Mussel-inspired silver-nanoparticle coating on porous titanium surfaces to promote mineralization

Jialong Chen [a,b,c], May Lei Mei [a], Quan-Li Li [c*], Chun-Hung Chu [a*]

[a] Faculty of Dentistry, The University of Hong Kong, 34 Hospital Road, Hong Kong, China

[b] College & Hospital of Stomatology, Anhui Medical University, Key Laboratory of Oral Diseases Research of Anhui Province,Hefei, China

[c] College of Pharmacy, Anhui Medical University, Hefei, China

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Corresponding to :

*E-mail: ql-li@126.com (Quan-Li Li), chchu@hku.hk (Chun-Hung Chu).

Tel: (+)86-551-65118677 (Quan-Li Li), (+) 852-28590287 (Chun-Hung Chu).

Fax: (+) 86-551-65121527 (Quan-Li Li), (+) 852-25599013 (Chun-Hung Chu).

Abstract

Objectives: *Biomaterials with high porosity for bone ingrowth facilitate the osseointegration of implants. However, this porosity structure is also favorable for bacterial colonization and biofilm formation, and hampers mineralization on implant surfaces. The objective of the study was to establish an antibacterial porous surface on titanium implants.*

Material and methods: *Auniform,3-dimensional, microporous structure was prepared by alkaline treatment on a titanium implant surface. Subsequently, the surface was treated with dopamine and silver nanoparticles by dopamine and silver nitrate solutions. Physicochemical properties were determined by SEM, EDS, XPS, and water contact angle tests. The antibacterial and mineralization properties of the modified titanium were evaluated in vitro.*

Results: *The results confirmed that the surface had been successfully coated with dopamine and silver nanoparticles. A mineralized layer formed on the surface after 1week in a calcification solution. Antimicrobial tests showed that the titanium implant with this surface structure inhibited the bacterial growth and biofilm formation of Escherichia coli, Staphylococcus aureus, and Streptococcus mutans.*

Conclusions: *An antibacterial porous surface was established on a titanium implant. This surface structure can enhance mineralization on porous titanium implants. This technique to prevent bacterial colonization and promote mineralization has great potential for clinical application in implants in orthopedics and dentistry.*

Introduction

Titanium and its alloys have been used extensively as implants in orthopedic and dental applications because of their specific combination of outstanding properties, such as excellent biocompatibility, high strength, good fatigue resistance and corrosion resistance (Zhao et al. 2009). However, bacteria that cause prosthetic joint and dental implant infections grow in highly structured biofilms (i.e., sessile communities of microorganisms adhering to the biomaterial embedded in a matrix of an extracellular polymeric substance that they produced) (Costerton 2005; Paquette et al. 2006). This protective environment enables bacteria to escape the host's defenses and antibiotic attacks. Moreover, the increased competence suggested for biofilm-embedded bacteria—which results in a higher degree of horizontal transfer of genes, including antibiotic resistance markers and the occurrence of persister cells—might further enhance biofilm-related antibiotic resistance (Darouiche 2004; Hetrick & Schoenfisch 2006). As a consequence, antibiotic treatment is often insufficient to eradicate biofilm-related implant infections, leading to potentially life-threatening systemic infections, tissue injury, device malfunction, and ultimately, a need to remove the implant. Biomaterial surfaces that are less prone to bacterial adherence and colonization have helped researchers make serious progress in reducing infection rates over the last few decades (Arciola et al. 2005). Surface modification with antibiotics and antimicrobial agents is an efficient way to reduce the risk of bacterial infection and biofilm formation, such as with gentamicin, vancomycin, and chlorhexidine (Antoci et al. 2008; Popat et al. 2007).

The use of silver has received increasing attention due to its lasting antibacterial effect against a very broad antimicrobial spectrum of bacterial and fungal species, including antibiotic-resistant strains, which have become a major public health concern (Fullenkamp et al. 2012). Silver had always been thought to be a promising alternative antibacterial agent (Marambio-Jones & Hoek 2010). However, silver can stain dental tissue black due to the oxidation process of ionic silver (Rosenblatt et al. 2009), as can silver diamine fluoride and amalgam, which has hindered its widespread use. Recently, silver nanoparticles (AgNPs) have drawn considerable attention because they have good color stability and large, active surface

areas, apart from broad spectrum antibacterial activity and the small possibility of resistant strains developing (Cao et al. 2011; Furuzono et al. 2013; Santos et al. 2014; Zheng et al. 2012). Biomaterials that contain AgNPs have been exhaustively investigated for the development of catheters, dental materials, orthopedic implants, and wound and burn dressings (Necula et al. 2012). To obtain satisfactory surfaces containing AgNPs, many methods have been performed to introduce silver on surfaces, such as plasma immersion ion implantation (Zhang et al. 2008), pulsed filtered cathodic vacuum arc deposition (Ewald et al. 2006), physical vapor deposition (Antad et al. 2014), and so on. Nevertheless, the major drawbacks to the methods mentioned above are their poor AgNP/material adhesion and the difficulty in controlling AgNPs' size (Esfandiari et al. 2014; Wang et al. 2015; Xie et al. 2014).Additionally, the special equipment required, the large amounts of energy consumed, and/or the complicated multistep procedures involved also have limited further applications. Given these problems, a meaningful approach would be to develop a simple and versatile strategy for surface modification with AgNPs. Smaller AgNPs with large surface areas can exhibit better antimicrobial activity than larger AgNPs, but the agglomeration of the AgNPs with small sizes is an important consideration and could result in a quick loss of antibacterial activity (Baker et al. 2005; Panáček et al. 2006). Many researchers have proposed that the aggregation state of immobilized nanoparticles on a rough surface could degrade (Mohammad et al. 2008).

Dopamine, a mussel-inspired biomolecule, contains unusually high concentrations of catechol and amine groups. The catechol side chain of dopamine readily oxidizes to form reactive species that can further undergo Michael-type additions or Schiff-base formations with nucleophiles and radical coupling with other catechols or amines (Waite 1987). Thus, dopamine offers a simple method of coating various organic and inorganic substrates (Chen et al. 2015; Fullenkamp et al.2012; GhavamiNejad et al. 2015). Another interesting feature is that dopamine, as a reducing and stabilizing agent, can reduce Au(III) or Ag(I) metal ions to form noble metal nanoparticles via catechol oxidation without the need for any toxic components, leading to in situ formation of AgNPs on the dopamine-modified surface (Fei et al. 2014; Luo et al. 2015). In addition, dopamine could promote cell adhesion, exhibit good biocompatibility (Hu et al. 2010), and induce mineralization (Zhou et al. 2012), which are of particular interest

in dental and orthopedic implantology for engineering surfaces with the ability to improve osseointegration.

Porous Ti structures or coatings are of special interest because they enable bone ingrowth into the porous structure, thus establishing a biological anchorage for the implant in the host bone. Osseointegration is strongly dependent on the structural characteristics of the surface, such as total open porosity and pore size. Porous structures with high porosity allow more bone ingrowth to support improved anchorage with the surrounding bone (Ryan et al. 2006), but the resulting large surface area renders the implant extremely susceptible to bacterial colonization and subsequent biofilm formation. Therefore, there is particular interest in dental and orthopedic implantology in designing surfaces that combine both the ability to improve osseointegration and simultaneously reduce infection risk.

The aim of the present study is to build a porous titanium surface carrying dopamine and uniformly distributed small AgNPs and then to evaluate whether this surface is able to exhibit antimicrobial activity and enhanced mineralization. For this, the porous titanium surface obtained by alkaline treatment was modified with dopamine using the dip-coating technique; then, AgNPs were coated onto the dopamine-modified surface in situ by reduction reaction between Ag(I) ions and dopamine. Finally, the antibacterial and mineralization properties of the modified titanium were evaluated in vitro. This method could have implications for dental- and orthopedic-related areas, because the efficient antibacterial activity and the high bioactivity of implant surfaces could be constructed by a simple method.

Materials and methods

Preparation and characterization of a multifunctional coating with dopamine and silver nanoparticles

Titanium discs were polished into a reflective, mirror-like surface. The discs were ultrasonically cleaned first in a detergent solution, then in acetone, ethanol and finally deionized water. After soaking in a 5 M NaOH solution at 60 °C for 48 h, the cleaned specimens

were soaked in deionized water at 80 °C for 8 h and were then denoted as **TiOH**. The specimens were immersed in a 2 mg/mL solution of dopamine (10 mM Tris buffer, pH 8.5) for about 24 h at room temperature in the dark. Then, the samples were sonicated for 10 min in deionized water (3 times) to remove the nonattached dopamine; these samples are denoted as **Ti-O-DA**. Then, 100 mg of silver nitrate ($AgNO_3$) was dissolved in deionized water (10 ml, adjust pH to 10 with NaOH). The dopamine-modified samples were then placed in a 24-well plate and incubated with a 600 µl $AgNO_3$ solution in an orbital shaker incubator at 80 rpm and 37 ºC for 24 hours. Then, the samples were rinsed vigorously for 10 min in deionized water and dried in a vacuum for further use; the samples are denoted as **Ti-O-DA-Ag.**

The surface topography of all of the samples was investigated using scanning electron microscopy (SEM, Hitachi S-4800). Energy-dispersive X-ray spectroscopy (EDS) analysis was also performed. The surface composition of the samples was analyzed by X-ray photoelectron spectroscopy (XPS, Thermo ESCALAB 250) with an Al Ka X-ray source (1486.6 eV photons). A wide-scan survey spectrum over a binding energy (BE) range of 0-1400 eV was recorded at a pass energy of 100 eV to estimate the chemical elemental composition and 30 eV for high-resolution detailed scans. The system was calibrated using the C1s peak at 284.8 eV. All spectra were recorded at a takeoff angle of 45 degrees. The maximum information depth of the XPS method was not more than 10 nm.

Water contact angle analysis was performed with a DSA100 drop-shape analysis system (DSA100, Krüss, Germany) using deionized water at room temperature. Five samples of each group were measured, and two separate measurements were made on each sample. All of the samples were sterilized with UV irradiation for 1 h prior to biological evaluation.

Mineralization of the multifunctional coating with dopamine and silver nanoparticles

The calcification solution was prepared according to the protocol described by Zhou (Zhou et al. 2012). The solution contained 2.58 mM calcium ($CaCl_2 \cdot 2H_2O$), 1.55 mM phosphate (KH_2PO_4), and 180 mM NaCl and was buffered by 50 mM of Tris-HCl. The calcification solution's pH was adjusted using 0.1 M HCl and 0.1 M NaOH. The samples were

placed in a 24-well tissue-culture plate and incubated with 1.5mL of calcification solution in an orbital shaker incubator at 80 rpm and 37 ºC. The calcification solution was replaced every day. The samples were taken out at 7 days, rinsed vigorously for 10 min with deionized water, and gradually dehydrated to a critical drying point prior to characterization.

Antibacterial test

Gram-negative bacteria, *Escherichia coli*, and gram-positive bacteria, *Staphylococcus aureus* and *Streptococcus mutans*, were used in the antibacterial tests. The numbers of both live and dead bacteria were used to indicate the antibacterial activity for the different materials. Samples were placed in a 24-well tissue-culture plate and incubated with different bacterial suspensions at a concentration of 10^7CFU/mL at 37 °C for different periods of time. Then, the samples were taken out and gently washed with phosphate-buffered saline. The viability of the bacteria on the samples was assessed using a combination dye (LIVE/DEAD ® *Bac*Light™ Bacterial Viability kit, Molecular Probes, Invitrogen, Carlsbad, CA). Viable bacterial cells were stained green, whereas dead cells were stained red.

The samples' antibacterial effect against the strains of gram-positive and gram-negative microorganisms was tested using zone of inhibition (ZOI) testing (Zhang et al. 2013). The samples were placed with face downward on a solid lysogeny broth medium agar plate surface, which was spread evenly with 20µlof the individual test-strain solutions (10^7CFU/mL). The inhibition zones were photographed after incubation for different times at 37 °C. The formation of a clear zone around the sample indicated antibacterial activity for the obtained surface.

The growth curve of the bacteria incubation with different samples was assayed to evaluate the samples' antibacterial properties. The samples were placed in a 24-well tissue-culture plate and incubated with 1.5ml of different bacterial suspensions at a concentration of 10^7CFU/mL at 37 °C. 100 µl of bacterial suspensions were taken out for optical density measurements at 660 nm (OD_{660}) using a UV/Vis spectrophotometer at a different time .A growth control with no samples was employed for each parameter.

Statistics

All of the experiments were performed at least 3independent times. All of the data were compared with one-way ANOVA tests to evaluate their statistical significance using SPSS software. Tukey multiple comparisons tests were performed to find significant differences between the pairs. Probability values less than .05 were considered statistically significant. In the figures, statistically significant differences ($p < .05$) were denoted with an asterisk (*).

Results

Characterization of multifunctional coating

The SEM images in Figure 1 show the different surfaces of TiOH, Ti-O-DA, and Ti-O-DA-Ag. As shown in Figure 1A and B, the surface of the NaOH-treated titanium was characterized by a uniform 3D microporous and mesh-like morphology. After dopamine functionalization (Figure 1C and D), the surface was no different from the TiOH surface. Some studies showed that the morphology of the samples did not change significantly after being coated with dopamine (Wang et al. 2015). The samples of Ti-O-DA were immersed in silver nitrate solution to obtain silver nanoparticles loaded on the surface as a hybrid. Figure 1E shows that the AgNPs were successfully attached and uniformly dispersed on the surface. The AgNPs were about 30–50 nm in size, and their shape was spherical. The high-magnitude image (Figure 1F) revealed that AgNPs were deposited on the top edges and the inner portion of the 3D microporous structures.

The chemical composition of the surfaces at various stages of surface functionalization was determined using EDS. As shown in Figure 2A, several types of peaks in the EDS spectrum were obtained from TiOH that corresponded to elemental titanium and oxygen. After dopamine functionalization (Figure 2B), the presence of elemental carbon, which was not detected in the surface of TiOH, and the decrease in the atomic percentage of titanium indicated that dopamine was successfully immobilized onto the surface of the titanium. As shown in Figure 2C, the atomic percentage of silver in the Ti-O-DA-Ag was 14.54%, indicating that a large amount of silver interlocked onto the dopamine-modified surface. The atomic percentages of titanium in

the TiOH (A), Ti-O-DA (B), and Ti-O-DA-Ag (C) were 73.04%, 51.13%, and 34.04%, respectively, and indirectly indicated that dopamine and AgNPs were successfully immobilized onto the titanium surface step by step.

The surfaces' chemical composition at various stages of surface functionalization was determined by XPS. The XPS wide-scan spectra and the high-resolution spectra of Ag3d of the TiOH, Ti-O-DA, and Ti-O-DA-Ag are shown in Figure 3. After dopamine functionalization (Ti-O-DA), the presence of N1s peak (~399eV) indicates that dopamine was successfully immobilized onto the surface of titanium due to the large amount of nitrogen in the dopamine. Meanwhile, the Ti2p peak disappeared, indicating that dopamine had completely covered the substrate materials. In addition, only the surface of Ti-O-DA-Ag exhibited two specific peaks, with binding energies of 368.45 eV and 374.45 eV (shown in Figure 3B), which were attributed to the $Ag3d_{5/2}$ and $Ag3d_{3/2}$ electrons of Ag^0, respectively. The spin energy separation was identified as 6.0 eV, which indicates that the silver on the dopamine-modified surface was metallic Ag^0 in nature (Luo et al. 2015); in turn, this further supported the conclusion that AgNPs had been successfully loaded onto the surface.

The measurement of the water contact angle (WCA) is well known as a useful technique to investigate surface characteristics. The WCA of the different surfaces is shown in Figure 4. Compared to the original titanium (Ti), the WCA of TiOH decreased significantly. After dopamine functionalization (i.e., Ti-O-DA), the WCA of the surface increased significantly. Relative to the dopamine-modified surface, the WCA of the AgNP-coated surface (i.e., Ti-O-DA-Ag) increased significantly further. These results indirectly indicated that dopamine and AgNPs were successfully immobilized onto the titanium surface.

Mineralization of the multifunctional coating

Because the dopamine could induce accelerated in vitro apatite formation (Kim & Park 2010; Zhou et al. 2012), here, the samples were immersed in the calcification solution to assess the material's osteoinductivity. After being soaked in the calcification solution alone for 1week, the uniform 3D microporous and mesh-like morphology on the TiOH, Ti-O-DA, and Ti-O-DA-

Ag was replaced by mineralized crystals. As shown in Figure 5A and B, this mineralized layer of TiOH consisted of loosely packed, needle-shaped crystals with a porous structure. Compared to TiOH, the mineralized layer on Ti-O-DA (Figure 5C and D) and Ti-O-DA-Ag (Figure 5E and F) consisted of a higher packing density of apatite crystals with a rod-like structure, which resembled a natural enamel structure with a high packing density of apatite crystals (Kim & Park 2010). The presence of AgNPs led to the rod-like crystals on the Ti-O-DA-Ag being thinner than on the Ti-O-DA. EDS revealed the presence of calcium ions on the surface of Ti-O-DA-Ag after it was soaked in the calcification solution alone for 1week (Figure 2D). These results indicated that the surface of Ti-O-DA-Ag could improve mineralization.

Antibacterial activity

The viability of the attached cells was evaluated using a confocal laser scanning micrograph via staining with a combination of dyes. As shown in Figure 6, the surface of the TiOH and Ti-O-DA supported rapid and extensive attachment of *Escherichia coli (E. coli)*, *Staphylococcus aureus (S. aureus),* and *Streptococcus mutans (S. mutans)*; however, attachment onto the AgNP-coated surface was reduced by more than 95% compared to TiOH or Ti-O-DA over the same time period. Most of the bacterial cells on the surfaces of the TiOH and Ti-O-DA were viable (stained green) throughout the immersion period, while the dead bacterial cells (stained red) observed on these surfaces were mainly attributed to cell death during the bacterial growth process rather than antibacterial activity. For the AgNP-modified surface (Ti-O-DA-Ag), the number of bacterial cells decreased very significantly, and the percentage of dead cells (stained red) was higher than on the TiOH. Even when prolonging the immersion time to 24 h, only a few sparsely distributed, single viable cells were observed, indicative of the high efficiency of AgNP conjugates in destroying the bacteria.

The antibacterial activity of the different surfaces was investigated by measuring the ability to inhibit *E. coli, S. aureus, and S. mutans* growth around samples on agar culture plates, as shown in Figure 7. After 24 h of incubation, bacterial colonies were clearly observed in contact with TiOH and Ti-O-DA, while clear transparent rings were obtained around Ti-O-DA-Ag, showing the killing effect on bacteria. The AgNP-modified surface demonstrated excellent

antibacterial properties, and its zone of inhibition (ZOI) did not significantly decrease during the first 2 days (data not shown).

To evaluate the samples' stability in air, all of the samples were stored in air for at least 1week and then used in the experiment below. The bacterial growth in the solution with different samples was monitored by measuring the optical density at 660 nm (OD660) to evaluate the antibacterial activity of the AgNP-coated samples on their local environment. The higher the OD, the greater the opacity based on the turbidity of the cell suspension. As shown in Figure 8, the surfaces without AgNPs (i.e., TiOH and Ti-O-DA) did not show noticeable antimicrobial activity against *E. coli, S. aureus, or S. mutans* growth, as the curve was similar to that of the control bacteria. The growth of the three types of bacteria was completely inhibited when the AgNP-coated samples occurred, suggesting strong inhibition of bacteria proliferation by the AgNPs. Previous research revealed that AgNPs could lose antibacterial activity in air within 5 days (Wang et al. 2012), but our data indicated that antibacterial activity of Ti-O-DA-Ag could be kept in air. It is reasonable to conclude that the surface of Ti-O-DA-Ag possess high and long-term antibacterial activity due to the high stability of AgNPs.

Discussion

It is well known that titanium, with its porous structure, has the merits of high bioactivity and lower elastic modulus (Chen et al. 2009; Crawford et al. 2007). Also, a 3D porous structure, which is a characteristic feature of native bone tissue, could increase the specific surface area to improve the osteointegration of orthopedic implants (Soumya et al. 2012). Dopamine could induce mineralization, which also can improve osteointegration (Zhou et al. 2012). Here, the surfaces containing dopamine (Ti-O-DA and Ti-O-DA-Ag) had mineralized within 1week (Figure 5). Although AgNPs on the surface led to the crystals with a rod-like structure being thinner than those on the Ti-O-DA in the mineralization process (Figure 5), these results indicated that dopamine on the Ti-O-DA-Ag could enhance mineralization.

All 3D porous structures with high porosity allow more bone ingrowth and therefore

support improved anchorage with the surrounding bone (Ryan et al. 2006), but such structures render the implant extremely susceptible to bacterial colonization and subsequent biofilm formation. Bacterial infections have always been an issue for metal implants, since they introduce a foreign material inside the human body (Darouiche 2004; Hetrick & Schoenfisch 2006). Silver nanoparticles were introduced to endow the 3D porous surface with antibacterial activity. To load AgNPs onto the surface, dopamine was used on the Ti-O-DA to reduce the Ag^+ in the silver nitrate solution to AgNPs, due to the catechol groups in the dopamine (Shi et al. 2015). After reduction, the AgNPs were tightly bound to the dopamine-modified surface without aggregation (Figure 1F). Compared to other reduction processes (Sharma et al. 2014), no additional reductant or heating is needed. Thus, this strategy of obtaining the AgNP-modified surface is simple, facile, and environmentally friendly. Compared to TiO_2 nanotubes (3D structure) (Guo et al. 2014), such a porous structure could permit more AgNPs to be uniformly deposited not only on the top edges but also the inner porous structures, leading to a large number of AgNPs being carried on the surface, as shown in the EDS result (Figure 2C). Good distribution and high-cover density of AgNPs on the substrate are important for surface-based applications (Li et al. 2013), so this strategy endows surfaces with excellent antibacterial activity, not only by inhibiting bacterial colonization on Ti-O-DA-Ag (Figure 6) but also by inhibiting the growth of a wide antimicrobial spectrum containing gram-negative bacterium and gram-positive bacteria around Ti-O-DA-Ag (Figure 7 and 8). Dopamine, as a reducing and stabilizing agent (Fei et al. 2014; Luo et al. 2015), could hinder the oxidation and/or aggregation of AgNPs in air, leading to a significant reduction of antibacterial activity (Lv et al. 2010). Thus, AgNPs on Ti-O-DA-Ag could sustain their high antibacterial activity after exposure to air for at least 1week (Figure 8).

Conclusion

Silver nanoparticles were successfully synthesized and uniformly dispersed onto a 3D porous titanium surface by alkaline treatment and immersion in a dopamine solution, followed by immersion in a silver nitrate solution. The results showed that such surfaces could enhance mineralization and inhibit bacterial colonization and subsequent biofilm formation. Therefore,

the AgNP-coated surface with 3D porous structure may not only facilitate osteointegration but also reduce the risk of infection of titanium implants. This simple, facile, and environmentally friendly technique is therefore believed to have great potential for clinical application.

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