ELSEVIER

Journal of Chromatography A, 944 (2002) 149–159

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Mass influences in the performance of oligomeric poly(diallyldimethylammonium chloride) as displacer for cation-exchange displacement chromatography of proteins

B. Schmidt, Ch. Wandrey, R. Freitag*

Department of Chemistry, Swiss Federal Institute of Technology Lausanne, 1015 Ecublens, Switzerland

Abstract

A novel type of linear polyelectrolyte, namely poly-DADMAC [poly(diallyldimethylammonium chloride)], was prepared and studied as a displacer for cation-exchange displacement chromatography of proteins. In contrast to the commercially available polymers of that chemistry, the novel type of poly-DADMAC introduced here is characterized by a homogeneous linear structure, a narrow distribution of the (adjustable) molar mass as well as by a defined and homogeneous affinity for the stationary phase. Five poly-DADMACs of different size (17 900 to 88 000 g/mol) were prepared and compared with regard to their stationary phase affinity and protein separation potential, taking a mixture of basic proteins, namely lysozyme, cytochrome C, and ribonuclease A (from bovine pancreas), as an example. The steric mass action model was employed to aid method development. Under the chosen conditions (low ionic strength of the mobile phase guaranteeing strong binding of both the proteins and the displacer) the poly-DADMAC with the lowest molar masses proved to be the most efficient displacers for the basic proteins with a stationary phase affinity constant of $5.3 \cdot 10^{16}$ and a steric factor of 224. Using this substance as displacer, a sample mixture containing up to three proteins was separated and the proteins recovered at high yields (80–97%) and in high purity and concentration.

Keywords: Adsorption; Steric mass action model; Surface; Displacement chromatography; Preparative chromatography; Proteins; Poly(diallyldimethylammonium chloride)

1. Introduction

Biotechnology is becoming a significant factor in the economy of many highly industrialized countries. Most products of modern biotechnology (e.g., recombinant proteins, peptides, antibodies, DNA) are produced in a rather complex environment. Particularly when they are to be purified to the highest possible degree (i.e. in the case of bio-pharmaceuticals), chromatography tends to play a major role in the downstream process [1].

The concept of displacement chromatography has repeatedly been suggested in this context as a powerful preparative technique (e.g., Ref. [2]). In displacement chromatography the substances are resolved into consecutive zones (''displacement train'') of the respective pure substances, which leave the column in the order of their stationary phase affinity, rather than into ''peaks'' as in elution chromatography. In the first phase of a displacement separation, the feed components are loaded onto the stationary phase (column) under conditions where they are well retained. A major part of the stationary phase's dynamic capacity can be exploited at this stage. In the next step, a so-called displacer is

*Corresponding author. Fax: +41-21-693-6030.
E-mail address: ruth.freitag@epfl.ch (R. Freitag).

0021-9673/02/$ – see front matter
PII: S0021-9673(01)01203-1

pumped through the column, under conditions where this substance binds more strongly to the stationary phase than the target substance(s). Hence the number of available binding sites for the feed components decreases steadily as the displacer front advances and competition increases. At this stage the less well bound substances are displaced from the stationary phase by the more strongly binding ones and those are in turn finally displaced by the displacer itself.

Various models have been proposed to simulate nonlinear preparative chromatography, including displacement chromatography [3–5]. In general, these models are quite advanced in considering mass transfer and kinetic limitations. They tend to use a simple Langmuir algorithm to describe the respective adsorption isotherms and hence the shape of the resulting band profiles [6]. As an improvement — originally developed for ion-exchange chromatography — the steric mass action (SMA) model was proposed in 1992 [7]. This model takes into consideration that the displacer will not only displace the feed compounds, but also to some extent the layer of adsorbed small counter-ions, which is found on the stationary phase surface. This induces a salt step in front of the displacer zone, which in turn influences the micro-environment in the displacement train and hence, in the end, also the shape and the concentration of the various substance zones.

For prediction of a displacement separation, the SMA model requires the determination of three parameters: the characteristic charge ν (the number of interaction points between the surface and the molecule), the steric factor σ (the number of salt counter-ions on the surface sterically hindered from free exchange with the mobile phase by the adsorbed target molecule) and the equilibrium constant K (a measure of the stationary phase affinity of the molecule) of all substances, including the displacer. To date, the SMA model has been used mainly to simulate the separation of proteins using low-molar-mass displacers [8,9], based on the so-called dynamic affinity and operating regime plots. The operating regime plot (salt concentration versus displacer concentration) defines the boundaries between displacement/desorption and displacement/elution, respectively [10]. The dynamic affinity plot ($\log K$ versus ν), on the other hand, can be used to illustrate and to predict the elution order in a displacement separation [11].

The displacer is of obvious importance in displacement chromatography. This preferably non-toxic substance should have a high but controllable stationary phase affinity. The latter becomes important where column regeneration is concerned. Especially for ion-exchange chromatography, various polyelectrolytes have been suggested as protein displacers. The rational design of an ion-exchange displacement separation of proteins under the influence of a polyelectrolytic displacer requires a good understanding of the interaction between the charged displacers and the oppositely charged stationary phase surface, which can be taken as one pertinent example for the adsorption of polyelectrolytes at interfaces. Such processes play an important role in many industrial processes besides chromatography, e.g. flocculation, colloidal stabilization and emulsification.

A comprehensive overview of our current understanding of polymer adsorption theory and adsorption models is presented in Ref. [12]. In general, polymer adsorption occurs if the difference between the free energy of the polymer segment/surface contacts and that of the solvent/surface contacts is sufficiently negative. Among the parameters that govern the adsorption are the molar mass of the polymer and the solvent quality. In the case of polyelectrolyte adsorption/interaction, additional parameters need to be taken into consideration, including the charge, the charge density (both of the polyelectrolyte and the surface) as well as the ionic strength of the solvent. In addition, we need to distinguish between highly charged polyelectrolytes (charge distance above the Bjerrum length) and polyelectrolytes possessing a low charge density. Only highly charged polyelectrolytes, for example, undergo counter-ion condensation. For both types of polyelectrolytes the charges can be permanent or non-permanent, that is, nearly independent of the pH of the solution or not. The terms strong and weak polyelectrolytes should be avoided in this context, since this classification sometimes causes confusion between polyelectrolytes with low charge density and those with a pH-dependent charge density, which may both be classified as ‘‘weak’’.

The ionic strength of the solution is an additional factor to consider, since it determines largely the solvent quality in aqueous polyelectrolyte (PEL) solutions. In polyelectrolyte solutions the ionic strength results from the combined concentration of the polyelectrolyte and whatever low-molar-mass salts are also present. Only when the salt concentration is much higher than the polyelectrolyte concentration may the latter be neglected. Considering the above classification, various limiting cases have been distinguished [12]:

(a) highly charged PEL at low ionic strength of the solution;

(b) lowly charged PEL at low ionic strength of the solution;

(c) highly charged PEL at high ionic strength of the solution;

(d) lowly charged PEL at high ionic strength of the solution.

Whereas the polyelectrolyte behavior of (a) and (b) is determined by electrostatic interactions and molar mass effects are nearly absent, the electrostatic contributions are screened in cases (c) and (d). Therefore, ''physico-chemical'' parameters such as the molar mass become important. In all cases the surface chemistry has an additional influence on the adsorption process. If the electrostatic adsorption, for example, is enhanced by a chemically mediated surface affinity ($\pi-\pi$ interaction, H-bridges), the system becomes less sensitive to the ionic strength [13,14]. This multitude of influencing factors may be the reason for the frequently contradictory experimental findings.

The poly(diallyldimethylammonium chloride) (poly-DADMAC) proposed in this paper as protein displacer in cation-exchange displacement chromatography may be classified as a highly and permanently charged cationic PEL [15]. The charge distance ($b \approx 0.5$ nm) is less than the Bjerrum length ($l_B = 0.712$ nm in water at 20°C). The molecules are characterized by a homogeneous structure, a narrow distribution of the (adjustable) molar mass and a defined affinity for the stationary phase. The influence of these parameters on the chromatographic performance and hence, presumably, on the strength and specificity of the stationary phase interaction were investigated taking the separation of up to three basic proteins on a conventional packed bed cation-exchange column as an example.

2. Materials and methods

2.1. Materials

Sodium phosphate, sodium chloride, ammonium sulfate, proteins and chemicals for buffer and eluent preparation were from Sigma (Deisenhofen, Germany). The strong cation-exchange column (Bio-Scale S2, column dimensions, 3.5×52 mm; column volume, 1.363 ml; column dead volume, $V_0 = 0.671$ ml; stationary phase volume, $V_{st} = 0.692$ ml; phase ratio, $\beta = 1.032$; stationary phase, 10 μm porous particles) was supplied by Bio-Rad (Munich, Germany). For analysis of the displacement fractions by reversed-phase liquid chromatography (RPC), a microparticulate 5 μm Agilent ZORBAX 300SB-C_{18} column (4.6×150 mm) from Agilent Technologies (USA) was used. Raw solutions of poly-DADMAC were prepared by ACIMA /Rohm & Haas (Buchs, Switzerland) following a synthesis protocol described previously [16].

2.2. Polymer analysis, purification and characterization

A Pelicon system (Millipore, Lausanne, Switzerland) was employed for polymer purification. Following purification, the polymers were freeze-dried with a Beta 1-16 lyophilisator (Christ, Osterode, Germany). Prior to the purification the monomer conversion during synthesis was determined by gel permeation chromatography (GPC) using a Shodex Ohpak SB-806M HQ column (Showa-Denko, Tokyo, Japan) at a mobile phase (0.2 *M* NaCl) flow-rate of 0.5 ml/min. The pure monomer was used for calibration. Using the same system and conditions, the purity, the molar mass and the molar mass distribution of the polymers were ascertained. Additionally, the molar mass was determined by dilution viscosimetry using a Vicologic TI1 instrument (SEMATech, Nice, France).

2.3. Column ion capacity

In order to determine the ion capacity of the stationary phase, the cation-exchange column was first equilibrated with a 75 m*M* sodium (monobasic) phosphate buffer, pH 7.2, for approximately 10 column volumes, followed by a step to 1.0 *M* ammonium sulfate. The sodium content of the column effluent was analyzed by atomic absorption spectrometry (Model 1100A, Perkin-Elmer, Norwalk, CT, USA). Effluent fractions were diluted 1000 times in UHP water prior to measurements. Na^+ standards (10, 20, 50, 100 μ*M*) were used for calibration. The ion capacity of the cation-exchange column was determined to be 367 m*M*.

2.4. Determination of the SMA parameters

2.4.1. Displacer

The characteristic charge of the displacer, ν_D, was determined from the induced salt gradient produced during a frontal chromatography experiment, according to:

$$\nu_D = n/n_D = \Delta C_s/C_D \quad (1)$$

where n is the total amount of sodium ions displaced during the frontal experiment, n_D is the amount of displacer retained on the stationary phase, ΔC_s is the step increase in the mobile phase counter-ion concentration, and C_D is the displacer concentration.

The steric factor, σ_D, was also determined by frontal chromatography at low mobile-phase salt concentration, i.e. when the displacer can be expected to completely saturate the stationary phase. The steric factor can then be calculated directly from the equation:

$$\sigma_D = (\Lambda/Q_D^{max}) - \nu_D \quad (2)$$

where Λ is the ion capacity of the column and Q_D^{max} is the maximum stationary phase capacity for the displacer.

The equilibrium constant of the displacer, K_D, was determined from another frontal experiment. This experiment was performed under conditions of elevated mobile-phase salt concentrations. The equilibrium constant was calculated directly from the breakthrough volume, V_B, using the expression:

$$K_D = (1/\beta)\Pi\{C_S/[\Lambda - (\nu_D + \sigma_D)(C_D/\beta)\Pi]\}^{\nu} \quad (3)$$

where β is the phase ratio of the column, Π is equal to $(V_B/V_0) - 1$, C_S is the initial salt concentration in the carrier and V_B and V_0 are, respectively, the breakthrough volume of the substance and the dead volume of the column.

2.4.2. Proteins

Isocratic linear elution experiments were carried out at various mobile-phase salt concentrations to determined the characteristic charge, ν_P, and the equilibrium constant, K_P, of the proteins using the equation:

$$\log k' = \log(\beta K_P \Lambda^{\nu_P}) - \nu_P \log C_S \quad (4)$$

where k' is the dimensionless retention time of the proteins.

A plot of $\log k'$ vs. $\log C_S$ yields a straight line with a slop of $-\nu_P$, and an intercept of $\log(\beta K_P \Lambda^{\nu_P})$.

The steric factors, σ_P, of the proteins were obtained from frontal experiments carried out for a single salt concentration. The steric factor was calculated from points in the nonlinear range of the respective adsorption isotherms according to the equation:

$$\sigma_P = \beta/(C_P\Pi)\{\Lambda - C_S[\Pi/(\beta K_P)^{1/\nu_P}]\} - \nu_P \quad (5)$$

2.5. Operating regime and dynamic affinity plots

The displacement line (boundary between displacement and desorption) of the operating regime plot was calculated by selecting values for C_D and substituting them into the following expression in order to obtain the corresponding salt concentration C_{SC}:

$$C_{SC} = (K_D/\Delta_D)^{1/\nu_D}\{\Lambda - [(\nu_D + \sigma_D)C_D\Delta_D]\} \quad (6)$$

where Δ_D corresponds to the partition coefficient of the displacer, which is kept constant (stationary phase concentration divided by mobile phase concentration). The elution line (boundary between displacement and elution) of the operating regime plot was calculated by modifying values for the displacer's partition coefficient Δ and substituting these into Eqs. (7) and (8). The critical displacer concentration for elution of the protein in the in-

duced gradient is given by expression (7), while the corresponding critical salt concentration is given by expression (8):

$$C_D = \{\Lambda[1 - (K_D/\Delta)^{1/\nu_D}(\Delta/K_P)^{1/\nu_P}]\}/\{(\Delta/K_P)^{1/\nu_P} \times [\Lambda - ((\nu_D + \sigma_D)\Delta)]\} \quad (7)$$

$$C_{SC} = (K_D/\Delta)^{1/\nu_D}\{\Lambda - [(\nu_D + \sigma_D)C_D\Delta]\} \quad (8)$$

For the dynamic affinity plot ($\log K$ as a function of ν, with Δ as the axial intercept), the Δ point of the system was simply connected to the points defined by the K/ν values of the individual substances. The Δ point was taken directly from the corresponding displacer adsorption isotherm taking the chosen displacer concentration in the mobile phase into account.

2.6. Chromatography

The system for displacement chromatography was assembled from a HPLC pump 422 (Bio-Tek Kontron Instruments, Basel, Switzerland) and a Valco 10-port valve (Valco, Houston, TX, USA). A 1-ml loop was used for sample injection. The displacer was introduced from a 5-ml preparative sample loop (Knauer, Berlin, Germany). A flow-rate of 0.2 ml/min was used unless mentioned otherwise. The mobile phase was a 75 m*M* phosphate buffer, pH 7.2. Isotherms were measured as suggested in Ref. [17] using the same chromatography system and two 5-ml preparative sample loops. The flow-rate was 0.5 ml/min in this case.

Displacement separations were monitored by collecting fractions (100 μl) twice per minute. Fractions were analyzed by reversed-phase HPLC. The analytical HPLC system was assembled from a degasser (ERC-3112, Ercatech, Bern, Switzerland), a pump 422 and an HPLC gradient former 425 (Bio-Tek Kontron Instruments) controlled by Chromatography Station for Windows (Bio-Tek Kontron Instruments). Detection (214 nm) was by a HPLC 535 UV detector (Bio-Tek Kontron Instruments). The fractions were diluted eight times in 75 m*M* phosphate buffer, pH 7.2. Sample injection (20 μl) was by autosampler (HPLC 560, Bio-Tek Kontron Instruments). Data collection and interpretation were carrier out with the Kontron chromatography software. Buffer A was deionized water with 0.1% (v/v) trifluoroacetic acid (TFA) added and buffer B was acetonitrile also with 0.1% (v/v) TFA added. The gradient was run from 10% B to 90% B (10 min, 1.0 ml/min, 60°C). All components of interest, including the displacer, were quantified by this method.

3. Results and discussion

3.1. Adsorption of poly-DADMAC to charged surfaces

Poly-DADMAC has a highly hydrophilic structure with a permanently charged quaternary ammonium group in each chain unit (Fig. 1). A priori, such a molecule may constitute a putative displacer for ion-exchange displacement chromatography. It is hence important to investigate how the molecular properties of the substance, especially its size, influence the performance (adsorption) under different chromatographic conditions. In the pertinent literature, several studies can be found where the adsorption of poly-DADMAC on charged surfaces was studied, for example with the aim to optimize solid–liquid separation processes, such as flocculation, in which the adsorption of the flocculant on particle surfaces is indispensable. Study of the adsorption of poly-DADMAC, having molar masses in the range 5000 to 428 000 g/mol, on negatively charged polystyrene latex gave isotherms of the high affinity type. The adsorbed amount increased with increasing

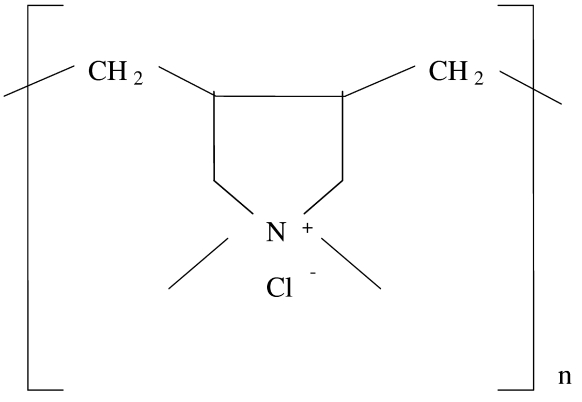

Fig. 1. Structure of poly-DADMAC [poly(diallyldimethylammonium chloride)].

ionic strength of the liquid phase. At high ionic strength the adsorbed amount increased with the molar mass of the poly-DADMAC [18]. In another related study the hydrodynamic layer thickness was found to increase with the amount of added salt (0.1–1.0 mol/l) and the molar mass of the dissolved poly-DADMAC [19]. Similar results have been reported for the adsorption of poly-DADMAC on colloidal silica particles [20].

While such studies cannot be ignored, they focussed on applications (i.e. mainly in flocculation) which require polymers and conditions (large mass, high salt) that may not be typical for applications of the same substance as protein displacer in ion-exchange displacement chromatography. However, in the pertinent literature on displacement chromatography and displacer design, the assumption is sometimes also encountered that larger polyelectrolytes show stronger binding (i.e. are ''better'' displacers) than corresponding smaller molecules of similar chemistry, while the smaller molecules have other advantages such as a higher flexibility in their binding behavior, which may translate in easier column regeneration, or better mass transfer properties. In addition, the investigations cited above were based on heterogeneous poly-DADMAC with broad mass and structural diversity, which makes the rigorous investigation of the influence of specific parameters, such as the mass, on the adsorption behavior difficult.

We have recently developed a method for the efficient synthesis and purification of a novel type of strictly linear poly-DADMAC [15], which leads to molecules characterized by a comparatively narrow molar mass distribution even at high monomer conversion during polymerization. Concomitantly, the average molar mass of a given preparation can be fine-tuned within a wide range by the synthesis conditions. A series of poly-DADMAC differing in molar mass were prepared according to this protocol. Synthesis conditions and molecular parameters are summarized in Table 1. In particular, the initiator concentration (0.002–0.04 mol/kg) and the monomer concentration (2–3 mol/kg) were used to adjust the molar mass, while a reaction temperature of 50°C was used throughout. Under these conditions, the molar masses varied between ca. 18 000 and 88 000 g/mol. It should be noted that the product parameters are influenced, to a certain extent, by the quality of the monomer solution and the process stability. Therefore, lab-grade quality of the monomer solution as well as precise process control is recommended.

Table 1
Synthesis conditions of the poly-DADMACs

Sample	Monomer conc. (mol/kg)	Initiator conc. (mol/kg)	Time (h)	Molar mass (g/mol)
1	2	0.040	2.5	17 900
2	2	0.010	8	18 600
3	2	0.010	8	23 000
4	2	0.005	15	32 400
5	3	0.002	8	88 000

Fig. 2 demonstrates how the adsorption isotherms of the poly-DADMACs in a low ionic strength buffer change as a function of the average molar mass. Table 2 summarizes the isotherm parameters (derived from a fit of the experimental data to the linearized Langmuir equation) and the column capacity for a given poly-DADMAC. According to these results, but contrarily to the above published

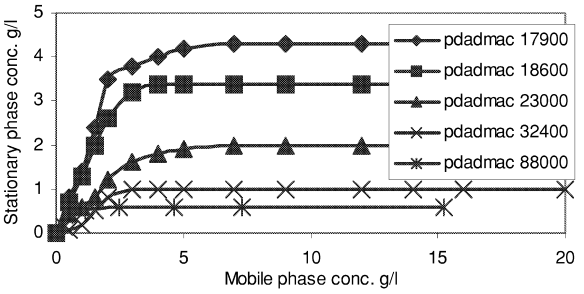

Fig. 2. Single component adsorption isotherms recorded for poly-DADMACs with molar masses of 17 900, 18 600, 23 000, 32 400 and 88 000 g/mol on a Bio-Rad S2 column in 75 m*M* phosphate buffer, pH 7.2.

Table 2
Isotherm parameters and column capacity of the polymers

Molar mass of poly-DADMAC (g/mol)	Fitted Langmuir isotherm $q = aC/(1 + bC)$, a,b constants	Column displacer capacity (g/l stat. phase)
17 900	$a = 3.73$, $b = 1.52$	4.3
18 600	$a = 3.54$, $b = 1.03$	3.4
23 000	$a = 1.22$, $b = 2.78$	2.0
32 400	$a = 0.15$, $b = 1.03$	1.0
88 000	$a = 1.51$, $b = 0.86$	0.6

results, it has to be concluded that, at least under the chromatographic conditions used here, the smaller poly-DADMAC molecules adsorb better to the porous stationary phase than the larger molecules as evidenced by a steeper initial slope (higher affinity) and a higher maximum capacity of the respective isotherms. Intuitively, it would follow from these data that short-chain poly-DADMAC constitutes a more powerful displacer than the corresponding molecules with high molar masses.

A putative displacer should bind more strongly to the stationary phase than the substances (proteins) to be displaced. Hence its isotherm should be above that of the proteins. When compared to the isotherms of three basic proteins, namely lysozyme, cytochrome C and ribonuclease (see Table 3 for details of the protein isotherms and adsorption capacities), this was the case only for the isotherms of poly-DADMAC with molar masses of 17 900 and 18 600 g/mol, but not for the isotherms of higher molar mass poly-DADMAC. Fig. 3 shows the results for two selected polymers and the three proteins.

Mobile phases with low ionic strength are commonly used in ion-exchange chromatography when strong binding of charged compounds is desired. From previous experiments [16], 75 m*M* phosphate buffer, pH 7.2, was assumed to be suitable for the intended protein separations by displacement chromatography. This buffer was therefore also used during the isotherm measurements. The ionic strength of this buffer is relatively low and probably not sufficient to screen the electrostatic interactions in the case of the smaller poly-DADMAC molecules, as lower molar masses require higher ionic strengths for efficient charge screening. It follows that, at a given low to moderate total ionic strength of the mobile phase and a given displacer concentration, the effective charge density would be higher in the shorter molecules than in the longer ones. This would explain the observed stronger binding of the smaller poly-DADMAC molecules to the stationary phase.

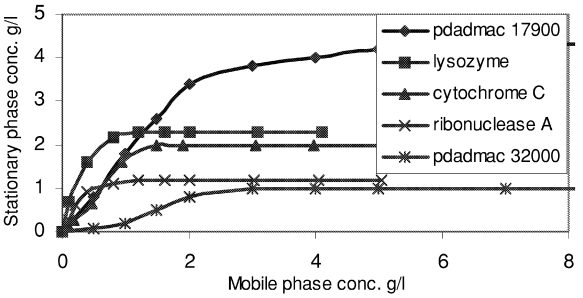

Fig. 3. Single component adsorption isotherms recorded for poly-DADMACs with molar masses of 17 900 and 32 000 g/mol in comparison with three basic proteins (lysozyme, cytochrome C and ribonuclease A) on a Bio-Rad S2 column in 75 m*M* phosphate buffer, pH 7.2.

Even at low salt concentration, however, literature data concerning the possible influence of the molar mass of polyelectrolytes on their adsorption behavior are somewhat diverse. Corroborating our results, it was observed, for example, that in the case of the adsorption of a variety of natural polyelectrolytes on activated carbon, components with small molecular size are adsorbed preferentially [21]. Since this behavior was found for a number of polyelectrolytes, it was suggested by the authors that it might be a rather general feature of the adsorption of polyelectrolyte mixtures from solution onto a porous material. Others have reported that the high molar mass fraction of polyelectrolytes is preferentially adsorbed in surface exclusion chromatography [22]. In yet another publication, a narrow fraction of polyelectrolytes with intermediate molar mass was found to be preferred in the particular case of adsorption on calcite particles [23]. Moreover, most models of polyelectrolyte adsorption on charged surfaces have

Table 3
Characteristic parameters of the proteins

Protein	Fitted Langmuir isotherm $q = aC/(1 + bC)$, a,b constants	Isoelectric point	Column capacity (g/l stat. phase)	Relative molar mass (g/mol)
Lysozyme	$a = 0.65$, $b = 1.07$	10.0	2.3	14 300
Cytochrome C	$a = 0.58$, $b = 0.20$	9.3–10.1	2.0	11 700
Ribonuclease A	$a = 1.86$, $b = 0.49$	9.3	1.2	14 200

been developed for flat surfaces and do not take the curvature of the surface into consideration, i.e. the geometry of the pores, which contain a major part of the adsorptive surface in our case. Under such conditions and especially when the ionic strength is relatively low, electrostatic exclusion could also become important.

3.2. Application of the SMA model

In a second attempt to characterize and predetermine the displacer potential of poly-DADMAC, the steric mass action model was employed. Again using the strong cation-exchange Bio-Scale S2 column as stationary phase and the conditions outlined in Materials and methods, the SMA parameters compiled in Table 4 were determined for two poly-DADMAC samples (molar masses 17 900 and 32 400 g/mol, respectively) and the three basic proteins, cytochrome C, lysozyme, and ribonuclease A. The SMA model was subsequently employed to search for the optimal conditions for a future displacement separation of the proteins and to investigate the effect of the operating conditions in these systems.

The SMA model parameters were employed to generate the dynamic affinity plots shown in Fig. 4 for the two poly-DADMACs (Fig. 4a, poly-DADMAC 17 900 g/mol; Fig. 4b, poly-DADMAC 32 000 g/mol). The Δ points in Fig. 4a and b were selected from the adsorption isotherms of the two polyelectrolytes. The Δ point of 0.65 (Fig. 4a) corresponds to a concentration of 0.4 m*M* of the poly-DADAMAC with a molar mass of 17 900 g/mol and the Δ point of 0.9 (Fig. 4b) to a concentration of 0.035 m*M* of a poly-DADAMAC with a molar mass of 32 000 g/mol. As seen from Fig. 4a and b, the dynamic affinity plots predict that the poly-DADMAC with a molar mass of 17 900 g/mol should indeed be able to displace lysozyme, cytochrome C and ribonuclease A under the chosen conditions (namely 75 m*M* phosphate buffer, pH 7.2), since its dynamic affinity line is in a region above the protein lines. On the contrary, the poly-DADMAC with a molar mass of 32 000 g/mol would most likely not be able to displace the proteins, since its dynamic affinity line is below the dynamic affinity lines of the proteins. Moreover, the dynamic affinity plot shown in Fig. 4a indicates that the elution order in a putative displacement separation would be ribonuclease A, cytochrome C, lysozyme and, finally, the displacer.

Table 4
SMA parameters for the proteins and two selected displacers

Substance	Characteristic charge, ν	Steric factor, σ	Equilibrium constant, K
Cytochrome C	1.67	4.75	0.67
Lysozyme	3.61	52.93	2.66
Ribonuclease A	0.24	5.19	0.62
Poly-DADMAC 17 900 g/mol	82	224	$5.3 \cdot 10^{16}$
Poly-DADMAC 32 400 g/mol	1.4	365	0.10

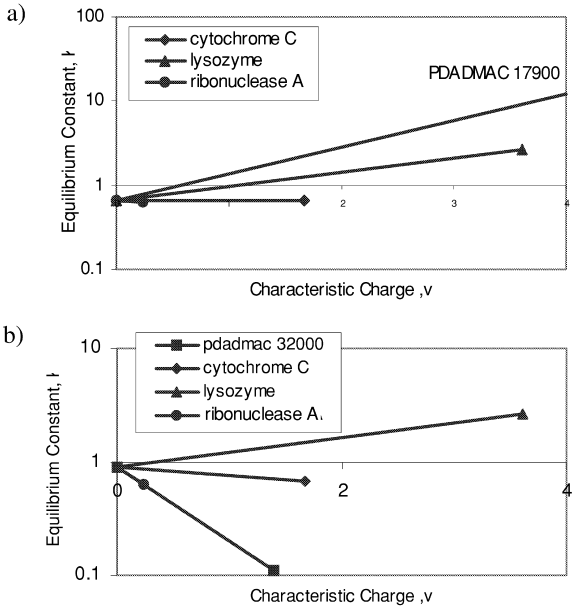

Fig. 4. Dynamic affinity plots for cytochrome C, lysozyme, and ribonuclease A and two poly-DADMACs with molar masses of (a) 17 900 g/mol ($K = 5.3 \cdot 10^{16}$, $\nu = 82$) and (b) 32 000 g/mol ($K = 0.10$, $\nu = 1.4$).

The operating regime plot for a system consisting of the three proteins and poly-DADMAC with a molar mass of 17 900 g/mol is shown in Fig. 5. In the case of the poly-DADMAC with a molar mass of 32 000 g/mol, even the construction of the operating regime plot proved to be impossible (no region of displacement existed between elution and the dis-

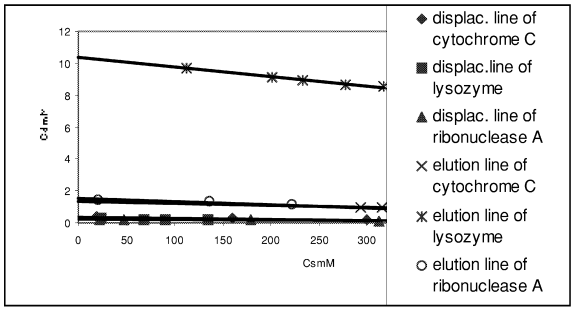

Fig. 5. Operating regime plots for lysozyme, cytochrome C, and ribonuclease A for poly-DADMAC (17 900 g/mol) as displacer and a mobile phase containing the indicated concentrations of phosphate buffer (pH 7.2).

placement line, or otherwise a negative displacer concentration in the mobile phase would be required). A closer examination of the operating plot shown in Fig. 5 indicates that a displacement separation of the three proteins should be possible with a displacer concentration between 0.35 and 1.35 m*M* depending on the salt concentration of the mobile phase. This result corresponds well with the recommendations for the dynamic affinity plot for the poly-DADMAC with the lower molar mass.

3.3. Protein separation in the displacement mode using oligomeric poly-DADMAC as displacer

To verify the predictions of the dynamic affinity and operating regime plots, several displacement experiments were performed. Separations of two of the model proteins, namely cytochrome C (isoelectric point between 9.3 and 10.1) and lysozyme (isoelectric point 10.0), were carried out to investigate the effect of the displacer concentration on the resolution. The results are compiled in Fig. 6a–d. In all experiments the displacer concentration and conditions in general were chosen in such a way that a satisfactory separation was to be expected from the prediction of the SMA model. In particular, 75 m*M* sodium phosphate buffer (pH 7.2) was chosen as mobile phase and the displacer concentration was varied between 0.35 and 1.35 m*M*, i.e. in the range suggested by the operating regime plot. All separations were repeated at least twice in order to ensure the overall reproducibility, although individual separations are shown.

A comparison of the results in Fig. 6 demonstrates that the performance improved when the displacer concentration increased from 0.559 to 1.12 m*M*.

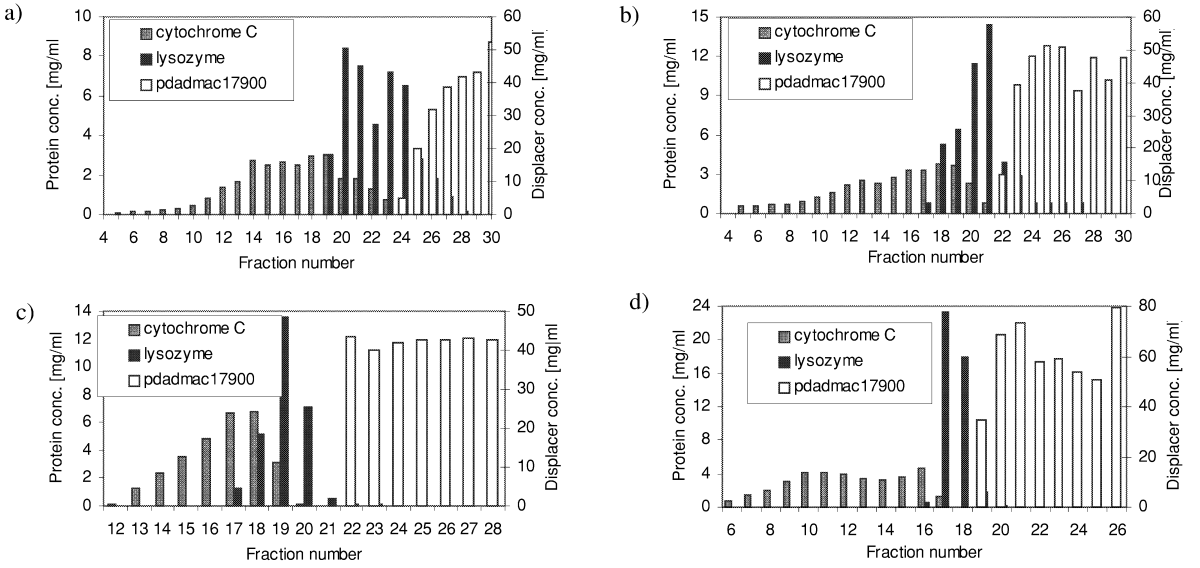

Fig. 6. Influence of the displacer concentration on the separation of lysozyme and cytochrome C under otherwise identical chromatographic conditions. Feed volume, 0.75 ml containing 0.343 m*M* lysozyme and 0.363 m*M* cytochrome C; flow-rate, 0.2 ml/min; mobile phase, 75 m*M* phosphate buffer, pH 7.2; fraction size, 100 μl; displacer, poly-DADMAC 17 900 g/mol; displacer concentration: (a) 0.559 m*M*, (b) 0.838 m*M*, (c) 0.978 m*M*, and (d) and 1.12 m*M*.

Shock layers tend to be narrower, while the concentration and the purity of the collected fractions improve. Apparently, even such comparatively small changes in displacer concentration have a significant influence on the separation. At the highest displacer concentration (i.e. 1.12 m*M* poly-DADMAC, Fig. 6d), both proteins are collected in a nearly pure and highly concentrated form. The latter effect is especially pronounced in the case of lysozyme, which is concentrated by almost a factor of 5. The recovery yield for cytochrome C was 87% and for lysozyme 88% under these conditions. When the displacer concentration was increased beyond 1.12 m*M*, however, the protein concentration in the fractions increased further until the protein zones became so narrow that recovery of the pure substances became impossible. This effect was especially pronounced in the case of lysozyme. For displacer concentrations higher than 1.5 m*M* the separation in terms of recovery and purity hence became worse, and a displacer concentration of approximately 1.2 m*M* must be considered as optimal for this particular separation of lysozyme and cytochrome C.

From the SMA model it also became evident that the separation of all three proteins, lysozyme, cytochrome C and ribonuclease A, in a single run should, in principle, be possible using poly-DADMAC (17 900 g/mol) as displacer. That this is indeed the case is shown in Fig. 7. The chosen mobile phase was again the 75 m*M* phosphate buffer, pH 7.2. In this case, 0.75 ml of a mixture containing 0.359 m*M* lysozyme, 0.363 m*M* cytochrome C and 0.282 m*M* ribonuclease A was loaded on the column at a flow-rate of 0.2 ml/min. The displacer concentration was adjusted to 1.42 m*M* poly-DADMAC (17 900 g/mol). The recovery yield for ribonuclease A was 80%, for cytochrome C 88% and for lysozyme 97% by weight. Cytochrome C was concomitantly concentrated by a factor of 2.5 and lysozyme by a factor of 4.

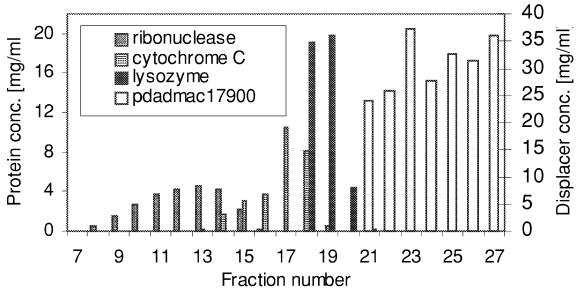

Fig. 7. Displacement separation of three proteins (lysozyme, cytochrome C and ribonuclease A) using poly-DADMAC (17 900 g/mol) as displacer. Feed volume, 0.75 ml containing 0.359 m*M* lysozyme, 0.363 m*M* cytochrome C and 0.282 m*M* ribonuclease A; flow-rate, 0.2 ml/min; mobile phase, 75 m*M* phosphate buffer, pH 7.2; fraction size, 100 μl; displacer concentration, 1.42 m*M*.

4. Conclusions

In view of the results presented in this paper, oligomeric poly-DADMAC can be considered an efficient displacer in the cation-exchange displacement chromatography of proteins. The stationary phase affinity, and hence the ability to displace more strongly bound substances, increases with decreasing molar mass of the polyelectrolyte. It is hence possible to choose a suitable displacer for a given separation. The steric mass action model was found to be a rapid and suitable way to aid process development.

5. Nomenclature

C_D	concentration of displacer in the mobile phase (m*M*)
C_P	concentration of a given protein in the mobile phase (m*M*)
C_S	initial salt concentration in the mobile phase (m*M*)
ΔC_s	step increase in the mobile phase counter-ion concentration (m*M*)
k'	dimensionless retention factor of a given protein
K_D	equilibrium constant of the displacer
K_P	equilibrium constant of a given protein
n	total amount of sodium displaced by the displacer (m*M*)
n_D	displacer adsorbed on the stationary phase (m*M*)
Q_D	stationary phase concentration of the displacer (m*M*)
Q_D^{max}	maximum stationary phase capacity of the displacer (m*M*)
V_0	dead volume of the column (ml)

V_B	breakthrough volume of a given substance front (ml)
β	column phase ratio
ν_D	characteristic charge of the displacer
ν_P	characteristic charge of a given protein
σ_D	steric factor of the displacer
σ_P	steric factor of a given protein
Λ	ion capacity of the column (mM)
Δ_D	partition coefficient of the displacer
Δ	variable partition coefficient of the displacer

Acknowledgements

This work is the result of cooperation between the Laboratory of Polyelectrolytes and Biomacromolecules and the Laboratory of Chemical Biotechnology (Center of Biotechnology), both part of the Department of Chemistry of the Swiss Federal Institute of Technology Lausanne, Switzerland, as well as ACIMA Rohm & Haas, a producer of fine chemicals (Switzerland). The research was supported by the Commission for Technology and Innovation, CTI (grant No. 3843.1). The help of David Espinosa with displacer characterization is most gratefully acknowledged.

References

[1] I.Y. Galaev, Biochemistry 63 (1998) 1258.
[2] R. Freitag, Displacement chromatography of biomolecules, in: H.Y. Aboul-Enein (Ed.), Analytical and Preparative Separation Methods for Biomolecules, Marcel Dekker, New York, 1999, p. 203.
[3] C. Brooks, S.M. Cramer, Chem. Eng. Sci. 51 (15) (1996) 3847.
[4] H. Kempe, A. Axelsson, B. Nilsson, G. Zacchi, J. Chromatogr. A 846 (1999) 1.
[5] V. Natarajan, S.M. Cramer, AIChE J. 45 (1) (1999) 27.
[6] B.J. Stanly, J. Krance, A. Roy, J. Chromatogr. A 865 (1999) 97.
[7] C.A. Brooks, S.M. Cramer, AIChE J. 38 (1992) 1969.
[8] S.D. Gadam, G. Jayaraman, S.M. Cramer, J. Chromatogr. 630 (1993) 37.
[9] G. Jayaraman, Y. Li, J.A. Moore, S.M. Cramer, J. Chromatogr. A 702 (1995) 143.
[10] S.R. Gallant, S.M. Cramer, J. Chromatogr. A 771 (1997) 9.
[11] A.A. Shucla, R.L. Hopfer, D.N. Chakravarti, E. Bortell, S.M. Cramer, Biotechnol. Prog. 14 (1998) 92.
[12] G.J. Fleer, M.A. Cohen Stuart, J.M.H.M. Scheutjens, T. Cosgrove, B. Vincent, Polymers at Interfaces, Chapman & Hall, London, 1993.
[13] F.W. Wiegel, J. Phys. A 10 (1997) 299.
[14] M. Muthukumar, J. Chem. Phys. 86 (1986) 7230.
[15] R. Freitag, Ch. Wandrey, European Patent Application No. 98810231.5, International Patent Application No. PCT/IB 99/00455.
[16] B. Schmidt, C. Wandrey, R. Freitag, H. Holzapfel, J. Chromatogr. A 865 (1999) 27.
[17] J. Jacobson, J. Frenz, Cs. Horvath, J. Chromatogr. 316 (1984) 53.
[18] R. Rehmet, E. Killmann, Colloids and Surfaces A 149 (1999) 232.
[19] A. Fuchs, E. Killmann, Colloid. Polym. Sci. 279 (2001) 53.
[20] D. Bauer, E. Killmann, Macromol. Symp. 126 (1997) 173.
[21] J.E. Kilduff, T. Karanfil, Y. Chin, W.J. Weber Jr., Environ. Sci. Technol. 30 (1996) 1336.
[22] C. Huguenard, J. Widmainer, A. Elaissari, E. Pefferkorn, Macromolecules 30 (1997) 1434.
[23] C. Geffroy, J. Persello, A. Foissy, P. Lixon, F. Tournilhac, B. Cabane, Colloids and Surface A 162 (2000) 107.