Bacterial Adhesion on Honeycomb-Structured Poly(L-Lactic Acid) Surface with Ag Nanoparticles

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Polymeric materials with ordered porous structure have received increasing interest due to their potential applications in biomaterials. However, the enhancement of cell proliferation by this porous structure also raises worries about the increase of bacterial adhesion. For their further use as biomaterials in vivo, it is essential to assess the bacterial adhesion on porous polymer films and find strategies to inhibit the bacterial retention. Honeycomb-structured poly(L-lactic acid) (PLLA) films with Ag nanoparticles were used in this purpose. The Ag release rate of Ag/PLLA films was analyzed. In vitro bacterial adhesions of S. aureus and E. coli on flat PLLA films, flat Ag/PLLA films, and honeycomb-structured Ag/PLLA films were compared. Ag nanoparticles activity appears to be more effective against bacteria in honeycomb-structured films compared with flat films of the same material. This activity is particularly significant when E. coli is used. The results suggest that the honeycomb surface topography, as well as the enhanced release of Ag+, can greatly contribute to the anti-adhesion property of PLLA surface. These honeycomb-structured Ag/PLLA films also have no significant cytotoxicity. The decoration of Ag nanoparticles in honeycomb structure provides an effective and safe strategy to reduce the bacterial adhesion on porous polymer surface.

Keywords: Ag Nanoparticles, Poly(L-Lactic Acid), Breath Figures, Honeycomb-Structured Film, Bacterial Adhesion.

1. INTRODUCTION

Microporous films with honeycomb structure have attracted intensive interest due to their potential applications as filtration membranes, catalytic templating, chemical sensors, optical apparatus, protein microarrays, superhydrophobic materials, and, most pertinent to this study, as scaffolds for tissue engineering. For biomedical application, polymers with good compatibility are usually chosen for fabrication of honeycomb surface. It is reported that the honeycomb structures exerted a strong influence on morphology, proliferation, and differentiation of cells. For normal cells (hepatocyte, endothelial cells, and cardiac myocytes), their adhesion on honeycomb structure leads to higher cell viability, higher cell growth, and higher cell functions including matrix production profiles. And the morphology, proliferation, and differentiation of neural stem/progenitor cells can be controlled by the pore size of the films. Hence, the honeycomb-structured films have potentials for tissue regeneration in a growth factor free proliferation process of stem cells. For cancer cells, their growth and cell motility on honeycomb-structured films are inhibited revealing their potential anticancer effect. Hence, it is expected that the honeycomb-structured films will provide an effective strategy for medical devices and implants.

However, if the honeycomb-structured films are utilized in vivo, the adhesion of bacteria to the porous surfaces in aqueous media or physiological environment forming biofilms might lead to medical implant infections. The retention of bacterial cells can be influenced by a number of surface properties, such as chemical composition, wettability, and topographical factors. Numerous papers have been published to study the effect of surface topography on bacterial adhesion. It is believed that if the surface roughness is of microbial dimensions or of
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nanodimensions,19,20 the microbial retention may increase. Hence, it is essential to find effective strategies to prevent the bacterial retention.

The decoration of nanoparticles on honeycomb structures is able to functionalize the surfaces of the spherical cavities. The number of publications on nanoparticles self-assembly at interfaces has steadily increased.21–24 For instance, silica nanoparticles21 and CdSe nanoparticles23 were used to decorate honeycomb structures on thin polymer films. In this work, Ag nanoparticles were introduced to functionalize these honeycomb holes. Ag nanoparticles are widely used in biomedical area,25–28 which have excellent antibacterial ability29–32 and optical properties as SERS.33 The application of Ag nanoparticles in honeycomb structure can not only provide the antimicrobial ability with the release of Ag+ but also can promote the formation of honeycomb structure as reported in our previous work.34 During the breath figure process, Ag nanoparticles are assembled at the liquid-liquid interface changing the interface behavior.

Honeycomb-structured poly(L-lactic acid) (PLLA) films with Ag nanoparticles were utilized in this current study. PLLA was selected for the current study due to its wide application in biomedical systems. The Ag+ release rate of Ag/PLLA films was analyzed. The adhesions of two bacteria species, S. aureus and E. coli, on flat PLLA films, flat Ag/PLLA films, and Ag/PLLA honeycomb-structured films were evaluated. Particular attention was given to the influence of the honeycomb morphology on the antibacterial ability of Ag nanoparticles. Furthermore, a possible mechanism of the improved antimicrobial property provided by microscale honeycomb pores and embedded nanoscale Ag particles was discussed.

2. EXPERIMENTAL DETAILS

2.1. Materials

Silver nitrate (AgNO3, 99%), sodium borohydride (NaBH4, 99%), absolute ethanol, chloroform (CHCl3, CA), sodium chloride (NaCl, 99%), and gluteraldehyde (25%) were purchased from Chemical Reagent Company (Shanghai, China). PLLA with Mw of 100,000 was purchased from Shanghai Institute of Medical Instrument (Shandong, China). S. aureus (ATCC25923) and E. coli (ATCC25922) were purchased from Jiangsu Provincial Center for Disease Prevention and Control (Nanjing, China). Beef extract and peptone were purchased from Aoboxing Biotech Company (Beijing, China). Dulbecco’s Modified Eagle Medium (DMEM) was purchased from Hyclone Biochemical Product Company (Beijing, China) and fetal calf serum (FCS) was purchased from Sijiqing Biological Engineering Materials Company (Hangzhou, China). All the other reagents were obtained from Chemical Reagent Company (Shanghai, China) and were of analytical grade, used without further purification.

2.2. Preparation of PLLA or Ag/PLLA Films

Ag nanoparticles-PLLA composite suspensions were prepared by in situ reduction method as reported in our previous work.34 First, 0.5 mL AgNO3 absolute ethanol solution (1 mmol·L−1) was mixed with 50 mL CHCl3 solution containing 5 g PLLA to obtain a PLLA/AgNO3 solution in CHCl3/ethanol. Then 0.5 mL freshly prepared NaBH4 ethanol solution (0.02 mol·L−1) was added to the above PLLA/AgNO3 solution with vigorous stirring to reduce Ag+ to Ag nanoparticles. The bright-brown suspension was obtained after the reaction was complete.

Then honeycomb-structured Ag/PLLA films were prepared by breath figure (BF) method which utilizes the volatile solvent of the polymer solution, causing condensation of water vapor on the surface of solution. The droplets of condensed water self-assemble and arrange into highly ordered arrays. As the concentration of the polymer increases with solvent evaporation, the precipitation of the polymer on the interface stabilizes the droplets and prevents coagulation. After the evaporation of solvent and water droplets, a well-ordered honeycomb structure is left on the film surface.35,36 In this work, the honeycomb-structured Ag/PLLA films were prepared by casting 100 μL of Ag/PLLA suspension on cover glasses in temperature- and humidity-controlled setup with controlled at room temperature (25 °C) with 90% relative humidity (RH). Meanwhile, flat Ag/PLLA films were obtained by drying 100 μL of Ag/PLLA suspension on cover glasses in dry conditions with 30% RH. After complete evaporation of the solvent, the films were washed with deionized water and then dried overnight. With the same procedure, the flat PLLA films also can be prepared from PLLA solution.

2.3. Characterization

The dispersion of Ag nanoparticles inside the PLLA composite was observed by transmission electron microscopy (TEM, JEOL, JEM2000EX). The samples were prepared by dropping 10 μL of Ag/PLLA suspension on TEM grids. Optical images of Ag/PLLA films were taken using a darkfield optical microscope (Zeiss, Axiovert 200). The surface morphologies of PLLA and Ag/PLLA films were investigated by scanning electron microscopy (SEM, JEOL, JSM-5610LV) and atomic force microscopy (AFM, Agilent, PicoPlus). The samples were coated with gold under reduced pressure with a sputter coater prior to SEM examination.

2.4. Ag+ Release Test

To study the kinetics of silver release from the prepared Ag/PLLA films, the flat or honeycomb-structured Ag/PLLA film was immersed in different mediums including deionized water, Luria-Bertani nutrient medium
(bacterial culture medium), and DMEM medium (cell culture medium), respectively. The films were cut into a square shape with 2.0 cm × 2.0 cm, after washing with deionized water for ten times and dried, and then the samples were immersed in 10 ml of medium solution at 37 °C without stirring, respectively. At the predetermined time (0.5, 5, 12, 24, 36, 72, and 108 h), the samples were taken out and the silver ion content of suspending fluids obtained from each time was analyzed by atomic absorption spectrometer (AA, Hitachi 180-80).

2.5. Assessment of Bacterial Adhesion

The adhesion of bacteria (gram-positive S. aureus and gram-negative E. coli bacteria) to PLLA films and Ag/PLLA films was examined. In detail, flat PLLA films, flat Ag/PLLA films, and honeycomb-structured Ag/PLLA films were utilized and cut into a square shape with 2.0 cm × 2.0 cm. Sterilization of the films was done by first immersing in 70% ethanol for an hour and then washing with sterile distilled water. Then each of the films was immersed in the bacteria suspensions with a concentration of 10^6 CFU·mL⁻¹ in Luria-Bertani nutrient medium (LB) (5 g L⁻¹ NaCl, 10 g L⁻¹ peptone powder, and 5 g L⁻¹ beef extract powder, pH 7.2) and incubated at 35 °C for 24 h without shaking. After incubation, the films were carefully removed from the medium, rinsed with sterile distilled water to remove non-adhered bacteria and placed into a centrifuge tubes containing 2 mL of sterile deionized water. The bacteria adhered on the surface of the films were removed by vigorous shaking of the centrifuge tube at 2000 rpm for 30 s and quantified by serial dilutions and spread plate technique. A 1 mL aliquot of the suspension was diluted decimally and from each dilution, 0.1 mL was transferred to a nutrient agar plate and the surviving bacteria were counted after 24 h of cultivation at 37 °C reported as CFU·cm⁻². Each experiment was repeated in triplicate, repeated at least three separate times.

For SEM characterization, the films plus retained bacteria were immersed in 4% (w/v) gluteraldehyde for 1 h at 4 °C. After fixing, films were washed gently with distilled water and passed down an ethanol gradient at 30, 50, 70, 90, and 100% each for 10 min. The samples were carefully removed from the medium, rinsed with sterile distilled water and passed down an ethanol gradient at 30, 50, 70, 90, and 100% each for 10 min. The samples were fixed with 2% osmium tetroxide and then dehydrated in a graded ethanol series and dried using a critical point dryer. The films were sputter-coated with gold and observed using a scanning electron microscope (JEM-2010, Jeol). The average diameter of the Ag nanoparticles in PLLA composite is shown in Figure 1(A). It is observed that Ag nanoparticles are monodispersed in the PLLA matrix without coagulation. Histograms (Fig. 1(A), inset) show that the average diameter of the Ag nanoparticles in PLLA is ± 22.09 nm. Controlling the spatial distribution of nanoparticles in composite materials usually poses significant challenges because nanoparticles can either disperse uniformly or aggregately.9 Various methods, many of which are complex and time-consuming, have been applied to design the ligand chemistry of nanoparticles to prevent aggregation and direct their assembly within polymers.40–43 In this work, the results demonstrate that the facile in situ reduction method can efficiently avoid the aggregation of Ag nanoparticles in PLLA. Thus, the nanoscale antibacterial effects of Ag can be maintained in Ag/PLLA films.

Darkfield optical microscopy images of Ag/PLLA films were also taken to investigate the status of Ag nanoparticles in PLLA films. Ag nanoparticles can be seen from darkfield images because of their optical properties of surface plasmons.44 Figure 1(B) shows a typical region where Ag particles display a variety of plasmon resonant colors in flat PLLA film. The optical images of Ag nanoparticles appear larger than their actual sizes because of the optical diffraction limit (~200 nm).45,46

Ag/PLLA films were prepared via the breath figure method from Ag/PLLA suspension under humid conditions and room temperature. Figures 1(C and D) represent the morphology of the honeycomb-structured Ag/PLLA film under brightfield and darkfield, respectively. As can be seen, the average pore size of the Ag/PLLA films is about 3 μm. In the darkfield image, plasmon resonant colors are observed on the walls of the holes, indicating the presence of Ag nanoparticles. As further investigation of the surface topography by SEM and AFM images (Fig. 2), Ag/PLLA films also show an ordered honeycomb structure with pores averaging 3 μm.

3. RESULTS AND DISCUSSION

3.1. Characterization of Porous PLLA Films and Ag/PLLA Films

A TEM image of Ag nanoparticles in the PLLA composite is shown in Figure 1(A). It is observed that Ag nanoparticles are monodispersed in the PLLA matrix without coagulation. Histograms (Fig. 1(A), inset) show that the average diameter of the Ag nanoparticles in PLLA is ± 22.09 nm. Controlling the spatial distribution of nanoparticles in composite materials usually poses significant challenges because nanoparticles can either disperse uniformly or aggregately.9 Various methods, many of which are complex and time-consuming, have been applied to design the ligand chemistry of nanoparticles to prevent aggregation and direct their assembly within polymers.40–43 In this work, the results demonstrate that the facile in situ reduction method can efficiently avoid the aggregation of Ag nanoparticles in PLLA. Thus, the nanoscale antibacterial effects of Ag can be maintained in Ag/PLLA films.
3.2. Effects of Honeycomb Structure on Ag$^+$ Release from Ag/PLLA Films

The antibacterial properties of Ag nanoparticles usually involve the release of Ag$^+$ and direct interaction with bacteria. Ag$^+$ has been reported to interact with cytoplasmic components and nucleic acids, inhibiting respiratory chain enzymes and interfering with membrane permeability. The Ag nanoparticles interact with bacteria and present a special physicochemical system, which confers antimicrobial activity via Ag$^+$. Hence, the antimicrobial activities of Ag-containing materials are often studied in terms of their Ag$^+$ release rate. Ag nanoparticles are highly sensitive to oxygen, and form partially oxidized Ag nanoparticles with chemisorbed Ag$^+$. Ag particles require oxidation to initiate Ag$^+$ release. This process will occur in aqueous media according to Reaction (1). The released Ag$^+$ is known to be effective against gram$^+$ and gram$^-$ bacteria.

\[ 4\text{Ag} + \text{O}_2 = 4\text{Ag}^+ + 2\text{O}^{2-} \rightarrow \text{Ag}_2\text{O} \]  

In contrast to colloid Ag nanoparticles directly explored in aqueous media, Ag nanoparticles in Ag/PLLA films are embedded in polymer. Since oxidation of Ag nanoparticles is required for antibacterial activity, whether Ag/PLLA films can contact with water to initiate oxidation and release Ag$^+$ to yield antimicrobial ability is particularly crucial. Flat Ag/PLLA films and honeycomb-structured Ag/PLLA films were utilized to test the Ag$^+$ release rate by atomic absorption spectroscopy. The amount of Ag$^+$ released from Ag/PLLA films in three different mediums (deionized water, LB medium, and DMEM medium) at different time periods (0.5, 5, 12, 24, 36, 72, and 108 h) is illustrated in Figure 3. The Ag$^+$ release of all Ag/PLLA films increases with time and the Ag/PLLA films with honeycomb structure exhibit a higher Ag$^+$ release. Moreover, Ag$^+$ releases fast in the first 3 days and the release slows down thereafter. The cumulative Ag$^+$ release in deionized water is higher than that in LB medium and DMEM medium. Honeycomb-structured Ag/PLLA films are more efficient in releasing Ag$^+$ than flat ones. This is mainly because the honeycomb structure on Ag/PLLA films provide a larger contact area between Ag nanoparticles and the aqueous environment, which is beneficial for the oxidation of Ag nanoparticles. Moreover, casting the Ag/PLLA films using the breath figure method can result in increased Ag nanoparticles assembly at the interface, which may also lead to increased Ag$^+$ release efficiency. The results suggest that polymer-impregnated Ag nanoparticles are able to form chemisorbed Ag$^+$ by oxidation, and the Ag/PLLA films are able to achieve sustained Ag ion release and enough antibacterial efficacy in different mediums.
3.3. Bacterial Adhesion on PLLA and Ag/PLLA Films

The bacterial adhesion degree for PLLA and Ag/PLLA films after 24 h incubation is presented as histograms in Figure 4. The number of bacteria adhered on flat Ag/PLLA films decreases compared with flat PLLA films implying the efficiency of the Ag nanoparticles modifications in reducing the adhesion of bacteria onto the surface. Moreover, in *S. aureus* group, a 58% inhibition compared to the flat PLLA films group is observed for honeycomb-structured Ag/PLLA films suggesting the capability of the combination of surface structure and Ag nanoparticles in hampering adhesion of gram-positive strain. As for the *E. coli* adhesion, fewer bacteria is retained on flat Ag/PLLA films, and they rarely absorb on honeycomb-structured Ag/PLLA surfaces with 83% inhibition compared to the flat PLLA films group. It is realized that the Ag/PLLA films is effective against both strains, while honeycomb-structured Ag/PLLA films with pore size of 3 μm are selective towards *E. Coli* for anti-adhesion.

The SEM images (Fig. 5) shows the detail of bacterial adhesion on flat PLLA films, flat Ag/PLLA films and honeycomb-structured Ag/PLLA films. The flat Ag/PLLA (Figs. 5(B and E)) films can prevent the adhesion of bacteria to some extent compared to those on flat PLLA films (Figs. 5(A and D)) by the antibacterial property provided by Ag nanoparticles. As seen in Figures 5(C and F), the retention of bacteria on honeycomb-structured Ag/PLLA films are greatly inhibited. Compared with the adhesive bacteria on PLLA films, *S. aureus* within the pores of honeycomb-structured Ag/PLLA films are smaller in size, and the bacterial cell membranes are broken, indicating the inactivity of bacteria on the Ag/PLLA surface. In the case of *E. coli*, the bacteria adhered on honeycomb-structured Ag/PLLA films are shorter in length compared with those on flat PLLA films. Moreover, on honeycomb-structured Ag/PLLA films, they can be found only on the flat ridge among pores (as noted by arrows in Fig. 5(F)), and are not observed inside the pores (inset in Fig. 5(F)). The findings suggest that the honeycomb-structured Ag/PLLA films are more efficient in preventing the adhesion of *E. coli* than *S. aureus*. This can be understood by considering that the pore size of 3 μm matches well with the length of *E. coli*. 

**Fig. 2.** (A) SEM and (B) AFM images of honeycomb-structured Ag/PLLA films cast at 90% RH.

**Fig. 3.** Amount of Ag⁺ released from flat and honeycomb-structured Ag/PLLA films in (a) deionized water, (b) LB medium, and (c) DMEM medium as a function of time.
3.4. Mechanism of Bacterial Adhesion on Ag/PLLA Films

The adhesion of bacteria to PLLA surfaces is the first step to biofilm formation. With the accumulation of bacteria on the film surface, adhesion proteins expressed on bacterial surfaces can bind bacteria to the PLLA surface or with other nearby bacteria. Then, a 3D community of bacteria and peptidoglycan envelope can form and lead to the development of the biofilm.\textsuperscript{56} Honeycomb-structured Ag/PLLA films can reduce bacterial adhesion and inhibit biofilm formation at the initial stage. The possible anti-adhesion mechanism of honeycomb-structured Ag/PLLA films is shown in Figure 6. In honeycomb-structured Ag/PLLA films (Fig. 6(B)), Ag nanoparticles assemble on the walls of pores by the breath figure method, while in flat Ag/PLLA films, they are dispersed inside the PLLA matrix (Fig. 6(A)). Hence, the amount of Ag nanoparticles at the surface of honeycomb-structured Ag/PLLA films is larger than that in flat films, which can enhance the release rate of Ag\textsuperscript{+}. Moreover, the honeycomb structure may partly store Ag\textsuperscript{+} on the surface of Ag/PLLA films inducing the death of either adhered bacteria or nearby bacteria. Hence, the attachment of bacteria to honeycomb-structured Ag/PLLA films can be restricted to an initial, rapid, and easily reversible interaction. This explanation is consistent with the experimental results of \textit{S. aureus} adhesion to flat Ag/PLLA films (Fig. 6(C)) and honeycomb-structured Ag/PLLA films (Fig. 6(D)).

3.5. Biocompatibility of Ag/PLLA Films

The biocompatibility and toxic problems of Ag have been greatly concerned and discussed recently.\textsuperscript{57, 58} It should be prudent to focus on the investigation of the effects of the honeycomb-structured Ag/PLLA films on tissue cytotoxicity with osteoblast cells as well as maintaining good bactericidal property. Flat PLLA films, flat Ag/PLLA films, and honeycomb-structured Ag/PLLA films were examined. The cell proliferation was evaluated by alamar-Blue\textsuperscript{TM} assay and summarized in Figure 7. There are no significant differences in osteoblast cells proliferations on all films and in control group, indicating that the PLLA-embedded

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**Fig. 4.** Histograms of bacterial adhesion degree for PLLA and Ag/PLLA films after 24 h incubation against two microorganisms.

![Image](image_url)

(also about 3 \( \mu \text{m} \)), which probably leads to more difficulties for \textit{E. coli} adhesion within the pores. In contrast, the cell size of \textit{S. aureus} is about 1 \( \mu \text{m} \), which is smaller than the pore size of honeycomb-structured Ag/PLLA films, resulting in the adhesion of \textit{S. aureus} inside the pores (inset in Fig. 5(C)).

**Fig. 5.** SEM images of (A, B, C) \textit{S. aureus} and (D, E, F) \textit{E. coli} adhesion on (A, D) flat PLLA films, (B, E) flat Ag/PLLA films, and (C, F) honeycomb-structured Ag/PLLA films after 24 h of culture, respectively.
Ag nanoparticles have little adverse effect on osteoblast proliferation. Initially, cell proliferation on PLLA films and Ag/PLLA films are almost the same. After 24 h of cell culture, cell proliferations on honeycomb-structured Ag/PLLA films start to be relatively faster than those on flat films. Facilitate cell proliferation ability of honeycomb Ag/PLLA might be mainly caused by the honeycomb structure. The increase of surface area of substrate assist and enhance cell contact, improving adhesion of cell on polymer substrate. The dose of released Ag ion is quite low to cell culture but efficient to achieve antibacterial ability. The in vitro cell culture tests prove that the PLLA-embedded Ag nanoparticles with honeycomb structure have no significant cytotoxicity. However, further biocompatibility tests and the mechanism study are needed to carried out for the honeycomb Ag/PLLA films.

The improved anti-adhesion property of honeycomb-structured Ag/PLLA films stems from two effects on different length scales: the nanoscale antibacterial effect by Ag nanoparticles and the microscale anti-adhesion effect of the honeycomb structure. Anti-adhesion ability can be efficiently improved by the combination of these two effects; this combination has been proven to perform better than single approach. Ag nanoparticles can provide honeycomb structure surfaces with long-term antibacterial ability, while the honeycomb structure can reduce interactions between each adhered bacteria by the sharp undulated topography and enhance the release of Ag\(^+\) from the Ag/PLLA film. Moreover, honeycomb-structured Ag/PLLA films can achieve controlled release of Ag\(^+\) via PLLA degradation.

Aside from the bactericidal property of Ag nanoparticles, other nanoparticles can also be decorated on microporous polymer films by the in situ reduction method, and corresponding multifunction can be obtained. This method introduces a straightforward route to modify surface functionality within breath figures to control structural, electronic and optical properties independently by tuning the core sizes of the particles\(^{59,60}\) and the chemistry of the attached ligands.\(^{61}\)
4. CONCLUSIONS

The bacterial adhesion on honeycomb-structured Ag/PLLA films were assessed. The combination of honeycomb structure and Ag nanoparticles in Ag/PLLA film greatly inhibit the bacterial retention for S. aureus and E. coli while showing good biocompatibility. The honeycomb structure with pore size of 3 μm is more selective for anti-adhesion of E. coli with an improvement of 83%. The Ag⁺ release test results suggest that honeycomb-structured Ag/PLLA films release more Ag⁺ than flat Ag/PLLA films by oxidation of impregnated Ag nanoparticles. The honeycomb structure can increase the efficiency of Ag⁺ release and might store Ag⁺ on the Ag/PLLA film surface, thereby inducing death of nearby and adhered bacteria. Honeycomb-structured Ag/PLLA films suggest a new route to inhibiting the bacterial adhesion on porous polymer films, and introduce a novel strategy for fabricating functionalized ordered surface structures.

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References and Notes

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