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Electrophoretic transfer of proteins across polyacrylamide membranes

D.B. Rylatt[a,*], M. Napoli[a], D. Ogle[a], A. Gilbert[a], S. Lim[b], C.H. Nair[a]

[a]*Gradipore Ltd., P.O. Box 1865, Macquarie Centre, Sydney, NSW 2113, Australia*

[b]*Macquarie University Centre for Analytical Biotechnology, Sydney, NSW 2109, Australia*

Abstract

The electrophoretic transfer of purified proteins has been examined in a Gradiflow ''Babyflow BF100'' unit. A number of factors affect protein separation within this preparative electrophoresis system. We established that the rate of protein transfer was proportional to the applied voltage. The transfer is slowest at the isoelectric point (p*I*) and increased the further away the pH was from the p*I* of the protein. Protein transfer was found to be independent of the ionic strength of the buffer, for buffers that excluded the addition of strong acids or strong bases or sodium chloride. Transfer decreased as the pore size of the membrane decreased. Finally, transfer was inhibited at high salt concentrations in the protein solution, but remained unaffected when urea and non-ionic detergents were added to the solution. To increase the speed of protein separations, buffers with low conductivity should be used. A pH for the optimal separation should be selected on the basis of the relative p*I* and size of the target proteins and that of the major contaminants.

Keywords: Membranes; Polyacrylamide membranes; Preparative electrophoresis; Proteins

1. Introduction

A variety of electrophoretic techniques have been developed for the processing of charged macromolecules with the most successful being polyacrylamide gel electrophoresis, isoelectric focusing and capillary electrophoresis. Attempts to translate this resolution to a more preparative scale have been less successful because the increasing volume of the porous matrix in a larger apparatus makes heat removal more difficult. Nevertheless, partial success has been achieved with some preparative systems including free flow electrophoresis [1], recycling isoelectric focusing [2], Righetti's multi-compartment electrolyser [3] and ''conventional'' gel preparative systems such as the Bio-Rad 491 preparative cell.

The Gradiflow apparatus was first described by Horvath et al. in 1994 [4]. This preparative electrophoresis system uses thin polyacrylamide membranes with defined pore sizes to enable separation of macromolecules on the basis of size or charge under ''native'' conditions. The novel design has overcome the heating problems usually associated with such systems by using a cooling buffer that continuously circulates through the system. Applications described previously include the purification of proteins from plasma and algal extracts [4], eggwhite [5], milk and recombinant proteins [6], pre-fractionation of human serum proteins prior to two-dimensional electrophoresis [7] and monoclonal antibodies from ascitic fluid [8]. The instrument as initially described with its peltier coolers, precision peristaltic pumps, computer control and in-built power remained a laboratory curiosity until the recent development of a simpler

*Corresponding author.

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ice cooled version termed the ''Babyflow''. This more portable version of the instrument uses a conventional peristaltic pump for the upstream and the downstream, a simple centrifugal pump for the buffer stream and a standard 200 V, 500 mA laboratory electrophoresis power supply.

Gradiflow technology allows the separation of native proteins by a combination of size and/or charge strategies. The effects of various parameters such as voltage, current, buffer strength and protein concentration has yet to be clearly defined. Here we describe a series of studies that define the mobility of proteins in the ''Babyflow'' apparatus. These investigations will assist in the development of a rationale for protein purification on the Gradiflow system.

2. Experimental

2.1. The instrument

Gradiflow technology has been described in detail previously [4,8]. The Babyflow BF100 unit is shown in Fig. 1. The separation unit is located on the front above the upstream and the downstream reservoirs. A disposable membrane cartridge is inserted into the separation unit, which is opened and closed by rotating the knob. The buffer reservoir is located behind the front panel of the Babyflow and is cooled by an ice-filled stainless steel beaker, which sits within the buffer reservoir. A small centrifugal pump circulates the buffer whilst a two channel peristaltic pump circulates the upstream and the downstream.

A schematic cross-section of the separation unit is shown in Fig. 2. The separation unit is an electrolytic cell consisting of four chambers. The two outer chambers house the electrodes and a flow of buffer passes through these chambers. The two inner chambers, which are the upstream and the downstream channels, are separated from the outer buffer chambers by restriction membranes. The restriction membranes consist of a tightly cross-linked polymer of pore size M_r 3500, which allows the movement of molecules and ions up to this molecular mass. A separation membrane, which is available in sizes ranging from M_r 3500 to $1 \cdot 10^6$, separates the upstream and the downstream chambers. The upper electrode is the cathode (negative) and the lower electrode is the anode (positive).

Fig. 3 shows an exploded view of the membrane cartridge. The upstream and the downstream channels are formed between the separating membrane and restriction membrane. The grid is a lattice structure, which supports the membranes at a fixed distance apart, while maximising the area available to the membrane. The thin lattice distributes the flow of liquid evenly over the membrane surface, inducing mixing of the liquid and preventing the formation of static zones. The components of the membrane cartridge are held together by the housing and the retaining clip. Both the electrodes and buffer chambers are incorporated into the body of the separation unit. The gaskets provide the sealing necessary to ensure that all streams remain separate once the cartridge is clamped into the unit.

2.2. Purified proteins

Purified proteins were, unless otherwise stated, obtained from Sigma (St. Louis, MO, USA). Each protein (2–10 mg/ml) was dissolved in the running buffer and 10 ml of the protein solution was placed in either the upstream or the downstream. Transfer at the stated pH was measured at 280 nm using a Cary 50 UV–visible spectrophotometer (Varian, Australia). Samples were diluted for protein determination when the absorbance at 280 nm was greater than 2.0.

2.3. Buffers

The choice of buffer is a major factor for ensuring the quality of separation in the Gradiflow. Buffers of low conductivity are recommended. The buffers used in the separations were as follows: pH 3.0–4.0, lactic acid–GABA (γ-aminobutyric acid); pH 4.0–5.5, acetic acid–GABA; pH 5.5–6.5, MES [2-(*N*-morpholino)ethanesulphonic acid]–histidine; pH 6.5–7.5, imidazole–HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulphonic acid]; pH 7.6–9.0, Tris–boric acid.

Unless otherwise stated the concentration of each buffer was between 10 and 100 m*M* depending on the required pH. Each buffer had conductivity less than 0.5 mS (measured on a RS 180-7127 conductivity meter). Titration to reach the required pH

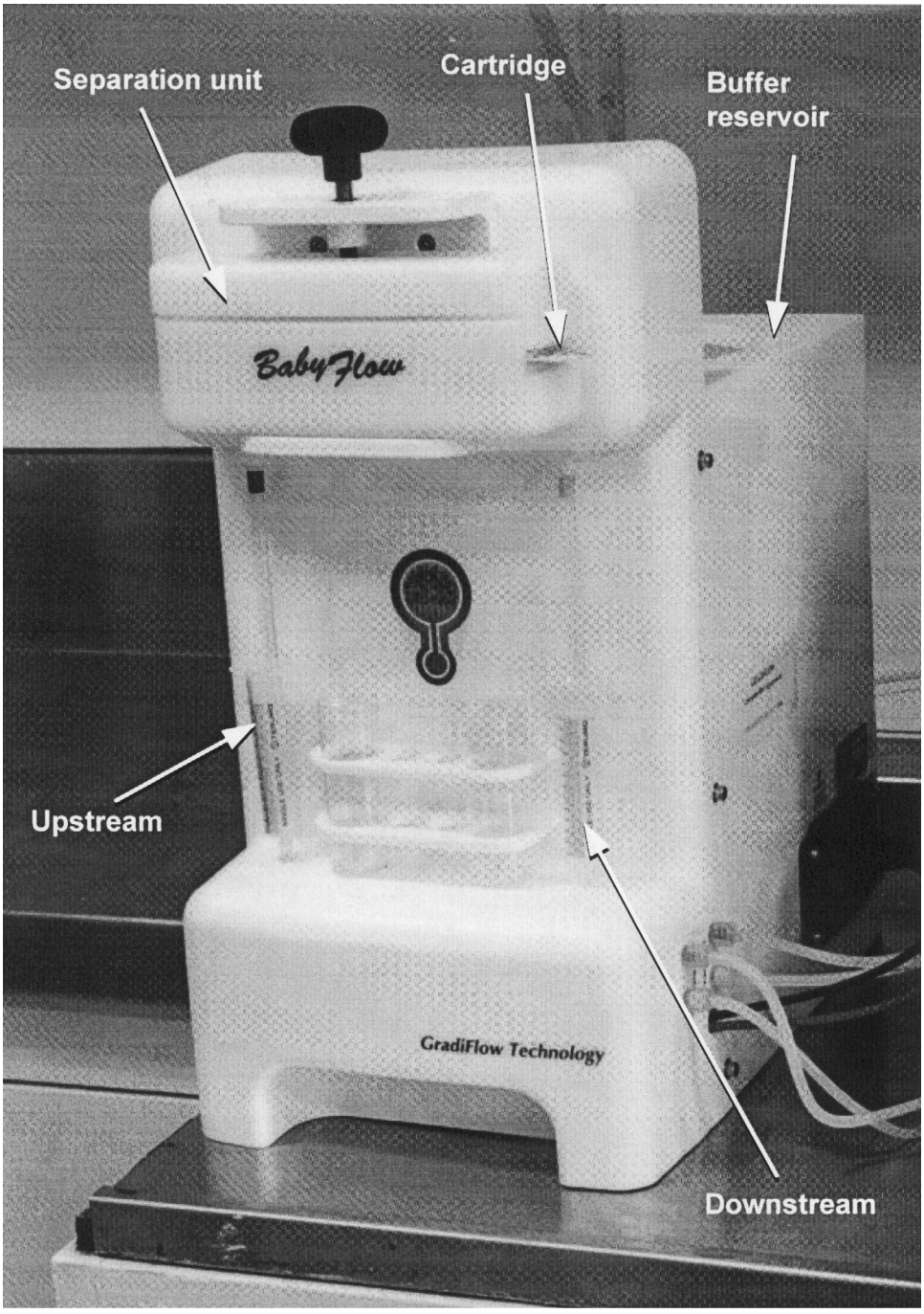

Fig. 1. Front view of Babyflow BF100. Note the location of the upstream and the downstream reservoirs, the separation unit, which houses the electrodes, and the cartridge inside the separation unit. The dimensions of the Gradiflow are 470 mm high, 250 mm wide and 390 mm deep. It weighs 6.6 kg.

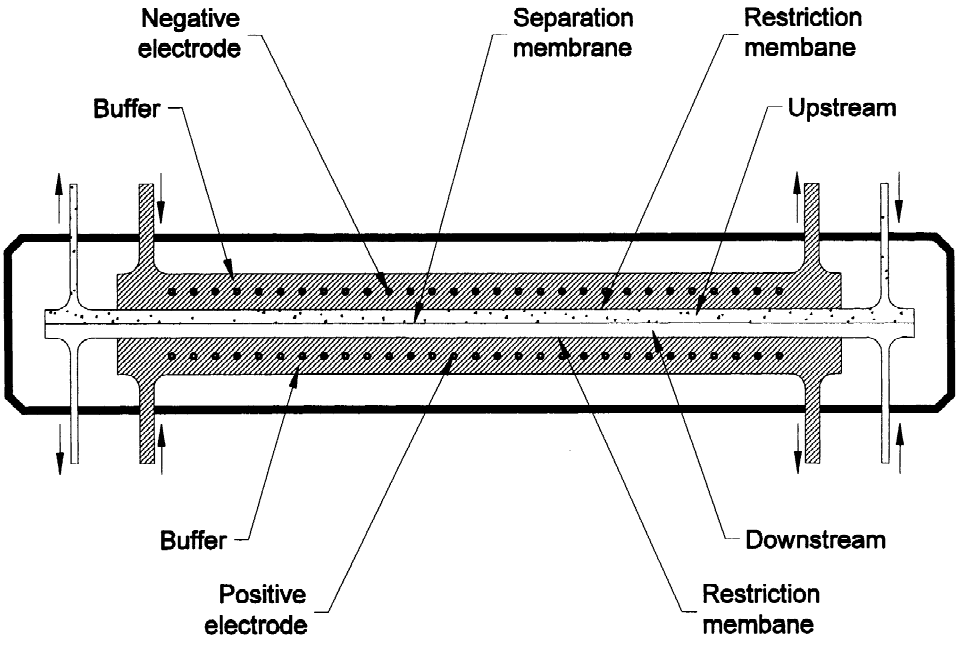

Fig. 2. Schematic cross-section of separation unit. The unit consists of four separate chambers, two of which are contained entirely within the membrane cartridge. The arrows show the direction of flow of the running buffer and of the upstream and the downstream.

was carried out by mixing together the required amount of acidic and basic components. For example, between pH 6.5 to 7.5, only HEPES or imidazole are mixed. It is important to avoid the addition of strong acids or bases, such as hydrochloric acid or sodium hydroxide, which will significantly (and undesirably) increase the conductivity of the buffer.

2.4. Membranes

Membranes are thin 0.1 mm polyester paper impregnated with polyacrylamide. These are prepared by a proprietary procedure and are available from Gradipore, Sydney, Australia. Variation in the porosity of different membranes is achieved by varying the ratio and amount of acrylamide and bis-acrylamide.

3. Results and discussion

3.1. Effect of voltage and buffer concentration

Fig. 4 shows the time dependent transfer of a 10 mg/ml solution of bovine serum albumin (BSA) across a M_r $1{\cdot}10^6$ membrane in a buffer of pH 7.2 containing 62 m*M* HEPES and 44 m*M* imidazole at varying applied voltages. The initial rate of transfer is directly proportional to the applied voltage. As the voltage was increased, there was a proportional increase in current flow (not shown). The heat generated at higher voltages imposes a practical upper limit to the voltage which can be applied to the Gradiflow. For the ice cooled Babyflow described here, this is around 200–300 V for the buffers described in this paper.

Fig. 5 shows BSA transfer at pH 7.2 carried out at a constant voltage (200 V) with a varying imidazole–HEPES buffer concentration of 20 m*M* to 160 m*M*, i.e., from one-quarter to two times the normal buffer strength. Very little change was observed in the rate of transfer of the protein with increasing buffer strength. This implies that there is no advantage in the rate of transfer by using a buffer with a higher ionic strength. However, the increased current at higher buffer strengths (see inset Fig. 5) imposes an upper limit on the ionic composition of buffers useful with the Gradiflow. Higher buffer strengths

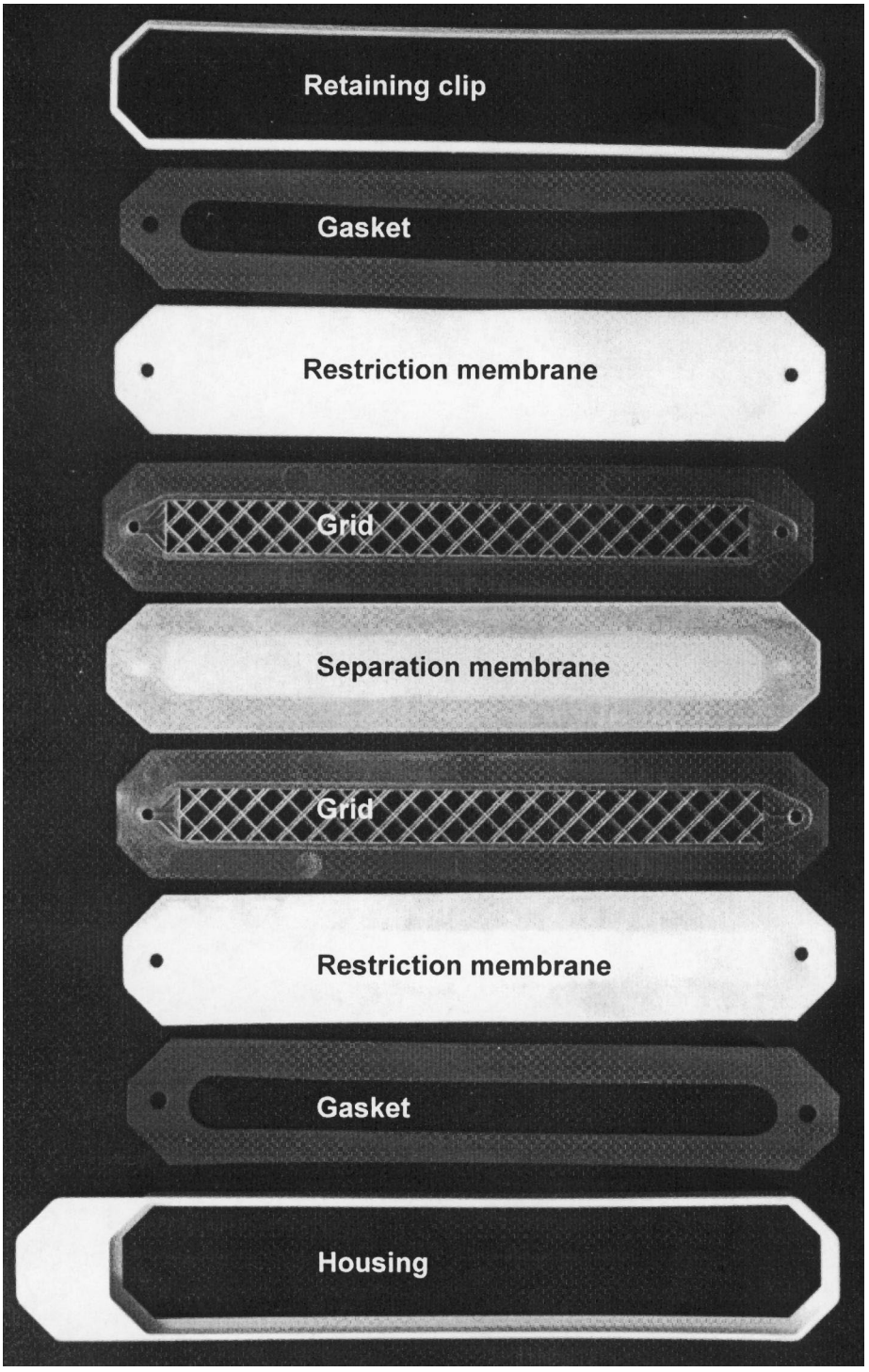

Fig. 3. Exploded view of membrane cartridge. The cartridge consists of three separate polyacrylamide membranes separated by grids, which provide support and maintain even buffer flow. The protein solution is contained between the separation and restriction membranes. The running buffer flows above and below the restriction membranes. The assembled cartridge is 15 cm long, 3 cm wide and 1 cm deep.

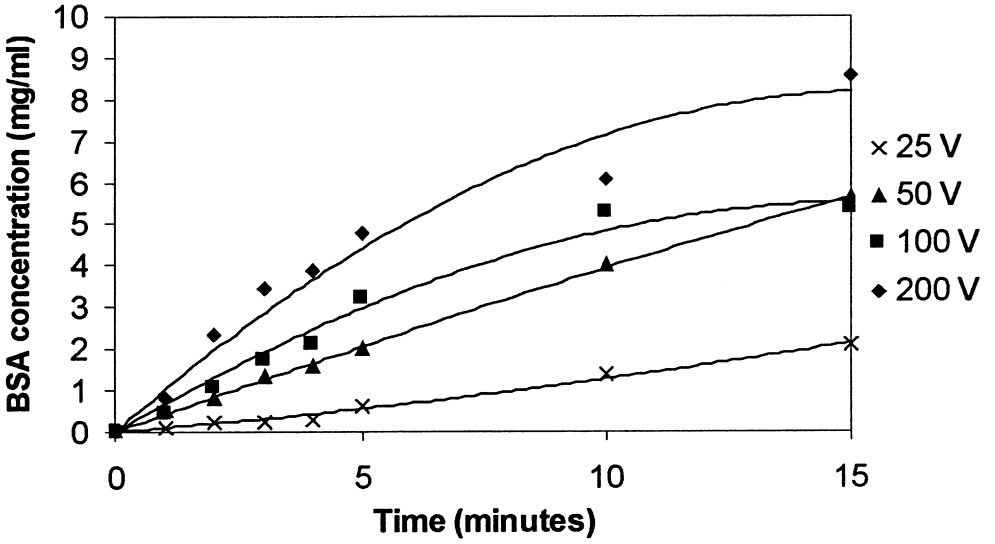

Fig. 4. Voltage dependence of protein transfer. Time dependent transfer of BSA at 25 V (×), 50 V (▲), 100 V (■) and 200 V (♦).

can lead to unwanted heat generation and currents above 500 mA to 1 A should be avoided. Low conductivity buffers allow the separation to be optimised between a high enough ionic composition to keep the protein in solution, conductivity and effective buffering capacity. Compositions of buffers suitable for most applications in the pH range 3 to 9 are shown in Section 2.3.

3.2. Effect of varying pH on transfer of proteins

Fig. 6a–e shows the rate of transfer at 200 V for a

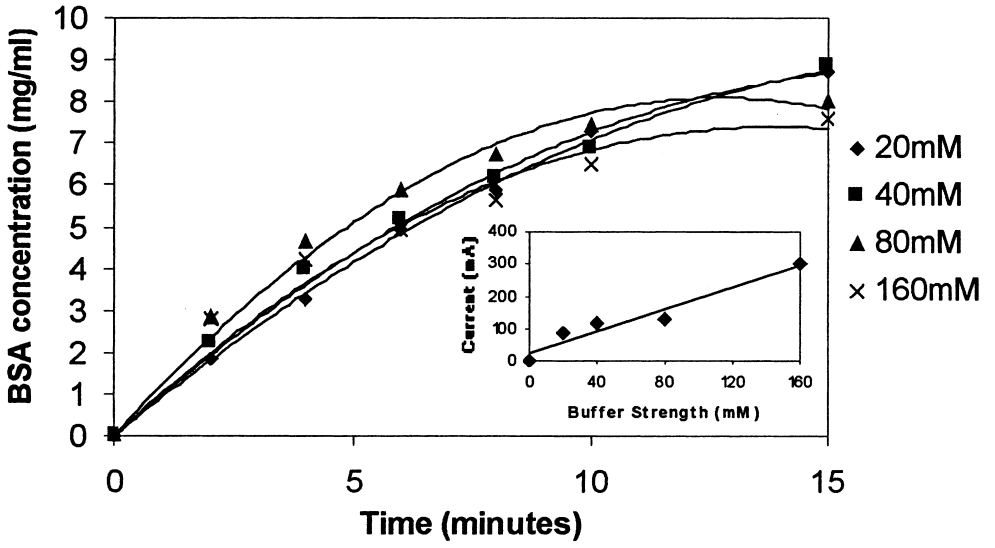

Fig. 5. Effect of increasing buffer concentration. Plot of time dependent BSA transfer at 20 m*M* (♦), 40 m*M* (■), 80 m*M* (▲) and 160 m*M* (×). (Insert: plot of current versus increasing buffer concentration).

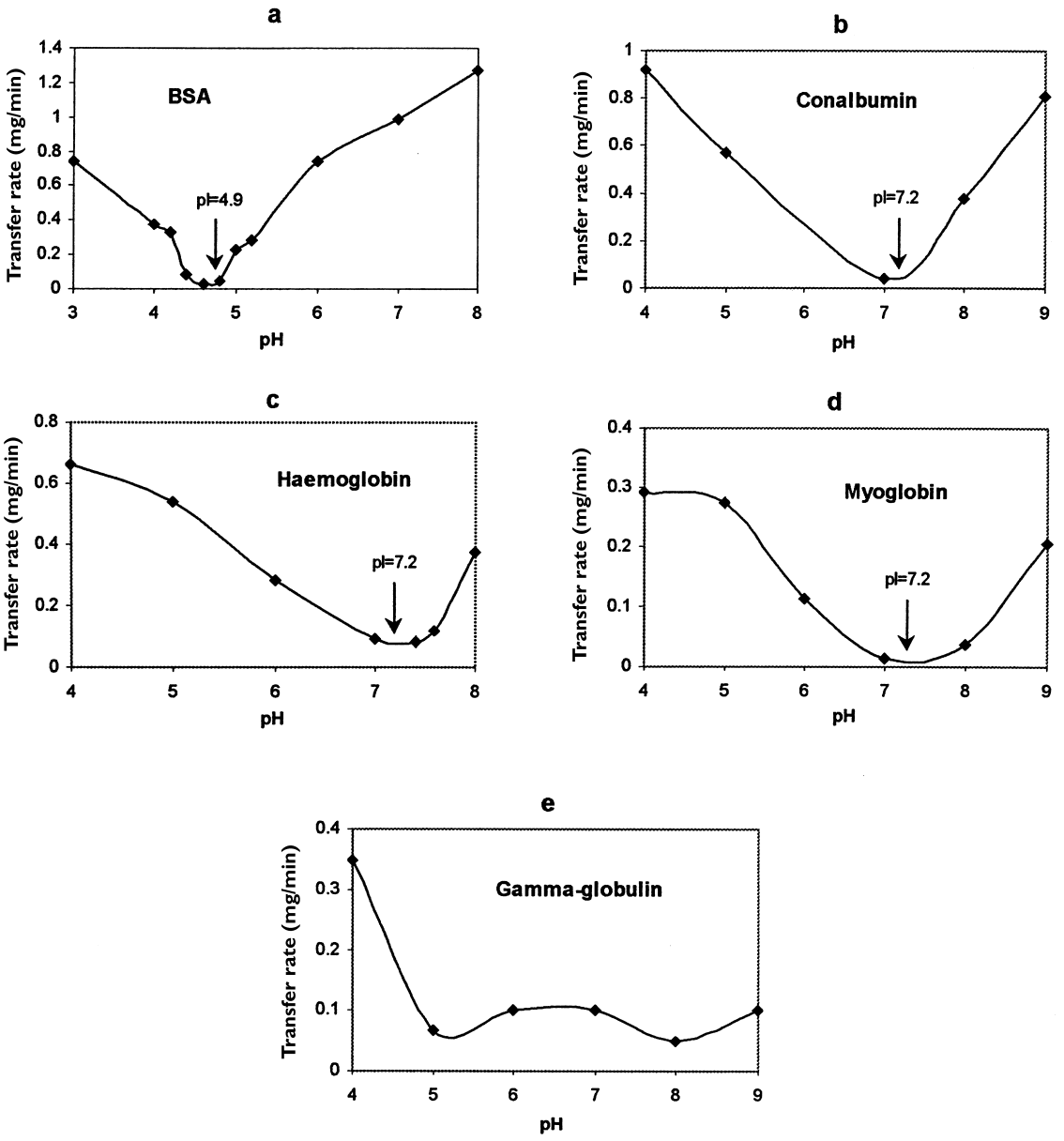

Fig. 6. Transfer of proteins at different pH. Plot of the rate of transfer (measured over 20 min) at different pH values for BSA (a), conalbumin (b), haemoglobin (c), myoglobin (d) and human immunoglobulin (e).

number of different protein preparations at different pH values across a M_r $1 \cdot 10^6$ membrane. The rate and direction of transfer was dependent on the buffer pH and the pI of the protein. For the purified proteins (a–d), there is no movement near the isoelectric point (p*I*) of the protein. For example, for BSA (Fig. 6a), no migration occurs at pH 4.9 which is the p*I* of albumin [8]. When pH values further away from the p*I* of albumin were selected, mobility increased with increasing charge. At lower pH values, serum albumin has a positive charge and moves towards the cathode. At higher pH values, the same protein has a

negative charge and moves towards the anode. Similar patterns were observed for the other proteins: conalbumin (Fig. 6b, p*I* 7.2); bovine haemoglobin (Fig. 6c, p*I* 7.2) and horse myoglobin (Fig. 6d, p*I* 7.2). Fig. 6e shows a similar profile with human gamma globulin. In this case the sample contains a range of p*I* values, with an average p*I* between pH 6 and pH 7. Little movement of immunoglobulin was noticed until the pH was brought down to pH 4. For larger proteins, like immunoglobulin, it is apparent that pH values of 2 or more pH units from the pI may be required to generate enough electromotive force to move the protein through the membrane.

Knowledge of the p*I* of the protein is one important determinant in working out a purification strategy for a particular target protein. A pH should be selected so that either: (a) the target protein of choice remains stationary while contaminants migrate across the separation membrane or (b) the protein of choice migrates across the membrane, leaving the contaminants behind in either the upstream or the downstream. The molecular mass of the target protein should also considered when determining transfer conditions.

3.3. Effect of concentration of protein on transfer

Fig. 7 shows the transfer of BSA at increasing concentrations of BSA at 100 V and pH 7.2 in imidazole–HEPES buffer across a M_r $1{\cdot}10^6$ membrane. The rate of transfer was proportional to the concentration of albumin up to 200 mg/ml. Results indicate that provided the protein remains in solution, the transfer of protein increases linearly with increasing protein concentration. It has been previously shown that the throughput of the Gradiflow was proportional to the surface area of the membrane [4], indicating that the Gradiflow technology appears to be scalable.

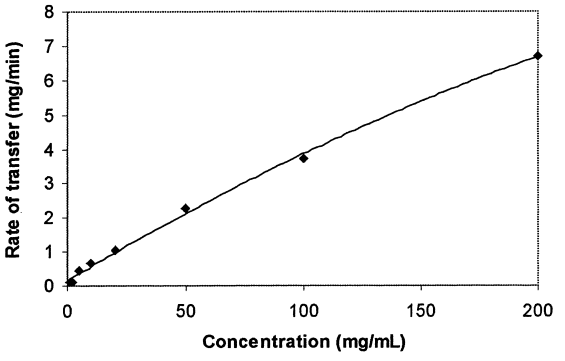

Fig. 7. Effect of protein concentration on protein transfer. Plot of rate of transfer over 20 min versus initial albumin concentration.

3.4. Effect of pore size of separation membrane on protein transfer

The rate of transfer of BSA through various pore sized separation membranes is shown in Fig. 8. Results show that the fastest transfer occurs with a membrane of M_r $1{\cdot}10^6$ cut-off which has the largest pores. As the membrane pore size approached the size of the protein, the rate of transfer slowed. There was no transfer across a membrane of M_r 50 000 (not shown).

3.5. Effect of salt, urea and non-ionic detergents in the sample

Fig. 9 shows the transfer of BSA at pH 7.2 in the presence and absence of 300 m*M* NaCl in the sample. In the absence of added salt, rapid transfer was observed. Transfer of BSA was inhibited in the presence of 300 m*M* NaCl. There is an initial lag phase in which the salt is removed by electrodialysis as shown by the decrease in conductivity of the sample before BSA transfer occurs. After 10 min, the rate of BSA transfer begins to approach the rate of transfer as determined previously in the absence of

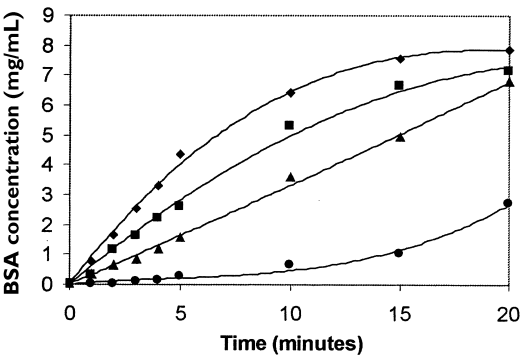

Fig. 8. Effect of pore size. The time dependent transfer of BSA across separating membranes with varying pore sizes: M_r 100 000 (●); 200 000 (▲); 500 000 (■) and $1{\cdot}10^6$ (♦).

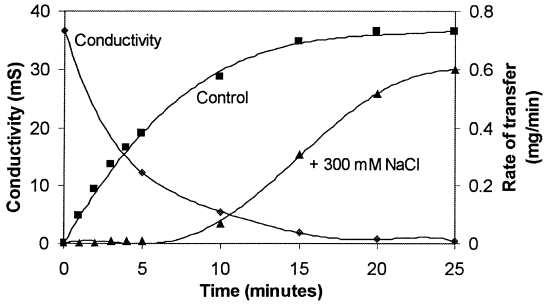

Fig. 9. Effect of salt. The time dependent transfer of BSA in the presence (▲) and absence (■) of 300 m*M* NaCl. The time dependent decrease in conductivity in the sample is also shown (♦).

added salt. The length of this lag phase depends on the salt concentration (not shown). The competing and inhibitory effect of salt is explained by the fact that the concentration of salt (300 m*M*) is 2000-fold higher than the concentration of BSA (0.15 m*M*). It is not surprising that BSA transfer is very slow until the concentration of salt equilibrates between the running buffer and the upstream and the downstream. Upon equilibration transfer proceeds at near normal rates, even though there remains a 10-fold molar excess of salt to protein under these conditions. Transfer was unaffected by the presence of uncharged molecules such as 8 *M* urea, 5% sucrose and the non-ionic detergent Triton X-100 (0.1%) in the sample (not shown).

Salt is less tolerated in the running buffer. The presence of 30 m*M* NaCl in the cooling buffer is sufficient to prevent the transfer of serum albumin (not shown). With 30 m*M* NaCl, the system contains 100 mg of protein but 3.6 g of added salt and this is sufficient to prevent the movement of protein through the system.

This paper describes some of the basic properties of electrophoretic transfer of proteins at a preparative scale using the Gradiflow. Understanding these properties will enable the rapid transfer of proteins with high yields. Importantly, proteins isolated by the Gradiflow have been shown to maintain their nativity and therefore their biological function [8]. Gradiflow technology has the potential to improve the rapidity and yields of a number of protein purification procedures in the basic research laboratory as well as for commercially important macromolecules.

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