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Separation of Proteins of Nearly the Same Size but Having Different Isoelectric Points by Convective Electrophoresis

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A method for separating proteins of nearly the same size but having different isoelectric points is presented. The method used is convective electrophoresis, which is based on the simultaneous effects of diffusion, electrical migration and opposing convective flow on the transport of proteins through a porous membrane. The pairs of model proteins used in this study were cytochrome c–lysozyme and human serum albumin–haemoglobin. The obtained results show that with a careful adjustment of pH these proteins can be separated by a continuous manner, and no coupling of protein fluxes could be detected in either case.

New genetic and protein engineering technologies have provided new powerful routes for the production of therapeutic and industrially significant products. The product of interest is often present only in small or trace quantities in a highly complex but relatively inexpensive starting material. Furthermore, these products, especially proteins, are in many cases labile.[1,2]

Continuous convective electrophoresis (CCE) through a porous membrane is a new separation strategy for protein purification. The basis for CCE is based on countercurrent electrolysis, which has been studied since 1920 for ionic separations in a batch process. The method, its realization in a continuous mode on a laboratory scale and its mathematical modelling for a continuous process have been presented for separation of small ions,[3–10] proteins[11] and pyrogenes.[12]

In this paper the presentation of this new approach to protein separation is continued. CCE is based on the simultaneous effects of diffusion, electrical migration and opposing convective flow on the transport of proteins through a porous membrane. A mathematical model based on the Nernst–Planck equation with appropriate material balances is derived in order to control the continuous separation process and to simulate the effects of many adjustable parameters involved. The model is examined and the separation efficiency is demonstrated by experiments with mixtures of model proteins.

The pairs of model proteins used in this study were cytochrome c (Cyt)–lysozyme (Lys) and human serum albumin (HSA)–haemoglobin (Hgb). Both of these pairs represent a case where the size of the proteins (manifested by almost the same diffusion coefficients) are about equal but the isoelectric points are different. The heme proteins (Cyt and Hgb) were chosen as the other protein in both pairs so that accurate quantitative protein analysis with a UV–VIS spectrophotometer could be performed.

Experimental

A schematic illustration of the experimental setup for CCE is given in Fig. 1. The symbols for the flow rates (V^j) and corresponding concentration (c_i^j) used in the modelling are shown in connection with each flow. A buffer solution is circulated in the electrode compart-

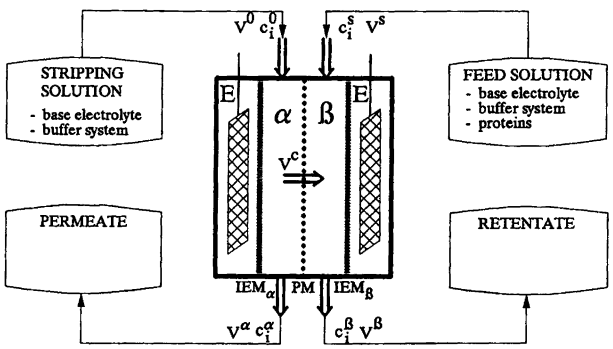

Fig. 1. Schematic drawing of the apparatus for CCE experiments. PM is a porous membrane, IEMs are ion exchange membranes and E denotes platinum electrodes.

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ments to neutralize the ionic products of the electrode reactions, i.e. H^+ and OH^- ions (these flows are not shown in Fig. 1). The compartments α and β are stirred with magnetic stirrers. The flows in the cell and permeate flow were controlled by a peristaltic pump. The retentate flow was a free outlet flow.

The 20% HSA solution was supplied by the Finnish Red Cross Blood Transfusion Service (purity 96.9%, containing 6.65 g/L^{-1} sodium caprulate as stabilizer). The other proteins were supplied by Sigma Chemical Co. (Cyt: C-2506 from horse heart, purity 95–100%; Lys: L-6876 from chicken egg white, purity 95%; Hgb: H-2500 from bovine). All proteins were used as supplied.

The apparatus was the same as presented earlier.[11,12] The membranes used were hydrophilic Durapore DVPP (Millipore, nominal pore diameter 0.65 μm, thickness ca. 0.15 mm, porosity 70%) as a porous membrane, Nafion-423 (DuPont) as cation exchange membrane and Tosflex IE-DF34 (Tosoh) as anion-exchange membrane. The protein concentration of each retentate and permeate sample were determined spectrophotometrically using the multicomponent analysis method (wavelengths 220 and 410 nm for Lys and Cyt, and 280 and 410 for HSA and Hgb) as described elsewhere.[11,12] The electric conductivities of samples were determined by means of a Philips PW9527 conductivity meter and a Radiometer CDC114 conductivity cell which was kept at the same temperature as the separation experiment. A Consort P207 pH-meter and an Ingold U402-M3-S7/60 combination electrode were used for the pH measurements.

In the case of Cyt–Lys experiments the cathode was separated from the α compartment with a cation-exchange membrane (IEM_α in Fig. 1), and the anode was separated from the β compartment with an anion-exchange membrane (IEM_β in Fig. 1). The polarity of the cell was chosen so that the flux of lysozyme would be greater than that of cytochrome c. Owing to the choice of the combination of the ion-exchange membranes, the electric current reduced the ionic strength of the solutions both in α and β compartments. This resulted in an increase of the electric field strength across the porous membrane in which the actual separations takes place. Moreover, the effect of lysozyme concentration on the flux of cytochrome c was studied at pH 10.7. The temperature was 10°C and the concentration of both proteins was 2.0 g L^{-1} in the feed stream, except in the cases where the effect of lysozyme concentration was studied, i.e. measurements 10–15 in Table 1.

HSA–Hgb experiments were carried out with the same apparatus and method as the previous experiments. The anode was separated from the α compartment with an anion-exchange membrane and the cathode with a cation-exchange membrane from the β compartment. The temperature was 8°C. The isoelectric point is 4.9 for HSA and 6.8 for Hgb.[14] The concentration of HSA and Hgb of each feed solution was 10 and 2.5 g L^{-1}, respectively.

The buffer system used in the case of Cyt–Lys was sodium tetraborate ($Na_2B_4O_7$ 10 H_2O and NaOH, Merck, p.a.) and in the case of HSA–Hgb phosphate (Na_2HPO_4 and KH_2PO_4, J. T. Baker, A.C.S.) and acetate (CH_3COOH and CH_3COONa, Merck, p.a.). The ionic strength of the feed and stripping solutions were adjusted with NaCl (Merck, p.a.). Phosphate buffer (pH 7) was circulated in the electrode compartments. For the sanitation of the cell and the tubing 0.5 M NaOH solution was pumped through them for 30 min followed by distilled and sterile filtered water for 2 h.

Mathematical modelling

A well defined, thin transition region between the retentate (β) and permeate (α) compartments of the cell is created by a hydrophilic microfiltration membrane (PM). The model presented in detail in Refs. 3–11 describes the transport processes in this region: diffusion due to a concentration gradient, migration due to an externally ap-

Table 1. The results for separation of cytochrome c and lysozyme. The concentrations of both proteins in the feed solution were 2 g L^{-1} except in runs 10–15, where the lysozyme concentration, c_L^s, is indicated in the table.

	V^α /$cm^3\ h^{-1}$	V^s /$cm^3\ h^{-1}$	V^c /$cm^3\ h^{-1}$	pH	κ /mS cm^{-1}	i /mA	G_{Cyt}	G_{Lys}	S	
1	0.726	0.772	0.1320	9.05	2.82	−6.0	0.3239	0.3530	1.09	
2	0.726	0.772	0.1320	9.70	5.47	−6.0	0.0645	0.1040	1.61	
3	0.532	0.728	0.204	9.75	4.43	−35.0	0.3532	0.5861	1.66	
4	0.733	0.742	0.1176	9.77	2.66	−3.5	0.1591	0.2112	1.33	
5	0.705	0.713	0.1068	10.12	3.12	−4.0	0.0892	0.1602	1.80	
6	0.624	0.752	0.171	10.32	10.16	−26.0	0.0640	0.2327	3.64	
7	0.624	0.752	0.171	10.32	8.13	−30.0	0.0280	0.2124	7.58	
8	0.585	0.751	0.182	10.49	7.61	−35.0	0.0797	0.2337	2.93	
9	0.766	0.763	0.1088	10.67	3.61	−4.5	0.0100	0.0904	9.02	
10	0.909	0.72	−0.0927	10.66	11.12	−14.0	0.1645	–	–	$c_L^s=0$
11	0.992	0.731	−0.1584	10.66	11.12	−14.0	0.2020	0.2507	1.24	$c_L^s=1.7$ g L^{-1}
12	0.835	0.735	0.0099	10.70	11.62	−14.0	0.0613	–	–	$c_L^s=0$
13	0.808	0.744	0.0226	10.70	11.62	−14.0	0.0462	0.1230	2.66	$c_L^s=1.7$ g L^{-1}
14	0.828	0.753	0.0214	10.86	11.98	−14.0	0.0314	0.1214	3.86	$c_L^s=2.9$ g L^{-1}
15	0.777	0.75	0.0394	10.62	7.38	−21.0	0.0119	0.2139	17.91	$c_L^s=2.9$ g L^{-1}

plied potential difference and forced convective flow created by controlling the flow rates of the stripping stream V° and permeate V^{α} across the porous membrane (Fig. 1). This transport problem is solved by applying the extended Nernst–Planck equations to each mobile species i excluding the solvent

$$j_i = -D_i c_i \left(\frac{\mathrm{d} \ln c_i}{\mathrm{d}x} + z_i \frac{F}{RT} \frac{\mathrm{d}\phi}{\mathrm{d}x} \right) + c_i v^{\mathrm{c}} ; \quad i = 1,2,...n \qquad (1)$$

where j_i, D_i, z_i and c_i are the flux, diffusion coefficient, effective charge number and concentration of the species i, ϕ is the electric potential, v^{c} is the linear convective flow velocity ($V^{\mathrm{c}} = v^{\mathrm{c}} A$, where A is effective surface area of the porous membrane), x is the spatial coordinate perpendicular to the membrane surface, F is the Faraday constant, T is the absolute temperature and R is the gas constant. In addition to the Nernst–Planck equations, the condition of electroneutrality and the relationship between fluxes and electric current density I ($I = i/A$, where i is electric current) are needed:

$$\Sigma z_i c_i = 0 \qquad (2)$$

$$\Sigma z_i j_i = \frac{I}{F} \qquad (3)$$

In our case now, we consider only the situation where the concentration of the supporting electrolyte is the same in both compartments α and β. Then the solution for the concentration ratio for the protein in α and β compartments is approximately according to eqn. (4):

$$\frac{c_i^{\beta}}{c_i^{\alpha z}} = \left(1 + \frac{V^{\alpha}}{\dfrac{z_i D_i F i}{\kappa RT} + V^{\mathrm{c}}} \right) \exp \left[\frac{1}{(A/l)} \left(\frac{z_i F i}{\kappa RT} + \frac{V^{\mathrm{c}}}{D_i} \right) \right] - \frac{V^{\alpha}}{\dfrac{z_i D_i F i}{\kappa RT} + V^{\mathrm{c}}} \qquad (4)$$

where κ is the electrical conductivity of the solution. The requirement for eqn. (4) to be established as a good approximation is that the contribution of the diffusion potential to the total electric field strength E across the membrane is negligible, i.e. $E \approx i/A\kappa$. In practice, this requirement is always fulfilled because of the high electric current densities used in separation experiments. This result can be utilized in the characterization of the separation efficiency with the aid of two parameters, the separation factor of a protein i (G_i) and the selectivity ratio (S) of two proteins, defined as

$$G_i = \frac{c_i^{\alpha}}{c_i^{\mathrm{s}}} \frac{V^{\alpha}}{V^{\mathrm{s}}} = \frac{1}{1 + \dfrac{V^{\beta}}{V\alpha}} \frac{c_i^{\beta}}{c_i^{\alpha}} \qquad (5)$$

$$S = \frac{G_2}{G_1} \qquad (6)$$

It should be emphasized that the effective charge number of a protein, z_i, is in fact an electrokinetic quantity. This means that its value, because of the electrophoretic effect,[13] is less than the net (i.e. thermodynamic) charge number. Furthermore, it is dependent on both the composition and concentration of the electrolyte solution.

Results and discussion

Cytochrome c–lysozyme. The isoelectric points of cytochrome c and lysozyme are 10.6 and 11.0, respectively.[14] Several separation experiments were carried out at pH values within the range 9–11 in order to demonstrate the separation efficiency when both proteins are in the cationic form, as well as when one is in the cationic and the other is in the anionic or isoelectric form.

The electro-osmotic water flow through ion exchange membranes, typically ca. 10 $\mathrm{cm}^{-3}\ \mathrm{A}^{-1}\ \mathrm{h}^{-1}$, is of the same order of magnitude as the convective flow rate when high electric current densities are used. Therefore, in the calculation of V^{c} electro-osmosis was taken into account by assuming that the water fluxes through both ion-exchange membranes were equal. The balance equation used was

$$V^{\mathrm{c}} = V^{0} - V^{\alpha} - \frac{1}{2} V^{\mathrm{eo}} \qquad (7)$$

where V^{eo} is the total water flow through the ion-exchange membranes. The possible effect of evaporation was ruled out by carrying out an experiment without electric current.

In the beginning of every experiment pH and conductivity in both compartments were adjusted first by a rough modelling and finally by trial and error, i.e. by changing the composition of the feed solution and measuring pH and conductivity, until they were about equal in both compartments. After the desired steady state had been reached, the proteins were added to the feed solution. When the steady state was again attained at least 12 h were allowed to pass before the samples were collected. Three consecutive samples from both permeate and retentate streams were collected during 48 h.

The results obtained are shown in Table 1. The pH and conductivity values (κ given in Table 1 are the average values in the α and β compartments. The difference between these values was always less than 10%.

As can be seen from Table 1, relatively high separation factors for lysozyme (G_{Lys}) as well as relatively high separation ratios (S) can be achieved using the experimental approach described above. Separation conditions giving a combination of high product flux and high separation factor were, however, not found. The reason for this was that too low an electric field across the porous membrane

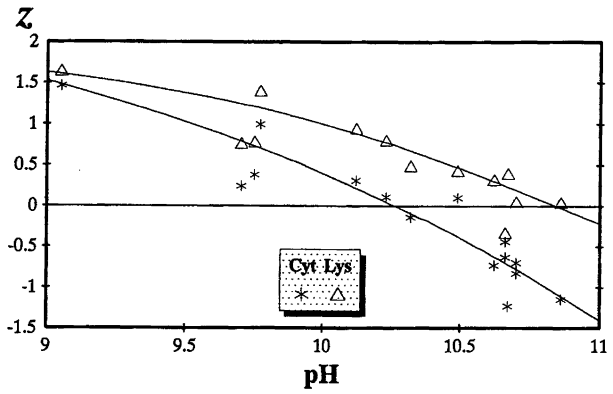

Fig. 2. The effective charge numbers (z) of cytochrome c (Cyt) and lysozyme (Lys) as a function of pH calculated from the separation experiments presented in Table 1.

was used in the experiments for such a small difference in the isoelectric points as in the case of cytochrome c and lysozyme. The highest currents which could be passed through the cell were restricted by the anion exchange membrane, where a limiting current density was reached causing variation in the pH of the solution.[15]

The diffusion coefficient for cytochrome c at 10°C is 1.16×10^{-10} m^2 s^{-1}, and this value is a reasonable approximation for lysozyme.[16] This is due to the fact that both proteins are globular and the molar masses of both of them are about equal. For the membrane constant A/l, the value of 26 cm was determined in a separate convective diffusion experiment in the system NaCl–H_2O. Using eqns. (4) and (5) the effective charge numbers can be calculated. The results obtained are shown in Fig. 2. As can be seen, the calculated effective charge numbers are in line with the measured values in Ref. 16. Furthermore, the isoelectric points (10.4 for Cyt and 10.9 for Lys as estimated from the curves in Fig. 2) are in agreement with literature[14] values, 10.6 and 11.0. The most probable reason for the distribution of the experimental points is the method for correcting of the effect of electro-osmosis.

The charge numbers of cytochrome c obtained from measurements 10–15 in Table 1 are −0.44, −0.63, −0.70, −0.83, −1.15 and −0.73. The relatively small differences in these values indicate the absence of coupling of the transport of cytochrome c and lysozyme even when the pH is fixed at a value between their isoelectric points. This result encourages us to continue developing this method. However, higher electric field strengths in the porous membrane should be employed. Experimentally this can be realized by increasing the ratio of the surface area of the ion-exchange membranes to that of the porous membrane.

Human serum albumin–haemoglobin. Before the actual separation experiments the diffusion coefficients and the effective charge numbers of these proteins as well as the membrane constant were determined by the method based on convective diffusion experiments.[17–19] The measurements were carried out in 0.1 M NaCl solution buffered at pH 6.5 and at 8°C. The results obtained were: membrane constant $A/l = 20.7$ cm, $D_{HSA} = 3.91 \times 10^{-11}$ and $D_{Hgb} = 4.47 \times 10^{-11}$ m^2 s^{-1} and the effective charge numbers $z_{HSA} = -2.8$ and $z_{Hgb} = -0.28$.

The results of the separation experiments for HSA–Hgb pair are given in Table 2. The separation factor of HSA increased with increasing electric field and pH, while the separation ratio was increased with increasing electric field and decreasing pH. Over 20% yield of HSA was obtained with a separation ratio of 20. A series of measurements with increasing electric currents (measurements 10–12, Table 2) was continued up to the point where $i = 38.7$ mA and $\kappa = 7.15$ mS cm^{-1}, giving $G_{HSA} = 0.4$ and $S = 29$. However, there was a rather large difference in pH and κ across the porous membrane (pH_α 5.5, pH_β 6.15, $\kappa_\alpha = 7.93$ mS cm^{-1}, $\kappa_\beta = 6.36$ mS cm^{-1}), and pH_α was extremely sensitive to changes, e.g. in flow rates. Evidently this instability indicates the attainment of a limiting current density at the surface of the anion-exchange membrane. This drawback could be eliminated by increasing the area of the ion-exchange membranes compared with the exposed area of the porous membrane.

The effective charge numbers calculated from the sepa-

Table 2. The results for separation of human serum albumin (HSA) and haemoglobin (Hgb).

	V^α /cm^3 h^{-1}	V^s /cm^3 h^{-1}	V^c /cm^3 h^{-1}	pH	κ /mS cm^{-1}	i /mA	G_{HSA}	G_{Hgb}	S
1	0.5921	0.6044	0.1033	5.20	8.65	20.3	0.114	0.0071	16.1
2	0.5741	0.6080	0.1207	5.70	11.32	20.3	0.120	0.0209	5.7
3	0.5619	0.5644	0.1275	5.79	10.94	20.3	0.140	0.0100	14.0
4	0.5741	0.6080	0.1207	5.80	11.55	20.3	0.095	0.0196	4.9
5	0.5619	0.5644	0.1275	5.82	10.55	20.3	0.149	0.0132	11.2
6	0.6022	0.6036	0.0849	6.35	15.98	20.3	0.229	0.0860	2.7
è	0.6063	0.6018	0.0851	6.40	13.29	20.3	0.255	0.0544	4.7
8	0.6147	0.6059	0.0894	7.22	13.92	20.3	0.236	0.0824	2.9
9	0.6056	0.5915	0.0882	7.76	15.06	20.3	0.240	0.1293	1.9
10	0.6009	0.5980	0.1244	5.43	16.62	20.3	0.045	0.0042	10.7
11	0.5619	0.5905	0.1497	5.50	12.35	27.0	0.099	0.0043	23.3
12	0.4754	0.5830	0.2276	5.76	8.05	33.7	0.209	0.0100	20.8

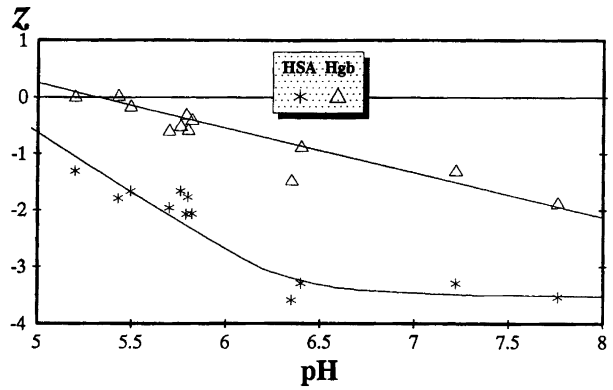

Fig. 3. The effective charge numbers (z) of human serum albumin (HSA) and bovine haemoglobin (Hgb) as a function of pH calculated from the separation experiments presented in Table 2.

ration experiments in Table 2 are given in Fig. 3. The value of the isoelectric point of Hgb is 5.5, estimated from the curve in Fig. 3. This value is substantially lower than the literature value of 6.8. This could be explained in terms of ion-binding phenomena because the literature values of isoelectric points have been determined using isoelectric focusing, which is carried out in isoelectric ampholyte buffer solution or gel in the absence of electrolytes. In our experiments the rather high ionic strength used gives rise to association of the proteins.

Therefore, it is probable that the separation efficiency could be improved if either the ionic strength is lowered or the buffer system is changed.

In order to study the interaction of two protein fluxes, separation experiments at pH 6.5 were performed: first without HSA, second without Hgb and third with both proteins present. The calculated charge numbers were for HSA -1.77 and -1.73 (without and with Hgb) and similarly for Hgb -0.26 and -0.08, respectively. This means that the low value for the isoelectric point of haemoglobin cannot be explained by the coupling of protein fluxes.

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