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Development of antibacterial polyacrylonitrile membrane modified with a covalently immobilized lysozyme

Abstract : A novel antibacterial polyacrylonitrile (PAN) membrane covalently immobilized with lysozyme was prepared. First, the virginal PAN membranes were prepared via the classic immersion precipitation method. After modification with NaOH, HCl, Ethylenediamine (EDA), lysozyme was covalently immobilized onto the surface of PAN membranes by glutaraldehyde. The chemical compositions of virginal and modified membranes were characterized by Fourier Transform Infrared spectroscopy (FT-IR) and Energy Dispersion X-ray (EDX). The morphology and performance of the immobilized membranes were characterized by Scanning Electronic Microscopy (SEM), filtration performance measurement, the amount of the bonded lysozyme, lysozyme activity measurement and flow cytometry method. The antibacterial tests confirmed that the immobilized lysozyme membrane displayed an excellent antibacterial performance against *staphylococcus aureus* (*S. aureus*).

KEYWORDS: PAN membrane, lysozyme, antibacterial, flow cytometry

1. Introduction

In recent years, membrane separation has been widely used in water treatment, food, pharmaceutical, and biotechnological industries. In all polymer membranes, polyacrylonitrile (PAN) membrane occupied the main part owing to its good solvent resistance, sufficient chemical stability, excellent mechanical properties, and superior thermal stability.[1] However, membrane fouling will shorten membrane lifetime，reduce membrane flux, increase energy cost and bio-deteriorating of membrane structures.[2]In food separation and water treatment, membrane fouling caused by the undesirable accumulation of microorganisms (mainly bacteria) is known as bio-fouling. And the build-up of biofilm is considered as a major obstacle in membrane separation. To decrease the bio-fouling due to biofilm by bacteria on the membrane surface, antibacterial treatment of the membrane surface with antibacterial agents is desired. Actually, a series of investigations [3-4]and antibacterial agents have been employed to construct bactericidal membranes, including N-halamine compounds,[5-7] silver ions/nanoparticles (AgNPs),[8-10] various polycations,[11-13] and enzymes.[14-16] Unfortunately, these methods either would lose their antibacterial properties as the antibacterial substance got released into the surrounding or the whole process was cumbersome, environmentally-unfriendly. With the growing public health awareness of the pathogenic effects, and stain formations caused by microorganisms, there is an increasing need for producing an antibacterial membrane that can not only eliminate the bacterial or even kill the bacterial, but also can keep excellent antibacterial properties after multiple uses.

Lysozyme is widely distributed in nature and well known for its antibacterial activity. It can break down bacterial cell walls via catalyzing the hydrolysis of 1, 4-beta-linkages between N-acetyl-dglucosamine and N-acetylmuramic acid residues in chitodextrins, which increased the permeability of bacteria and caused bacteria to burst.[17] Nevertheless, lysozyme as a soluble enzyme was very unstable and retained its activity only for a short period of time. Therefore, a lot of researches have focused on the conduct of molecular proteins that are immobilized on a solid support.[18,19] Howell et al improved the flux of ultrafiltration membrane by attaching enzyme onto UF membrane which can hinder the gel formation.[20] Shi et al developed anti-fouling and self-cleaning polyethersulfone membrane through immobilization of trypsin.[21] Compared with the free enzyme, immobilized enzyme showed more stability and more robust resistance to environmental changes. In addition, the immobilized enzyme system provided unique advantages in process control and continuous operation, which made the process more economical.[22]

In this study, virginal PAN membranes were prepared by the immersion precipitation phase-inversion method. After modification, lysozyme was covalently immobilized onto the surface of PAN membranes by glutaraldehyde. Morphologies, structures, water flux and antimicrobial efficacies (against *S. aureus*) of the membranes were investigated. The result indicated the activity of immobilized lysozyme was relatively high and could effectively prevent formation of biofilms. The highly antibacterial membranes have potential application in water treatment and food manufacturing.

2. Experimental

2.1 Materials

polyacrylonitrile (PAN) with an average molecular weight of 15,000 g/mol as the membrane-forming polymer was acquired from Sigma Chemical Co. Ltd. Lysozyme with a molecular weight of 14.4 kDa was supplied by Shanghai Sangon Biological Engineering Technology Service Co. Ltd. N-Methyl-2-pyrrolidone (NMP), Phosphoric acid, ethylenediamine (EDA), glutaraldehyde, sodium hydroxide, hydrochloric acid and other chemicals were purchased from Xilong Chemical Co. Ltd. All chemicals were used without further purification. *S. aureus* was preserved by our laboratory.

2.2 Preparation of virginal PAN membrane

The virginal PAN membranes were prepared via the classic immersion precipitation method from a NMP solution containing 16 wt% PAN powder and 4 wt% pore -forming agent H_3PO_4. The casting solution was stirred at 70 $^{\circ}$C for 4 h and kept another 8 h at ambient temperature to completely release air bubbles. Subsequently, the solution was cast on the glass plate (cleaned with acetone) with a casting knife. Then the glass plate was immediately immersed into a coagulation bath of deionized water at 25 $^{\circ}$C. After the primary membranes were peeled off from the substrate, the obtained membranes were left in deionized water overnight to completely remove the residual solvent.[23] The virginal membranes (V-PAN) were stored in deionized water for further utilization.

2.3 Surface Modification of PAN Membrane

For modification, the PAN membranes with 16 cm^2 surface area were first immersed into a 1 M NaOH solution and then the solution was left in a constantly shaking water bath at 40 $^{\circ}C$ for 1 hour until its color turned into a wine–red. The membranes were then rinsed with deionized water until neutral pH was reached and then immersed in to a 10% (v/v) HCl solution at room temperature for 2 h.[24] After re-rinsing with water until neutral pH was reached again, the modified membranes (M-PAN) were immersed in 150 cm^3 10% (w/w) solution of ethylenediamine for 1 h at room temperature. Finally, the gained membranes were taken out and washed thoroughly with deionized water to remove the unreacted ethylenediamine.

2.4 Immobilization of Lysozyme on Modified Membrane

The modified membranes (16 cm^2) were equilibrated with 10 mL of sodium phosphate buffer (pH 7.0) for 4 h. The equilibrated membranes were transferred to 10% (v/v) solution of glutaraldehyde for 1 h at 4 $^{\circ}C$, then the membranes were washed 3 times with 0.1 M sodium phosphate buffer (pH 7.0) to remove the excess glutaraldehyde.[25] The activated membranes were treated with lysozyme solution (1 g/L) for up to 20 h at 4 $^{\circ}C$.[26] Then immobilized membranes (I-PAN) were taken out and thoroughly rinsed with deionized water and 0.1 M solution of phosphate buffer. The preparation process of immobilized membrane (I-PAN) was shown in Figure 1.

---Figure 1---

2.5 Separation performance of membranes

A laboratory scale cross flow membrane system was used to characterize the performance of V-PAN, M-PAN, and I-PAN. The membranes were immersed in deionized water overnight at ambient temperature before filtration. Deionized water

was selected as feed solution. The sample membrane with an effective area of 10.48 cm^2 was installed on the membrane cell. The system was pre-compacted with deionized water at 0.2 MPa for 3 h to reach a steady state, followed by measuring water fluxes at pressures ranging from 0.08 to 0.16 MPa. And the feed flow rate 1.0 L/min. The water flux (J) was calculated using Equation (1)

$$J = \frac{V}{A\Delta t} \quad (1)$$

where V is the volume of permeate water (L), A is the effective area of the membrane (m^2), and Δt is the permeation time (h).

For bio-fouling investigations, after the pure water permeability J_0 was measured, the flux for bacterial suspension (*S. aureu,* 10^5 cells/ml) was measured at the same pressure. The filtration of the bacterial suspension was continued for 9 h and then the membrane was immersed in citric acid solution (pH 4.0) for 1 h and backwashed thoroughly with deionized water for 0.5 h. The water flux of the cleaned membranes J_R was measured again. Such a cycle of filtration was carried out continuously for three times and the operating pressure was 0.1 MPa for the whole process. In order to evaluate the anti-fouling property of membranes, the flux recovery ratio (FRR) was calculated as following Equation (2):

$$FRR = \frac{J_R}{J_0} \quad (2)$$

2.6 Immobilized lysozyme estimation

2.6.1 The amount of bound lysozyme

The amount of lysozyme bounded to the I-PAN was determined by measuring the

initial and final concentrations of lysozyme in the enzyme solution and washing via Brodford's method.[27] The method is based on spectrophotometric measurement of the blue color resulting from the Coomassie brilliant blue G-250 at 595nm. The amount of bound lysozyme can be calculated by the following Equation (3):

$$M_b=C_0V_0M-(C_1V_1+C_2V_2)\ M \quad (3)$$

where C_0, C_1, C_2 are enzyme concentrations for initial solution, final solution, and washing liquid respectively, V_0,V_1,V_2 are volumes of the enzyme solutions for initial , final and washing liquid, respectively, M is molar mass of lysozyme.

2.6.2 The Detection of Lysozyme Activity

The activity of the immobilized lysozyme was determined by the improved shugar's method.[28] The initial decrease in OD_{450} of the suspensions caused by the lysis of *M. lysodeikticus* cells was measured at 25 °C for 1 min. Decrease of 0.001 in OD by 1 cm^2 PAN membrane was defined as 1 unit of lysozyme activity. The lysozyme activity was measured for three samples and then were averaged.

The hydrolytic activity of the immobilized lysozyme can be calculated using the follow Equation (4):

$$U/cm^2=\triangle OD_{450}\times 1000/S \quad (4)$$

where: $\triangle OD_{450}$ was the initial decrease in OD_{450} of *M. lysodeikticus* cells for 1 min, S was the area of PAN membrane.

2.7 Membrane Characterization

2.7.1 FT-IR and EDX Analysis

The chemical compositions of V-PAN, M-PAN, and I-PAN were investigated by

Fourier Transform Infrared Spectroscopy Spectra (FT-IR) and Energy Dispersion X-ray (EDX). FT-IR micro-spectroscopic measurements were performed using a Nicolet 380 FT-IR spectrometer. And the diffuse reflectance spectra were scan over the range of 400–4000 cm^{-1}. The element composition and distribution of virginal membrane and immobilized membranes were determined by Energy Dispersion X-ray analysis employing the SEM (SH-4600) with a 20 keV energy beam.

2.7.2 SEM Analysis

In order to gather information about the cross-section of membranes, scanning electronic microscopy (Nova NanoSEM 230) measurements were carried out. The membrane samples for SEM analysis were initially dried at room temperature, then the membranes were immersed in liquid nitrogen and fractured into small pieces for examination of the cross-section. And then the samples were sputter-coated with gold-palladium alloy to a thickness of 5 nm before microscopic analysis.

2.8 Determination of Antibacterial Properties

2.8.1 The cultivation of staphylococcus aureus

The antibacterial activities of the V-PAN/I-PAN against the Gram-positive bacteria *S. aureus* were tested. *S. aureus* was incubated in 20 mL of broth medium (containing10 g/L peptone, 5 g/L beef extract, 5 g/L sodium chloride, at a pH of 6.8) at 36 $^{\circ}C$ until the exponential growth phase was reached. The bacteria-containing culture was centrifuged at 10000 rpm for 10 min. And after removal of supernatant, the cells were washed twice with sterile phosphate buffered solution (PBS, pH 7.4). Then the bacteria cells were re-suspended and diluted to 10^6 cells/ml using the standard serial

dilution method (based on standard calibration with the assumption that the optical density of 1.0 at 540 nm is equivalent to approximately10^9 cells/mL).[29]

2.8.2 Flow Cytometry

The Flow cytometry method was used to determine the antibacterial of the I-PAN. Flow cytometry could be used to count the number of microorganisms with high accuracy, stability. In this study, *S. aureus* solution used as sample solution for flow cytometry was collected from biofilm on membranes at the end of bio-fouling experiments. *S. aureus* were resuspended in PBS. And 50,000 events were detected each run and the rate of samples was 1000 events per second. The samples were stained with Propidium Iodide (PI) because PI binds to DNA and cannot cross an intact cell.[30] The antibacterial efficiency (E_b) was represented by detecting the percent of broken cells. The antibacterial efficiency was measured from Equation 5:

$$E_b=(W_2-W_1)\times 100\% \quad (5)$$

where W_2 and W_1 are the percent of broken cells of *S. aureus* corresponding to the V-PAN (as blank sample) and I-PAN, respectively.

3. Results and discussion

3.1 Surface Chemical Composition of the Membranes

V-PAN, M-PAN, and I-PAN are virginal PAN membrane, PAN membrane modified NaOH、HCl, PAN membrane immobilized lysozyme respectively. The V-PAN, M-PAN, and I-PAN were subjected to FT-IR analysis. The FT-IR spectrum of V-PAN

was shown in Figure 2(a). The spectrum of V-PAN shows characteristic peaks at 2930, 2240, 1470cm^{-1} were stretching vibration band of methylene (–CH_2–), stretching vibration band of nitriles (–CN–), and bending vibration band of methylene (–CH_2–), respectively. The characteristic spectra of virginal PAN was similar to a previous research of enzymatic surface modification of Polyacrylonitrile and Its Copolymers.[31,32] In the spectrum of Figure 2(b), there are two prominent peaks in the 3402 cm^{-1} region and 1699~1711cm^{-1} region respectively. This phenomenon was most likely to be ascribed to the presence of O–H bond and C=O bond of carboxylic acids in NaOH treated membrane. These two stretching peaks indicated the presence of -COOH in membrane after treated by NaOH. The two common bands of lysozyme 1658 and 1519 cm^{-1} belong to the amide I and amide II peaks have appeared in Figure 2(c), which corresponded to stretching vibrations of C=O bond and coupling of bending of N–H bond and stretching of C–N bond, respectively. However these peaks migrated to 1666 and 1560 cm^{-1} by the influence of the original amide group.[33,34] The appearance of these characteristic peaks indicated that lysozyme was immobilized on the PAN membranes by covalent bonding.

---Figure 2---

The changes in elemental composition between different membranes were analyzed by EDX. As shown in Table 1, the C element accounted for 63.98% (Wt) in V-PAN and 63.65% in M-PAN, then increased to 66.07% (Wt) in I-PAN. The EDX shows that no S element is existed in V-PAN and M-PAN. The S element accounted for 0.08% (Wt) in I-PAN because of the immobilized lysozyme. These changes further confirmed the successful grafting of lysozyme onto the PAN membrane.

---Table 1---

3.2 SEM Analysis

The SEM images of the cross section of V-PAN, M-PAN, and I-PAN were presented in Figure 3. It is apparent that all the membranes have typical asymmetric structures, which consist of a thin dense layer and a support layer with finger-like structure. The diameters of finger-like structure in modified /immobilized membrane were larger than its counterpart in V-PAN. These changes may be attributed to the damage in the process of modification by sodium hydroxide treatment.[35] Further, in the last step of immobilization of lysozyme, there was no evident change in the cross-sectional morphology of the M-PAN/I-PAN, which can be verified in the Figure 3(b) and Figure 3(c).

---Figure 3---

3.3 Membrane Filtration Performance

A cross flow membrane system was used to characterize the performance of the V-PAN, M-PAN and I-PAN. Figure 4 illustrates the pure water flux through the V-PAN, M-PAN and I-PAN membranes at different feed pressures. As the applied pressure increased, the pure water flux increased linearly. M-PAN had relatively higher pure water flux than V-PAN and I-PAN. This change can be attributed to that the finger-hole became a little bigger[35]and the surface hydrophilicity of V-PAN was enhanced after NaOH modification. Some enzyme was embedded in the holes when lysozyme was immobilized on the PAN membrane, which resulted in a reduction in the water permeability.

---Figure 4---

As shown in Figure 5, the flux of membrane decreased sharply in the initial few hours of operation. It is proposed that some *S. aureus* cells in the feed were deposited or adsorbed on the surface (biofilms formation).The flux recovery ratio (FRR) by cleaning with deionized water was analyzed for tested membranes. After first cycle, the FRR were 86.9%, 85.7%, and 93.1% for the V-PAN，M-PAN，I-PAN, respectively. The FRR decreased for both tested membranes with the increase in the operation time. The FRR of the second cycle were 75.4%, 77.8% and 86.2% for the V-PAN, M-PAN, I-PAN, respectively. Compared the data of V-PAN and M-PAN, the litter improvement of flux recovery may be attributed to enhance of hydrophility due the NaOH modification. Although immobilized membrane showed a fraction flux loss due to the fouling on the membrane surface, their higher recovery ratio proved that their absorbed fouling layers are relatively loose and easily removable. The phenomenon can be attributed to the higher hydrophility and reduce the biofilm formation of immobilized membrane due to lysozyme.

---Figure 6---

3.4 The Amount and Activity of the Immobilized Lysozyme

3.4.1 The Amount of the Immobilized Lysozyme

The amount of protein loading on I-PAN were measured and showed in Figure 6. The immobilized lysozyme reached a constant amount of 0.025 mg/cm^2 after 20 h of immobilization reaction. The effective of protein loading on the surface of membranes was restricted by the molecular size of lysozyme, density of aldehyde group, and the number of amino groups of lysozyme.

---Figure 6---

3.4.2 The Activity of the Immobilized Lysozyme

The activity of the immobilized lysozyme was determined by the improved shugar's method. The process included monitoring the degradation rate of *M. lysodeikticus* through the reduction in absorbance at 450 nm. In a sterilized beaker, 5 ml of the *M. lysodeikticus* solution (OD=1.0) in 0.1 M phosphate buffer (pH 6.24) and an immobilized PAN membrane was put quickly. The absorbance at 450 nm of the *M. lysodeikticus* solution was measured before/after treat 1 min of the immobilized membrane using a TU-1810 UV spectrophotometer. The free enzyme was measured by the similar strategy, 2.5 ml of the *M. lysodeikticus* solution in 0.1 M phosphate buffer and the same quality of enzyme were mixed quickly, and the reduction in the absorbance at 450 nm was recorded for 1 min. All the measurements were carried out at 25 $^{\circ}$C and pH 6.24. Compared with the free enzyme, the activity after immobilization was reduced from 33 to 17. The reason for this change may be attributed to the diffusional limitations resulting from the diminished molecular flexibility of the enzyme. What is more, during the immobilization process, spatial structure of the enzyme was changed and even some active sites were damaged in the immobilized form of the enzyme.

The preservation stability of the lysozyme immobilized on the membrane was also investigated. After preservation in 0.1 M PBS (pH 6.8) solution for 3 months, the immobilized lysozyme lost 24% of their initial activity. Some lysozyme was denatured and lost activity via hydrolysis though it was covalently immobilized.

3.5 Determination of Antibacterial Properties

The viable and broken cell counts of bacteria were measured by Flow cytometry method. At the predetermined time, 0.2 mL of bacteria culture was taken from sample solution and decimal serial dilutions with PBS were repeated with each initial sample. In total, 50,000 events were detected each run and the rate of samples was 1000 events per second. The antibacterial efficiencies (E_b) of the V-PAN/I-PAN were presented in Figure 7. In the image, X-axis refers to signal intensity of PI while Y-axis is number of cells. After being filtered by I-PAN, the number of intact of *S. aureus* was significantly less than those in the V-PAN. The percent of broken cells of *S. aureus* in control group was 19.3%, which was presented in Figure 7(a). When cells are in the exponential growth phase, it is a normal phenomenon that some cells would apoptosis. The percent of broken cells of *S. aureus* in experiment group was 56.6%, which was presented in Figure 7(b). The change of the percent of broken cells indicated that the immobilized lysozyme having a strong bacterial-killing capacity against *S. aureus* with an antibacterial efficiency (Eb) of 37.3%. This study showed that the immobilized membrane had the potential to reduce bio-fouling in water treatment and food separation.

---Figure 8---

4. **Conclusions**

In this study, a PAN ultrafiltration membranes prepared via the classic immersion precipitation method was modified by immobilized lysozyme on the surface of membranes, resulting in a novel PAN-immobilized membrane with anti-bacterial property. The membrane surface morphology, separation performance, immobilized lysozyme activity, and antibacterial activity were investigated. Compared to the virginal membrane, the membrane exhibited a moderate flux and a high antibacterial activity. In addition, the antibacterial activity analysis showed that the number of intact cells of *S. aureus* after filtered by the immobilized membrane was significantly less than those filtered by the virginal membrane, with an antibacterial efficiency of 37.3%. Therefore, membrane with effective antibacterial performances has been

prepared, which will have potential applications beyond water treatment or food manufacturing.

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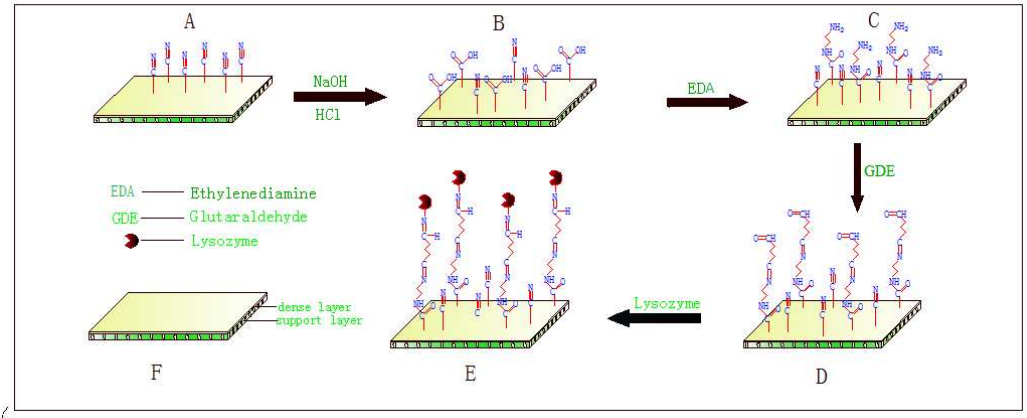


Figure 1. the route map for preparing of I-PAN.

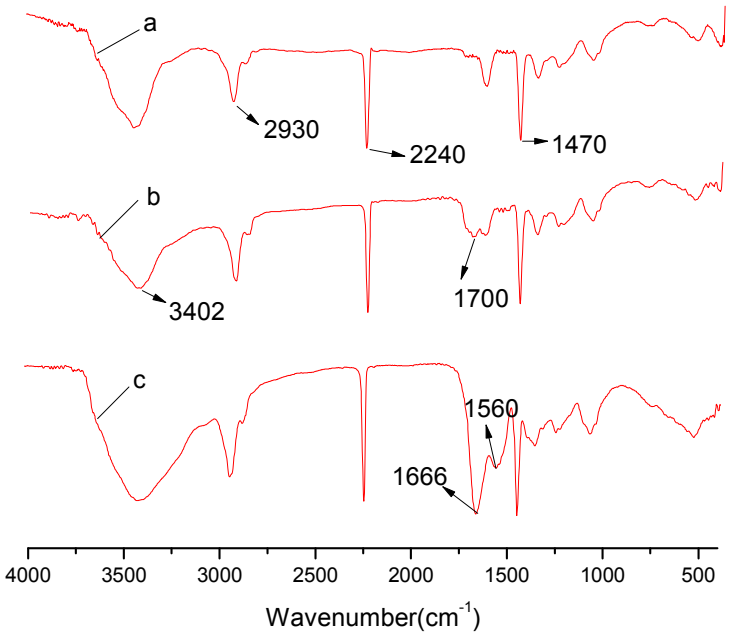


Figure 2. FT- IR spectra of (a) V-PAN (b) M-PAN and (c) I-PAN.

Table 1

Weight percent of elements based on EDX analysis.

Element	V-PAN	M-PAN	I-PAN
C	63.98	63.65	66.07
O	19.34	11.83	8.50
N	16.34	24.20	25.17
S	0.00	0.00	0.08
P	0.34	0.32	0.18

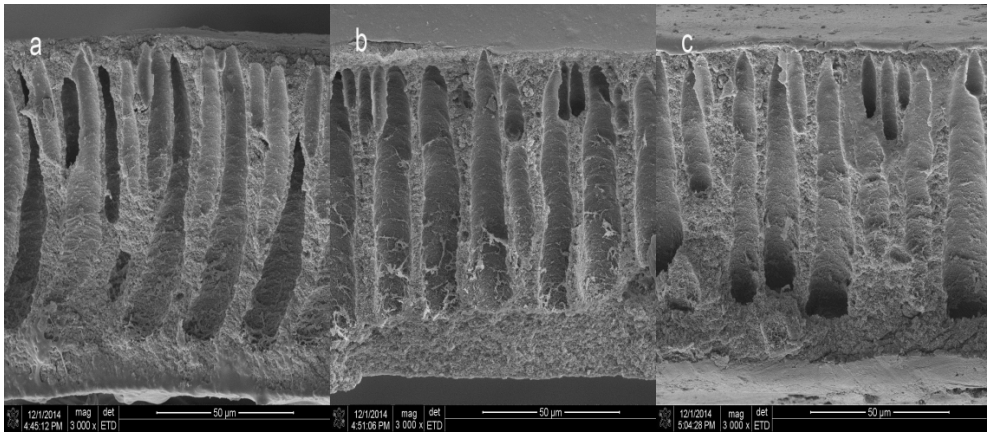


Figure 3. SEM images of cross-section morphology of membranes: a, V-PAN b, M-PAN and c, I-PAN.

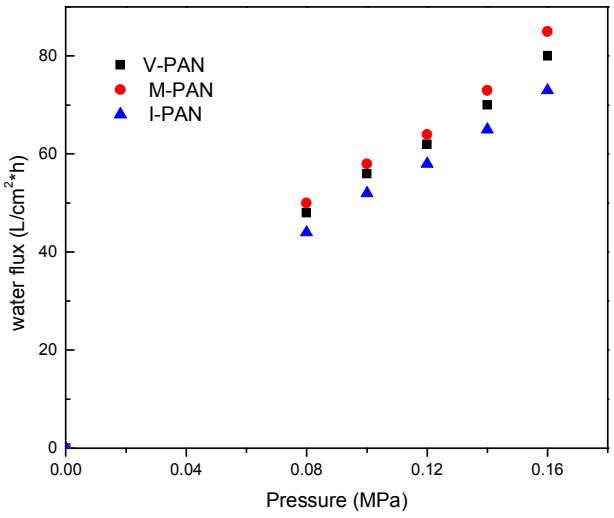


Figure 4. Pure water flux of the V-PAN, M-PAN and I-PAN as a function of feed pressure

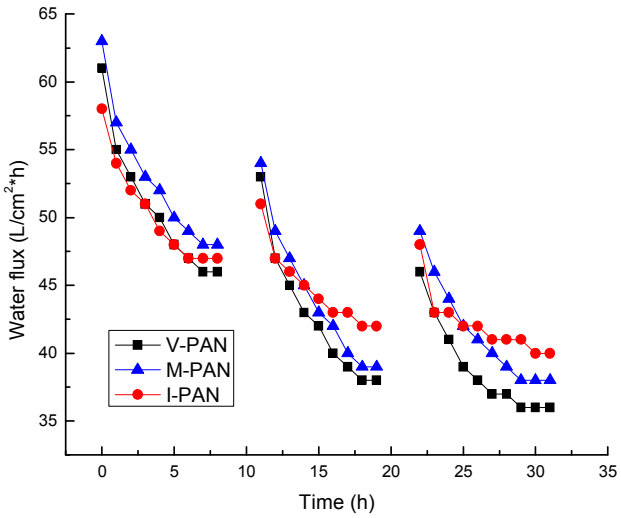


Figure 5. Water flux of the V-PAN, M-PAN and I-PAN during filtration of bacterial suspension.

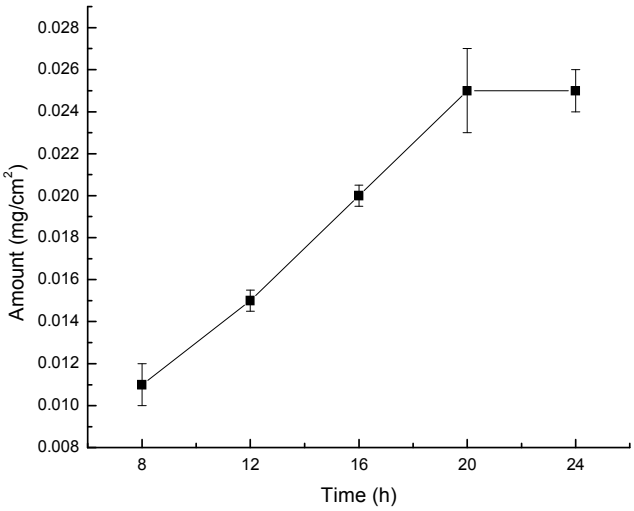
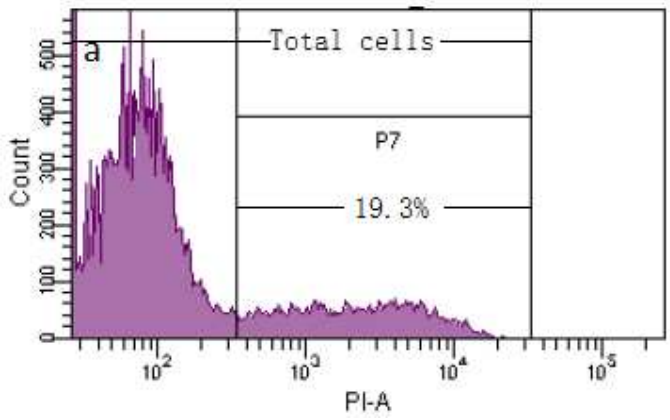


Figure 6. The amount of lysozyme loading on the I-PAN.



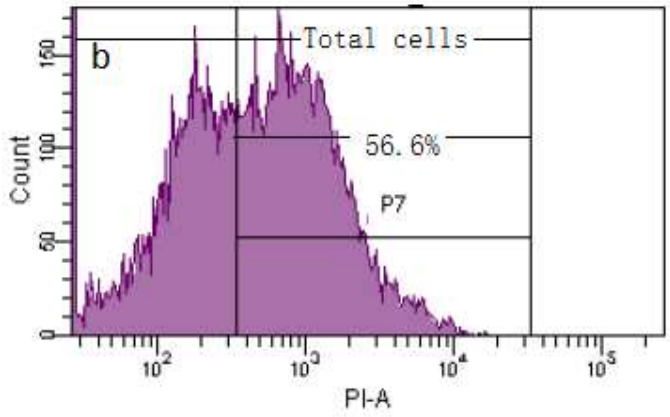


Figure 7. Effect of V-PAN/I-PAN on the cell membrane integrity of *S.aureus.* Because PI binds DNA and cannot cross an intact cell, the percent of broken cells was reflected by PI staining (showed as the right part P7 in each image). In the image, X-axis refers to signal intensity of PI while Y-axis is number of cells.