DICATIONIC IMIDAZOLIUM BASED IONIC LIQUID COATINGS ON ZIRCONIA SURFACES: PHYSICAL AND BIOLOGICAL CHARACTERIZATION

by

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To, my Family, Teachers and Friends for constant support and encouragement
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Osseointegration and soft tissue seal formation between a dental implant surface and host tissues are important steps in achieving successful implantation. However, factors such as bacterial biofilm adhesion, excessive stresses experienced by an implant during insertion and mastication, and other health-related factors may affect implant stability. Dicationic imidazolium-based ionic liquid (IL) coatings containing amino acids were previously developed for implant surface functionalization. These multi-functional ionic liquid coatings have demonstrated excellent results as surface coatings of titanium in terms of providing the material with anti-biofilm activity, lubrication and corrosion resistance while being compatible with host cells in vitro. The aim of this study was to investigate the possibility of using this technology on the surface of a ceramic material, zirconia, which has been recently introduced in the design of one and two components dental implants. In this work, the physical and biological performance of IL coatings on the surface of zirconia was investigated. In summary, zirconia surfaces coated with two IL compositions were
assessed for intermolecular interactions and coating morphology using X-ray photoelectron spectroscopy and optical microscopy. Coating stability was verified by release profiles using UV-vis spectroscopy. Mammalian and bacterial cell activity were studied on the surface of IL-coated zirconia using osteoblasts and fibroblasts cells and *S. salivarius* and *S. sanguinis*, respectively. Finally, wear tests were performed in simulated physiological conditions to determine coefficient of friction and wear volume loss in the presence of IL coatings. Results showed that ILs formed stable coatings on zirconia surfaces. IL containing phenylalanine demonstrated excellent anti-biofilm activity and sustained the conditions for growth and proliferation of host cells. The results of this study indicate that the investigated dicationic imidazolium-based IL coatings constitutes a potential technology for surface enhancement of zirconia dental implants.
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CHAPTER 1
SUMMARY

Zirconia dental implants were recently introduced in the market as an alternative to titanium implants. Only limited clinical data is available for this new implant system, and therefore, studies evaluating its performance in relevant physiological conditions are needed to support further development of this material for biomedical implant use. In the oral environment, long-term implant success is achieved through surface integration with surrounding soft and bone tissues [1, 2]. However, factors such as microbial biofilm formation, implant loading conditions, corrosion, systemic health and host immune-inflammatory responses may generate conditions that can affect implant performance [2, 3]. Biofilm formation is one of the major factors playing a role in early/late implant loss. This is important to consider because implant surfaces are prone to bacterial colonization immediately post-implantation, and this happens regardless of implant material or surface treatment [3, 4]. In the ‘race-for-the surface’, if bacterial biofilm formation occurs during early stages of implant healing, it may disrupt the normal progress of host tissue growth and sealing. In addition to this, stresses experienced by an implant due to insertion and mastication during its lifetime may create wear conditions compromising the integrity of the implant [3]. All the above factors determine the success/failure of an implant. Therefore mitigating these causal factors will help generate favorable conditions for host cells to win the race against bacterial cells.
Few surface modifications for zirconia such as sandblasting, selective ion etching and use of bioactive coatings have been reported in the literature [69]. However, these techniques are mostly focused at changing the surface roughness of the substrate. Antimicrobial coatings such as those including gentamycin with a hydroxyapatite layer are suggested for zirconia surfaces, however possible drawbacks such as toxicity and uncontrolled release have limited the use of such approach [105].

Previously, dicationic imidazolium-based ionic liquid coatings containing amino acids were developed for implant surface functionalization, and the behavior of these coatings was thoroughly evaluated on titanium surfaces [4, 7]. These multi-functional coatings are aimed at enhancing implant performance by mitigating several of the triggering factors that can affect implant performance. The proposed IL coatings demonstrated excellent results on titanium surfaces in terms of coating stability, anti-biofilm activity, corrosion resistance, wear performance and cell compatibility.

Motivated by these initial results, the goal of this study is to assess the performance of selected dicationic imidazolium-based ionic liquid coatings on the surface of zirconia. To address the research questions of this work, ILs were synthesized, deposited on the surface of zirconia and characterized for physical and biological behavior. In summary, ILs were deposited on zirconia specimens using a drop-casting technique. X-ray photoelectron spectroscopy was used to characterize the chemical environment and interaction of IL coatings on zirconia surfaces. The release profile of IL coatings into physiologically relevant media was studied using spectroscopy techniques. In vitro cytotoxicity assessment was performed with human gingival fibroblasts and mouse pre-osteoblast cells. Anti-biofilm activity of IL-coated zirconia specimens was tested.
against early colonizing oral bacterial strains, *S. salivarius* and *S. sanguinis*. Finally, lubricant activity was assessed through wear testing, and the coefficient of friction and wear volume losses were calculated. It was hypothesized that ILs would form stable coatings on zirconia surfaces and further improve wear behavior of these surfaces. In addition to this, IL coatings would prevent bacterial biofilm attachment on zirconia while maintaining conditions and cytocompatibility for growth of host cells.

This study is divided into the following chapters: Chapter 2 discusses the background information on factors leading to success or failure of dental implants and the use of zirconia as an alternative implant material. Previous work on dicationic imidazolium-based ionic liquid coatings on titanium surface is also discussed briefly. Chapter 3 provides the goals, rationale and hypotheses defining the present work. Chapter 4 is presented in manuscript format and discusses coating characterization, coating stability, cell viability, anti-biofilm activity and wear behavior of IL-coated zirconia samples. This includes an introduction, methodology, and results and discussion related to each experimental study. Chapter 5 gives a summary and conclusion of the present work. Chapter 6 discusses the prospective of future work following this study.

The goal of this research is to explore the possibility of using the proposed IL coatings as a surface treatment option for zirconia dental applications. Furthermore, there is currently limited literature describing the behavior of host and bacterial cells on the surface of this material. Thus, this study aims to contribute to the body of knowledge about zirconia as potential dental implant material, offering an alternative surface treatment to further improve the performance of the material *in vivo*. 

CHAPTER 2

BACKGROUND AND SIGNIFICANCE

This chapter discusses the prospects of zirconia as an alternative material for titanium dental implants. Factors responsible for success/failure of dental implants and current surface treatments available for enhancement of implant performance are discussed. Ionic liquids as a potential coating technology for implant surface enhancement is described.

2.1 GENERAL FACTS ABOUT DENTAL IMPLANTS

Dental implants is a well-accepted treatment method to compensate for a lost tooth in partial or complete edentulism [8]. Tooth loss may occur due to various reasons such as disease or trauma and statistical reports suggest that there is an increase in the number of implants placed every year. According to statistics from the American Academy of Implant dentistry (AAID), prosthodontics have reported that more than 35 million people in America have lost all their teeth in one or both jaws. The report also states that currently 3 million Americans have dental implants and the number of placed implants is expected to increase by 500,000 per year. The American Association of Oral and Maxillofacial Surgeons has reported that 69% of Americans lost at least one permanent tooth in the age group of 35 to 44. In addition to this, 26% of adults by the age of 74 have lost nearly all of their teeth. Overall, there has been a considerable increase in the number of dental implants placed in recent years. Although dental implants are successful devices, a 5-
10% of failure rates are reported, which results in a considerable financial and health burden to patients [9].

Dental implants are generally screw shaped devices, which are placed in the maxillary/mandibular bone. They serve as a root to help with the installation of dental prostheses. Usually dental implants are designed in combination with other components such as abutments and crowns as seen in Figure 2.1. Abutment is the intermediary component, which connects implant and crown together and provides support and stability to the implant system.

![Figure 2.1 Parts of a dental implant system comprising of crown, abutment and implant](Source: http://www.oxygenmedical.no/replacing-missing-teeth-dental-implants/).

The clinical procedure involving the placement of dental implants typically consists of two steps. In the first step, dental implant is placed in the jawbone and it is left to heal for a time period
ranging between several weeks to few months. Waiting time for implant healing depends on bone quality of the patient and region where implant is placed. During this healing period, osseointegration between the implant surface and jawbone bone takes place and an implant is secured in its position. Once an implant is osseointegrated properly, the abutment and crown are placed on top of the implant as a second and final step. This is a conventional protocol, however implant and prosthesis placement can also be done in one single step, which process is known as immediate loading of implant.

2.2 TITANIUM DENTAL IMPLANTS

Commercially pure titanium (cpTi) and titanium alloys (Ti6Al4V) have been used in the design of oral implants since decades. The long-term clinical survival rate of titanium implants have made them a gold standard for endosseous implants [10]. These implants exhibit biocompatibility, corrosion resistance, and good mechanical properties. The unique biocompatibility and chemical stability of titanium is provided by its surface oxide layer, which is formed as soon as the material is exposed to oxygen. This oxide film provides a protective barrier to the implant against corrosion and gives the basis for biocompatibility [11].

Although titanium implants have high clinical success rates, there are some drawbacks associated with this system, which have been vastly reported in the literature. Accumulation of metal particles in the nearby tissue regions and metal intolerance due to inflammatory responses mediated by titanium have been reported in a number of cases [20, 21]. Some studies also reported allergies, insensitivities and corrosion reactions towards titanium implants. It has been seen that metal ions released from implants in contact with body fluids, can form complexes with nearby
proteins causing allergy and hypersensitivity [20-23]. Presence of large concentrations of titanium metal degradation products in tissues at peri-implant regions has been detected in failed implants [11]. In addition to this, grey color of titanium can sometimes be seen through the peri-implant gingiva or in cases where gingival recession occurs as illustrated in Figure 2.2. This is aesthetically unappealing and leads to patient dissatisfaction [25].

![Figure 2.2 Left: Discoloration of the gums due to titanium implants, Right: Healthy teeth. (Source: https://www.dentalimplantsusa.com/metal-free-dental-implants/metal-free-implant-placement.html).](image)

2.3 **ZIRCONIA AS A DENTAL IMPLANT MATERIAL**

Dental implant designs have evolved drastically in the past few decades and there is an increased expectation for better aesthetics and performance. Because of the drawbacks associated with titanium, novel metal-free implant technologies using ceramics are being developed. Among these, zirconia ceramics (ZrO$_2$) have garnered maximum attention due to their tooth-like color and good mechanical and chemical properties. Zirconia is a crystalline dioxide of zirconium (ZrO$_2$). It has been used for orthopedic applications such as to manufacture ball heads for total hip replacement surgeries since 1969, and has recently become popular in dentistry. It has good
mechanical strength, toughness, and chemical and dimensional stability. All these properties make zirconia an excellent material choice for oral implants. There are many ceramic systems available today but the ones which are mostly used in dentistry are: magnesium cation-doped partially stabilized zirconia and yttrium cation-doped tetragonal zirconia polycrystals. Zirconia implants are available in a single piece design, where implant and abutment are fused into one component, as well as two piece designs where implant and abutment are separately available. Some of the commercially available designs are shown in Figure 2.3.

![Figure 2.3 Different designs of commercially available zirconia dental implants. From left: a one-piece implant; two-piece implant shown without abutment; one-piece implant; two-piece implant; and tapered one-piece implant. (Source: http://drshankland.com/ceramic-implants/).](image)

Given zirconia is a relatively new biomaterial in the field of dental implants, the behavior of host cells on the surface of this material has been only investigated in a small number of clinical and in vitro studies. However, these initial studies showed favorable cell response to zirconia
surfaces. In an *in vivo* study investigating bone tissue response, experimental sand blasted titanium implants and zirconia implants with three different surface roughness were inserted into jaws of nine beagle dogs. Tissue biopsies were obtained at the end of 3, 14 days, and 10 weeks and histological evaluation showed similar bone tissue response at both titanium and zirconia implant interfaces [26]. Within the zirconia dental implants group, an increase in surface roughness was seen to increase bone-to-implant contact (BIC) and rougher implants were seen to have higher BIC values after 14 days. However, this increase in BIC in implants with higher roughness as compared to implants with lower roughness was not found to be statistically significant.

In a clinical study, overall survival rates of titanium implants after 5 and 10 years were found to be 97.2% and 95.2% respectively [27]. In comparison, clinical success and survival rates of zirconia after 1 year of implantation was reported to be 92% for one and two-piece zirconia implants [28]. Based on clinical results found in the literature, zirconia ceramics are considered as potential alternative to titanium dental implants, however further studies are required to establish long-term performance.

*Hollander et al.* studied the clinical performance of zirconia dental implants comparing them to natural teeth [29]. The clinical study included 106 implants in 38 individuals after one year of loading. Parameters such as bleeding on probing (BOP), plaque index (PI), probing attachment level (PAL), probing pocket depth (PPD), and recession of gingiva were compared for zirconia implants in relation to control teeth. 100% survival rate was reported and no statistical significance was found between control natural teeth and zirconia implants with regard to BOP, PPD and PAL. This study also included a patient questionnaire and most of them were found to be satisfied with
the treatment. However, further long-term analysis and follow-up are required to strengthen the results of this clinical study.

Although dental implants have high success rates, relevant microbial adhesion on implant surfaces has been reported in a number of studies [30, 31]. It has been also shown that zirconia surfaces induce lower bacterial adhesion as compared to titanium surfaces; however, this topic remains debatable in literature [32-37]. In an in vivo human study, commercially pure titanium (cpTi) and zirconia specimens were glued to a removable acrylic device and were placed in molar-premolar region of 10 patients for 24 hours [35]. Significantly lower bacterial adhesion was found on the surface of zirconia specimens relative to cpTi (p=0.0001). Results showed that 19.3%±2.9% of the area of cpTi specimens was covered with bacteria as compared to 12.1%±1.96 of the area of zirconia specimens. It was concluded in that study that zirconia induces lower bacterial colonization than titanium surfaces.

In an experimental study involving in vitro and in vivo experiments, bacterial colonization of titanium and zirconia specimens by different strains of oral bacteria was investigated [36]. Among the oral bacteria tested, S. sanguinis was seen to attach more easily to titanium, whereas S. mutans had more affinity towards zirconia specimens. They also observed that in vitro, roughness had no impact on bacterial adherence. In vivo results showed that yttrium stabilized zirconia (Y-TZP) had less bacterial adhesion than titanium specimens and surface roughness did not influence the colonization on the surface of Y-TZP specimens.

In a recent study, biofilm formation on cpTi and zirconia surfaces was investigated in vitro in an anaerobic flow chamber model using 3-species biofilm (S. sanguinis, P. gingivalis, F. nucleatum) and human plaque samples [37]. Two different surface topographies including smooth
machined (Ti-M and ZrO₂-M) and micro-rough (Ti-SLA and ZrO₂-ZLA) were used. Zirconia surfaces were found to have significantly lower human plaque biofilm formation as compared to titanium surfaces.

Although these studies give an indication that lower bacterial adherence can be expected on the surface of zirconia implants, bacterial colonization still occurs on this material. Bacterial strains such as Streptococcus are found to inhabit implant surfaces as early as the implant is inserted and can lead to biofilm formation [38]. Bacterial biofilm formation on implant surfaces evokes inflammatory reactions from the surrounding host bone and soft tissues, which in turn can be one of the major factors for early or late implant failures. Therefore, it becomes important to protect implant surfaces, regardless their composition, from early colonizers during the initial healing period following implantation.

2.4 FACTORS INFLUENCING SUCCESS/FAILURE OF DENTAL IMPLANTS

There are a number of factors which are well described in the literature to contribute to the success or failure of dental implants. Osseointegration with bone and soft tissue seal formation around an implant neck are one of the key factors which are essential in determining success of placed implants [39]. However, failure factors include lack of primary stability, peri-implantitis, surgical trauma, corrosion, patient’s systemic and local factors and surgeon technique [3, 40, 41].

2.4.1 Understanding Implant Healing Process and Success Factors

Osseointegration was first described by Branemark in 1960 as the formation of a stable structural and functional connection between surrounding bone and the surface of an implant [42].
It is one of the most important factors which determines implant success. Successful osseointegration of an implant depends on proper integration of bone, epithelium and connective tissues. Peri-implant mucosa resembles the gingival tissue and consists of epithelial and connective tissues [43]. Unlike teeth, connective tissue fibers are arranged parallel to the surface of implant and do not penetrate the implant surface [43]. This arrangement resembles a formation of a cuff or a collar around implant neck as illustrated in Figure 2.4.

A sequence of molecular events is initiated after implantation, beginning with the formation of granulation tissue at the wound site. Bone formation starts during the first week in which primary bone, which consists of trabeculae of woven bone, is replaced by parallel-fibered and lamellar bone and marrow [17]. The bone tissues providing initial mechanical stability to the device are then replaced by new formed bone within 1-2 weeks [17]. A soft tissue seal formation around the neck of the implant protects the peri-implant bone from the oral cavity. It sits like a collar on the neck of the implant and protects the underlying osseous integration against oral pathogens. The formation of this biological seal is very important for the long-term success of an implant. Thus, integration of both soft and hard tissues is essential to maintain the stability and integrity of a placed implant as gingival connective tissue will form a seal around the implant neck and bone cells (osteoblasts) will help in bone formation.
2.4.2 Implant Failure Mechanisms

As mentioned earlier, there are a number of factors which can contribute to dental implant failure. Excessive mechanical stresses experienced by an implant during insertion or mastication [41], wear [41], surgical techniques [52], peri-implantitis [50, 51, 54], corrosion [53, 55] and patient conditions such as smoking habits, diabetes, and oral hygiene among [3, 9] are some of the factors which are often described in the literature. In general, dental implant failures can be distinguished as early or late-stage failures. Early failure occurs before an abutment is connected to an implant and is usually characterized by lack of proper integration. The number of early
failures reported in literature range from 1.5% to 21% [9]. The factors which contribute to early implant failure are bacterial adhesion, surgical trauma, premature loading, and improper sealing of soft tissues [3, 41, 47, 48].

Late stage failures occur after occlusal loading of an implant and are defined by the breakdown of an established osseointegration. These failures are reported in the range from 1% to 28% [9]. Occlusal overload and conditions such as peri-implantitis are seen as the main factors associated with late implant failures [41]. According to the 1st European Workshop on Periodontology (1993), peri-implantitis was defined as “the inflammatory process that leads to bone loss around an osseointegrated implant”. Peri-implantitis occurs later during implantation so it may be considered as a late stage clinical condition [13]. In a review study, incidence of peri-implantitis in titanium dental implants was reported in 10% of implants and 20% of patients after 5-10 years post implantation [101]. No instances of peri-implantitis associated with zirconia implants have been reported in literature at this point. This is due to the short clinical history available for this new implant system. In a clinical study, 49 two-piece zirconia implants were placed in 32 patients and survival outcomes were evaluated after 1 and 3 years of implantation. Cumulative survival after 1 and 2 years was found to be 87%, while no signs of peri-implantitis were observed [102]. However, long-term follow up is required to establish clinical success.

In both of the above mentioned failure modes, bacterial biofilm has been identified as one of the major causative factors [12-14]. It has been hypothesized in the literature that early colonization of implant surfaces before implant healing occurs can lead to creation of anaerobic conditions. Late colonizing anaerobic bacterial species can thrive on the favorable conditions created by early colonizers and destruct osseointegrated interfaces [106]. *Streptococcus and*
Actinomyces species are identified as early colonizers while Aggregatibacter actinomycetemcomitans and Fusobacterium nucleatum are classified as late stage colonizers [15,16]. Other factors such as mechanical forces experienced by an implant in the oral cavity may also contribute to instability of an implant. Frictional forces during insertion and mastication stresses experienced by an implant over its lifetime can also impair implant stability. Therefore, it can be assumed that by enhancing key surface properties such as reducing bacterial adherence and improving lubrication on implant surfaces may help the improvement of implant properties and performance.

### 2.4.3 Race-for-the-Surface

Zhao et al. recently described a race for implant surface between bacteria and host tissue cells following implantation [56]. There are two possibilities, which will decide the fate of an implant. In the first scenario, host connective tissue will first form on implant surfaces creating a protective seal around the implant neck, which will prevent invasion of oral bacteria towards bone. In the second scenario, bacteria will first colonize implant surfaces and a biofilm will be formed, which will prevent host cells to reach the surface of the implant. If host cells win the race and are able to form a protective seal first, this will provide protection against bacterial invasion and adhesion and help with the osseointegration process. On the other hand, if bacterial cells win the race and a biofilm is first formed, it will affect implant integration. Thus, conditions wherein biofilm formation can be inhibited will promote host cell adhesion and provide a better chance for implant osseointegration. Figure 2.5 illustrates race-to-the-surface post-implantation. It can be seen that both bacterial and host cells compete for attachment to implant surface, however in this
case, bacteria cells win the race and a biofilm is formed on implant surface. This minimizes the possibility of host cell attachment ultimately hampering soft tissues integration.

![Figure 2.5](image.png) A schematic representation of race-for-the-surface between host cells and oral bacteria after implantation. Host cells lost the race against bacterial cells in this case. (Source: http://www.intechopen.com/books/modern-surface-engineering-treatments/modern-orthopaedic-implant-coatings-their-pros-cons-and-evaluation-methods).

Early colonizers such as Streptococcus species attach themselves to implant surfaces soon after implantation [38]. In the study by Zhao et al., a co-culture model consisting of human gingival fibroblasts and Streptococcus sp was developed to investigate their competition for an implant surface. The study employed three Streptococcus sp and different implant materials characterized by roughness, hydrophobicity and elemental composition. Using the co-culture model, bacteria
was observed to win the race-for-the surface on all implant surfaces and within all bacterial species, while human gingival fibroblast could only win the race on the smoothest implant surface.

These examples from the literature further demonstrate that formation of soft seal by host cells is an essential step to achieve osseointegration and prevention of early implant loss. Therefore, it is of utmost importance that the surface of implant is protected from early bacterial colonization and biofilm adhesion during the healing period.

2.5 CURRENT APPROACHES IN SURFACE TREATMENT OF DENTAL IMPLANTS

Various physical and chemical properties of implant surfaces are believed to influence the behavior of host and bacterial cells on a surface. However, optimum surface characteristics for osseointegration of implants are still debatable [15]. In the literature, surface characteristics influencing implant osseointegration were found to be mainly surface chemistry, topography, roughness, surface energy, and hydrophilicity [15, 16]. Amongst these, free surface energy, roughness and hydrophilicity are considered the most crucial parameters [15-17]. Bone-to-implant contact (BIC) is one of the factors which determines the stability and long-term success, and increasing BIC and osseointegration is one of the main goals of surface treatments performed on implant surfaces. Fibroblasts and epithelial cells reportedly attach better to smooth surfaces, while osteoblasts are able to attach and differentiate better on rough surfaces [18]. Several studies have shown that surface roughness helps in increasing BIC and osseointegration as compared to smooth surfaces [16]. Surface hydrophilicity is another important determinant, which influences the interaction with physiological fluids and surface wetting. A hydrophilic surface has increased
protein adsorption and osteoblast adhesion as compared to hydrophobic surfaces, which may improve clinical performance of an implant [17, 19]. The differentiation of progenitor cells into osteoblasts is known to undergo 3 steps namely, cell proliferation, matrix maturation and matrix mineralization [12]. First, pre-osteoblasts attach to the surface and grow or proliferate to a significant amount forming multilayers of cells. Soon after, differentiation of pre-osteoblasts to osteoblasts occurs. This phase is marked by a high alkaline phosphatase (ALP) activity. *In vitro* studies have shown that hydrophilic surfaces favor cell differentiation and show high ALP activity [13]. Similarly, *in vivo* studies showed better BIC on hydrophilic surfaces during initial phase after implantation [14]. Several chemical, mechanical and physical surface modifications methods are available for enhancement of surface performance of titanium dental implants. Physical methods include sputtering, plasma spraying and ion deposition [15]. Sandblasting of zirconia particles on titanium surfaces to enhance osseointegration is also discussed in the literature [59]. Mechanical methods include sandblasting, grinding and machining [60]. Some of the chemical treatments available are hydrogen peroxide treatment, treatment with acids and alkali, chemical vapor deposition, anodization and sol-gel treatment [61]. A combination of blasting and acid etching (SLA) is another method which introduces topographical changes on titanium surfaces [62]. SLA surfaces are also used with additional modifications such as increasing the wettability and studies have shown that a hydrophilic SLA surface resulted in increased bone response versus conventional SLA surface [62, 63].

Since zirconia, a bioinert type I ceramic material, is a relatively new dental implant material, there is not a large literature body discussing methods to increase the material’s functionality for implant applications. Current modification methods available for zirconia mainly
include the following two approaches: (1) use of bioactive coatings using different ceramic materials such as calcium phosphates and collagen to enhance surface chemistry; (2) changing the microstructure and roughness of zirconia using different techniques. Sandblasting is a common technique used to increase roughness; however, the formation of grooves and scratches have been reported with this technique [65]. Selective infiltration etching (SIE) is a recent method, which has been used to add porosity to the surface of zirconia by heat-induced maturation and grain boundary diffusion techniques [66]. Delgado et al. have discussed another approach which is based on femtosecond laser microstructuring of zirconia surfaces. It is a precise technique which can be used to increase surface roughness with minimum damage to nearby areas [67]. Figure 2.5.1 shows the SEM images of zirconia surface after modifications using grit blasting, etching and silica-coating. It was observed that after grit-blasting zirconia surface, number of irregular structures were increased, however some micro-cracks were also produced [103]. Etching treatment created many crater-like structures due to dissolution of zirconia grains.
Figure 2.6 SEM images (original magnification Â 1000) after surface treatment of zirconia (A) Control zirconia; (B) Grit-blasting; (C) Etching with a mineral acid blend solution; (D) Etching with HF solution; (E) Silica-coating. 
(Source: https://www.researchgate.net/publication/273524456).

It is important to point out that most of these current techniques focus mainly on improving osseointegration of implant surfaces. There is not much evidence of methods for functionalization of the surface of zirconia to impart antimicrobial activity. Very few anti-microbial surface coatings
for implants are discussed in the literature and they include the use of gentamycin with a hydroxyapatite layer, essential oils, antimicrobial glassy coatings and chitosan-PVA-silver nanocomposite coatings [68, 69]. However, several limitations such as toxicity and uncontrolled release of antimicrobial agents are seen to be associated with these coatings [105]. As discussed, numerous surface treatment methods are available to improve the performance of titanium but not many modifications can be applied to zirconia surfaces. Thus, there is a need for innovative surface modification technologies that can be compatible with the properties of zirconia. Furthermore, it is important to provide the surface with multi-functional activities, meaning that more than one property can be targeted using a single surface treatment. Recent studies indicated that multi-functionalities such as anti-biofilm activity, improved wear performance and increased corrosion resistance can be imparted on the surface of dental implants using dicationic imidazolium-based ionic liquid coatings [4, 5, 6].

2.6 IONIC LIQUIDS

Ionic liquids (ILs) are a class of molten salts, which are liquid at room temperature. This unique property is achieved by combining an asymmetric large cation with a weakly coordinated anion. This asymmetric combination is not able to pack easily and renders a low melting point [70]. These compounds have excellent chemical and thermal stability, low vapor pressure and high conductivity [71, 72]. They also possess good solubility and catalytic properties [73]. One of the interesting features of ionic liquids is the tunability or flexibility in their structural design. By changing the structure of cations and anions, the properties of ILs can be tailored accordingly [74]. There are a large number of cation and anion combinations possible, which make them an
interesting class of chemicals for various industries. The unique properties of ILs, as compared to classic solvents, have encouraged their use across various fields such as catalysis, extraction and separation, electrochemistry, food and pharmaceutical industry [70, 72, 73]. ILs are considered as an alternative to traditional organic solvents as they present lesser environmental risk due to their negligible vapor pressure [75]. For example, imidazolium and pyridinium are the most common cations used in the design of ionic liquids. These cations are usually present in combination with chloride (Cl\(_2\)), bromide (Br\(_2\)), hexafluorophosphate (PF\(_6\)), and tetrafluoroborate (BF\(_4\)) [76]. Figure 2.6.1 shows the structure of dicationic imidazolium based ILs and some commonly used cations and anions in IL compounds.

ILs have been considered as “green solvents” due to their negligible vapor pressure and low inflammability [75]. However, concern has been raised about toxicity of these compounds. A pioneering study by Pernak et al. first discussed about the antimicrobial activity and ecotoxicity related to pyridinium and imidazolium-based ILs [76]. The study demonstrated that the cation plays an important role in the IL toxicity and the toxic effect increases with the length of alkyl chain. Following this, there were numerous studies to evaluate the toxicity associated with ILs. Literature also suggests the contribution and effect of anion in the toxicity of ILs. Biczak et al. compared the toxicity of five different anions (bromide [Br], nitrate [NO\(_3\)], \(p\)-toluenesulfonate (tosylate) [Ts], dimethylphosphate [dMP] and methanesulfonate [MS]) coupled with imidazolium [77]. It was found that anion had a pronounced effect in determining the overall toxicity of ILs. The main mechanism proposed to explain IL toxicity is the hydrophobic interactions between IL and biological membranes. This toxic effect particularly increases with increase in the length of alkyl chain.
Our group recently developed dicationic imidazolium-based ionic liquids with amino acid as anions as multi-functional coatings of titanium implants [4]. The series of ionic liquids developed are aimed at providing multi-functionailities to implant surfaces while being biocompatible with host cells. These compounds have been synthesized to provide anti-microbial activity, lubrication, anti-corrosive properties to the surface and were demonstrated to be...
compatible with host cells. With dicationic ILs, the toxicity related with the imidazolium group is combated using two strategies. First, the introduction of a second cationic moiety, dicationic head, traps the alkyl chain and increases the polarity of the IL structure. This will prevent penetration of the alkyl chain through cell membranes. Second strategy is the use of organic anions such as naturally occurring amino acids. Previously, Gindri et al. demonstrated the application of these ILs as surface coatings of cpTi titanium surfaces [5, 6, 79]. In the previous work, these ILs were demonstrated to form stable coatings on titanium surfaces and excellent results were obtained in regard to their anti-biofilm activity, anti-corrosive and lubrication behavior [4-6, 79]. The behavior of fibroblasts and pre-osteoblasts in the presence of these coatings was also observed. ILs allowed for the conditions for growth and proliferation of these cells while maintained effective antimicrobial activity for a period representative of early healing period [6].
CHAPTER 3
GOALS AND HYPOTHESIS

GOAL 1: To characterize dicationic imidazolium-based ionic liquids (ILs) on the surface of zirconia.

- Synthesize selected dicationic imidazolium-based ionic liquids using protocols previously established in the literature.
- Coat the surface of polished zirconia specimens with selected ILs using a drop casting technique and analyze surface morphology with optical microscopy.
- Study the interactions between IL and zirconia surface using X-Ray photoelectron spectroscopy.
- Evaluate stability and release of IL coating in simulated physiological environment using UV-visible spectroscopy.

RATIONALE: Dicationic imidazolium-based ionic liquids containing amino acids (phenylalanine and methionine) were designed and developed by us recently. This series of multifunctional ILs possess properties such as anti-biofilm activity, lubrication and anti-corrosive activity with low mammalian cell toxicity [4-6, 79]. We propose to use dicationic imidazolium based ILs, 1, 10-bis(3-methylimidazolium-1yl) decane diphenylalanine (IL1) and 1, 10-bis(3-methylimidazolium-1-yl) decane dimethionine (IL2), as surface coatings for zirconia. Before
analyzing the effect of coating on the surface properties, it was important to first study the molecular interactions between anionic and cationic moieties of the selected ILs with the surface of zirconia and analyze morphology of the surface coating. In previous work, the adhesion and interactions between a group of dicationic imidazolium-based ILs and the surface of commercially pure titanium was investigated. The potential application of zirconia in the design of dental implants requires further investigation of technologies to improve the performance of this material for the oral environment.

**HYPOTHESIS:** Dicationic imidazolium-based ionic liquids containing the amino acids phenylalanine and methionine will uniformly coat and interact with the surface of zirconia resulting in low IL release into immersion media after 7 days.

**GOAL 2:** To study the antimicrobial and anti-biofilm activity of IL-coated zirconia and control against bacterial strains of relevance in the oral environment.

- Cultivate early colonizing oral bacterial strains associated with *in vivo* biofilm formation to assess IL anti-biofilm activity.
- Select time points for immersion and optimize protocol for bacteria culture.
- Evaluate number of bacterial colonies in bacterial immersion fluid and on surface of specimens separately.

**RATIONALE:** Early colonizing bacteria such as *Streptococcus* species can colonize the implant surface soon after implantation [38]. Bacterial biofilm formation by these early colonizers may
hinder osseointegration and soft tissue seal formation during the healing period of an implant. Therefore, it is essential to protect the implant surface during the initial healing period. The second goal of this project evaluated bacterial cell activity of control zirconia and IL-coated zirconia against *S. salivarius* and *S. sanguinis* for 1 and 7 days.

**HYPOTHESIS:** The hydrophobic interaction between IL structure and bacterial cell membrane will inhibit biofilm adhesion on IL-coated zirconia surfaces.

**GOAL 3:** To assess host cell behavior and interaction with IL-coated zirconia.

- Develop a protocol to study attachment and growth of pre-osteoblasts and human gingival fibroblasts on control and IL-coated zirconia surfaces.
- Evaluate cell viability and differentiation after cell culture testing.

**RATIONALE:** Human gingival fibroblasts form a tight layer or seal around an implant neck while osteoblasts are responsible for secreting matrix and minerals for osseointegration. Both of these functions are essential to maintain long-term stability of an implant [39, 94]. Anti-microbial activity of imidazolium-based ILs is well discussed in the literature. However, cytotoxicity of these ILs has also been reported in many cases [77, 78]. The ILs proposed in this work have a dicaticonic moiety, which is aimed at reducing host cell toxicity of these compounds [4]. The third goal of this project investigates the interaction of mammalian cells with control and IL-coated zirconia surfaces. For this, 1 and 7-day experiments were conducted to evaluate growth and proliferation.
of pre-osteoblasts (MC3T3-E1) and human gingival fibroblasts (HGF-1) on zirconia surfaces using MTT assay. Differentiation of pre-osteoblasts to osteoblasts was verified using ALP assays.

**HYPOTHESIS:** IL alkyl chain will not be able to penetrate the cell membrane lipid bilayer and change its properties due to the presence of two cationic imidazolium heads at both ends of the chain. IL-coating containing amino acids will allow for proliferation and differentiation of host cells.

**GOAL 4:** To investigate the wear behavior of control zirconia and IL-coated zirconia surfaces
- Develop a protocol for tribological testing of control zirconia and IL-coated at 37°C.
- Evaluate coefficient of friction (COF) and total wear volume loss.

**RATIONALE:** Partially stabilized zirconia ceramics are wear resistant materials due to their improved toughness. However, the oral cavity is a complex environment subject to various factors such as friction forces during insertion, abrasion, fatigue, pH imbalance and mastication stresses, all of which may affect the tribological behavior of the material. The fourth goal of this project is to evaluate the lubrication behavior of IL-coated zirconia surfaces as compared to control zirconia.

**HYPOTHESIS:** IL coating will enhance lubrication of the zirconia surface reducing its coefficient of friction and total wear volume loss in comparison to control zirconia.
CHAPTER 4
DICATIONIC IMIDAZOLIUM-BASED IONIC LIQUID COATINGS ON ZIRCONIA SURFACES: PHYSICAL AND BIOLOGICAL CHARACTERIZATION

4.1 ABSTRACT

In the present work, performance of dicationic imidazolium-based ionic liquid coatings on zirconia surface is investigated. These multi-functional coatings were designed to prevent biofilm formation on zirconia surfaces while maintaining the material’s compatibility with host cells. In addition, the intrinsic lubrication characteristics of these ILs can be utilized to further enhance wear performance of zirconia. IL coatings containing phenylalanine and methionine were synthesized and deposited on zirconia surfaces. Intermolecular interactions driving the deposition of IL on the substrate were studied using X-ray photoelectron spectroscopy. Anti-biofilm activity and cell compatibility were tested *in vitro* for a period of 1 and 7 days, and wear performance was tested using pin-on-disk apparatus. Results indicated that ILs interacted with the substrate via strong hydrogen bonds and formed stable films on the zirconia surface. IL containing phenylalanine was found to be stable on the surface after 1 and 7 days of immersion in PBS and saliva and showed excellent anti-biofilm properties against *S. salivarius* and *S. sanguinis*. Compatibility with gingival fibroblasts and osteoblasts was maintained and conditions for growth and differentiation were preserved. A significantly lower coefficient of friction and wear volume loss were observed for IL-coated surfaces as compared to non-coated surfaces. Zirconia is seen as
an alternative to titanium dental implants and this study provides additional evidence of the materials’ behavior and a potential surface treatment technology for improvement of its properties.

4.2 INTRODUCTION

Commercially pure titanium has been a material of choice for oral implants since decades. Implants made of titanium have successful long-term clinical survival rates due to their biocompatibility, corrosion resistance, and good mechanical properties [11]. The unique biocompatibility and chemical stability of titanium implants is provided by the oxide layer, which provides a protective barrier for the implant surface against corrosion and has been also reported to help facilitate osseointegration [54]. However, titanium has a dark greyish color, which can sometimes be seen through the anterior peri-implant gingiva. It jeopardizes the esthetics and may lead to patient dissatisfaction in some cases [11]. In addition to this, concerns about accumulation of metal particles in peri-implant tissues, allergies, insensitivities, corrosion reactions, and metal intolerance have also been reported in some cases [22, 23, 80]. In recent years, high strength zirconia ceramics have garnered attention as novel metal-free implant technology and alternative material for dental implant design. One of the attractive aspects of zirconia for oral implants is its tooth-like color. Additionally, the material’s good mechanical and chemical properties, such as high fracture toughness, strength and resilience, and high corrosion stability make it a good choice for dental implant design [81].

Recent results from clinical and in vitro studies involving zirconia demonstrated comparable osseointegration as titanium implants [26]. In an in vivo study, similar bone-implant contact was found on both titanium and zirconia implants after 12 weeks [82]. In a review study, survival and success rate of zirconia was found to be 92% after one year of implantation [28].
Similar studies indicated biocompatibility and appropriate osseointegration of zirconia; however, these results are from short-term observations and extended studies and clinical follow up are needed to establish long-term performance.

For long-term success of any implant system, it is necessary that the implant surface achieves integration with soft and bone tissues, which will ensure sealing and stable connection. Furthermore, it has been discussed in the literature that soft tissue seal formation around an implant neck will provide a barrier against bacterial penetration toward the bone [2]. Despite high survival rate, failures observed with dental implants are expected to increase, given an estimated 500,000 dental implants are placed every year. There are many factors that influence the survival of an implant, which include implant loading procedures, microbial biofilm formation, corrosion, systemic health and host immune-inflammatory responses [3, 41, 50]. Recent studies have shown that bacterial biofilm formation is one of the major factors influencing both early as well as late stage implant failures [50, 51]. Thus, innovative methods that can provide protection of dental implant surfaces against bacterial adhesion may improve long-term performance.

Given zirconia is a relatively new alternative material for the design of dental implants, there are only a few studies reporting the biological and physical behavior of this material under circumstances relevant to the oral environment. Likewise, technologies aimed at improving its performance for this specific application are still at initial stages. Therefore, the successful introduction of zirconia as dental implant material requires more in depth investigation of the material’s behavior and properties, as well as investigation of new technologies aimed at improving the response of the material within the biological environment.
Although there are a number of approaches proposed for surface modification of dental implants, many of which apply to the surface of zirconia, there is a lack of approaches that provide multi-functionalities to an implant surface. Recently, dicationic imidazolium-based ionic liquid coatings were developed for dental implant applications. These IL coatings were functionalized to promote surfaces with antimicrobial activity, corrosion protection and improved lubrication [4-6, 79]. This series of ionic liquids consist of dicationic imidazolium head with an alkyl chain and an amino-acid based anionic moiety.

Ionic liquids (ILs) are a class of molten salts, which have an amphiphillic cationic moiety, and a weakly coordinated anion [4, 70]. Due to their structural flexibility and unique physical and chemical properties, there is a growing interest in their applications as lubricants, surfactants and solvents across many fields [72, 74, 75, 84]. In addition to these properties, anti-microbial activity of imidazolium, pyridinium and quaternary ammonium-based ILs has been described in the literature, however this activity comes with the cost of toxicity to host cells [70, 78]. As observed by Pernak et al., the main mechanism for this toxicity is the hydrophobic interactions between cell membrane and ILs [84]. The toxic effect increases with an increase in alkyl chain length due to the greater lipophilicity and increased probability to interact with the cell membrane. Therefore, it has been demonstrated in the literature that although monocationic IL structures have applications in many fields, these ILs are not suitable for biological applications due to their associated cytotoxicity [78]. On the contrary, the dicationic IL structures synthesized in previous studies exhibited good compatibility with host cells [4-6]. It was hypothesized that with these IL structures, the alkyl chain is trapped between two cationic heads and is therefore unable to interact and penetrate the cell membrane.
It has been demonstrated in previous studies that these dicationic imidazolium based compounds when added to the surface of cpTi formed stable films with high adhesion strength [5]. The coatings also promoted strong antimicrobial action against bacterial species relevant to the oral environment while maintained biocompatibility with host soft and hard tissues [6]. An improvement in anti-corrosive and tribological properties of titanium was also observed [56]. Based on these previous results, two ionic liquids were chosen for investigation with the surface of zirconia [4-6, 56].

This study presents a comprehensive characterization of the surface of zirconia coated with dicationic imidazolium based ionic liquids. Two ILs with same cation and alkyl chain length (10 carbons) but different anions (phenylalanine and methionine) were chosen for investigation. IL coatings were synthesized according to protocols described in the literature [4]. IL1 containing phenylalanine and IL2 containing methionine as anions were deposited via drop-casting on the zirconia surface. Surface-coating interactions and morphology were investigated with spectroscopy and microscopy techniques. Thereafter, the release of the coating was studied in simulated oral environment media consisting of saliva and phosphate-buffered saline (PBS). The antimicrobial activity of IL-coated surfaces against early colonizing bacterial strains was investigated with S. salivarius and S. sanguinis. Subsequently, cell studies were carried out with gingival epithelial cells (HGF-1) and mouse pre-osteoblast cell line MC3T3-E, wherein the viability and differentiation was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Alkaline Phosphatase (ALP) assays. Lastly, the tribological behavior of IL-coated zirconia was assessed in conditions simulating mastication stresses.
4.3 MATERIALS AND METHODS

4.3.1 Ionic Liquid Synthesis

The chemical products were of high grade purity and used as received from the manufacturers, without any further purification. They were as follows: L- phenylalanine (MP Biomedicals), L-methionine, 1-methylimidazole, 1,10-dibromodecane, ethyl ether, AMBERLITE IRN-78 OH (Acros Organics), ethanol and acetonitrile (Fisher Scientific). 1,10-bis(3-methylimidazolium-1-yl) decane hydroxide was prepared using anion exchange resin from the ethanolic solution of 1,10-bis(3-methylimidazolium-1-yl) decane bromide. To obtain IL1 and IL2, 10 mM of Phenylalanine and Methionine were added to 1,10-bis(3-methylimidazolium-1-yl) decane, respectively, and the mixture was stirred for 12 hours. The obtained solution was purified by adding 18 ml of acetonitrile and 2 ml of methanol while stirring and this mixture was then filtered. The purification step was done for a total of three times and the final filtrate was dried in vacuum for 72 hours at 60°C.

1,10-Bis(3-methylimidazolium-1-yl)decane diphenylalanine (IL1) and 1,10-bis(3-methylimidazolium-1-yl)decane dimethionine (IL2) were characterized by using H\textsuperscript{1} NMR (Nuclear Magnetic Resonance Spectroscopy) (500 MHz Bruker spectrometer) and the data was found in accordance with the literature [14]. For NMR experiments, 15 mg of IL was dissolved in 0.5 ml of DMSO-d6 and the experiment was carried out at 25°C. The structure of IL1 and IL2 is shown in Figure 4.1.
4.3.2 Sample Preparation

Wear resistant zirconia ceramic (ZrO₂) rods were obtained from McMaster-Carr. The rods were cut into disks with 0.95 cm diameter and 0.45 cm height and were subsequently mounted for polishing using epoxy resin. Mounted samples were polished using an automated polishing head (FEMTO 1100, Pace Technologies) with the polisher (NANO 1000T, Pace Technologies) using set force of 10 lbs. The polishing procedure involved the following sequence: (1) specimens were polished using 30 μm DIAMAT diamond paste on CERMESH metal mesh cloth; (2) followed by 6 um DIAMAT diamond paste on TEXPAN polishing pad; (3) followed by 1 um DIAMAT diamond paste on GOLDPAD polishing pad under at lbs force at a speed of 200 rpm for five minutes each; and (4) finished with SIAMAT colloidal silica on TEXPAN polishing pad, which
was used to polish the samples for five minutes at 10 lbs at a speed of 100 rpm. The polished samples were then removed from resin molds and cleaned by sonicating 15 minutes each with acetone, distilled water and ethanol and dried in the oven for 48 hours. To prepare coating solutions, 0.011 mM of IL1 and IL2 were dissolved in 500 µl of ethanol, respectively. 5 µl of the coating solution was pipetted onto specimens 10 times at an interval of 15 minutes. The coated specimens were then dried in the oven for 48 hours. Sample preparation steps are shown in Figure 4.2.

Figure 4.2 Schematic representation of sample preparation. (a) sample polishing; (b) cleaning by sonication; (c) oven drying; (d) control zirconia and pipette coating of IL1 and IL2; (e) oven drying.
4.3.3 Composition, Morphology and Release Profile of IL-coated Zirconia

Coating morphology on the surface of zirconia and surface elemental composition were analyzed using optical microscopy (VHX-5000 Digital Microscope, Keyence) and X-ray spectroscopy (PHI 5000 Versa Probe II X-ray Photoelectron Spectrometer), respectively.

For XPS experiments, one control (uncoated zirconia), one IL1-coated and one IL2-coated zirconia specimens were analyzed with 3 spots on each specimen and the spectra obtained were compared to understand IL-zirconia surface interactions. A monochromatic Al Kα source of 1486.6 eV was used and the measurements were taken at an angle of 45° with respect to the surface of the sample. The pressure in the analysis chamber was kept below 10^{-8} torr and the survey spectra was obtained using pass energy of 187.850 eV and 0.8 eV step size. The high resolution spectra was acquired using pass energy of 23.5 eV and step energy of 0.2 eV.

To study IL coating release release into the immersion media, triplicates of IL1-coated, IL2-coated and control zirconia were immersed in 1 ml each of PBS and artificial saliva, separately, in 24 well plates. The plates were maintained at 37°C for 1 day and 7 days to simulate the conditions found in the oral environment. After 1 and 7 days, absorbance was recorded from the aliquots obtained from each well using UV-Vis Spectrophotometer (Thermo Scientific Nanodrop 2000). The corresponding IL concentration in the immersion media was determined from the calibration curve of IL1 and IL2 in PBS and saliva [6].
4.3.4 Anti-biofilm Activity of IL-coated Zirconia

*Streptococcus* species are known to colonize an implant surface as soon as it is implanted and includes the majority of the early colonizing bacteria [38]. Two *Streptococcus* species, *Streptococcus sanguinis* 10556 and *Streptococcus salivarius* 13419 were chosen to evaluate the anti-biofilm activity of IL-coated zirconia specimens in comparison to control specimens against early bacterial colonizers. The bacterial strains were streaked on Brain Heart Infusion (BHI) agar plates and then incubated in microaerophilic atmosphere at 37° C. The bacterial inoculation was incubated and then diluted with 1.9:0.1 ratio of artificial saliva/BHI to a concentration of $10^5$ colony forming units per ml (CFU/ml). For 1-day test, triplicates of control zirconia and IL-coated zirconia specimens were immersed for 24 hours in the cell-density normalized bacterial culture. Thereafter, the specimens were taken out and washed 3 times gently with buffer to remove non-adherent bacterial cells. The specimens were then vortexed in buffer vigorously and the surface of the specimens was scraped with the help of a sterile spatula to remove any bacteria attached to the surface. The bacterial growth in immersion media and on specimen surface was then quantified in CFU/ml by plating the immersion fluid and buffer solution on BHI agar plates. To evaluate long-term stability of ILs during the initial implant healing period, triplicates of the control zirconia and IL-coated zirconia specimens were immersed in saliva/BHI medium for 7 days. Bacterial inoculation was done 24 hours before the final immersion and the CFU/ml on the surface and in the fluid was quantified as described above.
4.3.5 Evaluation of Mammalian Cell Behavior on IL-coated Zirconia Specimens

Human gingival fibroblasts (HGF-1), pre-osteoblast cells (MC3T3-E1), Dulbecco’s modified eagle medium, alpha minimum essential medium and trypsin were obtained from the American Type Culture Collection. Agar and brain heart infusion (BHI) were purchased from Fisher Scientific.

4.3.5.1 Cell Culture

To understand and evaluate host cell integration on IL-coated zirconia specimens (IL1 and IL2), cell culture tests were performed in vitro using 6 well plates for 1 and 7 days. Human gingival fibroblasts (HGF-1) and pre-osteoblasts (MC3T3-E1) were cultured in Dulbecco’s modified eagle medium with 10% fetal bovine serum and alpha minimum essential medium with 10% bovine serum respectively, at 37°C in a humid environment. Triplicates of control zirconia and IL-coated zirconia specimens were then placed individually in 6 well plates with seeding density of 350,000 cells per well for 1 day and 100,000 cells per well for 7-day tests. The plates were then incubated for 1 and 7 days and the culture medium was changed every 48 hours.

4.3.5.2 Cell Viability

Cell viability for fibroblasts and osteoblasts after being exposed to control zirconia and IL-coated zirconia for 1 and 7 days was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The cell medium was aspirated and the cells were washed with phosphate buffered saline (PBS) followed with the trypsinization of the cells in the wells as well as sample surface. Dissociated cells were then carefully transferred using cell medium into
individual centrifuge tubes. The cells were then centrifuged at 1200 rpm for 5 minutes. The supernatant was carefully aspirated and the cells were re-suspended by dissolving the pellet in 250 µ1 of cell medium.

From the cell suspension, 100 µ1 was transferred into 96 well plate and 10 µ1 of MTT reagent was carefully added to individual wells in the dark. The plate was then incubated for 4 hours at 37°C in a humid environment. After 4 hours, 100 µ1 of detergent reagent was added in each well and the plate was incubated overnight. The absorption was then measured at 570 nm using an automatic plate reader (Synergy mix, Biotek). Optical density values obtained were representative of the blue formazan product produced by viable cells. The cell viability percentages were then calculated with respect to the wells with control zirconia, after subtracting the blank value from each well.

4.3.5.3 ALP Activity

ALP expression was evaluated in two ways: (1) the amount of ALP produced by osteoblasts in contact with control zirconia as well as IL-coated zirconia was measured using a colorimetric based assay; and (2) differentiated cells were stained to observe their morphology under optical microscope.

For the colorimetric assay (Abcam), 50 µ1 of the cell suspension obtained in the procedure detailed in section 4.2.5.2 was transferred into a 96 well plate. After that, 30 µ1 of the ALP assay buffer followed by 50 µ1 of p-nitrophenyl phosphate (pNPP) were added in each well and the plate was incubated at 25 °C for 1 hour. After incubation, 20 µ1 of stop solution was added in each well and the optical density was measured at 405 nm using a plate reader. Optical density values were
used to obtain the corresponding pNP values by using the calibration curve made from the ALP enzyme provided in the assay kit. These values were representative of the amount of differentiated cells present in corresponding well of 96 well plate. A schematic illustration of cell study involving MTT and ALP is shown in Figure 4.3.

![Schematic of cell study](image)

**Figure 4.3** Schematic representation of cell study to investigate viability and differentiation of MC3T3-E1 cells on control zirconia and IL-coated zirconia.

Cell morphology, attachment and differentiation on control zirconia and IL-coated zirconia specimens was observed under optical microscopy after staining the differentiated cells. ALP activity can be detected by staining cells with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (MyBioSource) as a substrate. For performing the staining procedure, cells were first washed with PBS and 2 ml of neutral buffered formalin (10%) was used to wash each well for 60 seconds. Immediately after 60 seconds, the formalin was aspirated and buffer solution (0.05% Tween 20 to Dulbecco's PBS, w/o Ca2+/Mg2+) was used to wash the cells. After aspirating the buffer solution, 2 ml of BCIP/NBT was added to each well and the plate was incubated for 5
minutes at 37°C. The solution was then aspirated and 2 ml of washing buffer was added in individual wells followed by aspiration and addition of 2 ml of 1X PBS solution. After the staining procedure was complete, one control zirconia and one IL-coated zirconia specimens were observed under optical microscopy.

4.3.6 Investigation of the Tribological Behavior of the IL-coated Surfaces

The wear behavior of IL-coated zirconia specimens in comparison to non-coated (control) specimens was evaluated in artificial saliva at 37°C to simulate the conditions of the oral environment. A continuous sliding motion using a modified pin-on-disk apparatus mounted on a hybrid rheometer (DHR-3, TA Instruments) was used to perform wear testing. The method employed followed procedures described in ASTM G99 “Standard Test Method for Wear Testing with a Pin-on-Disk Apparatus” and ASTM G133 “Standard Test Method for Linearly Reciprocating Ball-on-Flat Sliding Wear” guidelines.

For this test, a stainless steel ball (12.7mm) was fixed in a modified ball specimen holder consisting of a semi-spherical fluid compartment as shown in Figure 4.4. 2 ml of saliva was then added into the fluid compartment which submerged the stainless steel ball completely. The radius of the circular wear scar on the zirconia specimens was maintained at 1.25 mm. 10 N of axial load was then applied on mounted specimens, which corresponds to the maximum elastic contact stress of 839 MPa. The relative speed between the contact point of the ball and specimens was maintained constant at 0.05 m/s for a distance of 50 m. The temperature of this setup was maintained at 37°C throughout the testing duration. The frictional and axial force values were measured at a 1 Hz sampling rate and the coefficient of friction was then calculated.
After the completion of wear testing, samples were cleaned with acetone to remove debris generated during testing and were observed under optical microscope (Digital Microscope VHX-5000, Keyence). The average width of wear scars was measured from six different points located on the circular wear scar. The wear volume loss was then calculated using Equations 1-3, which are applicable to a disk and spherical pin sliding against each other under elastic deformation conditions.

\[
\text{Disk Volume Loss} = 2\pi R \left[ r^2 \sin^{-1} \left( \frac{d}{2r} \right) - \left( \frac{d}{4} \right) \left( 4r^2 - d^2 \right)^{\frac{1}{2}} \right]
\]

Equation 1
Pin Volume Loss = \( \left( \frac{\pi h}{6} \right) \left( \frac{3d^2}{4} + h^2 \right) \) \hspace{1cm} \text{Equation 2}

\[ h = r - \left( r^2 - \frac{d^2}{4} \right)^{\frac{1}{4}} \] \hspace{1cm} \text{Equation 3}

where,

R = Radius of the wear track formed on zirconia specimen.

r = diameter of the spherical pin.

d = Width of the wear track on zirconia specimen or the diameter of the wear scar on the ball.

h = Wear depth of the flat wear scar on the ball.

4.4 RESULTS AND DISCUSSION

4.4.1 Coating Characterization

IL coatings were deposited on the surface of zirconia to investigate their adsorption and deposition profile. Spectroscopy and microscopy techniques were carried out to assess the coating morphology and to understand intermolecular interactions between IL and zirconia. The hypothesis was that carboxylate and amino groups in ILs would interact with zirconium (Zr) and oxygen (O) in the substrate. It was expected that a change in binding energies of constituent elements would be seen due to this interaction.
4.4.1.1 XPS Studies

X-ray photoelectron spectroscopy (XPS) is a widely-used technique to characterize the elemental makeup and chemical environment of atoms present within the first few nanometers of a material’s surface. Binding energy (BE) is the amount of energy required to remove an electron from a given orbital/shell of an atom, which is very specific to the elemental composition and oxidation state of atoms comprising a material. Any changes in the oxidation state due to the addition or removal of electrons or changes in the chemical environment surrounding an atom, such as bond formation, will alter the electrostatic shielding of nuclear charge leading to shifts in BE. Thus, BE can be used to assess the chemical interaction of ILs before and after the adsorption process for a given substrate. Shifts found in these BEs that are greater than the measurement error of the equipment (±0.1 eV) can be correlated to a change in the chemical environment of the constituent atoms on the material’s surface.

In a previous study of the interactions of IL coatings on the surface of titanium, it was observed that ILs containing amino acids as anionic moiety, including those investigated with the surface of zirconia (IL1 and IL2), demonstrated strong affinity for titanium surfaces [5]. The interaction of anionic moiety with hydroxyl groups on titanium surface resulted in a decrease in electron density of titanium (Ti) and oxygen (O) atoms and a shift towards higher BEs was observed. The study revealed strong interactions between carboxylate and amino groups in IL1 and IL2 and titanium surfaces.

The ILs selected for study on the surface of zirconia consisted of a dicationic moiety having the same alkyl chain length (n = 10) and different anionic moieties (phenylalanine and methionine). The cationic moiety in the ILs can interact with negatively-charged sites, and the anionic moiety
can interact with positively-charged sites. Similar to titanium, zirconia as a metal oxide also has two types of interaction sites: zirconium atoms act as an acidic site and can accept electrons, whereas oxygen acts as a basic site and can donate electrons. XPS data reported for zirconia is scarce in the literature. Therefore, it was even more interesting to evaluate these interactions between ILs and zirconia surface as this is the first study to report such data.

To understand the interaction IL-zirconia, BEs of elements comprising IL-coated zirconia were compared with BEs for pure ILs and control (non-coated) zirconia. In zirconia spectra, 3d orbital has the strongest peak, therefore for simplicity and to avoid redundancy, only the BE of the Zr 3d_{5/2} peak is discussed. XPS data for pure IL1 and IL2 is referenced based on our previous study [5]. IL1 and IL2 were used to coat one zirconia specimen each and three different areas were analyzed per sample. Spectra were calibrated using aliphatic C 1s photoemission peak at 285.0 eV per previous studies [87, 88] C 1s, Zr 3d, O 1s spectra for control zirconia, IL1-coated zirconia and IL2-coated zirconia is shown in Figure 5.

In the C 1s spectra of IL1-coated and IL2-coated zirconia, the aliphatic peak (representing C-C bonds in the alkyl chain, which are the primary type of C atoms in the IL structure) showed an increase in BE as compared to pure ILs. The intensity of more polar carbon was seen to increase after deposition of ILs on zirconia surface as compared to the aliphatic carbon of pure IL. However, an aliphatic carbon peak associated with pure IL not interacting with the surface was still present but at a lower intensity. It was hypothesized that most carbon atoms in the IL interacted with the substrate, resulting in the shift of the overall peak to higher BE. This behavior was similarly observed with IL-coated titanium surfaces, where a shift was seen in the aliphatic carbon peak after deposition of IL on titanium. It was hypothesized that this observation was due to a
change from a more hydrophobic environment in the pure ILs to a more hydrophilic environment of titanium surfaces containing hydroxyl groups. Despite not detecting a hydration layer, zirconia surfaces are still expected to exhibit a similar hydrophilic environment due to its metal oxide surface (ZrO₂).

In Figure 4.5, the dashed line represents the aliphatic carbon (285.0 eV) peak, which is the primary component of pure ILs [5]. After IL deposition on zirconia, it can be observed that there was an increase in intensity to more polar carbon (286.5 eV). Again, despite a lack of detecting a hydration layer on zirconia, it is hypothesized that hydrogen bonding occurred between hydrogen associated with the C atoms of the alkyl chain and oxygen present in ZrO₂ due to the covalent nature of the Zr-O bond.

Zr was detected on all surfaces of both IL1-coated and IL2-coated zirconia samples and the corresponding binding energies are shown in table 4.1. The binding energy of Zr 3d shifted to a higher BE for both ILs as compared to control zirconia (183.0 ± 1 eV). This illustrates the interaction between ILs and zirconia surface. The shift was found to be similar for IL1-coated (183.7 ± 0.2 eV) and IL2-coated (183.9 ± 0.0 eV) zirconia samples.

Table 4.1 Binding energies (eV) of Zr 3d5/2 for control zirconia, IL1-coated zirconia and IL2-coated zirconia.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Binding energy Zr 3d5/2 (±SD) eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control zirconia</td>
<td>183.0±0.1</td>
</tr>
<tr>
<td>IL1-coated zirconia</td>
<td>183.7±0.2</td>
</tr>
<tr>
<td>IL2-coated zirconia</td>
<td>183.9±0.0</td>
</tr>
</tbody>
</table>
The binding energy of O 1s in control zirconia samples was found to be in accordance with values reported in the literature [89, 90]. As seen in our previous study with titanium, apart from O 1s associated with TiO$_2$, a second species at a BE of 531.92 ± 0.03 eV was observed on control titanium samples [5]. This was attributed to the hydrated layer on the outer surface, which is usually 1.2-2.5 eV higher in BE than oxygen associated with metal oxide (ZrO$_2$). However, no such component could be readily distinguished in the O 1s spectra of control zirconia, suggesting that no significant degree of hydration occurred on its surface. Upon comparing O 1s spectra from control zirconia and IL-coated zirconia, a shift in BE was observed for both IL-coated samples. The BE of oxygen associated with ZrO$_2$ on IL1-coated (531.7 ± 0.2 eV) and IL2-coated (532.7 ± 0.0 eV) samples was found to be higher than control zirconia (530.8 ± 0.1 eV).

The N 1s spectra of IL1-coated and IL2-coated samples is shown in Figure 4.5. Dashed line represents the N 1s binding energy of pure ILs. The peaks from anionic nitrogen (N$_a$) and cationic (N$_c$) were seen to merge leading to overall broadening of the peak. However, in the spectra from pure ILs, both the peaks can be seen distinctly (illustrated in Appendix A.3). That is, there was a shift in binding energies of anionic nitrogen (N$_a$) and cationic nitrogen (N$_c$) when compared to pure ILs. The peak from anionic nitrogen had a pronounced change and was shifted to a higher BE. As noted in our previous study, this behavior can be attributed to hydrogen bonding occurring between the amine groups of the anionic moiety and oxygen atoms present in ZrO$_2$. Similarly, a shift in the BE of cationic nitrogen, although to a lesser extent, can be also attributed to the interaction between the cationic moiety and zirconia surface.
Overall, the results of XPS analysis indicated that interaction between ILs and zirconia surfaces occurred via strong hydrogen bonds formed between carboxylate and amino groups of ILs and substrate.

Figure 4.5 XPS spectra of (a) Zr 3d, (b) C 1s, (c) O 1s, and (d) N 1s of control zirconia (blue), IL1-coated (red), and IL2-coated (green) zirconia. Dashed lines (from left) represent BE of C 1s in IL1, BE of Zr3d in control zirconia, BE of O 1s in control zirconia, and BE of N 1s in IL1.
4.4.1.2 Release Profile

For successful application in dental implants, surface coatings should survive conditions of insertion forces and load bearing stresses. Coating stability is an essential requirement for the maintenance of surface functionalization during the initial healing period post-implantation. Studies have shown that matrix rich regeneration tissue starts to develop between the bone and implant surface during the first week of implantation [82]. Therefore, considering the initial healing period occurs within the first two weeks post-implantation, IL coatings were expected to survive on the surface of zirconia for at least 1-2 weeks. First week after implantation is particularly important because, in the race-for-the-surface, both bacterial and host cells compete to attach and grow on implant surfaces. Therefore, it is important that the coating remains on the surface during the initial healing period to provide antimicrobial activity and to protect the implant surface against biofilm formation. In this study, IL coating release in aqueous media was investigated after 1 and 7 days post-immersion to represent stability of coating during initial week after implantation. PBS and saliva were chosen to simulate relevant physiological conditions.

Figure 4.6 shows the concentration of IL (mM) released from coated specimens immersed in PBS and saliva. After one day of immersion in both media, lower IL release was observed for zirconia samples coated with IL1 (33.9±0.5% and 4.9±0.5%, in PBS and saliva, respectively) in comparison to samples coated with IL2 (43.2±4.6% and 39.2±1.5%, in PBS and saliva, respectively. This result is due to the fact that IL1 and IL2 contain phenylalanine and methionine as anionic moieties, respectively, and the presence of an aromatic ring in phenylalanine results in a more hydrophobic compound than methionine, which will not get easily solvated. It can be assumed that most of IL molecules in IL1 remained on the surface of zirconia and did not diffuse
into the immersion media. It is also assumed that besides the structural differences in IL composition, the difference in pH of the immersion media (saliva with a pH of 4.9 and PBS with a pH of 7.4) contributed to the pronounced effect on the release profile of ILs from zirconia. A significantly lower release was seen in saliva as compared to PBS for IL1-coated samples at both time points (1 day and 7 days) (p<0.05). Similar behavior was observed in our previous study with IL1-coated titanium specimens. In that study, IL1 showed lower release than IL2 on the surface of titanium in both immersion media and at both time points [6]. Particularly, lower release was seen in saliva than in PBS for all samples investigated. It has been observed in the literature that amino acids have a high affinity towards titanium surfaces in acidic environment [44]. Even after 7 days of immersion in PBS and saliva, the results showed that IL release was not significantly (p>0.05) increased when compared to the results obtained for 1 day of immersion in both solutions.

Figure 4.6 Concentration of IL 1 and IL 2 released from coated samples immersed in PBS and saliva. *Significantly different (p<0.05) n=3.
After 7 days, the percentage of IL1 concentration (mM) released in PBS and saliva relative to coated concentration (mM) was 41.9±8.3% and 8.7±1.9%, respectively, and for IL2 it was 64.4±8.7% and 59.9±19.5%, respectively. In our previous study, concentration release of IL1 and IL2 from coated titanium specimens was assessed in a similar experimental setup [6]. After 7 days, concentration release of IL1 in PBS and saliva was found to be 38.2±1.7% and 10.2±4.1% respectively, while for IL2, the values were 78.2±18.3% and 19.0±1.6% respectively. It can be seen that with IL1 coating, zirconia and titanium samples showed similar IL release percentages in PBS and saliva. IL2 showed similar release results in PBS for both zirconia and titanium samples, however IL release percentage in saliva was significantly lower for titanium samples as compared to zirconia (p<0.05). IL2 was seen to agglomerate on the surface of titanium after immersion in saliva, which is assumed to be the cause for its lower release in that immersion medium. Overall, in the present study, IL1 coating demonstrated high stability and significantly lower release in saliva as compared to IL2. This result is important as it supports our hypothesis of using these compounds as surface coatings for zirconia.

4.4.2 Mammalian and Bacterial Cell Activity

After implantation, a dental implant comes in contact with surrounding soft and bone tissue, where the implant screw is expected to integrate with bone while the top and abutment directly interfaces with gingiva and other soft structures. Thus, for long-term success of implant, it is essential that it integrates with soft and hard tissues and a secure connection at the implant-tissue interface is established [1, 2]. It has been proposed that formation of soft tissue seal at
implant neck contributes to the protection of alveolar bone from pathogenic attack at the initial healing period [1]. Fibroblasts and osteoblasts play an important role in maintaining biocompatibility and osseointegration at the implant site as they are the main components of soft tissues and bone, respectively.

The sequence of events post-implantation is expected to occur the same way with titanium and zirconia implants [82, 105]. Depprich et al. studied the osseous healing of titanium and zirconia dental implants in vivo and observed that healing in both types of implant surfaces was similar with no significant difference between them. Bacterial colonization on implant surface is a natural process and in most cases is similar to the microbiota present around natural healthy teeth [105]. However, when bacterial biofilms adhere at early stages post-implantation the normal progress of tissue growth and sealing may be interrupted. Therefore, it is essential that implant surfaces resist biofilm adhesion at the early healing period following implantation. All of this body of information has been gathered in the past few years for titanium dental implants. Although zirconia has been recently shown in the literature to exhibit less plaque adhesion than titanium surfaces, only a few studies have investigated how cells and bacteria behave on the surface of this material [34-36]. Further investigations in relevant physiological conditions are needed to understand this behavior. Therefore, the goal of this section of the study was to assess the fundamental behavior of mammalian host and bacteria cells on control zirconia and IL-coated zirconia surfaces in terms of growth and differentiation.
4.4.2.1 Mammalian Cell Activity

To better understand the cytocompatibility of IL-coated zirconia, cell viability was evaluated with Human gingival fibroblasts (HGF-1) and Mouse pre-osteoblasts (MC3T3-E1) after exposure to IL-coated (IL1 and IL2-coated zirconia) and control zirconia (non-coated) specimens and the results are summarized in Fig. 4.7. The cells were cultured on control zirconia and IL-coated zirconia samples for 1 and 7 days. Subsequently, MTT assays were carried out and cell viability was calculated relative to control zirconia. For HGF-1 cells, IL1-coated samples showed similar viability values (94.6%) as control zirconia (100%) after 1 day (p>0.05). However, IL2 resulted in significant reduction in cell viability (80.8%) when compared to control zirconia (p<0.05). After 7 days, both IL1 (82.2%) and IL2-coated zirconia specimens (90.1%) resulted in lower cell viability than control zirconia; however, these viability values were not statistically different than the control (p>0.05).

Figure 4.7 Cell Viability (%) relative to control zirconia for HGF-1 and MC3T3-E1 after 1 and 7 days. * Reduction was statistically significant (p<0.05) n=3.
For Pre-osteoblast cells, IL2-coated zirconia resulted in similar cell viability (92.7%) as control zirconia (100%) after 1 day, whereas IL1-coated zirconia displayed significantly lower viability values (82.2%) relative to control zirconia (p<0.05). After 7 days, viability values obtained for IL1-coated (87%) and IL2-coated (94.4%) samples were similar to control zirconia and no significant differences were observed between them (p>0.05). Overall, with all the compositions and at both time points investigated (1 day and 7 days), there was some decrease in the number of viable cells on IL-coated samples. According to ISO standards for assessment of cell viability on the surface of biomaterials, more than 30% of reduction in cell viability is considered a cytotoxic effect. Since cell viabilities obtained with the investigated samples were above 80% in all cases, minimum recommendations of ISO standards (ISO/En10993-5 2009) were met and the coatings are not considered cytotoxic. These results can be correlated with the release profile of IL1 and IL2-coated zirconia in PBS as discussed previously in section 4.3.1. It can be assumed that since a high concentration of IL1 and IL2 was released after the first day of immersion in this medium, lower viability was observed for IL-coated zirconia after 1 day. However, since the cell culture medium was changed every 48 hours, the effect of ILs was reduced and an increase in cell viability was observed.

In the previous study carried out with IL-coated titanium, IL1-coated samples showed more than 70% cell viability for both MC3T3-E1 and HGF-1 cells after 7 days, which was similar to the results found for zirconia coated with the same IL composition [6]. Interestingly, IL2-coating resulted in different cell viabilities on the surface of titanium and zirconia. It should, however, be noted that the amount of IL (µm) deposited on zirconia specimens (1.1 µm per specimen) was lower than the amount deposited on titanium (2 µm per specimen) [6]. This was expected to result
in differences in cell interactions with IL-coating between the two substrates. Higher concentration of IL2 on titanium specimens resulted in a reduction in viable cells. Nutritional and metabolic studies have shown that a high dose of methionine can be highly toxic and may result in fatal consequences [4]. However, low concentrations, such as the ones used for coating of zirconia specimens in this study, are not considered harmful for in vivo applications. Studies have shown that humans can metabolize phenylalanine, which is a safe amino acid [5]. Therefore, it can be concluded that cells exposed to IL1-coated surfaces, which had phenylalanine, exhibited higher viability than cells exposed to IL2-coated surfaces, which contained methionine.

Differentiation of pre-osteoblasts to osteoblasts correlates to a high ALP activity in cells. ALP is a common biochemical marker and is used to identify the differentiation of pre-osteoblasts to osteoblasts in vitro. Increase in the levels of ALP activity is often related to the initiation of bone mineralization [6]. The differentiation and proliferation of pre-osteoblasts on IL-coated and control zirconia were determined by their alkaline phosphatase activity and the results are summarized in Fig. 4.8. It was found that although progenitor cells exposed to both IL-compositions coated zirconia exhibited lower viabilities in relation to the control, the presence of IL on the surface did not affect their differentiation to osteoblasts. The ALP values of coated and non-coated zirconia were similar to each other both after 1 and 7 days (p>0.05).

To visualize the attachment and differentiation of pre-osteoblasts to osteoblasts, control zirconia and IL-coated specimens were stained to detect Alkaline Phosphatase enzyme after 1 and 7 days of culture. Stained cells were observed under optical microscopy and the differentiated cells were identified by the purple color obtained after staining. The staining results for the surfaces of control zirconia, IL1-coated as well as IL2-coated samples after 7 days is shown in Fig. 4.9. It was
observed that pre-osteoblast cells were able to attach and differentiate to osteoblasts on the surface of IL-coated specimens.

![Graph showing ALP activity of MC3T3-E1 cells](image)

**Figure 4.8** ALP activity of MC3T3-E1 cells after exposure to control zirconia, IL1-coated and IL2-coated zirconia samples for 1 and 7 days. (p>0.05) n=3.

![Images showing ALP staining](image)

**Figure 4.9** ALP staining in MC3T3-E1 cells on the surface of a). Control zirconia b). IL1-coated zirconia c). IL2-coated zirconia.

As demonstrated by Zhao *et al.*, the race for the surface is difficult to be won by host cells in the oral environment because of the enormous microflora present in the oral cavity [3]. The IL coatings proposed in this work are aimed at imparting anti-biofilm activity to zirconia surfaces
while being compatible with host cells. Thus, it becomes essential to determine the performance of these ILs on the surface of zirconia against bacterial strains of relevance in the oral environment.

### 4.4.2.2 Bacterial Cell Activity

Anti-biofilm activity of IL-1 and IL-2 coated zirconia was tested against *S. salivarius* and *S. sanguinis* for 1 and 7 days in culture broth containing BHI and saliva and the results are summarized in Fig. 4.10. After completion of the immersion period, concentration of bacteria in the immersion fluid as well as on the surface of zirconia specimens was quantified separately and reported in log CFU/ml. Bacterial concentration on the surface was determined by scraping the specimens in buffer solution and plating a small volume onto the BHI agar plates as explained in section 4.2.4. Bacteria present in the fluid was quantified by directly plating a small volume from the immersion fluid.

As shown in Figure 4.10, IL1-coated zirconia specimens produced significantly reduced bacterial count (log CFU/ml) in the immersion fluid of both *S. salivarius* (5.5±0.22) and *S. sanguinis* (4.3±0.38) as compared to control zirconia (6.7±0.21 and 5.9±0.31, respectively) after 1 day (p<0.05). The surface of specimens coated with IL1 was found to be almost devoid of bacteria for both *S. salivarius* (0.0±0.00) and *S. sanguinis* (0.0±0.00) and this reduction was significantly lower in relation to control zirconia (5.8±0.30 and 5.1±0.36, respectively) (p<0.05). However, IL2-coated zirconia specimens did not show anti-biofilm activity and had similar bacterial load as control zirconia. Bacterial count (log CFU/ml) in immersion fluid of IL2-coated specimens for *S. salivarius* (6.08±0.35) and *S. sanguinis* (6.3±0.19) was found similar to control zirconia (6.7±0.21 and 5.9±0.31, respectively) (p>0.05). Similarly, no significant difference was
found between the *S. salivarius* (5.2±0.15) and *S. sanguinis* (4.0±0.09) load on IL-2 coated surfaces in relation to control zirconia (5.8±0.30 and 5.1±0.36, respectively) (p>0.05). As discussed previously, the release of IL1 coating was only 4.9±0.5% after 1 day of immersion in saliva. This led to the decrease in CFU/ml measured in the immersion fluid, where the presence of the coating on the surface reduced the number of bacterial cells on the surface.

After 7 days of immersion, the anti-biofilm activity of IL1-coated zirconia was still retained, which resulted in significantly lower count of bacteria (log CFU/ml) in immersion fluid of *S. salivarius* (2.7±0.50) and *S. sanguinis* (4.4±0.26) as compared to control zirconia (6.2±0.02 and 6.1±0.13, respectively) (p<0.05). Interestingly, the surface of IL1-coated specimens showed absence of bacterial colonies and significantly lower bacteria count for *S. salivarius* (0.0±0.00) and *S. sanguinis* (0.0±0.00) after 7 days in relation to control zirconia (5.4±0.16 and 5.6±0.02, respectively) (p<0.05). Correlating these results with the coating release profile, 8.7±1.9% of IL1 was released in saliva from zirconia surface after 7 days. Since the amount released was relatively low, it helped to maintain the antimicrobial activity in fluid and on the surface. However, similar to 1 day results, IL2-coated zirconia specimens exhibited similar bacterial colonies as control zirconia after 7 days (p>0.05).
Figure 4.10 Log CFU/ml in fluid and surface of control zirconia and IL-coated zirconia after 1 day and 7 days of exposure to bacteria (*S. salivarius* or *S. sanguinis*). Reduction was statistically significant (p<0.05) n=3.

*Streptococcus* species are among the early colonizing bacteria in oral implants [8]. They attach and grow in a biofilm mode of growth and can hinder the process of initial osseointegration and soft tissue seal formation [97]. *Streptococcus* species have the ability to co-aggregate within the same genera as well as with other bacterial species [98]. This means they can bind with other early colonizers as well as with host molecules and form dental plaque readily. *S. salivarius* is one of the most prevalent oral bacterium. It is considered as a moderator as it colonizes the oral cavity first and creates favorable conditions for other bacterial species to colonize [98]. *S. sanguinis* attaches to oral surfaces and serves as an anchor for attachment of other oral bacteria [108]. Studies
have shown that early colonizers can generate favorable conditions for the growth of late colonizing bacteria such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans*, which can ultimately lead to bone loss and conditions such as peri-implantitis [9].

Observations of lower bacterial adhesion on zirconia surfaces as compared to titanium have generated debate in the literature. A few studies have reported lower bacterial load on zirconia than titanium [35-37], whereas others showed that the differences were not significant [32-33]. In an *in vivo* human study with 10 patients, significantly lower bacterial adhesion was found on zirconia as compared to titanium after 24 hours (p=0.0001) [35].

*Rimondini et al.* studied the colonization of titanium and yttrium-stabilized zirconia specimens by different strains of oral bacteria *in vitro* and *in vivo* [36]. Zirconia was found to have lower bacterial adhesion as compared to titanium *in vivo*. Specifically, only clusters or aggregates of bacteria could be found on zirconia surfaces while titanium specimens had more homogenous, continuous biofilm formation across the entire surface.

Despite studies demonstrating lower bacterial load on zirconia surfaces in physiological conditions, several other studies suggested similar bacterial adherence and biofilm formation on titanium and zirconia surfaces [32, 33]. However, the zirconia surface still undergoes colonization by oral bacteria, and the question of protection of implant surface during initial healing period still remains. Therefore, IL coatings selected in this study are relevant as they can provide anti-biofilm activity to the implant surface during the initial healing period.

Anti-biofilm activity of ILs against a wide range of bacterial sp has been investigated in the literature [78, 82]. This property is attributed to the hydrophobicity of the alkyl chain, which
can target and disrupt cell membranes. IL1 has been previously shown by Gindri et al. to have anti-biofilm activity against early colonizers on titanium surfaces [6]. Drawing conclusions from previous results, this coating was expected to present similar activity on the surface of zirconia. Zirconia specimens coated with IL1 demonstrated strong antimicrobial activity after 1 day; whereas, no such activity was observed for IL2-coated zirconia. These results demonstrate that IL1 coating constitutes an effective surface treatment to reduce biofilm formation on zirconia surfaces.

In addition to this, bacterial adhesion on titanium and zirconia can be directly compared from these two studies since polished titanium control was studied under the same experimental conditions as the zirconia specimens in the present investigation. It should be noted that concentration (mM) of the ILs used for coating was different between the studies. According to the results for control samples, control zirconia specimens showed lower bacterial adherence than control titanium specimens, which is in accordance with previous observations [36, 56]. A few studies in the literature have shown that surface hydrophilicity and material composition of titanium and zirconia surfaces appear to influence the degree of bacterial adhesion [10]. However, other studies mentioned that these parameters do not have an influence on bacterial adhesion [34]. Therefore, further investigations are required to better elucidate this behavior. Long-term clinical studies are expected to address these inconsistencies seem in short-term studies and with different *in vitro* experiments.
4.4.3 Tribology

High-strength zirconia ceramics are wear resistant materials and have excellent mechanical and chemical properties. However, in the complex oral environment, an implant may experience wear originating from one or combination of various factors. Factors such as friction forces during insertion, abrasion, fatigue, pH imbalance and mastication stresses may lead to implant wear [107]. The proposed IL coatings are aimed at further improving the wear resistance of zirconia surfaces by providing lubrication and reducing the wear volume loss. This is expected to provide protection to an implant surface during insertion, when an implant may experience excessive frictional stresses, which may disrupt surface morphology.

To verify the contributing effect of the two IL compositions investigated on the tribological behavior of zirconia, wear tests were performed at an axial load of 10 N and 37° C while samples were immersed in saliva. These experimental conditions were chosen to represent worst case scenarios for the implant experiencing stresses during insertion, mastication and micromotion [107, 112]. Coefficient of friction and wear volume loss were calculated, and the results are shown in Figure 4.11.

IL coatings were found to be stable throughout the testing and were able to reduce friction between the two surfaces in contact. A significant sustained decrease in the coefficient of friction values was observed for IL1-coated (0.17±0.00) and IL2-coated zirconia (0.2 ±0.00) compared to control zirconia (0.45±0.13) (p<0.05), as shown in Figure 4.11. The width of the wear scar formed on samples surface corresponded to the volume loss generated by the wear conditions. Wear volume loss for IL1-coated zirconia samples (0.0097±0.00 mm³) was significantly lower than control zirconia samples (0.0224± 0.00) (p<0.05) as can be seen in Figure 4.11. IL2-coated
zirconia samples (0.0142±0.00) also exhibited lower wear volume loss as compared to the control. However, this reduction was not significantly different (p>0.05).

Applied axial load, relative sliding speed between zirconia specimens and the stainless steel ball, and stability and thickness of the IL coating on the specimen surface were factors which influenced the coefficient of friction (COF) values. COF values between 0.2-0.3 for unlubricated metal-ceramic pairs and 0.55-0.75 for ceramic-ceramic pairs have been reported in literature [95, 109]. The COF values of control zirconia (0.45±0.13) found in this study were more than what is typically reported in literature for metal-ceramic sliding pairs. However, the metals used in other studies had lower hardness than the stainless steel counterpart used in this study.

The results showed that both IL compositions resulted in significant reduction in COF as compared to control samples. This reduction can be attributed to the presence of long alkyl chains in both IL structures. As discussed in the literature, longer alkyl chains help to form stable layers

![Figure 4.11](image-url)
on titanium surfaces, which in turn lead to lower wear loss [110]. Minami et al. described the effect of alkyl chain length on COF and concluded that increasing alkyl chain length leads to lower COF due to an increase in viscosity of IL [109]. Hydrophobicity of anions also plays an important role in determining lubrication performance of ILs. It has been reported that an increase in hydrophobicity results in lower COF and wear volume loss values [110]. This effect was seen in the present study as the anion in IL1 (phenylalanine), which is more hydrophobic than IL2 (methionine), resulted in slightly better lubrication performance as compared to IL2 [79].

In regards to lubricating film stability, ILs formed a stable layer on zirconia surfaces. COF values for IL1- and IL2-coated samples were found to be stable throughout test indicating hydrodynamic lubrication between the stainless steel ball and zirconia specimens. In this mode of lubrication, IL coatings on zirconia surfaces separated the sliding surfaces and prevented them from coming into direct contact with each other. As seen previously in the coating release profile study, selected ILs were found to be more stable in saliva and had a lower release in saliva as compared to PBS. The protective layer formed by IL was maintained in the solution throughout the testing, and stable COF values were obtained for both IL-coated samples as seen in Figure 4.12.
After completion of the wear test, a good amount of debris was observed on the samples. Even after wiping the samples with acetone, this dark colored accumulation was still visible in some areas. It can be assumed that parts of this debris were present on the surfaces due to material transfer from the wear of the stainless steel ball.

A similar study using stainless steel plates and ceramic balls with an applied load of 50 N and a relative velocity of 50 mm/s for 25 mm stroke length was performed [95]. A large amount of debris was generated during the process, and material transfer was seen in the wear areas of stainless steel plates and ceramic balls. It was found that the wear of the stainless steel plates occurred primarily by an abrasive behavior. Since the hardness of ceramics is higher than stainless steel, abrasion was found to be the major wear mechanism.
As seen in Figure 4.13, wear scars formed on control zirconia surfaces were found to be completely circular and prominent for all three samples. In IL-coated samples, the wear scars were not completely circular and particularly in IL1-coated samples, the wear track width was found to be comparatively thinner than control zirconia. This was due to slipping occurring on the surface during wear testing, demonstrating that the IL provided lubricant activity. Overall, IL1-coated samples were seen to have significantly less wear generation due to lower wear volume loss than control zirconia samples (p<0.05). Moreover, the total wear volume loss for IL-coated samples can mainly be attributed to wear generation from the stainless steel ball and not the zirconia specimen. That is, the appearance of relative large wear scars on zirconia was due to superficial scratching by the stainless steel ball as minimal indentation was visible on its surface. As stainless steel is less wear resistant than zirconia, the circular contact area between the ball and specimen became increasingly larger, allowing for more superficial damage on the zirconia surface. Still, a stable and protective layer of IL1 and IL2 was formed on zirconia surface depicting hydrodynamic lubrication, wherein stainless steel ball and zirconia surfaces were separated by a layer of IL in between them. This helped in minimizing direct contact between both surfaces resulting in lower wear total volume loss than control zirconia. Frictional forces during insertion of an implant, mechanical stresses and micromotion are factors contributing to early implant loss by hampering the surface stability of implant during initial phases after implantation [107]. Lubrication provided by ILs on implant surfaces can help minimize such frictional and wear effects, facilitating implantation.
In conclusion, dicationic imidazolium based ionic liquid coatings containing phenylalanine (IL1) demonstrated anti-biofilm activity and enhanced wear performance on zirconia surfaces while being compatible with host cells. The coatings were stable on zirconia and although some release was seen after 7 days, the coating was effective against bacterial adhesion for the tested time period. All these properties can be utilized by an implant during the initial healing period.

Putting all the observations together, it can be concluded that these ionic liquid coatings can be seen as a potential technology for surface modifications of dental implants. These coatings are expected to enhance the implant stability by: (i) preventing bacterial adhesion on implant surface, which would help host soft tissues to seal the implant; (ii) providing intrinsic lubrication to implant surfaces, thereby shielding it against mechanical forces during insertion.

In summary, the goals of this study were met and the hypothesis was proved correct. Some of the limitations of this study included the time periods for cell and IL release studies. A longer time duration of 14 days as compared to 7 days, would provide a better evaluation of performance of ILs during the initial healing period of implants. It will help understand the stability of ILs and cell adhesion on the coated surfaces during this period. In addition to this, wear testing under more
acidic crevice conditions would help understand the behavior of zirconia in severe oral conditions. Future studies involving extended \textit{in vivo} testing would help understanding the response of host cells and bacteria towards these coatings and to establish their role during the healing periods.
CHAPTER 5
SUMMARY AND CONCLUSIONS

Dicationic imidazolium-based ionic liquid coatings were deposited on the surface of zirconia, and interactions between IL-zirconia surfaces were determined using XPS. These results were particularly important as there is not much XPS data regarding zirconia present in the literature. As seen in XPS results, anionic moieties (phenylalanine methionine) in IL1 and IL2 interacted with the surface of the substrate, with amino and carboxyl groups forming strong hydrogen bonds with zirconia surface. This revealed a strong interaction between the IL coatings and zirconia surfaces and supports the idea of their use as surface coatings. Release profile of IL1 and IL2 coatings on zirconia in PBS and saliva showed that lower release was found for IL1 as compared to IL2 in both immersion media. IL1 has a more hydrophobic anion (phenylalanine) than IL2 (methionine), which caused a lower release than IL2. Therefore, IL1 would be a better candidate as a surface coating as coating stability is an important property for successful implementation. Cells and early bacterial colonizers associated with the oral environment were used to test the compatibility and anti-biofilm activity of IL-coated zirconia surfaces. Both IL1 and IL2 were found to be compatible with pre-osteoblasts and human gingival fibroblasts enabling their growth, proliferation, and differentiation (pre-osteoblasts) on zirconia surface. However, only IL1 demonstrated anti-biofilm activity towards the bacterial strains while IL2 did not show any such property and was not found to be useful against the oral bacteria strains (Streptococcus species) considered in the present study. This severely limited application of IL2 as a surface
coating for the proposed dental applications. Wear testing with control and IL-coated zirconia showed that a significant reduction in coefficient of friction and total wear volume loss was observed in IL1 coated zirconia samples as compared to control zirconia and to a lesser extent for IL2. Overall, IL1 exhibited excellent properties in terms of coating stability, cell compatibility, anti-biofilm activity and lubrication behavior. This supports our hypothesis regarding their use as coatings for dental implant applications. Therefore, it can be concluded that IL1 can be a potent strategy to provide multi-functionalities to zirconia surface and improve implant survival during the initial healing period post-implantation.
CHAPTER 6
FUTURE WORK

This work presented the performance of dicationic imidazolium-based ionic liquids on the surface of zirconia. This study is of importance as zirconia is a relatively new material and its behavior in relevant physiological conditions have not been explored much in literature despite it being used for dental application. The use of selected ILs helped enhance the surface properties of zirconia. However, this study can be carried forward and further investigations on IL behavior on zirconia surface can be investigated.

Firstly, longer time points depicting initial healing period of implant can be incorporated in different sections of this study to investigate IL coating behavior on zirconia. This will provide a better picture of IL performance in a complete implant healing scenario.

Secondly, co-culture studies can be designed using bacterial and mammalian cells in a single experimental setup to establish a race-for-the-surface model. It would be interesting to see the effect of IL coating on behavior of bacterial and host cells and their interaction with each other and whether IL coating would allow for cells to adhere or remain on the surface in the presence of bacteria.

Thirdly, in vivo experiments can be performed to understand the effect of IL coatings on osseointegration and soft tissue seal formation post-implantation. This will help understand host tissue response and protection against oral bacteria during healing period.
APPENDIX

Table A.1 Roughness and maximum adhesion strength of 1 sample of control zirconia, IL1-coated zirconia and IL2-coated zirconia.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Roughness (Ra)</th>
<th>Max Adhesion Strength (nN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control zirconia</td>
<td>2.05 nm</td>
<td>Control zirconia</td>
</tr>
<tr>
<td>IL1-coated zirconia</td>
<td>4.73 nm</td>
<td>IL1-coated zirconia</td>
</tr>
<tr>
<td>IL2-coated zirconia</td>
<td>3.11 nm</td>
<td>IL2-coated zirconia</td>
</tr>
</tbody>
</table>
Figure A.1 Atomic Force Microscopy (AFM) results for control zirconia and IL-coated zirconia. Top to bottom: 2D height; adhesion strength; and 3D height. Left to the right: column 1: control zirconia; column 2: IL1-coated zirconia; column 3: IL2-coated zirconia.
Figure A.2. Percentage release of IL concentration (mM) relative to coated concentration (mM) after immersion in PBS and saliva at 37ºC for 1 and 7 days.

Figure A.3. Percentage release of IL concentration (mM) relative to coated concentration (mM) after immersion in PBS and saliva at room temperature and 37ºC for 1 day.
REFERENCES


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VITA

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