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Isoelectric Buffers for Capillary Electrophoresis. 2. Bismorpholine Derivative of a Carboxylic Acid as a Low Molecular Weight Isoelectric Buffer

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A new compound class of synthetic isoelectric buffers is introduced, designed as a small molecule with one fully or prevailingly dissociated acidic group (such as sulfonic or carboxylic) and two partly pronated (buffering) basic amino groups attached onto a hydrophilic UV-transparent backbone. As an example, a new isoelectric compound 2,2-bis(4-morpholinylmethyl)propanoic acid (BMMPA) was synthesized by attaching two morpholine groups onto a molecule of pivalic acid. It was characterized as having an isoelectric point p*I* = 6.5 and exhibiting satisfactory buffering capacity at the p*I*. Solutions of BMMPA are transparent down to the low-UV spectral region, thus making it a potentially suitable buffer for a number of separation methods. Its use in capillary electrophoresis was demonstrated in a separation system for indirect photometric detection of anions based on an electrolyte with the anionic dye Orange G as the indirect detection probe and using BMMPA as a buffer. The use of an isoelectric buffering compound brings the advantages of a buffered electrolyte without the concomitant introduction of co-ions that would be detrimental to the indirect detection process. Submicromole per liter limits of detection for a number of inorganic and small organic ions were achieved. Optimal structural properties of the isoelectric buffer with respect to its buffering properties are discussed.

Buffering of pH is an essential concept in chemistry that is of great practical significance in most areas of analytical chemistry.[1,2] In capillary electrophoresis (CE) and other electroseparation methods, electrolysis is an accompanying phenomenon,[3−9] and therefore, pH buffering of the electrolytes is essential for robustness and reproducibility of the separation.[4,10−17] Buffers can be categorized according to their charge as anionic (such as phosphate or acetate), cationic (such as Tris or imidazole), and zwitterionic, which include some Good's buffers[18,19] (such as 2-(*N*-morpholino)ethanesulfonic acid) and isoelectric buffers (such as lysine or histidine).

Isoelectric buffers are zwitterionic compounds, which in addition to possessing an isoelectric point (p*I*) where the molecule's overall charge is zero also exhibit an appreciable buffering capacity at the p*I*. This is the case when at least two of the pK_a constants for protonation equilibria responsible for buffering are close to the p*I* (within ∼±1.5 pH units).[20] While there are many isoelectric compounds, such as most amino acids and peptides, the majority do not exhibit an appreciable degree of buffering at their p*I* because the two pK_a values closest to the p*I* are more distant from each other and the p*I* than desired; that is, |p*I* − pK_a| > 1.5 (for example, glycine, pK_{a1} 2.34, pK_{a2} 9.60, p*I* = 5.97). Because of this limitation, only a small percentage of isoelectric compounds can also function as isoelectric buffers.

Isoelectric buffers have been shown to be useful in 2D electrophoresis and in CE as low-conductivity buffering electrolytes.[21−43] Most of those reports involve separations of proteins

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conducted in acidic isoelectric buffers, such as glutamic, aspartic, or iminodiacetic acid. Other isoelectric buffers have been used as discrete ampholytes for generation of pH gradients in isoelectric focusing.[44−46]

In our laboratory, isoelectric buffers have been investigated with a view to developing buffered electrolytes for use in indirect spectrophotometric detection in CE, where an absorbing co-ion, also called the probe, is used to accomplish the indirect detection.[15,16,47] The options for electrolyte buffering are limited because co-ions in the electrolyte (i.e., ions having the same charge polarity as the analytes and the probe) must be avoided for optimal performance of the indirect detection.[15,16,47]

The complications arising from the combined use of one compound as both the electrolyte and the buffer provide an incentive for utilization of isoelectric buffers so the functions of the buffer and that of the electrolyte are independent. This outcome is possible if the buffer has an overall charge of zero and will therefore not act as a co-ion in the CE separation. Thus, in CE separations with indirect spectrophotometric detection, an electrolyte co-ion that is highly absorbing but not suitable as a pH buffer can be supplemented with an added low-conductivity isoelectric buffer.[15,16] In this case, it has to be emphasized that some previous criticism of the usage of isoelectric buffers as the sole electrolyte component in CE[43,48] does not apply because the isoelectric species functions solely as a buffer in this example.

A significant practical restriction to the use of isoelectric buffers in CE is their limited availability. Excluding those isoelectric buffers possessing undesirable properties such as high absorptivity across the UV spectrum (and hence interference in indirect detection), and those that are not easily available or are expensive, only a few remain, such as the amino acids lysine (pI = 9.7), histidine (pI = 7.7), and glutamic acid (pI = 3.2).[25] Importantly, the need for isoelectric buffers in the neutral pH region is not well satisfied with histidine because of its relatively low buffering capacity (|pI − pK_a| = 1.6). Therefore, new isoelectric buffers are needed, with particular emphasis on those that can be used in the neutral pH region.

It is pertinent to briefly review previous approaches to the design and synthesis of isoelectric buffers. Isoelectric buffers were first introduced in 1961 by Svenson[49,50] as mixtures of isoelectric ampholytes used as focusing media for isoelectric focusing.[51,52] In 1995, Hjerten and coauthors[25] proposed the following four types of very low conductivity or isoelectric buffers for CE: (i) high MW buffers with relatively few charged groups, (ii) a narrow pH fraction of carrier ampholytes for isoelectric focusing, (iii) buffering isoelectric ampholyte, for instance, with three properly spaced pK_a values (possessing either one acidic and two basic groups (e.g., lysine or histidine) or two acidic and one basic group (e.g., glutamic acid)), and (iv) an ampholyte with identical pK_a values for the acidic and the basic groups. In evaluating these suggestions, it has to be noted that while group (i) does not constitute isoelectric buffers, group (ii) buffers would be both difficult to prepare (e.g., by preparative isoelectric focusing of commercial carrier ampholytes) and very expensive, and group (iv) buffers lack suitable candidate molecules. Therefore, it is group (iii) buffers comprising low-MW isoelectric molecules (including glutamic acid, histidine, or lysine) that seem to possess the optimal design and so far have found most use as isoelectric buffers in electrophoresis.

Recently in our laboratory, carboxylated poly(ethylenimine) (PEI) was prepared as a high-MW isoelectric buffer by attaching carboxymethyl groups in a half molar ratio compared to the basic nitrogens of the poly(ethylenimine), resulting in a pI of ∼6.8.[53] While a high-MW material has the advantage of an easy purification by dialysis, a low-MW material of well-defined chemical composition would be desirable. The aims of the present study were to design a general class of new low-MW isoelectric buffers, to synthesize and characterize a new compound as a representative of this class and having a pI in the neutral pH range, and finally to investigate its usage as an isoelectric buffer in CE for indirect detection of anions.

EXPERIMENTAL SECTION

Instrumentation. The capillary electrophoresis instrument used in this study was an Agilent Technologies ^{3D}CE (Waldbron, Germany). This instrument was equipped with a deuterium lamp and a photodiode array detector. The separation voltage was applied as specified in the text, and the temperature was maintained at 25 °C. Injections were performed hydrodynamically with a pressure of 50 mbar used with various injection times.

Various lengths of fused-silica capillaries (Polymicro Technologies Inc., Phoenix, AZ) of 75-μm inner diameter and 375-μm outer

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diameter or 50-μm inner diameter and 375-μm outer diameter were used in conjunction with the appropriate capillary alignment interfaces.

Melting points were measured on a Reichert Thermopan hot-stage apparatus and are reported as uncorrected values.

NMR spectra were recorded on a Varian Mercury Plus 300 spectrometer operating at 299.90 (^{1}H) and 75.41 MHz (^{13}C) and are referenced to Me_4Si. Assignments of the spectra for BMMPA were obtained from further COSY, HMQC, and HMBC experiments using appropriate pulse programs from the Varian library.

The electrospray mass spectrum (ESMS) was recorded on a Finnigan LCQ spectrometer using negative ion mode and water as the mobile phase.

Microanalysis was carried out on a Carlo Erba 112 Series elemental analyzer using standard conditions.

Reagents. 2,2-Bis(chloromethyl)propanoic acid, morpholine, and cetyltrimethylammonium bromide (CTAB) were obtained from Aldrich (Milwaukee, WI). Orange G (1-phenylazo-2-naphthol-6,8-disulfonic acid, disodium salt) of Standard Fluka quality was obtained from Fluka (Buchs, Switzerland). Hydroxypropyl methyl cellulose (HPMC) and tris(hydroxymethyl)aminomethane (Tris, 99.9+% ultrapure grade) were obtained from Aldrich. A 50% (w/w) solution of poly(ethylenimine) of average molecular weight 50−60 000 was obtained from Acros Organics (Geel, Belgium). Sodium or potassium salts of anionic analytes (AR grade) were obtained from APS (NSW, Australia), Sigma-Aldrich, and BDH (Victoria, Australia). Water treated with a Millipore (Bedford, MA) Milli-Q system was used.

Procedures. (1) Synthesis and Characterization of 2,2-Bis(4-morpholinylmethyl)propanoic acid (BMMPA). A solution of 2,2-bis(chloromethyl)propanoic acid (5 g) in morpholine (20 mL) was stirred at 70 °C for 12 h under nitrogen. The excess morpholine was removed under vacuum, and the residue was dissolved in water (50 mL). The solution was acidified with concentrated hydrochloric acid, washed with chloroform (3 × 20 mL), made alkaline with a sodium hydroxide solution (10 M), and again washed with chloroform (3 × 20 mL). The pH of the aqueous solution was then carefully adjusted to 6.5 with dilute hydrochloric acid, and the solvent was removed under vacuum. Soxhlet extraction of the dry residue with anhydrous diethyl ether (150 mL) for 48 h gave the product as a white solid (6.12 g, 77%): mp 145 °C; ^{1}H NMR (D_2O) δ 1.06 (s, 3H, CH_3), 2.85−2.96 (m, 6H), 3.05 (dt, J = 12.8, 4.6 Hz, 4H), 3.13 (d, J = 14.0 Hz, 2H), 3.75 (t, J = 4.6 Hz, 8H, OCH_2); ^{13}C NMR (D_2O) δ 22.8 (CH_3), 44.6 (C), 54.2 (N*C*H_2CH_2), 65.2 (O*C*H_2), 65.4 (N*C*H_2C), 181.1 (CO); ESMS *m/z* 271.1 (M − 1). Elemental analysis calculated for $C_{13}H_{24}N_2O_4$: C, 57.33; H, 8.88; N, 10.29. Found: C, 57.25; H, 8.87; N, 10.18.

Following the synthesis and identification, the BMMPA was characterized to satisfy requirements for purity. Relative purity was determined as 97.6% by determination of anionic impurities by CE using indirect photometric detection at 254 nm and migration time normalization of peak areas in a separation system using Tris-buffered chromate electrolyte.[11] Experimental conditions were as follows: capillary, fused silica, 0.560-m length, 0.645 m to detector, 50-μm i.d.; voltage, −30 kV; electrolyte, 5 mM CrO_4/Tris, 0.1 mM CTAB, pH 8.5; injection, 2 s at 5 mbar of a 0.2 M (54 mg/mL) aqueous solution of BMMPA. Contents of chloride (Cl^-) relative to BMMPA was determined as 0.39% (mol/mol) by CE using external standard calibration and a separation system using Tris-buffered chromate electrolyte[11] and indirect photometric detection.

Buffering capacity (β), defined as the number of moles of a strong acid (HCl), n_{HCl}, required to be added to a volume of 1 mL (V(mL)) of buffer solution to cause a pH change (ΔpH) of one unit[54] was measured using a method similar to that of Veraart et al.[55] The determinations were conducted for 0.100 mol/L solutions of the investigated buffers, using an addition of such a volume of 0.1 M HCl to cause a change in pH in the range of approximately 0.1−0.2, the exact value of the pH change was measured, and the buffering capacity was calculated as follows: $b = n_{HCl}/(V(\text{mL}) \cdot \Delta\text{pH})$.

(2) Purification of Orange G. Orange G was purified by recrystallization from a 3:1 solution of ethanol−water.[56] Two successive recrystallizations were performed to ensure adequate purity. Determination of anionic impurities was performed by CE using a Tris-buffered chromate electrolyte[11] and indirect photometric detection at 254 nm. Purity of the dye after successive recrystallizations was found to be 99%, compared with an initial purity of 80%.

(3) CE Separations. Fused-silica capillaries were coated with poly(ethylenimine) by flushing with 1 M sodium hydroxide for 30 min and water for 30 min, followed by a 4% poly(ethylenimine) solution for 1 h, which was left to stand in the capillary for 30 min. The capillary was finally flushed with water for 30 min before use.

Detection limits were calculated for a signal-to-noise ratio of three (S/N = 3). Separation efficiencies were calculated from peak widths at half-height.

(4) Software. The pK_a prediction software used was ACD pK_a v.5 of Advanced Chemistry Development (Toronto, Canada).

RESULTS AND DISCUSSION

Buffer Molecule Design. The philosophy of the chosen approach is based on some of the zwitterionic Good's buffers.[18,19] These have an anionic group (such as CO_2^- or SO_3^-) and an amine group exhibiting a protonation equilibrium, which is responsible for the buffering. However, differing from the original Good's buffers, the proposed isoelectric buffer molecules would have two buffering amino groups attached to the backbone instead of one, as illustrated in Figure 1. Such a molecule has a pI that falls between the pK_a values of the two amino groups. While the zwitterionic Good's buffers (general formula R_1R_2N-(backbone)-SO_3H) range in their overall charge from 0 (in acidic solution at pH values well below pK_{a2} and pK_{a3}) to −1 (in alkaline solution at pH values well above pK_{a2} and pK_{a3}), a molecule possessing two amino groups would be isoelectric.

The choice of the backbone of the buffer molecule is relatively straightforward and has the requirements that is should be hydrophilic to provide aqueous solubility and should be UV-transparent to prevent excessive background absorbance of the electrolyte. Aliphatic backbones similar to those in the existing

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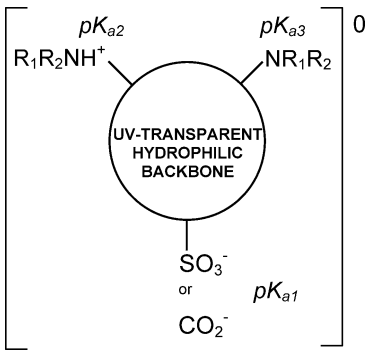

Figure 1. Design of IEB: generalized structures of two possible types.

zwitterionic Good's buffers are also suitable for the isoelectric ampholytes.

The choice of the amino groups can be guided by two criteria. First, the nature of the amino group determines the pK_{a2} and pK_{a3} and therefore also the pI. For an isoelectric ampholyte with pI in the neutral pH region, amino groups with relatively low pK_a value (such as morpholine, pK_a = 6.1) are suitable. Second, the two R_1R_2N groups should be similar or even identical so that pK_{a2} and pK_{a3} would be close ($|pI - pK_a| \leq 1.5$) for maximal buffering capacity at the pI. The choice of the anionic group is less critical. A sulfonate group is preferred as it has the advantage of ionization through the whole pH range, while exhibiting only relatively weak complexation equilibria with metal cations.[57] The use of a carboxylate group should also be possible in cases where the metal cation complexing properties of carboxylic groups would not pose a problem.

Model Compound BMMPA. In this work, a commercially available precursor dichloropivalic acid was chosen to simplify the synthesis of the isoelectric buffer compound used in this pilot study. A straightforward 1-step synthesis gives BMMPA, which has the protonation equilibria shown in Figure 2. BMMPA is a new compound, with structurally similar molecules previously reported as being only those with phenyl[58] or naphthyl[59,60] substituents in place of the methyl group and have been synthesized as target substances exhibiting choleretic activity.

There are two possible neutral forms of BMMPA, as indicated by the structures LH^0_1 and LH^0_2 in Figure 2. The structural properties of BMMPA indicate that the nitrogen atoms of the morpholine rings are likely to share the first associated proton (LH^0_2 in Figure 2), which will cause pK_{a2} and pK_{a3} to differ and will therefore affect the buffering capacity of BMMPA.

Useful structural information on BMMPA in aqueous solution was obtained by NMR investigations in deuterium oxide. The ^{1}H and ^{13}C NMR spectra of the neutral isoelectric compound can be fully assigned using COSY, HMQC, and HMBC experiments. The HMQC experiment showed that the H_B and H_B' hydrogens as well as the H_C and H_C' hydrogens are chemically nonequivalent (Figure 2). Although not unexpected, this is an important finding

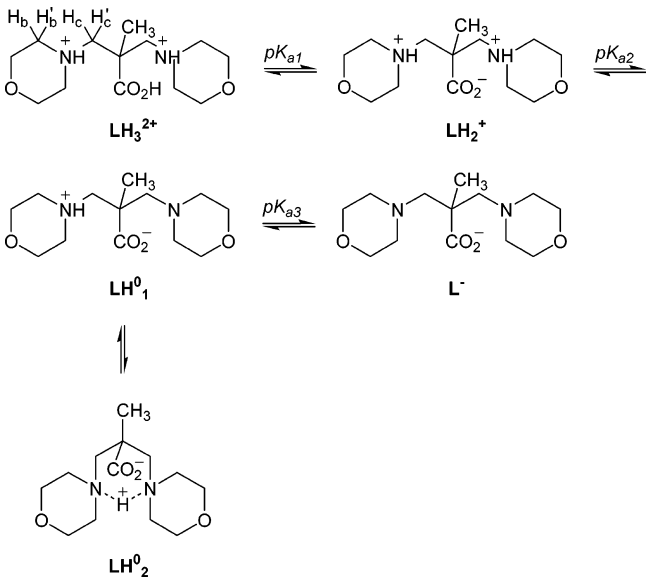

Figure 2. Structure of BMMPA and its protonation reactions.

as it confirms sharing of the H^+ of the isoelectric compound by both nitrogens (i.e., structure LH^0_2 in Figure 2), which blocks the free rotations and makes the molecule rigid. Further, using the integration of the ^{1}H NMR and the COSY experiment, the doublet at 3.13 ppm and part of the multiplet from 2.85 to 2.96 ppm were assigned as H_C and H_C', and the doublet of triplets at 3.05 ppm and part of the multiplet from 2.85 to 2.96 were assigned as H_B and H_B'. At lower pH, the signals for H_C and H_C' and H_B and H_B' collapse to a singlet and multiplet, respectively, indicating that the rigid form of the neutral molecule is due to the proton being shared by both nitrogens rather than to a steric hindrance to free rotation in the molecule.

pK_a and pI Values and Buffering Capacity. Estimation of the pK_a and pI values of the designed new compound using pK_a prediction software was investigated to determine whether this method could be used to assist in the design of a molecule having optimal pK_a and pI values. ACD pK_a DB software was used and is based on using Hammett-type equations, substituent constants, and literature references where available. An accuracy usually better than ±0.2 pH unit, but mostly not worse than ±0.5 pH unit, is claimed.[61] In the case of the BMMPA molecule, the constants pK_{a1}, pK_{a2}, and pK_{a3} were calculated as 2.70, 7.09, and 8.07, which would result in a pI value of 7.58, which is more than 1 pH unit higher than the experimentally determined pI (6.5, see below). This result can be attributed to significant shifts in the individual pK_a values resulting from sharing of the first associated proton by the two basic (amino) groups. Therefore, the isoelectric point (pI) has to be determined experimentally.

To determine the pI, a change in any of a range of physical parameters plotted versus pH can be used. Electrophoretic mobility should be particularly suitable, as the point where this parameter attains a zero value would also directly determine the isoelectric point. The determination of pK_a constants and pI by CE has been demonstrated for a series of isoelectric triazine derivatives, and it was shown that the pI values determined by

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Table 1. Buffering Capacities of BMMPA and Several Other Buffers, Together with Their pK_a and pI Values[a]

buffer	pK_a values	pI	concn (mM)	pH	buffering capacity β (mequiv L^{-1} pH^{-1}) expt	theory[b]
phosphate	2.12, 7.21, 12.67[63]	n/a	100	7.0	55	54.3
histidine	1.80, 6.04, 9.33[63]	7.7[25]	100	7.7	9.1	10.0
glutamic acid	2.13, 4.31, 9.76[64]	3.2[25]	100	3.3	31	32.2
BS albumin	n/a[c]	4.9[65]	1%	4.9	1.6	n/a
CMPEI	n/a[c]	6.8	100[c] (1.4%)	6.8	13	n/a
BMMPA	3.6, 4.9, 8.3	6.5	100	6.5	8.3	9.0

[a] Conditions: All buffering capacities were determined experimentally for the given experimental conditions (concentration and pH); for other conditions, see Experimental Section. BS albumin, bovine serum albumin; CMPEI, carboxymethylated poly(ethylenimine), concentration (in mM) expressed as of the corresponding monomer.[53] References are given for literature values of pK_a and pI. [b] Theoretical values were calculated according to the formula (eq 1)[67,68] $\beta = 2.303(([H^+] + (K_W/[H^+])) + (c_{H_2A}K_{a1}[H^+]([H^+]^2 + 4K_{a2}[H^+] + K_{a1}K_{a2}/([H^+]^2 + K_{a1}[H^+] + K_{a1}K_{a2}))))$. [c] Exact values not available for polymers.

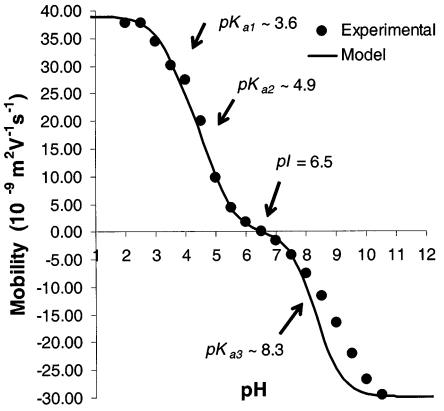

Figure 3. Change of BMMPA electrophoric mobility with pH. Conditions: capillary, α-chymotrypsinogen A-dextran sulfate (α-CA-DS) coated capillary,[66] (0.550 × 0.465) m, 50-μm i.d.; temperature, 25 °C; electrolyte, 10 mM phosphate; voltage, +30 kV; detection, 200 nm; injection 6 s at 50 mbar ~10.75 nL (1.37% capillary volume).

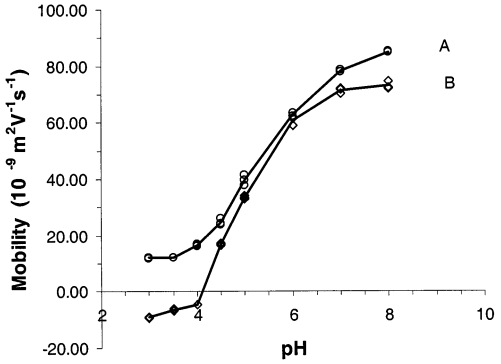

Figure 4. EOF vs pH profile for an electrolyte of 5 mM phosphate without BMMPA (A) and with 10 mM BMMPA (B) in a 75-μm-i.d. FS capillary. Conditions: capillary, fused silica (0.485 × 0.400) m, 50-μm i.d.; electrolyte, 5.0 mM phosphate, 0 (A) or 10.0 (B) mM BMMPA, pH 6.4; voltage, varying, −30 to +30 kV; detection, 254 nm; sample, ~1% (v/v) acetone in electrolyte; other conditions as in Figure 3.

CE agreed very well with those obtained from capillary isoelectric focusing.[62] While the pI is given directly by the pH corresponding to zero mobility, the pK_a constants are obtained by fitting a theoretical mobility versus pH curve calculated from first principles to the measured points. The measured BMMPA mobilities (dots) versus pH are plotted in Figure 3. The determined isoelectric point was pI = 6.5 ± 0.05.

Measurement of the individual pK_a constants can be performed by fitting a calculated (theoretical) curve based on the equation for effective mobility as a weighed average of mobilities of individual species (which depend on pH and pK_a values[62]). The fitted curve in Figure 3 resulted in values of pK_{a1} 3.6, pK_{a2} 4.9, and pK_{a3} ~8.3, but pK_{a3} could only be estimated approximately as the fitted curve in Figure 3 gave a poor fit in the alkaline region. It is clear that the constants pK_{a2} and pK_{a3} are set somewhat more apart ($|pI - pK_{a2}| \sim 1.6$) than desired optimally desired for a maximum buffering capacity. This is not unexpected because of the sharing of the first associated proton between both basic morpholine groups (structure LH^0_2 in Figure 2). However, the buffer capacity was determined experimentally to be 8.3 mequiv L^{-1} pH^{-1}, which is comparable to the isoelectric buffers histidine and carboxymethylated PEI (CMPEI) (see Table 1) with only glutamic acid having a substantially higher buffering capacity.

Effect of BMMPA in the Electrolyte on the Electroosmotic Flow (EOF). In CE, EOF and its characterization has great practical importance.[47] Any significant change in EOF caused by the addition of BMMPA would indicate a degree of adsorption onto the wall in fused-silica capillaries. Therefore, measurement of EOF versus pH in both the presence and absence of BMMPA in the electrolyte can be used to determine the extent to which BMMPA adsorbs onto the capillary wall. The results in Figure 4 show that there is some difference in the two EOF versus pH profiles, suggesting that there is some degree of interaction of BMMPA with the fused silica. However, this difference is not large, and the addition of BMMPA as a buffer to an existing electrolyte should not normally necessitate the complete redevelopment of an existing method.

UV/Visible Spectrum of BMMPA. Good low-UV transparency of a buffer has great practical importance in CE and is a major reason for the widespread usage of buffers such as

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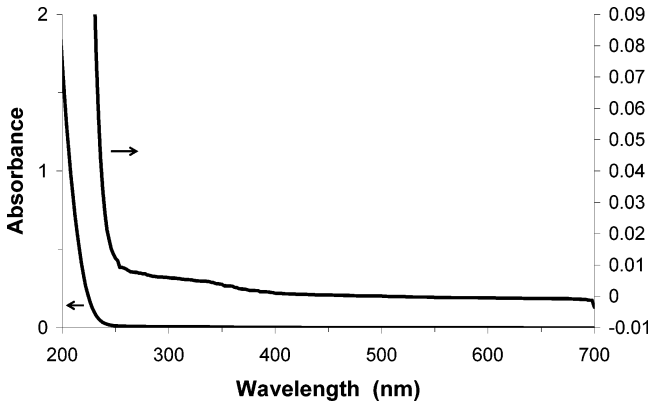

Figure 5. UV/visible spectrum of BMMPA. Conditions: 5 mM BMMPA in water, cuvette path length 1 cm.

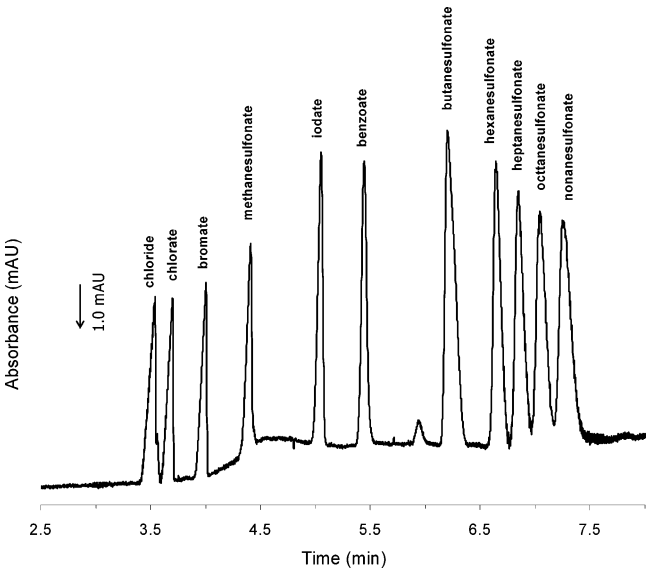

Figure 6. Electropherogram of 0.05 mM standard anions. Conditions: electrolyte, 4.0 mM Orange G, 5.0 mM BMMPA, 0.05% HPMC, pH 6.4; voltage, −10 kV; detection, 248 nm; sample, 0.05 mM each; other conditions as in Figure 3.

phosphate and borate. The UV/visible spectrum of BMMPA in water is presented in Figure 5 and shows that BMMPA has minimal UV absorbance at wavelengths above 240 nm, even with a path length of 1 cm, which is ~2 orders of magnitude larger than the typical path length used in CE and related capillary methods. Therefore, even at lower wavelengths (down to 190 nm), the background absorbance in a CE capillary is negligible. It can be anticipated that a major portion of the absorption in the low-UV region is to be ascribed to the carboxylate group, suggesting that the analogous buffer with a sulfonate group could offer somewhat lower absorption background.

Use of BMMPA as a Buffer in CE. BMMPA was used to buffer an electrolyte for high-sensitivity indirect detection of anions, based on the highly absorbing dye Orange G. A fused-silica capillary was coated with PEI to reduce the EOF and to minimize adsorption of the dye onto the capillary wall. An electrolyte containing 4.0 mM Orange G, 5.0 mM BMMPA, and 0.05% HPMC for EOF suppression (final pH 6.4) resulted in an EOF of $+4.0 \times 10^{-9}$ m^2 V^{-1} s^{-1}, and a typical separation of a model mixture of anions is presented in Figure 6. The analytical performance of the electrolyte was comparable with similar indirect photometric detection methods, and the use of BMMPA as a buffer did not lead to any negative side effects, such as introduction of system peaks due to additional co-ions. Importantly, BMMPA acted as an effective buffer and enabled the separation to be performed with a high degree of reproducibility.

CONCLUSIONS

A new class of synthetic low molecular weight isoelectric buffers has been presented possessing a general structure containing two identical (or very similar) basic groups and an acidic group (carboxylate or sulfonate). This structure results in an isoelectric compound, which should have pK_a values close to its pI and thus will provide buffering at the pI. By varying the basic groups, and also the structure of the backbone and especially its flexibility and ability to share the first associated proton between both the basic groups in the neutral molecule form, a wide variety of pI's should be available from the weakly acidic to strongly alkaline pH region. Sulfonate groups are the preferred functionality for applications where metal-complexing properties of the buffer are undesirable. These isoelectric buffers should primarily find use in CE, 2D electrophoresis, and related electroseparation methods, where their low conductivity or isoelectric properties can be best utilized, including use as low-conductivity buffers in preparative electroseparation methods. Further, they may find use as discrete ampholytes in isoelectric focusing and possibly also in liquid chromatographic methods, biochemistry, etc. It should be noted that, for their specific use as isoelectric buffers in CE with indirect detection, they should be free of ionic impurities.

BMMPA was prepared as a representative buffer, and it was found to have a pI of 6.5 and a moderate buffering capacity. Not unexpectedly, the pK_{a2} and pK_{a3} of BMMPA were somewhat different ($|pI - pK_a| \sim 1.6$) due to the sharing of a proton by the two morpholino nitrogen atoms. An optimal molecule would have the two basic groups attached to the backbone in such a way that they could not share a H^+, which should result in the pK_{a2} and pK_{a3} values being closer together, thereby providing a higher buffering capacity. BMMPA was used successfully as an isoelectric buffer for CE of anions with indirect detection of anions using a highly absorbing anionic dye Orange G as the indirect detection probe.

Molecular modeling could be helpful in the design of other isoelectric buffers by elucidating the conformations of the isoelectric molecule in aqueous solutions and also possibly in the prediction of the dissociation constants, the pI, and the buffering capacity.

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