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Surface modification of nanofibrous poly(acrylonitrile-*co*-acrylic acid) membrane with biomacromolecules for lipase immobilization

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ABSTRACT

In this work, poly(acrylonitrile-*co*-acrylic acid) (PANCAA) was electrospun into nanofibers with a mean diameter of 180 nm. To create a biofriendly microenvironment for enzyme immobilization, collagen or protein hydrolysate from egg skin (ES) was respectively tethered on the prepared nanofibrous membranes in the presence of 1-ethyl-3-(dimethyl-aminopropyl) carbodiamine (EDC)/*N*-hydroxyl succinimide (NHS). Confocal laser scanning microscopy (CLSM) was used to verify the surface modification and protein density on the nanofibrous membranes. Lipase from *Candida rugosa* was then immobilized on the protein-modified nanofibrous membranes by covalent binding using glutaraldehyde (GA) as coupling agent, and on the nascent PANCAA nanofibrous membrane using EDC/NHS as coupling agent, respectively. The properties of the immobilized enzyme were assayed. It was found that different pre-tethered biomacromolecules had distinct effects on the immobilized enzyme. The activity retention of the immobilized lipase on ES hydrolysate-modified nanofibrous membrane increased from 15.0% to 20.4% compared with that on the nascent one, while it was enhanced up to more than quadrupled (activity retention of 61.7%) on the collagen-modified nanofibrous membrane. The kinetic parameter, K_m and V_{max}, were also determined for the free and immobilized lipases. Furthermore, the stabilities of the immobilized lipases were obviously improved compared with the free one.

1. Introduction

Biotransformations catalyzed with enzymes have been pursued extensively, largely as a result of their chemio-, regio-, and stereoselectivity, mild reaction conditions and high level of catalytic efficiency [1–4]. While practical applications prefer immobilized enzymes since they offer easy catalyst recycling, feasible continuous operations and simple product purification. For enzyme immobilization, the support materials have a great impact on the performance of the immobilized enzymes. The improvement of biocatalytic efficiency can be achieved by manipulating the structure of supports for enzyme immobilization. Among various supports, nanofibrous membranes have gained widespread attentions due to their large surface area-to-volume ratio and fine porous structure. They can provide relatively high quantity of enzyme loading per unit mass and low diffusion resistance necessary for high reaction rate and conversion. What's more, in comparison with nanoparticles, nanofibrous membranes are easier to recycle from reaction media and thus benefit continuous operation [5–18].

Electrospinning has been recognized as an effective way to fabricate polymeric nanofibers with diameter ranging from several micrometers down to tens of nanometers. Among various polymers, acrylonitrile-based homo- and co-polymers were most recently fabricated into nanofibrous materials with reinforcing, superhydrophobic, and/or catalytic properties [17–25]. In our previous work [17], novel nanofibrous membranes that possess reactive carboxyl groups were fabricated from poly(acrylonitrile-*co*-maleic acid) (PANCMA) by the electrospinning process. Lipase was covalently immobilized onto these membrane surfaces via the activation of carboxyl groups. It was found that the enzyme loading and the activity retention of immobilized enzyme on the nanofibrous membrane were much higher than those on the PANCMA hollow fiber membrane. Therefore, the process described in our work presents a convenient approach to fabricate nanofibrous membranes with reactive groups for enzyme immobilization.

However, similar to many other synthetic polymer materials, the relatively poor biocompatibility of acrylonitrile-based polymers probably causes some non-biospecific interactions between enzymes and the supports surface, thus, resulting in partial denaturation of enzyme protein and loss of enzyme activity. Lipases have two forms, the closed form with its active site covered by

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a polypeptide chain called lid and the open form with the lid displaced and its active site exposed to the reaction medium. Some non-biospecific interactions between lipases and the supports surface may lead to the transition from the open form to the closed form. To suppress these unfavorable interactions, surface modification is a major approach that can enhance the surface biocompatibility of supports while keeping the bulk properties intact. It was demonstrated that surface modification with natural macromolecules could create a favorable microenvironment for the immobilized enzyme to retain its activity and enhance its stabilities. Collagen, one of the main proteins in extracellular matrix, has been successfully used to modify biomaterials such as polyurethane [26], polystyrene [27] and titanium [28]. Protein hydrolysate from egg skin (ES), which is easily available and low in cost, includes different molecular weight polypeptides and a little polysaccharide. Both biomacromolecules contain reactive groups (amino groups) and could be used to form biomimetic layers on the poly(acrylonitrile-*co*-acrylic acid) (PANCAA) membrane surface for enzyme immobilization. Furthermore, collagen is a triple helix structure called tropocollagen. Unlike the whole cylindrically shaped collagen, protein hydrolysate from egg skin is a mixture of various fragmental peptides with various lengths. Therefore, it may provide a possibility to directly compare the interaction difference of fragmental polypeptide-chain/enzyme and protein-unit/enzyme at similar condition by tethering them on the same support for enzyme immobilization. To create biofriendly surfaces for immobilized enzyme, these two biomacromolecules were tethered on the PANCAA nanofibrous membranes via covalent binding. Lipase was immobilized on the modified nanofibrous membranes and the performance of the immobilized enzymes was investigated.

2. Experimental

2.1. Materials

PANCAA with a viscosity-averaged molecular weight (M_v) of 2.4×10^5 g/mol was synthesized by a water phase precipitation co-polymerization process. The molar content of acrylic acid in this co-polymer is about 15.6% by elemental analysis method. Type I collagen (from bovine Achilles tendon), lipase (from *Candida rugosa*), 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *p*-nitrophenyl palmitate (*p*-NPP) were purchased from Sigma and used as received. Fluorescein isothiocyanate (FITC) (HPLC grade) was commercially obtained from Fluka. Coumassie brilliant blue G250 (CBBG) for the Bradford protein assay was purchased from Sinopharm Chemical Reagent Co. Ltd. and bovine serum albumin (BSA, BP0081) was obtained from Sino-American Biotechnology Co. *N*-Hydroxyl succinimide (NHS) and glutaraldehyde (GA) are biological grade. Other reagents are of analytical grade without further purification.

2.2. Preparation of PANCAA nanofibrous membranes by electrospinning

PANCAA was dissolved in dimethylformamide (DMF) at 60 °C with gentle stirring for 12 h to form a homogeneous solution of 4 wt.%. Electrospinning was carried out using a syringe with a 1.2 mm diameter stainless steel spinneret at an applied electrical potential difference of 13 kV over 15 cm gap between the spinneret and the collector. The feed rate of solution from the needle outlet was kept constant at 1.0 mL/h by a microinfusion pump (WZ-50C2, Zhejiang University Medical Instrument Co., Ltd., China). The collector is a flat plate wrapped with conductive aluminium film. It usually took 3 h to obtain nanofibrous membrane with sufficient thickness and uniform fiber diameter of about 180 nm. All the nanofibrous membranes were dried under vacuum at 80 °C for 24 h to remove residual solvent before use.

2.3. Preparation of ES hydrolysate

Hydrolysis of egg shells was followed Li's method with some modification [29]. Egg shells were collected and immersed in water for 2 h. ES was peeled from egg shell, thoroughly washed with de-ionized water, and dried in vacuum oven at room temperature. A sample of 2 g ES and 4 g $Ba(OH)_2{\cdot}8H_2O$ was dissolved in 40 mL de-ionized water and stirred for 5 h at 60 °C. After this, 10% H_2SO_4 solution was added into the mixture to terminate the hydrolysis and modulate the pH value of the solution to 4.0. The mixture was filtrated and the collected solution was concentrated by distillation and then dried under vacuum at 60 °C for 24 h to remove residual water before use. The molecular weight of ES hydrolysate was characterized by GPC.

2.4. Preparation of nanofibrous membranes modified with ES hydrolysate or collagen

An appropriate amount of nascent PANCAA nanofibrous membrane was thoroughly washed with de-ionized water, and then rinsed with phosphate buffer solution (PBS, 50 mM, pH 7.0). After this, the membrane was submerged into an EDC/NHS solution (10 mg/mL in PBS (50 mM, pH 7.0), the molar ratio of EDC to NHS = 1:1) and shaken gently for 2 h at room temperature. The activated membrane was taken out, washed several times with PBS (50 mM, pH 7.0) and then submerged into the ES hydrolysate solution (0.5 or 2 mg/mL in PBS (50 mM, pH 7.0)) or collagen solution (0.5 mg/mL or 2 mg/mL in 10 wt.% acetic acid water solution) and shaken gently for 3 h at 4 °C. The ES hydrolysate-modified membrane was washed with PBS for 10–12 times. The collagen-modified membrane was washed with acetic acid solution (10 wt.%) for 10–12 times and the residual acids were removed by rinsing with abundant PBS.

2.5. FITC labeling and CLSM examination

A common protocol described previously [30] was used for FITC labeling of amino group carrying nanofibrous membrane. Confocal laser scanning microscopy (CLSM, Leica, Germany) was then applied to verify the biomacromolecules (such as ES hydrolysate and collagen) located on the surface of PANCAA nanofibrous membrane. The modified membrane was immersed in 5.0 mL PBS (50 mM, pH 7.0), containing freshly dissolved FITC (0.1 mg/mL). The labeling was performed in a dark room and shaken gently in a water bath at 4 °C for 24 h. The same labeling protocol was also applied to the original PANCAA nanofibrous membrane for comparison. To remove physically adsorbed FITC, both nanofibrous membranes were extensively washed with PBS (50 mM, pH 7.0). The nanofibrous membranes labeled with FITC were observed by CLSM equipped with He-Ne laser. High-resolution images of the labeled membrane were taken with a 63× NA 1.4 lambda blue oil objective. These images were obtained by fixing the excitation wavelength at 488 nm. Relative fluorescent intensity was obtained with a 20× NA 0.7 dry objective at the xyz-scan mode.

2.6. Immobilization of lipase onto the modified nanofibrous membranes

Lipase solution (8 mg/mL) was prepared by adding appropriate amount of lipase powder to PBS (50 mM, pH 7.0). Lipase was immobilized onto the ES hydrolysate or collagen-modified membrane by