

Antibacterial activity of nanofibrous mats coated with lysozyme-layered silicate composites via electrospraying



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ABSTRACT

A mixture of positively charged lysozyme (LY) and rectorite (REC) composites was electrosprayed onto negatively charged electrospun cellulose acetate (CA) nanofibrous mats. The morphology and average diameter of CA mats and the mats coated with LY-REC were investigated by scanning electron microscopy. The composite mats were characterized by Fourier transform infrared spectroscopy, X-ray photoelectron spectroscopy and energy-dispersive X-ray spectroscopy, and the results confirmed that LY and REC were successfully immobilized on the surface of CA mats via electrospraying technique. The small-angle X-ray diffraction results showed that the silicate layers of REC were completely exfoliated. The enzyme activity and bacterial inhibition analysis verified that the antimicrobial effect of the composite fibrous mats was enhanced with the addition of REC. The protein delivery properties and the bound enzyme activity after removal of unbound lysozyme from fibers were measured and showed that the electrospraying technique was suitable for enzyme immobilization.

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1. Introduction

Advances in protein engineering and DNA technique made it possible to manipulate enzymes which exhibited the desired properties related to the substrate specificity, activity, selectivity, stability and pH optimum (Sheldon, 2007). Nevertheless, industrial application was often restricted by difficult recovery and reuse of enzymes and also lack of long-term operational stability. These drawbacks could often be overcome by enzyme immobilization (Sheldon, 2007). So recently enzyme immobilization has attracted interest in many areas, such as food production, pharmaceutical and chemical industries. Many physical enzyme immobilization methods, such as covalent attachment, semi permeable membrane or sol-gel entrapment, encapsulation and layer by layer assembly (Felipe Diaz, 1996) have been used, however, the loss of enzymes

could not be avoided or effectively controlled by using the above methods.

Under the driving force of physical deposition and electrostatic forces, the electrospraying (electrohydrodynamic spraying) technique was a method of liquid atomization (Jaworek & Sobczyk, 2008). During electrospraying, the liquid flowing out from a capillary nozzle under high electric potential was forced by the electric field to be dispersed into extremely small droplets (Jaworek & Sobczyk, 2008). The technique was regarded as a versatile, straightforward and powerful way to manufacture materials which could be well dispersed in solutions (Li et al., 2012a). In addition, there was almost no waste of the electrosprayed solutions, so the electrospraying technique could be well suited for industrial production and exactly coincided with our original design purpose.

Lysozyme (LY), with an isoelectric point of 10.7 (Croguennec, Nau, Molle, Graet, & Brule, 2000), is positively charged when dissolved in neutral aqueous solutions, and is an antibacterial enzyme able to hydrolyze the peptidoglycan layer of the cell wall of some Gram-positive bacteria, which was discovered by Fleming in 1922 (Jolles, 1964; Pellegrini, Bramaz, Klauser, Hunziker, & von Fellenberg, 1997). Besides, LY was perfect for antimicrobial application and played a key role in the innate immune system of most animals including human beings, so it could be ideal as a

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bacterial inhibitor in food packaging, food preservatives, wound dressing, etc. Hen egg white lysozyme exhibited antimicrobial activity against both Gram-positive and Gram-negative bacteria (Pellegriani et al., 1997), and it was found in many cells, tissues and secretions from a multitude of organisms (Hyslop, Kern, & Walker, 1974; Josephson & Greenwald, 1974; Torbeck & Prieur, 1979). However, attention should be paid to the relatively low stability of free LY. Immobilization of LY on solid substrate showed higher stability when facing environment changes, whereas its activity was restricted. For further improving LY activity after the immobilization, we tried to mix rectorite in LY solutions. According to the previous report (Wang et al., 2009), pure REC could not inhibit the growth of microbes, but when combined with antibacterial substance, the inhibition effect of the composites was much enhanced.

Rectorite (REC), with high surface area-to-volume ratio and water swelling property similar to that of montmorillonite, was a negatively charged layered silicate (Wang et al., 2009), which could be used to mix with some specific compounds according to its purpose. A few months ago, the European Food Safety Authority (EFSA) confirmed the safety of bentonite (another layered silicate) as food additives and verified bentonite was effective in reducing milk aflatoxin. EFSA had also pointed out that layered silicates were ubiquitous in the environment as natural soil components, which depicted their application could hardly do harm to the environment and human health (European Food Safety Authority, 2011). The interesting part was that layered silicates were also a kind of potent detoxifier, which could adsorb dietary toxins, bacterial toxins associated with gastrointestinal disturbance, hydrogen ions in acidosis, and metabolic toxins such as steroidal metabolites associated with pregnancy (Dong & Feng, 2005; Wang, Du, Luo, Lin, & Kennedy, 2007). The previous study reported that REC could contribute to controlling the protein release in the mats because of its large interlayer distance, separable layer thickness and layer aspect ratio (Li et al., 2012b).

As mentioned above, the electrospraying technique could be used to immobilize enzyme on a solid substrate without any waste, and nanofibrous mats here were also an ideal candidate of substrate due to their ultrafine fiber diameter, high surface area-to-volume ratio, three-dimensional (3D) structure, etc. (Deng et al., 2011; Ding, Kimura, Sato, Fujita, & Shiratori, 2004). As with fabricating nanofibers, electrospraying was regarded as a mature and simple way. The combination of the two techniques mentioned above was called electrospraying–electrospinning hybrid technique in our previous study (Li et al., 2012a). This modified method combined characteristics of different composites, and that could enable to fabrication of compounds on nanofibers in accordance with specific application.

The basic idea behind enzyme immobilization was to entrap the protein in a semi-permeable support material, and make it impossible for enzyme to leave while allowing substrates, products, and co-factors to pass through (Taqiuddin & Amiji, 2004). Cellulose acetate (CA), a kind of modified natural polysaccharide, was cheap and environmental-friendly. Interestingly, CA was negatively charged and could be easily fabricated into nanofibers with excellent mechanical properties. So the nanofibrous mats could be ideal substrate for the immobilization of LY (Huang et al., 2012b; Li et al., 2012a).

In this study, the mixture solutions consisted of lysozyme and rectorite, were electrosprayed on the negatively charged electrospun CA nanofibrous mats. The morphology and the composition analysis of the composite fibrous mats were investigated. Then the lysozyme activity of LY and antibacterial assay was conducted to study whether the inhibition effect of the composite nanofibrous mats was increased after adding REC. Finally, the protein delivery properties and the bound enzyme activity after removal of unbound lysozyme from fibers were measured.

2. Experimental details

2.1. Materials

The starting materials were used as follows: cellulose acetate (CA, M_n 3×10^4 , Aldrich Co., USA), calcium rectorite (REC, Mingliu Inc. Co., China), lysozyme (LY, activity 25,000 U mg^{-1} , Amresco Co., USA). Acetone and *N,N*-dimethylacetamide (DMAC) were supplied by the Aladdin Chemical Reagent Co., China. Bovine serum albumin (BSA, M_w 6.8×10^4 Da) was from Roche Diagnostics Co., USA, and Coomassie Brilliant Blue (G250) was supplied by Amresco Inc., USA. All other chemical reagents were of analytical grade. Distilled water with a resistance of 18.2 M Ω cm was used to prepare all aqueous solutions. Besides, *Micrococcus lysodeikticus* (*M. lysodeikticus*) powder was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) were obtained from China Center for Type Culture Collection, Wuhan University (Wuhan, China).

2.2. Fabrication of CA mats

16% CA solution was prepared by dissolving CA powder into a 2/1 (w/w) acetone/DMAC mixture under gentle magnetic stirring for 5 h. Then the solution was put into a plastic syringe driven by a syringe pump (LSP02-1B, Baoding Longer Precision Pump Co., Ltd., China). The metal needle tip of the syringe was clamped by the positive electrode of a high voltage power supply (DW-P303-1ACD8, Tianjin Dongwen High Voltage Co., China) and the diameter of the metal needle tip was 0.8 mm. A ground cylindrical layer was used as a collector which rotated with a linear velocity of 100 m/min. For electrospraying, the electric field was 16 kV/18 cm. Besides, the flow rate was set as 1 mL/h. The relative humidity and ambient temperature were maintained at 50% and 25 °C, respectively. Then the prepared fibrous mats were dried in vacuum to remove trace solvents at room temperature.

2.3. Assembling with LY-REC composites through electrospraying

LY was dissolved in 100 mM potassium phosphate buffer (PBS, pH 6.24) with different weight ratios at 2.5%, 5% and 10%, respectively. In addition, 5% and 10% LY solutions were mixed with REC, and the concentration of REC was maintained at 1%. All mixtures with gentle magnetic stirring for 2 h were fed into the electrospraying equipment mentioned before, and the consistent electric field was kept at 12 kV/10 cm. The flow rate was 0.25 mL/h. Each mixture was electrosprayed onto the collector covered by the previously obtained CA fibrous mats for 4 h.

2.4. Characterizations

The composite nanofibrous mats were processed by vacuum spray carbon, and then their surface morphology was observed by field emission scanning electron microscope (FE-SEM, S-4800, Hitachi Ltd., Japan). Image analyzer (Adobe photoshop 7.0) was used to calculate the average diameter of fibers. Energy-dispersive X-ray (EDX) spectroscopy (S-4800, Hitachi Ltd., Japan) was used to investigate the element composition of the composite nanofibrous mats. The ζ -potential analysis was determined by using Nano-25 zetasizer (Malven, England). Fourier transform infrared (FT-IR) spectra (170-SX, Thermo Nicolet Ltd., USA) were recorded in the wavenumber range from 4000 to 400 cm^{-1} , and the number of FT-IR scans was 64 times. The small-angle X-ray diffraction (SAXRD) was recorded by type D/max-rA diffractometer (Rigaku Co., Japan). X-ray photoelectron spectroscopy (XPS) was performed to identify

the existence of atoms on the surface of the composite nanofibrous mats by using an axis ultra DLD apparatus (Kratos, UK).

2.5. Lysozyme activity assay

The principle was that the lysis of *M. lysodeikticus* cells was in proportion to its turbidity. The turbidimetric method was used to determine the LY activity according to the previous reports (Charernsriwilaiwat, Opanasopit, Rojanarata, & Ngawhirunpat, 2012; Ibrahim, Matsuzaki, & Aoki, 2001), and the concentration of PBS used to dissolve *M. lysodeikticus* cells was 100 mM. And then the nanofibrous mats loaded with different weight ratios of LY, were cut into disks with the diameter of 6 mm and put into the *M. lysodeikticus* solutions. The decrease in absorbance at 450 nm (25 °C) was monitored each half minute. The activity of LY was obtained by calculating the rate of decrease in absorbance per minute.

$$\text{Activity (U cm}^{-2}\text{)} = \Delta \text{OD}_{450} \times \frac{1000}{S}$$

where ΔOD_{450} was the decrease in absorbance at 450 nm of *M. lysodeikticus* solution per minute, and S was the area (cm^2) of nanofibrous mats (Huang et al., 2012b).

The activity of free LY was measured by a modified approach as the previous literature (Huang et al., 2012c) and calculated as follows:

$$\text{Activity (U mg}^{-1}\text{)} = \Delta \text{OD}_{450} \times \frac{1000}{m}$$

where ΔOD_{450} referred to the reduction of the absorbance at 450 nm of *M. lysodeikticus* solution per minute, and m was the mass (mg) of LY in 200 μL enzyme solution.

2.6. Antibacterial assay

Disk-diffusion method was performed to investigate the inhibition activity of the composite nanofibrous mats. Gram-negative bacteria *E. coli* and Gram-positive bacteria *S. aureus* were selected as the representative microorganisms. The prepared nanofibrous mats, cut into disks with the diameter of 6 mm, were covered on the surface of the caky nutrient agar in Petri dishes which were inoculated with 100 μL bacterial solution taken from a diluted nutrient broth medium. The concentration of bacteria solutions was approximately 10^5 cfu/mL, counted by using the method of standard plate count. After incubated at 37 °C for 18 h, the inhibition zones were measured with a tolerance of 1 mm. The experiment was repeated three times for each sample.

2.7. Release profiles of LY from the composite fibrous mats

The LY release test was done in distilled water for 12 h (Pérez, De Jesús, & Griebenow, 2002). The composite mats, cut into squares with the area of $4 \text{ cm} \times 5 \text{ cm}$, were put into a conical flask containing 20 ml of distilled water, and then incubated on a constant temperature shaking bed at 37 °C and 100 rpm. After specific intervals, 1 mL of medium was withdrawn and immediately replaced with the same amount of fresh medium. The amounts of released LY were determined by Coomassie Brilliant Blue (G250) method (Li et al., 2012b). The non-loaded LY fibrous mats were taken as the control. All experiments were done in triplicate and mean values were reported. The LY encapsulation efficiency was calculated as follows:

$$\text{EE(\%)} = \frac{A - B}{A} \times 100\%$$

where A was the total amount of the LY given, and B was the amount of LY lost (Li et al., 2012b).

The enzyme activity test was also conducted in 20 mL distilled water for 2 h. The composite mats were cut into disks with the diameter of 6 mm, and 20 pieces of the disks were randomly selected for the enzyme activity test. After specific intervals, one piece of the disks was used to determine the activity of LY by using the turbidimetric method previously mentioned.

3. Results and discussion

3.1. Morphology of fibrous mats

In order to examine the morphology effect of LY and REC coated on the CA fibrous mats, the FE-SEM images of the composite fibrous mats were taken (Fig. 1). Apparently, all the mats maintained nanofibrous 3D structure and perfect fiber shape before/after the electrospraying. Compared with the morphology of CA fibrous mats, it could be observed that CA mats coated with LY or LY-REC composites had a few conglutinations marked with arrows in the images, which revealed that the electrosprayed LY or LY-REC solutions deposited on the surface of CA nanofibers successfully, and formed a thin layer of films but split into webs during the drying process, hence the surface of the composite mats was much rougher than that of CA mats. Besides, CA mats coated with LY-REC composites had more bundles, bigger junctions and thicker deposition films, and their morphology was obviously distinct from the mats coated with LY or pure CA mats. In addition, the number and size of the aggregations adhered to the CA fibers, affected by the addition of REC, were also approximately in proportion to the concentration of the sprayed solutions. Moreover, there was no apparent difference in morphology between CA mats coated with 5% and 10% LY-REC composites. According to the images of the diameter distribution, the average diameter of the CA mats was 375 nm, while that of the composite nanofibrous mats increased after coating with LY or LY-REC composites. Obviously, the average diameter of the composite mats enlarged in proportion to the concentration of the sprayed LY solutions and even affected by the addition of REC.

As we know, the driving forces during the electrospraying process included physical deposition and electrostatic adsorption, and the latter was predominant. The ζ -potential of the bulk materials was determined. In Table 1, the positively charged LY (+24.5 mV) was easily assembled on the surface of the negatively charged CA fibers (−28.3 mV). The ζ -potential of LY-REC at different pH value was also investigated, and when the solution was weakly acidic, the ζ -potential was positive. Although the ζ -potential of LY-REC solution (pH 6.28) was lower than that of the LY-REC solution (pH 5.81), LY-REC solution (pH 6.28) was chosen to be the electrosprayed solution for its positively charged property and better protection of LY activity, because the optimal pH of LY was around 6.2 according to the literature (Smolelis & Hartsell, 1952). From the table, the ζ -potential of LY-REC solution (pH 6.28) was approaching to zero, and it might be the reason for the phenomenon that the morphology of the samples containing REC had more bundles, bigger junctions and thicker deposition films.

3.2. FT-IR spectra analysis

The FT-IR spectra of the CA mats and the mats coated with LY or LY-REC composite was showed in Fig. 2. After electrospraying, all the mats still maintained the characteristic peaks of CA mats. According to the FT-IR spectrum of REC powder, the peaks for Si–O bending vibration at 467 and 546 cm^{-1} , –OH vibration at 3643 cm^{-1} (Deng et al., 2011; Wang et al., 2006, 2009), was characteristic of REC composition in the composite nanofibrous mats. The wide bands at 696.2 cm^{-1} and 817.7 cm^{-1} , found in the spectra of REC powder and CA mats coated with LY-REC, were assigned

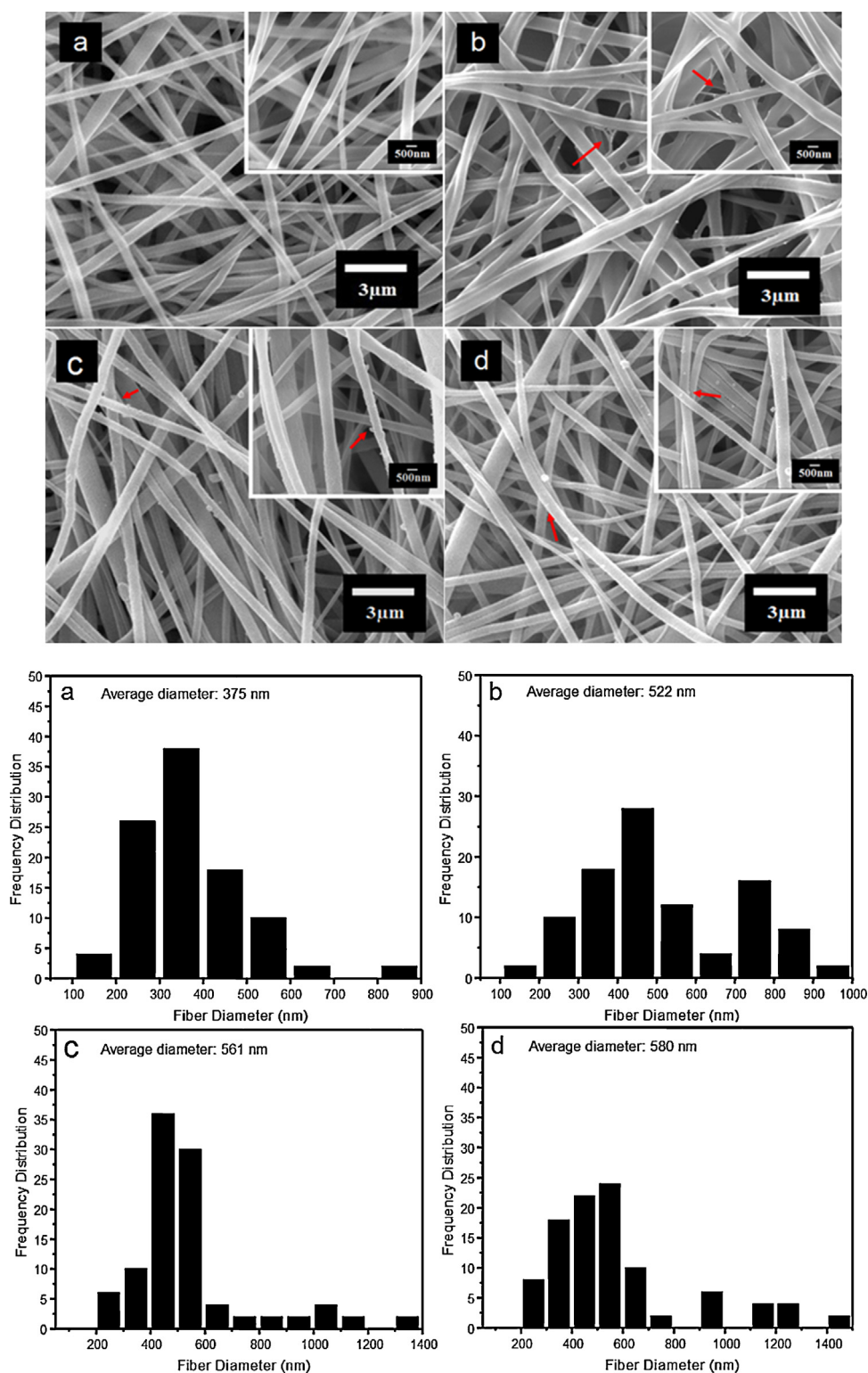


Fig. 1. FE-SEM images and the fiber diameter distribution of (a) CA fibrous mats and the CA mats coated with: (b) 5% LY, (c) 5% LY-REC composites and (d) 10% LY-REC composites.

Table 1
ζ-potential (mV) of CA, LY, REC and LY-REC composites.

	Samples					
	CA	LY	REC	LY-REC		
pH	6.79	6.26	7.31	5.81	6.28	7.23
ζ-potential (mV)	−28.31	+24.54	−15.72	+3.14	+1.45	−2.50

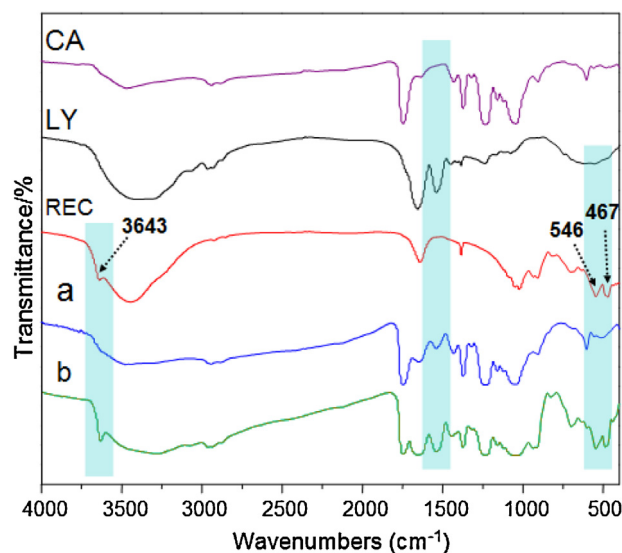


Fig. 2. FT-IR spectra of CA fibrous mats, LY powder, REC powder, CA fibrous mats coated with: (a) 5% LY and (b) 5% LY-REC composites.

to -NH- deformation out of plane vibrations (Masram, Kariya, & Bhawe, 2010) and the bending vibrations of the fundamental Si-O-Si bond (Kim, Hwang, Kim, Lee, & Kim, 2007), respectively. Likewise, it was also the evident that REC was successfully assembled on the surface of CA mats. The characteristic peaks at 1525 cm^{-1} and 1560 cm^{-1} represented for the secondary amide (peptide) groups, deriving from the coupled C-N vibration and N-H deformation frequency (Blout, Loze, & Asadourian, 1961). The strong absorbance at 1639.2 cm^{-1} was observed in the spectra of LY powder, REC powder, CA mats coated with LY and LY-REC composites, which corresponded to the C=O stretching, and the C=O bond in LY should belong to the peptide bond -NHCO- (Wu et al., 2007). Besides, this region of CA mats coated with LY-REC was wider than that of CA mats coated with LY, and it should be the synthetic effect of LY and REC.

3.3. Composition analysis

In order to investigate the surface composition of composite mats, EDX and XPS analyses were performed. Figs. 3 and 4 showed the EDX and XPS analysis of CA mats coated with 5% LY-REC, respectively. EDX spectra of CA mats coated with 5% LY-REC had the peaks of Si and Al, which were apparently from the REC

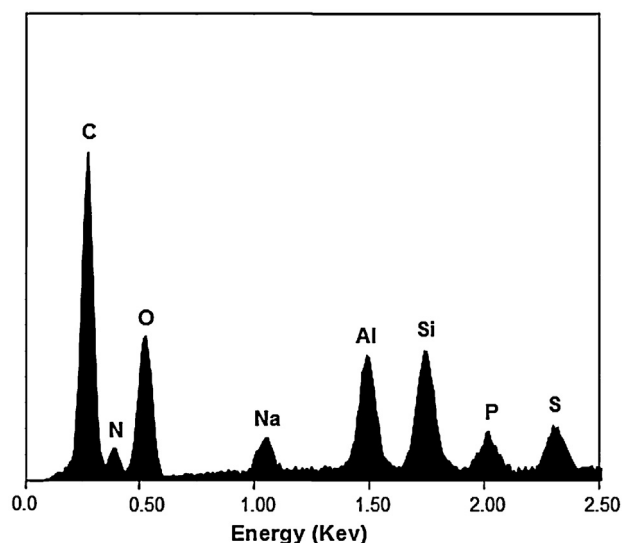


Fig. 3. EDX spectrum of the selected CA fibers coated with 5% LY-REC composites.

coating. Additionally, the characteristic elements of nitrogen and sulfur verified that LY was deposited on the surface of the fibers successfully. The element of P was from the PBS solution.

To further verify the EDX results, XPS analysis was used to investigate the elemental compositions at a depth of up to 10 nm from the surface of the fibers. The strong C 1s and O 1s peaks could be easily observed in CA mats (Fig. 4a) and the mats coated with LY-REC (Fig. 4b). The extra peaks, such as N 1s, S 2p, Si 2p and Al 2p, were in the composite mats and confirmed the deposition of LY and REC. The high-resolution signal of the C 1s contained two peaks described as follows: peak at 286.9 eV was characteristic of -C-O-, -C-OH and -C-OCH₃ of LY, peak around 284.6 eV was assigned to -C-C or -C-H of LY or CA, or -C-N of LY (Huang et al., 2012a; Ignatova et al., 2010). Additionally, the N 1s spectrum had one peak centered at 399.2 eV which was characteristic of pyridinic nitrogen (sp^2 hybridization) (Nath, Satishkumar, Govindaraj, Vinod, & Rao, 2000), and it attributed to the assignment of the binding energy of -C-N covalent bonds with almost no charge transfer between C and N (Ren et al., 1995) that should be from the LY and CA, which demonstrated LY had great interacted with CA. Moreover, the S 2p spectrum was appeared at 163.6 eV (unbound sulfur) which was identical to that of bulk disulfide bond (Ishida et al., 1999) from LY. The appearance of Si 2p peak at 101.9 eV (-Si-C) verified the incorporation existed between REC and LY. Furthermore, the Al 2p

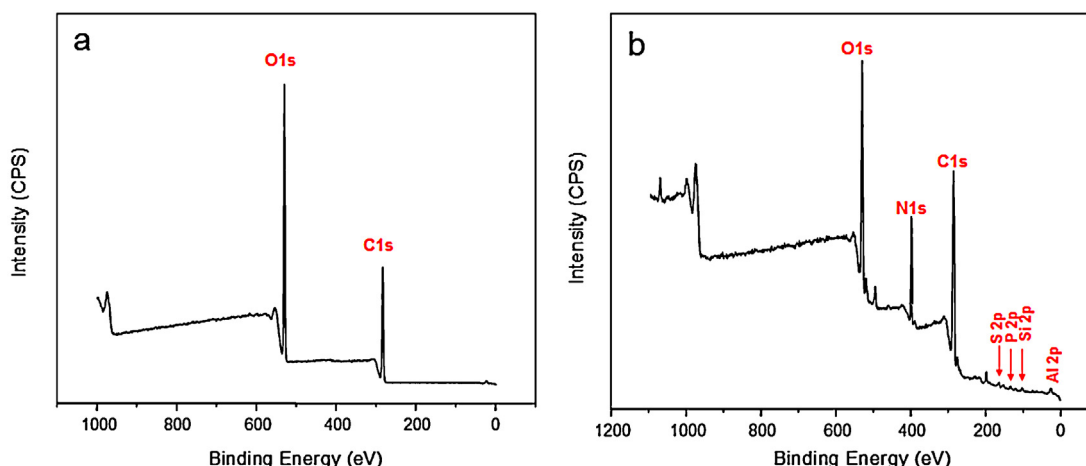


Fig. 4. XPS wide scans with the curve fit of: (a) CA nanofibrous mats and (b) CA nanofibrous mats coated with 5% LY-REC composites.

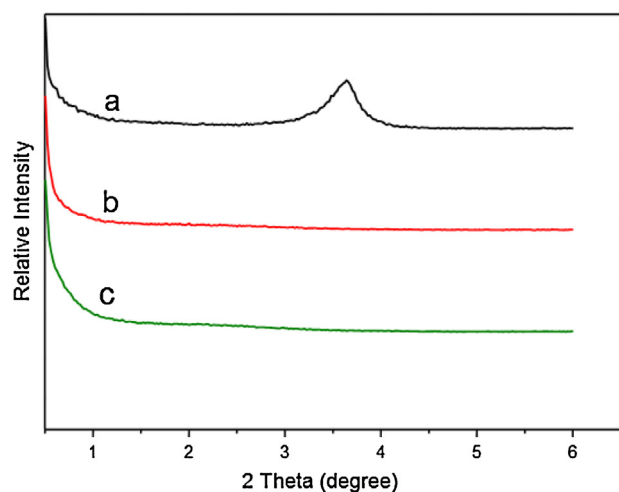


Fig. 5. SAXRD patterns of: (a) REC powder, (b) CA fibrous mats coated with 5% LY-REC composites and (c) CA fibrous mats coated with 10% LY-REC composites.

spectrum exhibited its peak at 73.8 eV (the metallic Al and oxidic peaks) was found in REC (Chang, Anderegg, & Thiel, 1996).

3.4. X-ray diffraction investigation

SAXRD patterns of mats were illustrated in Fig. 5 and the inter-layer distance of REC was calculated by Bragg's equation (Deng et al., 2011). There was a remarkable peak from the sample of REC powders observed in Fig. 5a which was at 3.64° and its interlayer distance was 2.43 nm. Interestingly, compared with REC powder, CA mats coated with LY-REC composites had no obvious peak. As mentioned above, all the FT-IR, EDX and XPS analysis could prove the incorporation existed between REC and LY. The cationic group of LY intercalated into the interlayers of REC generated electrostatic repulsion force and compelled the distance between the interlayers to become large enough to accomplish an overwhelming balance, which revealed the addition of REC enlarged the spacing distance too much, and at last the silicate layers of REC were completely exfoliated. The high content of LY strengthened the interaction between macromolecule chains and the REC particles, as well as promoted the capability of intercalation to exfoliation so that the crystallization of the LY-REC composites could not be investigated (Liu et al., 2012).

3.5. LY activity assay

The LY activity in the composite nanofibrous mats was shown in Fig. 6A, while the activity of free LY in solution was about 4700 U mg^{-1} . CA mats coated with 10% LY-REC composites showed the highest activity of 396.3 U cm^{-2} , which was slight higher than that of CA mats coated with 5% LY-REC composites. The results revealed that the activity of LY ascended as the concentration of LY increased, besides, the comparison between the results of CA mats coated with 2.5% LY and 5% LY was also the evidence of this conclusion. To compare the results of samples c and d, it could be observed that REC also had great effect on the activity of LY. When REC was added, the activity of LY increased significantly. The mainly reason was speculated as follows: Firstly, LY had strong antimicrobial effect and REC exhibited significant synergistic antibacterial ability. The combination of LY and REC could have an efficient inhibition on *M. lysodeikticus*, thus decreasing the turbidity of the *M. lysodeikticus* solution. Secondly, as to the mats coated with LY-REC, some LY molecules could be adhered to the surface of REC or absorbed in the interlayer of REC, and when the composites was immersed into the

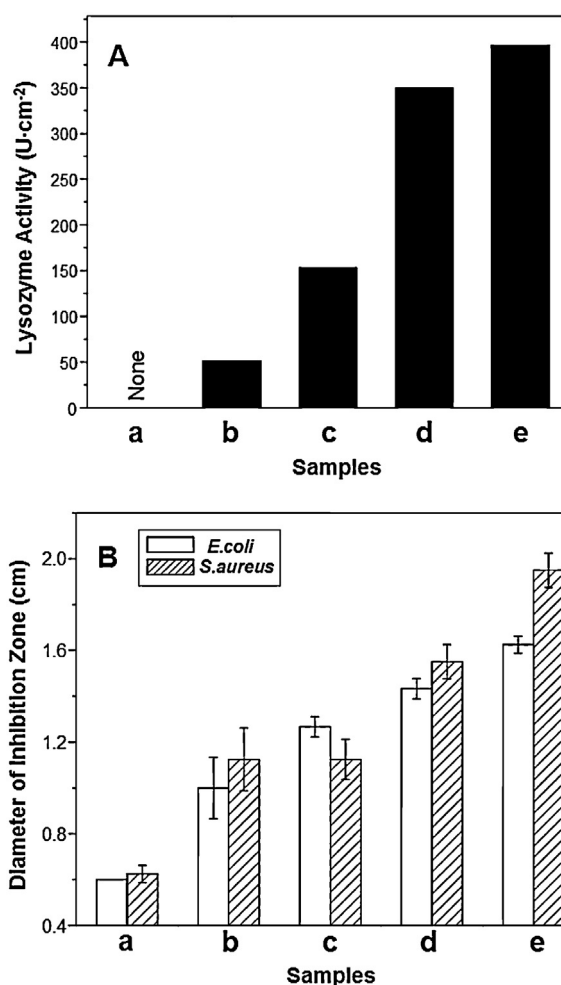


Fig. 6. (A) The activity of immobilized LY and (B) antibacterial activity against *E. coli* and *S. aureus* of (a) CA nanofibrous mats and the mats coated with: (b) 2.5% LY, (c) 5% LY, (d) 5% LY-REC composites and (e) 10% LY-REC composites.

M. lysodeikticus solution, more chances would be obtained to contact with *M. lysodeikticus*. Thirdly, REC had high surface-to-volume ratio and strong absorbability, and it could adsorb *M. lysodeikticus* powders, thus resulting in the decrease of the turbidity of the *M. lysodeikticus* solution.

3.6. Antibacterial assay

The antimicrobial effect of the composite mats against the Gram-negative bacteria (*E. coli*) and the Gram-positive bacteria (*S. aureus*) was shown in Fig. 6B, and the result was measured based on the diameter of zone surrounding circular films. CA mats, regard as the negative controls, showed little inhibitory effect and the diameter of inhibition zone was about 6.25 mm against *S. aureus* while 6 mm against *E. coli*, respectively. The CA film disks isolated oxygen and carbon dioxide from contacts with bacteria on the surface of the bacteria colonies, so the bacteria could not survive (Deng et al., 2012). All mats coated with LY or LY-REC composites exhibited antibacterial activity, which was affected by the concentration of LY and the addition of REC. LY could hydrolyze the peptidoglycan wall by breaking the β -1,4-glycosidic bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine, thus resulting in cell lysis (Vanderkelen et al., 2011). CA mats coated with 10% LY-REC composites had the strongest antibacterial activity and the diameter of inhibition zone against *S. aureus* almost reached 20 mm. This mats also showed the greatest antibacterial effect against *E. coli*.

Table 2Release profiles of LY including its content and activity from the composite fibrous mats. All data shown are the mean $\pm$ standard deviations ($n = 3$).

Samples	Release profiles of LY content from the composite mats					
	0.5 h	1 h	2 h	4 h	8 h	12 h
2.5% LY	8.59 $\pm$ 0.46%	11.76 $\pm$ 0.99%	11.97 $\pm$ 0.92%	14.51 $\pm$ 0.75%	18.75 $\pm$ 0.63%	19.68 $\pm$ 0.77%
5% LY	8.41 $\pm$ 1.02%	7.18 $\pm$ 0.93%	7.25 $\pm$ 0.89%	8.82 $\pm$ 0.38%	10.30 $\pm$ 0.58%	10.81 $\pm$ 1.13%
5% LY-REC	7.59 $\pm$ 0.98%	6.80 $\pm$ 1.01%	7.11 $\pm$ 1.33%	8.94 $\pm$ 0.62%	10.12 $\pm$ 0.39%	10.36 $\pm$ 0.81%
10% LY-REC	4.42 $\pm$ 1.23%	3.77 $\pm$ 0.97%	3.84 $\pm$ 0.82%	4.32 $\pm$ 0.48%	5.05 $\pm$ 0.67%	5.25 $\pm$ 0.71%
Samples (U cm ⁻²)	LY activity on the surface of the mats after release in distilled water					
	0.5 h	1 h	2 h			
2.5% LY	68.52 $\pm$ 8.06	91.67 $\pm$ 14.36	30.56 $\pm$ 7.84			
5% LY	201.85 $\pm$ 13.05	117.60 $\pm$ 10.06	29.63 $\pm$ 6.31			
5% LY-REC	206.48 $\pm$ 15.12	180.56 $\pm$ 10.15	58.33 $\pm$ 7.87			
10% LY-REC	209.26 $\pm$ 15.73	191.67 $\pm$ 11.11	90.74 $\pm$ 9.91			

Besides, the addition of REC was of great significance to the bacterial inhibition, and the result also coincided with the enzyme activity analysis mentioned above. The mechanism was the outstanding adsorption capacity of REC, which could inhibit the proliferation of bacteria. In addition, the interlayer distance of REC was enlarged by LY molecules, thus giving more chance in efficient contact and reaction between the composite mats and bacteria. The antibacterial capacity of almost all the samples against *S. aureus* was higher than that against *E. coli*, which was exactly in accordance with the conclusion that LY exhibited more effective antimicrobial capacity against Gram-positive bacteria than Gram-negative bacteria due to the protection of lipopolysaccharide layer surrounding their outermost membranes (Huang et al., 2012b).

In the twofold actions of REC and LY, the composites could destroy the cell wall, permeate through the cell membrane, and then disturb the ordinary vital movement and natural metabolism of the bacterial cell, thus resulting in the quick lysis of microorganisms.

3.7. *In vitro* release tests

In order to model the release system in antibacterial assay and investigate the controlled release properties of LY-loaded composite fibrous mats, *in vitro* release tests were performed in distilled water. The release results were shown in Table 2. Release of encapsulated proteins from films was characterized by an initial protein burst, due to the presence of pores in the matrix or liberation of surface-bound protein (Bezemer, Grijpma, Dijkstra, Feijen, & van Blitterswijk, 1999), and it could be seen in Table 2 that the initial protein burst did occur. Besides, the amount of released LY decreased after the initial protein burst, because some of free LY in the solution was reabsorbed onto the surface of the composite fibrous mats (Xu et al., 2012). In addition, REC had a little effect on the release rate and the protein release content of 5% LY-REC coated mats was depressed compared with that of 5% LY coated mats. It was because 5% LY-REC coated mats had larger distance between the REC interlayers which could entrap more LY, which indicated the protein release speed was affected by the addition of REC. Moreover, the release content profiles showed the release rate was low, especially in 10% LY-REC coated mats, which meant the enzyme loading and encapsulation were very successful and efficient. The activity of LY in distilled water was almost lost after 2 h (Caussette et al., 1997), so the release profiles of LY activity from the composite mats were determined within 2 h. Table 2 showed that the activity of LY was decreasing in 2 h for the effect of distilled water. As we knew, biological activity of enzyme was directly related to the substrate specificity, activity, selectivity, stability and pH optimum (Sheldon, 2007). The distilled water

could not provide various optimum conditions, and it also could affect the enzyme properties directly, leading to the composite fibrous mats losing the activity of LY.

4. Conclusions

The electrospraying technique, driven by physical deposition and electrostatic force, was successfully performed for the fabrication of CA mats coated with LY-REC. The morphology of the resultant samples with perfect fiber shape and 3D structure was significantly affected by the concentration of LY and the addition of REC, and the two components were nicely distributed on the surface of CA mats. The perfect intercalation of LY molecules into the interlayers of REC and the composites electrosprayed on the surface of CA mats was verified. The enzyme activity and antimicrobial effect assay depicted the excellent antibacterial capacity of the composite mats due to the immobilization of REC. Release profiles of LY content and LY activity over different period verified the enzyme loading was successful and efficient, especially suitable for industrialized application due to the properties of the long-term operational stability and reuse of enzymes. The novel method for the immobilization of enzyme onto substrates via electrospraying technique could be of great significance on food packaging, pharmaceutical application and antimicrobial wound dressing.

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