

ANTIBACTERIAL POLY(D,L-LACTIDE) (PDLLA) FIBROUS MEMBRANES MODIFIED WITH QUATERNARY AMMONIUM MOIETIES*

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Abstract Antibacterial poly(D,L-lactide) (PDLLA) fibrous membranes were developed *via* electrospinning, followed by surface modification which involved plasma pretreatment, UV-induced graft copolymerization of 4-vinylpyridine (4VP) and quaternization of the grafted pyridine groups with hexylbromide. The success of modification with quaternized pyridinium groups on the PDLLA fibrous membranes was ascertained by X-ray photoelectron spectroscopy (XPS). The antibacterial activities of these membranes were assessed against Gram-positive *Staphylococcus aureus* (*S. aureus*) and Gram-negative *Escherichia coli* (*E. coli*). The PDLLA fibrous membranes modified with quaternized pyridinium groups showed antibacterial efficiency against both bacteria as high as 99.999%. The results demonstrated that the antibacterial activity was based on the interaction of the positive charge of pyridinium group and negatively charged cell membrane of bacteria, resulting in loss of membrane permeability and cell leakage.

Keywords: Poly(D,L-lactide); Antibacterial; Electrospinning; Surface modification

INTRODUCTION

Poly(lactide) (PLA) has been the focus of significant research over the past 40 years and has a long track record of clinic applications due to its biodegradability, biocompatibility, good mechanical properties and ability to be dissolved in common solvents for processing^[1, 2]. The amorphous form, poly(D,L-lactide) (PDLLA), has successfully been applied in resorbable bone fixation devices^[3] and scaffolds that proved to be well suited for tissue engineering^[4, 5]. Recently, electrospinning technique has been recognized as an efficient processing method to manufacture nanoscale fibrous structures, and electrospun PLA based nanofibrous membranes have attracted a great deal of attention due to their potential applications in drug delivery, prevention of post-surgery adhesion and biomimetic scaffolds for tissue engineering, *etc*^[6–8]. Ko *et al.*^[9] developed nanofibrous organic and inorganic composite scaffolds containing nano-sized demineralized bone powders with biodegradable PLA by electrospinning, which may serve as a favorable matrix for the regeneration of bone tissue. PLA ultrafine fibers with calcium nitrate entrapment were also prepared by electrospinning and then incubated in phosphate solution to form in situ calcium phosphate on the polymer matrix^[10]. Blends of nano-TiO₂ and PLA nanofibers with the anticancer drug daunorubicin were explored to facilitate an efficient drug delivery in target cancer cells^[11]. However, adhesion and subsequent surface growth of bacteria is a commonly encountered problem with PLA

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based biomaterials which are hydrophobic in nature^[12].

A possible approach to prevent bacterial infections is to render the biomaterial surface antibacterial properties. In recent years researches on conferring antibacterial property to biomaterials have relied on surface functionalization techniques such as covalently attaching polycationic groups^[13], impregnating or loading chitosan nanoparticles with antimicrobial agents^[14] and coating of implant surfaces with quaternary ammonium compounds, iodine, or silver ions^[15–17]. Tiller *et al.*^[18] revealed that the surfaces of commercial polymers treated with *N*-alkylated PVP groups were lethal on contact to both Gram-positive and Gram-negative bacteria, and it was also shown that *N*-alkyl chain of six carbon units in length was one of the most effective. Since the functional groups are absent in the backbone of the PLA, it is difficult to modify the surface property by common chemical methods.

Plasma technique is a convenient and powerful method for modifying polymeric materials without altering their bulk properties. The free radicals and electrons created in the plasma treatment can be used to modify the polymer nanofibers chemically. Covalent attachment of functional compounds to polymer fiber surfaces provides long term protection against bacterial colonization, reducing the risk of developing antimicrobial resistance, as the concentration of antimicrobial groups is constantly above the minimal inhibitory concentration^[19].

The objective of the present study is to develop antibacterial poly(D,L-lactide) (PDLLA) fibrous membranes modified with poly(4-vinyl-*N*-hexyl pyridinium bromide). PDLLA fibrous membranes were prepared via electrospinning, followed by plasma pretreatment, UV-induced graft copolymerization and quaternization reaction. The surface chemical compositions of the modified PDLLA fibrous membranes were analyzed by X-ray photo-electron spectroscopy (XPS). The antibacterial activities of the modified PDLLA fibrous membranes were evaluated against Gram-positive *Staphylococcus aureus* (*S. aureus*) and Gram-negative *Escherichia coli* (*E. coli*).

EXPERIMENTAL

Materials

Poly(D,L-lactide) (PDLLA) ($[\eta] = 2.68$ dL/g, 25°C) was kindly provided by Dikang Co., Ltd. 4-Vinylpyridine (4VP) monomer was purchased from Aldrich Chemical Co. and freshly distilled under reduced pressure before use. Hexylbromide and solvents, such as acetone, *N,N*-dimethyl formamide (DMF), heptanes and 2-propanol, were of reagent grade and used as received from Aldrich Chemical Co. *Staphylococcus aureus* (*S. aureus*) (25923) and *Escherichia coli* (*E. coli*) (DH5 α) were obtained from American Type Culture Collection (ATCC).

Electrospinning of PDLLA

PDLLA was dissolved in a mixed solvent of acetone and DMF (1:1, *V/V*) to produce various spinning solutions with PDLLA concentrations ranging from 60 g/L to 120 g/L. The prepared solutions were electrospun with field strength of 1 kV/cm and delivery rate of 6 mL/h. The as-spun PDLLA fibrous membrane was then dried under vacuum and annealed at 60°C for 10 h. All electrospinning experiments were carried out at 20°C and relative humidity of 50%.

Surface Modification of PDLLA Fibrous Membrane

PDLLA fibrous membrane was subjected to argon plasma pretreatment in an Anatech SP100 plasma system for 60 s on both surfaces and immersed in 2-propanol solution of 20% (*V/V*) 4VP in a Pyrex glass tube. The tube was then exposed to UV irradiation in a Riko rotary photochemical reactor (RH400-10W) for 60 min on each surface. The graft copolymerized fibrous membrane was rinsed with copious amounts of 2-propanol and then placed in heptane solution containing 20% (*V/V*) hexylbromide at 50°C for 48 h. The fibrous membranes were thoroughly rinsed with heptane and dried under vacuum.

Surface compositions of PDLLA fibrous membranes were analyzed by X-ray photoelectron spectroscopy (XPS) on an AXIS HSi spectrometer (Kratos Analytical Ltd.) using a monochromatized Al K α X-ray source. To compensate the surface charging effect, all binding energies (BEs) were referenced to the C 1s hydrocarbon

peak at 284.6 eV. In the peak synthesis, the line width (full width at half-maximum) of the Gaussian peaks was maintained constant for all components in a particular spectrum.

Determination of Antibacterial Activity

S. aureus was incubated in 10 mL of a 3.1% yeast-dextrose broth (containing 10 g/L peptone, 8 g/L beef extract, 5 g/L sodium chloride, 5 g/L glucose, and 3 g/L yeast extract at a pH 6.8) for 6–8 h at 37°C until the exponential growth phase was reached. The bacteria-containing broth was centrifuged at 3000 r/min for 10 min, and after removal of supernatant, the cells were washed twice with sterile phosphate-buffered solution (PBS). The bacteria cells were resuspended to provide a final density of 10^7 cells/mL in PBS (based on standard calibration with the assumption that the optical density of 1.0 at 540 nm is equivalent to approximately 10^9 cells/mL^[20]).

PDLLA fibrous membranes electrospun from 60, 80 and 100 g/L solutions in acetone and DMF (1:1, *V/V*) (either pristine or modified, 8 mm × 8 mm) were sterilized with ultraviolet irradiation for 30 min, and then immersed in 10 mL of the bacterial suspension in an Erlenmeyer flask and shaken at 200 r/min at 37°C. The viable cell counts of bacteria were measured by surface spread plate method. At the predetermined time, 1 mL of bacteria culture was taken from the flask and decimal serial dilutions with PBS were repeated with each initial sample. A 0.1 mL drop of the diluted sample was then spread onto solid growth agar plate. After incubation of the plate at 37°C for 24 h, the number of viable cells (colonies) was counted manually, and expressed as mean colony-forming units per millilitre (CFU/mL) after multiplication with the dilution factor. The membrane was fixed by 3% (*V/V*) glutaraldehyde in PBS, followed by step dehydration with 25%, 50%, 70%, 95%, and 100% ethanol for 10 min each. The membranes were then dried under vacuum and examined under SEM (JEOL JSM 5600LV) to assess the adhesion and viability of the bacteria.

For *E. coli*, the same assay procedures were used as those described above for *S. aureus*.

RESULTS AND DISCUSSION

Surface Modification of PDLLA Fibrous Membranes

The surface compositions of PDLLA fibrous membranes were analyzed using X-ray photoelectron spectroscopy (XPS). Figure 1 shows XPS wide scan, C 1s and N 1s core-level spectra of the pristine PDLLA fibrous membranes, membranes grafted with poly(4-vinylpyridine) (4VP) and quaternized at 50°C for 48 h, respectively. The spectra of pristine PDLLA fibrous membranes showed two main signals corresponding to C 1s (284.6 eV) and O 1s (532 eV) (Fig. 1a). No nitrogen component was detected as shown in Figs. 1(a) and 1(c), which was consistent with the absence of nitrogen in the PDLLA structure. The C 1s core-level spectrum of the pristine PDLLA fibrous membrane was resolved into three component peaks with BE at 284.6 eV for C—C/C—H species, at 286.6 eV for C—O species and at 288.9 eV for —CO— species as shown in Fig. 1(b)^[21]. The success of UV-induced surface graft copolymerization of 4VP on the PDLLA fibrous membranes was deduced from the wide scan (Fig. 1d) which showed the presence of N 1s peak at around 400 eV. The C 1s core-level spectrum (Fig. 1e) showed an additional peak at BE of 285.4 eV attributable to the C—N species. The intensity of the peaks assigned to C—O and —CO— species of PDLLA chains was much reduced compared with Fig. 1(b). The corresponding N 1s spectrum (Fig. 1f) showed an intense peak at BE of 398.5 eV attributable to the imine moiety (—N) of pyridine rings^[22]. Figures 1(g)–1(i) show the XPS spectra of the graft copolymerized PDLLA fibrous membrane *N*-alkylated with hexylbromide. No significant difference was observed in C 1s core-level spectrum after *N*-alkylation (Fig. 1h). The corresponding N 1s core-level spectrum in Fig. 1(i) showed an additional peak at BE above 400 eV, attributable to the N⁺ groups of the pyridinium ions^[23], which confirmed the derivatization of the —N= groups by hexylbromide.

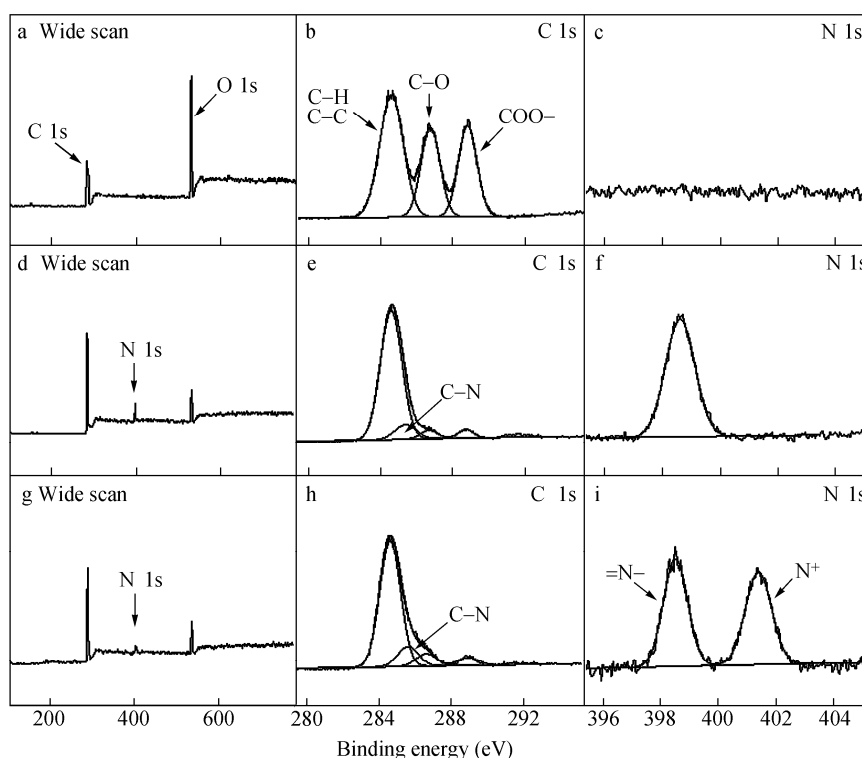


Fig. 1 XPS wide scans of (a) pristine PDLLA fibrous membrane, (d) PDLLA membrane grafted with poly(4-vinylpyridine) (PVP), and (g) PDLLA fibrous membrane modified with poly(4-vinyl-*N*-hexylpyridinium bromide); C 1s core-level spectra of (b) pristine PDLLA fibrous membrane, (e) PDLLA membrane grafted with PVP, and (h) PDLLA fibrous membrane modified with poly(4-vinyl-*N*-hexylpyridinium bromide); N 1s core-level spectra of (c) pristine PDLLA fibrous membrane, (f) PDLLA membrane grafted with PVP, and (i) PDLLA fibrous membrane modified with poly(4-vinyl-*N*-hexylpyridinium bromide)

PDLLA fibrous membranes were electrospun from 100 g/L solution in a mixed solvent of acetone and DMF (1:1, *V/V*).

The extent of surface grafting of poly(4-vinyl-*N*-hexyl pyridinium bromide) can be estimated from the sensitivity factor corrected ratio of the total N 1s peak over the total C 1s peak, expressed as $[N]/[C]$. As determined by XPS, the surface $[N]/[C]$ ratios of PDLLA fibrous membranes electrospun from 60, 80 and 100 g/L solutions were 0.057, 0.055, and 0.031, respectively. Besides, the degree of alkylation of the pyridine rings on these membranes surface, expressed as $[N^+]/[N]$, was 46.2%, 45.9%, and 45.0%, respectively. Apparently, the PDLLA fibrous membrane electrospun from 60 g/L solution was modified with more *N*-alkylated pyridinium groups. Previous work showed that concentration of electrospinning solution was the most effective variables for controlling the fiber morphology and lower concentration tended to fabricate fibers with smaller diameter^[24]. The electrospun fibrous membrane with smaller fiber diameter had a higher surface area-to-volume ratio, which could provide more reactive sites for graft copolymerization and quaternization. Furthermore, the PDLLA fibrous membrane modified with more *N*-alkylated pyridinium groups may provide higher antibacterial efficiency, as will be illustrated below.

Antibacterial Activity

Antibacterial efficiency of surface modified PDLLA fibrous membranes was evaluated by estimating the number of viable bacteria cells in the *S. aureus* and *E. coli* suspension after being in contact with membranes for various periods of time. Figure 2 shows the viable cell counts of *S. aureus* in contact with PDLLA fibrous membranes electrospun from 60, 80 and 100 g/L solutions. No significant decrease of viable bacteria was detected in the control experiment (without membrane). For pristine PDLLA fibrous membranes, the viable cell numbers in bacteria suspensions were similar to those for the control. The antibacterial efficiency of the PDLLA

fibrous membranes with poly(4-vinyl-*N*-alkylpyridinium bromide) grafted on the surface was significantly enhanced. As shown in Fig. 2, the viable *S. aureus* cell number in the suspension decreased by three and six orders of magnitude after 2 h and 4 h in contact with the modified PDLLA fibrous membranes, respectively. The surface modified PDLLA fibrous membranes electrospun from 60, 80 and 100 g/L solutions had high antibacterial efficiency for *S. aureus*, reaching 99.9998%, 99.9997% and 99.9994% after 4 h, respectively.

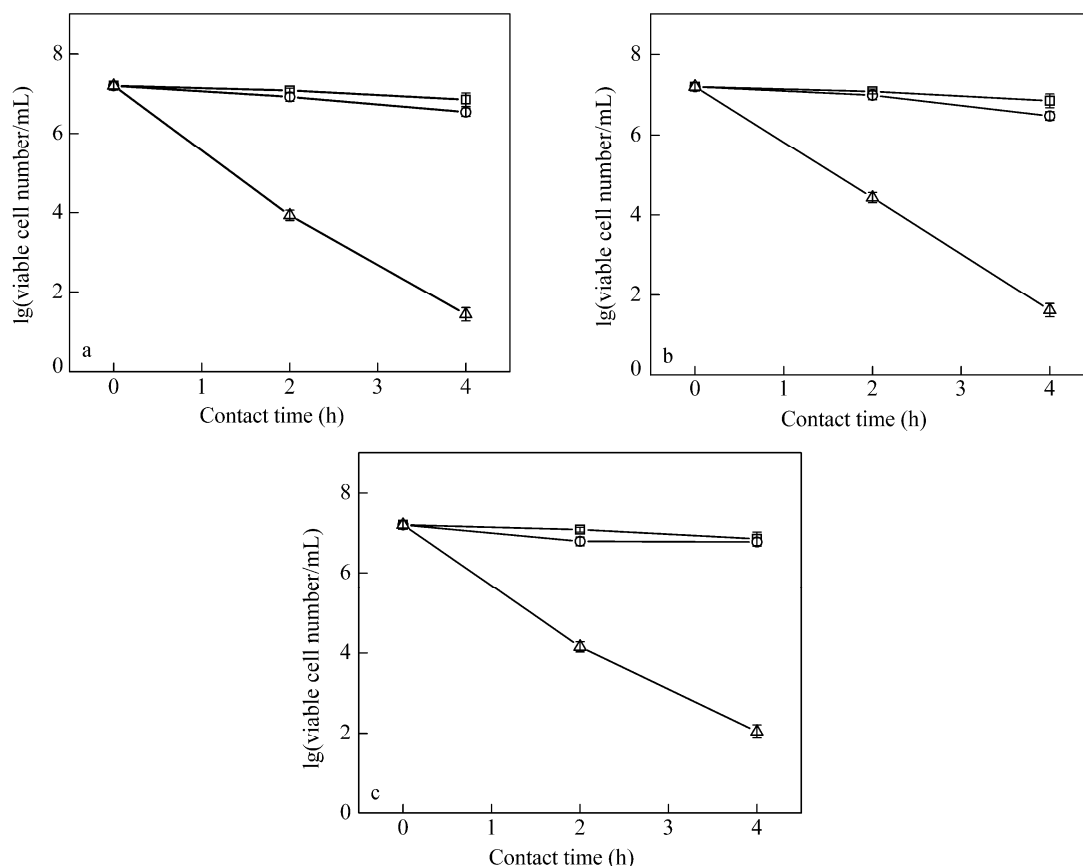


Fig. 2 Viable cell numbers of *S. aureus* as a function of time in contact with: (□) control, (○) pristine and (Δ) modified PDLLA fibrous membranes electrospun from (a) 60, (b) 80, and (c) 100 g/L solutions. The cell number was determined by surface spread plate method.

The antibacterial activity of the surface modified PDLLA fibrous membranes against *E. coli* (Fig. 3) was similar with that against *S. aureus*. For the modified PDLLA fibrous membranes electrospun from 60 and 80 g/L solutions, decreases in viable cell count were observed as shown in Figs. 3(a) and 3(b). The number of viable *E. coli* cell decreased to 40–50 cells/mL after 4 h in contact with modified membranes, which meant that approximately 99.9995% of the cells were no longer viable. In the case of the modified PDLLA fibrous membranes electrospun from 100 g/L solution, the antibacterial efficiency for *E. coli* was lower and the viable bacteria number in the suspension decreased by four orders of magnitude after 4 h.

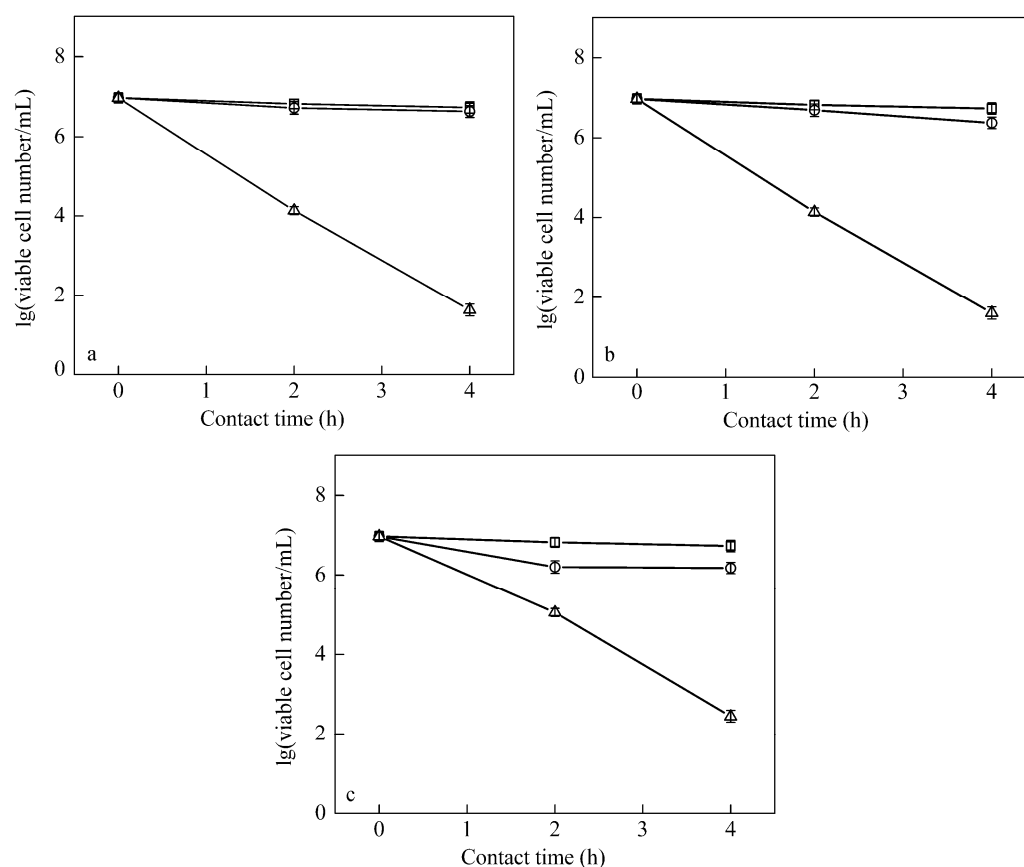


Fig. 3 Viable cell numbers of *E. coli* as a function of time in contact with: (□) control, (○) pristine and (Δ) modified PDLLA fibrous membranes electrospun from (a) 60, (b) 80, and (c) 100 g/L solutions. The cell number was determined by surface spread plate method.

Figure 4 shows the SEM images of the pristine and modified PDLLA fibrous membranes electrospun from 60, 80 and 100 g/L solutions after immersion in bacteria suspensions for 4 h. A few *S. aureus* cells can be observed on the pristine PDLLA fibrous membranes as shown in Figs. 4(a), 4(b) and 4(c). The bacteria cells were distributed not only on the upper surface of the pristine PDLLA fibrous membrane but also entrapped in the space between the thin fibers. So the viable cell numbers in bacteria suspensions were decreased after in contact with pristine PDLLA fibrous membranes which were not expected to have antibacterial activities (Figs. 2 and 3). In contrast, very few sparsely distributed bacteria cells could be spotted over the entire surface of the modified PDLLA fibrous membranes (Figs. 4d, 4e and 4f). Similar results were observed for *E. coli* on the surface of fibrous membranes as shown in Fig. 5. It is notable that the fragments of dead bacteria cells could be observed on the surface of the modified PDLLA fibrous membrane electrospun from 100 g/L solution as shown in Fig. 5(f). This may be ascribed to the positive charge of the pyridinium group which attracts the negatively charged cell membrane of bacteria. The cell membrane loses its electrical balance, and eventually the bacteria “explode” under their own osmotic pressure, similar to a bursting soap bubble^[25]. Since the PDLLA fibrous membrane electrospun from 100 g/L solution was modified with less antibacterial *N*-alkylated pyridinium groups than that from 60 and 80 g/L solution, the bacteria may adhere either on fibers with lower concentration of the *N*-alkyl pyridinium groups or on dead bacteria deposited on the fibers which provided a shielding effect, resulting in lower antibacterial efficiency.

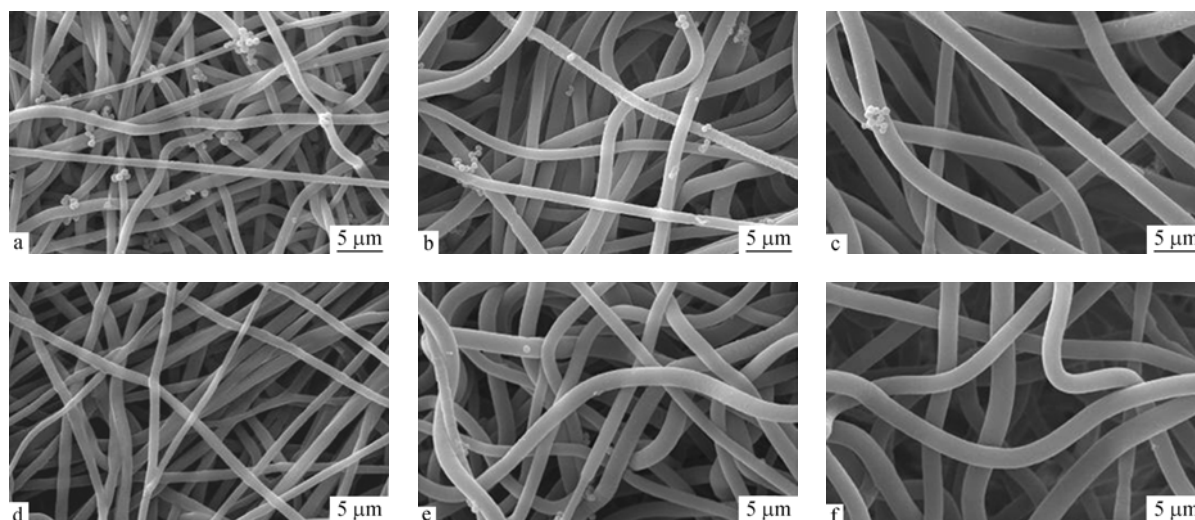


Fig. 4 SEM images of (a) (b) (c) pristine and (d) (e) (f) modified PDLLA fibrous membranes after immersed in PBS suspension of *S. aureus* at 10^7 cells/mL for 4 h

PDLLA fibrous membranes were electrospun from (a) (d) 60, (b) (e) 80, and (c) (f) 100 g/L solutions; Magnification: 3000

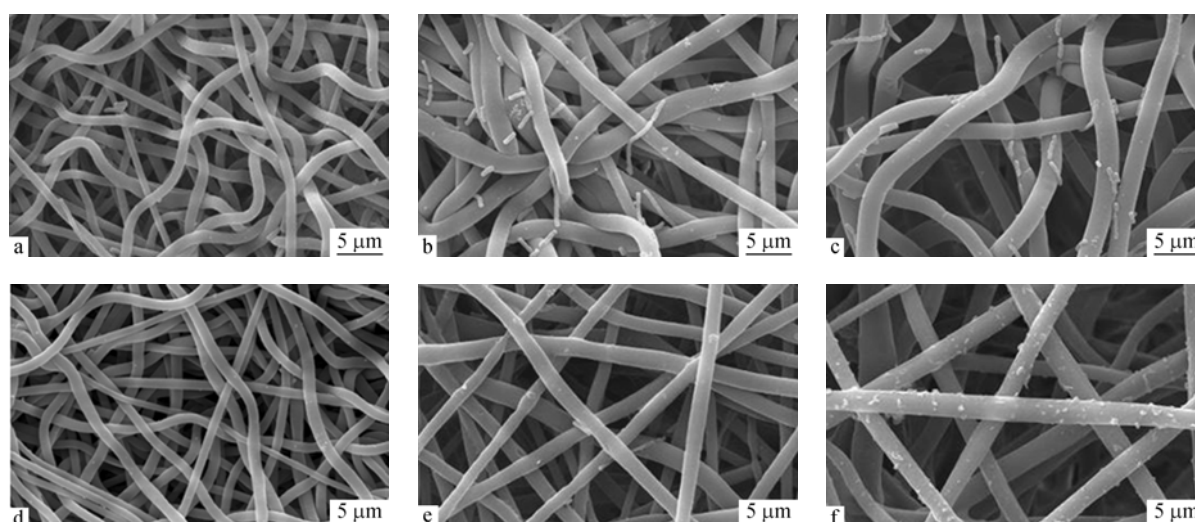


Fig. 5 SEM images of (a) (b) (c) pristine and (d) (e) (f) modified PDLLA fibrous membranes after immersed in PBS suspension of *E. coli* at 10^7 cells/mL for 4 h

PDLLA fibrous membranes were electrospun from (a) (d) 60, (b) (e) 80, and (c) (f) 100 g/L solutions; Magnification: 3000

CONCLUSIONS

The surface of electrospun PDLLA fibrous membranes was successfully modified with poly(4-vinyl-*N*-hexyl pyridinium bromide) using a method involving plasma pretreatment, UV-induced surface graft copolymerization and quaternization. PDLLA fibrous membranes electrospun from 60 g/L solution were modified with more *N*-alkylated pyridinium groups. Antibacterial assays showed that the modified PDLLA fibrous membranes possessed highly effective antibacterial activities against both Gram-positive *S. aureus* and Gram-negative *E. coli*. Very few sparsely distributed bacteria cells could be spotted over the entire surface of the modified fibrous membrane. The antibacterial PDLLA fibrous membranes may have a wide variety of potential applications in biomedical areas.

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