Construction of antibacterial multilayer films containing nanosilver via layer-by-layer assembly of heparin and chitosan-silver ions complex

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Abstract: Antibacterial multilayer films containing nanosilver were prepared via layer-by-layer fashion. PET film was aminolyzed with 1,6-hexanediamine to introduce amino groups on PET film surface; chitosan–silver nitrate complex and heparin were alternately deposited onto an aminolyzed PET film surface, and subsequently, the silver ions within the multilayer films were reduced with ascorbic acid to form silver nanoparticles. UV–visible spectroscopy and transmission electron microscopy confirmed the formation of well-dispersed nanosilver particles with sizes (10–40 nm) that depended on the initial concentration of silver ions in chitosan solution and the pH of ascorbic acid solution. The chitosan/heparin multilayer films were possessed of bactericidal effect on Escherichia coli (E. coli), and this antibacterial effect could be significantly enhanced by the incorporation of silver nanoparticles into the multilayer films. The multilayer films containing nanosilver were not only effective as antibacterial but also as anticoagulant coating. And cell toxicity evaluation suggested that the multilayer films containing nanosilver did not show any cytotoxicity. The multilayer films containing nanosilver may have good potentials for surface modification of medical devices, especially for cardiovascular implants. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 79A: 665–674, 2006

Key words: multilayer; anti-bacterial; nanosilver; chitosan; heparin

INTRODUCTION

Poly (ethylene terephthalate) (PET) has been widely used in cardiovascular implants because of its excellent mechanical properties and moderate biocompatibility. However, the infection and thrombogenicity are common to this kind of cardiovascular implants, which can lead to significant morbidity and mortality.1 Several strategies focusing on surface grafting have been developed to confer polymer films with antibacterial and anticoagulation properties.2–4 However, those methods involve a number of chemical steps that increase the difficulty in processing and controlling the concentration of antibacterial groups on the surface.

Layer-by-layer method, which is based on the alternating physisorption of oppositely charged polyelectrolytes, represents a new, alternative solution for biomaterial coating.5–9 The buildup is easy and the procedure can be adapted to almost any type of surface. Moreover, the method is valid whatever the shape of the solid. Recently, Boulmedais et al.10 constructed anti-adhesive multilayer films with pegylated polypeptides via layer-by-layer fashion. The adhesion of E. coli was reduced by 72% on films with one poly(1-lysine)/poly(1-glutamic acid)-g-poly (ethyl glycol) (PLL/PGA-g-PEG) bilayer and by 92% for films with three (PLL/PGA-g-PEG) bilayers compared with that of bare substrates. In our previous study, chitosan and heparin were alternatively deposited onto aminolyzed PET films to construct anti-adhesive and antibacterial multilayer films.11 The multi-
layer films not only reduced the bacterial adhesion significantly, but also killed a part of the bacteria adhered onto the surface. And the chitosan/heparin multilayer films had also been proved to have strong anticoagulant activity. The broad antimicrobial activity of silver is well known and it has been used in different fields in medicine for many years. Nanosilver has received considerable attention in recent years because of its sustained silver ions release. The layer-by-layer self-assembly of polyelectrolytes on charged surfaces offered a possibility to deposit nanosilver onto substrates.

Chitosan, which has natural antibacterial activity, may serve as a stabilizing ligand for silver ion and nanosilver particles because the amino groups of chitosan can chelate silver ions and nanosilver particles with coordination interactions. Heparin is a well-known sulfated polysaccharide that possesses strong anticoagulant activity. The layer-by-layer assembly of heparin and chitosan–silver ions complex was then explored to develop a novel nanosilver antibacterial coating in this study. The multilayer films containing nanosilver, which bound to the biocompatible polyelectrolyte, may limit the toxic effect of the silver metal toward the host organism while still allowing it to exercise infection controls.

**EXPERIMENTAL PROCEDURES**

**Aminolysis on PET membrane**

Aminolysis on PET film (Hangzhou Magnetic Tape Corporation of China) was performed by immersing a PET film in 0.06 g/mL of distilled 1,6-hexanediamine/propanol solution for 4 h at 37°C, and then rinsing with deionized water to remove free 1,6-hexanediamine, and dried under reduced pressure at 30°C for 24 h. The detailed description and the characterization of the aminolysis on PET membrane can be found in our previous research.

**Multilayer films preparation**

The aminolyzed PET films were treated with 0.1M acetic acid (HAc) solution for 2–3 h at room temperature and washed with a large amount of water. The substrates were dipped into a chitosan (average Mw = 410,000, 91% deacetylation; Qingdao Haihui Corporation of China) aqueous solution (1 mg/mL; pH = 3.8) with different concentrations of silver nitrate for 10 min and subsequently rinsed with pure water that was adjusted to the same pH as the polyelectrolyte dipping solution. And the substrates were blown dry with a flow of N2. The substrates were then dipped into 1mg/mL of heparin (Shanghai Chemical Reagent Company of China) aqueous solution (pH = 3.8) for 10 min followed by the same rinsing and drying procedures. Multilayer films were obtained by alternate deposition of chitosan–Ag+ and heparin. Finally, the substrates were immersed into 1 mM ascorbic acid solution for 3 h. The substrates were then washed with pure water and dried at 30°C under vacuum for 24 h.

**Multilayer films characterization**

UV–visible spectrum was recorded on a UV–visible spectrophotometer (CARY100 BIO, America). For transmission electron microscopy (TEM) imaging, the multilayer films were deposited onto an aminolyzed glass. The aminolyzed glass substrate was prepared by treating with poly(ethyleneimine) (PEI) at a concentration of 1 mg/mL to obtain a stable positively charged surface. The multilayers containing nanosilver were then constructed on the glass treated with PEI by the same procedure described earlier. The glass was immersed into hydrofluoric acid solution, and the multilayer films instantly broke off from the glass, and were immediately picked up with copper TEM grids and blown dry gently. The copper TEM grids were then coated with a thin layer of carbon in a thermal evaporator. TEM was performed with a JEOL JEM-2000FX operated at either 100 or 200 kV.

**Determination of antibacterial characteristics of PET substrates**

*Escherichia coli* BL21 strain was a gift from Jian Xu (Zhejiang University, Hangzhou, China). Waterborne and airborne tests were carried out according to the methods reported by Cen et al.3 For the waterborne assay, the broth containing *E. coli* was centrifuged at 3000 rpm for 10 min. After removing supernatant, the cells were washed twice with PBS and resuspended in PBS with 10⁷ cells/mL. The PET substrates were immersed into this suspension in a sterile plastic tube, and this tube was shaken at 200 rpm at 37°C for 2 h. The substrates were then removed and washed gently three times with sterile PBS and placed in petri dishes. This was followed by the immediate addition of solid growth agar (1.5% agar in yeast-dextrose broth). The petri dishes were then sealed and incubated at 37°C for 24 h. For the airborne assay, the broth containing *E. coli* was centrifuged at 3000 rpm for 10 min, and after the removal of the supernatant, the cells were washed twice with PBS and resuspended in PBS at a concentration of 10⁷ cells/mL. The PET substrates were then sprayed with the bacterial suspension using a commercial chromatography sprayer. After being dried in air for minutes, the substrates were placed in petri dishes. The petri dishes were sealed with growth agar and incubated at 37°C for 24 h. The substrates after waterborne or airborne tests were characterized by field-emission scanning electron microscope (FESEM; FEI, SiRion100). To prepare the sample for SEM investigation, the substrates were first immersed into 3 vol % glutaraldehyde solution in PBS at 4°C for 4 h. The glutaraldehyde solution was then removed and the substrates were washed with PBS, followed by step dehydration with 25, 50, 70, 95 and 100% ethanol for 10 min each. The substrates were then dried and sputter-coated with a thin film of platinum for imaging purposes.

The survival curves of E. coli onto the multilayer films with or without nanosilver were also investigated. The PET substrates were placed in well plates, and 1 mL of E. coli suspension in PBS with an initial cell concentration of 5 × 10⁵ cells/mL was pipetted onto the surface of the PET substrates. The well plates were incubated at 37°C. At predetermined times, cells were taken out and consecutive dilutions were prepared by taking 1 mL of the previous solution and mixing with 9 mL of PBS. From the solution, 0.2 mL was plated onto the triplicate solid agar using the spread plate method. After incubating for 24 h, the number of viable bacteria was then counted and the results after multiplication with the dilution factor were expressed as mean colony forming units (CFU)/mL. The survival ratio of bacteria was defined as the percentage of viable bacteria in the suspension relative to the total number of the initial bacteria in the suspension. Results represent mean ± SD of triplicates from three separate experiments (p < 0.05).

Plasma recalcification time test

Plasma recalcification time test was performed according to the method our group used previously. Briefly, 1 mL the fresh human plasma in which the Ca²⁺ was removed was warmed up to 37°C and was added into the silanized tube, and then the PET films were incubated in the tube for 1 min. After that, and finally, 1 mL of a previously warmed (37°C) CaCl₂ solution (0.025 mol/L) was added into the tube. At the same time, a small stainless steel hook was used to stir the recalcified plasma until the silky fibrin appeared. The time was recorded as plasma recalcification time (PRT). Data were presented as a mean standard deviation of five different experiments.

In vitro cytotoxicity testing

The mouse MC3T3 osteoblast cells were used to evaluate cell toxicity by cell morphological evaluation and cell activity test (MTT assay). The mouse MC3T3 osteoblast-like cells were maintained in α-MEM (α-minimum essential media, Hyclone.) supplemented with 10% heat-inactivated (30 min, 56°C) fetal bovine serum (Sijiqin Biotech, China, lot no. 020613.2), 80 units/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL gentamicin (Gibco) and kept at 37°C in a humidified 5% CO₂ atmosphere. The multilayer-modified PET films, sterilized in 75% ethanol and swollen in PBS, were placed into 96-well tissue culturing polystyrene (TCPS) plates (NUCLONTM, Cat. No.167008). The mouse MC3T3 osteoblast cells were then seeded in culture media to give a final density of 20 × 10⁴ cells/well and incubated for 12 h at 37°C in an atmosphere of 5% CO₂ in air. Cell activity was determined by MTT assay. The absorbance values were measured by using microplate reader (Bio-Rad, model 550) at wavelength 570 nm, blanked with DMSO solution. Five replicates were read for each sample, the mean value of the five was used as the final result.

After seeding for 2 days, the films were fixed in 2.5% glutaraldehyde solution for 15 min and washed with PBS at least three times. Cells’ morphology was characterized by FESEM. The cell monolayers on different substrates were also stained with fluorescein diacetate (FDA, Sigma) for confocal laser scanning microscopy (CLSM, Bio-Rad,
Radiance 2100; Zeiss Axiovert 200) investigation. FDA is an indicator of membrane integrity and cytoplasmic esterase activity. Stock solutions were prepared by dissolving 5.0 mg/mL FDA in acetone. The working solution was freshly prepared by adding 5.0 μL of FDA stock solution into 5.0 mL of PBS. FDA solution (20 μL) was added into each well and incubated for 5 min. The substrates were then washed twice with PBS and placed on a glass slide for CLSM examination. The 488 nm wavelength of the laser was used to excite the dye. Cells incubated into wells that did not contain materials were used as negative controls and cells incubated into wells that contained injurious agent (triton X100, 0.05%) were used as controls.

RESULTS AND DISCUSSION

UV measurement

Silver nitrate was added into chitosan solution to develop chitosan–silver nitrate complex. The silver ions are able to chelate with the amino groups of chitosan because of coordination interactions. The nanosilver particles with narrow size distribution are supposed to be developed under nature biomacromolecules stabilization within the multilayer films. The multilayer films were prepared by alternately depositing chito-
san–silver nitrate complex and heparin onto an aminolyzed PET film and subsequently reducing the silver ions with ascorbic acid to form silver nanoparticles (Scheme 1).

Figure 1 shows that the surface plasmon absorbance resulted from these nanosilver particles in the multilayer films centralizes at 414 nm and increases almost linearly with the number of layers in the film. It suggests that the nanosilvers are prepared successfully within the biocompatible natural polyelectrolyte multilayer films.

Figure 3. TEM images of 14-layer chitosan–Ag₀/heparin multilayer films reduced using ascorbic acid solutions with different pH during reduction process, the concentration of silver nitrate is 1 mM. (a) pH = 4.0, (b) pH = 6.0, and (c) pH = 10.0.

TEM characterization

 Figures 2 and 3 show that the nanosilver size depends on the initial concentration of silver ions in chitosan solution and the pH of the ascorbic acid solution. With 0.3, 1, 2 mM silver nitrate being used during deposition process, the average particle sizes are 12, 20, 42 nm, respectively. The UV absorbance shifts from 414, 418 nm to 424 nm, respectively. Ascorbic acid is selected as the reductant, because of its low toxicity and its ability to precipitate metallic
silver according to

\[ 2\text{Ag}^+ + \text{C}_6\text{H}_8\text{O}_6 \rightleftharpoons 2\text{Ag}^0 + \text{C}_6\text{H}_6\text{O}_6 + 2\text{H}^+. \]

With the pH of the ascorbic acid solution decreasing, the size of nanoparticles reduces from 24, 20 nm to 10 nm. According to the above equation, acidification of the ascorbic acid should shift the equilibrium, which affects the nucleation and growth stages in the precipitation of silver\textsuperscript{23} For this reason, the size of the nanosilver particles reduced with ascorbic acid solution at lower pH is smaller. It suggests that the
size of nanosilver particles can be controlled by adjusting the initial concentration of silver ions in chitosan solution and the pH of the ascorbic acid solution. Therefore, it is convenient to tune the release rate of silver ions by controlling the size of nanosilver particles.

**In vitro antibacterial test**

The waterborne test and airborne test to mimic the natural deposition of *E. coli* on substrates developed by Cen et al. are performed on the pristine PET films and multilayer-modified PET films. In the waterborne test [Figs. 4(a–c)], numerous distinguishable bacteria can be seen on the control PET films [Fig. 4(a)]. Meanwhile, the number of viable bacteria on the chitosan/heparin multilayer films decreases a lot [Fig. 4(b)], and this decrease is more substantial for multilayer films containing nanosilver [Fig. 4(c)]. A similar trend of antibacterial activities of these substrates can be observed in the airborne test. A larger number of bacteria exist on the PET films. However, almost no bacteria can be observed on the multilayer films containing nanosilver [Fig. 4(f)]. The only difference observed between the airborne and waterborne tests is more viable bacteria existing on the corresponding substrates in the airborne test, which is caused by the more initial bacteria deposition onto the substrates. Although the hydrophilicity of the multilayer films and antibacterial properties of chitosan within multilayer films reduce the number of viable bacteria on chitosan/heparin multilayer films, there are still a lot of bacteria observed on multilayer films. However, when the nanosilver particles were loaded into the multilayer films, the dual antibacterial effect of multilayer and nanosilver, especially the powerful antibacterial properties of nanosilver, make no bacteria survivable on the multilayer films.

The effect of multilayer films with or without nanosilver on the survival curves of *E. coli* is shown in Figure 5. When contacting with untreated PET film, the viable bacteria in the suspension reduces by only 5% after 6 h. This relatively small reduction may have resulted from natural apoptosis. With multilayer-modified PET film, the number of viable bacteria decreases rapidly; only about 36% of the cells are viable. As discussed earlier, because the chitosan on the surface of the multilayer films can kill the bacteria contacting with the surface, the number of viable bacteria decreases more rapidly. With multilayer films containin-
ing nanosilver, the antibacterial activity is further enhanced. After 1 h, the number of the viable bacteria has decreased by about 50%, and after 5 h, only about 10% of the cells remain viable; that is, a powerful anti-infection coating has been constructed on the PET film, which suggests that nanosilver within the multilayer films plays a key role in killing bacteria in the solution.

**Plasma recalcification time test**

The thrombogenicity is common to the cardiovascular materials, which can lead to significant morbidity and mortality. The chitosan/heparin multilayer films had been proved to have strong anticoagulant activity. Do the multilayer films still have a strong antico-
agulant activity when the nanosilver is loaded into the multilayer films? Figure 6 shows that the blood on the PET films is coagulated within 9 min, while anticoagulation activity is observed for the multilayer films and the blood on the multilayer films is not coagulated even within 30 min, which is consistent with the result reported by Takeshi et al. It is very interesting that, although silver ions released from the multilayer films containing nanosilver can kill the bacteria, the silver ions do not decrease the anticoagulant activity of multilayer films at all.

In vitro cytotoxicity testing

The mouse MC3T3 osteoblast-like cells were used to evaluate cell toxicity by cell morphological evaluation and cell activity test. Figure 7 shows the cell ac-
tivity of osteoblasts on various surfaces over a period of 48 h. It is found that the cell activities of osteoblasts cultured on multilayer film containing nanosilver do not differ significantly from those of cells cultured on virgin PET and multilayer-modified PET. The difference between the Triton group and the nontoxic control groups is significant. According to Figures 8 and 9, after 2 days, it can be seen that all the osteoblasts cultured on the nontoxic control groups have undergone some degree of proliferation in monolayer; the osteoblasts on these films’ surface maintain normal spreading morphology. However, addition of triton destroys the cells, and almost no vital cells are seen anymore. The above results suggest that the multilayer films containing nanosilver do not show any cytotoxicity.

CONCLUSIONS

Natural polyelectrolyte multilayer films containing nanosilver were successfully prepared on aminated PET film via layer-by-layer fashion. Well-dispersed nanosilber particles with sizes from 10 to 40 nm were formed upon reduction with ascorbic acid. The multilayer films containing nanosilver were effective in killing E. coli, and had good anticoagulation activity and low cell toxicity. The technique is easy and inexpensive, and the procedure can be adapted to almost any type of surface as long as surface charges are present. The biocompatible natural polyelectrolyte multilayer films containing nanosilvers may have good potentials for surface modification of medical devices, especially for cardiovascular implants.

References