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Effect of surfactants on the diffusion coefficients of proteins, measured by analytical SPLITT fractionation (ASF) in the diffusion mode

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Abstract: This work reports further studies of protein transport parameters in the new technique called analytical SPLITT fractionation. The SPLITT fractionation channel mimics a dialysis-like separation utilizing differential transport across an ultra-thin lamina flowing through a channel. The flowing stream is split into two sub-streams at the channel outlet, between which the protein is distributed. The cell is capable of rapidly measuring diffusion coefficients of proteins, based on their differential transport across the channel thickness, which affects the distribution between the two outlet sub-streams. The sub-stream next to the sample introduction port is more enriched than the sub-stream at the counter port when the diffusion coefficients is small. The smaller the diffusion coefficient, the higher the degree of retrieval of the protein at the sample side outlet. The entire process is realized within a short time, and a quantitative treatment of the signals of the two sub-streams yields a retrieval factor, which is used for the determination of the diffusion coefficient. The conditions under which the selectivity of the diffusion SPLITT cell is optimal were examined. Analytical SPLITT fractionation has proven successful, using a number of proteins under various conditions. Results showed that the technique can detect subtle changes in proteins diffusion coefficients, and confirm that addition of small concentrations of surfactants does not change their transport parameters significantly.

Keywords: *SPLITT; fractionation; ASF; diffusion coefficients; proteins; surfactants.*

Introduction

Split-flow thin (SPLITT) systems utilize ultra-thin, long, unpacked flow channels equipped with one or two flow splitters at the outlet and/or inlet ends, and the flow in them is laminar. These cells were recently developed as tools for the separation of suspended, or solubilized components entering the cell through one inlet port [1–12]. The separation process was termed SPLITT fractionation (SF). The bimodal populations to be fractionated by SF, are distributed laterally across different flow laminae by virtue of differential transport. Transport in SF is generally driven by an externally applied field (electrical [5], gravitational [6–8]), although the lateral transport can be driven merely by diffusion (the diffusion mode), as a result of a concentration gradient [8–12]. SPLITT fractionation has evolved from field-flow fractionation (FFF) [13–19], where the separation channel is also shaped as a long ultra-thin duct, however, the principle of separation is different. Differential elution along the thin channel is characteristic to FFF, whereas differential retrieval at the two outlets of the SPLITT channel characterize SF. Flow field-flow fractionation (FlFFF)is a method for the separation and characterization of proteins and other water soluble polymers using a semipermeable membrane as the accumulation wall [14–19]. The differential elution of the solutes in FlFFF is based on the differences in their diffusion coefficients, which may depend on the effects of additives to the fluid carrier.

The use of SPLITT fractionation (SF) for measurements of quantitative properties of sample components is termed analytical SPLITT fractionation (ASF). Examples of quantitative determinations include diffusion coefficients of proteins [10], the relative content of oversized particles above a cut-off diameter in particulate material, by gravitational sedimentation [7], from which size distribution can be deduced, and free drug released from colloidal drug carriers [11, 12].

The rapid measurements of diffusion coefficients of proteins by ASF involved the use of surfactants in the fluid carrier [10] in order to maintain clean surfaces inside the channel.

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However, the presence of surfactants in the fluid carrier may affect the conformation of the proteins under study when used at elevated concentrations. Preliminary measurements of diffusion coefficients by photon correlation spectroscopy as well as analysis by circular dichroism (CD) showed that small concentrations of Triton X-100 and FL-70 in the solution of several proteins had no significant affect on their diffusion coefficients [10]. The present paper demonstrates that the use of very low concentrations of surfactants with proteins does not cause significant variations on their diffusion coefficients measured by ASF.

Principle of SPLITT fractionation in the diffusion mode

The operation of the SPLITT channel in the diffusion mode is illustrated in Fig. 1, in which a side view of the channel is superimposed on a three dimensional presentation. A general schematic view of the flow system is illustrated in Fig. 2. The layer construction of the channel is given in details elsewhere [5, 6, 11]. Generally, each of the two incoming sub-streams and the two exiting sub-streams in the cell can have different flow rates, as shown in Figs 1 and 2; $\dot{V}(a')$, $\dot{V}(b')$ are the flow rates of inlet sub-streams a' and b', respectively; $\dot{V}(a)$, $\dot{V}(b)$ are the flow rates of outlet sub-streams a and b, respectively. It is, of course, necessary that the sum of the inlet sub-stream flow rates equals the sum of the outlet sub-stream flow rates:

$$\dot{V}(a') + \dot{V}(b') = \dot{V}(a) + \dot{V}(b). \qquad (1)$$

The sample-free carrier stream entering inlet b', has a greater flow rate than the sample-containing stream entering at a'. Consequently, the imaginary plane (termed the inlet splitting plane or ISP) dividing the fluid elements of these two sub-streams bends upwards, towards wall A, to accommodate the greater stream flow of b' (see Fig. 1). As a result, the sample stream is compressed into a narrow band against wall A. This band forms the sample region and becomes the reservoir for the transverse separative diffusive transport [9]. The outlet splitting plane, (Fig. 1 — OSP), is another imaginary plain which marks the division between fluid elements that will exit through a, from those that will exit through b. The position of either the inlet and outlet splitting planes (ISP and OSP) can be controlled by varying the ratio of the flow rates of the two sub-streams at each end. The effectiveness of the SPLITT process is optimized by simply varying the flow rates of inlet and outlet sub-streams, hence changing the positions of the ISP and the OSP and controlling the thickness of the transport region. In the diffusion mode the volumetric flow rate of the sub-stream entering b', $\dot{V}(b')$, considerably exceeds that of the sub-stream entering a', $\dot{V}(a')$, hence the ISP is closer to wall A and the sample feed is more compressed against this wall; the degree of compression is dictated by the flow rate ratio $\dot{V}(a')/\dot{V}$. Likewise, the OSP, dividing the flow streams in the cell into two laminae, which eventually emerge as sub-streams a and b, is controlled by the ratio of the outlet volumetric flow rates $\dot{V}(a)$ and $\dot{V}(b)$.

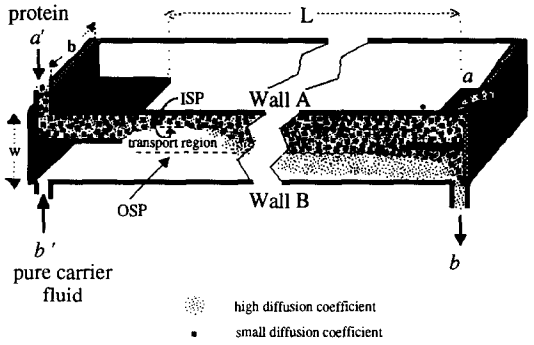

Figure 1
A schematic illustration of the principle of operation of analytical SPLITT fractionation (ASF). Samples are introduced through inlet sub-stream a', pure fluid carrier flows in through the inlet sub-stream b', and the adjustment of flow rates at sub-streams a and b affects the separation.

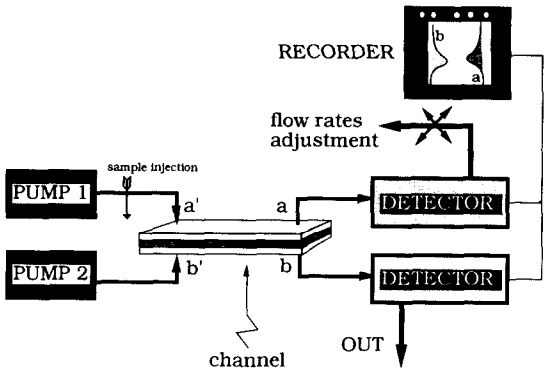

Figure 2
A schematic illustration of the complete flow system comprising analytical SPLITT fractionation. Two pumps deliver fluids into the two channel inlets, the protein is introduced into the channel through the injection port into inlet a', and it is retrieved through two outlet sub-streams a and b, to which two detectors are connected.

The ISP and the OSP are boundaries of a thin film of liquid, consisting of the fluid elements entering into b' and exiting through a, termed the transport lamina or the transport region (Fig. 1). As the sample components enter the cell and pass beyond the inlet splitter, they gradually dissipate into this transport lamina by lateral diffusion. Small molecular weight components that diffuse far enough can cross the outlet splitting plane (OSP) by the time they reach the outlet splitter, and emerge from both outlets a and b. On the other hand, larger molecules that diffuse slowly cannot easily cross the streamplanes and therefore remain mainly next to wall A (the sample side). As a result they are better retrieved near outlet a, next to the introduction wall. The larger the molecules, the higher the extent of retrieval at outlet a. A quantitative treatment of the fractions of a component emerging from both exits yields the corresponding diffusion coefficient [10].

The transport lamina can be compared to a thin dynamic membrane across which species must migrate to reach outlet sub-stream b. This virtual membrane is typically ~100 μm thick. Diffusion across this region is correspondingly fast. The unique advantages of SPLITT cells derive from the simple laminar flow and the narrow (sub-millimetre) geometry. The transport path is both short and homogeneous and extends across only a fraction of the thin dimension of the channel. Consequently, the high transport speed enables the short analysis time. For typical cells having a thickness ≤0.5 mm, the analysis time ranges from a few seconds to a few minutes. The simple geometry and flow patterns, as well as the uniform transverse transport, typical to SPLITT cells, facilitates the calculation of the fraction of a component in each of the outlet effluents, given the relevant transport coefficients [9]. By the same token, transport parameters can be calculated from the respective fractions of a component in the outlet sub-streams.

Analytical SPLITT fractionation is operated with the injection of discrete pulses, which, if desired can be made to follow one another in close sequence. In this study we utilize broad pulses, which have been used for the measurement of diffusion coefficients of proteins [9, 10, 12]. A sufficiently large plug of sample was injected to yield, at least momentarily, a steady-state operation that mimics continuous operation needed for the necessary theoretical treatment. The output, measured by a detector as a discrete flat top peak, can be interpreted quantitatively by measuring pulse height.

Theory

The theory of diffusional transport in SPLITT cells has been presented elsewhere by Williams *et al.* [9], thus only some fundamental aspects of the theory will be addressed here in order to understand the procedure by which diffusion coefficients are measured and calculated using ASF.

The simultaneous displacement by diffusion and parabolic flow govern the transport of a sample through the SPLITT channel. The mass balance equation is

$$\frac{\partial c}{\partial t} = -v(x)\frac{\partial c}{\partial z} + D\left(\frac{\partial^2 c}{\partial x^2} + \frac{\partial^2 c}{\partial z^2}\right),$$

where $\partial c/\partial t$ is the rate of change of concentration at any point within the channel, x is the transverse direction across the channel thickness, z is the coordinate along the channel length measured from the inlet, D is the diffusion coefficient, and $v(x)$ is the stream velocity at distance x from the wall.

There is no lateral convection transport in the channel, and thus all transverse transport occurs by diffusion, as can be seen from equation (2). However, axial transport is dominated mainly by convection, and longitudinal diffusion is negligible. Assuming that the system is operated under continuous steady state conditions, namely, $\partial c/\partial t = 0$, equation (2) reduces to

$$\frac{\partial c}{\partial z} = \frac{D}{v(x)}\frac{\partial^2 c}{\partial x^2}, \qquad (3)$$

where $c(x,z)$ is subject to the following boundary conditions:

$$c(x,0) = c_0 \quad \text{for } 0 \le x \le w_{a'}$$
$$c(x,0) = 0 \quad \text{for } w_{a'} \le x \le w$$
$$\partial c/\partial x = 0 \quad \textit{for } x = 0 \text{ and } w \text{ at all } z,$$

where $w_{a'}$ is the thickness of the sample stream and w is the channel thickness (see Fig. 1). Calculations of the transport parameters were based on a previous work, in which the bulk of the channel was divided into a regular grid and $c(x,z)$ at the points of intersection of the grid was solved numerically using the Crank–

Nicolson approximation [9]. The concentration profile over the lateral co-ordinate x at the outlet ($z = L$) is $c(x,L)$, which is governed by the distribution of the components between the two outlet sub-streams. The fraction of the component at each outlet sub-stream is the retrieval factor F_a, which is calculated from $c(x,L)$.

A dimensionless diffusion time $\tau D = Dt^0/w^2$ was developed from which the diffusion coefficient D is calculated, using known values of w (channel thickness) and t^0 (void time = the time of elution of a species dispersed in the total volume of the channel). The void time t^0 is related to the total flow rate $\dot{V}$ by

$$t^0 = V^0/\dot{V} = \mathbf{b}Lw/\dot{V}, \qquad (4)$$

where V^0 is the cell void volume expressed as a product of channel breadth **b**, length L, and thickness w. The dimensionless time parameter τ_D, is thus related to D, $\dot{V}$, and the channel dimensions by

$$\tau_D = Dt^0/w^2 = D\mathbf{b}L/w\dot{V}. \qquad (5)$$

Consequently D is given by

$$D = wV\tau_D/\mathbf{b}L. \qquad (6)$$

Calculated curves of F_a versus Dt^0/w^2 are presented in Fig. 3 (full curves, left axis) for different values of the fractional flow rate at the sample side outlet ($\dot{V}(a)/\dot{V}$). A specific fractional volumetric flow rate entering the sample inlet a' [$\dot{V}(a')/\dot{V} = 0.1$] was used for all the curves. To obtain experimental D values, the following steps are performed. Firstly, the experimental retrieval factor at outlet sub-stream a, F_a, is determined by the relative response of the detector signal at that sub-stream [see equation (7), results and discussion section]. The measured F_a is then used in Fig. 3 to obtain the value of τ_D, employing the specific curve corresponding to the flow ratio $\dot{V}(a)/\dot{V}$ used in the experiment. Finally, D can be calculated directly from the value of τ_D, using equation (6) and the geometrical dimensions of the apparatus w, **b**, L and the total flow rate $\dot{V}$.

Experimental

Instrumentation

The SPLITT flow system. Two pumps delivered flows into the channel; a syringe pump Varian 8500 (Palo Alto, CA, USA) and a single-line high pressure pump model 880-PU (Jasco, Tokyo, Japan). The sample was introduced through a Rheodyne injection valve, equipped with 500 μl loop. The channel, a generous gift from Prof. J.C. Giddings, was optimized by M.N. Myers from the University of Utah, and was built in the University of Utah machine shop. The thickness of the channel was either 0.0427 cm or 0.0627 cm, channel breadth and length were 3 and 15 cm, respectively. The two outlets were connected to two single wavelength UV detectors operating at 280 nm, a model 2138 UVICORD S of

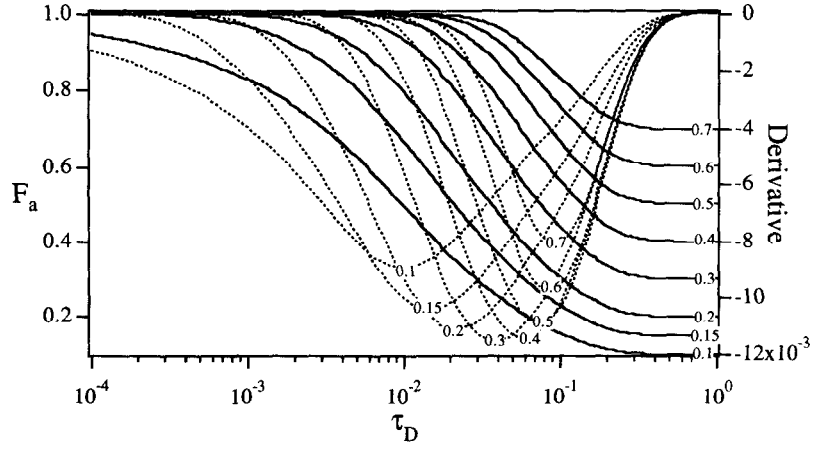

Figure 3
The retrieval factor F_a as a function of the dimensionless diffusion time $\tau_D = Dt^0/w^2$ (full curves, left axis). Superimposed are the derivatives of the curves (broken curves, right axis). The derivatives maxima show the points around which the selectivity of F_a to the diffusion coefficients is maximal.

LKB and a model UV-1 of Pharmacia (Bromma, Sweden), both equipped with HPLC flow cells. The output was recorded on a LKB-Pharmacia (Bromma, Sweden) dual pen chart recorder model REC-2. The flow-rate ratio at the outlets was adjusted by clamping a flexible silicone tubing, that was connected to one outlet. The clamp was adjusted to restrain the flow and divert a predetermined portion to the second outlet, until the required fractional flow rate was attained.

Materials

The following proteins were used in this study: human γ-globulins; serum albumin — bovine and human; turkey egg albumin and human haemoglobin. All were purchased from Sigma (St Louis, MO, USA). The solution of the acetate–sulphate buffer (pH 7.5) comprised of 0.08 M Na_2SO_4 (Merck, Darmstadt, Germany) + 0.01 M CH_3COONa (Merck) + 0.025% (w/v) sodium azide (Merck) with no surfactant or with sodium dodecylsulphate (BDH, Poole, UK) or FL-70 from Fisher Scientific (Fair Lawn, NJ). H_3PO_4 (Frutarom, Haifa, Israel), KH_2PO_4 (Merck) and K_2HPO_4 (Merck) were used in the preparation of the phosphate buffers. Guanidine hydrothiocyanate was purchased from Fluka (Buchs, Switzerland). All the solutions were filtered through a 0.2 μm filter before use.

Methods

Tests of flow rates. Volumetric measurements of the total flow rate, $\dot{V}$, the flow rates of inlet substreams $\dot{V}(a')$, $\dot{V}(b')$ and outlet substream, $\dot{V}(a)$, $\dot{V}(b)$, were performed occasionally, since the performance of the SPLITT system is based on the precise adjustment of flow rates. The flow rates were constant throughout the experiments, due to the high stability of both the syringe and the HPLC pumps, which provided constant flow rates regardless of the back pressure.

Normalization of the two detector signals. Two different detectors were connected to the two outlets *a* and *b*. Therefore, there was a need to normalize their response before measuring F_a of sample components. In order to do so, the two detectors were connected in series, and the ratio between the two signals was used as a correction factor, for the normalization of peak heights.

Laminar nature of the flow stream — measurements of diffusion coefficients. The system was routinely tested for laminarity of the flow. A routine test uses an intense colour indicator, injected into the channel to check the flow-lines. A uniform front and a smooth stream of the intensely coloured indicator solution indicated no turbulence.

Results and Discussion

The retrieval factor F_a

Diffusion coefficients of various albumins, γ-globulins and haemoglobins were measured from the fractional retrieval factor at substream *a*, F_a

$$F_a = \frac{\dot{V}(a)h_a}{\dot{V}(a)h_a + \dot{V}(b)h_b}, \qquad (7)$$

where h_a and h_b are peak heights (normalized for the differences between the two detectors, see experimental section); $\dot{V}(a)$ and $\dot{V}(b)$ are the flow rates of sub-streams *a* and *b*, respectively.

Optimization of the selectivity based on transport considerations

Of prime importance initially was testing the selectivity of the retrieval factor F_a to the diffusion time τ_D (Dt^0/w^2). High selectivity enables the detection of subtle changes in the diffusion coefficients. Plots of F_a versus log τ_D are shown in Fig. 3 for $\dot{V}(a')/\dot{V} = 0.1$ and various $\dot{V}(a)/\dot{V}$ (specified on the curves). Each curve can be divided into three main sections. One section corresponds to species having extremely small diffusion rates, namely, τ_D is relatively small and the curve levels off to a constant value of $F_a = 1$ where any change in *D* hardly affects F_a.

The second section in the curve is characterized by the steep slope, which describes the behavior of species with a relatively medium rate of diffusion, whose F_a is highly selective to their dimensionless diffusion rate τ_D. These are the species whose diffusion coefficients are measured under the present experimental conditions.

The third section, where the curve levels off again to a constant F_a, describes the dependency of F_a on the diffusion rate of relatively highly diffusing species. Due to the full dissipation of these molecules over the channel, F_a is not selective to the diffusion coefficients.

The curves in Fig. 3 were derivatized to obtain the inflection points, around which the selectivity of F_a is maximal. The derivatives were superimposed on Fig. 3 (broken curves, right axis). The range of transport parameters of proteins in the present diffusion SPLITT channel was $0.005 \leq \log \tau_D \leq 0.03$ at flow rates between 2 and 5 ml min^{-1} and channel thickness between 0.03 and 0.06 cm. Judging from the derivatives, this range of $\log \tau_D$ values requires adjustment of fractional flow rate to $0.1 \leq \dot{V}(a)/\dot{V} \leq 0.4$, which are the conditions typically used in the measurements of diffusion coefficients of proteins by ASF. The lower range of $\dot{V}(a)/\dot{V}$ setup is preferable when higher total flow rates (smaller t^0) and/or thicker channels are used.

Figure 4 shows the experimental measurements of F_a of four proteins that were studied here (markers) superimposed on the theoretical curve of F_a vs τ_D calculated for two different experiments. The values of D from which τ_D of the experimental points were calculated (equation 5) were taken from the literature [22, 23]. In both experiments the fractional flow of the sample inlet $\dot{V}(a')/\dot{V}$ was 0.1 and the total flow rate $\dot{V}$ was 3.3 ml min^{-1}. In one case the fractional flow at the sample side outlet $\dot{V}(a)/\dot{V}$ was 0.15 and the channel thickness was 0.0627 cm ($t^0 = 52$ s) and in the second case it was $\dot{V}(a)/\dot{V} = 0.2$ and the channel thickness was 0.0427 cm ($t^0 = 35$ s). The transport parameters in the two cases were different, reflecting the differences of t^0 and w between the two systems, nevertheless, the experimental F_a values were in good agreement with the calculations, indicating that the present ASF was workable and that the model of transport in the channel was valid. Day-to-day repeatability was up to 5% and the variability between different experimental setups ($\dot{V}(a)/\dot{V}$) was 10% [12].

Effect of surfactants

The protein may change its native conformation as a result of the presence of surfactants in the solution. A major cause for variations in the diffusion coefficient of a protein can be a change in its folding state [20, 21]. Surfactants, especially sodium dodecylsulphate (SDS), are used at high concentrations for denaturation of proteins in gel electrophoresis. However, lower concentrations may not be effective in changing the protein conformation dramatically, especially for relatively stable ones in a mild environment. Very small concentrations of surfactants are used in separations of macromolecules by field-flow fractionation and SPLITT systems, in order to maintain the channel walls as clean as possible, because clean walls remain smooth and do not perturb the streamplanes. The surfactants are added to the fluid carrier assuming that they do not affect the solute. This assumption was tested in the following experiments.

The effect of surfactants on the diffusion coefficients of various proteins was studied using SDS and FL-70 (a non-ionic mixture of polymeric surfactants). SDS was added to the sample (but not to the carrier fluid) at increasing concentrations to three proteins in a phos-

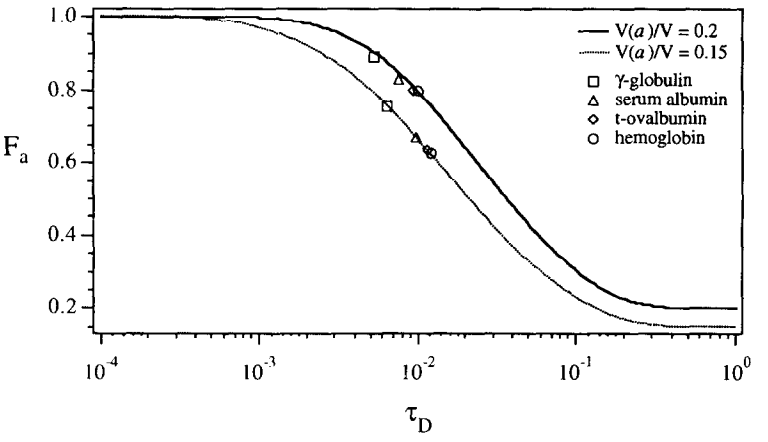

Figure 4
Experiments retrieval factors F_a of four proteins as a function of their Dt^0/w^2, in which D was taken from the literature [22, 23], superimposed on the theoretical curve of F_a vs Dt^0/w^2. Two cases are shown, one system ($w = 0.0627$ cm) was adjusted to $\dot{V}(a)/\dot{V} = 0.15$ and the other ($w = 0.0427$ cm) was adjusted to $\dot{V}(a)/\dot{V} = 0.2$. The total flow rate was 3.3 ml min^{-1} and $\dot{V}(a')/\dot{V} = 0.1$ in both cases.

phate buffer, as shown in Fig. 5. As can be seen in Fig. 5, the presence of the surfactants in the solution up to 0.02% (w/v) had little effect on the diffusion coefficients of the three proteins. Figure 6 shows the results of additional measurements of diffusion coefficients of several proteins. In this case an acetate–sulphate buffer with and without 0.01% (w/v) SDS in the sample was used. Again, no significant change in the diffusion coefficients was detected when 0.01% SDS was present in the solution. Since a change in protein conformation can be detected in its UV absorption [20, 21], it was measured with and without the surfactant in the acetate–sulphate and the phosphate buffers. Comparisons of the UV absorption spectra of the proteins in the acetate–sulphate as well as in the phosphate buffers with and without SDS revealed that there was no effect on the spectrum. An example of γ-globulins in the acetate–sulphate buffer with and without 0.01% (w/v) SDS is shown in Fig. 7.

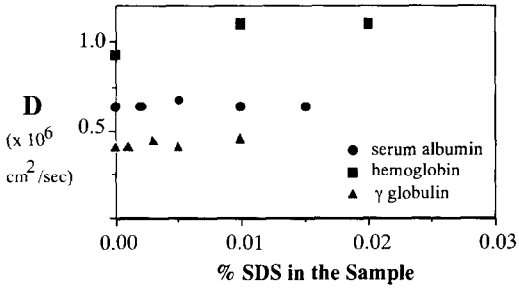

Figure 5
Diffusion coefficients of proteins ($\times 10^6$ cm^2 s^{-1}) with increasing concentrations of sodium dodecyl sulphate (SDS) in the sample. Carrier fluid was a buffer of 0.1 M phosphate pH 7.3; total flow rate was 3.3 ml min^{-1}; $\dot{V}(a')/\dot{V} = 0.1$; $\dot{V}(a)\dot{V} = 0.16$ for serum albumin and haemoglobin; $\dot{V}(a)/\dot{V} = 0.11$ for γ-globulins; concentration of the proteins was 0.25 mg ml^{-1}.

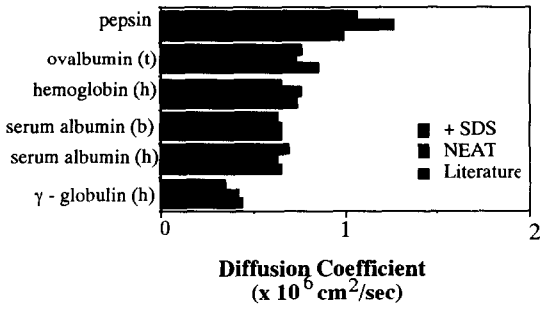

Figure 6
Diffusion coefficients of various proteins ($\times 10^6$ cm^2 s^{-1}) in acetate–sulphate buffer, with and without 0.01% (w/v) SDS in the sample, compared to the literature [22, 23]. Conditions as in Fig. 4.

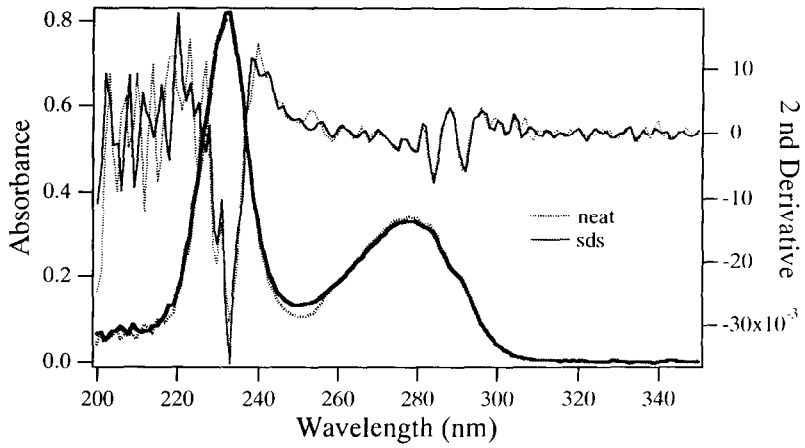

Figure 7
UV absorption spectrum of human γ-globulins with and without 0.01% SDS in the solution. The acetate–sulphate buffer was the same as in Fig. 6.

Table 1
Diffusion coefficients ($\times 10^7$ cm^2 s^{-1}) of proteins. The effect of addition of the surfactant FL-70 to the carrier fluid, which consisted of $NaCH_3COO$ and NA_2SO_4 at pH 7.5. The total flow rate was 2.2 ml min^{-1}, $\dot{V}(a'/\dot{V} = 0.1$, $\dot{V}(a)/\dot{V} = 0.3$. Concentration of the proteins was 0.25 mg ml^{-1}

Protein	$NaCH_3COO + Na_2SO_4$	$NaCH_3COO + Na_2SO_4$ + FL-70
Human haemoglobin	7.3	8.2
Pig γ-globulins	5.1	5.3
Bovine serum albumin	5.9	6.1
Human serum albumin	6.5	6.5

The effect of FL-70 on the diffusion coefficients of various proteins is shown in Table 1. The surfactant was added to the mobile fluid carrier (acetate–sulphate buffer), as used in previously reported measurements of diffusion coefficients [10]. Apart from haemoglobin (whose diffusion coefficient difference was just above the experimental error), the proteins studied here were not affected by the presence of the non ionic surfactant. Similar results were reported using photon correlation spectroscopy and CD measurements [10].

Effect of guanidine hydrothiocyanate

In order to verify that a change in D can be detected under the conditions used, γ-globulins were studied, using a fluid carrier containing phosphate buffer with and without guanidine hydrothiocianate (gd-SCN), a well known denaturation agent for proteins and peptides [21, 24]. The retrieval factor F_a of human γ-globulins was measured as a function of $\dot{V}(a)/\dot{V}$, in a phosphate buffer (7.5) and was compared to F_a, obtained using the same buffer containing 0.01 M gd-SCN. The results are shown in Fig. 8. F_a was significantly higher for all $\dot{V}(a)/\dot{V}$ setups, when gd-SCN was present in the solution. A higher retrieval factor F_a indicates a higher extent of enrichment at the sample introduction port due to a lower transport rate across the channel. A lower transport rate can serve as an indication for a possible change in conformation of the protein.

Additional measurement of diffusion coefficients of three proteins was done in an acidic phosphate buffer with and without the same amount of gd-SCN. The results are shown in Table 2. The diffusion rates were significantly lower in the presence of gd-SCN in all three proteins, demonstrating a sensitive response of the SPLITT cell to changes in their folding state. Comparison of the UV spectra of γ-globulins in the two different phosphate buffers with and without-SCN in Fig. 9 reveals that there were significant changes in the UV spectra when gd-SCN was present in the solution.

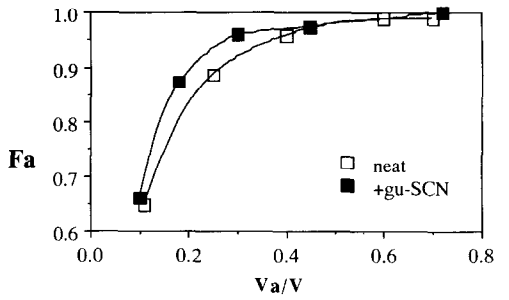

Figure 8
Retrieval factor of γ-globulins as a function of $\dot{V}(a)/\dot{V}$. Carrier fluid consisted of a 0.1 M phosphate buffer pH 7.3 with and without guanidine hydrothiocyanate; total flow rate 3.3 ml min^{-1}. $\dot{V}(a')/\dot{V}$ was 0.1; channel thickness was 0.0627 cm; concentration of the protein was 0.25 mg ml^{-1}.

Table 2
Diffusion coefficients ($\times 10^7$ cm^2 s^{-1}) of proteins in a phosphate buffer, with and without guanidine hydrothiocyanate (gd-SCN). Thickness was 0.0627 cm; total flow rate was 3.3 ml min^{-1}; $\dot{V}(a')/\dot{V} = 0.1$

	Phosphate + gd-SCN	Phosphate
γ-globulins (h)	3.2	4.1
Serum albumin (h)	4.9	6.4
Haemoglobin (h)	6.8	9.2

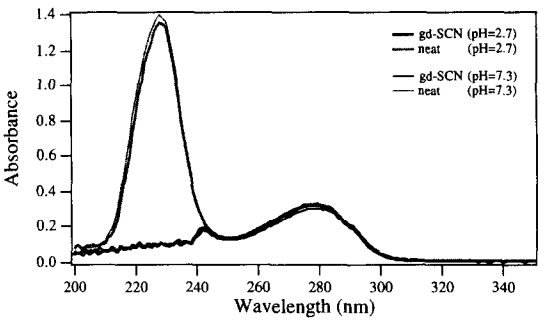

Figure 9
UV absorption spectrum of human γ-globulins with and without guanidine hydrothiocyanate in two different phosphate buffers. One buffer was the same as in Fig. 8 and the other the same as in Table 2.

Conclusion

Analytical SPLITT fractionation is a rapid and sensitive technique for the measurements of diffusion coefficients of proteins. It is essential to adjust the flow rates (the total flow rate and fractional flow rates at the inlet and outlet substreams) so that selectivity of the transport parameters to changes in the diffusion rate will be maximal. Once the appropriate experimental conditions are used, the effects of surfactants and denaturation agents can be studied quite easily from the change of transport parameters of the proteins in the SPLITT channel.

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Appendix: Nomenclature

a	outlet sub-stream at the sample side
a'	inlet sub-stream carrying the sample
b	outlet sub-stream — the counter port
b'	inlet sub-stream carrying pure fluid carrier
b	channel breadth
c_0	initial sample concentration
D	diffusion coefficient
F_a	retrieval factor at outlet sub-stream a
L	channel length
t^0	channel void time
τ_D	dimensionless transport parameter
V^0	channel void volume
$\dot{V}(a)$	flow rate of outlet sub-stream a
$\dot{V}(a')$	flow rate of inlet sub-stream a'
$\dot{V}(b)$	flow rate of outlet sub-stream b
$\dot{V}(b')$	flow rate of outlet sub-stream b'
$\dot{V}$	total flow rate
w	channel thickness
$w_{a'}$	sample region thickness
x	coordinate across the channel thickness
z	coordinate along the channel axis