nano-HA could increase protein loading efficiency considering that nano-HA has much larger surface area and much more exposed reaction sites for chemical bonding. For this purpose, nano-HA was synthesized by wet chemistry precipitation followed by hydrothermal treatment to gain control over desirable grain sizes and crystallinities. BMPs were chemically bonded to nano-HA through amino-

silane chemistry. Nano-HA/BMP conjugates were then dispersed in poly(lactide-co-glycolide) (PLGA) solutions to create an implantable scaffold by a solvent-casting technique. These scaffolds were characterized for drug loading efficiency and *in vitro* drug release profiles.

5.2. Model Drug Carriers and Model Drugs

5.2.1. The Choice of Model Ceramics: Nano-titania vs. Nano-HA

Previous *in vitro* studies reported the great potential of nanophase ceramic/polymer composites for orthopedic applications, particularly using nano-titania as a model ceramic. This chapter, however, will mainly focus on nanophase calcium phosphates and their derivatives such as nano-HA as model ceramics considering their advantages in controlled drug delivery applications [247,248]. Formulations of various phases of calcium phosphates will offer different chemical structure, density, crystallinity and subsequent properties of degradation, critical for serving as good carriers for BMPs and their derivatives [249]. Moreover, increased new bone formation has been observed on nano-HA compared to micron-HA coated scaffolds when implanted into rat calvarial bone [140,250], as shown in Figure 5.1. Enhanced new bone infiltration can be clearly seen as early as two weeks on the nano-HA coated tantalum scaffolds compared to micron-HA coated and uncoated tantalum scaffolds. Such results provided evidence of the promising translation of *in vitro* cell functions to *in vivo* bone growth.

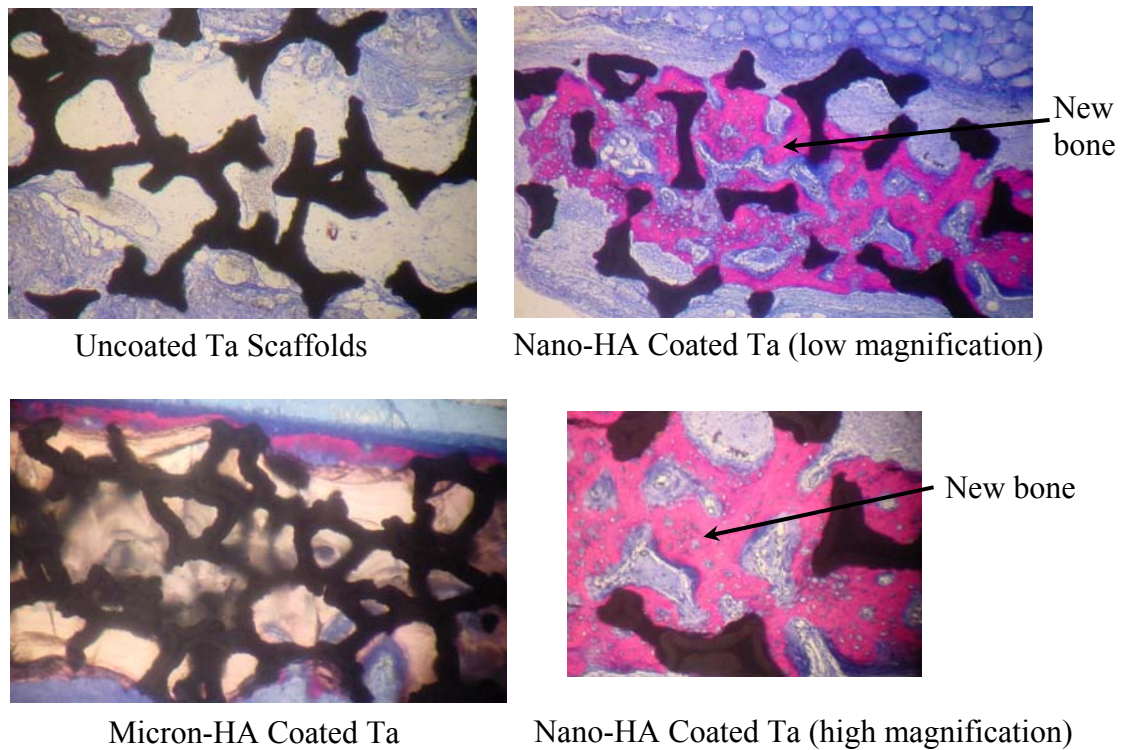


Figure 5. 1: Histology of rat calvaria after tantalum (Ta) scaffolds coated with either nano-HA or micron-HA which were implanted for 2 weeks. Red shows new bone infiltration which occurred in greater amounts on nano-HA coated Ta than either micron-HA coated Ta or uncoated Ta. (Adapted and redrawn from [140]).

In summary, these preliminary studies rationalized desirable properties of the nanocomposites *in vitro* and *in vivo* for better new bone regeneration. The objective of this chapter is to further expand the advantages of the nanocomposites by delivering BMPs or its derived peptides more efficiently to promote bone growth.

5.2.2. Bone Morphogenetic Proteins

Bone morphogenetic proteins, especially recombinant human bone morphogenetic protein-2 (or rhBMP-2) and BMP-7 (osteogenic protein-1), have the ability to induce new

bone formation [251,252]. It has been reported that rhBMP-2 is an osseointegrative protein that could effectively induce new host bone regeneration by guiding the modulation and differentiation of mesenchymal cells into bone forming cells [252,253]. Improved bone repair using rhBMP-2 was observed in the rat tibia, rabbit calvarium, dog mandible, and sheep tibia [254-258]. For example, rhBMP-2 could be adsorbed onto porous HA to enhance the osseointegration of implanted HA to the skull of adult white rabbits [259]. It was observed that rhBMP-2 increased the percentage of bone filling into microporous HA scaffolds compared to respective scaffolds without rhBMP-2 [260]. Moreover, rhBMP-2 mechanically strengthened HA implants as early as 4 weeks due to the induced faster healing. Specifically, when the initial fracture loads of HA scaffolds were 100 N, the strength of HA scaffolds combined with rhBMP-2 reached 650 N at 4 weeks and 800 N at 8 weeks, but the fracture load of HA implants without rhBMP-2 did not increase until 8 weeks after implantation [261]. Enhancing mechanical strength in an earlier stage allows patients to exert loading earlier after implantation, which could further accelerate the healing process [262]. In addition to all of these advantages, rhBMP-2 has recently received clearance from the Food and Drug Administration (FDA) for the specific clinical use.

However, these studies also exposed two problems with the current delivery systems for BMPs: low loading efficiency and lack of controlled drug release [263]. Recent studies demonstrated that a desired drug release profile could be achieved by controlling the fabrication method of nanocomposite-drug conjugates [264]. It has been demonstrated that when the enzyme (glucocerebrosidase) was pre-adsorbed onto nanophase calcium phosphate powders prior to dispersing in alginate, the initial burst of

drug release was significantly reduced and a slower prolonged release was achieved [264]. Nano-HA as a carrier for BMPs could be beneficial for a prolonged drug release compared to conventional (or micron) HA. Although the proposed drugs and carrier materials are different here, it is still very likely to achieve similar results for nano-HA with BMP growth factors dispersed in PLGA systems due to the same mechanism.

5.2.3. BMP-Derived Short Peptides

BMPs are the most potent growth factors for enhancing bone formation. Especially, BMP-2 and BMP-7 (osteogenic protein-1) promotes the formation and regeneration of bone and cartilage [265-267]. A single BMP, currently either BMP-2 or BMP-7, is usually chosen when bone regeneration is desired. However, when these BMPs are used in higher order mammals (such as human beings), a pharmacological dose rather than a physiological dosage has to be administrated because the efficacy of the BMPs is still dependent on the recruitment of local cells, additional BMPs, and other growth factors [268]. In higher order mammals, the BMPs need to be present in the targeted sites for a longer period of time in order to achieve desirable pharmaceutical effects. On the contrary, clinical studies demonstrated that they often quickly diffuse away from the currently used carriers before inducing a local effect [268]. Additionally, the number of progenitor cells that are responsive to BMPs may be more limited in the higher order mammals and human beings, particularly under clinical circumstances (such as non-unions and predominantly elderly patients) [268]. The amount of implanted protein exceeds by far the normal physiological concentration of this protein in the

fracture area. Therefore, it is not expected that an increase of the local dosage will lead to a higher efficacy [269].

Considering these problems associated with the delivery of BMPs, this study explored chemical bonding methods for immobilizing bioactive regions of BMPs to nanophase hydroxyapatite (nano-HA) to improve the drug delivery efficacy for local bone growth and interfacial bonding strength to juxtaposed bone. Moreover, the use of nano-HA could increase protein loading efficiency considering that nano-HA has a much larger surface area and much more exposed reaction sites for chemical bonding.

The BMPs have several hundred amino acids, approximately 2~3 nm, depending on the conformation, which are too large and complex to be chemically functionalized onto nanomaterials. These complex secondary structures of the proteins are prone to degradation and as a result, these proteins tend to lose their bioactivity quickly in aqueous physiological conditions. Moreover, short peptides can be attached to drug carriers more efficiently due to their small size. Therefore, it is proposed in this study to deliver short peptides that were derived from bioactive regions of BMP-7, instead of the whole BMP protein, by chemically functionalizing them onto nano-structured biomimetic materials.

Chen et al. investigated three short peptides derived from bioactive regions of BMP-7 [270]. These three peptides were composed of 10 amino acids and were designated as peptide a (SNVILKKYRN), b (KPCCAPTQLN) and c (AISVLYFDDS), as shown in Figure 5.2. The results showed that peptide b increased osteoblast proliferation while peptide a and c promoted osteoblast differentiation (e.g. mineralization) [270].

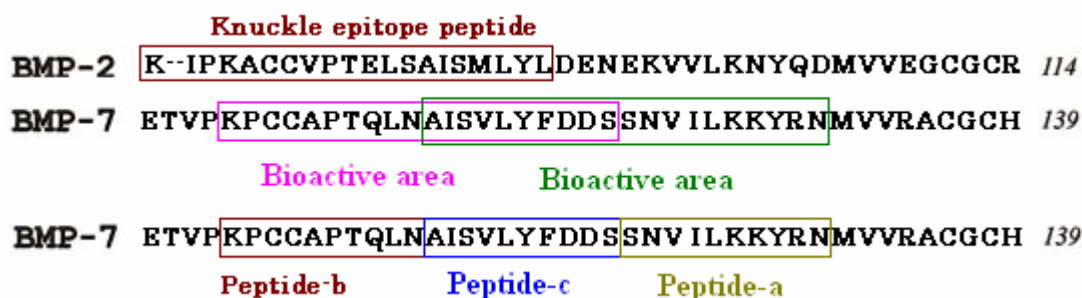


Figure 5. 2: Short peptides derived from BMP-7 and their amino acid sequences. (Adapted and redrawn from [270]).

In this study, peptide c was chosen and slightly modified as the model peptide for studying drug loading efficiency and long-term drug release, thus, promoting bone mineralization and healing. Combining BMP-7 short peptides with nano-HA/PLGA composites may provide a promising solution for designing and fabricating more effective nanotechnology-derived orthopedic implants that are capable of delivering growth factors in a controlled, tunable fashion.

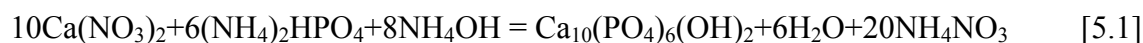
5.3. Materials and Methods

5.3.1. Material Preparation

5.3.1.1. Synthesis of Nanocrystalline Hydroxyapatite

Nanophase HA was synthesized using a wet chemistry precipitation method by mixing solutions of calcium nitrate and ammonium phosphate in an alkaline pH region [229]. Specifically, a 1 M calcium nitrate solution and a 0.6 M ammonium phosphate solution were prepared by dissolving their respective solid state powders in deionized (DI)

water separately. The produced ammonium phosphate solution was mixed with DI water which had been adjusted to pH 10 by ammonium hydroxide. The pre-made 1 M calcium nitrate solution was then added into the mixture of ammonium phosphate and ammonium hydroxide at a rate of 3.6 ml/min. Precipitation occurred as soon as the calcium nitrate was added. Chemically, the HA precipitation occurred through the reaction [5.1]:



Precipitation continued for 10 minutes at room temperature with constant stirring. The supernatant was collected, centrifuged (Eppendorf centrifuge, Model 5810 R) to reduce 75% of the solution volume and placed into a 125 ml Teflon liner (Parr Instrument). The Teflon liner was sealed tightly in a Parr acid digestion bomb 4748 (Parr Instrument) and treated hydrothermally at 200 °C for 20 hours to obtain nanocrystalline HA. The hydrothermal treatment has a great advantage to prepare a stoichiometric, ultrafine HA powder with a homogeneous shape and size distribution due to higher applied pressures than atmospheric [230,231]. After the hydrothermal treatment, nano-HA particles were rinsed with DI water and dried in an oven at 80 °C for 12 hours. Figure 5.2 shows the schematic procedures of HA synthesis followed in this study.

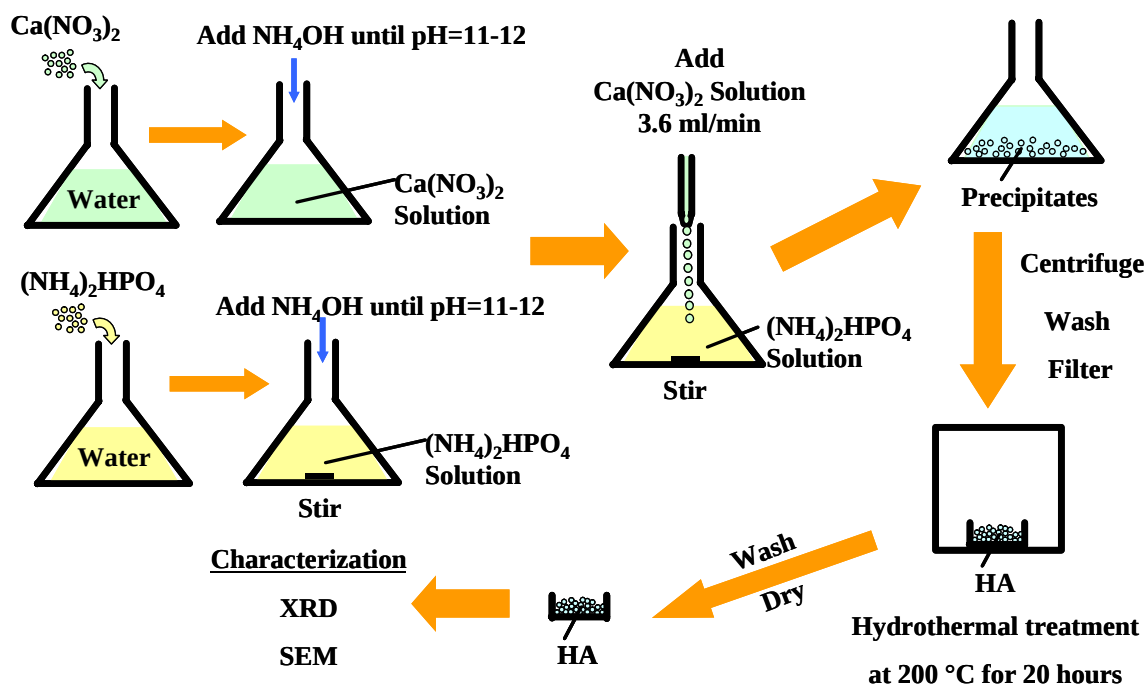


Figure 5. 3: The schematic diagram illustrating HA synthesis by a wet chemistry precipitation method.

5.3.1.2. Design and Synthesis of the Model Peptide

The peptide c (AISVLYFDDS) was further modified at its N-terminal with a cysteine-containing spacer to ease chemical conjugation onto the nano-HA particles using aminosilane chemistry followed by a maleimide cross-linker molecule. In this study, the peptide with a 12 amino-acid sequence of CKAISVLYFDDS was used as the model peptide and termed as DIF-7c.

The peptide DIF-7c was obtained as carboxyl terminal acids to more than 98.2% purity according to the HPLC profile provided by the manufacturer (GenScript Corporation, USA). The molecular weight of the peptide DIF-7c was 1360.56 g/mol.

5.3.1.3. Peptide Loading onto Nanophase Ceramic/Polymer Composites

As mentioned, the difficulties of drug delivery lie in the efficient loading and controlled release. In this study, two types of loading methods were used and compared for efficacy: chemical bonding and physical adsorption.

5.3.1.3.1. Immobilization of Peptide Using Aminosilane Chemistry

For chemical bonding, nano-HA was functionalized through aminosilane chemistry under dry conditions to avoid surface contamination and, thus, ensure stability of the peptide, as shown in Figure 5.4 [271,272]. First, nano-HA was silanized in 3-aminopropyltriethoxysilane (APTES; Sigma 440140) in anhydrous hexane (Sigma 296090). Second, for substituting a hetero-bifunctional cross-linker for the terminal amine, the silanized nano-HA was coupled with N-succinimidyl-3-maleimido propionate (SMP; also called 3-Maleimidopropionic acid N-hydroxysuccinimide ester, Sigma 358657) in anhydrous N,N-dimethylformamide (DMF; Sigma 494488). Third, the peptide DIF-7c was immobilized onto nano-HA in anhydrous DMF through a reaction between the outer maleimide group with the thiol group of cysteine present in the terminal of DIF-7c. The nano-HA and model peptide conjugates that were bonded using aminosilane chemistry were termed as HA_Ps.

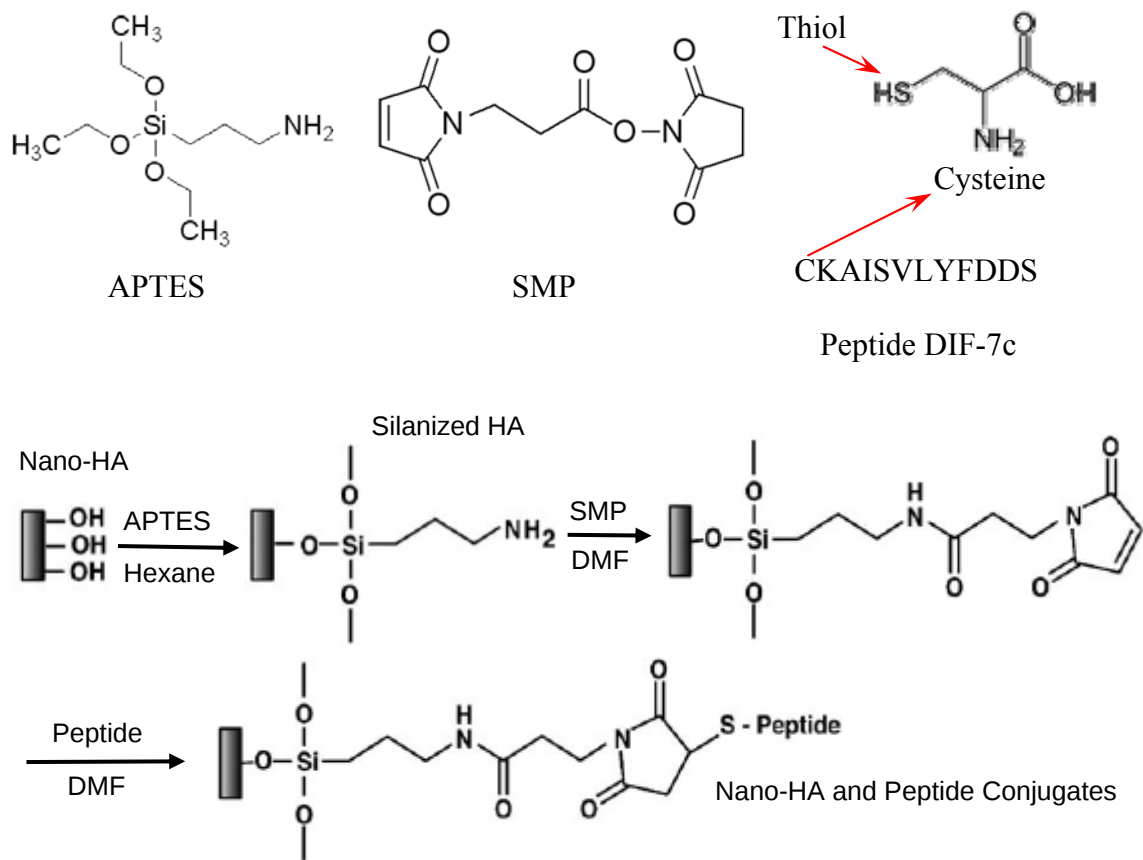


Figure 5. 4: The schematic illustrations of the chemical structures and the reactions that were used to bond the model peptide to nano-HA particles. (Adapted and redrawn from [271]).

Experimentally, immobilization of the peptide DIF-7c to HA nanoparticles was performed according to the following procedure, as shown in Table 5.1. To note, all these procedures were carefully carried out under dry conditions.

Table 5. 1: The detailed procedures that were followed for immobilization of the model peptide to nano-HA using aminosilane chemistry.

(1)	HA nanoparticles were dried and degassed at 40 °C in vacuum (10^{-5} Torr) for 24 hours.
(2)	HA nanoparticles were rinsed with anhydrous hexane using a vortex (Fisher Scientific) and a centrifuge (Eppendorf centrifuge, Model 5810 R).
(3)	HA nanoparticles were silanized by immersing in a solution of APTES (10 vol.%) in anhydrous hexane at 40 °C for 24 hours under continuous stirring.
(4)	After silanization, HA nanoparticles were rinsed with hexane for 3 times and dried at 40 °C in vacuum (10^{-5} Torr) for 24 hours.
(5)	Silanized HA nanoparticles were coupled with SMP (0.01 M) in DMF at 40 °C for 24 hours under continuous stirring.
(6)	After SMP attachment, HA nanoparticles were rinsed with DMF for 3 times and dried at 40 °C in vacuum (10^{-5} Torr) for 24 hours.
(7)	The model peptide DIF-7c was immobilized onto HA nanoparticles in anhydrous DMF (1mM) at 40 °C for 24 hours under continuous stirring.
(8)	The functionalized HA nanoparticles were rinsed 3 times with DMF followed by 3 washes with DI water
(9)	The HA-peptide conjugates (termed HA_Ps) were dried and degassed at 40 °C in vacuum (10^{-5} Torr) for 24 hours.

5.3.1.3.2. Immobilization of Peptide Using Physical Adsorption Methods

For physical adsorption, two possible ways were investigated in this study: (a) the peptide DIF-7c was pre-adsorbed to nano-HA particles and then was dispersed in PLGA solution, and (b) nano-HA and the peptide DIF-7c was dispersed individually in PLGA solution, as shown in Figure 5.5.

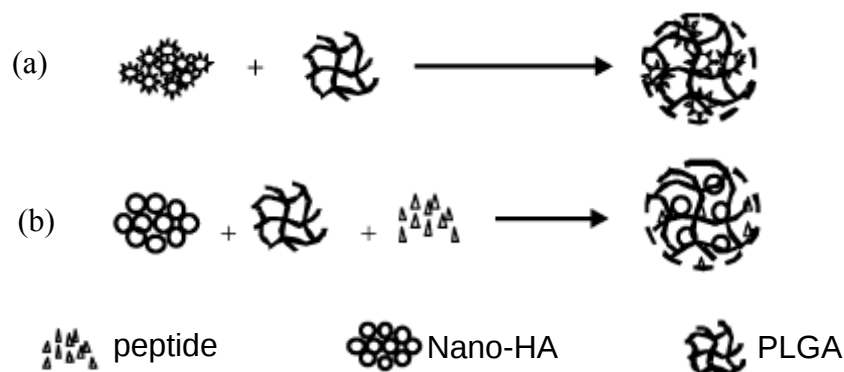


Figure 5. 5: Schematic illustrations of loading DIF-7c by physical adsorption. (a) Nano-HA particles with pre-adsorbed DIF-7c dispersed in PLGA solution. (b) Nano-HA and DIF-7c dispersed individually in PLGA solution.

For method (a), the peptide was first adsorbed to nano-HA particles in anhydrous DMF (1mM) at 40 °C for 24 hours under continuous stirring. Second, the nano-HA particles with adsorbed peptide were rinsed 3 times with DMF followed by 3 washes with DI water. The nano-HA and model peptide conjugates obtained by the physical adsorption method were termed as HA_Pa. Third, the HA_Pa nanoparticles were dispersed in the PLGA using controlled sonication. The detailed procedures will be described in a later section 5.3.1.4.

For method (b), PLGA was first dissolved in an organic solvent. Second, HA nanoparticles were added into the PLGA solution. Third, the peptide was added into the PLGA solution. Finally, controlled sonication was used to disperse nano-HA and the peptide in the PLGA. The detailed procedures will be described in a later section 5.3.1.4.

5.3.1.4. Nanophase Hydroxyapatite-Peptide-PLGA Drug Delivery Systems

The model peptide DIF-7c was loaded to nanophase HA/PLGA composites in 3 different ways as described in the previous section. Table 5.2 summarized the 3 types of nano-HA/Peptide/PLGA drug delivery systems, 6 types of controls and 2 references that were cultured in PBS for a prescribed period of time. Among them, HA_PLGA (Control 1), PLGA (Control 2), HA (Control 3) and 2 references were only used for assuring the success of the experiments, not for drug release tests. The detailed procedures used for preparing these drug delivery systems and controls are presented in the following sections.

Table 5. 2: A summarized list of nano-HA-peptide-PLGA drug delivery systems, controls and references of interest to this study.

Label		Abbreviations	Descriptions
Controls	1	HA_PLGA	Well dispersed nano-HA in PLGA composites, no peptide
	2	PLGA	PLGA only, no peptide
	3	HA	Nano-HA only, no peptide
	4	PLGA_P	PLGA with peptide
	5	HA_Pa	Nano-HA with pre-adsorbed peptide
	6	HA_Ps	Nano-HA with chemical functionalized peptide
Composites	7	HA_Pa_PLGA	nano-HA/PLGA composites, peptide was loaded by physical adsorption method onto HA
	8	HA_Pd_PLGA	nano-HA/PLGA composites, peptide was dispersed in HA/PLGA suspension using controlled sonication
	9	HA_Ps_PLGA	nano-HA/PLGA composites, peptide was loaded by silane chemistry method onto HA
References	Ref1	Glass	Borosilicate glass coverslips (Fisher Scientific; 1 cm in diameter)
	Ref2	PSTC	Polystyrene tissue culture plate (Corning, 12-well plates)

5.3.1.4.1. Preparation of Controls

PLGA with the peptide (control 2) and PLGA without the peptide (control 3) were used as polymer controls. For the PLGA (control 3), PLGA pellets (50/50 wt.% poly(DL-lactide/glycolide, Polysciences, Inc., Warrington, PA) were dissolved in chloroform at 40 °C in a water bath for 40 minutes, cast into a Teflon petri dish, evaporated in air at room temperature for 24 hours, and dried in an air vacuum chamber at room temperature for 48 hours. For the PLGA_P (control 2), 1.5 mg peptide was added into PLGA solution after PLGA was dissolved in chloroform at 40 °C. These PLGA films (0.3 mm in thickness) were then cut into 1 cm × 1 cm squares for use in material characterizations and *in vitro* studies.

For the HA/PLGA (control 1), nanocrystalline HA (synthesized in 5.3.1.1., average particle size 36 nm) was added into the PLGA solution to give a 30/70 ceramic/polymer weight ratio. The composite mixture was sonicated using a Misonix 3000 sonicator (Misonix, Inc.) with its microtip immersed in the mixture. After sonication, the composite suspension was cast into a Teflon dish, evaporated in air at room temperature for 24 hours, and dried in an air vacuum chamber at room temperature for 48 hours. The nano-HA/PLGA specimens were cut into 1 cm × 1 cm squares for use in material characterizations and *in vitro* studies.

HA, HA_Pa and HA_Ps were used as ceramic controls.

5.3.1.4.2. Preparation of HA/PLGA Composites Loaded with Peptides

The peptide DIF-7c was loaded to HA/PLGA composites by 3 methods, one was through chemical bonding, the other two were through physical bonding. Experimentally,

the HA_Pa_PLGA systems were prepared according to the procedures listed in Table 5.3. The HA_Pd_PLGA systems were prepared according to the procedures listed in Table 5.4. The HA_Ps_PLGA systems were prepared according to the procedures listed in Table 5.5.

Table 5. 3: The detailed procedures that were followed for preparing the HA_Pa_PLGA systems.

(1)	PLGA was dissolved in chloroform at 40 °C in a water bath for 40 minutes.
(2)	HA_Pa nanoparticles were added into PLGA solution. The weight ratio of HA_Pa to PLGA was 30/70.
(3)	The mixture was sonicated for 10 min at controlled powers to achieve a uniform dispersion of HA_Pa in PLGA.
(4)	After sonication, the mixture was cast into a Teflon mold, evaporated in air at room temperature for 24 hours, and dried in an air vacuum chamber at room temperature for 48 hours.

Table 5. 4: The detailed procedures that were followed for preparing the HA_Pd_PLGA systems.

(1)	PLGA was dissolved in chloroform at 40 °C in a water bath for 40 minutes.
(2)	HA nanoparticles were added into PLGA solution.
(3)	The peptide was added into PLGA solution. The weight ratio of (HA+peptide) to PLGA was 30/70.
(4)	The mixture was sonicated for 10 min at controlled powers to achieve a uniform dispersion of HA and peptide in PLGA.
(5)	After sonication, the mixture was cast into a Teflon mold, evaporated in air at room temperature for 24 hours, and dried in an air vacuum chamber at room temperature for 48 hours.

Table 5. 5: The detailed procedures that were followed for preparing the HA_Ps_PLGA systems.

(1)	PLGA was dissolved in chloroform at 40 °C in a water bath for 40 minutes.
(2)	HA_Ps nanoparticles were added into PLGA solution. The weight ratio of HA_Ps to PLGA was 30/70.
(3)	The mixture was sonicated for 10 min at controlled powers to achieve a uniform dispersion of HA_Ps in PLGA.
(4)	After sonication, the mixture was cast into a Teflon mold, evaporated in air at room temperature for 24 hours, and dried in an air vacuum chamber at room temperature for 48 hours.

5.3.2. Characterization of Nano-HA/PLGA Composites Loaded with the Model Peptide

5.3.2.1. Surface Characterization

Nano-HA/PLGA composites loaded with the peptide (such as HA_Pa_PLGA, HA_Pd_PLGA, and HA_Ps_PLGA) were characterized using a Field Emission Scanning Electron Microscope (FESEM, LEO 1530) at a 3 kV accelerating voltage. The nanocomposites and controls were sputter-coated with a thin layer of gold-palladium, using a Hummer I Sputter Coater (Technics) in a 100 mTorr vacuum argon environment for 3 min at 10 mA of current.

5.3.2.2. CBQCA Assay

A novel 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA, Molecular Probes) fluorescence technique was used to characterize the loading of the peptide onto the nano-HA. This technique could provide ultrasensitive detection of primary amines. Inherently CBQCA is a non-fluorescence molecule, but it becomes highly fluorescent

upon reaction with amine groups in the presence of cyanide molecules, as shown in Figure 5.6 [273]. CBQCA reacts specifically with primary amines to form conjugates that are highly fluorescent and the sensitivity of detection of CBQCA conjugates could reach the attomole range (10^{-18} moles).

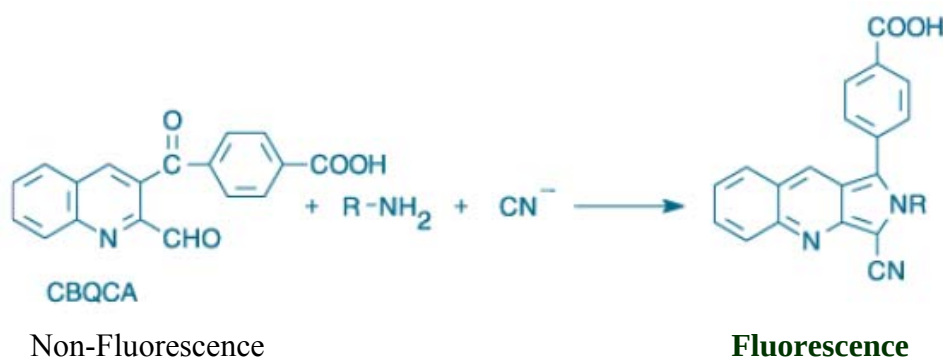


Figure 5. 6: The CBQCA reaction illustrates the transformation of the non-fluorescent CBQCA molecule into a fluorescent molecule when it reacts with amine groups in the presence of a cyanide catalyst.

CBQCA reagent solutions were prepared by dissolving the CBQCA (MW = 305.3 g/mol) in dimethylsulfoxide (DMSO, Sigma D2650) (10 mM). Potassium cyanide (KCN, MW = 65.1, Sigma 60178) was dissolved in DI water to give a 10mM working solution. Nano-HA particles with or without the peptide were exposed to CBQCA and potassium cyanide working solutions for 2 hours at room temperature. These Nano-HA particles were then carefully transferred onto a glass cover slip using a micropipette and visualized under a fluorescence microscope (LEICA DM5500B upright fluorescence microscope). Images were obtained using Image Pro software.

5.3.3. *In Vitro* Drug Release Profiles and Degradation of Drug Carriers

In vitro nano-HA/PLGA degradation and the peptide release kinetics were studied in PBS (pH=7.4). All samples of interest were incubated in PBS under standard cell culture conditions for 52 days. After 1, 3, 5, 7, 30, and 52 days, the supernatants were collected and analyzed. The appearance of specimen integrity was monitored and used to estimate the speed of degradation. The peptide release from scaffolds into culture solution was determined using a micro-BCA assay (Pierce). Briefly, the peptide DIF-7c standards were prepared by a serial dilution and the working reagent was mixed according to the established protocol [274]. Each standard and unknown sample were aliquoted in 150 μ L into a microplate well and mixed thoroughly with the working reagent on a plate shaker for 30 seconds. The reactions were incubated at 37 °C for 2 hours. The microplates were cooled to room temperature and read the absorbance at 562 nm using a spectrophotometer (SpectraMax[®] 340 PC, Molecular Devices). A standard curve was generated by plotting the average Blank-corrected 562 nm reading for each peptide standard versus its concentration in μ g/mL. The peptide concentration in the supernatants was calculated according to the standard curve.

5.4. Results and Discussions

5.4.1. Characterization of Drug Loading

5.4.1.1. Surface Characterization

Scanning electron micrographs suggest that the PLGA_P maintained a very smooth surface similar to the PLGA, as shown in Figure 5.7. The top and bottom surfaces

of the HA_Pa_PLGA scaffolds demonstrated that the HA_Pa nanoparticles were well dispersed in the polymer matrix, as shown in Figure 5.8. The HA_Pd_PLGA scaffolds had similar surfaces to the HA_Pa_PLGA (Images not shown). Scanning electron micrographs of the HA_Ps_PLGA demonstrated that HA_Ps nanoparticles had a finer dispersion in the polymer matrix compared to the HA_Pa_PLGA and HA_Pd_PLGA scaffolds, as shown in Figure 5.9. In general, the distribution of nano-HA particles was uniform in these drug delivery systems after controlled sonication, whether these HA nanoparticles were functionalized chemically or physically.

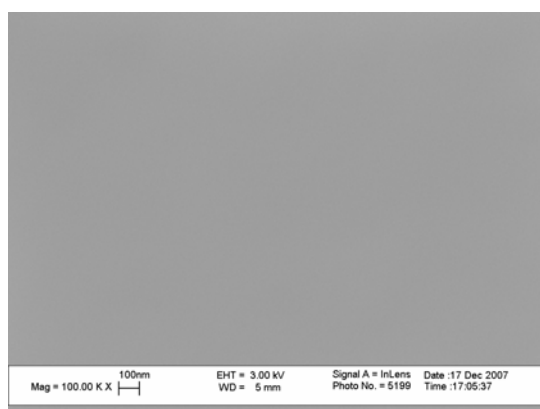


Figure 5. 7: SEM images of the PLGA_P. Original magnification is 100 kX. Magnification bar is 100 nm.

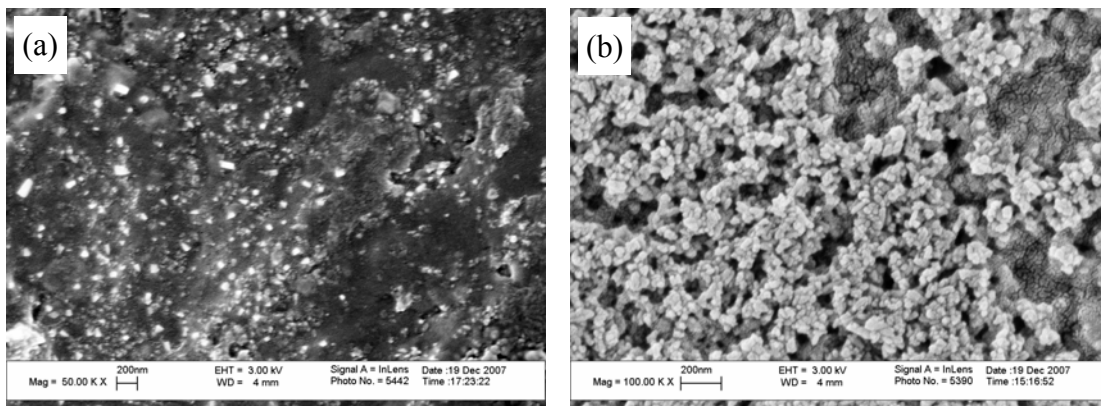


Figure 5. 8: SEM images of the HA_Pa_PLGA. Original magnifications are 50 kX for (a) and 100 kX for (b). Magnification bars are 200 nm.

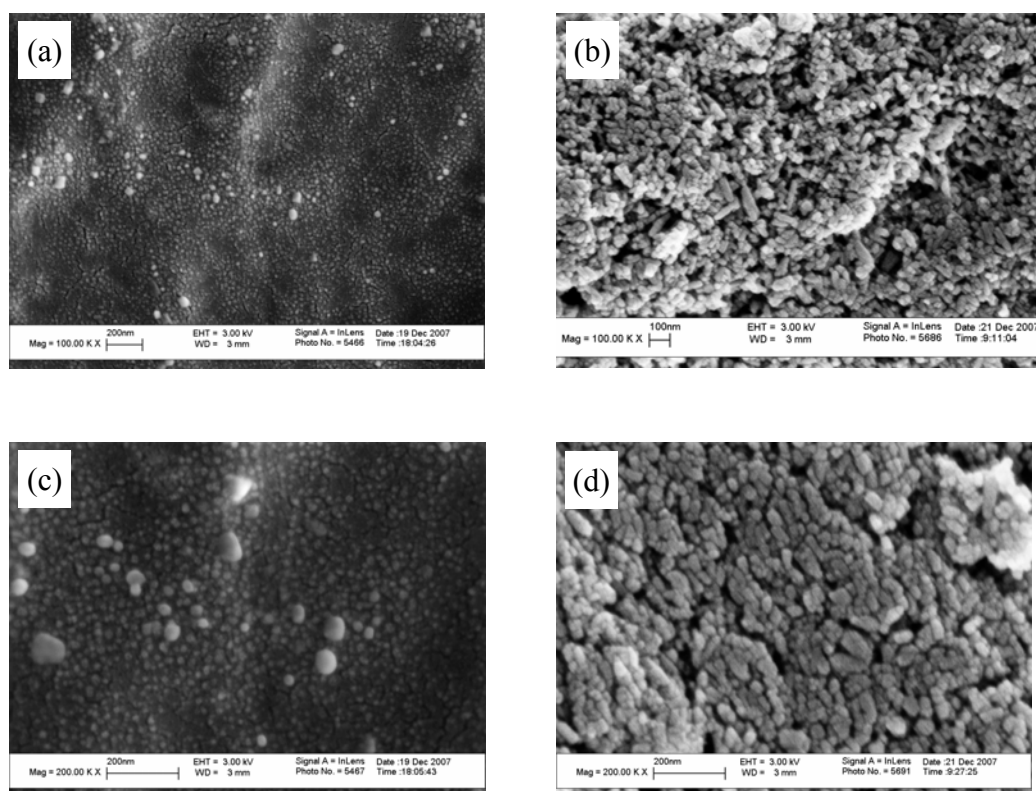


Figure 5. 9: SEM images of the HA_Ps_PLGA. Original magnifications are 100 kX for (a,b) and 200 kX for (c,d). Magnification bars are 200 nm for (a,c,d) and 100 nm for (b).

5.4.1.2. CBQCA Assay

The results of the CBQCA assay demonstrated the success of loading the peptide to nano-HA both chemically and physically, as shown in Figure 5.10 and Figure 5.11. In Figure 5. 10, nano-HA with chemically loaded peptide produced very good fluorescence (Figure 5. 10d), which indicated the successful attachment of the peptide to nano-HA. Moreover, in the absence of the CBQCA, APTES treated nano-HA did not fluorescence (image not shown). In contrast, in the presence of CBQCA, APTES treated nano-HA did fluorescence (Figure 5. 10b). Nano-HA after SMP reaction did not fluorescence (Figure 5. 10c), indicating that the amine groups were completely covered by the SMP. The nano-HA (without peptide) control did not show fluorescence (Figure 5. 10a), which provided evidence that the CBQCA did not react with HA and only reacted with the amino groups. In Figure 5.11b, nano-HA with physically loaded peptide (HA_Pa) produced detectable fluorescence at the same exposure conditions, although its signal strength was much weaker than that of nano-HA with chemically attached peptide (HA_Ps) as shown in Figure 5.10d. Figure 5.11a further confirmed the presence of fluorescence after CBQCA reacted with the peptide DIF-7c under the conditions defined in the CBQCA assay.

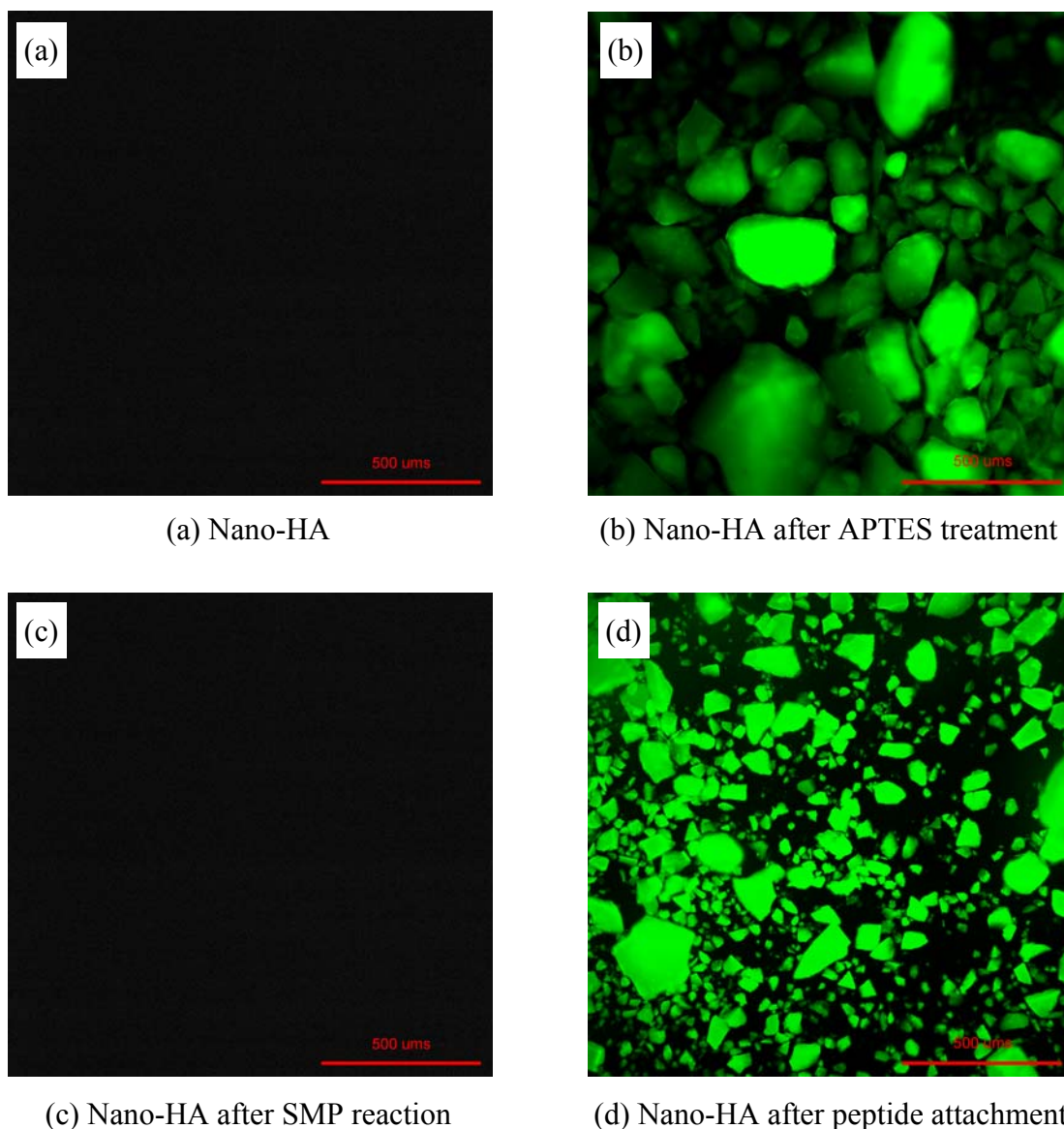


Figure 5. 10: The CBQCA analysis of nano-HA loaded with the model peptide DIF-7c by the chemical bonding method. Fluorescence images are: (a) nano-HA, (b) nano-HA after APTES treatment, (c) nano-HA after SMP reaction, and (d) nano-HA with the chemically attached peptide. Original magnifications are 10x. Scale bars are 500 μm . APTES: 3-aminopropyltriethoxysilane; SMP: N-succinimidyl-3-maleimido propionate; CBQCA: 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde.

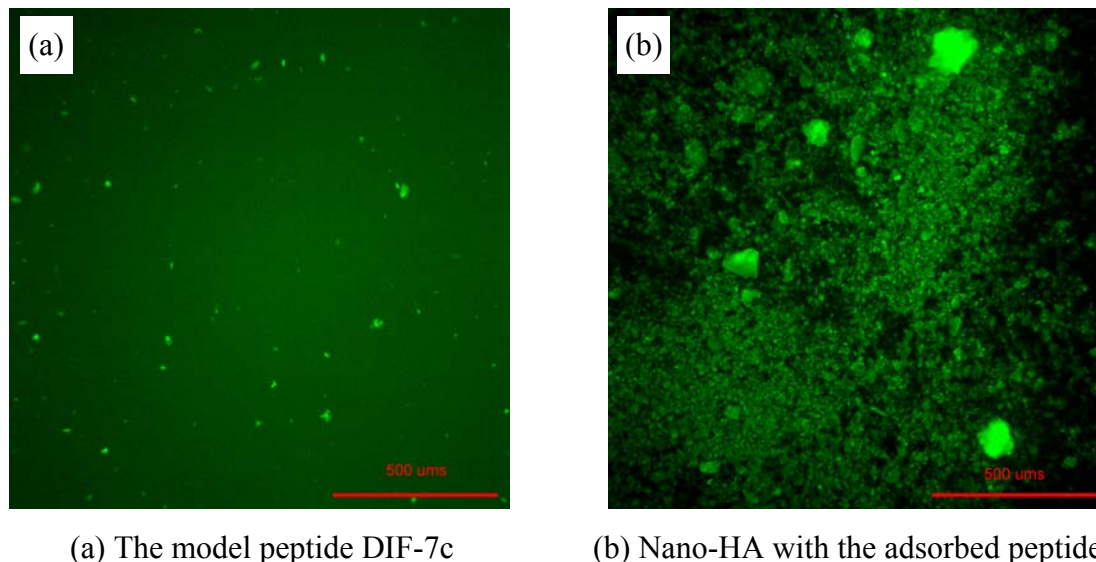


Figure 5. 11: The CBQCA analysis of nano-HA loaded with the model peptide DIF-7c by the physical adsorption method. Fluorescence images are (a) the peptide, and (b) nano-HA with the physically attached peptide. Original magnifications are 10x. Scale bars are 500 μ m.

5.4.2. *In Vitro* Drug Release and Degradation of Drug Carriers

5.4.2.1. *In Vitro* Drug Release Profiles

A series of drug therapies are usually necessary after orthopedic surgeries to prevent either infection or inflammation or to induce appropriate natural tissue integration with the implants. Currently, drugs (such as antibiotics, anti-inflammatory drugs and bone growth factors) are typically administered either orally or intravenously. These routes of drug delivery often result in limited bioavailability, thus, requiring high dosages for drugs to be effective at the site of implantation. The ideal situation is delivering drugs directly at the interface of the implant and tissue. In other words, drug carrying implants that are capable of controlled drug release may provide a promising approach for treating bone diseases at targeted sites.

The release of peptide DIF-7c *in vitro* was studied for up to 52 days, as shown in Figure 5.12. In Figure 5.12(a), the single phase drug carriers, including PLGA_P, HA_Pa, HA_Ps, all demonstrated one-phase release, although the major release happened at different time points for the HA carrier and the PLGA carrier. Specifically, the HA carrier (HA_Pa and HA_Ps) started the peptide release at day 1, while the PLGA carrier did not release any peptide until day 7. At day 30, the HA carrier stopped the peptide release, while the PLGA carrier showed evidence of peptide release. At day 52, the PLGA carrier continuously showed peptide release, while HA carrier did not release any peptide. The HA carrier demonstrated continuous peptide release from day 1 to 7. From day 1 to day 7, the total amount of peptide released by the HA_Ps was greater than the HA_Pa. It was speculated that the HA_Ps had higher peptide loading efficiency compared to the HA_Pa. That is, chemical functionalization permitted more peptide to be attached onto nano-HA compared to physical adsorption when the same peptide/HA ratio was used. The higher fluorescence intensity of the HA_Ps (Figure 5.10d) compared to the HA_Pa (Figure 5.11b) under the same exposure conditions also provided evidence for the higher peptide loading efficiency. In Figure 5.12(b), the composite drug carriers, including HA_Pd_PGA, HA_Pa_PLGA, and HA_Ps_PLGA, all demonstrated two-phase release. At phase I (from day 1 to 7), the HA_Ps_PGA demonstrated continuous peptide release, while the HA_Pa_PLGA and the HA_Pd_PLGA stopped releasing at day 5 and day 7. At phase II, the HA_Ps_PLGA demonstrated increased peptide release from day 30 to 52, while the HA_Pa_PLGA demonstrated decreased release from day 30 to 52. The HA_Pd_PLGA did not show any release at day 30, but showed peptide release at day 52.

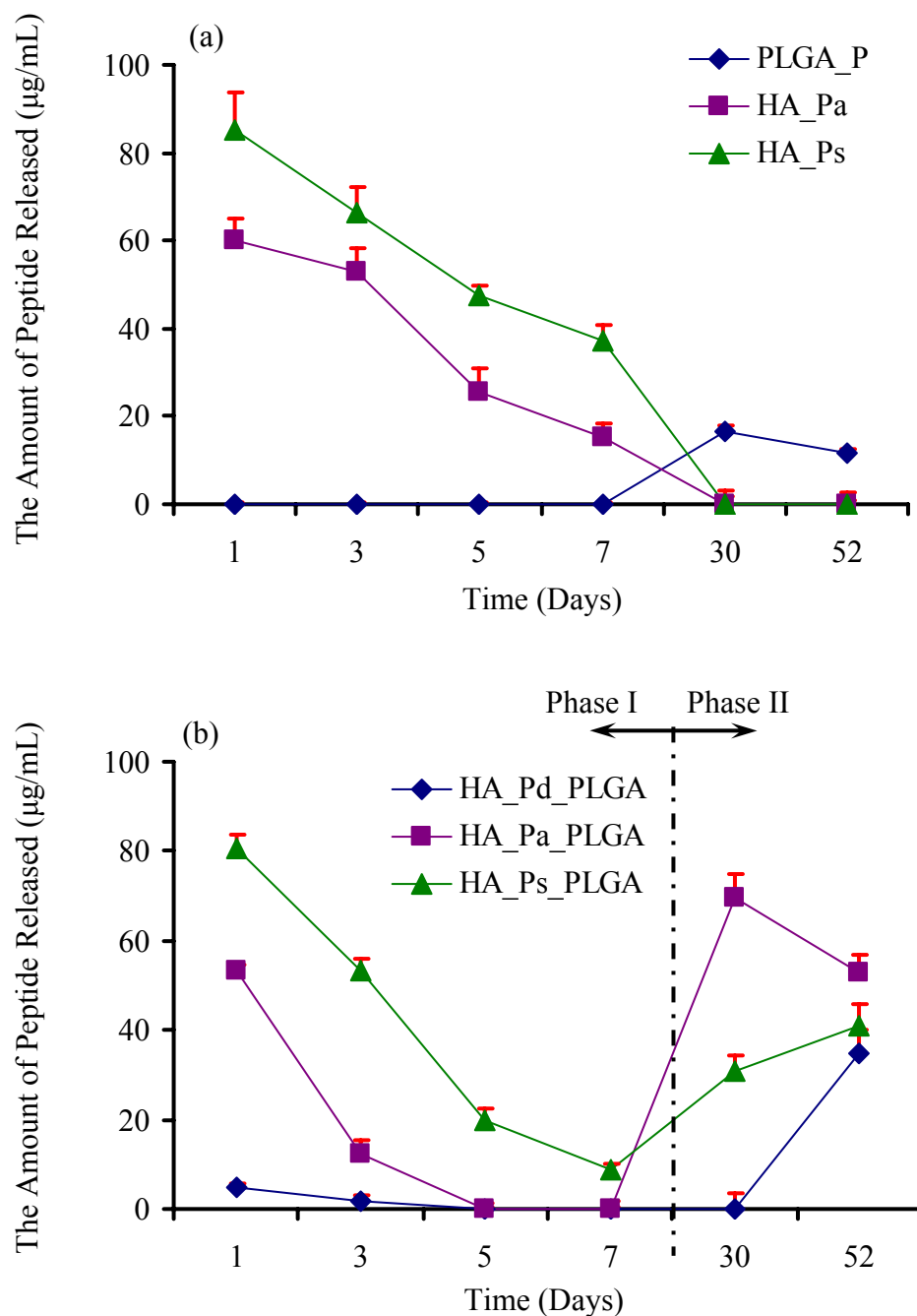


Figure 5. 12: The amount of peptide DIF-7c released from the drug delivery systems of interest to this study. The peptide concentration in the collected supernatant was determined by MicroBCA assay (Pierce). (a) Peptide released from the controls: PLGA_P, HA_Pa, and HA_Ps. (b) Peptide released from the nanocomposites: HA_Pd_PLGA, HA_Pa_PLGA, and HA_Ps_PLGA. Values are mean $\pm$ SEM; $N=3$. For each type of drug carriers, the amount of peptide released at the prescribed time points are significant different from one another, $p<0.05$, $N=3$.

Figure 5.13 summarized the total amount of peptide released from the various drug delivery systems during 52 days of culture *in vitro*. Among the single phase drug carriers, the total peptide released from the HA_Ps was the highest; the HA_Pa was the second; and the PLGA_P was the lowest. Among the composite drug carriers, the total peptide released from the HA_Ps_PLGA was the highest; the HA_Pa_PLGA was the second; and the HA_Pd_PLGA was the lowest.

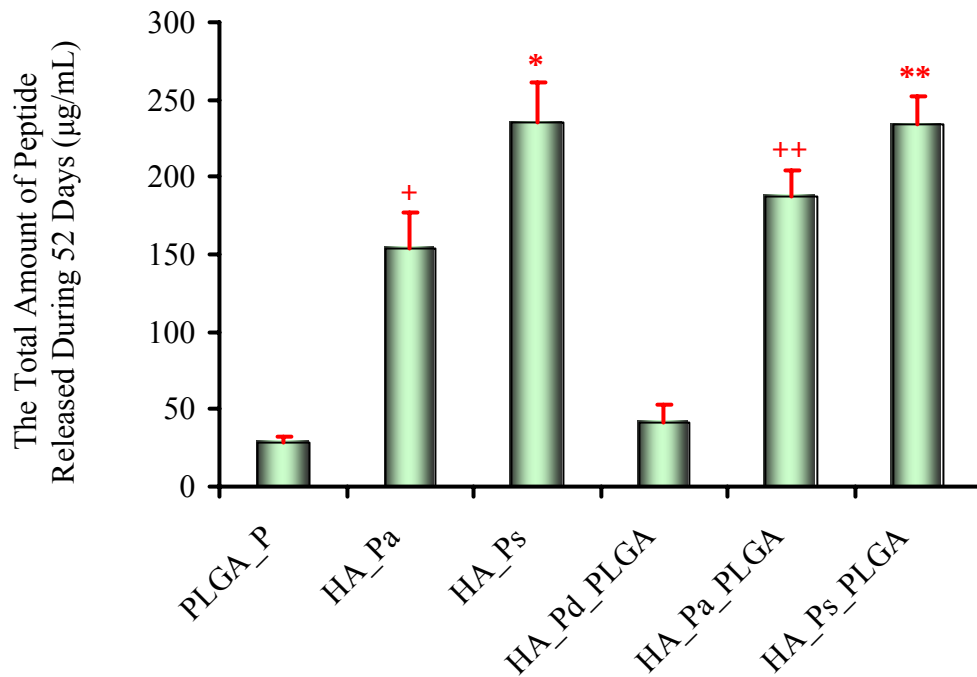


Figure 5. 13: The total amount of peptide DIF-7c released from the drug delivery systems during 52 days of culture *in vitro*. Values are mean $\pm$ SEM; N=3. * $p < 0.05$ compared to PLGA_P and HA_Pa; ** $p < 0.05$ compared to HA_Pd_PLGA and HA_Pa_PLGA; + $p < 0.05$ compared to PLGA_P; and ++ $p < 0.05$ compared to HA_Pd_PLGA.

Clearly, both drug carriers and drug loading methods played important roles in the drug release profiles. The drug carriers studied in this chapter provided a wide range of drug release profiles, from one-phase release to two-phase release. These results provided

important information for designing the drug carriers and drug loading methods for different biological applications. For example, if one application requires a short-term release (such as antibiotics release in a short time after surgeries), the single phase carriers such as HA_Ps or HA_Pa would be a good choice. If another application (such as growth factors for promoting bone regeneration) requires a long-term release, HA_Ps_PLGA would be a better choice. When selecting a drug carrier and a drug loading method, all the biological factors and characteristics of drug release should be considered and balanced for an optimal match with the appropriate orthopedic application.

5.4.2.2. Degradation of Drug Carriers

As reported in Chapter 2 that titania nanoparticles mediated PLGA degradation, nano-HA had similar effects on PLGA degradation. Moreover, the drug release profiles are related to the degradation kinetics of the nanocomposites and types of bonding formed between drug and carrier. Figure 5.14 showed the appearance of the drug carriers after 30 and 52 days of culture in PBS under standard cell culture conditions. In Figure 5.14(a), after 30 days, HA_PLGA maintained their integrity, while PLGA and PLGA_P lost their integrity and shape. In Figure 5.14(b), after 30 days, HA_Ps_PLGA maintained their integrity, while HA_Pa_PLGA and HA_Pd_PLGA showed apparent degradation trace around the specimens. In Figure 5.14(c), after 52 days, HA_PLGA, PLGA and PLGA_P completely degraded. In Figure 5.14(d), after 52 days, HA_Pd_PLGA had less than 10% of scaffolds left; HA_Pa_PLGA significantly shrank 50-60% and lost their original shape; and HA_Ps_PLGA shrank less than 20% and maintained their shape and integrity.

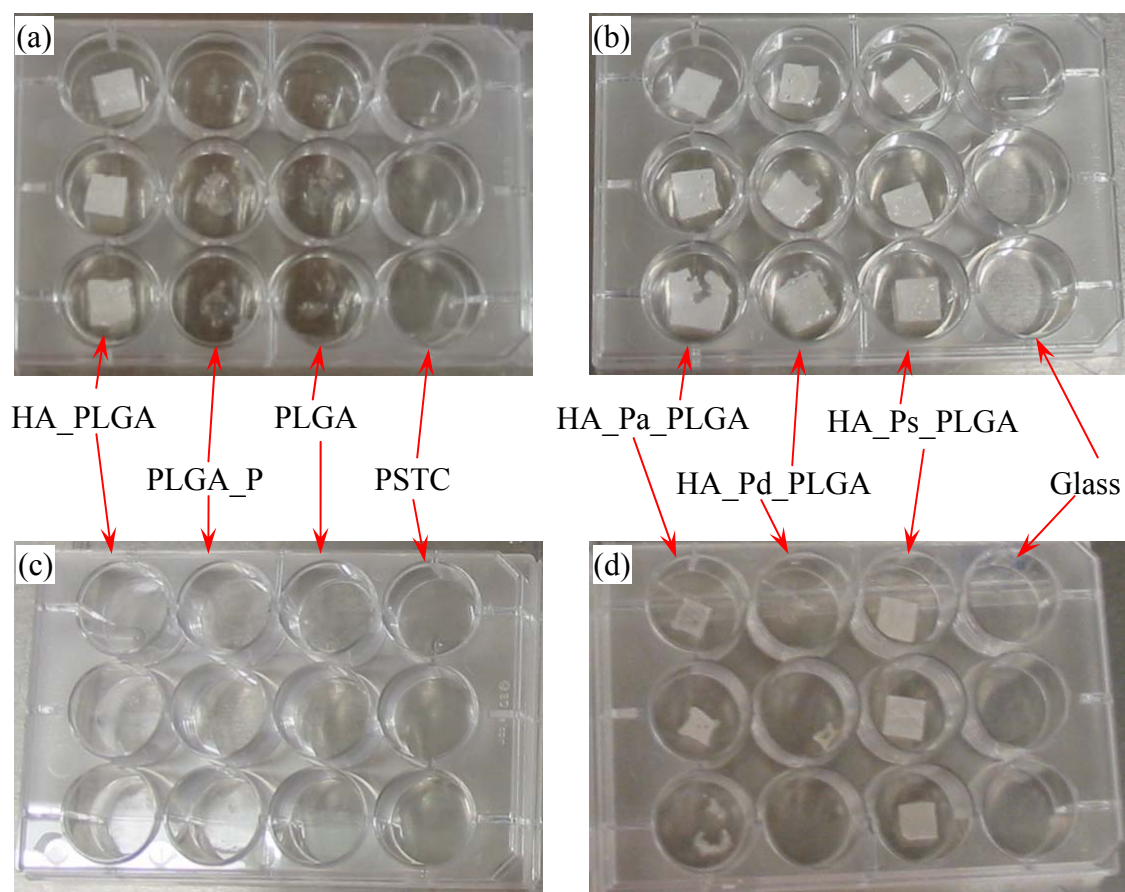


Figure 5. 14: The appearance of drug carriers after 30 and 52 days of culture *in vitro*. (a,b): after 30 days of culture. (c,d): after 52 days of culture.

5.5. Conclusions

Results of this chapter demonstrated a wide range of drug release profiles achieved by using various drug carriers and drug loading methods. The drug loading efficiency are also related to the drug carriers and the loading methods. Single phase drug carriers (such as HA_Pa, HA_Ps and PLGA) provided one-phase release profiles. The nanocomposite drug carriers demonstrated two-phase release profiles. Importantly, a

prolonged peptide release (up to 52 days) was achieved on the HA_Ps_PLGA drug delivery systems.

The drug carriers and the drug loading methods are very important factors that should be considered when designing the next generation of drug carrying orthopedic prostheses for various clinical applications. The appropriate drug carriers and drug loading methods should be carefully chosen for specific applications. Results of this chapter presented a useful guideline for designing more effective, controlled drug delivery systems.

CHAPTER 6. CONCLUSIONS AND PROPOSALS FOR FUTURE RESEARCH

6.1. Summary of Major Conclusions

This dissertation provided valuable information for designing and developing nanostructured ceramic/polymer composites as next generation orthopedic prostheses for more effective bone tissue regeneration.

Surface properties (such as topography, surface roughness and surface area) of nanostructured 2D titania/PLGA composites were successfully controlled by low- to high-power sonications and consequent dispersion states of nano-titania in PLGA composites. The results demonstrated a strong correlation between the surface properties of nanostructured composites and the *in vitro* osteoblast responses. Osteoblast adhesion and long-term functions (such as collagen synthesis, alkaline phosphatase activity, and calcium-containing mineral deposition) significantly increased on well-dispersed nano-titania in PLGA composites compared to single-phase PLGA and agglomerated nano-titania in PLGA composites. Moreover, well-dispersed nano-titania in PLGA composites decreased the harmful acidic pH changes of PLGA as it degrades, reduced weight loss of the nanocomposites, and prolonged the mechanical integrity of the nanocomposites necessary for matching bone growth.

Not only were surface properties investigated for bone cell functions, but also 3D structures built from nano-titania/PLGA composites. The aerosol-based 3D printing

technique produced well-ordered bone-like structures and successfully preserved nano-dispersion of ceramic in the final polymer composites, critical for optimizing bone cell functions. The results demonstrated that the 3D printed nanostructured titania/PLGA composites promoted bone cell infiltrations and subsequent bone ingrowth.

From the perspective of mechanical properties, well-dispersed ceramic nanoparticles (titania or HA) in PLGA composites promoted mechanical properties as compared to the PLGA and the agglomerated ceramic/PLGA composites. Specifically, well-dispersed nano-titania/PLGA composites promoted the tensile modulus, tensile strength at yield, ultimate tensile strength and compressive modulus as compared to PLGA and the more agglomerated nano-titania/PLGA composites. Nano-HA/PLGA nanocomposites also demonstrated greater tensile modulus, tensile strength and compressive modulus than the PLGA. Moreover, well-dispersed nano-HA in PLGA composites demonstrated a greater ductility than the more agglomerated nano-HA/PLGA composites.

Finally, nanophase ceramic/polymer composites were explored for controlled drug delivery applications. The results demonstrated a wide range of drug release profiles achieved by using various drug carriers and drug loading methods. The drug loading efficiency are also related to the drug carriers and the loading methods. Single phase drug carriers (such as HA_Pa, HA_Ps and PLGA) provided one-phase release profiles. Specifically, HA_Pa or HA_Ps provided an early stage release while PLGA offered a later stage release. All of the nanocomposite drug carriers demonstrated two-phase release profiles. Importantly, a prolonged peptide release (up to 52 days) was achieved on the HA_Ps_PLGA drug delivery systems.

6.2. Key Criteria and Considerations for the Next Generation of Orthopedic Prostheses

- (1) Surface properties: It is necessary to create more bone-like nanostructured surfaces in terms of topography, feature size, surface roughness, surface area, and etc.
- (2) 3D structures: It is critical to produce more bone-like 3D hierarchical structures using novel nanofabrication techniques.
- (3) Degradation properties: It is crucial to design materials with a tunable degradation rate to match the rate of bone regeneration.
- (4) Mechanical properties: It is essential to mimic mechanical properties of bone for load-bearing situations.
- (5) Controlled drug delivery: It is important to design drug-carrying implants to deliver necessary drugs to targeted sites at a desirable rate to facilitate bone healing.

6.3. Proposals for Future Research

6.3.1. Building 3D Tissue Constructs at the Patient Bedside by Rapid Prototyping Techniques

Previous studies in Chapter 3 demonstrated the feasibility of fabricating 3D nanocomposite scaffolds using an aerosol-based 3D printing technique. This 3D printing technique should be continuously investigated to build 3D tissue constructs that have tissue-like hierarchical inner architectures and outer structures of tissue defects. Future research should concentrate on designing CAD models for various tissue types, printing such structures, developing techniques that can potentially be used at the patient bedside

just before implantation, and investigating 3D interactions of such constructs with differentiated cell types (such as osteoblast, chondrocyte, endothelial cells, etc.) as well as stem cells.

6.3.2. Controllable Drug-Carrying Implants for Treating Bone Diseases at Targeted sites

The research goal is to conjugate bone substituting materials with bone morphogenetic proteins (BMPs), BMP-derived peptides and, for the future, other agents (such as antibiotics and anti-inflammatory drugs) to treat bone diseases (such as osteoporosis, osteomalacia, osteosarcoma, etc.) and promote bone healing. Drug-loading efficiency, drug release kinetics, and capability of targeting specific sites, are key problems that greatly influence the effectiveness of drugs. Therefore, future studies should focus on developing more effective drug delivery methods (such as combining current discoveries in this dissertation with magnetic nanoparticles) for enhancing drug-loading efficiency and prolonging drug release at targeted sites. Proteins, peptides, and anti-inflammatory agents (such as dexamethasone), among others, should be used as model drug molecules. *In vitro* drug release should be investigated using established tissue culture techniques and biochemical assays.

6.3.3. Stem Cell Differentiation on Nanocomposites Functionalized with Peptides

Previous studies in Chapter 5 demonstrated the success in functionalizing nanophase ceramic/polymer composites with a model peptide. Future research should also investigate the differentiation of mesenchymal stem cells (MSCs) in response to such

nanocomposites functionalized with short peptides since their differentiation to bone cells plays a crucial role in the success of bone regeneration. The short peptides can be specially designed or derived from growth factors that favor MSCs differentiating into osteoblasts or chondrocytes for various orthopedic applications. Future work should investigate the factors and mechanisms controlling the differentiation of mesenchymal stem cells *in vitro*. The long range goal of this proposed research should be to develop methods to direct the differentiation or development of stem cells along specific cell lineages to yield replacement cells for clinical use.

6.3.4. Animal Models for Preclinical Evaluations of Tissue Substitutes

It is necessary to develop animal models for testing tissue substitutes *in vivo* before clinical trials. Since this dissertation included *in vitro* studies, it is clear that *in vivo* drug release of some of the most promising materials here and their *in vivo* tissue integration should be conducted. Different animal models are needed for different orthopedic applications. For example, rabbit fracture models could be developed for testing low load-bearing bone substitutes. However, for large high load-bearing implant systems, sheep models should be developed.

6.4. Challenges, Promises and Ultimate Dreams

In this dissertation, nanostructured 2D to 3D ceramic/polymer composites have been designed, fabricated and evaluated for the goal of developing more effective

orthopedic prostheses with suitable biological and mechanical properties for bone regeneration and the capability of delivering drugs to the tissue-implant interface in a more controlled fashion. This dissertation has demonstrated how 2D surface properties (such as surface roughness and area) and well-ordered 3D structures can be controlled through the manipulation of fabrication techniques (such as sonication and 3D printing); how bone cells interact with these 2D to 3D nanostructures; how mechanical properties of these nanophase ceramic/polymer composites can be optimized through controlling the dispersion of nanophase ceramics in the polymer matrix; and how these nanocomposites can be used as drug carriers to achieve prolonged two-phase drug release profiles through regulating several variables (such as chemical or physical drug loading methods and degradation kinetics of drug carriers).

The potential undoubtedly exists to refine nanophase ceramic/polymer composites for the ultimate goal---replacing diseased or injured bone in a natural way, as shown in Figure 6.1. Figure 6.1 illustrates an ideal situation. That is, bone substitutes provide the appropriate support for the cells to proliferate and differentiate; their internal 3D structures allow for proper diffusion of oxygen and nutrients to cells as well as proper diffusion of waste from the cells; and their external architectures define the final shapes of newly regenerated bone. The eventual goal is to return full biological, physiological and mechanical functionality to a damaged bone tissue.

Over the past decade, it has been realized by researchers that the ideal situation illustrated in Figure 6.1 is truly difficult and challenging to achieve, but seemingly still very promising. The need for bone replacement will continuously increase as the population ages. This dissertation has revealed many factors that influence bone

regeneration and the complexity of ideal bone replacement; and it is still believed that this is an exciting and incredible research avenue.

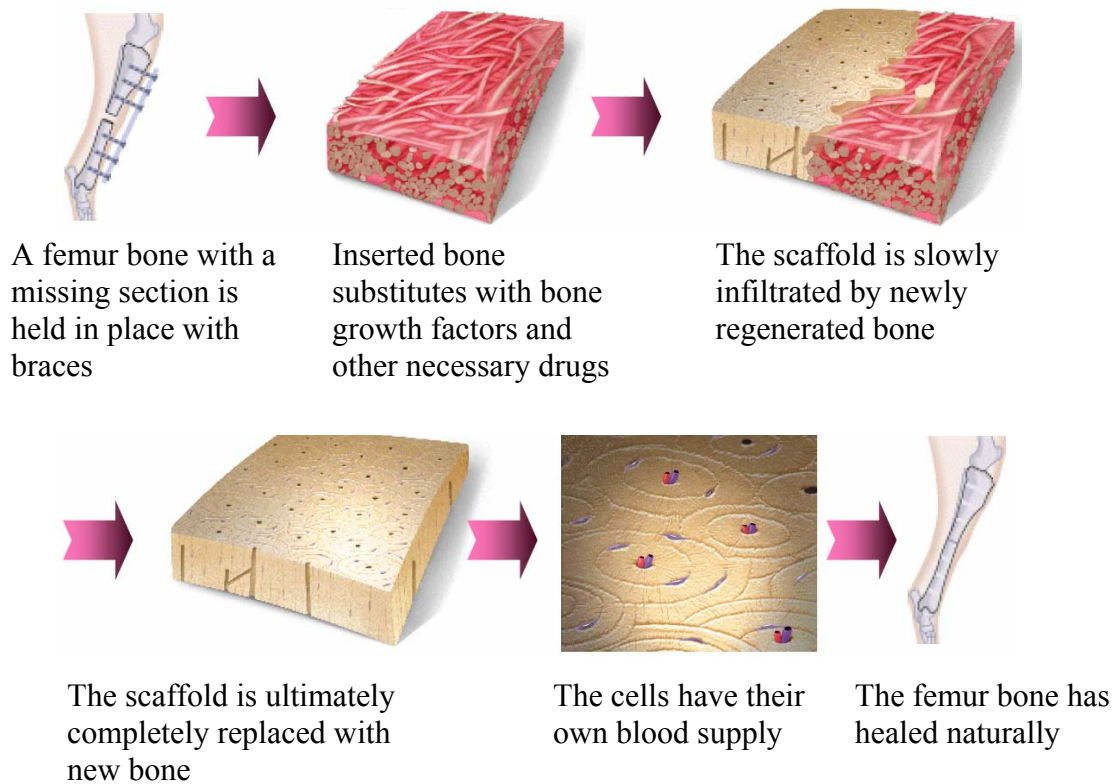


Figure 6. 1: Schematic diagram illustrating an ideal situation of bone regeneration. Bone substituting materials will resorb after fulfilling their initial tasks, thus, ideally, nothing foreign left in these patients. (Adapted and redrawn from [275]).

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