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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.

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 (Pellegrini 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, electrospinning was regarded as a mature and simple way. The combination of the two techniques mentioned above was called electrospinning–electrospraying 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 (Taqieddin & 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 MΩ 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 mental need 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 electrospinning, 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 electrospinning 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