A simple approach to constructing antibacterial and anti-biofouling nanofibrous membranes

Yan Mei, Chen Yao and Xinsong Li*

School of Chemistry and Chemical Engineering, Southeast University, Nanjing, PR China

(Received 3 September 2013; accepted 29 November 2013)

In this work, antibacterial and anti-adhesive polymeric thin films were constructed on polyacrylonitrile (PAN) nanofibrous membranes in order to extend their applications. Polyhexamethylene guanidine hydrochloride (PHGH) as an antibacterial agent and heparin (HP) as an anti-adhesive agent have been successfully coated onto the membranes via a layer-by-layer (LBL) assembly technique confirmed by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), energy-dispersive spectroscopy (EDS) and scanning electron microscopy (SEM). The antibacterial properties of LBL-functionalized PAN nanofibrous membranes were evaluated using the Gram-positive bacterium Staphylococcus aureus and the Gram-negative Escherichia coli. Furthermore, the dependence of the antibacterial activity and anti-biofouling performance on the number of layers in the LBL films was investigated quantitatively. It was found that these LBL-modified nanofibrous membranes possessed high antibacterial activities, easy-cleaning properties and stability under physiological conditions, thus qualifying them as candidates for anti-biofouling coatings.

Keywords: anti-biofouling; layer-by-layer; nanofibrous membranes; polyhexamethylene guanidine hydrochloride; heparin

Introduction

In recent years, polyacrylonitrile (PAN) nanofibrous membranes have been extensively investigated in the area of filtration (Zhang et al. 2010; Wang et al. 2012; Mei et al. 2013), wound dressing (Zhang et al. 2011) and vascular grafts, due to their striking properties including excellent permeability, high porosity and good stability (Nataraj et al. 2012; Troung et al. 2012). However, the absence of active functional groups on nanofibrous membranes means that they are unable to prevent the propagation of blocked microorganisms. This would result in a severe decline in their permeability and life span over a period of operation. Consequently, there is an urgent need to develop nanofibrous membranes that are capable of providing powerful anti-biofouling performance.

Strategies to prevent the cloning of microorganisms on the surfaces include the use of antimicrobial or anti-fouling coatings (Banerjee et al. 2011; Charnley et al. 2011; Neoh & Kang 2011; Busscher et al. 2012). The active approach is to kill bacteria with antimicrobial material, eg quaternary ammonium compounds (Fu et al. 2008; Yao et al. 2008; Asri et al. 2014), guanidine compounds (Chen et al. 2008), N-halamine (Ren et al. 2009), antibiotics (Aumuswan et al. 2007; Aumuswan et al. 2009; Guillame et al. 2012), antimicrobial peptides (Shukla et al. 2010; Nakamura et al. 2011; Kazemzadeh-Narbat et al. 2013), silver (Martinez-Gutierrez et al. 2013) and other metal ions (Gosau et al. 2010; Hwang et al. 2011; Xu et al. 2012). The passive approach is to resist bacteria by using antifouling (AF) coatings, such as polyethylene glycol (PEG), hyperbranched polyglycerol (HPG) (Weber et al. 2012), neutral hydrophilic brushes (Hook et al. 2012; Yang, Neoh et al. 2012), zwitterionic brushes (Jiang & Cao 2010; Yang et al. 2010; Smith et al. 2012) and DNase I (Swartjes et al. 2013). It is more desirable to combine the two approaches and use AF and antimicrobial substances simultaneously (Li, Chen et al. 2011; Lalani & Liu 2012; Peyre et al. 2012) to develop an effective coating, ie an ideal surface is able to kill attached bacteria through antibacterial materials initially and then release dead microbes via AF agents (Gao, Lange et al. 2011; Gao Yu et al. 2011; Ding et al. 2012; Liu et al. 2012; Mi & Jiang 2012).

Recently, anti-biofouling polyacrylonitrile (PAN) nanofibrous membranes containing both hydrophilic spacer and antibacterial agent were developed (Mei et al. 2012). Although these nanofibrous membranes have antibacterial and AF capabilities, the modification process cannot immobilize high concentrations of antibacterial groups because each grafted hydrophilic chain has only one functional end group (Li, Poon et al. 2011; Yu et al. 2011). The layer-by-layer (LBL) assembly technique is a simple and powerful method used for generating multifunctional surfaces (Nepal et al. 2008; Wong et al. 2010; Cerkez et al. 2011; Tang et al. 2012; Zhou et al. 2012). Multilayer thin films can be simply created by consecutive alternate deposition of positively and negatively charged species.

*Corresponding author. Email: lixs@seu.edu.cn

© 2014 Taylor & Francis
Heparin (HP) is a biocompatible polyanion and has many applications. The highly negative charge density of HP prevents the adhesion of bacterial cells and, therefore, this biopolymer is an excellent candidate to act as an anti-adhesive coating (Liu et al. 2010; Croes et al. 2011; Stevens et al. 2011). Polyhexamethylene guanidine hydrochloride (PHGH), a positively charged polymer, has broad-spectrum antimicrobial activities toward Gram-positive and Gram-negative bacteria, fungi and yeast (Guan et al. 2007; Qian et al. 2008; Sun et al. 2010). The excellent antimicrobial properties, low cost and low toxicity to human cells have enabled PHGH to be used extensively in diverse areas.

In the present report, a layer-by-layer assembly technique was used to develop antibacterial and easy-cleaning polyacrylonitrile nanofibrous membranes by combining the fouling-release property of heparin with the antibacterial property of PHGH. Some research groups have reported anti-adhesive and antibacterial flat surfaces based on the LBL technique (Fu et al. 2005; Vreuls et al. 2010; Follmann et al. 2012; Yang, Pranantyo et al. 2012). Unlike other flat templates, the deposition of functional materials on nanofibrous surfaces should be much higher due to their high specific surface area (Deng et al. 2011). After converting nitrile groups to negatively charged carboxylate ions on a PAN nanofibrous membrane, (PHGH/HP)-bilayer coatings built by LBL were deposited onto nanofibrous membranes and shown to be both anti-adhesive and antibacterial. It is worth highlighting that these novel nanofibrous membranes obtained by LBL techniques show potential in water filtration and biomedical devices.

Experimental
Materials
The polyacrylonitrile (PAN, Mw=150,000 g mol$^{-1}$) powder was obtained from Jiangsu Haide Group (Nanjing, Jiangsu, China). Polyhexamethylene guanidine hydrochloride (PHGH, Mn=800 g mol$^{-1}$) was supplied from Xianyang Huashenghuanneng Biological and Chemical (Xianyang, Shanxi, China). Heparin (HP, sodium salt) was purchased from Sigma-Aldrich (Shanghai, China). N,N-dimethyl formamide (DMF) was of reagent grade and received from Sinopharm Chemical Reagent (Shanghai, China). The Gram-positive bacterium Staphylococcus aureus ATCC 25,923, and the Gram-negative bacterium Escherichia coli ATCC DH5 with no known resistances, were obtained from Jiangsu Provincial Center for Disease Control and Prevention of China (Nanjing, Jiangsu, China).

Preparation of nanofibrous membrane
PAN powder was dissolved in DMF to prepare electrospinning solution at concentration of 11% (w v$^{-1}$, g ml$^{-1}$). The polymer solution was transferred into a 20 ml plastic syringe and electrospun through a needle with a tip diameter of 0.6 mm at a delivery rate of 4 ml h$^{-1}$ for 2 h. After high voltage ranging from 13 to 17 kV was applied to the needle, a positively charged jet of PAN solution formed from the Taylor cone and sprayed to a grounded drum, ~15 cm from the needle tip. The electrospun nanofibers were collected on a metal drum covered with non-stick aluminum foil. Finally, the as-spun nanofibrous membranes were then dried under a vacuum and annealed at 80 °C for 4 h. The electrospinning experiments were carried out at 20 °C and a relative humidity of 50%.

Fabrication of LBL-functionalized nanofibrous membranes
The PAN nanofibrous membranes were first hydrolyzed at 50 °C in 20% NaOH solution for 3 h (Liu & Hsieh 2006). Hydrolysis was terminated by rinsing the nanofibrous membranes in distilled water until the rinse became neutral. Hydrolyzed membranes (PAN-COO$^-$) were dried at 80 °C in vacuum oven for 4 h.

Stock solutions of PHGH and HP having a final concentrations of 1 mg ml$^{-1}$ were prepared in distilled water and adjusted to pH 4.0 with 1 M HCl or 1 M NaOH. In brief, multilayer thin films were formed by exposing the nanofibrous membranes to polyelectrolyte solutions, alternating between the PHGH and HP solutions, with 2-min distilled water rinses. Specifically, the PAN-COO$^-$ nanofibrous membranes, with negatively charged surfaces, were first immersed for 20 min in the prepared PHGH solution. Then, the membranes obtained were washed in distilled water for 2 min. This step aims to remove the PHGH weakly adsorbed on the PAN-COO$^-$ surfaces. After this process, the nanofibrous membranes PAN-COO$^-$-(PHGH/HP)$_{0.5}$ with PHGH layers absorbed on PAN-COO$^-$ surfaces were immersed for 20 min in the prepared HP solution and followed by washing, and PAN-COO$^-$-(PHGH/HP)$_{1}$ nanofibrous membranes were obtained. Repeating this cycle, multilayer thin films of PHGH/HP with 5, 5.5, 10, and 10.5 bilayers were prepared.

Scanning electron microscopy
The morphology of the nanofibrous membrane was investigated by using a scanning electronic microscope (SEM, JEOL JSM-6,360, Tokyo, Japan) after gold sputter coating. The images were taken by applying an electron accelerating voltage of 10 kV. The average diameters of the electrospun nanofibers were measured directly from SEM images, with an average value calculated from at least 100 measurements. A SEM equipped with an energy-dispersive spectroscopy X-ray (EDX)
spectrometer (EDAX Genesis-60S) was used to map the chemical composition of the nanofibrous membranes.

**Attenuated total reflectance Fourier transform infrared spectroscopy**

The chemical composition of the original and modified nanofibrous membranes was further characterized by attenuated total reflectance Fourier transform infrared spectroscopy (ATR/FT-IR) using a Nicolet 5700 spectrometer (Thermo, Madison, WI, USA) with an internal reflection accessory ZnSe crystal at an angle of 45°. All spectra were corrected for CO₂ using IR solution internal software.

**Porosity**

The porosity of the samples was measured by the following method: nanofibrous membranes were cut into squares with a length of 30 mm, followed by measurement of the thickness determined from the combination of SEM cross-sectional images and a micrometer. The porosity of the nanofibrous membranes is calculated by the following Equation:

\[
\varepsilon = 1 - \frac{W}{\rho \times Z}
\]

where \( W \) is the basic weight of the nanofibrous membrane, \( \rho \) is the density of PAN with a value of 1.184 g cm\(^{-3}\), and \( Z \) is the thickness of the sample.

**Determination of antibacterial properties**

The Gram-positive bacterium *S. aureus* and the Gram-negative *E. coli* were used to evaluate the bactericidal efficacy of the multilayer coatings. As described previously (Mei et al. 2012), *S. aureus* and *E. coli* were incubated in a nutrient-rich medium at 37°C until the exponential growth phase was reached. The bacteria-containing culture was centrifuged at 3,000 rpm for 10 min, and then the supernatant was removed. Bacterial cells were washed twice with sterile phosphate buffered solution (PBS, pH 7.4) and resuspended to provide a final density of \(10^9\) cells ml\(^{-1}\). The nanofibrous membranes (1 cm\(^2\)) were first washed with 75% ethanol to kill any live bacteria on their surfaces, and then immersed in 5 ml of the bacterial suspension in an Erlenmeyer flask and shaken at 200 rpm at 37°C. The viable cell counts of bacteria were measured by the surface spread plate method. At the predetermined time, 0.2 ml of bacterial culture was taken from the flask and decimal serial dilutions with PBS were repeated with each initial sample. A 0.1 ml diluent of the sample was then spread onto solid growth agar plates. After incubation of the plates at 37°C for 24 h, the number of viable cells (colonies) was counted manually, and expressed as mean colony-forming units per milliliter (CFU ml\(^{-1}\)) after multiplication by the dilution factor.

**Determination of anti-biofouling properties**

Two samples (1 cm\(^2\)) of each pristine and LBL-deposited nanofibrous membranes were placed in a 24-well cell culture plate. Then, 1.0 ml of *S. aureus* cells at a concentration of \(10^9\) cells ml\(^{-1}\) was added to each well and incubated under static condition at 37°C. After 24 h, the bacterial suspension was removed and the nanofibrous membranes were washed twice with PBS. One sample was used to fix the *S. aureus* cells. Adhered bacterial cells were investigated under a SEM after fixation and dehydration. The process was as follows: 3% glutaraldehyde in PBS was added for 5 h stored at 4°C. The glutaraldehyde solution was then removed and the nanofibrous membranes were washed with PBS, followed by step dehydration with 25, 50, 70, 95, and 100% ethanol for 10 min each. The membranes were dried under vacuum and gold sputter-coated before SEM observation. Another sample was used to quantify the viable cells on the nanofibrous membranes via ultrasonic processing to detach the viable cells from the surface.

**Determination of easy-cleaning properties**

Evaluation of easy-cleaning properties on these PAN nanofibrous membranes (both pristine and modified) was performed by direct SEM observation. Briefly, the membranes were first incubated in 1.0 ml suspension of *S. aureus* (\(10^9\) cells ml\(^{-1}\)) for 24 h. Then, the membranes were washed in PBS by agitation with a stirring speed of 200 rpm for 10 min to detach adhered bacterial cells. Finally, the adhered bacterial cells were investigated under a SEM after fixing with 3% glutaraldehyde solution for 5 h and dehydration with serial ethanol as described previously.

**Stability of multilayered nanofibrous membrane**

The stability of the nanofibrous membrane PAN-COO⁻(PHGH/HP)\(_{10.5}\) was evaluated by subjecting samples to the following usage conditions: (1) 2 weeks in pure water at room temperature; and (2) 2 weeks in 0.1 M PBS at 37°C. An EDX spectrometer was used to measure the S element of the nanofibrous membranes before and after each challenge.

**Results and discussion**

**Layer-by-layer assembly of PHGH and HP on PAN nanofibrous membranes**

The LBL-functionalized process of PAN nanofibrous membranes is schematically summarized in Figure 1.
After hydrolyzing the PAN nanofibrous membranes, overlapping layers of PHGH and HP having alternating positive and negative charges were assembled layer-by-layer to create multilayer coatings of PHGH/HP on substrata. The LBL-modified nanofibrous membranes were further characterized by ATR/FT-IR, EDX and SEM as follows.

**ATR/FT-IR spectra**

The conversion of nitrile groups on the surface of PAN nanofibrous membranes to carboxylate anions after alkaline hydrolysis was confirmed by ATR/FT-IR (the spectra of PAN and PAN-COO \(^{-}\) nanofibrous membranes are cited in supporting information, Figure S1; Supplementary material is available via a multimedia link on the online article webpage.). The peak associated with the nitrile groups of the PAN-COO \(^{-}\) nanofibrous membrane centered at 2,244 cm\(^{-1}\) was decreased significantly in its intensity compared to the original PAN nanofibrous membrane. Additionally, new adsorption bands at 1,407 cm\(^{-1}\), 1,565 cm\(^{-1}\), 1,658 cm\(^{-1}\) and 3,396 cm\(^{-1}\) in the spectrum of the PAN-COO \(^{-}\) nanofibrous membranes were attributed to native carboxylate anions (COO\(^{-}\)) and carboxylate groups (COOH). After repeating layer-by-layer assembly of PHGH and HP, the modified PAN nanofibrous membranes were evaluated by ATR/FT-IR as illustrated in Figure 2. The peaks at 1,030 cm\(^{-1}\) and 1,226 cm\(^{-1}\) due to C-O and S-O groups indicate the presence of HP after the LBL assembly process. In addition, the C=N stretching vibration peak at 1,633 cm\(^{-1}\) of the deposited PHGH was also observed in all groups, thus confirming the modification process illustrated in Figure 1.

**EDX spectra**

EDX spectra were recorded to further verify the surface composition of the modified PAN nanofibrous membranes. As shown in Supplemental Figure S2, the characteristic peak of the S element in the spectra was identified. Apparently, HP containing the S element was successfully deposited on the PAN nanofibrous membranes. Table 1 shows the surface element composition (wt.%) of the LBL-functionalized nanofibrous membranes obtained from the EDX results. The S peak in the EDX spectra increased from 1.1 wt.% to 2.2 wt.% as the number of bilayers increased from 5 to 10, indicating that the build-up of bilayers onto PAN nanofibrous membranes was linear. In addition, after introduction of a PHGH layer (from 5 bilayers to 5.5 bilayers or from 10 bilayers to 10.5 bilayers), the weight percentage of N increased while the S and O concentrations was slightly lowered. These changes are to be expected on the basis of the composition of the PHGH and HP.

**SEM images**

The morphology of the pristine and LBL-functionalized PAN nanofibrous membranes was observed by SEM images. In order to investigate the effects of the number of coating bilayers and the composition of the outermost layer on the formation LBL-coated nanofibrous membranes, four LBL structured PHGH/HP films were
deposited onto nanofibrous membranes. As indicated in Figure 3, the four structured nanofibers showed a larger diameter, bigger junctions together with more bundles compared to PAN and PAN-COO\textsuperscript{−} nanofibrous membranes. Additionally, the surface of LBL-modified nanofibrous membranes was much rougher than that of the original membrane, which could be attributed to the inhomogenous deposition of LBL films. Different from other flat templates, the deposition space among the adjacent nanofibers in membranes is limited and different from each other, which could result in the imbalance in the deposition driven force among the nanofibers.

As shown in Table 2, the PAN nanofibrous membrane contained loosely packed nanofibers with an average diameter of 670 nm. After alkaline hydrolysis, the average fiber diameter (672 nm) was similar to that in the original PAN nanofibrous membrane. These data indicated that successful hydrolysis of the PAN surface had occurred, with little damage of structural integrity during soaking in NaOH for 3 h. The layer-by-layer

| Sample                      | C (wt.%) | N (wt.%) | O (wt.%) | Na (wt.%) | S (wt.%) |
|-----------------------------|----------|----------|----------|-----------|----------|
| PAN                         | 82.7     | 13.0     | 3.9      | 0.2       | 0.2      |
| PAN-COO\textsuperscript{−}  | 81.8     | 11.2     | 5.4      | 1.4       | 0.2      |
| PAN-COO\textsuperscript{−}(PHGH/HP)\textsubscript{5} | 81.7     | 10.7     | 5.8      | 0.7       | 1.1      |
| PAN-COO\textsuperscript{−}(PHGH/HP)\textsubscript{5.5} | 81.6     | 11.5     | 5.8      | 0.2       | 0.9      |
| PAN-COO\textsuperscript{−}(PHGH/HP)\textsubscript{10} | 80.6     | 9.4      | 7.6      | 0.2       | 2.2      |
| PAN-COO\textsuperscript{−}(PHGH/HP)\textsubscript{10.5} | 80.3     | 10.8     | 6.7      | 0.3       | 1.9      |

Table 2. The physical characteristics of pristine and LBL-deposited nanofibrous membranes.

| Sample                      | Average diameter\textsuperscript{a} | SD  | Film thickness\textsuperscript{b} | Bilayer thickness |
|-----------------------------|--------------------------------------|-----|-----------------------------------|------------------|
| PAN                         | 670 nm                               | 85 nm |                                   |                  |
| PAN-COO\textsuperscript{−}  | 672 nm                               | 89 nm |                                   |                  |
| PAN-COO\textsuperscript{−}(PHGH/HP)\textsubscript{5} | 750 nm | 169 nm | 78 nm                             | 16 nm            |
| PAN-COO\textsuperscript{−}(PHGH/HP)\textsubscript{5.5} | 768 nm | 135 nm | 96 nm                             | 17 nm            |
| PAN-COO\textsuperscript{−}(PHGH/HP)\textsubscript{10} | 847 nm | 217 nm | 175 nm                            | 18 nm            |
| PAN-COO\textsuperscript{−}(PHGH/HP)\textsubscript{10.5} | 853 nm | 181 nm | 181 nm                            | 17 nm            |

\textsuperscript{a}determined by SEM; \textsuperscript{b}determined by average diameter.
coating process successfully placed PHGH/HP coatings onto nanofibrous membranes, leading to the increased diameter of the nanofibers. There was no obvious difference in diameter between 5 (750 nm) and 5.5 (768 nm) bilayer deposited membranes, as well as 10 (847 nm) and 10.5 (853 nm) bilayer deposited membranes. However, doubling the number of bilayers showed thicker deposition. Thus, it can be concluded that the number of coating bilayers plays a pivotal role in the formation of LBL-functionalized nanofibrous membranes.

**Porosity**

To determine whether the LBL-modified nanofibrous membranes possessed properties that could make them attractive as water filters or porous devices, an important index, the porosity of the nanofibrous membranes, was evaluated. It was found that the membrane porosity decreased slightly after modification, from 86% for pristine PAN nanofibrous membranes to 72–74% for LBL-deposited PAN nanofibrous membranes (for more details, see Figure S3). The decrease in the porosity as a result of deposited PHGH/HP bilayers partially occupied the pore space in the pristine nanofibrous membranes. Even though the porosity was slightly reduced, the porosity of the membranes after modification was still in the typical range for electrospun nanofibers.

**Properties of LBL-functionalized PAN nanofibrous membranes**

**Antibacterial activity**

As a first approach to investigating the antibacterial activities of PHGH/HP deposited nanofibrous membranes, the ability of the LBL-functionalized nanofibrous membranes to kill *S. aureus* cells was initially analyzed by surface spread plate method as revealed in Figure 4. It was found that no obvious loss of viable bacterial cells was detected in the blank control of PAN nanofibrous membranes (Figure 4a), indicating the original PAN surface was not capable of killing bacteria. For the nanofibrous membrane coated with (PHGH/HP)₅, the reduction rate was 99.665% and 99.960% after 0.5 h and 1 h exposure. When 10 bilayers of (PHGH/HP) were assembled on the surface, the retarding rate of bacterial growth reached 99.928% and 99.992% after 0.5 h and 1 h contact time. Overall, 99.999% of *S. aureus* cells were killed within 2 h contact time when the PHGH/HP bilayers were deposited on nanofibrous membranes, whereas those membranes with higher bilayers of PHGH/HP exhibited higher antibacterial activities.

The antibacterial activities of nanofibrous membranes consisting either of 10 or 10.5 bilayers, ie ending either with HP or PHGH, were further investigated. As shown in Figure 4a, the PAN-COO-(PHGH/HP)₁₀ nanofibrous membranes terminated with PHGH killed all *S. aureus* cells within 0.5 h. In the case of HP-ending nanofibrous membranes (10 bilayers), all bacteria were killed within 2 h, three times longer than with PHGH-ending nanofibrous membranes. Therefore, nanofibrous membranes ending with PHGH appear to be more effective at killing bacteria, thus indicating that the outer layer of the nanofibrous membrane plays a dominant role on the antibacterial activity of the membrane. Importantly, although the HP-ending nanofibrous membranes had lower antibacterial activities, they still approached the bacteria and had a killing effect on them. It is assumed that the outer layer of HP-ending nanofibrous membranes may also
contain some positively charged antibacterial agent – PHGH, as reported in the literature (Cui et al. 2010).

The antibacterial activities of the LBL-modified nanofibrous membranes against E. coli (Figure 4b) were similar to that against S. aureus. When 10 bilayers of PHGH/HP were assembled on the surface, the retarding rate of bacterial growth reached 92.836% and 99.383% after 0.5 h and 1 h contact time, lower than that for S. aureus.

Anti-biofouling properties

The anti-biofouling efficacy of the multilayer coatings was evaluated against the Gram-positive bacterium, S. aureus. Figure 5 (and Figure S4) shows the SEM images of a nanofibrous membrane after one anti-biofouling assay. As seen from Figure 5a, the pristine PAN nanofibrous membrane was highly susceptible to bacterial adhesion and growth. A large number of bacterial cells adhered readily on the surface and maintained their characteristic morphology. For the PAN-COO-(PHGH/HP)_{10.5} nanofibrous membranes (Figure 5b), a certain reduction in bacterial adhesion was observed compared with that of the original PAN nanofibrous membrane, indicating the AF properties of the denser polymer multilayer films. Meanwhile, the bacterial bodies on LBL-modified nanofibrous membranes were lysed into irregular shaped particles, which suggested the cells attached to the surfaces were dead. Hence, the multilayer films deposited on the nanofibrous membrane exhibited antibacterial properties.

Additionally, quantitative determination of the number of viable S. aureus cells on the LBL-functionalized surfaces was conducted. As shown in Figure 6, viable bacterial cells in all the LBL-functionalized nanofibrous membranes was greatly reduced by 98% compared with that of pristine PAN nanofibrous membrane, while PAN-COO-(PHGH/HP)_{10.5} surface exhibited the highest anti-biofouling efficacy – 99.62%. Apparently, the formation of thicker and denser LBL layers helps to account for higher anti-biofouling abilities. These results illustrate the superior anti-biofouling properties of the LBL modified nanofibrous membranes.

Easy-cleaning properties

Usually, a major problem with antibacterial surfaces is the adhesion of dead bacterial cells on the antibacterial surfaces, which can block the functional antibacterial groups. In addition, these dead cells would decrease permeate flux of the surface. It is desirable to have a coating with a fouling-release property to prolong the lifetime of the antibacterial material.

Figure 5. SEM images of (a) PAN, and (b) PAN-COO-(PHGH/HP)_{10.5} nanofibrous membranes treated with 1 ml of S. aureus suspension at 10^9 cells ml^{-1} for 24 h.

Figure 6. Number of viable adherent bacterial cells on (a) PAN, (b) PAN-COO-(PHGH/HP)_{5}, (c) PAN-COO-(PHGH/HP)_{5.5}, (d) PAN-COO-(PHGH/HP)_{10}, and e) PAN-COO-(PHGH/HP)_{10.5} nanofibrous membranes after exposure to S. aureus suspension at 10^9 cells ml^{-1} for 24 h. The cell number was determined by the spread plate method. Each error bar represents the SD calculated from three replicates.
LBL-functionalized nanofibrous membranes containing PHGH have proved to be able to efficiently kill bacteria. In order to evaluate the easy-cleaning properties of nanofibrous membranes, both pristine and the modified membranes were incubated in a bacterial suspension (10⁹ cells ml⁻¹) of *S. aureus* for 24 h. After the cleaning process, the bacteria remaining on these membranes were also observed by SEM. Figure 7 shows, before the cleaning process, a large number of viable bacteria were attached to the original PAN nanofibrous membranes, while a certain number of dead bacteria were adhered on the LBL-functionalized nanofibrous membranes.

Table 3. Remaining S elements on PAN-COO–(PHGH/HP)₁₀·₅ nanofibrous membranes after durability tests.

| Sample                                      | S (wt.%)|
|---------------------------------------------|---------|
| PAN-COO–(PHGH/HP)₁₀·₅                      | 1.9     |
| After 2 weeks immersion in pure water       | 1.8     |
| After 2 weeks immersion in PBS              | 1.2     |

Figure 7. SEM images of (a) PAN, and (b) PAN-COO–(PHGH/HP)₁₀·₅ nanofibrous membranes treated with 1 ml of *S. aureus* suspension at 10⁹ cells ml⁻¹ for 24 h followed by cleaning.

LBL-functionalized nanofibrous membranes containing PHGH have proved to be able to efficiently kill bacteria. In order to evaluate the easy-cleaning properties of nanofibrous membranes, both pristine and the modified membranes were incubated in a bacterial suspension (10⁹ cells ml⁻¹) of *S. aureus* for 24 h. After the cleaning process, the bacteria remaining on these membranes were also observed by SEM. Figure 5 shows, before the cleaning process, a large number of viable bacteria were attached to the original PAN nanofibrous membranes, while a certain number of dead bacteria were adhered on the LBL-functionalized nanofibrous membranes. Figure 7 (and Figure S5) shows *S. aureus* cells remaining on nanofibrous membranes after the cleaning process. Large numbers of live *S. aureus* cells on the original PAN nanofibrous membranes are observed in Figure 7a. In contrast, as shown in Figure 7b, no attached dead cells were left on the PAN-COO–(PHGH/HP)₁₀·₅ nanofibrous membranes. It appears that LBL-deposited surfaces released dead cells completely, while no release of the cells was observed on original PAN surfaces. It is supposed that the release of the attached dead bacteria is due to the presence of HP layers. Compared with the grafting method (Cringus-Fundeanu et al. 2007; Cao et al. 2013), layer-by-layer assembly of PHGH/HP is a simple and effective approach for developing easy-cleaning surfaces.

**Stability test**

The stability of the multifunctional nanofibrous membranes was investigated by measuring the change in the S element content before and after each challenge. As shown in Table 3 (and Figure S6), the nanofibrous membrane PAN-COO–(PHGH/HP)₁₀·₅ lost only 5% of the coated heparin after immersion in pure water at room temperature for 2 weeks. In PBS solution, the multifunctional nanofibrous membrane was successfully stored for at least 2 weeks at 37 °C, and retained 63% of the coated heparin after immersion. The results suggest that these novel antibacterial and easy-cleaning coatings via the layer-by-layer assembly exhibited good stability towards salts, which is similar to a previous study (Follmann et al. 2012).

**Conclusions**

Layer-by-layer assembly of PHGH/HP was explored to construct antibacterial and easy-cleaning multilayer films based on PAN nanofibrous membranes. The ATR/FT-IR and EDX results verified the progressive build-up of the multilayer film by alternate deposition of the polyelectrolytes. The novel modified nanofibrous membranes not only killed bacteria effectively but also released the dead cells through the cleaning process, thus making them candidates for anti-biofouling surfaces. The ability of the LBL-deposited nanofibrous membranes investigated herein to resist biofilm formation suggests their potential use as anti-biofouling materials ranging from water filters to biomedical devices. In addition, the easy-to-handle LBL technique provides the versatility to generate multifunctional surfaces for other applications.

**Acknowledgments**

This work was supported by the National Natural Science Foundation of China under Grants 51073036, 51373034; and the Industry and University Collaboration Foundation of Jiangsu under Grant BY2010143.
References

Asri LATW, Crismaru M, Roest S, Chen Y, Rudolf OIP, Tiller JC, Van der Mei HC, Loontjens TJA. 2014. A shape-adaptive, antibacterial-coating of immobilized quaternary-ammonium compounds tethered on hyperbranched polyurea and its mechanism of action. Adv Funct Mater. 24:346–355.

Aumsuwan N, Heinhorst S, Urban MW. 2007. The effectiveness of antibiotic activity of penicillin attached to expanded poly(tetrafluoroethylene) (ePTFE) surfaces: a quantitative assessment. Biomacromolecules. 8:3525–3530.

Aumsuwan N, McConnell MS, Urban MW. 2009. Tunable antimicrobial polypropylene surfaces: simultaneous attachment of penicillin (\textit{Gram}+) and gentamicin (\textit{Gram}-). Biomacromolecules. 10:623–629.

Banerjee I, Pangule RC, Kane RS. 2011. Antifouling coatings: recent developments in the design of surfaces that prevent fouling by proteins, bacteria, and marine organisms. Adv Mater. 23:690–718.

Busscher HJ, Van der Mei HC, Subbiahdoss G, Jutte PC, Van den Dungen JJAM, Zaat SAJ, Schultz MJ, Grainger DW. 2012. Biomaterial-associated infection: locating the finish line in the race for the surface. Sci Transl Med. 23:153rv10.

Cao B, Tang Q, Li LL, Humble J, Wu HY, Liu LY, Cheng G. 2011. Switchable antimicrobial and antifouling hydrogels with enhanced mechanical properties. Adv Healthcare Mater. 2:1096–1102.

Cerkez I, Kocer HB, Worley SD, Broughton RM, Huang TS. 2011. N-halamine biocidal coatings via a layer-by-layer Assembly Technique. Langmuir. 27:4091–4097.

Charnley M, Textor M, Ackigzo C. 2011. Designed polymer structures with antifouling-antimicrobial properties. React Funct Polym. 71:329–334.

Chen L, Bromberg L, Hatton TA, Rutledge GC. 2008. Electrospun cellulose acetate fibers containing chlorhexidine as a bactericide. Polymer. 49:1266–1275.

Cringus-Fundeanu I, Luijten I, Van der Mei HC, Busscher HJ, Schouten AJ. 2007. Synthesis and characterization of surface-grated polyacrylamide brushes and their inhibition of microbial adhesion. Langmuir. 23:5120–5126.

Croes S, Stobberingh EE, Stevens KNJ, Knetsh MLW, Koole LH. 2011. Antimicrobial and anti-thrombogenic features combined in hydrophilic surface coatings for skin-penetrating catheters. Synergy of co-embedded silver particles and heparin. ACS Appl Mater & Interfaces. 3:2543–2550.

Cui D, Szarpak A, Pignot-Paintrand I, Varrot A, Boudou T, Detrembleur C, Jérôme C, Picart C, Auzély-Velty R. 2010. Contact-killing polyelectrolyte microcapsules based on chitosan derivatives. Adv Funct Mater. 20:3303–3312.

Deng HB, Wang XY, Liu P, Ding B, Du YM, Li GX, Hu XW, Yang JH. 2011. Enhanced bacterial inhibition activity of layer-by-layer structured polycacahide film-coated cellulose nanofibrous mats via addition of layered silicate. Carbohyd Polym. 83:239–245.

Ding X, Yang C, Lim TP, Hsu LY, Engler AC, Hedrick JL, Yang YY. 2012. Antibacterial and antifouling catheter coatings using surface grafted PEG-b-cationic polycarbonatem diblock copolymers. Biomaterials. 33:6593–6603.

Follmann HDM, Martins AF, Gerola AP, Burgo TAL, Nakamura CV, Ruriba AF, Muniz EC. 2012. Antiadhesive and antibacterial multilayer films via layer-by-layer assembly of TMC/heparin complexes. Biomacromolecules. 13:3771–3772.

Fu GD, Yao F, Li ZG, Li XS. 2008. Solvent-resistant antibacterial microfibers of self-quaternized block copolymers from atom transfer radical polymerization and electrosprinning. J Mater Chem. 18:859–867.

Fu JJ, Ji J, Yuan WY, Shen JC. 2005. Construction of anti-adhesive and antibacterial multilayer films via layer-by-layer assembly of heparin and chitosan. Biomaterials. 26:6684–6692.

Gao GZ, Lange D, Hilpert K, Kindrachuk J, Zou YQ, Cheng JTJ, Kazemzadeh-Narbat M, Yu K, Wang RZ, Straus SK, et al. 2011. The biocompatibility and biofilm resistance of implant coatings based on hydrophilic polymer brushes conjugated with antimicrobial peptides. Biomaterials. 32:3899–3909.

Gao GZ, Yu K, Kindrachuk J, Brooks DE, Hancock REW, Kizhakkedathu JN. 2011. Antibacterial surfaces based on polymer brushes: investigation on the influence of brush properties on antimicrobial peptide immobilization and antimicrobial activity. Biomacromolecules. 12:3715–3727.

Gosau M, Prantl L, Feldmann M, Kokott A, Hahnel S, Burgers R. 2010. The effects of copper additives on the quantity and cell viability of adherent \textit{Staphylococcus epidermidis} silicone implants. Biofouling. 26:359–365.

Guan Y, Xiao HN, Sullivan H, Zheng A. 2007. Antimicrobial-modified sulphite pulps prepared by \textit{in situ} copolymerization. Carbohyd Polym. 69:688–696.

Guillaume O, Garric X, Lavigne JP, Van Den Bergh H. 2012. Multilayer, degradable coating as a carrier for the sustained release of antibiotics: preparation and antimicrobial efficacy \textit{in vitro}. J Control Release. 162:492–501.

Hook AL, Chang CY, Yang J, Luckett J, Cockayne A, Atkinson S, Mei Y, Baysron R, Irvine DJ, Langer R, et al. 2012. Combinatorial discovery of polymers resistant to bacterial attachment. Nat Biotechnol. 30:868–875.

Hwang SH, Song J, Jung Y, Kweon OY, Song H, Jang J. 2011. Electrospun ZnO/TiO2 composite nanofibers as a bactericidal agent. Chem. Comm. 47:9164–9166.

Jiang SY, Cao QZ. 2010. Ultralow-fouling, functionalizable, and hydrolysable zwitterionic materials and their derivatives for biological applications. Adv Mater. 22:920–932.

Kazemzadeh-Narbat M, Lai BFL, Ding C, Kizhakkedathu JN, Hancock REW, Wang RZ. 2013. Multi layered coating on titanium for controlled release of antimicrobial peptides for the prevention of implant-associated infections. Biomaterials. 34:5969–5977.

Lalani R, Liu LY. 2012. Electrospun zwitterionic poly(sulfobetaine methacrylate) for nonadherent, superabsorbent, and antimicrobial wound dressing applications. Biomacromolecules. 13:1853–1863.

Li D, Chen H, Wang SS, Wu QZ, Brash JL. 2011. Lysine–poly(2-hydroxyethyl methacrylate) modified polyurethane surface with high lysine density and fibrinolytic activity. Acta Biomater. 7:954–958.

Li P, Poon YF, Li WF, Zhu HY, Yeap SH, Cao Y, Qi XB, Zhou CC, Lamrani M, Beuerman RW, et al. 2011. A polycationic antimicrobial and biocompatible hydrogel with microbe membrane suctioning ability. Nat Mater. 10:149–156.

Liu CX, Zhang DR, He Y, Zhao XS, Bai RB. 2010. The effects of copper additives on the quantity and cell viability of adherent \textit{Staphylococcus epidermidis} silicone implants. Biofouling. 26:359–365.

Liu SQ, Yang C, Huang Y, Ding X, Li Y, Fan WM, Hedrick JL, Yang YY. 2012. Antimicrobial and antifouling hydro-
