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Antibacterial Silver Loaded Hydroxyapatite Coating Prepared Through the Reduction of Phytic Acid-Silver Ion Complex

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Abstract— **Hydroxyapatite coating was built on the surface of materialsby mineralization of phytic acid-metallic ion complex, whichfurther absorbed surplus phytic acidon the outer layer. Then, silver ions were stabilizedbased on their chelation with phytic acid. Finally, we obtained silver-loaded hydroxyapatite coating when the silver ions were reduced to nano silver particles under the effect of glucose. The successful loading of silver particles were proved by scanning electron microscopy, Raman and EDS measurement. This coating exhibited broad antibacterial properties against Escherichia coli (gram negative bacteria) and Staphylococcus aureus (gram positive bacteria). The antibacterial hydroxyapatite coating developed here might have potential applications in the area of biomedical, such as bone and dental implant.**

Index Terms—**phyticacid, hydroxyapatite, nano-Ag, antibacterial.**

I. INTRODUCTION

In recent years, with the development of biomedicine, biomaterials and artificial organs is more and more widely used in clinical treatment, however, with wide application of biological materials, biological materials related to bacterial infection has become a serious problem [1], every year close to 1 million people were affected by bacterial infection in implantation of biomaterials for implant materials[2][3]. Once infected, the material must be removed by operation and replaced by a new one, which not only delayed the disease, but also caused more pain to the patient[4]. In order to solve the problem of bacterial infection, it is of great significance to design materials for preventing bacterial infection for implant materials.

In all bacteria, Staphylococcus aureus (Staphylococcus aureus) and Escherichia coli (Escherichia coli) are the two common bacteria that cause the surface infection of implanted materials by non-specific and specific adhesion[5]. The adhesion of bacteria on the surface leads to the formation of the biofilm[6]. The formation of biofilms increases the viability of bacteria and tolerance to antibiotics[7]. Even if the surgical removal of an implant that has been infected by a biofilm can not completely solve this problem, the residual microorganism can lead to a repetitive infection[8]. Common bactericidal methods can be used in biological materials to introduce various fungicides[9] and kill bacteria with high bactericidal effect[10].

Hydroxyapatite (HA) is animportant component in natural bone. Therefore, it has good bioactivity and biocompatibility. It has been widely used in clinical bone defect repair, filling and shaping materials. However, the contagion caused by biological materials restricts their biomedical applications. It has been found that the phosphate ion of phytic acid can be combined with a variety of metal ions [11]. In recent years, Ag has attracted wide attention because of its excellent antibacterial and corrosion resistance[12]. Generally, silver or silver compounds of metal ions hinder microbial respiration, destruct the enzyme protein, thusexhibitbetter bactericidal effect compared with Cu^{2+}, Zn^{2+}[13][14].

The purpose of this study is to loadantibacterial Ag particles on the surface of biocompatible HA by the chelation of phytic acid-Ag^{+}, and the Ag^{+} was further reduced to Ag particles under the effect of glucose. This HA coating developed exhibited great antibacterial property which have potential application in bone and dental implant.

II. EXPERIMENTAL

A.Materials

Phytic acid (50% in water) was purchased from TCI Chemical Company.All other chemicals ($CaC1_2$,NaCl,$NaHCO_3$,KCl,$K_2HPO_4{\cdot}3H_2O$,$MgCl_2{\cdot}6H_2O$,HCl,Na_2SO_4,Tris,$AgNO_3$,Glucose) of analytical grade were purchased from Haihong Chemical Company and used as received. Titanium sheets of medical grade with thickness of 0.2 mm were obtained from Baoji Shengze Metal Co. Ltd. Medium of tryptone soybean agar and the medium of tryptone soybean broth were chemical pure. Hydroxyapatite (HA) is self-made in the laboratory.

B.Preparation of hydroxyapatite coating

This procedure was performed as follows. For the fabrication of phytic acid-Ca^{2+} multilayer, the cleaned substrates were soaked in phytic acid solution (0.255 μM/mL) for 10 minutes. Then, $CaCl_2$ solution (83.25 mM/mL) was added in above solution to make a final concentration of 12.49 mmol/L. After 2 minutes later, the substrates were taken out. This process was repeated for five times to obtain

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different layers of phytic acid-metal complex. Finally, the substrates were soaked in phytic acid solution for 10 minutes, taken out, rinsed with deionized water and dried by a stream of nitrogen. The substrates coated with phytic acid-metal complex were suspended in 1.5× SBF solution with the front side facing down and incubated at 37 ℃for 3 d. The 1.5× SBF solution was refreshed once a day.

C.Hydroxyapatite coating characterization

C.a. SEM and EDS observations

Used with field emission scanning electron microscopy and energy dispersive spectroscopy. The nano-silver loaded hydroxyapatite coating, gold sputter, the final analysis. The image was taken at 5 kV at the appropriate magnification.

C.b. Raman observations

Coating data was analyzed by a LabRam HR laser Raman spectrometer (Horiba, France, jsm-7500f). Hydroxyapatite coatings were excited with 522um incident light in the range of 200-4000 cm.

D..Preparation of hydroxyapatite coating loaded with nano silver

In order to prepare Ag nano hydroxyapatite coating, the hydroxyapatite coating immersed in 4.76mL phytic acid solution (0.255 uM/mL) for 10 minutes, and then immersed in 10 ml distilled water, then take 2 m L $AgNO_3$ (60 mg/mL) was added dropwise to the above solution, stirring for 10 min. In addition, 5 m L (36.032 mg/mL) glucose solution was added to the reaction system, and 12h was stirred at room temperature. After the reaction, three times were washed with distilled water and 24 h in vacuum drying. Meanwhile, a contrast experiment was set up. Under the same experimental conditions, hydroxyapatite coating with nano silver was prepared by reducing Ag NO_3 (20 and 5 mg/mL) with glucose solution (as shown in Figure 1).

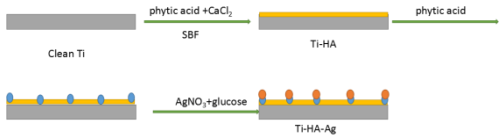

Figure 1. Schematic of a hydroxyapatite coating (Ti-HA-Ag) with nano-silver, an assembly method for Ti-HA-Ag.

E.Characterization of Hydroxyapatite Coatings Supported on Silver Nanoparticles

E.a.SEM and EDS observations

The field emission scanning electron microscope is used in combination with the energy spectrometer. The hydroxyapatite coating loaded with nanoscale silver was coated with gold, and the final analysis was made. The image is photographed at the appropriate magnification under 5kV.

E.b.Raman observation

The laser Raman spectrometer was used to analyze the coating data. The hydroxyapatite coating loaded with nanoscale silver was analyzed in the range of 200-4000 cm by 522 um incident light.

F.Colony Analysis

The Escherichia coli and Staphylococcus aureus solution were diluted 1000 times to reach the initial dosage of 10^5 CFU / mL. 300 μL bacterial solution was added to each hole of 48 hole plate, and titanium hydroxyapatite coating and hydroxyapatite coating with three concentrations of nano silver were placed respectively. At 37 ℃ for 24 hours. Each surface is washed three times with a sterile saline solution and carefully placed in each 10mL centrifuge tube containing 5 mL sterile saline. Each tube was treated by ultrasonic treatment for 8 seconds to separate bacterial cells, and the resulting suspension was removed from each tube and diluted with appropriate dilution factors. The diluted solution of bacteria was applied to the TSA agar medium. After incubating at 37 ℃ for about 16 hours, the number of colony forming unit (CFU) was counted. Each test was carried out in three copies[15].

G.Inhibition zone determination

The mixture of TSA nutrition agar solution and the empty culture plate were sterilized by high pressure. Then the TSA agar medium was poured into the culture dish and cooled by UV. About 10^7 CFU / mL Escherichia coli and Staphylococcus aureus were inoculated on each flat, then the surface of silver modified samples was buckled onto agar plate. All the plates were incubated at 37 ℃ for 16 hours, and then the inhibition area was measured.

III. RESULTS AND DISCUSSION

A.Hydroxyapatite and silver-loaded hydroxyapatite synthesis and characterization

A.a.SEM and EDS observations

Compared with titanium (a) and hydroxyapatite (b), C (d) and E (Ag), three kinds of Ag loaded silver hydroxyapatite, can be seen clearly in SEM (see Figure 2). Elemental analysis of the auxiliary EDS (see Figure 3 and Table 1) shows that there is no silver element present in titanium plate (a) and hydroxyapatite (b), whereas silver elements are evident in c, d and e.As the concentration of $AgNO_3$ added decreases, the percentage of elements in the layer decreases. It was sufficient proved that all three hydroxyapatite coatings were loaded with nano-silver at three $AgNO_3$ concentrations.

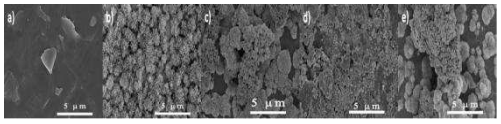

Figure 2. SEM image. a: clean titanium plate; b: hydroxyapatite coating: c-e: silver-loaded hydroxyapatite prepared at three AgNO $_3$ concentrations.

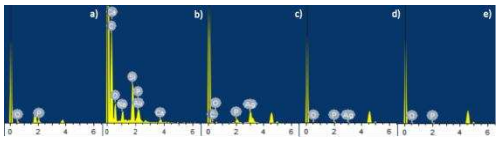

Figure 3. Energy Spectrometer (EDS) Elemental Analysis Image. a: clean titanium plate; b: hydroxyapatite coating: c-e: silver-loaded hydroxyapatite prepared at three $AgNO_3$ concentrations.

Table 1. The atomic percent of Ag in the sample

	Ti	Ti-HA	Ti-HA-Ag-1	Ti-HA-Ag-2	Ti-HA-Ag-3
Agatomic percent/%	0	0	19.78	8.06	1.62

A.b. Raman observations

Analysis by Raman spectroscopy data (see Figure 4) revealed that there were four more peaks in the three $AgNO_3$ concentrations compared to titanium and hydroxyapatite, which were 360 、 565.5 、 903 and 1543.2 cm^{-1}, respectively.This result proves that all hydroxyapatite coatings are loaded with nano-silver particles.

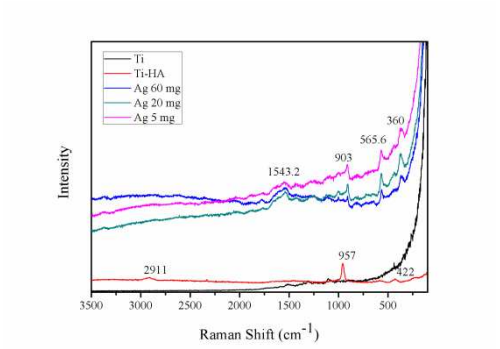

Figure 4. Raman spectrum of the sample.

B.Antibacterial test.

B.a. 24 h antibacterial experiment

In order to quantitatively examine the situation of bacterial adhesion and growth on the surface of hydroxyapatite on loaded and unloaded nano silver, the number of viable bacterial cells on the surface after 24 hours incubation was measured by plate counting method. As shown in Figure 5, the unloaded silver nanoparticles support the attachment of a large number of living cells to the two bacterial strains. For Staphylococcus aureus, 4.5 x 10^5 and 6.4 x 10^5 CFU were found on the surface of the original titanium and hydroxyapatite, and 1.4 x 10^6 and 2.2 x 10^6 CFU for E. coli, respectively. After silver nanoparticles were loaded on hydroxyapatite surface (Ti-HA-Ag), all three Staphylococcus aureus and Escherichia coli were killed. This shows that the hydroxyapatite loadedwith nano silver loaded in three concentration has similar bactericidal effect.

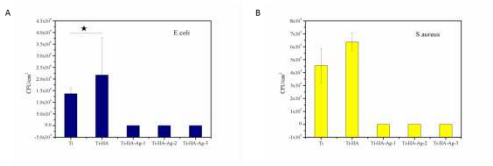

Figure 5. Antibacterial activity against E. coli (A) and Staphylococcus aureus (B). Bacteria colonies on titanium, hydroxyapatite and silver-loaded hydroxyapatite coated surfaces of three different Ag concentrations were incubated for 24 hours.

B.b.Bacteriostatic ring experiment

The antibacterial effect was evaluated by bacterial inhibition ring for Escherichia coli and Staphylococcus aureus. No obvious inhibition area was observed on the surface of titanium and hydroxyapatite on unloaded nanosilicon, but there was an obvious inhibitory area on the surface of hydroxyapatite coated with nano silver (Fig. 6).

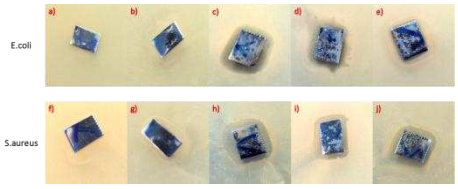

Figure 6. Inhibition regions of silver-modified regions observed. a-e samples of bare titanium, hydroxyapatite, and silver-loaded hydroxyapatite at three different concentrations of Ag on TSA agar plates inoculated with E. coli. f-j shows the sample of titanium, hydroxyapatite, and silver-loaded hydroxyapatite of three different Ag concentrations on TSA agar plates inoculated with S. aureus.

IV. CONCLUSION

Nano silver is widely used in biomaterial coating because of its broad and nonspecific biological properties. However, the preparation method of hydroxyapatite coated with nanoscale silver has not studied a simple method before. In order to obtain antibacterial hydroxyapatite surface, silver nanoparticles are first loadedon hydroxyapatite by chelation of phytic acid and further reduction of glucose. The structure of hydroxyapatite and Ti-HA-Ag were studied by EDS, SEM and Raman. The overall antibacterial performance was analyzed by 24 hour plate counting and bacteriostasis test to show the colony analysis of living bacteria cells.This silver loaded hydroxyapatite coating shows great broad antibacterial property and can be further used in bone and dental implant.

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