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The Application of the Ultrafine Technology in Improving the Biocompatibility and Osteo-inductivity of Dental Implants

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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THE APPLICATION OF THE ULTRALFINTE TECHNOLOGY IN IMPROVING
THE BIOCOMPATIBILITY AND OSTEO-INDUCTIVITIVY OF DENTAL
IMPLANTS

(Thesis format: Integrated Article)

by

Nicholas Y. Hou

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Degree Doctor of Philosophy

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Abstract

Dental implants are very effective medical devices. However, although stable, the conventional titanium implants are not very bioactive which in some instances could reduce their efficacy. This thesis described the research progress of using polymeric ultrafine-particles with bioactive additives to enrich the surface of titanium substrate, thereby increasing the biocompatibility and osteo-inductivity of the biomaterial. During this doctoral project, three different types of polymers were examined, initially the conventional polyester, and later the novel epoxy as well as the epoxy/polyester hybrid polymers. Physical characterizations confirmed that all of the coating powders were ultrafine particles, and homogeneous surfaces were constructed from these particles with the correct incorporation of the functional and flow additives. The biocompatibility of the enriched surfaces were confirmed after examining their water contact angles, cell attachment/proliferation abilities, and their mitochondrial activities. After proliferation and differentiation, the osteo-inductivity of the surfaces were studied by labelling the mineral deposits that were formed on the surfaces. In addition to the biological performance, the adhesive strength of the enrichment layer was also assessed. After comparing between calcium oxide and calcium phosphate as the bioactive additive, and a series of different epoxy and polyester based polymers in the presence/absence of micron-sized TiO$_2$, the results showed that the calcium oxide and micron-sized TiO$_2$ containing epoxy/polyester hybrid surface was the best candidate for future clinical in vivo studies. From this study, a better understanding in the factors that affect the biocompatibility and osteo-inductivity of titanium substrate were obtained in order to create more effective biomaterials.

Keywords

Ultrafine particles, electrostatic powder coating, biocompatibility, titanium biomaterial, osteo-inductivity, calcium oxide, calcium phosphate, mesenchymal stem cells, TiO$_2$, polyester, epoxy, polyester/epoxy hybrid, in vitro cell studies, particle analysis
Co-Authorship Statement

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Author: Hou, Nicholas Yue; Zhu, Jesse; Zhang, Hui; Perinpanayagam, Hiran

All experimental works were conducted by Nicholas Yue Hou under the supervision of Dr. Jesse Zhu and Dr. Hiran Perinpanayagam. All drafts and modifications of this manuscript were also completely by Nicholas Yue Hou under the supervision of Dr. Jesse Zhu and Dr. Hiran Perinpanayagam. This manuscript has already been respectively accepted and published by Surface and Coatings Technology on April 15th, 2014 and July 25th, 2014.

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# Table of Contents

Abstract .............................................................................................................................................. ii
Co-Authorship Statement ....................................................................................................................... iii
Acknowledgments ................................................................................................................................. v
Table of Contents ............................................................................................................................... vii
List of Tables ......................................................................................................................................... xi
List of Figures ......................................................................................................................................... x

Chapter 1: Application of ultrafine powder coating on dental implants ............................................. 1
  1.1 Development of ultrafine powder coating .................................................................................. 2
  1.2 Components of powder coating particles ................................................................................. 6
  1.3 Dental implants ............................................................................................................................ 7
  1.4 Calcium phosphate & calcium oxide ......................................................................................... 9
  1.5 Mesenchymal stem cells & cell differentiation ........................................................................... 10
  1.6 Biocompatibility & osteo-inductivity ......................................................................................... 11
  1.7 Objectives .................................................................................................................................. 13
  1.8 Thesis structure ........................................................................................................................... 14
  1.9 Major contributions ..................................................................................................................... 16
  1.10 References ................................................................................................................................ 18

Chapter 2: Ultrafine calcium-titania-polyester dry powder coatings promote human mesenchymal cell attachment and biomineralization ................................................................. 22
  2.1 Introduction ................................................................................................................................. 22
  2.2 Materials and methods ............................................................................................................... 25
  2.3 Results ........................................................................................................................................ 30
  2.4 Discussion ................................................................................................................................... 41
  2.5 Acknowledgements ..................................................................................................................... 45
List of Tables

Table 1.1: Ultrafine enriching particle formulation .................................................................6
Table 2.1: Composition of ultrafine powders ........................................................................26
Table 2.2: Elemental composition of coating surfaces ............................................................32
Table 4.1: Composition of ultrafine powders ......................................................................80
Table 5.1: Composition of ultrafine particles ......................................................................106
Table A1: An example of the enhancement particle formulation ......................................138
List of Figures

Figure 1.1: In powder coating, particles are first charged and dispersed using a Corona Gun, sequentially the particles are attracted and temporarily attached onto the grounded substrate through electrostatic forces. The particles are then permanently cured onto substrate in a curing oven resulting in a stable coated surface ........................................... 3

Figure 1.2: Geldart's classification of powders according to their sizes and fluidization properties. At smaller sizes, the particles become cohesive such the group C powders. Such particles have a low flowability, when used in dry powder coating, such particles can clogged the spraying instruments ......................................................... 4

Figure 1.3: This image demonstrates the principle behind the ultrafine-particle technology. For cohesive particles, nano-particles can be added to increase the flowability of the particles by increasing the interparticular distance and hence, effectively reduce the intermolecular forces between two particles and their cohesiveness ........................................................................................................ 5

Figure 1.4: In dental implants, the implant itself is anchored into the patient’s jaw bone using its titanium screw. Image obtained with permission from: http://melaniebachdds.com/ ............................................................................. 8

Figure 2.1: Surface coatings were created by an ultrafine dry powder coating process. Base polymer (polyester resin) containing filler (CR822), pigment (micron-sized TiO2), flow modifiers (nTiO2), and curing (TGIC), degassing (BEN) and bioactive agents (CaO/Ca3(PO4)2) were combined in a high-shear mixer, sprayed onto a grounded metal substrate (Ti), and cured in a furnace (200 °C, 10 min). ................................................................. 26

Figure 2.2: Powder formulations were analyzed for particle-size distribution. PPC, PPC + 1%CaO, PPC + 3%CaO, PPC + 5%CaO and PPC + 5%CaP had relatively similar particle-size distributions with a D50 of under 30 μm ......................................................... 31

Figure 2.3: Coating surfaces were analyzed by elemental mapping. Calcium was undetectable beyond background levels in the PPC and PPC + 5%CaP. There were a few
large calcium clusters in the PPC + 1%CaO, and several small evenly distributed clusters in PPC + 3%CaO and PPC + 5%CaO. There were some small phosphorus clusters in the PPC + 5%CaP.

Figure 2.4: Water contact angles were measured on the coatings. PPC, PPC + 1%CaO and PPC + 5%CaP coatings were hydrophilic and had contact angles that were similar (A). PPC + 3%CaO, PPC + 5%CaO and titanium (Ti) were progressively more hydrophilic and had progressively smaller contact angles that were different from each other (B, C, D). (n=5, each alphabet represents a statistical group, p<0.05).

Figure 2.5: Human mesenchymal cells were seeded onto the surface coatings and examined by optical microscopy. After 24 h, low power (120 μm scale bar) showed few cells on PPC and titanium (Ti), more cells on PPC + 1%CaO, even more cells and cell spreading on PPC + 3%CaO, and the most cells on PPC + 5%CaO. There was a moderate amount of cells on PPC + 5%CaP. High power (30 μm scale bar) showed cell spreading and elongated morphologies on all surfaces.

Figure 2.6: Cell cultures grown on the surface coatings were stained with Alizarin Red S. After 2 weeks, there were few deposits on titanium, more on PPC and PPC + 5%CaP, moderate amounts on PPC + 1%CaO and PPC + 3%CaO, and large amounts on PPC + 5%CaO. Then after 4 weeks, there were some deposits on titanium and PPC, moderate amounts on PPC + 1%CaO, PPC + 3%CaO and PPC + 5%CaP, and very large amounts on PPC + 5%CaO.

Figure 3.1: An ultrafine dry powder coating technique was used to create novel biocompatible surface coatings enriched with bioactive agents, which were then evaluated in cellular assays. Polyester resin, micron-sized titanium dioxide (TiO2), nanoparticles of titanium dioxide (nTiO2), and functional additives that included calcium oxide (CaO) or calcium phosphate (Ca3(PO4)2), were ground and mixed in a high-shear mixer to create ultrafine particles. The ultrafine powders were then sprayed onto commercially pure titanium (cpTi) surfaces and cured in a furnace. The surface coatings were sterilized and used as substrates in cellular assays.
Figure 3.2: The surface coatings were analyzed by energy dispersive x-ray spectroscopy (EDX) to confirm their chemical composition. Titanium (Ti) was detected at high levels in all of the coatings. Calcium (Ca) was detected at much lower levels in only the PPC+1%CaO, PPC+3%CaO, PPC+5%CaO and PPC+5%CaP surfaces, and in proportion to their formulation. Phosphorus (P) was detected in at very low levels in only the PPC+5%CaP surfaces.

Figure 3.3: Human mesenchymal cells were seeded onto coatings and control surfaces that were suspended in tissue culture medium in an incubator. After 24 hours, cells (white arrows) that had attached and spread out onto the surfaces were examined by scanning electron microscopy (SEM). Spreading cell morphologies were seen on all of the coatings and controls, and elongated morphologies were seen on the PPC surfaces.

Figure 3.4: Human mesenchymal cells were seeded onto coated and control surfaces that were suspended in tissue culture medium in an incubator. After 24 hours, cells that had attached and spread out onto the surfaces were collected and counted. Cell numbers were higher on the PPC+5%CaO coatings than on any other surfaces. (n=12, each alphabet represents a statistical group, p<0.05)

Figure 3.5: Human mesenchymal cells were seeded onto coated and control surfaces that were suspended in tissue culture medium in an incubator. After 24 hours, cells that had attached and spread out onto the surfaces were collected and counted. Cell numbers were highest on the PPC+5%CaO coatings, followed by the PPC+3%CaO coatings, the PPC+1%CaO coatings and the titanium (Ti) controls. (n=3, the alphabet represents the same statistical group)

Figure 3.6: Human mesenchymal cells were seeded onto coatings and control surfaces that were suspended in tissue culture medium in an incubator. After 72 hours, the cells that had attached, spread out and proliferated on the surfaces were collected and counted. Cell numbers were highest on the PPC+5%CaO coatings and lowest on the PPC controls, but mostly similar on all surfaces. (n=12, each alphabet represents a statistical group, p<0.05)
Figure 3.7: Human mesenchymal cells were seeded onto coated and control surfaces that were suspended in tissue culture medium in an incubator. After 24 hours, the cells that had attached and spread out onto the surfaces were measured for metabolic activity (MTT). Their activities were higher on the PPC+1%CaO, PPC+3%CaO and PPC+5%CaO coatings, than on the PPC+5%CaP, PPC and titanium (Ti) surfaces. (n=5, each alphabet represents a statistical group, p<0.05)..............................65

Figure 3.8: Human mesenchymal cells were seeded onto coated and control surfaces that were suspended in tissue culture medium in an incubator. After 2 and 4 weeks of growth and biomineralization, the surfaces were stained with Alizarin Red-S. Numerous mineral deposits were detected on the PPC+3%CaO and PPC+5%CaO coatings within 2 weeks, and few deposits on other surfaces after 4 weeks..........................................................67

Figure 4.1: An ultrafine dry powder coating technique was used to create novel biocompatible surface coatings that are enriched with bioactive agents. Both epoxy and epoxy/polyester resins, calcium oxide (CaO) and nanoparticles of titanium dioxide (nTiO2) were ground and mixed, either with or without micron-sized titanium dioxide (TiO2), in a high-shear mixer. The powder mixtures were passed through a sieve to obtain ultrafine particles, which were confirmed by particle-size analysis. The ultrafine powders were then sprayed onto grounded titanium (cpTi) disks and cured in a furnace.79

Figure 4.2: The powder mixtures were analyzed for their particle-size distribution. The Epoxy-TiO2 and the Epoxy-Polyester-TiO2 had similar particle-size distributions with a relatively small D50 that was around 20µm. The Epoxy and Epoxy-Polyester had very similar particle-size distributions with a larger D50 that was around 30µm ..................80

Figure 4.3: The coated surfaces were analyzed by elemental mapping. Carbon (C) was detected at high levels, and oxygen (O) at moderate levels on all of the surfaces. Titanium (Ti) was detected at high levels on the Epoxy-TiO2 and Epoxy-Polyester-TiO2 surfaces, and at much lower levels on the Epoxy and Epoxy-Polyester surfaces. Calcium (Ca) was detected at very high levels on the Epoxy-Polyester-TiO2 surfaces, in clusters at moderately high levels on Epoxy-Polyester, and unevenly at lower levels on Epoxy and Epoxy-TiO2 surfaces. Each dot represents the presence of respective element.................81
Figure 4.4: The flowability of the ultrafine particles were lastly confirmed by examining their angle of repose. This assay illustrated that all of the particle formulations were highly flowable. The pure epoxy particles were found to have the best flow, followed by the micron-sized TiO2 containing hybrid ultrafine particles, whereas the micron-sized TiO2 containing epoxy and pure epoxy/polyester hybrid particles exhibited similar flow properties. (n=3, each alphabet represents a statistical group, p<0.05) ............................88

Figure 4.5: The flow properties of the ultrafine particles were examined by assessing their avalanche angles. This assay showed that the micron-sized TiO2 containing particles were less flowable by exhibiting a higher avalanche angle comparing to their pure counter-parts. Nevertheless, the different types of polymer backbones did not have any significant effects on the flowability of the particles. (n=199, p>0.05) ......................89

Figure 4.6: The flowability of the ultrafine particles were further studied by obtaining their resting angle after each avalanche. The resting angle study also showed the micron-sized TiO2 containing particles were less flowable compared to their pure counter-parts. However, the resting angle assay also suggested that the epoxy particles were significantly more flowable than the epoxy/hybrid particles due to their smaller angles. (n=199, p>0.05) ........................................................................................................90

Figure 4.7: The biocompatibility of all of the coated surfaces were examined by assessing the cellular morphology of the cultures that were seeded on each surfaces. In lower power optical microscopy (40x), the images showed that all of the surfaces were able to support cell cultures; while in high power optical microscopy (100x), the cellular morphology clearly supported the biocompatibility of the surfaces by exhibiting a spreading morphology with their extended filopodia, showing the intimate contact between the cells and the biomaterial........................................................................................................91

Figure 4.8: Human mesenchymal cells were seeded onto coated and control surfaces that were suspended in tissue culture DMEM medium. After growth and biomineralization, the surfaces were stained with Alizarin Red-S. (A) After 2 weeks, a few mineral deposits were detected on the Epoxy-Polyester and the Epoxy-Polyester-TiO2 coated surfaces,
and on the Epoxy-TiO2. (B) After 4 weeks, much more mineral deposits were detected on the Epoxy-Polyester and the Epoxy-Polyester-TiO2 coated surfaces ..........................93

Figure 5.1: The patented ultrafine particle coating technology was employed to develop this novel biocompatible surface enrichment. This surface layer is enriched with bioactive agents. Both epoxy and epoxy/polyester resins, as well as calcium oxide (CaO) were sheared and sieved (35 µm); the sequential mixture was then mixed again with nano-sized titanium dioxide (nTiO2) to improve this flowability. The ultrafine surface enhancing particles were then sprayed onto grounded titanium (cpTi) disks, cured in a furnace and prepared for biological assays to examine its biological performance ........105

Figure 5.2: EDX illustrated that majority of the surface enrichment layer is composed of carbon, followed by oxide which existed in much smaller quantity. The presence of titanium fluctuated according to the presence of micron-sized TiO2, more was detected in the Hybrid-TiO2 and Epoxy-TiO2 compared to the Hybrid pure and Epoxy pure surfaces. Lastly, there was also small variation in the presence of Calcium amongst the different surfaces. (n=3) ........................................................................................................................................112

Figure 5.3: Hydrophilicity assay suggested that although all of the surfaces were hydrophilic, there was a noticeable trend where hybrid surfaces exhibited a smaller contact angle than the epoxy coated biomaterial, and the micron-sized TiO2-containing surfaces were more hydrophobic than their counterparts. (n=5, each alphabet represents a statistical group, p<0.05) ..........................................................................................................................113

Figure 5.4: The 7, 14 and 28 days after cell culture media immersion adhesion test showed that all of the surfaces achieved the highest adhesion rating (5B). (n=4, p>0.05) .................................................................................................................................114

Figure 5.5: The cell attachment assay indicated that the HEPM cells were able to attach significantly faster to the micron-sized TiO2-containing surfaces than the rest of the biomaterials. Similarly, the hybrid surfaces also significantly outperformed their epoxy counterparts. The conventional titanium surfaces had the lowest cell attachment rate. (n=12, each alphabet represents a statistical group, p<0.05) .........................................................................................115
Figure 5.6: After 7 days of incubation, the proliferation results suggested although there was no statistical significance, there was a noticeable trend where the hybrid surfaces had a higher cell proliferation rate than the epoxy surfaces. In addition, the micron-sized TiO2-containing surfaces also outperform their none-containing counterparts. (n=5, each alphabet represents a statistical group, p<0.05)........................................................................116

Figure 5.7: The 24 hour MTT assay indicated that the conventional titanium and epoxy-pure surfaces exhibited the highest mitochondrial activities, where hybrid surfaces had similar and lower metabolic activities. On the other hand, the cells raised on micron-sized TiO2 containing epoxy surfaces demonstrated the least amount of mitochondrial activity. (n=7, each alphabet represents a statistical group, p<0.05)........................................................................118

Figure 5.8: The 7 day MTT assay indicated that the HEPM cells that were seeded on titanium, micron-sized TiO2-containing Epoxy and the non-TiO2 containing surfaces had similar mitochondrial activity, whereas the micron-sized TiO2-containing hybrid surfaces exhibited the least amount of activity. (n=7, each alphabet represents a statistical group, p<0.05)........................................................................119

Figure 5.9: HEPM cells were plated onto enriched and control surfaces. These cultures were incubated for 24 hours in DMEM medium, then switched to differentiation medium for reminder of the assay to induce biomineralization. The surfaces were stained with Alizarin Red-S to visualized mineral deposits. (A) After 2 weeks, some mineral deposits were detected on the Epoxy/Polyester Hybrid and the Epoxy/Polyester Hybrid-micron-sized TiO2 coated surfaces, and in smaller extent on the Epoxy-TiO2. (B) After 4 weeks, much heavily stained areas were observed on the Epoxy-Polyester and the Epoxy-Polyester-TiO2 coated surfaces ........................................................................................................120

Figure A1: Ultrafine particle coating technology was utilized to construct these novel biocompatible, bioactive enriched surfaces. The base polymer and the functional additive were sheared and sieved (35 µm) to obtain ultrafine particles; the sequential mixture was then mixed again with nano-sized titanium dioxide (nTiO2) to improve this flowability. The surface enhancement particles were then electrostatically sprayed onto grounded
titanium (cpTi) disks, cured in a furnace and prepared for physical and biological examinations .................................................................138

Figure A2: Laser particle analysis indicated that all of the particle mixtures constructed using the ultrafine particle technology had a D50 of less than 35 microns, confirming the ultrafine nature of the particles. In addition, all of the coating particles exhibited a similar size distribution, illustrating the reliability of the ultrafine particle technology.............145

Figure A3: EDX demonstrated that the surface coating particles were composed mostly out of carbon, oxygen, and titanium. However, there is smaller quantities of aluminum, silicon and sulfur that came from the polymer backbone. In addition, the calcium presence increased with the increasing incorporation of calcium oxide, and phosphors was only detected in calcium phosphate-containing mixtures. Collectively, these findings confirmed the accurately of the ultrafine particle technology in incorporation functional additives .................................................................................................146

Figure A4: Element mapping showed that calcium existed in smaller clusters while under low concentration, whereas it is more evenly spread out at higher concentration. In addition, element mapping also demonstrated that the phosphorus was co-localized with calcium in calcium phosphate surfaces........................................................................147

Figure A5: Water wettability assay indicated that by increasing the % of surface calcium oxide, its hydrophilicity can be significantly increased. Furthermore, although all of the surfaces were hydrophilic, the 5% calcium oxide and the conventional titanium surfaces were significantly more hydrophilic than rest of the biomaterials .........................149

Figure A6: Atomic force microscopy was used to assess the surface roughness of the biomaterial. The images showed the smooth surface of the conventional titanium substrate; however, after polymeric coating, intricate nano-sized topographies can be observed on the surface of biomaterial .........................................................................................149

Figure A7: Immunochemistry was employed to study the cytoskeletal integrity of the attached cells. The images showed very organized actin filaments in all of the cells, and their stretch morphology ........................................................................................................151
Figure A8: Scanning electron microscopy illustrated a high cell confluency of the coated biomaterial and a spreading morphology where cells exhibited extended filopodia, which consequently suggested an outstanding degree of the surface biocompatibility ............152

Figure A9: Optical microscopy suggested that all of biomaterials were able to support HEPM cell cultures. However, under lower power (100x), the images suggested that the cellular confluence correlated with the incorporation of calcium oxide. While at high magnification (400x), a spreading morphology was observed on all of the surfaces, indicating the close association between the cells and their underlying substrate ..........153

Figure A10: Biomineralization assay suggested even though all of the surfaces showed increased signs of mineral deposits over time, 5% CaO-containing surfaces exhibited the great degrees of Alizarin Red staining after 2 week and 4 weeks of proliferation and differentiation. However, conventional titanium showed least amount of mineral deposits amongst all of the surfaces..........................................................155

Figure A11: The cellular attachment ability was examined after 24 hours of incubation, while the cellular proliferation ability was studied after 72 hours of incubation. The hemocytometry assay suggested that polymer coated could achieve a similar cell confluency as the conventional titanium surfaces ..........................................................157

Figure A12: After 24 hours of incubation, MTT assay illustrated that the mitochondrial activities varied between surfaces. However, polymer coated biomaterial could potentially have a higher metabolic activity compared to commercially pure titanium..158

Figure A13: PRC demonstrated all of the constitutional genes that were expressed within the cells raised on commercially pure titanium was also detected on polymer coated surfaces ..........................................................................................................................159
Chapter 1

Application of ultrafine powder coating on dental implants

The development of ultrafine powder coating and functional additives has opened a new realm allowing researchers to develop multipurpose and multifunctional surfaces to accommodate today’s demanding needs. Compared to liquid coating, the use of powder coating is much more cost-efficient and environmental-friendly. These qualities are archived through recycling over-sprayed particles and eliminating toxic solvents. However, one disadvantage that has been saddling the field of powder coating is the poor fluidizability of the particles. The use of small coating particles results in the dominating interparticular attractive force render the flowability of the coating particles. Fortunately, the invention of ultrafine particle technology effectively overcame this drawback. With utilization of nano-sized flow-additives which minimized the interparticular attractive forces, the ultrafine particle technology created by the Particle Technology Research Center at The University of Western Ontario allowed the development of highly flowable ultrafine-coating particles.

Furthermore, with the incorporation of functional additives, surfaces can now be tailored towards their specific needs, such as enriched biocompatible and osteo-inductive orthopedic implants. Biocompatibility and osteo-inductivity often are the two most sought after qualities in dental or bone implants. Although, much research interest has been invested in promoting these two characteristics, most of the currently explored methods were either too complex, or too expensive for commercialization. This project is aimed to further improve a less expensive and more effective novel alternative technique which has been shown to improve the biocompatibility and osteo-inductive of bone implants via the ultrafine particle technology. The effects of different concentrations of calcium oxide and calcium phosphate were examined and compared on enriched
commercially available titanium surfaces; similarly, the effects of different polymer backbones were also studied. The results demonstrated that not only were the coated biomaterials biocompatible and osteo-inductive, but their results also far exceeded the performance of current conventional surfaces. In the future, animal studies should be conducted to examine the effective in vivo effects of ultrafine particle-enhanced bone implants and biomaterial.

1.1 Development of ultrafine powder coating

In recent history, dry powder coating has been steadily gaining popularity in a field that was once primarily occupied by liquid coating. There are many reasons contributed to this shift in coating preference; however, there were two major driven forces behind this change. First, compared to liquid coating, which often contains toxic solvents and have high levels of wastage, dry powder coating has the qualities of being environmentally-friendly and economical-effective by eliminating the need for solvents and allowing the reuse of over sprayed particles (1). Typically, powder coating involves the use of dry particles, which are dispersed using an electrostatic sprayer, such as a Corona Gun. The Corona Gun electrically charges the paint particles then disperse them towards a grounded substrate. Once the charged dry particles are temporarily attached onto the grounded substrate by electrostatic forces, the coated substrate will then undergo a rapid high temperature curing process to solidify the attachment (Figure 1.1). From start to finish, a substrate can get coated in 10 – 20 minutes. The substrate can take various forms ranging from wood, plastic, and to metal. Although dry powder coating has the advantage over liquid painting by offering qualities such as environmentally friendliness, economical efficiency and a rapid curing period, there was one bane that has troubled powder coating since its initial development.
Figure 1.1: In powder coating, particles are first charged and dispersed using a Corona Gun, sequentially the particles are attracted and temporarily attached onto the grounded substrate through electrostatic forces. The particles are then permanently cured onto substrate in a curing oven resulting in a stable coated surface.

The surfaces coated using conventional coating powders would often result in a poor appearance. Smaller particles have been suggested to overcome this problem; however, with the decrease in particle size, the intermolecular forces such as van der Waals and electrostatic forces began to increase exponentially. As a result, even though coating particles within the ultrafine range, D50 of less than around 30 µm, can be easily obtained using different techniques, such particles would exhibit a much larger apparent particle size due to particle cohesion and agglomeration (Figure 1.2). This agglomeration can make coating particles difficult to fluidize, which can eventually result in the uneven distribution of the particles during the spraying process and the clogging of the electrostatic spraying instruments. Thus, in order for powder particle coating to become popular, a new technique is needed to overcome the strong interparticular forces between those fine coating particles.
Figure 1.2: Geldart’s classification of powders according to their sizes and fluidization properties. At smaller sizes, the particles become cohesive such the group C powders. Such particles have a low flowability, when used in dry powder coating, such particles can clogged the spraying instruments.

Recently, the Particle Technology Research Center (PTRC) at the University of Western Ontario has developed a method which can effectively create highly flowable ultrafine particles for the industry of dry powder coating (2)(3). The researchers from PTRC proposed that using nano-particles as spacers, the inter-particular distance between the dry particles can be effectively increased, thereby effectively decrease the dominance of inter-particular forces and consequently increase the flowability of the ultrafine particles (Figure 1.3). As a result, this now a patented technique known as ‘the ultrafine-particle technology’, allowed for the use of ultrafine particles in dry powder coating which significantly improved the coating performance.
Figure 1.3: This image demonstrates the principle behind the ultrafine-particle technology. For cohesive particles, nano-particles can be added to increase the flowability of the particles by increasing the interparticular distance and hence, effectively reduce the intermolecular forces between two particles and their cohesiveness.

However, the research progress did not just conclude after the invention of flow additives. The researchers at the PTRC also proposed that similar to using nano-flow additives to improve the flowability of coating particles, various functional additives can be incorporated to supplement different attributes to the surface of substrates. Using this idea, researchers have constructed unique surfaces which exhibited super-hydrophobicity, extraordinary hardness, anti-microbial, biocompatible and osteo-inductive properties (4)(5)(6)(7)(8).

In conclusion, with the invention of nano-flow additives, the ultrafine-particle technology not only allowed us to coat surfaces using ultrafine particles, but with the introduction of functional additives, it is now also possible to construct tailored surfaces according to their specific needs, such as anti-microbial functions to prevent the spreading of diseases and highly biocompatible and osteo-inductive surfaces to improve the effectiveness of orthopedic implants (Table 1).
Table 1.1: Ultrafine enriching particle formulation.

<table>
<thead>
<tr>
<th>Polymer backbone</th>
<th>Flow additive</th>
<th>Functional additive</th>
</tr>
</thead>
<tbody>
<tr>
<td>94.5 weight %</td>
<td>0.5 weight %</td>
<td>Up to 5 weight %</td>
</tr>
</tbody>
</table>

1.2 Components of powder coating particles

Typically, the powder coating particles are composed of a polymer backbone, filler, flow agent, degassing agent, curing agent, and colour pigments. The small quantities of the flow, degassing, and curing agents in the coating powders allow the smooth finish and the proper curing of the final coated surface. The larger volumes of filler and colour pigments reduced the price of the coating powders and allowed them to achieve any desirable colour. Lastly, the common polymer backbone used in powder coating uses a base resin which can be either one of polyester, polyurethane, polyester-epoxy (hybrid), or pure epoxy.

Polyester, and polyurethane have been shown to be very biocompatible (5)(19)(20)(21); in addition, such polymers offered a flexible scaffold which possess design flexibility, and studies have showed bone formations when various calcium containing molecules were incorporated into the coating (22)(23). While the polyester coatings demonstrated their ability in promoting desirable cellular responses, the recent health concerns regarding triglycidylisocyanurate (TGIC), the essential curing agent in the conventional polyester-based polymer backbone, severely hindered its future application in the medical field. Thus, our research group has redirected our focus onto the alternative polyester-epoxy hybrid and pure epoxy base polymers. The pure epoxy coating particles require only a minute amount of an alternative curing agent, dicyandiamide. Dicyandiamide is a compound that is used in the pharmaceutical industry and has been thoroughly researched and found to pose no food safety concerns. Moreover, the polyester/epoxy hybrid base
polymers have the ability of inter-curing between the epoxy and polyester, thus consequently completely eliminating the need of curing agents.

During this PhD project research, the use of conventional polyester base polymers with the incorporation of bioactive additions were further explored to improve the biocompatibility and osteo-inductivity of bone implants. In addition, the use of polyester/epoxy hybrid and pure epoxy base polymers were also examined for their feasibility as alternative polymers to the conventional polyester base polymer in the field of bone implants.

1.3 Dental implants

Dental and orthopedic implants are very effective medical devices for reinforcing or replacing damaged tissue. Due to their efficacy, the market of bone implants has been increasing at a remarkable pace. In the US alone, the field of orthopedic implants has already comprises a market worth an astonishing $14.3 billion while still maintaining a consistent annual growth at a rate of 8.9 percent (9). The source for all these accomplishments can be traced to the on-going research for improving the performance of bone implants.

Dental and orthopedic implants are most commonly made of titanium. Titanium offers bone implants not only an extraordinary strength to weight ratio, but also an impressive inertness once it has been implanted into the patients’ body. This inertness is the result of the formation of a titanium dioxide layer after the titanium is exposed to oxygen and bodily fluid. This passive out layer granted titanium its anti-corrosion and rust-proof characteristics. Orthopedic and dental implants both function in a similar fashion, where a
portion of the implant is anchored within the patient’s bone, while rest of the implant act to enhance or replace damaged body tissue (Figure 1.4).

Figure 1.4: In dental implants, the implant itself is anchored into the patient’s jaw bone using its titanium screw. Image obtained with permission from: http://melaniebachdds.com/

Even though bone implants are known for their simplicity and effectiveness, like any medical devices, there are always areas that require improvements. In the case of bone implants, the post-surgical success can sometimes be compromised by a faulty tissue-implant interface, which can then result in hypersensitive immune-responses and leading to the loosening of the implant (10);(11). More importantly, the post-surgical stability of a bone implant can also be affected by the poor bone quality or the lack of bone quantity near the surgical site. Experienced surgeons can often predict the probability of loosening of the implant by examining the severity of osteoporosis at the implantation site (12). As a result, even though bone implants already occupied a very astounding market and
proved its effectiveness as a medical device, there is still much research is needed to improve their efficacy.

1.4 Calcium phosphate & calcium oxide

A bone can be classified into 2 categories, the living cells, and the mineralized organic matrix. The latter portion can be described as mineralized and organic due to its organic component consisting of collagen fibers and the mineralized portion which is mainly composed of hydroxyapatite formed from salts of calcium and phosphate. As a result, calcium phosphate has often been used to enrich the surface of biomaterials so they can better mimic the natural tissue environment (13);(14);(15);(16). Through these studies, researchers noted that such materials can be perfectly biocompatible, less erodible than hydroxyapatite, able to reduce dentin hypersensitivity, showed good cell viability, proliferation and differentiation, and they also found to be in excellent agreement in physical properties between the native bone and artificial scaffold. As a result, calcium phosphate has presented itself as a very beneficial molecule to increase the efficacy of biomaterials.

Another material which has also showed much promise in the field biomaterial is a class called bioactive glasses. A commercially available family of the bioactive glasses is called Bioglass, which can be divided into two categories, Class A and Class B. Both Class A and B bioglasses are osteoproductive; however, the Class A materials bind to both soft and bone tissues and were able to induce the formation of hydroxycarbonate apatite layer within several hours, while the Class B bioglasses do not bond to soft tissues and the hydroxycarbonate apatite layer can take up to several days to form (17). Although there are variations on the specific chemical composition of bioglasses, they all contained various levels of SiO₂, and CaO (18). Although CaO is contained in essentially all of the
bioglasses, its effects on the biocompatibility and osteo-inductivity of bone implants were not as well understood.

During this doctorial research, the efficacy of calcium phosphate, which mimics the bone environment, and calcium oxide, which is found in bioactive glasses, in improving the biocompatibility and osteo-inductivity of conventional titanium substrate were assessed. Both compounds were incorporated into polymeric coating particles and applied onto commercially available titanium sheets. After curing, the coated surfaces underwent various in vitro assays to examine the biological performance of the respective surfaces.

1.5 Mesenchymal stem cells & cell differentiation

During this doctoral project, the biocompatibility and osteo-inductivity of coated titanium substrates will be assessed through the response of mesenchymal stem cells. Mesenchymal stem cells are multipotent stromal cells that have the ability of differentiation into a variety of cells such as the osteoblasts, chondrocytes and the myocytes (24);(25);(26);(27). Due to the presence of mesenchymal stem cells in the bone marrow and their abilities in differentiating into bone formatting osteoblasts, much bone-implant research interest has been invested in elucidating the parameters that encourage their osteogenic-differentiate, thereby improving the osseointegration of such implants (28);(29);(30).

Thus far, studies have shown that the HEPM cells were able to attach, spread, proliferate and mineralize on surfaces that were coated using ultrafine powder coating (5);(6);(7);(31);(32). Furthermore, other studies have suggested that mechanical loading, the presence of calcium phosphate particles, stimulated the osteogenic differentiation of the HEPM cells (33);(34). In addition, HEPM cells have been found to attach and
proliferate well on various titanium-based alloys and calcium phosphate-based scaffolds (35);(36). Moreover, it also have been found that the rigidity and the flexibility of a polymeric surface also participated in promoting the growth of fibroblasts and osteoblasts (37).

Consequently, the human embryonic palatal mesenchymal cells (HEPM, ATCC CRL-1486) was used to examine the biological performances of the conventional titanium substrates that were coated using the ultrafine particle technology. The HEPM presented itself to be an excellent study model for this doctoral study due to its immortal nature and their ability in exhibiting myriad cellular responses that occur on implants surfaces, including attachment, spreading, proliferation, metabolic activity, differentiation and bone-like mineral formation (6);(7);(31);(32). Thus, a variety of in vitro assays were conducted to examine the biocompatibility and osteo-inductivity of coated titanium substrate.

1.6 Biocompatibility & osteo-inductivity

Biocompatibility and osteo-inductivity are arguably the two most sought after properties in bone implants. Biocompatibility is a term that was first mentioned in 1970 (38); currently, biocompatibility is considered to be but not limited to the mutual acceptance and the proper co-existence between the biomaterial and the bodily tissue (39). Osteo-inductivity can be described as the ability of a biomaterial in inducing de novo bone formation, biomineralization and osteo-genesis (40).

After realizing the importance of these two characteristics, much of the recent research interest has been invested in promoting the biocompatibility and osteo-inductivity of biomaterials. Thus far, studies have found that the biocompatibility of the biomaterial and
the metabolism of the adsorbed cells can be encouraged by increasing surface roughness (41);(42);(43). Similarly, by incorporating various molecules onto the surface of the biomaterial, its osteo-inductivity can also be effectively stimulated. Researchers have also noted that by modifying the surface of commercially pure titanium or titanium alloy (Ti6Al4V or Ti64) using discrete crystalline deposition of calcium phosphate, the resultant biomaterial showed an improved bone growth and suggested an increased osteoconduction (44). Moreover, after enriching surfaces with electrochemically deposited hydroxyapatite, the researchers demonstrated through histologic observations that such surfaces exhibited the ability of encouraging new bone growth and bone-to-implant contact (45).

In other studies, researchers found that those implant surfaces which were enriched with bioactive silica-calcium phosphate or solely calcium phosphate were able to promote the enzymatic activities of the attached cells, as well as to support osteointegration (46);(47) (48). Most recently in 2014, Anitua et al. examined the in vitro and in vivo effects of calcium-ion (Ca$^{2+}$) modified surfaces (49). They noted that in vitro, such surfaces were superhydrophilic and were able to induce surface clot formation, platelet adsorption and activation upon the exposure to blood plasma. While in vivo, Ca$^{2+}$ enriched surfaces in rabbits showed significantly improved implant bone volume and density, and with the incorporation of growth factors, a significantly intimate bone contact was formed after 2 weeks of implantation.

In conclusion, the biocompability and osteo-inductivity of a bone implant can be effectively improved using various techniques. Thus, this doctorial project attempted to further refine the ultrafine particle technology and tailored it towards improving the biocompability and osteo-inductivity of dental implants.
1.7 Objectives

During this PhD project, the application of ultrafine particle technology on dental implants and the factors which affect the biocompatibility and osteo-inductivity of such implants were refined and studied. The detailed overall objectives are outlined as follow:

Incorporating calcium oxide and calcium phosphate as functional additives:

- To study the stability of calcium phosphate and calcium oxide as functional additives and their feasibility as being components of ultrafine-particle coating on titanium substrate using EDX and elemental mapping respectively
- To compare the hydrophilicity of the coated surfaces against the commercially available titanium substrate using goniometry
- To examine the ability of calcium phosphate and calcium oxide in supporting cell cultures by studying the cell morphology using optical and scanning electron microscopy
- To assess the biocompatibility of the surfaces by quantifying the in vitro cell culture density of human mesenchymal stem cells using hemocytometer and mitochondrial metabolic activity using MTT assay
- To study the in vitro effects of using calcium oxide versus calcium phosphate in inducing biomineralization through short term and long term mesenchymal cell studies

Examining the feasibility of using a TGIC-free polymer backbone to enhance dental implants:

- To provide an alternative TGIC-free polymer backbone that replaces the conventional TGIC-based coating particles
To examine the particle characteristics of the potential pure epoxy and epoxy/polyester hybrid polymer backbones using EDX, cross-hatch, goniometry as well as elemental mapping

To study the ability of surfaces coated using pure epoxy and epoxy/polyester hybrid based polymers in supporting mesenchymal cell cultures via their cell morphology using optical and scanning electron microscopy

To compare the in vitro ability of pure epoxy or epoxy/polyester hybrid coated titanium surfaces in inducing biomineralization through short term and long term mesenchymal cell studies

Examining the effects of micro-sized Titanium oxide in the performance of dental implants

To examine the particle characteristics and the surface properties of surfaces that were enhanced with and without the presence of micron-sized titanium dioxide using techniques including EDX, cross-hatch, goniometry, laser particle size analysis, flowability assays, as well as elemental mapping

To compare the in vitro cellular biocompatibility and osteo-inductivity of surfaces that were enhanced using coating particles under the presence and absence of micron-sized titanium dioxide using hemocytometry, short term and long term biomineralization assays

1.8 Thesis structure

This thesis is consisted of 6 chapters and following the “Integrated Article” format as outlined in the ‘Thesis Regulation Guide’ by the School of Graduate and Postdoctoral Studies (SGPS) of the University of Western Ontario. At the beginning of this PhD research, the research goals and strategies were decided and planned for course of this doctoral program. Consequently, this thesis is structured so that Chapter 1 provides
background information, chapters 2 to 5 presents the research progress of the ‘Biocompatible and osteo-inductive dental implant’ project. To conclude, Chapter 6 offers a summary and presents the limitations of this project, as well as provides recommendations for future studies. Appendix also gives an overview of the technology achievement since the beginning of the development to this date. A brief summary of the all the chapters included in this thesis is outlined as follow:

Chapter 1 provides a background on the benefits of dry powder coating and the development of ultrafine powder coating. It also introduces the fundamentals for dental and orthopedic implants, and the current development progress of bone implants. Furthermore, research objectives, thesis structure as well as the major contributions from this doctoral program are described.

Chapter 2 reports the comparison of the surfaces constructed using calcium phosphate versus various concentrations of calcium oxide as functional additive in the presence of a polyester backbone. Particle analysis, surface characterization as well as in vitro studies were conducted to examine which molecule would be the better candidate as the functional additive in surface-enhancing formulation.

Chapter 3 presents the additional surface characterizations as well as more in-depth in vitro studies conducted on human mesenchymal stem cells. These studies focuses on comparing the effects of using calcium phosphate versus calcium oxide as the functional additives in promoting the biocompatibility and osteo-inductivity of enhanced surfaces.

Chapter 4 describes the two new polymer backbones that were used for ultrafine-particle surface coating, pure epoxy and epoxy/polyester hybrid. Particle and surface character analysis were conducted to examine the performance and the feasibility of these polymer
backbones in replacing the conventional pure polyester backbones. In addition, the effects due of micron-sized titanium dioxide in coating particles were also explored.

Chapter 5 outlines the additional surface analysis as well as the in-depth in vitro studies on the biocompatible and osteo-inductive effects of using pure epoxy or epoxy/polyester hybrid as the backbone polymer, and the effects of micron-sized titanium dioxide. Furthermore, the conclusion whether pure epoxy or epoxy/polyester hybrid would be a suitable alternative candidate to pure polyester as the backbone polymer in ultrafine-particle coating.

In chapter 6, a summary of the results obtained from this doctoral program was provided. In addition, the limitations as well as potential experiments were presented for future research.

In appendix, a summary of the entire ultrafine-particle enriched dental implant research was provided. This chapter overviewed all of the characteristics of the enriched substrates, and the respective cellular effects to date including the previous studies conducted in the group.

1.9 Major contributions

Throughout this doctoral project, the ultrafine-particle technology was utilized to improve the biocompatibility and osteo-inductivity of the commercially available titanium substrate. Sequentially, this research led to the better understanding allowing the following contributions:
Two calcium-containing molecules, calcium oxide and calcium phosphate were examined for their efficacy in promoting biocompatibility and osteo-inductivity on titanium substrate. Coating particles containing calcium oxide and calcium phosphate were able to reach the sizes of ultra-fine particles and those two molecules were able to be successfully incorporated onto titanium surfaces. The coated biomaterials were found to be hydrophilic and exhibited increased biocompatibility and the osteo-inductivity through demonstrating a higher cell count, more intimating cell-substrate association, metabolic activity and biomineralization. Furthermore, it has also been found that calcium oxide was able to create significantly more biocompatible and osteo-inductive surfaces than calcium phosphate.

The feasibility of utilizing epoxy and epoxy/polyester hybrid polymers as the TGIC-free alternative backbone polymers was examined. The constructed particles were characterized to be ultrafine-particles and the coated surfaces demonstrated the ability of the two polymer to successfully incorporate functional additives. Such surfaces were also determined to be hydrophilic and were able to reach the highest adhesion strength rating in the American Society for Testing and Materials (ASTM) standard class of 5B. In addition, both types of coated surfaces exhibited a higher biocompatibility and osteo-inductivity than the conventional titanium substrates through a series of hemocytometry, cell-substrate association, metabolic and biomineralization in vitro cell studies. The data confirmed the feasibility of using epoxy and epoxy/polyester hybrid polymers as the TGIC-free alternatives to replace the conventional polyester coating particles.

The effects of micron-sized titanium dioxide on biocompatibility and osteo-inductivity were also studied. Surfaces of similar composition with the exception of presence or absence of micron-sized titanium dioxide were constructed. Laser particle-size analysis confirmed the ultrafine-particle classification of the coating
particles and goniometry confirmed the hydrophilicity of the surfaces. In addition, all of the surfaces were tested for their biocompatibility and osteo-inductivity using *in vitro* cell studies. The results demonstrated that all of the coated surfaces were better at supporting cell cultures and encouraging biomineralization than the conventional titanium surfaces. However, the surfaces that contained micron-sized titanium dioxide were found to be more biocompatible and osteo-inductive than their counterparts.

- Consequently, this PhD research improved the previous formulation that was used in this novel surface enhancing technology to further increase the biocompatibility as well as the osteo-inductivity of conventional titanium substrates.

### 1.10 References


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Chapter 2

2 Ultrafine calcium–titania–polyester dry powder coatings promote human mesenchymal cell attachment and biomineralization

Abstract

An electrostatic ultrafine dry powder coating technique was developed, which utilizes nanoparticles to prevent agglomeration. When polyester resin, TiO$_2$ (25%), nano-TiO$_2$ (0.5%), filler, flow, degassing and curing agents were combined in a high-shear mixer, then sprayed (20 kV) onto metal sheets and cured (200 °C), biocompatible polymeric powder coatings (PPC) were created. This study's objective was to determine if these surface coatings could be successfully augmented with calcium-rich functional additives. Thus, formulations were enriched with CaO (1, 3 and 5%) and Ca$_3$(PO$_4$)$_2$ (5%, CaP), and their coatings characterized. Particle size analysis of the powders confirmed their ultrafine dimensions. Volume diameters for 50% (D 0.5) were under 30 μm, and 90% (D 0.9) under 50 μm. Energy dispersive X-ray (EDX) analysis confirmed integration with minimal modification to the PPC base. Carbon (61–67%), oxygen (15–25%) and titanium (9–15%) dominated all surfaces, whereas calcium (0.5–1.0%) and phosphorus (0.3%) were only detected in the CaO and CaP enriched coatings respectively. Elemental mapping showed calcium clusters that were smaller but more abundant with more CaO (PPC + 3%CaO, PPC + 5%CaO), and colocalized to phosphorus in the PPC + 5%CaP. Contact angle measurements indicated hydrophilic coatings (62–81°) and significantly (P < 0.05) increased surface wetting with CaO. Optical microscopy showed human mesenchymal cell (HEPM, ATCC CRL-1486) attachment and spreading on all coatings within 24 h, and increasing confluence with CaO incorporation. Alizarin Red-S staining detected the biomineralization of cultures on all coatings that increased with CaO and time. Ultimately, the PPC + 5%CaO had the most cells and biomineralization. This ultrafine dry powder coating technology can create biocompatible surfaces from polyester resins and TiO$_2$, which can be readily augmented with functional additives such as calcium, to titrate optimal cellular responses.

2.1 Introduction

The coating process has been widely used to improve the appearance of surfaces, and more recently to enhance their properties with desirable qualities for highly specialized
applications such as impact protection [1], hydrophobicity [2], antimicrobial activity [3], and even biocompatibility [2], [4], [5] and [6]. However, traditionally the process of surface coating is accomplished by liquid painting, which involves over-spraying paints that are then wasted, and drying toxic solvents that release harmful by-products [7].

Accordingly, electrostatic dry powder coating technology has become an increasingly popular alternative to traditional liquid painting, due to its superior cost-effectiveness and environmental friendliness. This is achieved by recycling the unused dry powder particles and by eliminating toxic solvents from the coating process. However, the conventional electrostatic dry powder coating technique creates surfaces that are significantly rougher in appearance than their liquid painted counterparts, which is unfavorable when a smooth finish is desired. Fortunately, these shortcomings have now been overcome by the recent development of an advanced ultrafine powder coating technique [8] and [9]. This patented technology utilizes nano-sized flow modifiers to prevent the agglomeration of ultrafine powders, and thereby creates coatings that have much smoother surfaces and reduced thicknesses. Additionally, we have recently shown that the ultrafine powder coating formulations can be readily modified to incorporate biocompatible agents for biomaterial applications [2], [4], [5] and [6].

The biomaterial surfaces that have been most extensively studied are dental implants and orthopedic prostheses [10] and [11]. They are usually fabricated from commercially pure titanium (cpTi) and titanium alloys (Ti₆AlV₄), and are covered in an inert TiO₂ surface layer. However, their immediate and long-term retention and success are dependent on optimal integration with the surrounding tissues [12] and [13]. This involves intimate interactions between the biomaterial surfaces and the surrounding tissues at the cellular and molecular level, in a process known as implant osseointegration within bone. This is particularly important for many of the patients who receive these prostheses, due to the presence of comorbid conditions such as alveolar ridge atrophy and osteoporosis that
compromise the quantity and quality of bone at the surgical site [14]. Therefore several strategies have been developed to modify the biomaterial surface in an effort to enhance the cellular response. These include topographical features such as roughness and hydrophilicity, as well as chemical inserts such as hydroxyapatite to enhance protein adsorption, cell attachment, proliferation, differentiation and biomineralization [15] and [16].

This osseointegration depends on the cellular behavior of bone forming cells, osteoblasts and their cellular precursors that originate from undifferentiated mesenchymal stem cells. Therefore, numerous studies have examined the response of human mesenchymal cells to modified implant surfaces, in the hope of identifying features that might enhance this process [17], [18] and [19]. In particular, the human embryonic palatal mesenchymal cells (HEPM, ATCC CRL1486) offers a convenient study model, because they are an immortalized cell line that was derived from the developing palate of a human fetus. In addition, HEPM presents a clinically relevant phenotype for dental implants, we have found that they are also an excellent model for the myriad cellular interactions that occur on implant surfaces, including attachment, spreading, metabolic activity, proliferation, differentiation and in vitro bone-like mineral formation [2], [4], [5] and [6].

The human mesenchymal cells responded favorably to ultrafine dry powder coated surfaces, when we modified their formulation to increase the biocompatible components and topographical features [2], [4] and [5]. There was cell attachment, spreading, proliferation and mineralization on these surfaces. Furthermore, we found that the incorporation of small amounts of bioactive agents on their surfaces served to further induce the cellular response [6]. More specifically we found that the incorporation of commercially available mineral trioxide aggregates (ProRoot® MTA) within the coating led to an increase in human mesenchymal cell attachment, growth and differentiation. This MTA that is used in clinical dentistry is actually an assorted mixture of mineral
oxides \((\text{CaO})_3\cdot\text{SiO}_2 + (\text{CaO})_2\cdot\text{SiO}_2 + (\text{CaO})_3\cdot\text{Al}_2\text{O}_3 + (\text{CaO})_4\cdot\text{Al}_2\text{O}_3\cdot\text{Fe}_2\text{O}_3 + \text{CaSO}_4\cdot2\text{H}_2\text{O} + \text{Bi}_2\text{O}_3\) with a ceramic-like composition. Accordingly, we postulated that a more refined selection of metal ceramics would better serve as functional additives in our biocompatible coatings, and thereby provide an opportunity to carefully titrate desirable cellular responses that include attachment, growth and mineralization. Consequently, the purpose of this study was to evaluate the feasibility of incorporating calcium oxide (CaO) and calcium phosphate (Ca\(_3\)(PO\(_4\))\(_2\)) into the ultrafine powder coating formulations, to verify the success of their integration into the coatings, and to examine the biocompatibility of the modified surfaces.

### 2.2 Materials and methods

#### Preparation of ultrafine powders

The ultrafine powders were prepared as previously described [2], [4], [5] and [6] (Fig. 2.1), and their formulations were modified to include bioactive agents as described in Table 2.1. They were prepared by using Avalanche White polyester (LinksCoating, London, Canada) as the base resin polymer. This commercially available product contains small quantities of filler (CR822), flow (P10), degassing (BEN) and curing agents (TGIC), as well as larger amounts of micron-sized TiO\(_2\) (25\%) pigment. These powders were mixed together and ground in a high-shear grinder to obtain the initial particles that were then refined by passage through a sieve (35 μm). Then, TiO\(_2\) (0.5\%) nanoparticles (Degussa, USA) were added as flow additives, and either CaO (0, 1, 3, or 5\%) powders (Sigma-Aldrich Canada Co. Oakville, Ontario) or Ca\(_3\)(PO\(_4\))\(_2\) (5\%) powders (Sigma-Aldrich Canada Co. Oakville, Ontario) were added as bioactive agents. They were combined in the high-shear mixer and again passed through a sieve (35 μm) to obtain the final ultrafine powders.
Figure 2.1: Surface coatings were created by an ultrafine dry powder coating process. Base polymer (polyester resin) containing filler (CR822), pigment (micron-sized TiO2), flow modifiers (nTiO2), and curing (TGIC), degassing (BEN) and bioactive agents (CaO/Ca3(PO4)2) were combined in a high-shear mixer, sprayed onto a grounded metal substrate (Ti), and cured in a furnace (200 °C, 10 min).

<table>
<thead>
<tr>
<th>Coating</th>
<th>Base polymer</th>
<th>Flow additive (wt.%</th>
<th>Bioactive agent (wt.%)</th>
<th>Bioactive calcium (mmol/100 g)</th>
<th>Bioactive phosphate (mmol/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPC</td>
<td>Polyurethane</td>
<td>None</td>
<td>CaO (1%)</td>
<td>17.83</td>
<td>0.00</td>
</tr>
<tr>
<td>PPC + 1%CaO</td>
<td>Polyester resin (Avalanche White)</td>
<td>nTiO2 (0.5%)</td>
<td>CaO (3%)</td>
<td>53.50</td>
<td>0.00</td>
</tr>
<tr>
<td>PPC + 3%CaO</td>
<td>Polyester resin (Avalanche White)</td>
<td>nTiO2 (0.5%)</td>
<td>CaO (5%)</td>
<td>89.16</td>
<td>0.00</td>
</tr>
<tr>
<td>PPC + 5%CaO</td>
<td>Polyester resin (Avalanche White)</td>
<td>nTiO2 (0.5%)</td>
<td>CaO (5%)</td>
<td>89.16</td>
<td>0.00</td>
</tr>
<tr>
<td>PPC + 5%CaP</td>
<td>CaPO4 (5%)</td>
<td>CaO (5%)</td>
<td>0.29</td>
<td>0.29</td>
<td></td>
</tr>
</tbody>
</table>

The ultrafine dimensions of these powders were measured for verification by particle size analysis with a BT-9300s Laser Particle Analyzer (Ningbo Yinzhou Hybers, China). The powders were suspended in water and a laser stream was used to create a diffraction pattern that was then reflected onto a detector and analyzed to measure particle size.
Creation of surface coatings

The surface coatings were prepared by using ultrafine powder-coating technology [8] and [9], as previously described [2], [4], [5] and [6]. The PPC, CaO-enriched PPC (PPC + 1%CaO, PPC + 3%CaO, PPC + 5%CaO) and Ca3(PO4)2-enriched PPC (PPC + 5%Ca3(PO4)2) coatings were created by the application of ultrafine powders onto titanium substrates (Fig. 1). The ultrafine powders were sprayed onto smooth sheets (Grade 2, thickness = 0.5 mm, McMaster-Carr, Cleveland, OH) of commercially pure titanium (cpTi). A Corona Gun (Nordson, Westlake, OH) was used to apply voltage (20 kV) and ionize the powder particles that were then sprayed onto the titanium sheets that had been grounded. Subsequently, the thin layers on titanium were cured (200 °C, 10 min) in a high performance air flow oven (Sheldon manufacturing, Inc., Cornelius, OR). The coated sheets were then cut into regular sized disks (diameter = 24 mm) for further analysis.

Characterization of coatings

The coatings were characterized for their surface composition and homogeneity as previously described [6]. The surface coatings were analyzed by energy dispersive X-ray spectroscopy (EDX) with a Hitachi S-4000 SEM (Hitachi, Pleasanton, CA). The coated disks were mounted onto metal stubs, secured with adhesive carbon tape and sputter coated with nano-sized (10 nm) gold particles. The working voltage (15 kV) and working distance (15 mm) were set. Each element was identified (minimum detection limit = 0.0%), and its presence calculated (wt.%). The EDX analyses were repeated at three separate locations on each surface, and the mean surface concentrations of key components calculated. Additionally, the EDX analyses were repeated across the entire surface of each disk to identify the presence and distribution of the elements. This elemental mapping showed the distribution of key elements across each surface.
The surface coatings were compared for wettability by measuring their water contact angles. Water droplets (80 μl) were dripped onto each coating, and onto unmodified titanium control surfaces, and their water contact angles measured with a Ramé-Hart Model 100 goniometer (Ramé-Hart Instrument Co., Succasunna, New Jersey). These measures were repeated 5 times to ensure reproducibility.

**Cell attachment to coatings**

The surface coatings were scrutinized for their capacity to support cellular responses as previously described [5] and [6]. In preparation for cell culture, the coated disks and unmodified titanium controls were disinfected and sterilized. For this the disks were rinsed twice with ethanol (70%), washed thrice with phosphate buffered saline (PBS, Gibco, pH 7.4, free of calcium and magnesium), and then rinsed thrice with trypsin (0.25%, Gibco). They were placed in centrifuge tubes (50 ml, BD Falcon), submerged in fresh trypsin and sonicated for 60 min, then submerged in sodium hypochlorite (50%) and sonicated for another 60 min. They were rinsed 10 times in distilled water, twice in sterile distilled water, thrice in ethanol (70%) and thrice in PBS. Finally the disks were placed into sterile 6-well tissue culture plates (1 disk/well, BD Falcon) within a tissue culture hood, and exposed to UV light for 30 min on each side.

Following their disinfection and sterilization, the coated disks and unmodified titanium controls within tissue culture plates were submerged in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with fetal bovine serum (10% FBS, Gibco), l-glutamine (2 μmol/ml), penicillin G (100 U/ml), streptomycin sulfate (100 μg/ml) and amphotericin B (0.25 μg/ml). The centers of the disks were seeded (50,000 cells/disk) with human embryonic palatal mesenchymal cells (HEPM, ATCC CRL-1486), and the plates then transferred to a tissue culture incubator (Sanyo 37 °C, 5% CO2).
After 24 h of culture the disks were carefully removed from the incubator and rinsed three times in PBS (5 min/rinse). Cells that remained attached to the disks were fixed with paraformaldehyde (4% PFA, Fisher Scientific) over 24 h at room temperature. The disk surfaces were then examined with an optical microscope at 100 × and 400 × magnification, and digital images captured.

**Biomineralization of cultures on coatings**

The coatings were assessed for their capacity to induce the biomineralization of cell cultures as previously described [5]. For this, additional coated disks and unmodified titanium controls were again submerged in culture media, seeded with HEPM cells and incubated for 24 h as outlined before. Then, after 24 h the regular culture media were replaced with enriched media containing that contained ascorbic acid (50 μmol/ml) and β-glycerophosphate (10 μmol/ml) as additives to induce osteogenic differentiation. The osteogenic media were replenished every 3 days and the cultures maintained for either 2 weeks or 4 weeks. The 2 and 4 week time points were selected to study the extent of initial and the long term mineralization abilities of the cell cultures that were seeded on each surface.

Following 2 and 4 weeks of growth and biomineralization, the media were discarded and the cultures gently rinsed in PBS and fixed in formalin (4%) over 1 h. They were rinsed twice in calcium-free Nanopure water and stained with Alizarin Red-S (2%, EMD) for 10 min at room temperature. Their surfaces were examined for Alizarin Red stained deposits and digital images captured.

**Statistical analyses of data**

All of the quantitative data were analyzed by SigmaPlot 12.0 (Systat Software Inc., Chicago, IL, USA). The values for mean and standard error were calculated, examined
graphically and analyzed statistically. Differences were identified by one-way ANOVA, and post hoc comparisons were performed by the Holm–Sidak method with the significance level set at $\alpha = 0.05$.

2.3 Results

**Surface coatings were created from ultrafine powders**

The application of advanced ultrafine dry powder coating technology (Fig. 1) was successful in creating surface coatings on metal substrates. This technique utilized a mixture of powders that included polyester resin as the base polymer, micron-sized TiO2 pigment as a biocompatibility agent, CR822 as filler, TGIC as a curing agent, BEN as a degassing agent, nano-sized TiO2 as a flow additive, and in some instances either CaO or Ca3(PO4)2 as bioactive agents (Table 1). A particle size analysis of these mixtures confirmed that they were ultrafine powders, since 50% of the particles (D 0.5) had a volume diameter that was less than 30 μm (Fig. 2.2). Furthermore, 90% of the particles (D 0.9) had a volume diameter that was less than 50 μm. Indeed, all of the formulations had relatively similar particle size distributions, although PPC + 3%CaO had a slightly higher volume fraction of particles between 17 μm and 32 μm, and PPC + 5%CaP had a slightly smaller D 0.5 than the others.
Figure 2.2: Powder formulations were analyzed for particle-size distribution. PPC, PPC + 1% CaO, PPC + 3% CaO, PPC + 5% CaO and PPC + 5% CaP had relatively similar particle-size distributions with a D50 of under 30 μm.

When these ultrafine powders were electrostatically sprayed onto grounded sheets of titanium and then cured in an oven, their constituents were incorporated into surface coatings. EDX analyses of the coating surfaces showed that their elemental composition reflected their ultrafine powder constituents (Table 2.2). The predominant element was carbon (C) that accounted for about two-thirds (61–67%) of the surface, and originated from the polyester resin base polymer. The second most common element was oxygen (O) that accounted for about 15 to 25%, and also originated from polyester. The third major constituent was titanium (Ti) that accounted for about 9 to 15% of the surface, and originated from the TiO2 biocompatibility agent and flow additive.
Table 2.2: Elemental composition of coating surfaces.

<table>
<thead>
<tr>
<th>Element</th>
<th>PPC (wt.%)</th>
<th>PPC + 1%CaO (wt.%)</th>
<th>PPC + 3%CaO (wt.%)</th>
<th>PPC + 5%CaO (wt.%)</th>
<th>PPC + 5%CaP (wt.%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon (C)</td>
<td>65.28 ± 0.91</td>
<td>67.25 ± 0.04</td>
<td>61.44 ± 0.25</td>
<td>62.85 ± 0.23</td>
<td>65.25 ± 0.19</td>
</tr>
<tr>
<td>Oxygen (O)</td>
<td>17.58 ± 0.53</td>
<td>15.89 ± 0.09</td>
<td>18.83 ± 0.07</td>
<td>25.71 ± 0.12</td>
<td>18.51 ± 0.15</td>
</tr>
<tr>
<td>Titanium (Ti)</td>
<td>14.09 ± 0.39</td>
<td>15.24 ± 0.17</td>
<td>14.16 ± 0.01</td>
<td>9.09 ± 0.30</td>
<td>14.19 ± 0.10</td>
</tr>
<tr>
<td>Aluminum (Al)</td>
<td>0.21 ± 0.04</td>
<td>0.17 ± 0.01</td>
<td>0.25 ± 0.01</td>
<td>0.31 ± 0.00</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>Silicon (Si)</td>
<td>0.45 ± 0.06</td>
<td>0.38 ± 0.01</td>
<td>0.53 ± 0.01</td>
<td>0.54 ± 0.04</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>Sulfur (S)</td>
<td>0.56 ± 0.06</td>
<td>0.50 ± 0.06</td>
<td>0.56 ± 0.03</td>
<td>0.49 ± 0.04</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>Calcium (Ca)</td>
<td>ND</td>
<td>0.47 ± 0.15</td>
<td>0.61 ± 0.02</td>
<td>0.95 ± 0.01</td>
<td>0.58 ± 0.07</td>
</tr>
<tr>
<td>Phosphorus (P)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.34 ± 0.03</td>
</tr>
</tbody>
</table>

(ND) Not detected.

**Titania–polyester coatings were readily enriched with calcium**

When the ultrafine powder formulations were marginally modified to include bioactive agents, coating surfaces were created that were enriched with functional additives and enhanced properties. EDX analyses of the coating surfaces showed that their elemental composition was largely similar despite the inclusion of small amounts of bioactive agents (Table 2.2). In all instances their elemental composition was dominated by carbon, oxygen and titanium in the same order of frequency, and in largely the same proportions. Yet, the EDX analyses did show that functional additives had become incorporated into their surfaces, as small amounts (0.5–1.0%) of calcium (Ca) were detected when either CaO or Ca₃(PO₄)₂ were used as bioactive agents, and trace amounts (0.3%) of phosphorus (P) were detected when Ca₃(PO₄)₂ was used (Table 2.2). Additionally, elemental mapping of the coatings verified the incorporation of calcium and phosphorus, and showed their distribution across the surfaces (Fig. 2.3). Whereas there were only background levels of calcium in the PPC and PPC + 5%CaP coatings, there were a few
large calcium clusters in the PPC + 1%CaO coatings, and several small and evenly distributed calcium clusters in the PPC + 3%CaO and PPC + 5%CaO coatings. Similarly, there were a few small phosphorus clusters in the PPC + 5%CaP coatings.
Figure 2.3: Coating surfaces were analyzed by elemental mapping. Calcium was undetectable beyond background levels in the PPC and PPC + 5% CaP. There were a few large calcium clusters in the PPC + 1% CaO, and several small evenly distributed clusters in PPC + 3% CaO and PPC + 5% CaO. There were some small phosphorus clusters in the PPC + 5% CaP.
These slight modifications of coating composition were accompanied by distinct and progressive changes in their surface properties. Measurements of the water contact angle showed that there were differences in their hydrophilic capacity and surface wettability (Fig. 2.4). The PPC + 5%CaP coatings had the largest contact angle (80.7 ± 0.7°) which indicated that they were the least hydrophilic. Then there was a progressive decrease in the water contact angle which indicated a progressive increase in the hydrophilic capacity, from PPC, to PPC + 1%CaO, to PPC + 3%CaO, and PPC + 5%CaO respectively. The slight decreases in contact angle from PPC + 5%CaP, to PPC and PPC + 1%CaO respectively, were not significant (P > 0.05). However, the contact angles on PPC + 3%CaO and PPC + 5%CaO were significantly (P < 0.05) different from each other, and all of the other surfaces. Yet, ultimately the uncoated titanium controls had the smallest contact angle (56.4 ± 0.9°) which indicated that they were the most hydrophilic surfaces.
Figure 2.4: Water contact angles were measured on the coatings. PPC, PPC + 1% CaO and PPC + 5% CaP coatings were hydrophilic and had contact angles that were similar (A). PPC + 3% CaO, PPC + 5% CaO and titanium (Ti) were progressively more hydrophilic and had progressively smaller contact angles that were different from each other (B, C, D). (n=5, each alphabet represents a statistical group, p<0.05)

Calcium-rich surfaces promoted cell attachment and biomineralization

All of the titania-polymeric coatings supported human mesenchymal cell attachment and spreading, and the enriched coatings supported enhanced cellular responses. Optical microscopy showed that the cells had attached and spread out onto all of the coatings within 24 h of plating (Fig. 2.5). They had an elongated morphology with cellular extensions and projections that were directly attached to the coating surfaces. The cells were least confluent on the titanium controls and PPC coatings. Then, there was a
progressive increase in confluency from the PPC + 1%CaO to the PPC + 3%CaO, and ultimately the PPC + 5%CaO which were the most confluent. There was also a moderate amount of confluency on the PPC + 5%CaP coatings.
Figure 2.5: Human mesenchymal cells were seeded onto the surface coatings and examined by optical microscopy. After 24 h, low power (120 μm scale bar) showed few cells on PPC and titanium (Ti), more cells on PPC + 1% CaO, even more cells and cell spreading on PPC + 3% CaO, and the most cells on PPC + 5% CaO. There was a moderate amount of cells on PPC + 5% CaP. High power (30 μm scale bar) showed cell spreading and elongated morphologies on all surfaces.
These favorable cellular responses seen in short-term culture were then verified in long-term cultures that showed evidence of biomineralization on all of the coatings. The Alizarin Red staining showed that there were calcified mineral deposits in the cultures after 2 and 4 weeks of growth and biomineralization on all of the surfaces (Fig. 2.6). After 2 weeks, there were few deposits on titanium control surfaces, a few more on PPC and PPC + 5%CaP coatings, a moderate amount on PPC + 1%CaO and PPC + 3%CaO, and a large amount on PPC + 5%CaO. Then after 4 weeks, there were some deposits on titanium and PPC, a moderate amount on PPC + 1%CaO, PPC + 3%CaO and PPC + 5%CaP, and a very large amount on the PPC + 5%CaO coatings.
Figure 2.6: Cell cultures grown on the surface coatings were stained with Alizarin Red S. After 2 weeks, there were few deposits on titanium, more on PPC and PPC + 5%CaP, moderate amounts on PPC + 1%CaO and PPC + 3%CaO, and large amounts on PPC + 5%CaO. Then after 4 weeks, there were some deposits on titanium and PPC, moderate amounts on PPC + 1%CaO, PPC + 3%CaO and PPC + 5%CaP, and very large amounts on PPC + 5%CaO.
2.4 Discussion

In this study, biomaterial surface coatings were created by using a novel and patented electrostatic ultrafine dry powder coating technique, which was recently developed in the Particle Technology Research Centre at the University of Western Ontario [8] and [9]. The ultrafine dry powder formulations consisted mainly of polyester resin and TiO$_2$, as they were found to create biocompatible surface coatings in prior studies [2], [4], [5] and [6]. The polyester resin was the base polymer that formed a polymeric backbone, and the micron-sized TiO$_2$ pigment (25%) served to increase biocompatibility. These powders were deemed to be ultrafine particles because the particle size analysis showed that 50% of the particles (D 0.5) had a volume diameter under 30 μm (Table 2). Prior studies showed that these ultrafine particles could be utilized to create nano-scale surface topographies and roughness, which induced the cellular response [2], [4], [5] and [6]. Therefore, TiO$_2$ nanoparticles (0.5%) had to be included as flow modifiers to prevent the ultrafine particles from aggregating, and thereby ensuring an adequate flow. The nTiO$_2$ has the added benefit of providing additional biocompatibility [2]. Furthermore, since these applications involved an electrostatic dry powder coating technique, the toxic hazards of solvents and volatile organic compounds were eliminated from the process, and thereby from the final products.

These biocompatible coatings [5] were further enhanced by including ceramic inserts as bioactive agents or functional additives. These included either CaO (1, 3 or 5%) or Ca$_3$(PO$_4$)$_2$ (5%), that were added alongside nTiO$_2$ flow additives during the ultrafine powder preparation process. The modified mixtures differed nominally from the base formulations (PPC), since the additives accounted for as little as 1% or as much as only 5% by weight, and they were all processed through the same electrostatic ultrafine dry powder coating technique. This ensured that the functional additives did not disrupt the continuity and homogeneity of the base coating.
The actual enrichment of the coatings with bioactive agents was confirmed by careful chemical analyses of their surfaces. EDX spectroscopy showed that calcium (Ca) could not be detected in the base PPC, but was clearly detected in the CaO and Ca₃(PO₄)₂ enriched coatings, and progressively higher amounts were measured when more additives were used. Similarly, phosphorous (P) was only detected in the Ca₃(PO₄)₂ enriched coating. The EDX analyses also confirmed that the overall composition of the coatings were largely unaffected by their enrichment, since the additives only made up a minor component of the formulations. There were similarly high proportions of carbon (C), oxygen (O) and titanium (Ti) as the predominant elements, which is consistent with polyester resin and TiO₂ being the major constituents. Even the trace amounts of Al, Si and S detected were consistently low, and likely originated from the CR822 filler used in Avalanche White polyester. These agreed with prior analyses of similar coatings [5].

Furthermore, elemental mapping provided additional confirmation that the additives were present, and that they were evenly distributed across the coating surfaces. The mapping showed that above background noise, calcium was only detected on the CaO and Ca₃(PO₄)₂ enriched coatings, and its detection increased when increasing amounts were used. There were fewer but larger clusters of calcium when less CaO (1%) was used, and more small clusters when additional CaO (3 and 5%) was used. Interestingly, the moderate number of medium-sized calcium clusters in the PPC + 5%CaP coatings appeared to co-localize with phosphorus, suggesting that they were present together as hydroxyapatite-like entities. Similarly, our prior use of mineral trioxide aggregates (MTA) showed that they were successfully incorporated into the coatings, as small clusters of calcium-rich areas that were evenly dispersed across their surfaces [6].

The mineral trioxide aggregates (ProRoot® MTA) that were used as ceramic inserts in our prior coatings, are commercially available products that are widely used in dentistry [20], [21] and [22]. They are mixtures of calcium silicates and metal oxide powders that form interlocking crystals upon hydration and setting [23]. We showed that their
integration into polymeric coatings induced the cellular response, but our ability to enhance and titrate these effects was limited by the amorphous composition of the MTA mixtures. Therefore, pure compounds were used alone as additives in this study, and their concentration modified so as to verify a dose–response. These included CaO in three progressively higher concentrations by weight (1, 3 and 5%) and Ca$_3$(PO$_4$)$_2$ at a single concentration (5%). For CaO, there is one calcium for every oxygen, so that ample calcium was detected at the surface when even minimal amounts (1%) were used. However, for Ca$_3$(PO$_4$)$_2$ there are three calcium and two phosphorus for every eight oxygens, which account for a much higher molecular weight. Therefore with Ca$_3$(PO$_4$)$_2$, much less calcium or phosphorus was available when even high concentrations (5% by weight) were used.

Now despite the relatively modest amounts of these functional additives in the coatings, they appeared to have had a profound effect on the physical and biological properties of the surfaces. Goniometric data showed that there was a progressive increase in hydrophilic surface wetting with an increase in the additives. The water contact angle measurements showed that the PPC base coatings were the least hydrophilic, since their predominant constituent the polyester resin is a non-polar polymer. Then, with increasing concentrations (1, 3 and 5%) of CaO additive there was a progressive increase in hydrophilic tendency such that the PPC + 5%CaO surfaces were the most hydrophilic coatings. In contrast, there was little decrease in hydrophobicity when Ca$_3$(PO$_4$)$_2$ was used, given the smaller amounts of calcium and phosphorus, and the relative insolubility of the compound in water. Therefore PPC + 5%CaP surfaces were as hydrophobic, if not more so, than the PPC base coatings. In contrast, unmodified titanium controls provided the most hydrophilic surfaces, since their TiO$_2$ outer layer readily forms hydrogen bonds with water [24]. These hydrophilic wetting characteristics are one of the features that account for the biocompatibility of titanium, since surface wetting and the contact angle appear to correlate with the biocompatibility of a material [25], [26], [27] and [28].
The biocompatibility of these coatings was clearly demonstrated in the study. All of their surfaces showed human mesenchymal cell attachment and spreading. Optical microscopy showed numerous cells attached and spread out on the coating surfaces within 24 h of seeding. High power magnification showed cells that were intimately associated with their underlying substrates. They had elongated morphologies with numerous cellular extensions and projections. Furthermore, there were more cells observed on the enriched surfaces than on the base coatings and titanium controls, and there appeared to be even more cells on the coatings that contained more additives. Accordingly the PPC + 5%CaO surfaces appeared to have the highest confluence and have the most cells. This abundance of cells was most likely an indication of increased cell attachment and survival rather than cell growth and proliferation, since only 24 h had lapsed since their initial seeding. Therefore it appears that the inclusion of modest amounts of functional additives created surfaces that actually promoted human mesenchymal cell attachment, survival and spreading. Interestingly these effects appeared to be more evident with CaO compared to Ca$_3$(PO$_4$)$_2$, that is likely due to the greater importance of calcium over phosphate for both protein binding [29] and cell attachment [30], and the inhibitory effects of Ca$_3$(PO$_4$)$_2$ on cell spreading [31].

In addition to biocompatibility, their osteo-inductive potentials were verified by examining in vitro bone-like mineral formation. Indeed all of the coatings were able to support the biomineralization of human mesenchymal cell cultures growing on their surfaces. The mineralization was detected by Alizarin Red-S stain which binds specifically to the calcium found in mineral deposits, through its sulfonic acid and hydroxyl groups [32]. The staining showed mineral formation within 2 weeks, and an increase in deposition over 4 weeks. The least amount of mineralization was seen on the titanium controls, and more moderate amounts were seen on the PPC base and Ca$_3$(PO$_4$)$_2$ enriched coatings. Whereas much higher levels of mineralization were seen with increasing amounts of CaO enrichment, such that the PPC + 5%CaO cultures were clearly the most heavily mineralized. Likewise, prior studies showed enhanced biomineralization on polymeric coatings that had been enriched with commercially
available mineral trioxide aggregates (MTA) [6]. Since these somewhat amorphous mixtures are rich in calcium containing minerals, and it was the calcium that was detected on their enhanced coating surfaces, it is likely that the calcium is what accounted for these effects.

Collectively the data shows that this novel ultrafine dry powder coating technique can be used to create biocompatible surfaces from polymeric resins and TiO₂ pigment [2], [4], [5] and [6]. Furthermore, these preparations can be readily modified to include small amounts of amorphous minerals [6], large molecular weight compounds like Ca₃(PO₄)₂, and small molecules like CaO, to induce the cellular response. When small amounts of CaO (1–5%) were added to the formulations, calcium was integrated into the coating surfaces, hydrophilic wetting was increased, and their biocompatibility clearly enhanced. There was an increase in human mesenchymal cell attachment and biomineralization of cultures grown on the calcium-enriched polymeric coatings, and this increased with additional enrichment. This simple process creates highly biocompatible surfaces that support enhanced cellular responses.

2.5 Acknowledgements

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2.6 References

Chapter 3

3 Human mesenchymal cell attachment, growth and biomineralization on calcium-enriched titania-polyester coatings

Abstract

Titanium implant osseointegration can be enhanced by surface modifications that include hydroxyapatite from Ca$_3$(PO$_4$)$_2$. However, CaO may provide more surface calcium (w/w) to induce cellular responses. Therefore, the purpose of this study was to compare responses to novel CaO and Ca$_3$(PO$_4$)$_2$-enriched titania-polyester (PPC) nanocomposite coatings, which were created by an electrostatic ultrafine powder coating technique. EDX confirmed the presence of a base polymer scaffold, biocompatible titanium, and CaO or Ca$_3$(PO$_4$)$_2$. SEM showed that human embryonic palatal mesenchymal cells (ATCC CRL-1486) had attached and spread out onto all of the surfaces within 24 hours. Cell attachment assays showed that there was a progressive increase in cell numbers with surface CaO incorporation (0-5%), such that the PPC+5%CaO coatings supported the most cells. Furthermore, the PPC+5%CaO had significantly more (P=0.006) cells attached to their surfaces than the PPC+5%CaP coatings and titanium controls, at 24 hours. The PPC+5%CaO also had more cells that had proliferated on their surfaces over 72 hours, although these differences were not significant (P>0.05). Similarly, MTT assays showed that the cells had sustained metabolic activity on all surfaces. Again, metabolic activities were highest on the PPC+5%CaO, and they were significantly higher (P<0.05) on all of the CaO-enriched surfaces (1/3/5% CaO) than on the PPC+5%CaP. Subsequently, Alizarin Red-S staining detected the initiation of biomineralization within 2 weeks, and abundant mineral deposits after 4 weeks of growth on PPC+5%CaO and PPC+3%CaO. These nanocomposite coatings have shown that CaO enrichments may provide a heightened cell response when compared to conventional hydroxyapatite.

3.1 Introduction

There has been a growing demand for dental implants and orthopedic prostheses to replace diseased, damaged and missing tissues. Accordingly, dental implants and orthopedic prostheses made of commercially pure titanium (cpTi) and titanium alloys (Ti6A1V4) respectively, have been widely studied to maximize clinical success. Their
Immediate and long-term retention and function are dependent on intimate interactions between the biomaterial surfaces and surrounding tissues, in a process known as osseointegration. Implant surfaces that mimic the surrounding extracellular bone matrix may promote favorable cellular responses, which enhance osseointegration. Therefore strategies were developed to enhance titanium surfaces by incorporating chemical inserts such as hydroxyapatite \((\text{Ca}_{10} \text{(PO}_4)_6 \text{(OH)}_2)\), calcium phosphate \((\text{Ca}_3 \text{(PO}_4)_2)\) and various ceramics \((1–8)\).

Since hydroxyapatite is the naturally occurring inorganic component in bone and teeth, it has been incorporated onto implant surfaces to enhance protein adsorption, cell attachment, proliferation, differentiation and biomineralization \((9)\). However, hydroxyapatite surface coatings are usually made by the plasma spray technique \((10,11)\), which creates thick layers that lack surface homogeneity and have low bonding strength \((12)\). They can also be made by sputter coating techniques \((13,14)\), but these are costly and time consuming. Furthermore, hydroxyapatite alone mimics only the inorganic mineral, and lacks the collagenous matrix that is present in teeth and bones. Therefore, polymer/ceramic hybrid coatings have now been developed to combine organic polymers with inorganic minerals \((15)\). The polymer provides a continuous scaffold with design flexibility \((16)\), and the ceramic inserts promote bone formation \((17)\).

Accordingly, we developed novel polymer/ceramic composite coatings that contain polymeric scaffolds and ceramic inserts \((18)\). They were created by a novel, simple and inexpensive electrostatic ultrafine dry powder coating technique \((19,20)\). This method generates uniform, continuous, homogenous and highly adherent polymer coatings with intricate nano-topographies, surface roughness and a high degree of biocompatibility \((21–23)\). We augmented their formulation by including small amounts \((5\% \text{ w/w})\) of commercially available mineral trioxide aggregates \((\text{ProRoot® MTA})\), which are a ceramic-like mixture of mineral oxides \(((\text{CaO})_3 \cdot \text{SiO}_2 + (\text{CaO})_2 \cdot \text{SiO}_2 + (\text{CaO})_3 \cdot \text{Al}_2 \text{O}_3 + (\text{CaO})_4 \cdot \text{Al}_2 \text{O}_3 \cdot \text{Fe}_2 \text{O}_3 + \text{CaSO}_4 \cdot 2\text{H}_2 \text{O} + \text{Bi}_2 \text{O}_3)\) \((19,24)\). Now, we have refined this
formulation and shown that either calcium oxide (CaO) or calcium phosphate (Ca$_3$(PO$_4$)$_2$) alone can be readily incorporated into their surfaces, without compromising the composition and properties of the base coating (25).

The calcium in these surface coatings may be particularly beneficial, since calcium can promote bone formation around implants. Indeed, a recent study showed that merely the pretreatment of their surfaces with calcium ion containing solutions, promoted bone formation around implants in rabbit femora (26). Other studies have shown that Ca$_3$(PO$_4$)$_2$ coated titanium implants have enhanced bone formation in rat femora (27), promoted the healing of intrabony defects in dog mandibles (28), and increased their resistance to dislodgement in rabbit tibia (29). Furthermore, silica-Ca$_3$(PO$_4$)$_2$ nanocomposite coatings increased the alkaline phosphatase activity of bone marrow mesenchymal stem cells that were attached to their surfaces (30).

These cellular responses are often studied in mesenchymal stem cells that are the undifferentiated precursors to bone forming osteoblasts. Several studies have used a human embryonic palatal mesenchymal cell line (HEPM, ATCC CRL1486) that was derived from the developing palate of a human fetus, which has provided a clinically relevant model to study the cellular response to implant surfaces (31–33). We found that the human mesenchymal cells attached, spread out, proliferated and differentiated on the surfaces of polymeric coatings, and initiated biomineralization in extended culture (21–23). Furthermore, these cells responded favorably to the polymer/ceramic composite coatings (18), and to the CaO- and Ca$_3$(PO$_4$)$_2$-enriched coatings that were recently created in our refined formulation (25). However, despite widespread study of Ca$_3$(PO$_4$)$_2$ coatings on implants, the cellular response to CaO-enriched surfaces is largely unknown. The CaO additives may in fact deliver more calcium (w/w), although Ca$_3$(PO$_4$)$_2$ provides both calcium and phosphorous in a hydroxyapatite-like layer. Therefore, the purpose of this study was to examine the response of human mesenchymal cells to the CaO-enriched
coatings, and to compare them to the Ca$_3$(PO$_4$)$_2$-enriched coatings, unmodified surface coatings and titanium controls.

### 3.2 Materials and methods

**Formulation of Ultrafine Powders**

The ultrafine powders were prepared as previously described (Figure 3.1) (18,21–23,25). This involved the use of a base powder formulation that was then enriched with bioactive ingredients. To prepare the base powder, commercially available White Avalanche polyester (LinksCoating, London, Canada) that contained micron-sized TiO$_2$ (25% w/w) was enriched with small amounts (0.5% w/w) of nano-sized TiO$_2$ (nTiO$_2$; Degussa, USA) that served as a flow additive to prevent agglomeration of the ultrafine particles (20). These base powders were then enriched with either CaO (Sigma-Aldrich, Oakville, Ontario) at progressively higher concentrations (1, 3 or 5% w/w), or Ca$_3$(PO$_4$)$_2$ (Sigma-Aldrich, Oakville, Ontario) at only the higher concentration (5% w/w), to serve as bioactive agents. All of these powders were combined in a high-shear mixer and passed through a sieve (35 µm) to obtain ultrafine particles. Their ultrafine dimensions were then verified through a particle size analysis that was performed by a BT-9300s Laser Particle Analyser (Ningbo Yinzhou Hybers, China). The laser beam interacted with particles suspended in water to create a diffraction pattern that was then analyzed to determine particle size.

**Preparation of Surface Coatings**

Surface coatings were created with the formulations, by using an ultrafine dry powder coating technique (19,20), as previously described (18,21–23,25) (Figure 3.1). The base powder formulation (PPC), CaO-enriched PPC (PPC+1%CaO, PPC+3%CaO,
PPC+5%CaO) and Ca₃(PO₄)₂-enriched PPC (PPC+5%Ca₃(PO₄)₂) were sprayed onto sheets of cpTi (Grade 2, thickness = 0.5 mm, McMaster-Carr, Cleveland, OH). They were sprayed with a Corona Gun (Nordson, Westlake, OH) at a set voltage (20 kV), so that the ultrafine particles became ionized and electrostatically attracted to grounded cpTi. The coated surfaces were then cured (200°C, 10 minutes) in a high performance air flow oven (Sheldon manufacturing, Inc., Cornelius, OR). The PPC-coated cpTi sheets were then cut into circular disks (diameter = 24 mm) for analysis.

To confirm their composition, the surface coatings were chemically analyzed as previously described (18,23,25). The coated titanium disks were mounted onto metal stubs, secured with adhesive carbon tape and sputter coated with nano-sized gold particles (10 nm). They were then analyzed by energy dispersive X-ray spectroscopy (EDX) using a Hitachi S-4000 SEM (Hitachi, Pleasonton, CA) with a working voltage (15 kV) and distance (15 mm). The EDX analyses were repeated at three separate locations at roughly 0°, 120°, and 240° edge of each disk surface, and a representative chromatogram selected.
Figure 3.1: An ultrafine dry powder coating technique was used to create novel biocompatible surface coatings enriched with bioactive agents, which were then evaluated in cellular assays. Polyester resin, micron-sized titanium dioxide (TiO2), nanoparticles of titanium dioxide (nTiO2), and functional additives that included calcium oxide (CaO) or calcium phosphate (Ca3(PO4)2), were ground and mixed in a high-shear mixer to create ultrafine particles. The ultrafine powders were then sprayed onto commercially pure titanium (cpTi) surfaces and cured in a furnace. The surface coatings were sterilized and used as substrates in cellular assays.

**Cell Culture Studies**

The surface coatings and their controls were prepared to serve as the substrate layer in cell culture studies. The coated and uncoated titanium disks were rinsed (x3) with phosphate buffered saline (PBS, Gibco, pH 7.4, calcium chloride and magnesium chloride free), and then rinsed (x3) with trypsin (Gibco, 0.25% with EDTA and phenol red). They were then placed in polypropylene tubes (BD Falcon, 50 ml), submerged in fresh trypsin (0.25%) and sonicated (60 minutes), and then submerged in sodium hypochlorite (2.5%) and sonicated (60 minutes). Following each sonication, the surfaces were washed (x10) with ddH2O and then washed (x2) with autoclaved ddH2O. Finally,
the surfaces were rinsed (x3) with ethanol (70%) and PBS, and sterilized by UV light (30 minutes on each side) in a tissue culture hood.

The coated and uncoated titanium disks were then placed individually within the wells of 6-well tissue culture plates (BD Falcon) with the prepared surfaces on top to serve as substrates for cell culture. They were covered with Dulbecco’s modified eagles medium (DMEM, Gibco) supplemented with fetal bovine serum (10% FBS, Gibco), L-glutamine (2 µmol/ml), penicillin G (100 U/ml), streptomycin sulfate (100 µg/ml) and amphotericin B (0.25 µg/ml). Human embryonic palatal mesenchymal cells (HEPM, ATCC CRL-1486) were seeded onto the center of the discs within the tissue culture plates, and incubated (37°C, 5% CO2) with culture media that was replenished every 3 days.

**Cell Morphology and Surface Interactions**

Cell morphology and surface interactions with the coatings and their controls were examined by scanning electron microscopy (SEM). The cell cultures on coated and uncoated titanium disks were harvested 24 hours after seeding. The culture media was carefully removed by suction and the surfaces gently rinsed (x3) with PBS. They were fixed (20 minutes) with glutaraldehyde (2.5%, Sigma-Aldrich) in sodium cacodylate trihydrate buffer (100 mM, Sigma-Aldrich) and rinsed (x2) with PBS. Finally they were dehydrated by placement (10 minutes each) in progressively higher concentrations of ethanol (25, 50, 75, 95 and 100%). The coated and uncoated titanium disks were then mounted onto metal stubs, secured with conductive carbon tape and sputter coated with nano-sized gold particles (10 nm). Their surfaces were examined with a Hitachi S-2600 SEM (Hitachi, Pleasanton, CA).
Cell Attachment and Growth

The cell attachment and growth on surface coatings and their controls were measured in cell attachment assays. The cell cultures on coated and uncoated titanium disks were harvested 24 and 72 hours after seeding. The culture media, detached and dead cells were carefully removed by suction and the surfaces gently rinsed (x2) with PBS. The disks were then transferred individually to wells in fresh 6-well tissue culture plates and rinsed (x2) again with PBS. Cells that remained attached to the surfaces were released by a trypsin (300 µl/well) incubation (37°C, 5 minutes), mixed with DMEM (300 µl/well), and counted in a hemocytometer. The cultures for were harvested at 24 hours to ensure there was sufficient time for cell attachment while avoiding cellular proliferation. The proliferation ability of cell cultures were examined after 72 hours of seeding. The seeding cell densities for the attachment and proliferation assays were 50,000 and 10,000 cells per disk respectively (11,000 cells/cm², 2,200 cells/cm²).

Mitochondrial Enzyme Activity

The metabolic activity of cells grown on surface coatings and their controls were measured by MTT assay. The cell cultures on coated and uncoated titanium disks were harvested 24 hours after seeding. The culture media was carefully removed by suction and replaced with fresh media (2 ml/well) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent solution (300 µl/well). Following 4 hours of incubation (37°C), MTT solubilizing agent (1 ml/well) was added and an aliquot of the mixture in each well transferred to a 96-well plate. Their absorbance (570 nm) readings were measured in a Safire Multi-Detection Microplate Reader (Tecan, San Francisco, CA).
**Biomineralization of Cultures**

The biomineralization of cultures on surface coatings and their controls was detected by Alizarin Red-S staining. The cell cultures on coated and uncoated titanium disks were harvested 2 and 4 weeks after seeding. The culture media was carefully removed by suction and the surfaces gently rinsed (x2) with PBS. They were fixed (1 hour) in formalin (4%) and rinsed with ddH2O (calcium-free). The calcified mineral deposits in the cultures were stained (10 minutes) with Alizarin Red-S (2%, EMD) and rinsed with ddH2O (calcium-free). 2 and 4 weeks were selected to respectively examine the extent of initial biomineralization and the long term mineralization and the mineral deposit maintaining ability of the surfaces.

**Data Analyses**

Cell counts from the attachment assays and absorbance levels from the MTT assay were analyzed by SigmaPlot 12.0 (Systat Software Inc., Chicago, IL, USA). Their mean values and standard errors were calculated, charted and statistically analyzed. Differences were identified by one-way ANOVA, and post hoc comparisons were performed by the Holm-Sidak method with the significance level set at P=0.05.

### 3.3 Results

**Bioactive surface coatings were created**

The surface coatings were created by an ultrafine dry powder coating technique and enriched with bioactive agents (Figure 3.1). They all had a very similar chemical composition when analyzed by EDX (Figure 3.2). All of their surfaces contained an abundance of carbon (C), oxygen (O) and titanium (Ti). Additionally, those coatings that had been prepared with enriched ultrafine powder formulations contained small amounts
of the bioactive agents within their surfaces. The PPC+1%CaO, PPC+3%CaO and PPC+5%CaO coatings contained progressively higher levels of calcium (Ca), which reflected the progressively higher amounts of CaO that had been added to their formulations. Similarly, the PPC+5%CaPO4 coating contained small amounts of both calcium (Ca) and phosphorous (P), whereas the PPC coating control had none of these bioactive additives.
Figure 3.2: The surface coatings were analyzed by energy dispersive x-ray spectroscopy (EDX) to confirm their chemical composition. Titanium (Ti) was detected at high levels in all of the coatings. Calcium (Ca) was detected at much lower levels in only the PPC+1% CaO, PPC+3% CaO, PPC+5% CaO and PPC+5% CaP surfaces, and in proportion to their formulation. Phosphorus (P) was detected in at very low levels in only the PPC+5% CaP surfaces.
Surface coatings supported cell attachment and spreading

The surface coatings supported human mesenchymal cell attachment and spreading on their surfaces. All of the coatings had cells that had attached and spread out onto their surfaces within 24 hours of seeding, when viewed by SEM (Figure 3.3). The cells had attached and spread out onto the coatings as they had on the titanium controls. They were firmly attached, with cytoplasmic extensions and projections onto the underlying surfaces. There were wide and spreading cellular morphologies on all of the CaO- and Ca$_3$(PO$_4$)$_2$-enriched surfaces, and an elongated appearance on the PPC coating control.
Figure 3.3: Human mesenchymal cells were seeded onto coatings and control surfaces that were suspended in tissue culture medium in an incubator. After 24 hours, cells (white arrows) that had attached and spread out onto the surfaces were examined by scanning electron microscopy (SEM). Spreading cell morphologies were seen on all of the coatings and controls, and elongated morphologies were seen on the PPC surfaces.
Calcium-enrichment promoted cell attachment

The calcium-enrichment of the coatings enhanced the initial human mesenchymal cell attachment to their surfaces. The PPC+5%CaO coatings had many more cells attach within 24 hours, than any of the other surfaces, when counted in cell attachment assays (Figure 3.4). They had significantly (P=0.006) more human mesenchymal cells that attached to their surfaces within 24 hours, than the PPC+5%CaPO4 coatings or the PPC and titanium controls.

Figure 3.4: Human mesenchymal cells were seeded onto coated and control surfaces that were suspended in tissue culture medium in an incubator. After 24 hours, cells that had attached and spread out onto the surfaces were collected and counted. Cell numbers were higher on the PPC+5%CaO coatings than on any other surfaces. (n=12, each alphabet represents a statistical group, p<0.05)
All of the CaO-enriched coatings had more cells that attached to their surfaces within 24 hours, than the titanium controls, when counted in cell attachment assays (Figure 3.5). Furthermore, the progressive increase in CaO enrichment from the PPC+1%CaO to the PPC+3%CaO and the PPC+5%CaO coatings was reflected in a progressive increase in the cells counted on their surfaces.

Figure 3.5: Human mesenchymal cells were seeded onto coated and control surfaces that were suspended in tissue culture medium in an incubator. After 24 hours, cells that had attached and spread out onto the surfaces were collected and counted. Cell numbers were highest on the PPC+5%CaO coatings, followed by the PPC+3%CaO coatings, the PPC+1%CaO coatings and the titanium (Ti) controls. (n=3, the alphabet represents the same statistical group)
Cell proliferation and metabolism were sustained

All of the coatings supported human mesenchymal cell proliferation on their surfaces. All of the coatings and their controls had more cells growing on their surfaces after 72 hours, than in the first 24 hours, when counted in cell attachment assays (Figure 3.6). The PPC+5%CaO coatings had the most cells on their surfaces, followed by the PPC+5%CaPO4 and titanium, and the PPC control coatings had the least. However, these differences were not statistically significant (P>0.05).
Figure 3.6: Human mesenchymal cells were seeded onto coatings and control surfaces that were suspended in tissue culture medium in an incubator. After 72 hours, the cells that had attached, spread out and proliferated on the surfaces were collected and counted. Cell numbers were highest on the PPC+5%CaO coatings and lowest on the PPC controls, but mostly similar on all surfaces. (n=12, each alphabet represents a statistical group, p<0.05)

All of the coatings supported human mesenchymal cell metabolism on their surfaces. All of the coatings and their control surfaces had cell cultures with active mitochondria, when measured in MTT assays (Figure 3.7). The CaO-enriched coatings had the most active cultures that had significantly (P<0.05) more activity than the PPC+5%CaPO₄ coatings, or the PPC and titanium controls. However, the differences between the PPC+3%CaO coating and titanium controls were not significant (P>0.05).
Figure 3.7: Human mesenchymal cells were seeded onto coated and control surfaces that were suspended in tissue culture medium in an incubator. After 24 hours, the cells that had attached and spread out onto the surfaces were measured for metabolic activity (MTT). Their activities were higher on the PPC+1% CaO, PPC+3% CaO and PPC+5% CaO coatings, than on the PPC+5% CaP, PPC and titanium (Ti) surfaces. (n=5, each alphabet represents a statistical group, p<0.05)

**High-calcium coatings promoted biomineralization**

Higher levels of calcium enrichment in the coatings promoted the initiation of biomineralization in human mesenchymal cell cultures growing on their surfaces. The PPC+3%CaO and PPC+5%CaO coatings showed numerous mineral deposits in their cell cultures within 2 weeks, when stained with Alizarin Red-S (Figure 3.8). Similarly, they showed widespread mineralization in their cultures after 4 weeks of growth. However, the PPC+1%CaO and the PPC+5%CaPO4 coatings, as well as the PPC and titanium
controls had no mineral deposits after 2 weeks, and only sparse mineralization after 4 weeks of growth.
Figure 3.8: Human mesenchymal cells were seeded onto coated and control surfaces that were suspended in tissue culture medium in an incubator. After 2 and 4 weeks of growth and biomineralization, the surfaces were stained with Alizarin Red-S. Numerous mineral deposits were detected on the PPC+3\% CaO and PPC+5\% CaO coatings within 2 weeks, and few deposits on other surfaces after 4 weeks.
3.4 Discussion

This study has confirmed our prior research, which showed that a novel electrostatic ultrafine dry powder coating technique (19,20) can be used to create biomaterial surfaces (21–23). This dry powder coating technique has an advantage over conventional surface coating processes by eliminating the need to use toxic solvents and volatile organic compounds in the coating process, and thereby from the final product. Additionally, it can create highly biocompatible coatings that contain nano-scale surface topographies and roughness, which induce favorable cellular responses.

Furthermore, this research has confirmed that the surface coatings can be readily enriched with bioactive agents, which then serve as functional additives. This was initially done with ProRoot® MTA powders (18), which are a ceramic-like mixture of mineral oxides that are widely used in dentistry (24,34). However, we have now shown that either CaO or Ca$_3$(PO$_4$)$_2$ alone can be used (25). These are added alongside other additives in the process of preparing ultrafine powders, and account for only a nominal modification to the base formulation (PPC). They were limited to a maximum of 5% by weight, so as to ensure the continuity and homogeneity of the base coating. Whereas the original preparations were restricted by the amorphous composition of MTA mixtures, the refined formulations with CaO and Ca$_3$(PO$_4$)$_2$ can deliver calcium and phosphorous at higher levels.

Remarkably, these small enrichments were readily detected within the coating surfaces. There were small amounts of calcium in the CaO-enriched coatings, and both calcium and phosphorous in the Ca$_3$(PO$_4$)$_2$ coatings, when analyzed by EDX. Furthermore, their calcium content reflected their formulation, so that it increased progressively from the PPC+1%CaO, to the PPC+3%CaO and the PPC+5%CaO surfaces. For CaO, there is one calcium for every oxygen, so that ample calcium was detected when even minimal
amounts (1% w/w) were used. However, for Ca$_3$(PO$_4$)$_2$ there are three calcium, two phosphorus and eight oxygen that account for a much higher molecular weight. Therefore, these additives delivered less calcium or phosphorus when even high concentrations (5% w/w) were used. Yet, despite these modifications, overall chemical compositions were largely unchanged, and were very similar in all of the coatings (25).

All of the coatings supported human mesenchymal cell attachment and growth, as they did on the commercially pure titanium (cpTi) surfaces. The cells attached and spread out onto coatings and control surfaces within 24 hours of seeding, as demonstrated by SEM. They attached firmly and spread out with cellular filopodia that suggest intimate surface interactions. Additionally, the coatings sustained ongoing cell metabolism on their surfaces, which was measured as mitochondrial enzyme activity in the MTT assays. Indeed, prior studies found that similar surface coatings were able to support human mesenchymal cell attachment, metabolic activity and proliferation (21–23), and that their enrichment with ceramic-like additives, did not reduce their biocompatible features (18,25).

These favorable cellular responses were amplified by enriching the surface coatings with bioactive agents. Cell counts were higher on all of the CaO-enriched coatings than on the titanium controls, within 24 hours of seeding. Furthermore, their counts increased progressively with the increase in CaO content in the formulations (1, 3, 5% w/w). Although some of these increases were not statistically significant, a distinct trend was evident, and the PPC+5%CaO coatings had the most cells on their surfaces. PPC+5%CaO coatings had significantly (P=0.006) more cells on their surfaces within 24 hours of seeding, than any of the other enriched or unenriched coatings or titanium controls. Yet, after 72 hours of growth and proliferation, cell counts became similar on all surfaces. Therefore it appears that the small amounts of calcium in the surface coatings had actually promoted human mesenchymal cell attachment and spreading, without altering their subsequent rates of proliferation.
This increase in cell attachment and spreading on CaO-enriched coatings was accompanied by an increase in metabolic activity and downstream events. Cultures grown on CaO-enriched coatings had higher metabolic activities than those on the unenriched PPC coatings and titanium controls. Furthermore, the cultures grown on PPC+5%CaO surfaces had the very highest levels of activity. Subsequently, the PPC+3%CaO and the PPC+5%CaO coatings were the first to show initiation of mineralization within 2 weeks, and widespread mineral deposits after 4 weeks of extended culture. This biomineralization was detected by Alizarin Red-S staining, which binds specifically to mineral deposits through its sulfonic acid and hydroxyl groups (35).

These experiments showed that CaO-enriched coatings had induced differentiation of human mesenchymal cells into a pre-osteoblast-like phenotype, which could initiate bone-like mineral formation. Similarly, a prior study showed that the coating enrichment appeared to promote mineralization, and that the PPC+5%CaO surfaces had a plethora of mineral deposits after 4 weeks (25). Another study found that silica-calcium phosphate coatings increased alkaline phosphatase activity in bone marrow mesenchymal stem cells, which indicated an increase in osteogenic differentiation (30).

These effects may have been mediated by the surface calcium alone. Since a recent study found that merely the pre-treatment of implant surfaces through sonication with calcium solutions increased bone formation (26). They found increased bone volume and density within 2 weeks, and bone contact after 8 weeks of implantation in rabbit femora. They found that calcium-treated implant surfaces promoted clot formation, platelet adsorption and activation when exposed to blood plasma. Therefore, the enhanced human mesenchymal cell responses that were discovered in our in vitro studies may become amplified in an in vivo setting, with the added presence of plasma, platelets and clot formation.
In contrast to this enhanced response to the CaO-enriched surfaces, the response to Ca$_3$(PO$_4$)$_2$-enriched coatings was largely subdued. The human mesenchymal cell attachment, spreading, proliferation, metabolic activity and biomineralization on PPC+5% Ca$_3$(PO$_4$)$_2$ coatings were comparable to those on unenriched PPC surfaces and titanium controls. These coatings were clearly biocompatible, but did not possess the inductive effects of the CaO-enriched surfaces, particularly the PPC+5%CaO. This may have been due to low calcium and phosphate incorporation within their surfaces, given the high oxygen content of high molecular weight Ca$_3$(PO$_4$)$_2$ additives. It may also have been due to the greater importance of calcium over phosphate for protein binding (36) and cell attachment (37), and an inhibitory effect of Ca$_3$(PO$_4$)$_2$ on cell spreading (38). Yet, several other studies have reported enhanced effects from Ca$_3$(PO$_4$)$_2$ modifications. They include increased bone formation in rat femora (27), enhanced healing in dog mandibles (28), and better retention in rabbit tibia (29), when Ca$_3$(PO$_4$)$_2$-coated titanium implants were used. Thus, additional animal studies will need to be performed to determine if the enhanced performance of CaO-enriched surfaces are sustained in vivo.

In conclusion, this study reported on an innovative surface modification for titanium implants that clearly warrants further investigation. It has shown that surface enrichments with CaO additives alone can markedly enhance the human mesenchymal cell response to biocompatible surfaces. These surface coatings can be prepared by a novel ultrafine dry powder coating technique that uses polyester resins and TiO$_2$ powders to create a polymeric base, which can be readily enriched with functional additives. The bioactive additives include ceramic-like mixtures of mineral oxide powders, and Ca$_3$(PO$_4$)$_2$ or CaO alone. Whereas most prior studies reported on the beneficial response to hydroxyapatite-like Ca$_3$(PO$_4$)$_2$ surfaces, this study documented a more heightened response from CaO additives alone.
3.5 References

Chapter 4

4 Biocompatible and osteo-inductive alternatives for polyester-based biomaterial surface enhancements

Abstract

In recent history, the use of epoxy-based coating has achieved much success in enhancing the biocompatibility and osteo-inductivity of orthopedic implants. However, with recent concerns of potential harmful effects regarding triglycidyl isocyanurate (TGIC), an essential component of polyester-based surface coating particles, an alternative coating polymer is needed to continue the research of polymer-based particles for enriching orthopedic implants. In this study, two different polymers that function without the presence of TGIC, epoxy and epoxy/polyester hybrid, were studied for their potentials in replacing the use of conventional epoxy-based coatings. While constructing coating particles using epoxy and epoxy/polyester hybrid polymers, the same previously described ultrafine-particle technology was used. This technique involves the shearing of polymer chips into ultrafine particles using a high speed shear mixer, followed by the addition of bioactive calcium oxide and nano-sized TiO2 respectively as the functional and flow additive. In addition, each of the polymer, epoxy and epoxy/polyester hybrid, was divided into two groups, either with or without the incorporation of micron-sized TiO2. This was to examine the importance of purity in the polymers. The resultant ultrafine particles were then placed under thorough examination of their flow properties by studying their avalanche angles, resting angles and the angle of repose. After confirming their flowability, such particles were then electrostatically applied onto titanium substrate and cured to form enriched biomaterial. Elemental mapping was sequentially employed to confirm the surface chemical homogeneousness of the constructed biomaterial. in vitro microscopy studies then suggested that the surfaces were compatible and were able to support cell attachment and proliferation. In addition, Alizarin Red staining suggested although all of the biomaterials possess osteo-inductive characteristics, micron-sized TiO2 containing-epoxy/polyester hybrid surfaces exhibited the great extent of mineralization. In conclusion, this study confirmed the feasibility of using epoxy and epoxy/polyester polymers as the alternatives for TGIC-reliant polyester polymers in enhancing the biocompatibility and osteo-inductivity for orthopedic implants.
4.1 Introduction

Orthopedic implants archived much success in the medical field, a success can be traced to their efficacy in enhancing or replacing damaged tissue. In the US alone, the field orthopedic implants has already occupied a $14.3 billion market while still maintaining a steady annual growth of 8.9 percent (1). Nevertheless, like any other medical devices, there are always areas which can be improved to make bone implants more effective. One such area is the integration of implant into patients’ body. For example, the success of bone or orthopedic implants can often be affected by complications such as hypersensitivity and immune-response, which all can trace their source to a compromised tissue-implant interface (2) (3). In addition, the post-surgical stability of an orthopedic implant is also highly dependent on not only the quality, but also the quantity of the surrounding bones for providing anchorage. It is said that a surgeon can often predict the probability of loosing of the implant before the surgery by visually examining the severity of osteoporosis at the surgical site (4). In conclusion, even though orthopedic implants have proven to be as a successful in replacing damaged tissue, there are still areas for improvements to make orthopedic implant safer and more effective.

Consequently, much of the recent effort has been invested in understanding the tissue-implant interaction and in encouraging the differentiation of absorbed cells, to improve the biocompatibility and the osteo-inductivity of the biomaterial respectively (5)(6). Thus far, studies have shown that various surface-molecules were able to exert beneficial effects such as encouraging cellular survival, metabolism, proliferation, attachment and differentiation of absorbed cells (7)(8)(9)(10); and similarly, various surface modification techniques such as grounding and chemical etching have demonstrated their effectiveness in improving the biocompatibility of implants and the metabolism of the surrounding cells (11). Moreover, our research group has also been improving a new surface modifying technique where the surfaces of implants were enriched using polyester-based ultrafine particles. These enhanced surfaces showed significant improvements in their
biocompatibility and osteo-inductiveness when comparing to commercial available titanium (10,12–15).

An essential component of polyester-based coating particles is 1,3,5-Triglycidyl Isocyanurate (TGIC), which acts as the curing agent for dry powder coating. However, with the recent concerns of TGIC, authorities have recommended employers to implement proper ventilation in the spraying area and provide their workers with sufficient body protections (16). Although authorities indicated TGIC is not harmful to the general public due to its low concentration, to avoid any further potential complications, an alternative surface-enhancing polymer is needed for the further development of this technology (17).

During this study, two different polymer backbones that do not rely on TGIC were selected, and their biocompatibility and osteo-inductivity were assessed. The first polymer examined was pure epoxy, due to its chemical stability and the use of an only minute amount of an alternative curing agent, dicyandiamide. Dicyandiamide is a compound that is used in the pharmaceutical industry and has been thoroughly researched and found to pose no food safety concerns. Similarly, the second polymer assessed was an epoxy/polyester (50:50) hybrid. This material was selected due to its inter-curing ability between the epoxy and polyester polymers which completely eliminate the need of curing agents.

Furthermore, in addition to comparing between the two polymer backbones, we also studied the effects of the purity within polymer-backbone on biocompatibility and osteo-inductivity. This was accomplished by comparing pure epoxy and epoxy/polyester hybrid polymers against epoxy and epoxy/polyester groups that also contained 50 weight % TiO2. Lastly, after incorporating calcium oxide (CaO, 5 weight %) which has been proven to be very biocompatible and osteo-inductive (12), a series of particle- and surface-property assessments were conducted to examine the physical properties and the
feasibility of such biomaterial. Similarly, osteo-inductivity assays were also conducted to test the biological properties of such enhanced surfaces. Through this studying, we hoped to continue using our polymer-enhancement technology to develop a more suitable biomaterial for orthopedic implants and to gain a better understanding of the biocompatible and osteo-inductive effects that various polymer-backbones would exert on in vitro cultures.

4.2 Materials and methods

**Preparation of ultrafine-particle enhancement**

The ultrafine enhancement particles were created in a similar fashion to previous studies (12,14,13,15,10) (Figure 4.1). Flow and bioactive additives were incorporated to the coating polymers as listed to improve particle flowability, as well as the surface biocompatibility and the osteo-inductivity respectively (Table 4.1). The two backbone-polymers for the surface enhancements were specially developed and were divided into either one of the two experimental groups, pure polymer, or micro-sized TiO2-containing. All of the formulation groups were then enhanced with nano-sized TiO2 (0.5 mass percent) (Degussa, USA) as the flow additive and 5 mass percent of calcium oxide (CaO, Sigma-Aldrich Canada Co. Oakville, Ontario) as the bioactive agent. Lastly, the final coating particles were obtained by mixing the recipe in a high-shear mixer and passing through a sieve (35 µm).
Figure 4.1: An ultrafine dry powder coating technique was used to create novel biocompatible surface coatings that are enriched with bioactive agents. Both epoxy and epoxy/polyester resins, calcium oxide (CaO) and nanoparticles of titanium dioxide (nTiO2) were ground and mixed, either with or without micron-sized titanium dioxide (TiO2), in a high-shear mixer. The powder mixtures were passed through a sieve to obtain ultrafine particles, which were confirmed by particle-size analysis. The ultrafine powders were then sprayed onto grounded titanium (cpTi) disks and cured in a furnace.

*Base polymer composition: epoxy/epoxy-polyester, or epoxy/epoxy-polyester + 25 wt% TiO2 pigment
** Base polymers with Functional additive (CaO)
Table 4.1: Composition of ultrafine powders.

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Ultrafine particle formulation

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Epoxy resin - Anhui Shanfu New Material Technology Co., LTD., China
Polyester resin - Haining Guanghua Chemicals Inc., China
Dicyandiamide - Hubei Nice Chemicals and New Materials Inc., China
2-Methylimidazole - Source Chemical (China) Co., LTD., China
TiO\textsubscript{2} micron-sized - Luanchuan Yuxing Chemicals Inc., China
CaO - Sigma-Aldrich Canada Co. Oakville, Ontario
nTiO\textsubscript{2} - Degussa, USA

The dimensions of ultrafine-particles were then verified via particle size analysis using a BT-9300s Laser Particle Analyser (Ningbo Yinzhou Hybers, China). During this process, the enhancement particles were suspended in water media and a laser beam was used to create a diffraction pattern that was then reflected onto a detector and analyzed using computer program to determine their sizes.
The flowability of the newly developed particles were assessed by examining their angle of repose, avalanche angle and their resting angles. The angle of repose (AOR) for each powder was measured by using a powder characteristic tester (PT-N Powder Characteristic Tester, Hosokawa Micron Powder Systems Co., Summit, NJ, USA). To measure the AOR, the powder sample was slowly dispensed onto a flat surface to form a pile. The AOR was then taken as the angle between the surface of the pile and the flat surface. Six AOR measurements were completed for each sample and the average was recorded. Sequentially, the avalanche angle (AVA) and resting angle (RA) were measured using a rotating drum (Revolution Powder Analyzer, Mercury Scientific Inc., Sandy Hook, CT, USA). This instrument works by placing a known amount of powder into a transparent drum and monitoring how the powder flows as the drum is rotated. The AVA was determined by rotating 120 mL of powder (tapped volume) at 0.6 rpm and measuring the maximum angle that the powder achieves before it avalanches (collapses) to the bottom of the drum, and RA was the angle of the pile formed after each avalanche. This tests were conducted until 200 avalanches occur and the average was calculated.

**Coating of implant substrate**

The surface of implant substrates were coated using a novel patented ultrafine particle-coating technology (18)(19). The coating particles were electrostatically applied onto consistent sized grounded commercially pure titanium disks (Figure 4.1) (diameter = 24 mm, Grade 2, thickness = 0.5 mm, McMaster-Carr, Cleveland, OH) using a Corona Gun (Nordson, Westlake, OH). The voltage of the Corona gun was set at 20kV during the spraying application process, which charges the particles allowing them to be electrostatically attracted onto grounded titanium disks. Subsequently, the ultrafine particle coated titanium substrates were cured at 200 °C in a high performance air flow oven for 10 minutes (Sheldon manufacturing, Inc., Cornelius, OR). The resultant surfaces then analyzed for surface characteristics and *in vitro* biological performance.
**Characterization of coatings**

The coated surfaces were characterized for their surface composition as described in previous studies (12,10). The surfaces of biomaterials were analyzed via energy dispersive X-ray spectroscopy (EDX) with a Hitachi S-4000 SEM (Hitachi, Pleasanton, CA). The enhanced surface were firstly mounted onto metal stubs, secured with adhesive carbon tape and sputter coated with nano-sized gold particles (10nm). After configuring the SEM (the working voltage = 15 kV, working distance = 15 mm), each surface element was identified (minimum detection limit = 0.0%), and its presence was calculated (weight percent). The EDX analyses were repeated at three different locations for each surface, and the mean surface concentrations of key components calculated. Furthermore, the EDX analyses were also repeated across the entire surface of each disk to identify the presence, localization and distribution of the key elements, proving us with an elemental mapping across each surface.

**Surface preparation for in vitro studies**

Before seeding cells onto the biomaterials to assess their in vitro performance, the surfaces were thoroughly sterilized. The biomaterials were initially rinsed three times using phosphate buffered saline (PBS, Gibco, pH 7.4, calcium chloride and magnesium chloride free) and then rinsed three times again using 0.25% trypsin (Gibco, with EDTA and phenol red). The surfaces were then submerged in fresh trypsin and sonicated for 60 minutes in a 50mL polypropylene conical tube (BD Falcon). After discarding the used trypsin, the surfaces were sonicated again for 60 minutes in a 50:50 bleach:water solution. After the sonications, the surfaces were washed 10 times using distilled water and twice more using autoclaved distilled water. Finally, the surfaces were rinsed three times with firstly 70% EtOH and PBS, followed by UV sterilization of both sides for 30 minutes in a tissue culture hood.
**Cell culture maintenance**

To examine the osteo-inductivity of the various biomaterial surfaces, the control (Ti) and coated (epoxy-pure, epoxy-TiO2, hybrid-pure, hybrid-TiO2) disks were placed in one of the wells of a 6-well tissue culture plate (BD Falcon MULTIWELLTM 6 well, polystyrene). These disks were then covered by tissue culture media which contained DMEM (Gibco, DMEM high glucose 1x, with 4.5g/L D-Glucose, L-Glutamine, and 110 mg/L sodium pyruvate), 10% fetal bovine serum (FBS, Gibco) and 1% Anti-Anti (Gibco, Antibiotic-Antimycotic). Sequentially, human embryonic palatal mesenchymal cells (HEPM, ATCC CRL-1486) were seeded onto the center of each disc. The tissue culture plates were maintained in a Sanyo CO2 incubator that was set at 37°C and 5% CO2. The culture media were refreshed every 3 days and the cell cultures were allowed to proliferate.

**Cellular attachment and morphology assessment**

After 24 h of incubation, the disks with the cell cultures were carefully removed from the incubator and rinsed three times in PBS (5 min/rinse). Cells that remained attached to the disks were fixed with paraformaldehyde (4% PFA, Fisher Scientific) for 24 hours at room temperature. The disk surfaces were then examined with an optical microscope at 100 × and 400 × magnification for cell attachment and morphology, and the images were digitally captured.

**Osteo-inductive ability assay**

The biomaterial surfaces were also assessed for their ability to support the biomineralization of HEPM cells as previously described (12,15). These cell cultures were then allowed to proliferate and biomineralize in DMEM for either 2 or 4 weeks. During the 2- or 4-week biomineralization period, the culture media were replenished every 3 days. After the respective biomineralization periods, the culture media was
removed using vacuum suction, the biomaterials were rinsed in PBS and fixed for 1 hour in 4% formalin followed by two more washes in calcium-free Nanopure water. To visualize the osteo-inductive ability of the surfaces, mineral deposits were stained using 2% Alizarin Red S for 10 minutes at room temperature. Lastly, the cultures were washed again using Nanopure water to remove the excess stain, and the surfaces were then examined visually for mineral deposits and the images were also digitally captured. The 2 and 4 week time points were selected to individually examine the extent of the initial biomineralization and the long term mineralization abilities of the cell cultures that were seeded on each surface.

**Statistical analyses of data**

All of the quantitative data were analyzed by SigmaPlot 12.0 (Systat Software Inc., Chicago, IL, USA). The values for mean and standard error were calculated, examined graphically and analyzed statistically using SigmaPlot. Differences were identified by one-way ANOVA, and post hoc comparisons were performed by the Holm–Sidak method with the significance level set at $\alpha = 0.05$.

### 4.3 Results

**Analogous ultrafine particles were created from pure and hybrid formulae**

The pure epoxy, epoxy mixed with micron-sized TiO$_2$, epoxy/polyester hybrid, and epoxy/polyester hybrid mixed with micron-sized TiO$_2$ were created into largely analogous ultrafine powder particles. These pure and hybrid powder formulae were mixed with CaO (5% w/w) as a bioactive agent, and nanoparticles of TiO$_2$ (0.5% w/w) as a flow modifier, and they were all ground and sieved through the same process (Figure 4.1). The resulting dry powder mixtures had very similar properties and dimensions, despite the differences in their chemistry and composition. Particle size analyses of these
mixtures confirmed that they were ultrafine powders, since 50% of the particles (D 0.5) had a volume diameter that was less than 35 µm (Figure 4.2). The Epoxy and Epoxy-Polyester powders had an almost identical particle size distribution, whereas the Epoxy-TiO$_2$ powders were clearly smaller (D0.5= 18.71), and the Epoxy-Polyester-TiO$_2$ were even smaller (D0.5= 16.34).

Figure 4.2: The powder mixtures were analyzed for their particle-size distribution. The Epoxy-TiO$_2$ and the Epoxy-Polyester-TiO$_2$ had similar particle-size distributions with a relatively small D50 that was around 20µm. The Epoxy and Epoxy-Polyester had very similar particle-size distributions with a larger D50 that was around 30µm.

**Surface calcium-enrichment increased on polyester-containing coatings**

When these ultrafine powders were electrostatically sprayed onto grounded sheets of titanium and then cured in an oven, their constituents were incorporated into surface coatings. Elemental mapping of their surfaces showed that the predominant element was
carbon (C), which originated from the epoxy and polyester resin base polymers (Figure 4.3). The other common element was oxygen (O), which also originated from the base polymers. There were also high levels of titanium (Ti) on the coatings that had been enriched with micron-sized TiO$_2$ coatings, and much lower levels on the remaining surfaces, which only contained nTiO$_2$ that served as a flow additive in all of the formulae. Similarly, CaO (5%) had been added to all of the formulae as a bioactive additive. However, surface calcium levels varied widely from being very high on the Epoxy-Polyester-TiO$_2$ and Epoxy-Polyester coatings, and lower on the Epoxy-TiO$_2$ and Epoxy surfaces.

![Elemental mapping images](image_url)

Figure 4.3: The coated surfaces were analyzed by elemental mapping. Carbon (C) was detected at high levels, and oxygen (O) at moderate levels on all of the surfaces. Titanium (Ti) was detected at high levels on the Epoxy-TiO$_2$ and Epoxy-Polyester-TiO$_2$ surfaces, and at much lower levels on the Epoxy and Epoxy-Polyester surfaces. Calcium (Ca) was detected at very high levels on the Epoxy-Polyester-TiO$_2$ surfaces, in clusters at moderately high levels on Epoxy-Polyester, and unevenly at lower levels on Epoxy and Epoxy-TiO$_2$ surfaces. Each dot represents the presence of respective element.
Physical differences were induced by the addition of micro-sized TiO2 and the different polymer-backbone

With the introduction of micro-sized TiO2, and the use of different polymer backbones (pure epoxy versus epoxy/polyester hybrid), significant physical differences as well as non-significant but noticeable trends were observed between the different particle mixtures. The flowability for all of the enhancement particles were examined by studying their angles of repose, avalanche angles, as well as their resting angles. Even though the angle of repose data did not suggest the different base polymers or the addition of micro-sized TiO2 was able to contribute to any specific significant difference, avalanche angles and resting angles hinted that the micro-sized TiO2 containing particles exhibited a significantly lower flowability than their counterparts (Figure 4.4, 4.5, 4.6). In addition, when comparing pure epoxy and epoxy/polyester-hybrid particles, a non-significant nevertheless clear and observable trend of lower flowability were demonstrated by the hybrid particles.
Figure 4.4: The flowability of the ultrafine particles were lastly confirmed by examining their angle of repose. This assay illustrated that all of the particle formulations were highly flowable. The pure epoxy particles were found to have the best flow, followed by the micron-sized TiO2 containing hybrid ultrafine particles, whereas the micron-sized TiO2 containing epoxy and pure epoxy/polyester hybrid particles exhibited similar flow properties. (n=3, each alphabet represents a statistical group, p<0.05)
The flow properties of the ultrafine particles were examined by assessing their avalanche angles. This assay showed that the micron-sized TiO2 containing particles were less flowable by exhibiting a higher avalanche angle comparing to their pure counter-parts. Nevertheless, the different types of polymer backbones did not have any significant effects on the flowability of the particles. (n=199, p>0.05)
Figure 4.6: The flowability of the ultrafine particles were further studied by obtaining their resting angle after each avalanche. The resting angle study also showed the micron-sized TiO2 containing particles were less flowable compared to their pure counter-parts. However, the resting angle assay also suggested that the epoxy particles were significantly more flowable than the epoxy/hybrid particles due to their smaller angles. (n=199, p>0.05)

All of the coated surfaces showed biocompatibility and were able to support cell cultures

After careful optical microscopy examination, under lower power (40x), all of the surfaces showed the numerous cell clusters on each biomaterial, indicating their capabilities of supporting the attachment and proliferation of HEPM cultures. In addition, when the cellular morphology of the attached cells were studied using high power optical microscopy (100x). The images showed that the HEPM cells exhibited a ‘spreading’ morphology with extended filopodia, suggested a close association with the underlying biomaterial and the biocompatible nature of the constructed surfaces (figure 4.7).
Figure 4.7: The biocompatibility of all of the coated surfaces were examined by assessing the cellular morphology of the cultures that were seeded on each surfaces. In lower power optical microscopy (40x), the images showed that all of the surfaces were able to support cell cultures; while in high power optical microscopy (100x), the cellular morphology clearly supported the biocompatibility of the surfaces by exhibiting a spreading morphology with their extended filopodia, showing the intimate contact between the cells and the biomaterial.
Biomineralization was supported on polyester-containing coatings

The human mesenchymal cell cultures that were grown on Epoxy-Polyester and Epoxy-Polyester-TiO2 coatings showed the initiation of *in vitro* biomineralization. The Alizarin red staining showed that there were numerous calcified mineral deposits in the cultures grown on Epoxy-Polyester and Epoxy-Polyester-TiO2 surfaces after 4 weeks of growth in differentiation media (Figure 4.8). There were moderate amounts of mineral deposits on the Epoxy-Polyester and Epoxy-Polyester-TiO2 within 2 weeks of growth, and also on the Epoxy-TiO2 surfaces. There were much smaller amounts of mineral on all of the coatings that were in control media that did not contain any cells, and no calcified deposits on the titanium surfaces.
Figure 4.8: Human mesenchymal cells were seeded onto coated and control surfaces that were suspended in tissue culture DMEM medium. After growth and biomineralization, the surfaces were stained with Alizarin Red-S. (A) After 2 weeks, a few mineral deposits were detected on the Epoxy-Polyester and the Epoxy-Polyester-TiO2 coated surfaces, and on the Epoxy-TiO2. (B) After 4 weeks, much more mineral deposits were detected on the Epoxy-Polyester and the Epoxy-Polyester-TiO2 coated surfaces.
4.4 Discussion

The feasibility of using pure epoxy and epoxy/polyester hybrid to replace conventional pure-polyesters for ultrafine-particle coating was confirmed during this study. In addition, by examining the difference due to the presence and absence of micron-sized TiO$_2$ pigment (25 weight percent), the effects of purity in the surface enhancement formulation was also studied. The development of such particles involves size-reduction of the larger polymer chips and the incorporation of flow and bioactive functional additives. The construction and the application of such ultrafine particles utilized a recently developed technique by the Particle Technology Research Centre at the University of Western Ontario (18,19), yet this technique has already been well explored by numerous studies (12,14,13,15,10).

The polymeric backbones used in this surface enhancement were composed of either 100% epoxy, or a mixture of 50:50 epoxy:polyester hybrid. With the use of these two polymers, the need of TGIC, a substance that recently has been shown to impose health concerns, was effectively eliminated. These biocompatible coatings (15) were further enhanced through the incorporation of a ceramic insert, CaO (5 weight percent) as the bioactive agent to encourage osteo-inductivity and addition biocompatibility (12). The low quantity of functional additives guaranteed the continuity and homogeneity of the polymer backbone. In addition, because the biomaterials in this study were constructed using electrostatic dry particle coating, the hazardous solvents and volatile organic compounds which were usually associated with liquid coating were also eliminated, consequently making these surfaces even safer and more reliable. After the development of these novel alternative ultrafine polymer particles, their physical characteristics and the biological performance were analyzed.
From the first assessment, particle-size analysis deemed these particles as ultrafine due to their D0.5 of under 32 µm (Table 2). The ultrafine characteristic was sought after because previous studies demonstrated that ultrafine-sized particles were able to form nano-scale topography and increase the surface roughness of conventional titanium substrate (14,13,15,10). Surface roughness can improve the performance by encouraging osteointegration in bone deficient bone environment and increasing cell attachment, which has been well demonstrated in surfaces enriched using ultrafine particles (20)(21)(22)(23)(24)(25)(26).

Nevertheless, there are also problems associated with using ultrafine particles. By decreasing the size of particles, the inter-particular forces begin to dominate, thus reducing the flowability of such particles and their usefulness (27). Fortunately, the ultrafine particle technology allowed the industrial application of ultrafine particles by incorporating flow modifiers. These modifiers essentially act as spacers between coating particles, allowing them to effectively minimize the inter-particular attractions between the coating particles and thereby improving the flowability of coating particles. More specifically, nano-sized TiO$_2$ (nTiO$_2$) particles (0.5 weight %) was used in this study to ensuring the adequate flow needed for the electrostatic coating particle application. Furthermore, besides its ability to improve flowability, n TiO$_2$ has also been found to have the additional benefit of providing further increased biocompatibility to enhanced biomaterial (13). In addition to n TiO$_2$, such biocompatible polymeric coatings typically involved the incorporation of micron-sized TiO$_2$. Nevertheless, the mechanism behind additional biocompatibility is unknown. Thus, this study also examined the physical and biological effects of micron-sized TiO$_2$ were also explored. Immediately, through particle size analysis, the results suggested the particles which contained micro-sized TiO$_2$ was able to achieve a smaller D0.5 than the pure polymer surfaces, thus making TiO$_2$ particle more desirable.
By altering the type of the base polymer and the incorporation of micron-sized TiO$_2$, other notable physical differences were observed. One of the most important physical characteristics that the ultrafine technology that to offer is its ability to improve the flowability of coating particles. A poor flowability can cause a variety of problems ranging from an uneven distributed surface to even the clogging of the distribution apparatus, which would completely halt the entire enhancement process; thus, the flowability of the newly constructed particles had to be assessed prior to the actual application of the particles. The flowability assays suggested that the enhancement-particles which contained micro-sized TiO$_2$ were less flowable by exhibiting a significantly higher avalanche and resting angle. This phenomenon could be caused by the smaller particle sizes achieved shown in the particle size analysis with the incorporation of micro-sized TiO$_2$. It has been well-established that the decrease in the size of particles would lead to an increase in the inter-particular forces, and thereby decreasing the flowability of the corresponding particles. Consequently, by including micro-sized TiO$_2$, the smaller diameters were obtained as shown by the particle size analysis also caused a decrease in flowability which were shown by their avalanche and resting angles. However, with the addition of flow additive, all of the particles were expected to be flow-able for electrostatic application.

Sequentially, after coating the implant substrate, Energy dispersive x-ray (EDX) were used to confirm the success of bioactive additive incorporation of the constructed surfaces by examining their chemical composition. EDX spectroscopy showed that the majority of the enhanced layer was composed of carbon while there was a small quantity of oxygen present, which complies with the organic nature of the base polymer. Furthermore, there was also minute concentrations of calcium and titanium, which can also be explained by the incorporation of TiO$_2$s and CaO. However, different levels of titanium was detected by EDX between the biomaterial surfaces. As expected, the results showed that the titanium presence increased with the incorporation of micro-sized TiO$_2$. In conclusion, the finding from EDX agreed with prior analyses of similar coatings (15)(12) and suggested that all of the intended chemicals were indeed correctly
incorporated onto the surface of the titanium substrate. Moreover, elemental mapping also provided additional confirmation that not only the additives were present, carbon, oxygen and titanium were also evenly distributed, forming a homogenous coating surfaces. However, calcium was distributed in more of a cluster fashion, a phenomenon that has also been noted in other studies (12,10).

Lastly, *in vitro* experiments were conducted to study the biocompatibility and osteo-inductive potentials of the coated surfaces using HEPM cell cultures. Using optical microscopy, the images indicated all the biomaterial demonstrated high levels of biocompatibility by allowing the attachment and proliferation of HEPM cells. In addition, the attached cells exhibited a spreading morphology, suggesting a close association between the cells and the biomaterial. In addition to biocompatibility, the biomineralization activity was visually assessed using Alizarin Red-S dye. Alizarin Red has long been shown to accurately detect mineralization by binding specifically to the mineral deposits through its sulfonic acid and hydroxyl groups (28). The findings demonstrated that with the only exception for the control surfaces which only exhibited minimal amount of staining, rest of the surfaces showed various degrees of mineral formations. In addition, all of the coated biomaterials also showed greater extend of staining after 4 weeks of incubation comparing to their respective 2 week period, indicating that all of the surfaces were highly osteo-inductive by continuously forming mineral deposits over time. Nevertheless, differences were observed amongst the coated biomaterial surfaces. The data suggested that the micro-sized TiO$_2$-containing surfaces showed a greater signs of mineralization than its counterpart and the hybrid polymers outperformed the pure epoxy groups. These results corresponded to the previous studies where researchers found that the incorporation of TiO$_2$ was able to improve the cell responses to its attached surface (12,14,13,15,10). Similarly, the higher biocompatibility observed in hybrid surfaces could due to the presence of 50% polyester which has been shown to exhibit excellent biocompatibility and has been intensively studied for various medical applications (12)(29)(30)(31). In addition, the greater polarity of polyester
making the biomaterial more hydrophilic which has also been shown to improve biocompatibility (32)(33)(34).

Consequently, this study confirmed that the two novel ultrafine particle polymer backbones, epoxy and epoxy/polyester hybrid, can be effectively used to host functional additive in creating biocompatible and osteo-inductive surfaces. Furthermore, this study also suggested that different polymer backbones were also able to induce different physical characterizations and biological behaviors. More specifically, the incorporation of micro-sized TiO$_2$ although reduced flowability of coating particles, it also allowed the creating of smaller particle size, which has been demonstrated through in vitro studies to encourage favourable cellular responses and promoting biocompatibility. In addition, although all of the coated surfaces showed high degrees of osteo-inductivity, the micro-sized TiO$_2$ containing epoxy/polyester hybrid surfaces outperformed rest of the surfaces. As a result, this study shined lights on a new potential alternative to the pure polyester-based surface enhancement with micro-sized TiO$_2$ containing epoxy/polyester hybrid surfaces being the best candidate, and basic knowledge to how various combinations of additives helped to contribute to a better implant material. In the future, in vitro studies should be conducted to gain a more in-depth understanding of the mesenchymal cellular behaviour to these novel surface coatings.

4.5 References

23. Lincks J. Response of MG63 osteoblast-like cells to titanium and titanium alloy is dependent on surface roughness and composition. Biomaterials. 1998 Dec;19(23):2219–32.
Chapter 5

5 Biological analysis of TGIC-free polymers for enhancing the biocompatibility and osteo-inductivity of orthopedic implants

Abstract

Due to the recent health concerns regarding the safety of triglycidyl isocyanurate (TGIC), an essential component of polyester-based surface coating particles, two plausible alternative polymers were developed to continue the research of using ultrafine-particles to encourage the biocompatibility and osteo-inductivity of orthopedic implants. Epoxy and epoxy/polyester ultrafine particles, able to function in the absence of TGIC, are highly flowable. This characteristic allowed such particles to be applied onto implant substrates in the same manner as conventional polyester-based coating particles, presenting them as potential replacement candidates. In addition, surfaces enriched with such particles have also demonstrated biocompatibility and osteo-inductivity; however, with all these exciting findings, there have not been any quantifiable biological assay reports. Thus, this study aimed to thoroughly examine the biological performance of implant substrates that were enriched using such particles. Energy dispersive x-ray spectroscopy (EDX) conducted on the enriched surfaces illustrated the incorporation of the flow and bioactive additives, confirming the reliable of the particle application technique. Water wettability assays indicated the hydrophilic and the biocompatible nature of the resultant surfaces by showing contact angles of less than 90°. Through a cross hatch cutter, all of the coatings achieved an adhesion rating of 5B, demonstrating the excellent adhesion between the enrichment and the underlying substrate. In in vivo assays, the cells seeded on micron-sized TiO₂-containing epoxy/polyester hybrid surfaces illustrated the highest cellular attachment and proliferation rate. Lastly, through MTT assay and Alizarin Red staining, again the cells plated on the micron-sized TiO₂-containing epoxy/polyester hybrid surfaces exhibited the highest level of biomineralization. Consequently, although all of the constructed TGIC-free surfaces demonstrated feasibility in replacing the conventional polyester coating particles, micron-sized TiO₂-containing epoxy/polyester hybrid particles appeared to be the best candidate.

5.1 Introduction

Orthopedic implants were recognized to be very effective medical devices for replacing and enhancing damaged tissues. However, the effectiveness of such implants can be
saddled by complications such as inflammation or even the loosening of the implant, in which the source of both problems can be traced to a compromised implant-tissue interface (1)(2). As a result, researchers postulated that an ideal implant would be one that can mimic and interact with the surrounding bone environment, through a process known as osteo-integration.

Osteo-integration has been described as being highly biocompatible and osteo-inductive; thus, to overcome the obstacle of faulty implant-tissue interface, the biocompatibility and osteo-inductivity of orthopedic implants would need to be improved. Conventional orthopedic implants are typically made of commercially available titanium or titanium alloys. Titanium is the choice for bone implants due to the passive titanium oxide layer that it forms under the exposure to moisture, which effectively acts as a barrier isolating the implant from the surround tissue. This isolation in turn effectively minimized the potential negative interactions between the implant and the body. Although bio-inert, such surfaces are often not very bio-active. As a result, conventional titanium implants typically do not actively participate in the post-surgical healing, nor does it aid in strengthening of the surrounding bone tissues (3).

Consequently, different strategies were developed in an attempt to improve the biocompatibility and osteo-inductivity of bone implants. Studies have found that the various techniques which alter the topographic features of the implants such as increased hydrophilicity, roughness and the incorporation of chemical inserts have shown success in improving the cell attachment, proliferation, and differentiation as well as biomineralization abilities of the implant (4) (5). More particularly, numerous studies have found calcium to be very biocompatible and osteo-inductive; thus implant surfaces which were enriched with calcium exhibited increased protein activity and were very effective in promoting bone formation around the implant, respectively (6–10). Consequently, researchers have previously attempted incorporating various minerals onto the surface of implants using plasma spraying or sputter coating (11–14). Nevertheless,
these techniques are often expensive and time consuming, and the surfaces constructed from such techniques lacked homogeneity and exhibit low bonding strength (15). To overcome these difficulties, our research group has been developing a novel technique which utilizes the patented ultrafine particle technology. Our technique enriched the surface of conventional titanium substrate via a polyester backbone scaffold which contained bioactive minerals. This method was able to create homogenous surfaces with intricate nano-topographies and exhibited high degrees of biocompatibility and osteo-inductivity.

However, the recent potential health concern raised regarding the safety of triglycidyl isocyanurate (TGIC), an essential component of polyester-based surface coating particles, has placed a temporary halt on the development of this very promising technology which relies on using such particles. Thus, with the potential hazard of TGIC, an alternative methodology is needed. Fortunately, two potential replacement polymers have been found which possess the same ability as polyester in acting as the polymer scaffold for enriching the surface of orthopedic implants with bioactive molecules. These two alternatives, epoxy and epoxy/polyester hybrid function in the complete absence of TGIC and have primarily demonstrated their biocompatibility and osteo-inductivity, nevertheless, no quantifiable data was available.

Thus, this study was designed to comprehensively examine the physical characteristics and the in vitro performance of such enriched surface through studying their hydrophilicity, chemical composition, adhesion strength, as well as metabolic activity, cell count and mineralization ability. From this study, we are hoping to confirm the feasibility of using epoxy and epoxy/polyester hybrid as the alternative polymer backbone for enriching the surface of orthopedic implants. The findings sequentially could allow our research group to continue using the ultrafine particle technology as a way to inexpensively and conveniently enhance the biocompatibility and osteo-
inductivity of orthopedic implants, consequentially making bone implants more clinically effective.

5.2 Materials and methods

Preparation of Ultrafine-particle enhancement

The ultrafine enhancement particles were developed as previously described (11;18;19; 21; 26) (Figure 5.1). Flow and functional additives were added to each of the base polymer as listed (Table 5.1). These two types of backbone-polymers of the surface enhancements was specifically developed and each were divided into either one of the two sub-groups, micro-sized TiO2-containing, or pure-polymer. The four enhancement groups were then all enhanced with nano-sized TiO2 (0.5 mass percent) (Degussa, USA) to improve the flowability of the ultrafine enhancement particles and 5 mass percent of calcium oxide (CaO, Sigma-Aldrich Canada Co. Oakville, Ontario) as a bioactive agent. Lastly, the recipe was mixed in a high-shear mixer and passing through a sieve (35 µm) which results in the final ultrafine surface enhancement particle mixture.
Figure 5.1: The patented ultrafine particle coating technology was employed to develop this novel biocompatible surface enrichment. This surface layer is enriched with bioactive agents. Both epoxy and epoxy/polyester resins, as well as calcium oxide (CaO) were sheared and sieved (35 µm); the sequential mixture was then mixed again with nano-sized titanium dioxide (nTiO₂) to improve this flowability. The ultrafine surface enhancing particles were then sprayed onto grounded titanium (cpTi) disks, cured in a furnace and prepared for biological assays to examine its biological performance.
Table 5.1: Composition of ultrafine particles.

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The dimensions of ultrafine particles were then verified through particle size analysis using a BT-9300s Laser Particle Analyser (Ningbo Yinzhou Hybers, China). During this process, the enhancement particles were suspended in water media and a laser beam was used to create a diffraction pattern which was then reflected onto a detector and analyzed using computer program to determine their sizes.

Development of enhanced biomaterial

The surface-enhancement particles were developed by using a patented ultrafine particle-coating technology (16);(17). This technique has been well described by previous studies (18;19;21;26). The four enhancement formulations were electrostatically sprayed onto commercially pure titanium substrates (Figure 5.1) (Grade 2, thickness = 0.5 mm, McMaster-Carr, Cleveland, OH) using a Corona Gun (Nordson, Westlake, OH). The voltage of the Corona gun was set at 20kV during the spraying process, which ionizes the particles allowed them to be electrostatically attracted onto grounded titanium sheets. Sequentially, the enhancement particles were cured onto the commercially available
titanium substrate (200°C, 10 min) in a high performance air flow oven (Sheldon manufacturing, Inc., Cornelius, OR). The resultant sheets were then cut into consistent sized disks (diameter = 24 mm) for biocompatibility analysis.

**Characterization of coatings**

The cured surface coatings were characterized for their surface composition as described by previous studies (18;26). The surfaces of biomaterials were analyzed by energy dispersive X-ray spectroscopy (EDX) using a Hitachi S-4000 SEM (Hitachi, Pleasonton, CA). The enhanced biomaterials were mounted onto metal stubs, secured with adhesive carbon tape and sputter coated with nano-sized gold particles (10nm). The working voltage and working distance were set at 15kV and 15 mm respectively. Each element on the surface was identified (minimum detection limit = 0.0%), and its presence calculated (weight percent). The EDX analyses were repeated at three separate locations on each surface, and the mean surface concentrations of key components calculated.

**Adhesion to Substrate**

The adhesion strength of the surface enhancement to the underlying substrate was studied with an Elcometer 107 Cross Hutch Cutter (Elcometer Ltd., Windsor, Canada) using the ASTM protocol, D3359. The coatings were cut down to the substrate with a blade (11*1.5mm²), as recommended by ASTM D3359, and several perpendicular cuts were made to create a grid of small squares. This lattice was brushed to remove debris, covered with adhesive tape (SEMicro CHT, direct substitute for Permacel P99, M.E. Taylor Engineering, INC., Rockville, Maryland, US.), and firmed with a pencil eraser. The tape was then withdrawn by a single smooth pull, and the remaining grid squares compared for retention. The lattice was then assessed for adhesion by using the ASTM D3359 standards.
**Surface preparation**

Before cell plating, the surfaces were thoroughly sterilized. The biomaterials were rinsed three times using phosphate buffered saline (PBS, Gibco, pH 7.4, calcium chloride and magnesium chloride free) and then rinsed three times again using 0.25% trypsin (Gibco, with EDTA and phenol red). The surfaces were then submerged in fresh trypsin and sonicated for 60 minutes in a 50mL polypropylene conical tube (BD Falcon). After discarding the used trypsin, the surfaces were sonicated again for 60 minutes in a 50:50 bleach and water ratio solution. After the sonications, the surfaces were washed 10 times using distilled water and twice more using autoclaved distilled water. Lastly, the surfaces were rinsed three times with firstly 70% EtOH and then PBS, and followed by UV sterilization of both sides for 30 minutes in a tissue culture hood.

**Cell cultures**

To examine the biocompatibility of the various surfaces, the control (Ti) and enhanced (epoxy-clear, epoxy-TiO2, hybrid-clear, hybrid-TiO2) discs were firstly placed in one of the wells of a 6-well tissue culture plate (BD Falcon MULTIWELLTM 6 well, polystyrene). These discs were then covered by tissue culture media which contained DMEM (Gibco, DMEM high glucose 1x, with 4.5g/L D-Glucose, L-Glutamine, and 110 mg/L sodium pyruvate), 10% fetal bovine serum (FBS, Gibco) and 1% Anti-Anti (Gibco, Antibiotic-Antimycotic). Human embryonic palatal mesenchymal cells (HEPM, ATCC CRL-1486) were then seeded onto the center of each disc. The tissue culture plates were maintained in a Sanyo CO2 incubator that was set at 37°C and 5% CO2. The culture media were refreshed every 3 days and the cell cultures were allowed to proliferate.

**Cell attachment and proliferation assays**

To study the attachment-ability of HEPM cells onto the biomaterial surfaces, the cultures were firstly removed from the incubator after 24 hours of seeding. DMEM media along
with detached and dead cells were then removed from the culture plate through vacuum suction. The biomaterials were sequentially rinsed twice using PBS and transferred into a new tissue culture plate where they were rinsed twice more with PBS. 300µL of trypsin were then added to each well and the new tissue culture plate was placed back to the incubator for 5 minutes to allow remaining cell to disassociate from the surfaces. Following by mixing the cells with another 300µL of DMEM medium, a hemocytometer was used to examine the cell-abundance of each surface under an optical microscope. The cultures for were harvested at 24 hours to ensure there was sufficient time for cell attachment while avoiding cellular proliferation. To study the HEPM proliferation-ability on these various biomaterials, the HEPM cells were allowed to culture for 7 days before the same hemocytometric assay. The seeding cell densities for the attachment and proliferation assays were 50,000 and 10,000 cells per disk respectively (11,000 cells/cm², 2200 cells/cm²).

**Cellular metabolic activity**

After 24 hours of seeding, the old culture media was removed from the culture plate using vacuum suction and replaced with 2 mL of fresh media along with 300 µL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent solution. After a 4-hour incubation period, 1 mL of MTT solubilizing agent was added to each well. After solubilization, the solution from each well was transfer to a 96-well plate and analyzed using a Safire Multi-Detection Microplate Reader (Tecan, San Francisco, CA) at 570 nm to examine the MTT intensity.

**Osteo-inductive ability assay**

The biomaterial surfaces were assessed for their ability to support the biomineralization of HEPM cells as previously described (11;21). During these studies, HEPM cells were allowed to proliferate for 24 hours on biomaterials in DMEM media after which the cultures were switched to enriched media (DMEM, 50 µmol/mL of ascorbic acid, and 10
µmol/mL of β-glycerophosphate). During the 2 or 4 week biomineralization period, the culture media were replenished every 3 days. 2 and 4 weeks were selected to examine the extent of initial biomineralization and the long term mineralization abilities of the cell cultures that were seeded on each surface. After mineralization periods, the enriched media was removed using vacuum suction, the biomaterials were rinsed in PBS and fixed for 1 hour in 4% formalin followed by two more washes in calcium-free Nanopure water. To visualize the osteo-inductive ability of the surfaces, mineral deposits were stained using 2% Alizarin Red S (EMD) for 10 minutes at room temperature. Lastly, the cultures were washed again using nanopure water to remove the excess stain, and the surfaces were then examined visually for mineral deposition.

Statistical analyses of data

All of the quantitative data were analyzed by SigmaPlot 12.0 (Systat Software Inc., Chicago, IL, USA). The values for mean and standard error were calculated, examined graphically and analyzed statistically using SigmaPlot. Differences were identified by one-way ANOVA, and post hoc comparisons were performed by the Holm–Sidak method with the significance level set at $\alpha = 0.05$.

5.3 Results

Surfaces enriched using ultrafine-particles were readily incorporated with functional additives

The novel ultrafine particle coating technology was able to successfully create biocompatible and bioactive surfaces on titanium substrate. This technique relied on two types of chemically stable polymers, pure epoxy and epoxy/polyester hybrid, acting as the scaffold, as well as micro- and nano-sized TiO$_2$ as the biocompatible and flow additives respectively; in addition, CaO was also incorporated as the bioactive agent.
After enhancement particles were electrostatically applied onto grounded titanium substrates and cured in an oven, their constituents were examined using energy dispersive x-ray spectroscopy (EDX). EDX analysis confirmed the reliability of using electrostatic application in enriching the orthopedic implants by illustrating the elemental compositions of each surface reflected their respective enhancement formulation (Table 5.1). The two most predominant elements were carbon (C) and oxygen (O) that accounted for over 90%, originated from the epoxy as well as the polyester polymer resins. The two minor constituents were titanium (Ti) and calcium (Ca), their presence fluctuated according to whether the enrichment formulation contained micron-sized TiO$_2$, and their origins can be traced to the bioactive CaO and the biocompatible and flow additives micro- and nano-sized TiO2. Thus, with the marginal modifications of the ultrafine particle formulation to include bioactive agents, EDX showed that their chemical compositions were largely similar despite the small variations of bioactive agents (Figure 5.2).
Figure 5.2: EDX illustrated that majority of the surface enrichment layer is composed of carbon, followed by oxide which existed in much smaller quantity. The presence of titanium fluctuated according to the presence of micron-sized TiO$_2$, more was detected in the Hybrid-TiO$_2$ and Epoxy-TiO$_2$ compared to the Hybrid pure and Epoxy pure surfaces. Lastly, there was also small variation in the presence of Calcium amongst the different surfaces. (n=3)

**Hydrophilicity was altered by the addition of micro-sized TiO$_2$ and the different polymer-backbone**

With the introduction of micro-sized TiO$_2$, and the use of different polymer backbones (pure epoxy versus epoxy/polyester hybrid), observable trends were noted between the hydrophilicity of respective particle formulations.

While studying the hydrophilicity between the various enhanced surfaces, a non-statistically significant but nevertheless a clear trend were noted. The water contact angle
study suggested that the micro-sized TiO$_2$ containing surfaces were less hydrophilic than their pure counter parts by having a smaller contact angle, and epoxy surfaces were more hydrophilic than the epoxy/polyester-hybrid surfaces. However, all of the surfaces are hydrophobic due to their contact angles of less than 90° (Figure 5.3).

![Figure 5.3: Hydrophilicity assay suggested that although all of the surfaces were hydrophilic, there was a noticeable trend where hybrid surfaces exhibited a smaller contact angle than the epoxy coated biomaterial, and the micron-sized TiO$_2$-containing surfaces were more hydrophobic than their counterparts. (n=5, each alphabet represents a statistical group, p<0.05)](image-url)
**Surface enhancements were adequately adhered onto biomaterial substrate**

The long term adhesive ability of the polymer layer was assessed using a cross hatch cutter. This was accomplished by examining the adhesion of the surface layer over three time periods, 7, 14, and 28 days after being submerged in culture media. The results suggested that all of the surfaces at three times points remained tightly attached onto the titanium substrate, and achieved the highest adhesion rating, 5, according to ASTM D3359 (Figure 5.4).

![Figure 5.4: The 7, 14 and 28 days after cell culture media immersion adhesion test showed that all of the surfaces achieved the highest adhesion rating (5B). (n=4, p>0.05)](image)

**HEPM cells were able to attach and proliferate on all of the surfaces**

All of the biomaterial surfaces supported the attachment and the proliferation of human embryonic palatal mesenchymal cell (HEPM) cells. However, the enhanced surfaces with
the exception of epoxy-pure, were all able to exhibit significantly improved cellular responses.

The 24 hour hemocytometric data suggested that all of the micro-TiO$_2$ containing surfaces had a higher cell attachment compared to their pure counterparts. In addition, hybrid-pure surfaces also exhibited a significantly higher cell count compared to epoxy-pure surfaces. However, even though there was no significant difference between hybrid-TiO$_2$ and epoxy-TiO$_2$ surfaces, a slightly higher cell count was recorded on the hybrid-TiO$_2$ surfaces (Figure 5.5).

Figure 5.5: The cell attachment assay indicated that the HEPM cells were able to attach significantly faster to the micron-sized TiO$_2$-containing surfaces than the rest of the biomaterials. Similarly, the hybrid surfaces also significantly outperformed their epoxy counterparts. The conventional titanium surfaces had the lowest cell attachment rate. (n=12, each alphabet represents a statistical group, p<0.05)
After 72 hours of seeding, HEPM cells were allowed to proliferate on the various biomaterial surfaces. The proliferation study indicated that even though the results were not statistically significant, a trend similar to the 24 hour cell-attachment assay was observed. The data suggested that the micro-sized TiO$_2$ surfaces and hybrid-based surfaces demonstrated a higher proliferation rate compared to the pure and epoxy-based surfaces. And again, the commercially available titanium surfaces had the slowest proliferation rate (Figure 5.6).

Figure 5.6: After 7 days of incubation, the proliferation results suggested although there was no statistical significance, there was a noticeable trend where the hybrid surfaces had a higher cell proliferation rate than the epoxy surfaces. In addition, the micron-sized TiO$_2$-containing surfaces also outperform their none-containing counterparts. (n=5, each alphabet represents a statistical group, p<0.05)
Cells were metabolically active and biomineralization was observed on enhanced surfaces

The initial metabolic activity of the cells seeded on the various biomaterial surfaces were studied though a MTT assay. The MTT assay suggested that after 24 hours, all of the cells that were seeded onto the biomaterial exhibited high levels of metabolic activity. Furthermore, the results revealed that the commercially available titanium and epoxy-pure surfaces exhibited the higher metabolic activities than the cells raised on hybrid surfaces. However, the addition of micro-size TiO$_2$ did not appear to cause any effects on the metabolic activities of the cells (Figure 5.7). Similarly, after 7 days of incubations, the cells raised on conventional titanium again showed high levels of mitochondrial activities while the cells from the hybrid surfaces were comparatively less active (Figure 5.8).
Figure 5.7: The 24 hour MTT assay indicated that the conventional titanium and epoxy-pure surfaces exhibited the highest mitochondrial activities, where hybrid surfaces had similar and lower metabolic activities. On the other hand, the cells raised on micron-sized TiO$_2$ containing epoxy surfaces demonstrated the least amount of mitochondrial activity. (n=7, each alphabet represents a statistical group, p<0.05)
Figure 5.8: The 7 day MTT assay indicated that the HEPM cells that were seeded on titanium, micron-sized TiO$_2$-containing Epoxy and the non-TiO$_2$ containing surfaces had similar mitochondrial activity, whereas the micron-sized TiO$_2$-containing hybrid surfaces exhibited the least amount of activity. (n=7, each alphabet represents a statistical group, p<0.05)

In addition, various degrees of biomineralization were noted amongst the different biomaterial surfaces. The results suggested the hybrid group outperformed rest of the surfaces, followed by the epoxy; on the other hand, the conventional titanium discs showed the least amount of Alizarin Red staining, suggesting minimal levels of mineral deposits. However, all of the surfaces were able to accumulate mineral deposits over time by showing an increased Alizarin Red staining at 4 weeks compared to 2 weeks (Figure 5.9).
Figure 5.9: HEPM cells were plated on enriched and control surfaces. These cultures were incubated for 24 hours in DMEM medium, then switched to differentiation medium for reminder of the assay to induce biomineralization. The surfaces were stained with Alizarin Red-S to visualize mineral deposits. (A) After 2 weeks, some mineral deposits were detected on the Epoxy/Polyester Hybrid and the Epoxy/Polyester Hybrid-micron-sized TiO2 coated surfaces, and in smaller extent on the Epoxy-TiO2. (B) After 4 weeks, much heavily stained areas were observed on the Epoxy-Polyester and the Epoxy-Polyester-TiO2 coated surfaces.
5.4 Discussion

Numerous studies have illustrated potential of using functionalized ultrafine polyester particles to enhance the biocompatibility and osteo-inductivity of orthopedic implants (18–22). However, after the discovery of possible hazards of triglicydyl isocyanurate (TGIC), an essential component in the polyester coating particles, an alternative coating formulation is needed for continuing the developing of this promising technology. Recently, two new polymers, epoxy and epoxy/polyester hybrids, were found to be plausible candidates for replacing pure polyester coating particles. This study comprehensively studied the physical characteristics as well as the biological performances of epoxy and epoxy/polyester hybrid surfaces to examine their suitability for improving conventional orthopedic implants.

These enhanced biomaterial surfaces were constructed from conventional biomaterial using a novel and patented electrostatic ultrafine dry powder coating technique. This technique was a product from the Particle Technology Research Centre at the University of Western Ontario (31;30), although novel, its reliability and performance already has been well studied (18;19;21;26;11). The polymeric backbones used in this ultrafine particle surface enhancement were either epoxy, or 50:50 epoxy:polyester hybrid, which neither of them rely on the use of TGIC. In addition, micron-sized TiO$_2$ pigment (25 weight percent) was also incorporated to increase the biocompatibility of the enhancement particles.

Previous studies showed that ultrafine particles have the beneficial characteristics creating nano-scale surface topographies and roughness on conventional titanium, which in turn improve the biocompatibility and osteo-inductivity of the biomaterial (11;18;19;21; 26). Consequently, nano-sized TiO2 (nTiO2) particles (0.5%) were included as flow modifiers to prevent the ultrafine particles from aggregating and ensure
the proper dispersion of coating particles; also, the nTiO$_2$ has been found to have the additional benefit of providing further increased biocompatibility to the coated surfaces (18). These biocompatible coatings (21) were further enhanced by including a ceramic insert, CaO (5 weight percent), as the bioactive agent, which has been found to encourage osteo-inductivity and biocompatibility (11). This low amount of functional additives guarantees the continuity and homogeneity of the base coating. Moreover, because these biomaterials were developed using an electrostatic dry powder coating technique, the toxic hazards of solvents and volatile organic compounds which were usually associated with liquid coating process were eliminated, consequently, making these surfaces even safer and more reliable.

After the final coating particles were developed, the actual enrichment composition of the surfaces including bioactive agents was confirmed by thorough chemical analyses using energy dispersive X-ray spectroscopy (EDX). EDX concluded that all of the surfaces exhibited similarly high proportions of carbon (C) and oxygen (O) as the predominant elements, calcium in (Ca) in smaller proportions and the minimal presence of gold (Au), which is consistent with polymer resin being the major constituents, calcium oxide as the bioactive additive and gold sputtering before EDX. Consequently, these data confirmed that the overall composition of the coatings remained largely unaffected after enrichment, which is expected since the additives only occupied a minor component of the formulations. Nevertheless, EDX detected varying concentrations of titanium, which correlated whether the surface enhancement formulation contained micro-sized TiO$_2$. In conclusion, these findings agreed with prior analyses of similar coatings and the reliability of using the ultrafine particle technology in enriching conventional titanium substrates (21)(18).

Sequentially, the adhesive strength of the surface enhancements were also examined using the protocol suggested by ASTM D3359. This series of assays were conducted over a span of 28 days, where the coating adhesions were examined at 7, 14 and 28 days after
the biomaterial was submerged in DMEM media. The results showed that there were no
significant differences in adhesive strength between the different surfaces; even after 28
days of being submerged in DMEM media, the adhesion of the surface enrichments did
not significantly change over time. Similarly adhesion abilities were also found in
surfaces coated with other polymeric particles (21)(23). Consequently, the adhesive
strength assays concluded that all of the surfaces were tightly attached onto the
underlying titanium substrate and all of them achieved an adhesion rating of 5B
according to the ASTM D3359 protocol.

Furthermore, it has been well established that hydrophilic wetting characteristics, such as
the surface wetting and water contact angle, can reveal information and account for the
biocompatibility of a material (Hu et al., 2006; Menzies and Jones, 2010; Tsuji et al.,
1998; Altankov et al., 1996). Thus, the hydrophilicity of enriched biomaterials were
studied using a goniometer. Although all of the surfaces were deemed to be hydrophilic
and expected to be biocompatible, none statistical significant trends were observed. The
goniometric data suggested that there was greater hydrophobicity, a larger contact angle,
on the surfaces that contained micro-sized TiO$_2$. Similarly, epoxy polymers were also
more hydrophobic than epoxy/polyester hybrid surfaces. The incorporation of micron-
sized TiO$_2$ might contribute to the surfaces’ hydrophobicity of biomaterial because
surfaces that contained micron-sized TiO$_2$ had smaller size distribution. Previous studies
have found that the surfaces which were coated with ultrafine particles exhibited nano-
topographies that were able to trap air bubbles which in turn increased the hydrophobicity
of the subsequent surfaces (28). Thus, with the smaller size distribution, micron-sized
TiO$_2$ surfaces might have been more effective at trapping air bubbles and thereby making
them more hydrophobic. Furthermore, the increased hydrophilicity observed in
epoxy:polyester hybrid surfaces could be the result of the incorporated polyester.
Polyesters are more polar than epoxies because of the carbon double-bonded oxygen in
their ester group; since water is also a polar molecule, the ester groups in the
epoxy:polyester hybrid surfaces helped attracted water, consequently improving the
hydrophilicity of the resultant surface. As a result, the water contact angle studies
suggested that by modifying the enhancement formulation, the physical properties of the surfaces were also altered.

The *in vitro* performance of the enriched biomaterials were firstly studied by examining the metabolic activity of the HEPM cultures that were plated on each of the biomaterial surface. Through MTT assays, the results showed that after 24 hours of seeding the highest mitochondrial activities were observed on the titanium substrate and the pure epoxy surfaces, whereas the epoxy/polyester hybrid surfaces exhibited lower activities. Similarly, after 7 days of incubation, the mitochondria were again more active in the cultures that were raised on titanium and epoxy surfaces, while the epoxy/polyester hybrid metabolic activities remained fairly low. This phenomenon could be explained by the early cellular differentiation that had occurred in the cells seeded onto the hybrid surfaces; previous studies have found that mature osteoblasts realize more heavily on glycolysis for energy, which might have caused this decrease in mitochondria activity (29)(30).

In addition, the biocompatibility of these coatings was also clearly demonstrated in this study. Firstly, after the 24 hours of plating, HEPM cells have already began to attach onto their respective biomaterial. Similarly, the cell proliferation ability of the surfaces were also confirmed after each of the cultures were left in the incubation for 7 days. Through hemocytometry, both of the attachment and proliferation results showed that the conventional titanium substrate had the lowest performance, whereas the micron-sized TiO$_2$ coatings were able to significantly improve the surfaces’ cell attachment ability. Again, a noticeable trend of enhanced performance in epoxy/polyester and micron-sized TiO$_2$ containing surfaces were observed in both 24 hour and 7 day assays. These finding correlated with the previous findings which noted that smaller particle size distribution could be achieved with the incorporation of micron-sized TiO$_2$ (18)(22); in addition, water contact angle assays also concluded that the epoxy/polyester hybrid surfaces were
more hydrophilic, which could contribute to the attachment and proliferation of cell culture.

In addition to biocompatibility, the osteo-inductive potentials of the surfaces were also verified by examining in vitro bone-like mineral formation using Alizarin Red staining. The results confirmed that all of the coatings were able to support the biomineralization of HEPM cells. The mineralization was visualized using Alizarin Red-S, which is a stain that binds specifically to the mineral deposits through its sulfonic acid and hydroxyl groups (31). The staining showed detectable mineral formation within 2 weeks, and an increase in mineral accumulation over 4 weeks. The least amount of mineralization was observed on the titanium controls, and more moderate amounts were seen on the pure epoxy surfaces. Whereas much higher levels of staining, suggesting great extent of mineralization were seen on the epoxy/polyester hybrid surfaces. Likewise, prior studies showed enhanced biomineralization on polymeric coatings that had been enriched with commercially available MTA minerals (22). The heightened biomineralization observed on epoxy/polyester hybrid surfaces under enriched media could the results of the better biocompatibility that such surfaces were able to offer as seen in the cell count, MTT, and hydrophilicity assays.

Collectively, this study confirmed the possibility of using epoxy and epoxy/polyester hybrid polymers as TGIC-free alternatives for replacing the conventional polyester particles. These two types of polymers can be applied without any modifications of the current application apparatus and the resultant surfaces are also highly compatible and osteo-inductive. Through careful physical surface analysis such as water contact angle and in vitro examinations which include cell attachment and proliferation, metabolic activity and biomineralization assays, the micron-sized containing epoxy/polyester surfaces appeared to be the best candidate. In the future, animal studies should be conducted to study the in vivo performance of such enriched biomaterial to reveal their clinic potentials.


5.5 References


Chapter 6

6 Conclusions and recommendations

6.1 Conclusions

During this doctoral project, the utilization of ultrafine particle coating technology in enhancing titanium biomaterial substrates was optimized. This was accomplished through the investigations of several ultrafine particle formulations to elucidate the factors that enhance the biocompatibility as well as osteo-inductivity of titanium substrates for bone implants. Three different areas of improvements on ultrafine-particle formulation were individually studied, they include the refining of functional additives, the examination of alternative backbone polymers and the effects of purity of backbone. During the surface enhancing process, ultrafine particles of D0.5 at 30 µm with the aid of flow modifiers were applying onto conventional titanium surfaces using a corona electrostatic spray gun and then cured in a curing oven.

Functional additives were optimized to effectively promote the biocompatibility and osteo-inductivity of conventional orthopedic implant substrates. The candidate functional additives were selected from various biomaterials such as mineral trioxide aggregate (MTA) and Bioactive glass, which all have been shown to be highly effective in clinical. These functional additives were organized into different sets of concentrations, in vitro cellular experiments were then conducted to examine their results on mesenchymal cell cultures. Consequentially, the optimal candidate for improving the biocompatibility and osteo-inductivity of conventional implants was selected based on cellular response. After thorough examinations of the physical and as well in vitro properties, 5 weight percent calcium oxide enhanced substrates exhibited highly hydrophilic surfaces; in addition, such surfaces demonstrated the highest ability in supporting cell cultures and encouraging
biomineralization. Thus, 5 weight percent of calcium oxide was deemed to be the better functional additive.

In addition, this project also confirmed the possibility of using TGIC-free polymers, such the utilization of pure epoxy and epoxy/polyester hybrid polymers to replace the conventional pure polyester backbone. These alternative polymer backbones have the benefits of not replying on curing agents such as TGIC, which has been shown to be toxic and consequently banned in the European Union. During the search of a safer polymer backbone, this study demonstrated that not only pure epoxy and epoxy/polyester hybrid polymers can be easily reduced to the size of ultrafine particles, but also still exhibited good flowability. Moreover, the resultant backbones after curing were proven to possess excellent substrate adhesion ability. The in vitro cell studies suggested the epoxy/polyester hybrid based surfaces outperformed pure epoxy surface in being the more biocompatible through hemocytometry and the osteo-inductive through mineral-staining; nevertheless, both surfaces exhibited comparable or better cellular responses when compared to pure epoxy-based surfaces.

Furthermore, the effects of backbone polymer purities were also determined. The surface analysis results suggested the incorporation of micro-sized titanium dioxide allowed for smaller sized ultrafine particles to be developed, which previously has been found to increase surface roughness (22) (23); however, with the smaller particle sizes, the micro-sizes titanium dioxide containing formulations also exhibited a reduced flowability. Nevertheless, the in vitro cell studies showed that the micro-sized titanium oxide enriched surfaces exhibited higher compatibility through cell counts and higher osteo-inductivity through mineral staining while compared to their respective pure counterparts. As a result, this study confirmed the benefits of incorporating micron-size titanium oxide.
In summary, while this doctoral project confirmed the feasibility of applying the ultrafine particle technology on titanium based biomaterial, it also refined the ultrafine particle formulation to ensure biocompatibility while encouraging biomineralization. 5 weight percent of calcium oxide has been demonstrated to be the ideal bioactive additive and concentration. In addition, epoxy/polyester-based hybrid backbone polymers have found to be TGIC-free alternatives for the conventional pure polyester-based polymers. Furthermore, the introduction of micro-sized titanium oxide also further improved the biocompatibility and the osteo-inductivity of the sequential surfaces compared to their pure counterparts.

6.2 Recommendations and limitations

Throughout this doctoral project, although the utilization of ultrafine-particle technology on dental implants have thoroughly examined and the different aspects of the surface enhancement formulation have been optimized, there is more experimental work that need to be conducted before this technology can be transferred to clinic. Thus, to build on the content of current study, the following recommendations should be considered for future work:

➢ Although all of the three objectives that were planned during this program have been met, this Ph.D. project was still limited to in vitro studies. Thus, in vivo experiments could be conducted in the future to better understand how enhanced bone implants would perform within one’s body to offer more convincing results for the transition of such enhanced implants to the clinical.

➢ For any load-bearing bone implants, it is crucial that not only there is a sufficient amount of bones at the site of implantation, but it is also necessary to have high quality of surrounding bones anchoring the implant. Thus, during future in vivo
surveys, histological studies could be included to examine the bone-implant interface. From such studies, information such as the intimacy of bone-implant contact, as well as the bone morphology and density can be revealed. In addition, mechanical examinations such as the pull-out test could be conducted to further verify the stability of the implant.

- In addition to examining the anchorage of the implant, the body’s immune responses to implants may be studies in the future. This doctoral project demonstrated the cellular biocompatibility of the enhanced commercially available titanium biomaterial that were constructed using the ultrafine-particle technology, however such results do not reveal on the possible responses of a body’s immune system. Thus, *in vivo* examination of an intact organism’s immune system response of such bone implants would be conducted to reveal a more in-depth understanding of the immune-biocompatibility.

- Furthermore, although previous studies have shown that such polymeric biomaterial surfaces were able to induce the differentiation of mesenchymal cells, this studies only examined the extent of mineral deposits on the constructed surfaces. Thus, future studies should be conducted to examine the expression of osteogenic genes such as BSP and RUNx2 to confirm the correct differentiation of the mesenchymal cells and the source of mineral deposits. Moreover, the MTT assays conducted during this doctoral project only measured the overall metabolic activity in each of the cultures, the claims of the biocompatibility could be strengthened of the individual cellular metabolic activity was measured.

- Titanium has typically been used as the substrate for bone implants due to its strength and its ability in forming a highly inert titanium oxide layer once it is inside one’s body. However, with the utilization of our surface-enhancement technology, cheaper metals that normally are not as inert in the bodily environment can be coated using enhanced ultrafine-particles to replace the use of
conventional titanium substrate. Consequently, the cost of dental and orthopedic implants can be significantly reduced.
Appendix

An overview on the novel development of biocompatible coatings for bone implants
- A review of our research progress up to date

Introduction

Orthopedic and bone implants enjoyed much success in the recent medical history due to its efficacy in enhancing or replacing damaged tissues. The material of choice of such implants are typically commercially available pure titanium or titanium alloy. Titanium has been proven to be an excellent candidate for bone implants because of its inert titanium dioxide layer which forms under the exposure to moisture; this passive layer then essentially act as a barrier between the implant and tissues, effectively minimizing the chances of the implant from further oxidation, thereby minimizing the chances of immuno-response. However, in addition to promoting biocompatibility, an ideal orthopedic implants would also need to ensure the appropriate integration of implant into the patient’s body in a process known as osteo-integration. Only with a sufficient amount of osteo-integration, can the host then provide proper archer to retain the implant, and consequently decreasing the chances for loosing of the implant and increasing its post-surgical success. Thus, much of the current research interest has been invested to encourage the biocompatibility and the osteo-integration of orthopedic implants.

Many different approaches have been proposed to improve the performance of bone implants. Studies demonstrated that the implant surfaces which mimic the bodily physiological environment exhibited the best potential for promoting desirable cellular responses. As a result, much effort has been spent on incorporating chemical molecules such as hydroxyapatite (Ca_{10}(PO_{4})_{6}(OH)_{2}), calcium phosphate (Ca_{3}(PO_{4})_{2}) and calcium oxides (CaO), as well as other arrays of mineral oxides onto the surfaces of biomaterials to better mimic bodily tissue (49–56). Amongst the different minerals that have been incorporated and studied, researchers found that calcium is an especially beneficial surface enriching molecule because it has been proven to be...
very osteo-inductive and is very effective at encouraging bone formation around the implants. A recent study demonstrated that the incorporation of calcium ions onto the surfaces of biomaterials promoted bone growth around the implant in rabbits’ femora (21). Similarly, other animal studies have also confirmed the beneficial effects of calcium such as improved bone formations in rat femora, increased healing of intrabony defects in dog mandibles, and enhanced resistance to dislodgement in rabbit tibia (16,19,20). Furthermore, silica-calcium phosphate nano-composite coatings also showed improved alkaline phosphate activity of bone marrow stem cells that were attached onto the surfaces of biomaterials (18).

To enrich the surface of biomaterials, researchers utilized techniques such as plasma spraying and sputter coating (28,40–42). However, the surfaces created from these methods were often either too thick, lacked surface homogeneity, exhibited poor bonding strength, or the techniques were too expensive and time consuming (43). Thus, although research has shown promising results in improving the biocompatibility and osteo-integration of bone implants, a more effective technique is needed to realistically incorporate various mineral oxides onto the surface of biomaterials. To overcome this obstacle, we adopted our patented ultrafine particle coating technique, which allowed us to effectively construct highly adherent while thin surface film to improve the biocompatibility and osteo-inductivity of bone implants (5–7,22,23).

Powder coating has two major advantages over the conventional liquid coating techniques, one of which is the environmental-friendliness through the elimination of toxic solvents, while the other advantage is the economical-efficiency achieved from the ability to recycle over-sprayed particles. Nevertheless, the field of dry powder coating has been saddled by one bane, the poor surface smoothness that is caused by the large coating particles. Ultrafine particles have been postulated as the solution to this phenomenon; however, due to the decreasing particle size, intermolecular forces eventually begin to dominate, resulting in the aggregation of coating particles. To overcome this drawback, our research group has developed a novel ultrafine particle technology where nano-additives were incorporated into the coating particles to act as spacers, thereby increasing the intermolecular distances and decreasing the intermolecular forces between the particles, consequently minimizing the aggregation of ultrafine paint particles (1)(2). Similarly, based on this idea, we demonstrated that not only we were able to develop smoother
surfaces using ultrafine powder coating, but the use of functional additives also opened a realm which allows research to enrich surface with various properties. During this study, we will discuss the uses of this technology along with functional additives to improve the biocompability and the osteo-inductivity of orthopedic implants (5–7,22,23).

During the coating of the titanium bone implant substrate, a polyester base layer is created which acts as a scaffold and has been shown to possess design flexibility, and various calcium containing functional additive mixtures are incorporated to the coating, forming a surface that promotes bone formation (57,58). Over the last few years, we demonstrated that through this technique, we were able to construct uniform, continuous, homogenous and highly adherent as well as biocompatible polymer coatings. Studies have also demonstrated that such surface also contained intricate nano-topographies and surface roughness, which were also proven to enhance biocompability (7,22,23). The functional additive consisted of only a small fraction of the surface coating particles to ensure the composition and integrity of the base polyester (5,7). Nevertheless, significant physical and biological differences were observed, confirmed using cell cultures (5,7). The cell line used was a human embryonic palatal mesenchymal cell line (HEPM, ATCC CRL1486), which was derived from the developing palate of a human fetus. This cell line has historically provided a clinically relevant model to study the cellular response to implant surfaces (59–61). Our cell studies demonstrated that the human mesenchymal cells attached, spread out, proliferated and differentiated on the surfaces of modified biomaterials, and initiated biomineralization in extended culture (5–7,22). Consequently, our research group hopes to use ultra-fine particle technology to develop more biocompatible and osteo-inductive biomaterial surfaces making orthopedic and bone implants more effective.

**Materials and Methods**

**Sample preparation**

**Bone implants**
In clinical, bone implants, typically composed from commercially available pure titanium or titanium alloy, are anchored into the tissue through its screw-portion. Thus, the stability of the implant is highly depended on the contact between the surface of implant’s screw portion and the surrounding bones. To simulate the *in vivo* environment, one side of titanium substrate will be coated using functionalized ultrafine particles, and HEPM cells are then seeding onto the enriched side.

**Preparation of ultrafine coating particles and the modification of biomaterial**

The ultrafine particles were prepared as previously described [2], [4], [5] and [6] (Figure A1), which were modified using bioactive agents (Table A1). The basis of this coating mixture is composed of a polyester resin and micron-sized TiO$_2$ (25%) pigment. These powders were mixed and grounded in a high-shear grinder to obtain ultrafine particles (>30 μm) that were then refined passing through a sieve (35 μm). To aid the flowability of such ultrafine particles, nano-TiO$_2$ (0.5%) particles (Degussa, USA) were added, and bioactive functional additives were added to promote biocompatibility and osteo-inductive. The coating mixture was then combined in the high-shear mixer and again passed through a sieve (35 μm) to obtain final ultrafine particles.

The implant surfaces were then modified using the ultrafine powder-coating technology [8] and [9], as previously described [2], [4], [5] and [6]. The coatings were spraying ultrafine particles onto smooth commercially pure titanium substrates (cpTi, Grade 2, thickness = 0.5 mm, McMaster-Carr, Cleveland, OH) (Fig. 1). A Corona Gun (Nordson, Westlake, OH) was used to apply a voltage (20 kV) which ionizes and sprays the ultrafine particles onto grounded titanium substrates. Finally, the thin layer of particles on the titanium substrate were cured (200 °C, 10 min) in a high performance air flow oven (Sheldon manufacturing, Inc., Cornelius, OR). The coated titanium substrates were then cut into regular sized disks (diameter = 24 mm) for further analysis.
Ultrafine particle coating technology was utilized to construct these novel biocompatible, bioactive enriched surfaces. The base polymer and the functional additive were sheared and sieved (35 µm) to obtain ultrafine particles; the sequential mixture was then mixed again with nano-sized titanium dioxide (nTiO2) to improve this flowability. The surface enhancement particles were then electrostatically sprayed onto grounded titanium (cpTi) disks, cured in a furnace and prepared for physical and biological examinations.

Table A1: An example of the enhancement particle formulation.

<table>
<thead>
<tr>
<th>Coating</th>
<th>Base polymer</th>
<th>Flow additive (wt. %)</th>
<th>Bioactive agent (wt/ %)</th>
<th>Bioactive calcium (mmol/100g)</th>
<th>Bioactive phosphate (mmol/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPC</td>
<td>Polyester resin</td>
<td>nTiO2 (0.5%)</td>
<td>None</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>PPC + 1% CaO</td>
<td></td>
<td></td>
<td>CaO (1%)</td>
<td>17.83</td>
<td>0.00</td>
</tr>
<tr>
<td>PPC + 5% CaO</td>
<td></td>
<td></td>
<td>CaO (5%)</td>
<td>89.16</td>
<td>0.00</td>
</tr>
<tr>
<td>PPC + 5% CaP</td>
<td></td>
<td></td>
<td>CaPO4 (5%)</td>
<td>0.29</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Physical characterizations

Particle size analysis

Particle size analysis was conducted to ensure the ultrafine nature of the coating particles. The dimensions of these ultrafine particles powders were examined using a BT-9300s Laser Particle
Analyzer (Ningbo Yinzhou Hybers, China). The powders were suspended in water and a laser stream was used to create a diffraction pattern that was then reflected onto a detector and analyzed to measure particle size.

**Surface analysis**

The modified biomaterial surfaces were characterized for their surface composition and homogeneity as previously described [6]. The surfaces were analyzed using energy dispersive X-ray spectroscopy (EDX) with a Hitachi S-4000 SEM (Hitachi, Pleasanton, CA). The coated biomaterials were mounted onto metal stubs, secured with adhesive carbon tape and sputter coated with nano-sized (10 nm) gold particles. The working voltage and working distance of the SEM were set at 15 kV and 15 mm, respectively. Sequentially, each element was identified (minimum detection limit = 0.0%) and its presence calculated (wt.%). The EDX analyses were repeated at three separate locations on each surface, and the mean concentrations of the key elements were calculated. Additionally, the EDX analyses were repeated across the entire surface of each disk to identify the presence and distribution of the key elements, providing an elemental mapping which demonstrates the distribution of key elements across each surface.

**Hydrophilicity assay**

The different modified biomaterial surfaces were compared for their wettability which has been associated with biocompatibility through measuring their water contact angles. Water droplets (80 μl) were dripped onto each surface, and their water contact angles measured using a Ramé-Hart Model 100 goniometer (Ramé-Hart Instrument Co., Succasunna, New Jersey). These measures were repeated 5 times to ensure reproducibility and an average was taken.

**Surface roughness analysis**

The roughness of coated surfaces, which has been shown to aid cell attachment, were analysed with a dynamic force mode XE-100 atomic force microscope (AFM) (Park Systems, Suwon, Korea). The spring cantilever had a length of 125 μm, a width of 40 μm and a thickness of 4 μm.
The spring constant was ~40 N m$^{-1}$ and the nominal tip radius of the silicon cantilever was 10 nm. The biomaterials were examined at room temperature, and 256 × 256 pixel resolution images were taken. These images were analysed with image processing and analysis software (XEI 1.7, Park Systems) to exhibit the roughness of each individual surface.

**Biological performance**

**Protein localization and visualization**

Protein localization and intracellular features were visualized using immunochemistry. Human embryonic palatal mesenchymal cells (HEPMs, ATCC CRL-1486) were seeded onto various biomaterials in 24-well tissue culture plates (50 000 cells/well). The cultures were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with fetal bovine serum (FBS, 10%), L-glutamine (2 μmol ml$^{-1}$), penicillin G (100 U ml$^{-1}$), streptomycin sulphate (100 μg ml$^{-1}$) and amphotericin B (0.25 μg ml$^{-1}$)) at 37 °C within an incubator. After 24 h, the surfaces were harvested and washed three times using phosphate buffered saline (PBS). The cells that were attached to the surfaces were fixed in paraformaldehyde (4% for 10 min) and permeabilized by Triton X-100 (0.1% for 5 min). The actin filaments of the cytoskeleton were labelled (2 h) with rhodamine phalloidin (Cytoskeleton, Denver, CO). The surfaces were then mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and sequentially examined by an inverted fluorescence microscope (Axiovert 40 CFL, Carl Zeiss Canada Ltd, Toronto, Canada).

**Optical and scanning electron microscopy**

Cell morphology can often reveal information about the biocompatibility of the surface; thus, optical and scanning electron microscopy were conducted to examine the morphologies of the HEPM cells raised on various biomaterials. After 24 h of incubation, the biomaterials were removed from the incubator and rinsed three times in PBS. Cells that remained attached to the disks were fixed in paraformaldehyde (4% PFA, Fisher Scientific) for 24 hours at room temperature. The disk surfaces were then examined using an optical microscope at 100 × and 400 × magnification, and images were captured digitally.
In addition, cell morphologies and surface interactions were visualized by SEM. After 24 h of cell attachment and spreading, the surfaces were collected and washed three times using PBS. They were then fixed with glutaraldehyde (2.5% for 20 min) in cacodylate buffer (100 mM), dehydrated in ascending grades of ethanol (25%, 50%, 75%, 95% and 100%) and immersed in hexamethyldisilazane. Finally, the surfaces were air dried, mounted on metal stubs, sputtered with nano-sized gold particles (20 nm) and examined with a Hitachi S-2600 SEM (Hitachi, Pleasanton, CA).

**Biomineralization assay**

The surfaces of biomaterials were assessed for their capacity to support osteogenic differentiation and the biomineralization of cell cultures. Biomaterials were incubated in culture media, seeded with HEPM cells and for 24 hours. After the initial 24 hour period, DMEM were replaced with enriched media containing that contained ascorbic acid (50 μmol/ml) and β-glycerophosphate (10 μmol/ml) as additives to induce osteogenic differentiation. The osteogenic media were replenished every 3 days and the cultures maintained for either 2 weeks or 4 weeks.

Following the 2 and 4 week of differentiation period, the media were discarded and the cultures gently rinsed in PBS and fixed in formalin (4%) for 1 h. They were then rinsed twice in calcium-free Nanopure water and stained with Alizarin Red-S (2%, EMD) for 10 min at room temperature. Lastly, the surfaces were examined for Alizarin Red stained mineral deposits and images were captured digitally.

**Cellular assays**

**Cell proliferation assay**

The ability of cellular attachment and growth were quantified by cell attachment assays. Human mesenchymal cells were seeded onto various surfaces in 24-well tissue culture plates (50 000 cells/well). After 24 and 72 h of cell attachment and growth, the biomaterials were transferred to separate 24-well tissue culture plates and triplicate cultures harvested from each surface. They were then rinsed with PBS to remove unattached cells. Trypsin (150 μl) was added to each well
and the culture plates were incubated (37 °C for 5 min) to release the attached cells. These cells were then collected and counted using a hemocytometer.

**Metabolic activity**

Cell viability and metabolic activity were measured using the MTT assay. After 24 and 72 h of cell attachment and growth, the surfaces were rinsed with trypsin to release attached cells. They were then collected and reseeded into multiple 48-well tissue culture plates. After 24 h, MTT reagent (tetrazolium (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) was added to each cultures before they were incubated (37 °C) for an hour in the dark. The reagents were then replaced with an MTT solubilizing solution (acid-isopropanol), and the absorbance (570 nm) were measured in a Safire Multi-Detection Microplate Reader (Tecan, San Francisco, CA).

**Gene expression**

The attached cells were analyzed for their expression of key marker genes through RT-PCR analysis. HEPM cells (200000 cells/well) were seeded onto replicate of biomaterial surfaces in 6-well tissue culture plates. After 24 h, ascorbic acid (50 μmol ml⁻¹) and β-glycerophosphate (10 μmol ml⁻¹) were added to the media to induce osteogenic differentiation.

At incubating in osteogenic media, cell cultures were harvested to analyze their gene expression. The cultures were rinsed three times with PBS to remove unattached cells and the cells’ RNA was extracted with an RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The cells were then re-suspended in lysis buffer and homogenized by passage through a QIA shredder column (Qiagen). The homogenized lysate was then applied to the RNeasy column, rinsed repeatedly with a series of buffers (RLT), and eluted into RNase-free deionized water. These RNA extracts were kept at −70 °C for storage. Then, aliquots of the RNA extracts were diluted in deionized water and examined using a bioanalyzer (Agilent Technologies, Wilmington, DE) to measure concentration and ensure purity.
During analysis, RNA extracts were subjected to conventional RT-PCR Analysis with human-specific primers for Runt-related transcription factor 2 (RUNX2), type I collagen (COL1A1), alkaline phosphatase (ALP), bone sialoprotein (BSP) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as previously reported [38]. The RNA was reverse transcribed into cDNA with Oligo(DT) primer and SuperScript TM II RNase H Reverse Transcriptase at 42°C for 50 min. The specific transcripts were then amplified in separate tubes using PCR with gene-specific primers and Platinum R© Taq DNA polymerase. The thermal cycling parameters were set at 94 °C for 2 min to activate the polymerase, followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min. The RT and PCR reactions were performed in an AmplitronR© II thermocycler (Barnstead Thermolyne, Dubuque, IA). The PCR products were separated on an agarose gel (1%) containing ethidium bromide (0.05 μg ml\(^{-1}\)), visualized on a trans-illuminator (Fisher, Pittsburgh, PA) and recorded digitally (Panasonic, Japan).

Results and Discussion

Through the incorporation of bioactive functional additives via ultrafine particle technology, our research group demonstrated that this technology can be successfully utilized to improve the biocompatibility and osteo-inductivity of titanium orthopedic and bone implants (5,7). Titanium and its alloys were typically the choice for bone implants due to the inert passive titanium dioxide that acts as an insulation layer which reduces the chances of inflammation. However, the success of orthopedic implants is also heavily dependent on a process of osteo-integration. Previous studies have suggested that such process can be affected by various characteristics of the implant surface such as surface roughness, hydrophilicity and surface chemical composition.

Our research team postulated that we can use the ultrafine particle technology to incorporate various bioactive mineral additives onto the surface of implant substrate and thereby to find the optimal additive for increasing the biocompatibility and the osteo-inductivity of orthopedic implants. This technology relies on the use of nano-flow additives to reduce the aggregation of ultrafine particles, and the incorporation of bioactive-functional additives to enhance the biocompatibility and the osteo-inductivity of orthopedic implants. Prior to the application of our
surface enhancement particles, particle size analysis was conducted to ensure the ‘ultra-fine’ nature of our formulation particles. The resultant biomaterials were then assessed for their physical characteristics, biological performances, as well as cellular assays for their efficacy to determine the best potential surface coating for orthopedic implants.

**Physical characterizations**

Prior to coating the implant substrate, the sizes of coating particles were analyzed using a laser particle size analyser. Though laser analysis, the results confirmed the ultrafine classification of the coating particles by exhibiting a D50 less than 30 microns (Figure A2). In addition, particle size analysis also indicated the uniformity of the particles through their size-distribution, demonstrating the reliability of our technique. After the implant substrate has been coated, the resultant surfaces were further studies and compared. The coated biomaterials were then examined for their surface composition using EDX. The results demonstrated that the majority of the surface coating is composed of carbon and oxygen, which complies with the chemical composition of polyester. EDX also revealed that by varying the bio-additives, the sequential changes in the chemical composition can be effectively detected. Specifically, the results from EDX demonstrated that the presence of calcium increased with the proposed enrichment formula (Figure A3). Similarly, specific elements were only detected in their respective designated formulation groups, such as phosphorous was only detected in calcium phosphate coated surfaces. These findings demonstrated that the surfaces were coated according to our anticipation and mineral formulation, and confirmed the accuracy of the ultrafine coating technology.
Figure A2: Laser particle analysis indicated that all of the particle mixtures constructed using the ultrafine particle technology had a D50 of less than 35 microns, confirming the ultrafine nature of the particles. In addition, all of the coating particles exhibited a similar size distribution, illustrating the reliability of the ultrafine particle technology.
Figure A3: EDX demonstrated that the surface coating particles were composed mostly out of carbon, oxygen, and titanium. However, there is smaller quantities of aluminum, silicon and sulfur that came from the polymer backbone. In addition, the calcium presence increased with the increasing incorporation of calcium oxide, and phosphors was only detected in calcium phosphate-containing mixtures. Collectively, these findings confirmed the accurately of the ultrafine particle technology in incorporation functional additives.

Sequentially, elemental mapping was utilized to examine the localization and the distribution of bioactive functional additives. Like EDX, the results suggested that calcium, component of the bioactive additives, was absent in the titanium and polyester control surfaces and the presence of calcium also increased with its formulation, nevertheless elemental mapping also showed that calcium existed as small clusters throughout mineral containing surface. Furthermore, phosphorus was only observed in calcium phosphate enriched biomaterials; in addition, the location of calcium correlated with the location of phosphorus in calcium phosphate enriched surfaces, indicating that the electrostatic spraying process can safely maintain the integrity of the functional
additive, and demonstrating the non-destructive nature of the powder application technique (Figure A4). Together with EDX and elemental mapping, the reliability of our coating technique in accurately enrich titanium surfaces is confirmed.

Figure A4: Element mapping showed that calcium existed in smaller clusters while under low concentration, whereas it is more evenly spread out at higher concentration. In addition, element mapping also demonstrated that the phosphorus was co-localized with calcium in calcium phosphate surfaces.

Moreover, previous studies have suggested that the biocompatibility of the implant surface is highly depended on its hydrophilicity; thus, the water-wettability of enriched biomaterials were compared to the conventional titanium substrate. To examine the hydrophilicity of enriched
biomaterial, the water wettability of each surface was examined by measuring the water contact angle using a goniometer. Although all of the surfaces were hydrophilic, the results suggested that the commercially pure titanium exhibited highest hydrophilicity, followed by calcium oxide-containing surfaces and polymer-only surfaces, whereas the calcium phosphate-enriched surfaces were found to be the most hydrophobic (Figure A5). More interestingly, the results also illustrated a correlation between the hydrophilicity and the quantity of incorporated of calcium oxide. The outstanding hydrophilicity demonstrated by the titanium control substrate could be the result of the passive titanium dioxide layer that’s formed on the surface, which increased the polar nature of the surface. However, the weaker water-wettability exhibited by the polymer control surfaces could be the consequence of the extensive of carbon, a very neutral element. Nevertheless all of the surfaces were expected to be biocompatible due to their hydrophilicity. In addition to hydrophilicity, surface roughness has been shown to aid cell attachment and improve biocompatibility. Thus, the enriched biomaterials were also compared to the commercially available titanium substrate using AFM. Through AFM, we noticed that not only the enriched surfaces had a greater surface roughness at a micron level, when examined at a nano-level, a series of complex and intricate convexities and concavities were noted on enriched surfaces (Figure A6), which could in turn aid in the attachment of tissue cells and thereby increasing the biocompatibility of biomaterial.
Figure A5: Water wettability assay indicated that by increasing the % of surface calcium oxide, its hydrophilicity can be significantly increased. Furthermore, although all of the surfaces were hydrophilic, the 5% calcium oxide and the conventional titanium surfaces were significantly more hydrophilic than rest of the biomaterials.

Figure A6: Atomic force microscopy was used to assess the surface roughness of the biomaterial. The images showed the smooth surface of the conventional titanium substrate; however, after polymeric coating, intricate nano-sized topographies can be observed on the surface of biomaterial.
In conclusion, through physical assessments demonstrated the ultrafine-particle technology can be used not only reliably to produce ultrafine coating particles, but such particles can be accurately applied onto commercially pure titanium. In addition, the coated surfaces were found to be hydrophilic and coated complicated surface topographies which could aid in cellular attachment and improve their biocompatibility.

**Biological performances**

In order to obtain a more in-depth and relevant understanding, the biological performance of cells that were raised on such coated surfaces were examined to study the biocompatibility and osteo-inductivity of each biomaterial. The extent of such two quality were elucidated by an array of biological *in vitro* assays were conducted that examines the cell morphology and biomineralization activities. Using immunochemistry, the nucleus was stained using DAPI and the actin filaments were labelled with phalloidin. The fluorescent microscopy images illustrated that the cell cultures existed in clustered cultures as shown by DAPI, suggesting a high cell density, and the phalloidin staining demonstrated the stretched out filopodia, which is often associated with an intimidate cell-surface association as a result of the biocompatibility of the surface. Immunohistochemistry also revealed that there were numerous DAPI stains across all of the surfaces, suggesting the attachment of HEPM cells and the formation of cell clusters; furthermore, monoclonal antibodies illustrated the elongated morphology of actin filaments, demonstrating the intimate association between the HEPM cells and the biomaterial surfaces, and again, the compatibility of various biomaterial surfaces (Figure A7).
Figure A7: Immunochemistry was employed to study the cytoskeletal integrity of the attached cells. The images showed very organized actin filaments in all of the cells, and their stretch morphology.

Moreover, the associations between the biomaterial and the cellular filopodia were studied in more detail using optical and high power electron scanning microscopies. Again, the microscopy images illustrated the cell clusters and the close association between cell cultures and the underlying biomaterial, confirming the biocompatibility of enriched titanium surfaces (Figure A8). In addition, SEM images illustrated that all of surfaces were able to support cells and all of the cells exhibited a spreading morphology, suggesting the biocompatibility of all the examined surfaces. Furthermore, using optical microscopy, the same biocompatible characteristics of the coated surfaces were observed; however, optical microscopy also suggested that the cellular confluency of the biomaterial was increased through the increasing incorporation of calcium oxide resulting in the highest cellular confluence in 5% CaO surfaces, whereas the commercially pure titanium exhibited the least amount of cell confluency (Figure A9).
Figure A8: Scanning electron microscopy illustrated a high cell confluency of the coated biomaterial and a spreading morphology where cells exhibited extended filopodia, which consequently suggested an outstanding degree of the surface biocompatibility.
Figure A9: Optical microscopy suggested that all of biomaterials were able to support HEPM cell cultures. However, under lower power (100x), the images suggested that the cellular confluence correlated with the incorporation of calcium oxide. While at high magnification (400x), a spreading morphology was observed on all of the surfaces, indicating the close association between the cells and their underlying substrate.
As previously mentioned, in addition to being biocompatible, a successful orthopedic implant should possess sufficient osteo-inductivity to ensure proper integration between the implant and surrounding tissue. To study the effects of the coating on the osteo-inductiveness of the biomaterials, the biomineralization capability of each surface was assessed using Alizarin Red. Alizarin Red S dyes are known for their ability to bind specifically with mineral deposits through its sulfonic acid and hydroxyl groups; thus, it is often used to label and assess the presence of minerals (31).

The Alizarin Red staining showed that although all of the surfaces were able demonstrate an increased amount of biomineralization over time from 2 weeks of incubation to 2 weeks, there were differences between the levels of mineralization on each surface within the same time group (Figure A10). More specifically, a general trend of increasing mineralization with the increasing level of calcium oxide incorporation was observed in both times groups. On the other hand, although calcium phosphate and the polymer control surfaces also showed increased osteo-inductivity, their efficacies were not nearly as pronounced as calcium oxide. Lastly, the commercially pure titanium demonstrated the least amount of mineralization. The greater extend of biomineralization observed on enriched surfaces can be the result of the functional additive, calcium, acting as a seedling for mineralization, which increased the accumulation of mineral deposits. While the greatest extend of biomineralization which was observed on calcium oxide surfaces could be the result of a greater number moles of calcium that can be incorporated in calcium oxide compared to calcium phosphate, since the functional additives were measured by mass; similarly, studies have also suggested that the presence of phosphate has been shown to decrease cellular activity, resulting in the reduced biomineralization.
Figure A10: Biomineralization assay suggested even though all of the surfaces showed increased signs of mineral deposits over time, 5% CaO-containing surfaces exhibited the great degrees of Alizarin Red staining after 2 week and 4 weeks of proliferation and differentiation. However, conventional titanium showed least amount of mineral deposits amongst all of the surfaces.
As a result, through cellular assays such as immunochemistry, optical and SEM microscopy, as well as biomineralization assays, it is evident that all of the examined surfaces displayed levels of biocompatibility and osteo-inductivity. Nevertheless, the coated surfaces, especially the calcium oxide containing surfaces demonstrated the greatest level of biocompatibility and osteo-inductivity, placing it as the optimal candidate for enhancing orthopedic and bone implants.

**Cellular assays**

To confirm the positive biological responses induced by the coated biomaterial surfaces as illustrated by the *in vitro* cellular experiments, relevant quantifiable cellular assays were conducted to study the inductive effects of surface coating on gene expression. Firstly, noticeable cell confluency observed on coated surfaces during optical and SEM assessments were confirmed using cell counts. During this study, hemocytometry was employed to quantify the surface cellular confluency. The results suggested that the enriched surfaces can achieve a similar cell density as the cultures that were raised on commercially pure titanium surfaces. This phenomenon could be result of the biocompatibility polymers, where previous studies demonstrated that polymers such as polyester and polyurethane is highly biocompatible (Figure A11).
The cellular attachment ability was examined after 24 hours of incubation, while the cellular proliferation ability was studied after 72 hours of incubation. The hemocytometry assay suggested that polymer coated could achieve a similar cell confluency as the conventional titanium surfaces. In addition, the effects of bioactive coatings on cellular metabolic activity was also quantified using MTT assay. MTT is a colorimetric assay that reveals information regarding the cellular mitochondrial activity; thus, it is often used to assess the cellular metabolic activity. Through MTT assays, a similar trend to hemocytometry was observed. The data suggested that the cell cultures that were seeded onto enriched surfaces were able to exhibit higher metabolic activities than commercially pure titanium (Figure A12). By exhibiting encouraging the cellular metabolic activities, the attached cultures could be more efficient in encouraging bone formations which were observed on coated surfaces in Alizarin Red assay and also noted by various previous studies. Furthermore, the expression of constitutional and the genes that are responsible for bone formations were also analyzed to examine the viability of cell cultures using PCR. The results showed that the genes that are essential for cellular survival such as COL1 was observable in all of the cultures; similarly, the bone forming genes such as BSP, RUNX2 and ALP were expressed in similar levels in all of the cultures. The use of polyester and other polymer based coatings on biomaterials have been well reported by previous studies. Thus, it is commonly agreed that the
use of polymer coating on biomaterial possessed no negative effects and would not deteriorate cell integrity (Figure A13).

Figure A12: After 24 hours of incubation, MTT assay illustrated that the mitochondrial activities varied between surfaces. However, polymer coated biomaterial could potentially have a higher metabolic activity compared to commercially pure titanium.
Figure A13: PRC demonstrated all of the constitutional genes that were expressed within the cells raised on commercially pure titanium was also detected on polymer coated surfaces.

In conclusion, through cellular assays, hemocytometry demonstrated the biocompatibility of mineral-enriched surfaces, MTT assay suggested the source for increased biomineralization activity in coated biomaterial and gene expression confirmed the safety of polymer based coatings. Thus, our research team demonstrated that not only can we employ the ultrafine particle technology to improve the biocompatibility of orthopedic implants, it can also safely increase the osteo-inductivity of implant substrate.
Conclusion

In conclusion, our research has demonstrated that through the utilization of ultrafine particle technology and bioactive functional additives, the biocompatibility and osteo-inductivity can be effectively improved. Studies have shown that coated surfaces were able to facilitate cell attachment, promote biomineralization, encourage cell proliferation, increase metabolic activity, while does not offer any negative side effects. Consequently, our research team hope to provide the medical field with a new generation of bone and orthopedic implants that are not only highly biocompatible, but also exhibit outstanding osteo-inductivity.

References

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