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Sodium dodecyl sulfate capillary electrophoresis of proteins in entangled solutions of poly(vinyl alcohol)

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Abstract

A novel polymer network is described for efficient sieving of sodium dodecyl sulfate (SDS)–protein complexes: poly(vinyl alcohol) (PVA; average M_r 133 000). The entanglement threshold of these solutions was found to be at 3% (w/v) PVA. Solutions from 4 to 6% PVA offer excellent resolution in the 14 400–94 000 protein molecular mass interval. Ferguson plot analysis showed that the separation is indeed based on mass discrimination, as it should in SDS electrophoresis, with extrapolated (at 0% polymer) limit values of mobility for all particles in the range $(2.34–2.87) \cdot 10^{-8}$ m^2 $V^{-1}s^{-1}$. The advantages of PVA are full transparency in the UV region down to 200 nm and extremely low viscosities (e.g., a 5% PVA solution has a viscosity 25 times greater than that of buffer at 30°C). A unique wall effect was found, by which, on decreasing the inner diameter of the capillary from 75 to 25 μm, the apparent entanglement threshold was shifted to extremely dilute PVA solutions, since in 25-μm capillaries efficient sieving was obtained below 1% PVA, i.e., at concentrations well below the entangled regime. It is hypothesized that residual, free silanols present (even in a coated capillary) act as nucleation sites for H-bond formation and aggregation of free PVA molecules.

1. Introduction

In 1983, Hjertén [1] was the first to demonstrate the use of capillaries filled with gels while separating the components of bovine serum albumin. Later, Cohen and co-workers [2,3] showed the value of gel-filled capillaries in high-resolution analysis of charged macromolecules by capillary zone electrophoresis (CZE). Typically, for mass evaluation of sodium dodecyl sulfate (SDS)–protein complexes, a polyacrylamide gel was the matrix of choice also in CZE [2–4], but separations in 10–25% HydroLink gels were also reported [5]. However, in contrast to slab-gel techniques, gel-filled capillaries have so far met with only limited success. This is due to a variety of reasons, such as pore-size limitations, formation and trapping of air bubbles during gel polymerization, denaturation and collapse of the matrix due to local overheating, sample trapping and precipitation at the injection port.

Soon chemically cross-linked gels were

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abandoned in favour of viscous solutions of linear (or branched) polymers, according to original observations of De Gennes [6] and Bode [7] that polymer networks, above a critical concentration (the "entanglement threshold") would be efficient in sieving macromolecules. Linear polyacrylamide, as a viscous polymer solution, was soon proposed for mass discrimination of SDS–protein complexes in CZE [8]. Sieving dynamic matrices are immune from the noxious problems of air-bubble formation (which would automatically open the electric circuit in such tiny channels) and from sample precipitation at the injection port. Owing to the lack of a fixed-pore geometry, also very large macromolecules (or aggregates) can open a pore in their wake, while they would inevitably precipitate at the deposition site in cross-linked polyacrylamide gels. This allows repeated use of the same matrix (typically >30 runs). Even on matrix fouling, the viscosity of a 6% polyacrylamide network still allows refilling of the capillary at the normal pressures utilized in CZE for, e.g., sample injection [9]. However, even this solution did not prove optimal. As shown by Ganzler et al. [10], the ideal detection system for proteins in transit in a capillary would be by UV absorption at 214 nm, where the molar absorptivities of proteins are 20–50 times larger than at 280 nm. However, at this low wavelength, polyacrylamide, which also contains amido bonds, absorbs 20–50 times more light than other UV-transparent polymers at comparable concentrations.

A search was therefore started for efficient UV-transparent polymers, ideally also exhibiting low viscosity, so as to allow replacement and refilling even after each run, if needed. Ganzler et al. [10] reported successful SDS runs in two such formulations: a 10% solution of dextran (M_r $2 \cdot 10^6$) and a 3% solution of polyethylene glycol (PEG, average M_r 100 000). The applicability of such an approach was later confirmed by Lausch et al. [11]. Soon, several articles appeared reporting efficient protein sieving in other types of polymers. Thus, Guttman et al. [12] described the use of lower M_r dextrans (M_r 72 000, 10% branching, at a 15% concentration) and of polyethylene oxide (PEO, typically a 3% solution of average M_r 100 000). They also studied the influence of temperature on the sieving effect in SDS–protein complexes and found the best separations at 50°C (in dextrans, whereas a deterioration of separation was apparent in the same temperature interval in PEO). Recently, SDS electrophoresis was reported for other types of UV-transparent polysaccharides, such as pullulan [a branched polysaccharide composed of α-(1–6)-linked maltotriose] [13]. Typically, separations were carried out in a 7% polymer solution (having average M_r 50 000–100 000), although good resolution was achieved for proteins standards (in an M_r range from 14 400 to 116 000) from as low as 1% up to 7% pullulan.

In this work, we investigated the possibility of using poly(vinyl alcohol) (PVA, average M_r 133 000) as a dynamic sieving matrix for SDS–protein complexes. This investigation stems from the observation of Righetti and Snyder [14] that the viscosity of PVA solutions could be greatly modulated by adding to this polymer short PEG chains, to the point of forming a thermally reversible gel. The use of PVA has been reported previously by Schomburg's group, but mostly for dynamic coating of bare fused silica [15] and in some attempts at DNA sieving [16]. The same group ultimately rejected the use of PVA, on the grounds of its deterioration on storage due to strong self-aggregation and potential precipitation.

2. Experimental

2.1. Reagents

Poly(vinyl alcohol) (PVA) and hydroxyethylcellulose (HEC) were obtained from Poly-Sciences (Warrington, PA, USA), polyethylene glycol (PEG), SDS, 2-mercaptoethanol and sulfuric acid from Merk (Darmstadt, Germany) and 2-amino-2-methyl-1,3-propanediol (AMPD) and cacodylic acid (CACO) from Sigma (St. Louis, MO, USA). Low-molecular-mass protein standards (α-lactalbumin, M_r 14 400, trypsin inhibitor, M_r 20 100, carbonic anhydrase, M_r 30 000,

ovalbumin, M_r 43 000, bovine serum albumin, M_r 67 000, phosphorylase *b*, M_r 94 000, dextran T-2000, dextran T-500 and dextran T-250 (Dex) were purchased from Pharmacia–LKB (Uppsala, Sweden). Cerium (IV) sulfate tetrahydrate was obtained from Fluka (Buchs, Switzerland) and acrylamide, ammonium peroxodisulfate and N,N,N′,N′-tetramethylethylendiamine (TEMED) from Bio-Rad Labs (Richmond, CA, USA). N-acryloylaminoethoxyethanol (AAEE) was synthesized according to Chiari et al. [17].

2.2. Capillary electrophoresis

CZE was performed with a Waters Quanta 4000 apparatus from Millipore (Milford, MA, USA) and in parallel with a Model 270 A-HT capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA). Capillaries (34 cm × 100–25 μm I.D.) coated by a slight modification of Hjertén's protocol utilizing AAEE as monomer [18] and filled with different types of polymers at various concentrations were used. For separations in dextran polymers the capillaries were additionally coated with Ce^{IV} salts as described by Ganzler et al. [10]. Before sample injection the baseline and current were monitored at 5 kV for 20 min until constant values were reached. The samples were injected at 5 kV for 2 s. The absorbance was monitored at 214 nm.

2.3. Sample preparation

The low-molecular-mass markers were dissolved (to a final concentration of 4 mg/ml) in 100 μl of 60 m*M* CACO–AMPD buffer containing 1% SDS and 1% 2-mercaptoethanol (pH 8.8), heated at 100°C for 5 min and immediately injected. The same buffer was used in all CZE runs, except that the concentration of SDS was lowered to 0.1% and 2-mercaptoethanol was omitted.

2.4. Polymer preparation

PVA and HEC were dissolved in the AMPD–CACO running buffer at 70°C, then sonicated to eliminate air bubbles. PEG and Dex, at various concentrations, were dissolved in AMPD–CACO buffer at room temperature. Polyacrylamide was polymerized in capillaries for 1 h at room temperature. All capillaries were conditioned with separation buffer for 20 min at 5 kV prior to the initial protein separation run.

2.5. Viscosimetry

Viscosity measurements on polymer solutions were performed on a Bohlin VOR rheometer (Bohlin Rheology, Lund, Sweden), with the sample thermostated at 30°C. The shear rate chosen was in the interval 1.86–2.34 s^{-1}. A concentric cylinder measuring system was adopted, with a 14 mm diameter for the inner cylinder and a 15.4 cm diameter for the outer cylinder. Viscosity is expressed in mPa s, the viscosity of water at 25°C being 1 and that of the running buffer being 1.68.

2.6. Calculation of global resolution

The global resolution (R_{sg}) was calculated as the product of each individual resolution divided by the average of individual resolutions. By this method too large differences in individual resolutions are minimized and data are more easily compared. A global resolution as a plain average of individual resolutions could give erroneous data [19].

3. Results

We first evaluated the overall resolution of the six denatured protein markers as a function of the percentage of polymer in the Dex-2000 system of Ganzler et al. [10], and also in shorter dextran chains (M_r 500 000 and 250 000, Dex-500 and Dex-250, respectively). As shown in Fig. 1A, Dex-2000 appears to offer the highest R_{sg} at the lowest concentration. Both Dex-500 and Dex-250 exhibit lower R_{sg} and have to be used at high concentrations, well above the 10% value found to be optimum with Dex-2000 [10]. However, the situation is more complex than that: if

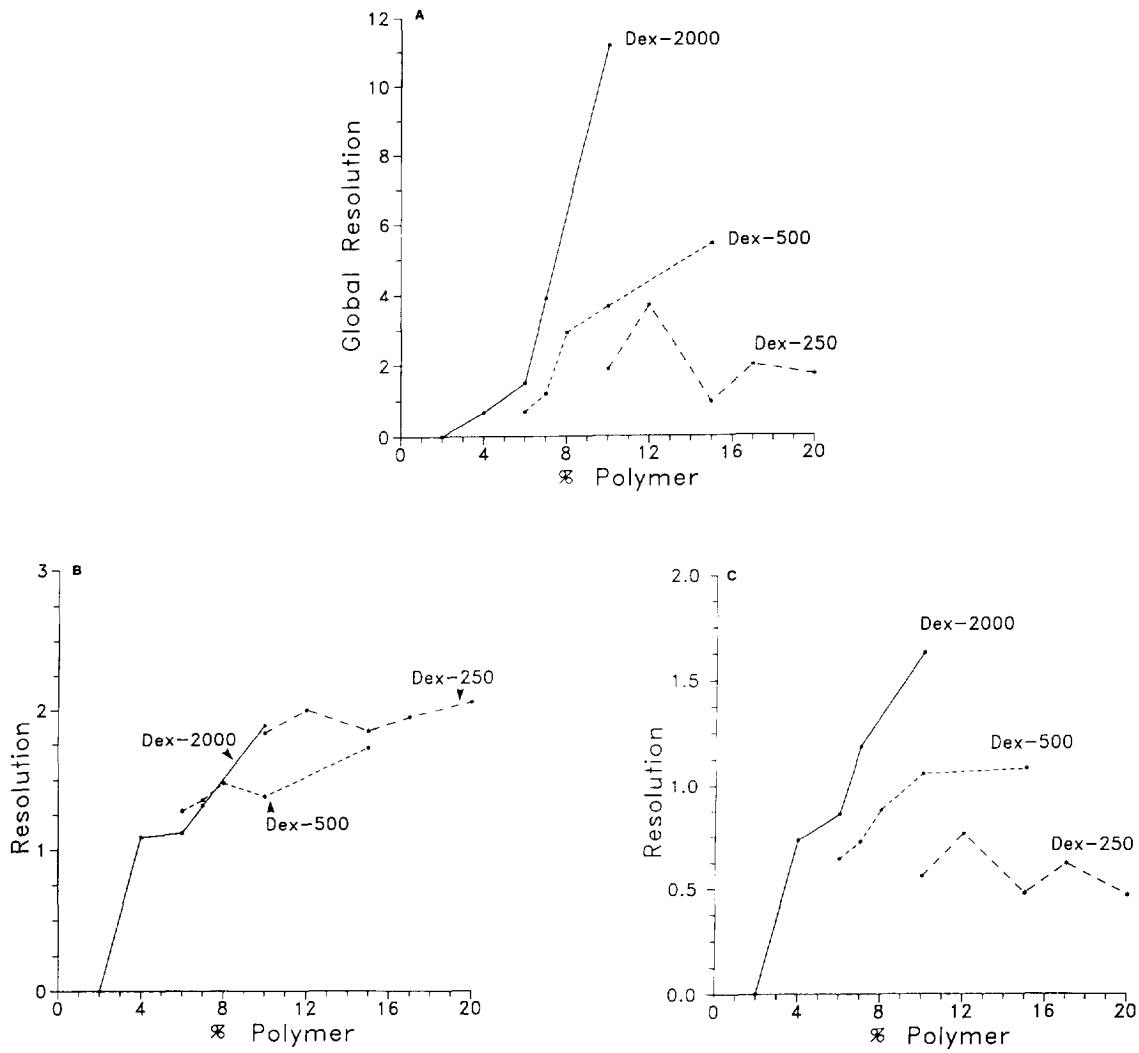

Fig. 1. Resolution vs. percentage of polymer in the separation of six protein markers in three different dextran solutions: Dex-2000, Dex-500 and Dex-250. (A) Global resolution; (B) resolution of an adjacent pair of polypeptide chains of relatively low M_r (14 400 and 20 100); (C) resolution of an adjacent pair of polypeptide chains of relatively high M_r (67 000 and 94 000). Experimental conditions: Waters Quanta 4000; capillary, 34 cm × 75 μm I.D.; buffer, 60 m*M* AMPD–CACO (pH 8.8) in 0.1% SDS; sample load, 5 kV, 2 s (protein concentration 4 mg/ml); run, 8 kV, 11.5 μA. Analogous results were obtained with the Model 270 A-HT capillary electrophoresis system.

we now evaluate resolution in terms of adjacent pairs, it is found that for the smaller proteins (the pair of M_r 14 400 and 20 100), the highest resolution is indeed obtained in the shorter dextran chains (Dex-250; see Fig. 1B). Conversely, if we now evaluate the resolution for the highest M_r pair (67 000 and 94 000), the best performance is again offered by Dex-2000 (Fig. 1C). However, attempts at otpimizing the resolution over the entire analyte M_r range by blending Dex-250 with Dex-2000 in appropriate ratios did not give significant improvements in performance (data not shown).

The 10% Dex-2000 sieving matrix, as proposed by Ganzler et al. [10], was found to offer a good performance coupled with good run repro-

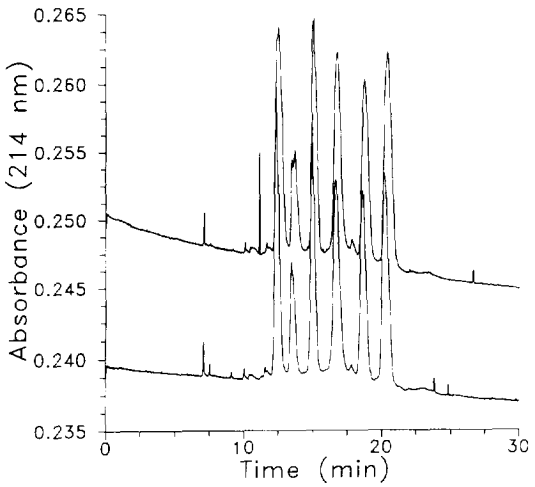

Fig. 2. SDS electropherogram (214-nm readings) of six protein standards. Peaks (from left to right): α-lactalbumin, M_r 14 400; trypsin inhibitor, M_r 20 100; carbonic anhydrase, M_r 30 000; ovalbumin, M_r 43 000; bovine serum albumin, M_r 67 000; phosphorylase *b*, M_r 94 000. Sieving matrix, 10% Dex-2000. All other conditions as in Fig. 1. Two consecutive runs are displayed.

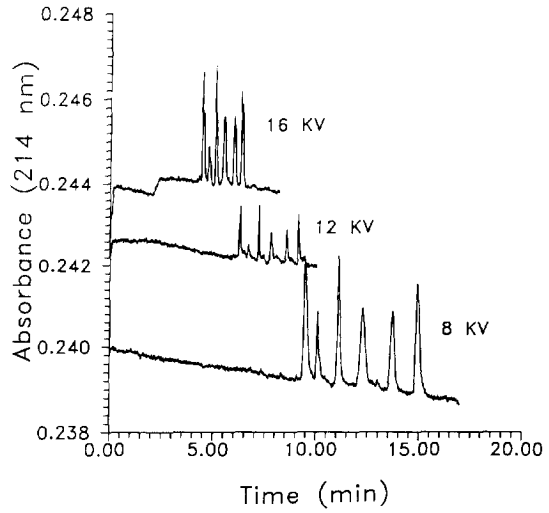

Fig. 3. SDS electropherograms (214-nm readings) of six protein standards. Peaks (from left to right): α-lactalbumin, M_r 14 400; trypsin inhibitor, M_r 20 100; carbonic anhydrase, M_r 30 000; ovalbumin, M_r 43 000; bovine serum albumin, M_r 67 000; phosphorylase *b*, M_r 94 000. Sieving matrix, 4% PVA; capillary, 34 cm × 50 μm I.D. Upper trace, 16-kV run; middle trace, 12-kV run; lower trace, 8-kV run.

ducibility, as shown in Fig. 2. Attempts at using Dex-2000 at concentrations >10% failed for two reasons: the viscosity is too high and the resolution drops markedly (possibly also owing to too long transit times with concomitant peak diffusion; not shown).

We next assessed the performance of PVA as a function of applied voltage at fixed percentage of polymer (4%) and fixed capillary I.D. (50 μm). As shown in Fig. 3, the set of six protein markers is fully resolved at all applied voltages, with baseline resolution even at 16 kV. As expected, at the highest applied voltage the train of peaks is eluted much earlier, between 4 and 8 min. The sieving properties of PVA, however, vary dramatically with concentration. As shown in Fig. 4, when evaluating resolution as a function of polymer concentration, the series of profiles shown, covering the 3–8% interval, suggest a loss of resolution at both ends of the range, with a peak of performance centred at 5–6% polymer. However, while the loss of resolution at the lowest polymer concentration explored (3–4%) is not so pronounced (the six marker peaks are still there), the higher PVA concentration values

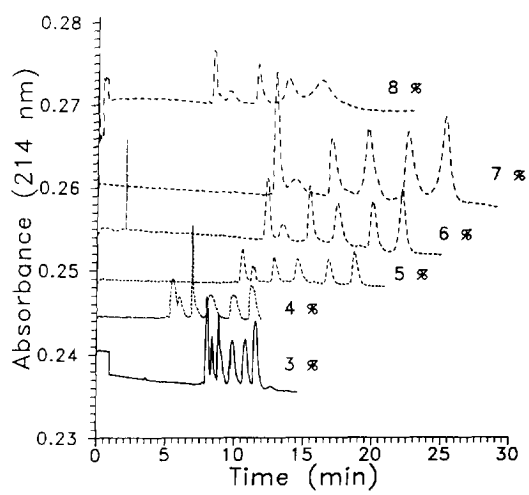

Fig. 4. SDS electropherograms (214-nm readings) of six protein standards. Peaks (from left to right): α-lactalbumin, M_r 14 400, trypsin inhibitor, M_r 20 100; carbonic anhydrase, M_r 30 000; ovalbumin, M_r 43 000; bovine serum albumin, M_r 67 000; phosphorylase *b*, M_r 94 000. Capillary, 34 cm × 75 μm I.D. Sieving matrix (from bottom to top): 3%, 4%, 5%, 6%, 7% and 8% PVA. All other experimental conditions as in Fig. 1.

(7–8%) show a marked decrease in performance, with loss of some analyte peaks and unacceptably high zone spreading.

In order to demonstrate that the separation is based on protein M_r values, we constructed a Ferguson plot with the data in Fig. 4. Ferguson graphs are elaborated by plotting the logarithm of mobility (in $m^2\ V^{-1}\ s^{-1}$) as a function of the concentration of sieving polymer. As shown in Fig. 5, these plots give quasi-linear curves, having a slope (K_R) proportional to the effective molecular surface area and thus, ultimately, to M_r. It is also seen that, when extrapolated to 0% PVA, the mobilities tend to converge to limiting values in the range $\mu = (2.34\text{–}2.87) \cdot 10^{-8}\ m^2\ V^{-1}\ s^{-1}$.

A unique effect of the capillary diameter on resolution is reported in Fig. 6A. Curiously, if one adopts a 75 μm I.D. capillary, the overall resolution is diminished and the R_{sg} peak is shifted at higher PVA values (6% PVA, Fig. 6A). Conversely, when a 50 μm I.D. capillary is utilized, two phenomena are evident: R_{sg} is five times higher and the highest resolution is found at much lower PVA concentrations (barely 4%). An even more dramatic effect is found if a 25 μm I.D. capillary is adopted: the highest R_{sg} is found centred below 1% PVA (not shown, owing to difficulties in obtaining a proper UV absorbance signal). This effect is unique, as apparently such separations are obtained well below the entanglement threshold, as will be discussed below. If the results are expressed in terms of

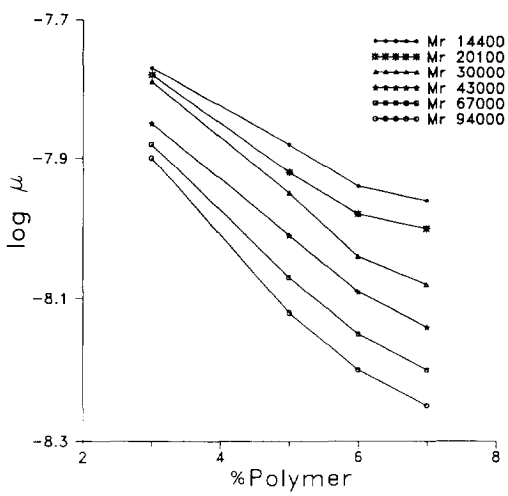

Fig. 5. Ferguson plot analysis of the data in Fig. 4. The peak transit times in Fig. 4 (at different PVA concentrations) are transformed into mobilities (μ, expressed in $m^2\ V^{-1}\ s^{-1}$). Finally, for each M_r marker, the log M_r is plotted against PVA concentration (in the 3–7% range).

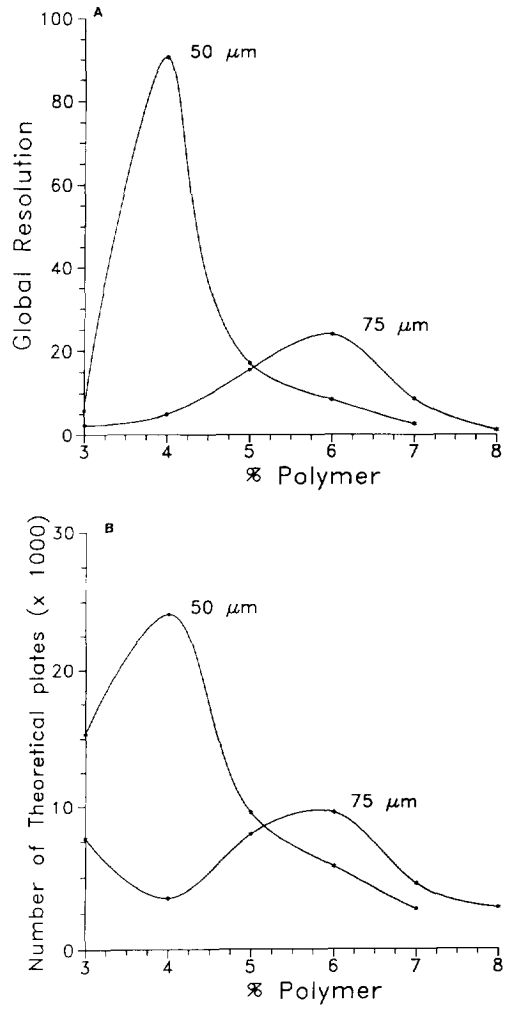

Fig. 6. (A) Global resolution and (B) number of theoretical plates vs. PVA concentration in two different capillaries of 50 and 75 μm I.D.

theoretical plate number, analogous data are obtained (Fig. 6B) for the two capillary diameters investigated. Hence it appears that, with PVA solutions, resolution is strongly dependent on some peculiar wall effect (see Discussion). We could exclude temperature effects on R_{sg}, as shown in Fig. 6A, because, at the applied voltage used (8 kV), the temperature difference between the two runs was barely 1°C, as calculated with the aid of the thermal theory and a computer program we have developed for assessing the precise inner temperature in a capillary [20,21].

We finally adopted a 50 μm I.D. capillary, as it represents a good compromise between sensitivity and the "wall effect" reported in Fig. 6A. With this system, we investigated global resolution as a function of increasing PVA concentration and different applied voltages. As shown in Fig. 7, the highest R_{sg} is obtained at 8–12 kV, with a peak centred at relatively low PVA concentration (4%). When the applied voltage is increased to 16 kV, two phenomena become apparent: R_{sg} is substantially lowered and maximum resolution is obtained only at higher PVA concentrations (here slightly above 5%). It should be noted that there are no appreciable viscosity changes, because on going from 8 to 16 kV the temperature increment of the buffer inside the capillary is only 1°C.

In a recent report, Barron et al. [22] described a unique phenomenon occurring in hydroxyethylcellulose (HEC, M_n 90 000–105 000): DNA separations seem to occur at HEC concentrations ($\leq$0.002%) well below the entanglement threshold. Since in principle an SDS-coated portein could behave, in electrophoretic migration, much like a small DNA fragment, we tried to evaluate whether such a phenomenon could apply also in the present instance. We therefore assessed the viscosity of the PVA solutions at progressively higher concentrations. As shown in Fig. 8, departure from linearity occurs at precisely 3% (w/v) polymer concentration. According to Barron et al. [23], this point represents the entanglement threshold. Hence it appears that all SDS–protein separations are only operative above this critical threshold, in agreement with De Gennes' [6] theory. The fact that in describing Fig. 6A and B we have hinted that in a 25

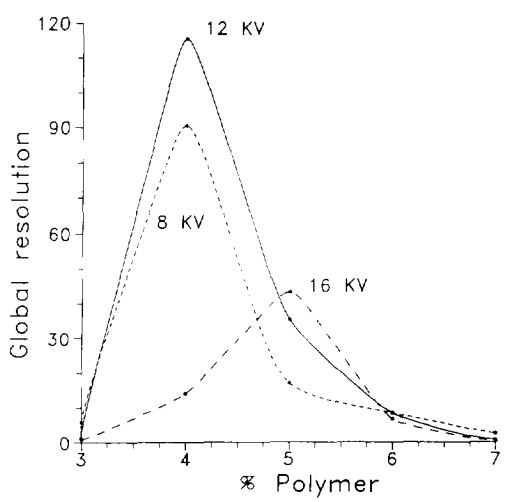

Fig. 7. Global resolution vs. PVA concentration in a 50 μm I.D. capillary as a function of three different applied voltages: 8, 12 and 16 kV.

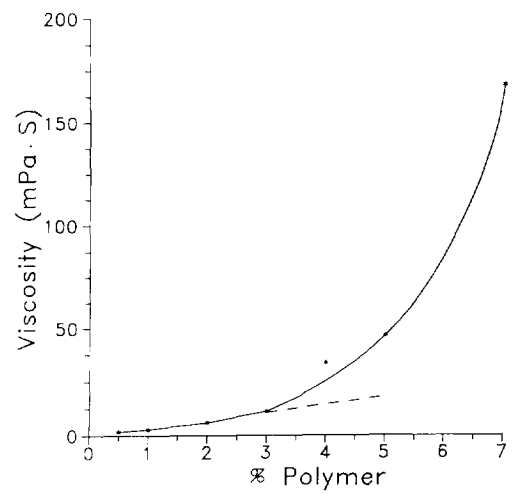

Fig. 8. Viscosity of PVA solutions. The point of departure from the initial linear slope is taken to represent the entanglement threshold (in this case 3% PVA solution). Viscosities measured at 30°C with a Bohlin VOR rheometer.

μm I.D. capillary separations could occur at PVA values as low as 1% is interpreted by us by assuming a completely different mechanism (see Discussion).

4. Discussion

4.1. Use of viscous polymer solutions

As now amply shown in the literature, viscous polymer networks represent a unique solution for proper macromolecular sieving in capillaries, where gel polymerization is besieged by severe problems. For routine operations, the search has been extended to polymers possessing some unique characteristics, such as UV transparency (to as low as 214 nm for peptide bond analysis) and low viscosity, so as to allow refilling even after each run, if needed. The solution proposed by Ganzler et al. [10], of using Dex-2000, satisfies both requirements and appears to work very well in SDS electrophoresis. In a search for other polymers, we propose here PVA, which also is transparent to as low as 200 nm and which also exhibits low viscosity. In fact, at the concentrations routinely used of 4–5% polymer, above the entanglement threshold, the viscosity is extremely low, 25 times higher than that of plain buffer (see Fig. 8). This is very advantageous, as replenishing the capillary after each run does not in fact present any problem, as opposed to polyacrylamide solutions, which are so viscous as to be unyielding to any pumping process above a critical 6% concentration [9,24]. However, such low-viscosity solutions would represent a problem in uncoated capillaries: the electrosmotic pump would quickly push them out of the capillary, thus impairing resolution. Therefore, even though, in principle, in separations of highly negative charged species, such as DNA and SDS-laden proteins, coating of the capillary inner surface would per se not be necessary, it is in fact essential in PVA, dextran and any other low-viscosity polymer solution. In order to enhance reproducibility, we have additionally adopted coating with the novel monomer N-acryloylaminoethoxyethanol, coupling a high hydrophilicity with extreme hydrolytic stability. This coating was found to be stable even up to 200 h of operation at pH 10.5 [17].

4.2. Entanglement threshold

According to De Gennes' theory [6], a fundamental distinction exists between dilute polymer solutions where the coils are separated and more concentrated solutions where the coils overlap. At the overlap threshold, the coils begin to be densely packed and thus begin to entangle. As a result, a kind of a "porous" structure is formed, with a correlation length, or average mesh size, that decreases rapidly with increasing concentration. When biological macromolecules are driven through this meshwork of coils, sieving occurs, much like in a chemically cross-linked gel. Thus, up to the present time, by general consensus, it was believed that sieving in viscous polymer solutions would occur only near and above this entanglement threshold. This view has now been challenged by Barron and co-workers [22,23], who demonstrated that, with DNA fragments, sieving can occur in extremely dilute HEC solutions ($\leq$0.002%), i.e., well below this threshold, set at 0.35% polymer in the case of HEC. In reality, these data can be fully reconciled with De Gennes' theory simply by invoking a "transient entanglement coupling mechanism". A good example is given in ref. [22]: HEC (M_n 90 000–105 000) behaves in solution much like a rigid rod, with a Porod–Kratky radius of gyration (R_g) of 56.5 nm. If, in a dilute HEC solution, one attempts separation, by a sieving mechanism, of a small DNA fragment, say of 118 base pairs (bp) (having an R_g of 35 nm and a contour length of 40 nm, i.e., below the 45-nm persistence length of ds DNA), essentially no entanglement will result between this small DNA and HEC and no sieving will occur. Conversely, if one takes a long restriction fragment (e.g., a 9416 bp DNA, having an R_g of 533 nm, i.e., ten times larger than that of an HEC molecule), it will be this long DNA filament (which, if extended, would have an end to end length of 3201 nm) which will screen a large number of HEC coils and become entangled.

Sieving will thus occur through this transient "entangled" regime. In agreement with this, we have found that in our solutions of PVA coils (which have an average size similar to that of HEC) we could not obtain a satisfactory separation of PCR fragments in the 100–400 bp range. How is it, then, that we can have efficient sieving of proteins in the M_r 14 400–94 000 range? This could be due to two additive mechanisms. First, protein–SDS co-micelles are much larger than a protein filament alone. In addition, PVA chains might have a unique entanglement regime, owing to extensive hydrogen bonding, as explained below.

4.3. Use of Ferguson plots

The Ferguson plot analysis (Fig. 5) is presented at the request of a referee and was not in our original paper. Fortunately, analysis of Fig. 5 could lead to some interesting conclusions. It should first be recalled that, according to the original theory [25], proteins having the same surface charge, but differing in mass, should have different slopes (K_R), but the same y-intercept (corresponding to free mobility, μ). This should be the case for DNA and SDS–protein micelles alike. However, if one analyses most of the Ferguson plots presented in the literature, one can see that, although the K_R values almost always agree with expectations, very rarely does this occur for the y-intercepts. Our data are no exception to this general trend. Going back to Fig. 5, one can see that there are at least three different "intercepts": (a) the smaller molecules (M_r 14 400–30 000), coalesce at 2.2% PVA (at which they exhibit an apparent common value of free mobility of $1.9 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}); (b) larger molecules (M_r 43 000–94 000) coalesce at 1.2% PVA (at which they exhibit the same apparent common value of free mobility of $1.9 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}); finally, if one extrapolates to 0% PVA, all free μ values diverge again, in the range $(2.34–2.87) \cdot 10^{-8}$ m^2 V^{-1} s^{-1}. What is the meaning of all this? We should like to propose two different scenarios, as follows.

(α) The α scenario goes back to the concept of relativity. Perhaps the "entanglement" threshold, so much under debate today, is not really an absolute value, but a relative one. It is an intrinsic property of the polymer solution, per se, but it is a relative value because it depends also on the size of the object to be sieved. Thus, while the "entanglement threshold" of pure PVA, per se, is 3% (see Fig. 8) in reality, for smaller proteins (the M_r 14 400–30 000 group) the solution becomes fully disentangled at 2.2% concentration (where all species exhibit the same free mobility), whereas for larger particles (in our case the M_r 43 000–94 000 polypeptides) the solution is fully disentangled at 1.2% concentration (common μ value).

(β) The β scenario regards the common extrapolated value of μ at 0% PVA (which is anything but common). What is the meaning of this? We have at the moment only one explanation: perhaps it is not completely true that the ratio of protein to SDS (believed to be in general 1:1.4, w/w) is as constant as we believe it to be, but it depends to some extent also on the mass of the protein (exceptions due to the amino acid composition and to prosthetic groups are well known already). If this is true, one can never really expect a single μ value corresponding to the free mobility of the particle under investigation. Of course, one could argue that larger particles experience a greater viscous drag in free solution, so that the free μ value will not be the same, anyhow. However, this can only apply (assuming that the charge-to-mass ratio is constant in protein–SDS micelles) if the shape of such micelles is not the same, whereas in general it is believed that polypeptides swamped by SDS have the same shape, resembling a prolate ellipsoid, with a constant minor axis and a major axis proportional to the length of the polypeptide chain [26].

4.4. Use of different sieving polymers

It is now apparent that different polymers cannot be used interchangeably for DNA and protein separations alike. For example, although HEC has been found to perform extremely well in DNA separations, especially for larger fragments, it did not give any acceptable separation

of protein–SDS complexes in all ranges of concentrations explored (from 0.35% up to 1.5%, i.e., the highest possible concentration compatible with injecting the solution in a capillary). Also PEG (M_n 35 000) did not produce any acceptable result up to 40% concentration. Conversely, polyacrylamide, which is one of the best viscous polymer solutions for DNA separations, also performed very well in sieving SDS–protein co-micelles. However, owing to its very high absorbance, it cannot be proposed as the matrix of choice when monitoring at 214 nm. PVA seems to have a unique behaviour, producing different types of entangled regimes, possibly as a result of a wall effect originating inside the capillary. We have seen, in fact, that on decreasing the capillary I.D. from 75 to 25 μm, the peak resolution is progressively lowered from 6% to 4% PVA to as low as <1% PVA in the narrowest bore explored (25 μm). This unique behaviour (which would seem to contradict the fact that sieving should occur above the entanglement threshold, set at 3% polymer in the case of PVA) might be explained through an observation made by Schomburg and co-workers [16]: "PVA could be subjected to strong self-aggregation by intermolecular interaction between the polymer chains via hydrogen bonding between the hydroxyl groups present in high concentration in the PVA molecule. The hydroxylic character of this molecule also affects the strong adsorptive interaction with the fused-silica surface and therefore the modifying properties of PVA. Ageing of PVA solutions in fused-silica capillaries could probably be initiated and accelerated by the silanol at the silica surfaces. The adsorbed PVA molecules may undergo a conformational rearrangement on the silica surface, which may facilitate the fixation of additional PVA layers on this surface". In agreement with these observations, we found, when attempting to measure the viscosity of PVA solutions directly in a CZE unit, as recently proposed by Bello et al. [27], that in each successive measurement (performed in bare silica capillaries) the viscosity of the same PVA solution increased markedly until complete clogging of the capillary bore occurred. In addition, recent observations suggest that, owing to the high shear rate existing in the double layer, polymer solutions could undergo a transition from a collapsed globule to extended filaments, which would greatly favour their adsorption into the Debye–Hückel layer and potentially facilitate the build-up of successive strata [28]. Hence we believe that the unique sieving properties shown in narrow-bore capillaries (25 μm), by which PVA solutions well below the entanglement threshold can efficiently separate protein–SDS complexes, are indeed an apparent phenomenon: at such a high surface-to-volume ratio, the wall effect becomes very pronounced and probably forces the PVA solution to assume an "entangled" regime. Hence we suggest that the wall, in very narrow-bore capillaries, provides nucleation sites for entanglement of very dilute PVA solutions, forcing them to assume an "entangled" regime (i.e., here the mechanism proposed by Barron et al. does not apply). In agreement with this, Righetti and Snyder [14] have shown that even minute amounts of sodium tetraborate, when added to a 5% PVA solution, can induce a transition from a viscous liquid to a gel phase. For example, in the presence of only 0.1% sodium tetraborate, a 5% PVA solution forms a thermally reversible gel, with a melting point of 50°C. As an additional proof of the formation of extensive hydrogen bonds in PVA solutions, we performed viscosity measurements on 8% PVA solutions as such and in the presence of 6 *M* urea. Whereas the viscosity of 8% PVA alone was 566 mPa s, the viscosity of 8% PVA in the presence of 6 *M* urea (the latter having a viscosity of 1.68 mPa s) was drastically diminished by 40%, down to only 341 mPa s.

5. Conclusions

PVA solutions [in the 4–6% (w/v) range] offer a unique dynamic sieving matrix for SDS electrophoresis. Such solutions are transparent down to 200 nm and have an extremely low viscosity, thus allowing replenishment of the capillary after each run. It is recommended that the capillary inner wall should be coated, not only for suppressing the electrosmotic flow, but also for minimizing the unique ageing and aggregation

phenomenon described above, induced by free silanols at the silica surface. Even in coated capillaries, it is suggested that when using PVA solutions, replenishment is routinely adopted after each run. In the absence of this precaution, separations start to deteriorate after the third run, possibly owing to the fact that, even with the best coating procedure, some silanols are still free and available for interaction.

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Note added

After this paper had been accepted, and during its revision, we found a few more articles just published dealing with SDS-CZE, which will be briefly mentioned here. Shieh et al. [29] have studied more thoroughly the sieving system composed of polyethylene oxide (PEO)–SDS. They found that, if the capillary is rinsed with 1 *M* HCl between each run, the coating (made of linear polyacrylamide) is stable for more than 400 runs (as a criticism of this, we can add that such strongly acidic conditions will hardly remove traces of contaminant proteins from run to run, as these conditions are precipitating, not solubilizing, for proteins). They also reported relative standard deviations (over 19 runs) of only 0.35–0.45%. We regret, in this paper, the continuing use of the term "gel" for viscous polymer solutions, which can only add to the confusion already existing. With the same PEO–SDS system, Guttman et al. [30], proposed an automated Ferguson plot analysis which can correct for the non-ideal behaviour of glycoproteins and lipoproteins in SDS electrophoresis due to anomalous binding of SDS. The possibility of performing Ferguson plot analysis, in the same PEO–SDS system, was simultaneously and independently proposed by Benedek and Thiede [31].

References

[1] S. Hjertén, in H. Hirai (Editor), *Electrophoresis '83*, Walter de Gruyter, Berlin, 1983, pp. 71–79.
[2] A.S. Cohen and B.L. Karger, *J. Chromatogr.*, 397 (1987) 409–417.
[3] A.S. Cohen, D.R. Najarian, A. Paulus, A. Gutman, A.J. Smith and B.L. Karger, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 9660–9663.
[4] K. Tsuji, *J. Chromatogr.*, 550 (1991) 823–830.
[5] V. Dolnik and M.V. Novotny, *Anal. Chem.*, 65 (1993) 563–567.
[6] P.G. De Gennes, *Scaling Concepts in Polymer Chemistry*, Cornell University Press, Ithaca, NY, 1979.
[7] H.J. Bode, *Anal. Biochem.*, 83 (1977) 204–210.
[8] A. Widhalm, C. Schwer, D. Blass and E. Kenndler, *J. Chromatogr.*, 546 (1991) 446–451.
[9] M. Chiari, M. Nesi, M. Fazio and P.G. Righetti, *Electrophoresis*, 13 (1992) 690–697.
[10] K. Ganzler, K.S. Greve, A.S. Cohen, B.L. Karger, A. Guttman and N.C. Cooke, *Anal. Chem.*, 64 (1992) 2665–2671.
[11] R. Lausch, T. Scheper, O.W. Reif, J. Schlosser, J. Fleischer and R. Freitag, *J. Chromatogr. A*, 654 (1993) 190–195.
[12] A. Guttman, J. Horvath and N. Cooke, *Anal. Chem.*, 65 (1993) 199–203.
[13] M. Nakatani, A. Shibukawa and T. Nakagawa, *J. Chromatogr. A*, 672 (1994) 213–218.
[14] P.G. Righetti and R.S. Snyder, *Appl. Theor. Electrophoresis*, 1 (1988) 53–58.
[15] M. Gilges, H. Husmann, M.H. Kleemiss, S.R. Motsch and G. Schomburg, *J. High Resolut. Chromatogr.*, 15 (1992) 452–457.
[16] M.H. Kleemiss, M. Gilges and G. Schomburg, *Electrophoresis*, 14 (1993) 515–522.
[17] M. Chiari, C. Micheletti, M. Nesi, M. Fazio and P.G. Righetti, *Electrophoresis*, 15 (1994) 177–186.
[18] M. Chiari, M. Nesi and P.G. Righetti, *Electrophoresis*, 15 (1994) 616–622.
[19] J.R. Torres-Lapasiò, R.M. Villanueva-Camanas, J.M. Sanchis-Mallos, M.J. Medina-Hernández and M.C. García-Alvarez-Coque, *J. Chromatogr. A*, in press.
[20] M.S. Bello, P. de Besi and P.G. Righetti, *J. Chromatogr. A*, 652 (1993) 317–327.

[21] M.S. Bello, P. de Besi and P.G. Righetti, *J. Chromatogr. A*, 652 (1993) 329–336.
[22] A.E. Barron, H.W. Blanch and D.S. Soane, *Electrophoresis*, 15 (1994) 597–615.
[23] A.E. Barron, D.S. Soane and H.W. Blanch, *J. Chromatogr. A*, 652 (1993) 3–16.
[24] M. Chiari, M. Nesi and P.G. Righetti, *J. Chromatogr. A*, 652 (1993) 31–39.
[25] H.R. Maurer, *Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis*, Walte de Gruyter, Berlin, 1971, pp. 8–20.
[26] B.D. Hames, in B.D. Hames and D. Rickwood (Editor), *Gel Electrophoresis of Proteins*, IRL Press, Oxford, 1990, pp. 1–148.
[27] M.S. Bello, R. Rezzonico and P.G. Righetti, *J. Chromatogr. A*, 659 (1994) 199–204.
[28] M.S. Bello, P. de Besi, R. Rezzonico, P.G. Righetti and E. Casiraghi, *Electrophoresis*, 15 (1994) 623–626.
[29] P.C.H. Shieh, D. Hoang, A. Guttman and N. Cooke, *J. Chromatogr. A*, 676 (1994) 219–226.
[30] A. Guttman, P. Shieh, J. Lindahl and N. Cooke, *J. Chromatogr. A*, 676 (1994) 227–231.
[31] K. Benedek and S. Thiede, *J. Chromatogr. A* 676 (1994) 209–217.