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Separation of proteins using hydrophobic interaction membrane chromatography

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Abstract

Membrane chromatography can overcome some of the problems associated with packed bed chromatography. In most membrane chromatographic studies reported so far, ion-exchange and affinity interactions have been utilised. In this paper the use of hydrophobic interactions for chromatographic separation is described. A polyvinylidene fluoride membrane was identified which could bind specific proteins in the presence of high ammonium sulphate concentration. The separation of CAMPATH-1G monoclonal antibody and bovine serum albumin using this membrane is discussed. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Hydrophobic interaction membrane chromatography; Membranes; Proteins; Albumin; Monoclonal antibodies

1. Introduction

Chromatography is widely used for the separation and analysis of proteins and nucleic acids. Chromatographic processes are traditionally carried out using packed beds. However, packed bed chromatography using conventional (soft) chromatographic media has several major disadvantages. The pressure drop across the column is high even at low flow rates. The pressure drop also tends to increase during the process due to bed consolidation. In addition to this there are major diffusional limitations to the transport of solute molecules to their binding sites within the pores of the chromatographic media. With these soft materials, the particle size distribution is broad and this affects the axial and radial dispersion of solutes within the column. Employing monodisperse, non-porous, rigid particles that have all the binding sites on the surface of the chromatographic media can solve some of these problems [1,2]. The use of rigid macroporous beads for chromatographic separation of proteins has also been reported [3]. However, these media are expensive and with non-porous beads there is drastic lowering of the binding capacity since binding takes place only on the surface. Also the problem of high-pressure drop still persists and there are problems associated with reproducibility.

An alternative approach to solving some of these problems is to use synthetic membranes as chromatographic media [4–21]. In membrane chromatography, the transport of solutes to the binding sites takes place by convection and hence the process is very fast. Such membrane adsorbers are also referred to as convective interaction media (CIM). Membrane

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chromatography has been used for analytical and preparative separations. Most of the problems associated with soft chromatographic media are solved using membranes. The only drawback is the slightly lower binding capacity of membrane adsorbers compared with packed beds.

Different types of interactions (e.g. ion-exchange, affinity, hydrophobic interaction and reversed phase) have been utilised in membrane chromatography. Some of the work done in this area has been reviewed in Refs. [4–6]. Ion-exchange and affinity interactions have been more widely used [7–17]. There are relatively fewer references on the use of hydrophobic and reverse phase interactions [18–21]. A general discussion on hydrophobic interaction chromatography of proteins is available in the book by Gooding and Regnier [22]. In this paper, hydrophobic interaction chromatographic separation of proteins (CAMPATH-1G monoclonal antibody and bovine serum albumin) using polyvinylidene fluoride (PVDF) membrane is discussed.

2. Experimental

2.1. Material

CAMPATH-1G monoclonal antibody (batch no. 60, concentration 5.882 mg/ml) was kindly donated by the Therapeutic Antibody Centre, Oxford, UK. CAMPATH-1G is a rat IgG2b antibody, which was earlier used to treat kidney graft rejection. It is now used to treat recipients of bone marrow transplants, especially where the risk of rejection is very high. Bovine serum albumin (BSA) (catalogue no. A8022) was purchased from Sigma (St. Louis, MO, USA). Disposable PVDF syringe filters (13-mm diameter, 0.2-μm pore size, catalogue no. 6777 1302) were purchased from Whatman, Maidstone, UK. The membrane used in the syringe filter was found to have a thickness of ~0.09 mm. All buffers and sample solutions were prepared using ultra-pure water (18.2 MΩ cm) obtained from a Simplicity (Millipore) water purification unit.

2.2. Chromatographic equipment

A fast protein liquid chromatography (FPLC) system was used for carrying out the chromatographic studies. The syringe filters were integrated with the FPLC unit using standard PEEK tubing and tubing connectors. The dead volume was kept at a minimum.

2.3. Buffers and protein samples

The monoclonal antibody was found to bind to the PVDF membrane only in the presence of high ammonium sulphate concentration. Preliminary experiments indicated that the minimum concentration of ammonium sulphate required for binding to take place was 1.8 *M*. Therefore, 20 m*M* sodium phosphate buffer, pH 7, containing 2 *M* ammonium sulphate was used as the binding buffer in all the subsequent experiments. The eluting buffer was 20 m*M* sodium phosphate, pH 7. The monoclonal antibody and BSA solutions were prepared in binding buffer. Prior to chromatographic runs these feed solutions were centrifuged at 13 000 rpm for 20 min and the concentration of monoclonal antibody/BSA remaining in solution was measured using the Bradford assay method [23].

3. Results and discussion

3.1. Dynamic adsorption of monoclonal antibody on PVDF membrane

Dynamic adsorption studies were carried out in the pulse mode using different concentrations of the monoclonal antibody. The sample pulse volume was maintained constant at 200 μl. In these dynamic adsorption studies the flow rate was maintained fixed at 1 ml/min and this gave a superficial velocity of 0.753 cm/min through the membrane. The monoclonal antibody samples were injected after 2 ml of binding buffer flow through the membrane. After the sample injection, the binding buffer flow was maintained for another 2 ml after which a linear gradient from binding to eluting buffer was carried out over 2 ml of flow. The eluting buffer flow was maintained for another 2 ml to ensure complete elution of bound material. The amount of monoclonal antibody bound to the membrane was determined from the bound and unbound peak areas:

$$M_b = M_i \cdot \left(\frac{AUC_b}{AUC_b + AUC_u} \right) \quad (1)$$

For the monoclonal antibody concentration range examined, the *AUC* was found to vary linearly with the concentration and the line passed through the origin. Therefore this correlation could be used.

Fig. 1 shows the amount of monoclonal antibody bound per unit membrane volume at different applied concentrations. The amount bound increased almost linearly up to an applied concentration value of 0.631 mg/ml. The corresponding amount bound to the membrane was ~10 mg/ml. With further increase in applied concentration the amount bound did not increase significantly, i.e. a kind of saturation value was reached.

An adsorption isotherm based on the equilibrium solution phase concentration often gives more meaningful information about the nature of the binding process. Fig. 2(a) shows a plot of the amount bound to the membrane (q) versus the dynamic equilibrium solution concentration (y). These dynamic equilibrium concentration values were obtained from the *AUC* of the unbound monoclonal antibody peaks. Fig. 2(b) shows a double reciprocal plot of q and y.

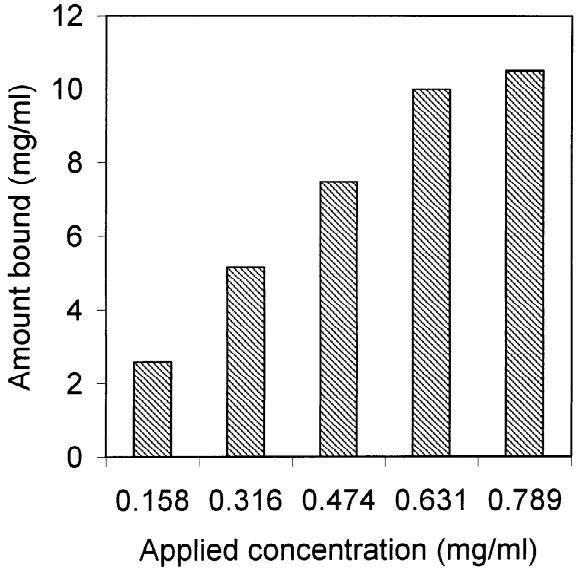

Fig. 1. Adsorption of CAMPATH-1G monoclonal antibody on PVDF membrane: effect of applied concentration on amount bound.

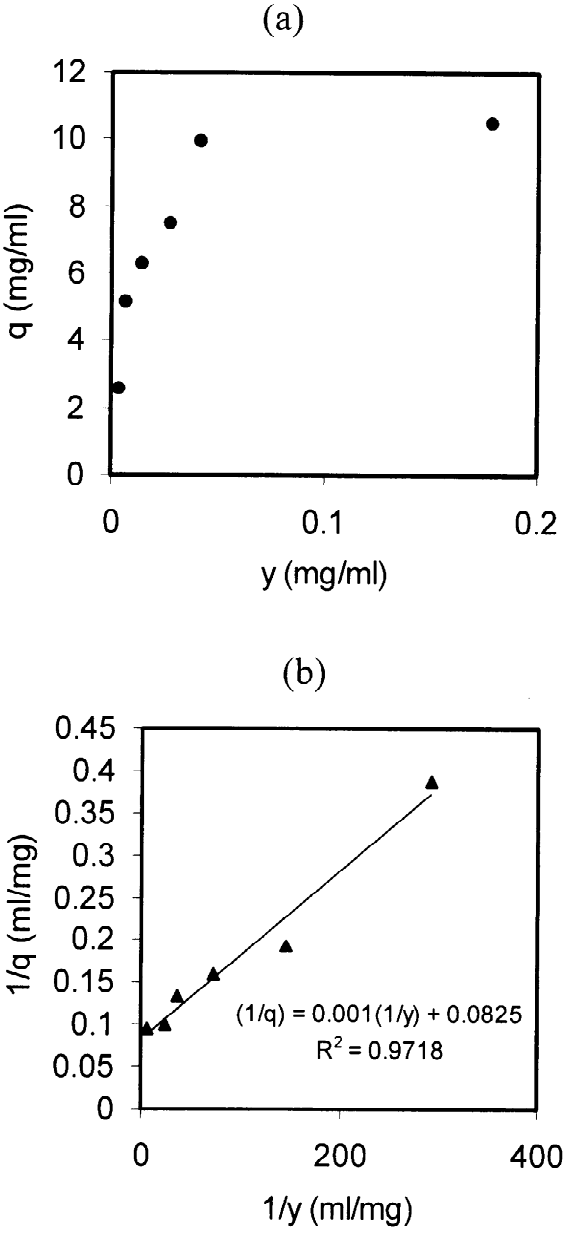

Fig. 2. Adsorption isotherm for CAMPATH-1G monoclonal antibody based on dynamic equilibrium solution concentration: (a) normal plot; (b) double reciprocal plot.

The points on this double reciprocal plot show reasonably good linear fit ($R^2 = 0.9718$). Therefore the adsorption isotherm for the binding of monoclonal antibody onto the PVDF membrane was of the Langmuir type:

$$q = \frac{q^* y}{y^* + y} \quad (2)$$

From a linear regression $q^* = 12.121$ mg/ml and $y^* = 0.0121$ mg/ml. Therefore the peak monoclonal antibody binding capacity of the membrane was 12.121 mg/ml. The low y^* value indicates that the amount of the protein bound to the membrane would be very high even at very low concentrations.

Fig. 3 shows the effect of applied concentration on the fractional binding of monoclonal antibody. The fraction bound (f) is defined as the amount bound divided by the amount applied:

$$f = \frac{AUC_b}{AUC_u + AUC_b} \quad (3)$$

The binding of monoclonal antibody to the PVDF membrane was also examined in the step input mode. This experiment was carried out at a flow rate of 1 ml/min and the concentration of monoclonal antibody in the feed solution was 0.3 mg/ml. The feed solution was prepared using the binding buffer. This was passed through the column as step input. From the absorbance–effluent volume profile, the breakthrough curve (Fig. 4) was constructed using an

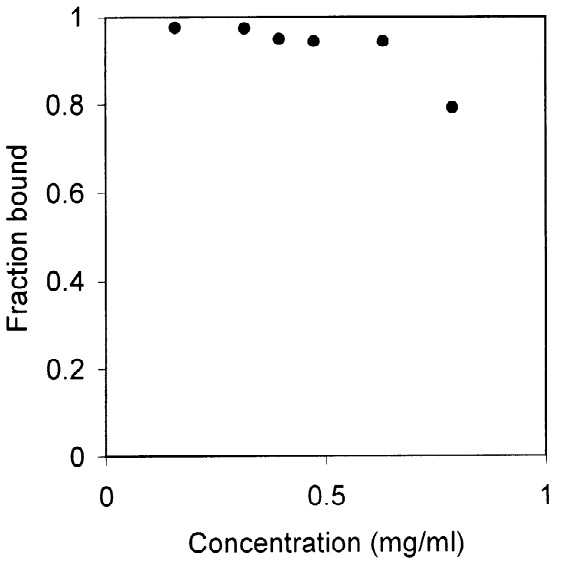

Fig. 3. Effect of monoclonal antibody concentration on fraction bound.

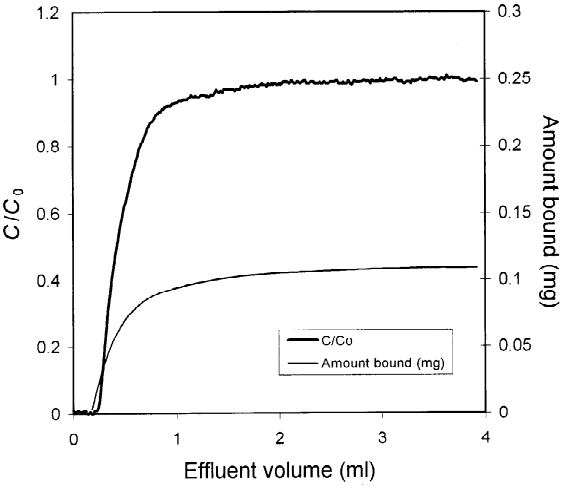

Fig. 4. Breakthrough curve for CAMPATH-1G monoclonal antibody.

appropriate calibration for the monoclonal antibody. The cumulative amount of antibody bound to the membrane (Fig. 4) was calculated from the breakthrough curve using material balance. The amount of monoclonal antibody bound to the membrane after 3.92 ml of feed flow was 0.1086 mg. The corresponding q value was 9.091 mg/ml.

3.2. Effect of flow rate on the binding of monoclonal antibody

Sarfert and Etzel [24] and Tennikov et al. [25] have discussed the mass transfer phenomenon of membrane chromatography. In the present study the effect of flow rate on the fractional binding of monoclonal antibody was examined in the range of 1–9 ml/min. Fig. 5 shows the fractional binding at different superficial velocities. For the experimental range examined, the fractional binding was found to be independent for the superficial velocity. This indicates that even at the highest flow rate examined the protein molecules travelling through the pores had sufficient time to be transported to the binding sites on the pore wall. The average Reynolds number of the fluid flowing through the membrane pores is given by:

$$Re_{pore} = \left(\frac{u_s d_{pore}}{\nu \epsilon} \right) \quad (4)$$

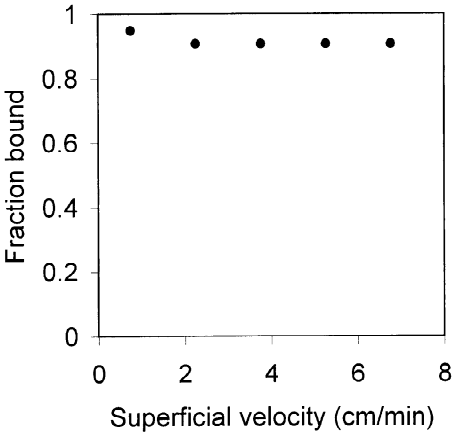

Fig. 5. Effect of flow rate on fraction bound (antibody concentration = 0.395 mg/ml; sample volume = 200 μl).

The smaller the value of ϵ the larger the pore Reynolds number. Even for a small value for ϵ (say 0.2) the pore Reynolds number (assuming $d_{pore} = 0.2$ μm) at a flow rate of 9 ml/min was insignificantly small. Therefore the flow was expected to be laminar at all the flow rates examined. For laminar flow the centreline velocity is twice the average velocity. Therefore the residence time of a fluid element in the centreline of the fluid flowing through a membrane pore is given by:

$$\theta_C = \frac{\delta_m \tau \epsilon}{2u_s} \quad (5)$$

The greater the value of τ the greater the centreline residence time. For the minimum value, i.e. $\tau = 1$, the centreline residence time at a flow rate of 9 ml/min would be $7.96 \cdot 10^{-3}$ s. The time taken for a monoclonal antibody molecule to diffuse from the centreline to the pore wall is given by:

$$\theta_D = \left(\frac{d_{pore}^2}{4D} \right) \quad (6)$$

For a diffusivity value of $4.2 \cdot 10^{-11}$ m^2/s θ_D is $2.38 \cdot 10^{-4}$ s. Therefore, even at the highest flow rate the centreline molecules at the pore inlet had sufficient time to diffuse to the pore wall.

3.3. Separation of BSA and monoclonal antibody

The chromatographic separation of BSA and the monoclonal antibody was carried out at a flow rate of 1 ml/min. BSA and BSA/monoclonal antibody solutions were prepared in binding buffer. A 100-μl sample loop was used for sample injection. After sample injection, 2 ml of the binding buffer was allowed to flow through the membrane, followed by a linear gradient from binding buffer to eluting buffer which was carried out over 12 ml of liquid flow. Fig. 6 shows the chromatogram (a) obtained by injecting BSA solution having a concentration of 0.2 mg/ml. The BSA did not bind to the membrane. A small double peak was observed around 7-ml effluent volume. This was probably due to trace amounts of bovine IgG, which are normally present in commercially available BSA. Fig. 6 also shows the chromatogram (b) obtained by injecting a mixture of BSA (0.3 mg/ml) and monoclonal antibody (0.238 mg/ml). Quite clearly these two proteins could be separated using the PVDF membrane by salt induced hydrophobic interaction chromatography.

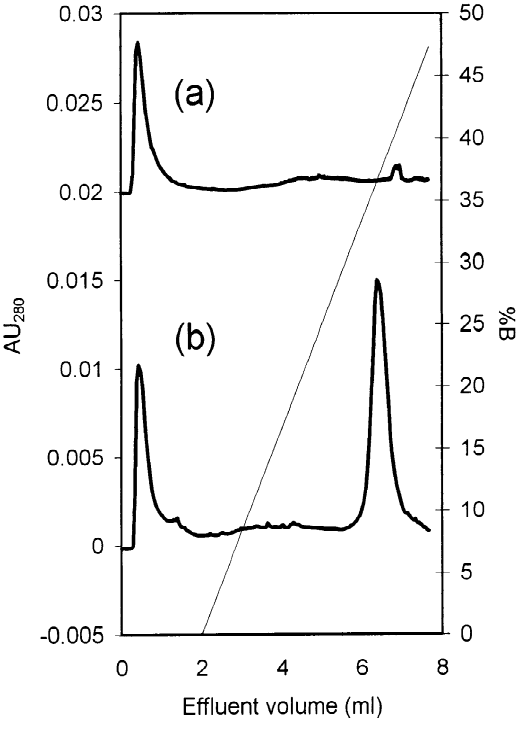

Fig. 6. Separation of BSA and CAMPATH-1G monoclonal antibody using hydrophobic interaction membrane chromatography: (a) BSA; (b) BSA + monoclonal antibody.

4. Conclusions

From the experimental results the following can be concluded.

(a) The PVDF membrane used in the experiments could selectively bind CAMPATH-1G monoclonal antibody at high ammonium sulphate concentrations.

(b) The adsorption isotherm indicated a peak binding capacity of ~12 mg/ml. The low y^* value obtained from the isotherm indicated that the amount of the protein bound to the membrane would be high even at very low concentrations.

(c) The flow rate did not affect the fraction of monoclonal antibody bound in the flow rate range examined.

(d) BSA did not bind to the membrane.

(e) Monoclonal antibody and BSA could thus be separated using a gradient chromatographic programme.

5. Nomenclature

AUC_b	Area under curve of bound peak (AU-m^3)
AUC_u	Area under curve of unbound peak (AU-m^3)
C_0	Feed concentration (kg/m^3)
d_{pore}	Average pore diameter (m)
D	Diffusion coefficient (m^2/s)
M_b	Mass bound to membrane (kg)
M_i	Mass injected (kg)
q	Amount bound per unit membrane volume (kg/m^3)
q^*	Saturation constant (kg/m^3)
Re_{pore}	Pore Reynolds number (–)
u_s	Superficial liquid velocity (m/s)
y	Dynamic equilibrium liquid phase concentration (kg/m^3)
y^*	Saturation constant (kg/m^3)

Greek symbols

δ_m	Membrane thickness (m)
ϵ	Porosity (–)
ν	Kinematic viscosity (m^2/s)
θ_C	Centreline residence time (s)
θ_D	Diffusion time (s)
τ	Tortuosity (–)

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