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Quantitative studies on the adsorption of proteins to the bare silica wall in capillary electrophoresis
II. Effects of adsorbed, neutral polymers on quenching the interaction

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Abstract

A novel method is reported for quantifying protein adsorption to naked silica tubings and for assessing the efficacy of polymers added to the background electrolyte as dynamic wall modifiers. It consisted of flushing a fluorescently-labelled protein (myoglobin) into a capillary equilibrated in Tris–acetate buffer, pH 5.0, until full saturation of the potential adsorbing sites. Desorption was then affected by electrophoretically driving sodium dodecyl sulphate micelles into the capillary from the cathodic reservoir: the peak of eluted material is quantified by using a dual laser beam instrument able to read the fluorescein isothiocyanate-derivatized myoglobin at 520 nm and the internal standard (sulphorodamine) at 630 nm. Four polymers have been assessed as potential quenchers of interaction of proteins with the silica wall: hydroxypropylmethylcellulose (HPMC, M_r=1 000 000), hydroxyethylcellulose (HEC, M_r=27 000), poly(vinyl alcohol) (PVA, M_r=49 000) and short-chain poly(dimethylacrylamide) [poly(DMA)] (average M_r ca. 150 000). HPMC, poly(DMA) and PVA were effective in the 0.005 to 0.02% (w/v) range, whereas HEC was active in the 0.1 to 0.8% concentration range. All polymers, however, except for poly(DMA), exhibited a rather poor performance in suppressing protein interactions with the siliceous surface, and could inhibit adsorption only by, at most, 50% (contrary to oligoamines which can quench such interactions by >90%). It is hypothesized that dynamically adsorbed polymers leave ample regions of the capillary inner surface unmasked, thus allowing strong interactions of proteins with the silica wall. This is also confirmed by the modest reduction of electroendoosmotic flow upon polymer adsorption, as compared with an untreated silica surface. Although poly(DMA) can inhibit protein adsorption by as much as 85%, its hydrophobic nature could in turn provide more adsorption sites for less hydrophilic proteins than myoglobin.

Keywords: Buffer composition; Capillary columns; Adsorption; Proteins

1. Introduction

There is an urgent need to develop more scientifically sound methods for the evaluation of column efficacy!

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This plea for ''law and order'' was issued by Regnier and Lin [1] in a recent review summarizing the progress made in capillary zone electrophoresis (CZE) of proteins. In a way, this phrase is quite disturbing, since it implies that we have not quite done our homework in properly implementing protein and peptide separations in CZE and that the technique, notwithstanding the rosy pictures to be found in the innumerable papers on the topic, is far from satisfactory.

The well-known problem in CZE of proteins is that silanol groups cause fused-silica columns to act as a weak cation exchanger at any operative pH>3.0. Severe band spreading and diminished recovery occur when proteins with an isoelectric point (p*I*) higher than the buffer pH interact with the capillary wall. When analysing, e.g., an entire cell lysate, it should be remembered that, at pH 7, approximately one-third of all proteins are positively charged [2] and therefore could potentially be adsorbed by the silica wall, causing not only a dramatic loss of performance, but also altering the zeta potential at the capillary wall. Up to the present, protein binding (or the presumed absence of binding) has been evaluated via a set of visual parameters on the electropherogram: peak shape, absence (or presence) of tailing, improved resolution, number of theoretical plates. Recent reports [3,4] suggest that all these indicators are inadequate measures of column efficacy. Recovery of proteins can be poor and the peaks still be sharp and symmetrical; likewise, plate counts for column evaluation suffer from the problem that variables other than surface silanols determine efficiency.

A vast number of remedies have been described in the literature for minimizing or abolishing peptide/protein interaction with the silica wall. In one approach, dynamic modifications are adopted for modifying the surface of capillaries by using mobile phase (buffer) additives. A typical remedy is to add competing ions, such as salts, amines or zwitterions. Amines appear to function by ion pairing with anionic silanolic groups at the silica surface. This increases both efficiency and resolution by (i) decreasing electroendoosmotic flow (EOF) and (ii) competing with proteins for anionic groups at the surface. A large number of amines have been tabulated by Regnier and Lin [1]. In addition to modification of composition of the background electrolyte, another strategy consists in using polymers, either dynamically or covalently bound to the capillary wall. An extensive literature exists on the use of polymers for deactivating the capillary wall, following an early report dating back to 1967, when Hjertén first described the treatment of thin quartz tubes with methylcellulose at low pH in presence of formaldehyde, for cross-linking this polymer onto the wall [5]. Two, in depth reviews cover this field quite adequately [1,6]. Briefly, these coatings can be divided in the following categories:

(i) *Neutral, hydrophilic polymers*: they are typically, but not exclusively, bonded to the silica wall. Among them: polyacrylamide [7], poly(acryloyl aminoethoxyethanol) [8], poly(acryloyl aminopropanol) [9]; celluloses and dextran [10–13]; poly(vinyl alcohol) [14,15]; epoxy polymer [16–18]; poly(ethylene–propylene glycol) [19,20]; polyethylene oxide [21]; poly[*N*-(acryloylaminoethoxy)-ethyl-β-D-glycopyranoside [22].

(ii) *Neutral, hydrophobic polymers*: cellulose acetate [23] and highly cross-linked poly(styrene–divinylbenzene) layers [24]. The latter forms a ''tube-in-the-tube'' structure, but it is so hydrophobic that it has to be rendered hydrophilic with a second layer of, e.g., polyoxyethylene oligomer.

(iii) *Anionic polymers*: 2-acrylamido-2-methylpropane sulphonate [25]. This type of coating stabilizes the EOF for improved reproducibility since, by incorporating a strong acid, the latter becomes pH independent.

(iv) *Cationic polymers*: polyethyleneimine [26,27]; a copolymer of vinylpyrrolidone and vinylimidazole [28]; polybrene, poly(methoxyethoxyethyl)ethylenimine, poly(diallyldimethylammonium) chloride [29]; chitosan [30]; a copolymer of *N*-(polyethyleneglycol monomethyl ether)-*N*-methylmorpholinium [31].

(v) *Anionic/cationic and zwitterionic polymers*: this coating is based on the chemistry of Immobilines, i.e., the acrylamido weak acids and bases used for creating immobilized pH gradients [32]. Due to the nature of these chemicals (10, with p*K* values ranging from 1.0 up to 13.0) it is possible to impart ''tunable'' positive and negative charges (or, if needed, zwitterionic) to the capillary wall.

(vi) *Neutral surfactants*. Towns and Regnier [33]

derivatized capillaries with octadecyltrichlorosilane to form a covalently bonded hydrophobic phase. An aqueous solution of a non-ionic oxyethylene-based surfactant (e.g., Brij-35), above its critical micellar concentration, was then introduced for forming an adsorbed hydrophilic layer. Pluronics have also been used as an alternative family of surfactants [34].

(vii) *Cationic surfactants*: Emmer et al. [35] reported a fluorinated cationic additive (Fluorad FC134) as a dynamic surfactant coating. CTAB (cetyltrimethylammonium bromide) has also been suggested as a quencher of protein adsorption [36,37].

We have recently evaluated a number of additives (mono- to oligoamines) currently adopted for improving CZE analysis of proteinaceous material [38]. As potential quenchers, a series of monoamines have been investigated (triethylamine, triethanolamine, ethylamine), followed by diamines (putrescine, cadaverine and hexamethonium bromide) and finally by oligoamines [spermidine, spermine and tetraethylenepentamine (TEPA), i.e., a tri-, a tetra- and a pentaamine, respectively]. Oligoamines, especially spermine and TEPA, were found to be most effective, since they strongly inhibited protein adsorption to silica already in the sub-millimolar up to 1 m*M* range.

In the present report, we evaluate the efficacy of dynamically adsorbed neutral polymers in quenching protein adsorption onto siliceous surfaces. This investigation has been spurred by the current notion that a number of such neutral polymers are effectively adsorbed at the Stern layer at the interface of the silica wall, thus drastically diminishing EOF and analyte adsorption. For example, in a recent article, Chiari et al. [39] suggested that small amounts (0.03–0.05%) of poly(dimethyl acrylamide), added to the background electrolyte, would provide comparable performance to permanently coated capillaries, and even longer life times, in DNA separations.

2. Materials and methods

2.1. Chemicals

Horse skeletal muscle myoglobin was obtained from Sigma (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC), dimethylformamide (DMF), dimethylacrylamide (DMA), hydroxylamine, sodium dodecyl sulphate (SDS) and poly(vinyl alcohol) (PVA), M_r=49 000, were from Fluka (Buchs, Switzerland). Hydroxyethylcellulose (HEC), M_r=27 000, was purchased from Polysciences (Warrington, PA, USA). Hydroxypropylmethylcellulose (HPMC), M_r=1 000 000, was from Aldrich (Milwaukee, WI, USA). Fused-silica capillaries (100 μm I.D.×375 μm O.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Protein labelling

Labelling of myoglobin with FITC has been performed according to the protocol in Ref. [40]. Briefly, myoglobin (5 mg/ml) is dissolved in 0.1 *M* sodium hydrogencarbonate, pH 9.0. FITC (10 mg/ml) is dissolved in DMF under vigorous stirring. Gently add 100 μl of FITC to 1 ml of protein solution, under constant stirring. Incubate for 1 h at room temperature. The reaction is blocked by adding 1 ml of 1.5 *M* hydroxylamine–HCl, pH 8.5, per ml of reaction mixture. The derivatized protein is then purified by gel filtration on a Sephadex G-25 column, equilibrated with PBS (phosphate-buffered saline, pH 7.4). The FITC-derivatized protein can be dispensed in aliquots and stored frozen at −70°C for at least 6 months.

2.3. Electrophoretic protein desorption

In order to measure the amount of protein bound to the capillary wall at any pH value of the background electrolyte, two methods have been reported: the one described by Towns and Regnier [3] and that of Verzola et al. [38]. In the first case (illustrated in Fig. 1), protein recovery is measured by using a capillary with two detectors 50 cm apart: the peak decrement at the second detector will give the amount of protein adsorbed by the silica wall along the path between the two sensors. In the second case (depicted in Fig. 2), we exploited a method first reported by Barberi et al. [41], consisting of desorption of bound material via sweeping the silica surface with SDS micelles, driven electrophoretically into the lumen from the cathodic reservoir. Verzola et al.

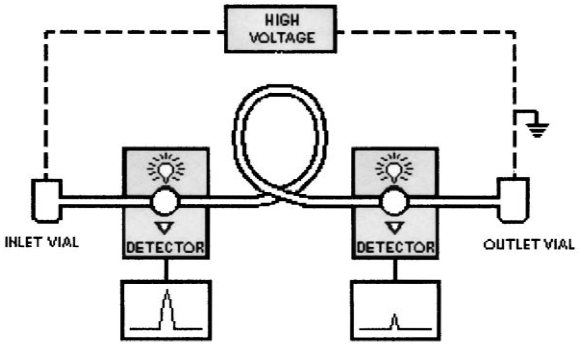

Fig. 1. Scheme of the method of Towns and Regnier [3] for monitoring protein adsorption to the capillary wall. A CZE instrument equipped with two detectors, placed 50 cm apart, is built: the decrement of peak area as measured at the second detector gives a quantitation of the amount of protein adsorbed along the migration path.

[38] have clearly demonstrated that this method is most effective in desorbing any proteinaceous material bound to silica, and it is even more efficient than drastic washings, such as those in 1 *M* NaOH or 1 *M* HCl. For the experiments given here, a final pH of 5.0 was chosen for the background electrolyte, since at this pH myoglobin is well below the p*I* (=7.6), so it has a substantial positive charge, whereas the silica is strongly negatively charged, since this pH value is near to the average p*K* of silanols, given by Bello et al. as 6.3 [42]. The experiments proceed as follows: a 4 μ*M* solution of FITC-derivatized myoglobin is made up in 40 m*M* Tris–acetate buffer, pH 5.0. This solution is fluxed into the naked silica capillary for 100 s (total volume circulated ca. 0.5 ml). Under these conditions, preliminary runs had established that the silica wall was fully saturated with protein. The capillary thus conditioned is then washed for 6 min with the same Tris–acetate buffer, pH 5.0, in order to eliminate all unbound protein and/or additional layers of protein physically bound to the first adsorbed layer. Protein desorption is affected by placing in the cathodic vessel a 25 m*M* phosphate buffer, pH 7.0, containing 60 m*M* SDS (the same buffer, but devoid of SDS, is used as anolyte). The electrophoretic run is performed at 25°C at a voltage drop of 180 V/cm (typical current of 25 μA) in a 24.6 cm (20 cm to the detection window)×100 μm I.D. capillary. Quenching of protein adsorption was obtained via addition of a series of neutral polymers, in concentrations given under the relevant figures. In this last case, the capillary was first equilibrated with the same pH 5.0 buffer solution, containing the desired amounts of polymer, so as to induce any dynamic adsorption of such polymers to the capillary wall prior to the exposure to the protein solution. Finally, when the protein solution was flushed into the capillary (100 s) also this last solution contained the same amount of polymer used to pre-condition the silica tubing.

2.4. Peak quantitation

The instrumentation used for the experiments was a Bio-Rad (Hercules, CA, USA) Bio Focus 3000, equipped with two laser detectors, one an argon-ion beam (488 nm excitation), the other a helium–neon beam (594 nm excitation wavelength). In order to obtain a precise quantitation of the eluted FITC-derivatized myoglobin peaks, the sample was added with an internal standard of sulphorodamine, supplied as a tester kit by Bio-Rad. Thus, within each run, the FITC-derivatized myoglobin peak was detected at 520 nm, whereas the internal standard peak was revealed at 630 nm. The internal standard was used for correcting for any variation in peak area resulting from fluctuations in the injected volume.

2.5. Polymer preparation

A PVA stock solution was freshly prepared by dissolving 0.1% (w/v) PVA in boiling water. HPMC and HEC stock solutions were prepared by slowly adding the powders to water at room temperature and stirring overnight. All solutions were degassed by sonication prior to use. Short-chain poly(DMA) was prepared by the chain-transfer method of Gelfi et al. [43]. The polymer was precipitated in ethanol and extensively washed, so as to remove traces of catalysts and ungrafted monomers.

3. Results

Figs. 1 and 2 depict the principles of quantitation of protein adsorption as devised by Towns and Regnier [3] and by Verzola et al. [38]. The second method is clearly more prone to implementation in

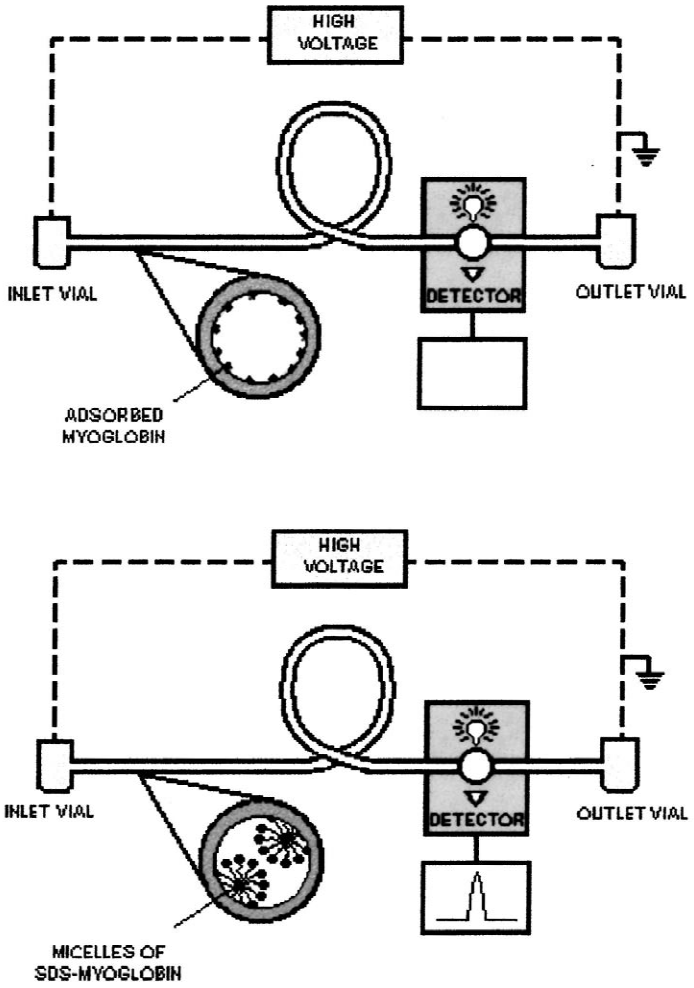

Fig. 2. Scheme of the protein method for assessing protein adsorption. Upper drawing: the capillary is conditioned by flushing with the desired protein, until full saturation of any potential binding site. Lower drawing: a 60 m*M* SDS solution is used as catholyte and protein desorption affected by electrophoretically driving SDS micelles into the lumen of the capillary. Any desorbed protein appears as a peak at the detector.

any laboratory, since it does not require modifications of commercial CZE instruments. Ample evidence is given in Ref. [38] on the efficacy of this last procedure, which is based on the principle of frontal elution: SDS micelles, added only to the cathodic reservoir, migrate electrophoretically into the silica tube, and completely desorb any bound proteinaceous material by incorporating it into the surfactant micelle. Since the desorbed protein travels with the SDS micelles front, a sharp elution peak is monitored at the detector window, permitting quantitation of the total amount of protein previously adsorbed along the migration path. Precise peak quantitation is made possible by dual monitoring with a double laser beam of both, the eluted protein and internal standard admixed with the injected sample.

Fig. 3 gives a measure of the potency of HEC, used to condition the capillary and added to the background electrolyte upon sample injection, in preventing protein adsorption to the silica wall. Contrary to data obtained in the case of amines, where an exponential decay is observed at increasing amine concentrations, here only 50% inhibition of binding is obtained at the first level of HEC used

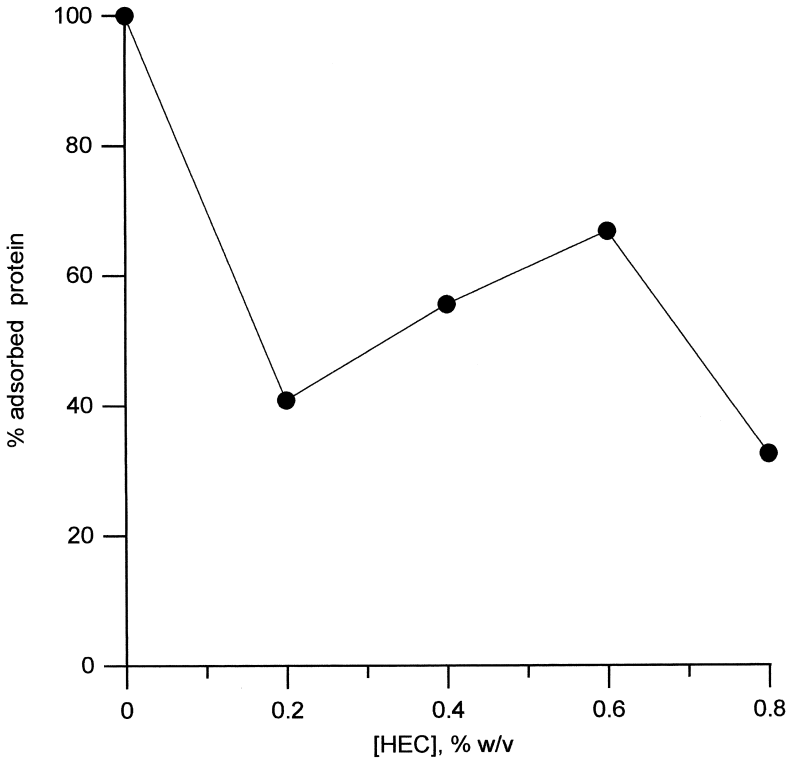

Fig. 3. Efficacy of HEC (in the 0.1 to 0.8% concentration range) in preventing protein adsorption to the capillary wall. Electrophoretic desorption is implemented by placing in the cathodic vessel a 25 m*M* phosphate buffer, pH 7.0, containing 60 m*M* SDS (the same buffer, but devoid of SDS, being used as anolyte). The electrophoretic run is performed at 25°C at a voltage drop of 180 V/cm (typical current of 25 μA) in a 24.6 cm (20 cm to the detection window)×100 μm I.D. capillary.

(0.2%). Nothing is gained when augmenting the amounts of HEC, up to 0.8%. In fact, there seems to be a tendency, at intermediate HEC levels, to desorb less protein. The same plateauing phenomenon seems to occur also with HPMC, here used at much lower concentrations, in the 0.005% to 0.02% (this additive should be used at such high dilutions, due to the very high viscosity of long-chain polymers): there is nothing to be gained in protein desorption when doubling the level of HPMC from 0.01% to 0.02% (Fig. 4). Very similar profiles are also obtained with PVA, used in the same concentration range: here too ca. 60% inhibition of protein adsorption is obtained, but it seems to be impossible to prevent further such adsorption phenomenon at progressively increasing PVA concentrations (Fig. 5). The only polymer that deviates from this general trend is poly(DMA), an additive claimed to cling tightly to the silica wall [39]: although this polymer exhibits the typical plateauing phenomenon of all other polymers, by which inhibition of adsorption becomes quickly independent from concentration (Fig. 6), its potency seems to be quite superior, since it can inhibit protein adsorption by as much as 85%. Fig. 7 summarizes the data with the four polymers here studied and shows the very similar behaviour for all the set, at least in terms of plateauing, independently from the length and hydrophilicity/hydrophobicity of the different molecules.

4. Discussion

The data here presented are quite unexpected, given the popularity of dynamically adsorbed polymers in CZE analysis. The ability of such polymers in preventing protein adsorption to the capillary wall is vastly inferior to that of amines, even the simplest

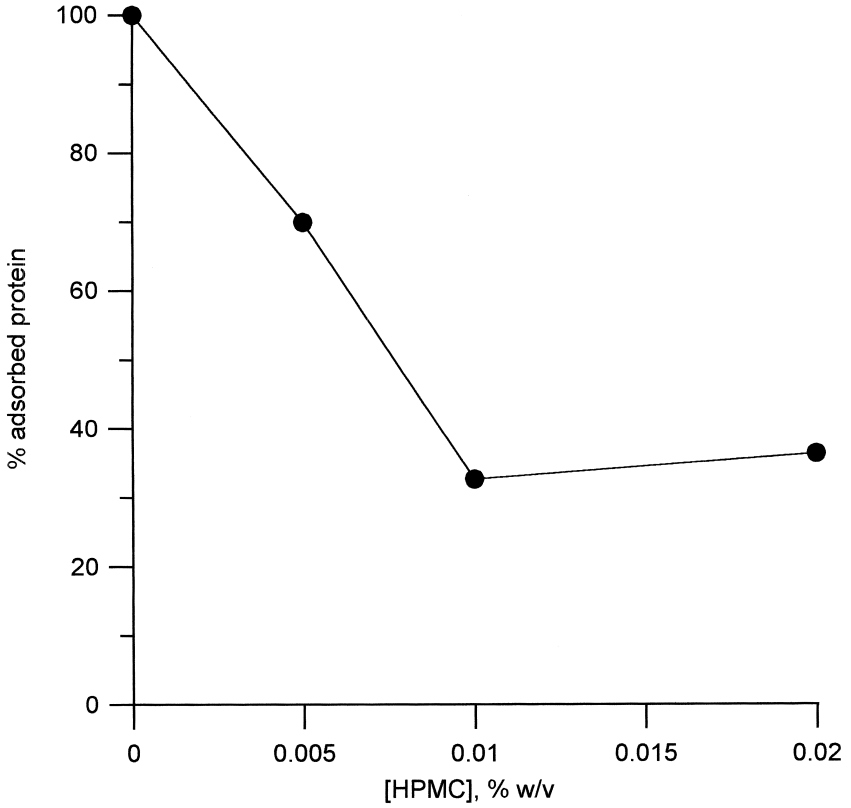

Fig. 4. Efficacy of HPMC (in the 0.005 to 0.02% concentration range) in preventing protein adsorption to the capillary wall. All other conditions as in Fig. 3.

monoamines. In this last case, all compounds are able to inhibit protein adsorption by at least 90%, albeit with widely differing efficacy. Thus, while for a monoamine, such as triethanolamine, 90% inhibition is obtained at a level of 560 m*M* (an exaggerated concentration for a proper CZE separation), for oligoamines, such as spermine and TEPA, the same effect is obtained at concentrations of only 1 m*M*. At somewhat higher concentrations, even 95% inhibition can be secured. Curiously, not only the efficacy of adsorbed polymers is modest [they can inhibit, at best, adsorption of proteins up to 50%, with the only exception of poly(DMA)], but their behaviour is peculiar too, in that this phenomenon reaches a plateau and is, at this point, concentration independent.

On the contrary, the inhibition behaviour of amines follows an exponential decay approaching asymptotically the value of 100% desorption at progressively higher concentrations. Clearly, the mechanism of attachment of such additives to the silica must be quite different. In the case of amines, it is believed that they are bound to the wall via ion pair formation with anionic silanol groups. Not much is gained in efficacy in going from a mono- to a diamine, but as soon as oligoamines are used (e.g., spermine, with four, and TEPA, with five amino groups) the phenomenon becomes cooperative and strong binding, with strong inhibitory effects in the sub-millimolar range, is quickly reached. In addition, the strength of such amines seems to be dependent on three other parameters: the total number of $CH_2(CH_3)$ groups, the CH_2/NH ratio and the molecular mass (on all these accounts spermine is thus slightly more efficient than TEPA). This would suggest that, perhaps, more than one mechanism of attachment is operative: the initial ionic interaction must be followed by a hydrophobic binding to the

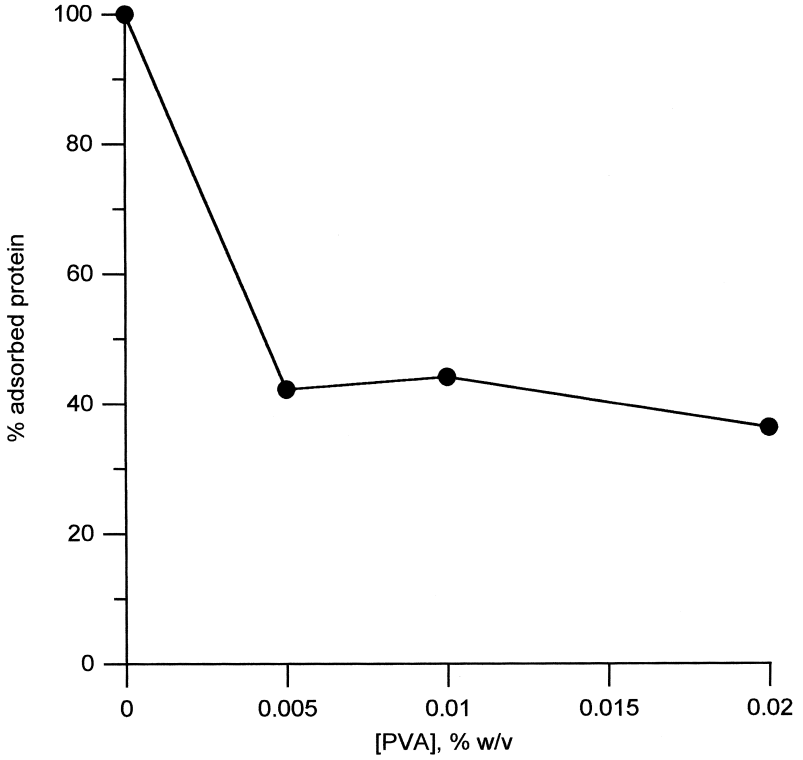

Fig. 5. Efficacy of PVA (in the 0.005 to 0.02% concentration range) in preventing protein adsorption to the capillary wall. All other conditions as in Fig. 3.

silica wall, brought about by charge neutralization. On the contrary, neutral polymers can only be adsorbed to the silica either by hydrogen bonding or by some hydrophobic interaction or both. Two phenomena then must be responsible for the poor shielding of the siliceous surface by polymers: one is the extent of patching of the surface, the other is lack of neutralization of ionized silanols. According to our data, it would appear that all polymers, independently from their molecular mass and relative hydrophilicity/hydrophobicity, can only cover the silica surface to no more than 50% of the available area, leaving thus a large ''naked'' portion to which proteins can adhere. Although we have no direct evidence for that, there exists plenty of indirect corroboration in the literature, as shown by the data collected in Table 1. This table shows how efficient are different types of polymers, once adsorbed onto the silica surface, in diminishing the value of EOF. Considering that the EOF of a control, uncoated surface, is of the order of $7.2 \cdot 10^{8}$ ($m^2\ V^{-1}\ s^{-1}$), these data clearly show that the quenching of EOF is typically by a factor of 3 to 5 and rarely, if at all, reaches one-order of magnitude. On the contrary, a permanently-coated capillary, if properly reacted, gives a value of suppression of EOF which reaches three- to four-orders of magnitude, so vanishingly small that we had to devise a new method for assessing it [44]. Such properly coated capillaries are known not to adsorb proteins [9]. It is thus clear that, up to the present, scientists have taken the wrong parameter for assessing the merits of adsorbed polymers in quenching protein interaction with silica: decrements of EOF do not guarantee their efficacy in shielding proteins to the wall. Or, better, we should have been aware that such modest decrements of EOF, as brought about by adsorbed polymers, should have rung an alarm bell in the case of extrapolating

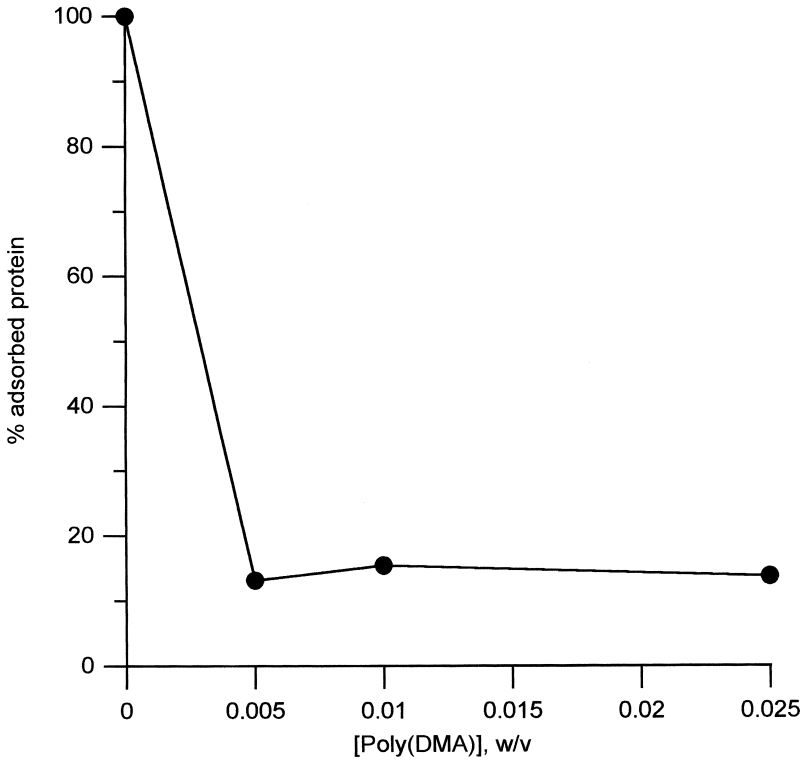

Fig. 6. Efficacy of poly(DMA) (in the 0.005 to 0.025% concentration range) in preventing protein adsorption to the capillary wall. All other conditions as in Fig. 3.

these data to protein protection from the silica wall. The other problem with adsorbed, neutral polymers is that they will not neutralized the negative charges of ionized silanol, but merely shield them. Proteins adsorbed onto nearby, naked surfaces, could still feel the attraction of such negative charges. It is of interest to note that the only polymer that deviates from this general behaviour is poly(DMA), which is able to inhibit protein adsorption by as much as 85%. We can only speculate that its hydrophobic nature offers a stronger anchorage to the silica surface, thus preventing its peeling off upon washing or when the background electrolyte is devoid of it. However, its increased hydrophobicity (the partition coefficient in water–*n*-octanol of DMA is 0.5, as opposed to 0.2 for acrylamide and 0.01 for such a hydrophilic monomer as trisacryl) [48], could render problematic its use in the general case of protein separations, where mixtures of proteins ranging from fully hydrophilic to rather hydrophobic have to be analyzed. In 1989–1990 we had used a pure poly(DMA) matrix for isoelectric focusing of proteins and, much to our dismay, we noticed that all the sample was adsorbed at the deposition site and no protein could be focused (Gelfi and Righetti, unpublished results). Thus, poly(DMA) might only be suitable for analysis of DNAs, when only partial suppression of EOF is needed, rather than for prevention of adsorption to the siliceous surface; in fact, up to the present, poly(DMA) has been advocated only for this use [39].

At this point, the *mea culpa* should come from our laboratory, since we have promulgated for years the notion of using HEC as a quencher of peptide/protein interaction with the silica wall [45–47]. As it turned out, although we were not aware of the poor performance of such polymers, we were quite right, in that we recommended this procedure only when performing peptide/protein separations in acidic,

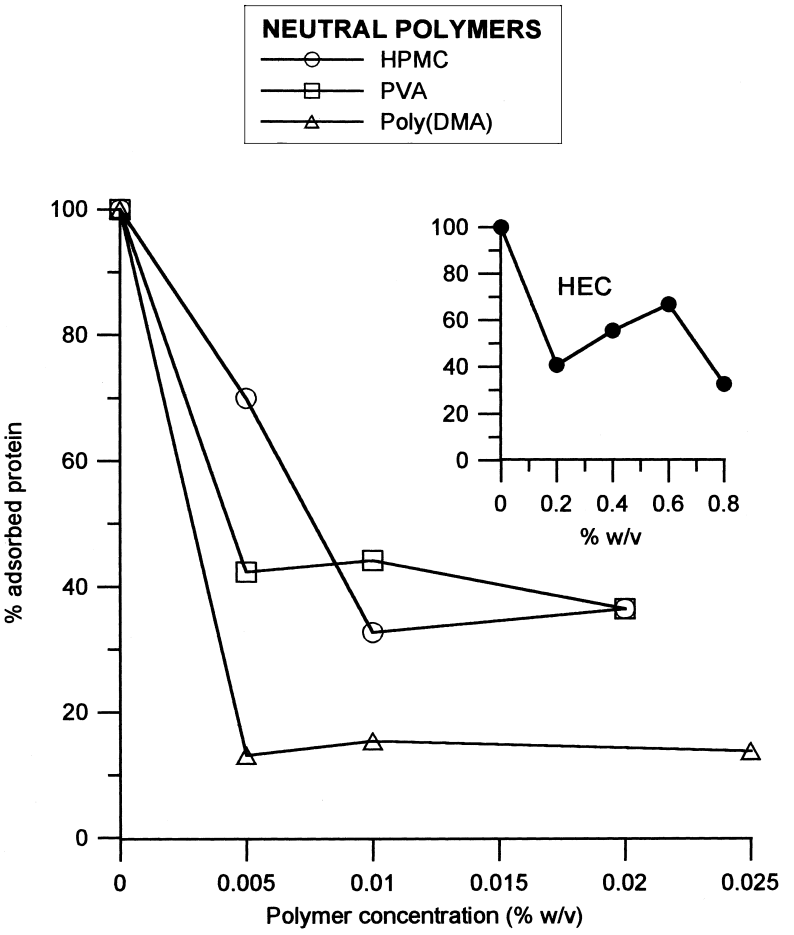

Fig. 7. Comparison of the behaviour of the four polymers [HPMC, HEC, PVA and poly(DMA)] in quenching adsorption of proteins to the silica wall.

Table 1
Effect of dynamically adsorbed polymers on EOF at the silica wall[a]

Adsorbed polymer	EOF ($m^2\ V^{-1}\ s^{-1}$) $\cdot 10^8$
Tween-20	2.030
Brij-35	1.650
Methyl cellulose 15	0.722
Poly(vinyl pyrrolidone)	0.672
Poly(vinyl alcohol) 2000	0.645
Methylcellulose 25	0.586
Methylcellulose 400	0.574
Methylcellulose 1500	0.428
Poly(vinyl alcohol) 124 000	0.363
Methylcellulose 4000	0.320

[a] The data refer to a 50 μm I.D. capillary, with EOF measured at pH 8.0. The typical EOF of a control is $7.2 \cdot 10^8$ ($m^2\ V^{-1}\ s^{-1}$).

isoelectric buffers, at prevailing pH values below pH 3.0. At such acidic pH values, HEC (and other polymers) are quite effective in quenching protein/wall interactions, due to the very sparse silanol ionization. But, as the operational pH value becomes greater than 3, the risk of protein adsorption becomes quite real (Olivieri et al., manuscript in preparation).

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