The Impact of Column Technology on Protein Analysis by Capillary Electrophoresis: Surface Coatings and Analytical Approaches for Assessment

To avoid unwanted interactions between proteins being analyzed and the surface of the fused silica CE capillary, the surface must be deactivated. Four approaches to surface deactivation for protein analysis are presented. A method for determining the extent of protein adsorption is discussed.

by Sally A. Swedberg and Monika Dittmann

In 1986, Henk Lauer and Doug McManigill of HP Laboratories published an article on the capillary electrophoresis of proteins on untreated fused silica.[1] In an attempt to solve the problem of untoward interactions of protein solutes with the high-energy fused silica surface, they took the approach of using organic additives in the electrolyte solution to deactivate the surface dynamically. They concluded that the approaches taken for dynamic deactivation were limiting for protein analysis in general for a variety of reasons. In conclusion, they wrote that "… the development of chemically bonded wall deactivators remains an important topic."

To give some insight into the impact of this study, it is important to understand what impact the CE project at HP was perceived to have at this time. Because of a number of publications by J.W. Jorgenson and others,[2] CE was thought to have the potential to revolutionize the way analysis of biopolymers would be done in the future. Therefore, protein analysis by CE was considered extremely significant. The conclusion of Lauer and McManigill that still more work on the capillary chemistry needed to be done to provide routine analysis across a range of protein samples drew new attention to research on coating chemistries for surface deactivation of fused silica capillaries.

This article describes research at HP Laboratories and the Waldbronn Analytical Division on coating chemistries effective for protein analysis by CE.

In Search of the Biocompatible Surface

Based on biophysical studies of the interactions of proteins at interfaces, it is known that one attribute of a surface that is necessary for reversible protein/surface interactions to occur is that the surface must be highly hydrated. Though this condition is necessary, it is not sufficient. Further, selection rules for surface attributes that promote reversible, low protein/surface interactions are not well-understood. The complexity of the heterogeneous polymer known as the protein makes rational approaches to such selection rules quite difficult. In addition, the Gibbs free-energy interaction term for protein/surface interactions is also

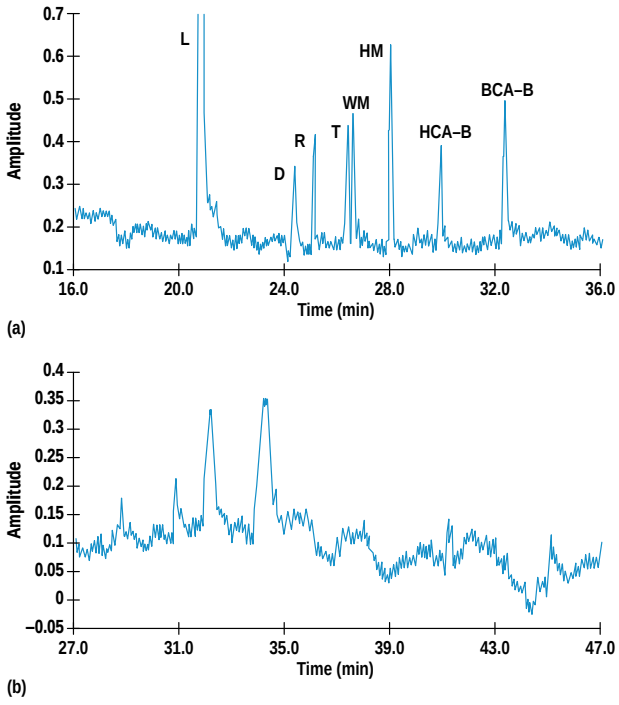

Fig. 1. Elution profile of seven protein markers and DMSO on (a) an arylpentafluoro-treated column and (b) an untreated fused silica column. Conditions: 200 mM phosphate, 100 mM KCL solution, pH 7, in 20-μm capillary tubing using an applied voltage of 250 V/cm. Detection: on-column UV at 219 nm. Length of column to detection: 100 cm. Peaks: L = hen egg white lysozyme, D = DMSO, R = bovine ribonuclease A, T = bovine pancreatic trypsinogen, WM = whale myoglobin, HM = horse myoglobin, HCA-B = human carbonic anhydrase B, BCA-B = bovine carbonic anhydrase B. (Reprinted from reference 3. ©1990 Academic Press. Reproduced with permission.)

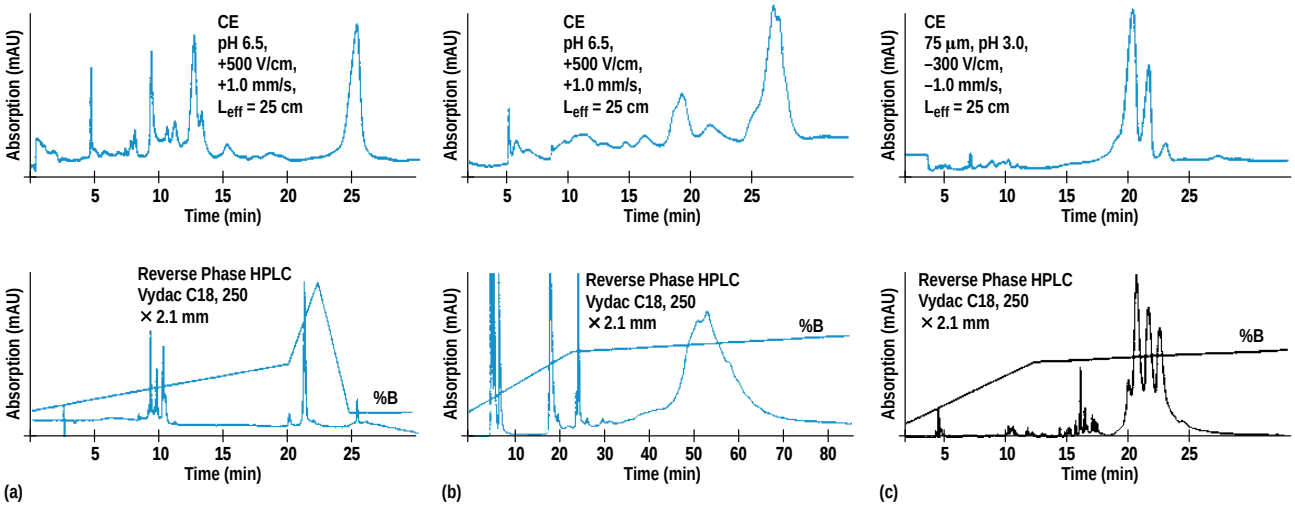

Fig. 2. Comparison of CE analysis on a bovine serum albumin modified capillary with HPLC analysis for (a) trypsin inhibitor, (b) ovalbumin, (c) α-chymotrypsinogen. With respect to the resolution of the number of species in the commercial protein preparations, the two methods appear to be macroscopically comparable, overall. In the case of trypsin inhibitor, CE separated more components than HPLC. For ovalbumin, HPLC gave more indication of the microheterogeneity in the major peak for this glycoprotein. For α-chymotrypsinogen, the leading shoulder peak of the major peak appeared to be more clearly resolved by LC. The total analysis time was considerably shorter for CE. Even in the case of α-chymotrypsinogen, CE does not require the reequilibration time required for reverse phase LC columns.

dependent on the solvent conditions used during analysis, not just on the chemistry of the surface alone.

Solving the problem of untoward protein/surface interactions requires both insights into biocompatible surfaces and a means for assessing the best solvent conditions in conjunction with a suitable surface. The next section gives an overview of some of the work done at HP Laboratories on surface coatings for analysis of proteins by CE. Then, work done at the Waldbronn Analytical Division to develop an analytical tool for assessing the extent of protein/surface interactions under various conditions is described. The specific example of a very promising surface, polyethylene glycol, is presented.

Surface Modified Capillaries

Arylpentafluoro Modified Capillaries. Fig. 1a shows the separation of seven protein standards on an arylpentafluoro-treated surface. This was one of the first surface modifications tried, and was based on attributes that were known from affinity and hydrophobic interaction chromatography (HIC) to be desirable for reducing protein/surface interactions.[3] Shown in Fig. 1b is the control, in which the same protein sample was run on a fresh fused silica surface. This separation, which is the result of untoward protein/surface interactions, is illustrative of the problems frequently encountered in the analysis of complex samples of proteins by CE on unmodified fused silica surfaces. As can be seen from the conditions, fairly high concentrations of phosphate buffer are required for this analysis. The second paragraph below discusses how the prolonged contact of phosphate with the silica surface produces an interesting surface modification that is also protein-compatible.

Bovine Serum Albumin (BSA) Modified Capillaries. Fig. 2 shows examples of protein samples analyzed by both CE and high-performance liquid chromatography (HPLC).[4] There are two interesting features of the BSA surface for the purpose of protein analysis. First, it is a highly hydrated surface, which in part explains the low sorptive nature of a variety of proteins to this surface, even at very low ionic strength of the buffer (10 mM citrate, in these examples). Second, since proteins are amphoteres, it displays a sigmodal electroosmotic flow profile as a function of pH (Fig. 3). It is therefore possible to tune both the magnitude and the direction of the electroosmotic flow as a function of the pH of the solution. The impact of this feature is demonstrated in Fig. 3.

Phosphosilicate Modified Capillaries. In 1988, McCormick demonstrated that with prolonged contact with phosphate buffers, a stable phosphosilicate surface is formed.[5] He demonstrated that successful protein separations could be done in fused silica capillaries after the phosphosilicate surface had been formed. His work was done at pH 2, which is an undesirable pH for many proteins, promoting denaturation and aggregation. Based on his studies, other groups have demonstrated that this approach can be used across a wide range of pH (Fig. 4).[6,7] Further, since these surfaces form readily, thereby obviating the need for specially manufactured capillaries, this is a useful method for protein analysis that can be easily integrated into the bioscience laboratory.

While physical means for assessing the extent of protein/surface interactions were used in conjunction with the separations discussed above,[8] a more robust approach using frontal analysis was developed at Waldbronn. This frontal analysis approach was used to assess still another promising surface, polyethylene glycol. That study is described in the following section.

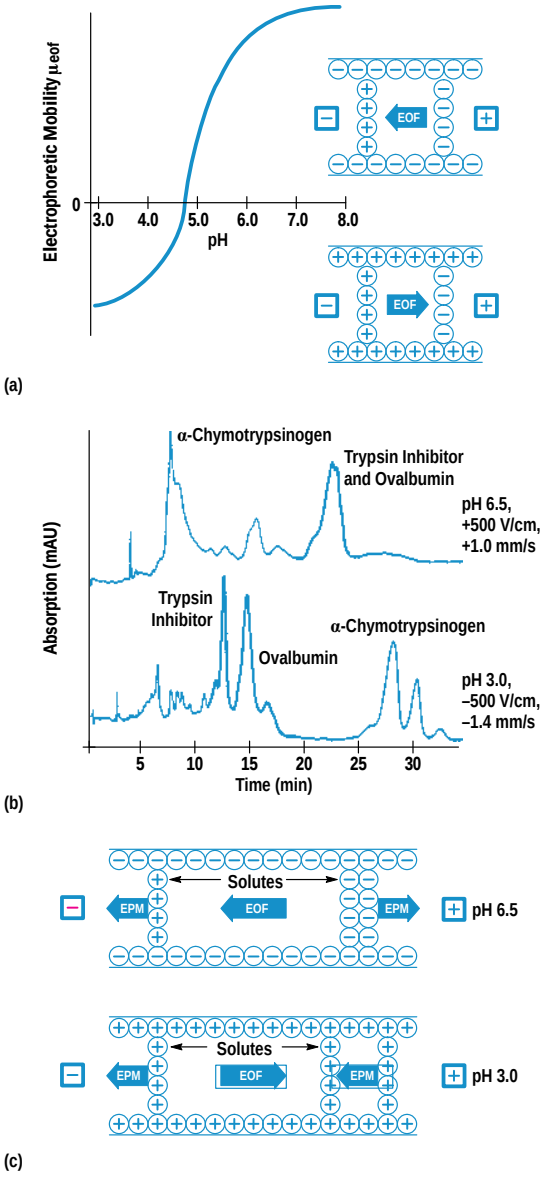

Fig. 3. Tuning selectivity of amphoteric phases. (a) Effect of pH on mobility. For amphoteric surfaces, the electroosmotic flow (EOF) changes predictably in both magnitude and direction as a function of pH. For the BSA surface the useful working range is between pH 3.0 and 8.0. (b) Control of resolution and elution order. For the same capillary, by changing the pH of the solution and the polarity of the voltage supply, it is possible to change the elution order and selectivity of the protein species. For these three proteins, pH 3.0 gives the best separation of the parent peaks.
(c) Since the net charge (both magnitude and sign) of a protein solute will also change as a function of pH, the solute electrophoretic mobility (EPM) changes with respect to the changing electroosmotic flow (EOF).

Polyethylene Glycol Surface Coatings

In 1989, the Waldbronn Analytical Division became involved in the development of biocompatible coatings for the CE separation of proteins. As previously mentioned, a surface that provides reversible protein/surface interactions must be

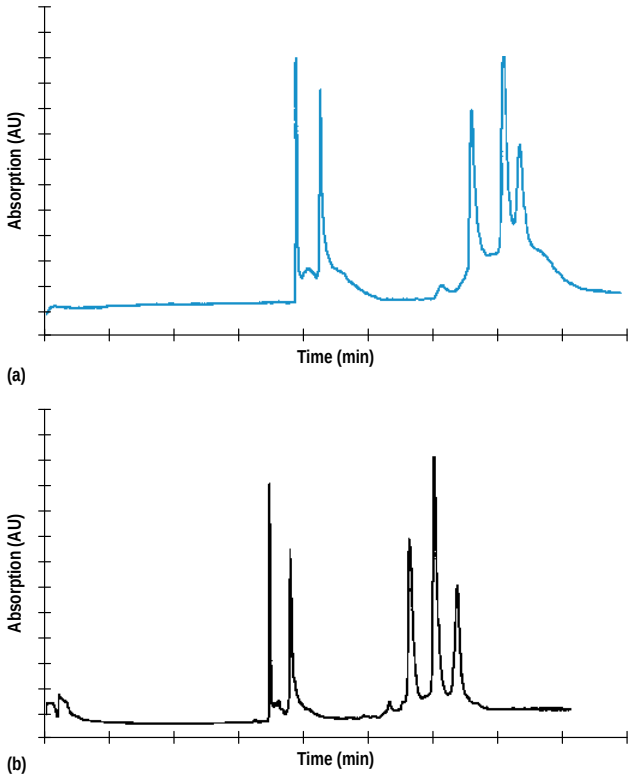

Fig. 4. Effects of sodium sulfate and ammonium sulfate on the separation of five protein standards. Buffer = 150 mM phosphate, pH 7.0, with (a) 200 mM sodium sulfate at 118 microamperes and (b) 200 mM ammonium sulfate at 138 microamperes. Conditions: 15°C, 400 V/cm, L = 49.5 cm, L_{eff} = 41 cm. Peaks (left to right): ribonuclease, myoglobin, B-lactoglobulin A, trypsin inhibitor, B-lactoglobulin B.

highly hydrated. One class of materials that fulfill this condition is the class of hydrophilic polymers known as polyethylene glycol polymers. Polyethylene glycol polymers as surface coatings are known to have little interaction with proteins.[9] Polyethylene glycol coated capillaries have long been available for gas chromatographic separations. This type of coating was tested at Waldbronn for feasibility in the separation of proteins.

Determination of Protein Adsorption by Frontal Analysis

A method that allows direct determination of the adsorption of a solute on a surface is frontal analysis. Frontal analysis is essentially a chromatographic method for measuring the amount of protein adsorbed and the strength of the protein/surface interactions. A solution of the protein of interest is pumped through the coated capillary and the breakthrough of the protein front is observed (Fig. 5).The amount of protein adsorbed at the surface under given conditions can be calculated from:

$$q(c) = c(V_f - V_d)/A_s,$$

where q(c) is the amount of protein adsorbed per unit area, c is the concentration of protein, V_f is the retention volume of the protein front (flow rate times retention time of the

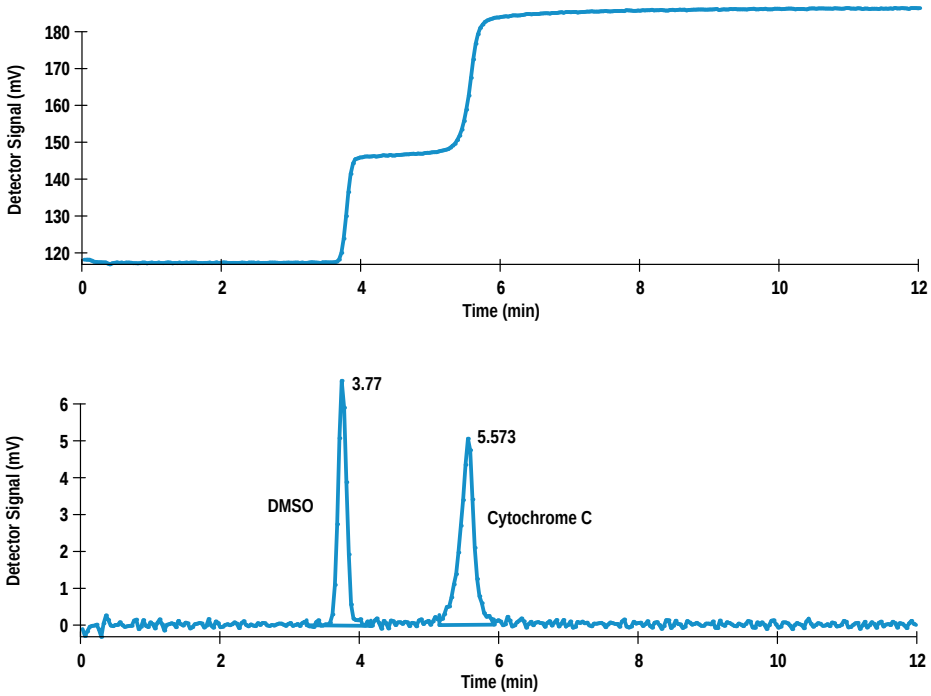

Fig. 5. Example of a frontal analysis curve of a mixture of DMSO (dead volume marker) and cytochrome C. The upper trace is the detector signal. The lower trace is the derivative of the detector signal.

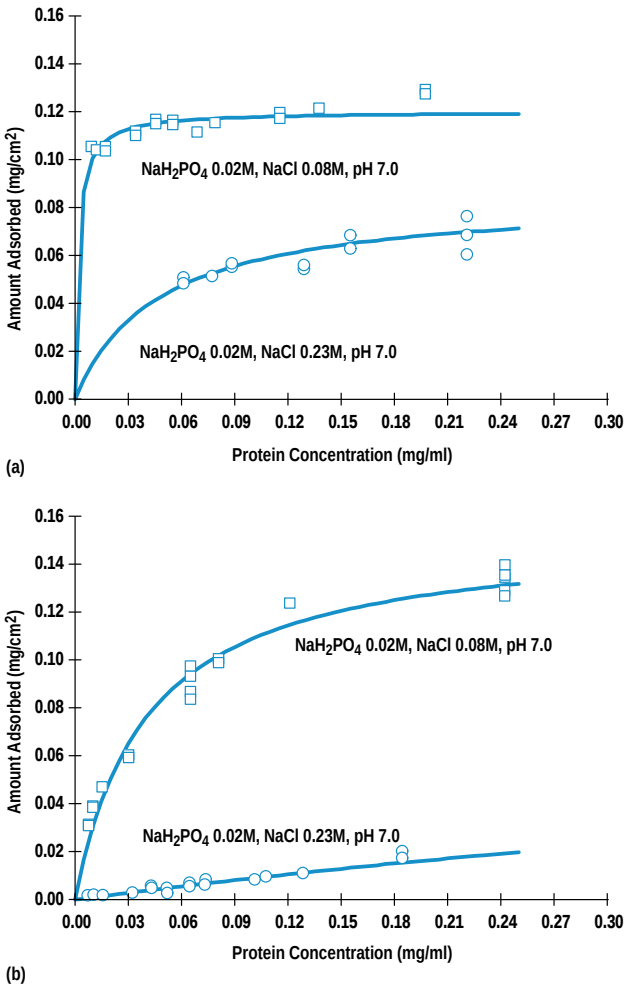

Fig. 6. Adsorption isotherms for (a) cytochrome C and (b) lysozyme on an uncoated fused silica capillary. Buffer: 0.02M NaH_2PO_4, pH 7.0, plus 0.08M NaCl and 0.23M NaCl.

protein front), V_d is the capillary dead volume (flow rate times retention time of the marker front), and A_s is the inside surface area of the capillary.

If q(c) is determined for a number of different protein concentrations, an adsorption isotherm is obtained. For many solutes, these adsorption isotherms are of the Langmuir type and can be fit to the equation:

$$q(c) = ac/(1 + bc),$$

where a is the slope of the isotherm at low concentration and b is an empirical parameter. The parameter a is related to the retention coefficient k′ by the equation $k' = a\beta$, where β is the phase ratio.

If adsorption isotherms of proteins are determined under different conditions (temperature, solvents, buffer concentrations, etc.) conclusions can be drawn as to the type and strength of protein/surface interactions. In this regard, proper separation conditions can be determined from frontal analysis experiments.

Adsorption isotherms of lysozyme and cytochrome C on an uncoated fused silica capillary at two different salt concentrations in the buffer (0.08M NaCl and 0.23M NaCl) are shown in Fig. 6. An increase of salt concentration reduces both the maximum amount of protein adsorbed at the surface and the initial slope of the isotherms at low protein concentrations. This indicates the presence of electrostatic protein/surface interactions on the uncoated fused silica surface that are suppressed by the addition of NaCl. Fig. 7 shows adsorption isotherms of lysozyme and cytochrome C on a polyethylene glycol coated capillary with no NaCl added to the buffer. On this surface, the amount of protein adsorbed is very small, indicating that this surface might be suited for protein separations. An addition of 0.03M NaCl completely suppressed protein adsorption on this surface.

Fig. 8 shows a separation of four proteins that cannot be eluted from an uncoated capillary unless a high pH or a very

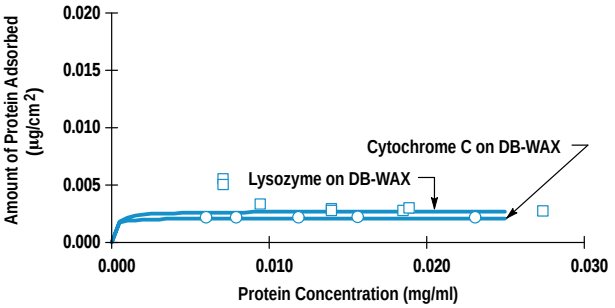

Fig. 7. Adsorption isotherms of lysozyme and cytochrome C on DB-WAX capillary at low protein concentrations. Conditions: 20 mM NaH_2PO_4, pH 7, 25°C.

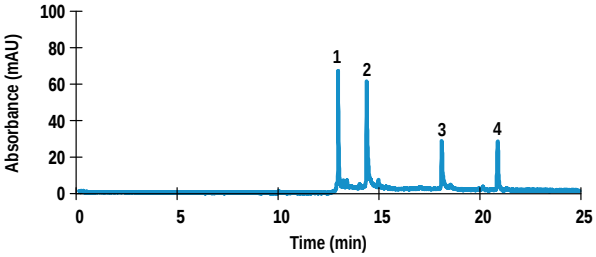

Fig. 8. Separation of (1) lysozyme, (2) cytochrome C, (3) ribonuclease A, and (4) chymotrypsinogen A on a polyethylene glycol coated capillary, L = 57.8 cm, L_{eff} = 51.3 cm, i.d. = 100 µm. Conditions: 20 mM phosphate buffer, pH 5.0, plus 30 mM NaCl, constant-voltage mode, 15 kV, 25°C, detection at 214 nm.

high salt concentration is used in the eluting buffer. On the polyethylene glycol coated capillary, a highly efficient separation can be obtained under moderate conditions.

Further studies concerning the stability of the coating and separation of real-life samples are under way.

Acknowledgments

The authors would like to thank Fred Strohmeier for his continued support and helpful discussions. Additional acknowledgments are expressed to Gerard Rozing and Doug McManigill for their helpful discussions and support on the frontal analysis experiments.

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