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Capillary Electrophoresis Separation of Neutral Organic Compounds, Pharmaceutical Drugs, Proteins and Peptides, Enantiomers, and Anions

by

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TABLE OF CONTENTS

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ABSTRACT

Addition of a novel anionic surfactant, namely lauryl polyoxyethylene sulfate, to an aqueous-acetonitrile electrolyte makes it possible to separate nonionic organic compounds by capillary electrophoresis. Separation is based on differences in the association between analytes and the surfactant. Highly hydrophobic compounds such as polyaromatic hydrocarbons are well separated by this new surfactant. Migration times of analytes can be readily changed over an unusually large range by varying the additive concentration and the proportion of acetonitrile in the electrolyte. Several examples are given, including the separation of four methylbenz[a]anthracene isomers and the separation of normal and deuterated acetophenone.

The effect of adding this new surfactant to the acidic electrolyte was also investigated. Working at acidic condition at pH 2.4 has the advantage that electroosmotic flow is virtually eliminated. Neutral compounds are dragged by their association with the surfactant, moving as the same direction as the surfactant, resulting in fast separations. Basic compounds are also investigated under the same conditions. Excellent resolution of a mixture of 19 polyaromatic hydrocarbons was obtained in only 20 min.

Incorporation of cetyltrimethylammonium bromide in the electrolyte is shown to dynamically coat the capillary and reverse electroosmotic flow. The coating prevents basic proteins and peptides adsorption into the capillary wall, resulting in high separation efficiencies. A systematic study of experimental parameters demonstrated the importance of selecting a suitable buffer and an appropriate pH.

Chiral recognition mechanism is studied using several novel synthetic surfactants as chiral selectors, which are made from amino acids reacting with alkyl chloroformates. It was found that enantiomeric resolution can be readily manipulated by varying the alkyl groups (different chain lengths), amino acids and surfactant concentrations. Sulfonated ß-cyclodextrin is also employed as a chiral selector to compare chiral selectivity with these synthetic surfactants. A duo-chiral selectors system is further investigated for the possibility of eliminating some tedious steps in chiral compounds method development.

A satisfactory separation of both inorganic and organic anions is obtained using electrolyte solutions as high as 5 M sodium chloride using direct photometric detection. The temperature inside the capillary that resulted from Joule heating is calculated, and used to explain the unexpected fast and efficient separations. Since electroosmotic flow is suppressed, a quaternary ammonium additive (Q^+), which is normally used to reverse the electroosmotic flow in anion separations, is not utilized in our study. The effect of various salts on electrophoretic and electroosmotic mobility is further discussed. Several examples are given under high-salt conditions.

CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation begins with a general introduction containing a review of pertinent literature. This is followed by three research papers that are published or have been accepted for publication. The fourth paper is under preparation and the fifth paper has been submitted for publication. Permission from the publisher extending reproduction and distribution rights has been obtained. A general conclusion section follows these five papers. Each paper is similar to the published version, although additional figures and tables have been added. Figures and tables are contained in the text of the paper at the appropriate location. References cited within each paper are listed after the conclusions of each paper.

Capillary Electrophoresis

Capillary Electrophoresis (CE) is a technique for separating charged molecules based on their movement through a medium under the influence of an applied electric field. It was first introduced by Jorgenson and Lukacs in 1981 [1]. Over the last 20 years CE has demonstrated to be a fast and versatile analytical technique that combines simplicity with high reproducibility [2]. Narrow-diameter polyimide-coated fused silica capillaries assures flexibility and allows efficient heat dissipation, thereby permits the use of high field strength resulting in separation efficiencies over 10^5-10^6 theoretical plates. Complex mixtures of analytes can be resolved and recorded as sharp peaks due to the lower risk of zone

broadening. In its diverse modes of operation (capillary free zone electrophoresis (CZE) [3-5], micellar electrokinetic chromatography (MEKC) [6-10], capillary gel electrophoresis (CGE) [11-14], capillary isotacophoresis (CITP) [15-17], capillary isoelectric focusing (CIEF) [18-20], and capillary electrochromatography (CEC) [21-23]), CE can be applied to analyze a wide variety of analytes ranging from low molecular weight analytes such as inorganic anions [24-28], metal cations [29-32], drugs [33-35] to larger molecules such as oligosaccharides [36-40], peptides [11,41-43], proteins [5,44-46], DNA [46-50], bacteria [51,52], and single cells [53-55].

Separation by CE is based on different electrophoretic mobilities of ions (μ_{ep}, $cm^2/V \cdot s$), which are governed by their charge/size ratio [56],

$$\mu_{ep} = \frac{q}{6\pi\eta r} \tag{1}$$

where q is the net charge, η is the viscosity of the buffer, and r is the hydrated radius. According to Eq. 1, electrophoretic mobilities are independent of electric field (E) and capillary length (L). However, both mobilities (μ) and velocities (ν) can be measured experimentally:

$$\nu = \frac{L_d}{t_m} \tag{2}$$

$$\mu = \frac{\nu}{E} = \frac{L_d \cdot L_t}{t_m \cdot V} \tag{3}$$

where L_d is the length of the capillary to the detector, L_t is the total length of the capillary, t_m is the migration time, and V is the applied voltage.

A prominent phenomenon in CE is electroosmosis (EO). Electroosmosis occurs due to the surface charge on the wall of the capillary. An anionic charge on the capillary surface presumably owing to the ionization of silanol groups at most pH conditions results in the formation of an electrical double layer. When an electric field is applied, the layer of positive charge migrates toward the negative electrode. Since ions are solvated by water, the fluid in the buffer is mobilized as well and dragged along by the migrating cations, resulting in the bulk flow of liquid in the direction of the cathode, known as electroosmotic flow (EOF). The electroosmotic mobility (μ_{eo}) as defined by Smoluchowski in 1903 is given by

$$\mu_{eo} = \frac{\varepsilon_0 \xi}{4\pi\eta} \tag{4}$$

where ϵ_0 is the dielectric constant, η is the viscosity of the buffer, and ξ is the zeta potential on the surface. The magnitude of the EOF is largely affected by the pH of the solution. This is because the degree of dissociation of the silanol groups (which has a pK_a of 6-7) on the capillary wall is dependent upon the pH of the solution, and so is the zeta potential. Other experimental conditions, such as temperature, the buffer concentration, organic solvents concentration, and chemical additives, can also be manipulated to vary both magnitude and direction of the EOF.

The measured mobilities according to Eq. 2 are truly the sum of the electrophoretic (μ_{ep}) and electroosmotic mobilities (μ_{eo}):

$$\mu = \mu_{ep} + \mu_{eo} \tag{5}$$

Micellar Electrokinetic Chromatography

Neutral solutes cannot be separated by CZE due to their zero electrophoretic mobilities. It was in 1984 when Terabe and co-workers [6] introduced micellar electrokinetic chromatography (MEKC), and both neutral and charged solutes can be separated simultaneously using the same CZE instrumentation. An anionic surfactant, such as sodium dodecyl sulfate (SDS), is added in the buffer system. When the concentration of the surfactant exceeds its critical micelle concentration (cmc), surfactants incline to self-aggregate, such that hydrophobic tails form a nonpolar core while hydrophilic heads extend on the outer shell. This association of surfactant molecules is referred to as a micelle. For an anionic micelles, its electrophoretic mobility is toward the anode. However, under most conditions the micelle's electrophoretic velocity is not large enough to overcome the EOF, thus the micelle still migrates slowly toward the detector. This micellar medium provides a pseudostationary phase upon which analytes can interact. A cationic surfactant can be employed as well although a reversed electrode polarity has to be used.

The differential partitioning of solutes between the aqueous phase and the micelle results in different retention factors k', which is the ratio of the number of moles of solute in the micelle and aqueous phase. The migration time (t_m) for neutral compounds in MEKC can be expressed in term of retention factor k' [57]:

$$t_m = \frac{1+k'}{1+\frac{t_0}{t_{mc}}k'}t_0 \qquad (6)$$

Similarly, the resolution (Rs) is given by

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k'}{1+k'}\right)\left(\frac{1-\frac{t_0}{t_{mc}}}{1+\frac{t_0}{t_{mc}}k'}\right) \quad (7)$$

where N is the number of theoretical plates, α is the selectivity, t_0 is the migration time for unretained solutes, and t_{mc} is the time for the micelles to pass the column. The difference between conventional column chromatography and MEKC is the extra term t_0/t_{mc} in the denominator. When t_{mc} equals to infinity, Equation 6 and 7 reduce to conventional chromatographic expressions.

t_{mc}/t_0 is also called elution range and has a large impact on both peak capacity (n_c) and resolution (R_s). As the elution range increases, so do the peak capacity and the resolving power. A variety of conditions have been examined to evaluate their effect on elution range, including electroosmotic flow [58], buffer concentration [59], pH [60,61], surfactant concentration [58,59,62], surfactant type [63-65], organic modifier content [62,66-68], surfactant counterion [60,61,65,69,70], and mixed micelle composition [71,72].

Type of surfactants

The development of new micelle phases continues to be a very active area of research. Since sodium dodecyl sulfate (SDS) was introduced in 1984, a large variety of surfactants have been utilized in CE, including anionic, cationic, and zwitterionic, etc. The research presented in Chapter II involved the separation of neutral compounds using a novel synthetic anionic surfactant, namely lauryl poly(oxyethylene) sulfate. This new surfactant is shown to be very effective for the separation of compounds range from highly hydrophobic polycyclic hydrocarbon compounds to small polar compounds.

Anionic

Whereas sodium dodecylsulfate (SDS) is a widely used anionic surfactant in MEKC [73-75], other anionic surfactants have been utilized including sodium pentanesulfonate, sodium octanesulfonate, sodium octyl sulfate [76], sodium decyl sulfate [64], to name a few. Generally, surfactants with alkyl tails of C8 or less do not form micelles under conditions found in capillary electrophoresis and are added as ion-pairing reagents. Alternatively, surfactants with alkyl tails greater than C14 have poor aqueous solubility.

Ahuja and Foley [70] studied the influence of dodecyl sulfate counterion on efficiency, elution range, and resolution. They found that the elution range increases in the order from lithium, sodium, to potassium. Cole and Sepaniak [77] reported that use of bile salts such as sodium cholate or sodium deoxycholate as pseudostationary phases improved separations of hydrophobic compounds, and Nishi et al. [78] utilized bile salts to separate lipophilic corticosteroids and benzothiazepine analogues.

A double-chain surfactant, disodium 5,12-bis(dodecyloxymethyl)-4,7,10,13-tetraoxa-1,16-hexadecanedisulfonate, was employed by Tanaka and co-workers [79] for several substituted naphthalene and benzene derivatives, and it was found to exhibit remarkably different selectivity and a wider migration time window compared with SDS. Likewise, Shi and Fritz [80] utilized sodium dioctyl sulfosuccinate (DOSS) in an aqueous solution containing 40% (v/v) acetonitrile. They reported that different electrophoretic mobilities for nonionic organic compounds were created as a function of the strength of the analyte/DOSS association in a nonmicellar environment.

The work of Smith and El Rassi [81,82] focused on MEKC with in situ charged

micelles. The surface charge on micelles formed by complexation between octylglucoside surfactant and alkylborate could be varied by changing the operation parameters such as borate concentration and/or pH of the buffer. This in turn created micelles with a range of hydrophobic character.

Cationic

Cetyltrimethylammonium bromide (CTAB) [63] was found to be useful for the separation of phenolic carboxylic acids [83], beta-blockers [84], flavonoids [85], and nucleic acid constituents [86]. Other cationic surfactants with similar structure, such as dodecyltrimethylammonium bromide (DTAB), dodecyltrimethylammonium chloride (DTAC), and cetyltrimethylammonium chloride (CTAC), have been evaluated in MEKC by Burton et al. [64] and Otsuka et al. [87]

Jorgenson and co-workers [88] studied electrophoretic medium consisting 0.025 M tetrahexylammonium perchlorate in 1:1 (v/v) acetonitrile/water solvent. Electrophoretic separations of polycyclic aromatic hydrocarbons (PAHs) can be accomplished by solvophobic association with tetraalkylammonium ion. Shi and Fritz [89] utilized a tetraheptylammonium salt in an aqueous acetonitrile buffer to separate nonionic organic compounds.

Nonionic and Zwitterionic

A non-ionic surfactant (Tween 20) was found to be useful for the separation of dansylamino acids by Matsubara and Terabe [90]. A zwitterionic surfactant N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (SB-12) was utilized near its cmc for a peptide separation reported by Greve and co-workers [91].

Mixed systems

Foley's group studied an anionic-zwitterionic mixed micelles system, which consists of sodium dodecyl sulfate (SDS) and N-dodecyl-N,N-dimethylammonium-3-propane-1-sulfonic acid (SB-12) [72]. The Joule heating contributed by surfactants can be minimized by partially substituting a zwitterionic surfactant for the original charged surfactant. The same group [71] reported that a nearly infinite elution range in MEKC is feasible by using a nonionic/anionic mixed micellar system, which comprised of a mixture of nonionic poly(oxyethylene)(23) dodecanol (Brij-35) and anionic (SDS) surfactants.

Separation of hydrophobic cations under the mixed micellar system composed of SDS and Tween 20 was investigated by Esaka and co-workers [92]. Likewise, a mixed micellar system of SDS and sodium cholate was evaluated for the separation of highly hydrophobic compounds such as corticosteroids [93].

Cyclodextrins (CDs) have also been used with SDS micelles to separate corticosteroids [94], aromatic hydrocarbons [94], and polyaromatic hydrocarbons [95]. This separation mode is called CD-modified MEKC. Sepaniak et al. [96] employed a duo-CD system consisting of a neutral CD and a charged CD for separation of nonionizable solutes. Neutral CDs acted as a primary phase which transported with electroosmotic flow, and charged CDs as an electrophoretically mediated secondary phase. The specificity associated with solute-CD inclusion complexation provides unique elution orders that do not follow the hydrophobicity trends of MEKC. Moreover, capacity factors can be altered in a convenient and predictable fashion simply by changing the CD phase ratio. Likewise, Szolar and co-workers [97] used a mixture of neutral and anionic ß-CDs to demonstrate the principle of

separation based on differential partitioning of analytes between the cyclodextrins, and achieved good separations for PAH isomers, including benzo[a]pyrene and benzo[e]pyrene.

Polymers

Palmer et al. [98] utilized oligomerized sodium 10-undecylenate to form a stable monomolecular pseudostationary phase. Tanaka and co-workers [99] employed starburst dendrimers (SBDs) (poly(amidoamines)) as a pseudostationary phase. The separation of neutral aromatic compounds was found to be influenced by the size and charge state of the SBDs.

Terabe and Isemura [100] used poly(diallyldimethylammonium chloride) (PDDAC) and (diethylamino)ethyldextran (DEAE-dextran) to separate isomeric ions having identical electrophoretic mobilities, for instance, 1- and 2- naphthalenesulfonate.

Resorcarenes, macrocyclic molecules built up by four alkylidene-bridged resorcinol units, have been employed as new pseudostationary phases to separate 12 PAHs by Bachmann and co-workers [101]. Similarly, Sun et al. [102] reported that calixarenes, a class of macrocyclic phenolic compounds with a basket-like shape, were used for separations of native and substituted polycyclic aromatic hydrocarbons.

Other types of alternate stationary phase include microemulsions [103], suspensions [104], and packed capillary [105].

pH

In chapter III of this dissertation, the use of extremely low pH ($\sim$ 2.4) for separation of neutral compounds is discussed. Again, the synthetic surfactant, Brij-S, is utilized as a pseudostationary phase.

Usually MEKC is done at basic pH, this is because stronger electroosmotic flow (EOF) is required to overcome the micellar electrophoretic mobility. Moreover, pH higher than 8 is necessary to achieve reproducible migration times. This is conformed by Shi and Fritz [80]. They found that electroosmotic flow reaches a maximum plateau above pH 8.0.

Some other pHs were occasionally utilized in MEKC depending on nature of the analytes. Fujiwara and Honda [65] employed a carrier containing 0.05 M SDS and 0.02 M phosphate at pH 11 to separate principal ingredients of antipyretic analgesics. While in another paper by Fujiwara and co-workers [64], pH 9.0 was found to be optimum to separate water-soluble vitamins.

Acidic pH system is seldom tried on MEKC. Sepaniak et al. [106] pointed out that separations at low pH (about pH 5) are impractical because the EOF is reduced, and is counterbalanced by the micelle electrophoretic mobility in the opposite direction, resulting in excessively long migration times. Terabe et al. [107] found that the poor reproducibility of migration times in bare-silica columns below pH 5.0 causes unreproducible results.

Recently, Janini and co-workers [108] demonstrated that separation of some hydrophobic compounds can be achieved at acidic pH on a polyacrylamide coated capillary (eliminates the EOF) by MEKC. Mclaughlin et al. [109] applied this mode of MEKC to separate linear alkyl benzene sulfonate (LAS) samples.

Organic modifiers

Gorse et al. [68] studied effects of organic mobile phase modifiers in MEKC. They found that the addition of 1-20% (v/v) of methanol or acetonitrile to the mobile phase is shown to extend the elution range and thus increase the peak capacity of a given system.

The efficiency of these MEKC systems is also increased with the addition of either organic modifier. Moreover, the two solvents show differing influences on selectivity depending on solute polarity. Sepaniak and co-workers [110] observed a reversal in elution order for substituted phenolic compounds using an acetonitrile step gradient. Fujiwara and Honda [111] examined the effect of addition of organic solvent on the separation of positional isomers in CZE.

Many organic modifiers are useful in MEKC. These include methanol, propanol, acetonitrile, tetrahydrofuran, dimethylformamide, and others. Adding an organic modifier to the buffer is also necessary for solutes of moderate-to-high hydrophobicity. The percent of a modifier that can be added is limited by the impact of the solvent on the micellar aggregate. Features such as the CMC, aggregation number, and micellar ionization are affected by the percent organic modifier. Since high percentage of organic solvent can disrupt micelle formation, the amount of organic solvent that can be employed in MEKC is usually limited to less than 30%. However, the elimination of micelles does not mean that separations will not occur. There are many examples [88,89,112] showing that even ionic surfactant monomers and neutral solutes can form charged solvophobic complexes that could be separated electrophoretically.

In Chapter II, the use of high percentage of acetonitrile in the separation of PAH compounds is discussed, while in Chapter III, the use of a mixture of two organic solvents is compared with one solvent alone.

Separation of Peptides and Proteins

Since the beginning of modern CE (early 1980s), the protein application was one of the first to be fully explored in the technique development. There are three basic CE techniques for protein and peptides, i.e., free solution capillary electrophoresis (FSCE), capillary gel electrophoresis (CGE), and capillary isoelectric focusing (CIEF).

FSCE

CZE is the predominant mode in FSCE for protein and peptide applications due to its simplicity, speed, and low reagent costs. Separation is based on differences in charge density. Surfactant-based MEKC is also used in this application. Although large protein molecules are too bulky to fully partition into micelles, surfactants might help to improve separation to some extent, possibly by an ion-pair or/and hydrophobic interaction between the surfactant molecule and the protein.

Proteins are polyelectrolytes, and a major concern in protein separations is that adsorption usually occurs because of coulombic attractions between the negatively charged capillary surface and the positive charges on the protein molecules. This results in either tailing peaks or even no peaks at all. There are several ways to suppress or eliminate protein adsorption, i.e., pH extremes, either dynamically or permanently coating the surface, and ion-pairing.

Luer and McManigil [113] performed protein separations at high pH. When pH is above the isoelectric point (pI) of a protein, the protein becomes negatively charged and tend to be repelled from the capillary surface.

McCormick [45] utilized low pH buffers to separate peptides and proteins. Most of

the negative charge on the capillary surface will be suppressed at low pH below 2. This means that the protein will not be electrostatically attracted to the neutral wall. A disadvantage of the extreme pH approach is that there is a wide rage of pHs which are unusable. Moreover, since proteins are either fully protonated or deprotonated at extreme pH, the separation for proteins varying only in subtle differences would be difficult.

Okafo et al. [114] utilized phytic acid as an ion-pairing reagent in order to suppress peptide adsorption. This anionic ion-pairing reagent reacts with cationic sites on the peptide molecule, making it electrophoretically negatively charged, thus eliminating its adsorption on the same charged capillary wall.

Another effective approach is to coat the capillary surface either dynamically or covalently. Covalent coating is achieved by attaching hydrophilic polymers to the silanol groups by using chemical derivatization procedures. The coatings which have appeared in the literature include methylcellulose, polyacrylamide, polyethyleneglycol, polyvinylpyrrolidone, 3-glycidoxypropyltrimethoxysilane, polyether, α-lactalbumin, etc.

Dynamic coating is performed by rinsing the capillary with an electrolyte in which various additives can be added. Green and Jorgenson [115] minimized adsorption of proteins by addition of alkali metal salts to the buffer. Since the salt competes for adsorption sites on the capillary wall with proteins, the higher the salt concentration, the higher the probability of salt adsorption. A drawback of this approach is that a high current and temperature are induced with a high concentration of the salt. Bushey and Jorgenson [116] later utilized zwitterionic salts such as tricine, CAPS, and CHES instead of ionic salts to reduce Joule heating. Since the zwitterions yield very low conductivity, up to more than

1 M of these salts may be used.

Addition of divalent amines to the buffer was found to reduce the EOF and suppress solute-capillary wall interactions [113]. This phenomenon seemed specific to diamino structure because the corresponding monoamino compounds were not effective. 1,4-diaminobutane, 1,5-diaminopentane, and 1,3-diaminopropane have been used for this purpose [117,118].

By decreasing the zeta potential on the capillary surface, protein adsorption can also be suppressed. Diverse cellulose derivatives (e.g., methylcellulose, hydroxyethylcellulose, and hydroxypropylmethylcellulose), nonionic surfactant, polyethylene oxide, polyvinyl alcohol, or polyethyleneglycol have been employed for this purpose. Moreover, these additives increase the viscosity of the buffer, therefore decrease the inclination for protein adsorption.

Cationic surfactants such as cetyltrimethylammonium bromide (CTAB) [119] may be used to reduce protein adsorption by creating a positively charged surface. Fuerstenau and co-workers [120] discovered that the zeta potential on quartz beads could be reversed in alkyltrimethylammonium salt solutions with alkyl chain lengths between C10 and C18. They postulated a concentration-dependent formation of a bilayer on the surface, or so-called hemimicelles, to explain the charge reversal.

Lucy and Underhill [121] investigated the characterization of the cationic surfactant induced reversal of EOF. They reported that nature of the anionic counterion in the solution has a strong effect on the magnitude of the reversed EOF observed.

Tsuda et al. [122] utilized the reversed electroosmotic flow by CTAB for the

separation of anionic analyte. Later, Zare and co-workers [123] investigated another additive, tetradecyltrimethylammonium bromide (TTAB) for separation of a mixture of fast and slow anions.

Zemann and Volgger [124] studied coelectroosmotic MEKC of phenols using CTAB as pseudostationary phase and acetonitrile as organic modifier. Fast separations for phenol isomers were shown.

In a study by Cifuentes et al. [125], different buffer additives, e.g., potassium chloride, morpholine, CTAB, poly(vinyl alcohol) and polyethyleneimine, were compared for the separation of basic proteins. CTAB was found to give the best separations.

Cordova and co-workers [126] examined four polycationic polymers for limiting the adsorption problem; polyethylenimine (MW=15,000), polybrene (MW=25,000), poly-(methoxyethoxyethyl) ethylenimine (MW=64,000), and poly(diallyldimethylammonium chloride) (MW=10,000). Detection of proteins with high pI was readily achieved using the first three of these polycationic polymer coating but not with the poly(diallydimethyl-ammonium chloride).

The research presented in Chapter IV dedicated to this CTAB approach. A low concentration of CTAB below its cmc was utilized to avoid protein adsorption. The experimental parameters, such as concentration of the buffer ions, types of buffer ions, organic solvent, were found to be critical in optimizing the protein resolution in our study.

CGE

In 1987 Cohen and Karger [11] first introduced crosslinked polyacrylamide gel in the capillary column to separation proteins. The gel structure creates a molecular sieving effect,

allowing the separation based on different size and charge. One drawback in the use of crosslinked polyacrylamide gels is the lack of low-UV transparency for on-column detection.

Later on, linear polymer matrices were explored, such as linear polyacrylamide, dextrans, polyethylene oxides, and polyvinyl alcohols. Not only these polymer networks allow the use in the low-UV region, they also can be easily replaced by a simple rinse. Compared to conventional SDS-PAGE slab gel electrophoresis, capillary gel electrophoresis (CGE) has roughly equivalent resolution, however it can be fully automated, the separation is fast, and electropherograms are stored permanently.

CIEF

In capillary isoelectric focusing (CIEF), proteins are separated by electrophoresis in a pH gradient [18], which is provided by carrier ampholytes consisting of polyamino-polycarboxylic acids with slightly different pI values.

The technique is developed on the basis of the difference in pI of different proteins. During a positively charged protein migrates toward the cathode, it encounters gradually higher pH, hence picking up more negative charges. Eventually, the protein stops when the net charge is zero. At this point (pI), the sample is focused in a narrow zone. Routinely, proteins differing by 0.01 pI units can be separated by CIEF.

The next step consist of transporting the focused zones past the detection point in the capillary, using either one of the three modes, e.g., chemical mobilization, hydrodynamic mobilization, and electroosmotic mobilization.

Separation of Enantiomers

Stereochemical resolution of optically active molecules remains an important and essential stage in many fields of study concerning the life sciences, as well as in the development of biological active chemical entities as potential drugs. The current analytical methods for the separation of enantiomers can be either gas chromatography (GC), high performance liquid chromatography (HPLC), thin layer chromatography (TLC), supercritical fluid chromatography (SFC), or capillary electrophoresis (CE).

Regardless of the separation technique employed, chiral recognition is obtained in one of three ways:

1. formation of diastereomers by additives to the mobile-phase or carrier electrolyte;

2. formation of diastereomers through integration with a stationary phase or heterogeneous carrier electrolyte; or

3. pre-column (capillary) derivatization with an optically pure derivatizing reagent.

In the first two cases, the techniques rely on the formation of transient metastable diastereoisomers between each enantiomer of an optically active compound and a chiral component, occurring by electrostatic and/or hydrophobic mechanisms. Chiral resolution of the two enantiomers is possible when there are sufficient differences in the free energies of formation of the two diastereoisomeric complexes. In the third case, covalently bound diastereomers are resolved on an achiral phase as a result of differences in their physicochemical properties.

Mostly, chiral resolution in CE involves the addition of chiral compounds to the buffer system. Alternatively, precapillary derivatization is also employed to create synthetic

diastereomers.

The most commonly used chiral additives in CE include: cyclodextrins (native and derivatized), metal-ion complexes, chiral crown ethers, chiral surfactants (naturally occurring and synthetic), acyclic carbohydrates (neutral oligosaccharides and charged polysaccharides), protein additives, and macrocyclic antibiotics (rifamycins and glycopeptide antibiotics).

Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides composed of between six and eight glucopyranosyl units to form a hollow cone structure. The outer surface are linked by hydrophilic primary and secondary hydroxyl groups, while the interior cavity consists of hydrophobic glycosidic bonds and carbon skeleton. Naturally occurring cyclodextrins contain either six (α-CD), seven (ß-CD), or eight (γ-CD) glucose units. There are two requirements for which the chiral discrimination can take place. One is that the analyte must have the appropriate structure geometry to fit into the CD cavity. The other is that polar groups should be in close proximity to the chiral carbon center.

Furuta and Doi [127] demonstrated that the size of the aromatic group in the analytes and the nature of the attached substituents influence the inclusion within the CD cavity. For instance, enantiomeric resolution of thiazole derivative was achieved using ß-CD and γ-CD, however, attempt to use α-CD was unsuccessful due to the poor fit of the molecule within the cavities of α-CD. As a general rule, analytes with unsubstituted aromatic rings fit perfectly in α-CD (cavity diameter, 5 to 6 Å); compounds with naphthalene groups or substituted phenyl rings suit in ß-CD (cavity diameter, 7 to 8 Å), while most complex polycyclic structures are able to include in γ-CD (cavity diameter, 9 to 10 Å).

Chemically modifying the hydroxyl groups at the 2, 3, and/or 6 positions by introducing a range of charged and uncharged functional groups can alter the physicochemical properties of CDs, such as the aqueous solubility, the overall hydrophobic character, the shape and size of their cavities, their hydrogen-bonding ability, and ultimately the enantioselectivity.

Uncharged CD derivatives can be synthesized by selective alkylation of hydroxyl groups at 2-, 3-, 6-, 2,3- or 3,6- positions by methyl, ethyl, hydroxypropyl, naphthylcaramoyl and glycosylation. Yoshinaga and Tanaka [128] compared native ß-CD with 3-methyl-ß-CD, 2,3-dimethyl-ß-CD, and 2,3,6-trimethyl-ß-CD for the separation of dansyl-DL-leucine, and found that a significant enhancement in the enantioselectivity was achieved by those derivatized CDs.

Charged CD derivatives are prepared by carboxyl-, succinyl-, sulfonyl-, aminoalkyl-, or sulfoalkylation of hydroxyl groups. One primary advantage of these charged CDs over their neutral relatives is that the separation window can be increased due to a larger difference between the electrophoretic mobilities of the analyte and that of the charged CD. Another benefit from using charged CDs is that different modes can be used depending on the pH of the buffer.

Ingelse and co-workers [129] utilized a soluble neutral ß-cyclodextrin polymer to enantiomeric resolve a range of cationic pharmaceutical racemates, e.g., selegine, clenbuterol, and beta-blockers. They found that the resolution is influenced by pH, temperature, organic solvents, and concentration of polymeric additive, as in the circumstance of their monomeric counterparts.

Chiral crown ethers

The first chiral crown ether, known as (2R, 3R, 11R, 12R)-(+)-1,4,7,10,13,16-hexaoxacyclooctadecane-2,3,11,12-tetracarboxylic acid, was reported by Kuhn et al. [130] for the separation of aromatic amino acids racemates. Enantiomeric discrimination by chiral crown ethers involves either a steric or stereoselective hydrogen-bonding interaction between the analyte and the carboxylic acids on the crown ether. A primary amino functional group in the analyte is required to allow the hydrogen bonding. This is why all enantioseparations involving chiral crown ether are carried out at low pH conditions.

Chiral surfactants

The research presented in Chapter V of this dissertation involved enantiomeric separation of pharmaceutical drugs using synthetic chiral surfactants, and studies of a mixed chiral selector system. An optically active surfactant usually possesses a chiral functional headgroup and an alkyl hydrophobic chain. The chiral discrimination mechanism is presumed to involve two steps, e.g., the first step consists of stereoselective interactions between functional groups on the chiral analyte and the optically active surface of the micelle, and the second step involves inclusion of the preferred isomer into the interior of the micelle. Bile salts [131,132], digitonin [133] and saponins [134] are examples of naturally occurring optically active detergents which have been used as chiral additives in CE.

The headgroups of synthetic chiral surfactants can either be enantiomers of amino acid derivatives, other chiral acids, or sugar derivatives. Dobashi et al. [131] reported the utilization of N-dodecanoyl-L-valinate and SDS to resolve the enantiomers of N-(3,5-

dinitrobenzoyl)-O-isopropyl esters (DNB) of four amino acids racemates. Besides L-valine, other amino acids have been used in the synthesis of chiral surfactants, such as L-threonine [136], L-alanine [137], and L-serine [137]. However, these surfactants showed lower α values for DNB-amino acids than the valine derivative.

Mazzeo and co-workers [138] introduced another class of synthetic chiral surfactants, e.g., (R)- and (S)-N-dodecoxycarbonyl-valines. These compounds have been found to have a broader enantioselectivity than N-dodecanoyl-L-valine counterpart, and shown to resolve a range of twelve pharmaceutical drugs including beta-blockers, bupivacaine, and homatropine. Reversal of the migration order of related isomers was simply accomplished by replacing an L-valine derived surfactant with the corresponding surfactant from D-valine. This is particularly critical in the determination of a small quantity of chiral impurities in a sample consisting predominantly of another isomer.

Other classes of synthetic chiral surfactant include dodecyl-ß-D-glucopyranosyl derivatives [139] and 2-undecyl-4-thiazolidine carboxylic acid [140].

Carbohydrates

These chiral selectors can be classified into two groups: noncyclic neutral oligosaccharides, e.g., maltodextrins, and charged polysaccharides, including dextran sulfate, heparin, and chondroitin sulfate C. D'Hulst and Verbeke [141] were the first to use maltodextrins to separate enantiomers of coumarinic anticoagulant drugs. Maltodextrins are formed by D-(+)-glucose units linked linearly through 1-4α glycosidic bonds. The conformation of these linear dextrins is thought to be a flexible random coil, which can change to a complete helix in the presence of an interacting analyte and buffer salts. The

flexibility of this structure allows them to interact with a wider range of molecular structures than cyclodextrins. It was found that the degree of enantiomeric resolution and selectivity is basically dependent on the degree of polymerization and molecular weight of the oligomer. Increasing the concentration of the chiral additive was also utilized to enhance the enantiomeric resolution.

Metal-ion complexes

Zare et al. [142] were the earliest to recognize the addition of Cu(II) and L-histidine to the buffer solution to resolve dansyl amino acids via a trimolecular complex. Later, aspartame, L-proline, and L-histidine were found to possess the structural components for chelation with the metal ion. This chiral separation method has mainly limited to the enantiomeric resolution of dansyl-DL-amino acids.

Proteins

The advantage in protein-based chiral separation is that there are many stereoselective bonding interactions involved, including hydrogen bonding, pi-pi, and steric effects. The term affinity electrokinetic chromatography (AEKC) has been applied to this mode of CE. Bovine serum albumin, α1-acid glycoprotein, human serum albumin, conalbumin, fungal cellulases, and ovomucoid have been used as protein additives in the resolution for enantiomers.

Antibiotics

Used as chiral selectors for LC, TLC, GC, SFC, and CE, macrocyclic antibiotics compounds were first introduced by Armstrong et al. [143-145]. This class of compound falls into two categories: ansamycin antibiotics (rifamycin B and SV) and the amphotiric

glycopeptide antibiotics (vancomycin, ristocetin A, and teicoplanin). Most of these antibiotics have multiple chiral carbon centers, and the presence of multiple stereogenic sites and functional groups makes them ideal candidates for stereoselective interactions.

High Salt Capillary Electrophoresis

It has been long believed that Joule heat, which is a result of the electric current passing through the electrophoresis buffer within the capillary, can have negative affect on the quality of the separation. The temperature gradients can cause natural convection, which will remix separated sample zones, distort the peak shapes, and damage the separation performance. There are several papers that devote to discussing the temperature profile inside the capillary, the thermal effects, and the zone spreading [146-148].

However, using a lower salt concentration in the sample than in the electrolyte to achieve electrostacking effect is used in many cases to improve separation efficiency and detectability. In a study by Chien and Burghi [149], an enhancement factor ~ 100 was achieve for the sample injection using electrostacking method.

Thornton and Fritz [28] separated inorganic anions employing an acidic buffer system, where the buffer contains 0.024 M hydrochloric acid. They reported that the greatest peak height occurred for $AuCl_4^-$ when they used a three to one ratio between the chloride concentrations of the carrier electrolyte and the sample.

Mclaughlin et al. [150] demonstrated the effect of the ionic strength of the running buffer on the migration and peak height of a standard mixture of bioactive peptides. The running buffer was sodium phosphate at pH 2.5. When its concentration went from 0.025

M to 0.125 M, a significant increase in peak efficiency and peak height was observed.

Jones [151] utilized a water jacket which surrounds a capillary to study temperature effect on seven inorganic anions. The ambient temperature around the capillary was readily changed by controlling the thermostatted water, however, the actual temperature inside the capillary was not measured. Temperature from 25°C to 60°C was studied. The fundamental effect of temperature is through the change of viscosity. It is estimated that temperature-induced changes in electrophoretic mobility are approximately 2%~3% per degree. The sequence of migration remains unchanged over the entire temperature range, and apparently the resolution does not deteriorate.

Improvements in separation efficiency at a higher temperature have been observed in MEKC by Balchunas and Sepaniak [152]. NBD-amines were separated at two different temperatures (e.g., 27 and 35°C) using a mobile phase containing 0.015 M SDS and 0.005 M Na_2HPO_4. Peaks were reported to be extremely sharp at the elevated temperature.

Jorgenson and co-workers [114] utilized high concentration of metal ions to suppress protein adsorption. However, low voltage was applied in order to avoid excess Joule heat, resulting in long migration times.

Chapter VI of this dissertation discusses the impact of high concentration of buffer ions on the separation of both inorganic and organic anions. The temperature rise inside the capillary is calculated. Direct analysis of samples with high salt content such as seawater is presented.

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CHAPTER 7. GENERAL CONCLUSIONS

A novel surfactant (lauryl polyoxyethylene sulfate) was developed to separate neutral organic compounds, especially for highly hydrophobic compounds. Its unique structure consisting of several oxyethylene groups, making it more hydrophilic than commonly used surfactant sodium dodecyl sulfate (SDS). It offers a significant advantage over SDS, such that it is suitable for a large variety compounds ranging from polyaromatic hydrocarbons (PAHs) to small polar compounds. Retention factor can be greatly increased by adding an organic solvent such as acetonitrile. A largest separation window is found where acetonitrile concentration is about 15-25%. Nonetheless, surfactant concentration is another experimental parameter that can be varied to manipulate migration times. Excellent separations are obtained for PAHs as well isomers, normal and deuterated acetophenones.

Separation of neutral compounds at acidic condition is successfully done for the first time. At pH 2.4, electroosmotic flow is virtually eliminate due to deprotonation of silanol groups on the capillary wall. This separation mode has several advantages over conventional MEKC (basic) mode. Fast migration times can be achieved because there is no counter-migration. Large, hydrophobic compounds migrate first owing to strong association with the surfactant. Second, acidic condition may add another dimension to basic compound separation owing to ion-pair interaction. The method gives excellent results for the separation of PAHs, basic compounds, alkyl substituted phenols, and acidic drugs and basic drugs. Using two organic solvents together, such as acetonitrile and isopropanol, different selectivity can be attained.

Protein or peptides adsorption on the capillary surface is effectively suppressed by incorporation of a cationic surfactant, namely cetyltrimethylammonium bromide (CTAB) in the running electrolyte. The surfactant can be employed at a low concentration below cmc so that peak broadening caused by protein-micelle interaction can be eliminated. High resolution and efficiency can be obtained after a suitable buffer and pH is carefully chosen. This method is proven to be simple and give excellent reproducibility.

Chiral selectivity in capillary electrophoresis can be manipulated easily by changing chiral surfactant structures, including in both chiral functional group and achiral hydrocarbon tail. Chiral resolution increases with the surfactant concentration before it reaches a maximum. An enhanced chiral resolution can be obtained by adding two totally different selectors in the same electrolyte. Baseline resolution of DL-dansyl amino acids enantiomers are attained using this mixture of selectors. It is concluded that combination is not confined to two selectors, more chiral additives can be used together to achieve some difficult enantiomeric separations.

It is found that good electropherograms can be obtained even with samples and electrolytes containing a very high concentration of salt, provided the electrolyte solution has a higher salt content (three times higher) than that of the sample. One advantage of the use of high salt content in electrolyte is that electroosmotic flow is reduced to a small value. A flow modifier, usually a quaternary ammonium (Q^+), is not needed in anion separations. This is particularly important for some anions that will precipitate with Q^+. Anions in seawater such as bromide and nitrate can be determined directly using the same salt (sodium chloride) in the electrolyte.

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