Electrosprayed Chitosan-Calcium Phosphate Nanoshells Composite Coatings on Silanated Titanium Plates

Andrew Blass Watson

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ELECTROSPRAYED CHITOSAN-CALCIUM PHOSPHATE NANOSHELLS COMPOSITE COATINGS ON SILANATED TITANIUM PLATES

By

Andrew Blass Watson

A Thesis

Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

Major: Biomedical Engineering

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ABSTRACT

The aim of this work was to take advantage of the electrospray technology and the osteocompatibility of both chitosan and calcium phosphate (CaP) to fabricate chitosan coatings loaded with CaP nanoshell particles. The key electrospray parameters of capillary diameter, voltage, and pressure were identified and adjusted for creating uniform chitosan-CaP coatings on titanium surfaces. Coatings containing from 0 to 1 wt% CaP particles to chitosan mass were electrosprayed on to silanated titanium to create composite coatings bonded to the metal surface. Scanning electron microscope (SEM) and energy dispersive X-ray spectroscopy (EDS) showed that CaP particles were homogenously distributed throughout the chitosan coating. Mechanical tensile testing demonstrated that there was no statistically significant loss of coating adhesion strength up to 1.0 wt% incorporation of CaP particles. Furthermore, the incorporation of CaP particles was shown to support growth of bone cells on the coating. We have demonstrated that CaP particles can successfully be incorporated into electrosprayed chitosan coating.
# TABLE OF CONTENTS

LIST OF FIGURES ....................................................................................................................... ix  
LISTS OF TABLES ........................................................................................................................ xi  
LIST OF ABBREVIATIONS .......................................................................................................... xii  

CHAPTER I ........................................................................................................................................ 1  
INTRODUCTION ............................................................................................................................. 1  
Statement of Clinical Problem ....................................................................................................... 1  
Research Aims ............................................................................................................................... 6  

CHAPTER II ....................................................................................................................................... 8  
LITERATURE REVIEW ................................................................................................................... 8  
Implant materials ........................................................................................................................... 8  
Bone ............................................................................................................................................... 10  
Osseointegration ............................................................................................................................ 11  
Coatings ......................................................................................................................................... 12  
Chitosan ......................................................................................................................................... 14  
Surface Coating Methods ............................................................................................................. 16  
Electrospraying ............................................................................................................................. 20  

CHAPTER III .................................................................................................................................... 23  
Electrosprayed Chitosan -Calcium Phosphate Nanoshells Composite Coatings on Silanated  
Titanium Plates ............................................................................................................................. 23  
Abstract .......................................................................................................................................... 23  
Introduction ...................................................................................................................................... 23  
Materials ......................................................................................................................................... 26  
Methodology ..................................................................................................................................... 27
Sample Preparation .............................................................................................................................................. 27
Characterization .................................................................................................................................................. 30
Results ..................................................................................................................................................................... 33
Optimization of Electrospraying Parameters .............................................................................................. 33
Particle size, charge determination and surface morphology of CaP nanoshells........................................... 36
Determination of silane deposition on titanium samples ................................................................................. 38
Determination of surface morphology of chitosan coatings ......................................................................... 39
Determination of bond strength of the chitosan coating ............................................................................... 43
Determination of hydrophilic nature over chitosan coating ......................................................................... 45
In-vitro evaluation of chitosan coatings ........................................................................................................ 46
Discussion ......................................................................................................................................................... 48

CHAPTER IV ..................................................................................................................................................... 52

CONCLUSION .................................................................................................................................................. 52

CHAPTER V ....................................................................................................................................................... 53

FUTURE WORK AND RECOMMENDATION ............................................................................................... 53

References ......................................................................................................................................................... 55

APPENDIX ....................................................................................................................................................... 60
LIST OF FIGURES

Figure 1. Parts of a total hip replacement. A) The titanium femoral stem. B) Co-Cr femoral head. C) UHMWPE articulation surface. D) Titanium acetabular cup. ....................................................... 2

Figure 2. A 250 µm capillary tube with CaP arrows indicating aggregate clogs. ......................... 34

Figure 3. Titanium sample that had coating process stopped due to capillary clogging mid-spray. Arrow A indicates where coating is, arrow B indicates where coating is not. ......................... 34

Figure 4. Titanium sample that had coating process halted after oversaturation (flooding) with chitosan. ........................................................................................................ 35

Figure 5. Successful Chitosan CaP Coating ................................................................................. 36

Figure 6. SEM of Calcium Phosphate nanoshells ......................................................................... 37

Figure 7. SEM with EDS overlay of Calcium Phosphate nanoshells surface ................................. 38

Figure 8. Prediction of surface chemistry and bonding of silane and chitosan to titanium using FTIR spectra. Spectra 1 corresponds to a cleaned titanium. Spectra 2 corresponds to titanium that has undergone NaOH treatment. Spectra 3 corresponds to silanated titanium. ......................... 39

Figure 9. Surface morphology recorded using SEM of electrosprayed chitosan coatings having (A) no added CaP shells, (B) 0.25 wt % CaP shells, (C) 0.5 wt% CaP shells, and (D) 1.0 wt% CaP .... 40

Figure 10. SEM with false-color, EDS overlay of chitosan (pink) coating surface on titanium loaded with CaP particle (green). Carbon-pink, green-oxygen, yellow-calcium and blue-phosphorous. (A) 0.25 wt % CaP shells, (B) 0.5 wt% CaP shells, and (C) 1.0 wt% CaP .......... 41

Figure 11. SEM with false-color, deep-scanned EDS overlay of cross-section of Ti (green) with chitosan coating (blue and red) with incorporated CaP particle (yellow and pink) ..................... 42

Figure 12. EDS of the cross-section is separated into respective components. (A) Cross-section of Titanium (green). (B, C) Carbon and oxygen of the chitosan coating (blue and red). (D, E)
Phosphorus and calcium of incorporated CaP particle (pink and yellow). (F) Silicon from TESBA on titanium................................................................................................................................................. 43

**Figure 13.** Results of tensile bond strength (MPa) of silanated electrosprayed coatings with and without Calcium Phosphate nanoshells. Error bars correspond to standard deviation. Letters correspond to groups with no statistically significant differences. .................................................. 44

**Figure 14.** Coating post tensile test failure. (A) electrosprayed, silanated, no CaP loaded; (B) electrosprayed, non-silanated, no loaded CaP; (C) electrosprayed, silanated, 0.25 wt% CaP loaded; (D) electrosprayed, silanated, 0.5 wt% CaP loaded; and (E) electrosprayed, silanated, 1.0 wt% CaP loaded..................................................................................................................................................... 45

**Figure 15.** In-vitro Cytotoxicity study on Chitosan electrosprayed Titanium implant using W20-17 cells Non-normalized.................................................................................................................................................. 47

**Figure 16.** Day 1 of Coating ........................................................................................................................................................................ 48
LISTS OF TABLES

Table 1: Original electrospraying parameters for chitosan ................................. 30

Table 2. Revised electrospraying parameters for chitosan loaded with CaP nanoshells ........ 36

Table 3. ZETA Potential Results ................................................................................. 37

Table 4. Tensile bond strength (MPa) of silanated electrosprayed coatings with and without Calcium Phosphate nanoshells ................................................................. 44

Table 5. Table indicating the average results of the contact angle coatings as well as the 95 % confidence interval. ........................................................................................................ 46

Table 6: Two-Factor with Replication ANOVA for tensile testing of silanated vs test groups ..... 60

Table 7: Two-Factor with Replication ANOVA for tensile testing of non-silanated vs test groups. ......................................................................................................................... 60

Table 8: All pairwise multiple comparison procedures Holm-Sidak post-hoc test of tensile test. Overall significance level = 0.05. ........................................................................................................ 60

Table 9: Two-Factor with Replication ANOVA for Cell study. .................................................. 61

Table 10: Day 1 pairwise multiple comparison procedures (Student-Newman-Keuls Method) post-hoc test of tensile test. Overall significance level = 0.05. .................................................. 61

Table 11: Day 3 pairwise multiple comparison procedures (Student-Newman-Keuls Method) post-hoc test of tensile test. Overall significance level = 0.05. .................................................. 62

Table 12: Day 5 pairwise multiple comparison procedures (Student-Newman-Keuls Method) post-hoc test of tensile test. Overall significance level = 0.05. .................................................. 62
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<tr>
<th>Abbreviation</th>
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<td>µm</td>
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<td>2D</td>
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<td>Three dimensional</td>
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<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>CH</td>
<td>Chitosan</td>
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<td>cm</td>
<td>Centimeter</td>
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<tr>
<td>Co-Cr</td>
<td>Cobalt-chrome</td>
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<tr>
<td>DDA</td>
<td>Degree of deacetylation</td>
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<tr>
<td>DI water</td>
<td>Deionized water</td>
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<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
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<td>EDS</td>
<td>Energy-dispersive X-ray spectroscopy</td>
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<td>et al.</td>
<td>and others</td>
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<td>FTIR</td>
<td>Fourier-transform infrared spectroscopy</td>
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<tr>
<td>MPa</td>
<td>Megapascal</td>
</tr>
<tr>
<td>Non-silanated</td>
<td>Not treated by silane</td>
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<tr>
<td>Nm</td>
<td>nanometers</td>
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<tr>
<td>NSIADs</td>
<td>Nonsteroidal Anti-inflammatory Drugs</td>
</tr>
<tr>
<td>-OH</td>
<td>Hydroxyl</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic) acid</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
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<tr>
<td>rpm</td>
<td>Round per minute</td>
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<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
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<td>Silane-based treatment</td>
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<td>Tri-ethoxy-silylbutyraldehyde (TESBA) silane</td>
</tr>
<tr>
<td>Std dev</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>THR</td>
<td>Total Hip Replacement</td>
</tr>
<tr>
<td>Ti</td>
<td>Titanium</td>
</tr>
<tr>
<td>TJR</td>
<td>Total Joint Replacement</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>TKR</td>
<td>Total Knee Replacement</td>
</tr>
<tr>
<td>UHMWPE</td>
<td>Ultra-high-molecular-weight polyethylene</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
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<td>v/v</td>
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CHAPTER I
INTRODUCTION

Statement of Clinical Problem

Arthritis is the leading cause of disability among adults in the U.S. and, according to the CDC, about 54 million adults in the United States have been diagnosed with arthritis. Osteoarthritis is the most common type of arthritis, affecting an estimated 31 million Americans. Osteoarthritis occurs due to deterioration/degradation of the cartilage in the joint that can ultimately lead to direct contact between articulating bones of the joint and causing severe pain limiting mobility and function. Arthritis is more common among people with other chronic conditions and according to the CDC, after age-adjustment, adults who are obese, had diabetes, or heart disease are approximately 1.5, 1.7, and 1.9 times more likely than those without the corresponding condition to have arthritis. Economic cost of arthritis are far-reaching, affecting almost two-thirds of working age adults in the U.S. (18-64 years). According estimates by the CDC in 2005 the total costs from arthritis and related rheumatic conditions were about $128 billion. By the year 2040 it is expected that more than 78 million will be diagnosed with arthritis in general making arthritis one of the leading causes of disability among adults in the U.S.

The initial treatments therapies for arthritis typically use medication to treat the symptoms caused by the joint wear. Analgesics ease mild to moderate pain and Nonsteroidal Anti-inflammatory Drugs (NSAIDs) relieve pain and inflammation. While helpful in treating the symptoms of arthritis, these drugs do not address the degradation of the joints. In patients with severe cartilage loss/damage, and ongoing pain, limited function and diminished life
quality, biomedical total joint implant devices have become a reliable and effective means for replacing and restoring joint function. [4]

Total joint devices are typically composed of metal components, that are implanted into bone and metal, polymer or ceramic components to provide articulation of the devices. Figure 1 shows the components of a total hip device. Ti-based alloys such as Ti-6Al-4V widely used for the components implanted into due to their high corrosion resistance, biocompatibility and since their modulus is closer to that of native bone to reduce stress shielding. [5] Articulating components of total joint devices are commonly composed of Co-Cr alloys due to their high hardness and wear resistance and UHMWPE due to its low coefficient of friction. [6,7] In total joint devices like total hip and shoulder replacements that have a ball-and socket type joint, high strength ceramic materials like alumina can be used for the articulating components. [8]

Figure 1. Parts of a total hip replacement. A) The titanium femoral stem. B) Co-Cr femoral head. C) UHMWPE articulation surface. D) Titanium acetabular cup
While most implants will perform well over the span of 15-20 years about 5% of implants will suffer some type of failure. [1] The most common causes of total joint replacement (TJR) failure include polyethylene wear, infection, and implant component loosening. [9,10] Polyethylene particles come from wear debris from the lining of the joint articulating surface the or how they occur, and their accumulation in adjacent tissues leads to inflammatory processes leading to bone resorption and implant failure. Implant associated infections are a serious condition that leads to inflammatory responses, poor healing, or loss of integration of implants in the bone. Bacteria are then able colonize implant surfaces, create biofilms leading to implant associated infections. Bones can become infected in several ways: Infection in one part of the body may spread through the bloodstream onto the implant, or an open fracture or surgery may expose the bone and implant devices.

According Kim, Kyung Tae, et al. implant associated infections account for 26-40% of implant failures depending on the age group of the patients. Infection-related failures of total knee replacements (TKR) typically occurring within 2 years of implantation. [3] It was also found that there were higher failure rates of aseptic loosening in young patients which may be associated with the higher level of activities and functional demands, and longer remaining life expectancy in young patients. [3] In the case of other total joint replacements, in 2004 it was reported that of the over 250,000 THRs performed in the US, 94% were performed for osteoarthritis. [11] Deep infection occurred in around 0.5–2% of total hip replacements (THR) and was the cause for revision surgery in at least 7.5% of failures. Infection is more common in inflammatory arthritis, psoriatic arthritis, patients taking corticosteroid treatment, chronic renal failure, diabetes mellitus, high risk surgical patients, malnutrition, and older age. [1] This can be attributed to the formation of biofilms and the possibility of compromised immune system of the
patient. [4] However, the most common cause of revision in total hip replacements is aseptic loosening which causes 75% of failures. The main culprit of aseptic loosening is increased wear of early generation polyethylene, especially in thin and low-conformity inserts, associated with some implant designs such as discontinuous porous-coating cementless components. Additionally, surgical factors such as malalignment, malposition, and uncorrected instability may contribute to aseptic loosening. [11]

It is important to note that even though there are a relatively low numbers of implant failures, the costs can be disproportionately high. [12] The total inpatient cost of primary and revision total hip replacement exceeds $8 billion annually, 50% of THRs recipients discharged to inpatient care facilities for further rehabilitation, and the remainder generally have rehabilitation services provided at home, adding another several billion dollars to the total national annual cost of total hip replacements. [13]

Because of the past success of total joint replacement procedures, their use is expanding into demographically different groups. Today's population most likely to receive total joint replacements is demographically different than in the past. Compared with a few decades ago, patients currently receiving total joint replacements are more physically active, almost 20% heavier, live more than 25% longer, and are three times more likely to have a high school or college education.[14] Because of the expansion of implants into younger and more active patient groups with the expectation for longer use, osseointegration remains of critical importance to device success.

Key to the success of these implants to the ability to osseointegrate into host bone tissues. Osseointegration is defined as the direct structural and functional connection between living bone and the surface of a load-bearing artificial implant. An implant is considered
osseointegrated when there is no progressive relative movement between the implant and the bone with which it has direct contact. [15] This is important as micro-motion between the implant and adjacent tissues can lead to fibrous tissue formation and implant loosening causing implant failure. [16] The ability to osseointegrate is important to securely fixing devices in host bone tissues to prevent these types of failure.

Implant coatings could improve patient outcomes and minimize the need for revisions of TJRs due to septic or aseptic loosening. Bioactivity of orthopedic implants is essential for the development of early and long-term stability of the implant in contact interfaces with the bone tissue. [15, 17] Prosthesis-associated infections are one of the main causes of implant failure. [3] The ability to deliver therapeutic drugs like bone growth factors and antibiotics to the implant-bone interface would help alleviate these problems and improve osseointegration.

Chitosan, a cationic linear polysaccharide biopolymer derived from chitin, exhibits properties of biocompatibility and biodegradability, as well as its bacteriostatic and drug delivery capabilities. [18] Chitosan has been widely studied in both bone tissue engineering and antimicrobial delivery applications as it possesses two of the most important characteristics of a local therapeutic delivery system: biodegradability and biocompatibility. [18] When chitosan degrades it breaks down into nontoxic products without causing any inflammatory reaction or producing any toxic end-products when the new tissues are formed. [18]

Researchers have also examined calcium phosphate coatings for increasing bioactivity of implants since CaP affects adhesion, proliferation, and new bone formation in osteoblasts. [19] While CaP has shown excellent ability to support bone cell attachment, growth and differentiation, the material is brittle, can suffer from delamination from substrates and have exhibited higher rates of bacterial infection. [20] The opportunity to combine the bioactivity,
antimicrobial, and drug delivery capabilities of chitosan that with the osseointegration properties of CaP has the potential to result in an implant coating that leads to and enhanced osseointegration of implant devices and with the potential to prevent infections and/or delivery therapeutics.

**Research Aims**

The goal of this work is to determine conditions for the manufacture of chitosan-CaP composite coatings using electrospraying technology for potential use in enhancing osteointegration of orthopedic implants.

**Aim 1: Identify electrospray parameters for creating chitosan-CaP coatings on titanium surfaces.**

*Capillary size, voltage and pressure will be adjusted to create a visibly stable aerosol spray for depositing uniform chitosan-CaP composite coatings.*

**Aim 2: Determine the effects of incorporating CaP particles on the mechanical adhesive strength of electrosprayed chitosan coatings on Ti samples.**

*Composite coatings will be electrosprayed from chitosan solutions containing 0, 0.25, 0.5 and 1wt% CaP nanoparticles on to silanated Ti samples. Coatings will be examined by SEM/EDS for amount of CaP deposited in coatings and in tensile tests for effects on coatings adhesive strengths. Coating fracture surfaces will be examined by SEM and FTIR for adhesive-cohesive failures of the coatings.*

**Aim 3: Determine effects of incorporating CaP particles into electrosprayed coatings on coating hydrophilic properties and on ability to support bone cell attachment and growth.**
Coatings will be electrosprayed from chitosan solutions containing 0, 0.25, 0.5 and 1wt% CaP nanoparticles. Coatings will be examined using water contact angle measurements to determine effects of CaP nanoparticle incorporation on the hydrophilic characteristics of the coatings. The effects of CaP nanoparticle incorporation on the attachment and growth of bone cells will be evaluated over 5 days.
CHAPTER II
LITERATURE REVIEW

Implant materials
Implants can be constructed out of many different types for materials such as metals or polymers. Metals utilized in orthopedic implants include surgical grade stainless steel (commonly 316L), cobalt-chromium (Co-Cr) alloys and pure commercial titanium (Ti) or titanium alloys.

Stainless steel is one of the most common metallic alloys used in orthopedic applications. Type 316L cold-worked stainless steel is one of the most common types used in implant applications. The “L” denotes that this steel is a low carbon alloy for improved corrosion resistance and the “316” denotes that the steel is an austenitic class of stainless. Type 316L stainless steel selected for surgical implants contains approximately 17 to 19% of chromium, for corrosion resistance, and 14% nickel, to stabilize the austenite to stainless steel. [5, 21] Additionally, molybdenum is added to the stainless-steel alloy that forms a protective layer sheltering the metal from exposure to an acidic environment. [5]

Co-Cr alloys are well known for their biocompatibility due to a durable chromium oxide surface layer and the formation of Co- Cr- and Mo- carbides provide these alloys with higher wear resistance compared to Ti alloys. [22] Co-Cr also exhibits mechanical properties similar to stainless steel, a result of a multiphase structure and precipitation of metal carbides, which increase the hardness of Co-Cr alloy and gives high wear resistance. The alloy composition used in orthopedic implants is described in industry standard ASTM-F75: cobalt with 27 to 30% chromium, 5 to 7% molybdenum, and other important elements such as less than 1% manganese and silicon, less than 0.75% iron, and less than 0.5% nickel. Moreover, tensile and fatigue
strength increases radically as they are heat-treated. [23] However, Co-Cr alloys tend to have low ductility, which can cause component fracture, and Co-Cr’s high modulus can create a mismatch between the implant and bone which can contribute to stress shielding. Co-Cr alloys are most commonly used to make articulating components of total joints including knee and hip joints due to high wear-resistance and biocompatibility.[6] Co-Cr alloys tend to be corrosion resistant and chemically inert. This minimizes the possibility of irritation, allergic reaction, and immune response from surrounding tissues. However, despite these implants’ good corrosion resistance, release of chromium, cobalt and nickel ions in vivo are a major concern as these metals are known carcinogens.

The most common Titanium alloy used in total joint devices is Ti-6Al-4V since it has sufficient mechanical strength with a modulus close to native bone. This two-phase alloy contains 6% Al and 4% Va. (Ti 6 Al 4V). [5] These added elements act as Phase- condition stabilizers. Aluminum is alpha-phase condition stabilizer and it also increases the strength and decrease the weight of the alloy. Vanadium acts as beta-phase stabilizer. The alloys most commonly used for orthopedic implants are of the alpha-beta variety. [24]

Titanium and its alloys are more corrosion resistant than Co-Cr alloys and 316L stainless because of the formation of titanium oxide on the surface. [24] However, the surface oxide layer is not very resistant to wear and thus is not optimal for articulating surfaces. Abrasion of the oxide layer can lead to the release of particles into the surrounding tissues causing undesirable tissue response such as aseptic loosening. However, because of excellent corrosion resistance, high strength and a modulus closer to bone than stainless steel or CoCr alloys, titanium is used where integration into bone is important like the stem of the femoral component of total hip implant. [24]
Bone

Bone is the framework of the body and is responsible for a host of functions including aiding in motion, housing the internal organs, providing structure, storing and distributing ions, and assisting in blood cell formation. It is a composite of organic products of cellular synthesis and mineral deposited by extracellular matrix calcification. There are two main types of bone known as cortical and cancellous bone.

The hard, outer layer of bones is composed of cortical bone, also called compact bone as it is much denser than cancellous bone. It forms a hard-dense layered exterior (cortex) of bones and functions to support the whole body, protect organs, provide levers for movement, and store and release chemical elements. [25] Cortical bone is very important for maintaining body structure and weight bearing because of its high resistance to bending and torsion.

Typically found at the ends of long bones, near joints and in the interior of vertebrae, cancellous bone, also called trabecular or spongy bone, is a meshwork of weaker and more flexible spongy tissue known as trabeculae which is made of mature adult bone. It is typically found at the core of vertebral bones in the spine and the ends of the long bones such as the femur or thigh bone. [25]

After it is formed bone is constantly undergoing remodeling to adapt to new loads and repair damage. The three different types of cells responsible for bone formation and remodeling are osteoblast, osteoclast, and osteocytes. [25] Osteoblast are mononucleate bone-forming cells derived from mesenchymal stem cell lines. The osteoblast creates and repairs new bone depositing osteoid which mineralizes to become bone. Osteoid is the unmineralized, organic portion of the bone matrix that forms prior to the maturation of bone tissue and is primarily composed of Type I collagen. The collagen fibers are used as a framework for mineralization.
Osteoclasts are considered bone reabsorption cells and oversee the removal of bone matrix. They secrete enzymes and hydrogen ions that acidify the local environment and breakdown the bone matrix. There is a constant cycle of bone formation and reabsorption that is used to remodel the bone. Lastly, most common cell type in bone osteocytes are the least metabolically active mature bone cells. However, they are responsible for the physical attributes of bone and are important regulators of bone mass by controlling the activity of both of osteoblasts and osteoclasts. They play are a key endocrine regulator of phosphate metabolism and biomineralization. [25]

**Osseointegration**

Osseointegration is defined as direct interface of living bone tissue with surgical implants without the formation of interpositioned connective tissue. [26] The osseointegration process securely fixes the implant in the living bone to allow for load bearing functions. An implant is considered osseointegrated if there is no relative motion between the implant and bone. Osseointegration can be compared with direct fracture healing, in which the fragment ends become united by bone, without intermediate fibrous tissue or fibrocartilage formation. [17] A fundamental difference, however, is that osseointegration unites bone not to bone, but bone to a foreign material, the implant.

Current standards state that an implant is considered osseointegrated when there is no progressive relative movement between both the implant and the bone with which it has direct contact. [26] The process of osseointegration reflects an anchorage mechanism whereby non-vital components can be reliably incorporated into living bone and which persist under all normal conditions of loading. [17]

Following implant placement, two stages of healing need to occur for the implant to begin osseointegration. According to Misch the two stages in osseointegration each can stage
split into two substages. [27] Stage one is the surface modeling where first woven bone is formed at implant site. This bone has numerous irregular shaped osteocytes, a relatively low mineral density characterized by random, felt-like orientation of collagen fibrils. As this woven callus matures it is replaced by lamellar bone to achieve sufficient strength for loading.

The second stage is remodeling and maturation which begins at the same time when woven callus is completing lamellar compaction. [17] During this stage callus starts to resorb, and remodeling of implant interface begins. The interface remodeling helps in establishing a viable interface between the implant and original bone. This remodeling decreases callus volume and interface remodeling continues based on loading. [17,27]

The clinical term osseointegration describes a state that provides for long-term stability of a prosthesis; however, this is not a biological property of any implant system or metal. In vivo and in vitro research have also been performed to evaluate the biology of the healing response to the implant surface and how the material's characteristics, such as surface preparations, chemical composition, coatings and sterilization procedures may affect the short- and long-term stability of the metallo-biological interface and achieve osseointegration. [28] One strategy to improve osseointegration is to coat implants to provide surfaces that accelerate bone cell attachment, growth and differentiation.

**Coatings**

Common biomaterials used as coatings on implants include bioactive glass, calcium phosphates, and poly(lactic) acid (PLA). [8,19,20,29,30,31]

Bioglass has been investigated for use as coatings due to its ability for direct bonding with host tissues. Bioglass is a silicate-based (SiO2-CaO-P2O5) bioactive glass, that when implanted, forms a hydroxyapatite layer on the bioglass surface. This layer allows for the
bioglass to bond to living bone. Bioglass has the potential to bond to hard, as well as soft tissue, depending on composition and structure. Studies have reported that coatings of bioglass on metallic and ceramic implants improve the rate of osseointegration. [29]

Calcium phosphate (CaP) materials have also been widely investigated as coating materials due to their chemical similarity to native bone mineral. Calcium phosphate is a family of compounds containing calcium Ca2+ and phosphate (PO4)3-. There are many types of CaP each with its own unique chemical, structural, mechanical and degradation properties with hydroxyapatite (HA) being the most widely used CaP in implant coatings. [19] Due to its chemical similarity to human bone and teeth, hydroxyapatite (HA) is the most widely used biomaterials in implant coatings. Hydroxyapatite is a mineral of the apatite group that is the main inorganic constituent of tooth enamel and bone. It has been shown to have excellent biocompatibility, good osteoconduction and osseointegration. While both bioglass and CaP have shown excellent ability to support bone cell attachment, growth and differentiation, both materials are brittle, suffer delamination from the titanium substrate and have exhibited higher rates of bacterial infection which have limited their use clinically. [20] Thus, there is a need to modify these materials or search for other new biomaterials to circumvent these drawbacks. [30]

PLA has also been investigated for use as implant coatings. PLA is the polymeric form of lactic acid and is the natural product of fermentation of sugars in sugarcane and corn by microorganisms. [32] It is a biodegradable thermoplastic with good mechanical strength and excellent biocompatibility. PLA-based materials are extensively used as biomedical materials in the fields of controlled drug delivery systems and tissue regeneration. These materials have also been used as alternatives for ceramic based polymeric materials in order to reduce impact on environment. [8] In composite scaffolds, PLA has been shown to speed up the degradation of
other materials causing faster overall degradation. Some disadvantages of PLA include excessive brittleness and slow crystallization; leading it to often be copolymerized with hydroxyalkanoic acid (a biodegradable thermoplastic), or blended with other polymers, fillers (include talc, mica, kaolin, starch and more), or plasticizers.[31]

Past research efforts have been dedicated to developing a suitable implant coating materials that would improve osseointegration and have the potential to locally deliver drugs to stimulate bone formation and/or to inhibit or prevent bacterial attachment/biofilm formation on implant surfaces. Chitosan, a naturally derived polysaccharide, has piqued the interest of researchers as an implant coating due to its biocompatibility, biodegradability, osteoconductivity, bioadhesivity, bacteriostatic and drug delivery capabilities. [33]

**Chitosan**

Chitosan is a cationic, linear polysaccharide made of randomly distributed β-(1→4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) derived from chitin, an abundant biopolymer in insect and crustacean exoskeletons. Chitin is widely distributed in nature and serves as an inexpensive readily available resource. [18] Chitosan has been widely studied in both bone tissue engineering and antimicrobial delivery applications as it possesses two of the most important characteristics of a local therapeutic delivery system: biodegradability and biocompatibility. [18] When chitosan degrades it breaks done into nontoxic products usually oligomeric units without causing any inflammatory reaction or producing any toxic end-products when the new tissues are formed. [18]

The two most commonly cited factors for determining the properties of chitosan are its degree of deacetylation (DDA) and molecular weight (MW). Chitosan DDA has often been cited as an important parameter that determines many physiochemical and biological properties of
chitosan like cell response, crystallinity, degradation, and hydrophilicity. Chitosan with 50% DDA has the most rapid degradation rate; whereas, 0% DDA chitin and 100% DDA chitosan exhibit the slowest degradation rates. [34] These differences can be explained by the crystallinity of the polymers as chitosan’s with the greater the percentage of DDA are more crystalline the chitosan. Highly crystalline polymers are more difficult to degrade compared to amorphous polymers. Some methods to modify DDA are changing processing conditions such as the concentration of sodium hydroxide, reaction temperature, reaction time, and number of successive reactions. [34]

MW has been shown to be an important factor in chitosan properties such as crystallinity, degradation, tensile strengths and moisture content. Typically, the molecular weight of commercially produced chitosan is between 3.8 and 20 kilo-Daltons (kDa). However, chitosans with MW as high as 710 kDa have been commonly tested in bone implants. Some studies have reported that chitosans with high MW’s, usually 600-1000 kDa, have a significantly higher tensile strength and moisture adsorption when compared to chitosans with similar DDA’s but lower MW’s, 50–60 kDa. [35]

Because of the versatility that chitosan provides it has been developed into many different technologies that take advantage of these properties and utilize them. Some examples include hydrogels, nanoparticles, paste, tissue scaffolds, and sponges. [36] Coatings and thin films have been investigated in order to tune the surface properties of various medical devices. [37]
**Surface Coating Methods**

There are variety of techniques used to coat implant metals and ceramics with chitosan. Some methods include plasma spraying, sputter coating, solution casting and spin coating and electrostatic methods are layer-by-layer, electrolytic and electrophoretic deposition.

Plasma Spray coating is a technique were the spraying of molten or heat softened material onto a surface provides a coating. Material in the form of powder is injected into a very high temperature plasma flame, where it is rapidly heated and accelerated to a high velocity. The hot material impacts on the substrate surface and rapidly cools forming a coating. The spray greatly increases the surface area of implants by increasing surface roughness. The plasma spraying coating technique has been successfully used to adhere HA to commercial titanium implants. [38] It provides porous implant surfaces greater bone contact and can be used to coat 3 dimensional objects. However, one of the disadvantages of plasma spraying is the high fabrication temperature as it may decompose and alter the structures organic materials, like chitosan.

Sputter coating is a physical vapor deposition process used to apply a very thin, functional coating on a substrate. The process starts by electrically charging a sputtering cathode which in turn forms a plasma causing material to be ejected from the target surface. The stable plasma created provides a more uniform deposition making coatings more consistent and durable as the material is now a permanent part of the substrate rather than an applied coating or plating of the surface. Like plasma spray, the high energy nature of this method is not conducive to coating chitosan.
Spin coating rotates the substrate deposited with chitosan solution at a constant angular velocity. The centripetal force will spread the chitosan uniformly on the substrate and excessive solution will be thrown off.

Solution casting involves casting chitosan solution on the substrate surface and allowing the solvent to dry under room temperature or in a heated environment. The thickness of the coating can be controlled by using carrier tape or doctor blade to spread or cast the chitosan solution in certain thickness, which is not conducive to create even coating. Bumgardner et al used solution casting to coat implant materials with chitosan and the first to use silane linkers to improve adhesion of the coatings to the implant surfaces. [39] Using silane covalent linking strategy, they bonded the chitosan to the surfaces of flat titanium coupons and thin (2 mm diameter) titanium rods [39,40] They showed that the silane bonding resulted in a significant 3-fold increase in coatings bonding strengths of the chitosan as compared to the simple solution cast method without silane bonding. [39] The coatings used a high DDA chitosan that showed low degradation and remained adhered to substrates over 12 weeks in vitro. [40] Their studies also showed that the coatings supported bone cell attachment and growth in vitro and osseointegrated similar to traditional CaP sputter coated Ti pins. [40] It was noted that while the silane bonding mechanism increased coating adhesion, the bond strengths were much lower than those for clinically used CaP coatings. To overcome that issue and to improve coating adhesion strength, Martin et al modified Ti surfaces using surface hydroxylation methods and an organic solvent system to enhance the deposition of the silane linker molecules to enhance bonding strengths. [41] Their results showed that they were able to increase bond strength to be on par with that of the CaP coatings. Several studies have used modified coating strategies to create chitosan coatings incorporating antibiotics or growth factors for local delivery. [42,43] Studies
incorporating antibiotics in the chitosan coatings showed that the coatings provide short term (<1 week) delivery of antibiotics at levels that were inhibitory to both orthopedic and dental pathogens, were not cytotoxic to cultured cells and that the coatings remained attached to implant material surfaces in mock surgical screw insertion tests. [43] Leedy et al incorporated VEGF, a growth factor important to both angiogenesis and osteoblast differentiation, into the chitosan coatings using an adsorption process to avoid loss of expensive growth factor during solution casting. [44] While their results showed that the coatings released VEGF from the coatings over 3 days, this short rapid release did not have any effect on terminal mineralization of the bone cells on the chitosan coated specimen. However, the chitosan coatings, both with and without VEGF, did support enhanced mineralization of the cells as compared to uncoated Ti providing indicating potential of chitosan coatings to enhance bone healing and osseointegration processes. [44]

While these studies demonstrate the potential of solution cast chitosan coatings for promoting osseointegration and for local delivery of drugs like antibiotics, there remain significant shortcomings to this method of coating. These passive methods are difficult to uniformly coat 3D object with irregular contour, difficult to control the thickness of the coating and the excessive chitosan solution used is not recoverable. Furthermore, if other bioactive compound like growth factors or antibiotics are included, the large amount of volume loss to drying can lead to waste. Although carrier tape or medical tape can be used to control the thickness and uniformity of coating, this method is effective on 2D plain surfaces but not 3D surfaces with complex contours. [45]
Another way of coating 3D objects with complex contours are with electrostatic methods by utilizing electric charge to make layer-by-layer coats on the substrate by joining alternating layers of polycation and polyanion using electrostatic interaction.

Electrolytic deposition passes electrical current through chitosan solution between anode and cathode where the substrate acts as cathode. Due to the chitosan molecule’s positive charge nature, it will be attracted towards the cathode/substrate and precipitate on the cathode/substrate surface forming a layer of coating. However, electrophoretic deposition technique deposits electrically charged particles suspended in liquid medium onto a substrate using external electric field. There are several studies that have used electrolytic methods to create different composite chitosan on many different substrates. Some of the most common materials used for these electrophoretic deposition chitosan composites are made using bioglass or hydroxyapatite. Many other materials have also been paired with chitosan including graphene-related materials, and carbon nanotubes. A variety of nanoparticles such as TiO2, ZnO, Fe2O3, Cu, Ag, nano-clays, and halloysite nanotubes have been used in combination with chitosan to develop functional composite coatings. However, electrophoretic deposition of composite coatings is challenging due to the differences in electrophoretic mobility of the two (or more) constituents involved.

Although electrostatic methods are better at coating 3D surfaces with complex contour when compared to passive methods like solution casting, they still are associated with several disadvantages. Firstly, it is difficult to control the thickness of the coating due to the decrease in chitosan concentration over time as more chitosan gets precipitated, meaning it will require precise and accurate timing to achieve the desired thickness of coating. Secondly, the coating process requires precise voltage control to achieve optimum coating morphology or high voltage
will induce surface crack. Post processing on the coating may be required to get thin and uniform surfaces. Furthermore, the process will generate excessive wastage of chitosan and any therapeutics that are being incorporated since some of the coating solution is not recoverable. [51]

**Electrospraying**

In order to overcome the drawbacks in other chitosan coating techniques, electrospray deposition has been investigated as a method to apply coatings. Also known as electrohydrodynamic atomization, electrospraying utilizes the principle of particle/droplet charging to produce coating. The advantages of electrospray are: easy to coat 3D surfaces with complex and irregular contour by utilizing electric charge, precise control on coating thickness, capable of controlling the size distribution of the sprayed droplets, resulting in less waste of coating material as compared to solution casting and other electrophoretic deposition methods. [52] Furthermore, electrospraying has been shown to allow for the easy to incorporation of therapeutic molecules or other components into coatings. Studies have investigated the application of electrospray deposition method to create chitosan-based coatings on implant materials to improve the osseointegration and the local delivery of bioactive materials such as growth proteins and therapeutics. [53,54]

In electrospraying chitosan, the chitosan solution is pushed out from the nozzle by the applied pressure from the syringe pump. The nozzle is connected to positive high voltage source to charge the chitosan solution as well as creating a high electric field between the nozzle and substrate. As the chitosan droplet emerges from the nozzle, the electric field applied will deform the interface of the droplet [52]. The electric charge generates electrostatic repulsion force within the droplet. The maximum amount of electric charge a droplet or particle could carry is called
Rayleigh limit. If the number of charge in a droplet exceeds its Rayleigh limit, electrostatic repulsion force overcomes the surface tension of the droplet, the excess charge will be dissipated through the breakup of large droplet into micro to nano-sized droplets. Since the micro/nano sized droplets carry similar charge, the Coulomb repulsion between the droplets will disperse and not reaccumulate together. The electric field between the nozzle and substrate drives the micro/nano sized droplets towards the substrate.

Chng et al used electrospraying to create chitosan coatings on Ti materials. [52] Their study examined the potential to bond electrosprayed chitosan coatings to surfaces of titanium via silane linker molecules. Their results demonstrated that the electrosprayed coatings were well retained during simulated implantation into sawbone materials as compared to non-bonded electrosprayed coatings. While this work demonstrated that electrosprayed chitosan could be covalently bonded to surfaces of titanium via silane linker molecules, the compatibility of the coatings was not evaluated.

This work will further evaluate the silane bonded electrosprayed chitosan coatings and to examine the impact of incorporating CaP particles into the coatings. Calcium phosphate (CaP) is a major component of bone and have been widely used bone substitutes in bone tissue engineering. When used as implant coating, CaP has been shown to improve osteointegration of implants into the bone tissues. CaP nanoparticles have been studied due to their high bioactivity, biocompatibility, chemical stability and strong adsorption ability under physiological conditions. [55] Some of these properties can be further tailored through both surface modification and structural design to achieve a sustained, controlled, or targeted release of a therapeutic. By incorporating CaP nanoparticles into a chitosan coating, it may be possible to improve both implant integration and deliver therapeutics such as antimicrobials to prevent bacterial infection.
The focus of this work will be on the ability to create chitosan-CaP composite coatings using electrospray methodology and evaluate coating properties and initial in vitro compatibility.
CHAPTER III

Electrosprayed Chitosan -Calcium Phosphate Nanoshell Composite Coatings on Silanated Titanium Plates.

Abstract

The aim of this work was to take advantage of the electrospray technology and the osteocompatibility of both chitosan and calcium phosphate (CaP) to fabricate chitosan coatings loaded with CaP nanoshell particles. The key electrospray parameters of capillary diameter, voltage, and pressure were identified and adjusted for creating uniform chitosan-CaP coatings on titanium surfaces. Coatings containing from 0 to 1 wt% CaP particles to chitosan mass were electrosprayed on to silanated titanium to create composite coatings bonded to the metal surface. Scanning electron microscope (SEM) and energy dispersive X-ray spectroscopy (EDS) showed that CaP particles were homogenously distributed throughout the chitosan coating. Mechanical tensile testing demonstrated that there was no statistically significant loss of coating adhesion strength in coating up to 1.0 wt% CaP. Furthermore, the incorporation of CaP was shown to support growth of bone cells on the coating. We have demonstrated that CaP can successfully be incorporated into electrosprayed chitosan coating.

Introduction

Arthritis is the leading cause of disability in the U.S. and, according to the Center of Disease Control (CDC), between 2013–2015, an estimated 54.4 million US adults (22.7%) and 300,000 juveniles have been diagnosed with arthritis.[1,2,56] In patients with severe arthritis, ongoing pain, limited function, and diminished life quality, biomedical total joint implant devices have become a reliable and effective means for replacing and restoring joint function. In
2014 there were 370,770 total hip replacements and 680,150 total knee replacements in elderly or children. [4,13,14]

While most of these orthopedic implants perform well during their operational lifetimes, reports indicate about 5% of implants experience some type of failure. [1, 57, 58] Common causes of failure include mechanical loosening, infection, and dislocation. [9] Early fixation of implants in bone has been identified as a critical step in obtaining long-term success of total hip replacement. [59] Titanium is widely used as components of orthopedic implant devices due to its capacity for osseointegration, as well as its excellent corrosion resistance and high fatigue limit.[5] To further improve osseointegration of titanium implants, surface coatings using different materials including calcium-phosphates, bioglass, and polymers such as chitosan have been explored. [20, 29, 31, 55, 60]

Calcium phosphate (CaP) is a highly biocompatible inorganic biomaterial. CaP coatings have been extensively investigated as implant coatings due to their similarity to native calcium-phosphate mineral found in bone. [19, 20, 55, 60] CaP coatings have demonstrated excellent biocompatibility, osteoconduction, and osseointegration, but are observed to be brittle, suffer delamination problems, and can exhibit higher rates of bacterial infection compared to non-CaP coated implants, limiting their use clinically. [20, 30] In addition, calcium-phosphate coatings made by traditional sputter coating, plasma spraying, or other high energy coating methods limit ability to incorporate of bioactive agents including antimicrobial and growth factors. [19, 60]

Bioglass has also been used as a coating. Bioglass is a silicate-based (SiO2-CaO-P2O5) bioactive glass, that when implanted, forms a hydroxyapatite layer on the bioglass surface. [29] Studies have reported that coatings of bioglass on metallic and ceramic implants improve the rate
of osseointegration. [29] However, due to bioglass’s brittleness and relatively poor mechanical properties, clinical applications have been limited to non-load bearing implants. [61]

Natural polymers have been used in medical applications for decades thanks to their biocompatibility, biodegradability, and tunable properties. Chitosan, a linear polysaccharide derived from the deacetylation of chitin polymer, has attracted interest as an implant coating material due to its biocompatibility and biodegradability, as well as its bacteriostatic and drug delivery capability. [18, 34, 39, 51] Many techniques have been used to fabricate chitosan coatings including solution casting with silane bonding, sputter coating, freeze-drying, layer-by-layer electrolytic deposition, and electrospraying. [37, 39, 41, 51, 52] These coating methods have been reported to create chitosan coatings that are able to support bone cell attachment, growth and differentiation, osseointegrate in animal models and or ability to incorporate and release bioactive agents to stimulate bone healing and or prevent microbial complications in vitro and or in vivo models. However, each method has its drawbacks. In solution casting it is difficult to coat complex shapes uniformly. High energy coating methods like sputter coating can damage/limit incorporation of expensive therapeutics. Layer-by-layer electrolytic deposition can require large volumes of coating solution as the entire substrate must be submerged in solution.

Electrospraying is a method, based on the principle of particle/droplet charging, that overcomes many of the limitations of other chitosan coating fabrication methods. The advantages of electrospray are: easy to coat 3D surfaces with complex and irregular contour by utilizing electric charge, precise control on coating thickness, capable of controlling the size distribution of the sprayed droplets and process results does in less waste of coating material as compared to solution casting and other electrophoretic deposition methods. [52]
Furthermore, electrospraying is not damaging to therapeutic molecules and makes it easy to incorporate therapeutic molecules or other components into coatings. Several studies have investigated the application of electrospray deposition method to create chitosan-based coatings on implant materials to improve the osseointegration and the local delivery of bioactive materials such as drugs for drug delivery application with. [52, 53, 54]

The aim of this work is to take advantage of the electrospray technology and the osteocompatibility of both chitosan and calcium phosphate mineral to fabricate chitosan coatings loaded with CaP nanoparticles. The key electrospray parameters of capillary diameter, voltage, and pressure were identified and adjusted for creating chitosan-CaP composite coatings on titanium surfaces. Composite coatings were then evaluated and compared to plain chitosan coated and uncoated titanium controls for changes in hydrophobic/hydrophilic properties, coating adhesive strengths, and ability to support bone cell attachment and growth. Composite coatings were also evaluated for distribution of CaP particles within the electrosprayed coatings.

**Materials**

Titanium (Titanium Industries, commercially pure ASTM F67 grade 2, Hillsboro, TX) was used as substrates for fabricating electrosprayed coatings. Tri-ethoxy-silylbutyraldehyde (TESBA) silane, purchased from Gelest (product code: SIT8185.3, Morrisville, PA), was used for the silanation of the titanium substrates. Carbon nanospheres were obtained from the Physics Department of the University of Memphis. Chitosan powder (92.6 DDA, molecular weight 300-700kDA) purchased from Heppe Medical Chitosan GmbH, Germany, product no.: 24711, was used for making the chitosan coatings. All other reagents and chemicals used were obtained from Thermo Fisher Scientific, USA.
Methodology

Sample Preparation

Cleaning and Hydroxylation of Titanium Substrate

Titanium substrates were wet ground with a sequence of 400, 600, 800, and 1200 grit silicon carbide grinding paper and then cleaned with dilute soap and warm water. Next, the substrates were ultrasonically (FS60H, Thermo Fisher Scientific, Waltham, MA) cleaned with acetone, 100% ethanol, and de-ionized water for 10 min each. After cleaning, samples were submerged in 5M sodium hydroxide (NaOH) at 60°C for 24 h to enhance surface hydroxide formation.[41] Samples were washed in fresh deionized distilled water (DI) water and stored in DI Water to prevent drying until silanation step. [41]

Silanation of Titanium Substrate

The silanation process of the titanium substrate was adopted from Chng et al (2019). [52] First, titanium substrates were placed in a glass pyrex dish on a magnetic stir plate and stirred in a 5:95 (v/v) de-ionized water/ethanol solution (pH = 4.5). The solution pH was maintained to 4.5 using pH meter (Accumet BASIC, AB15 pH meter, Thermo Fisher Scientific) and 1M acetic acid and 1M sodium hydroxide solution. [52] Tri-ethoxy- silylbutyraldehyde (TESBA) silane was added to make a 2 % (v/v) solution of silane in ethanol solution. The titanium substrates were placed on a belly dancer (IBI Scientific, The Belly Dancer, Thermo Fisher Scientific, Waltham, MA) for 10 mins to react with silane which the ethoxy groups of the silane molecules are organized via H-bonding with the hydroxide group on the titanium substrate surface. [52] The silanated substrates were then rinsed with ethanol to remove non-adhered silane and cured at 110 °C in oven (Thermo Fisher Scientific, Waltham, MA) for 10 mins to convert the H-bond between
silane and titanium to covalent Si-O-Ti bonds. The silanated substrates were immediately stored
in a vacuum chamber after being cured in the oven. [52]

Chitosan Solution Preparation

For electrospraying 3 parts of a 1 wt% chitosan in 0.5v% acetic acid solution was mixed
with 1 part of a 95v% ethanol 5v % 2-propanol solution. The alcohol solution is used to reduce
the viscosity and surface tension of the chitosan solution to make it sprayable [52]. The solution
was stirred using a magnetic stirrer to ensure thorough mixing. After mixing, the pH of the
solution was adjusted to 5.1 and then sonicated for 7 minutes (Sonic probe, CL-18, Thermo
Fisher Scientific, Waltham, MA) to remove microbubble in the solution before being
sprayed.[52]

Synthesis of CaP Nanoshells

The procedure for the synthesis of the CaP nanoshells was adapted from Wu et al. [62]
Briefly, CaP nanoshells were prepared via calcium phosphate condensation reaction around
carbon nanosphere precursors. Sodium chloride (NaCl) was added to a volume of DI water and
stirred for 15 minutes, followed by potassium chloride (KCl), Carbon Nanospheres, calcium
chloride dihydrate (CaCl2 · 2H2O), magnesium chloride hexahydrate (MgCl2 · 6H2O),
disodium phosphate (Na2HPO4), sodium bicarbonate (NaHCO3) are added sequentially at 10-
minute intervals and continuously stirred using a magnetic stir plate (Isotemp, Fisher Scientific).
Immediately, 20 % of the final volume of DI water was added to the solution. The pH was
adjusted using tris(hydroxymethyl)aminomethane base and hydrogen chloride solutions to 7.4.
The solution was stirred about 15 minutes, and then remaining DI water was added to bring the
final volume (1000 ml). The entire solution is placed in an oven at 37 °C for 3-4 days for
particles to settle out. The precipitate was washed until the product turned into light grey.
Finally, the product was dried and ground (Cole-Parmer Mortar and Pestle) and baked in oven at 280°C for 5 h to remove the carbon nanosphere precursors from the final CaP shells. [62] The obtained CaP Nanoshells were stored in an airtight container until further use.

**Incorporation of CaP shells into chitosan solution**

Calcium phosphate shells were incorporated into the chitosan electrospraying solution at 0.25, 0.5, 1.0 wt% of CaP shells to wt% chitosan powder. Solutions were ultrasonicated (Sonic probe, CL-18, Thermo Fisher Scientific, Waltham, MA) for 5 minutes using an ultrasonic probe to break apart any CaP aggregates in solution and then vortexed to ensure homogenous mixing of CaP shells in the solution prior to spraying.

**Electrospraying Parameters**

The initial spray parameters used were based on those previously developed using the same in-house custom electrospray set up. [52] These parameters were subsequently modified to achieve spray flow rate, spray stability, and spray conditions of the chitosan - CaP shell solution to achieve a stable uniform coating on the titanium surfaces. The spray flow rate refers to the rate of volume of the chitosan solution that can be ejected from the electrospray and spray conditions refer to dry or wet deposition of the chitosan droplets on the substrate. The spray stability refers to the ability of the electrospray to sustain the stability of the spray without interruption such as large droplet formation or accumulation of spray solution on the nozzle. The spray parameters were adjusted by trial and error based on original conditions established for spraying chitosan developed by Chng et al (2019).[52] After being sprayed, coatings were neutralized in a 0.25 M phosphate buffer solution then rinsed with DI water and dried at room temperature in a laminar flow hood.
**Characterization**

*Particle size and charge determination*

The particle size distribution of CaP nanoshells was measured using dynamic light scattering (Nano-ZS, Malvern Instruments, UK). From the measured distributions, Z-average diameters of the nanoshells were calculated. A micro-electrophoresis device (Nano-ZS, Malvern Instruments, UK) was used to determine the surface charge of the CaP nanoshells and acquire zeta-potential. For both the measurements, the samples were diluted with deionized water to avoid or reduce the multiple scattering effects.

*Electrospraying parameter determination*

The initial starting parameters for electrospraying chitosan can be seen in Table 1. The parameters were adjusted one by one to determine what which of the variables were key for a successful coating. Order of which parameters were adjusted were capillary size, pressure, voltage, substrate-capillary distance.

**Table 1: Original electrospraying parameters for chitosan.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary size</td>
<td>250 µm</td>
</tr>
<tr>
<td>Capillary material</td>
<td>Silica</td>
</tr>
<tr>
<td>Voltage</td>
<td>6.3 kV</td>
</tr>
<tr>
<td>Pressure</td>
<td>45 PSI</td>
</tr>
<tr>
<td>Substrate-Capillary distance</td>
<td>3 mm</td>
</tr>
<tr>
<td>Base Chitosan Solution</td>
<td>3:1 (95 % DDA, 1 % wt chitosan 0.5 % acetic acid + ethanol)</td>
</tr>
</tbody>
</table>

*Shape and Surface morphology of CaP nanoshells*

To determine the surface morphology, prepared CaP nanoshells were observed using scanning electron microscopy (SEM) (NovaNanoSEM650, FEI/ThermoFisher Scientific, Waltham, MA, USA). [62] Briefly, the nanoshells were evenly mounted on to the metallic stub
and further sputter gold coated. All images were recorded at an acceleration voltage of 15-18kV. The images were recorded with and without false color EDS overlay.

**Determination of silane deposition on titanium samples**

Fourier transform infrared (FTIR, Perkin Elmer Frontier, Waltham, MA) in attenuated total reflectance (ATR) mode was used to examine the surface chemistry, bonding of silane and chitosan to the titanium surface. The samples were examined with clean polished titanium as background run after sodium hydroxide (NaOH) and silanation treatment.

**Determination of surface morphology of chitosan coatings loaded with CaP nanoshells**

To confirm and evaluate the incorporation of CaP nanoshells into the chitosan coatings, the samples were examined using SEM and EDS. SEM was used to examine surface morphology of the coatings with and without CaP nanoshells. Coatings were further evaluated in SEM using EDS to map Ca and P distribution as an indicator of CaP nanoshell distribution in coatings. Sample were also evaluated from the edge using SEM and EDS to examine CaP distribution through the depth of the coating. Edge view was taken by cutting a piece off of an already coated sample so it could be placed on its side in the SEM.

**Determination of bond strength of the chitosan coating**

To test the bond strength of the coatings to the Ti surfaces, aluminum studs were attached to the coatings using GorillaWeld Steal Bond epoxy (Gorilla Glue, United States). Glued specimens were loaded into an Instron 33R test frame (Series 4465, Instron, United States) using custom fixtures and tested in tension using 5 kN load cell at 0.5 mm/min until failure. The maximum tensile stress before failure was recorded. The test specimens were examined visually to determine the failure mode either adhesive failure of the glue, or cohesive failure of the coating. The test groups were control groups of electrosprayed coatings on silanated and non-
silanated Ti, and electrosprayed coatings with 0.25, 0.5, 1.0 wt % CaP on silanated Ti. The 95% confidence intervals and ANOVA with post-hoc testing using Holm-Sidak method for statistical comparison. All groups were compared to each other.

**Determination of hydrophilic nature on chitosan coating**

To assess the change in hydrophilic or hydrophobic properties of the chitosan coating after incorporation of CaP nanoshells water contact angle measurements were performed using Contact Angle Measurement (CAM) (VCA Optima, AST Products, INC, Billerica, MA, USA). The samples examined were cleaned and polished non-silanated titanium (control) and electrosprayed chitosan coatings with 0.0, 0.25, 0.5, and 1.0 wt % CaP on silanated titanium respectively. The droplet size used for testing the nature of coatings was 3 μL for all the samples. Four replicate (n=4) samples per test group were evaluated. The 95% confidence intervals of the angles were calculated for statistical comparison.

**In-vitro cytocompatibility studies of the chitosan coatings**

A cytocompatibility study was performed on the chitosan coatings using W-20-17, ([W-20 clone 17] (ATCC® CRL-2623™)), preosteoblast mouse bone marrow stromal cells. The cells were first cultured in Dulbecco's modified Eagle's medium (DMEM), with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5 % CO2 incubator. Cells were seeded on the Ti control and test chitosan-CaP coated specimen (9.5 mm diameter disks) in 48 well plates at 3x10⁴ cells/well in 0.5 ml of medium and allowed to attach overnight. Medium was removed and replaced with fresh medium every 2-3 days over the experimental time frame. Viability was measured after 1, 3, and 5 days after seeding. Viability of the cells was determined using the Cell Titer glo assay (CellTiter-Glo® Luminescent Cell Viability Assay, Cat.# G7570, Promega). The Cell Titer glo assay is a homogeneous method of determining the
number of viable cells in culture based on quantitation of the ATP present. Results were
normalized to tissue culture plastic control. There were three replicates per group for each time
point. Data was then analyzed statistically using two-factor ANOVA with replication. Due to
interactions data were analyzed separately based on days.

Cells on a single sample from each test group were stained using Alexa Fluor and
methylene blue for qualitative imaging of cell attachment and spreading. After 1 day or 3 or 5
days, one sample was removed, rinsed with PBS and fixed with 1.5 % glutaraldehyde followed
by washing with PBS and rinse with 0.5 % Triton X buffer. Samples were stained in imaged on
microscope.

**Results**

**Optimization of Electrospraying Parameters**

The initial starting parameters for electrospraying chitosan can be seen in Table 1.

In initial trials a 250 µm capillary tube was used for electrospraying. However, the
solutions with as little as 0.25 wt% CaP particles showed formation of aggregates inside the
capillary tube. (Figure 2) These aggregates were formed by CaP particles building up on the
walls of the capillary tubes. The accumulation of the aggregates caused clogging of the tubes and
lead to incomplete coatings (Figure 3). To overcome this issue, the capillary size was increased
to the next size up, 320 µm inner diameter. Aggregates were not observed to form in capillary
tubes with 320 µm with 1 wt% CaP particles and complete coatings were able to be made on test
substrates.
Figure 2. A 250 µm capillary tube with CaP arrows indicating aggregate clogs.

Figure 3. Titanium sample that had coating process stopped due to capillary clogging mid-spray. Arrow A indicates where coating is, arrow B indicates where coating is not.

With the increased diameter of the capillary tube the initial pressure of 45 PSI was found to be too high and pushed too much material out of the capillary leading to loss of spray cone. This resulted in coatings being oversaturation (Figure 4). The pressure was gradually reduced to 15 PSI, for the 320 µm capillary tubes at which a stable spray cone was visually observed.
Pressure was further reduced to 13 PSI to prevent flooding of the substrate during longer coatings.

![Image](image.jpg)

**Figure 4.** Titanium sample that had coating process halted after oversaturation (flooding) with chitosan.

Changing the substrate-capillary distance and voltage of the electrospraying apparatus proved to hold no visually discernible improvements on coating quality versus the initial parameters. The distances between 2-7 mm were adjusted and voltages between 4-8 kV were also tried to determine if there were improvements in coating quality. Changes in these factors in the ranges evaluated resulted in no improvements in coating quality. Figure 5 shows the successful results of the adjusted electrospray parameters. The revised parameters for the chitosan-CaP solutions are shown in Table 2.
Table 2. Revised electrospraying parameters for chitosan loaded with CaP nanoshells.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary size</td>
<td>320 µm</td>
</tr>
<tr>
<td>Capillary material</td>
<td>Silica</td>
</tr>
<tr>
<td>Voltage</td>
<td>6.3 kV</td>
</tr>
<tr>
<td>Pressure</td>
<td>11-15 PSI</td>
</tr>
<tr>
<td>Substrate-Capillary distance</td>
<td>3 mm</td>
</tr>
<tr>
<td>Base Chitosan Solution</td>
<td>3:1 (95 % DDA, 1 % wt chitosan 0.5 % acetic acid + ethanol)</td>
</tr>
</tbody>
</table>

**Particle size, charge determination and surface morphology of CaP nanoshells**

Figure 6 is an SEM image of CaP particles made using the Wu process. [62] The particles were observed to be spherical in shape. DLS analyses indicated that the CAP particles were in range from 1.1-2 µm in diameter. The surface charge of the CaP shells was determined to be 26.28 mV. The electrophoretic mobility for the CaP shells was recorded as 2.34 E-04 cm²/Vs. Results are shown in Table 3. The CaP nanoshells were spherical in shape with rough surfaces
when examined by SEM (Figure 6). The EDS overlay image in Figure 7 showed the primary elements present on the surface of the CaP shells. The most abundant elements observed were Sodium, Phosphorous, Oxygen and Calcium. The lack of carbon in present in the shells indicates that the carbon precursors have been removed.

**Table 3. ZETA Potential Results**

<table>
<thead>
<tr>
<th>DLS</th>
<th>Surface Charge</th>
<th>Electrophoretic Mobility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1-2 um</td>
<td>26.28 mV</td>
<td>2.34 E-4 cm²/Vs</td>
</tr>
</tbody>
</table>

*Figure 6. SEM of Calcium Phosphate nanoshells*
Determination of silane deposition on titanium samples

The result of FTIR analysis of the silane deposition on the titanium samples is depicted in Figure 8. In the figure, spectra 1 is the FTIR of the cleaned titanium. Spectra 2 is the FTIR after the NaOH treatment of the Ti sample, there is a noticeable increase in the broad peak between 3100 and 3600 cm\(^{-1}\) indicative of an increase in the presence of -OH groups on the surface of the material. Spectra 3 corresponds to silanated titanium with peaks from the TESBA molecule at 1050 cm\(^{-1}\) and 1150 cm\(^{-1}\) corresponding to -Si-O bonds and peaks at 1700 cm\(^{-1}\) corresponding to -C=O bonds.
Figure 8. Prediction of surface chemistry and bonding of silane and chitosan to titanium using FTIR spectra. Spectra 1 corresponds to a cleaned titanium. Spectra 2 corresponds to titanium that has undergone NaOH treatment. Spectra 3 corresponds to silanated titanium.

Determination of surface morphology of chitosan coatings

Figures 9 are SEM images of each of the test composite coatings and the control chitosan coatings. As compared to the control chitosan coating, all CaP coatings showed more rough textured coatings. Qualitatively, CaP increased from 0.25 to 1wt%, the texture/roughness of the coatings increased reflecting the increase in CaP particle content.

Figure 10 shows EDS mapping of Ca and P for identifying presence of CaP particles in the coatings. In the image SEM with false-color, EDS overlay of chitosan, carbon of chitosan shown in pink, coating surface on titanium loaded with CaP particles, shown in green due to the yellow calcium and blue phosphorous color combination. With the EDS CaP particles can be seen inside of the chitosan coating.
Figure 9. Surface morphology recorded using SEM of electrosprayed chitosan coatings having (A) no added CaP shells, (B) 0.25 wt % CaP shells, (C) 0.5 wt% CaP shells, and (D) 1.0 wt% CaP
Figure 10. SEM with false-color, EDS overlay of chitosan (pink) coating surface on titanium loaded with CaP particle (green). Carbon-pink, green-oxygen, yellow-calcium and blue-phosphorous. (A) 0.25 wt% CaP shells, (B) 0.5 wt% CaP shells, and (C) 1.0 wt% CaP

Figure 11 is a SEM image with a false-color, EDS overlay of cross-section of Ti (green) with chitosan coating (blue and red) loaded with CaP particle (yellow and pink). This image is the interface between the chitosan coating to the titanium substrate were the bottom of chitosan coating can be seen. In Figure 12 the EDS of cross-section is separated into its respective components for better observation of the titanium (green) with chitosan coating (blue and red) with incorporated CaP particle (yellow and pink).
Figure 11. SEM with false-color, deep-scanned EDS overlay of cross-section of Ti (green) with chitosan coating (blue and red) with incorporated CaP particle (yellow and pink)
**Figure 12.** EDS of the cross-section is separated into respective components. (A) Cross-section of Titanium (green). (B, C) Carbon and oxygen of the chitosan coating (blue and red). (D, E) Phosphorus and calcium of incorporated CaP particle (pink and yellow). (F) Silicon from TESBA on titanium.

**Determination of bond strength of the chitosan coating**

Results of tensile testing are in Figure 13, and Table 4. Results showed that all the silanated groups exhibited significantly greater tensile bond strengths than the no-silanated group. Using ANOVA and Holm-Sidak post-hoc to compare the samples with CaP to controls showed that all groups were statistically significant from the non-silanated controls with p-values less than 0.05 (Table 5). There was no statistically significant difference between 0.25 wt % CaP, 0.5 wt % CaP, 1.0 wt% CaP and control chitosan coatings.
Table 4. Tensile bond strength (MPa) of silanated electrosprayed coatings with and without Calcium Phosphate nanoshells.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Average ± standard deviation</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrosprayed Silanated (n=12)</td>
<td>5.62 ± 1.70</td>
<td>4.66 to 6.59</td>
</tr>
<tr>
<td>Electrosprayed Non-silanated (n=16)</td>
<td>2.51 ± 0.96</td>
<td>2.04 to 2.98</td>
</tr>
<tr>
<td>Electrosprayed Silanated + 0.25 wt% CaP (n=6)</td>
<td>5.49 ± 1.34</td>
<td>4.42 to 6.56</td>
</tr>
<tr>
<td>Electrosprayed Silanated + 0.5 wt% CaP (n=6)</td>
<td>4.97 ± 0.92</td>
<td>4.23 to 5.71</td>
</tr>
<tr>
<td>Electrosprayed Silanated + 1.0 wt% CaP (n=5)</td>
<td>4.31 ± 0.68</td>
<td>3.71 to 4.91</td>
</tr>
</tbody>
</table>

Figure 13. Results of tensile bond strength (MPa) of silanated electrosprayed coatings with and without Calcium Phosphate nanoshells. Error bars correspond to standard deviation. Letters correspond to groups with no statistically significant differences.
**Figure 14.** Coating post tensile test failure. (A) electrosprayed, silanated, no CaP loaded; (B) electrosprayed, non- silanated, no loaded CaP; (C) electrosprayed, silanated, 0.25 wt% CaP loaded; (D) electrosprayed, silanated, 0.5 wt% CaP loaded; and (E) electrosprayed, silanated, 1.0 wt% CaP loaded.

Images for the pin and fractured coatings for the different test chitosan groups is shown in Figure 14. In Figure 14A electrosprayed, silanated coatings remained mostly attached to the Ti substrate, only coming of the titanium in a few small pieces. The electrosprayed, non-silanated coatings, as seen in Figure 14B showed complete removal of the chitosan coating from the titanium substrate. Figure 14C-E shows the post-failure image of silanated, coatings incorporated with CaP. These coatings did not peel off completely but started to rip off in sections in which some sections of the coatings remained adhered to the Ti surface and other parts were removed from surface. As the CaP loading percentage was increased, the coating adhesiveness to the titanium substrate was weakened in larger proportions. Both Figure 14C and 14D show that most of the coating was still intact to the titanium substrate but still failed in places, with the size of the failure area increasing as the CaP percent increases. This was more evident from the coatings loaded with 1.0 wt% of CaP nanoshells seen in Figure 14E.

**Determination of hydrophilic nature over chitosan coating**

Table 6 depicts the results of the five test groups of cleaned and polished non-silanated titanium and electrosprayed chitosan coatings with 0.0, 0.25, 0.5, and 1.0 wt% CaP on silanated titanium with the accompanying 95% confidence intervals. It was observed that addition of CaP
nanoshells in the chitosan coating caused an increase in the standard deviation of all coatings. The 95% confidence intervals of all chitosan coatings with incorporated CaP do cross over the 95% confidence interval of the plain chitosan coatings. TiCH and TiCH 0.25 wt% CaP 95% confidence intervals do not overlap with that of Ti, indicating that they are more hydrophilic than the Ti. The 0.5 wt% CaP and 1.0 wt% CaP 95% confidence intervals do overlap with that of Ti, indicating they are not different from Ti. Furthermore, none of the chitosan coatings are different from each other as all their intervals overlap.

Table 5. Table indicating the average results of the contact angle coatings as well as the 95% confidence interval.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Average ± standard deviation (n=4)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti</td>
<td>75.6 ± 1.7°</td>
<td>74.0 to 77.3°</td>
</tr>
<tr>
<td>Ti + CH</td>
<td>71.6 ± 1.3°</td>
<td>70.3 to 72.9°</td>
</tr>
<tr>
<td>Ti + CH + 0.25 wt% CaP</td>
<td>68.0 ± 4.8°</td>
<td>63.2 to 72.7°</td>
</tr>
<tr>
<td>Ti + CH + 0.5 wt% CaP</td>
<td>74.7 ± 3.6°</td>
<td>71.2 to 78.2°</td>
</tr>
<tr>
<td>Ti + CH + 1.0 wt% CaP</td>
<td>69.6 ± 4.6°</td>
<td>65.1 to 74.1°</td>
</tr>
</tbody>
</table>

In-vitro evaluation of chitosan coatings

The results for the in vitro viability and growth of cells on the test chitosan coatings and the control Ti are shown in Figure 15. Result of statistical analyses using two-factor ANOVA indicated that there were statistically significant differences in the growth of cells on the different test coatings (p=4 E-30), significant differences over time (p= 8 E-9), and there was a significant interaction between the factors of test coatings and time (p= 6E-7). Due to interactions data were analyzed separately based on days.
When all groups were compared using pairwise SNK testing, at day 1 TiCH, TiCH + 0.5 wt% CaP, and TiCH + 0.5 wt% CaP coatings were all statistically similar. Furthermore, TCP and TiCH + 0.5 wt% CaP coatings were statistically similar, and TiCH + 0.5 wt% CaP and TiCH + 1.0 wt% were also statistically similar. On day 3 all coatings were found to be statistically different from each other. At day 5 the TCP control was statistically higher than the coated samples. However, the TiCH, TiCH + 0.5 wt% CaP, and TiCH + 1.0 wt% CaP were all statistically similar with the TiCH + 0.5 wt% CaP being different from all other coatings. All groups showed a net increase in viable W2017 cells over the 5-day culture period. While all coatings had positive cell growth and showed statistical difference from each other, no particular group could be determined as superior.

**Figure 15.** In-vitro Cytotoxicity study on Chitosan electrosprayed Titanium implant using W2017 cells Non-normalized
Due to the high density of cells on the test samples, it was not possible to evaluate the spreading of the cells on the surfaces. Nevertheless, the staining did show many cells to be well attached to test composite coatings. (Figure 16)

Figure 16. Day 1 of Coating

Discussion

From the ZETA potential results shown in Table 3, with a surface charge of 26.28 mV it was evident that the CaP particles would be prone to aggregate formation causing moderate instability. A zeta potential values lower than −30 mV or greater than +30 mV are considered to have sufficient repulsive force to attain better physical colloidal stability, small zeta potential values result in particle aggregation and flocculation due to the van der Waals attractive forces act upon them which may result in physical instability. [63] This was evident during the optimization of the electrospraying process, as the loading of CaP nanoshells in the spray solution resulted in aggregation in the 250 µm capillary tube necessitating an increase to 350 µm tube size up to 250 µm.
The FTIR spectra (Figure 8) confirms the development of a -OH rich surface on the Titanium and subsequent attachment of silane molecules. The attachment of the silane is crucial for electrospray/solution casting of chitosan to obtain good adhesion strength between the Ti and chitosan itself. The ethoxy groups of this silane coupling agent react with Ti-OH group, and the aldehyde group is expected to chemically react with amino group of chitosan. The cleaned Ti coupon spectra reveal no specific peaks indicating no pre-existing chemical bonds (spectra 1). The NaOH treatment (spectra 2) and aldehyde silanation process (spectra 3) indicates the formation of surface bonds. Peaks in curve 2 that appear at wavenumbers 3600-3000 nm\(^{-1}\) (corresponding to OH stretching) and 1640-1555 nm\(^{-1}\) (that shows OH bending) confirm the formation of the Ti-OH bond on the surface. Further, the absorbances of -CH\(_2\), C=O (aldehyde), and Si-O-Si are shown by peaks at 2925-2850, 1720, and 1200-1000 nm\(^{-1}\), respectively in spectra 3, confirming the success of the silanation process at the Ti surface. This is comparable to what is seen in the study performed by Martin. [41]

Using SEM/EDS we were able to observe spherical CaP nanoshells produced using calcium phosphate condensation. In the Figures 9 and 10, SEM and deep-scanned EDS showed that CaP were homogenously distributed throughout the surface of the chitosan coating. In Figure 11 and 12 the SEM and EDS image illustrates that the CaP particles have been incorporated throughout the full depth of the chitosan coating. In comparison to the standard chitosan coating samples, the surface roughness of chitosan coating loaded with CaP nanoshells was increased. This could be helpful in providing a larger surface area for contact between the bone and the implant. The cross-section images from SEM/EDS (Figure 11 and 12) of the chitosan coated samples indicated in-depth loading of CaP nanoshells.
The electrosprayed, non-silanated coatings showed very low adhesive strengths associated with simply physical adsorption phenomena. The adhesion strength of the silanated coatings were 1.72 to 2.24 times that of the non-silanated coatings, confirming the formation of chemical bonds between silane and chitosan as the main source of the adhesion strength. Table 4 indicates no statistical significance between 0.25, 0.5 and 1.0 wt % CaP loading with respect to the standard chitosan coating (control). The tensile strength of the silanated control group was compared to the reported values obtained by Chng in which the coatings produced had a tensile strength of 5.87 MPa ± 2.03 MPa for electrosprayed, silanated chitosan on titanium. [52] ANOVA showed that there were differences in coating adhesion between the program p< 0.001 and that post-hoc testing using Holm-Sidak showed that all silanated coatings had significantly higher adhesion properties than the non-silanated but there was no statistically significant effect of the amount of CaP nanoshells on coating adhesion strengths. However, the 1.0 wt% CaP was close to be different from control silanated chitosan with a p=0.4. Based off the mean values, as the loading percent of CaP nanoshells increases the strength of the chitosan coatings gradually decreases. This could be because as the CaP nanoshells do not bond to the silane and hence as the amount of nanoshells increase, there may be less bonding between the chitosan molecules and the silane molecules on the surfaces.

All coatings were found to have water contact angles less than 90° indicating that the coatings were hydrophilic. Previous studies have reported chitosan as hydrophilic, with chitosan-coated surfaces having a contact angle of 76.4 ± 5.1°. [64] This is slightly higher to the values obtained for this study’s chitosan coatings. The higher hydrophilicity in this study than others might be due to differences in how coatings are treated to remove or neutralize residual acidic acetate salts. Contact angle measurements did not identify any differences in hydrophilic
properties between the plain chitosan coatings and the coatings containing CaP particles. It is possible that the chitosan could be effectively covering the surface of the coatings hindering major effects that the CaP shells would have on the coatings’ wettability. It was also observed that addition of CaP nanoshells in the chitosan coating caused an increase in the standard deviation of all coatings. This could be caused by an increase in surface roughness, possibly due to the formation of CaP aggregates creating surface topography. This roughness can be observed in Figure 9. Regardless, the hydrophilic characteristics of the coatings lead to a high degree of cell attachment as indicated by cytoskeletal staining which showed very high numbers of cells on the surfaces.

In the cell study all groups showed a high cell viability and attachment was observed at the earliest time point to the coating. We theorize that this was majorly due to the biocompatibility of chitosan coating towards cells and the structure of chitosan resembles glycosaminoglycan, which is the key molecule in the extracellular matrix to modulate cell morphology and function enhancing the cell attachment. [65] Also, earlier reports indicated that calcium phosphate on alkaline chitosan coatings dissolve fast and released Ca ions stimulating faster osteogenic cells. [66]
CHAPTER IV
CONCLUSION

Our research shows that electrospraying technique can be used to successfully electrospray chitosan with incorporated CaP nanoshells onto a Ti substrate. Our results indicate that electrospraying technique can be utilized to incorporate up to a concentration of 1.0 wt% CaP into chitosan solutions and still be sprayable. Using tensile testing it was found that there was no statistically significant loss of coating adhesion strength in 0.25 wt%, 0.5 wt%, and 1.0 wt% CaP coatings. However, as the CaP percentage increased in the coatings the mean strength of the coating did seem to decrease. Coatings were more hydrophilic than other reported chitosan surfaces but contact angle measurements did not identify any differences in hydrophilic properties between the plain chitosan coatings and the coatings containing CaP particles. The incorporation of CaP did not significantly affect cell growth relative to plain chitosan coatings.

This work demonstrated that electrosprayed chitosan – CaP nanoshells are able to be bonded to implant surfaces and are cytocompatible. These coatings may have potential to incorporate therapeutic agents into the CaP nanoshells and or chitosan electrosprayed coatings for local drug delivery to enhance osseointegration processes.
CHAPTER V

FUTURE WORK AND RECOMMENDATION

The prominent factor in controlling the CaP particle size are the carbon nanosphere precursors. Smaller carbon nanosphere precursors with a smaller size distribution would allow for the creation of smaller CaP particles. This should help alleviate aggregate formation in the electrospraying set up and help keep coating more uniform. More even distribution of CaP throughout the coatings should cause less surface roughness.

With the decrease in spray pressure due to the larger capillary size, there arises an issue of system throughput. The speed of the coating process is largely limited by single capillary electrospray. This can be overcome using multi-nozzle electrospray set-ups to increase the spray flowrate.

While ANOVA and post-hoc testing of the tensile testing showed all silanated coatings had no statistically significant difference in strength with of the amount of CaP nanoshells on coating adhesion strengths, the 1.0 wt% CaP was close to be different from control silanated chitosan with a p=0.4. Furthermore, there was a noticeable mean decrease coating adhesion strength as the CaP percentage increased. This is further noticed by the increased coating failure area as the CaP percentage increases. It is recommended that additional tensile testing is warranted to confirm if there is or is not an effect. It would also be recommended to examine the degradation characteristics of the Chitosan CaP verses silanated and non-silanated titanium. If the increase in CaP is shown to cause a decrease in coating adhesion, then a more aggressive silanation process, such as, the piranha solution sanitation used by Martin et al, could be investigated. [41] Otherwise, the titanium could be roughened to increase the surface area between the chitosan and the substrate increasing bond strength.
During the cellular compatibility testing, titanium samples were not used because titanium has been tested before and chitosan has been shown to be comparable. Further studies should be made comparing chitosan CaP coatings to other implant materials and coating such as stainless steel and calcium phosphate.

The drug loading and release characteristics of the CaP nanoshell particles need to be examined. Future studies may determine elution kinetics of antimicrobials, such as vancomycin and amikacin since they are commonly used in the prophylaxis of complex musculoskeletal trauma and in combination have activity against Gram positive and Gram negative bacteria. Coating degradation may play a part in elution as the coatings degrade CaP particles inside the coating will then be exposed to their surroundings. Whether this will increase the total elution time or volume should be investigated.

Future studies should evaluate combination chitosan CaP coatings on titanium versus both chitosan coated titanium and uncoated titanium will incorporate in vivo evaluations of biocompatibility and osteointegration. A rodent model could be used to evaluate the safety of the device when incorporated into a living system without infection present. If the chitosan CaP coatings are proven to be biocompatible, its efficacy can be evaluated in an in vivo model of infection prevention. Early studies will focus on preventing infection from occurring by placing the delivery system alongside bacteria before they establish an infection.
References

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### APPENDIX

**Table 6:** Two-Factor with Replication ANOVA for tensile testing of silanated vs test groups.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>6.8791</td>
<td>3</td>
<td>2.293033</td>
<td>1.21887</td>
<td>0.323487</td>
<td>2.991241</td>
</tr>
<tr>
<td>Within Groups</td>
<td>47.03194</td>
<td>25</td>
<td>1.881278</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>53.91104</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 7:** Two-Factor with Replication ANOVA for tensile testing of non-silanated vs test groups.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>53.32641</td>
<td>3</td>
<td>17.77547</td>
<td>17.76637</td>
<td>9.74E-07</td>
<td>2.93403</td>
</tr>
<tr>
<td>Within Groups</td>
<td>29.01485</td>
<td>29</td>
<td>1.000512</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>82.34126</td>
<td>32</td>
<td></td>
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</tr>
</tbody>
</table>

**Table 8:** All pairwise multiple comparison procedures Holm-Sidak post-hoc test of tensile test. Overall significance level = 0.05.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Es, Silanated vs. Es, Non-Silanated</td>
<td>3.115</td>
<td>6.607</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>CaP 0.25 wt% vs. Es, Non-Silanated</td>
<td>2.98</td>
<td>5.042</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>CaP 0.5 wt%, vs. Es, Non-Silanated</td>
<td>2.463</td>
<td>4.168</td>
<td>0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>CaP 1.0 wt%, vs. Es, Non-Silanated</td>
<td>1.802</td>
<td>2.849</td>
<td>0.047</td>
<td>Yes</td>
</tr>
<tr>
<td>Es, Silanated vs. CaP 1.0 wt%,</td>
<td>1.313</td>
<td>1.998</td>
<td>0.277</td>
<td>No</td>
</tr>
<tr>
<td>CaP 0.25 wt% vs. CaP 1.0 wt%,</td>
<td>1.178</td>
<td>1.575</td>
<td>0.481</td>
<td>No</td>
</tr>
<tr>
<td>Es, Silanated vs. CaP 0.5 wt%,</td>
<td>0.652</td>
<td>1.056</td>
<td>0.756</td>
<td>No</td>
</tr>
<tr>
<td>CaP 0.5 wt%, vs. CaP 1.0 wt%,</td>
<td>0.661</td>
<td>0.884</td>
<td>0.764</td>
<td>No</td>
</tr>
<tr>
<td>CaP 0.25 wt% vs. CaP 0.5 wt%,</td>
<td>0.517</td>
<td>0.725</td>
<td>0.722</td>
<td>No</td>
</tr>
<tr>
<td>Es, Silanated vs. CaP 0.25 wt%</td>
<td>0.135</td>
<td>0.219</td>
<td>0.828</td>
<td>No</td>
</tr>
</tbody>
</table>
Table 9: Two-Factor with Replication ANOVA for Cell study.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>4.97E+13</td>
<td>2</td>
<td>2.48E+13</td>
<td>1346.751</td>
<td>4.26E-30</td>
<td>3.31583</td>
</tr>
<tr>
<td>Columns</td>
<td>1.71E+12</td>
<td>4</td>
<td>4.28E+11</td>
<td>23.23018</td>
<td>8.01E-09</td>
<td>2.689628</td>
</tr>
<tr>
<td>Interaction</td>
<td>1.56E+12</td>
<td>8</td>
<td>1.95E+11</td>
<td>10.5887</td>
<td>6.44E-07</td>
<td>2.266163</td>
</tr>
<tr>
<td>Within</td>
<td>5.53E+11</td>
<td>30</td>
<td>1.84E+10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5.35E+13</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A result of “Do Not Test” occurs for a comparison when no significant difference is found between two means that enclose that comparison. It should be noted that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

Table 10: Day 1 pairwise multiple comparison procedures (Student-Newman-Keuls Method) post-hoc test of tensile test. Overall significance level = 0.05.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>p</th>
<th>q</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti-CH vs. Control (TCP)</td>
<td>54100</td>
<td>5</td>
<td>5.999</td>
<td>0.012</td>
<td>Yes</td>
</tr>
<tr>
<td>Ti-CH vs. TiCH 1.0 wt% CaP</td>
<td>53501.67</td>
<td>4</td>
<td>5.932</td>
<td>0.009</td>
<td>Yes</td>
</tr>
<tr>
<td>Ti-CH vs. TiCH 0.5 wt% CaP</td>
<td>22060</td>
<td>3</td>
<td>2.446</td>
<td>0.242</td>
<td>No</td>
</tr>
<tr>
<td>Ti-CH vs. TiCH 0.25 wt% CaP</td>
<td>7549.667</td>
<td>2</td>
<td>0.837</td>
<td>0.567</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>TiCH 0.25 wt% CaP vs. Control (TCP)</td>
<td>46550.33</td>
<td>4</td>
<td>5.162</td>
<td>0.02</td>
<td>Yes</td>
</tr>
<tr>
<td>TiCH 0.25 wt% CaP vs. TiCH 1.0 wt% CaP</td>
<td>45952</td>
<td>3</td>
<td>5.095</td>
<td>0.012</td>
<td>Yes</td>
</tr>
<tr>
<td>TiCH 0.25 wt% CaP vs. TiCH 0.5 wt% CaP</td>
<td>14510.33</td>
<td>2</td>
<td>1.609</td>
<td>0.282</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>TiCH 0.5 wt% CaP vs. Control (TCP)</td>
<td>32040</td>
<td>3</td>
<td>3.553</td>
<td>0.073</td>
<td>No</td>
</tr>
<tr>
<td>TiCH 0.5 wt% CaP vs. TiCH 1.0 wt% CaP</td>
<td>31441.67</td>
<td>2</td>
<td>3.486</td>
<td>0.034</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>TiCH 1.0 wt% CaP vs. Control (TCP)</td>
<td>598.333</td>
<td>2</td>
<td>0.0663</td>
<td>0.964</td>
<td>Do Not Test</td>
</tr>
</tbody>
</table>
Table 11: Day 3 pairwise multiple comparison procedures (Student-Newman-Keuls Method) post-hoc test of tensile test. Overall significance level = 0.05.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>p</th>
<th>q</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (TCP) vs. TiCH 0.5 wt% CaP</td>
<td>675232.333</td>
<td>5</td>
<td>18.92</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Control (TCP) vs. TiCH 0.25 wt% CaP</td>
<td>504200.667</td>
<td>4</td>
<td>14.127</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Control (TCP) vs. TiCH 1.0 wt% CaP</td>
<td>287447.333</td>
<td>3</td>
<td>8.054</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Control (TCP) vs. Ti-CH</td>
<td>158560.333</td>
<td>2</td>
<td>4.443</td>
<td>0.11</td>
<td>Yes</td>
</tr>
<tr>
<td>Ti-CH vs. TiCH 0.5 wt% CaP</td>
<td>516672</td>
<td>4</td>
<td>14.777</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Ti-CH vs. TiCH 0.25 wt% CaP</td>
<td>345640.333</td>
<td>3</td>
<td>9.685</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Ti-CH vs. TiCH 1.0 wt% CaP</td>
<td>128887</td>
<td>2</td>
<td>3.611</td>
<td>0.029</td>
<td>Yes</td>
</tr>
<tr>
<td>TiCH 1.0 wt% CaP vs. TiCH 0.5 wt% CaP</td>
<td>387785</td>
<td>3</td>
<td>10.866</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>TiCH 1.0 wt% CaP vs. TiCH 0.25 wt% CaP</td>
<td>216753.333</td>
<td>2</td>
<td>6.073</td>
<td>0.002</td>
<td>Yes</td>
</tr>
<tr>
<td>TiCH 0.25 wt% CaP vs. TiCH 0.5 wt% CaP</td>
<td>171031.667</td>
<td>2</td>
<td>4.792</td>
<td>0.007</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 12: Day 5 pairwise multiple comparison procedures (Student-Newman-Keuls Method) post-hoc test of tensile test. Overall significance level = 0.05.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>p</th>
<th>q</th>
<th>P</th>
<th>P&lt;0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (TCP) vs. TiCH 0.25 wt% CaP</td>
<td>1238673.667</td>
<td>5</td>
<td>9.475</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Control (TCP) vs. TiCH 0.5 wt% CaP</td>
<td>790774.333</td>
<td>4</td>
<td>6.049</td>
<td>0.008</td>
<td>Yes</td>
</tr>
<tr>
<td>Control (TCP) vs. Ti-CH</td>
<td>731031.667</td>
<td>3</td>
<td>5.592</td>
<td>0.007</td>
<td>Yes</td>
</tr>
<tr>
<td>Control (TCP) vs. TiCH 1.0 wt% CaP</td>
<td>572468</td>
<td>2</td>
<td>4.379</td>
<td>0.011</td>
<td>Yes</td>
</tr>
<tr>
<td>TiCH 1.0 wt% CaP vs. TiCH 0.25 wt% CaP</td>
<td>666205.667</td>
<td>4</td>
<td>5.966</td>
<td>0.021</td>
<td>Yes</td>
</tr>
<tr>
<td>TiCH 1.0 wt% CaP vs. TiCH 0.5 wt% CaP</td>
<td>218306.333</td>
<td>3</td>
<td>1.67</td>
<td>0.49</td>
<td>No</td>
</tr>
<tr>
<td>TiCH 1.0 wt% CaP vs. Ti-CH</td>
<td>158563.667</td>
<td>2</td>
<td>1.213</td>
<td>0.411</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>Ti-CH vs. TiCH 0.25 wt% CaP</td>
<td>507642</td>
<td>3</td>
<td>3.883</td>
<td>0.05</td>
<td>Yes</td>
</tr>
<tr>
<td>Ti-CH vs. TiCH 0.5 wt% CaP</td>
<td>59742.667</td>
<td>2</td>
<td>0.457</td>
<td>0.753</td>
<td>Do Not Test</td>
</tr>
<tr>
<td>TiCH 0.5 wt% CaP vs. TiCH 0.25 wt% CaP</td>
<td>447899.333</td>
<td>2</td>
<td>3.426</td>
<td>0.036</td>
<td>Yes</td>
</tr>
</tbody>
</table>