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Regenerated Cellulose Membrane with Different Spacer Arm Length of Diamine Monomer for Membrane Chromatography

Yue Wei Lee and Syed M. Saufi

Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang, LebuhrayaTunRazak, 26300 Kuantan, Pahang, Malaysia.

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ABSTRACT

Packed bed column chromatography is widely used for protein separation. However, it has several limitations such as high pressure drop and long processing times. Membrane chromatography is an alternative technique used for protein separation. Specific monomer can be grafted to uncharged membrane to transform into membrane chromatography material. Optimization of parameters involved during this chemical modification is crucial for the development of high performance membrane chromatography for protein separation. The purpose of this research is to develop anionexchanger membrane chromatography from regenerated cellulose membrane by attaching different spacer arm lengths of diamine monomer. Regenerated cellulose membrane was activated in a solution containing sodium hydroxide (NaOH) and epichlorohydrin (EPI). The membrane was grafted with diamine solution of 1,2-diaminoethane or 1,4-diaminobutane to produce positively charged membrane chromatography. The concentration of NaOH activation from 0.05M to 0.50M and diamine monomer concentration from 0.25M to 2.0M during grafting were studied. The optimum concentration of NaOH was 0.20M which produced anion exchange membrane chromatography with capacity of 0.310±0.033 mgBSA/cm^2 membrane. High concentration of diamine monomer at 2.0M 1,4-diaminobutane showed a binding capacity of 0.385±0.027mgBSA/cm^2 membrane.

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INTRODUCTION

Conventional techniques for protein separation involve several steps such as impurities removal, isolation, purification and polishing. More than 60% of the total cost of bioproduct manufacturing is contributed by downstream processing for recovery and purification of bioproduct. High efficiency and high productivity separation techniques were essential to pharmaceutical industry. Besides that, increasing industrial demand of food products for large-scale extraction and purification had caused the separation process to further develop and exploit.

Chromatography technique is widely used for protein separation in the packed bed column configuration. However, it had several limitations such as high pressure drop, long processing times due to slow pore diffusion and complicated scale up procedures [1]. Sometimes, channeling can occur due to cracking of packed bed which caused a major problem. Membrane chromatography is becoming more popular as an alternative to the packed bed chromatography. It is a combination of membrane filtration and chromatographic principle. Membrane chromatography shows several advantages such as low pressure drop, fast protein accessible to the specific functionality in the membrane by bulk convection, easy to scale up and set up [2, 3].

Anion exchanger membrane chromatography with high binding capacity has potential for commercial application in industries. It can be prepared by chemical grafting, UV-grafting, photo-grafting and polymer grafting [4]. Negatively charged protein can be separate selectively and effectively by using positively charged membrane. Spacer arm lengths of diamine, which use as positive charged functional ligand on membrane surface, have strong effect on the protein binding capacity [5, 6]. Different spacer arm length, determined by the number of alkyl groups between membrane and functional ligand, hasdifferent protein binding and behavior.

Cellulose and its derivative has been widely used membrane material for protein adsorption. Liu et al. prepared regenerated cellulose-based immobilized metal affinity membrane (IMAM) for penicillin G acylase purification [7].Another type of IMAMs were prepared by Wu et al. [8] by immobilizing copper ions on

Corresponding Author: Syed M. Saufi, Faculty of Chemical and Natural Resources Enginering, Universiti Malaysia Pahang, LebuhrayaTunRazak, 26300 Kuantan, Pahang, Malaysia.
E-mail: smsaufi@gmail.com

microporous regenerated cellulose membranes through different types of chelating agents (dentate and triazine dye). This affinity membrane was tested for adsorption of g-globulin, BSA and lysozyme. Chitosan/cellulose acetate blend hollow fiber membrane was prepared by Liu and Bai [9] to adsorp bovine serum albumin (BSA) and copper. Similar chitosan–cellulose composite membrane was prepared through coating chitosan on filter paper [10]. The effect of polyethylene glycol (PEG) contents in the chitosan preparation solution and evaporation time of the solution after coating was studied. The composite membrane was then immobilized with Protein A ligand to adsord human IgG.

The objective of this study is to develop anion exchanger membrane chromatography from regenerated cellulose membrane by attaching different spacer arm lengths of diamine monomer. Regenerated cellulose (RC) membrane was activated in a solution containing sodium hydroxide (NaOH) and epichlorohydrin (EPI). Then, the membrane was immersed in diamine solution of 1,2-diaminoethane or 1,4-diaminobutane to produce positively charged membrane chromatography. The concentration of NaOH activation solution from 0.05M to 0.50M and diamine monomer concentration from 0.25M to 2.0M during grafting were studied.

MATERIALS AND METHODS

Materials:

Regenerated cellulose (RC) membrane with pore size of 0.45µm was purchased from Whatman Ltd. Epichlorohydrin (EPI), 1,2-diaminoethane and 1,4-diaminobutane were purchased from Merck. All reagents were analytical grade and used without any further purification.

BSA with purity range of 95 to 99% was supplied from Sigma-Aldrich and used as model protein for binding. 20mM sodium phosphate buffer pH 7.0 was used as binding buffer.Elution buffer was a 1.0M of sodium chloride (NaCl) dissolved in binding buffer.

Modification of RC Membrane:

Modification process was performed on 47mm diameter of RC membrane disk. The membrane disk was handled carefully with limit contact to membrane edges to avoid any damage on the membrane. Each membrane was soaked in ultrapure water for at least 45 minutes to clean the membrane surface. The water was replaced for two times during this cleaning process. The membrane was sonicated in an ultrasonic cleaner for 15 minutes to remove any impurities bond to the membrane. The membranes were then stored in 0.1M NaOHsolutions for membrane swelling.

Membranes disk were removed from NaOH and cleaned with soft tissue to remove excess solution on the surface. The reaction chemistry was adapted from Mehta and Zydney [5]as shown in Figure 1. Clean membrane was immersed in solution containing 10mL of 0.1M NaOH and 5mL of EPI for cross-linking reaction. The reaction was carried out in an incubator shaker with temperature adjusted to 45°C and agitated at 150rpm for 2 hours. The membrane was removed and rinsed with ultrapure water. Then, the membrane was wiped with soft tissue to remove excess water on the membrane surface.

Cross-linked membranes disk were immersed in 20mL of a 1.0M diamine solution with pH adjusted to 11.0±0.2 by addition of small amount of 1.0M HCl as needed. The reaction was allowed to progress at 45°C with agitation at 150rpm for 12 hours. Membrane was removed from the solution and rinsed with ultrapure water for at least 1 hour.

The above procedure was repeated using different concentration of NaOH from 0.05M to 0.50M (step 1 Figure 1) in order to study the effect of hydrolysis on the membrane binding capacity. In another experiment, the concentration of 1,4-diaminobutane monomer was varied from 0.25M to 2.0M (step 2 Figure 1) to study the effect of monomer concentration.

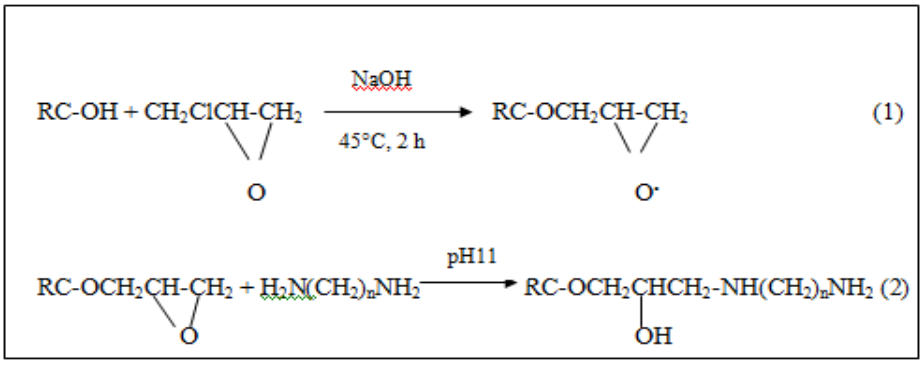

Fig. 1: Schematic of reaction chemistry used to couple EPI and different diamines on RC membrane

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Weight of Membrane:

The weight of dried membrane before and after modification was measured to calculate the weight change. Biotron model Cleanvac 12 freeze dryer was used to completely dry the wet membrane. Original weight of membrane was taken for the membrane after washing step. The weight change was calculated according to Eq.(1):

$$\text{Weight change (\%)} = \frac{b - a}{b} \times 100\% \quad (1)$$

where*b* is dried weight of modified membrane and *a* is dried weight of original membrane.

Protein Binding and Elution:

Membrane disk was cut into rectangular shapes of 1cm x 2cm dimension for binding experiment. The weight of membrane was measured. Modified membrane was equilibrated for 3 hours in 1.5ml of 20 mMsodium phosphate pH 7.0 binding buffer. Equilibrated membrane was then incubated with 1.5ml of 2mg/ml BSA solution dissolved in binding buffer for 12 hours at room temperature. The equilibrium protein solution concentration was then measured using uv-vis spectrophotometer as explain in later section. The binding capacity was calculated using Eq. 2.

$$\text{Binding Capacity (mg BSA/cm}^2\text{ Membrane)} = \frac{V_b(C_o - C_e)}{A} \quad (2)$$

whereV_b is volume of protein solution in ml, C_o and C_e are initial and equilibrium of protein concentration respectively in mg/ml and A is the area of membrane in cm^2.

The bound membrane was then incubated with 1.5ml of elution buffer for 3 hours to elute the bound BSA from the membrane. The elution buffer was prepared by adding 1 M NaCl in binding buffer.All binding and elution steps were carried out using 2.0 ml centrifuge tube and mixed on the rotator. The protein concentration in elution solution was measured using the uv-vis method. The elution recovery was calculated according to the Eq. 3.

$$\text{Elution Protein Recovery (\%)} = \frac{\text{mass of bound protein on membrane}}{\text{mass of protein in elution solution}} \times 100\% = \frac{V_b(C_o - C_e)}{V_{el}C_{el}} \times 100\% \quad (3)$$

whereV_b is volume of protein solution in binding experiment in ml, C_o and C_e are initial and equilibrium of protein concentration in binding experiment respectively in mg/ml, V_{el} is volume of elution buffer in ml and C_{el} is protein concentration after elution in mg/ml.

Protein Concentration Analysis:

UV-vis spectrophotometer was used for quantitative determination of BSA proteins concentration. The protein absorbance at 280nm was measured using Hitachi U-1800 model UV-vis spectrophotometer. The BSA concentration was calculated based on the absorbance-concentration curve developed from known BSA concentrations range from 0.03125 to 1 mg/ml. Triplicate samples were prepared in standard curve experiment.

Water Flux Test:

Original membrane and modified membrane were cut into 2.6cm diameter circle shape for water flux measurement. Membranes were immersed in ultra pure water and cleaned in ultrasonic bath around 2 minutes before the test. The membranes were fixed tightly into Amicon stirred cell Model 8010.

The stirred cell was filled in with clean de-ionized water from the upper inlet hole (gas inlet hole) and stirred around 200 to 300 rpm speed. No pressure is needed during this test due to the membrane used was in microfiltration (MF) range. Water could penetrate through MF membrane without pressurized condition. The time taken to permeate 5 ml water through the membrane was taken. The flux was calculated using Eq.(4):

$$Flux, J = \frac{V}{A.\,t} \quad (4)$$

where$J$ is flux in ($L/m^2.h$), A is the area of membrane in m^2, $V$is the volume of permeated in L and t is the time taken in hour.

RESULTS AND DISCUSSION

Membrane Performance Modified with Different Diamine Monomer:

Table 1 shows the BSA binding capacity and elution recovery of various types of RC membranes.Unmodified RC membrane showed relatively low binding capacity of 0.022±0.008 mg BSA/cm^2 membrane. RC membrane does not have any positively functional group to interact with negatively charged BSA. In addition, it had negatively hydroxyl (OH) group that can repelled the BSA. Therefore, small amount of binding in unmodified membrane is due to the nonspecific bindinginteraction of the membrane. The hydroxyl groups are still available in modified membrane, the amine group are however much more reactive than the hydroxyl groups [11].

Table 1: Protein binding capacity and elution recovery of various types of RC membrane

Monomer Type	Binding capacity (mg BSA/cm^2 membrane)	Protein recovery (%)
Unmodified RC membrane	0.022±0.008	81.6±14.5
1,2-diaminoethane	0.192±0.014	62.0±8.7
1,4-diaminobutane	0.298±0.041	53.3±10.9

The binding capacity of RC membrane grafted with 1,4-diaminobutane monomer is higher than 1,2-diaminoethane monomer as shown in Table 1. The binding capacity increased about 55.21% when the number of carbon increased by 2 carbons chain from 1,2-diaminoethane to 1,4-diaminobutane.

Compared to unmodified RC membrane, both modified membranes showed an increase about 88.51% and 92.62% BSA binding capacity for 1,2-diaminoethane and 1,4-diaminobutane monomer respectively. The capacity increased due to the addition of positive functional group of primary amines in modified RC membrane which able to bind negatively charged BSA.

The binding capacity achieved in this study is higher compared to charged membrane prepared by Wang et al. [12] through blending of carboxylic polyethersulfone (CPES) with PES at different ratiosof CPES to PES. The BSA binding capacity obtained was 9.50±0.4 µg/cm^2 and 7.61±0.2 µg/cm^2 for CPES to PES ratio 1:4 and 1:2 respectively [12].

Characterization of Modified RC Membrane Chromatograph:

The change in membrane weight represents the progress of modification steps involve in preparing membrane chromatography. Figure2 shows the weight of unmodified membrane and membrane modified with 1,4-diaminobutane monomer.

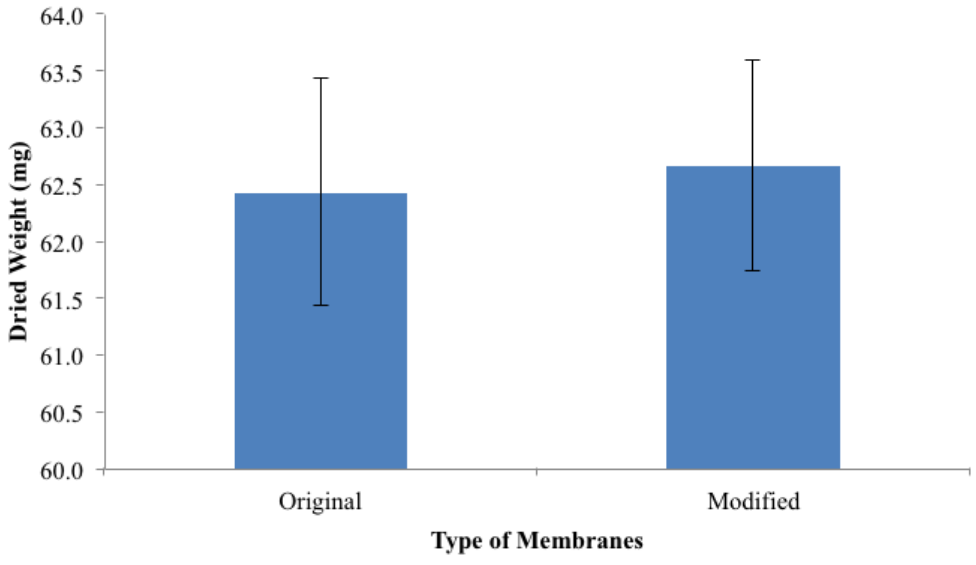

Fig. 2: Weight of unmodified membrane and membrane modified with 1.0M 1,4-diaminobutane

Modified membranes gained weight increment about 0.1mg to 0.4mg which was about 0.36±0.17%. Although this increment was low, but it proved that some new functionalgrouphas been successfully attached on the membrane structure after the modification.

Water flux of the membrane was showedin Figure3. For a modified membrane, a layer of cross-linker or grafted monomerprobably was formed on the membrane surface or modified the pore size of the membrane.

This will add more resistance for water to pass through the membrane. Hence, the water flux for modified membrane isreduced about 10% compared to theunmodified membrane.

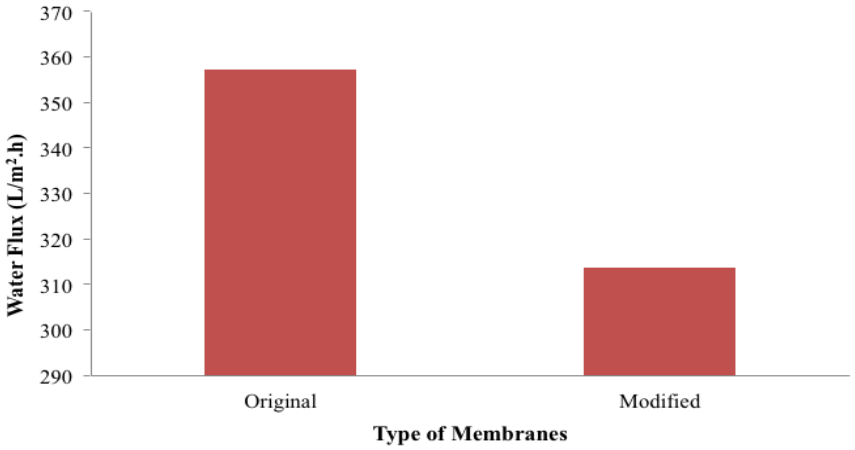

Fig. 3: Water flux of original membrane and membrane modified with 1,4-diaminobutane

Effect of NaOH Concentration during Activation on the Performance of Membrane Chromatography:

During activation step, NaOH functioned as hydrolysis and swelling agent. In NaOH hydrolysis, H^+ ion was removed from the RC membrane to create an active RCO^- site for coupling with EPI. EPI cross-linked RC membrane later was grafted with 1.0M of 1,4-diaminobutane. In this study, the concentration of NaOHwas varied from 0.05M to 0.50M, while other parameters were keep constant. Figure4 shows the performance of anion exchanger membrane chromatography activated using different concentration of NaOH.

The binding capacity was increased as the concentration of NaOH increased from 0.05M to 0.20M, and then decreased from the concentration of 0.25M to 0.50M. Optimum NaOH concentration achieved was at 0.20M which gave the highest binding capacity of 0.310±0.033 mg BSA/cm^2 membrane. After optimum concentration, the binding capacity did not show much impact as the concentration increased from 0.25M to 0.50M. Higher concentration of NaOH created more active site on membrane surface for reaction with cross-linker EPI, however it was limited to the available OH group in the specific area of RC membrane used.

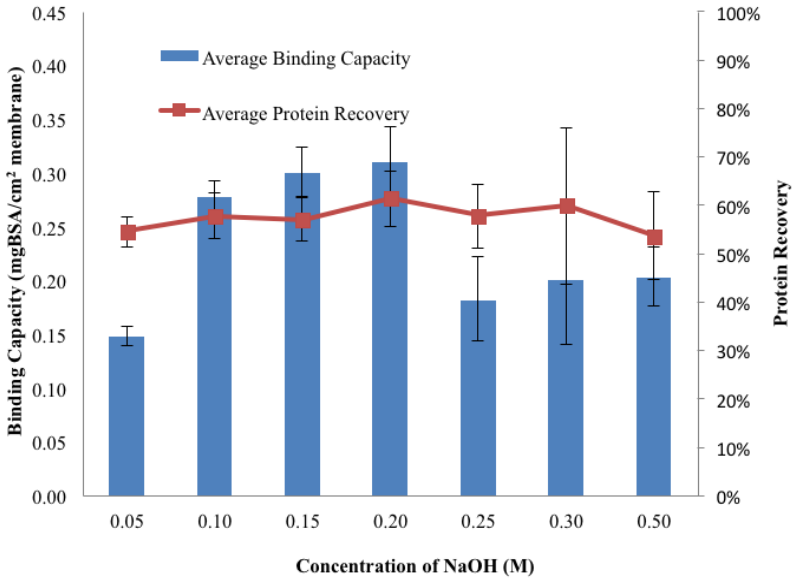

Fig. 4: Binding capacity and protein recovery 1,4-diaminobutane membrane chromatography activated using different concentration NaOH.

Effect of 1,4-Diaminobutane Concentrations on the Performance of Membrane Chromatography:

The protein binding at different diamine monomer concentration 0.50M to 2.00M was shown in Figure 5. Protein binding was increased significantly when the monomer concentration increased from 1.0M to 2.0M.

This drastic change occurred due to the available active site (RCO-) on the membrane were progressively attached with the monomer molecules. When all the available active site was reacted, it will reach the maximum capability of monomer grafting onto the membrane. An increase on monomer concentration after this point will not increase significantly the binding capacity. The optimum concentration of 1,4-diaminobutane was found at 2.0M which gave the highest binding capacity of 0.385 mg BSA/cm^2 membrane.

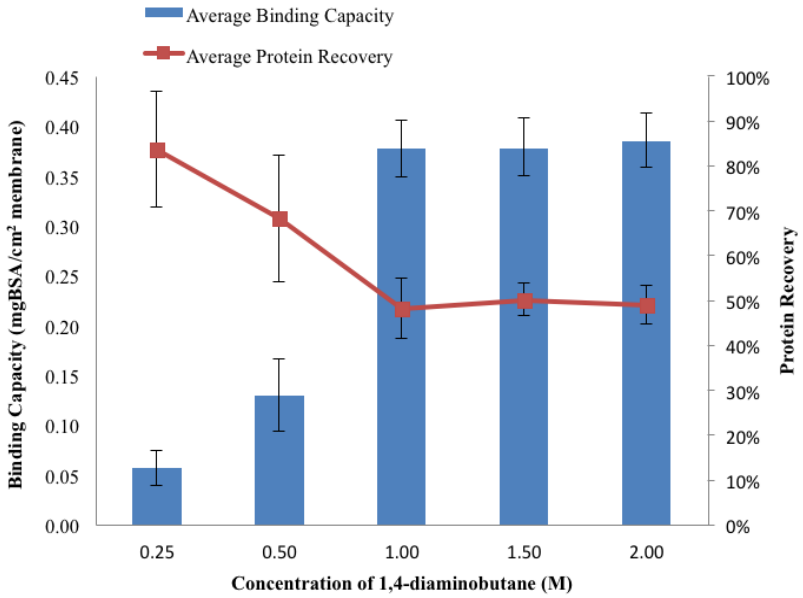

Fig. 5: Binding capacity and protein recovery for different concentration 1,4-diaminobutane

Conclusions:

Regenerated cellulose membrane modified with 1,2-diaminoethane and 1,4-diaminobutane was compared during this study. Membrane modified with longer spacer arm length 1,4-diaminobutane showedhigh average binding capacity of 0.298±0.041 mgBSA/cm^2 membrane. Optimum concentration of 1,4-diaminobutane monomer was achieved at 2.0M which showed a binding capacity of 0.385±0.027mgBSA/cm^2 membrane.

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