POLY (2-METHYL-2-OXAZOLINE) (PMOXA) AND ANTIMICROBIAL PEPTIDE GKH17 AS POTENTIAL ANTIMICROBIAL COATINGS FOR CONTACT LENSES

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Abstract

Microbial contamination on contact lens surfaces can occur during handling, storage, and even cleaning with contaminated solutions. Several severe consequences of this event include microbial keratitis, contact lens-induced peripheral ulcer, and inflitrative keratitis. In this study we used SiO_2 as a model substrate for silicon-based contact lenses. The antimicrobial activities of two polymers, poly(2-methyl-2-oxazoline) (PMOXA) and antimicrobial peptide GKHKNKGKKNGKHNGWK (GKH17), on surfaces, were tested against two types of bacteria, Gram-positive *Staphlyococcus sp*. and Gram-negative *Neisseria sp*., isolated from used contact lenses. The inhibition activity of the polymers was first tested using the Kirby-Bauer method, and the properties of the polymers immobilized on SiO_2 surface were investigated by means of viable plate count and DNA absorbance measurements. Our results show that both types of bacteria are susceptible to both PMOXA and GKH17, however with a different degree of susceptibility. Both polymers are thus potential antimicrobial coatings for contact lenses.

Keywords: Bacteria, Contact Lens, GKH17, PMOXA, Surface Coating

Introduction

Rigid (hard) contact lenses made of glass (SiO_2) were first introduced between 1888 and 1940, while the ones made of plastics like poly(methyl methacrylate) (PMMA) or silicone acrylate materials were produced later. Soft contact lenses made of polymeric hydrogel have had a massive impact on the global contact lens market since their introduction in the early 1970s. Combining silicone with conventional hydrogel monomers has been a goal for polymer scientists since the late 1970s. Over the past 50 years, the number of soft contact lenses being prescribed around the world has steadily increased. An industry producing contact lenses is estimated to be worth around USD 8 billion annually (this figure does not include the sale of contact lens cleaning solutions) [1]. Nowadays contact lenses are classed as a medical device regulated by the U.S. Food and Drug Administration (FDA), in which they must satisfy the requirements of not having toxic or injurious effect for eyes.

Studies on microbial contamination of contact lenses, lens care solution, and their accessories have shown that lens handling greatly increases the occurrence of microbial contamination to the lens surface, while the ocular surface has a tremendous ability to destroy microorganisms. However, even when removed aseptically from the eye, lens surfaces are found to anchor pathogenic microorganisms, almost exclusively bacteria. The occurrence of microbial contamination is also pronounced during storage. In addition, care solutions can become contaminated [2].

Serious eye infections that can lead to blindness affect up to 1 out of every 500 contact lens users per year. Bacterial keratitis, a painful eye infection caused by bacteria due to

improper contact lens use, leads to 1 million doctor-and-hospital visits annually, at a cost of $175 million to the US healthcare system [3]. Both Gram-positive and Gram-negative types of bacteria often infect contact lenses. Willcox *et al*.[4] reported a list of bacteria isolated from contact lenses at the time of adverse responses that include microbial keratitis, contact lens induced acute read eye, contact lens induced peripheral ulcer, infiltrative keratitis, and asymptomatic infiltrative keratitis. Among the bacteria are *Staphylococcus aureus, Aeromonas hydrophila, Enterobacter sp.,* and *Neisseria sp.*[4, 5]

Currently, several approaches have been developed to protect biomaterials, such as contact lenses, from bacterial contamination [6, 7]. Based on the type of interaction with bacteria, one may distinguish between 'biopassive' and 'bioactive' biomaterial surfaces. Biopassive surfaces prevent the adhesion of bacteria but do not actively interfere with them. In contrast, bioactive surfaces aim at killing bacteria upon contact or at interfering with the quorum sensing between bacteria preventing further proliferation and maturation of biofilms. A further distinction can be made between stably surface-immobilized antimicrobials and active release systems. Appropriate choice of systems depends on the type of antimicrobials and their mechanism of action. Regulatory aspects are also to be considered, in particular in regard to whether approval for a medical device of drug is required. Recent research suggests that effective protection of biomaterial surfaces may be achieved by dual-functional surface coatings that impart both biopassive and bioactive properties [8]. In addition, the coating must be as thin as possible (at a nano-scale) so that it does not interfere the bulk properties of the material.

Singh *et al*.[9] incorporated hyaluronic acid, either with or without poly(ethylene glycol) (PEG) spacer, to contact lens surfaces and found that the hydrophilic polymers enhance water retention and prevent the adsorption of proteins and microorganisms to the surface. Lin *et al*.[10] immobilized PEG on a hydrophobic acrylic intraocular lens and found that the PEG-modified surface conveys biopassive properties, *i.e*. repels platelets, macrophages, and lens epithelial cells. Similarly, D'Sa *et al*.[11] immobilized PEG on a silicon elastomer and found biopassive properties of the modified surface. Another emerging polymer is poly(2-methyl-2-oxazoline) (PMOXA). PMOXA with brush configuration has been reported to convey biopassive properties to the same extent, however, with significant higher stability compared to PEG [12-15].

Bazzaz *et al*. [16] incorporated silver nanoparticles to a hydrogel contact lens material. The modified hydrogel showed bioactive (killing) properties against bacteria. Another emerging bioactive compound is cationic antimicrobial peptides (AMPs) that are small peptides (12-50 amino acid residues) with an overall positive charge and a broad spectrum of antimicrobial activity [17]. Currently, AMPs have been of rapidly growing interest in the field of antibacterial surfaces. These peptides are abundant components of the innate immune system present in most living organisms [18]. Therefore, application of AMPs can allow preparation of biologically compatible, nonimmunogenic antimicrobial coatings. Haynie *et al*.[19] immobilized the antimicrobial peptide magainin-2 onto a water-insoluble resin and proved its antimicrobial activities. Gabriel *et al*.[20] reported antibacterial activity of AMP cathelin LL37 covalently bonded to a PEG spacer with molecular weight 5.4 kDa. This coating showed a high efficiency against *E. coli K12* when grafted onto a titanium surface. The flexible PEG spacer is believed not only to render the surface biopassive, but also to facilitate contact with the bacterial membrane. Pasupuleti *et al*.[21] found that GKHKNKGKKNGKHNGWK (GKH17) was a potent bioactive compound against *Pseudomonas aeruginosa*, one of major pathogens in keratitis, in a contact lens model.

Our study focused on investigation of the potential of PMOXA and GKH17 as biopassive and bioactive coating, respectively, on SiO_2 surfaces as model contact lens surfaces. Both types of coatings were tested against Gram-positive *Staphylococcus sp.,* and

Gram-negative *Neisseria sp.* Willcox *et al*. reported the presence of *Staphylococcus aureus* on contact lens surfaces at the time of microbial keratitis and contact lens-induced peripheral ulcer adverse responses, and the presence of *Neisseria sp*. at the time of inflitrative keratitis.

Materials and Methods

Polymers

PMOXA grafted to poly(L-lysine) (PLL) that results in PLL-*g*-PMOXA graft copolymer was previously synthesized and characterized according to the published protocols [22, 23]. The PLL molecule acts as an anchor to negatively charged surfaces [23, 24]. GKH17 was purchased from custom synthesis service by Bachem AG, Switzerland.

Bacteria Rejuvenation and Subculture

Bacteria were previously isolated and characterized from a used contact lens and the discrete colonies of *Staphylococcus sp.* and *Neisseria sp.* were isolated using the strake plate method [25]. Each isolate was rejuvenated by aseptically taking and growing 1 oz of isolate on Nutrient Agar (NA) media by means of c*ontinous streak method* at 37°C for 24 hours, until a discrete colony was obtained. A stock culture was prepared by inoculating the discrete colony on a NA slant. The stock culture was kept in 4°C. A working culture for experiments was prepared by inoculating the discrete colony on a medium, followed by incubation at 37°C for 24 hours.

Bacteria Solution

Bacteria solution was prepared by aseptically adding 1 oz of working culture into a culture tube containing 20 ml liquid medium Nutrient Broth (NB), followed by incubation in a shaking (100 rpm) incubator at 37°C for 24 hours. Before exposure to surfaces, the bacteria solution was adjusted to 1 optical density (1 OD) at 600 nm.

Inhibition Activity of Polymers

Inhibition activity of the polymers was tested by means of Kirby-Bauer method, *i.e.* clear zone determination using paper discs. Paper discs with 5 mm diameter were prepared from Whatman paper No. 41. The paper discs were incubated in polymer solutions with varied concentration of 1000 µg/ml, 500 µg/ml, 250 µg/ml, 100 µg/ml, 50 µg/ml, and 25 µg/ml, for 2 hours.

20 ml of sterilized Mueller Hinton Agar (MHA) was poured into a petri dish and left until it forms a solid agar medium at room temperature. 0.1 ml of bacteria solution (1 OD at 600 nm) was then added covering the solid agar medium surface. After 15 minutes, the prepared paper discs were put on the solid agar surface, and incubated at 37°C. After 24 hours, the diameters of clear zones around the paper discs were measured. Inhibition zones were then calculated by substracting the diameters of clear zones with that of the paper discs.

Investigation of Polymer Coating Properties on SiO_2 Surfaces

SiO_2 substrates (microscope slide cover glasses) were cleaned by means of piranha cleaning procedure [26]. The cleaned substrates were then exposed to either PMOXA or GKH17 polymer solution with a concentration of 0.1 mg/ml, for 2 hours, followed by rinsing using sterile aquadest. The coated SiO_2 surfaces were then exposed to 250 µl of the prepared bacteria solutions, kept in a parafilm-sealed petri dish, and incubated at 37°C for

12 hours. As controls, bare (un-coated) SiO_2 surfaces were also included during the experiments.

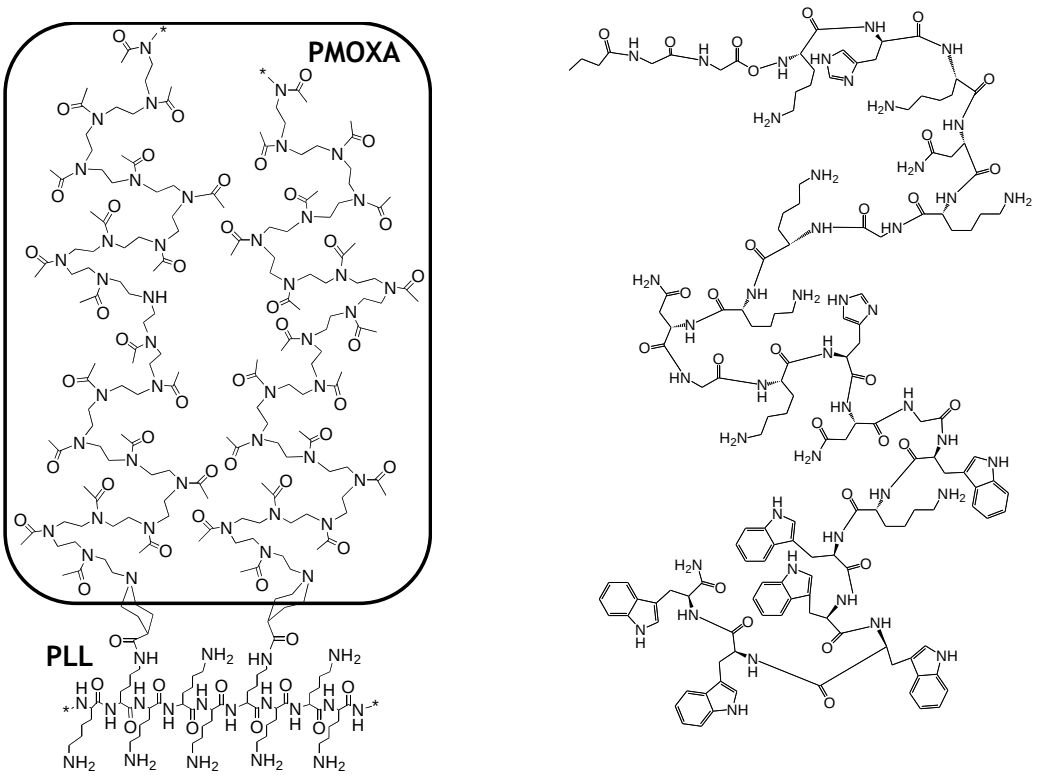
To investigate the numbers of alive (surviving) bacteria after exposure to the surfaces, 100 µl of bacteria solutions were carefully pipetted from the SiO_2 surfaces, followed by dilutions and viability test (viable plate count). The viable colonies were then multiplied by the dilution factor divided by the sampling volume (100 µl) to obtain numbers of viable cells per ml bacteria solution.

Furthermore, cell leakage analysis was performed to determine the biopassive and bioactive properties of the coatings. After 12 hours of exposure to the surfaces, 100 µl of bacteria solutions were pipetted, followed by dilution in 1900 µl of phosphate buffer. The diluted bacteria solution were centrifuged at 3500 rpm for 15 minutes, and the supernatant was carefully pipetted and placed in spectrophotometer cuvetes. The DNA absorbances were measured using a UV-Vis spectrophotometer (Biorad) at 260 nm.

Results and Discussions

Previous characterizations of PMOXA (molecular mass of 4 kDa), both in bulk and on oxide surfaces, have shown successful synthesis and PLL-anchored surface immobilization, and will thus not be discussed in this report [22-24]. In this case, PLL-*g*-PMOXA (α = 0.33) as described in the previous publication [23] was used during the whole study.

HPLC and MALDI characterization of GKH17 from the custom synthesis showed a pure substance at molecular mass of approximately 3 kDa. The chemical structures of PMOXA and GKH17 in un-charged forms are shown in Figure 1.



(a) (b)

Figure 1. Chemical structure of a) PMOXA and b) GKH17 in uncharged forms. The PLL and GKH17 will be positively charged in buffer solution at neutral pH and will thus immobilize on negatively charged substrates, such as SiO_2, due to electrostatic interactions

Inhibition Activity of Polymers

Prior to investigation of the polymer coating properties on SiO_2, the inhibition activity of the polymers were investigated against contaminating bacteria that were isolated from used contact lenses, *i.e.* Gram-positive *Staphylococcus sp.*, and Gram-negative *Neisseria sp*. The inhibition zones of PMOXA and GKH17 against the bacteria at various polymer concentration for disc incubation are shown in Figure 2.

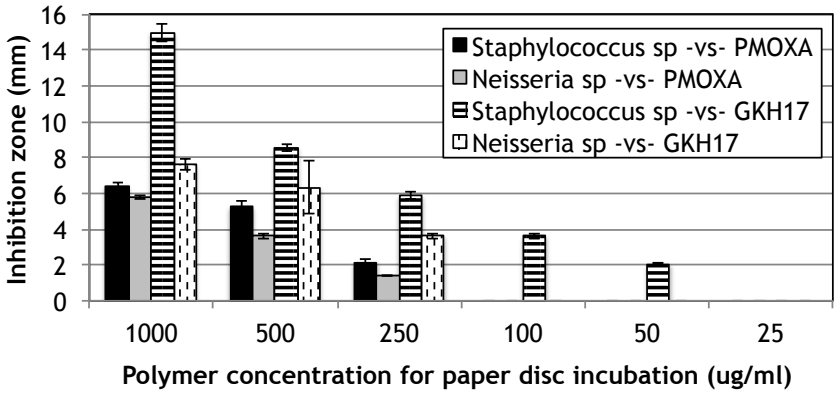


Figure 2. Inhibition zone of PMOXA and GKH17 against *Staphylococcus sp*. and *Neisseria sp*. after 24 hours of exposure, as a function of polymer concentration for paper disc incubation

It is seen in Figure 2 that incubation of paper discs in PMOXA concentration of 25, 50, and 100 µl/ml did not show any inhibition activity for both *Staphylococcus sp*. and *Neisseria sp*., while that of 250, 500, and 1000 µl/ml show increasing area of inhibition zone for both bacteria types. The paper discs were prepared from Whatman filter paper No. 41 that is made of cellulose material carrying numerous hydroxyl (-OH) groups. The paper discs were thus expected to carry negative charges upon incubation in polymer solution with neutral pH, while the PLL-*g*-PMOXA molecules carry positive charges from the protonated amine groups of the PLL. This should result in immobile PMOXA molecules on the paper discs. In addition, the molecular mass of the whole PLL-*g*-PMOXA molecule is approximately 140 kDa. Diffusion of the molecules into the agar medium should thus be unlikely. The activity of the PMOXA is expected to be sensed by bacteria around the paper disc surfaces only. At low polymer concentration on paper disc surfaces, the PMOXA shows no inhibition zone, probably due to insuficient PMOXA density to inhibit the bacterial growth around the surface. In addition, the paper disc surfaces are expected to carry an approximately neutral charge due to the "shielding" effect of un-charged PMOXA layer [23]. The (low) increasing inhibition zone at polymer concentration of 250, 500, and 1000 µl/ml indicates that the PMOXA in fact gives a weak bioactivity (inhibition effect) to the bacteria.

Compared to PMOXA, GKH17 shows a higher inhibition effect for both bacteria types. Like PLL, GKH17 contains amine groups that are protonated in solution with neutral pH. The GKH17 will thus be immobile in the cellulose fibers of the paper discs. The significant difference of molecular mass between PLL-*g*-PMOXA and GKH17 molecules, *i.e.* approximately 140 and 3 kDa, respectively, should result in different diffusion profile of the molecules into the cellulose fibers of the paper discs. We hypotesized that most of the big molecules of PLL-*g*-PMOXA immobilized on the paper disc surface, while the significantly smaller molecules of GKH17 could easily diffuse and immobilize into the cellulose fibers. Furthermore, the presence of GKH17 also switches the charge of the paper disc surface from negative to positive. Since the bacterial cell walls carry a net negative

charge, there will be electrostatic interaction between the bacteria cells and the positively charged GKH17 on the paper disc surface. Combined with the action from hydrophobic groups of the GKH17 molecules, this interaction is believed to be responsible for the lysis of the bacterial cells [27; 28]. For *Staphylococcus sp*., the inhibition zone increases with increasing GKH17 concentration, starting from 50 µl/ml, while for *Neisseria sp.* starting from 250 µl/ml. The difference indicates different defense mechanism between Gram-positive and Gram-negative bacteria against GKH17, probably due to the different cell wall structures. Gram-negative bacteria are surrounded by a thin peptidoglycan cell wall that itself is surrounded by an outer membrane containing lipopolysaccharide (LPS). Gram-positive bacteria are surrounded by a much thicker peptidoglycan layer, however, lack an outer membrane [29]. From Figure 2 it is seen that the Gram-positive *Staphylococcus sp*. bacteria are more susceptible to the action of GKH17, compared to the Gram-negative *Neisseria sp*. bacteria. It is reported that LPS on the outer membrane of Gram-negative bacteria plays a critical role in the barrier function, in which the tight packing of LPS molecules is a very effective barrier for hydrophobic molecules [29].

Investigation of Polymer Coating Properties on SiO_2 Surfaces

After the rigorous piranha cleaning, the surfaces of SiO_2 substrates are expected to form a layer of hydroxyl groups (-OH) that will be deprotonated at neutral pH. The deprotonation results in negatively charged SiO_2 surfaces, which will in turn, attract positively charged polymer molecules. As shown in Figure 1 and described above, the PLL (as the PMOXA anchor) and GKH17 contain amine groups ($-NH_2$) that will be protonated at neutral pH. The protonation results in positively charged molecules, which will immobilize on negatively charged SiO_2 due to electrostatic interactions.

Investigation of the properties of the polymer coating on SiO_2 surfaces was performed by analyzing the cell viability and the cell leakage of bacteria upon exposure to the polymer-coated surfaces, by means of viable plate count and measurement of DNA absorbance, respectively. Figure 3 shows the values of log (numbers of viable cells/ml) and DNA absorbance in bacteria solutions, after exposure to bare- and polymer-coated SiO_2 surfaces for 12 hours.

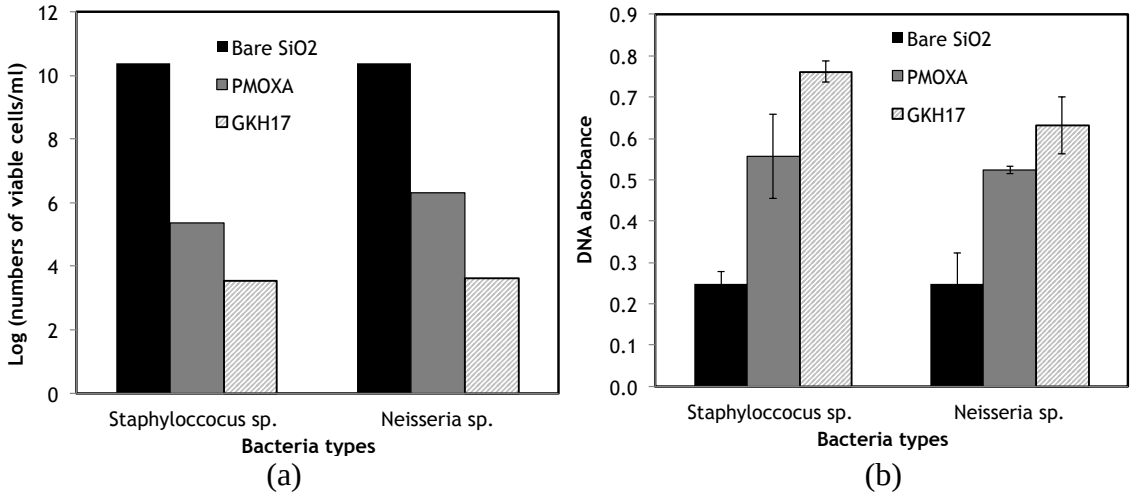


Figure 3. Values of a) log (numbers of viable cells/ml) and b) DNA absorbance in bacteria solutions, after a 12 h exposure to bare and polymer coated surfaces, for *Staphylococcus sp*. and *Neisseria sp*.

Figure 3a) clearly shows that the numbers of viable (alive) bacteria in the bacteria solution after exposure to both PMOXA- and GKH17-coated surfaces are significantly lower than those after exposure to bare SiO_2. This phenomenon indicates bioactive properties of surface-attached PMOXA and GKH17 to both Gram-positive and Gram-negative bacteria types. Although we hypothesized that PMOXA layers conveys only biopassive properties, meaning that it prevents the adhesion/contact between the bacteria and the underlying surface without interfering (killing) the bacterial cells as reported in our previous publication [30], Figure 3a) shows for the first time that PMOXA also contributes to bioactive properties (killing effect), thus reducing the numbers of viable cells. However, the bioactivity of the PMOXA is weaker than that of GKH17, as shown by lower numbers of viable cells after exposure to GKH17-coated surface. An alternative explanation for the bioactive properties observed on PMOXA-coated surface is the presence of amine groups of the PLL that are not conjugated with the PMOXA chains, nor attached to the surfaces. The presence of these groups creates a slightly positive charge on the surface [31], which can attract and disrupt the bacterial membranes.

DNA absorbance values in Figure 3b) indicates the level of bacterial cell leakage (lysis). As DNA is located inside the bacterial cytoplasm, lysis of the bacterial cell walls is expected to transport the DNA out of the cells to the bulk bacteria solution. Centrifugation of the bulk bacteria solution results in separation between low molecular mass molecules such as DNA and other small proteins in the supernatant and other high molecular mass molecules in the settled solid part. At 260 nm wavelength, DNA absorbance values that indicate the DNA concentration in the supernatant could be detected using a UV-Vis Spectrometer. In agreement with Figure 3a), Figure 3b) shows that DNA absorbance values for bare SiO_2, PMOXA, and GKH17 are low, medium, and high, respectively. This indicates that both surface-attached GKH17 and PMOXA convey bioactive properties, however, with a different degree where GKH17 conveys higher degree of bioactive properties.

Conclusions and Outlook

We have investigated the reponse of Gram-positive *Staphylococcus sp*. and Gram-negative *Neisseria sp*. bacteria upon exposure to PMOXA and GKH17 polymers. Apart from the biopassive properties of the un-charged PMOXA as previously reported [12; 23; 30; 32], our study showed that PMOXA conveys a weak bioactive properties, indicated from a decrease of viable bacteria cells and an increase of cell lysis (DNA absorbance) upon exposure to PMOXA-coated surfaces, compared to those upon exposure to bare SiO_2 surfaces. Meanwhile, the cationic antimicrobial peptide GKH17 showed a higher degree of bioactive properties compared to PMOXA. The inhibition zone data indicated that the Gram-positive and Gram-negative bacteria might undergo a different defense mechanism against the GKH17 polymer. Based on these findings, both polymers are potential candidates as antimicrobial coatings to protect the surface of contact lenses from microbial contamination, which will in turn minimize the adverse reactions for the contact lens users. The next focus of our research will be on covalent conjugation between PMOXA and GKH17 that will result in dual-functional surface coatings that comprise both biopasive and bioactive properties.

Acknowledgement and Competing Interests

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