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Chitosan/phosvitin antibacterial films fabricated via layer-by-layer deposition

Bin Zhou [a,b], Ying Hu [a,b], Jing Li [a,b], Bin Li [a,b,*]

[a] College of Food Science and technology, Huazhong Agriculture University, Wuhan 430070, China
[b] Key Laboratory of Environment Correlative Dietology (Huazhong Agricultural University), Ministry of Education, Wuhan, China

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ABSTRACT

Negatively charged phosvitin (PV) and positively charged chitosan (CS) were alternately deposited on negatively charged cellulose mats via layer-by-layer (LBL) self-assembly technique. The deposition of PV and CS was confirmed by X-ray photoelectron spectroscopy (XPS), Fourier transform infrared spectra (FT-IR), and wide-angle X-ray diffraction (XRD). Morphologies of the LBL films coating mats were observed by scanning electron microscope (SEM). Thermal degradation properties were investigated by differential scanning calorimetry (DSC) and thermo-gravimetric analysis (TGA). Additionally, the result of microbial inhibition assay indicated that the composite nanofibrous mats had excellent antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, which could be used for antimicrobial packing, tissue engineering, wound dressing, etc.

1. Introduction

Electrostatic layer-by-layer (LBL) self-assembly technique, introduced by Decher and Hong, has been one of the most frequently utilized process for preparation of functional multilayer films [1–3]. The process is commenced by adsorbing a polyelectrolyte onto an oppositely charged surface, thereby reversing the surface charge. Further layers are added by repeating the process until the desired film thickness is reached [3,4]. In general, multilayer films with oppositely charged polyelectrolytes have enabled the engineering of functional coatings for a wide range of applications, from sensors and solar cells to biomaterial and tissue engineering [5]. Recently, nanofibrous mats obtained via electrospinning were used as templates for LBL deposition because of their unique characteristics such as ultra-thin fiber diameter, small pore size, high specific surface, three-dimensional structure, etc [6,7].

Up to now, various deposition materials have been utilized to fabricate the functional LBL structured composite films including proteins, polysaccharides, metal irons, particles, etc [8,9]. Composite materials could be more effective and attractive than single material. Nowadays, polyanion/polycation composites, include protein–polysaccharide, polysaccharide–polysaccharide, protein–other polymers, and others, attracted intensive attentions and they could be good candidates as deposition materials to form LBL structured films to improve the properties of the templates.

In the past decades, lots of antibacterial agents are selected to add into the nanofibrous mats. As antibacterial agents, the safety should be considered firstly, which would limit their application area greatly [10]. Chitosan is an attractive natural material owing to its good properties and abundance in nature, it is useful in many different applications. One of the most valuable properties of chitosan is its inherent antimicrobial activity which is mainly due to its polycationic nature. Its cationic amino group associates with anions on the bacterial cell wall, suppressing biosynthesis; moreover, CS disrupts the mass transport across the cell wall accelerating the death of bacteria [11]. In addition, phosvitin (PV) is a polyanionic phosphoglycoprotein present in egg yolk and represents about 7% of yolk proteins, of the known proteins, it is the most phosphorylated one [12,13]. This unique primary structure makes this protein one of the strongest metal (iron, calcium, etc.) chelating agents. And the iron binding capabilities contribute to the antimicrobial properties of phosvitin [14]. It can damage the outer membrane and kills *E. coli*, a model strain of Gram-negative bacteria, under thermal stress [15,16].

In this work, novel LBL films on nanofibrous polysaccharide template, specifically cellulose nanofibers hydrolyzed from electrospun cellulose acetate nanofibers. Chitosan and phosvitin were selected as deposition materials to be coated on cellulose nanofibers via electrostatic LBL self-assembly technique by alternate adsorption of positively charged chitosan and negatively charged phosvitin

* Corresponding author at: College of Food Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, China.
Tel.: +86 27 6373 0040/+86 27 8728 6847; fax: +86 27 8728 8636.
E-mail address: libinfood@mail.hzau.edu.cn (B. Li).

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in aqueous media. The effect of the outermost layer variation, the number of deposition bilayers, and the composition of the multilayer on the formation of the LBL structured nanofibrous mats was investigated. Meanwhile, the bacterial inhibition experiments were performed under different temperature to determine the antimicrobial property of the resultant samples.

2. Materials and methods

2.1. Materials

Cellulose acetate (CA, $M_n = 3 \times 10^4$) was purchased from Sigma–Aldrich Co., USA. Chitosan (CS, $M_w = 2.0 \times 10^5$ kDa) from shrimp shell with 92% deacetylation was provided by Zhejiang Yuhuan Ocean Biochemical Co., China. The other reagents were analytical grade purchased from China National Pharmaceutical Group Industry Corporation Ltd. All aqueous solutions were prepared using purified water with a resistance of 18.2 MΩ cm. *E. coli* and *S. aureus* were obtained from China Center for Type Culture 140 Collection, Wuhan University (Wuhan, China).

2.2. Preparation of PV from hen eggs

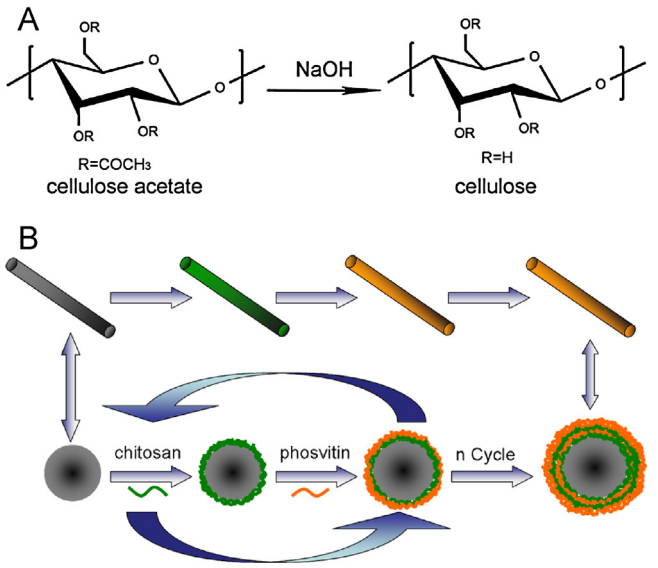
Hen egg yolk phosvitin was isolated by modifying the Losso and Nakai method [17]. Egg yolk was lightly washed with distilled water and rolled on filter paper to remove adhering albumen. The yolk membrane was punctured with a needle and the contents were collected. Then, 100 g of yolk were diluted with 0.5 L of cold water at pH 5.0 and stirred at 4 °C for 1 h. The precipitate was collected by centrifugation at 10,000*g* for 20 min at 4 °C. The pellet was dissolved in 200 mL distilled water, stirred for 1 h and centrifuged at 10,000*g* for 20 min at 4 °C. The pellet was extracted with 400 mL of hexane:ethanol (3:1, v:v) at 4 °C for 3 h and centrifuged. The resulting cake was dried and extracted with 200 mL of 1.74 M NaCl overnight at 4 °C. Then the suspension was centrifuged at 10,000*g* for 20 min at 4 °C and the supernatant was dialyzed against distilled water for 24 h at 4 °C and freeze dried.

2.3. SDS-PAGE

PV was dissolved in double distilled water, loaded and electrophoresed on a 12.5% SDS-PAGE gel to check its purity. SDS-PAGE was performed in 1.0 M Tris–HCl buffer (pH 6.8) for stacking gel (5% acrylamide) and 1.5 M Tris–HCl buffer (pH 8.8) for separating gel (12.5% acryamide). Migration was performed at 80 and 120 V in the stacking gel and separating gel, respectively. After electrophoresis, the gel was stained using the modified staining solution containing 0.05% Coomassie brilliant blue R-250, 0.1 mol/L aluminum nitrate, 25% isopropanal,10% acetic acid and 1.0% Triton X-100 [18,19].

2.4. Electrospinning of CA nanofibrous mats and their hydrolysis

Nanofibrous CA mats were fabricated by Deng's method [20]. Briefly, 17 wt% CA solution prepared in a 2/1 (w/w) acetone/DMAc mixture. The CA solution was fed into a plastic syringe, which was driven by a syringe pump (LSP02-1B, Baoding Longer Precision Pump Co., Ltd., China). The positive electrode of a high voltage power supply (DW-P303-1ACD8, Tianjin Dongwen Co., China) was clamped to the metal needle tip of the syringe. A grounded cylindrical layer was used as a collector which rotated with a linear velocity of 100 m/min. The applied voltage was 16 kV and the tip-to-collector distance was 20 cm. The ambient temperature and relative humidity were maintained at 25 °C and 45%, respectively. The prepared fibrous mats were dried at 80 °C in vacuum for 24 h to remove the trace solvent. Hydrolysis of the CA mats was performed in a 0.05 M NaOH aqueous solution at ambient temperature for 7 days following the previous report. And the schematic diagram of hydrolysis of CA was shown in Scheme 1A.



Scheme 1. (A) Schematic diagram illustrating the hydrolysis of CA nanofibrous mats and (B) schematic diagram illustrating the fabrication process of the LBL film-coating cellulose mats.

2.5. Formation of LBL structured multilayer on nanofibrous mats

The concept for fabrication of LBL structured fibrous mats was shown in Scheme 1B. The concentration of the positively charged CS solutions was fixed as 1 mg/mL by dissolving them in a 0.5% (V/V) aqueous acetic acid solution and the pH of solutions was controlled at 5.0. The negatively charged PV solution was 1 mg/mL in pure water and the pH was adjusted to 6. The ionic strength of all dipping solutions was regulated by the addition of NaCl at a concentration of 0.1 mg/mL.

First, nanofibrous cellulose mats were immersed into CS for 20 min followed by 2 min of rinsing in three pure water baths [21]. The mats were then immersed into the PV solution for 20 min, followed by the identical rinsing steps. The adsorption and rinsing steps were repeated until the desired number of deposition bilayers was obtained. Here, $(CS/PV)_n$ was used as a formula to label the LBL structured films, where *n* was the number of the CS/PV bilayers. The outermost layer was CS composite when n equaled to 5.5 and 10.5. The LBL films coated fibrous mats were dried at 80 °C for 24 h under vacuum prior to further characterizations.

2.6. Characterization

The morphology and composition of fibrous mats were examined by scanning electron microscopy (SEM) (S-4800, Hitachi Ltd., Japan). The diameters of the fibers were measured using an image analyzer (Adobe Photoshop CS5.0). Fourier transform infrared (FT-IR) spectra were recorded using a Nicolet170-SX instrument (Thermo Nicolet Ltd., USA) in the wavenumber range of 4000–400 cm^{-1}. The surface elemental composition of the samples was identified by X-ray photoelectron spectroscopy (XPS) using an axis ultra DLD apparatus (Kratos, U.K.). X-ray diffraction (XRD) was carried out using a diffract meter type D/max-rA (Rigaku Co., Japan) with Cu target and Ka radiation ($\lambda = 0.154$ nm). ξ-potential analysis was preformed using a Nano-25 zetasizer (Malven, England).