DYE AFFINITY NANOFIBER MEMBRANE FOR ADSOPRTION OF LYSOZYME

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Abstract - Polyacrylonitrile (PAN) nanofiber membrane was prepared by electrospinning technique. After heat treatment and alkaline hydrolysis, the ion exchange membrane (P-COOH) was grafted with chitosan molecule. The chitosan modified membrane (P-CS) was then covalently immobilized with Reactive orange 4 to be used as a dye ligand affinity membrane (P-Dye). The dyed membrane was used to evaluate the binding capacity of lysozyme under various operating parameters (e.g., adsorption pH, chitosan and dye concentrations, and temperature) were studied in batch modes. The results revealed that the optimal adsorption pH for lysozyme was pH 8 and the optimal concentration of chitosan for use in preparation of membrane was 1.0%. Moreover, the optimal immobilization density of dye was 90.52 (μmol dye/g membrane). The results showed that the dye affinity nanofiber membrane may be an ideal support for the adsorption and purification of lysozyme because of its low cost, simplicity of its preparation method, and high binding capacity.

I. INTRODUCTION

Membrane chromatography has been commonly used in separation and purification of proteins[1-3]. The selection and preparation of the membrane are key factors affecting the adsorption performance in chromatography techniques[4-5]. Various types of the membrane supports have been applied in the purification of proteins[1-5], such as cellulose membrane, hollow fiber membrane, modified chitosan membrane, and nanofiber membrane. Recently, polymer nanofiber membrane prepared by electrospinning technique is of interest to many applications in biotechnology fields[6-11], e.g., tissue engineering, bio-catalysis, filtration, protein adsorption, and antibacterial materials. In this work, PAN nanofiber membrane was chosen as a matrix. After heat and hydrolysis steps, the modified nanofiber with carboxyl groups was then grafted with chitosan molecule. A reactive dye ligand (Reactive Orange 4) was immobilized covalently onto the chitosan modified membrane via nucleophilic substitution reaction under alkaline conditions. The aim of this work was to investigate the adsorption characteristics of pure lysozyme by the dyed nanofiber membrane. The experiments were carried out under various conditions, such as pH, chitosan and dye concentrations and temperature.

II. EXPERIMENTAL

A. Materials

Polyacrylonitrile (PAN) yarn (Mw 120, 000, containing 93% acrylonitrile and 7% vinylacetate) was purchased from Fortune Industries Inc. (Tao-Yuan, Taiwan). Polyethyleneterephthalate (PET) spunbond fabric (basis weight 15 g/m^2, thickness 90 μm, fiber diameter 300-500 μm) was supplied from Freudenberg Far Eastern Spunweb Co., Ltd. (Tao-Yuan, Taiwan). Water soluble chitosan (molecular weight 50,000 g/mol) and Reactive Orange 4 dye were obtained from Charming & Beauty Co., Ltd. and First Chemical Manufacture Co., Ltd. (Taipei, Taiwan), respectively. All chemicals and solvents were used without further purification. Electrospinning device was purchased from Falco Enterprise Co., Ltd. (New Taipei City, Taiwan) and electrospinning process was conducted at room temperature. The pH of the adsorption buffer was monitored with a pH meter (Mode SP-2200, Suntex Ins. Co., Taiwan). A UV-Vis spectrophotometer (Model Ultrospec 3100 pro, GE Healthcare Biosciences, Uppsala, Sweden) was used to measure the absorbance of lysozyme samples.

A.Preparation of PAN nanofiber membrane When PAN

Yarn (15 g) was completely dissolved in 100 mL dimethylacetamide (DMAc). A 10 mL prepared PAN solution was loaded into a syringe with 21-gauge stainless steel nozzle. The electrospinning process was performed at 25oC and the operating parameters were determined based on the previous study by Chiu et al.8-9. The PAN nanofiber mat was collected on nonwoven PET, which was wrapped around the grounded steel drum. The PAN-PET-PAN membranes (the number of PAN nanofiber layer was 2, designated as PAN) were subjected to heat pressing process at 100oC for 1 h and further used in this study.

B. Preparation of ion exchange membrane

The PAN membrane (diameter 25 mm) was placed in 5 mL 3 M NaOH at 85^{o}C for 25 min. After alkaline treatment, the membrane was removed and successively washed with H_2O to remove the excess NaOH. The hydrolyzed membrane was then treated with 0.1 M HCl (designated as P-COOH), followed by washing and drying in an oven at 60oC before use.

C. Preparation of chitosan modified membrane

The carboxylated membrane was submerged into 5 mL 0.1 M MES (4-Morpholineethanesulfonic acid

hydrate) solution (pH 6.0), containing 100 μmol 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 100 μmol) and chitosan (0.2-1.2 mg/mL). The mixture was shaken at 100 rpm and 25°C for 3 h. Finally, the chitosan (CS) modified membrane was taken out, washed with H_2O to remove the free chitosan and then dried in an oven at 60°C.

D. Immobilization of dye onto chitosan modified membrane

A piece of activated CS membrane was placed in each flask, containing 3.0 mg/mL Reactive Orange 4, 20% NaCl in 5 mL 0.1 M Na2CO3 at pH 11.0. The reaction mixture was heated at 60 °C for 6 h in a sealed flask. After the reaction period, all uncoupled dye was removed. The dyed membranes (designated as P-Dye) were thoroughly washed with a series of solutions (i.e., warm H_2O, 10% ethanol, 1 M NaCl, and 6 M urea) until no dye molecule in the washing solution was detected by UV-vis spectrophotometer at 489 nm. Finally, the dyed membranes were stored at room temperature before use. The dye ligand density on the membrane can be determined by the difference of initial and final concentration of dye. The dye concentration in solution was measured by spectrophotometry at 489 nm.

E. Effect of operating parameters on lysozyme adsorption performance

Adsorption of lysozyme from aqueous medium on dyed membrane (3.14 cm^2) was firstly studied at various pH values (5 mL, 20 mM buffer solutions) in batch studies, including sodium acetate buffer (pH 4), sodium phosphate buffer (pH 6-8), glycine-NaOH buffer (pH 9-10), and sodium carbonate buffer (pH 11). The initial concentration of lysozyme was 1.0 mg/ml in each buffer solution. The adsorption experiments were conducted at 298 K and 100 rpm for 3 h. After this period had elapsed, the amount of adsorbed lysozyme on the membrane (mg lysozyme/g membrane) was determined by measuring the initial and final concentrations of lysozyme. Different sets of batch experiments were performed to investigate the effects of chitosan (0.05-1.2 mg/mL), dye (0.1–4.0 mg/ml), NaCl (0.1-1.0 M) concentrations during the adsorption process at 298 K. The effect of temperature on adsorption of lysozyme was studied in 5 mL 1.0 mg/mL lysozyme (pH 9) at different temperatures (4–37 °C). The lysozyme concentration in liquid phase was measured by UV-vis spectrophotometer at 280 nm. The extinction coefficient for lysozyme is 2.65 $(ml/mg \cdot cm)$[12]. The amount of adsorbed lysozyme on the membrane (mg lysozyme/g membrane) was calculated according to the following equation:

$$q = \frac{v(C_o - C)}{w} \quad (1)$$

where C_o and C (mg lysozyme/ml solution) are the initial and final concentrations of lysozyme in the aqueous, respectively. q (mg lysozyme/g membrane) is the final concentration of lysozyme in the membrane solid phase. v is the volume of aqueous phase (mL) and w is the weight of membrane (g). All experiments were performed in triplicate with SD < 5% and the data presented are the mean value calculated from these independent experiments.

F. Determination of lysozyme concentration

The total protein concentration was measured by the Bradford method using lysozyme as the standard.

III. RESULTS AND DISCUSSION

A. Morphology and physical properties of dye affinity nanofiber membrane

The physical properties and morphologies of the modified nanofiber membranes are observed by SEM and the results are shown in Fig. 1.

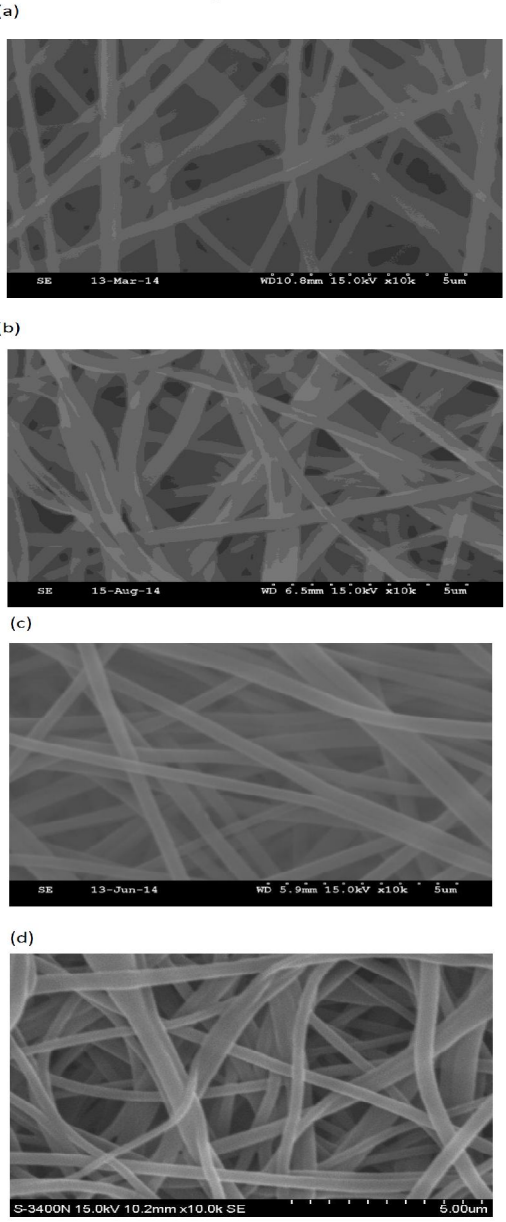


Fig. 1.SEM images of electrospunnanofiber membrane. (a) PAN, (b) P-COOH, (c) P-CS, (d) P-Dye

The prepared PAN membrane would be partially soluble as the hydrolysis time was longer than 25 min. On the contrary, as the hydrolysis time < 25 min, the membrane structure, the average diameter and mechanical strength were thereby maintained. Hence, the hydrolysis time was performed at 3M NaOH and 85oC for 25 min in this study. It has been observed that the P-COOH membranes are randomly oriented nanofibers after alkaline hydrolysis step. After chitosan and dye modification processes, the modified membranes have not significant influence on their overall morphology.

The PAN membrane (designated as PAN) used in the following experiments includes two-layer PAN nanofiber and one supporting layer (PET) to form a sandwich framework structure. After heating treatment and alkaline hydrolysis, the weak cationic exchange membrane (P-COOH) was obtained. The modified ion exchange membrane with carboxylic groups was then grafted with chitosan and further covalently immobilized with Reactive Orange 4 to be as a dye affinity nanofiber membrane.

B. Influence of adsorption pH

Studies on adsorption of lysozyme with dyed membrane in this work were carried out at pH ranges of 4-12 in a reciprocal shaking water bath at 25 ^{o}C. As shown in Fig. 2, the optimal adsorption pH for lysozyme was pH 8 and the maximum binding capacity was 334.2 mg/g. A number of studies have shown similar results that adsorption of proteins cannot be satisfactorily explained by the net charge concept. The adsorption process is primarily governed by the distribution of charges on the proteins[13-14].

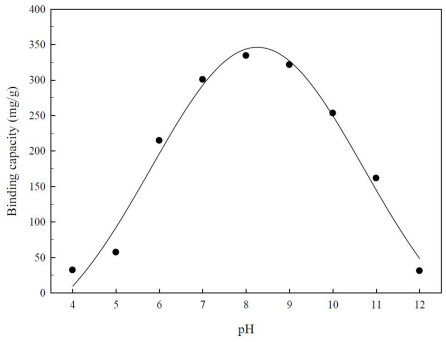


Fig. 2.The effect of adsorption pH on binding capacity of lysozyme.

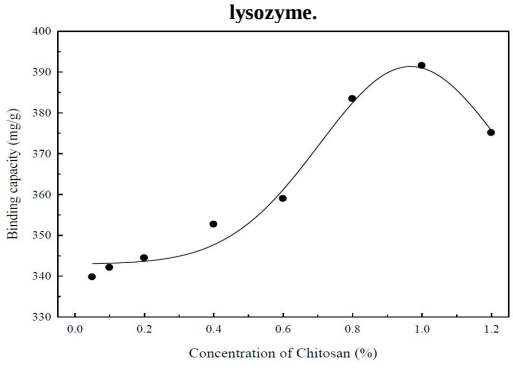


Fig. 3.The effect of chitosan concentration on binding capacity of lysozyme.

C. Influence of chitosan concentration

Chitosan chains have three reactive sites, including two hydroxyl groups (primary and secondary) and one primary amine (more active). The amide linkage between chitosan molecule with primary amino group and activated membrane with carboxylic group can be achieved by EDC mediated coupling reaction. In this work, the optimal coupling pH for EDC-mediated immobilized schemes was 6.0. The residual amino and hydroxyl groups of activated chitosan membrane can be easily modified with reactive dye molecules. The immobilized dye onto the activated chitosan membrane was carried out in the range of chitosan concentration from 0.05 to 1.2% (w/v) as shown in Fig. 3. The results showed that the optimal concentration of chitosan for use in preparation of chitosan modified membrane was 1.0%.

D. Influence of initial dye concentration

The degree of dye immobilized onto the membrane has been considered as an important parameter in adsorption of proteins. The results are shown in Fig. 4. The relationship between lysozyme binding capacity and immobilized dye concentration was shown in Fig. 5. At immobilized dye density of 90.52 (μmol dye/g-M), the maximum binding capacity for lysozyme was achieved (369.9 mg/g-M). The differences in binding capacity for lysozyme may be caused by one or more of the following factors, such as variation in immobilized and charge density of dye molecules, variation of charge distribution and arrangement of dye molecules, or interactions between lysozyme and dye molecules (e.g., ionic, hydrophobic, or specific interaction)[13-14].

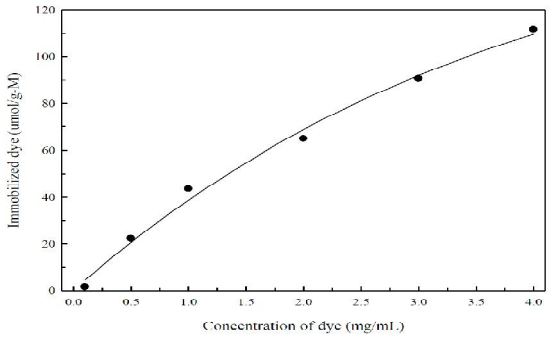


Fig. 4. Variation of the amount of dye immobilized onto chitosan membrane with various initial dye concentration.

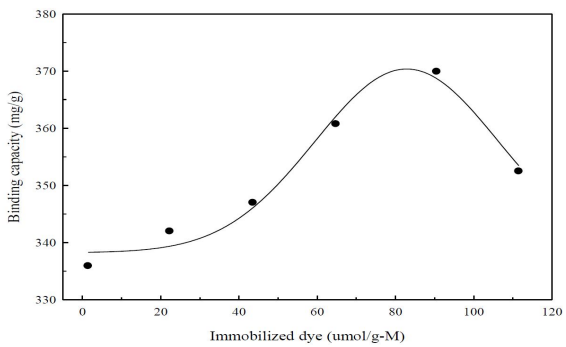


Fig. 5.The effect of immobilized dye density on binding capacity of lysozyme.

The effects of operating temperature on the binding capacity of lysozyme were investigated in the range of 277-323 K (Fig. not shown). The operating temperature at 25oC was to achieve the maximum binding capacity of 354.42 mg/g membrane. An increase in temperature caused an increased in binding capacity, which indicated that the adsorption of lysozyme exhibited positive temperature cooperativity. Similar results were also observed in the adsorption of lysozyme by other dyed membranes[13-14]. The increase in the binding capacity of the membrane would be related to the conformational changes of lysozyme molecule. In this case, this could be attributed to an increase in the hydrophobic interaction as the temperature increased[13].

CONCLUSION

In this work, lysozyme was chosen as a model protein and its binding characteristics onto the dye affinity nanofiber membrane was investigated by varying the adsorption pH, chitosan concentration for coupling reaction, initial lysozyme concentration for immobilization, salt concentration of NaCl, and operating temperature. The results showed that the optimal adsorption at pH 8, chitosan concentration of 1.0 mg/ml, initial dye concentration of 3.0 mg/ml and operating temperature at 25^{o}C were to achieve the highest binding capacity of 391.5 mg/g membrane. The molecular forces contributed to the interactions between lysozyme and dye molecules may be mainly ionic and hydrophobic interactions.

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