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## Novel Zwitterions as Buffer Additives for Improving Efficiency and Reproducibility in Capillary Electrophoresis by Minimizing Protein Adsorption

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## Abstract

Zwitterions comprised of sulfonic acid and quaternary amine groups were investigated as buffer additives for use in capillary electrophoresis. These zwitterions are effective in reducing the interaction of proteins with fused silica capillary surfaces under conditions of neutral pH. Over 300,000 theoretical plates were obtained in less than 15 minutes for the difficult case of lysozyme at pH 7 using the trimethylammoniumpropylsulfonate zwitterion. The low conductivity of these buffers also permitted the use of 75  $\mu$ m **Poster No. PT-69** 

NOVEL ZWITTERIONS AS BUFFER ADDITIVES FOR IMPROVING EFFICIENCY AND REPRODUCIBILITY IN CAPILLARY ELECTROPHORESIS BY MINIMIZING PROTEIN ADSORPTION

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### Effect of a novel **bis-zwitterion** on Lysozyme migration time, peak shape and efficiency, EOF

The effectiveness of the bis-zwitterion, N,N'-bis-(sulfopropyl)-N,N,N',N'tetramethylhexanediamine 2, was compared to the trimethylammoniumpropylsulfonate 1.



On the basis of equal numbers of charges (i.e. comparing 0.5 M bis-zwitterion to 1.0 M monozwitterion), there was little difference in the effectiveness of these two compounds in improving lysozyme efficiency. At pH 7 in 120 mM phosphate, 87,000 plates were obtained with lysozyme. However, the bis-zwitterion caused a greater reduction in electroosmotic flow indicating that it is more strongly adsorbed to the capillary surface. The electroosmotic flow was  $2.8*10^{-4}$  cm<sup>2</sup>/V-sec and the electrophoretic mobility of lysozyme was  $4.5*10^{-5}$  cm<sup>2</sup>/V-sec in the presence of 0.5 M bis-zwitterion (100 mM phosphate, pH 7).

#### Reproducibility

The ability of the zwitterion to compete with lysozyme and prevent fouling of the capillary surface is very important. Over the course of ten runs, the migration time of lysozyme, 12.89 minutes, had a standard deviation of 0.23 minutes or 1.8%. The area of the peak was also very reproducible with a standard deviation of 2.4%. The high efficiency of the separation was reproducible, average plates were 75,000 with a standard deviation of 9.5%.

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## Appendix Guidelines for performing high efficiency CE

Assume a migration time of 15 minutes, a sample diffusion coefficient of  $1*10^{-6}$  cm<sup>2</sup>/sec, and a capillary length of 50 cm.

In an ideal system, this gives a peak width, 4  $\sigma$ , of 3.1 seconds or 1.7 mm and a theoretical efficiency of 1,400,000 plates.

If the goal is 1 million plates actual, then P.W. = 2 mm Thus,  $2^2 = det^2 + inj^2 + 1.7^2$  and if inj=det then inj = 0.74 mm

Issues involving lengths:

injection length: 0.74 mm (2 sec hydrostatic injection for a 75 µm capillary with a 10 cm head, viscosity 1 cp) detector length: 0.74 mm (length of capillary illuminated)

Issues involving time: peak width is 3.6 seconds detector time constant: 0.1 sec sampling rate: 20 pts/sec

# ELECTROLYTE MODIFICATION VERSUS MODIFICATION OF THE CAPILLARY SURFACE

As the field of capillary electrophoresis advances, one wonders how the dynamic approach to surface modification, electrolyte modification, will compete with or complement static surface modifications (i.e. coated capillaries). The advantage of coated capillaries is that they simplify buffer preparations. If a capillary is available that provides both the desired electroosmotic flow and the inert surface required for a separation, the use of the capillary, itself, is straightforward. However, at the moment, the choices of coated capillaries are limited, and the lifetime of the capillaries uncertain. Under these circumstances, dynamic surface modification which provides opportunities for the control of analyte adsorption on the capillary surface and electroosmotic flow allows the user maximum flexibility in separation development. Furthermore, prospects for the development of a totally nonadsorptive surface for all sample matrices seem remote. Since adsorption in electrophoresis not only results in the inability to analyze the adsorbed species, but also results in changes in electroosmotic flow and migration times of all the analytes, dynamic modifications which create self-cleaning surfaces are extremely useful.

#### CONCLUSIONS

Zwitterions containing quaternary amines and sulfonic acids can be used to inhibit protein adsorption to capillary surfaces.

Very high efficiency separations may be obtained even in difficult cases (lysozyme at pH 7).

High electroosmotic flow rates may be obtained even when high concentrations of zwitterion are used.

To optimize a particular separation, the analyst may trade off zwitterion concentration, buffer concentration and pH.

Run to run reproducibility is improved because of the ability of the zwitterion to maintain a "clean" capillary surface.

THE COMPANION POSTER, PT-25: NEW DEVELOPMENTS IN THE CAPILLARY ELECTROPHORESIS OF BASIC PROTEINS CONTAINING PROTEINS, MIKE MERION ET. AL. DISCUSSES THE USE OF THE TRIMETHYLAMMONIUMPROPYLSULFONATE ZWITTERION WITH REAL SAMPLES. lit code R-25

## AN INITIAL DEMONSTRATION OF THE EFFECTIVENESS OF TRIMETHYLAMMONIUMPROPYLSULFONATE IN MINIMIZING THE INTERACTION OF LYSOZYME WITH CAPILLARY SURFACES

At pH 7.0, in 100 mM phosphate buffer, lysozyme interacts strongly with the capillary wall and elutes as a broad, asymmetric peak after the neutral marker.

In the same buffer with 1 M zwitterion, lysozyme elutes as a narrow (117K plates), symmetric peak well before the neutral marker.



## Electrophoresis

All separations were performed using the Waters Quanta<sup>TM</sup> 4000 and monitored at 214 nm. The capillaries were nominally 75  $\mu$ m x 60 cm (52.5 cm to detector). The capillaries were cleaned with 0.5 M KOH when changes in electroosmotic flow were observed. Lysozyme, chicken egg white (Sigma L-6876)

MW 14,400 pI 11.0  $D_{20,w}$  1.2\*10<sup>-6</sup> cm<sup>2</sup>/sec

## Zwitterions containing Quaternary Amines and Sulfonic Acids



trimethylammoniumpropylsulfonate

high dipole moment

zwitterionic over the pH range from 3 to 10

good solubility — up to 3 M

(note that because of significant volume changes (ca. 10% for a 1 M zwitterion solution), the zwitterion cannot simply be added to a 100 mM buffer solution without altering the buffer concentration)

low conductivity — in theory the conductivity of a zwitterion is zero, in practice 10% solutions of the zwitterions used for this work all had conductivities less than 50  $\mu$ S/cm. (The conductivity of a pH 7.0, 0.1 M phosphate buffer is 9250  $\mu$ S/cm. The same buffer containing 1 M trimethylammonium-propylsulfonate has a conductivity of 6660  $\mu$ S/cm.)

low UV absorbance Abs=0.2 AU for a 1.0 M solution, 1 cm path at 214 nm (useful at 185 nm in 75 $\mu$ m capillary)

## General principles of capillary electrophoresis

Efficiency is proportional to field strength:

 $N \mu V/L$ 

Heat generated is proportional to field strength, capillary radius, buffer concentration and buffer conductance:

$$P/L = KCr^2(V^2/L^2)$$

P is power L is capillary length r is capillary radius C is buffer concentration K is molar conductance of the buffer

Although, very small (10-25 $\mu$ m) capillaries will allow the use of high field strengths even with relatively conductive solutions, this is usually accomplished at the expense of detector sensitivity.

Therefore, it is desirable to work with low conductivity buffers so that 50 to 75  $\mu$ m capillaries can be employed.

#### Goals of this work

Identify novel zwitterionic compounds that have:

- high dipole moments,
- zwitterionic character over a broad pH range,
- good solubility in aqueous buffers and
- low UV absorbance

We chose to test the efficacy of these compounds in a CE system using lysozyme as the probe for interaction between the capillary surface and proteins at pH 7. Because lysozyme is strongly basic, at pH 7 it is strongly adsorbed to silica surfaces and has frequently been used as a probe for adsorption in the area of HPLC as well as in HPCE.

#### Background

A long standing problem in the area of protein chemistry is the propensity of proteins to adsorb, sometimes very strongly, to surfaces. This problem is especially severe when low concentrations of proteins are present as is often the case in separation systems. In the context of capillary electrophoresis using fused silica capillaries, this adsorption is largely related to the coulombic attraction of positively charged regions of the protein to the capillary surface. Since the fused silica capillary walls are negatively charged above pH 3, this problem must be confronted with many important buffer systems.

## Approaches to Protein Separation by CE

The first "solution" to this problem follows from the discussion above, at low pH (<3) the capillary wall is neutral and the problem of adsorption can be reduced. A related approach is to work at high pH (>9) and alter the charge on the protein so that no significant regions of positive charge exist. (see Lauer and McManigill, *Anal. Chem.* **1986**, *58*, 166.) These approaches have both been useful, but they greatly restrict the use of pH to control electrophoretic mobility and selectivity in developing separations. Of course, they also require that the molecules of interest be stable under extreme pH conditions. Thus, there is a need for capillary electrophoresis systems which allow protein and other macromolecule separations at neutral pH (5-8). One approach to this problem is to coat the capillary surface, an area of active research and the other is to work with modified buffers, the subject of this poster.

## Modification of pH 7 buffers to minimize protein interactions

Increasing the ionic strength of the buffer minimizes protein interaction with the capillary surface and allows high efficiency separations. Unfortunately, the increased conductivity of the solution makes it necessary to use small capillaries and low voltages ( $25 \mu m$ , 50V/cm). Jorgenson and Green, J. Chromatogr. 1989, 478, 63.

Increased ionic strength coupled with amino acid zwitterions also can be used to minimize adsorption. This approach places some limitations on detection (background absorbance) but allows the use of increased voltages in 25  $\mu$ m capillaries. Jorgenson and Bushey, J. Chromatogr. 1989, 480, 301.

The adjacent electropherogram shows that under optimized conditions — 200 mM phosphate, 1 M zwitterion — lysozyme can be eluted as an extremely narrow, symmetrical band at pH 7. For this analysis N=350,000 and the asymmetry at 10% peak height is 1.2.



HPCE '91 Poster PT-69 p. 11 R-18 Portions of the work in this poster are the subject of a pending patent. Effect of **pH** on Lysozyme migration time, peak shape and efficiency, EOF (buffer (100mM phosphate), zwitterion (1 M) concentration held constant, sodium concentration varies)

Lysozyme mobility and migration time — as pH increases, lysozyme mobility increases because of decreasing interactions between lysozyme and the wall. In this system, an overall reduction of lysozyme retention time is observed as pH increases.

Lysozyme efficiency and peak shape — as pH increases, lysozyme efficiency and peak shape improve.

EOF — little change in the electroosmotic flow rate is observed in this system as a function of pH over the range from 6 to 8.



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## A THREE DIMENSIONAL VIEW OF THE EFFECTS OF ZWITTERION AND BUFFER CONCENTRATIONS

As shown in the three dimensional graph, improvements in efficiency and peak shape can be obtained through increased concentration of zwitterion or buffer. Increases in buffer concentration are, of course, limited by the ability of the electrophoretic system to dissipate heat. The degree to which concentration increases are required to give acceptable separations, depends on the strength of the analyte interaction with the capillary surface. Lysozyme represents a difficult case because of the strong positive charge this protein bears at neutral pH.

## Effect of Zwitterion and Phosphate concentrations on efficiency



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Effect of **Buffer concentration** (sodium phosphate) on Lysozyme migration time, peak shape and efficiency, EOF (pH 7.0, zwitterion concentration, 1.0 M, held constant)

EOF — as has been widely reported, increasing buffer concentration decreases electroosmotic flow and this effect is also observed in the presence of zwitterion.

Lysozyme mobility and migration time — as the concentration of buffer increases, lysozyme mobility increases because of decreasing interactions between lysozyme and the wall. Since electroosmotic flow decreases as buffer concentration increases, the migration time of lysozyme increases with increasing buffer concentrations

Lysozyme efficiency and peak shape — as the concentration of buffer increases, lysozyme efficiency and peak shape improve. However, even at 200 mM phosphate, in the absence of zwitterion, the lysozyme peak tails badly.



HPCE '91 Poster PT-69 p. 8 R-18 Portions of the work in this poster are the subject of a pending patent.

Effect of **Trimethylammoniumpropylsulfonate Zwitterion Concentration** on Lysozyme migration time, peak shape and efficiency, EOF (pH 7.0, buffer concentration held constant, 100 mM phosphate)

EOF — increasing the zwitterion concentration up to 2.5 M had little effect on the electroosmotic flow rate. At 1.0 M the electroosmotic flow was reduced 10%. (Note that the degree to which EOF changes depends on pH and ionic strength. We have observed reductions of EOF from 5 to 20% while working with the trimethyl zwitterion under various conditions.)

Lysozyme mobility and migration time — as the concentration of zwitterion increases, lysozyme electrophoretic mobility increases because of decreasing interactions between lysozyme and the wall. In the absence of zwitterion, the interaction is so strong that lysozyme actually elutes after the neutral marker.

Lysozyme efficiency and peak shape — as the concentration of zwitterion increases, lysozyme efficiency and peak shape improve. At 1.0 M, the lysozyme peak has an efficiency of 75K plates and an asymmetry of 1.2 (half height).





HPCE '91 Poster PT-69 p. 7 R-18 Portions of the work in this poster are the subject of a pending patent.



### **COMPARISON OF**

## TRIMETHYLAMMONIUMPROPYLSULFONATE TRIETHYLAMMONIUMPROPYLSULFONATE TRI-n-PROPYLAMMONIUMPROPYLSULFONATE

First we examined the effect of increasing alkyl group size on the electrophoretic behaviour of lysozyme

EOF — Increasing alkyl group size decreases electroosmotic flow. This reduction is due to increased viscosity and increased association of the tripropylzwitterion with the capillary wall. Lysozyme mobility and migration time — the electrophoretic mobility of lysozyme is reduced by a factor of 6 in the transition from trimethyl to tripropyl. The reduced mobility results from increased viscosity and may also be an indication of association of the hydrophobic tripropyl group with the protein. Association of the zwitterion with the protein surface increases the effective stokes radius of the protein. Lysozyme migration times are greatly increased in the transition from methyl to propyl due to the combination of reduced electroosmotic flow and lysozyme mobility.

**Lysozyme efficiency** — All three compounds were effective in minimizing lysozyme interaction with capillary walls and provided high efficiency separations.

Zwitterion	Efficiency 1/2 height	EOF μeo cm <sup>2</sup> /V-sec	Lysozyme Mobility µeo cm <sup>2</sup> /V-see	Current µA c	Viscosity cp @ 23°C
Trimethyl-Z	185K	3.46*10-4	6.65*10-5	150	1.45
Triethyl-Z	254K	2.00*10-4	3.42*10 <sup>-5</sup>	116	1.90
Tri-n-propyl-Z	250K	1.04*10-4	1.08*10-5	75	2.96

#### Conditions

Capillary:	75μm x 60 cm fused silica
Elelectrolyte:	1.0 M Zwitterion, 200 mM phosphate pH 7.0
Run Voltage:	10 kV
Sample:	Lysozyme (100 µg/mL), 10 second hydrostatic injection

On the basis of hydrophobicity, solubility considerations and its limited impact on electroosmotic flow, additional investigations were performed with trimethylammoniumpropylsulfonate.



Without zwitterions, macromolecules with regions of positive charge are adsorbed to capillary walls. Reversible adsorption leads to low efficiency separations and tailing peaks. Irreversible adsorption changes the zeta potential of the capillary surface, the electroosmotic flow and the migration times of all the analytes.





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Zwitterions compete with macromolecules and prevent adsorption to capillary walls



The use of the AccuPure<sup>m</sup> Z1-Methyl reagent is the subject of a pending patent application.