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Critical conditions for separating the microheterogeneous components of glycoproteins by capillary electrophoresis

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Abstract

To separate the microheterogeneous components of glycoproteins, capillary electrophoresis (CE) conditions were systematically investigated with ovalbumin from chicken egg white as the main testing sample. In addition to the well-known adsorption effect, a reversible capillary wall partitioning effect was found to contribute greatly to the separation and the favorable tubing wall was that coated with polyacrylamide. The separation media, especially the buffer composition, its concentration and pH, were also found to be critical. Buffers composed of boric acid and alcohol amines were demonstrated to be more effective media than gels and other solutions, but the selection of the buffer concentrations and pH was dependent on samples. By using free-solution CE with polyacrylamide-coated capillaries and boric acid–alcohol amine (BA) buffers, ovalbumin of agarose electrophoresis purity can be split into more than twenty peaks at pH 8.0±0.3 (0.2–0.4 mol/l BA). In contrast, free-solution CE with uncoated capillaries and gel electrophoresis with polyacrylamide-filled capillaries yielded only five or less peaks.

Keywords: Coating; Capillary columns; Buffer composition; Glycoproteins; Proteins; Ovalbumin

1. Introduction

Glycoproteins are now known to be involved in a number of important biological functions including antigenicity, transport, folding, recognition and/or other biological activity [1]. Some of the glycoproteins (hormones) have been produced by biological engineering techniques [2]. The analysis of glycoproteins has hence become an important or attractive topic. This is also a great challenge to analytical chemistry because, in contrast to proteins, a "pure" glycoprotein is normally composed of a number of sub-components, termed as microheterogeneity. To elucidate the biological function of the microheterogeneity and to identify the biological engineering products of glycoproteins, a highly efficient separation method is required. Traditional and modern analytical tools such as slab or column chromatography and electrophoresis have been explored, of which chromatographic focusing and isoelectric focusing [3] showed the highest ability to separate the microheterogeneous components. However, these tools are normally labor-intensive, time-consuming and need large amounts of samples; in addition, their resolution is still poor [3]. A theoretically ideal way is to use the newly developed capillary electrophoresis (CE) which has been demonstrated to be fast and extremely powerful in the separation of proteins, DNA, carbohydrates and other biological molecules [4]. Minute samples can easily be manipulated because nanoliter injection volumes are an inherent feature of a ($\leq$100 μm I.D.) capillary.

CE of glycoproteins was firstly reported in 1989 [5,6]. Recently, some improved methods have been

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developed [1–4,7–11]. Since 1990, we have also started a similar research program. The performance was carried out by using uncoated capillaries and borate buffer [7]. In theory, borate will increase the resolution of the microheterogeneous components because of its ability to form complexes with the hydroxy groups of the glycan moieties. Unfortunately, the resulting separation was not as ideal as we hoped [8]. It seems that there is something special or critical which has not yet been found out.

In this paper, we will discuss what factors are critical or special. After a systematic investigation, we have found that most of the CE conditions such as voltage, temperature, injection method and tube length etc. do not show any special effect, but the capillary wall and the separation support do. In addition to the well-known adsorption effect, reversible partition of analytes between the capillary wall and buffer (shortened as wall partition that is normally negligible) contributes greatly to CE of glycoproteins. A favorable way is using polyacrylamide-coated tubes. The separation media, especially the buffer composition, its concentration and pH, are also critical. Free solutions composed of boric acid and alcohol amines will be demonstrated to be more effective media than gels and other solutions. The selection of the buffer concentrations and pH is mainly dependent on samples.

Ovalbumin from chicken egg white is mainly used as a testing sample. This is a well-known glycoprotein composed of 385 amino acids with Asn_{292} and/or Asn_{312} glycosilated [12–14]. Although there are only two possible glycosylation sites within the peptide chain, ovalbumin is highly heterogeneous [15] and nine different glycan strands have already been identified [16–20]. Its complexity in composition and its negligible adsorption effect on silica surface make it an ideal testing sample for our investigations.

By using free-solution CE with polyacrylamide (PA)-coated capillaries and boric acid–alcohol amine buffers (BA), abbreviated as PABA–CE, ovalbumin of agarose electrophoretic purity can easily be split into five clusters of peaks (with about five peaks in each cluster) at pH 8.0±0.3 (0.3±0.1 mol/l boric acid–Tris buffer). In contrast, common free-solution CE and capillary polyacrylamide gel electrophoresis (cPAGE) yield only five or less peaks.

2. Experimental

2.1. Chemicals

Tris [tris(hydroxymethyl)aminomethane], AMP (2-amino-2-methyl-1,3-propandiol), MAP (2-methyl-3-amino-1-propanol) and polyvinylpyrrolidone K15 (M_r~10 000) were from Fluka (Buchs, Switzerland). Tricine [N-tris(hydroxymethyl)methylglycine], HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), MES (morphorlinoethanesulfonic acid) and other biological buffer reagents were from Dojindo Chemical Laboratory (Tokyo, Japan). γ-MPS (γ-methacryloxypropyltrimethoxysilane) and HPMC (hydroxypropylmethylcellulose, viscosity of a 2% aqueous solution is about 4000 cP) were from Sigma (St. Louis, MO, USA). Acrylamide and Bis [N,N′-methylenebis(acrylamide)] were obtained from Beijing Purchasing Station, Chinese Pharmacy (Beijing, China). TEMED (N,N,N′,N′-tetramethylethylenediamine), ethanolamine, TEA (triethanolamine), ethylenediamine and HDA (hexanediamine) were analytical reagent grade from Xi'an Chemical Works (Xi'an, China). Boric acid, borax, APS (ammonium persulfate) and other chemicals were all of analytical reagent grade from Beijing Chemical Works (Beijing, China). The water used was triple distilled.

2.2. Sample preparation

Ovalbumin from chicken egg white was of agarose electrophoretic purity from the Institute of Biochemistry, Chinese Academy of Sciences (Shanghai, China), and was dissolved in water each morning at the concentration of 1 mg/ml except where mentioned otherwise in the text.

2.3. Preparation of polyacrylamide-coated capillaries

A new capillary (J&W, distributed by Composite Metal Services, Worcestershire, UK) was silanized at room temperature by filling it with 0.6% (v/v) γ-MPS and 0.5% (v/v) acetic acid in dichloromethane. After 40–50 min, the capillary was washed with methanol and water for 10 min each, it was then

filled with 1.5% T+5% C gelling solution[1] and kept at room temperature for 1 h. The capillary was washed again with water for 10 min and finally dried with a hot air flow (>70°C) for >1 h. This coated capillary can be stored at room temperature or in a vacuumed desiccator. The detection window of the capillary was created, just before use, by removing the polyimide over-coating for about 2 mm with a scalpel [12].

The coated capillaries can normally be used for more than three months if dried after electrophoresis every day. In cases where the coating is damaged (dramatically prolonging the migration time), the capillary should be re-coated. To perform re-coating, the capillary is washed first with 2 mol/l NaOH for more than 1 h, then with water and methanol for 20 min and 5 min, respectively. After dried with the hot air flow, the capillary is treated as a new capillary. This procedure is also suitable for coating other used tubes.

2.4. Preparation of low background gel-filled capillaries

The low background polyacrylamide gel-filled capillaries were prepared according to Ref. [12]. A polyacrylamide-coated capillary was filled with 10 cm of buffer followed by 30 cm of 3–10% T+5% C gelling solutions. Both ends of the filled capillary were plugged into one sealed vial which was then pressurized by injection of ice-cooled water. The capillary with the vial up was hung at room temperature for 5 h and then stored at room temperature by plugging both its ends into a running buffer. Just before use, a detection window was created at the buffer-filled part, 2 cm from the gel.

2.5. Electrophoresis

All electrophoresis was performed with the Beckman P/ACE system (model 2050, Beckman Instruments, Fullerton, CA, USA). The operation was controlled by an IBM compatible computer (586 CPU) with the System Gold software. For free-solution CE, capillaries of 67 cm (effective length 60 cm)×50 μm I.D. (375 μm O.D.) were used. Except where mentioned in the text, the samples were introduced by pressure for 1 s and separated at −400 V/cm for coated tubes or at +400 V/cm for uncoated tubes. The separated bands were detected at 200 nm and 1 s rise-time and the peak data were acquired at 1 Hz.

For cPAGE, a low background capillary of 37 cm (effective length 30 cm)×75 μm I.D. (375 μm O.D.) with 28 cm of gels and 9 cm of running buffer was used. Before injection, the capillary was equilibrated with the running buffer at −200 V/cm until the current became stable. The samples were introduced into the gel tip at −5 V for 2 s. Other conditions were the same as mentioned above.

3. Results and discussion

3.1. Critical conditions

3.1.1. Capillary wall partitioning effect

In CE of proteins, the well-known problem is the capillary wall adsorption which leads to a decrease in resolution. In a serious case, no separation or even no peak may result. For the separation of glycoproteins, such a wall effect is also a serious problem and has to be eliminated. However, we found that, even when the adsorption effect is negligible, the resolution of glycoproteins still depends on the wall nature.

Fig. 1 shows two pairs of comparisons between the capillaries with and without polyacrylamide coating. None of the electropherograms exhibits an evident adsorption effect but the resolution or separating profiles are very different: the polyacrylamide-coated capillary can split the ovalbumin into five groups of peaks, with about five peaks per group (Fig. 1A and C), while the uncoated capillary can only yield five "peaks" (Fig. 1B and D). This phenomenon was also observed in the separation of RNase or transferrin (data not shown).

It seems that the improved separation of the ovalbumin with the coated tubes is a result from the

[1] %T=grams of acrylamide and Bis in a 100 ml solution, %C=grams of Bis over 100 g of acrylamide and Bis. A gelling solution is a monomer solution with TEMED and APS which are added just before filling. Their final concentrations are 0.1% (v/v and w/v, respectively) for coating a capillary, or 0.04–0.05% for preparing a gel-filled capillary.

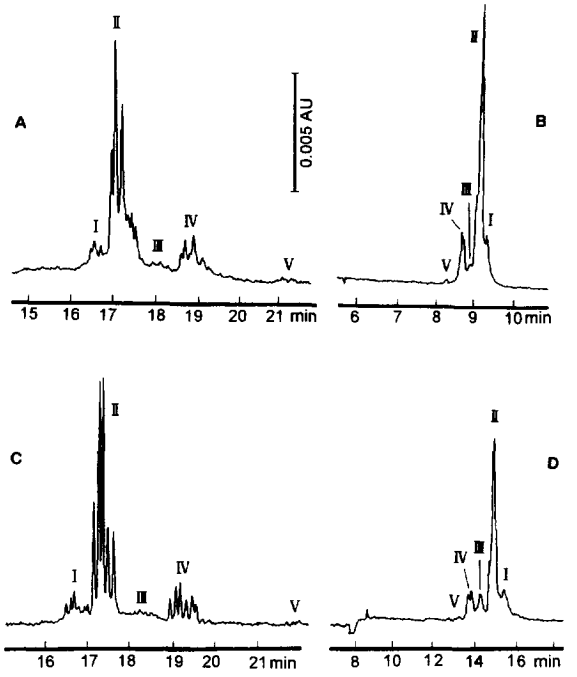

Fig. 1. Electropherograms of ovalbumin obtained from polyacrylamide-coated (A and C) and uncoated capillaries (B and D) at 24°C, with a running buffer of 50 mmol/l borax (A and B) or 200 mmol/l boric acid–200 mmol/l Tris (C and D). The Roman numbers indicate the migration order of the same seperands in different capillaries. Other conditions were described in Section 2.

increase in running time, which can be "demonstrated" by comparing within Fig. 1 A with B and C with D (or D with B). Landers et al. and Oda et al. have also suggested that increasing the separation time by using electroosmotic modifiers such as alkyldiamines was an effective way to improve the resolution of glycoproteins [1,2,9]. We have also tried to prolong the running time by another method, that is, by increasing the separation length of the uncoated capillary from 60 to 90 cm, and although the resolution between each "peaks" (see Fig. 1B or D) was improved, further splitting of each "peak" has not yet been observed. In contrast, if we reduce the separation length of a polyacrylamide-coated capillary from 60 cm to 30 cm, the peak clusters can be maintained though their resolution decreases. Furthermore, when a newly coated tube is specially dried in a vacuumed desiccator for one week (to further reduce the electroosmosis) and run at a higher temperature, the elution time of ovalbumin can be reduced to the same order as in Fig. 1D, but the resolution is even slightly improved as shown in Fig. 2A. A reasonable explanation for this phenomenon should thus not be based on the running time but on the coating or partitioning effect. Using the partition concept, we can also explain the results obtained by Lander et al. and Oda et al.: their modifiers dynamically created a new surface on the tubing wall so that a favorable partitioning effect occurred.

We have tried to replace the polyacrylamide coating with cyclodextrins or polyethylene glycol or to dynamically modify it with a buffer additive such as cellulose (HPMC), polyvinylpyrrolidone or HDA, but the resolution was reduced. Fig. 2B shows an electropherogram obtained from a buffer containing HDA, which is somehow different from literature results [1,2]. The reasons are not yet clear. Interestingly, long term washing of the polyacrylamide-

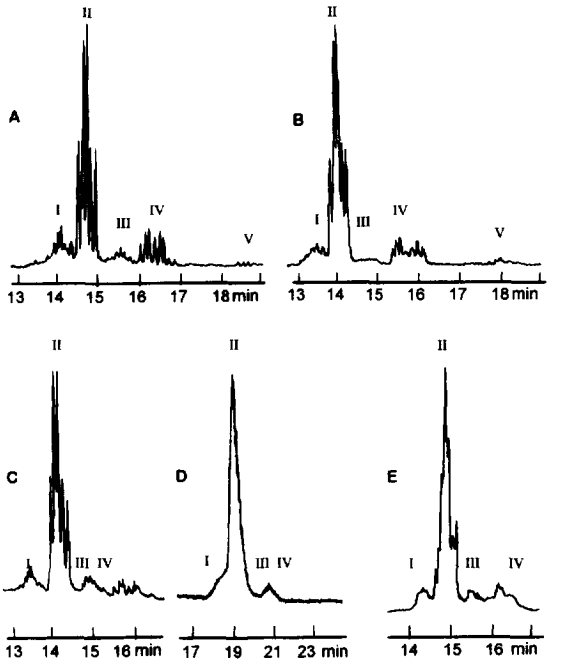

Fig. 2. Electropherograms of ovalbumin from free-solution CE (A–C and E) and cPAGE (D). A–C: capillary newly coated with polyacrylamide and dried in a vacuumed desiccator for one week. E: the same capillary as in A–C but used for more than six weeks. D: low background capillary filled with 5% T+5% C gel. Buffer: 200 mmol/l boric acid–Tris, pH 8.25 (A and D), containing 0.1 mmol/l HDA (B) or 0.5% T+5% C (C), and 200 mmol/l boric acid–borax, pH 8.23 (E). Temperature: 28°C. Voltage: −40 V/cm (A–C, E) and −200 V/cm (D).

coated tubes with phosphate buffer, which has a low speed of equilibration with the silica surface, will also reduce the splitting power of the capillary (see Fig. 3D). These experiments demonstrate that polyacrylamide is at present the most effective coating and further modification of it may be harmful.

3.1.2. Sieving media

Unexpectedly, although polyacrylamide is an ideal coating material, it is not suitable as a separation medium even at a very low concentration of 0.5% T+5% C (Fig. 2C). When the gel concentrations increase to 3% T+5% C or higher (up to 10% T+5% C), only two or three broad peaks can be measured (Fig. 2D). It seems that sieving media equalize the microheterogeneity because non-gels such as HPMC also decrease the resolution of ovalbumin as mentioned above. Therefore, cPAGE will not be suggested.

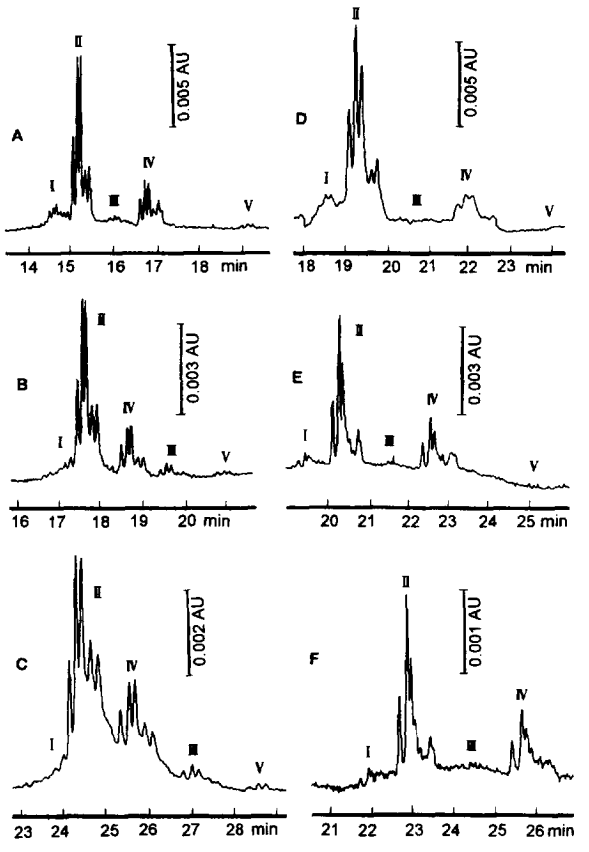

Fig. 3. Influence of electrolytes on the resolution of ovalbumin at 24°C, with a polyacrylamide-coated capillary. Buffer: 200 mmol/l boric acid adjusted to pH 8.20 with AMP (A), ethylenediamine (B), HDA (C) and Na_3PO_4 (D), or 100 mmol/l Tris adjusted to pH 8.20 with Tricine (E) and MOPS (F).

3.1.3. Electrolytes

Buffer electrolytes, especially those forming counter-ions to the samples, are clearly an important factor because they may interact with the analytes by electrostatic force, coordination or other principles. Considering that a glycoprotein contains amino and hydroxy groups, alcohol amines of structural similarity should be an ideal sort of electrolyte which can be used for adjusting the buffer pH or just as the buffer reagents. As expected, ovalbumin can easily be split in boric acid buffers with Tris or AMP to adjust their pH (Fig. 1C, Fig. 2A and Fig. 3A). Further studies show that quite a lot of alcohol amines can generate similar separations of ovalbumin, including Tris, AMP, MAP, TEA and ethanolamine etc, of which Tris and AMP are the most convenient as they are crystals.

When the alcohol amines are replaced by alkyldiamines such as ethylenediamine (Fig. 3B) and HDA (Fig. 3C), the peak group I will be integrated into II and the elution order of peak groups III and IV is also reversed (Fig. 3B and C), which clearly shows the interaction occurring between the sample and the alkyldiamines. The resolution will further be reduced with Na_3PO_4 (Fig. 3D) or borax as the replacing reagents, especially when the coated capillaries are used for more than one month (Fig. 2E). This possibly results from the fact that the small positive ions adsorb onto and/or penetrate into the coating and then lead to further damage of the coating because the resulted capillaries are not recoverable. NaOH and KOH show similar effects.

Acidic electrolytes, normally used as buffer reagents, mainly influence the resolution by coordination. We have investigated several kinds of organic and inorganic acids, including boric acid, phosphoric acid, tartaric acid, and biological buffer reagents such as HEPES, MOPS, Tricine and MES. Boric acid, able to form complexes with the hydroxy groups of the glycan strands, yielded the best resolution (Fig. 3A) while the other acids generate a similar but fairly poor resolution (Fig. 3E–F).

3.1.4. Buffer pH and concentration

As known, pH controls the dissociation degree of analytes and hence greatly influences their migration behavior or separation. Clearly, the pH should be optimized according to the samples. For ovalbumin, the optimum pH found is 8.0±0.3 (Fig. 4 and Fig. 1A). It is interesting that lower pH (out of the optimum range) yields worse separation than higher. When the pH is lowered to 7.30, no evident separation of the microheterogeneous composition can be observed (Fig. 4A). In contrast, acidic buffers are much better than basic ones for the separation of RNase or transferrin. In this case, tartaric acid is suggested as an additional pH adjusting reagent, which will be discussed elsewhere.

Buffer concentrations are not as critical as pH but should also be optimized according to samples. For ovalbumin, the best separation is obtained at 0.30±0.10 mol/l of boric acid or 0.1–0.2 mol/l of alcohol amines. Again, lower buffer concentrations yield worse separations than higher ones at the same pH. If the concentration is lowered to 0.05 mol/l of boric acid, no separation of ovalbumin may also be obtained.

3.2. Other conditions

Several other conditions have also been examined including buffer solvents, voltage applied, column temperature, injection methods etc. We have tried to improve the resolution of ovalbumin by modifying

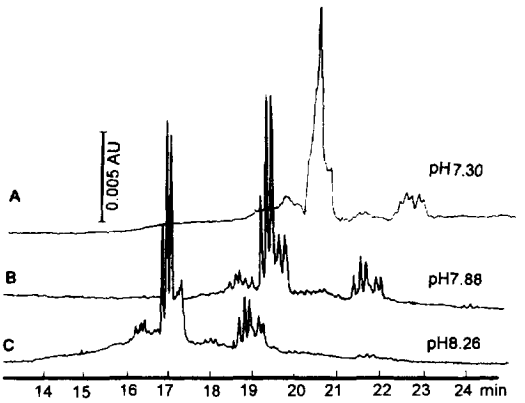

Fig. 4. Influence of pH on the resolution of ovalbumin at 20°C, with a polyacrylamide-coated capillary and 100 mmol/l AMP–boric acid buffers. The buffer pH was adjusted from high to low.

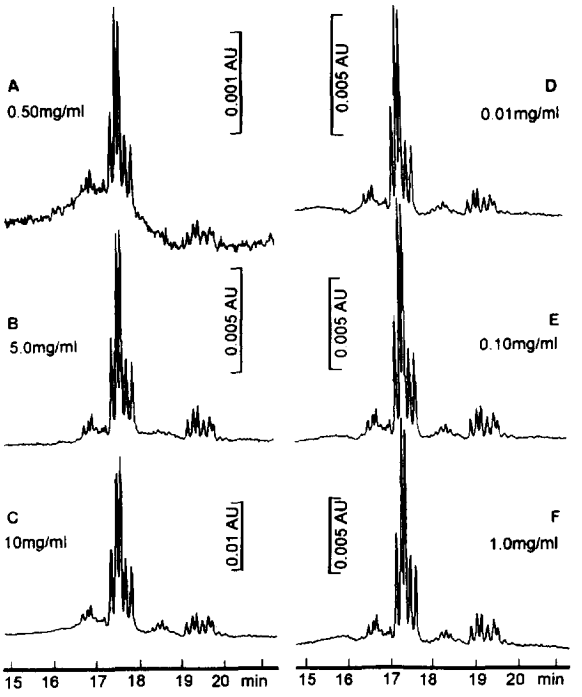

Fig. 5. Reproducible separation of ovalbumin with buffer of 200 mmol/l boric acid and 200 mmol/l Tris at 24°C. The sample was injected by pressure for 1 s (A–C) or by electromigration at 10 kV for 2 s (D), 10 kV for 1 s (E) and 3 kV for 1 s (F) following a pressure introduction of water for 2 s.

the pure water solvent with 5–20% (v/v) of methanol, ethanol, propanol, acetonitrile, DMSO or acetone but have not yet observed any evident effect. In contrast, these binary solvents (except for water–acetonitrile) greatly increase the detection background. The voltage applied among 200 and 400 V/cm and the temperature set at 20–30°C only slightly affect the resolution (comparing Fig. 1C and Fig. 3A). Sample introduction methods such as electromigration and pressure give similar resolution, but if the sample is injected by electromigration just after the pressure introduction of a plug of water, the resolution and reproducibility can be maintained over quite a wide range of sample concentrations (comparing Fig. 5A–C with D–F).

4. Conclusions

CE performing conditions have been discussed

fairly systematically and wall partition and separation media, especially the buffer composition, its concentration and pH, have been demonstrated to be critical and have to be carefully selected. The optimum conditions obtained include: (1) using the free buffers composed of boric acid and alcohol amines as the separation media; (2) coating the capillaries with polyacrylamide; (3) selecting the buffer concentration and pH according to the samples to be separated. The first two conditions have been stated as free-solution CE with polyacrylamide-coated capillaries and boric acid–alcohol amine buffers or PABA–CE. By using PABA–CE, ovalbumin has been separated into more than twenty peaks at pH 7.7–8.3 within 20 min, which is comparable to or better than reported results [1]. Other glycoproteins can also be separated using PABA–CE combined with a re-optimization of the buffer concentration and pH, which will be discussed elsewhere.

Acknowledgments

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