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Quantitative studies on the adsorption of proteins to the bare silica wall in capillary electrophoresis III: Effects of adsorbed surfactants on quenching the interaction

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Abstract

The efficacy of two classes of surfactants, non-ionic and zwitterionic, in quenching the interaction of proteins with the naked silica wall in capillary electrophoresis, is evaluated. The class of non-ionic detergents is found to be rather inefficient in preventing protein binding to the fused-silica surface, since large amounts (up to 10%) are required for reducing such interactions by 90%. Conversely, zwittergents appear to be much more efficient, since, in the case of sulphobetain SB-16, 90% binding inhibition is achieved at a concentration of surfactant of only 0.3%. In this last case, it is found that the binding inhibition closely follows the values of critical micellar concentrations (CMCs) of the various surfactants, those having the lowest CMC value exhibiting the highest inhibition power. The CMC values also follow a hydrophobicity scale, suggesting that the most hydrophobic zwittergents are the ones that shield more efficiently the silica surface.

Keywords: Buffer composition; Capillary columns; Adsorption; Proteins; Surfactants

1. Introduction

Separations of peptides and proteins by capillary zone electrophoresis (CZE) can be achieved with a very high plate number and excellent resolution in the absence of macromolecule–wall interaction. If such molecules collide statistically with the capillary wall, they may weakly or strongly interact with the surface and thus be retarded in their migration through the capillary under band broadening and symmetry distortion, depending on the type of interaction and the velocity of diffusion back from the wall into the bulk of the separation medium. This retentive type of band broadening by analyte interaction is, according to chromatographic theory, a consequence of non-equilibrium zone broadening and resistance to mass transfer in the stationary phase, which strongly decreases the separation efficiency, especially in the case of large molecules such as proteins and peptides. In the absence of binding, large analytes can be separated by CZE with very high efficiency, because of their rather slow axial diffusion. These phenomena have been treated theoretically by, e.g. Ermakov and co-workers [1,2] and Schure and Lenhoff [3] who have concluded that the presence of adsorption is particularly devastating

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for large macromolecules, even when the amount adsorbed is minute.

Unfortunately, adsorption of proteins and peptides is an ever present hazard, due to progressively higher silanol ionization at any pH value above 2.5. The initially ionically-bound protein can be denatured onto the silica surface and favour additional deposition of other macromolecules. Thus, the inner silica surface could rapidly become saturated by proteinaceous material, with strong alterations of the electroendoosmotic flow (EOF) and dramatic loss of resolution. In order to minimize or abolish this phenomenon, a large number of coating procedures have been proposed, as reviewed, e.g. by Chiari et al. [4], Schomburg [5], Regnier and Lin [6] and Rodriguez and Li [39]. Among the surface modification protocols, particularly attractive appear the so-called 'dynamic coating' procedures, i.e. compounds which can interact in a transient fashion with the wall, by an adsorption mechanism, since they do not require complex and costly chemical treatments of the inner silica surface. In the previous papers of this series, we have been able to assess quantitatively the efficacy of different dynamic coatings, and to provide figures of merit for each of these compounds, by exploiting a frontal elution method able to fully desorb bound proteins and quantify the amount of released material. In the first paper [7], the efficacy of different amino compounds, ranging from simply monoamines up to oligoamines (notably tetra- and pentaamines) has been evaluated. It was found that oligoamines can provide 90% binding inhibition, at pH 5.0, in the submillimolar range, thus confirming the strong affinity of such compounds for ionized silanols. However, these species become useless in alkaline milieus, due to progressive deprotonation of the oligoamino backbone and this led to the discovery of a remarkable new molecule, a quaternarized piperazine, highly performing in the entire pH scale [8,9]. In a second article [10], the efficacy of adsorbed neutral polymers was assessed. Contrary to amino quenchers, which act by exponentially suppressing protein binding at progressively higher concentrations, asymptotically tending to 100% inhibition, adsorbed polymers inhibit protein adsorption by at most 60% and then quickly reach saturation levels. After a critical concentration, the inhibitory power reaches a plateau and becomes independent of the polymer concentration in the background electrolyte (BGE). In the present report, we investigate the binding inhibition of a third class of potential quenchers: neutral and zwitterionic surfactants.

2. Materials and methods

2.1. Chemicals

Palmityl sulfobetaine (SB-16), caprylyl sulfobetaine (SB-10), lauryl sulfobetaine (SB-12), Nonidet P-40, Triton X-100 and horse skeletal muscle myoglobin were from Sigma (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC), dimethylformamide (DMF), sodium dodecylsulphate (SDS), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and hydroxylamine were from Fluka (Buchs, Switzerland). Brij 35 and Tween 20 were from Carlo Erba (Milan, Italy). Tables 1 and 2 give the chemical names, formulas, M_r and critical micellar concentrations (CMCs) of the various surfactants used. Fused-silica capillaries (100 μm I.D.× 375 μm O.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA).

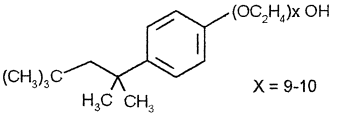
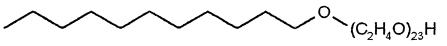
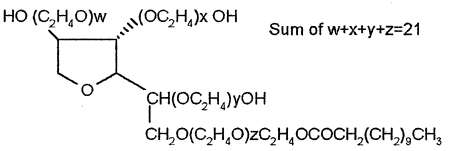
2.2. Protein labelling

Labelling of myoglobin with FITC has been performed according to the protocol in [11]. 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 each 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

Table 1
Non-ionic surfactants

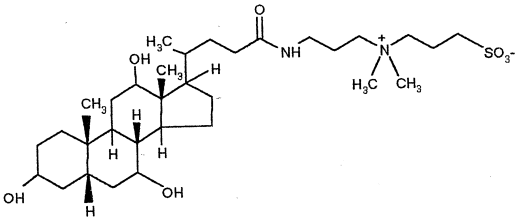
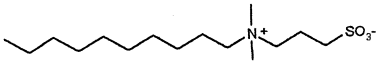
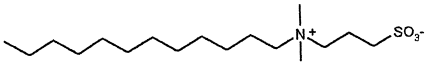
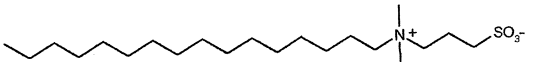
Detergent	Formula	CMC (m*M*)	M_r
Triton X-100 (polyethylene glycol *tert.*-octylphenyl ether)		0.02–0.09	625
Brij 35 (polyoxyethylene lauryl ether)		0.05–0.1	1225
Tween 20 (polyoxyethylene sorbitanmonolaurate)		0.06	1228

to the capillary wall at any pH value of the BGE, we exploited the method of Barberi et al. [12], consisting on desorption of bound material via sweeping the silica surface with SDS micelles, driven electrophoretically into the lumen from the cathodic reservoir. For the experiments given here, a final pH of 5.0 was chosen for the BGE, since at this pH myoglobin is well below the isoelectric point (p*I* = 7.6), so it has a substantial positive charge, whereas the silica is strongly negatively charged, since this pH value is in the neighborhood of the average p*K* of silanols, given by Bello et al. as 6.3 [13] and by Schwer and Kenndler as 5.3 [14]. The experiments proceed as follows: a 4 μ*M* solution of FITC–myoglobin is made up in 30 m*M* Tris–acetate buffer, pH 5.0. This solution is fluxed into the a naked silica capillary for 100 s (total volume circulated ~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 effected by placing in the cathodic vessel 100 μl of a 25 m*M* phosphate buffer, pH 7.0, containing 60 m*M* SDS (the same buffer, but devoid of SDS, is used as analyte). 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 surfactants, 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 detergent, so as to induce binding (if any) of these micelles to the capillary wall prior to the exposure to the protein solution. Finally, when the protein solution was flushed into the capillary (100 s) this last solution also contained the same amount of surfactant used to precondition 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–myoglobin peaks, the sample was added with an

Table 2
Zwitterionic surfactants

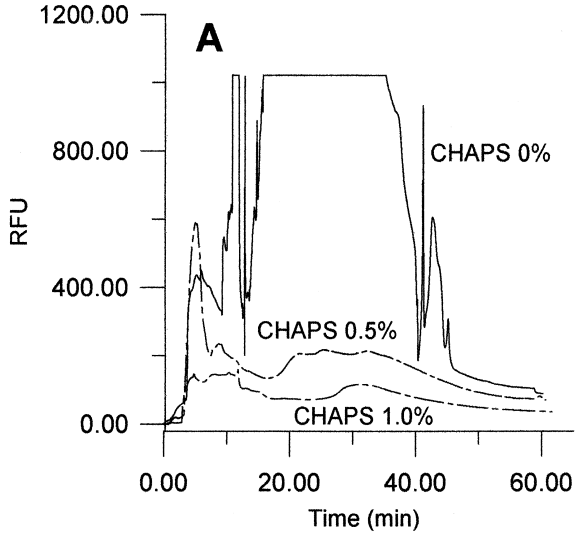
Detergent	Formula	CMC (m*M*)	M_r
CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate)		6–10	614.9
Caprylyl sulfobetaine SB-10 (N-Decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate)		25–40	307.6
Lauryl sulfobetaine SB-12 (N-Dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate)		2–4	335.6
Palmityl sulfobetaine SB-16 (N-Hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate)		0.01–0.06	391.6

internal standard of sulphorhodamine, supplied as a tester kit by Bio-Rad. Thus, within each run, the FITC–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.

3. Results

Fig. 1B gives an example of a typical run of a mixture of sulphorhodamine and FITC–myoglobin, as utilized for measuring adsorption of the injected protein to the silica surface and its prevention via a variety of additives. Fig. 1A displays a series of successive runs, in the absence and presence of a surfactant (in this example the zwitterionic species CHAPS), so as to obtain a visual impact of the process. The three tracings are plotted on the same fluorescence scale: in the control run (upper tracing), it can be seen that, upon frontal elution with SDS micelles, a huge peak of adsorbed material is released from the wall. However, when the capillary is preconditioned with 0.5% CHAPS, and the FITC–myoglobin flushed in the capillary in presence of the same amount of CHAPS, subsequent desorption with 0.5% CHAPS releases much less material from the wall. An additional run in presence of 1.0% CHAPS (lower tracing) shows further decrements of protein adsorption.

On the basis of a series of experiments performed as shown in Fig. 1A, we have derived plots giving the efficacy in inhibiting protein adsorption by any additive present in the BGE. Fig. 2 summarizes the



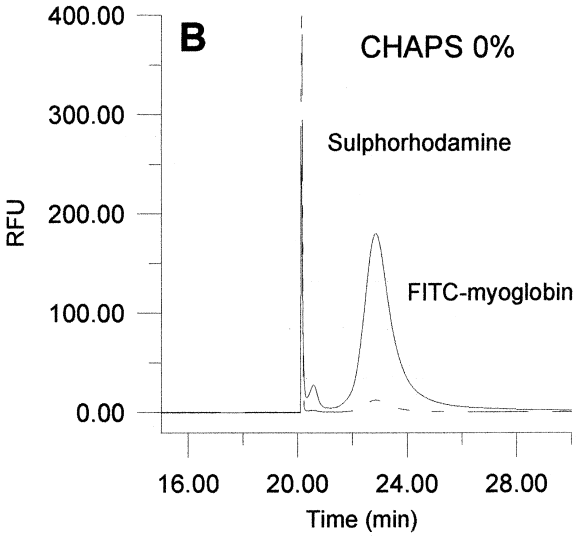


Fig. 1. Demonstration of the procedure for assessment of protein binding. (B) Typical run of a mixture of FITC–myoglobin and sulphorhodamine. In this run, the internal standard, added for precise quantitation purposes, can be seen as a sharp peak eluting at ~20 min. (A) Typical example of an electrophoretic desorption profile, as elicited by an SDS micelle electrophoretic elution step, after adsorbing FITC–myoglobin at pH 5.0. Upper tracing: elution of FITC–myoglobin in the absence of any additive in the BGE; middle profile: amount of protein adsorbed when preconditioning the capillary with 0.5% CHAPS; lower tracing: in presence of 1.0% CHAPS.

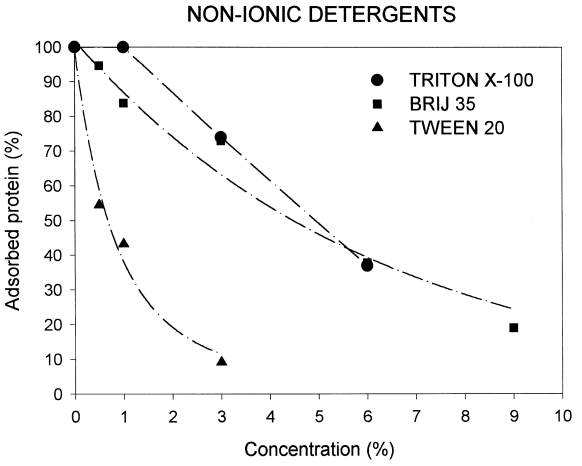


Fig. 2. Quantitation of the efficacy of three non-ionic surfactants (Brij 35, squares; Triton X-100, circles and Tween 20, triangles) in inhibiting FITC–myoglobin binding to the silica wall, as a function of their respective concentrations in the BGE (30 m*M* Tris–acetate, pH 5.0).

data obtained with the neutral surfactants listed in Table 1 (Nonidet P-40 is omitted, since its structure and behaviour is essentially identical to that of Triton X-100). Triton X-100 and Brij 35 are rather poor quenchers of protein binding to the wall and that 80% adsorption inhibition can only be obtained at very high levels (10%) of these species in solution. Such levels are unacceptable, since, among other problems, the solution viscosity becomes very high and handling of solutions problematic. Among all the neutral surfactants, only Tween 20 appears to exhibit a reasonable inhibitory power, since it is already quite effective at a 3% concentration. Fig. 3 gives the overall efficacy of each additives expressed with two concentration values, that achieving 50% and that inducing 90% binding inhibition. If one refers only to the adsorbed protein as representing the total amount of applied sample, then the 50% value represents the dissociation constant, since it gives the concentration of additive inducing an equimolar equilibrium between the amount of adsorbed and released protein.

By the same token, we could construct curves of competitive binding for the four zwitterionic surfactants listed in Table 2. Fig. 4 summarizes the behaviour of these four species: it can be appreciated

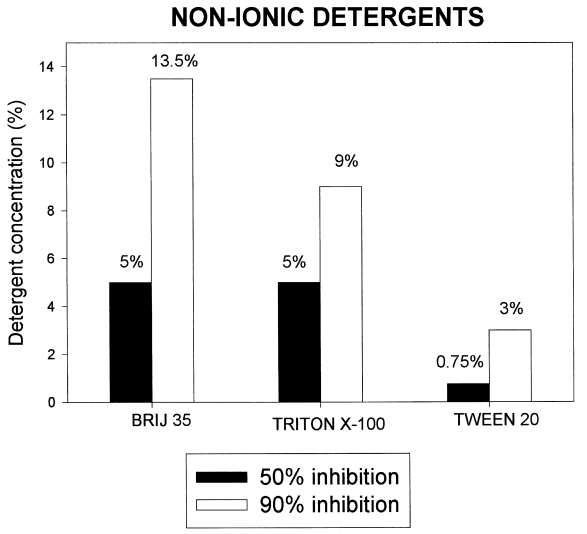


Fig. 3. Figures of merit for the binding inhibition of the 3 surfactants of Fig. 2. The two bars for each compound represent the concentrations eliciting 50% (black filling) and 90% (empty bars) inhibition of binding of FITC–myoglobin to the naked silica, when added to a BGE composed of 30 m*M* Tris–acetate, pH 5.0.

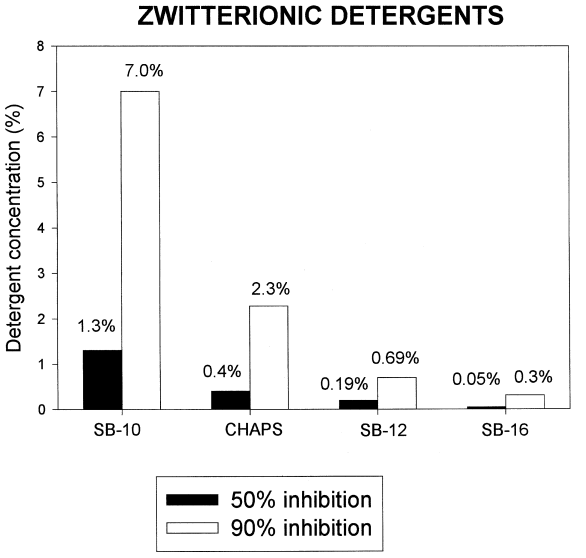


Fig. 5. Figures of merit for the binding inhibition of the four surfactants of Fig. 4. The two bars for each compound represent the concentrations eliciting 50% (black filling) and 90% (empty bars) inhibition of binding of FITC–myoglobin to the naked silica, when added to a BGE composed of 30 m*M* Tris–acetate, pH 5.0.

that their efficacy (except for SB-10, which resembles Tween 20) is much higher than that of the neutral surfactants. Fig. 5 gives figures of merit for the binding inhibition, again expressed as 50 and 90% inhibitory power.

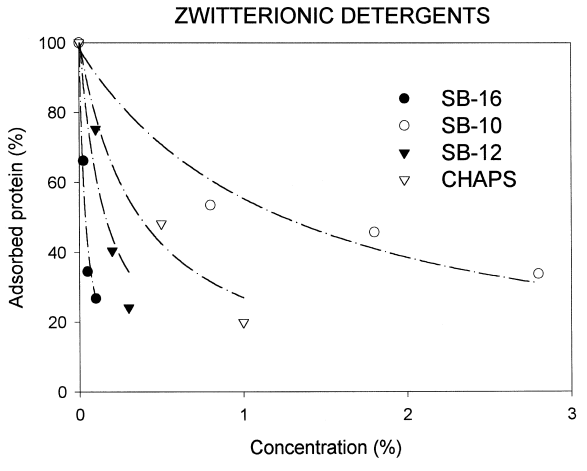


Fig. 4. Quantitation of the efficacy of four zwittergents (SB-10, open circles; CHAPS, open triangles, SB-12, solid triangles and SB-16, solid circles) in inhibiting FITC–myoglobin binding to the silica wall, as a function of their respective concentrations in the BGE (30 m*M* Tris–acetate, pH 5.0).

4. Discussion

4.1. On the use of surfactants in CZE

Although a vast literature exists on the effect of a variety of additives in quenching protein binding to the silica wall, not much has been reported on the direct use of the two categories of surfactants reported in this paper. The strategy adopted by, e.g. Town and Regnier [15] and Ng et al. [16], has been a coating mediated by hydrophobic interaction. The idea was to covalently sequester many of the surface silanols with octadecyl- or dimethylsilane and then dynamically coat the hydrophobic surface of the column via nonionic surfactants. It had previously been shown, in RP-HPLC, that such species stereospecifically adsorb to hydrophobic surfaces and create a hydrophilic surface layer [17]. Alkyl groups of the surfactant are directed into the alkylsilane layer, while the hydrophobic portion of the surfactant

projects into the aqueous phase, thus shielding the surface from the approach of proteins. In these studies, adopting the strategy of hydrophobically-mediated binding of surfactant to the treated silica wall, mostly the Tween (20, 40, 80) and Brij (35, 78) series where studied, although other reports have dealt with pluronic surfactants [16,18]. Only in a few cases has direct adsorption of neutral compounds (notably Triton X-100) to uncoated capillaries been studied [19,20], but more in terms of its effect on the electroendoosmotic flow rather than on its potential capability of impeding analyte adsorption. A number of reports have dealt with adsorption of both, anionic (e.g. sodium deoxycholate and SDS) [19,20] and cationic [21] surfactants, but these compounds have not been studied in the present report, also due to their strong denaturing activity on proteins. In addition, several communications have appeared dealing with the use of both neutral and zwitterionic surfactants, but here too the accent was on micellar electrokinetic chromatography, i.e. on the ability of these compounds to modulate the separation of peptides (and in general of hydrophobic compounds) via adsorption/interaction of the analyte with the detergent micelle. Thus Greve et al. [22], Issaq et al. [23], van de Goor et al. [24] and Yeung and Lucy [40] have used zwitterionic surfactants for improving the separation of different peptide mixtures; Swedberg [25] adopted non-ionic and zwitterionic detergents with heptapeptides; Matsubara et al. [26] and Matsubara and Terabe [27] utilized Tween 20 and Polysorbate 20 for optimizing the separation of closely related angiotensin II analogues.

4.2. On the efficacy of the two classes of detergents

In the past, there has been a hot debate on the solubilizing power of neutral vs. zwitterionic detergents. Whereas on the one hand Gonenne and Ernst [28] and Hjelmeland and co-workers [29,30] reported a superior solubilizing properties of zwittergents, on the other hand opposite conclusions were reached by Burghes et al. [31] and Booz and Travis [32], who favoured non-ionic surfactants. This prompted a thorough investigation by Satta et al. [33] and Navarrete and Serrano [34] who proved conclusively, by running two-dimensional maps of plasma membranes, that zwittergents had indeed the best solubilizing power. Among them, the sulfobetaines (SBs) with 12- and 14-carbon atoms tails (SB-12 and SB-14) were found to be by far the best compounds, except for the fact that they were precipitated out of solutions in >4 *M* urea solutions. As a spin-off of these investigations, Gianazza et al. [35] and Rabilloud et al. [36] proposed a new family of detergents with much improved solubilizing properties: amido sulfobetaines. These compounds differ from the conventional sulfobetaines by the presence of an amido group bridging the hydrophobic linear tail and the polar head, which substantially increases their water solubility and urea tolerance. This novel class of surfactants, and a family of derivatives, have now become standard additives in two-dimensional maps of highly hydrophobic proteins [37,38].

There is a striking parallelism between our data and those discussed above on the properties of these two families of surfactants. If we equate solubilizing power, as highlighted above, with the ability of shielding proteins from the silica wall, as demonstrated in the present report, then our data fully agree with those in the literature, since they amply support the much higher efficacy of zwitterionic surfactants over neutral detergents. In contrast with the huge amounts of Triton X-100 needed for providing 90% shielding from the silica surface ($>10\%$), at the opposite extreme the same results are obtained with only 0.3% of SB-16, a sulfobetaine with a 16-carbon atoms tail. It is quite possible that this shielding effect occurs via the concomitant action of adsorption of surfactants to the silica wall, on the one hand, with the binding to hydrophobic sites on the protein surface, on the other hand. It is known that, at low concentrations, detergents are adsorbed onto the surface of proteins without denaturation, increasing the Stoke radius and, in case of ionic surfactants, also the charge. Fewer than ten surfactant molecules are probably bound [6]. It is also of interest to note (see Fig. 4) that, in the case of zwittergents, their ability of shielding proteins from the silica surface closely follows the value of their CMC, which in turn should depend on the hydrophobicity of the molecule. The smaller is the CMC value, the higher the efficacy of the surfactant. In a way, this mechanism resembles that of mono- to oligoamines, where

the shielding efficacy, per equal number of charged nitrogens, closely follows a hydrophobicity scale [7].

5. Conclusions

At the end of this series of articles, we would like to underline an important conclusion, often overlooked by all those who proclaimed (in a vast body of reports) that their novel shielding procedure would be the best of all those previously reported: no matter how effective the additive present in the BGE and adsorbed onto the silica surface is, we have to live, in protein analysis, with some adsorption. It is seen, in all of our three reports, that achieving more than 90% inhibition of protein binding is a mere dream, and even such a high level of quenching comes at great effort. Thus, it is to be expected that, after a series of runs, as more and more protein accumulates on the silica wall, carpeting its full length up to the detector, the system will break down and results will suddenly become highly erratic. Thus, we suggest that, in any protein run, no matter how good is the additive adopted, frequent, rather strong washing procedures be adopted, so as to free the surface from accumulated proteinaceous material. Which procedure to adopt, then, is a matter of personal choice: we much prefer, as reported in our papers, a desorption step with SDS micelles, since this seems to be even more thorough than drastic washings with strong NaOH or HCl solutions. The extra benefit of an SDS-washing step is that it occurs at neutral pH values, thus avoiding the strong hysteresis loops generated by washing at pH values far removed from operative pH conditions. As a note of warning, since the present data have been elicited by using a single, small and rather hydrophilic protein, they cannot be extrapolated to the realm of all possible expressed phenotypes, since there will be large differences in size and hydrophobicity. Nevertheless, the present report offers a new, simple and most efficient tool for a rapid evaluation of protein adsorption when using any possible protein concoction.

Another comment is appropriate here. Although it might sound strange, it appears that the efficacy of all the additives so far investigated is closely related to their hydrophobicity: this was quite clear in the case of amines, where the CH_2/N ratio seems to be the dominant theme [7]; it became then apparent in the case of neutral polymers, where the most effective one was found to be poly(N,N-dimethylacrylamide) (a quite hydrophobic polymer) [10] and it is now true also in this investigation, in which we have clearly demonstrated that the shielding efficacy of surfactants closely follows a hydrophobicity scale.

Acknowledgements

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