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Influence of injected mass and ionic strength on retention of water-soluble polymers and proteins in hollow-fibre flow field-flow fractionation

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Abstract

The retention behaviour of water-soluble polymers and proteins in hollow-fibre flow field-flow fractionation was studied. For a charged polymer such as polystyrene sulphonate (PSS), a low ionic strength of the carrier solution can completely disturb the retention mechanism by overloading, resulting in early-eluting, deformed peaks. Overloading was observed at polymer concentrations far below the so-called semidilute region. Under conditions where overloading effects could be observed, no evidence was found for a threshold value of the amount injected below which overloading did not occur. For proteins the influence of the ionic strength on overloading is less than for PSS, although there is an effect when the pH of the eluent is not at the isoelectric point of the protein. In contrast, non-charged pullulan is not effected by the ionic strength of the eluent and much larger amounts can be injected before significant overloading occurs.

Keywords: Retention behaviour; Ionic strength; Overloading; Field-flow fractionation; Polymers; Proteins; Polystyrene sulfonate; Pullulan; Ferritin

1. Introduction

Field-flow fractionation (FFF), a separation method for macromolecules and particles, was first described by Giddings [1]. The technique has many subtechniques of which flow-FFF is the most universal one [2]. Flow-FFF has been used mainly for the separation and characterization of water-soluble polymers [3–5], particles [6,7], and biological materials [5,8]. Most separations are carried out in aqueous media; only a few articles report on the use of organic solvents (e.g., [9,10]).

In flow-FFF separation is carried out in an open rectangular channel or a hollow fibre with a laminar flowing liquid. Perpendicular to this flow there is a cross-flow through the wall(s) of the channel or fibre which compresses all solutes to the accumulation wall. In principle, flow-FFF can be used for every solute provided that a suitable solvent and membrane are available. Since diffusion is the only opposing force against the cross-flow, large molecules, with a low diffusion coefficient, move close to the accumulation wall (Fig. 1). Small molecules have a high diffusion coefficient, so, on the average, they can move in the faster streamlines of the axial flow. Small molecules thus elute earlier than large ones. As can be concluded from the above, the separation

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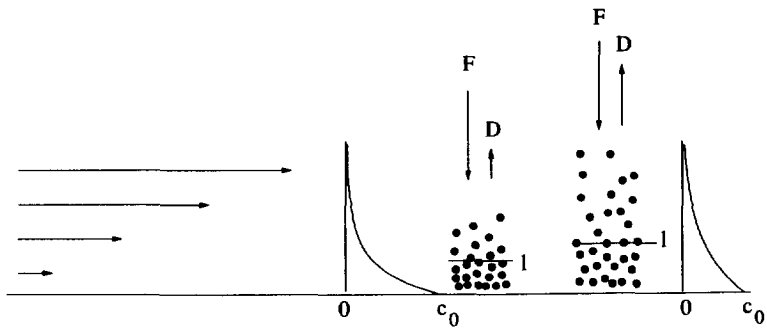

Fig. 1. Schematic representation of the accumulation of solute at the wall and the resulting separation process in flow-FFF.

in flow-FFF is only based on differences in the diffusion coefficient of the solute.

There are three main types of flow-FFF which differ in the shape of the separation channel and in the way the cross-flow is formed. The type most often applied is the symmetrical system in which a rectangular channel with a permeable upper and lower wall is used. The lower wall is covered with a membrane. A second pump is delivering the cross-flow [11]. In the asymmetrical system also a channel is used but the upper wall is not permeable and the cross-flow is therefore a part of the axial flow [8]. The type developed first is the hollow-fibre flow-FFF [12], the technique on which is reported here. In hollow-fibre flow-FFF the separation is performed in a semi-permeable hollow fibre. The cross-flow is a part of the axial flow, making the technique a form of asymmetric flow-FFF.

It is inherent to the FFF separation principle that the sample is concentrated in the channel. During the relaxation phase, after the injection, the sample is compressed in a solute layer, with a characteristic thickness l. The concentration profile of the analyte close to the accumulation wall can be described by:

$$c = c_0 \exp\left(-\frac{x}{l}\right) \quad (1)$$

where c_0 is the wall concentration and x the distance from that wall (Fig. 1).

Because of this concentration effect, which does not occur in most other separation techniques, overloading is frequently a problem in FFF. Especially polymer separations are very sensitive to overloading, which forces one to inject a small amount of sample. This can become a problem for detection. When leaving the channel the sample zone is diluted with solvent from all streamlines of the flow profile, and thus the sample concentration in the detection cell will be much lower than that in the sample zone in the channel.

The problem of overloading in flow-FFF was first recognized by Giddings et al. [13], soon after the first articles on flow-FFF were published [11,14]. It is still an important experimental requirement imposed on practical FFF operation to find a proper balance of factors that provide an adequate detector signal while avoiding observable overloading. For charged polymers the problem is even more complicated: the ionic strength is also influencing the overloading concentration. A low ionic strength will decrease the critical concentration (c^*) at which the individual chains start to overlap considerably.

Since the first article in 1978 a number of publications mentioned the problem of overloading and the influence of the ionic strength on it. Litzén and Wahlund [15] studied the influence of the ionic strength on the sample loadability for the cow pea mosaic virus and for the protein ferritin. At a low ionic strength an increasing sample load caused an increasing apparent diffusion coefficient. At a high ionic strength the opposite result was obtained. Besides, the peak shape was influenced by the overloading. In a study on charged water-soluble polymers Benincasa and Giddings [16] found that high-load peaks elute earlier than those generated by small sample loads. This cannot be explained by

chain entanglement or a modification in the viscosity of the solution, as in organic solvents, and must be due to electrostatic repulsion effects. Carlshaf and Jönsson [17] also mention electrostatic repulsion as one of the causes of deviating retention times for polystyrene latex particles at low ionic strength. At high ionic strength other effects due to a closer packing of the particles make the retention times more unpredictable.

In general, for a charged polymer two effects can be expected when the ionic strength of the solvent is decreased. Firstly, because the thickness of the double layer increases, the chain is less flexible and has a larger hydrodynamic radius. Secondly, due to a decreased shielding of the charges on the chain, chains will repel each other at lower sample concentrations. In flow-FFF the first effect would lead to longer retention times; a larger hydrodynamic radius gives a lower diffusion coefficient and molecules with a lower diffusion coefficient elute late in flow-FFF. The second effect, the mutual repulsion of the chains, causes shorter retention times. Due to that repulsion it will be more difficult to compress the molecules in the thin solute layer, l. With increasing amount of solute the layer will become thicker and the molecules move with faster streamlines and thus have shorter retention times.

To study these two effects of the solvent ionic strength, we have compared the behaviour in hollow-fibre flow-FFF of three types of water-soluble polymers: polystyrene sulphonate (a synthetic linear polyelectrolyte), ferritin (a protein), and pullulan [a linear polysaccharide produced by the fungus *Aureobasidium pullulans* (de Bary) Arnaud; *Pullularia pullulans* (de Bary) Berk [18]]. While for PSS both effects of the ionic strength may play a role, for ferritin an effect on the hydrodynamic radius is not expected to occur, due to the rigid tertiary structure of the protein. The behaviour of the neutral pullulan is not expected to be influenced by the ionic strength.

2. Experimental

2.1. Apparatus

The hollow-fibre flow-FFF equipment has been described before [6,19]. The system consisted of two pumps (P-6000, Pharmacia, Uppsala, Sweden) providing the axial- and the cross-flow. The pumps were connected to a LCC-500-plus controller (Pharmacia) to regulate the flow-rates. The injection was done with a 5-μl valve (Rheodyne, Berkeley, CA, USA). Fibres used were PM-100 polysulphon hollow fibres (Romicon, Woburn, MA, USA), which had a length of 20 cm and an I.D. of 1.1 mm. Their molecular-mass cut-off was 100 000. For the experiments with sodium polystyrene sulphonates and proteins an ABI 575 UV detector was used (Applied Biosystems, Ramsey, NJ, USA). The wavelengths used were 235 nm (PSS) and 280 nm (proteins). A refractive index detector (Shodex, RI 71, Showa Denko K.K., Tokyo, Japan) was used for the detection of pullulans.

2.2. Chemicals and solutions

PSS standards were obtained from Polymer Laboratories (Church Stretton, Shropshire, UK). Their polydispersity was stated as less than 1.1. Pullulan standards were also from Polymer Laboratories, with polydispersities between 1.09 and 1.14. Proteins used are listed in Table 1. Unless stated otherwise,

Table 1
Proteins used in this study.

Protein	Molecular mass	Isoelectric point	Supplier
β-Glucuronidase	75 000	5.5	Sigma
Conalbumine	79 000	6.0	Pharmacia
Catalase	234 000	6.7	Pharmacia
Ferritin	445 000	5.0	Sigma
Thyroglobuline	662 000	4.5	Sigma

injected masses were 0.5 μg for PSS and 5 μg pro injection for proteins and pullulan. Eluents for the polymers were demineralized water, ammonium acetate or sodium chloride solutions. Ammonium acetate was dissolved in demineralized water in concentrations between 0.0001 and 0.1 mol/l. Sodium chloride was used as a 1 mol/l solution. For the proteins two different buffers were used as eluent. For pH 7.7, Tris-hydroxymethylaminomethane was used in concentrations of 0.001 and 0.01 mol/l; HNO_3 was added to adjust the pH. For pH 5.0, KH_2PO_4 was used in a concentration of 0.01 mol/l; KOH was added to adjust the pH.

2.3. Procedures

Before starting a run the fibre was always flushed with eluent at an axial flow-rate of 1 ml/min to remove possible contamination from the former run. After 1 min cleaning, the cross-flow was set at the relaxation value of 0.4 ml/min, and the axial flow was diminished to 0.03 ml/min. Injection took place 0.2 min later. After 5 min, the cross-flow and the axial flow were set to the elution values (e.g. 0.2 and 1.0 ml/min) and the elution starts.

3. Results and discussion

In hollow-fibre flow-FFF the diffusion coefficient D of a compound with a moderate to high retention ($t_R > 10t_0$), can be calculated from the retention time t_R by [6]:

$$t_R = \frac{R^2}{8D} \ln \frac{F_{in}}{F_{out}} \quad (2)$$

where R is the fibre radius and F_{in} and F_{out} are the flow entering the fibre and the flow-rate at the end of the fibre, respectively.

The relation between the molecular mass (M_r) of a polymer and its diffusion coefficient is generally expressed by the formula [20]:

$$D = A M_r^{-b} \quad (3)$$

where A and b are empirical constants. The value of b is found as the slope of the line in a log D–log M_r plot. For a random-coil polymer the value of $-b$ is between 0.50 for a poor solvent and 0.59 for an average good solvent [20]. For spherical particles the slope is 0.33; for other rigid particles such as proteins the slope is larger when the particles are more elongated [21].

These relations were verified for the three types of polymer samples. Retention times of standards were measured with a 0.01 mol/l ionic strength solvent. Injected amounts were 0.5 μg for PSS and 5 μg for the proteins and for the pullulan standards. In Fig. 2 log D–log M_r plots for PSS, pullulan and proteins are shown. Diffusion coefficients were calculated from retention times using Eq. 2. For PSS and pullulan straight lines were found with slopes of 0.56±0.02 and 0.53±0.03, respectively. The value for PSS is close to the theoretical value as was discussed in previous work [19]. The value for pullulan is somewhat lower than the value stated in literature (0.57) [18], which was found by light scattering, but close to the theoretical value for random coils. The deviation can be caused by the quite low cross-flow (0.1 ml/min) which was used. In a former article [19] we already mentioned that for early eluting peaks the deviation in the flow-rates just after the relaxation phase is relatively important. A higher cross-flow was not possible because of detection problems.

The diffusion coefficients of PSS and pullulan

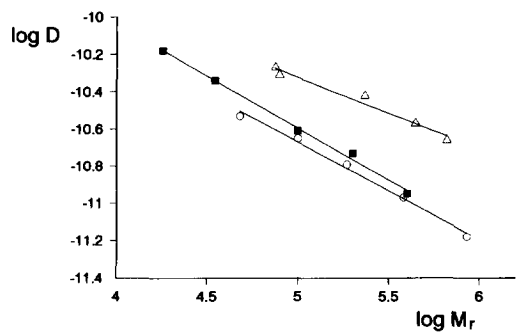

Fig. 2. Log D–log M_r plots for PSS and pullulan standards and proteins. Axial flow, 1 ml/min; cross-flow, 0.1, 0.2, and 0.3 ml/min (PSS, diffusion coefficients are mean values) or 0.1 ml/min (pullulan and proteins); injected mass, 0.5 μg (PSS) or 5 μg (pullulan and proteins); carrier ionic strength, 0.01 mol/l; (■) PSS, (○) pullulan, (△) proteins.

polymers with the same molecular mass are almost equal. For a given molecular mass proteins have a larger diffusion coefficient, and thus a smaller hydrodynamic radius, than the corresponding random coil polymers. Moreover, it is not possible to compare proteins of different molecular masses with each other in the way a polymer molecule can be compared with an other polymer molecule of the same kind and a different molecular mass. The slope of the line in the $\log D$–$\log M_r$ plot for the proteins is 0.38 ± 0.04, which is somewhat higher than 0.33 as predicted by theory for hard spheres. The value matches with the value of 0.38 as found by Litzén [22] with asymmetrical flow-FFF. From the above it can be concluded that the results match well with the theory.

As said before, the shape and the hydrodynamic volume of polyelectrolytes such as PSS is strongly influenced by the ionic strength of the solution [23,24]. This was investigated by varying the ionic strength of the eluent in hollow-fibre flow-FFF. Fig. 3 shows a plot of the logarithm of the apparent diffusion coefficient and the logarithm of the molecular mass for six different ionic strengths. Data for the smallest standards (18 000 and 35 000) cannot be given for an ionic strength of 1 mol/l because the fibre is permeable for those standards under the conditions used. This is not so much caused by the radius of the molecule: diffusion coefficients for the other molecular masses do not change much when the ionic strength is varied from 0.001 to 1 mol/l. It is probably due to the fact that the pores of the fibre are easier accessible at very high ionic strength.

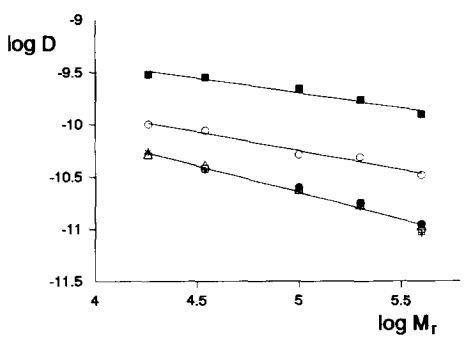

Fig. 3. Log D–log M_r plots for PSS at different ionic strengths. Axial flow, 1.0 ml/min; cross-flow, 0.1, 0.2 and 0.3 ml/min (diffusion coefficients are mean values); injected mass, 0.5 μg; ionic strength: (■) 0, (○) 0.0001, (*) 0.001, (□) 0.01, (△) 0.1, and (●) 1 mol/l.

One would expect that because a low ionic strength causes a larger hydrodynamic radius, a low ionic strength would yield a lower diffusion coefficient. However, as can be seen from Fig. 3, the diffusion coefficients of PSS standards in solutions with ionic strengths 0.001–1 mol/l do not differ very much. PSS is behaving as a random coil in that region. We do not find any influence of the ionic strength at these high salt concentrations (and low sample loads). In contrast, at very low ionic strength (0.0001 and 0 mol/l) we even see a strong increase in the apparent diffusion coefficients. An explanation for this can be found in the separation mechanism of FFF, which is based on compression of the analyte into a thin layer on the wall. However, when the charged individual molecules start to repel each other it is impossible to bring the polymer molecules together in such a thin solute layer. Solutes are moving with the faster streamlines, and thus have short retention times.

Another indication that overloading plays a role is found in the slopes of the lines in Fig. 3. For ionic strengths of 0.001 to 1 mol/l the average value of the slope is 0.56; for an ionic strength of 0.0001 mol/l it is 0.35 and without added salt it is 0.28. A smaller slope indicates that for the molecules with a high molecular mass the apparent diffusion coefficients have increased the strongest. This is in accordance with the general observation [25] that the mutual repulsion of charged random coil molecules, in solutions with a certain mass fraction of the polymer, increases with the length of the chains.

The effect of overloading is twofold. Fig. 4 shows the detector signals at three different injection loads. The first thing to be mentioned is the shape of the peak. The peak shape changes from a gaussian curve to a triangle with increasing sample load; this is similar to what is observed in capillary electrophoresis and HPLC. At high loads the front of the peak is sharp while the back is tailing. Apart from the peak shape the retention time of the peak maximum also changes. A higher injection load results in a shorter retention time. These effects are also mentioned by Giddings et al. [13] and are typical for charged molecules in aqueous solutions.

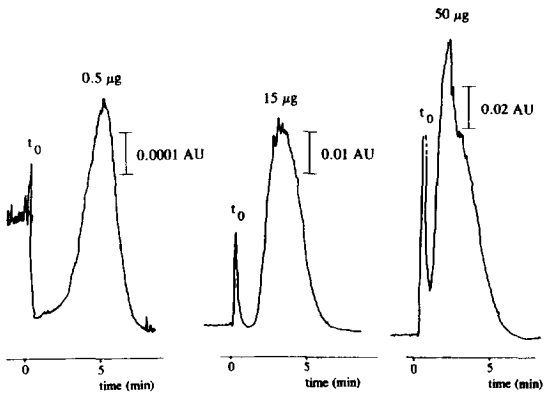

Fig. 4. Effect of the injected mass of PSS 100 000 on the detector signal. Axial flow, 1 ml/min; cross-flow, 0.1 ml/min; ionic strength, 0.01 mol/l. Injected mass, from left to right: 0.5, 15, and 50 μg, respectively.

Overloading effects for polymers in organic solvents are just the opposite: fronting peaks and longer retention times [26]. When uncharged polymers are compressed much they will get entangled and the viscosity of the eluent will increase, leading to the results mentioned above.

Overloading was studied further by injecting different sample loads of PSS 100 000 at different ionic strengths (Fig. 5). Sample loads of 0.025 μg up to 50 μg were used. The influence of the sample load on the retention at an ionic strength of 0.0001 mol/l is dramatical. Even the lowest sample load gives an apparent diffusion coefficient of 3.6×10^{-11} m^2/s, which is 1.4 times the expected value of 2.6×10^{-11} m^2/s [19]. Higher loads result in apparent diffusion coefficients up to 10.4×10^{-11} m^2/s for 1 μg injected. With ionic strengths of 0.01 and 0.001 mol/l, retention times are much less influenced by the sample load, although the apparent diffusion coefficients almost double when going from a sample load of 0.5 μg to 50 μg. The diffusion coefficients for the two highest ionic strengths used (0.1 and 1 mol/l) do not differ by much even at high loads. We do not see an evidence of a threshold value of the injected mass above which the overloading starts. This is in contrast with the results of dynamic light scattering experiments, where such a threshold value, below which the apparent diffusion coefficient did not depend on the polymer concentration, was found [25].

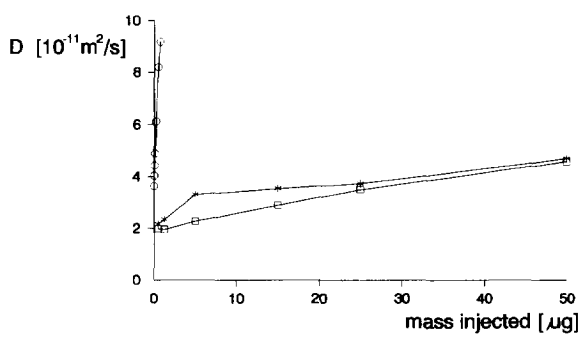

Fig. 5. Influence of the injected mass on the apparent diffusion coefficient for PSS 100 000. Axial flow, 1.0 ml/min; cross-flow, 0.1 or 0.2 ml/min; ionic strength: (○) 0.0001 mol/l, (*) 0.001 mol/l, and (□) 0.01 mol/l.

When extrapolating the lines for different ionic strengths to zero injected mass, one finds the same values for the retention time (within the experimental error). From this it can be concluded that the influence of the ionic strength on the hydrodynamic radius of the sample molecule is small. Moreover, it shows that particle–wall interactions are not significant.

When overloading is discussed, it is illustrative to calculate the relevant solute concentration during the separation process. Van Asten et al. [27] described the concentration of the solute peak in thermal-FFF as a function of the traversed distance in the channel. Initially the solute concentration decreases very rapidly, but after that the peak dispersion is relatively small. At one tenth of the channel length from the relaxation point, the concentration is only twice the concentration at the end of the channel. Similar reasoning is valid for hollow-fibre flow-FFF. Therefore, the peak concentration over almost the entire length of the fibre is of the same order of magnitude as that at the end of the fibre. The latter can be derived from the concentration in the detector. For this, two dilution effects have to be taken into account. First, the solute zone will be accelerated when it leaves the fibre, because the retention is lifted. This is accompanied by a dilution of the zone or peak with the carrier inversely proportional to the retention factor, which in hollow-fibre flow-FFF is equal to 4λ. Here, λ is the scaled layer thickness or l/R. Secondly, the detector gives a cross-section averaged signal, while for overloading the (accumulated) concentration at the wall of the fibre is

relevant. When Eq. 1 is used to describe the radial concentration profile inside the fibre, it follows that the wall concentration is larger than the cross-section averaged concentration by a factor of 2λ. Taking these two effects together, we can find the wall concentration of the solute at the end of the fibre ($c_{0,end}$) from the detector concentration (c_{det}) as:

$$c_{0,end} = \frac{c_{det}}{8\lambda^2} \quad (4)$$

In Table 2 wall concentrations are given calculated for the peak maxima obtained after injections of various amounts of PSS 100 000. The detector concentration at the peak maximum was calculated from the peak height using an extinction value of 30 $cm^{-1}\ g^{-1}\ l$ for PSS. The value of λ was calculated from the retention time t_R of the peak and the time of the unretained peak t_0:

$$\lambda = \frac{t_0}{4t_R} \quad (5)$$

The wall concentrations vary from 0.04 mg/ml with an injected mass of 0.5 μg to a ceiling value of approximating 0.3 mg/ml for injected amounts of 15 μg and higher. These values are far below the c^* value of 6.3 mg/ml given by Koene and Mandel [25] as the lower limit of the semi-dilute region.

In Fig. 6 the influence of the injected mass on the apparent diffusion coefficient for PSS (100 000), ferritin, and pullulan (180 000) are compared. Measurements were carried out with an eluent with an ionic strength of 0.01 mol/l. As was discussed before, the charged polymer PSS is influenced strongly by the injected mass. For pullulan there is only a small influence. Ferritin is charged at a pH of 7.7, but the charge density is not as high as the

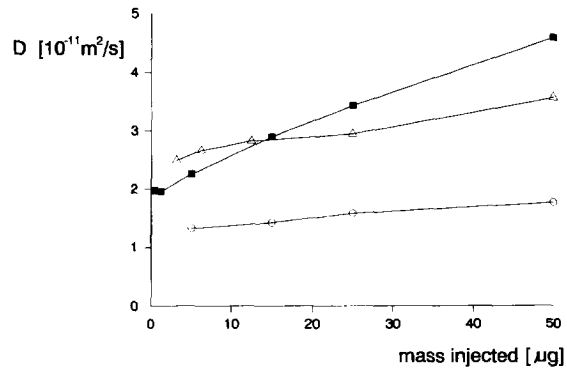

Fig. 6. Influence of the injected mass on the apparent diffusion coefficient for PSS (M_r 100 000), pullulan (M_r 180 000) and ferritin. Axial flow, 1.0 ml/min; cross-flow, 0.1 ml/min; ionic strength, 0.01 mol/l; (■) PSS, (○) pullulan, (△) ferritin.

charge density on PSS. For ferritin there is also hardly any influence of the injected mass. At first sight, it seems somewhat surprising that the influence of the injected mass on the retention time is not larger for ferritin than for the uncharged pullulan. However, the molecular mass of ferritin is 2.5 times that of the pullulan standard used. This means that the number of molecules of pullulan is 2.5 times that of ferritin, for the same injected mass. Moreover, the hydrodynamic radius of a pullulan molecule of 180 000 is larger than that of ferritin.

By using proteins, it is possible to study both the effect of the injected mass and the solute charge on the retention in one component. By varying the pH of the eluent it is possible to change the charge on the protein without significantly changing its radius. Changing the ionic strength does not change the protein radius either, both due to the protein tertiary structure. Experiments in the pH range 4.5–9.9 have

Table 2
Wall concentration in the sample zone at the end of the fibre for PSS 100 000

Amount injected (μg)	λ	c_{det} (mg/ml)	c_0 (mg/ml)
0.5	1.86×10^{-2}	0.11×10^{-3}	0.04
1.25	1.83×10^{-2}	0.21×10^{-3}	0.08
5	2.12×10^{-2}	0.58×10^{-3}	0.16
15	2.73×10^{-2}	1.9×10^{-3}	0.32
25	3.30×10^{-2}	2.3×10^{-3}	0.26
50	4.30×10^{-2}	4.2×10^{-3}	0.37

Axial flow, 1.0 ml/min; ionic strength, 0.01 mol/l.

shown that the pH does indeed not influence the retention, provided that the ionic strength is high enough. Although the radius of the protein does not change, the ionic strength can influence the retention in FFF (Fig. 7). Experiments in Fig. 7 were carried out with ferritin which has a mass of 445 000 and an isoelectric point of 5.0. At a pH of 5 the injected mass (up to 50 μg) does not have any influence on the retention time. At pH 7.7 and an ionic strength of 0.01 mol/l there is a slight decrease in the retention time above an injected mass of 25 μg. When the ionic strength is diminished to 0.001 mol/l at the same pH, the retention time starts to deviate at 10 μg injected mass.

At the lowest injected mass, there is, within experimental error, no influence of the pH or the ionic strength on the apparent diffusion coefficient. From this it can be concluded that the hydrodynamic radius of these molecules is unchanged and that the deviation of the lines at higher injected masses must be due to mutual repulsion of the molecules. The slope of the line for the ionic strength of 0.001 mol/l is 1.6 times lower than the slope of the line for PSS at an ionic strength of 0.001 mol/l (Fig. 5). The lower value is due to the lower charge density on the ferritin and to the lower number of particles injected. Both will lead to a smaller repulsion of the chain and thus a smaller slope. The diffusion coefficient for ferritin observed at low injected mass (2.8×10^{-11} m^2/s) is lower than the value reported by Litzén and Wahlund (4.0×10^{-11}), who used a flat, asymmetrical channel for flow-FFF [15,28].

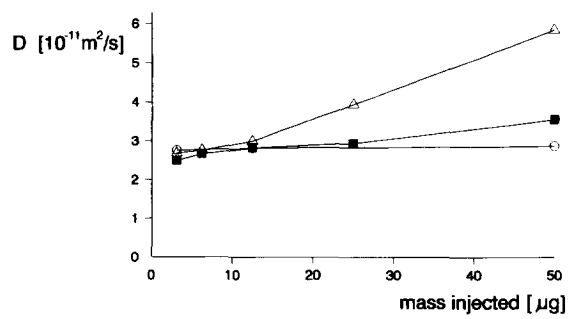

Fig. 7. Influence of the injected mass on the retention of ferritin. Axial flow, 1.0 ml/min; cross-flow, 0.2 ml/min; carrier: (△) Tris-HNO_3 buffer pH 7.7, ionic strength 0.001 mol/l; (■) Tris-HNO_3 buffer pH 7.7, ionic strength 0.01 mol/l; (○) phosphate buffer pH 5.0, ionic strength 0.01 mol/l.

In the introduction two possible effects of lowering the ionic strength were mentioned: the increase of the radius of the molecules and the influence that the molecules exercise on each other. From the experiments described in this article it appears that the changes in the molecules radii are too small to be detected here. Influences of the chains mutually (overloading), on the other hand, are very pronounced: retention times can decrease dramatically. A sharp increase of the overloading effects was observed when changing the ionic strength of the solution from 0.001 to 0.0001 mol/l for PSS. From this it may be clear that, when working with charged samples, it is necessary to inject small amounts of sample. Moreover, the ionic strength should be higher than 0.001 mol/l.

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