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Dispersion effects accompanying pressurized zone mobilisation in capillary isoelectric focusing of proteins

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Abstract

A theory of peak dispersion in capillary isoelectric focusing arising when mobilisation is carried out by pressure is presented and proved experimentally. Contributions of flow profile and wall adsorption to zone broadening are evaluated, varying the viscosity and surface activity by addition of a polymer (derivatized cellulose). The chromatographic model of the contribution of adsorption to the plate height is applied. The plate height is determined for a protein (myoglobin) as a function of pressure, voltage and concentration of polymer additive. According to the theoretical model, due to adsorption, increasing peak broadening is found with increasing velocity of mobilisation and decreasing polymer concentration. The predicted influence of the direction of pH gradient on peak dispersion during mobilisation is demonstrated.

Keywords: Dispersion; Adsorption; Coating; Zone mobilisation; Proteins

1. Introduction

In isoelectric focusing (IEF), amphoteric compounds, e.g. proteins, are separated according to their isoelectric points (p*I*) in a pH gradient generated by applying an electric field onto carrier ampholytes; migration stops at a region where the respective components become uncharged, the pH being identical with the p*I*.

Different approaches for IEF in capillaries (cIEF) have been described [1]. In order to minimize electroendoosmosis, coated capillaries are generally used [2–5]. For detection, the separated components have to be mobilized and driven past the detector. This can be realized either by chemical mobilization (by replacing the catholyte or anolyte with a salt solution), or by a hydrodynamic flow applied after completion of focusing [6–8].

cIEF in uncoated capillaries is easy to perform, it shows, however, poor migration time reproducibility. Another severe limitation of uncoated fused-silica capillaries is adsorption, which is particularly crucial for proteins [9]. Generally, a small amount of a neutral polymer e.g. derivatized cellulose, like methylcellulose or hydroxypropylmethylcellulose, is employed for dynamic column conditioning. The polymer reduces protein interactions with the capillary walls as well as the electroosmotic flow. The residual electroosmosis can be used to mobilize the entire pattern of separated bands past the detector

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point [10–16]; focusing and elution are thereby accomplished in one step. Migration time reproducibility is still poor. If necessary, faster elution can be achieved by hydrodynamic means.

Although the theoretical separation performance of cIEF is very high [17,18], the experimental results often do not fulfil these expectations. The narrow bands of the focused zones are jeopardised by dispersion effects accompanying the post-separation mobilisation in the capillary.

Two of these dispersion effects contribute significantly to zone broadening: (i) the parabolic hydrodynamic flow profile due to the applied pressure, and/or (ii) adsorption at the capillary walls. The present work concentrates on a theoretical model of these two effects and evaluates the model experimentally.

The contribution of slow linear adsorption of an analyte moving in a parabolic radial velocity profile was expressed in terms of the theoretical plate height, H. The derived plate height depends, besides other variables, on the migration velocity and the distribution coefficient. This dependence was experimentally proved for myoglobin as the adsorbed protein, varying the migration velocity by the application of various pressures for zone mobilisation after focusing, and by influencing the extent of adsorption by various concentrations of a polymer, added to the electrolyte.

2. Experimental

All experiments were performed on a CE system (HP3D, Hewlett-Packard, Palo Alto, CA, USA) with UV/Vis detection at 214 and 406 nm, equipped with an uncoated fused-silica capillary, 75 μm I.D., 80.5 cm/72 cm (Polymicro Technologies, Bloomfield, NJ, USA). For conditioning, the capillary was washed for 5 min with 0.1 mol/l NaOH, 2 min with water and 7 min with catholyte, prior to each run. The catholyte consisted of 20 mmol/l NaOH containing between 0 and 0.16% (w/w) hydroxypropylmethylcellulose (HPMC, Sigma, St. Louis, MO, USA, viscosity of 2% solution at 25°C: 4000 cP). The capillary was then filled with 4% ampholyte Pharmalyte™ (pH 6–8, Pharmacia, Sweden) containing 0.2 mg/ml equine myoglobin (pI=7.3) (Serva, Germany) by a pressure of 50 mbar applied for 40 s. The anode vessel was filled with 10 mmol/l phosphoric acid as the anolyte. Focusing was at 20 kV for 5 min, resulting in a decrease in the current from approximately 25 μA to <3 μA. The power was disconnected and the zones were mobilised by application of pressure between 25 and 50 mbar. In some experiments a voltage of between 1 and 5 kV was maintained during the mobilisation (see Section 3. In capillary zone electrophoresis (CZE) experiments, the background electrolyte consisted of 10 mmol/l phosphate buffer (pH 6.3 or 7.3) or 10 mmol/l borate buffer (pH 8.3), respectively. In these cases, myoglobin was injected for 3 s at 50 mbar. The driving voltage was set at 20 kV.

3. Results and discussion

3.1. Contribution of adsorption and parabolic flow profile to plate height

If the movement in the hydrodynamic radial velocity profile is accompanied by linear adsorption of the analyte at the wall of the cylindric capillary, the resulting contribution to the plate height, H, has the form [19–21]:

$$H = \frac{6R^2 - 16R + 11}{24D} r_c^2 v + \frac{2R(1-R)}{k_d} v \qquad (1)$$

where r_c is the capillary radius, D is the diffusion coefficient of the analyte, k_d is the rate constant of desorption and v is the velocity of the unretained analyte. R is the fraction of the analyte in solution defined as:

$$R = \frac{n_l}{n_l + n_s} \qquad (2)$$

where n_l and n_s represent the total molar amount of the free sample in solution and adsorbed on the wall, respectively.

The following expression relating the fraction R to the distribution coefficient K can be derived [22]:

$$R = \pi r_c^2 c_{eq} / (\pi r_c^2 c_{eq} + 2\pi r_c a_{eq}) = r_c / (r_c + 2K) \qquad (3)$$

where the distribution coefficient is defined as K=

a_{eq}/c_{eq}; c_{eq} and a_{eq} are the respective equilibrium concentrations of the analyte in the liquid solution and on the solid surface. It should be pointed out that due to adsorption the mean velocity of the sample, v_m, is given by $v_m = v \cdot R$.

Substituting $r_c/(r_c+2K)$ for R and v_m/R for v in Eq. 1 gives:

$$H = \frac{r_c^2 + 12Kr_c + 44K^2}{24D} \frac{r_c}{(r_c + 2K)} v_m + \frac{4K}{(r_c + 2K)k_d} v_m \quad (4)$$

Eq. 4 expresses the complete contribution to the plate height of the slow linear adsorption for an analyte moving in a parabolic radial velocity profile. The two terms on the right hand side of Eq. 4 indicate that both radial diffusion and kinetics of adsorption contribute to the dispersion of analyte peaks.

The contribution of the axial diffusion to the plate height is given by $2D/v_m$; under the conditions considered here (i.e., $D = 1 \cdot 10^{-10}$ $m^2 s^{-1}$, $v_m = 0.5$ mm/s, $r_c = 40$ μm) it results in a plate height about several thousand fold less than the plate height given in Eq. 4 and can thus be neglected here.

It is obvious that, apart from the axial diffusion, other effects that are not dependent on the velocity should also be taken into account as, for instance, the initial sample profile or electromigration dispersion. The contribution of these latter effects can be expressed as H_{ind}, which is the initial plate height independent of the migration velocity v_m. The overall plate height as a function of v_m can be expressed by:

$$H_{tot} = H_{ind} + \frac{r_c^2 + 12Kr_c + 44K^2}{24D} \frac{r_c}{(r_c + 2K)} v_m + \frac{4K}{(r_c + 2K)k_d} v_m \quad (5)$$

For a non-adsorbed sample, K is zero and Eq. 5 simplifies to the form:

$$H_{tot} = H_{ind} + \frac{r_c^2}{24D} v_m \quad (6)$$

which expresses the plate height of the sample undergoing Taylor dispersion [23].

The diffusion coefficient, D, is approximately indirectly proportional to the viscosity of the solution. An increase of the viscosity, e.g. by addition of a polymer additive, decreases D and thus results in a steeper slope of the H versus v_m dependence, due to slower radial mass transport at higher viscosity (Eq. 6). In the absence of adsorption the slope of the plot thus should increase with polymer concentration.

3.2. *H versus v_m for pressurized zone mobilisation of adsorbed solutes*

The dispersion of proteins during cIEF was investigated under conditions where the selected protein, myoglobin, is adsorbed on the fused-silica surface [9]. Myoglobin dissolved in the ampholyte solution was injected into the capillary and subjected to cIEF. Both adsorption effects and the hydrodynamic flow profile thus contribute to the overall zone spreading at pressurized mobilisation. Upon completion of the focusing, the voltage was disconnected and the gradient was mobilized hydrodynamically. Use of various pressures resulted in different migration velocities, v_m, of the focused myoglobin. The positions of the resulting peaks depending on the pressure applied are shown in Fig. 1. For each peak the corresponding plate height was calculated.

According to Eq. 5, adsorption of the protein results in a stronger dependence of H_{tot} on the velocity v_m as compared to a non-adsorbed solute,

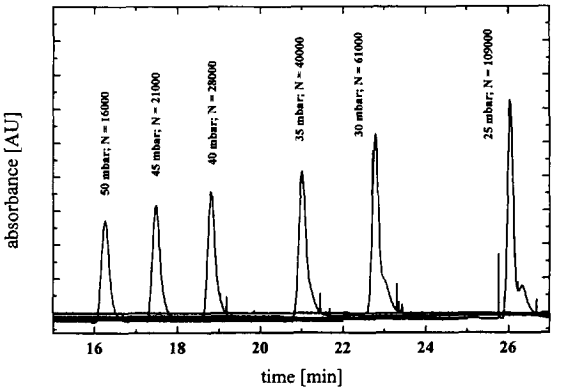

Fig. 1. cIEF of myoglobin. Conditions: uncoated fused-silica capillary, 75 μm I.D., 80.5 cm/72 cm length; pressure injection, 40 s/50 mbar; focusing, 5 min at 20 kV; mobilisation, from 50 to 25 mbar at 2.5 kV; detection, UV–Vis (406 nm); N = plate number.

which would be dispersed only by the flow profile (Eq. 6). Covering the active surface of the capillary wall by a polymer [24] should cause a decrease in the distribution coefficient, K, and should thus result in lower values of the plate height, i.e., increase in efficiency (see Eq. 5).

For myoglobin, the contribution of adsorption was evaluated by varying the concentrations of HPMC. Obviously, addition of HPMC also modifies the viscosity of the solution. In order to avoid changes of the diffusion coefficient caused by changing the viscosity, HPMC was added to the catholyte only; no polymer was added to the ampholyte solution. It was assumed that HPMC forms a layer on the surface which more or less remains when the catholyte is replaced by the sample solution, at least for the time that the mobilisation lasts. Such a memory effect is well known and has been applied for a long time in isotachophoretic practice. During mobilisation, the protein quickly reaches the capillary region with the polymer coating. It was assumed that the extent of the shielding of the active sites on the surface increases with increasing initial polymer concentration.

For each concentration of HPMC, the plate heights at various pressures, as used for mobilisation, and, consequently, at various velocities, were calculated. Plots of H_{tot} versus v_m are shown in Fig. 2. It can be seen that the results of the experiments agree reasonably well with the theoretical predictions; the slope is in fact found to be steepest in the absence of polymer additive and is gradually decreasing with polymer concentration.

3.3. H versus v_m for pressurized zone mobilisation under focusing conditions

It has previously been shown that maintaining the high voltage (and thus focusing conditions) during pressure mobilisation enhances the efficiency of cIEF [6–8]. It can be expected that focusing during mobilisation leads to lower values of the plate height than those predicted by Eq. 6. The H versus v_m curves for myoglobin, at different voltages applied during the mobilisation, are shown in Fig. 3. Indeed, the plate height decreases significantly (at the same velocity) with increasing voltage; concomitantly, the slopes of the curves increase by almost one order of

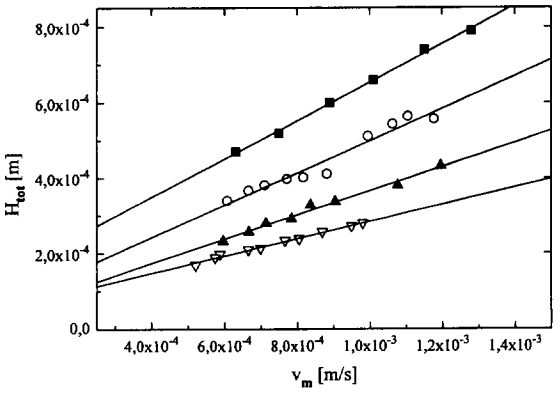

Fig. 2. Dependence of the plate height H_{tot} on the velocity of mobilisation v_m for myoglobin at different concentrations of polymer (HPMC) used for covering the capillary wall. Conditions: uncoated fused-silica capillary, 75 μm I.D., 80.5 cm/72 cm length; pressure injection, 40 s/50 mbar; focusing, 5 min at 20 kV; HPMC concentrations (w/w), 0% (■; slope = 0.53), 0.04% (○; slope = 0.43), 0.08% (▲; slope = 0.32) and 0.16% (▽; slope = 0.23); mobilisation, from 25 to 50 mbar.

magnitude from 0 to 5 kV during mobilisation. The beneficial effect of continued focusing during mobilisation is also seen in Fig. 4, where the plate height as a function of the voltage applied during the mobilisation step is presented at different polymer concentrations. It can be seen that applying a voltage of 5

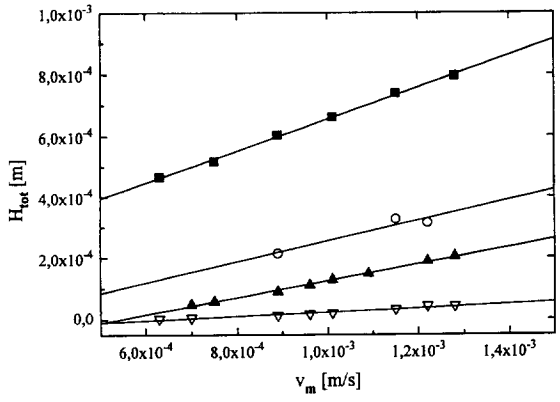

Fig. 3. Dependence of the plate height H_{tot} on the velocity of mobilisation v_m for myoglobin at different voltages maintained during the mobilisation. Conditions: uncoated fused-silica capillary, 75 μm I.D., 80.5 cm/72 cm length; no polymer added to the electrolyte solutions; pressure injection, 40 s/50 mbar; focusing, 5 min at 20 kV; mobilisation, from 25 to 50 mbar; Voltage applied during the mobilisation, 0 kV (■; slope = 0.52), 1 kV (○; slope = 0.34), 2.5 kV (▲; slope = 0.27) and 5 kV (▽; slope = 0.07).

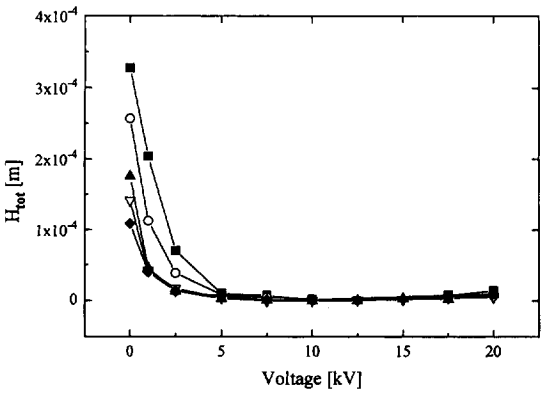

Fig. 4. Dependence of the plate height H_{tot} on the voltage maintained during mobilisation at different concentrations of HPMC used for covering the capillary wall. Conditions: uncoated fused-silica capillary, 75 μm I.D., 80.5 cm/72 cm length; pressure injection, 40 s/50 mbar; focusing, 5 min at 20 kV; mobilisation, 30 mbar; HPMC concentrations (w/w), 0% (■), 0.04% (○), 0.08% (▲), 0.12% (▽) and 0.16% (◆).

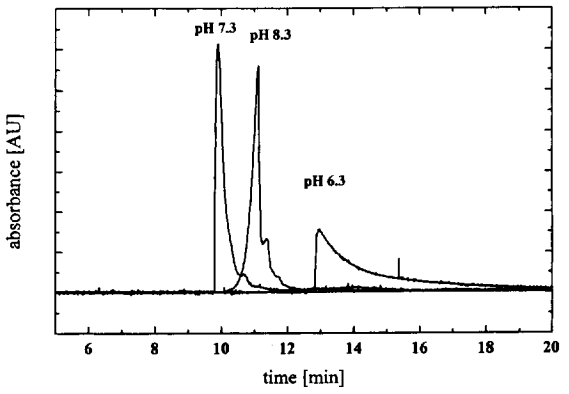

Fig. 5. CZE of myoglobin in buffers with pH at the p*I* of the protein (7.3) and at p*I*±1. Conditions: uncoated fused-silica capillary, 75 μm I.D., 80.5 cm/72 cm length; pressure injection, 3 s/50 mbar; driving voltage, 20 kV (normal mode); detection, UV–Vis (406 nm); Buffers: 0.010 mol/l sodium phosphate (pH 6.3 and 7.3) and 0.010 mol/l sodium borate (pH 8.3), respectively. No polymer added to the buffer solutions.

kV almost completely suppresses the dispersion effects.

3.4. Influence of the pH gradient direction– "displacement" effect

The influence of the pH on the extent of adsorption is well known [24–26]. Assuming that adsorption predominantly originates from the attractive electrostatic forces between positively charged protein sites and the negative silica wall, it can be expected that charging the protein positively by lowering the pH in the range where silica is still negatively charged (its p*K* is about 5.5 [27]) leads to stronger adsorption. This effect is clearly reflected by the shapes of the peaks observed in CZE of myoglobin (p*I* about 7.3) at pH 6.3, 7.3, and 8.3 (see Fig. 5). The significant peak broadening due to adsorption at lower pH values is evident.

As the extent of protein adsorption is dependent on the distribution coefficient *K*, it consequently depends on the pH [9]. For the considered case, the partition coefficient, *K*, should increase with decreasing pH. Therefore, a "*K*-gradient", originating from the pH gradient along the capillary must exist in cIEF. Adsorption accompanying focusing and pressurised mobilisation of the zones in cIEF is thus dependent on the direction of the pH gradient with respect to the direction of mobilisation. Usually, the detector is positioned at the cathode side (filled with hydroxide solution). The pH gradient (as well as the *K*-gradient) thus extends from the injection to the detection side of the capillary.

The influence of the *K*-gradient on peak dispersion becomes evident when the focused zone is mobilised by applying pressure. If the pressure is applied from the anodic side, the focused sample is replaced by the low pH solution, which results in higher *K* values, thus leading to stronger adsorption of the protein. If, on the contrary, the pressure is applied from the cathodic side, the sample is replaced by high pH solution, resulting in lower *K* values; adsorbed protein is thus better displaced from the capillary wall. In the same way as a gradient of the mobile phase acts in HPLC, the separation efficiency should be enhanced in the latter case. For these reasons, the direction of the pH (and *K*-) gradient during mobilization should lead to different peak dispersions in those cases where adsorption on the walls is not negligible.

The influence of the mutual directions of the pressurised mobilization and the pH gradient on peak dispersion is shown as *H* versus v_m plots in Fig. 6. It can be seen indeed that displacement of the sample from the cathodic side results in higher separation efficiency than displacement from the anionic side.

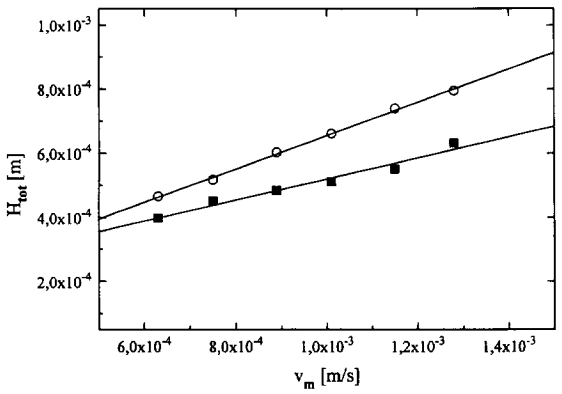

Fig. 6. Plate height, H_{tot}, of myoglobin as a function of the velocity of mobilisation, v_m, obtained at the two different directions of the pH gradient. Mobilizing pressure applied from the anodic (○; slope = 0.52) or cathodic (■; slope = 0.33) side. Conditions: uncoated fused-silica capillary, 75 μm I.D., 80.5 cm/72 cm length; pressure injection, 40 s/50 mbar; focusing, 5 min at 20 kV; mobilisation, from 25 to 50 mbar.

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