Selectivity control in micellar electrokinetic chromatography of small peptides using mixed fluorocarbon–hydrocarbon anionic surfactants

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Abstract

Electrophoretic mobilities and capacity factors for a group of Trp-containing small peptides were determined by micellar electrokinetic chromatography (MEKC) using mixtures of a fluorocarbon anionic surfactant, lithium perfluorooctane sulfonate, and a hydrocarbon anionic surfactant, lithium dodecyl sulfate. Upon mixing these two surfactants, which have different microenvironments and interactive characteristics, greater control over migration of solutes is achieved. The changes in the composition of mixed micelles such as the mole fraction of the surfactants result in different solute–micelle binding as well as migration times of the micelles (t_{mc}). Consequently, capacity factor, selectivity and elution window (t_{mc}/t_0) change with the composition of the mixed micellar system. Another characteristic of the mixtures of fluorocarbon–hydrocarbon surfactants is the possibility of forming two different types of micelles which offers an additional partitioning process for each solute in the MEKC system. Such a unique phenomenon offers a higher degree of selectivity control. This mixed MEKC system is quite effective for the separation of small peptides. It provides an alternative to the free-solution capillary zone electrophoresis system for the separation of charged solutes with nearly identical electrophoretic mobility.

1. Introduction

The rapid development of capillary zone electrophoresis (CZE) during the past decade has shown that it is one of the most powerful separation techniques. As one of the early demonstrations of the analytical power of CZE [1], peptide separation will continue to be among the most common applications of this technique. Since CZE is a method strongly dependent on charge differences between analyte species, it has been considered as a complementary method to RP-HPLC for peptide mapping. The primary

factor for manipulation of selectivity of CZE separations is the buffer pH which alters the analytes' charges and, thus, their mobilities [2]. However, for the separation of peptides with high structural similarities, optimization of pH may not be adequate due to the nearly identical mobilities over the entire pH range.

Meanwhile, by introducing micelle-forming surfactants to the buffer medium, a dynamic partition mechanism of solutes into the micellar pseudo-stationary phase is established to supplement the differences in the electrophoretic mobilities of ionic analytes such as peptides [3]. Micellar electrokinetic chromatography (MEKC) incorporates both aspects of electrophoretic and chromatographic separations, so that subtle dif-

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ferences in the hydrophobicity, charge, size, and shape of analytes can be effectively utilized to enhance separation [4–7].

Since the first description by Terabe et al. [3], considerable attention has been paid to the effects of the composition of the micellar solutions on MEKC separations [8]. Greater control of migration behavior and selectivity has been achieved through manipulating several compositional parameters such as type and concentration of surfactants, addition of modifiers such as cyclodextrins, urea, or organic solvents, as well as pH and temperature. Among these approaches which have often resulted in the enhancement of resolution in MEKC separations, the type of surfactants generally influences migration patterns and exhibits great selectivity effects in MEKC. Partition coefficients of analytes into micelles and mobility of micelles depend upon the nature of the hydrophobic moiety, the charged head group, and perhaps even the counterion of the micelle-forming surfactants. Thus, one can tailor the structure of surfactants in MEKC in order to solve different separation problems.

One alternative to tailoring micellar microenvironments is to mix surfactants with different structural properties in order to manipulate solute–micelle interactions. By mixing two surfactants with different microenvironments and interactive characteristics, such as hydrocarbon and fluorocarbon functional groups, one can achieve a greater control over migration of solutes in electrokinetic chromatography (EKC). We refer to this mode as mixed micellar electrokinetic chromatography (MMEKC). The changes in the composition of mixed micelles (e.g. mole fraction of surfactants) would result in different solute–micelle partitioning. The use of mixed surfactants has attracted attention only recently. The majority of the reported use of mixed surfactants in MEKC, however, has involved addition of a non-ionic surfactant (such as Brij 35) to an ionic surfactant [9–11]. An extensive study has been initiated in this laboratory in exploring the usefulness of a wide variety of mixed micellar systems in EKC. Several mixed micellar systems such as the mixture of a fluoro-

carbon and a hydrocarbon micelles, the ternary mixture of these two micelles with Brij 35, mixed bile salt surfactants, and the binary, ternary, and even quaternary mixtures of a hydrocarbon [sodium dodecyl sulfate (SDS)] and different bile salts have already been explored [12–14].

The aim of this study was to evaluate a MMEKC system using mixtures of a fluorocarbon surfactant, lithium perfluorooctane sulfonate (LiPFOS) and a hydrocarbon surfactant, lithium dodecyl sulfate (LiDS), for the separation of charged solutes such as small peptides. Liu et al. have demonstrated successful separation of structurally similar small peptides and tryptic digests by using anionic (SDS) and cationic [dodecyltrimethylammonium bromide (DTAB), hexadecyltrimethylammonium bromide (HTAB)] surfactants as well as cyclodextrins [15]. Another interesting example of selectivity in MEKC separation of small peptides is the use of an anionic surfactant taurodeoxycholic acid (TDC) [16]. For large peptides, on the other hand, their interactions with micelles become too strong and the separation cannot be readily achieved. Using non-ionic surfactant (Tween 20) [17] as well as combining organic solvent with the cationic surfactant cetyltrimethylammonium bromide (CTAB) [18] were two successful approaches which reduced such interactions between peptides and micelles and led to the separations of angiotensins and motilins.

Liquid mixtures of fluorocarbon and hydrocarbon solvents exhibit large positive deviations from ideal solutions described by Raoult's Law. The abnormal interactions of fluorocarbon and hydrocarbon surfactants in micellar systems were first pointed out by Mukerjee et al. [19]. In addition to the intra-hydrophobic interactions between hydrocarbon–hydrocarbon, and fluorocarbon–fluorocarbon functional groups, there exists a hydrocarbon–fluorocarbon mutual phobia effect. This often results in the coexistence of two types of micelles (demicellization) in the range of the mole fraction where the critical micelle concentration (CMC) goes through a maximum (so called azeotropic point): one type of micelle is fluorocarbon-rich and the other one is rich in hydrocarbon surfactant. Some evi-

dences for demicellization in the mixture of LiPFOS and LiDS were reported by Asakawa et al. [20]. If such a condition exists under the operating conditions of MMEKC, higher degrees of selectivity control are expected to yield because of the presence of an additional partitioning process for each solute [12].

2. Experimental

2.1. Apparatus

All experiments were carried out on a laboratory-built CE system. It consisted of a 0–30 kV high-voltage power supply (Series EH; Glassman High Voltage, Whitehouse Station, NJ, USA), a variable-wavelength UV–Vis detector (Model 200; Linear Instruments, Reno, NV, USA) operating at 214 nm, and 52 μm I.D. × 375 μm O.D. fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ, USA). The total length of the capillary was 55.7 cm, and the polyimide coating was burned off 42.3 cm from the injection end to form the detection window. The capillary temperature was maintained at 40°C by jacketing in light mineral oil using a constant-temperature circulator (Type K2-R; Lauda, Germany). Electropherograms were recorded with an electronic integrator (HP3394A; Hewlett-Packard, Avondale, PA, USA).

2.2. Materials

Nine test peptides (from Sigma, St. Louis, MO, USA), listed in Table 1, were used as received. LiDS was also purchased from Sigma and LiPFOS was a gift from 3M (St. Paul, MN, USA).

2.3. Procedure

In order to maintain relatively low electrical current (especially at high surfactant concentrations), a pH 8 borate buffer with an ionic strength of only 5 mM was used in all MEKC

Table 1
Peptides separated

Peak	Peptide
1	Ala–Trp (AW)
2	Trp–Ala (WA)
3	Trp–Tyr (WY)
4	Glu–Trp (EW)
5	Trp–Gly–Gly (WGG)
6	Trp–Leu (WL)
7	Leu–Trp (LW)
8	Trp–Phe (WF)
9	Trp–Trp (WW)

experiments. The total surfactant concentration was varied from 75 to 150 mM and mixtures of LiDS and LiPFOS were prepared from stock solutions at different mole fractions. The sample injection end was raised manually to 3 cm high for hydrodynamic injection from the anode end of the capillary for 5 s.

A new capillary was flushed with 1 M LiOH for 1 h, 1 M HCl for 15 min and water for 15 min before the running buffer was introduced into the tube. The capillary was rinsed with the running buffers for 3 min and then with power applied for another 3 min between each run.

2.4. Determination of t_0 and t_{mc}

The micellar migration time (t_{mc}) and the migration time of the electroosmotic flow marker, i.e. "unretained" solute, (t_0) were obtained through an indirect method using homologous series of alkylphenones [21].

The electrophoretic mobility μ was determined by measurement of the migration times (t) relative to that of the electroosmotic flow marker (t_0) according to the following equation [1]:

$$\mu = \frac{L}{E} \cdot \left(\frac{1}{t} - \frac{1}{t_0} \right) \tag{1}$$

where L is the length of the capillary to the detector and E is the electric field strength.

3. Results and discussion

The high resolving power of CZE facilitates the separation of peptides that differ slightly in their mobilities. Depending on a particular peptide mixture, pH can be adjusted to control the net charge, and consequently the quality of separation as well as the analysis time. In Fig. 1a, the amino groups of the N-terminal of the nine Trp-containing small peptides are completely deprotonated at pH 12.4 while the carboxyl groups bear an identical negative charge, thus separation is mainly based on their size. As the pH of the buffer is decreased to 8.0, the net charges on the molecules are close to zero, so most peptides migrate near t_0 and the separation deteriorates. When the pH is further decreased to 2.5 (Fig. 1b), which is close to the pK_a values of the carboxyl group of C-terminal, the resolution is improved at the expense of the longer analysis time as compared to the previous two conditions. However, EW (peak 4) and WY (peak 3) co-migrate as well as peaks of WF (peak 8) and WW (peak 9) overlap. One benefit from choosing a low or a high pH is that the unwanted coulombic interaction between the peptides and the capillary wall is reduced. At low pH, the silanol groups on the capillary surface are protonated and there is little interaction between the positively charged peptides and the silica wall [22]. At high pH, on the other hand, peptides are negatively charged which results in an electrostatic repulsion between wall and analyte [23].

Fig. 2 shows the separations of peptides by MMEKC carried out at pH 8.0 at different LiPFOS mole fractions in a mixed micellar system of LiPFOS and LiDS at a constant total micelle concentration of 125 mM. Much better separation, reasonable peak shape, and analysis time are observed in the MMEKC system as compared to that obtained in the CZE mode (Fig. 1a and b). Interestingly large variations in selectivity are achieved as a result of changing the composition of the mixed micellar solution as evidenced by changes in the relative retention of the peaks and the frequent changes in the migration order of the bands. For example, EW
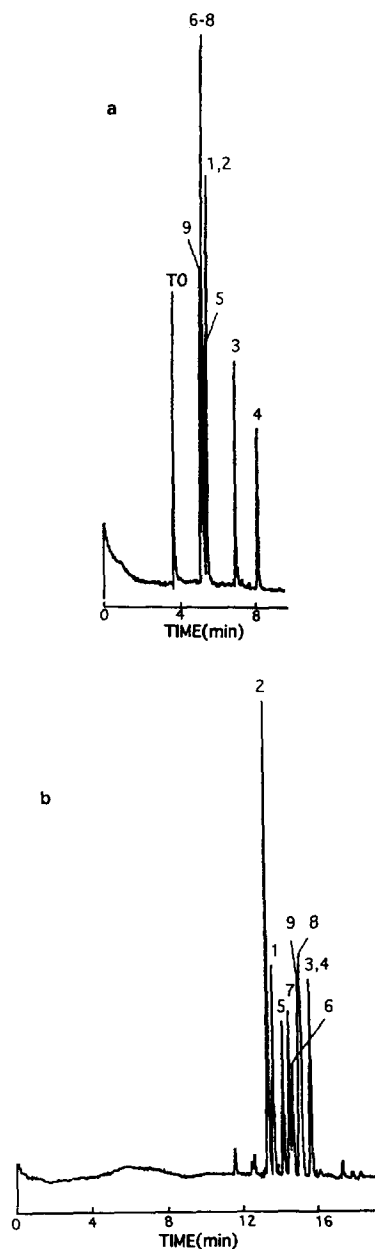

Fig. 1. CZE of peptides. Separation buffers and operating voltages are: (a) 100 mM phosphate–borate buffer (pH 12.4), 9 kV; (b) 100 mM phosphate buffer (pH 2.5), 15 kV. Peak numbers correspond to Table 1.

(peak 4), which migrates in the middle of chromatogram in a mixture of 100 mM LiDS–25 mM LiPFOS (Fig. 2a) is the last compound in 25 mM LiDS–100 mM LiPFOS mixed micelles (Fig. 2c).
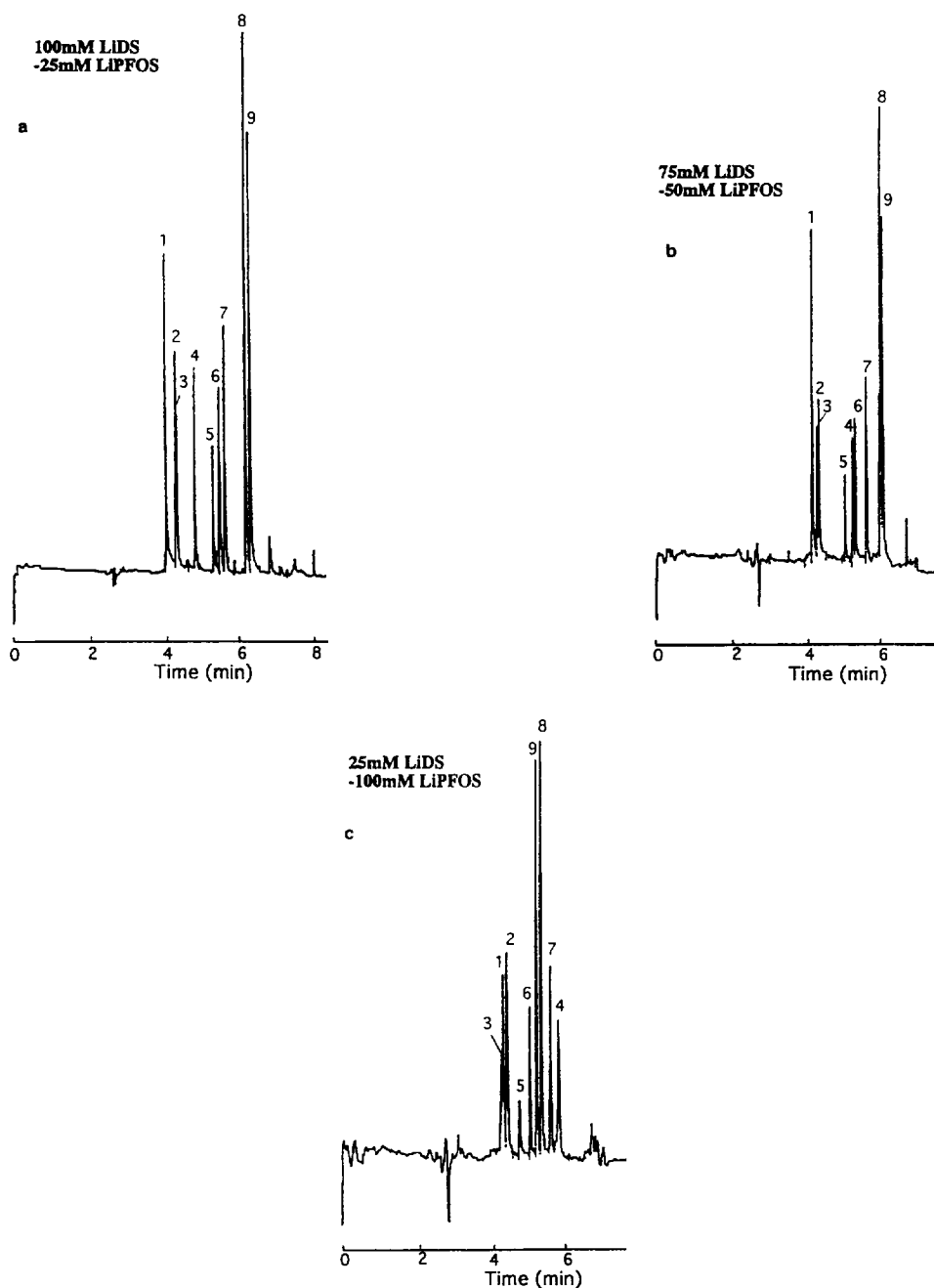
Fig. 2. MMEKC separation of peptides at 125 mM total micelle concentration. Mole fraction of LiPFOS: (a) 0.2, (b) 0.4 and (c) 0.8. Peak numbers correspond to Table 1. Operating voltage: 20 kV.

Capacity factor k' is an important migration parameter for both neutral and charged solutes in MEKC. It is defined as the ratio of the number of moles of solute associated with micelles to that in the aqueous phase. In other words, k' is the parameter that directly reflects

solute–micelle interactions. For charged solutes, it can be calculated from Eq. 2 [5,6]:

$$k' = \frac{\mu - \mu_0}{\mu_m - \mu} \qquad (2)$$

where μ_m is the mobility of the micelles and μ_0 is the mobility of the charged solute in the absence of micelles. Fig. 3 illustrates the effect of the mole fraction of the LiPFOS surfactant in the LiDS–LiPFOS mixed micelles on the capacity factor at 125 mM total micelle concentration. A similar trend was observed at other micelle concentrations. The overall k' values of these peptides in the fluorocarbon system are smaller than that in the hydrocarbon system which means that the solutes interact less with the LiPFOS micelles. This agrees with the phobia effect between the hydrocarbon and the fluorocarbon functional groups. Thus, the capacity factors of the majority of the peptides decrease upon increasing the mole fraction of the fluorocarbon surfactants. Among these peptides, capacity factors of the most hydrophobic compounds like WW, WF decrease to a larger extent with the increase in the LiPFOS mole fraction [X(LiPFOS)]. On the other hand, the capacity factors of the polar ones such as EW, AW, WA and WY change slightly over the entire mole fraction range because the k' values are less than

Fig. 3. Effect of changing micellar phase composition on capacity factor, k', of peptides. Total micelle concentration: 125 mM. pH 8.0. Experimental conditions as in Fig. 2. ■ = EW; □ = AW; ● = WA; ▽ = WY; ○ = LW; + = WGG; × = WL; | = WF; △ = WW; – – – = micelle.

1 which indicates these peptides prefer the aqueous over the micellar phase. For LW, WL and WGG with intermediate hydrophobic characteristics, the extents of reduction in k' are smaller than those for the hydrophobic peptides but larger than those for the polar ones.

Note that the capacity factors for most of the peptides (except hydrophobic WW and WF) either increase or remain constant in the region of X(LiPFOS) = 0–0.2. The exact reason for this behavior which has also been observed for uncharged aromatic compounds [12] is not known. However, this can be attributed to the dual nature of solutes interactions with the fluorocarbon micelles: first is the favorable hydrogen bonding interactions between the polar functional groups with the fluorocarbons and the second is the unfavorable phobia effect between the hydrocarbon moieties of the solutes and the fluorocarbon groups of micelles [24]. Apparently, the second effect is predominant at X(LiPFOS) > 0.20 for all solutes. This turning point as well as X(LiPFOS) = 0.4 separates the entire mole fraction range to three regions of A, B and C as shown in Fig. 3. In the region A, where the mole fraction of fluorocarbon surfactants varies from 0 to 0.2, WW and WF, which are very hydrophobic, interact less with the micellar phase of increasing fluorocarbon content, i.e., k' decreases with X(LiPFOS). On the other hand, polar compounds such as EW, AW and WA apparently favor the interaction with the fluorocarbon containing mixed micelles. When X(LiPFOS) further increases from 0.2 to 0.4 (region B), the phobia interaction between hydrocarbon and fluorocarbon functional groups becomes dominant and thus capacity factors of all peptides decrease. When the mole fraction of the fluorocarbon surfactant reaches 0.4, the capacity factors, k', of all peptides are below 2. Consequently the effect of changing composition by increasing fluorocarbon surfactants in micellar phases becomes less significant. The extent of reduction in k' upon increasing X(LiPFOS) in the region C becomes smaller than that in region B.

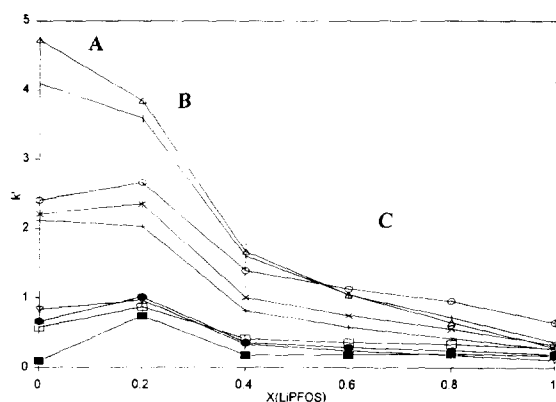It is important to note, however, that using k' as the migration parameter might be associated

with some uncertainties. One source of such uncertainties comes from the accuracy of determination of t_{mc} which is essential on obtaining k' values (Eq. 2). The problem of accurate measurement of t_{mc} in MEKC due to the lack of a reliable method is even more complicated in the MMEKC systems. This is especially true for the situation where there might be two distinctive types of micelles formed. Intuitively, one can conclude that net mobility is a more reliable parameter than capacity factor in MMEKC systems.

The net electrophoretic mobility μ of a charged solute in MEKC is the weighted sum of two mobility terms and can be expressed as [6]:

$$\mu = \frac{k'}{k'+1} \cdot \mu_m + \frac{1}{k'+1} \cdot \mu_0 \qquad (3)$$

In Eq. 3, the ratio $k'/(k'+1)$ represents the fraction of the solute that associates with the micelles and migrate at μ_m while $1/(k'+1)$ is the fraction of the charged solute in the aqueous phase and moving at μ_0. Therefore, the first term on the right side of the Eq. 3 can be regarded as an electrokinetic term and the second one an electrophoretic term. Fig. 4 illustrates the effect of mixed micellar composition on these two terms. The electrokinetic terms decrease for the peptides (except EW) upon increasing the concentration of the fluorocarbon surfactant after $X(\text{LiPFOS}) = 0.20$ (Fig. 4a), which indicates that these solutes interact more with the hydrocarbon surfactants than the fluorocarbon surfactants. The electrophoretic term, on the other hand, remained nearly unchanged or even increased (Fig. 4b). Obviously, upon the reduction of peptide–micelle interactions (i.e., decrease of the electrokinetic term) with an increase in $X(\text{LiPFOS})$, the electrophoretic effect plays a larger role at higher $X(\text{LiPFOS})$. The contribution from the electrokinetic term to overall mobility, however, is predominant as compared with that from the electrophoretic term.

The variations in peptides net mobilities as a function of the mole fraction of LiPFOS are nearly linear (Fig. 5). In spite of the increase in the mobility of the mixed micellar phase with the $X(\text{LiPFOS})$, the net mobilities for all peptides
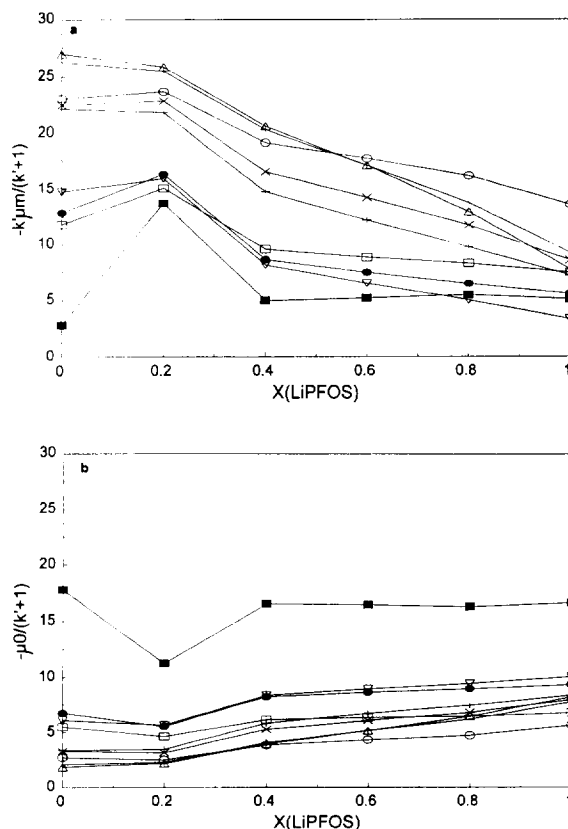

Fig. 4. Effect of changing micellar phase composition on (a) $-[k'/(k'+1)]\mu_m$ term and (b) $-[1/(k'+1)]\mu_0$ term in Eq. 3. Other conditions as in Fig. 3.
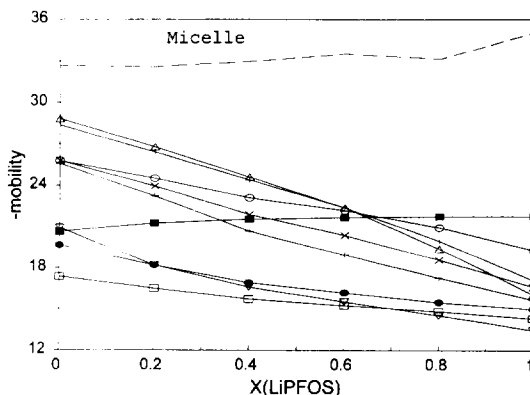

Fig. 5. Effect of changing micellar phase composition on the net mobility of peptides. Other conditions as in Fig. 3.

(except EW) decrease. At pH 8, these solutes carry net negative charges and move with the anionic micelles towards the anode, therefore, more negative values indicate larger mobilities. Meanwhile, the electroosmotic mobility μ_{EOF} is larger than the mobilities of both micelles and the peptides which results in their net migration towards the cathode where the detector is located. EW which apparently has the largest negative charge among these peptides is probably electrostatically repelled from the anionic micelles and is almost insensitive to the changes of the micellar composition.

Elution window defined as the ratio of t_{mc} and t_0 is an important factor that influences resolution and depends on the composition of the mixed micelle in MMEKC. Since the LiPFOS micelle has a larger negative mobility than the LiDS micelle, the t_{mc}/t_0 ratio increases with X(LiPFOS) (Fig. 6). Note that μ_{EOF} is little affected by the compositional variations. Consequently, the resolution can be enhanced without an adverse effect of longer analysis times. This demonstrates another advantage of this approach over adding other modifiers such as organic solvent where the resolution is improved at the expense of longer analysis times.

One other important factor that influences mobility variations with the mixed micellar composition in MMEKC is the micelle-induced pK_a shift. The extent of ionization of ionizable solutes in the MMEKC system depends on the apparent ionization constant in micellar solution, $K_{a,app}$, which is different from the aqueous ionization constant, K_a, and can be expressed as [25]:

$$K_{a,app} = K_a \cdot \frac{K_{A^-}^m[M] + 1}{K_{HA}^m[M] + 1} \qquad (4)$$

where K^m values are the respective binding constants of the acid, HA, and its conjugated base, A^-, to the micelle. Because the electrostatic repulsion between the A^- and the head group of anionic micelles such as LiPFOS and LiDS greatly reduces solute–micelle interactions, the term $K_{A^-}^m[M]$ is negligible as compared to 1. So $K_{a,app}$ is a function of the binding constant of the fully protonated acid to micelle K_{HA}^m, micelle concentration [M], and aqueous K_a. In fluorocarbon–hydrocarbon mixed micellar system, the extent of pK_a shift caused by the compositional variation of the micellar phase is dependent on the difference in binding constants of the acid,
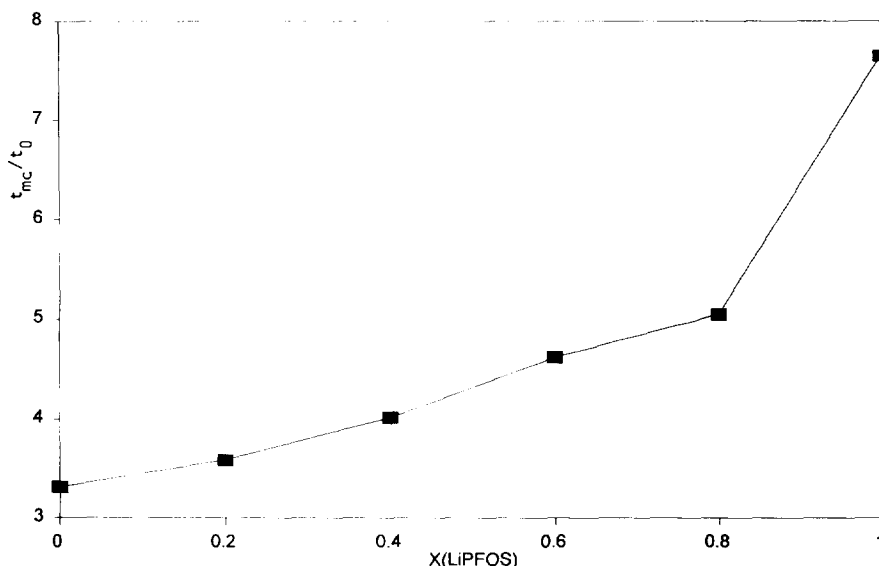

Fig. 6. Effect of changing micellar phase composition on elution window (t_{mc}/t_0) at 125 mM total micelle concentration.
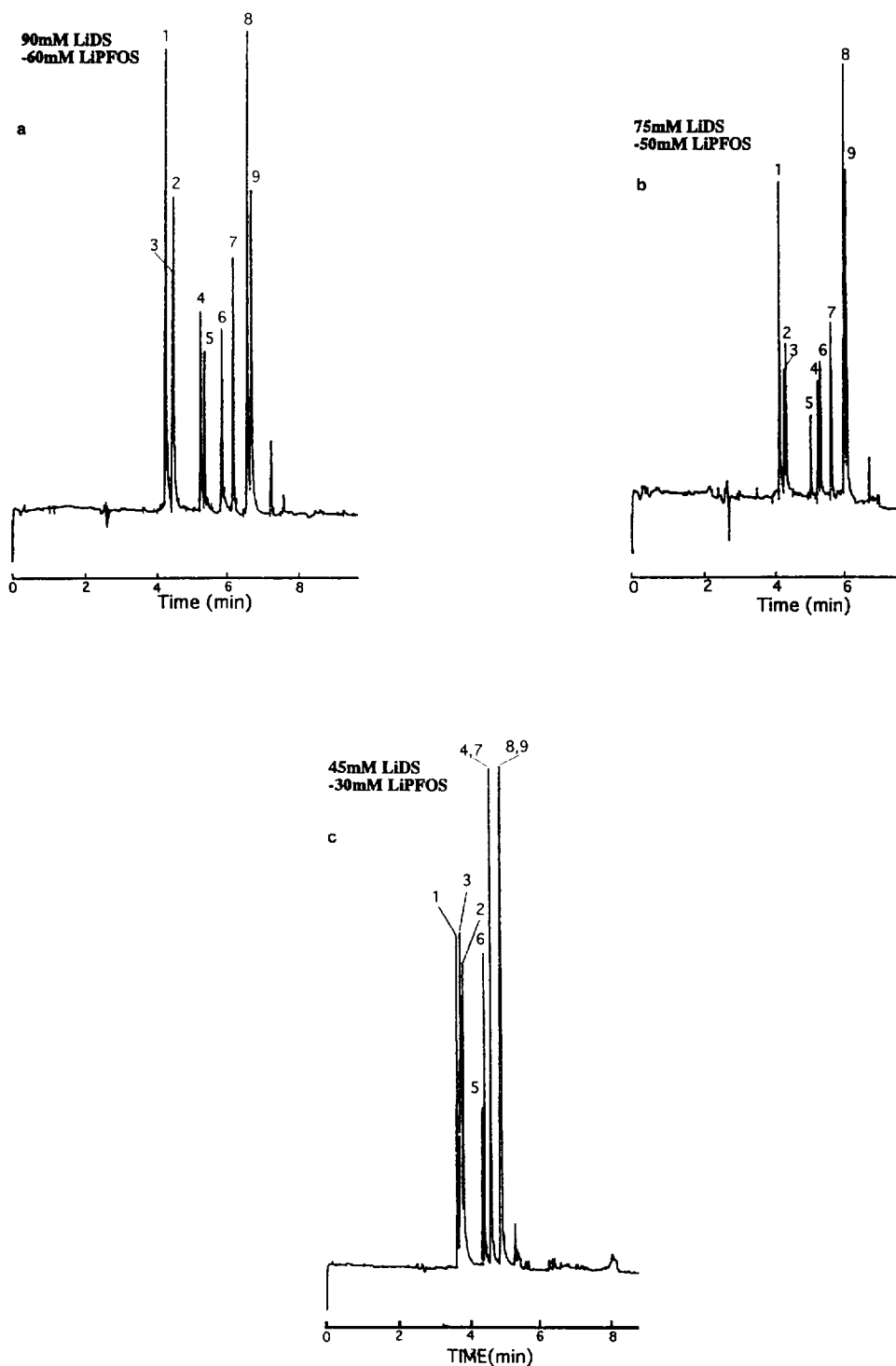
Fig. 7. MMEKC separation of peptides at X(LiPFOS) = 0.4. Total micelle concentration: (a) 150 mM, (b) 125 mM and (c) 75 mM. Peak numbers correspond to Table 1. Other conditions as in Fig. 2.

HA, to the hydrocarbon micelle (HC) and the fluorocarbon micelle (FC) and can be derived from Eq. 4 as:

$$pK_{a,app}^{HC} - pK_{a,app}^{FC} = \log (K_{HA}^{HC}[M] + 1)$$
$$- \log (K_{HA}^{FC}[M] + 1) \qquad (5)$$

According to Eq. 5, at a constant [M], the larger the difference between K_{HA}^{HC} and K_{HA}^{FC}, the larger the difference in $pK_{a,app}^{HC}$ and $pK_{a,app}^{FC}$. Therefore hydrophobic peptides such as WW and WF that have a larger difference in their binding to the hydrocarbon micelles and the fluorocarbon micelles are expected to have larger pK_a shifts with the mixed micellar composition. Consequently, the degree of variation of their electrophoretic terms with X(LiPFOS) for these compounds is greater (Fig. 4b).

Fig. 7 shows the effect of the total micelle concentration on the separation of the peptide mixture at X(LiPFOS) = 0.4. Except EW (peak 4), changes in the overall migration patterns are relatively small compared to the previous set of separation (Fig. 2). For uncharged solutes, total micelle concentration only affects k' and elution window (t_{mc}/t_0) with little or no impact on selectivity. For charged molecules such as peptides, the effect of total surfactant concentration on selectivity can take place through the different magnitude of the micelle-induced pK_a shift (Eqs. 4 and 5). Apparently, this phenomenon is not much pronounced for the mixtures at the operating conditions.

Acknowledgement

References

[1] J.W. Jorgenson and K.D. Lukacs, Anal. Chem., 53 (1981) 1298.

[2] S.C. Smith and M.G. Khaledi, Anal. Chem., 65 (1993) 193.

[3] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, Anal. Chem., 56 (1984) 111.

[4] S.C. Smith and M.G. Khaledi, J. Chromatogr., 632 (1993) 177.

[5] J.K. Strasters and M.G. Khaledi, Anal. Chem., 63 (1991) 2503.

[6] M.G. Khaledi, S.C. Smith and J.K. Strasters, Anal. Chem., 63 (1991) 1820.

[7] C. Quang, J.K. Strasters and M.G. Khaledi, Anal. Chem., 66 (1994) 1646.

[8] M.G. Khaledi, in J. Landers (Editor), Handbook of Capillary Electrophoresis: A Practical Approach, CRC Press, Boca Raton, FL, 1993, p. 43.

[9] E.L. Little and J.P. Foley, J. Microcol. Sep., 4 (1992) 145.

[10] H.T. Rasmussen, L.K. Goebel and H.M. McNair, J. Chromatogr., 517 (1990) 549.

[11] B.R. Thomas and S. Ghodbane, J. Liq. Chromatogr., 16 (1993) 1983.

[12] M.G. Khaledi and M.R. Hadjmohammadi, in preparation.

[13] J.G. Bumgarner and M.G. Khaledi, Electrophoresis, 15 (1994) 1260.

[14] M.G. Khaledi, R.S. Sahota, C. Quang, J.K. Strasters and S.C. Smith, in N. Guzman (Editor), Capillary Electrophoresis Technology, Marcel Dekker, New York, 1993, p. 185.

[15] J. Liu, K.A. Cobb and M. Novotny, J. Chromatogr., 519 (1990) 189.

[16] I. Beijersten and D. Westerlund, Anal. Chem., 65 (1993) 3484.

[17] N. Matsubara and S. Terabe, Chromatographia, 34 (1992) 493.

[18] T. Yashima, A. Tsuchiya, O. Morita and S. Terabe, Anal. Chem., 64 (1992) 2981.

[19] P. Mukerjee and A.Y.S. Yang, J. Phys. Chem., 80 (1976) 1388.

[20] T. Asakawa, K. Johten, S. Miyagishi and M. Nishida, Langmuir, 4 (1988) 136.

[21] D.M. Smith, J.K. Strasters and M.G. Khaledi, unpublished results.

[22] R.M. McCormick, Anal. Chem., 60 (1988) 2322.

[23] H.H. Lauer and D. McManigill, Anal. Chem., 58 (1986) 166.

[24] S. Yang and M.G. Khaledi, Anal. Chem., in press.

[25] M.G. Khaledi and A.H. Rodgers, Anal. Chim. Acta, 239 (1990) 121.