Titanium dioxide nanoparticle-imbedded polyelectrolyte multilayers as an osteoconductive and antimicrobial surface coating

Submitted by
Matthew Rothpan

Biomat’X Laboratories
Faculty of Engineering
McGill University
Montreal, Quebec, Canada

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Abstract

Bioactive surface coatings have retained the attention of researchers and physicians for their versatility and range of applications in the field of orthopedics, particularly in the context of infection prevention. Antibacterial metal nanoparticles (mNPs) are a highly disputed, but promising therapeutic, with equally vast application opportunities. The current research aims to construct a polyelectrolyte multilayer (PEM) on semiporous titanium disks using alternating thin film coatings of chitosan and alginate using the Layer-by-Layer (LbL) self-assembly technique, along with the incorporation of silver nanoparticles (AgNPs) or titanium dioxide nanoparticles (TiO2NPs), for antibacterial and osteoconductive activity.

LbL PEM deposition was validated using Quartz Crystal Microbalance with Dissipation (QCM-D), followed by surface characterization and visualization with atomic force microscopy (AFM) and scanning electron microscopy (SEM). Tests of cell viability and differentiation were performed on murine pre-osteoblast cell line MC3T3-E1 cells using AlamarBlue and Alkaline Phosphatase (ALP) assays, respectively. Antibacterial activity of the functionalized disks was evaluated after 24-hour liquid culture exposure, via standard colony counts of adherent methicillin-sensitive staphylococcus aureus (MSSA).

Surface characterization confirms stepwise multilayer deposition and overall nanoparticle incorporation, validating this iteration of LbL assembly. Mammalian tests for viability show enhanced MC3T3-E1 proliferation at moderate (13.33mg/ml) doses of TiO2NPs compared to all other groups after 1 and 4 days. Upper concentrations of AgNPs (4mg/ml) show marked cytotoxicity (approximately 30% viability relative to control) after 4 and 7 days of culture. ALP tests performed after 7 days demonstrate improved differentiation in a positive dose-response for TiO2NPs groups. At day 7, ALP activity of AgNPs show highest relative response in the low concentration group (0.4mg/ml) compared to others, though not significantly different from the high concentration group of TiO2NPs (26.67mg/ml). Dose-response trends for AgNPs are inconclusive. Antibacterial activity of this coated structure against MSSA shows no significant main effect, however regression analysis of TiO2NPs groups show a significant dose-dependent antibacterial effect. These results may serve to motivate further refinement of this surface coating for the eventual implementation into in-vivo models of periprosthetic joint infection (PJI).
Résumé

Les revêtements de surface bioactifs ont retenu l'attention des chercheurs et des médecins pour leur polyvalence et leur gamme d'applications dans le domaine de l'orthopédie, en particulier dans le contexte de la prévention des infections. De plus, l’usage des nanoparticules métalliques antibactériennes (mNP) dans le cadre thérapeutique est très controversé, mais prometteuse, avec des possibilités d'application tout aussi vastes. La recherche actuelle vise à construire une multicouche de polyélectrolytes (PEM) sur des disques de titane semi-poreux en utilisant des couches minces alternées de chitosane et d'alginate, en utilisant la technique auto-assemblage couche par couche (LbL), ainsi que l'incorporation de nanoparticules d'argent (AgNPs) ou des nanoparticules de dioxyde de titane (TiO2NPs), pour une activité antibactérienne et ostéoconductrice.

Le dépôt LbL de la PEM a été validé à l'aide d'une microbalance à cristal de quartz avec dissipation (QCM-D), suivi d'une caractérisation et d'une visualisation de surface avec un microscope à force atomique (AFM) et un microscope électronique à balayage (SEM). Des tests de viabilité et de différenciation cellulaire ont été effectués sur des cellules de lignée pré-ostéoblaste murine MC3T3-E1 en utilisant respectivement les essais AlamarBlue et Phosphatase Alcaline (ALP). L'activité antibactérienne des disques fonctionnalisés a été évaluée après une exposition à la culture liquide pour 24 heures, via des dénombrements de colonies adhérentes de Staphylocoques dorés sensible à la méthicilline (MSSA).

La caractérisation de surface confirme le dépôt multicouche par étapes et l'incorporation de nanoparticules, validant cette itération de l'assemblage LbL. Les tests de viabilité montrent une prolifération accrue de MC3T3-E1 à des doses modérées (13,33 mg/ml) de TiO2NPs par rapport à tous les autres groupes après 1 et 4 jours. Les concentrations supérieures d'AgNPs (4 mg/ml) présentent une cytotoxicité marquée (environ 30 % de viabilité par rapport au témoin) après 4 et 7 jours de culture. Les tests ALP effectués après 7 jours démontrent une différenciation améliorée dans une dose-réponse positive pour les groupes TiO2NPs. Au jour 7, l'activité ALP des AgNPs montre la réponse relative la plus élevée dans le groupe à faible concentration (0,4 mg/ml) par rapport aux autres, bien que non-significativement différente du groupe à forte concentration de TiO2NPs (26,67 mg/ml). Les tendances dose-réponse pour les AgNPs ne sont pas concluantes. L'activité antibactérienne de cette structure revêtue contre MSSA ne montre aucun effet principal.
significatif, mais l'analyse de régression des groupes TiO2NPS montre un effet antibactérien significativement dose-dépendant. Ces résultats peuvent servir à motiver le peaufinement de ce revêtement de surface pour la mise en œuvre éventuelle dans des modèles *in-vivo* d'infection articulaire périprothétique (PJI).
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Firstly, it is my privilege to thank my co-supervisors Drs. Maryam Tabrizian and Adam Hart who have, for the last 2 years, provided me with the endless support and guidance necessary to prepare and successfully complete my Master’s project. They have both been integral in my coming this far. Dr. Michael Tanzer has also been a steadfast resource and advisor throughout my research, encouraging and challenging me to bring out the best in my work. I also want to thank Dr. Ling Li, the lab manager of BiomatX Research Laboratories, for the prompt contribution of her expertise and guidance whenever called upon.

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<td>PJI</td>
<td>Periprosthetic joint infection</td>
</tr>
<tr>
<td>TKHA/TKA</td>
<td>Total Knee/Hip Arthroplasty / Total Knee Arthroplasty</td>
</tr>
<tr>
<td>mNPs</td>
<td>Metal nanoparticles</td>
</tr>
<tr>
<td>DAIR</td>
<td>Debridement, antibiotic, and implant retention</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant Staphylococcus Aureus</td>
</tr>
<tr>
<td>TNT</td>
<td>Titanium nanotubes</td>
</tr>
<tr>
<td>LbL</td>
<td>Layer-by-Layer</td>
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<tr>
<td>TiO2NPs</td>
<td>Titanium dioxide nanoparticles</td>
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<tr>
<td>AgNPs</td>
<td>Silver nanoparticles</td>
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<tr>
<td>PEM</td>
<td>Polyelectrolyte multilayer</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>QCM-D</td>
<td>Quartz crystal microbalance with dissipation</td>
</tr>
<tr>
<td>MSSA</td>
<td>Methicillin-sensitive Staphylococcus Aureus</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>mNPs</td>
<td>Metal nanoparticles</td>
</tr>
<tr>
<td>PNAG</td>
<td>Poly-b(1e6)-N-acetylglucosamine</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>APTES</td>
<td>3-aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>NTA</td>
<td>Nanoparticle tracking analyzer</td>
</tr>
<tr>
<td>pNPP</td>
<td>Para-Nitrophenylphosphate</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
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</table>
Chapter 1: Introduction and Research Objectives

Introduction and research rationale

Total hip and knee arthroplasty (TKHA) are performed on over 150,000 Canadians each year\textsuperscript{1}, and rates are projected to increase in the coming years\textsuperscript{2}. Periprosthetic joint infection is the most common cause implant failure following joint replacement surgery\textsuperscript{3,4}. Treatments for septic implant failure exist, such as Debridement, antibiotics, and implant retention (DAIR) protocols, one-and two-step revisions, and more\textsuperscript{5}. Unfortunately, these procedures are highly morbid, patient outcomes are poor, and eradication of the infection is not always possible\textsuperscript{5}. As well, multi-step, and long-term care results in grossly heightened costs for both medical institutions and patients\textsuperscript{6}. A change toward available, cost-effective preventative measures is much needed.

Bioactive surface coatings have been researched extensively for the various benefits they can provide by imparting antibacterial and other properties onto select substrates\textsuperscript{7}. Layer-by-Layer (LbL) self-assembly of polyelectrolytes is a technique for the application of charged polymers onto a biomaterial\textsuperscript{8}. This process forms what is known as a polyelectrolyte multilayer (PEM), which can, depending on the polymers used, improve osteoblast adhesion and proliferation, while reducing bacterial colonization and growth\textsuperscript{9}. Chitosan and alginate are well-studied, abundant, naturally derived, and biocompatible polyelectrolytes. They have previously been used in PEMs and have demonstrated promising innate antimicrobial and osteoconductive effects\textsuperscript{10,11}.

Metal nanoparticles (mNPs) have also received attention for their antimicrobial effects\textsuperscript{12}. To further enhance antibacterial activity of the PEM, while reducing the risk of antibiotic resistance, mNPs can be integrated into the multilayer. Silver nanoparticles (AgNPs) have long been considered for their antibacterial capacity, though their cytotoxicity has greatly halted progression toward clinical application\textsuperscript{7,13}. Titanium dioxide nanoparticles (TiO2NPs), on the other hand, have shown antibacterial activity, while demonstrating better cytocompatibility\textsuperscript{14,15}, and even enhanced bone formation\textsuperscript{16}.

As such, the research presented herein aimed at developing a novel nanoparticle-imbedded PEM on a semi-porous titanium substrate using alternating layer depositions of chitosan and alginate, with the incorporation of either AgNPs or TiO2NPs. The LbL method was employed using chitosan and alginate. These polysaccharide-based polyelectrolytes have been extensively
researched due to their high biocompatibility, porous microstructure, and antimicrobial activities\textsuperscript{10,11}. To further enhance the antibacterial effects of the chitosan/alginate coating, AgNPs or TiO2NPs were incorporated into the multilayer at increasing concentrations to observe their dose-effect relationships, as well as to compare the biocompatibility and antimicrobial activity of the different nanoparticles and overall formulations.

**Hypothesis and research objectives**

The goal of this research was to fabricate a nanoparticle-incorporated PEM using the LbL self-assembly technique for quantitative assessment of their antibacterial activity and cell proliferation once used as a coating on porous titanium substrates. The use of the specific nanoparticles mentioned herein related to their respective bioactive properties, seeking to discern which of the two offered overall superior antibacterial and biocompatible effects, as literature lacks a direct and comprehensive comparison between these two materials. It was hypothesized that substrates coated with the PEM and either nanoparticle would show improved antibacterial activity compared to uncoated counterparts, or those with coatings but without nanoparticles, while silver and titanium nanoparticles would show comparable effects. Further, it was hypothesized that TiO2NPs would demonstrate superior biocompatibility to all other coated, or uncoated groups. To meet this end, we established the following objectives:

1. Develop and characterize a polyelectrolyte multilayer with imbedded TiO2NPs and AgNPs on porous titanium substrates.
2. Study *in-vitro* cell viability and differentiation analyses on MC3T3-E1 pre-osteoblast cells.
3. Assess antimicrobial activities of coated titanium substrates against *S. aureus* bacteria.
4. Perform side-by-side comparison of TiO2NPs and AgNPs effects encapsulated in PEM on cell viability and antibacterial activities.

**Thesis outline**

Chapter 1 provides a brief introduction, rationale, and research objectives for the project. Chapter 2 describes the background literature on TKHA, periprosthetic joint injection, treatments, and challenges.
Chapter 3 summarizes the LbL self-assembly technique, and the methods & materials commonly used.

Chapter 4 presents a background of nanoparticles, specifically the mNPs, silver and titanium dioxide, in the context of antimicrobial activity.

Chapter 5 denotes the methods and materials employed to fulfil the aforementioned research objectives.

Chapter 6 presents the collected data and figures collected from experimentation.

Chapter 7 discusses the results presented earlier in the context of the research questions, and considers the limitations and future directions.

Chapter 8 provides a brief recapitulation and summary of the research questions and results in context.
Chapter 2: Periprosthetic Joint Infection

State of the science and prospective prevalence

Total knee/hip arthroplasty (TKHA) boasts high efficacy as a surgical intervention to restore and ameliorate function and quality of life to patients suffering from arthritis or other diseases or malformations of those joints\textsuperscript{2-4,17}. With over 150,000 TKHAs performed annually in Canada\textsuperscript{1}, and 800,000 worldwide, projections estimate increases in incidence to above 4 million by the year 2030\textsuperscript{17}. Others estimate based on multiple projection models an annual increase in primary TKHA rates by an average of 43\%\textsuperscript{2}.

Implant design, surgical approach, materials, and additional instrumentation used for these procedures will vary depending on the case. The fixation method, for example, is a subject of consideration, and has previously undergone extensive comparative research examining the differences between cementless and cemented fixation on patient outcomes\textsuperscript{18}. As well, the material that composes the components of the implants can vary, and the decision to use a particular type depends on a number of factors, like allergy or hypersensitivity to metals\textsuperscript{19,20}. As such, in the cases where traditional materials like cobalt-chromium (CoCr) and titanium are unsuitable, they can be treated to produce titanium niobium nitride, titanium nitride, or zirconia nitride coated surfaces. Alternatively, they can also be substituted for ceramic components, which are composed of aluminum oxide (Al\textsubscript{2}O\textsubscript{3}) or zirconium oxide (ZrO\textsubscript{2})\textsuperscript{20}. Further, off-the-shelf versus patient-specific instrumentation (PSI) (Figure 1) is also a subject of contention, as personalized medicine continues to be an important consideration, especially when personally-fitted equipment or prostheses could make surgery easier, faster, and potentially improve patient outcomes\textsuperscript{21}. Figure 2 depicts an x-ray of one such implanted prosthesis.

Unfortunately, despite the numerous surgical advances in implant design, surgical technique, and perioperative care, component failure remains a major concern and represents a revision rate of approximately 5\% at 20 years\textsuperscript{2,17,22}. Revision surgeries require extensive treatment and place an immense burden on the healthcare system and on patients, both economically and psychologically\textsuperscript{17,23,24}. Today, the most common cause for revision is periprosthetic joint infection (PJI), accounting for 15.3\%\textsuperscript{4} of complications and is responsible for approximately 25\% of revision TKHAs within the first 2 years of primary intervention\textsuperscript{3}. The risk of PJI is even higher
upon revision, with rates between 3.3 and 5.6\%\textsuperscript{3}. Figure 3 shows a graphic image of a TKA infection case.

![Image](image_url)

Figure 1. Image depicting a PSI distal femoral cutting guide and a 3D model of a patient’s distal femur. Mattei et al. (2016), with permission from Annals of Translational Medicine.

Collectively, with the projected increases in the number of TKHAs, growing antimicrobial resistance, and the growing rates of infection, the need for preventative methods is of paramount importance.

**Infection and biofilm**

Infection is the most common cause of implant failure\textsuperscript{25}, and therefore necessitates understanding of bacterial colonization mechanisms, as well as the means by which bacteria can be eliminated.

Of the causative infectious organisms responsible for PJI, the gram-positive bacteria *Staphylococci* account for approximately 75\% of infections\textsuperscript{26}. *Staphylococcus Aureus* is a Gram-positive, facultative anaerobic bacteria, and is an essentially ubiquitous member of natural human flora. In contrast, virulent and pathogenic strains of this bacteria also exist. It can infect just about any human tissue, ranging from bones, to skin, to visceral organs, and more, leading to its
classification as a Level 2 Risk Group. Owing to its high level of adaptability, and its frequent exposure to antibiotic therapies, there has been inadvertent selection for drug-resistant strains, leading to therapeutic failures\textsuperscript{27}. \textit{S. Aureus}, as well as \textit{S. epidermidis}, are the most commonly responsible organisms for PJI, with 53\% of \textit{Staphylococci} isolates found to be methicillin-resistant\textsuperscript{23}, such as methicillin-resistant \textit{Staphylococcus aureus} (MRSA). These infections can be particularly problematic, as upon bacterial colonization, they may proliferate at 0.5cm\textsuperscript{2} per hour, and can create a robust protective biofilm on the implant surface within 24 hours\textsuperscript{26}.

The mechanism of this biofilm formation is illustrated in Oliveira \textit{et al}. and Chourifa \textit{et al}.\textsuperscript{7,26}. Bacterial invasion may initially be caused by transfer via contact with contaminated devices, hands, or other surgical equipment, after which biofilm-producing bacteria like \textit{S. aureus} adhere to other cells and/or implants\textsuperscript{26}. This initial bacterial adhesion is considered the first of the three-step biofilm formation process. Importantly, this first stage corresponds to a reversible interaction consisting of van der Waals and electrostatic forces between the bacteria and the surface to which they are adhered, contrary to hydrogen- and ionic bonding, as well as dipole interactions formed in subsequent stages\textsuperscript{7,26}. This poses an interesting timeframe for early/preventative intervention. The second stage is microcolony formation, along with the activation of the biofilm-forming phenotype. Finally, the maturation of the biofilm, referred to also as microcolonies, occurs\textsuperscript{7,26}.

Figure 2. Frontal radiograph of left total hip arthroplasty (a) preoperative and (b) postoperative. Images taken by Dr. Adam Hart.
These biofilms adhere to the substrate with the help of extracellular polymeric substances, which mainly consist of polysaccharides, but also lipids and proteins. One such polysaccharide is the intercellular adhesin poly-b(1e6)-N-acetylglucosamine, or PNAG – the major contributor to adhesion of common Staphylococci species to medical devices26.

The major issue that results from biofilm formation is its effect on bacterial resistance to antimicrobials. Through delayed or inhibited penetration of the biofilm matrix, dormant cells, and development of resistant variants, biofilm-protected bacteria may enjoy a 500- to 5000-fold increase in their antimicrobial resistance, compared to their planktonic counterparts26. Major efforts in the medical field are focused on finding ways to combat infection by preventing inoculation of the bacteria onto the implant and subsequent biofilm formation.

**Treatments and challenges**

Debridement, antibiotics, and implant retention (DAIR) is widely used and is considered a safe and effective treatment for acute presentation of PJI5,28–31, when the biofilm is presumably immature. Byren et al. describes the DAIR protocol as performed based on 112 cases of PJI treated
with this method\textsuperscript{32}. Briefly, management was split into two categories: antibiotic management, and surgical management. Treatments generally began with intra-operative tissue and microbiological sampling for identification of the pathogens, as well as margin excision and necrotic tissue (and other debris) removal. The integrity of the implant and its integration was assessed and retained if sufficiently fixed. Loose grafts or modular components were removed or replaced if necessary. Tissues were finally irrigated, and the wound closed over drains. For six weeks following surgery, patients were administered intravenous antibiotic therapy targeted to the causative pathogen(s). Subsequently, patients received oral follow-on antibiotic treatment for a minimum of 12 months, or on a case-by-case basis. \textit{S. aureus} was isolated from 42\% of the examined infections, with 8\% of the total infections containing MRSA.

Other treatments for PJI include long-term suppressive antibiotic treatment (when further surgery is not possible), one- or two-stage revision, resection arthroplasty, arthrodesis, or amputation; the latter two considered only as a last resort\textsuperscript{5}.

Many other studies and reviews have been performed to examine the efficacy of DAIR, and have found widely varying success rates, ranging anywhere from 11\% to nearly 100\%\textsuperscript{5,30,32}. Such a wide range in clinical outcomes is dependent on a number of factors, including geographic location where treatment is given, age, joint type, type of infection, and duration of DAIR treatment, to name a few explicitly investigated\textsuperscript{5}. Yet, regardless of the treatment outcome, the presence of an infection and its subsequent treatment with DAIR imposes a significant financial burden on patients and healthcare systems. For example, research done by Morcos \textit{et al}. found that overall costs to patients are five-fold higher in those who undergo two-stage TKA revision\textsuperscript{6}. Additionally, the cost of revision for infection cases are two-fold higher than their aseptic counterparts\textsuperscript{33}.

\textbf{Alternative treatments / Preventative measures}

In response to the issues presented above, one of the best options to combat PJI would be to reduce the risk of its occurrence or development in the first place. In other words, prevent infection. Using preventative methods as opposed to curative solutions may effectively eliminate the requirement for considering complex biological mechanism and processes, such as the chemistry involved in managing bacterial adhesion to implants, as well as dealing with issues in antibiotic usage against multidrug resistant infections. This alternative, preventative approach would have to address two
aspects relating to infection: bacterial repulsion from the implant surface, and killing of the bacteria. These concepts are known also as bacteriostatic or bactericidal properties, respectively. The application of these properties can then be performed by chemical or physical modification of the implant surface itself, or by application of a coating bearing those properties to the implant. It may also be possible to use materials that attain these goals, but which also impart additional characteristics that facilitate or ameliorate the recovery process following surgery. These additional benefits could include enhanced bone growth onto and into the implant surface (osteointegration and osteoconduction), improved osteogenesis by stimulation of pre-osteoblast recruitment (osteoinduction), and ultimately improve implant stability.

**Surface modification**

Bacteriostatic modifications describe the changes in surface properties designed to inhibit bacterial adsorption to the implant surface. There are multiple mechanisms by which this can be achieved, including reduced surface energy, electrostatic repulsion, or steric repulsion. In such cases, bacteria are prevented from colonizing the surface of the implant, though they are not killed by the modification. Though importantly, non-selective bacteriostatic modifications tend to also influence host cells, and so it is advised to use combined approaches that also promote their proliferation. Presented below (Table 1) is a table summarizing some recent research into coating materials and their respective effects on cell adhesion and viability. Some examples here that show promise are chitosan, hyaluronic acid, and titanium dioxide for their abilities to promote proliferation, differentiation, and adhesion of bone cells and fibroblasts. As can also be noted, the application of these modifications can be performed on different substrates, further detailing the versatility of these materials.
Table 1. Recent development of osteogenic coatings on orthopedic and dental implants to improve osteointegration. Adapted from Liu et al. (2021), with permission from Biotechnology Journal.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Composition of surface coating – associated coating strategy</th>
<th>Effects on surface characteristics</th>
<th>Biological effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaP-based coatings</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure Ti</td>
<td>HA – electrochemical deposition</td>
<td>Controllable and homogeneous coating</td>
<td>Induced in vitro mineralization</td>
</tr>
<tr>
<td>SiO₂/MgO doped HA – plasma spraying</td>
<td></td>
<td></td>
<td>↑ In vivo osteogenesis and osteointegration</td>
</tr>
<tr>
<td>Chitosan/gelatin/HA – LBL assembly</td>
<td></td>
<td></td>
<td>↑ Adhesion, proliferation and osteogenic differentiation of MSCs</td>
</tr>
<tr>
<td>TNT – electrochemical anodization</td>
<td></td>
<td></td>
<td>↑ Adhesion and mitigation of HUVECs</td>
</tr>
<tr>
<td>Ti6Al4V</td>
<td>HA – plasma spraying</td>
<td>↑ Surface roughness</td>
<td>↑ Adhesion and attachment of osteoblast</td>
</tr>
<tr>
<td></td>
<td>HA – magnetron sputtering</td>
<td>Homogeneous crystalline coating</td>
<td>↓ Osteosarcoma</td>
</tr>
<tr>
<td></td>
<td>Si doped HA – dip coating</td>
<td></td>
<td>↑ In vivo osteointegration</td>
</tr>
<tr>
<td></td>
<td>VEGF – physical adsorption</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biophasic CaP</td>
<td>nHA – hydrothermal deposition</td>
<td></td>
<td>↑ Viability and osteogenic differentiation of MSC</td>
</tr>
<tr>
<td>PLA</td>
<td>HA – biomimetic coating</td>
<td>Nanoroughened</td>
<td>↑ Adhesion, proliferation and osteogenic differentiation of hMSCs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ Hydrophilicity</td>
<td></td>
</tr>
<tr>
<td>TiO₂-based coatings</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure Ti</td>
<td>TiO₂ – thermal atmospheric oxidation</td>
<td>↑ Hydrophilicity</td>
<td>↑ Attachment of fibroblasts</td>
</tr>
<tr>
<td></td>
<td>TiO₂ – thermal oxidation</td>
<td></td>
<td>↑ In vitro apatite formation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Expression of osteogenic genes and new bone formation</td>
</tr>
</tbody>
</table>

Bactericidal modifications would perform the antimicrobial function necessary for sure-fire infection prevention. The mechanisms that can be employed to this end are through contact killing, or by the release of biocidal molecules. As mentioned just above, one major consideration regarding modifications of this nature is ensuring that native cells/tissue are not harmed in the process. The variety of methods that can be used to forward this objective are depicted in Table 216.
Table 2. Recent development of antibacterial coatings on orthopedic and dental implants. Adapted from Liu et al. (2021) with permission from Biotechnology Journal.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Composition of surface coating - associated coating strategy</th>
<th>Effects on surface characteristics</th>
<th>Biological effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure Ti</td>
<td>PEG/GRD – plasma polymerization, electrodeposition, silanization</td>
<td>† Hydrophilicity</td>
<td>† Fibroblast adhesion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>† Albumin adsorption and adhesion of S. sanguinis and L. salivarius</td>
</tr>
<tr>
<td></td>
<td>PEG – electrodeposition RGD/LF1-11 – covalent immobilization</td>
<td>† Hydrophilicity</td>
<td>† Osteoblast adhesion and spreading</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>† Adhesion and viability of S. sanguinis and protein adsorption</td>
</tr>
<tr>
<td>GL13K (AMP) – covalent immobilization</td>
<td>† Hydrophilicity Slightly † surface roughness</td>
<td>† Viability of P. gingivalis</td>
<td>† Fibroblast viability and attachment</td>
</tr>
<tr>
<td>Osteogenic growth peptide/ciprofloxacin – covalent immobilization</td>
<td>† Hydrophilicity → Surface roughness</td>
<td>† Osteoblast spreading and osteodifferentiation</td>
<td></td>
</tr>
<tr>
<td>NO releasing coating – silanization, diazeniumdiolate functionalization</td>
<td>† Hydrophilicity † Surface roughness</td>
<td>† Adhesion and colonization of S. aureus and P. aeruginosa</td>
<td>No cytotoxicity to osteoblasts</td>
</tr>
<tr>
<td>Ag/PDA – physical adsorption</td>
<td>-</td>
<td>† Growth and colonization of S. mutans and P. gingivalis</td>
<td></td>
</tr>
<tr>
<td>Ag NPs/GO – electroplating</td>
<td>Microstructured</td>
<td>† Adhesion and viability of S. mutans and P. gingivalis</td>
<td>Cytotoxicity with higher GO and Ag NPs</td>
</tr>
<tr>
<td>GL13K/TNT – physical adsorption/electrochemical anodization</td>
<td>-</td>
<td>† Growth of F. nucleatum and P. gingivalis</td>
<td>Proliferation and no cytotoxicity to MC3T3-E1 cells</td>
</tr>
<tr>
<td>Ag NPs/ vancomycin/TNTs – plasma immersion ion implantation (PIII) – functionalization</td>
<td>-</td>
<td>† Adhesion of S. aureus No cytotoxicity to fibroblasts</td>
<td></td>
</tr>
<tr>
<td>Ag/TNT – electrochemical anodization Chitosan/divalent cation – LBL assembly</td>
<td>Nano-roughened † Hydrophobicity after LBL</td>
<td>† Osteoblast growth</td>
<td>Delayed Ag release † Adhesion and growth of S. aureus and E. coli</td>
</tr>
</tbody>
</table>

Similar to the above modification options, some of the listed materials have adhesion- and proliferation-promoting effects toward mammalian cells. The intended antimicrobial activity is also simultaneously achieved in most of the presented cases, where reduced bacterial adhesion is seen in multiple strains, along with a reduction in bacterial growth. Some of the major active components of these coatings include titanium nanotubes, AgNPs, and polymer multilayers. Care should be taken when using some materials however, as sufficiently high doses of AgNPs for example, do demonstrate cytotoxicity. This dose-dependent cytotoxicity of nanoparticles will be further elaborated upon in the “Metal nanoparticles” section.

**Porous metal implants**

Another type of modification creates pores on the surface of the material, usually titanium. Initially, this process was a physical surface modification for fabrication of titanium nanotubes (TNTs). In such a process, nanotubes were synthesized by a number of different approaches,
including sol-gel, hydrothermal or template-assisted synthesis, or by electrical anodization, the latter being the simplest and most economical of the fabrication methods\textsuperscript{25}. In experiments examining the effects of the surface treatment, it was found that bacterial adhesion to the material was reduced compared to a control surface, explained by a few mechanisms, but most notably the surface charge\textsuperscript{25}. The effect that this modification had on the materials surface roughness also led to more reduced adhesion, as increases in surface roughness in the nanometer range generally results in decreased adhesion\textsuperscript{25}.

Additionally, with the majority of hip-, and a growing proportion of knee replacement implants being cementless, fabrication of innately porous titanium implants with defined specifications have been developed. These structures have been shown to have good biocompatibility, and offer enhanced implantation stability, while also serving to facilitate osteogenesis and integration\textsuperscript{35}. For these reasons, a porous titanium substrate was also used for this project.

Given the possibilities described above, it can be suggested that a combinatorial approach using such methods may prove to be an effective multifunctional preventative measure to combat PJI. One common methodology for assembling these different modifications is known as the layer-by-layer (LbL) self-assembly technique.
Chapter 3: Layer-by-layer Self Assembly of Polyelectrolytes

**LbL technique**

LbL self-assembly is a simple, easy, and versatile method for building up multilayers using alternating coating applications of oppositely charged polyelectrolytes\(^8,9,36\), usually with the intention of imparting their characteristics onto the whole. Figure 4, from Elizarova & Luckham depicts the concept and simplicity of this method\(^8\). Primary interest here is in part “(a)” of this figure, as the current project focuses on thin film application to flat surfaces.

![Figure 4. Conventional electrostatic layer-by-layer assembly on (a) flat substrate and (b) colloidal substrates. Elizarova & Luckham (2018) with permission from Elsevier.](image)

As the authors describe, the LbL technique enlists the materials’ intrinsic electrostatic properties to assemble multilayers, where the procedure can be summarized as briefly as: immerse-rinse-repeat. The dilute polyelectrolyte must simply be allowed time to adsorb to the surface, before rinsing off excess. Aside from its simplicity, the method boasts high reproducibility, efficiency\(^8\), and requires no expensive sophisticated equipment\(^37\). The materials that are most often used for LbL assembly are also diverse, can be used with different substrates\(^38\), and generally abundant and low-cost\(^39\).

There are also different means by which the polymers can be applied; dip- or spin-coating, as well as spraying or perfusion. Almeida *et al.* describe the differences in surface morphology and characteristics between the dip- and spin-coating methods using catechol-modified chitosan and hyaluronic acid, with or without incorporation of bioactive glass nanoparticles\(^9\). The dip-coating protocol used was effectively identical to the one previously mentioned. Spin-coating was performed with specialized equipment, and layers were deposited as a defined volume of
alternating polyelectrolyte over a specified period on three different substrates: glass, stainless steel, and titanium foils. Their results denote an overall “smoother and more homogenous surface morphology” (Almeida et al., 2020) in the spin-coating group compared to the dip-coating group, though this difference was less pronounced in the metal samples compared to the glass. Surface wettability was almost always improved by the coating procedure (except in the titanium groups), especially when incorporated with the nanoparticles. The coating thickness was also greater in groups coated by dipping. The authors claimed that greater surface roughness like that observed on the dip-coated multilayered titanium samples has been shown to promote superior osteoblast anchoring, survival, and differentiation.

Major considerations for this approach are therefore the choice of polyelectrolytes used to suit the intended purpose, which in turn, may also be influenced by the substrate to be coated. In the context of the medical application mentioned previously, titanium would be the base material used as the substrate for these applications, for its ubiquitous presence in orthopedic surgery. Then, the application method must also be determined, to fit the needs of the formulation as well as the resources available.

**Chitosan**

There are many different compounds that can be used as coating materials for the purposes of antimicrobial and osteoconductive activity. Chitosan is one of the most popularly researched polymers in the area of bioactive materials for coating, drug delivery, and tissue engineering. Derived from chitin via deacetylation, chitosan is one of the most abundant natural polymers, and can be harvested and prepared relatively cheaply. A cationic polymer, chitosan is a polyelectrolytic compound, capable of forming polyelectrolyte films and complexes with other charged materials.
There are a number of reasons for chitosan’s popularity in research areas involving tissue engineering, wound healing, and orthopedics. Firstly, its cationic nature facilitates cell adhesion, which is further enabled by its nontoxic and biocompatible qualities. Owing in part to its high (tunable) porosity and biomechanical compatibility, it is also osteoconductive and capable of promoting bone formation and enables cell ingrowth. In addition, it has demonstrated innate antimicrobial activity. For drug delivery, chitosan also proves useful due to its biodegradation. Given these characteristics and versatility, chitosan is a prime candidate for use as a component in the design of an antimicrobial and biocompatible surface coating.

**Alginate**

Alginate is another natural polysaccharide, derived often from seaweed and brown algae, and is negatively charged. Alginate, like chitosan, also supports a large variety of modifications, and is capable of forming microcapsules, hydrogels, and more. Alginate shares many of the beneficial characteristics described above with chitosan, such as its abundance, low cost, non-toxicity, and biocompatibility. It has been used in conjunction with other biomaterials for applications in drug delivery, wound dressing, bone, and other tissue engineering therapies.

In particular, previous research using the LbL technique with both chitosan and alginate (or hyaluronic acid) have shown promise in their combined usage toward antimicrobial activity and biocompatibility. Though, thin films are not the only type of polyelectrolyte complex that can be formed. Figure 7 depicts other forms that complexes can take for a variety of different uses.
Surface functionalization

The LbL protocol is quite straightforward when both the layering and base materials are charged. Base materials used as the anchor for the multilayer are known as template materials\textsuperscript{8}, and are generally electrically charged. However, even if the base material is not electrostatically active, it can still be used for LbL purposes, on the condition that the substrate can be treated in some ways, including surface functionalization enabling the bonding between the layering and template materials. Surface functionalization of template material offer a stronger, more stable, and durable bond between the substrate and the coating, as well as permit, facilitate, or strengthen the subsequent modifications. Surface functionalization may include grafting material to or from the substrate\textsuperscript{7} or the application of the polyelectrolyte multilayers, among many others.

Chouirfa \textit{et al.} describe two general surface functionalization techniques, “grafting from” and “grafting to”\textsuperscript{7}. The “grafting from” technique is a direct method to produce a surface chemistry
that more readily and covalently binds the desired coatings. In one example, researchers managed to graft a bioactive polymer poly(sodium styrene sulfonate) to titanium substrates in a two-step procedure. First, the titanium surface was treated with a sulfuric acid and hydrogen peroxide mixture, producing titanium hydroxide and peroxide functional groups on the surface. The substrate was then heated and submerged in a sodium styrene sulfonate monomer solution. This led to peroxide decomposition and catalysis of the sodium styrene sulfonate polymerization onto the substrate surface. This chemically treated substrate was then used for direct grafting of the desired compound.

The “grafting to” approach adds an additional step to the process by first functionalizing the substrate with an anchor molecule for the subsequent covalent attachment of the layering compounds. This method often requires a crosslinking agent to ensure sufficient reactivity and bonding. Some examples of anchor molecules used for this purpose include silane, catechol, and phosphate. The silanization protocol functionalizes the metal base material with the covalent attachment of organofunctional alkoxysilane. The subsequent bonding of proteins, peptides, or polymers is then achieved with the use of bifunctional linker molecules like glutaraldehyde or maleimide. Alternatively, catechol-based modification can be achieved using three methods. In all cases, catechol acts as the linker molecule. The major differences between the three methods are whether the catechol group is first attached to the substrate or the polymer. Phosphor-based modifications function on the principle that these compounds act as the crosslinker between the substrate and layering material. It has also been found that these phosphonate linkers are more stable than some other anchor molecules which may suffer from instability when under certain conditions.

Other research has also implemented a different surface functionalization methodology that involves the adhesion of a protein film onto a substrate surface for the purpose of imparting additional hydrophilicity, positive charge, and anticorrosion characteristics. This “phase-transited lysozyme” has been used previously as a linker and surface functionalization for the application of polymer multilayers. This method, which is 100% reversible, also simply achieves its intended purpose with a one-pot protein immobilization.

The implantation process of orthopaedic surgery is a physically aggressive one, and there exist risks of damage to the bioactive coating applied to the implant surface. Should this coating be damaged, it may be expected to observe a reduction in its desired effects, such as reduced
osseointegration, disorderly bone regrowth, or even the loss of any antimicrobial activity. In order to reduce these risks and ensure sufficient bond strength between the titanium implant substrate and its bioactive coating, we opted for crosslinking using a homobifunctional crosslinking agent, glutaraldehyde. This allowed to enhance the interaction between the substrate and coating, via covalent linkage.

**Surface characterization techniques**

For all the complexities involved in these processes, methods have been developed to validate and confirm the techniques applied. As this project was interested in LbL buildup, surface morphology, and cell assaying, the following are some of the most common methods employed to assess such characteristics.

Quartz Crystal Microbalance with Dissipation (QCM-D) is a measurement tool that allows real-time assessment of changes in frequency ($\Delta f$) and energy dissipation ($\Delta D$) as a substance is flowed over a resonating conductive quartz crystal disc which may be thinly coated with a given material. Simply, the principle is based on measuring the vibrational deviations of the crystal from its fundamental (and overtone) resonance frequency, which is known. As a substance is flowed across the disc surface, and material adheres/adsorbs to or desorbs from its surface, detectible changes in the frequency of its vibration are observed and measured. The dissipation, or dampening measurement represents the cumulative loss in energy of the system over the oscillation cycle. The effect of this dampening is particularly present with soft thin films, compared to more rigid films. Together, this technology allows us to observe and measure in real-time the performance and quality of our proposed layer-by-layer polyelectrolyte coating as it is being formed.

Atomic Force Microscopy (AFM) is an imaging tool used to gather high resolution data of surface characteristics. Using an electrical current, a conductive probe is made to resonate while being moved along the surface of a substrate. As a laser contacts the probe tip, and reflects onto a detector, the diffraction of that incident beam due to surface-related changes in the probe’s vibration provides topographical information, which is translated into an image representing a map of the surface characteristics. With a number of different modes, including Contact, Tapping, and PeakForce HR, it is possible to collect different forms of information from a sample, such as surface structure and roughness, elastic modulus, and coating thickness.
MC3T3-E1 cell assays

AlamarBlue is a well-documented and widely used assay for testing cell viability and cytotoxicity. The major purpose of using the AlamarBlue assay as opposed to other options was for its ease of use, sensitivity, and its lack of interference in cellular processes, allowing for a single sample to be tested multiple times, at different time points.

Alkaline phosphatase (ALP) activity is a well-known early genetic marker for osteoblast differentiation, and its timewise production profile for MC3T3-E1 cells is well documented. Thus, the above two assays were chosen to document the changes in MC3T3-E1 metabolism and viability resulting from exposure to the formulated coating.

This above chapter denoted the major research and most used methods for surface functionalization and analysis for the purpose of LbL surface modification using polyelectrolytes. In reality, there are a variety of options for this method, including the choice of materials for functionalization, coating, or additional additives to the multilayer. While each prospective component offers its respective characteristics and advantages, the choice of material combinations chosen are made in response to the specific challenges and problems that researchers have sought to overcome. For the purposes of enhancing bactericidal activity, for example, one can find an additive that can supplement those effects. This enhancing of the antimicrobial activity of the multilayer is often attempted with the implementation of incorporated particles into the multilayer structure. These may include the incorporation of antibiotics, antimicrobial peptides, or nanoparticles. Since antibiotic resistance is a concern in modern medicine, and nanoparticles are a popular topic in this area of research, we focus our attention on the use of these nanoparticles, namely silver and titanium dioxide nanoparticles for antimicrobial activity in this context.
Chapter 4: Metal Nanoparticles

Nanoparticles

Nanomaterials are defined as materials with at least one dimension in and whose basic unit is in the nanometer scale, generally between 10-100nm\(^6\). Nanoparticles are the unit materials that fit within this definition. They are classified by their composition and structure, while their size and properties dictate their characteristics and possible interactions with organisms and biomaterials. Figure 8 illustrates the categorization of different nanoparticle types, and includes examples and some details on their properties\(^1\). Some of these nanostructures can be used as carriers, moving drugs or other cargo to sites of interest with high bioavailability, and reduced toxicity. Others may act themselves as therapeutics against microbes.

![Figure 8. Nanoparticle Types and Examples. Thambirajoo et al. (2021). Open access from MDPI.](image)

In most cases, the appeal of nanoparticles comes from their small size, high surface-to-volume ratio, increased reactivity (compared to bulk material), and good stability\(^1\). However, the shift to a focus on nanoparticles as antimicrobial agents originates from an increased need for an alternative to antibiotics, especially in cases where antibiotic resistance has become a persistent
problem. Research has shown that the use of nanocoatings with particle enrichment can reduce bacterial adhesion and growth, while also improving the adhesion and growth of other cell types, like osteoblasts\textsuperscript{64}.

**Metal nanoparticles**

As described briefly in Figure 8, mNPs exist as nanometer-sized, often spherical units of elemental metal or metal oxides\textsuperscript{12}. Some examples that have been researched most commonly are silver, copper, zinc oxide, gold, and TiO2NPs. Overall, metal-based nanoparticles have received much attention for their apparent ability to circumvent current issues in bacterial drug resistance, by targeting the bacterial cell wall, while directly and indirectly initiating processes resulting in the bacteria’s demise. The activity and reactivity of these nanoparticles are highly influenced by their size and shape, as these are defining characteristics for determining the particle's surface area to volume ratio. It is this relation that effectively dictates binding and ion release properties; important factors for antimicrobial activity. This relationship between size and reactivity (or antimicrobial activity) is inversely related. That is, the smaller the nanoparticle, the more potent it will be. This potency is a measure of metal ion release rate, where the higher relative surface area (and thus smaller size) allows for a greater rate of ion release\textsuperscript{7,12,64,65}. The next section describes in more depth the mechanisms of action employed by mNPs for antimicrobial activity.

**Antimicrobial mechanisms**

Figure 9 depicts the antimicrobial mechanisms of AgNPs. However, they are similar enough to assist in the graphical understanding of the general antibacterial mechanisms of mNPs\textsuperscript{52}. Wang \textit{et al.} classify the antibacterial mechanisms of mNPs into 3 overarching categories: metal ion release, oxidative stress, and non-oxidative mechanisms\textsuperscript{64}. They also describe each of these categories in depth. The following explanations are adapted from these authors, though other research corroborates these descriptions\textsuperscript{12,16,52,57,66,67}.
For metal ions, they simply penetrate the bacterial cell wall and interact with the functional groups of intracellular proteins. This directly influences enzymatic activity, which leads to downstream cell damage through interruption of normal cellular processes, and alteration of bacterial physiology.

Oxidative stress refers to the loss of balance in intracellular redox potential as a result of excessive reactive oxygen species (ROS) production. These ROS, which consist of the hydroxyl radical, the superoxide radical, hydrogen peroxide, and diatomic oxygen, are reactive intermediates involved in normal cells processes. However, as a result of excess, this stress leads to disruption of cellular processes and damage of integral structures. Specifically, oxidative stress can result in loss of membrane integrity, DNA damage, altered gene expression, protein and enzyme disruption or destruction, and ultimately bacterial cell apoptosis. Interestingly, the excess production of particular ROS is influenced by the specific nanoparticle used. For instance, copper

Figure 9. Graphical representation of proposed AgNPs antibacterial mechanism of action, at the surface and intracellular levels. Qing et al. (2018). Open access from Dove Press.
oxide nanoparticles promote generalized ROS production, while zinc oxide may preferentially lead to overproduction of hydrogen peroxide and the hydroxide radical.

Non-oxidative antimicrobial mechanisms of mNPs are less studied, but are supported through the antibacterial activity of magnesium oxide nanoparticles. In these cases, analysis tools such as transmission electron microscopy, mass spectrometry, Fourier transform infrared spectroscopy, and more have demonstrated little-to-no increases in ROS linked to magnesium oxide nanoparticle usage. Although, other bacterial metabolic processes are affected, suggesting a non-oxidative mechanism is at play.

The specific interactions that take place between nanoparticles and bacteria are further described by Wang et al. They discuss how the bacterial cell wall acts as the primary barrier to nanoparticle effects. The differences between the cell walls of gram-positive and gram-negative bacteria play a role by changing the efficacy with which nanoparticles to adhere to the surface. Additionally, some nanoparticles, like titanium dioxide can alter membrane integrity and permeability, causing leakage of cell contents. The cellular membrane also participates in cellular respiration, and so changes and damage can result in dysregulation of the respiratory pathway. Penetration of the bacterial membrane is performed primarily by released metal ions, though is also achieved by sufficiently small nanoparticles (<10nm). From induction of ROS, to disruption of protein function, synthesis, and DNA, nanoparticles also influence metabolic function and gene expression. In particular, authors suggest that this metabolic dysregulation also primarily inhibits biofilm formation.

**Silver**

AgNPs have grown in popularity in the field of bioactive materials and tissue engineering for their non-specific biocidal activity against many organisms, including even multi-drug resistant bacteria.

While commercially available, many often choose to synthesize their own AgNPs. This synthesis can be performed using either a top-down or bottom-up approach, represented in Figure 10. The top-down approach achieves nanoparticle synthesis with size ranges from 10-100nm, using physical means of milling or grinding bulk materials. Though straightforward, this approach requires complex machinery, and yields nanoparticles that are less stable and less resistant to agglomeration, as no capping or stabilizing agents are used in these methods. Alternatively, the
bottom-up approach is more commonly used, and can employ either chemical or biological methods, often growing the nanoparticles by chemical reduction of its ionic form (Ag⁺). As mentioned, this method allows for the use of stabilizers to prevent undesired nanoparticle aggregation. Some of such stabilizers include trisodium citrate, sodium borohydride, and polyvinylpyrrolidone (PVP). The reason that prevention of aggregation of nanoparticles is of such importance relates to the inversely proportional relationship between the nanoparticle size and its potency, mentioned earlier. Should AgNPs aggregate, it can result in a complete loss of their intended properties.\cite{65,69}

![Figure 10. AgNP synthesis approaches and methods. Xu et al. (2020). Open access from Theraonotics.](image)

AgNPs have been researched both alone, and as an additive to PEMs or the like. Samples showed a clear (dose-\cite{68} and size-dependent\cite{65,66}) antibacterial effect on zone of inhibition (ZOI)\cite{70}, viable plate count\cite{71} and MTT\cite{65} toxicity tests, which can be traced back to almost exclusively silver ion release\cite{71}. Effective concentrations in these experiments were within the μg/ml or μg/cm² range.

However, AgNPs are toxic to mammalian cells. Kirmanidou et al. performed fluorescent live/dad staining, MTT, and BrdU assays on SaOS-2 osteoblast cells to determine the effect of AgNPs on their viability and proliferation, respectively\cite{72}. Results showed a reduction in viability of cells exposed to 5nm AgNPs after 24 hours, but a subsequent recovery afterwards, explained by a compensatory cell cycle arrest in response to oxidative stress. For 30nm nanoparticles, this
initial reduction was not observed, but authors did record a delay in cell proliferation at higher nanoparticle concentrations.

Conversely, Belteky et al. showed a 50% viability reduction of human keratinocytes and prostate cancer cells after 24 hour exposure to nanoparticles. In their tests of antibacterial activity, they also observed high toxicity to three different strains. A review by Xu et al. also cite many articles that have performed in-vitro and in-vivo AgNP toxicity tests on vertebrates (and cell lines) and invertebrates using different routes of exposure, demonstrating significant toxic effects, such as developmental abnormalities, DNA damage, neuroinflammation, necrosis, and apoptosis, to name a few.

Taken together, it is clear that the promising antibacterial activity of AgNPs are contraindicated for progress into clinical use due to their significant cytotoxicity risks. If developments with AgNPs are to progress, a strategy must be employed to mitigate, or balance these undesirable effects.

**Titanium dioxide**

Like AgNPs, nano-sized titanium has been studied for its biomedical applications (Table 1 and Table 2). The form and application of titanium for these purposes varies quite widely, due to several factors. Firstly, titanium does not necessarily need to be in the form of nanoparticles to exert their biological effects. One such formulation is TNTs. Using the process of electrochemical anodization, solid titanium surfaces can be modified to introduce pits with specific geometries so as to increase surface area, add drug loading ability, facilitate mammalian cell growth, and reduce bacterial adhesion. When in the form on nanoparticles, TiO2NPs have also shown antibacterial activity, increased mammalian cell adhesion, osteogenic differentiation, and osteointegration.

Additionally, the crystal structures of TiO2NPs have an influence on its bioactive properties. Titanium dioxide primarily exists as one of three crystalline polymorphs, anatase, rutile, and brookite. Depending on the forms, TiO2NPs may have different properties, and exert differential bioactive effects. However, results in this area are inconsistent. For instance, some have found that rutile nanoparticles have less cytotoxic consequences compared to anatase TiO2NPs, though some cytotoxicity is still observed. In the case of antibacterial activity, some researchers found that rutile titanium showed equivalent antibacterial activity to anatase, before and after photoactivation. Other research demonstrated that rutile had significantly less
antibacterial activity compared to anatase or a rutile:anatase mix\textsuperscript{73}. This information together, may allude to the rutile crystal phase as being a better candidate nanomaterial for the purposes of this project.

In some \textit{in-vivo} and \textit{in-vitro} trials, incorporation of TiO2NPs into a nanocomposite wound dressing yielded enhanced healing time, evaluated by wound closure rate, and no cytotoxicity, while simultaneously showing wide ZOIs on antibacterial tests against 5 different strains\textsuperscript{63,78}. Additionally, when compared with other nanoparticles, particularly silver and zinc oxide nanoparticles, TiO2NPs have demonstrated less cytotoxicity, immune activation, and oxidative stress responses\textsuperscript{14,15}. However, direct comparisons of nanoparticle effects while incorporated into PEMs seems limited, as much of the previously cited research examined effects of direct nanoparticle exposure.

\textbf{The importance of incorporation into the multilayer}

In considering the ability of mNPs to exert these antibacterial and osteoconductive effects, the benefit of using the PEM in encapsulating them is related to the PEM ability for releasing nanoparticles or their ions to exert their effects. This nanoparticle and/or ion release occurs in two stages, 1 – burst release, and 2 – sustained release. The burst release phase refers to the initial release of particles from the surface at a high rate over a short period following initial application. The sustained release phase takes place after the first stage, when the nanoparticle release rate becomes slower and more consistent over a longer duration\textsuperscript{79}. The use of a multilayer within which the nanoparticles could be housed, permits a more controlled burst release phase that can mitigate the release of a cytotoxic dosage in the early stages of use. Figure 11 depicts the effect that additional coating layers has on the initial burst release phase of a coated copper composite\textsuperscript{79}. As can be seen, once a PEM is deposited, the burst release phase is immediately crippled to a much slower rate, compared to that without coating. Along these lines, the nanoparticle release profile may also be further controlled by modification of the PEM porosity, often managed by chemical crosslinking of the multilayers\textsuperscript{38}.

While these factors do contribute to the release profile of this project’s nanocoating, we do not discuss them here in depth, as it is outside the scope of the present research.

Finally, given the information summarized in Chapters 2-4, the overarching goal of this master thesis was the fabrication of a chitosan/alginate-based PEM with incorporated nanoparticles.
of silver or titanium dioxide, with the intention of determining and comparing their antibacterial activity and cytocompatibility, in the context of infection prevention for TKHA and related PJI. The following chapter described the specific methodology used to conduct the relevant experiments to achieve this goal.

Figure 11. Cupric ion release rates from a copper substrate coated with increasing layers of a chitosan multilayer. Tian et al. (2013) with permission from Elsevier.
Chapter 5: Materials and Methods

Materials

High molecular weight Chitosan (>90% deacetylated) was purchased from MP Biomedicals (Ohio, United States). Alginic acid sodium salt (Alginate), Silver nanopowder (<100nm particle size, contains PVP as dispersant), glacial acetic acid, sodium hydroxide pellets (for production of basifying Sodium Hydroxide solution), 25% EM-grade glutaraldehyde solution, 3-aminopropyltriethoxysilane (APTES), 0.25% Trypsin/EDTA solution, and Dulbecco’s Phosphate Buffered Saline without calcium (PBS) were purchased from Sigma Aldrich (Missouri, United States). TiO2NP dispersion (Rutile, 40-wt%, 30-50nm) was purchased from Nanostructured and Amorphous Materials Inc. (Katy, Texas). 15ml Corning or Falcon tubes, reagent grade acetone, ethanol, 30% hydrogen peroxide solution, concentrated sulfuric acid, Alpha MEM media without phenol red (αMEM-), 48-well microtiter plates, Gibco Penicillin-Streptomycin (10,000 U/mL), AlamarBlue reagent, and Fetal Bovine Serum (FBS) were purchased from Thermo Fisher Scientific (Massachusetts, United States). MC3T3-E1 murine pre-osteoblast cells, as well as MSSA (DNC274, ATCC 29213) was purchased from ATCC (Virginia, United States). Gibco Alpha MEM cell culture medium with nucleosides & no ascorbic acid (αMEM+) was purchased from Invitrogen (Massachusetts, United States). T-75 adherent cell culture flasks with vented cap were purchased from Sarstedt (Numbrecht, Germany). Alkaline Phosphatase Assay Kit (Colorimetric) was purchased from Abcam (Cambridge, United Kingdom). Agar, ultrapure for bacteriology was purchased from Alfa Aesar (Massachusetts, United States). Luria-Bertani broth (LB Broth Miller) was purchased from BioShop Canada (Burlington, Ontario). Semi-porous titanium rods were purchased from Amplify Inc. (Scarborough, Maine). Ultra-pure water was used for all experiments where aqueous dilutions were made.

LbL solution preparation

Preparation of polyelectrolyte solutions

0.2% Chitosan solution was prepared by adding 20mg/ml (0.2g/100ml) of chitosan powder to 1% glacial acetic acid, and let to stir overnight until completely dissolved. Immediately prior to all experiments, aliquots of appropriate volume were isolated, and pH was neutralized using 10M and
1M sodium hydroxide until a pH of 6.0 was reached. The final concentration of the chitosan solution was then adjusted to 0.1% with ultrapure water.

0.2% Alginate solution was prepared by adding 20mg/ml (0.2g/100ml) of sodium alginate powder into double distilled water, and let to stir overnight. For experimental conditions where alginate was used without nanoparticles, aliquots were further diluted to a concentration of 0.1%.

**TiO2NP dispersions in alginate solution**

To produce 3 different concentrations of Alginate-TiO2NPs suspensions, 40 wt% aqueous stock suspension of TiO2NPs (calculated to an equivalent of 666.67mg/ml), was first diluted using ultrapure water to a concentrations double that of the final desired concentration of the most concentrated dispersion group. An aliquot of this original stock suspension was diluted to a concentration of 53.33mg/ml by adding 1.2ml of stock suspension to 13.8ml of ultrapure water. Into 3 15ml tubes, we mixed 7ml of 0.2% Alginate, 1.75, 3.5 or 7ml of the diluted TiO2NPs, and then filled with ultrapure water to a final volume of 14ml, to achieve a final alginate concentration of 0.1%, and TiO2NPs concentrations of 6.67, 13.33, and 26.67mg/ml. Suspensions were vortexed vigorously for 1 minute each to attain even dispersions.

**AgNP dispersions in alginate solution**

To produce 2 different concentrations of Alginate-AgNPs suspensions, PVP-coated nanosilver powder was dispersed in ultrapure water at a concentration double that of the highest intended AgNPs group concentration. Stock dispersions were made to a concentration of 8mg/ml. To ensure even dispersion, the solution was vortexed vigorously for 1 minute, and then bath-ultrasonicated for 1 hour. Meanwhile, 7ml of 0.2% Alginate was added to 2 separate 15ml tubes. Once ready, AgNPs suspensions were added to tubes containing alginate solutions, and filled to a final volume of 14ml with ultrapure water, to finally obtain alginate concentrations of 0.1%, and AgNPs concentrations of 0.4, and 4mg/ml.

**Nanoparticle analysis**

Prior to experimentation, AgNPs and TiO2NPs were suspended in water and examined for their size distribution using the Nanosight NS300 Nanoparticle Tracking Analyzer (NTA) (Salisbury, United Kingdom). Nanoparticles were studied using a NTA for the purpose of properly characterizing and ensuring that the purchased material met the specifications indicated on their labels. Suspensions were prepared as described above, though using water as the diluent. 1mg/ml
suspensions were made, and diluted 500-fold to enable effective and accurate measurements by the equipment. Before running each sample, a 1ml syringe filled with ultrapure water was installed on the NTA pump. Water was flowed into the channel to flush the system of impurities or previous sample residues. Once the video monitor showed no remaining impurities in the chamber, the nanoparticle dispersion was aspirated into a clean 1ml syringe, and attached to the pump. The pump was programmed to record the flow of nanoparticles for a 60 second duration, before a subsequent 5 second delay, followed by another 60 second recording period. This process was repeated for a total of 5 sets of recordings per sample. Feeding the sample suspension into the channel began with an introduction of 200 units per minute, and was incrementally increased by 200 units per minute until a final flow rate of 600 units per minutes was reached. During this increase, the camera focus was adjusted. Recordings began once this final flow rate was reached, and nanoparticles were in focus and could be seen flowing through the channel. NTA measured particle size distribution, and no further analysis was performed on the retrieved data.

**Titanium substrate preparation**

The substrates used for this project were cylindrical semiporous 3D-printed titanium rods made according to the specifications outlined in Figure 12A. That is, rods were 4.5mm in diameter, with a length of 25mm. Titanium rods were cut transversely to produce approximately 3mm thick disks. Viewed from the top (Figure 12B), one half of the substrate was solid titanium, while the other half had a porous microstructure with a 400-micron average pore size, 300-micron strut diameter, and was approximately 65% porous.

**Surface functionalization**

**Experimental procedure**

Chemical crosslinking was adapted from Martin *et al.* to achieve surface functionalization as described below. All cut semiporous titanium disks were washed 3 times, 10 minutes at a time, in acetone, ethanol, then ultrapure water to remove any impurities, and then blown dry using inert nitrogen gas. Next, a 3:1 mixture of concentrated sulfuric acid and 30% hydrogen peroxide, producing Piranha solution, was prepared. Disks were carefully submerged under constant stirring for up to 1 hour in this highly acidic mixture to further clean and hydroxylate specimens. Disks were then removed from the solution and washed 3 times in ultrapure water.
To produce amino-functionalized disks, samples were immersed in a 2% solution of APTES for 1 hour. Samples were then washed 5 times with acetone to remove any residual silane groups.

To facilitate and enable the deposition and crosslinking of the primary polymer layer, samples were then treated in 4% glutaraldehyde solution under stirring for 8 hours. Samples were then removed and washed thrice in ultrapure water. The primary chitosan layer was applied by immersing samples in 0.1% chitosan solution for 8 hours, and then rinsing non-adhered chitosan from the surface with ultrapure water (Figure 13).

Figure 12. Titanium substrate details. (A) Semi-porous titanium coupon specifications; (B) Substrate cross-section.

Figure 13. Reaction Steps Involved in the Binding of Chitosan to Titanium Substrates. (1) 3-Aminopropyltriethoxy-silane (APTES) Deposition, (2) Reaction of APTES with Glutaraldehyde, and (3) Reaction of Glutaraldehyde with Chitosan. Martin et al. (2007). Open access from American Chemical Society.
Layer-by-layer polyelectrolyte deposition and characterization

Experimental procedure
After surface functionalization, the subsequent application of multilayers began with the deposition of alginate atop the primary chitosan layer, completing the first bilayer. The remaining 4 bilayers were applied according to the following protocol, adapted from the layer-by-layer deposition method demonstrated by Zhong et al.36.

Briefly, each polymer layer was applied in a similar manner to the above layers, where samples were submerged in 15ml centrifuge tubes containing the appropriate mixture, then fitted onto a rotator to ensure consistent flow and even coverage of the polymer throughout the disks. Submerged samples were allowed to immerse under rotation for 15 minutes. After, samples were removed from their respective tubes, washed twice in ultrapure water to remove unadsorbed polymer from the sample surface. Specimens were then added to tubes containing the appropriate next solution/suspension. This process was repeated until 5 bilayers were applied, at which point the disks were washed one last time, then let to dry in air overnight.

Samples coated with alginate containing 6.67, 13.33, and 26.67mg/ml of TiO2NPs were labeled T1, T2, and T3, respectively. Samples coated with alginate containing 0.4, and 4mg/ml of AgNPs were labeled as S1 and S2, respectively. Samples coated using alginate without any nanoparticle additives were labeled as CA, representing a PEM made of otherwise unmodified chitosan and alginate. Control samples that were not coated (or bare) were labeled as B.

Before any cell-related experiments, samples were sterilized according to a process detailed below in section “Sample preparation for experimental cell culture”.

In-situ assessment of layer build-up using QCM-D
To conduct tests of in-situ LbL buildup observation, we used a QSense QCM-D Analyzer (Biolin Scientific Inc., Gothenburg, Sweden) instrument, with titanium QCM-D crystals (QSX 310) (Biolin Scientific Inc., Gothenburg, Sweden), to best mimic the surface properties of the semiporous titanium implant samples that were used in our other experiments. It should be noted that we did not surface functionalize the crystals for these tests, as the acid treatment would damage them. As a result, we simply flowed the chitosan and alginate solutions through the system to observe solely the build-up of the PEM due to the electrostatic interactions between the polyanion and polycation layers.
For QCM-D analysis, samples were cleaned using UV-Ozone (UV–ozone chamber Bioforce Nanosciences, Inc., Virginia, United States) treatment for 10 minutes, followed by a 5-minute wash in 5:1:1 mixture of ultrapure water, 25% ammonia, and 30% hydrogen peroxide at 75°C, followed finally by another 10-minute UV-Ozone treatment. The experiment was set up to oscillate the crystals at their fundamental resonance frequency \( f = 4.95 \text{MHz} \), and their odd overtones (3-11) using electrodes supplying a radiofrequency voltage. The LbL process began by flowing water into the chambers at a rate of 400µl/minute for 5 minutes to establish a baseline measurement. Then, chitosan was flowed in at the same rate for 3 minutes to ensure that the entire crystal was covered with the polymer. At that point, the flow pump was stopped for 15 minutes, to allow the polymer to adsorb onto the crystal surface. Water was then flowed for 3 minutes to remove unadsorbed polymer. After, the tubing was switched to the alginate solution, and the same process was followed. The procedure was repeated until a total of 5 bilayers was applied. Frequency and dissipation measures were performed in real time with QSoft QCM-D software, while viscoelasticity and thickness calculations were performed using the Voigt-based viscoelastic model in the QTools software.

**AFM analysis of layer deposition**

We used a MultiMode 8-HR AFM purchased from Bruker (Massachusetts, Unites States). The surface roughness and morphology were assessed for non-functionalized bare titanium disks (labeled “Bare”), disks coated with two bilayers of chitosan and alginate (“CA/2B”), and disks coated with four bilayers, both with TiO2NPs (“T3/4B”) and without TiO2NPs (“CA/4B”). Labels refer to their corresponding coating (as described above in “Experimental procedure”), and the number of bilayers applied, such that “Group / # of bilayers”. All samples were prepared according to the same preparation procedure as those for cell culture and other experiments. PeakForce mode in air was used for all imaging, using a silicone probe with a spring constant \( k = 0.35 \text{ N/m} \), and a resonance frequency \( f_0 = 65 \text{ kHz} \). Images were taken in sections of 20 X 20 µm.

**ESEM analyses of coating before and after cell culture**

FEI Quanta450 Environmental Scanning Electron Microscope (ESEM) instrument was used to further confirm the deposition of the PEM, as well as to examine its porous microstructure before cell culture. The SEM was set to a full vacuum, and samples were lifted on the platform to a distance of 10mm from the camera. Samples from each group were imaged at 5-10 kV, final images were taken over 10 seconds for heightened resolution.
Samples after MC3T3-E1 cell culture assays were also imaged with SEM for visualization of mineralization deposited on the substrate surface. To prepare samples for this set of images, culture media was removed from sample wells, and samples were washed thrice with phosphate buffered saline (PBS), before fixation using 4% paraformaldehyde for 1 hour. Samples were washed with PBS and processed for dehydration, by immersing them in incrementally higher concentration ethanol washes (30%, 50%, 70%, 80%, 90% 100%) for 15 minutes at a time. Subsequently, samples were dried using critical point drying, and then coated using a platinum sputter coater.

**MC3T3-E1 cell culture**

**MC3T3-E1 pre-osteoblast culture**

All cell culture media was supplemented with 10% FBS and 1% Penicillin/Streptomycin before use with cells. MC3T3 cells were cultured and expanded in T-75 flasks with αMEM+ prior to all experiments to produce sufficient numbers for seeding experimental conditions. Initial cell culture began by adding approximately 1x10^6 MC3T3-E1 cells to 10ml of αMEM+ in a T-75 flask. Every 2-3 days, cell culture media refreshed until cells reached approximately 80% confluency, at which point they were ready for experimental use. Collection of adherent cells from the surface of the flask was performed by removing culture media, washing gently with PBS, then incubating for 5 minutes in 2ml of 0.25% trypsin/EDTA. 5ml of αMEM+ was added to the flask to resuspend the detached cells and inactivate the trypsin, and then everything was transferred to a 15ml centrifuge tube. At this point, cell density of the suspension was calculated using an automatic cell counter for determination of required volumes for experimental seeding densities. Cells were pelleted using a centrifuge, and were resuspended with fresh αMEM-. While there is minimal interference of media containing phenol red on either AlamarBlue or ALP assays, we nevertheless opted to use colourless media for these tests.

**Sample preparation for experimental cell culture**

The experimental setup was as follows. First, all titanium disks were sterilized as adapted from Holmes et al. Briefly, samples were immersed in 70% ethanol for 1 hour, and then further washed in an ethanol series of 35%, 17.5%, 8.75% for 30 minutes each. The disks were then washed 3 times for 10 minutes at a time in sterile PBS (pH 7.4) to remove any residual ethanol. Specimens were then placed in triplicate into wells of a 48-well microtiter plate such that there was only 1 disk per well. The wells with no titanium disks placed inside were used as positive...
growth control. MC3T3-E1 cells were then seeded into all experimental wells at a volume of 400µl and a density of about 3x10^4 cells/ml. Cells were incubated for the predefined timepoints in a CO_2-controlled incubator which was kept at 37° C. αMEM- medium was refreshed every 2-3 days, and 24 hours before collection/testing timepoints for cell viability and differentiation tests. The duration of the cell culture portion of this experiment was 21 days. AlamarBlue assays were run for 7 days, and ALP activity assays for 14 days.

**AlamarBlue assay for cell viability assessment**

On days 1 (24 hours after initial seeding), 4, 7, and 14 of the cell culture experiment, media from wells was collected into appropriately labeled cryotubes and immediately stored at -80° C for later assays of alkaline phosphatase (ALP) activity. In its place, 400µl of fresh αMEM- media containing 10% AlamarBlue (9:1 ratio between cell culture medium and AlamarBlue) was added to the wells. The well plate was then covered and placed back in the incubator in the dark for 4 hours to allow the Resazurin to react. After incubation, 100µl of media from each well was withdrawn in triplicate, and added to wells of a 96 Well Clear Flat Bottom UV-Transparent Microplate (Sigma Aldrich, Missouri, United States). The wells of the 48-well plate were once again replenished with 400µl of αMEM- , and returned to the incubator.

Using a Spectramax i3 spectrophotometer (Molecular Devices, California, United States), the absorbance values of media samples were measured at 570nm and 600nm. Percent Difference in AlamarBlue reduction was calculated from the absorbance data and extinction coefficients of resazurin according to the formula presented in Equation 1.

**Equation 1. AlamarBlue calculation for percent difference.**

\[
\text{Percent Difference} = \frac{(\varepsilon_{OX})_{\lambda_2} \cdot A_{\lambda_1} - (\varepsilon_{OX})_{\lambda_1} \cdot A_{\lambda_2}}{(\varepsilon_{OX})_{\lambda_2} \cdot A^*_{\lambda_1} - (\varepsilon_{OX})_{\lambda_1} \cdot A^*_{\lambda_2}}
\]

Where,

- \( \varepsilon_{ox} \) = molar extinction coefficient of AlamarBlue oxidized form (Table 3)
- \( A \) = absorbance reading of test wells
- \( A^* \) = absorbance reading of positive growth control well
- \( \lambda_1 = 570\text{nm} \) (540nm may also be used)
- \( \lambda_2 = 600\text{nm} \) (630nm may also be used)
<table>
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<td>80,586</td>
</tr>
<tr>
<td>600nm</td>
<td>117,216</td>
</tr>
</tbody>
</table>

**Osteogenesis assessment with ALP assay**

On days 1, 4, 7, and 14 of cell culture with coated disks, \( \alpha \)MEM- cell culture media was extracted from each well, and ALP assays were conducted on said media extracts. The method used for the ALP assay was performed as instructed using the ab83369 Alkaline Phosphatase Assay Kit (Colorimetric) manual. All reagents were thawed or reconstituted, and standard curve was prepared as described. All samples were assayed in duplicate (note: each experimental condition was also run in technical triplicates). Briefly, 80\( \mu \)L of each sample was loaded into well of a 96-well microtiter plate, as well as 80\( \mu \)L of media only as a background control, and 120\( \mu \)L of each standard dilution. The ALP reaction was executed according to the following steps: 1) 20\( \mu \)L of stop solution was added to the background control wells, and mixed by pipetting. 2) 50\( \mu \)L of 5mM para-Nitrophenylphosphate (pNPP) was added to sample and background control wells. 3) 10\( \mu \)L of ALP enzyme was added to each of the standard dilution wells and mixed by pipetting. 4) Plates were covered in foil to protect from light, and incubated at 25\( ^\circ \)C for 60 minutes. 5) Added 20\( \mu \)L of stop solution to sample and standard wells. 6) Plates were gently shaken, and colorimetric measurements were taken on a Spectramax i3 spectrophotometer (Molecular Devices, California, United States) at 405nm. Standard curve measurements were made and plotted, and ALP activity was calculated using Equation 2.

Equation 2. ALP activity

\[
ALP\ Activity = \left( \frac{B}{\Delta T \ast V} \right) \ast D
\]

Where,

B = amount of pNP in sample well calculated from standard curve (\( \mu \)mol).
\( \Delta T \) = reaction time (minutes).
V = original sample volume added into the reaction well (mL).
D = sample dilution factor.
**Bacterial cell culture**

**Cell culture protocol**

Bacterial cell culture liquid media and agar plates were prepared under sterilizing flame using LB broth and agar mixtures with ultrapure water. Solutions were autoclaved before use and plate pouring.

It should be noted that all work with *S. aureus* (DNC274, ATCC 29213) was conducted inside of a Biosafety Cabinet (BSC) with capabilities suitable for handling Biosafety Level 2 (BSL2) organisms, and all required PPE was worn at all times.

From frozen stock, *S. aureus* was streaked onto an LB agar plate using a sterile loop, and let to incubate overnight so that isolated colonies could be observed and sampled. Between 4-5 separate colonies were then transferred into 1 15ml culture flask and let to incubate under stirring at 200rpm overnight. The next day, triplicate samples from this bacterial suspension were removed and diluted into LB broth to between 0 and 1 optical density units. Serial dilutions were prepared for these sample suspensions and dot spotted on LB agar plates overnight for subsequent colony counts. Colony counts were determined, and correlated back to their respective optical densities from the previous day. Since bacterial cell counts for *S. aureus* are relatively linear at optical densities between 0 and 1, it is possible to calculate cell density of a stock suspension using a simple linear equation. Using this method, we calculated the required dilution factor necessary to create a $1 \times 10^4$ colony forming units (CFU) per milliliter stock suspension for use in our experiments. Serial dilutions were also made from the inoculation suspension, plated and colonies counted, in order to confirm inoculum concentration.

Once bacteria suspensions were sufficiently diluted, $400\mu l$ of LB broth *S. aureus* suspensions at $1 \times 10^4$ CFU/mL were added to each well of a 48-well microtiter plate, over each titanium disk sample. A control group for bacterial growth (containing no titanium disk) was also included at this step. The prepared multi-well plate was then placed in an incubator at 37°C for 24 hours without shaking. Normally, these bacteria grow better under shaking at 200rpm, due to increased media aeration. However, this shaking would negatively influence biofilm formation, and we decided to eliminate this potential source of error.
**Antimicrobial activity**

After 24 hours of incubation at 37°C, planktonic and adherent bacteria samples were collected for colony formation and counting.

*Test of planktonic bacteria*

To sample planktonic bacterial growth, 100μl aliquots of suspension from each sample well were removed and transferred in triplicate to a 96-well microtiter plate. 10-fold serial dilutions were made using sterile PBS and plated on LB Agar in an array of 10μl dot spots. Plates were incubated at 37°C for 20 hours, ensuring colonies did not grow into each other and prevent accurate counting. After, colonies were counted.

*Test of adherent bacteria*

After necessary aliquots of planktonic bacteria were sampled from wells, the remaining suspensions were pipetted out and discarded. The wells and disks within were then gently washed three times with PBS, taking care to not disturb or remove adhered bacteria during the process. Disks were then transferred to individual 1.5ml centrifuge tubes containing 1ml of PBS. These tubes were then vortexed at high intensity for 1 minute each to detach adhered bacteria and suspend them in solution. As in the test for planktonic bacteria, triplicate serial dilutions were made from each tube, and plated via dot spotting. Plates were incubated as described above, and colonies were counted.
Chapter 6: Results

Nanoparticle characterization

The following data (Figure 14A) describe the characterization results obtained about PVP-coated AgNPs dispersed in ultrapure water. The average size of AgNPs was 77.9nm, where at least 50% are below 100nm. Though the mean was higher than intended, likely due to some aggregation creating larger particles, the distribution of particle sizes was effectively unimodal at about 45.6nm. These results offer sufficient support for the method used to suspend the purchased AgNPs, and showed that they are within a range of sizes that are acceptable for our purposes.

Data represented in Figure 14B describe the distribution of TiO2NPs sizes from the purchased nanoparticle suspension. With a narrow unimodal peak at 39.2nm, and an average diameter of 39.4nm, the purchased rutile TiO2NPs are within the advertised range of sizes, and within the range desired for this research.

In-situ LbL observation using QCM-D

LbL self-assembly of the PEM was performed on titanium dioxide coated quartz crystals for simulation and in-situ observation of the coating method, for its validation. As seen in Figure 15A, the frequency decreases linearly along the LbL process, indicating the consistent sequential buildup of the PEM on the titanium dioxide quartz crystal. This interpretation is further confirmed by assessment of dissipation measurements, changes in surface viscoelasticity, and increases in

Figure 14. Nanoparticle size distribution as measured by NTA. (A) AgNPs; (B) TiO2NPs.
layer thickness (Figure 15B-D, respectively). Dissipation measurements follow from frequency changes, while viscoelasticity and thickness increase stepwise with each bilayer. According to plot readings from the thickness graph (Figure 15D), when read during the first and last water flow stages respectively, the smallest thickness measurement was $5.4982 \times 10^{-14}$ m, and the greatest thickness was $1.8529 \times 10^{-8}$ m, meaning that the overall thickness of the multilayer applied to the chip was $1.8528945 \times 10^{-8}$ m, or 18.53nm.

![QCM-D plots](image)

Figure 15. QCM-D plot of (A) the change in frequency (Hz) over time of a titanium oxide-coated quartz crystal undergoing LbL application of 5 bilayers of chitosan and alginate polyelectrolytes. Arrows denote time points at which specific solutions began being administered. Black - ddH2O, Blue - Chitosan, Red – Alginate. (B) Change in dissipation. (C) Changes in viscoelastic properties. (D) Change in film thickness over coating period.
Characterization of sequential LbL surface using AFM

AFM images were taken of titanium substrates in 4 different states. Both 2D and 3D images for samples are depicted in Figure 16, while measurements of their surface roughness characteristics are denoted in Table 4. Firstly, it can be noted that the changes in surface characteristics between bare and 2 or 4 bilayers of coating confirm the PEM deposition onto the titanium substrate. This is particularly apparent when considering the drastic smoothing of the surface upon multilayer adsorption, where high average roughness in the Bare sample (Ra = 91.3nm) is no longer reflected in samples with even only 2 bilayers (Ra = 11.2nm). When comparing groups with 4 bilayers, those with TiO2NPs (Ra = 71.2nm) showed greater surface roughness than other (Ra = 17.0nm) coated samples. In these nanoparticle-incorporated PEMs, it is also easy to see both on the 2D and 3D images, the even distribution of nanoparticles along the surface, most of which appear to be of similar size. The large peak on the top left of Figure 16D (denoted with an arrow) may likely also be a nanoparticle that has undergone aggregation, though this is a rare occurrence given the presented images.

Table 4. AFM roughness measurements, in correspondence with Figure 16, such that “Condition – Figure 16 numeration”. Bare – (A); CA/2B – (B); CA/4B – (C); T3/4B – (D).

<table>
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<tr>
<th>Group description</th>
<th>Label</th>
<th>Rq (nm)</th>
<th>Ra (nm)</th>
<th>Rmax (nm)</th>
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<td>638</td>
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<td>93.0</td>
<td>71.2</td>
<td>859</td>
</tr>
</tbody>
</table>
Figure 16. 2D and 3D AFM images of different coating configurations. (A) Bare; (B) CA 2 bilayers; (C) CA 4 bilayers; (D) T3 4 bilayers. Arrow denotes a large potential agglomeration of TiO2NPs.
**SEM of coated substrate**

The electron microscopy micrographs of differently coated titanium substrates are shown in Figure 17A-D. Using a dotted line as a visual aid, the top-down view of the sample in Figure 17A denotes the structural distinction between the solid and porous sides of the substrate. These images demonstrate the successful coating of the titanium substrates with the PEM, as well as the porous structure formed by the polymers (Figure 17B and C). Figure 17D shows a coated sample where incorporated TiO2NPs are uniformly monodispersed across the surface.

![Figure 17. SEM images of PEM-coated titanium substrates. (A) Top-down view of semi-porous titanium shows both the solid and porous sides of the structure. (B) Close-up view into a pore of a sample coated with chitosan and alginate, shows successful PEM application and penetration into the pore. (C) Interface between a coated portion of the substrate and a section where the sample was cut, showing the bare titanium surface. (D) View into the pore of a coated sample with TiO2NPs. Note: Scale bars vary with image.](image)

Images post-experimentation with pre-osteoblast cells are pictured in Figure 18A-C. All samples show mineral deposition across the surface of the titanium substrates, both coated and non-coated. In some images, this mineral layer is more densely distributed than in others, and some fixed cells can be seen scattered across the surface.

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Pre-osteoblast viability was evaluated at various time points (1 day, 4 days, 7 days) using AlamarBlue to determine the relative cytotoxicity of the samples produced. Percent Difference represents a measurement of relative cell viability of experimental groups compared to control wells seeded with preosteoblasts without sample exposure (group E). The calculation provides a quantitative description of how much more/less cell growth took place in our experimental groups,
compared to how much cell growth was observed in positive control wells. Using this method, it is possible to determine the relative efficacy of the treatment.

A 2-way analysis of variance (ANOVA) with Tukey’s post-hoc comparisons was performed based on the Percent Difference results. This analysis showed a main effect of Day ($p < 0.0001$), Group ($p < 0.0001$), and an interaction between the two ($p < 0.0001$). That is, the coating had a significant effect on the viability of MC3T3-E1 cells at a given time, as well as across days. Data was also compartmentalised according to test day, demonstrated in Figure 19. For each of these data sets, we conducted a one-way ANOVA with Tukey’s post hoc comparisons. After 1 day, samples coated with 13.33mg/ml of TiO2NPs showed higher cell viability compared to all other test groups ($p < 0.0003$), while other groups scored similarly to each other. On day 4, these same cells retained their superior viability ($p < 0.05$) to all other groups. Additionally, cells exposed to samples coated with 4mg/ml of AgNPs experienced a reduction in relative viability, leading to those cells showing less viability compared to all other groups ($p < 0.0001$). At day 7, the most significant remaining difference between the groups was the sustained reduction in viability in the 4mg/ml AgNP group, which was still lower than all other groups ($p < 0.0001$).

![Figure 19. Plots of AlamarBlue tests of percent difference across test days. D1 - after 1 day; D4 - after 4 days; D7 - after 7 days. Dotted line at y = 100 refers to the measured AlamarBlue results from the reference group (cells only, no samples). Stars denote significant differences, * - <0.05; ** - <0.01; *** - <0.001; **** - <0.0001. Group labels are defined as CA – chitosan/alginate without nanoparticles; T1 – PEM with 6.67mg/ml TiO2NPs; T2 - PEM with 13.33mg/ml TiO2NPs; T3 - PEM with 26.67mg/ml TiO2NPs; S1 - PEM with 0.4mg/ml AgNPs; S2 - PEM with 4mg/ml AgNPs; B – uncoated sample. All coated samples are composed of 5 bilayers.](image-url)
MC3T3-E1 cell differentiation

Tests of ALP activity were conducted on extracted cell culture media to determine the extent of preosteoblast differentiation using alkaline phosphatase as a biomarker. In these tests, groups were also separated based on the polymer layer that was applied as the topmost coating; nanoparticle-incorporated alginate, or chitosan. One-way ANOVAs were conducted on samples from each test day to determine the significance of this top coating. Despite a significant effect of top-coat on day 7 ($p = 0.0001$), this difference was not seen on any other test day, and so it was determined that the top coat was a non-significant factor in the ALP activity of cells exposed to samples across the experimental period. Therefore, MC3T3-E1 analyses were conducted with those groups combined. A two-way ANOVA was performed on the combined ALP data, and it was found that there was a main effect of Day ($p < 0.0001$), of Group ($p < 0.0001$), and an interaction of Group x Day ($p < 0.0001$). Tukey’s post-hoc comparisons were used to elucidate the differences between specific groups. There were no statistical differences between groups on days 1 or 4. On day 7, cells exposed to samples containing 26.67mg/ml of TiO2NPs showed marginally greater ALP activity compared to those exposed to 6.67mg/ml of TiO2NPs ($p = 0.0453$). Tests on day 14 showed a marked increase in ALP activity in all groups compared to their respective counterparts on previous days. Within day 14, cells exposed to 0.4mg/ml of AgNPs showed one of the highest absolute means of ALP activity (0.01528μmol/min/ml), second only to the cells-only control group (0.01530μmol/min/ml). This heightened ALP activity was greater than that of groups without nanoparticles ($p < 0.05$), with 13.33mg/ml TiO2NPs, 26.67mg/ml TiO2NPs ($p < 0.001$), but not statistically different from those exposed to 6.67mg/ml TiO2NPs and 4mg/ml AgNPs. These data are graphically summarized in Figure 20.
Figure 20. ALP activity of coated samples across tests days where differently top-coated groups are combined. Stars denote significant differences, * - <0.05; ** - <0.01; *** - <0.001; **** - <0.0001. Group labels are defined as CA – chitosan/alginate without nanoparticles; T1 – PEM with 6.67mg/ml TiO2NPs; T2 - PEM with 13.33mg/ml TiO2NPs; T3 - PEM with 26.67mg/ml TiO2NPs; S1 - PEM with 0.4mg/ml AgNPs; S2 - PEM with 4mg/ml AgNPs; B – uncoated sample; E – empty well, cells only. All coated samples are composed of 5 bilayers.
We also performed a simple linear regression on an isolated segment of the data, focusing on the potential nanoparticle dose-dependent responses that cells may have to the construct. These data are presented in Figure 21. Here, a difference was observed in the responses of cells depending on the nanoparticle concentration incorporated into the PEM. For samples containing TiO2NPs, a significant positive relationship was found between nanoparticle concentration and ALP activity in the chitosan-topped group ($R^2 = 0.0832$, $F(1,7) = 35.74$, $p = 0.0006$) but not the alginate-topped group. In AgNP-containing samples, a negative relationship was observed between concentration and ALP activity ($R^2 = 0.7328$, $F(1,4) = 10.97$, $p = 0.0296$) in the alginate group. This suggests that PEMs with greater concentrations of TiO2NPs, especially when coated with chitosan, may promote greater ALP activity, while PEMs coated with alginate with increasing concentrations of AgNPs show diminishing ALP activity in response.

![Figure 21. Linear regression plots of nanoparticle concentration on ALP activity of MC3T3-E1 cells exposed to nanoparticle-loaded PEMs.](image)

A

**TiO2NP Concentration effects on ALP Activity of MC3T3-E1 Cells on Day 7 of culture**

- Alginate-topped
- Chitosan-topped

B

**AgNP Concentration effects on ALP Activity of MC3T3-E1 Cells on Day 7 of culture**

- Alginate-topped
- Chitosan-topped
Antimicrobial activity

Plate counts of dot-spotted adherent bacteria were performed after a 24-hour liquid culture with coated samples. Counts of planktonic bacteria were also conducted, to control for error in cell seeding. No statistical differences were found in planktonic bacteria counts, validating a consistent seeding density across groups. As groups in this experiment were also distinguished by their top coat (alginate with nanoparticles, or chitosan), a two-way ANOVA was done to extract any differences. From this, we noted a main effect of top-coat ($p = 0.0070$) and of group ($p = 0.0412$), and an interaction between these factors ($p = 0.0168$). Bonferroni’s multiple comparisons revealed that these differences were pulled out due to the unique differences in the 26.67mg/ml TiO2NPs group’s topmost polymer layer ($p = 0.0007$), and that between the chitosan-topped 13.33mg/ml and 26.67mg/ml TiO2NPs groups ($p = 0.0041$) (Figure 22).

Figure 22. Estimated bacterial growth based on cell counts of adherent bacteria exposed to samples in liquid media for 24 hours. Stars denote significant differences, ** - $<0.01$. Group labels are defined as CA – chitosan/alginate without nanoparticles; T1 – PEM with 6.67mg/ml TiO2NPs; T2 - PEM with 13.33mg/ml TiO2NPs; T3 - PEM with 26.67mg/ml TiO2NPs; S1 - PEM with 0.4mg/ml AgNPs; S2 - PEM with 4mg/ml AgNPs; B – uncoated sample. All coated samples are composed of 5 bilayers.
In the interest of understanding the potential dose-effect characteristics of the antibacterial activity of the nanoparticles, we also isolated the nanoparticle-containing groups into their respective datasets, and performed a simple linear regression on each of them (Figure 23). For samples containing TiO2NPs, a significant negative relationship was found between nanoparticle concentration and colony counts in the alginate-topped group ($R^2 = 0.6798$, $F(1,7) = 14.86$, $p = 0.0063$) but not the chitosan-topped group. In AgNP-containing samples, a positive relationship was observed between concentration and bacterial growth ($R^2 = 0.7667$, $F(1,4) = 13.14$, $p = 0.0222$) in the alginate group. Together, these data suggest a trend toward dose-dependent antibacterial activity in alginate-topped TiO2NP-incorporated PEMs.

Figure 23. Linear regression plots of nanoparticle concentration on antibacterial activity against S. aureus cells exposed to nanoparticle-loaded PEMs.
Chapter 7: Discussion

In order to fully appreciate the value of the data in question, we consider them in the context of the proposed research objectives, reiterated here:

1. Develop and characterize a polyelectrolyte multilayer with imbedded TiO2NPs and AgNPs on porous titanium substrates.
2. Study *in-vitro* cell viability and differentiation analyses on MC3T3-E1 pre-osteoblast cells.
3. Assess antimicrobial activities of coated titanium substrates against *S. aureus* bacteria.
4. Perform side-by-side comparison of TiO2NPs and AgNPs effects encapsulated in PEM on cell viability and antibacterial activities.

**Assembly and characterization of PEM**

In the present research, we conducted *in-situ* LbL deposition of chitosan and alginate on a titanium dioxide-coated quartz crystal to assess the real-time buildup of the PEM, for validation of the methodology. Based on measurements of frequency and dissipation changes, it was clear that the polyelectrolytes were being adsorbed onto the chip surface, and built upon each other. Thickness measurements also confirmed this multilayer deposition, although it appeared that the final PEM was thinner than was anticipated. This might have been due to the methods employed with the QCM-D. It could have been that the flow rate was set too high, and during the wash phases, water may have flowed strongly enough to have sloughed off some of the polyelectrolyte layer that had just been laid down. In other research³⁷, polyelectrolytes and water were flowed at 100µL/min, while we used 400µL/min. This assumption is supported by Figure 15, where the frequency change immediately following the beginning of the wash stage undergoes a sharp increase, suggesting that some polymer may be removed. Alternatively, the absence of the crosslinking surface functionalization could have contributed to reduced adsorption of the initial layer, potentially destabilizing its, and subsequent polymer depositions.

Regarding the topography of the substrate, particularly the deposition of an increasing number of layers, we chose to observe changes in surface roughness, using AFM. From 2 to 4 bilayers, it was found that overall roughness increased slightly, though Rmax (the distance from the lowest trough to the highest peak) decreased. This may be interpreted to mean that the coating smoothed
out the highly variable topographic mapping of the native titanium substrate, as also evidenced between the Bare and 2 bilayer groups. While the greater roughness of the Bare group was smoothed by the application of a PEM, the subsequent increase in Ra after additional PEM deposition may be attributable to an increase in porosity of the multilayer. The large increase in surface roughness of the 4 bilayers samples with or without nanoparticles is expected, and the difference between Ra values of the 26.67mg/ml TiO2NP-containing 4 bilayer group and the unmodified PEM 4 bilayer group is consistent with the added peaks that the nanoparticles would impart on the coating surface. This increase in surface roughness is also a beneficial characteristic, as it has been shown that cell adhesion and bone-implant contact is improved with higher roughness\textsuperscript{81}.

Similar to AFM, in order to visually confirm the deposition of the PEM, as well as to examine its porous microstructure, which was not tested with AFM, Scanning Electron Microscopy (SEM) was used. It was also important to use this technique in the earliest stages of this research so as to ensure that the method used for application of the PEM coating was in fact permitting the coating’s entrance into the pores of the semi-porous titanium rods. The major goal and expectation at this stage was to observe the porous microstructure of the applied PEM. There was also great interest in the dispersion and aggregation of the nanoparticles, as it is known that these characteristics affect the intended effectiveness of the attributes of the bioactive coating. Based on the images in Figure 17, we can conclude that the PEM was adequately applied and took on the appropriate porous structure that was hypothesized to promote bone ingrowth. In images taken after experiments with pre-osteoblasts (Figure 18), it was noticed that mineralization occurred on the substrate surface regardless of modification, though those that were coated with nanoparticles displayed visibly more complete coverage. This may suggest improved growth and differentiation of bone cells when exposed to this formulated coating, and this finding is in accordance with the described cell data. In the future, it may be useful to perform quantitative analyses of both the porosity of the PEM, and the magnitude of mineralization on the implant surface. Some assays that could be used for this purpose is the Alizarin Red or Von Kossa assays.

Together, these characterization data satisfy our first research objective, and confirm the successful application of the LbL PEM, with or without the incorporation of nanoparticles. Nanoparticles were within the appropriate size range, and suspensions were sufficiently monodispersed. This monodispersity was also effectively transferred into the coating, resulting in
the even coverage of the entire substrate (including within the porous titanium lattice) with the PEM.

**MC3T3-E1 preosteoblast cell response**

One of the most important features of the proposed coating was its capacity for promoting pre-osteoblast viability and differentiation. To study the effect of different coating formulations on MC3T3-E1 viability *in-vitro*, we used the AlamarBlue assay.

The results from AlamarBlue showed an enhancement in cell viability in the 13.33mg/ml TiO2NP group compared to all other groups across the first 4 days of culture. After the 4th day, the cells appeared to recover from any initially reduced viability caused by the coatings or otherwise, and differences were lost, except for the group with 4mg/ml of incorporated AgNPs. In this group (4mg/ml AgNPs), reduced viability persisted between days 4 and 7, denoting its marked cytotoxic effect on these cells. The lower concentration of AgNPs did not show this cytotoxic effect, and it may be reasoned that 0.4mg/ml is below the cytotoxic threshold for MC3T3-E1 cells in these conditions. At all other concentrations of TiO2NPs, and in the absence of nanoparticles or PEM, cells still grew at reasonable levels, though there was a clear optimal concentration of TiO2NPs to promote viability.

ALP activity results are consistent with literature\textsuperscript{51,82}, showing no notable increases or differences until day 7, while ALP activity increases significantly thereafter. In this case, increases in ALP activity on day 7 were low, but enough to elicit statistical significance between select groups. However, the differences measured after day 14 more clearly characterize the differences in induced differentiation between groups. It was suspected, based on the viability data, that cells of the 13.33mg/ml TiO2NPs group would demonstrate the highest ALP activity, due to that group having shown the most promising results in that regard. However, the 0.4mg/ml AgNPs group instead showed significantly greater ALP activity, comparable to the positive control group. In comparison with TiO2NP groups, this was an unexpected outcome, as it was hypothesized that the same decrement seen in viability would be observed in differentiation, linked by the supposed increase in ROS production. These data are at odds with each other, though this might be an artifact of the differential activity of these nanoparticles. Perhaps there exists a concentration of AgNPs such that both viability and differentiation are enhanced. This may well also be the case of TiO2NPs. Unfortunately, the array of concentrations used in these experiments did not suffice to
touch on what that optimal concentration may be, though it was outside of the scope of the project to focus on optimizing this aspect. In the future, more resources can be applied toward identifying these doses, and a more appropriate comparison can be made. As well it can be useful to include an additional ALP test day after 10 days, as it is possible that some other interesting differences may exist between the day 7 and day 14 timeframe.

**Antimicrobial activity**

For examination of the antibacterial activity of the coated substrates (Aim 3), we exposed samples to a liquid culture of *S. aureus*. Despite a lack of significant differences in the mean adherent bacterial growth inhibition between groups, it was still possible to extract meaningful information from these data. Data retrieved from each set of groups containing various nanoparticles were isolated, and group by type. A simple linear regression revealed an effect of nanoparticle concentration. In the case of TiO2NPs, this effect showed that increasing concentrations of nanoparticles resulted in less bacterial growth of adherent bacteria on the surface of the coated substrate. In AgNP groups, this relationship was not present, and in one case, showed the opposite trend, where a greater concentration resulted in more bacterial growth. This was an unexpected outcome, as it was assumed that the same concentrations of AgNPs that were toxic to MC3T3-E1 cells would demonstrate comparative toxicity toward bacteria. While these trends did exist, the magnitude of growth inhibition was less than anticipated. The absolute colony counts were nevertheless relatively high (in the range of $10^7$ CFU/ml), considering a seeding density of $1x10^4$ CFU/ml.

There are two likely reasons for this to have been the case. Firstly, it may be attributed to the interaction between the elements of the overall construct. That is, the PEM may have succeeded in lessening the burst release phase, though to such an extent so as to diminish its antibacterial activity. This possibility is supported by the mammalian cell viability data. Percent Difference results (Figure 19) show that cell viability in the higher AgNP concentration group only experience a marked decline after 4 days of exposure. This delay in the response to the presumed increase in ROS may also be at play here. Although, the rapid growth of *S. aureus* may diminish the effect of this, particularly at this supposed slower rate of nanoparticle/metal ion release.

The second potential explanation relates to the bacterial culture methodology. The infectious dose of *S. aureus* is estimated to be about 100,000 organisms. However, with a doubling time of
20 minutes\textsuperscript{27}, seeding at that number of CFUs was predicted to quickly become overwhelming for the samples. We reduced that seeding density 10-fold, down to 10,000 CFU/ml for liquid culture. However, based on the counts of surviving planktonic bacteria from each sample well (not shown), as well as the counts of adherent bacteria, it is likely the case that this cell density also rapidly overwhelmed the samples, and diminishing their potential antibacterial effects. Another source of this issue could have been that cells were cultured with samples in a 48-well culture plate, with samples set inside each well. While it can be said that cells were exposed to the coating, there was in fact a large proportion of liquid culture that allowed for bacteria to grow without direct contact with the coating. As such, this could have allowed the bacteria the opportunity to multiply to a number that would become unmanageable by the coating.

Potential solutions to this are numerous. On one hand, the amount of time that the samples are left in culture with bacteria could be shortened from 24 hours, to prevent this overwhelming growth. Another option would be to use this shorter incubation time, and also remove samples from initial culture after a few hours, transferring them to a new multi-well plate with fresh media, so that only cells that adhere to the disks can be cultured, and there is reduced risk for planktonic growth to become a significant confound. Other alternatives may include a type of well-diffusion antibacterial test, though it is unsure how well the nanoparticles and metal ions would be able to diffuse through/across agar, given the coating.

**Limitations and future directions**

The solutions presented immediately above respond to a primary limitation experienced in the antibacterial portion of this research, which was the overwhelming growth of the bacteria in liquid media, which seemed to diminish the observable antibacterial effects of the proposed construct. In previous research exploring antibacterial activity against *S. aureus* of nanoparticle-containing coated structures, or other formulations containing nanoparticles, both the methods used and seeding densities (from 10\textsuperscript{5} CFU/ml to 10\textsuperscript{9} CFU/ml) of the bacteria varied widely\textsuperscript{44,53,60,62,68,70,74,78,84–86}. There is a clear link between the assay method and the required seeding density, though some preliminary tests using a variety of different method might be necessary to identify the most appropriate methodology for the materials presented herein. For example, the disk diffusion assay places samples in an agar plate with plated bacteria to be incubated together. In this case, it is possible to see a zone of inhibition develop around a sample,
denoting the extend of its bactericidal ability. This method would eliminate the issue of overwhelming growth, though there would also be a highly reduced contact area for the bacteria to meet the substrate, making assessment of its bacteriostatic ability more difficult.

Other limitations also presented themselves in MC3T3-E1 testing. Assays of mineralization are a common indirect test for osteoblast growth and differentiation. When attempted in this research using Alizarin Red, the samples themselves disrupted the monolayer of mineralized cells formed on the surface of cell culture plates. This created significant difficulty at the time of testing, as the possible methods for sample extraction were affected by this issue. In future research, it may be useful to attempt a different test where this is not a problem. Another solution may be to instead culture cells while samples are held within cell culture plate inserts, so that samples do not sit on the floor of the well and potentially disturb the monolayer. In such a case however, the cells would not have the opportunity to come in contact with the samples, and we would therefore lose a valuable aspect of the analysis.

Other research into this area relating to the use of mNPs for antimicrobial purposes, specifically with regard to titanium nanoparticles, more often use the Anatase form, as opposed to the Rutile form, which was used in the present research. Such research suggests that nanoparticles of the Anatase form may be more suitable for use for the purposes described here. However, they did not compare the antimicrobial effects of these two compositions while embedded in multilayers as described in this thesis, nor did they examine their effects on mammalian cell lines to examine their cytotoxicity.

Compared to previous research (AgNPs, TiO2NPs) using either type of nanoparticle, this research used higher concentrations when formulating the multilayer, in the microgram range compared to milligrams per millilitre, though many experiments using TiO2NPs generally used concentrations higher than those with AgNPs. Such research consisted of suspended nanoparticles, or of nanoparticles contained in some other PEM or complex. Across these articles, nanoparticles concentrations varied widely. The main reasoning in using higher doses in this case began as a test to assess the upper limits of MC3T3-E1 tolerance for the specific compounds being used. This was also the reasoning with regard to antibacterial experiments. As it turned out, the cytotoxic and osteointegrative action are lower than what was initially expected from these doses. One hypothesis is that the PEM diminishes the peak activity of the nanoparticles. This could result from an inhibition of nanoparticles to exit the multilayers,
an extreme effect resulting from the attempt at controlling the burst release phase of the PEM. In the future, it may be worth examining an even wider range of concentrations in order to establish a true dose-response curve for this specific formulation. Additionally, it would be worthwhile to quantify the nanoparticle release profile of the PEM.
Chapter 8: Conclusions

This project sought to design a novel bacteriostatic, bactericidal, and osteoconductive surface coating for use as an anti-infective surface modification and preventative measure against periprosthetic joint infection. We used the LbL technique to assemble PEMs with incorporated nanoparticles of silver or titanium dioxide, and examined and compared their effects on pre-osteoblast viability and differentiation, as well as on *S. aureus* growth inhibition. Using multiple techniques for surface characterization, it was confirmed that the PEM and its imbedded nanoparticles were successfully applied to the substrate. Mammalian cell viability was enhanced in the early stages at defined nanoparticle concentrations, and tests of antimicrobial activity show a dose-effect trend of TiO2NPs toward growth inhibition.

Until now, research has been lacking on simultaneous and direct comparisons between the more extensively researched AgNPs and other potential antibacterial candidates, like TiO2NPs. And although much more research is necessary, the observations made here are a testament to the care and time that must be taken in selecting the right therapeutic, as well as the doses that best exert their desired antibacterial effect, but do not pose substantial harm to host cells. It is believed that further research into this coating formulation will serve to refine the testing procedures to best elucidate the properties that it is designed to have. All together, these data are of particular significance as they begin to shed light on the potential of other options to the field of medicine and biomedical engineering for the purposes of infection prevention and enhanced bone growth.
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