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Quantitation of protein binding to the capillary wall in acidic, isoelectric buffers and means for minimizing the phenomenon

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Abstract

Notwithstanding the use of acidic, amphoteric, isoelectric buffers with isoelectric points (p*I*) in the pH 2–3 range, adsorption of proteins to the naked silica wall can be non-negligible. Two such buffers have been tested: iminodiacetic acid (IDA; p*I* 2.23, apparent pH 3.2 in 7 *M* urea) and aspartic acid (p*I* 2.77, apparent pH 3.7 in 7 *M* urea). Three potential quenchers of such interactions have been tested: hydroxyethylcellulose (HEC; number average molecular mass, M_r 27 000), TEPA (tetraethylenepentamine) and a novel, quaternarized piperazine [*N*(methyl-*N*-ω-iodobutyl)-*N′*-methylpiperazine] (Q-Pip), either alone or in binary and ternary mixtures. Human α- and β-globin chains have been used as test proteins in capillary electrophoresis separations. It has been found that mixtures of these compounds are the worst possible remedy. E.g., a ternary mixture comprising 0.5% HEC, 0.5 m*M* TEPA and 1 m*M* Q-Pip still leaves behind 4.5% adsorbed protein onto the silica surface in runs in IDA buffer and 7 *M* urea (pH 3.2). Conversely, 0.5 m*M* TEPA or 1 m*M* Q-Pip, when used alone, minimize adsorption down to only 1.8% and 0.5%, respectively. When the same globin chain separations are performed in Asp and 7 *M* urea (pH 3.7), the situation is much worse: 44% protein is adsorbed in a ternary mixture of 0.5% HEC, 1 m*M* Q-Pip and 0.5 m*M* TEPA. However, when used alone, 0.5 m*M* TEPA and 1 m*M* Q-Pip reduce globin adsorption to levels of 8% and 5%, respectively. TEPA and Q-Pip are found to be in all cases the best quenchers of protein interaction to naked fused-silica; in addition they exhibit the unique property of smoothing the base-line and giving reproducible runs. The best method for desorbing bound protein was found to be an electrophoretic step consisting in driving sodium dodecylsulphate micelles from the cathodic reservoir.

Keywords: Buffer composition; Capillary columns; Adsorption; Proteins; Hydroxyethylcellulose; Tetraethylenepentamine; (Methyliodobutyl)methylpiperazine

1. Introduction

Capillary zone electrophoresis (CZE) of peptides and proteins in isoelectric, acidic buffers is a technique of growing importance in the field of analysis of macromolecules. There are at least two distinct advantages inherent to this methodology: first of all, at the low operative pH (pH 2–3) silanol ionization is abolished, so that naked capillaries can be adopted; secondly, due to the fact that these buffers are quasi stationary, high-voltage gradients can be applied. The first report appeared in 1997, when Nembri and Righetti [1] suggested isoelectric aspartic acid as the sole background electrolyte, operating

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at pH=isoelectric point (p*I*)=2.77 (at 25°C). These authors could produce peptide maps of casein in only 10–12 min (as opposed to >70 min in standard phosphate buffer, pH 2.0) at voltage gradients as high as 800 V/cm, with much increased resolution. Adsorption of some larger peptides to the wall was minimized by adding to the background, isoelectric Asp buffer, 0.5% hydroxyethylcellulose (HEC; number average molecular mass, M_n 27 000), and 5% trifluoroethanol (TFE).

The method has been successfully used for a number of applications: for generating peptide maps of α- and β-globin chains from tryptic digests of human adult hemoglobin [2], for analysis of gliadins in the screening of wheat cultivars [3] and of zeins in maize [4,5] and for human globin chain separation and quantitation [6,7]. The fundamental properties of such buffers have been enunciated [8] and a number of reviews have already covered the field [9,10]. Notwithstanding the increasing importance of such isoelectric buffers, some limitations are apparent. As the charge of the peptides plays a pivotal role in their separation by CZE, other pH values might be necessary in order to avoid co-migration of peptides having cross-over points at a given pH value in their titration (pH/mobility) curves. A case in point is offered by the example of α- and β-globin tryptic digests [2]: the β-T2/T9 coeluted at the pH=p*I*= 2.77 of 50 m*M* Asp and could only be split at an operative pH of 3.0, as elicited by diluting the running buffer to 30 m*M*. It was thus apparent that more than one amphoteric buffer was needed, in order to explore larger pH windows along the titration curves of proteins and peptides. In search for additional background electrolytes, Bossi and Righetti [11] reported the use of iminodiacetic acid (IDA), possessing a remarkably low apparent p*I* (2.23 at 100 m*M* concentration) and of cysteic acid, with an apparent p*I* of 1.85 (at 100 m*M* concentration) and an extraordinary buffering power, given by its remarkably low Δp*K* value [12].

Although we now have a number of buffers covering a pH 2–3 window which seem to be highly suitable for peptide and protein separations, there still remains a major point to be evaluated: whether protein adsorption indeed occurs to some extent in this pH interval. Bello et al. [13] have reported an average p*K* value for silanols of 6.3, with a point of zero electroosmotic flow (EOF) assessed as pH 2.3. However, Schwer and Kenndler [14] have given a value of 5.3, which means that the point of wall neutralization would be below pH 2.0. Given these findings, it is to be expected that some silanol ionization would occur in the pH 2–3 interval, and this could be sufficient to induce binding of some classes of proteins (e.g., those having a high content of basic amino acids, such as human α- and β-globin chains). Although we have always warned researchers about this risk in our papers, we have never been able so far to evaluate the existence of this phenomenon and quantify it. In the present paper, we report a method for the precise assessment of this event and we propose efficient methods for minimizing it.

2. Experimental

2.1. Reagents

Aspartic acid (Asp) and IDA were obtained from Sigma (St. Louis, MO, USA). Urea, sodiumdodecylsulphate (SDS), phosphoric acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany). HEC (M_r, 27 000) was from Polysciences (Warrington, PA, USA). The trisubstituted piperazine [*N*(methyl-*N*-ω-iodobutyl)-*N′*-methylpiperazine] (Q-Pip) was synthesized in the laboratory of Professor A. Citterio at the Polytechnic Institute of Milan. Tetraethylenepentamine (TEPA) was from Riedel-De Haen (Seelze, Hannover, Germany). Fused-silica capillaries (27 cm×50 μm ID.×375 μm O.D.) were from Polymicro Technologies (Phoenix, AZ, USA) and were used as such, without inner coating. Globin chains (Hb Aubenas) were prepared as reported in Righetti et al. [6].

2.2. Capillary electrophoresis

CZE was carried out with a Beckman P/ACE System 2100 instrument. Uncoated capillaries of 27 cm (20 cm to the detector)×50 μm I.D. were used. The wall adsorption of the proteins was measured using three different substances in presence of IDA (pH=p*I*=2.30 at 25°C) or of Asp (pH=p*I*=2.77 at 25°C) and in both electrolytes in 7 *M* urea. In all

cases, the samples were loaded by hydrostatic pressure for 5 s. Separations were performed at different voltage gradients (500 V/cm in IDA, 700 V/cm in Asp and 300 V/cm in SDS buffer). Ultraviolet absorbance was monitored at 214 nm. At rest, overnight, the capillary was filled with water. In order to study the efficiency of the three substances (HEC, Q-Pip and TEPA) for creating a good dynamic coating, a sample of human globin chains (Hb Aubenas) was adopted for testing potential wall adsorption. Six different combinations of these substances were prepared: one ternary mixture (0.5% HEC, 0.5 m*M* TEPA, 1 m*M* Q-Pip), two binary mixtures (0.5 m*M* TEPA with 0.5% HEC and 1 m*M* Q-Pip with 0.5% HEC) and three solutions of each single agent: 1 m*M* Q-Pip, 0.5% HEC and 0.5 m*M* TEPA alone. Each of the above mentioned combinations was analyzed either in 7 *M* urea and 50 m*M* IDA or in 7 *M* urea and 40 m*M* Asp as background electrolytes. We made 10 consecutive runs of each sample (negative electrode at the detection side), separated by 2 min washing with the same buffer between the runs. In order to measure the quantity of protein adsorbed to the wall, we made a run (reverse polarity) with a solution of 25 m*M* sodium phosphate pH 7 and 7 *M* urea at the anode and with the same buffer with 60 m*M* SDS at the cathode. It was previously demonstrated that, as the SDS micelles would be electrophoretic transported from the cathodic side into the capillary lumen, they would displace any bound proteinaceous material, in an electrophoretic desorption process analogous to displacement chromatography [15]. In order to evaluate the amount of protein adsorbed to the wall, the area of the peaks resulting from the ten consecutive protein runs was integrated and summed up to the area of all the previous individual runs; this final value was assumed to represent 100% of all protein material injected in the capillary. This global value was then compared to the area obtained with the desorbing SDS run, thus deriving the percentage of adsorbed protein onto the wall.

3. Results

Figs. 1A-C show three representative runs of α- and β-globin chains in a background electrolyte consisting of 50 m*M* IDA, in presence of 7 *M* urea (apparent pH 3.2), added with different, single quenchers. Each panel represents the overlay of the first and 10^{th} consecutive runs. In Fig. 1A, B and C only single additives, 0.5 m*M* HEC, 0.5% TEPA and 1 m*M* Q-Pip, respectively, are shown. From these data (and from the runs of the various binary and ternary mixtures, here not shown) some general patterns emerge: (a) first of all, it would appear that all mixtures (ternary and binary) of quenchers are definitely worse than the single additives utilized alone; (b) secondly, some additives ensure good run to run reproducibility and repeatability of transit times, whereas in other concoctions the subsequent runs are shifted either to the left (earlier eluting) or to the right (late eluting) of the initial run; (c) thirdly, some additives give errant, whereas others ensure smooth baselines. In particular it would appear that HEC, either alone or in a mixture, is unable to ensure flat baselines (Fig. 1A). On the contrary, both the diamine and the oligoamine (TEPA) produce smoother baselines (cf. Figs. 1B and 1C). The three single additives, when used alone, ensure also more reproducible transit times, with shifts of the order of 3–4 s over a total running time of ca. 5 min. The significance of these findings can be better appreciated by comparing the data in the bar graph of Fig. 2, which plots the percentage of protein adsorbed to the wall after any set of 10 runs in the conditions given in the legend. It is thus seen that the ternary mixture of additives is the worst, since it gives the highest protein adsorption (4.5%). The two binary mixtures fare a bit better (3.2% for TEPA-HEC and 2.5% for Q-Pip-HEC). The individual quenchers appear to have the best performance, with a score of 1.9% for TEPA, 1.8% for HEC and only 0.5% for Q-Pip, which thus appears to be the best additive for quenching any interaction of proteins to the silica wall.

Figs. 3A-F display a similar set of data as in Fig. 1, but in this case by adopting 40 m*M* Asp, added with 7 *M* urea (apparent pH 3.7), as background electrolyte. In this case, the worst possible mixture of quenchers is that containing 0.5% HEC and 0.5 mM TEPA (A), since in this case not only the baseline is drifting, but also the α- and β-peaks (which should approach a ratio of unity) give strongly unbalanced zones, suggesting preferential adsorption of some of

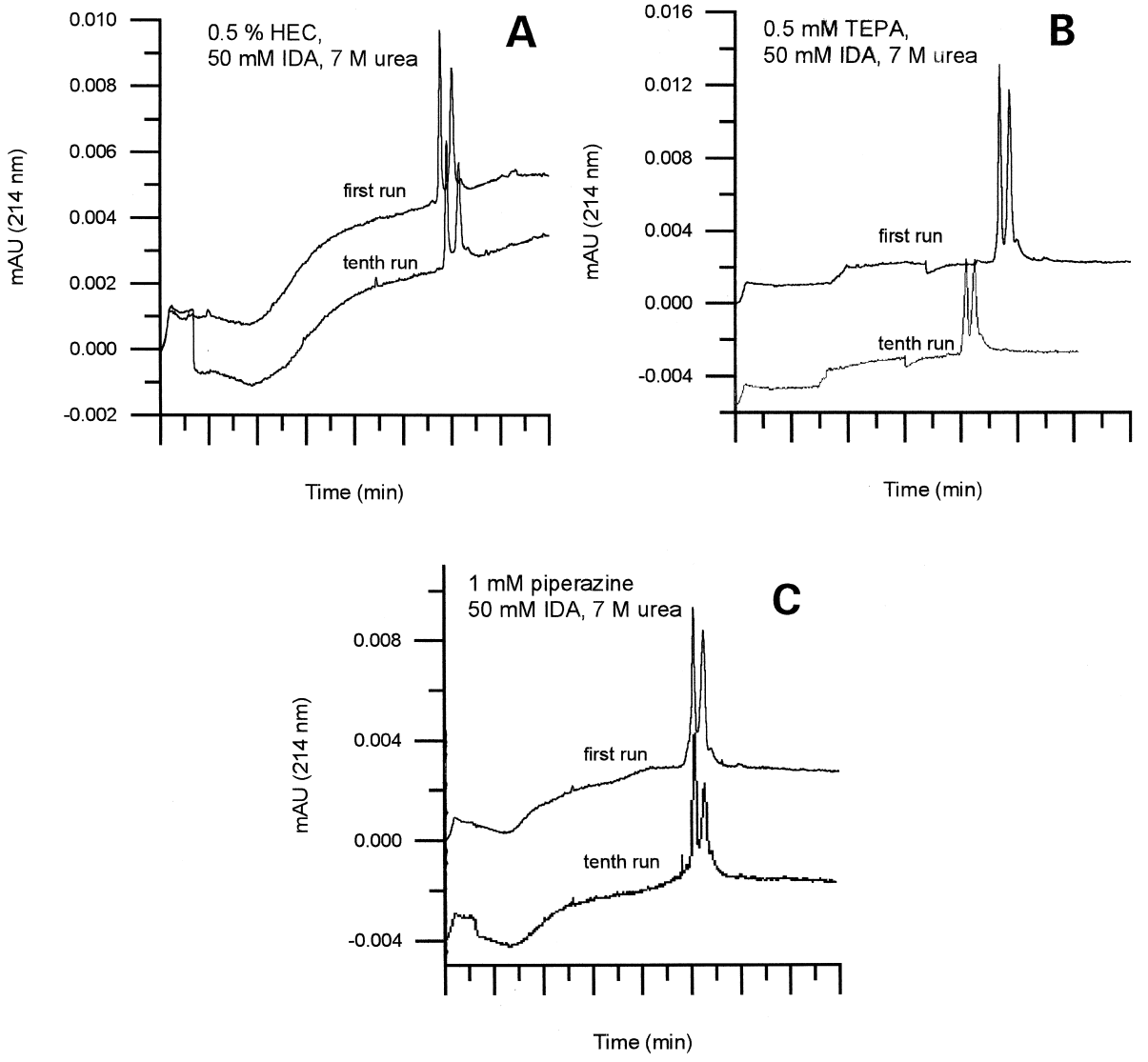

Fig. 1. Separation of α- and β-globin chains in 50 m*M* IDA, 7 *M* urea buffer, pH 3.2, in presence of a number of quenchers. (A) Added with 0.5% HEC; (B) in 0.5 m*M* TEPA and (C) in 1 m*M* Q-Pip. In all cases, the samples were loaded by hydrostatic pressure for 5 s and the separations were performed at 500 V/cm. The ultraviolet absorbance was monitored at 214 nm.

the chains. The second worst concoction is the other binary mixture 0.5% HEC, 1 m*M* Q-Pip (Fig. 3B), closely followed by the tertiary mixture 0.5% HEC, 0.5 m*M* TEPA, 1 m*M* Q-Pip (Fig. 3C). Just like in Fig. 1, the single components appear to be the best additives, in order of increasing performance: 0.5% HEC (Fig. 3D), 0.5 m*M* TEPA (Fig. 3E) and 1 m*M* Q-Pip (Fig. 3F). In the last two cases, one should again notice both the reproducibility in transit times and the rather smooth baselines. The reasons for this behaviour are evident from the bar graph of Fig. 4, which plots the percentage of protein adsorbed to the wall after any set of 10 runs. It can be appreciated that now the amount of protein adsorbed after a total of 10 runs is quite severe: it ranges from as high as 43% for the mixture 0.5% HEC, 0.5 m*M* TEPA and 1 m*M* Q-Pip (bar No. 1) to only 5% in the case of Q-Pip alone (bar No. 6). It is thus evident that the

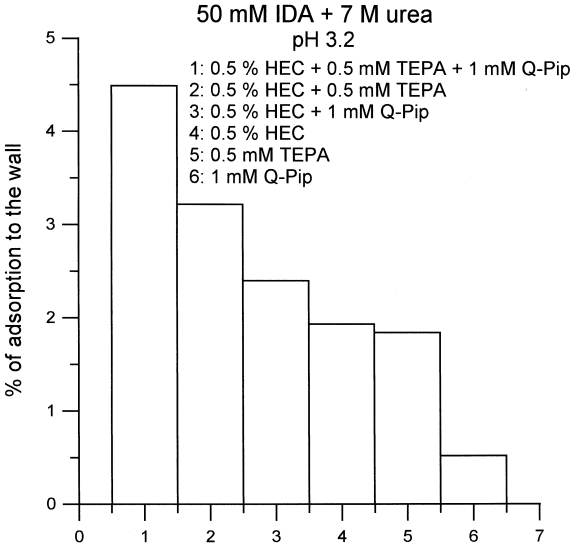

Fig. 2. Quantitative evaluation of protein adsorption in runs in presence of 50 m*M* IDA and 7 *M* urea, with different additives. Quantitation was obtained by electrophoretic elution in SDS micelles of any protein adsorbed to the silica wall after 10 consecutive separations of α- and β-globin chains.

amount of protein adsorbed is dictated by the pH of the buffer conditioning the silica wall and that, in both cases (IDA and Asp buffers) the best quencher is the mono-quaternarized piperazine. The possible reasons for these phenomena are discussed below.

4. Discussion

It had been debated for a long time whether our isoelectric, acidic buffers would allow proper protein separations in the absence of adsorption to the naked fused-silica wall. Although we always gave out a warning on this potential hazard, we had been unable to quantify this phenomenon and to give figures of merit to any potential additive admixed to the background electrolyte for minimizing or even abolishing such adsorption. It must be stated that we encountered this phenomenon even in the case of peptides separated in Asp buffer in the absence of urea (pH 2.77), but in the case of these tryptic digests of casein, the wall adsorption seemed to be eliminated by the simple addition of 0.5% HEC [1]. The present situation, however, is considerably more complex: now we deal with entire polypeptide chains, which, due to their size, are potentially prone to much stronger interactions with any ionized silanol on the capillary wall. Moreover, in order to keep these (and many other) proteins in solution, we have to resort to the addition of 7 *M* urea, which drastically changes the pH of the solution, typically raising it by as much as 1 pH unit. This in turn implies a more pronounced risk of protein adsorption to the wall.

The novelty of the present data rests on the fact of having found a robust method for quantifying protein adsorption. It consists in desorbing any bound protein via a frontal mechanism, by which electrophoretically driven SDS micelles enter the capillary from the cathodic side and displace any bound protein by adsorbing in into the micelle. This method appears to give an accurate evaluation of protein adsorption. In fact, first of all, repeated runs in SDS buffer do not appear to desorb additional proteinaceous material from the wall, as also previously reported by Barberi et al. [15]. Secondly, no other harsh method, be it a washing step in strong acid (HCl) or in strong base (NaOH), as recommended in all washing procedures, after the SDS-desorption process, is able to release any additional material [19]. Thus, we are forced to conclude that all proteins adsorbed to the wall have been efficiently released, the other alternative being that they are permanently bound to the wall, a most unlikely event indeed.

Some important conclusions can be drawn from the present results. First of all, and contrary to amply held belief, mixtures of different quenchers do not produce a cooperative effect in releasing bound material; on the contrary, they are least efficient. It could be that ternary and binary mixtures of such additives compete for the silanol binding sites, producing a poor and non-homogenous dynamic coating. Thus, single additives are to be preferred. Among them, the two bases (the bibasic, mono-quaternarized piperazine and the oligoamine TEPA) appear to be by far the best performers. In fact, oddly, the first one has a somewhat better performance than the second, even though the oligoamine, at the operative pH, bears from 4 to 5 positive charges. Q-Pip, bearing only one quaternary nitrogen, should be most effective at such low

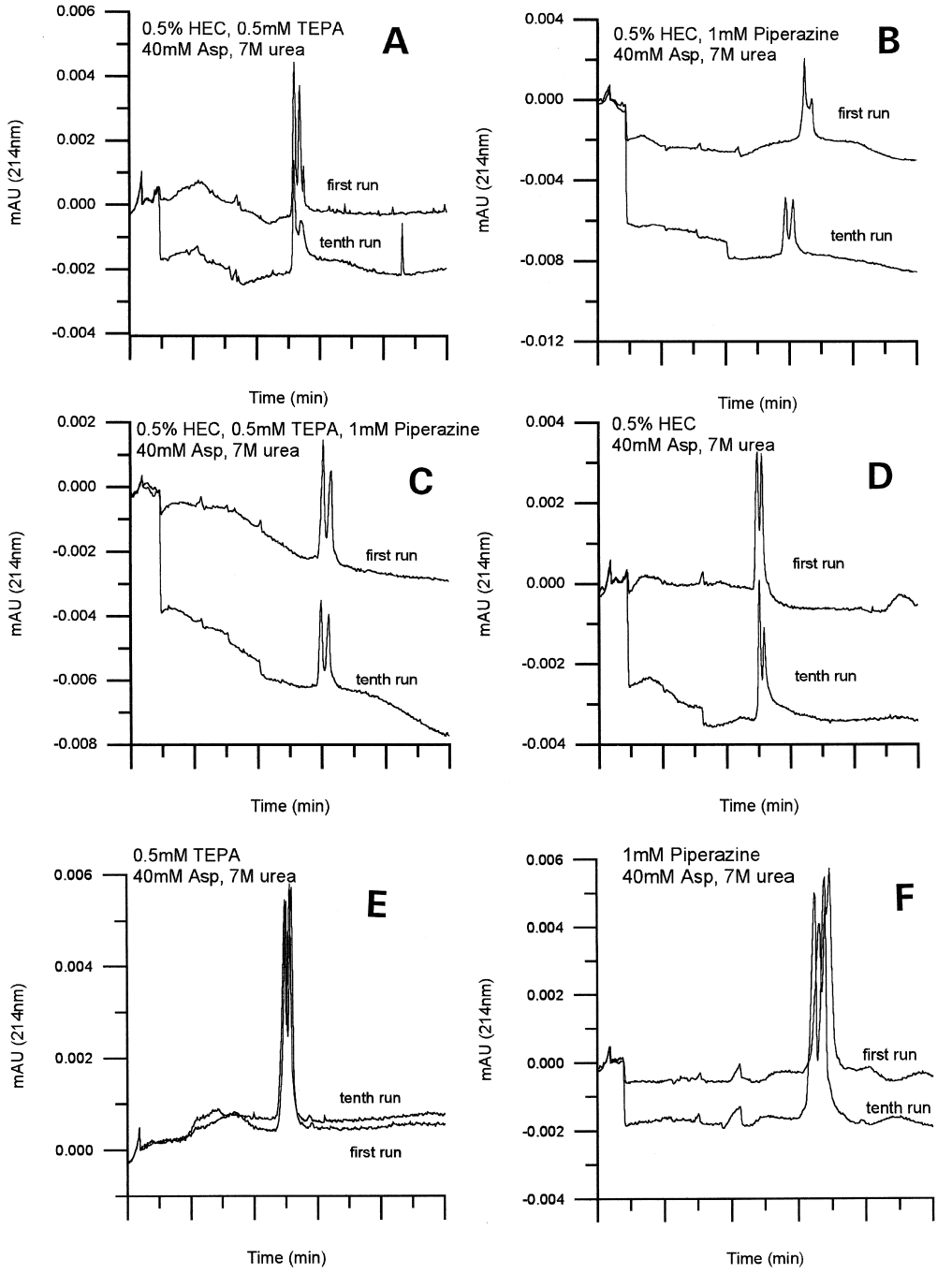

Fig. 3. Separation of α- and β-globin chains in 40 m*M* Asp, 7 *M* urea, buffer, pH 3.7 in presence of a number of quenchers. (A) Added with 0.5% HEC and 0.5 m*M* TEPA; (B) added with 1 m*M* Q-Pip and 0.5% HEC; (C) in 1 m*M* Q-Pip, 0.5 m*M* TEPA and 0.5% HEC; (D) in 0.5% HEC; (E) in 0.5 m*M* TEPA and (F) in 1 m*M* Q-Pip. In all cases, the samples were loaded by hydrostatic pressure for 5 s and the separations were performed at 700 V/cm. The ultraviolet absorbance was monitored at 214 nm. The order of elution is: α-, followed by β-globin.

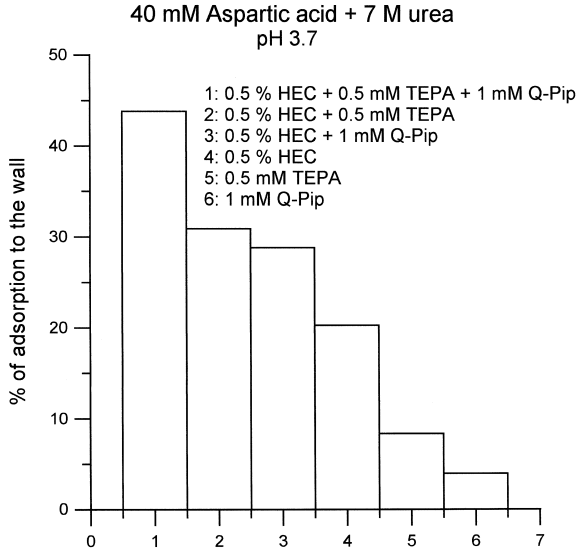

Fig. 4. Quantitative evaluation of protein adsorption in runs in presence of 40 m*M* Asp and 7 *M* urea, with different additives. Quantitation was obtained by electrophoretic elution in SDS micelles of any protein adsorbed to the silica wall after 10 consecutive separations of α- and β-globin chains.

operative pH values, since also the second, weaker group (p*K* ca. 6) should be fully protonated. Nevertheless, with a total of only two positive charges, it should in principle be less effective than TEPA, which has a chance of much stronger, cooperative interaction with ionized silanols. We believe that the reason for the better quenching ability could rest on the type of alkane substituent placed onto the quaternarized nitrogen: the short alkyl chain, while not dramatically increasing the hydrophobicity of the site, could help the piperazine moiety to be strongly adsorbed to the wall via additional Van der Walls interactions with non-ionized silanols adjacent to the ionized ones, coupled via charge-charge interaction with the protonated amines. Fig. 5 gives the chemical formula of the compound (left side), whereas the right side gives a view of what we believe is the mechanism of action of this derivative. At alkaline pH values, we hypothesize that three different types of bonds occur: (a) an ionic interaction with the quaternary amine; (b) a hydrogen bond interaction via the tertiary amine (now deprotonated) and (c) a covalent link via reaction between the silanol on the wall and the terminal iodine molecule on the alkyl

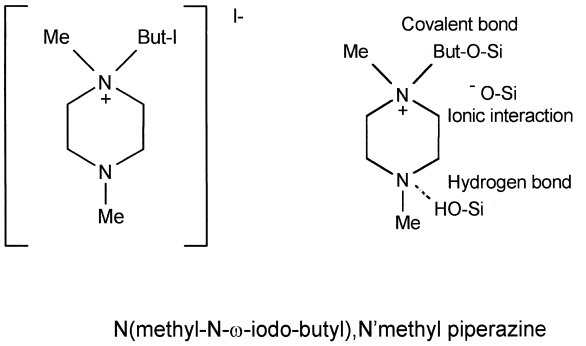

Fig. 5. Chemical formula (left side) and mechanism of action at alkaline pH (right side) of the novel modifier here reported.

chain [20]. It is a fact, indeed, that in separations at alkaline pH values the presence of the modifier is not needed in the running buffer, but solely in the equilibration procedure prior to the electrophoretic run. Additional evidence for the mechanism of covalent interaction can be derived from Fig. 6: here the conditioning of the capillary was performed at pH 9.0 and then EOF measured at pH 2.23 in plain IDA buffer. It is seen that a strong, reversed EOF flows ensues, which reaches stabilization after ca. 24 h of operation. After that, and for a total of seven days of monitoring, this reversed EOF remained

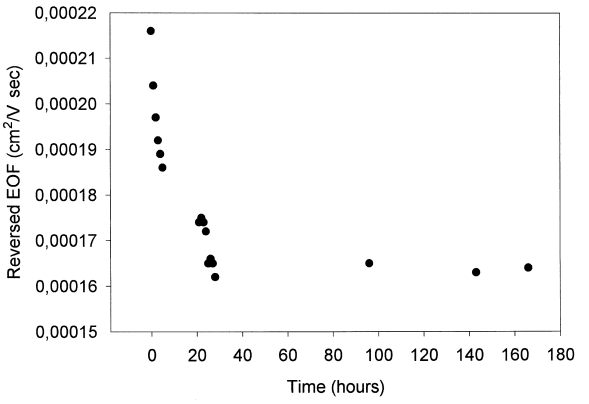

Fig. 6. Reversed EOF flow in capillaries treated with Q-Pip. The silica wall was first conditioned at alkaline pH (2 mm Q-Pip in 40 m*M* hydrogencarbonate buffer, pH 9.5, for 15 min, room temperature), then bathed in 50 m*M* IDA buffer, pH 2.23. Measurements of EOF were continued for up to 1 week at this low pH value, by injecting 8 m*M* acrylamide as neutral marker. Note that the EOF is reversed, indicating the presence of positive charges on the wall.

constant, indicating not only that the Q-Pip had to be covalently bonded to the silica surface (being the latter fully protonated, a mechanism of ionic interaction would be most unlikely) but that this bond was stable and not hydrolyzed under our rather strong acidic pH values. However, since all the experiments here reported were performed by direct equilibration of Q-Pip in the IDA buffer, only the ionic mechanism of interaction of the piperazine moiety with the wall could be operative, and this could account for its lower efficiency and for the need of the presence of the modifier in the background electrolyte. Nevertheless, even at this low operative pH the novel molecule here reported appears to be still the best additive of all those tested. As for TEPA, it is one of the classical oligoamines adopted by Vesterberg [16] for the synthesis of carrier ampholytes, the soluble, amphoteric buffers able to create and maintain a pH gradient in an electric field [17]. It should be noted that TEPA is a mixture of many different compounds (positional isomers), since, when longer than four nitrogens, such oligoamines are both linear and branched. The five p*K* values of TEPA are given as: 9.9, 9.1, 7.9, 4.3 and 2.7. The ability of TEPA to interact so strongly with the silica wall might be facilitated by its heterogeneity, allowing a better match of different compounds with the sparsely ionized silanol surface.

As a final remark, it should be stated that the best way for desorbing proteinaceous material from silica surfaces is not via HCl or NaOH treatments, as amply described in the present literature (as surveyed in [18]), but via treatment with SDS micelles under an electric field. In fact, whereas a silica surface, after treatment with SDS, is unable to release any more material upon subsequent NaOH or HCl washings, the opposite does not apply. When proteins are desorbed via NaOH or HCl conditioning, an additional treatment with micellar SDS-buffer is able to further release proteinaceous material [19].

Acknowledgements

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