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Dye-Affinity Nanofibrous Membrane for Adsorption of Lysozyme: Preparation and Performance Evaluation

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SUMMARY

Polyacrylonitrile (PAN) nanofibrous membrane was prepared by an electrospinning technique. After heat treatment and alkaline hydrolysis, the weak ion exchange membrane was grafted with chitosan molecule and then covalently immobilized with a Cibacron Blue F3GA (CB). Fibre diameter, porosity and pore size of the membrane and immobilized dye density were characterized. Furthermore, the membrane was applied to evaluate the binding performance of lysozyme under various operating parameters (pH, chitosan mass per volume ratio, dye concentration, ionic strength and temperature) in batch mode. The experimental results were directly applied to purify lysozyme from chicken egg white by membrane chromatography. The results showed that the capture efficiency, recovery yield and purification factor were 90 and 87 %, and 47-fold, respectively, in a single step. The binding capacity remained consistent after five repeated cycles of adsorption-desorption operations. This work demonstrates that the dye-affinity nanofibrous membrane holds great potential for purification of lysozyme from real feedstock.

Key words: nanofibre, polyacrylonitrile, dye-affinity membrane, lysozyme, adsorption

INTRODUCTION

Various nanofibrous membranes can be prepared by using an electrospinning process and they have been widely applied in diverse fields, such as tissue engineering (*1,2*), drug delivery (*3*), biosensors (*4*), antibacterial membranes (*5*), filtration and adsorptive membranes (*6,7*), and wastewater treatment (*8*). Column chromatography is an effective technique for purification of proteins. However, there are many disadvantages, such as compressibility of the packed beds, fouling of the adsorbent beads, and slow operating flow rate. To overcome these problems, membrane chromatography may be an alternative technique (*9-12*).

Nanofibrous membranes designed for use in purification of proteins should feature high porosity, large specific area, high chemical, biological and mechanical stabilities, high hydrophilic properties, low non-specific adsorption, and fast binding kinetics (*6,10,12*). The high specific surface gives the nanofibrous membrane higher adsorption capacity and a faster adsorption rate than conventional membranes.

The polyacrylonitrile (PAN) membrane is less suitable for use in adsorption of proteins than hydrophilic cellulose membranes due to its hydrophobicity (*13,14*). Hence, the hydrophilic group must be introduced on the membrane surface to combine the advantages of both hydrophilic and hydrophobic membranes. Some advantages of the PAN membrane are its strong mechanical strength and chemical stability (*15,16*). Therefore, the PAN membrane was chosen for use in this work.

The chitosan molecule is a non-toxic, biodegradable and biocompatible biopolymer, and can be used to improve the hydrophilicity of the PAN membrane. It has high chemical reactivity and can be effective for use in the adsorption of proteins and heavy metals due to the presence of amino and hydroxyl groups in the chain (*17-19*). In recent years, various dye ligands have been immobilized on different types of membranes, such as PAN (*19*), cellulose (*20*), chitosan composite (*21*) and polyethersulfone (*22*). One of the most utilized dye ligands

is Cibacron Blue F3GA (CB) reactive dye, which is commonly used in the adsorption of proteins (*16,22*). There are many advantages of using biomimetic dyes as the ligand for purification of proteins, such as low cost, ease of immobilization, high binding capacity and medium specificity (*23*). The dye molecule can be directly coupled to hydroxyl and/or amino groups on the membrane surface. Therefore, chitosan molecule can be directly used to covalently bind CB molecules. In this work, PAN nanofibrous membranes were prepared by an electrospinning technique. The surface of PAN membrane was first hydrolysed with 3 M NaOH to convert nitrile group into carboxylic group, and chitosan molecule was then covalently bound to the membrane surface. Finally, the dye molecule was immobilized onto the chitosan membrane.

Chicken egg white contains a variety of proteins, often as the main source of lysozyme. It is the ideal model for use in the purification of lysozyme. In egg white proteins, ovalbumin, ovotransferrin and ovomucoid are the main components, accounting for 54, 12-13 and 11 %, respectively. Lysozyme is a minor component, accounting for 3.5 % and is a commercially valuable enzyme (*24*). It has considerable potential in the pharmaceutical and food industries (*25*). Until now, the adsorption behaviour of lysozyme on dyed nanofibrous membranes has not been studied in detail (*26*). Conventional dye-affinity membrane chromatography has been used to purify lysozyme from egg white (*27,28*). The objective of this study is to investigate the adsorption characteristics of pure lysozyme on dyed nanofibrous membranes. The batch experiments were carried out under various adsorption and elution conditions, such as pH, chitosan mass per volume ratio, dye concentration, ionic strength, temperature and hydrophobicity. Based on the results from batch studies, the lysozyme was purified from chicken egg white by dye-affinity nanofibrous membrane chromatography.

MATERIALS AND METHODS

Materials

Polyacrylonitrile (PAN) yarn (M=1.2·10^5 g/mol, containing 93 % acrylonitrile and 7 % vinyl acetate) was purchased from Fortune Industries Inc. (Taoyuan, Taiwan). Polyethylene terephthalate (PET) spunbond fabric (basis mass 15 g per m^2, thickness 90 μm, fibre diameter 300-500 μm) was supplied from Freudenberg Far Eastern Spunweb Co., Ltd. (Taoyuan, Taiwan). Water-soluble chitosan (M=5.0·10^5 g/mol) was obtained from Charming&Beauty Co., Ltd. (Taipei, Taiwan). Cibacron Blue F3GA reactive dye was obtained from the First Chemical Manufacture Co., Ltd. (Taipei, Taiwan). *Micrococcus luteus* cells and other chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of PAN nanofibrous membrane

Electrospinning device was purchased from Falco Tech Enterprise Co., Ltd. (New Taipei City, Taiwan) and consisted of syringe pump, high voltage DC power supply, spinning polymer solution, and rotating cylindrical collector, as shown in **Fig. 1**.

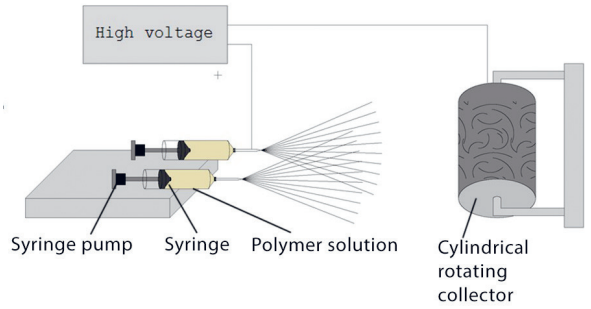

Fig. 1. Schematic representation of cylindrical rotating electrospinning assembly, including two-syringe pump, DC high voltage, and cylindrical rotating collector

PAN yarn (15 g) was dissolved in 100 mL of dimethylacetamide (DMAc). A volume of 10 mL of prepared PAN solution was loaded into a syringe with a 21-gauge stainless steel nozzle. The electrospinning process was performed at 298 K and the operating parameters were determined based on the previous study by Chiu *et al.* (*6,29*). The applied electric voltage, the distance between the nozzle tip and the collector, the feeding rate of the polymer solution, and the rotation rate of the collector were fixed at 26.5 kV, 15.8 cm, 1.0 mL/h and 24.0 cm/s, respectively. The nozzle moved along the y-axis (20.0 cm) at a frequency of 12 times per min. The PAN nanofibre mat was collected on PET fabric and wound on a ground steel drum. The PAN-PET-PAN membranes (one PET fibre and two PAN nanofibre layers, designated as AEA) were subjected to a heat pressing process at 373 K for 1 h and then further used in this study.

Preparation of ion exchange membrane

An amber glass bottle with safety seal (SV-50A, 50 mL) was obtained from Nichiden-Rika Glass Co., Ltd. (Kobe, Japan). The glass bottle containing one sheet of AEA membrane (effective area 4.91 cm^2, diameter 25 mm) in 5 mL of 3 M NaOH was placed in a constant temperature oven (model Risen PID-96T; Chemist Scientific Corp., New Taipei City, Taiwan) at 358 K for 25 min. After alkaline treatment, the membrane was removed and successively washed with water to remove the excess NaOH. The hydrolysed membrane was then treated with 0.1 M HCl (designated as AEA-COOH), followed by washing and drying in an oven at 333 K before use. The surface density of carboxylic groups on the AEA-COOH membrane was determined by dyeing with toluidine blue O (TBO; Sigma-Aldrich) as described by Yang and Lin (*30*).

Preparation of chitosan membrane

One sheet of AEA-COOH membrane (effective area 4.91 cm^2) was submerged into 5 mL of 0.1 M 4-morpholineethanesulfonic acid (MES) hydrate solution (pH=5.0)

containing 200 mmol of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and chitosan (0.1-1.0 %, mass per volume ratio). The mixture was shaken using an orbital shaker (model KS/OS-300; Chemist Scientific Corp.) at 100 rpm and 298 K for 3 h. Finally, the chitosan membrane was taken out, washed with water to remove the free chitosan and then dried in an oven (model Risen PID-96T; Chemist Scientific Corp.) at 333 K. The surface density of the amino groups on the chitosan membrane (designated as AEA-COOH-CS) was determined by dyeing with Acid Orange 7 (AO7) as described by Yang and Lin (*30*).

Immobilization of dye on the activated chitosan membrane

One sheet of chitosan membrane (effective area 4.91 cm^2) was placed in each glass bottle (50 mL), containing 0.5 mg/mL of Cibacron Blue 3GA (CB), 20 % NaCl (mass per volume ratio) in 5 mL of 0.1 M Na_2CO_3 at pH=11.0. The reaction mixture was heated at 333 K for 6 h in a sealed bottle. After the reaction period, all uncoupled dye was removed. The dyed membranes (designated as AEA-COOH-CS-dye) were thoroughly washed with a series of solutions (*i.e.* water at 333 K, 10 % ethanol (by volume), 1 M NaCl and 6 M urea) until no dye molecules were detected in the washing solution by a UV-Vis spectrophotometer (model Ultrospec 3100 Pro; GE Healthcare Biosciences, Uppsala, Sweden) at 620 nm. Finally, the dyed membranes were stored at room temperature before use. The dye ligand density on the membrane was determined by the difference between initial and final dye concentrations. The dye concentration in solution was measured by spectrophotometer at 620 nm (*31*).

Effect of operating parameters on lysozyme adsorption

The adsorption of lysozyme on the dyed membrane (one sheet of membrane, effective area 4.91 cm^2) was first monitored in buffer solutions of different pH, including sodium acetate buffer (pH=4), sodium phosphate buffer (pH=6-8), glycine-NaOH buffer (pH=9-10) and sodium carbonate buffer (pH=11). In each buffer solution, the initial concentration of lysozyme was 1.0 mg/mL and the adsorption experiments were done at 298 K and 100 rpm for 3 h. After completion of the adsorption, the initial and final concentrations of lysozyme were determined to calculate the amount of lysozyme adsorbed on the membrane. All experiments were performed under different batch conditions, including chitosan (0.05-1.0 %), dye (0.1-4.0 mg/mL), and NaCl (0.1-1.0 M). In addition, the effect of temperature on the adsorption of lysozyme (5 mL, 1.0 mg/mL of lysozyme, pH=9) was investigated in the temperature range between 277 and 323 K by using a shaking water bath (model B603DL; Firstek, New Taipei City, Taiwan). The lysozyme concentration in the liquid phase was measured by a UV-Vis spectrophotometer (model Ultrospec 3100 Pro; GE Healthcare Biosciences) at 280 nm. The molar absorptivity of lysozyme is ε=2.65 mL/(mg·cm) (*31*). The amount of adsorbed lysozyme on the dyed membrane (mg of lysozyme per g of membrane) was calculated by the following equation:

$$q = \frac{V \cdot (c_0 - c)}{m} \qquad /1/$$

where q (mg of lysozyme per g of membrane) is the binding capacity of the membrane for lysozyme, V is the volume of aqueous phase (mL), c_0 and c (mg of lysozyme per mL of solution) are the initial and final concentrations of lysozyme, respectively, and m is the mass of the membrane (g). All experiments were performed in triplicate with standard deviation (S.D.) and the data presented are the mean values calculated from these independent experiments.

Purification of lysozyme from chicken egg white

Nanofibrous membrane chromatography is a relatively new technique based on the integration of nanofibre filtration and liquid membrane chromatography into a single-step operation. In this experiment, one sheet of the dyed membrane placed in a membrane holder (diameter 25 mm, effective area 3.7 cm^2; Pall Corporation, Ann Arbor, MI, USA) integrated with an AKTAprime chromatographic system (GE Healthcare Biosciences) was applied to purify the lysozyme from clarified chicken egg white.

A volume of 10 mL of homogenized chicken egg white was diluted with 90 mL of 20 mM glycine/NaOH buffer (pH=9), followed by centrifugation (centrifuge model Allegra X-22R; Beckman Coulter, Inc., Fullerton, CA, USA) and filtration (filter pore size 0.45 μm) at 10 000×*g* for 20 min. A volume of 10 mL of supernatant was then pumped through the CB membrane filter holder at 0.1 mL/min, followed by washing with 10 mL of adsorption buffer at 1.0 mL/min. A two-step elution scheme with NaCl (*i.e.* 0.6 and 1.0 M) at 0.1 mL/min was further used to investigate the elution efficiency of adsorbed lysozyme. The eluents were collected and total protein and lysozyme activity were assayed.

Total protein and lysozyme activity assay

The total protein was measured using the Bradford method (*32*). The activity of lysozyme was assayed by lysis of 0.25 mg/mL of *M. luteus* cells in 100 mM sodium phosphate (pH=6.24) at 298 K. One unit of lysozyme activity was defined as the decrease of $A_{450\ nm}$ at 0.001 per minute (*33*).

RESULTS AND DISCUSSION

In this study, lysozyme was chosen as the model protein, and the dye-affinity nanofibrous membrane was used to assess the feasibility of the specific adsorption of protein under various operating parameters (pH, chitosan mass per volume ratio, dye concentration, ionic strength and temperature). Furthermore, the dyed membrane was used to directly purify lysozyme from chicken egg white as a complex feedstock.

Morphology and physical properties of dye-affinity nanofibrous membrane

It is well known that the morphology, mechanical properties and surface characteristics of the PAN nanofibre are significantly influenced by the polymer solution properties, processing parameters, and environmental conditions (*29*). The operating parameters for preparation of the PAN nanofibrous membrane used in this study were based on Chiu *et al.* (*29*). The PAN membrane (designated as AEA) used in this study includes a two-layer nanofibre (PAN) and one supporting layer (PET) to form a sandwich framework structure. After heating treatment and alkaline hydrolysis, the weak cationic exchange membrane (designated as AEA-COOH) was obtained. The modified ion exchange membrane with carboxylic groups was then grafted with chitosan and further covalently immobilized with Cibacron Blue F3GA (CB) to serve as the dye-affinity nanofibrous membrane. The scheme of the modification process of PAN membrane is shown in **Fig. 2**.

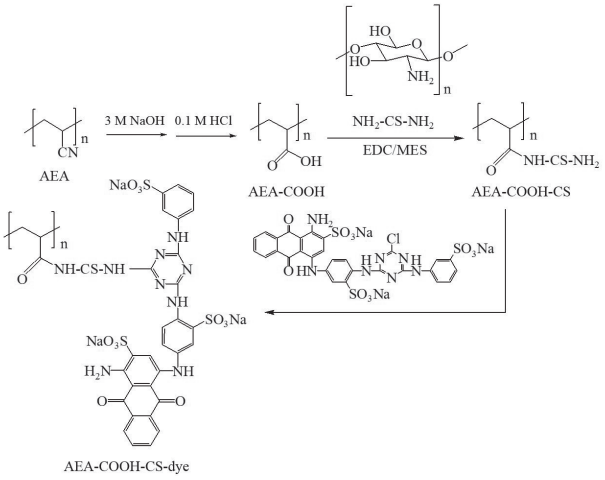

Fig. 2. Schematic representation of synthetic route for the AEA--COOH-CS-dye nanofibre. EDC=1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, MES=4-morpholineethanesulfonic acid

The physical properties and morphologies of the modified nanofibrous membranes were observed by scanning electron microscopy (SEM) and the results are shown in **Fig. 3** and **Table 1**, respectively. The prepared PAN membrane was partially soluble when the hydrolysis time was longer than 30 min. On the contrary, with hydrolysis time <30 min, the membrane structure, the average diameter, and the mechanical strength remained. Hence, the hydrolysis time of 25 min at 3 M NaOH and 358 K was used in this study. It has been observed that the AEA-COOH membranes consist of randomly oriented nanofibres after the alkaline hydrolysis step. The modified PAN membrane became rough and the degree of entanglement also increased. Similar observations have also been reported by other authors (*8,19,34*). After chitosan and dye modification, the modified membrane caused the nanofibre to be thicker without any significant influence on their overall morphology. Similar preparation of dyed nanofibre membrane was investigated by Zhang and Sun (*14*). However, their modification was more complex and consumption costs were higher.

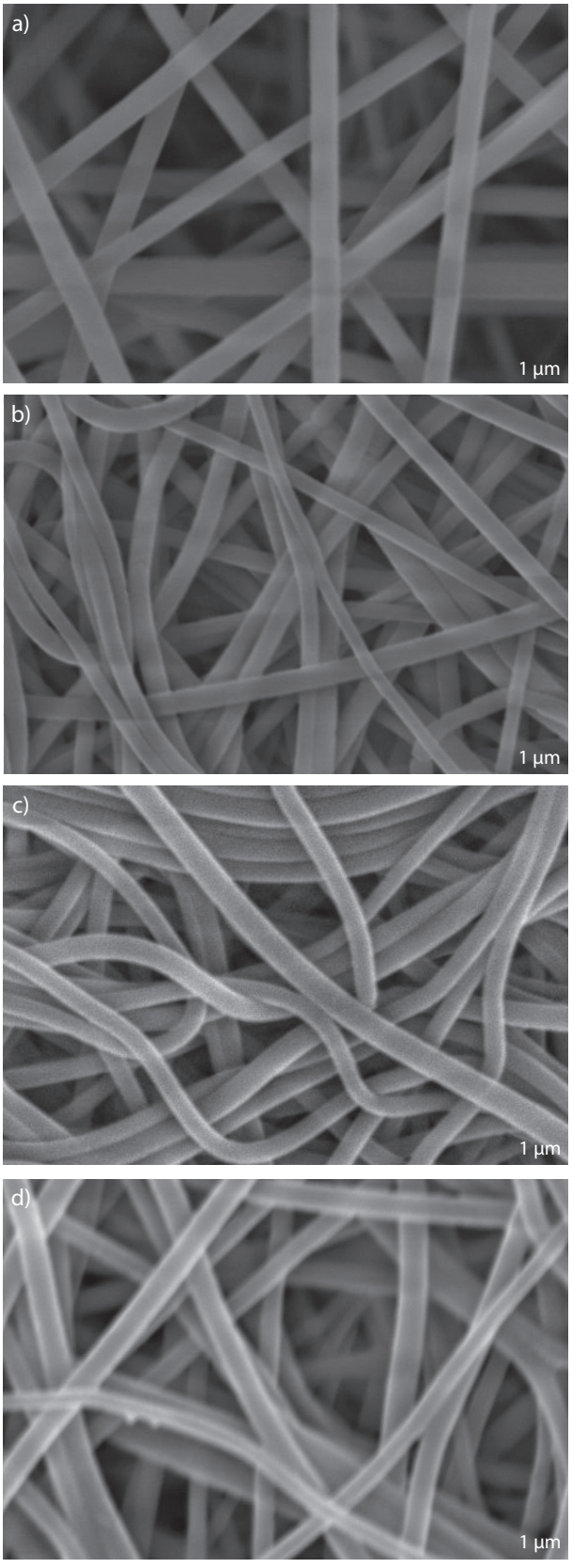

Fig. 3. Scanning electron microscopy (SEM) micrograph of electrospun fibre: a) polyacrylonitrile (PAN), b) hydrolysed PAN (PAN-COOH), c) with chitosan (PAN-COOH-CS) and d) with dye (PAN-COOH-CS--dye)

Table 1. Physical properties of the modified PAN nanofibrous membrane used in this work

Type of membrane	Thickness/m	Nanofibre diameter/nm	Surface porosity/%	Average pore size/μm	*b*(ligand)/(μmol/g)
AEA	236±20	492±30	85.69	0.472	-
AEA-COOH	206±25	480±25	84.32	0.461	425.23 (-COOH)
AEA-COOH-CS-NH_2	231±20	557±20	83.65	0.457	408.78 (-NH_2)
AEA-COOH-CS-dye	239±30	570±35	82.63	0.446	170.65 (-dye)

Effect of pH on adsorption

Studies of the adsorption of lysozyme with a dyed membrane were carried out in a pH range of 4-11 in a reciprocal shaking water bath at 298 K. As shown in **Fig. 4**, the maximum binding capacity of lysozyme was at pH=9. In the range of pH=4-8, the amount of adsorbed lysozyme increased with increasing pH and the binding capacity increased from 49.2 to 209.3 mg of lysozyme per g of membrane. In this pH range, the dye molecule carries negative charges; however, the positive charge density of the lysozyme molecule decreases with increasing pH. As indicated by the binding capacity, this may be due to the asymmetric distribution of the surface charge on the lysozyme molecule. In this case, the positively charged groups may be located in one region of the lysozyme molecule, even though there are more positively charged amino groups at lower pH values. A number of studies have shown similar results (*35,36*). The binding capacity of protein may not be satisfactorily explained by the net charge concept, and the adsorption is mainly controlled by the charge distribution on the protein.

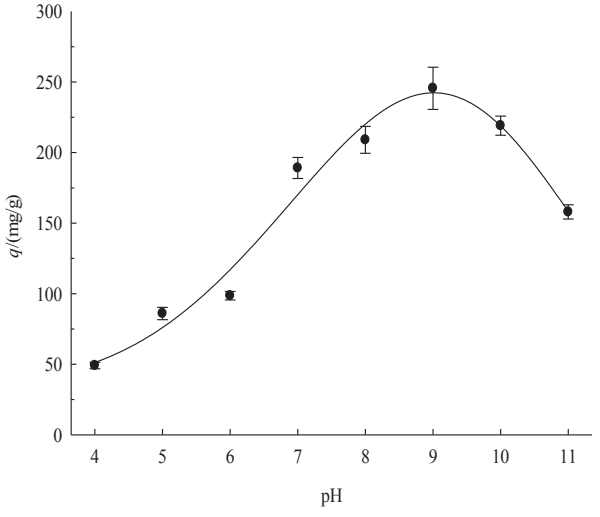

Fig. 4. The effect of pH on the binding capacity (*q*) of lysozyme

As the pH increased to 11 (near the pI value of lysozyme) (*37*), the adsorption capacity decreased to 158.2 mg/g. This may be due to the fact that more lysozyme molecules are less protonated and thus form less positively charged groups. This could contribute to having weaker electrostatic effect on the positively charged lysozyme and negatively charged membrane.

Based on these results, it was suggested that the optimal pH for the adsorption of lysozyme was pH=9 and the maximum binding capacity was 245.6 mg/g. Therefore, this pH value was used for further experiments.

Effect 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 the primary amino group and the AEA-COOH membrane with the carboxylic group can be achieved by an EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide)-mediated coupling reaction (*22,30*). In the study of Ma *et al.* (*22*), the coupling pH for EDC-mediated immobilized schemes was 5.0. The residual amino and hydroxyl groups of an activated chitosan membrane can be easily modified with reactive dye molecules. The immobilized mass of dye on the chitosan membrane in the range of chitosan mass per volume ratio from 0.05 to 1.0 % was found to be almost the same (59.8 mg of dye per g of membrane), resulting in similar binding capacity of lysozyme (*i.e.* 245.6 mg of lysozyme per g of membrane), as shown in **Fig. 5**. Hence, the optimal mass per volume ratio of chitosan for use in preparation of dyed membrane was 0.2 %.

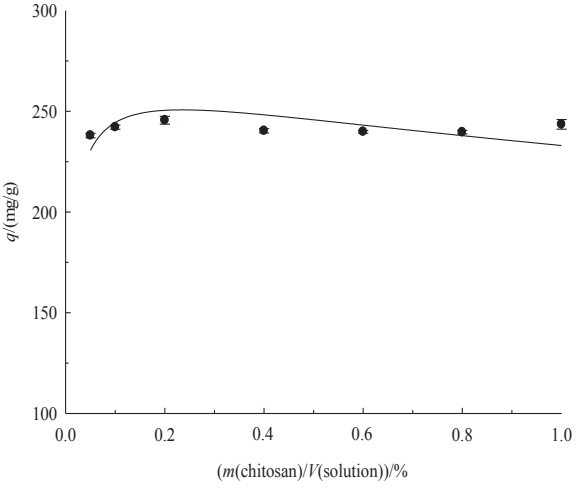

Fig. 5. The effect of chitosan (mass per volume ratio) on the binding capacity (*q*) of lysozyme

Effect of dye concentration

The amount of dye immobilized on the membrane has been considered as an important parameter in adsorption of proteins (*14,23,38*). **Fig. 6** shows the relationship between lysozyme binding capacity and initial dye concentration. The difference in the adsorption capacity of the membrane to lysozyme may be caused by one or more of the following factors, such as changes in the mass of the immobilized dye molecule, changes in the charge distribution and arrangement of the dye molecule, or changes in interactions between lysozyme and the dye molecule (*e.g.* ionic, hydrophobic or specific interaction) (*27*). The maximum binding capacity (245.6 mg of dye per g of membrane) was observed when 0.5 mg/mL of dye was loaded, which corresponded to the mass of the immobilized dye of 59.8 mg per g of membrane, as shown in **Fig. 7**. However, the increase of dye loading (>0.5 mg/mL) led to the reduced binding capacity. This decrease may be due to the steric effects at a larger mass of dye on the membrane. In such a case, the dye molecule would not easily approach the active binding sites on the lysozyme molecule. Hence, the degree of steric hindrances between the dye and lysozyme molecules on the membrane was a significantly important parameter for the binding capacity. It has been shown that protein molecules adsorbed under a higher dye coupling density are more difficult to elute (*23*). This tighter binding has been attributed to multivalent interactions between lysozyme and immobilized dye (*39*). Hence, it was suggested that the optimal mass of immobilized dye was 59.8 mg per g of membrane, which corresponds to the molal concentration of 71.2 mmol per g of membrane.

Effect of salt concentration

The binding capacity of lysozyme on the membrane under different salt concentrations (0.1-1.0 M NaCl, pH=9.0) is presented in **Fig. 8**. The results show that the increase of NaCl concentration caused a remarkable decrease in binding capacity. As the concentration increased from 0.1 to 1.0 M NaCl, the binding capacity decreased from 214.9 to 33.5 mg/g. Also, the adsorption of lysozyme decreased by 84 % as the NaCl concentration increased from 0.1 to 1.0 M. This may be mainly attributable to the competition of positively charged lysozyme for binding to negative ions in the salt buffer or negatively charged membrane framework. The results indicated that part of adsorbed lysozyme could not be eluted by 1.0 M NaCl, due to the presence of hydrophobic interactions. This was caused by the presence of aromatic structures on the immobilized dye and hydrophobic chains of the lysozyme molecule. Similar results have been found for the adsorption of lysozyme by other different types of dyed membranes (*27,28*). Hence, the combination of electrostatic and hydrophobic interactions is likely involved during adsorption.

Influence of temperature

The effects of operating temperature on the binding capacity of lysozyme were investigated in the range of 277-323

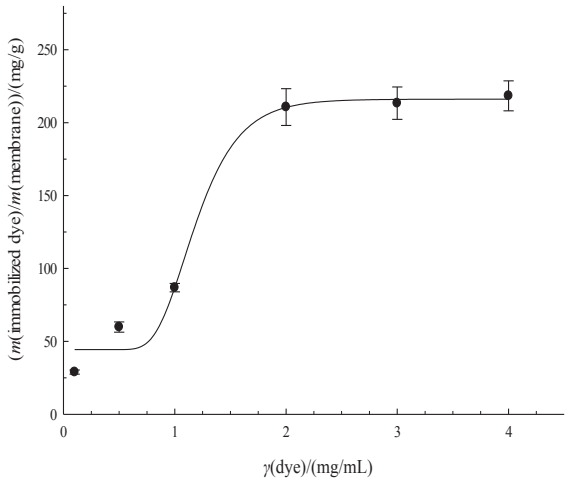

Fig. 6. Variation of the amount of dye immobilized on chitosan membrane with various initial dye concentrations

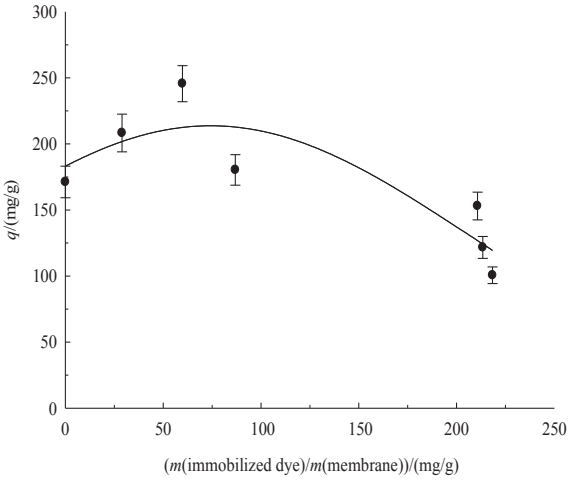

Fig. 7. The effect of immobilized dye density on the binding capacity (*q*) of lysozyme

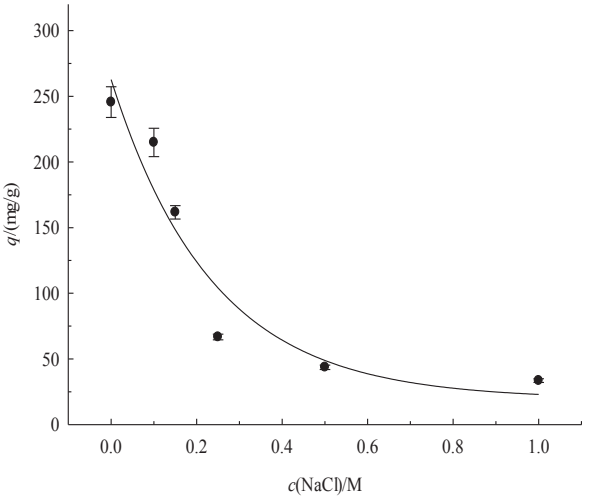

Fig. 8. The effect of sodium chloride on the binding capacity (*q*) of lysozyme

K. As shown in **Fig. 9**, it was observed that the binding capacity was increased from 154.3 to 309.2 mg/g as the temperature increased from 277 to 323 K. An increase in temperature caused an increase in binding capacity. Similar results were also observed in the adsorption of lysozyme by other dyed membranes (*27,39-43*). The increase in binding capacity of the membrane was related to the conformational changes of the lysozyme molecule. Moreover, this could be attributed to an increase in the hydrophobic interaction as the temperature increased (*41*).

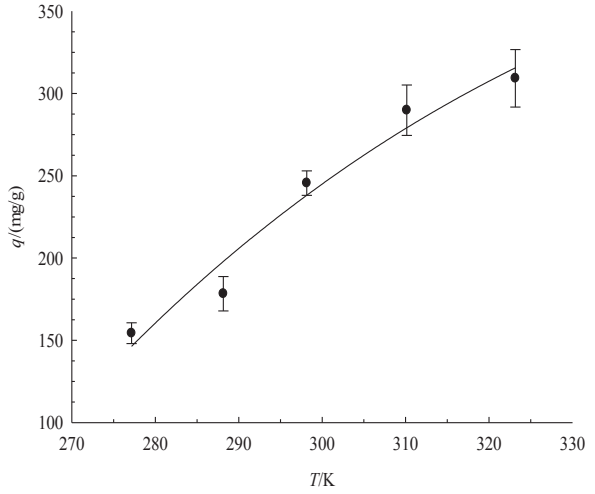

Fig. 9. The effect of operating temperature on the binding capacity (*q*) of lysozyme

Comparison with related membrane techniques

The binding capacity of the dye-affinity nanofibrous membrane for pure lysozyme adsorption (expressed in mg/g, mg/cm^2 or mg/cm^3) was compared to other types of membranes investigated in previous studies, as shown in **Table 2**. It was found that the dye nanofibrous membrane developed in this work resulted in much higher binding capacity (245.6 mg/g, 50.1 mg/cm^2 or 131.9 mg/cm^3) than the other dyed membranes. It has been demonstrated that the nanofibrous membrane techniques are far more economically advantageous than other conventional membrane techniques due to the high surface area per mass unit resulting from the small diameter and porous structure. This may lead to a fast adsorption rate and high binding capacity. The nanofibrous membrane appears to be more effective for the adsorption of lysozyme when processing samples of large volume.

Membrane chromatography for purification of lysozyme from chicken egg white

One sheet of dyed membrane (effective adsorption area 3.7 cm^2), connected with the AKTAprime chromatographic system (GE Healthcare Biosciences) was used to obtain the purification profile of lysozyme from chicken egg white solution, as shown in **Fig. 10**. At the end of the adsorption phase, the breakthrough curves of the contaminating proteins and lysozyme activity increased to approx. 95 and 30 % of the inlet values, respectively. In the adsorption and washing stages,

Table 2. Comparison with conventional membranes for binding capacity of pure lysozyme

Membrane	Maximum binding capacity			Reference
	mg/g	mg/cm^2	mg/cm^3	
AEA-COOH-CS-dye nanofibre membrane	245.58	50.12	131.86	This work
Reactive Red 120 immobilized pHEMA/chitosan composite membrane	32.30	1.32	40.80	(*27*)
Reactive Green 19 immobilized pHEMA/chitosan composite membrane	48.25	1.97	60.80	(*28*)
Procion Green H-E4BD immobilized pHEMA ion exchange membrane	11.22	0.42	14.14	(*35*)
Procion Brown MX-5BR immobilized pHEMA/chitosan IPNs membrane	101.75	3.33	128.20	(*36*)
Procion Brown MX-5BR immobilized pHEMA/chitosan composite membrane	96.43	3.16	121.50	(*39*)
Procion Green H-4G immobilized pHEMA membrane	63.65	2.19	80.20	(*41*)
Procion Green H-4G immobilized pHEMA/chitosan composite membrane	16.10	0.53	20.28	(*42*)
Cibacron Blue F3GA immobilized regenerated cellulose membrane	-	0.41	51.25	(*43*)

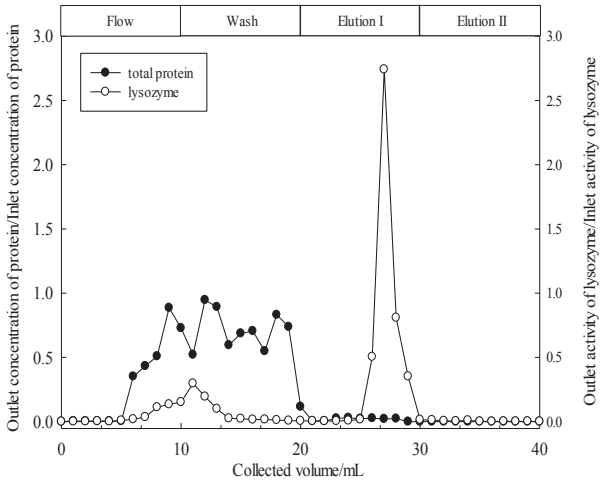

Fig. 10. Purification of lysozyme from chicken egg white by dye-affinity nanofibrous membrane chromatography

the degree of protein removal was as high as 95 %, but the loss of lysozyme activity at these stages was only 12 %. The purification results of the experiment are shown in **Table 3**.

The results showed that the capture efficiency of the membrane for lysozyme was approx. 89 %. The purification factor of lysozyme was about 24-fold and recovery yield about 57 % in a single step. The total yields of contaminating proteins and lysozyme calculated by adding up all individual stages were 97 and 56 %, respectively. It was found that a small amount of egg white protein (3 %) and a large amount of lysozyme (44 %) were not recovered during the purification of lysozyme. This may be due to the presence of multi-type relational interactions between the lysozyme molecule and the membrane, such as ionic charge interaction, hydrophobic interaction and specific interaction. Hence, the adsorbed lysozyme was not completely eluted by 0.6 M NaCl from the dyed membrane.

Our results demonstrated that the binding strength of the dyed membrane for lysozyme was much stronger than those of contaminating proteins. Therefore, optimization of the elution scheme for lysozyme should be further developed.

Improvements of elution schemes for lysozyme

Based on the experimental results as described above, the adsorbed lysozyme was not completely eluted by using 0.6 M NaCl (pH=9). The results showed that there was still 34 % of adsorbed lysozyme retained on the dyed membrane. These findings suggested that the binding mechanism of lysozyme to dyed adsorbent may result from the combined effects of electrostatic and hydrophobic forces. Hence, elution efficiency by 0.6 M NaCl (pH=9) may be enhanced by the addition of a polarity-reducing agent (*e.g.* ethanol or ethylene glycol). In this work, 25 % ethanol or ethylene glycol in 0.6 M NaCl (pH=9) was chosen to elute the lysozyme from the dyed membrane.

As described in experimental section, the operating procedures were carried out with diluted chicken egg white solution (5 mL, lysozyme content 19750 U/mL, total protein content 16.0 mg/mL) at 150 rpm in batch studies. After adsorption and wash stages, the adsorbed lysozyme was completely eluted by 25 % ethanol in 0.6 M NaCl (5 mL, pH=9). However, only 63 % of adsorbed lysozyme was eluted by 25 % ethylene glycol in 0.6 M NaCl (5 mL, pH=9) from the dyed membrane. Other remaining lysozyme on the membrane was further eluted by 25 % ethylene glycol in 1.0 M NaCl (5 mL, pH=9). Hence, one-step elution with 25 % ethanol in 0.6 M NaCl (pH=9) was highly effective for recovery of lysozyme. The results revealed that the electrostatic and hydrophobic interactions between lysozyme and immobilized dye may play an important role in the elution.

Improved purification of lysozyme

It has been shown that lysozyme could be completely eluted from the dyed membrane by 25 % ethanol in 0.6 M NaCl (pH=9) as described previously. The operating conditions obtained from the batch experiments were used in membrane chromatography. The experiment was carried out to purify the lysozyme from complex chicken egg white solution (10 mL, lysozyme content $1.05 \cdot 10^4$ U/mL, total protein content 6.64 mg/mL, specific activity $1.58 \cdot 10^3$ U/mg). The CB membrane holder (one sheet of membrane, effective area 3.7 cm^2) was integrated with an AKTAprime chromatographic system. In the purification procedures, the liquid flow rates selected for the

Table 3. Purification of lysozyme from chicken egg white by dye-affinity nanofibrous membrane chromatography

Purification step	*V*/mL	*m*(total protein)/mg	Total activity/U	*w*(total protein)/%	*w*(lysozyme)/%	Specific activity/(U/mg)	Purification fold (lysozyme)
Clarified chicken egg white	10	66.18	$1.03 \cdot 10^5$	100.00	100.00	$1.56 \cdot 10^3$	1.0
Flow and wash	20	62.88	$1.19 \cdot 10^4$	95.01	11.55	-	-
Elution (I) (0.6 M NaCl)	10	1.23	$4.59 \cdot 10^4$	1.86	44.56	$3.73 \cdot 10^4$	24.0
Elution (II) (1.0 M NaCl)	10	$1.79 \cdot 10^{-2}$	$3.91 \cdot 10^2$	$2.70 \cdot 10^{-2}$	0.38	$2.19 \cdot 10^4$	14.0
Total				96.90	56.51		

Data are expressed as the mean value of triplicate measurements

adsorption (10 mL), wash (10 mL) and elution (20 mL) steps were 0.1, 0.5 and 0.1 mL/min, respectively. In adsorption and wash stages, the adsorption efficiency of lysozyme was as high as 90 %, and the amount of contaminating protein was only 5 %. The experimental results of the elution with 25 % ethanol in 0.6 M NaCl showed that the recovery yield of lysozyme was 87 % and the purification factor was up to 47-fold. After the change of the elution scheme, this experiment was very successful. Therefore, it was concluded that the application of nanofibres in dye-affinity chromatography for purification of proteins is quite feasible and could replace the conventional membrane chromatography.

Reusability of dyed membrane

To evaluate the possibility of the repeated use of a dyed membrane, the adsorption-desorption operations were repeated for five cycles by using the same dyed membrane in batch studies. The time of cleaning-in-place (CIP) procedure was carried for one hour by using a cocktail solution containing 1.0 M NaCl, 25 % ethanol and 4 M urea. The dyed membrane was readily cleaned and the binding capacity for lysozyme was not significantly reduced (<3 %). The results showed that this CIP procedure for the dyed membrane was very effective.

CONCLUSIONS

In this study, lysozyme was chosen as a model protein and its binding characteristics on the dye-affinity nanofibrous membrane were extensively investigated by varying the pH, chitosan mass per volume ratio for coupling reaction, initial dye concentration for immobilization, salt concentration and operating temperature. The results showed that optimal adsorption occurred at pH=9, chitosan mass per volume ratio of 0.2 %, immobilized dye of 59.8 mg per g of membrane, and operating temperature of 298 K. Under these conditions, a high binding capacity of 245.6 mg/g was achieved. The molecular forces contributing to the interactions between the lysozyme and dye molecules may be mainly the ionic and hydrophobic interactions. The capture efficiency, recovery yield, and purification factor of lysozyme using dye-affinity chromatography were found to be 90 and 87 % and 47-fold, respectively, in a single step.

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REFERENCES

1. Huang ZM, Zhang YZ, Kotaki M, Ramakrishna S. A review on polymer nanofibers by electrospinning and their applications in nanocomposites. Compos Sci Technol. 2003;63:2223-53. https://doi.org/10.1016/S0266-3538(03)00178-7
2. Sill TJ, von Recum HA. Electrospinning: Applications in drug delivery and tissue engineering. Biomaterials. 2008;29(13):1989-2006. https://doi.org/10.1016/j.biomaterials.2008.01.011
3. Mei Y, Yao C, Fan K, Li X. Surface modification of polyacrylonitrile nanofibers with superior antibacterial and easy-cleaning properties through hydrophilic flexible spacers. J Membrane Sci. 2012;417-418:20-7. https://doi.org/10.1016/j.memsci.2012.06.021
4. Yoo HS, Kim TG, Park TG. Surface-functionalized electrospun nanofibers for tissue engineering and drug delivery. Adv Drug Deliv Rev. 2009;61:1033-42. https://doi.org/10.1016/j.addr.2009.07.007
5. Ren X, Akdag A, Zhu C, Kou L, Worley SD, Huang TS. Electrospun polyacrylonitrile nanofibrous biomaterials. J Biomed Mater Res A. 2009;91:385-90. https://doi.org/10.1002/jbm.a.32260
6. Chiu HT, Lin JM, Cheng TH, Chou SY, Huang CC. Direct purification of lysozyme from chicken egg white using weak acidic polyacrylonitrile nanofiber-based membranes. J Polym Sci. 2012;125:E616-21. https://doi.org/10.1002/app.36764
7. Wang R, Liu Y, Li B, Hsiao BS, Chu B. Electrospun nanofiber membranes for high flux microfiltration. J Membrane Sci. 2012;392-3:167-74. https://doi.org/10.1016/j.memsci.2011.12.019
8. Huang F, Xu Y, Liao S, Yang D, Hsieh YL, Wei Q. Preparation of amidoxime polyacrylonitrile chelating nanofibers and their application for adsorption of metal ions. Materials. 2013;6:969-80. https://doi.org/10.3390/ma6030969
9. Charcosset C. Purification of proteins by membrane chromatography. J Chem Technol Biotechnol. 1998;71:95-110. https://doi.org/10.1002/(SICI)1097-4660(199802)71:2<95::AID-JCTB823>3.0.CO;2-J
10. Orr V, Zhong L, Moo-Young M, Chou CP. Recent advances in bioprocessing application of membrane chromatography. Biotechnol Adv. 2013;31:450-65. https://doi.org/10.1016/j.biotechadv.2013.01.007
11. Thömmes J, Kula MR. Membrane chromatography - An intergrative concept in the downstream processing of proteins. Biotechnol Progr. 1995;11:357-67. https://doi.org/10.1021/bp00034a001
12. Zhu J, Sun G. Facile fabrication of hydrophilic nanofibrous membranes with an immobilized metal-chelate affinity complex for selective protein separation. ACS Appl Mater Interfaces. 2014;6:925-32. https://doi.org/10.1021/am4042965
13. Ye P, Xu ZK, Wu J, Innocent C, Seta P. Nanofibrous poly(acrylonitrile-co-maleic acid) membranes functionalized with gelatin and chitosan for lipase immobilization. Biomaterials. 2006;27:4169-76. https://doi.org/10.1016/j.biomaterials.2006.03.027

14. Zhang S, Sun Y. Further studies on the contribution of electrostatic and hydrophobic interactions to protein adsorption on dye-ligand adsorbents. Biotechnol Bioeng. 2001;75:710-7. https://doi.org/10.1002/bit.10067
15. Kim J, Grate JW, Wang P. Nanostructures for enzyme stabilization. Chem Eng Sci. 2005;61:1017-26. https://doi.org/10.1016/j.ces.2005.05.067
16. Ma Z, Lan Z, Matsuura T, Ramakrishna S. Electrospun polyethersulphone affinity membrane: Membrane preparation and performance evaluation. J Chromatogr B. 2009;877:3686-94. https://doi.org/10.1016/j.jchromb.2009.09.019
17. Haider S, Park SY. Preparation of the electrospun chitosan nanofibers and their applications to the adsorption of Cu(II) and Pb(II) ions from an aqueous solution. J Membrane Sci. 2009;328:90-6. https://doi.org/10.1016/j.memsci.2008.11.046
18. Sencadas V, Correia DM, Areias A, Botelho G, Fonseca AM, Neves IC, Gomez Ribelles JL, Lanceros-Méndez S. Determination of the parameters affecting electrospun chitosan fiber size distribution and morphology. Carbohydr Polym. 2012;87:1295-301. https://doi.org/10.1016/j.carbpol.2011.09.017
19. Zhang H, Nie H, Yu D, Wu C, Zhang Y, White CJB, Zhu L. Surface modification of electrospun polyacrylonitrile nanofiber towards developing an affinity membrane for bromelain adsorption. Desalination. 2010;256:141-7. https://doi.org/10.1016/j.desal.2010.01.026
20. Ma Z, Kotaki M, Ramakrishna S. Electronspun cellulose nanofiber as affinity membrane. J Membrane Sci. 2005;265:115-23. https://doi.org/10.1016/j.memsci.2005.04.044
21. Zhang H, Wu C, Zhang Y, White CJB, Xue Y, Nie H, Zhu L. Elaboration, characterization and study of a novel affinity membrane made from electrospun hybrid chitosan/nylon-6 nanofibers for papain purification. J Mater Sci. 2010;45:2296-304. https://doi.org/10.1007/s10853-009-4191-3
22. Ma Z, Masaya KM, Ramakrishna S. Immobilization of Cibacron blue F3GA on electrospun polysulphone ultra-fine fiber surfaces towards developing an affinity membrane of albumin adsorption. J Membrane Sci. 2006;282:237-44. https://doi.org/10.1016/j.memsci.2006.05.027
23. Boyer PM, Hsu JT. Effects of ligand concentration on protein adsorption in dye-ligand adsorbents. Chem Eng Sci. 1992;47:241-51. https://doi.org/10.1016/0009-2509(92)80218-2
24. Awadé AC, Efstathiou T. Comparison of three liquid chromatographic methods for egg-white protein analysis. J Chromatogr B. 1999;723:69-74. https://doi.org/10.1016/S0378-4347(98)00538-6
25. Li-Chan E, Nakai S, Sim J, Bragg DB, Lo KV. Lysozyme separation from egg white by cation exchange column chromatography. J Food Sci. 1986;51:1032-6. https://doi.org/10.1111/j.1365-2621.1986.tb11226.x
26. Ma J, Wang X, Fu Q, Si Y, Yu J, Ding B. Highly carbonylated cellulose nanofibrous membranes utilizing maleic anhydride grafting for efficient lysozyme adsorption. ACS Appl Mater Interfaces. 2015;7:15658-66. https://doi.org/10.1021/acsami.5b04741
27. Arıca MY, Bayramoğlu G. Purification of lysozyme from egg white by Reactive Blue 4 and Reactive Red 120 dye-ligands immobilized composite membranes. Process Biochem. 2005;40:1433-42. https://doi.org/10.1016/j.procbio.2004.06.028
28. Yılmaz M, Bayramoğlu G, Arıca MY. Separation and purification of lysozyme by Reactive Green 19 immobilized membrane affinity chromatography. Food Chem. 2005;89:11-8. https://doi.org/10.1016/j.foodchem.2004.01.072
29. Chiu HT, Lin JM, Cheng TH, Chou SY. Fabrication of electrospun polyacrylonitrile ion-exchange membranes for application in lysozyme adsorption. eXPRESS Polym Lett. 2011;5:308-17. https://doi.org/10.3144/expresspolymlett.2011.31
30. Yang MC, Lin WC. Surface modification and blood compatibility of polyacrylonitrile membrane with immobilized chitosan-heparin conjugate. J Polym Res. 2002;9:201-6. https://doi.org/10.1023/A:1021347810130
31. Suen SY, Lin SY, Chiu HC. Effects of spacer arms on Cibacron blue 3GA immobilized and lysozyme adsorption using regenerated cellulose membrane discs. Ind Eng Chem Res. 2000;39:478-87. https://doi.org/10.1021/ie990421t
32. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54. https://doi.org/10.1016/0003-2697(76)90527-3
33. Shugar D. The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. Biochim Biophys Acta. 1952;8:302-9. https://doi.org/10.1016/0006-3002(52)90045-0
34. Saeed K, Haider S, Oh TJ. Park SY. Preparation of amidoxime-modified polyacrylonitrile (PAN-oxime) nanofibers and their applications to metal ions adsorption. J Membrane Sci. 2008;322:400-5. https://doi.org/10.1016/j.memsci.2008.05.062
35. Hashim MA, Chu KH, Tsan PS. Effects of ionic strength and pH on the adsorption equilibria of lysozyme on ion exchangers. J Chem Technol Biotech. 1995;62:253-60. https://doi.org/10.1002/jctb.280620307
36. Kaçar Y, Arıca MY. Procion Green H-E4BD-immobilized porous poly(hydroxyethyl methacrylate) ion-exchange membrane: Preparation and application to lysozyme adsorption. Colloids Surf B Biointerfaces. 2001;22:227-36. https://doi.org/10.1016/S0927-7765(01)00194-1

37. Bayramoğlu G, Kaya B, Arıca MY. Procion Brown MX-4BR attcched and Lewis metals ion-immobilized poly(hydroxyethyl methacrylate)/chitosan IPNs membranes: Their lysozyme adsorption equilibria and kinetics characterization. Chem Eng Sci. 2002;57:2323-34. https://doi.org/10.1016/S0009-2509(02)00141-0

38. Suen SY, Tsai YD. Comparison of ligand density and protein adsorption on dye-affinity membranes using different spacer arms. Sep Sci Technol. 2000;35:69-87. https://doi.org/10.1081/SS-100100144

39. Liu YC, Stellwagen E. Zonal chromatographic analysis of the interaction of alcohol dehydrogenase with blue-sepharose. J Chromatogr. 1986;376:149-55. https://doi.org/10.1016/S0378-4347(00)80832-4

40. Bayramoğlu G, Yılmaz M, Arıca MY. Affinity dye-ligand poly(hydroxyethyl methacrylate)/chitosan composite membrane for adsorption lysozyme and kinetic properties. Biochem Eng J. 2003;13:35-42. https://doi.org/10.1016/S1369-703X(02)00098-0

41. Chen TX, Nie HL, Li SB, Branford-White C, Su SN, Zhu LM. Comparison: Adsorption of papain using immobilized dye ligands on affinity membranes. Colloids Surf B Biointerfaces. 2009;72:25-31. https://doi.org/10.1016/j.colsurfb.2009.03.012

42. Arıca MY, Yılmaz M, Yalçın E, Bayramoğlu G. Affinity membrane chromatography: Relationship of dye-ligand type to surface polarity and their effect on lysozyme separation and purification. J Chromatogr B. 2004;805:315-23. https://doi.org/10.1016/j.jchromb.2004.03.021

43. Bayramoğlu G, Arıca MY. Procion Green H-4G immobilized on a new IPN hydrogel membrane composed of poly(2-hydroxyethyl methacrylate)/chitosan: Preparation and its application to the adsorption of lysozyme. Colloids Surf A Physicochem Eng Asp. 2002;202:41-52. https://doi.org/10.1016/S0927-7757(01)01055-X