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METAL-DOPED FLUROAPATIE FOR THE PREVENTION OF BIOFILM FORMATION

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Abstract— Bone anchored percutaneous implants are used as an alternative docking system for exoprostheses attachments. Although successful, 5% of the dental implants and 7-20% of percutaneous osseointegrated prostheses (POP) are explanted due to device failures related to infection. Although these implants can restore the functionalities after the loss of a tooth or limb, the potential to become infected is attributed to the inherent inability of the skin to form a seal around the metallic implant surface. As a result, opportunistic bacteria can infiltrate into the periprosthetic tissues, adhere to the implant surface, and form a biofilm. The antimicrobial coating could prevent bacterial adhesion on the device and limit infection. However, such coatings also should have the ability to promote skin, particularly the epidermal cells, to complete the wound-healing at the interface. Previously data from our lab showed that fluorapatite (FA) could promote epidermal cells to implant adhesion. Thus, this study was designed to improve the antimicrobial properties of FA by doping it with known antimicrobial metals. Various apatites were made in-house, and their antibiofilm properties were tested using a CDC-biofilm reactor. The data showed that 2% Zn-doped FA could limit biofilm formation by a log-fold on the surface. However, due to variation data, there was no significant difference in biofilm formation between metal-doped FA and FA disks. Future studies should expand the sample sizes and percentages of other metallic apatites.

Keywords: antibacterial, fluorapatite, metallic ions, percutaneous osseointegrated device, infection.

I. INTRODUCTION

Percutaneous osseointegrated implants (POIs) are bone-anchored devices that protrude through the skin into the external environment. In dentistry, approximately 450,000 are implanted annually[1]. Another type of POI device is the percutaneous osseointegrated prosthetic (POP) device, which is slowly emerging as an alternative to a socket-type prosthetic docking system. Though dental implants are commonly used, POP devices have the potential to become an effective option for amputees. A major concern for both devices is high rates of infection-related implant failures. These implants are commonly made from titanium or titanium alloys and pass through the skin, leaving an open stoma that is continually trying to heal to re-establish a protective skin barrier against the external environment [2]. Because of this, the skin begins to downgrow away from the implant and form a sinus tract, which exposes the periprosthetic tissue to the potential external bacterial environment. Infection of the dental implants is a common cause of their failures, with approximately 5% of dental implants being removed yearly [3], [4]. Likewise, between 7-20% of POP devices fail due to infection after 12 months[5]–[7].

In dentistry and orthopedic applications, it is common practice to modify the surface properties of the implants to promote bone integration (osseointegration), which includes osteoconductive or antimicrobial coatings. One of the most common classes of osteoconductive coatings is the calcium phosphate bioceramics [8]–[10], which includes hydroxyapatite (HA)[2]. HA has been shown to improve the outcomes in bone-anchored hearing aids, certain dental implants, and the ITAP (a POP device designed and tested in the UK) devices[11]–[13]. Such commonly used calcium phosphate coatings take no proactive steps to prevent infection or promote healing. It should be noted that slight modifications to the chemical composition of such

coating could reduce both the rate of downgrowth and infection.

Previous work has shown that these HA coatings are biocompatible. However, they can readily be absorbed. It was realized that the strength and crystallization properties could be improved by replacing the hydroxy ions with fluoride ions to form fluorapatite (FA)[4], [11], [12]. FA is stoichiometrically similar to the mineral composition of natural bone, and thus, it can readily be resorbed and reused by bone cells. FA has been shown to have higher mechanical strength and lower *in-situ deg*radation properties, leading to an overall more suitable bone substitute [2], [15]. FA scaffolds have also been fabricated and, when sintered at high temperatures, have been shown to have a similar mechanical strength as cancellous bone[3]- [4]. It has also been demonstrated that these apatite-based bioceramics allow for the bone ingrowth and regeneration of bone tissue in both bone scaffolds and apatite-coated orthopedic implants *in vivo* [2], [16]. Incorporation of various metals, such as zinc (Zn), strontium (Sr), and cesium (Cs), have been shown to improve the antibacterial properties of fluorapatite in slurry [14], [15], [17]. However, the effect of these metal-doped apatites on the formation of biofilms on their surface remains unknown. Biofilms form naturally on implanted devices as bacteria adhere and adsorb onto the surface of the implant due to hydrophobic interactions. This creates a multicellular, three-dimensional structure enclosed by polysaccharides. This provides an ideal environment where the bacteria to avoid attack from the host immune system and antibiotic agents[18].

Therefore, the purpose of this research is to determine if the incorporation of metal ions into the crystal structure of fluorapatite would provide some antimicrobial protection against the formation of biofilms. To do this, FA was synthesized in the lab with differing molar substitutions of Zn, Sr, Cs, and Ag, ranging from 0-10% molar substitution. These modified FAs, referred to as metal-doped FAs, were then pressed into disks and sintered at high temperatures to improve their crystallinities. The surface antimicrobial properties were determined using a biofilm reactor with *S. aureus* as the inoculum. The formation of biofilms on the surface of these disks was quantified by counting the number of bacteria using serial dilutions. The number of bacteria counted from each disk will then be analyzed to determine the statistical significance of the data. This information will then facilitate the future development of antimicrobial, osteogenic implant coatings. These enhanced implant coatings would be a significant step toward preventing infections associated with POI implants.

II. BACKGROUND

With an aging population, the need for POI dental devices is increasing, along with the need to develop an effective solution to prevent infection in these devices[19]. Additionally, POP devices are of interest because they allow for an alternative prosthetic attachment to the patient in a way that transfers the load of the patient's body directly into the skeletal system while avoiding the skin issues prevalent in socket prosthetics[2]–[4]. However, the high infection rate in POI prosthetics is a significant barrier to the common clinical use of POP devices. Currently, the primary method of treatment for these types of infections is the use of antibiotics, though this is less-than-desirable due to potential antibiotic-resistant bacteria [9], [10], [21], [22].

Bacterial colonization during continuous healing is a major contributing factor in the delayed repair of skeletal injuries in dental and orthopedic implants. The formation of biofilm on the implant or scaffold surface makes the removal of the bacteria and cleaning of the implants more difficult. A biofilm is a layer of bacterial colonies protected with an extra-cellular matrix formed by the bacteria on the surface of artificial materials or body tissue [18]. The bacteria are attracted to the implant's surface due to hydrophobic interactions and adsorb onto the surface to form a film-like coating. This bacterial layer is exceptionally resistant to both antibiotics and immune system infiltration due to the tightly enclosed nature of the film of metabolically inactive bacteria[23].

Certain metals such as Zn, Sr, Ag, and Cs have been found to possess inherent antibacterial properties [24], [25]. Ag, for example, has been found to have strong antibacterial properties, although the exact mechanism is not fully understood. It is thought that the interaction of the bacterial cell membrane with silver ions could disrupt cell membrane functionality, or perhaps the silver ions cause the formation of reactive oxygen

species, which damage the cell membrane[24]. Stanić *et al.* demonstrated that during FA synthesis, metallic ions could be incorporated into the crystal structure to potentially modify the antimicrobial properties of FA[25]. It has also been demonstrated that certain molar percentages of Cs^{+} and Sr^{2+} can be incorporated into FA during synthesis and help to prevent bacterial growth in slurry[26]. It has also been shown that the antibacterial properties of FA could be improved by incorporating Ag nanoparticles into the crystal structure[27]. However, the effect of metal-doped FA on antibiofilm formation has not been extensively studied.

The purpose of my work was to improve the antibacterial properties of FA, thereby reducing the rate of infection in POI devices. This was done by studying the effect of Zn-, Sr-, and Ag-doped fluorapatite on biofilm formation *in vitro*. We synthesized FA powder doped with Zn (0-10% molar substitution of Ca ions), Sr (0-10% molar substitution of Ca ions), and Ag (0-10% molar substitution of Ca ions). Then, a known amount of powder was pressed at 3,000 kg to make uniform size, 10mm in diameter disks. These disks were sintered to temperatures ranging from 1100ºC to 1200ºC. They were then placed in a CDC biofilm reactor with *S. aureus* as the bacterial inoculum. Under the shear condition, the bacteria can form biofilms on the disks, and the number of adhered bacteria on each disk was counted and compared to titanium standard and non-doped FA disks. This knowledge can also be used to determine the utility of metal-doped FA as bone substitutes as scaffolds for orthopedic and dental applications. Using the T-tests, the number of adhered bacteria (i.e., colony-forming unit (CFU)) data from each disk was statistically compared to the titanium control group.

III. METHODS

In order to test the effect of different concentrations of metallic ion replacement in the fluorapatite, 0%, 1%, 5%, and 10% Ca ion deficient FAs were synthesized in-house using published methods to achieve pure powders [11-12]. Briefly, an aqueous precipitation technique was used, adding 2.4 M calcium nitrate ($CaNO_3$), 1.2 M sodium phosphate (Na_3PO_4), and 0.54 M sodium fluoride (NaF). The method used for the metallic-doped FA was different from the published method. The corresponding molar percentage of each respective metallic nitrate was used to replace a proportional amount of the calcium nitrate. The two solutions were added dropwise to a heated 12 L reaction flask containing 10 L of deionized water at 100ºC. The reaction flask was mixed at 200 rpm, and the pH was maintained a 9.0 using the pH-STAT controller and AUTO burette (TIM 856 Titration Manager; Radiometer Analytical, Copenhagen, Denmark) with 2 M NaOH. After letting the reaction solution precipitate and settle overnight, the precipitate was filtered, rinsed, then dried at 60ºC for 48 hours. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

The solid FA was then ground into fine particles and sieved to obtain samples of consistent particle size. For this project, particles between 106 and 250 micrometers were used. Using 0.3 g of each doped-FA powder and 65 microliters of water, a paste was formed and inserted into a 10 mm die press. The disks were each pressed at three metric tons for 2 minutes before sintering at 1150ºC. Once sintered, the disks were cleaned with ethanol and autoclaved. They were then loaded into a CDC biofilm reactor by following the ASTM standard method (E3161 – 18 - Preparing a *Pseudomonas aeruginosa* or *Staphylococcus aureus* Biofilm using the CDC Biofilm Reactor. Briefly, the reactor was kept at 37ºC with 30 g/L tryptic soy broth (TSB) stirring at 60 rpm and *S. aureus* as the bacterial species. After 24 hours, a 1 g/L TSB flow broth was run through the reactor for an additional 24 hours. After which, disks were removed from the reactor, rinsed with PBS, and sonicated for 30 minutes to disrupt the biofilm. The obtained bacterial solutions were then serially diluted and plated onto agar Petri dishes. After allowing the bacteria to grow for an additional 24 hours, the resultant colonies were counted and used to calculate the original concentration of adhered bacteria on the disks. Once the data for each disk was collected, it was normalized to the titanium disks that were used as the control. The data was then processed in MATLAB to determine the statistical significance of the data using an unpaired student's t-test.

IV. RESULTS

The numbers of CFU per unit area were calculated from 4 samples/disk types. Representative set images of 5% Zn disks are shown in Figure 1. The average CFUs/disk type is given in Figure 2 and represented as a fold change compared to the titanium control disks. This was to compare data from different biofilm runs, CFU data were normalized to the control Ti disks. Although some surfaces seemed to be promising, the variations within the groups were more extensive that resulting in statistically insignificant findings between most groups and the non-doped FA group. Within the tested surfaces, 1% Cs and the 5% Cs-doped disks were found to have statistically significant numbers of adhered bacteria than the non-doped FA disks.

Figure 1. A representative set of 5% Zn discs showing variations in disk surface morphology, including chips, divots, and cracks imaged at 50x magnification using a light microscope.

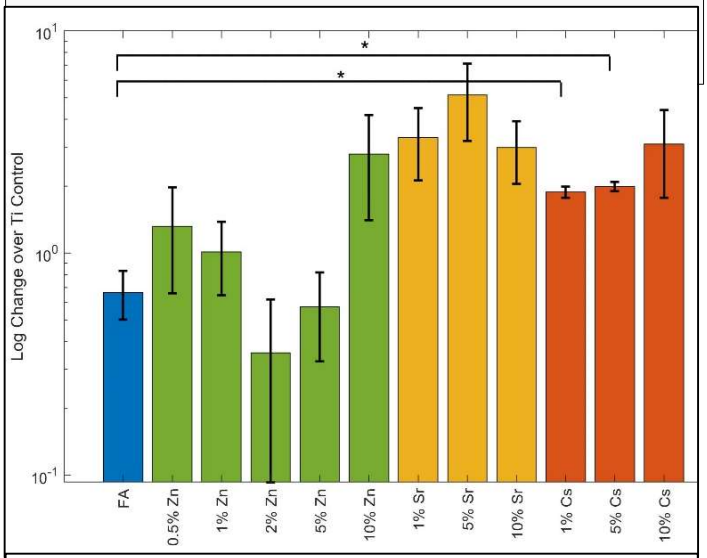

Figure 2. A bar chart showing the average and standard error of the mean of the log fold change over the titanium control. For the data that have been collected so far, the most prominent trend is that the results have a very high standard error of the mean.

Although statistically significant, this result was contrary to what was originally expected.

V. DISCUSSION

For POI devices, such as dental implants and POP devices, bacterial colonization and infection are primary concerns. While having the benefit of integrating directly into the bone, these devices also leave a chronic opening through the skin (stoma), where pathogens can infiltrate. Once colonized, bacteria may attach to the surface of the implant to begin to form a biofilm which can be challenging to treat. It has been reported that up to 20% of POP devices will become infected within 12 months of implantation, and up to 23% of dental implant failures are caused by infection [2-4]. Because of such a high rate of infection associated with these devices, there has been significant research done to prevent bacterial colonization of the open stoma. One area of research is in the modification of the implant coating to prevent the formation of a biofilm on the implant's surface. Certain metals, such as Zn, Sr, and Cs, have antibiotic properties in slurry, but their effect on biofilm formation is unclear[24]–[26].

For this study, the purpose was to understand how the incorporation of varying percentages of Zn, Sr, and Cs metal in FA would affect the formation of a biofilm *in vitro*. This was accomplished by synthesizing FA with various levels of metal incorporated into the chemical structure and then forming it into small disks for use in a CDC biofilm reactor with S. *aureus* as the bacterial species. This allowed the bacteria to adhere to the surface of each disk and begin to form a biofilm. Once the biofilm had formed, the disks were removed from the biofilm reactor and sonicated to remove the adhered bacteria, then counted using typical laboratory procedures.

This process showed a high rate of variability in the results. However, 1% Cs and 5% Cs-doped FA had significantly higher biofilm formation; these results are still inconclusive due to the smaller sample sizes and high variability within the data. Although the cause of the high variability is unclear, it could likely be attributed to irregularities in the surface of the disks (Figure 1). The method of forming the disks was subject to creating small pits, cracks, and divots on the surface of the disks, and it was difficult to control these deviations. Such surface imperfections (Figure 1) increase the surface area of the disk and could allow for bacteria to more easily replicate and form biofilm networks. These surface variations could have acted as confounding

variables. For future work, data from this study can be used to calculate ideal sample sizes and improve disk fabrication techniques to enhance surface quality and uniformity.

VI. CONCLUSION

Though the results from this preliminary study have been largely inconclusive, these findings will help to develop proactive methods for preventing the formation of biofilms in POI devices. Infection-free POI devices would have the ability to reduce the current drawbacks and complications associated with these implants, and significantly improve the quality of life for patients post-implantation. Future work should include further investigate into the incorporation of additional metals that may possess antibacterial properties, such as silver and copper.

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