FLUORESCENT REAGENTS TO IMPROVE THE ANALYTICAL INFRASTRUCTURE OF CAPILLARY ELECTROPHORETIC SEPARATIONS

A Dissertation

by

MING-CHIEN LI

Submitted to the Office of Graduate Studies of Texas A&M University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Chemistry
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Approved by:

Chair of Committee, Gyula Vigh
Committee Members, Coran Watanabe
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Head of Department, David H. Russell

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ABSTRACT

Fluorescent Reagents to Improve the Analytical Infrastructure of Capillary Electrophoretic Separations.

(May 2012)

Ming-Chien Li, B.S., Tankang University;
M.S., National Tsing Hua University
Chair of Advisory Committee: Dr. Gyula Vigh

Two types of fluorescent molecules had been designed and synthesized to improve the analytical infrastructure of capillary electrophoretic separations. First, a hydrophilic version of the permanently cationic acridine-based fluorophore, HEG₂Me₂-DAA was synthesized. HEG₂Me₂-DAA has a $\lambda_{\text{ex}}^{\text{max}}$ of 490 nm which matches the 488 nm line of the commonly used argon ion laser. The emission spectra of HEG₂Me₂-DAA are pH-independent. HEG₂Me₂-DAA was used in capillary electrophoresis with an aqueous background electrolyte and was found to be free of the detrimental peak tailing of the acridine orange-based fluorophore that was caused by adsorption on the inner wall of the fused silica capillary. Bovine serum albumin was labeled with excess of the designed amine reactive reagent and the lowest concentration at which the tagged bovine serum albumin was tested was 15 nM. Chicken ovalbumin was also labeled with FL-CA-PFP and analyzed by capillary isoelectric focusing (cIEF) with LIF detection. The pI values of the tagged proteins shifted in the alkaline direction by about 0.02 compared to the pI
values of the non-tagged proteins. A tri-functional probe intended to enable selective enrichment and selective detection of a variety of molecules (e.g., natural products, pharmaceuticals, inhibitors, etc.) was also designed and synthetized by combining FL-CA with biotin and an azide group in a “proof-of-principle” level experiment.

In cIEF, the profile of the pH gradient can only be determined with the help of pI markers. A large set of pyrene-based fluorescent pI markers was rationally designed to cover the pI range 3 to 10. To prove the feasibility of the proposed synthetic approach, the subgroup of the pI markers having the greatest structural complexity was synthesized and characterized. The classical zone electrophoretic pI determination methods failed due to severe chromatographic retention of the APTS based pI markers on the capillary wall. Exploratory work was done to design a new pI value determination method that combines the advantages of the immobilized pH gradient technology of the OFFGEL instrument and the carrier-ampholyte-based IEF technology. The method aspects of cIEF have also been improved in this work. The new segmented loading method yielded a more linear pH gradient than the previously known cIEF methods. To exploit a unique property of the newly developed fluorescent pI markers, we used them as pyrene-based ampholytic carbohydrate derivatizing reagents. The pI4 carbohydrate derivatization reagent proved advantageous over 8-aminopyrene-1,3,6-trisulfonic acid (APTS): the pI4 conjugates have higher molar absorbance at 488 nm than the APTS conjugates and become detectable in positive ion mode of MS affording better detection sensitivity.
I would like to express my sincere gratitude to all my committee members for their help.

I am heartily thankful for the generous support and resources provided by Dr. Daniel Romo and his group which enabled the completion of this work.

I want to thank Dr. Roy Estrada who has been a great mentor to me.

Lastly, I want to thank my advisor, Dr. Gyula Vigh. Without his guidance, I could not have achieved my dream.

“A teacher affects eternity; he can never tell where his influence stops.”

– HENRY ADAMS
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1. INTRODUCTION

1.1 Fluorescent Labeling Reagents in Capillary Electrophoresis (CE)

1.1.1 Capillary Electrophoresis for Bioanalysis

In 1981, Jorgenson and Lukacs [1] performed the first high performance zone electrophoretic experiment in an open-tubular capillary of 75 µm i.d. with a separation potential of 30 kV. They successfully separated fluorescent derivatives of standard amino acids, dipeptides and amines, as well as components of a human urine sample, and detected them with a homemade on-column fluorescence detector. They demonstrated a separation efficiency of over 400,000 theoretical plates. CE is known as a rapid and high-resolution separation technique that can handle limited sample amounts such as those encountered in proteomic analysis, especially when coupled with on-line capillary separation-MS detection, allowing high-sensitivity detection, wide dynamic range and high throughput [2].

1.1.2 Fluorescence Detection in CE

Sensitivity is an important issue in bioanalysis where nanomolar or lower sample concentrations are common. The sensitivity of UV absorbance detection in CE suffers from the short light path length across the capillary: detection sensitivity is in the micromolar range. Laser-induced fluorescence (LIF) is one of the alternatives for

This dissertation follows the style and format of Electrophoresis.
superior detection sensitivity compared to UV absorbance detection [3]. In 1995, Yeung’s group [4] was able to monitor the reaction of a single molecule with LIF detection in a capillary.

1.1.3 Examples of Fluorescent Labels Used in CE

Compared to lasers that operate in the ultraviolet region, lasers that operate in the visible region are usually less expensive and more reliable. The argon-ion laser emitting at 488 nm is the most commonly used laser source with the commercially available CE instruments. A considerable variety of fluorescent dyes had been designed and synthesized [5-9]. One of the most common ways of labeling biomolecules with fluorescent dyes is by reacting them with the amino groups of the lysine residues since lysine is a relatively prevalent amino acid and is most often located at the surface of the protein. Here are some examples of amine-reactive fluorescent labels which can be excited with the argon ion laser [10].

1.1.3.1 Fluorescein [11]

![Figure 1.1 Structures of fluorescein-based amine-reactive labeling reagents [11].](image)
Fluorescein possesses high absorptivity and quantum yield which are ideal for highly sensitive detection. Fluorescein-based amine-reactive labeling reagents, structures shown in Figure 1.1, include fluorescein isothiocyanate (FITC), carboxyfluorescein succinimidyl ester (CFSE) and 5-(4,6-dichrolotriazinyl) aminofluorescein (DTAF): they have different labeling reactivities and efficiencies.

1.1.3.2 NBD Dyes [12]

![NBD Dyes Reaction Scheme]

Figure 1.2 Reaction scheme for labeling an amine with NBD-F [12].

The structure of 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) is shown in Figure 1.2. It has been reported that the fluorescence of NBD derivatives is independent of pH in the 2 to 9 range. However, the fluorescence intensity is sensitive to solvent polarity.
1.1.3.3 FQ and CBQCA [13, 14]

3-(2-Furoyl)quinoline-2-carboxyaldehyde (FQ) and 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) showed in Figure 1.3 are both quinolone based fluorogenic labels that react with amines forming fluorescent isoindoles.

1.2 Requirements and Limitations of Existing Fluorescent Labeling Reagents

High fluorescence intensity and good quantum yield basically define a good fluorophore, however, it’s not enough for fluorescent labeling reagents used in CE. The required properties and limitations of the current fluorescent labeling reagents for CE are discussed as follows.

1.2.1 Good Water Solubility

Labeling reagents with good water solubility are crucial in CE since most of the CE experiments are carried out in aqueous background electrolytes (BGEs). Water miscible organic solvents and detergents can be used to improve the solubility of the fluorophore but it doesn’t work for tests in which ionic strength and pH are critical. Moreover, if not
used in a hydrophilic coated capillary, there is hydrophobic interaction between the hydrophobic fluorophore core and the wall of the fused silica capillary that results in adsorption and seriously tailed peaks. It’s been reported that the solubilities of proteins were altered by labeling due to the hydrophobicity of Cy-dyes [16]. One way of making a more water soluble dye is by modifying the fluorophore with strong electrolyte functional groups. For example, introduction of sulfonate groups on cyanine dyes had been reported [17, 18].

1.2.2 pH-Dependent Fluorescence

pH is an important parameter in CE; some measurements will be hindered or limited if the fluorescent spectral properties of a fluorophore vary with pH of the BGEs. This property is especially critical in capillary isoelectric focusing (cIEF) analysis where the pH changes in the capillary, e.g., from 3 to 10. Fluorophores that have weak acid or weak base functional groups conjugated to an aromatic ring structure often display pH-dependent fluorescence. For instance, the fluorescence of rosamine showed in Figure 1.4 is less pH sensitive than that of fluorescein in acidic solutions due to the removal of the carboxylate group from the conjugation [19].

![Figure 1.4 Structures of fluorescein and rosamine [19].](image)
1.2.3 Changes in Analyte Properties After Labeling

Often, the properties of the analytes change after labeling. For example, the size, overall charge, hydrophobicity, etc. can change. A change in the charge-to-size ratio influences the electrophoretic mobility. If the degree of modification is relatively high and derivatization is random, a mixture of labeled products can be obtained leading to a group of distributed peaks in CE analysis. A more selective and specific labeling mechanism is the solution to the problem. In amine-reactive labeling, an amino group is replaced with a label and the overall charge of the analyte can be altered accordingly. Exchanging a basic amino group by an acidic moiety on a protein may lead to a noticeable change in the pI value which alters the results in cIEF analysis. In this case, a better approach would be a basic label which minimizes the change in the pI value of the analyte [20].

1.3 Capillary Isoelectric Focusing (cIEF)

1.3.1 Principle of cIEF

Isoelectric focusing (IEF) is a separation technique which separates ampholytic components based on differences in their isoelectric points. IEF is frequently used for the separation of peptides and proteins. Isoelectric point is defined as the pH at which the ampholyte is immobile in an electric field [21]. In IEF, since the surface charge of an ampholyte depends on the pH, the ampholyte moves with its corresponding mobility along the pH gradient until it reaches a position where its net mobility decreases to zero: the pH at this position indicates the pI value of the ampholyte. Capillary IEF (cIEF) is
an IEF process taking place in a capillary. A pH gradient can be created in a capillary by using a mixture of carrier ampholytes (CAs), i.e., a mixture of polyamino polycarboxylic acids. Before applying voltage, the pH is uniform in the capillary and it is set by the mixture of CAs. Once the voltage is switched on, electrophoretic transport causes the CAs to migrate based on their transient mobility determined by the environment. For example, take a CA with a pI value of 3 in a CA mixture of pI 3 to 10: it remains anionic throughout most of the capillary and tends to move toward the anode. It becomes neutral and stops moving until it gets protonated by a more acidic molecule. A higher degree of protonation is also possible: it converts the pI 3 CA into a cationic specie and makes it to move toward the cathode. This pendulum-like motion keeps the pI 3 CA in a confined band and if the concentration of the CA is high enough, sets the local pH equal to the pI of the CA. The same behavior applies to the rest of the CAs: the CAs get focused and stacked according to their pI values forming a pH gradient [22].

1.3.2 cIEF Technologies

1.3.2.1 Mobilization Mode and Detection

When a cIEF separation is monitored with a fixed, single-point detector, the focused, isoelectric, non-moving components need to be moved past the detector to obtain the cIEF trace. There are two types of mobilization techniques: “hydrodynamic” and “electrophoretic”. Hydrodynamic mobilization can be generated by either pressure [23, 24] or gravity [25]. An electric field is sometimes maintained during hydrodynamic mobilization to minimize the band broadening caused by the laminar flow [25-27].
Electrophoretic mobilization is also called chemical or salt mobilization: it can be done by exchanging the anolyte with the catholyte or *vice versa* [28]. For instance, by replacing the hydroxide ion in the catholyte by another anion, (*e.g.*, acetate), the pH will decrease in the cathodic end of the capillary, the high *pI* components become protonated and move past the detection window toward cathode. It is a cationic isotachophoretic (ITP) process with hydronium acting as the leading electrolyte [29]. In these “two-step” cIEF experiments, mobilization is initiated after focusing is finished. A moderately strong EOF had also been utilized in cIEF for mobilization. In this manner, focusing and mobilization occur simultaneously yielding what is considered a “one-step” cIEF process. One of the challenges in this case is to balance electrophoretic transport (focusing) and electroosmotic displacement to avoid incomplete focusing before the components are pumped out of the capillary [30, 31].

1.3.2.2 Capillary

Coated capillaries are used in most of the cIEF experiments for two main reasons, to minimize the adsorption of analytes on the capillary wall, and to control the EOF. Proteins and peptides are polyvalent ampholytes and their surface charges change depending on the pH: the resulting electrostatic interaction can cause adsorption on the bare fused silica wall. Depending on the structure of proteins, hydrophobic interactions are also possible with the wall of the fused silica capillary. A coating with neutral and hydrophilic surface properties is the most suitable choice for cIEF use. A linear polyacrylamide (LPA) coating was developed by Hjerten [32] which includes the
coupling of 3-methacryloxypropyltrimethoxysilane to the silanol groups of the capillary wall, and is followed by copolymerization with acrylamide. LPA-coated capillaries have been widely used in cIEF [33-35]. Gao and Liu [36] later improved the stability of the LPA coating by incorporating a cross-linking reagent to make crosslinked polyacrylamide (CPA). This coating covers the capillary wall more completely, leads to better suppression of the EOF and is more resistant to hydrolytic degradation. Hydrophilic polymers are also used to dynamically coat bare fused silica capillaries, alone or in combination with covalently coated capillaries [37]. For example, soluble cellulose derivatives [38, 39], poly(vinyl alcohol) (PVA), linear PAA [40] and poly(ethylene glycol) [31, 41] were used as dynamic coating agents in cIEF.

1.3.2.3 Additives

In cIEF, the analyte is concentrated during focusing. In addition, its solubility decreases when it reaches its pI. Sample precipitation after focusing is more pronounced for proteins. The additives used in the cIEF mixture to improve the solubility of proteins and avoid their precipitation are called solubilizers. The solubilizers have to be neutral or zwitterionic components: for example, urea [42, 43], glycerol [33, 44], ethylene glycol, propylene glycol [45], etc. Another type of additive used in cIEF is called a blocker or a sacrificial agent [46, 47]. Bidirectional isotachophoresis (ITP) in cIEF can cause the loss of CAs at both the acidic and basic edges of the pH gradient. To overcome this problem, one can add ampholytic components that have higher and lower pI values than the pI values of the CAs in the pH gradient. In this case, instead of losing
the CAs which participate in building the pH gradient, the blocker moves out of the capillary first by ITP leaving behind the CAs of interest in the capillary.

1.3.2.4 Carrier Ampholytes

“Two ampholytes, e.g. proteins can only be separated by stationary electrophoresis if there is a third ampholyte with an intermediate isoelectric point,” stated Vesterberg [48], based on Svensson’s work in 1962 [21]. Ampholytes are characterized by the number of protolytic groups and their corresponding pK_a values. For most of the low-molecular ampholytes, the pl value can be calculated from the two pK_a values that exert the greatest influence on the ionization of the molecule around the pl value according to the following equation (1.1) [21]:

$$pl = \frac{1}{2} (pK_{a1} + pK_{a2}) \quad (1.1)$$

where pK_a1 < pl < pK_a2.

For an ampholyte focused at its own pl, if its pK_a1 and pK_a2 values are close enough to each other, the focused band will contain - along with the zwitterions - an appreciable amount of cations and anions. These contribute to conductance over the pH gradient. Also, the buffering capacity of an ampholyte is proportional to the slope of the titration curve. In this case, the closer the two pK_a values, the more their dissociation steps overlap, which results in a steeper slope in the titration curve [49].
A good carrier ampholyte should have $\Delta pK_a$ ($pI-pK_a$) values as low as possible and no greater than 2 pH units. Righetti [50] called it the iron law of Svensson-Rible and this law can be understood by considering Figure 1.5 in which the buffering power ($\beta$) of both lysine and bicine are plotted against pH. Lysine has a $pI$ of 9.8 and exhibits a high buffering power at its $pI$. On the other hand, bicine which is one of Good’s buffers has a $pI$ of about 5.1 where the buffering capacity is close to zero because, as can be seen in the graph, the two $pK_a$ values of bicine are at least 6 pH units apart.

![Figure 1.5 The relationship of buffering power ($\beta$) and the $pK_a$ values which determine the $pI$ of lysine and bicine [50].](image)

Vesterberg [48] summarized the desired properties of carrier ampholytes as follows: (1) The carrier ampholytes should have a buffering capacity of at least 0.3 $\mu$equiv/ mg pH. (2) The adjacent carrier ampholytes should have $pI$ value differences of less than 0.1 to permit a reasonable resolution. (3) The $pI$ values of carrier ampholytes should cover the 2.5-11 range allowing the separation of most proteins. (4) Carrier ampholytes must have good water solubility to avoid their precipitation after focusing. (5) The carrier
ampholytes should have minimum light absorption. (6) The carrier ampholytes should be hydrophilic to prevent their adsorption on to proteins through hydrophobic interactions. (7) Their molecular weight should be low enough to permit their separation from proteins after focusing with simple methods, for instance, with molecular sieving. (8) The conductivity distribution created by carrier ampholytes should allow proteins to focus well in the pH gradient.

The first batch of CAs was designed and synthesized by Vesterberg in 1969 [49] by reacting acrylic acid with different linear oligoethylenimines making a series of homologues and isomers of polyamino polycarboxylic acids with different pI values. Vesterberg’s reaction scheme is shown in Figure 1.6.

\[
\begin{align*}
R_1 - \hat{\text{NH}}_4 - (\text{CH}_2)_n - \hat{\text{NH}}_4 - R_2 & \quad + \quad \text{CH}_2 = \text{CH} - \text{COO}^- \\
& \quad \overset{\text{=}}{=} \\
R_1 - \hat{\text{NH}}_4 - (\text{CH}_2)_n - \hat{\text{NH}} - R_1 \\
& \quad \overset{\text{=}}{=} \\
\text{CH}_2 - \text{CH}_2 - \text{COO}^-
\end{align*}
\]

Figure 1.6 Reaction scheme of making CAs according to Vesterberg [49].

Righetti et al. [50] developed a 3-D methodology to examine the carrier ampholytes (Pharmalyte, Ampholine, Bio-Lyte and Servalyte) produced by three companies. The first dimension was a “focusing” fractionation by a preparative isoelectric focusing device, the Rotofor [51, 52]; the second dimension was a capillary electrophoretic (CE) separation at very acidic pH providing all CAs with maximum net positive charge. All components eluted from the capillary were then analyzed by a mass spectrometer (MS)
as the third dimension. Based on the analysis, the molecular weights of CAs were found to be between 200 and 1,200. For a given molecular weight, the CAs in the acidic range had higher numbers of isoforms than in the basic range. The focusing behavior of CAs was correlated by the number of Rotofor fractions in which they were found: a decrease in focusing efficiency was seen from the acid (pH 2-4) to the basic (pH > 8) end of CAs.

1.4 pI Markers

A pI marker is an ampholytic compound with a known pI value and is used to profile the pH gradient. The theoretical background for the selection and design of pI markers is discussed in [21, 27, 53, 54]. The focusing ability of an ampholytic component in IEF dependents on its -dµ/d(pH) value at its pI, i.e. [-dµ/d(pH)]pI, where µ denotes the effective electrophoretic mobility of the ampholyte. Since µ is roughly proportional to the charge, [-dµ/d(pH)]pI is approximately proportional to [-dz/d(pH)]pI, where z is the net charge of an ampholyte at a given pH. The relation of [-dz/d(pH)]pI to the pKₐ values of the ampholyte can be described by Equation (1.2) [27]:

$$[-dz/d(pH)]_{pI} = 2.303 \sum \frac{K_{a_i}10^{-pI}}{(K_{a_i}+10^{-pI})^{2}} \quad (1.2)$$
The length of the focused ampholytes zones, expressed as the zone variance, $\sigma^2$, can be calculated as [54]:

$$\sigma^2 = \frac{RT}{FE[-dz/d(pH)]_{pI} d(pH)/dx}$$  \hspace{1cm} (1.3)

where $R$ is the universal gas constant, $F$ is the Faraday constant, $E$ is the electric field strength and $d(pH)/dx$ is the steepness of the pH gradient at the $pI$ value. Based on the above equations, the value of $[-dz/d(pH)]_{pI}$ becomes large when the $pK_a$ value is close to the $pI$ of the ampholyte. Since it is inversely proportional to $\sigma^2$, the greater the value of $[-dz/d(pH)]_{pI}$ the narrower the focused ampholyte band. It has been reported that an ampholyte should have a $[-dz/d(pH)]_{pI}$ value larger than 0.045 to focus into a band. The types of $pI$ markers are discussed in 3.1.1.
2. DEVELOPMENT OF A HYDROPHILIC ACRIDINE-BASED AMINE-REACTIVE FLUORESCENT LABELING REAGENT THAT HAS pH-INDEPENDENT SPECTRAL PROPERTIES

2.1 Design and Synthesis

2.1.1 Background and Objective

An ideal fluorescent labeling reagent for capillary electrophoresis should exhibit the following properties [55]: (1) high purity; (2) high solubility in water; (3) high stability of the conjugate; and (4) strong and pH-independent fluorescence emission of the conjugates. Acridine orange can be obtained in high purity and it has a visible-range $\lambda_{\text{ex}}^{\text{max}}$ around 490 nm making it compatible with the 488 nm line of the argon ion laser. In addition, it possesses pH-independent fluorescent properties. An acridine orange-based amine-reactive monocationic fluorescent labeling reagent [56], shown in Figure 2.1 (a), synthesized in our laboratory, has most of the desired properties mentioned above, except for good aqueous solubility. Acridine orange is not appreciably soluble in water.

Figure 2.1 (a) Acridine orange-based and (b) Me$_2$HEG$_2$-DAA-based amine-reactive fluorescent labeling reagent.
When an acridine orange-based fluorescent label was used in capillary electrophoresis with an aqueous background electrolyte, serious peak tailing was observed due to hydrophobic adsorption of the acridine orange core on the silica wall of the capillary. At least 20% of a water-miscible organic solvent, such as acetonitrile, dimethylformamide or dimethylsulfoxide had to be added to the background electrolyte for the acridine orange-based fluorophore to have a reasonably shaped peak. To improve the aqueous solubility of the core fluorophore while maintaining the desired spectral properties of the acridine-based fluorescent reagent, we designed a hydrophilic version of the acridine-based fluorophore by connecting oligo(ethylene glycol) groups to the acridine core structure (Figure 2.1 (b)).

Figure 2.2 Calculated logP of [(EtO)ₙ]₂-DAA as a function of the mer-number of the ethoxy groups.
Log$P$ serves as an indication of the hydrophobicity of a component as shown in Equation 2.1:

$$logP_{octanol \over water} = log\frac{[solute]_{octanol}}{[solute]_{water}}$$  \hspace{1cm} (2.1)

where $P$ is the partition coefficient, a ratio of the concentrations of a component partitioned between n-octanol and water. The calculated log$P$ values are plotted in Figure 2.2 as a function of the mer-number of the ethoxy groups on DAA. Acridine orange has a calculated log$P$ value of about 4.65. By replacing a methyl group on both amino groups with a tetra(ethylene glycol) (n = 4) group, calculated log$P$ drops to 2.33, representing a solubility in water that is about 200 times higher. To improve the solubility of the fluorescent label in aqueous solutions and decrease its hydrophobic adsorption on surfaces, we decided to make a hepta-ethoxy substituted DAA, n = 7, for which the calculated log$P$ value becomes -0.18.
Figure 2.3 Synthesis scheme for making HEG$_2$Me$_2$-DAAPS.

Figure 2.4 Synthesis scheme for elongation of the tether and activation of the HEG$_2$Me$_2$-DAA-based amine-reactive fluorescent labeling reagent.
The Me<sub>2</sub>HEG<sub>2</sub>-DAA-based amine-reactive fluorescent labeling reagent was synthesized according to the reaction schemes shown in Figures 2.3 and 2.4. First, (AcO)<sub>2</sub>-DAA was quaternarized with 1,3-propane sultone, followed by the hydrolysis of the amide groups to get DAAPS. Two mono-tosylated tetra(ethylene glycol) groups were connected to DAAPS through reductive amination of DAAPS and the tetra(ethylene glycol) chains were elongated with 2-(2-(2-methoxyethoxy)ethoxy)ethanol to make (HEG)<sub>2</sub>-DAAPS. To maintain the desired spectral properties of acridine orange, DAAPS needed to be tetraalkylated. Therefore, formaldehyde was used to alkylate the two secondary amino groups and make Me<sub>2</sub>(HEG)<sub>2</sub>-DAAPS. The fluorophore was then connected to methyl 6-aminohexanoate, followed by hydrolysis of the ester group to get FL-CA. FL-CA was then activated with pentafluorophenyl trifluoroacetate to form amine reactive FL-CAPFP.

2.1.2 Materials and Method

2.1.2.1 Chemicals and Equipment

3,6-Diaminoacridine (DAA), tetra(ethylene glycol), acetonitrile, dimethyl sulfoxide, dichloromethane, phosphorus pentoxide, triethylamine, sodium cyanoborohydride, 4-dimethylaminopyridine, pyridine, dimethylformamide, hydrochloric acid, sodium hydride, anhydrous tetrahydrofuran, methanol, oxalyl chloride, dichloroethane and pentafluorophenyl trifluoroacetate were purchased from Sigma-Aldrich Co. (St. Louis, MO). Water used in the HPLC and CE experiments was from Mallinckrodt Chemical Co. (St. Louis, MO). The source of calcium hydroxide used in this work was hydrated
lime (Voluntary Purchasing Group, Inc., Bonham, TX). Methyl 6-aminohexanoate used in the synthesis was made in-house by refluxing 6-aminocaproic acid with methanol with a catalytic amount of hydrochloric acid [57]. An HPLC system containing a solvent delivery module Model 126, an auto injector Model 508 and a photodiode array detector Model 168 operating under 32 Karat software control (Beckman-Coulter, Fullerton, CA) was used for reaction monitoring. RP separations were obtained on a 4.6 mm I.D., 75 or 150 mm long analytical column packed with a 3 µm Gemini C18 stationary phase (Phenomenex, Torrance, CA). LC-MS analysis was performed with an ion trap LC-MS 6300 system operating under ChemStation software control (Agilent Technologies, Santa Clara, CA). The column used in the LC-MS analysis was a 2 mm I.D., 150 mm long analytical column packed with a 3 µm Gemini C18 stationary phase (Phenomenex, Torrance, CA). The CE experiments were carried out with a ProteomLab PA 800 system coupled with a LIF detector using a 488 nm argon ion laser module (Beckman-Coulter, Fullerton, CA). Separations were obtained in 50 µm I.D., 360 µm O.D. bare fused silica capillary columns (Polymicro Technologies, Phoenix, AZ) having total and inlet-to-detector lengths of 30 and 20 cm, respectively.

2.1.2.2 Synthesis of Mono-Tosylated Tetra(Ethylene Glycol) (mTs-TEG)

![Chemical structure](image.png)

Figure 2.5 Reaction scheme for making mono-tosylated tetra(ethylene glycol).

```text
2.1.2.2 Synthesis of Mono-Tosylated Tetra(Ethylene Glycol) (mTs-TEG)

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**Figure 2.5 Reaction scheme for making mono-tosylated tetra(ethylene glycol).**
Reaction scheme for making mono-tosylated tetra(ethylene glycol) is shown in Figure 2.5. An 18.60 g portion of hydrated lime was mixed with 50 ml of acetonitrile and heated at 70 °C with stirring. 38.84 g of tetra(ethylene glycol) and 40.04 g of tosyl chloride were dissolved in 40 ml acetonitrile and carefully added into the heated acetonitrile slurry of lime. The reaction was carried at 70 °C with good stirring for 15 min, then the mixture was cooled with an ice bath. Progress of the reaction was monitored by gradient RP-HPLC with a PDA detector (3 µm, Gemini-C18 stationary phase, 75×4.6 mm column, 1 ml/min flow rate, initial mobile phase composition 60:40 A:B, gradient time 18 min, final mobile phase composition 100 % B, A: water; B: methanol, both with 5 mM ammonium formate, pH 4.5). Based on the integration of the peaks at 260 nm in the chromatogram, approximately 10 % of bis-tosylated tetra(ethylene glycol) was formed. Once the reaction was complete, the solids were removed from the reaction mixture by filtration. Then, the acetonitrile filtrate was diluted sixfold with water and the mixture was centrifuged. Bis-tosylated tetra(ethylene glycol) was separated from the mono-tosylated tetra(ethylene glycol) as an oil phase that formed at the bottom of the centrifuge tube. The tosylation reaction was done with 50 g tetra(ethylene glycol) batches and had a 67 % final, isolated yield with about 99 % purity (based on the integration of the peaks at 260 nm).

The conventional way of making mono-tosylated tetra(ethylene glycol) calls for reacting tosyl chloride with excess tetra(ethylene glycol) at low temperature (0 °C) under basic conditions. The reaction time is hours-to-days long and excess unreacted tetra(ethylene
glycol) makes purification troublesome [58]. In this work, the tosylation reaction was carried out at 70 °C and was completed in 15 min with only 1.05 equivalent of tetra(ethylene glycol). In this work, Ca(OH)$_2$ from hydrated lime was used as the base, and the reaction byproduct, CaCl$_2$ remained insoluble in the reaction mixture making for very easy removal by simple filtration. The chromatogram for the reaction mixture is shown in Figure 2.6 (a): about 88 % of the product and 12 % bis-tosylated byproduct were formed after 15 min. The work-up procedure included sixfold dilution of the acetonitrile reaction mixture with water, followed by centrifugation to force phase separation of the approximately 12 % bis-tosylated tetra(ethylene glycol) from the majority of mono-tosylated tetra(ethylene glycol). Both phases from the workup were analyzed by RP-HPLC and the chromatograms are shown in Figure 2.6 (b) and (c), respectively. The $^1$H NMR spectrum and EIS mass spectrum of structure of mono-tosylated tetra(ethylene glycol) is shown in Figure 2.7 and 2.8. Mono-tosylated tetra(ethylene glycol) was obtained with approximately 99 % purity. Compared to the conventional methods summarized in Table 2.1, we were able to develop an unique synthesis and purification scheme to obtain mono-tosylated tetra(ethylene glycol) inexpensively, and with high purity.
Figure 2.6 RP-HPLC of (a) mono-tosylated TEG reaction mixture, (b) aqueous phase obtained after work-up, (c) oil phase obtained after workup.

Figure 2.7 $^1$H NMR spectrum of mono-tosylated tetra(ethylene glycol) in CDCl$_3$. 
Figure 2.8 ESI mass spectrum of mono-tosylated tetra(ethylene glycol).
Table 2.1 Comparison of the mono-tosylated tetra(ethylene glycol) synthesis and purification methods.

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>Literature [58-61]</th>
<th>This work</th>
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<tbody>
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<td>Eq of TsCl</td>
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<tr>
<td>Base</td>
<td>NaOH, pyridin, Ag$_2$O</td>
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<td>Reaction temperature</td>
<td>0 ºC to RT</td>
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<td>Cost</td>
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<td>&lt; $ 0.5 / g</td>
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*A quote received on August 28, 2008 from Sinochemexper.

2.1.2.3 Synthesis of Mono-Tosylated Tetra(Ethylene Glycol) -Aldehyde [62]

![Reaction scheme for making mono-tosylated TEG-aldehyde.](image)

Figure 2.9 Reaction scheme for making mono-tosylated TEG-aldehyde.
Reaction scheme for making mono-tosylated TEG-aldehyde is shown in Figure 2.9. 1.33 g of dimethyl sulfoxide was dissolved in 1 ml of dichloromethane and added into 10 ml dichloromethane with 2.43 g of P$_2$O$_5$ under stirring at -10 °C. Dimethyl sulfoxide was activated by P$_2$O$_5$ to form a complex [63] which facilitates the conversion of alcohol to aldehyde. After 1 min, 1.28 g of mono-tosylated tetra(ethylene glycol) in 3 ml dichloromethane was added into the dimethyl sulfoxide /P$_2$O$_5$ reaction mixture. After the addition of mono-tosylated tetra(ethylene glycol), the -10°C bath was removed and the reaction mixture was stirred at room temperature. Progress of the reaction was monitored by gradient elution RP-HPLC with a PDA detector (3 µm, Gemini-C18 stationary phase, 75×4.6 mm column, 1 ml/min flow rate, initial mobile phase composition 60:40 A:B, gradient time 18 min, final mobile phase composition 100 % B, A: water; B: MeOH, both with 5 mM ammonium formate). 7 ml of triethylamine was added slowly to the reaction mixture under -10 °C to quench the reaction. The final yield of aldehyde was 65 % based on the integration of the peaks at 260 nm in the chromatogram. Aldehyde formation was confirmed by $^1$H NMR as shown in Figure 2.10 using the signature chemical shift of the aldehyde group at around 10 ppm.
2.1.2.4 Synthesis of DAAPS (4) [64]

![Reaction scheme for making (AcO)$_2$-DAAPS.]

Reaction scheme for making (AcO)$_2$-DAAPS is shown in Figure 2.11. Our first attempt to make Me$_2$HEG$_2$-DAAPS was to quaternize Me$_2$HEG$_2$-DAA with 1,3-propane.
sultone. This route failed to give fair conversion: probably, the oligo(ethylene glycol) groups hindered reaction with the ring nitrogen atom. We took an alternate approach by quaternarizing AcO₂-DAA first, then building the oligo(ethoxy)methyl chains. AcO₂-DAA (2) was synthesized by refluxing DAA (1) with acetic anhydride, 4-dimethylaminopyridine and pyridine.

![Figure 2.12 LC-MS analysis of the AcO₂-DAA and 1,3-propane sultone reaction mixture after 7 minutes of heating in a microwave oven. (a) TIC, (b) UV absorbance trace at 250 nm.](image)

Quaternarization of AcO₂-DAA was tried in a conventional microwave oven first. After heating the reaction mixture of AcO₂-DAA and 1,3-propane sultone for 7 minutes in the microwave oven, LC-MS analysis as shown in Figure 2.12 indicated that the conversion
rate was only about 50% and byproducts were formed with repeating $\Delta m/z$ of 122, which indicates oligomerization of 1,3-propane sultone.

![RP-HPLC diagram](image)

Figure 2.13 RP-HPLC of the reaction mixture of AcO$_2$-DAA with CaO and without base.

Quaternarization of AcO$_2$-DAA was improved by base addition as shown in Figure 2.13. K$_2$CO$_3$, Cs$_2$CO$_3$ and CaO were tested during reaction optimization. CaO gave higher conversion rates than the other two bases tested.

The final optimized quaternarization conditions used 3.5 g of AcO$_2$-DAA and 0.25 g of calcium oxide in 40 g of melted 1,3-propane sultone at 75 °C with stirring. After 40 min of heating, the reaction mixture was washed 5 times with 80 ml of dichloromethane (total volume of 400 ml) to remove excess 1,3-propane sultone. Next, 100 ml of 0.3 M aqueous solution of acetic acid was added to dissolve CaO, then the reaction mixture
was filtered. The remaining solid was washed three times with 40 ml of dimethylformamide (total volume of 120 ml) to get AcO₂-DAAPS (3). Reaction progress and the work-up steps were monitored by RP-HPLC with a PDA detector. Chromatograms for the reaction mixture and the product after work-up are shown in Figure 2.14. ESI mass spectrum of AOc₂-DAAPS is shown in Figure 2.15. AcO₂-DAAPS was hydrolyzed by dissolving it in 1 M HCl and heating the solution at 50 °C for 1 h to get DAAPS (4).

![Figure 2.14 RP-HPLC](image)

Figure 2.14 RP-HPLC of (a) AcO₂-DAA, (b) quaternization reaction mixture of AcO₂-DAA, (c) product after workup.
Figure 2.15 ESI mass spectrum of AOc$_2$-DAAPS.

Figure 2.16 Reaction scheme for making DAAPS.
Once AcO$_2$-DAAPS was made, it was treated with 1 M HCl at 50 °C to get DAAPS as shown in Figure 2.16. The reaction was monitored with RP-HPLC as shown in Figure 2.17. The main byproducts were unreacted AcO$_2$-DAA and DAA before and after the hydrolysis reaction, respectively.

2.1.2.5 Synthesis of TsTEG$_2$-DAAPS (5)

Figure 2.17 RP-HPLC of AcO$_2$-DAAPS and of the reaction mixture during hydrolysis of AcO$_2$-DAAPS.
Mono-tosylated tetra(ethylene glycol)aldehyde was mixed with DAAPS in a 1 : 1 mixture of dimethylformamide and H₂O that also contained 0.75 M HCl (aq). The same equivalents of NaBH₃CN and aldehyde were used in the reductive amination reaction. Progress of the reaction was monitored by RP-HPLC with a PDA detector. Figures 2.18 and 2.19 show the reaction scheme and monitoring of the making of TsTEG₂-DAAPS. It required about 15 equivalents of aldehyde and NaBH₃CN to push the formation of TsTEG₂-DAAPS to completion. A small amount of tri-alkylated products was also observed: they were eluted with a retention time of 23 min. TsTEG₂-DAAPS was purified from the reaction mixture by preparative HPLC.

Figure 2.19 RP-HPLC of DAAPS and of the reaction mixture that contains TsTEG₂-DAAPS.
2.1.2.6 Synthesis of HEG$_2$-DAAPS (6)

![Reaction scheme for making HEG$_2$-DAAPS.](image)

Reaction scheme for making HEG$_2$-DAAPS is shown in Figure 2.20. 2 ml of 2-(2-(2-methoxyethoxy)ethoxy)ethanol was mixed carefully with 2 g of NaH in 35 ml of anhydrous THF while stirring in an ice bath. After 7 min, TsTEG$_2$-DAAPS in anhydrous THF was added into the above mixture. Progress of the reaction was monitored by RP-HPLC with a PDA detector: the chromatogram is shown in Figure 2.21. An intermediate appears in the chromatogram with a retention time of 17.5 minutes: it corresponds to (TsTEG)(HEG)-DAAPS. The reaction was carried out at room temperature and went to completion in 4 hours. Unreacted NaH was quenched with excess of methanol.
2.1.2.7 Synthesis of Me$_2$HEG$_2$-DAAPS (FL) (7)

Formaldehyde is the shortest aldehyde that can be used to alkylate the two secondary amino groups to red-shift the $\lambda_{\text{ex max}}$ making the fluorophore more compatible with the 488 nm line of the argon ion laser while minimizing the increase in overall
hydrophobicity. Reaction scheme for making $\text{Me}_2\text{HEG}_2$-DAAPS (FL) is shown in Figure 2.22. Formaldehyde was mixed with $\text{HEG}_2$-DAAPS in a 1 : 1 mixture of dimethylformamide and $\text{H}_2\text{O}$ that also contained 0.75 M HCl. The same equivalents of NaBH$_3$CN and aldehyde were used in the reductive amination reaction. Progress of the reaction was monitored by RP-HPLC with a PDA detector. The insert in Figure 2.23 shows that the spectrum was red-shifted after tetra-alkylation. Figure 2.24 shows the ESI mass spectrum of $\text{Me}_2\text{HEG}_2$-DAAPS: it confirms the expected structure.

![Figure 2.23 RP-HPLC analysis of $\text{HEG}_2$-DAAPS and the reaction mixture of $\text{Me}_2\text{HEG}_2$-DAAPS. (Insert: UV absorbance spectra of $\text{HEG}_2$-DAAPS and $\text{Me}_2\text{HEG}_2$-DAAPS).](image-url)
2.1.2.8 Synthesis of FL-CA (9)

The reactive carboxylic acid group was connected to the core fluorophore by sulfonamide formation between the sulfonyl chloride derivative of FL and methyl 6-aminohexanoate as shown in Figure 2.25. 3 equivalents of oxalyl chloride and a catalytic amount of dimethylformamide were added to FL in dichloroethane while stirring in an ice bath to produce the sulfonyl chloride derivative. The chlorination
reaction was monitored by analyzing a stable sulfonamide product which was formed by reacting the sulfonyl chloride derivative with morpholine. Methyl 6-aminohexanoate and TEA in dichloroethane were mixed with the sulfonyl chloride derivative of FL to form FL-Me-CA (8). FL-Me-CA was hydrolyzed by dissolving it in 0.1 M HCl\textsubscript{(aq)} at 50 °C for 1 h to obtain FL-CA (9). The reaction was monitored by CE with LIF detection. FL was a zwitterion and moved with the electroosmotic flow in the CE analysis and could be differentiated from the cationic FL-Me-CA as shown in Figure 2.26.

![Figure 2.26 CE-LIF analysis of the conversion of FL to FL-Me-CA.](image)

Figure 2.26 CE-LIF analysis of the conversion of FL to FL-Me-CA.

![Figure 2.27 Reaction scheme for making FL-CA.](image)

Figure 2.27 Reaction scheme for making FL-CA.
FL-Me-CA was then treated with HCl to get FL-CA as shown in Figure 2.27. Its RP-HPLC analysis and ESI mass spectrum is shown in Figure 2.28 and 2.29.

Figure 2.28 RP-HPLC analysis of the reaction mixture during the hydrolysis of FL-Me-CA to FL-CA.

Figure 2.29 ESI mass spectrum of FL-CA.
2.1.2.9 Synthesis of FL-CA-PFP (10)

The carboxylic acid group of FL-CA was activated by forming a pentafluorophenyl ester by reacting FL-CA with 5 equivalents of pentafluorophenyl trifluoroacetate (PFP-TFA) in dichloroethane in the presence of triethylamine, at 55 °C for 30 min as shown in Figure 2.30. The reaction was monitored by RP-HPLC and the target m/z was confirmed with LC-MS as shown in Figure 2.31 and 2.32.

Figure 2.30 Reaction scheme for making FL-CA-PFP.

Figure 2.31 RP-HPLC analyses of FL-CA and its activated form, FL-CA-PFP.
2.2 Spectral Properties

2.2.1 Background and Objective

In the visible range, the $\lambda_{\text{ex}}^{\text{max}}$ of acridine orange is around 490 nm making it compatible with the 488 nm line of the argon ion laser. To preserve this advantage in the hydrophilic version of the acridine-based fluorophore, we made the electronic configuration of the fluorescent core as similar as possible by converting both primary amino groups of proflavine into tertiary amino groups.

Figure 2.33 Comparisons of $\lambda_{\text{ex}}^{\text{max}}$ for proflavine, acridine orange and HEG$_2$Me$_2$-DAA
Comparisons of $\lambda_{\text{ex}}^{\text{max}}$ for proflavine, acridine orange and HEG$_2$Me$_2$-DAA is shown in Figure 2.33. Fluorescent labels used in CE and cIEF usually are subjected to buffers with varied pH values. It is important and necessary to ascertain that the fluorescence properties of the designed label are pH-independent allowing both qualitative and quantitative analysis.

2.2.2 Materials and Method

The UV absorbance spectra were obtained using a Mod 168 photodiode array detector (Beckman-Coulter, Fullerton, CA). Fluorescence spectra at different pH values were taken by using an Aminco Bowman Series 2 Luminescence Spectrometer. Phosphoric acid-based buffers were made by titrating phosphoric acid with sodium hydroxide to make pH 2.4, 7.3 and 10.9 solutions with 10 mM ionic strength for the fluorescence measurements.

2.2.3 Results and Discussion

![Figure 2.34 UV absorbance spectra of differently alkylated 3,6-diaminoacridines.](Image)

Figure 2.34 UV absorbance spectra of differently alkylated 3,6-diaminoacridines.
Proflavine has its visible-range $\lambda_{\text{ex}}^{\text{max}}$ around 450 nm as shown in Figure 2.34, spectrum number 0. With increasing degree of alkylation on the anilinic amino groups, $\lambda_{\text{ex}}^{\text{max}}$ is red-shifted, on average, by about 10 to 12 nm per alkyl group. For the tetra-alkylated HEG$_2$Me$_2$-DAA, $\lambda_{\text{ex}}^{\text{max}}$ reaches 491 nm.

![Figure 2.35](image)

Figure 2.35 Comparison of the fluorescence excitation (A) and emission (B) spectra of acridine orange (dashed line) and HEG$_2$Me$_2$-DAA (solid line).

The fluorescence excitation and emission spectra of acridine orange and HEG$_2$Me$_2$-DAA are compared in Figure 2.35. Concentrations for both components were adjusted to obtain identical absorbances at $\lambda=280$ nm. $\lambda_{\text{ex}}^{\text{max}}$ remains the same for both the tetramethyl and the tetraTEG substituted proflavines. $\lambda_{\text{em}}^{\text{max}}$ of HEG$_2$Me$_2$-DAA is about 5 nm blue-shifted compared to acridine orange. Notice that though the solutions had similar concentrations, HEG$_2$Me$_2$-DAA has about two times higher intensity than acridine orange in both the excitation and emission spectra. This result could be due to solvatochromic effects: HEG$_2$Me$_2$-DAA has oligo(ethoxy)methyl chains that alter the
hydrophilic/hydrophobic balance around the molecule compared to that of acridine orange.

Figure 2.36 Fluorescence emission spectra of HEG$_2$Me$_2$-DAA in H$_3$PO$_4$/NaOH aqueous buffers from pH 2.4 to 10.9 with 10 mM ionic strength.

The emission spectra of HEG$_2$Me$_2$-DAA were tested in aqueous buffers obtained by titrating H$_3$PO$_4$ to pH 2.34, 7.3 and 10.9 with NaOH and an ionic strength of 10 mM. As shown in Figure 2.36, neither the intensity nor $\lambda_{\text{em}}^\text{max}$ change throughout the entire pH range.

To sum up the spectral studies of HEG$_2$Me$_2$-DAA, we were able to preserve the desirable $\lambda_{\text{ex}}^\text{max}$ at about 490 nm, making the fluorophore compatible with the 488 nm
line of the argon ion laser. The emission spectra remain pH-independent and the emission intensities are higher than in acridine orange due, presumably, to the presence of oligo(ethoxy)methyl chains.

2.3 Chromatographic Properties

2.3.1 Background and Objective
In CE, with purely aqueous background electrolytes, acridine orange-based fluorescent reagents exhibit a tailing peak shape indicating hydrophobic interactions with the wall of the bare fused silica capillary. The chromatographic properties of Me$_2$HEG$_2$-DAAPS were tested and compared with those of acridine orange propane sulfonate in CE.

2.3.2 Materials and Method
The background electrolyte used in the CE analysis was 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) titrated with TEA to pH 10.1. A 2% (w/w) aqueous poly(vinylpyrrolidone) (PVP) solution was used to dynamically coat the wall of the capillary between runs by rinsing the capillary at 30 psi for 2 minutes [65]. Concentrations for both acridine orange propane sulfonate and Me$_2$HEG$_2$-DAAPS were adjusted to obtain identical absorbances at $\lambda=280$ nm. The CE experiments were carried out with same instrument and type of capillary as described in 2.2.2.1. CE analysis was run in free zone mode with 30 kV applied potential.
2.3.3 Results and Discussion

CE tests for both acridine orange propane sulfonate and Me$_2$HEG$_2$-DAAPS were done with the same capillary as the first and second run. In Figure 2.37, the shape of the Me$_2$HEG$_2$-DAAPS peak is Gaussian while the peak of acridine orange propane sulfonate shows a 1.5 minutes long tail. Based on these results, Me$_2$HEG$_2$-DAAPS does show improved chromatographic behavior.

![Figure 2.37 CE analyses of acridine orange propane sulfonate and Me$_2$HEG$_2$-DAAPS.](image)

2.4 Labeling Reactions Using FL-CA-PFP

2.4.1 Background and Objective

FL-CA-PFP was designed as an amine-reactive fluorescent labeling reagent to tag amines and improve their detection sensitivity in CE analysis. Arginine, histidine, glutamic acid and glycine were labeled with FL-CA-PFP and analyzed by CE-LIF.
Bovine serum albumin and chicken ovalbumin were also labeled with FL-CA-PFP and analyzed by SDS-CEG-LIF and cIEF, respectively.

2.4.2 Materials and Methods

2.4.2.1 Labeling of Amino Acids and Their Analysis by CE-LIF and RP-HPLC.
Arginine, histidine, glutamic acid and glycine were labeled with FL-CA-PFPF in a pH 8.2, 0.1 M aqueous sodium bicarbonate buffer. The CE experiments were carried out with the same instrument and type of capillary as described in 2.2.2.1. A 2 % (w/w) aqueous poly(vinylpyrrolidone) (PVP) solution was used to dynamically coat the wall of the capillary between runs by rinsing it at 30 psi for 2 minutes. The pH 2 background electrolyte used in the CE analyses of labeled arginine, histidine and glycine was obtained by adding 15 mmol tetrabutylammonium hydroxide to 5 mmol H₃PO₄ in 1 L water. The background electrolyte used in the CE analyses of labeled glutamic acid was 25 mM boric acid titrated to pH 8.3 with NaOH. HPLC analyses and LC-MS analyses were done with the instrument and column as described in 2.2.2.1.

2.4.2.2 Labeling of Bovine Serum Albumin (BSA) and Its Analysis by SDS-CGE-LIF
Bovine serum albumin was labeled with excess of FL-CA-PFP in a pH 8.2, 0.1 M aqueous sodium bicarbonate buffer at room temperature for 1 hour. An aliquot of the labeling reaction mixture was diluted with the Beckman SDS-MW sample buffer. SDS-CGE was carried out with the same CE instrument and type of capillary as described in 2.2.2.1. The capillary was preconditioned as recommended by Beckman for their SDS-
MW analysis. Briefly, the capillary was rinsed with an aqueous 0.1 M NaOH solution at 50 psi for 5 minutes. This was followed by a rinse with an aqueous 0.1 M HCl solution at 50 psi for 2 minutes, then a rinse with deionized water at 50 psi for 2 minutes. The capillary was then filled with the SDS-MW gel buffer solution at 40 psi for 10 minutes. A 15 kV potential was applied (negative-to-positive polarity), with a ramp-up time of 5 minutes and 20 psi nitrogen blanket on both the inlet and outlet vials.

2.4.2.3 Labeling of Chicken Ovalbumin (Ov) and Its Analysis by cIEF

Chicken ovalbumin was labeled with FL-CA-PFP in a pH 8.2, 0.1 M aqueous sodium bicarbonate buffer with a protein-to-fluorophore ratio of approximately 1 to 7 at room temperature for at 1 hour. The labeling reaction mixture was filtered with a membrane having a 1000 Da cut-off to remove excess FL-CA and NaHCO₃. An aliquot of the labeled protein solution was serially diluted with the cIEF separation solution which contained 2% of carrier ampholytes 3 < pI < 10, 15 mM of arginine (cathodic blocker), 2.5 mM of iminodiacetic acid (anodic blocker), 2.5 M urea and the pI makers in cIEF gel until the desired LIF signal strength was achieved. The carrier ampholytes and the cIEF gel solution were provided by Beckman-Coulter (Fullerton, CA). The fluorescent pI markers used in this work included the acridine orange conjugate of aspartic acid (pI 4.11) and the trisulfonamide obtained by reacting 8-hydroxypyrene 1,3,6-trisulfonyl chloride with 4-(2-aminoethyl)morpholine (AEM) (pI 6.38), both synthetized earlier in our laboratory [56]. cIEF analyses were carried out with the same CE instrument as described in 2.2.2.1. The 50 µm I.D., 360 µm O.D. capillary columns with a neutral
coating and having total and inlet-to-detector lengths of 30 and 20 cm, respectively, were provided by Beckman-Coulter. The capillary was prepared by rinsing it with 10 mM H$_3$PO$_4$ at 30 psi for 2 minutes, followed by a rinse with water at 30 psi for 2 minutes. The capillary was then filled with the mixture of the labeled protein and the cIEF separation solution at 30 psi for 2 minutes. The anolyte solution was 200 mM H$_3$PO$_4$ in cIEF gel with 2.5 M urea. The catholyte was 300 mM NaOH in deionized water. Focusing was done with an applied potential of 25 kV for 12 minutes and the components were mobilized by applying a 0.5 psi pressure while maintaining a potential at 21 kV. An aqueous solution of 2 mM sodium acetate in 4.3 M urea was used to clean the capillary between runs by applying, simultaneously, 30 psi pressure and 25 kV potential for 5 minutes.

2.4.3 Results and Discussion

2.4.3.1 Labeling of Amino Acids and Their Analysis by CE-LIF

FL-CA-tagged amino acids were analyzed by CE-LIF in the free zone mode: the results are shown in Figure 2.38. For tagged glutamic acid, the CE analysis was done with a pH 8.3 background electrolyte and 30 kV applied potential (negative-to-positive polarity). At pH 8.3, the two carboxylic acid groups of FL-CA-glutamic acid were fully deprotonated making the total charge of FL-CA-glutamic acid equal to -1. FL-CA at pH 8.3 was a zwitterion and appeared as a neutral marker during CE analysis and could be differentiated from the anionic FL-CA conjugate of glutamic acid. Within 15 minutes analysis time, FL-CA had not passed the detector window, because the direction of the
electroosmotic flow pointed away from the detector. For tagged arginine, histidine and glycine, the CE analyses were carried out at pH 2 with 30 kV applied potential (positive-to-negative polarity). In the pH 2 BGE, both FL-CA-glycine and FL-CA appeared to be cationic with a total charge of +1. They could be separated by CE due to their size differences. As shown in Figure 2.38 (c), FL-CA-glycine has a slightly slower mobility than FL-CA. Both FL-CA-arginine and FL-CA-histidine carried a total charge of +2 at pH 2. Thus, they moved faster than FL-CA, as shown in Figure 2.38 (a) and (b).

Figure 2.38 CE analyses of FL-CA-PFP tagged amino acids. (a) tagged arginine, (b) tagged histidine, (c) tagged glutamic acid and (d) tagged glycine.
The same set of tagged amino acids was also analyzed by RP-HPLC. The signature UV absorbance spectrum of FL served as a diagnostic tool to help assign the peaks in the chromatograms. The chromatograms shown in Figure 3.39 were acquired at a detection wavelength of 500 nm. Only the FL-related components would appear at this wavelength. FL-CA, a hydrolysis product of FL-CA-PFP, was observed in all the tagging tests as shown in Figure 3.39. At pH 2.7, all four tagged amino acids were retained less in the RP column than FL-CA-PFP. The ESI mass spectra for the four tagged amino acids are shown in Figure 2.40.

![Figure 2.39 RP-HPLC analyses of FL-CA-PFP tagged amino acids. (a) tagged arginine, (b) tagged histidine, (c) tagged glutamic acid and (d) tagged glycine.](image-url)
2.4.3.2 Labeling of Bovine Serum Albumin (BSA) and Its Analysis by SDS-CGE-LIF

Bovine serum albumin was labeled with excess of FL-CA-PFP and analyzed by SDS-CGE with LIF detection. As shown in Figure 2.41, the lowest tested concentration for tagged bovine serum albumin was 15 nM which is one-to-two orders of magnitude lower than what can be achieved for the CE system used with UV absorbance detection.
2.4.3.3 Labeling of Chicken Ovalbumin (Ov) and Its Analysis by cIEF

Chicken ovalbumin was labeled with FL-CA-PFP with a protein-to-label mole ratio of 1 to 7 in a 0.1 M sodium bicarbonate buffer. The hydrolyzed label and sodium bicarbonate were removed by centrifuging the samples through 10 kD permeation cut-off dialysis membranes in order to avoid interferences during cIEF. The detector traces obtained during cIEF analyses of non-tagged and tagged chicken ovalbumin are overlaid in Figure 2.42 for the UV absorbance and the LIF detectors, respectively. The composition of the cIEF separation solutions (concentrations of pI markers, carrier ampholytes, cathodic and anodic blockers) for both the non-tagged and the tagged samples were the same in order to minimize the changes which are caused by the system but not by the nature of the proteins. The concentration of pI markers needed to get decent signals with the UV absorbance detector overwhelmed the LIF detector. A common problem with amine-reactive protein labels is the acidic shift in the pI value of the protein after labeling due to the loss of a buffering amino group to an amide group.

Figure 2.41 SDS-CGE analysis of labeled bovine serum albumin using LIF detection.
In the overlay, the pI of the tagged proteins was shifted in the alkaline direction by about 0.02 compared to the non-tagged protein. It is expected that this minimal basic shift in pI caused by tagging with the monocationic FL-CA-PFP is due to the replacement of an amino group with a higher pKa quaternary ammonium group. The result suggests that derivatization using monocationic FL-CA-PFP does not change the pI of the band of the tagged chicken ovalbumin in a noticeable manner, though the general validity of this assumption over the entire pI range must be experimentally verified once suitable fluorescent pI markers become available. The combined reproducibility of the cIEF analysis and the labeling of chicken ovalbumin was also investigated. As shown in Figure 2.43, the mobilization traces for samples taken from three independent labeling reactions are almost identical demonstrating the reproducibility of the procedure.

Figure 2.42 Mobilization traces obtained in the cIEF analysis of chicken ovalbumin with UV absorbance detection and labeled chicken ovalbumin with LIF detection.
2.5 Design and Synthesis of a FL-CA-based Trifunctional Probe

2.5.1 Background and Objectives

The objective of this work was to briefly test the feasibility, on a “proof-of-principle” level, of combining biotin, FL-CA and an azide group into a single, quasi-universal probe to facilitate selective enrichment and selective detection of a variety of molecules (e.g., natural products, pharmaceuticals, inhibitors, etc.) that carry an alkyne anchoring group that minimally perturbs their biological function. The alkyne anchoring group can be, presumably, readily coupled to the azide-containing trifunctional probe using Cu(I)-mediated click chemistry [66]. Based on log$P$ values calculated by ALOGPS 2.1 (http://www.vcclab.org) [67], the log$P$ value of our trifunctional probe is about 6 units lower than Tate’s [68]. The 10$^6$-fold improvement in hydrophilicity implies better
biocompatibility and hopefully helps to reduce the degree of aggregation and precipitation of the probe-protein conjugates.

The trifunctional probe was synthesized according to the reaction schemes shown in Figures 2.44 and 2.45. The probe was built by a converging approach: a biotin-azide conjugate, compound 6 and FL-CA-PFP were built independently, then connected into one piece via amide formation.

Figure 2.44 Synthesis scheme for making compound 6.
2.5.2 Materials and Method

FL-CA-PFP was made as described in Section 2.1.2. Compound 6 was synthesized by Dr. Robles of Professor Romo’s group at TAMU. RP-HPLC and LC-MS analyses were carried out as described in Section 2.1.2.1. Sodium hydride, sulfur trioxide pyridine, diisopropylethylamine and 1H-thieno[3,4-d]imidazole-4-pentanamide were purchased from Sigma-Aldrich Co. (St. Louis, MO).

2.5.2.1 Synthesis of Biotin-Azide Compound 6

Tetra(ethylene glycol) was mixed with 1 equivalent of sodium hydride and 2 equivalents of tosyl chloride in dimethylformamide and reacted at room temperature. Monotosylated tetra(ethylene glycol) was obtained through flash column chromatography with an approximate yield of 76%, then reacted with sodium azide in dimethylformamide
while heating at 60 °C to make compound 3, ethanol, 2-[2-(2-azidoethoxy)ethoxy]-. Compound 3 was mixed with sulfur trioxide pyridine and diisopropylethylamine in dimethyl sulfoxide to make compound 4, acetaldehyde, 2-[2-(2-azidoethoxy)ethoxy]-[69]. After flash column chromatography, compound 4 was reacted with 1H-thieno[3,4-d]imidazole-4-pentanamide in a mixture of dichloroethane and dimethylformamide to make compound 6. The same equivalents of sodium triacetoxyborohydride [70] and aldehyde were used in the reductive amination reaction. Finally, compound 6 was purified by RP-HPLC using an eluent of 7 % acetonitrile and 93 % water mixture, in isocratic mode. The result of the LC-MS analysis of compound 6 is shown in Figure 2.46.

Figure 2.46 LC-MS analysis of compound 6.
2.5.2.2 Synthesis of the Desired Trifunctional Probe

FL-CA-PFP was mixed with triethylamine and compound 6 in acetonitrile and heated at 50 °C with stirring. After 1 hour, the reaction mixture was analyzed with LC-MS: the results are shown in Figure 2.47. Based on the integration of the peaks at 450 nm in the chromatogram, conversion in this batch was about 35 %. FL-CA was recovered and converted back to FL-CA-PFP. The UV absorbance spectrum of the trifunctional probe, shown in the insert, agrees with that of FL-CA.

Figure 2.47 LC-MS analysis of the reaction mixture obtained during the final assembly of the trifunctional probe. (Insert: UV absorbance spectrum of the trifunctional probe.)
An attempt was made by Dr. Robles to couple a cyclopropanated derivative of parthenin with the trifunctional probe through click chemistry (reaction scheme shown in Figure 2.48). The reaction mixture was analyzed by LC-MS: its results are shown in Figure 2.49. A small peak with a retention time of 24 minutes was observed in the m/z 2074 extracted ion chromatogram (target m/z) as shown in Figure 2.49(b). It was retained slightly longer than the trifunctional probe itself shown in Figure 2.49(c).

Figure 2.48 Reaction scheme of coupling a cyclopropanated derivative of parthenin with the trifunctional probe.
2.6 Concluding Remarks

A hydrophilic, monocationic, fluorescent, amine-reactive labeling reagent having pH-independent spectral properties has been made to facilitate the LIF-CE analysis of compounds having primary or secondary amino groups. Each synthetic step was monitored and characterized by RP-HPLC, LC-MS and CE. This hydrophilic version of the acridine orange-based fluorophore is about 30,000 time more water soluble than the original acridine orange-based fluorophore allowing CE analysis with a decent peak.
shape under purely aqueous conditions. FL-CA has its visible-range $\lambda_{\text{max}}^{\text{ex}}$ around 490 nm making it compatible with the 488 nm line of the argon ion laser. The spectral properties of FL-CA were independent of pH in the $2.4 < \text{pH} < 11$ range. The monocationic charge of FL-CA helped to minimize the change in the pI value of chicken ovalbumin after derivatization. In a proof-of-principle experiment, FL-CA was incorporated into a trifunctional probe that contained FL-CA to facilitate selective, sensitive LIF detection, a biotin group to permit selective enrichment from dilute solutions (via affinity interactions with avidine), and an azido group to facilitate coupling to target molecules labeled with a minimally disruptive alkyne group. The trifunctional probe was successfully synthesized and Cu(I)-mediated coupling to an alkyne-labeled cyclopropanated derivative of parthenin had been tried on a microgram scale and the reaction mixture was analyzed by LC-MS.

Though traditional gel-based electrophoresis suffers from low resolution and low sensitivity compared to CE-based separations, it has been widely used in proteomics [71]. Because of the selection of the unique reporting group, the trifunctional probe has the potential to become a quasi-universal affinity probe in CE-mediated analysis. Its compatibility with cIEF is especially significant considering that cIEF is one of the widely used techniques for pI characterization and simultaneous sample concentration in bio-analysis. We demonstrated that labeling of chicken ovalbumin with FL-CA-PFP lead to a pI shift of only 0.02 after derivatization. The designed FL-CA-PFP is a suitable derivatizing agent for cIEF-LIF due to its high water solubility, pH-independent spectral
properties and the minimal shift it caused in the pI value of the test protein, chicken ovalbumin. However, the validity of this claim could not be demonstrated across the entire pI range, because the number of available LIF-compatible pI markers having reasonably narrow pI gaps and allowing the accurate determination of pI values is very limited. The lack of this experimental proof currently prevents the widespread use of FL-CA-PFP in cIEF and renders it to the status of just another “me too” derivatizing agent. Another important reason why fluorescent pI markers are necessary is that the low sensitivity of UV absorbance detection requires the use of very high (at least three orders of magnitude higher) sample loadings than LIF detection. Too high analyte concentration alters the pH gradient locally in an uncontrolled manner and leads to false pI values. Very low sample loads that do not distort the pH gradient and do not cause loss of resolution are only possible when highly sensitive detection methods, such as LIF are available. In order to facilitate and maximize the application of FL-CA-PFP in highly sensitive and accurate cIEF-LIF analysis, it is necessary to develop optimized, closely-spaced fluorescent pI markers. Consequently, we decided to work on the design and synthesis of LIF-compatible pI markers as discussed in Chapter 3.
3. DESIGN AND SYNTHESIS OF A FAMILY OF PYRENE-BASED FLUORESCENT pI MARKERS

3.1 Design and Synthesis

3.1.1 Background and Objective

According to one of the cIEF reviews written by Righetti [22], “in cIEF, there is no other way for charting the course of pH than to use pI markers.” Proteins with known pI values can be used as pI markers in cIEF with UV absorbance detection. Ten commercially available protein pI markers and seven UV-absorbing tryptic digest peptides derived from cytochrome c were used as pI markers in cIEF by Mohan and Lee [72]. These proteins and peptides cover a pI range from 3.67 to 10.25. Native protein pI markers have disadvantages: their solubility is low in isoelectric state, they tend to aggregate, denaturing reagents can not be present in the cIEF system, their purity is often insufficient and the choice of their pI values is limited by nature [54]. To avoid the disadvantages of using proteins as pI markers, modifications and synthetic approaches had been tried. Arai et al. [27] labeled both tryptic peptides and ampholytes with dansyl chloride to enhance their detectability at 280 nm to profile the pH gradient. Synthetic oligopeptides as pI markers had also been designed by Shimura et al. [26, 27]. The C-terminal carboxyl group, and the side chains of aspartic acid and glutamic acid were utilized to obtain acidic pI markers. On the other hand, tyrosine and lysine were essential constituents for markers in the alkaline pI region. For pI markers around the neutral pH, the N-terminal amino group and the histidine residues were utilized.
Moreover, each peptide was designed to contain a tryptophan residue to facilitate detection by UV absorbance at 280 nm. The pI values were determined by slab-gel IEF: the pH values of the focused bands were measured directly with a microprobe glass electrode. Some characteristic properties of the synthetic peptide pI markers are summarized in Figure 3.1. The 16 pI markers cover the 3.38 to 10.17 range and the measured pI values are reasonably close to the calculated ones. Their focusing behavior was characterized by the \([-dz/d(pH)]_p\) and Vh/cm values (the integral of the required focusing potential and time for unit gel length). The \([-dz/d(pH)]_p\) value is inversely proportional to Vh/cm. Rapid focusing was achieved with less than 120 Vh/cm for the markers whose \([-dz/d(pH)]_p\) values were greater than 1.

<table>
<thead>
<tr>
<th>No.</th>
<th>Peptides</th>
<th>pI det.</th>
<th>SD</th>
<th>pI cal.</th>
<th>Δp</th>
<th>(-dz/dpH)</th>
<th>Vh/cm</th>
</tr>
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<tr>
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<td>H-Trp-Tyr-Lys-Arg-OH</td>
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<td>95</td>
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<tr>
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<td>32</td>
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<td>8.42</td>
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<td>0.52</td>
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<tr>
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<td>1.31</td>
<td>120</td>
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<tr>
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<td>0.12</td>
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<td>5.48</td>
<td>-0.04</td>
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<td>270</td>
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<tr>
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<td>0.07</td>
<td>0.53</td>
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<td>70</td>
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<tr>
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<td>3.38</td>
<td>0.00</td>
<td>1.70</td>
<td>120</td>
</tr>
</tbody>
</table>

- a) pI det., determined pI values (average of five independent experiments)
- b) SD, standard deviations of determined pI values
- c) pI cal., calculated pI values using Eq. (1)
- d) Δp, differences between pI det. and pI cal
- e) \(-dz/dpH\), \([-dz/dpH]_p\) values calculated using Eq. (2)
- f) Vh/cm, the sum of the products of time and applied field strength required for focusing (see Section 3.2)

Figure 3.1 Peptide sequences and characteristic properties of the synthetic peptide pI markers [27].
Compared to protein and peptide pH markers, it is easier to obtain synthetic low-molecular-mass markers in pure form and they are less susceptible to change over time. The pH values of a series of ampholytic dyes (Patent Blue V, Fast Green FCF, Methyl Blue, Evan’s Blue and Congo Red) were measured in gel IEF: they range from 3 to 5.8 [73]. They were used as internal pH standards to obtain the pH of bovine serum albumin, ovalbumin and bovine insulin which resulted in a good agreement with the literature values. Four different colored ferrous complexes of phenanthroline [74] were tested in gel IEF. It was reported that they focused relatively sharply in the gel and had a pH range from 5.45 to 6.82. In 1994, Slais et al. [54] designed 19 UV absorbing pH markers by varying the substituents on an aminomethylphenol core. The structures of these pH markers are listed in Figure 3.2. The substitution patterns were selected to optimize the [-dz/d(pH)]_pH values and minimize the hydrophobicities of the markers. The properties of these markers are summarized in Figure 3.3. The pH values were determined by potentiometric titration: they ranged from pH 5.3 to 10.4. As for [-dz/d(pH)]_pH, all markers had a value greater than 0.045, except markers numbered 6 and 7, and thus were acceptable focusers. The pH markers also had satisfactory molar absorbance values in both the UV and the visible regions. LogP is an indication of how hydrophobic a molecule is. Most of the pH makers had good water solubilities with calculated logP values below 1. The design took the acid-base, spectroscopic and hydrophobic properties into consideration and set an example of how to build desirable pH markers.
## Structures of Suggested \( pI \) Markers of General Formula I

<table>
<thead>
<tr>
<th>No.</th>
<th>( R_1 )</th>
<th>( R_2 )</th>
<th>( N(\bar{R}_2, \bar{R}_4) )</th>
<th>( M_r )</th>
</tr>
</thead>
<tbody>
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</tr>
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<td>HPipe</td>
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</tr>
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<td>H</td>
<td>DEA</td>
<td>261</td>
</tr>
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<td>H</td>
<td>PIP</td>
<td>273</td>
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<td>MOR</td>
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<td>Cl</td>
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<td>HPipe</td>
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<tr>
<td>15</td>
<td>NO(_2)</td>
<td>H</td>
<td>MOR</td>
<td>275</td>
</tr>
<tr>
<td>16</td>
<td>Cl</td>
<td>NO(_2)</td>
<td>MOR</td>
<td>309</td>
</tr>
<tr>
<td>17*</td>
<td>4-CH(_2)N(\bar{R}_2, \bar{R}_4)</td>
<td>2-Cl-6-NO(_2)</td>
<td>MPIPE</td>
<td>359</td>
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<tr>
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<td>2-Cl-6-NO(_2)</td>
<td>HPipe</td>
<td>389</td>
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<tr>
<td>19*</td>
<td>4-CH(_2)N(\bar{R}_2, \bar{R}_4)</td>
<td>2-Cl-6-NO(_2)</td>
<td>MOR</td>
<td>309</td>
</tr>
</tbody>
</table>

*PIP = 1-piperidyl; MPIPE = 1-(4-methylpiperazinyl); HPipe = 1-(4-hydroxyethylpiperazinyl); DEA = diethylamino; MOR = 4-morpholinyl
* Aminomethyl group in position 4- and substituents \( R_i \) and \( R_j \) in position 2- and 6-, respectively.

Figure 3.2 List of the structures of the 19 UV absorbing \( pI \) markers designed by Slais et al. [54].
There is a significant limitation of using UV absorbance as the detection method in cIEF: the detection sensitivity is poor because the currently available carrier ampholytes also have absorbance in the UV range. Using laser induced fluorescence (LIF) as the detection method can improve the detection sensitivity in cIEF; mass detection limits in cIEF coupled with LIF detection had reached the attomole range [75]. Only a few fluorescent pI markers are available, they are (1) fluorescent proteins, (2) fluorophore-labeled ampholytes, and (3) fluorescent small organic molecules. The problem with fluorescent proteins as pI markers is that the choices are very limited by the low numbers of proteins that can be excited with visible-range lasers.

Figure 3.3 Characteristic properties of the synthetic UV absorbing pI markers [54].
Shimura and Kasai made 5 fluorescent pH markers ranging from pH 3.95 to 8.20 by labeling peptides with 5-carboxytetramethylrhodamine succinimidyl ester on the N-terminal amino group [75]. The spectral properties of 5-carboxytetramethylrhodamine are pH independent: fluorescence can be excited with the green light (543.5 nm) from an He-Ne laser. The potential detection limit of the labeling dye was reported to be $10^{-21}$ mol in capillary zone electrophoresis [76]. The problem with the fluorophore-labeled ampholytes as pH markers is that the presence of multiple labeling sites leads to a mixture of products with different pH values. Reversed phase HPLC was used to purify the labeled peptides with a recovery rate of 40% - 70% (based on the initial number of moles of peptides used in the synthesis). Shimura et al. [77] continued the work of making tetramethylrhodamine-labeled peptides as fluorescent pH markers in 2002. In this work, each peptide was designed to include a cysteine residue which was coupled with an iodoacetylated derivative of tetramethylrhodamine to avoid multiple labeling. 19 fluorescent pH markers were made covering the pH range from 3.64 to 10.12. The maximum pH gap was 0.68 as shown in Figure 3.4. To mitigate the effects of microbial or enzymatic degradation, the peptide-based pH markers were stored under acidic conditions: this, in turn, caused hydrolytic degradation after 3 days under a fluorescent lamp.
Figure 3.4 Peptide sequences and characteristic properties of the tetramethylrhodamine-tagged peptide pI markers [77].

Slais et al. [39] synthesized four fluorescein-based pI markers by reacting fluorescein methylester with mono-alkylated piperazines at 80°C for 20-60 hours under reflux. The reaction scheme is shown in Figure 3.5. This series of low-molecular-mass markers covers a relatively narrow pI range from 5.4 to 6.6. Fluorescein-based pI markers are compatible with argon ion laser-induced fluorescence detection. However, their spectral properties are pH dependent: the detection limits are more favorable in the basic pH region.
Figure 3.5 Synthetic scheme (left) and the summarized properties (right) of fluorescein-based pI markers [39].

The need for LIF-compatible pI markers remains unsatisfied. Slais [78] summarized the criteria a good colored pI marker should fulfill as follows: (1) high color intensity in the visible range, (2) good focusing ability, (3) high water solubility, (4) chemical and hydrolytic stability, (5) purity and (6) accessibility.

Up to now, compounds selected as pI markers had to be found via extensive screening [79], because there were no known correlations between the structure of an ampholyte and its pI value. It was discovered in our laboratory that the pKa values of two identical protic groups used as substituents of the sulfonamido groups on a pyrene core structure differ by about 0.7. The same was found for protic groups attached to bis-sulfonamido groups of certain other aryl core structures as well. The significance of this finding is that it permits the development of ampholytes with \[-dz/d(pH)\]pI values greater than 0.9 in a methodical and systematic way. (The significance of the \[-dz/d(pH)\]pI value of an ampholyte was discussed in Chapter 1.4.)
The generic structure of the rationally designed pyrene-based fluorescent pH markers is shown in Figure 3.6. The pH markers contain two identical buffering groups as part of the sulfonamido substituents attached at the 1 and 3 positions and a charge-balancing (titrating) group attached as part of an alkoxy substituent at the 6 position of the pyrene core.

![Diagram of pyrene-based pH marker structure](image)

Figure 3.6 One of the possible generic structures of the pyrene-based pH markers.

The effective charge versus pH curve of a designed pH marker can be calculated using the SPARC software, Ver. 4.6 (available at sparc.chem.uga.edu/sparc/index.cfm). The calculated pH value can be found at the pH where the effective charge of the molecule equals zero as in the example shown in Figure 3.7. A large set of pyrene-based fluorescent pH markers with predicted excellent focusing properties covering the pH range 3 to 10 is listed in Tables 3.1 to 3.7.
Figure 3.7 An effective charge versus pH curve generated by SPARC for a pI marker utilizing β-alanine as the source of the buffering groups and N,N-dimethylamino bromoethane as the source of the titrating group.
<table>
<thead>
<tr>
<th>$p_I$</th>
<th>Sulfonamido buffering group derived from</th>
<th>Titrant group derived from</th>
<th>$[-dz/dpH]_{p_I}$</th>
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<tbody>
<tr>
<td>3.04</td>
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Table 3.1 (continued)

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<td>2-Hydroxy-4-aminobutyric acid</td>
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<td>Aspartic acid</td>
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<td>3.95</td>
<td>Glutamic acid</td>
<td>Dimethylaminopropanol</td>
<td>1.20</td>
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Table 3.2 A list of the possible pyrene-based fluorescent pH markers (4 < pH < 5)

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<th>Titrant group derived from</th>
<th>[-dz/dpH]_{pH}</th>
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<td>1.22</td>
</tr>
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<td>3-Aminopentanedicarboxylic acid</td>
<td>Choline</td>
<td>1.22</td>
</tr>
<tr>
<td>4.06</td>
<td>Iminodiacetic acid</td>
<td>Choline</td>
<td>1.22</td>
</tr>
<tr>
<td>4.10</td>
<td>3-Hydroxy-4-aminobutyric acid</td>
<td>Choline</td>
<td>1.15</td>
</tr>
<tr>
<td>4.17</td>
<td>Aspartic acid</td>
<td>Dimethylaminopropanol</td>
<td>1.16</td>
</tr>
<tr>
<td>4.18</td>
<td>Aspartic acid</td>
<td>Choline</td>
<td>1.17</td>
</tr>
<tr>
<td>4.26</td>
<td>β-Alanine</td>
<td>Choline</td>
<td>1.15</td>
</tr>
<tr>
<td>4.26</td>
<td>Iminodi(3-propionic acid)</td>
<td>N,N’N’-trimethylpiperazine</td>
<td>1.30</td>
</tr>
<tr>
<td>4.32</td>
<td>5-Aminocaproic acid</td>
<td>Isethionic acid</td>
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</tr>
<tr>
<td>4.52</td>
<td>4-Aminoheptanedicarboxylic acid</td>
<td>Choline</td>
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</tr>
<tr>
<td>4.53</td>
<td>4-Aminobutyric acid</td>
<td>Choline</td>
<td>1.15</td>
</tr>
<tr>
<td>4.55</td>
<td>4-Aminoheptanedicarboxylic acid</td>
<td>Dimethylaminopropanol</td>
<td>1.13</td>
</tr>
<tr>
<td>4.56</td>
<td>Iminodiacetic acid</td>
<td>N,N’N’-trimethylpiperazine</td>
<td>1.23</td>
</tr>
<tr>
<td>4.65</td>
<td>Glycine</td>
<td>Choline</td>
<td>1.15</td>
</tr>
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</table>
Table 3.3 A list of the possible pyrene-based fluorescent pH markers (5 < pH < 7)

<table>
<thead>
<tr>
<th>pH</th>
<th>Sulfonamido buffering group derived from</th>
<th>Titrant group derived from</th>
<th>([\text{-d}z/\text{d}p\text{H}]_{\text{pH}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.06</td>
<td>Piperazine-N-ethanesulfonic acid</td>
<td>N-(2-hydroxyethyl)-N-methyl-N'(2-sulfoethyl)-N'-methylmethylenediamine</td>
<td>0.73</td>
</tr>
<tr>
<td>5.52</td>
<td>N-(2-Hydroxyethyl-(ethoxy(ethoxy))-piperazine</td>
<td>Isethionic acid</td>
<td>1.13</td>
</tr>
<tr>
<td>5.61</td>
<td>Piperazine-N-ethanesulfonic acid</td>
<td>N-(2-hydroxyethyl)-N-methyl-N'(2-sulfoethyl)-N'-methyl-propylenediamine</td>
<td>1.19</td>
</tr>
<tr>
<td>5.69</td>
<td>Histidine</td>
<td>Isethionic acid</td>
<td>0.76</td>
</tr>
<tr>
<td>5.98</td>
<td>Piperazine-N-3-propanesulfonic acid</td>
<td>N-(2-hydroxyethyl)morpholine</td>
<td>0.74</td>
</tr>
<tr>
<td>6.09</td>
<td>Piperazine-N-3-propanesulfonic acid</td>
<td>N-(2-hydroxyethyl)-N-methyl-N'(2-sulfoethyl)-N'-methyl-propylenediamine</td>
<td>0.90</td>
</tr>
<tr>
<td>6.20</td>
<td>Piperazine-N'-ethanesulfonic acid</td>
<td>Dimethylaminopropanol</td>
<td>0.93</td>
</tr>
<tr>
<td>6.20</td>
<td>5-Hydroxymethyl imidazole</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>6.25</td>
<td>4-Hydroxymethyl imidazole</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>6.29</td>
<td>Aminomethylphosphonic acid</td>
<td>Dimethylaminopropanol</td>
<td>1.14</td>
</tr>
<tr>
<td>6.46</td>
<td>Imino (diethylene glycol)</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>6.47</td>
<td>2-Hydroxypropylmorpholine</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>6.53</td>
<td>Imidazole</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
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</table>
Table 3.3 (continued)

<table>
<thead>
<tr>
<th>pI</th>
<th>Sulfonamido buffering group derived from</th>
<th>Titrant group derived from</th>
<th>([-dz/dpH]_{pI})</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.73</td>
<td>Morpholine</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>6.75</td>
<td>4-Imidazolecarboxylic acid</td>
<td>Dimethylaminopropanol</td>
<td>1.15</td>
</tr>
<tr>
<td>6.87</td>
<td>4-Imidazoleacetic acid</td>
<td>Dimethylaminopropanol</td>
<td>1.17</td>
</tr>
</tbody>
</table>
Table 3.4 A list of the possible pyrene-based fluorescent pH markers (7 < pH < 9)

<table>
<thead>
<tr>
<th>pH</th>
<th>Sulfonamido buffering group derived from</th>
<th>Titrant group derived from</th>
<th>[-dz/dpH]_pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.01</td>
<td>2-Aminoethylmorpholine</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>7.31</td>
<td>Histidine</td>
<td>Ethylene glycol</td>
<td>0.39</td>
</tr>
<tr>
<td>7.50</td>
<td>N-(2-Aminoethyl)-tris(hydroxymethyl)aminomethane</td>
<td>Isethionic acid</td>
<td>1.14</td>
</tr>
<tr>
<td>7.78</td>
<td>Tris(hydroxymethyl)amino methane</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>8.00</td>
<td>N,N-di(2-hydroxyethyl)-N’-methyl-ethylenediamine</td>
<td>Isethionic acid</td>
<td>1.14</td>
</tr>
<tr>
<td>8.23</td>
<td>Diethanolamine</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>8.38</td>
<td>Lysine</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>8.66</td>
<td>(2-Hydroxyethyl-(ethoxy-(ethoxy))amine</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>8.70</td>
<td>Glycine</td>
<td>Dimethylaminopropanol</td>
<td>1.20</td>
</tr>
<tr>
<td>8.87</td>
<td>N-Ethyl-N-(2-Hydroxyethyl-(ethoxy-(ethoxy))amine</td>
<td>Isethionic acid</td>
<td>1.14</td>
</tr>
<tr>
<td>8.89</td>
<td>5-Aminocaproic acid</td>
<td>Dimethylaminopropanol</td>
<td>0.48</td>
</tr>
<tr>
<td>8.93</td>
<td>N-Propyl(α-alanine)</td>
<td>Dimethylaminopropanol</td>
<td>0.68</td>
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Table 3.5 A list of the possible pyrene-based fluorescent pH markers (9 < pH < 10)

<table>
<thead>
<tr>
<th>pH</th>
<th>Sulfonamido buffering group derived from</th>
<th>Titrant group derived from</th>
<th>[-dz/dpH]_pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.04</td>
<td>Di(3-propanol)amine</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>9.09</td>
<td>Glycine</td>
<td>Choline</td>
<td>1.10</td>
</tr>
<tr>
<td>9.13</td>
<td>2-Hydroxy-4-aminobutyric acid</td>
<td>Dimethylaminopropanol</td>
<td>0.92</td>
</tr>
<tr>
<td>9.20</td>
<td>N,N-(Di(2-hydroxyethyl)ethylenediamine</td>
<td>Isethionic acid</td>
<td>1.17</td>
</tr>
<tr>
<td>9.26</td>
<td>Dimethylamine</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>9.27</td>
<td>2-Hydroxy-4-aminobutyric acid</td>
<td>Dimethylaminopropanol</td>
<td>0.36</td>
</tr>
<tr>
<td>9.31</td>
<td>Iminodi(4-butyric acid))</td>
<td>Dimethylaminopropanol</td>
<td>0.34</td>
</tr>
<tr>
<td>9.33</td>
<td>N-ethyl-N-(2-hydroxyethyl)amine</td>
<td>Isethionic acid</td>
<td>1.14</td>
</tr>
<tr>
<td>9.39</td>
<td>Diethylamine</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>9.43</td>
<td>N-Methyl-N',N'(dimethyl)ethylenediamine</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>9.45</td>
<td>Lysine</td>
<td>Dimethylaminopropanol</td>
<td>0.75</td>
</tr>
<tr>
<td>9.48</td>
<td>N,N'-Di(2-hydroxyethyl)ethylenediamine</td>
<td>Isethionic acid</td>
<td>1.17</td>
</tr>
<tr>
<td>9.59</td>
<td>N-Propylamine</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>9.61</td>
<td>Lysine</td>
<td>Dimethylaminopropanol</td>
<td>0.75</td>
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Table 3.5 (continued)

<table>
<thead>
<tr>
<th>pI</th>
<th>Sulfonamido buffering group derived from</th>
<th>Titrant group derived from</th>
<th>[-dz/dpH]_{pI}</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.63</td>
<td>Ethanolamine</td>
<td>Isethionic acid</td>
<td>1.15</td>
</tr>
<tr>
<td>9.65</td>
<td>Piperazine</td>
<td>Isethionic acid</td>
<td>1.17</td>
</tr>
<tr>
<td>9.80</td>
<td>3-Hydroxy-4-aminobutyric acid</td>
<td>Choline</td>
<td>1.09</td>
</tr>
</tbody>
</table>

Seven pyrene-based pI markes with the highest structural complexity were chosen to demonstrate that the proposed synthetic approach is feasible and that the pI markers are good focusers, as predicted. Three amino acids (aspartic acid, histidine and lysine) were used to introduce the buffering groups and amino, alcohol and sulfate functional groups were selected as the charge balancing (titrating) groups (see Table 3.6). A general synthetic scheme of making the pyrene-based fluorescent pI markers is shown in Figure 3.8. The final pI marker structures are shown in Figure 3.9.
Table 3.6 Seven synthesized pyrene-based pI markers.

<table>
<thead>
<tr>
<th>pI</th>
<th>Buffering Group</th>
<th>Titrating Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.07</td>
<td>Asp</td>
<td></td>
</tr>
<tr>
<td>4.16</td>
<td>Asp</td>
<td></td>
</tr>
<tr>
<td>5.73</td>
<td>His</td>
<td></td>
</tr>
<tr>
<td>7.45</td>
<td>His</td>
<td></td>
</tr>
<tr>
<td>8.34</td>
<td>Lys</td>
<td></td>
</tr>
<tr>
<td>9.42</td>
<td>Lys</td>
<td></td>
</tr>
<tr>
<td>10.50</td>
<td>Lys</td>
<td></td>
</tr>
</tbody>
</table>

* pI values were calculated using SPARC (SPARC Performs Automatic Reasoning in Chemistry) Online Calculator v4.6.
Figure 3.8 Synthesis scheme of making the pyrene-based fluorescent PI markers.
Figure 3.9 Structures of the pyrene-based pI markers synthesized in this work and their calculated pI values.
3.1.2 Materials and Method

3.1.2.1 Chemicals and Equipment

8-Amino-1,3,6-pyrenetrisulfonic acid (APTS), trisodium salt was provided by Beckman Coulter Inc. (Fullerton, CA). 3-Aminopropionaldehyde diethyl acetal, N-Boc-L-lysine methyl ester hydrochloride and dimethyl-L-aspartate hydrochloride were purchased from TCI America Co. (Portland, OR). Chlorosulfonic acid, methyl iodide, cesium carbonate, N,N-dimethyl propanolamine, histidine methyl ester dihydrochloride, tetrahydrofuran, acetonitrile, dimethyl sulfoxide, tetra(ethylene glycol), triethylamine, sodium cyanoborohydride, potassium hydroxide, sodium sulfate, hydrochloric acid and sulfur trioxide pyridine complex were purchased from Sigma-Aldrich Co. (St. Louis, MO). HPLC analyses and LC-MS analyses were done with the instrument and column described in 2.1.2.1.

3.1.2.2 Synthesis of Compound 2 [56]

![Reaction scheme for making compound 2](image)

Figure 3.10 Reaction scheme for making compound 2.
Reaction scheme for making compound 2 is shown in Figure 3.10. A 60 mg portion of APTS was mixed with chlorosulfonic acid at a rate of 18 ml acid per gram of APTS in a dry round bottom flask, placed into an ice bath, then heated to 50°C with stirring. After 20 minutes, the reaction mixture was removed from the 50°C oil bath and carefully added into 20 mL of iced water forming a red precipitate, chlorinated APTS. The slurry was centrifuged and the supernatant was decanted to get a pasty solid. 250 µL (15 equivalents) of 3-aminopropionaldehyde diethyl acetal and 810 µL (50 equivalents) of triethylamine were premixed in 2 mL acetonitrile and the solution was mixed with chlorinated APTS at room temperature for 5 minutes. The insert in Figure 3.11 shows that the absorbance maximum of the product was red-shifted compared to that of APTS as a result of sulfonamidation. Work-up of the reaction mixture containing compound 2 was done by concentrating the reaction mixture about tenfold, then mixing it with a 20 times larger volume of water to precipitate out the target. The precipitate was used in the following methylation step without further purification.
Figure 3.11 RP-HPLC analysis of compound 2. (Insert: overlay of the UV absorbance spectra of APTS and compound 2).

3.1.2.3 Synthesis of Compound 3

Figure 3.12 Reaction scheme for making compound 3.
Reaction scheme for making compound 3 is shown in Figure 3.12. Methylation of compound 2 was done by mixing 17.4 mg of compound 2 with methyl iodide in the presence of 80 mg (12 equivalents) of cesium carbonate in 100 µL tetrahydrofuran. The extent of methylation of compound 2 was monitored by RP-HPLC as shown in Figure 3.13. Methylation was complete after adding 20 equivalents of methyl iodide. The residues of 3-aminopropionaldehyde diethyl acetal, triethylamine and water from the previous synthetic steps and the work-up procedures consumed the excess amount of methyl iodide. An overmethylated byproduct was also found during LC-MS analysis of the methylation reaction mixture. Work-up of the reaction mixture containing compound 3 was achieved by removing most of the left-over methyl iodide and tetrahydrofuran with a stream of nitrogen gas, followed by addition of 200 µL of water to precipitate out the methylated target.
Figure 3.13 (a) ESI mass spectrum (negative ion mode) of compound 3 and (b) RP-HPLC analysis of the reaction mixture and compound 3.
3.1.2.4 Synthesis of Compound 4

It was discovered earlier in our laboratory that when trisulfonamido APTS was treated with sodium hydroxide in a 50% solution of alcohols in dimethyl sulfoxide [56], one of the sulfonamido groups was replaced by an alkoxy group causing a 10 nm red shift in \( \lambda_{\text{ex max}} \) compared to that of the trisulfonamido APTS. Therefore, dimethylamino propanol and tetra(ethylene glycol) were used to react with compound 3 to make the corresponding alkoxy derivatives as shown in Figure 3.14. Both alcohols were mixed with dimethyl sulfoxide at a 1 to 1 volume ratio and mixed with compound 3. Finely ground potassium hydroxide was added to the reaction mixture as a base depot. The reactions were carried out at 80°C overnight with good stirring. The alkoxy substituted
reaction products and the starting material were analyzed by RP-HPLC as shown in Figure 3.15. The UV absorbance spectra of both derivatives were the same and agreed with the reported analogous spectra [56]. ESI mass spectra of compounds 4a and 4b are shown in Figure 3.16. The work-up procedure included a tenfold dilution of both reaction mixtures with water, followed by three successive extractions of the solution with equal volumes of ethyl acetate. Compounds 4a and 4b were obtained as oily concentrates after solvent removal and used, without further purification, in the next step.

![Figure 3.15 RP-HPLC analysis of compound 3 and its alkoxy derivatives. (Insert: UV/VIS absorbance spectra of compounds 3, 4a and 4b.)](image)
To make the desired pI marker with a calculated pI value of 10.5 as described in Figure 3.9, compound 4c carrying an alkoxy substituent with a terminal quaternary ammonium group was synthesized by methylating the dimethylamino group of compound 4a as shown in Figure 3.17. 10 mg of compound 4a was dissolved in 100 µL of tetrahydrofuran with a depot of cesium carbonate and 2 µL (3 equivalents) of methyl iodide were added at room temperature. The reaction mixture was analyzed by LC-MC after 2 hours. The results are shown in Figure 3.18. Cesium carbonate was removed.
from the reaction mixture by centrifugation and the solution obtained was used, without further purification, in the next step.

Figure 3.18 LC-MS analysis of the reaction mixture of compound 4c.

3.1.2.5 Synthesis of Compounds 5 and 6

Figure 3.19 Reaction scheme for making compounds 5 and 6.
The conversion of compound 4 (diacetal) to 5 (aldehyde) as shown in Figure 3.19 was performed on the 10 mg scale by adding 2.2 equivalents of hydrochloric acid to compound 4 which was dissolved in a mixture of 75% (v/v) acetonitrile and 25% (v/v) water. Conversion to the aldehyde form was monitored by RP-HPLC. The conversion reaction was complete within 15 minutes at 50°C for the tetra(ethylene glycol) derivative and 30 to 45 minutes for both the dimethylamino propanol and quaternary ammonium derivatives. As shown in Figure 3.20, the peak of the aldehyde compound was broadened by the moderately fast (on the time scale of the separation) equilibration rate between the diol and aldehyde forms of compound 4 in the column. Once compound 4 was converted into 5, an aldehyde, the pH of the solution was adjusted to around 4~6...
with acetic acid and a 5% sodium bicarbonate solution. Water from the reaction mixture was then removed by passing the mixture through, twice, a Pasteur pipet column that was packed with about 1 gram of anhydrous sodium sulfate, using acetonitrile as the eluent. Compound 5 in acetonitrile was then concentrated and used in the following reductive amination step. The free base form of dimethyl-L-aspartate was obtained by neutralizing dimethyl-L-aspartate hydrochloride with sodium hydroxide in water, then extracting it into dichloromethane. The same procedure was used to get N-Boc-L-lysine methyl ester from its hydrochloride salt. Histidine methyl ester dihydrochloride was neutralized with 2 equivalents of sodium hydroxide in methanol and used directly in the reductive amination step.

The general reductive amination procedure was to mix compound 5 with the corresponding amino acid esters at a mole ratio of 1 to 20 in acetonitrile or methanol with a depot of anhydrous sodium sulfate, at room temperature, followed by, after 30 minutes, the addition of sodium cyanoborohydride in tetrahydrofuran as the reducing agent. RP-HPLC analysis of the reaction mixtures obtained during reductive amination to make the respective compound 6 derivatives are shown in Figure 3.21.
Figure 3.21 RP-HPLC analysis of the reaction mixtures obtained during reductive amination to make the respective compound 6 derivatives. (a) general structure of compound 6. (b) to (f) chromatograms.
3.1.2.6 Sulfation of the TEG Derivatives of Compound 6

![Figure 3.22 Sulfation of the TEG derivative of compound 6.](image)

The APTS-based pI markers with calculated pI values of 5.73 and 7.45 have the same buffering groups (histidine) on the APTS, but the charge-balancing (titrating) groups are different: by changing the charge-balancing group from a neutral alcohol group to a sulfate group shifts the pI value from 7.545 to 5.73. The same is true for the markers with pI values of 9.42 and 8.34. The sulfation reaction was done by reacting both the histidine and lysine derivatives of compound 6 with excess sulfur trioxide pyridine complex in dimethylformamide in the presence of a catalytic amount of pyridine as shown in Figure 3.22. LC-MS analysis of the sulfation reaction mixture of the histidine derivative of compound 6 is shown in Figure 3.23.
Figure 3.23 LC-MS analysis of the sulfation reaction mixture of the histidine derivative of compound 6.

3.1.2.7 LC-MS Analysis of APTS-Based pI Makers after Purification

5 to 10 mg amounts of all 7 APTS-based pI makers were purified with semipreparative-scale RP-HPLC to get the final products and analyzed by LC-MS as shown in Figures 3.24 to 3.30.
Figure 3.24 ESI mass spectrum of the APTS-based pI marker having a calculated pI value of 3.07 (Insert: chromatogram of the purified compound)

Figure 3.25 ESI mass spectrum of the APTS-based pI marker having a calculated pI value of 4.16 (Insert: chromatogram of the purified compound)
Figure 3.26 ESI mass spectrum of the APTS-based pI marker having a calculated pI value of 5.73 (Insert: chromatogram of the purified compound)

Figure 3.27 ESI mass spectrum of the APTS-based pI marker having a calculated pI value of 7.45 (Insert: chromatogram of the purified compound)
Figure 3.28 ESI mass spectrum of the APTS-based pI marker having a calculated pI value of 8.34 (Insert: chromatogram of the purified compound)

Figure 3.29 ESI mass spectrum of the APTS-based pI marker having a calculated pI value of 9.42. (Insert: chromatogram of the purified compound.)
3.2 pI Value Determination

3.2.1 Background and Objective

There are basically two ways of determining the pI values of ampholytic compounds: directly, by isoelectric focusing (IEF) or by indirectly, finding the respective pKα values and calculating the pI value. IEF can be performed in a slab-gel format and the pH of the carrier ampholyte-filled gel segment where the analyte is focused can be measured directly with a miniaturized glass electrode. The main disadvantage of this method is that a large sample amount is needed. Performing IEF in a capillary [25] helps solve the problems encountered in slab-gel IEF and allows the use of higher applied potential for better separation efficiency. Factors affecting the measurement and pros and cons of the
cIEF determination of the pI values were discussed clearly in one of Righetti’s IEF reviews [22]. Poor engineering of the pH gradient and instability of the pH gradient are the major problems in cIEF. Shimura et al. [27] found that the calibration curve obtained by plotting the pH values as a function of detection time was not linear in most cases and pointed out that “pI values of samples should be estimated by assuming a linear relationship for pH against detection time only between two flanking marker peptides.” Another approach to determine the pI value is, in principle, to obtain the respective pKa values since the pI value can be calculated as $pI = 1/2 \left( \frac{1}{pK_{a1}} + \frac{1}{pK_{a2}} \right)$ where $pK_{a1}$ and $pK_{a2}$ are the neighboring pK values and $pK_{a1} < pI < pK_{a2}$, for a diprotic ampholyte [80]. In capillary zone electrophoresis (CZE), the pKa value of an analyte can be obtained by appropriately fitting the curve of the effective mobilities of the analyte as a function of the pH of the background electrolytes [81-83]. The pI values of the APTS based pI markers synthesized in this work were determined by pressure-mediated capillary electrophoresis (PreMCE) [84] and cIEF.

3.2.2 Materials and Method

An APTS-based molecule used as fluorescent electroosmotic flow marker (neutral marker) and hydroxypyrenetrisulfonate-based (HPTS-based) pI makers were synthesized in our laboratory [56]. Dextran sulfate sodium salt (Mw~ 500, 000), 25% (w/w), poly(vinylsulfonic acid, sodium salt), hexadimethrin bromide (Polybrene) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Pharmalyte pI 3-10 carrier ampholytes and the cIEF kit were purchased from Beckman-Coulter (Fullerton, CA).
Both PreMCE and cIEF experiments were carried out with a ProteomLab PA 800 system coupled with a LIF detector using a 488 nm argon ion laser module (Beckman-Coulter, Fullerton, CA).

3.2.2.1 Pressure-Mediated Capillary Electrophoresis (PreMCE)

The Polybrene (PB)-coated capillary used in PreMCE experiment was prepared from 25 µm I.D., 360 µm O.D. bare fused silica capillary column stock (Polymicro Technologies, Phoenix, AZ). The capillary was first rinsed with methanol for 3 minutes, water for 3 minutes and then with 1 M sodium hydroxide for 45 minutes, at 70 psi pressure. After this preconditioning step, the capillary was treated with a 10 % (w/w) Polybrene solution for 15 minutes to form the first cationic layer. A 3 % (w/w) dextran sulfate (DS) or 25 % (w/w) poly(vinylsulfonate) solution was next rinsed through the PB-coated capillary for 15 minutes to form an anionic layer on top of PB. The final PB-coated capillary had a total of 3 PB layers deposited by sequential rinses with PB and DS [85]. The same preconditioning procedure was used to make the anionic poly(vinylsulfonate)-coated capillary. After depositing the first layer of PB onto the capillary, a poly(vinylsulfonate) solution was rinsed through the capillary twice, with water rinses in between. The BGEs were prepared with HPLC grade water and the details of their preparations are listed in Table 3.7. The pH values of the BGEs were measured at 25 °C with a sympHony pH meter (VWR International, Radnor, PA) and a MicroProbe pH sensor (Thermo Fisher Scientific Inc., Waltham, MA ).
Table 3.7 Background electrolytes used in the PreMCE experiments

<table>
<thead>
<tr>
<th>pH</th>
<th>Compositions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5-3.0</td>
<td>H$_3$PO$_4$ – LiOH (Ionic strength = 5 mM)</td>
</tr>
<tr>
<td>3.5-4.0</td>
<td>β-alanine – MSA (Ionic strength = 5 mM)</td>
</tr>
<tr>
<td>4.3</td>
<td>13 mM HOAc and 5 mM LiOH</td>
</tr>
<tr>
<td>4.7</td>
<td>6.5 mM HOAc and 5 mM LiOH</td>
</tr>
<tr>
<td>6.2</td>
<td>9 mM MES and 5 mM LiOH</td>
</tr>
<tr>
<td>6.9</td>
<td>14 mM MOPS and 5 mM LiOH</td>
</tr>
<tr>
<td>8.1</td>
<td>10 mM TRIS and 5 mM MSA</td>
</tr>
<tr>
<td>8.8</td>
<td>20 mM morpholine and 5 mM MSA</td>
</tr>
<tr>
<td>9.9</td>
<td>9 mM β-alanine and 5 mM LiOH</td>
</tr>
<tr>
<td>11.4</td>
<td>0.45 mM H$_3$PO$_4$ and 4.5 mM LiOH</td>
</tr>
</tbody>
</table>

*The ionic strength of all listed BGEs was 5 mM.

PreMCE was performed according to the procedures described by Williams and Vigh [84]. The programmed potential ($V_{prog}$) was set at 30 kV with normal or reversed polarity, depending on the surface coating of the capillary. Electrophoretic separation times were varied from 2 to 3.5 minutes with linear voltage ramp-up and ramp-down times of 0.17 minutes each. The pressure mobilization velocity was about 0.1 cm/sec. Injection times ($t_{inj}$) were 4 seconds, and transfer times ($t_{tr}$) were set for 45 seconds, both at 4 psi. The analyte tested was injected first and transferred a distance into the capillary
by applying 4 psi pressure for time $t_{tr}$. Second, the EOF marker was injected and transferred a distance into the capillary by applying 4 psi pressure for the same time $t_{tr}$. Third, another EOF marker was injected and transferred, again for time $t_{tr}$. After loading the analyte and EOF markers, a potential $V_{prog}$ was applied to cause electrophoretic migration. Finally, a neutral marker was injected into the capillary at the end of the electrophoretic process and the bands were mobilized with 4 psi pressure to pass them by the detector. Examples of the detector traces obtained for both the load and the electrophoretic experiments are shown in Figure 3.31. The effective mobility ($\mu_{eff}$) values of the analyte can be determined accurately with PreMCE.

Figure 3.31 Detector traces obtained in a PrEMCE experiment.
3.2.2.2 Effective Mobility of a Polyprotic Electrolyte as a Function of pH

The pH value of an ampholyte can be determined by measuring and analyzing its effective mobility versus pH curve. According to Tiselius [22], the effective mobility of an analyte, \( \mu_{\text{eff}} \), is calculated as [86]:

\[
\mu_{\text{eff}} = \sum_{i} \alpha_{i} \mu_{i} \quad (3.1)
\]

where \( \alpha_{i} \) is the mole fraction and \( \mu_{i} \) is the ionic mobility of the \( i \)th species of the analyte.

For a monoprotic weak acid, the acid dissociation equilibrium can be schematically described by:

\[
\text{HA} + \text{H}_2\text{O} \rightleftharpoons \text{H}_3\text{O}^{+} + \text{A}^{-}
\]

The corresponding concentration-based equilibrium coefficient \( K_a \) is:

\[
K_a = \frac{[\text{A}^{-}][\text{H}_3\text{O}^{+}]}{[\text{HA}]} \quad (3.2)
\]

The analytical concentration of HA is:

\[
C_{\text{HA}} = [\text{A}^{-}] + [\text{HA}] \quad (3.3)
\]
From Equation 3.2, the molar concentration of \( A^- \) can be expressed as Equation 3.4 and substituted in Equation 3.3:

\[
[A^-] = \frac{K_a}{[H_3O^+]} [HA] \quad (3.4)
\]

\[
C_{HA} = [A^-] + [HA] = \frac{K_a}{[H_3O^+]} [HA] + [HA] = [HA] \left( \frac{K_a}{[H_3O^+]} + 1 \right) \quad (3.5)
\]

With Equation 3.4 and 3.5, the mole fraction \( \alpha_{HA} \) of HA can be expressed as:

\[
\alpha_{HA} = \frac{[HA]}{C_{HA}} = \frac{[HA]}{[HA] \left( \frac{K_a}{[H_3O^+]} + 1 \right)} = \frac{[H_3O^+]}{K_a + [H_3O^+]} \quad (3.6)
\]

Similarly, the mole fraction \( \alpha_{A^-} \) of \( A^- \) is expressed as in Equation 3.9:

\[
[HA] = \frac{[H_3O^+]}{K_a} [A^-] \quad (3.7)
\]

\[
C_{HA} = [A^-] + [HA] = [A^-] + \frac{[H_3O^+]}{K_a} [A^-] = [A^-] \left( 1 + \frac{[H_3O^+]}{K_a} \right) \quad (3.8)
\]

\[
\alpha_{A^-} = \frac{[A^-]}{C_{HA}} = \frac{[A^-]}{[A^-] \left( 1 + \frac{[H_3O^+]}{K_a} \right)} = \frac{K_a}{K_a + [H_3O^+]} \quad (3.9)
\]
For a polyprotic acid, H\textsubscript{n}A, all \( \alpha_i \) values can be expressed as:

\[
\alpha_0 = \frac{[H_n A]}{C_{HA}} = \frac{[H_3O^+]^n}{D} \tag{3.10}
\]

\[
\alpha_1 = \frac{[H_{n-1}A^-]}{C_{HA}} = \frac{K_{a1}[H_3O^+]^{n-1}}{D} \tag{3.11}
\]

\[\ldots\]

\[
\alpha_n = \frac{[A^{n-}]}{C_{HA}} = \frac{K_{a1}K_{a2}\ldots K_{an}}{D} \tag{3.12}
\]

where the denominator \( D \) is:

\[
D = [H_3O^+]^n + K_{a1}[H_3O^+]^{n-1} + K_{a1}K_{a2}[H_3O^+]^{n-2} + \ldots + K_{a1}K_{a2}\ldots K_{an} \tag{3.13}
\]

Therefore, the effective mobility of a polyprotic acid varies with the molar concentration of hydronium as:

\[
\mu_{eff} = \frac{K_{a1}[H_3O^+]^{n-1}}{D} \mu_1 + \frac{K_{a1}K_{a2}[H_3O^+]^{n-2}}{D} \mu_2 + \ldots + \frac{K_{a1}K_{a2}\ldots K_{an}}{D} \mu_n \tag{3.14}
\]

3.2.2.3 Capillary Isoelectric Focusing (cIEF)

Two different ways of performing cIEF were tested in this work. In the conventional cIEF runs, the settings followed the instructions of the cIEF kit from Beckman-Coulter. A cIEF mixture containing 2 % carrier ampholytes (CAs), 15 mM arginine, 2.5 mM
iminodiacetic acid as the anodic and the cathodic blocker, respectively, and 7 APTS-based pI markers dissolved in Beckman Coulter’s cIEF polymer solution was prepared. 200 mM H$_3$PO$_4$ and 300 mM NaOH were used as the anolyte and the catholyte. Once the capillary was filled with the cIEF mixture, focusing was done by applying 25 kV potential for 9 minutes, followed by mobilization of the content of the capillary by 0.5 psi pressure while maintaining 21 kV potential. The other way of performing cIEF was tested when we tried to optimize the cIEF runs. Instead of mixing the two blockers with CAs, they were loaded into the respective ends of the capillary sequentially. Arginine and triethanolamine were both tested as cathodic blockers, and IDA and acetic acid as anodic blockers. Lengths and concentrations of the blockers and the CAs were optimized as stated in the results and discussions section. Focusing was done by applying 30 kV potential for 9 minutes, followed by 1 psi pressure mobilization.

3.2.3 Results and Discussion

3.2.3.1 pI Value Determinations by PreMCE

We planned to determine the pI values of the APTS based pI markers by first obtaining the effective mobilities of the APTS based pI markers in background electrolytes of different pH values using pressure-mediated capillary electrophoresis (PreMCE) described in 3.3.2.1. The pKa values of the APTS based pI markers were then to be determined by nonlinear curve fitting to Equation 3.14 described in 3.3.2.2. Unfortunately, we encountered severe adsorption due to electrostatic and hydrophobic interactions between the APTS based pI markers and the capillary wall in the low ionic...
strength background electrolytes, which makes accurate measurement of the effective mobilities in certain pH ranges impossible. A detector trace obtained in a PrEMCE experiment where no chromatographic retention is evident is shown in Figure 3.32: the distance between the analyte and the first neutral marker (d_{AN}) is equal to the distance between the first and second neutral marker (d_{NN}).

![Figure 3.32 The LIF detector trace recorded in a PrMCE experiment with the pI marker having a calculated pI value of 9.42 and loaded as the first band in a background electrolyte of pH 9.6, in a Polybrene-coated capillary.](image)

Based on its structure, the APTS-based pI marker with a calculated pI value of 4.16 is considered to have the weakest chromatographic retention from among all the seven markers synthesized in this work. The pI 4.16 (calculated) marker was loaded as the
first band in a PreMCE experiments carried out in a background electrolyte of pH 3.6 in four capillaries that had different surface coatings: a cationic coating (Polybrene), an anionic coating (poly (vinylsulfonate)) and two types of hydrophilic neutral coating (cross-linked polyacrylamide and GuarantTM). The respected detector traces are shown in Figures 3.33 to 3.36. At pH 3.6, all three of the amino groups of the pI marker are protonated and at least two of its carboxylate groups are dissociated. This makes the pI marker having a calculated pI value of 4.16 a cation. Due to the presence of multiple anionic and cationic groups on the pI marker, the marker was chromatographically retained on both the cationic and the anionic surface coatings via electrostatic interactions. The APTS based pI marker having a calculated pI value of 4.16 was then tested in two other capillaries that had different types of neutral surface coatings. In these capillaries, retention was reduced but not eliminated. We also checked the behavior of the APTS based pI marker having a calculated pI value of 7.45 (considered to be the most hydrophobic APTS-based pI marker among all seven of the markers synthesized in this work due to the presence of the two histidine-derived buffering groups). As shown in Figures 3.37 to 3.40, chromatographic retention was found in all four of the coated capillaries at pH 9.0, even though the pH of the BGE was at least 1.5 pH units above its calculated pI value.
Figure 3.33 The LIF detector trace recorded in a PrMCE experiment with the pI marker having a calculated pI value of 4.16 and loaded as the first band in a background electrolyte of pH 3.6, in a Polybrene-coated capillary.

Figure 3.34 The LIF detector trace recorded in a PrMCE experiment with the pI marker having a calculated pI value of 4.16 and loaded as the first band in a background electrolyte of pH 3.6, in a poly (vinylsulfonate)-coated capillary.
Figure 3.35 The LIF detector trace recorded in a PrMCE experiment with the pI marker having a calculated pI value of 4.16 and loaded as the first band in a background electrolyte of pH 3.6, in a cross-linked polyacrylamide coated capillary.

Figure 3.36 The LIF detector trace recorded in a PrMCE experiment with the pI marker having a calculated pI value of 4.16 and loaded as the first band in a background electrolyte of pH 3.6, in a Guarant™ capillary (ALCOR Biosystems).
Figure 3.37 The LIF detector trace recorded in a PrMCE experiment with the pI marker having a calculated pI value of 7.45 and loaded as the first band in a background electrolyte of pH 9.0, in a poly (vinylsulfonate)-coated capillary.

Figure 3.38 The LIF detector trace recorded in a PrMCE experiment with the pI marker having a calculated pI value of 7.45 and loaded as the first band in a background electrolyte of pH 9.0, in a Polybrene-coated capillary.
Figure 3.39 The LIF detector trace recorded in a PrMCE experiment with the pI marker having a calculated pI value of 7.45 and loaded as the first band in a background electrolyte of pH 9.0, in a cross-linked polyacrylamide coated capillary.

Figure 3.40 The LIF detector trace recorded in a PrMCE experiment with the pI marker having a calculated pI value of 7.45 and loaded as the first band in a background electrolyte of pH 9.0, in a Guarant™ capillary (ALCOR Biosystems).
Due to the fact that strong chromatographic retention was observed even on the best commercially available coated capillaries, we were not able to use the classical zone electrophoretic pI determination method for the APTS-based pI markers. Therefore, we designed and tested in proof-of-principle experiments a new pI value determination method that combines the advantages of the immobilized pH gradient technology used in the OFFGEL instrument and the carrier-ampholyte based IEF technology [87].

The OFFGEL instrument shown in Figure 3.41 consists of two parts: an immobilized pH gradient (IPG) gel strip and, on top of it, a frame that contains the collection compartments and is tightly sealed against the IPG gel strip. For the separation, the sample is typically prepared in a mixture of carrier ampholytes and is loaded into all wells. Since the compartments are isolated from each other by ion-impermeable walls, the sample components are forced to migrate through the IPG gel where the IEF process
takes place. The analytes become focused in the gel then move into solution in the corresponding compartments by diffusion.

The pyrene-based pI marker having a calculated pI value of 7.45 was selected to test whether the OFFGEL system could be used to determine the pI value of a pI marker. First, 100µL of a 4% w/w solution of 3-10 carrier ampholyte mixture (Beckman-Coulter, Fullerton, CA) was loaded into each of the 24 compartments of the well frame positioned over the pH 3-10 IPG strip and the carrier ampholytes were pre-focused to form the pH gradient. Once the pH gradient became stable (determined by repeated measurement of the pH of the solution in each compartment), the pyrene-based pI marker was loaded into one of the wells in the OFFGEL system. Specifically, the pI marker sample having a calculated pI value of 7.45 was loaded into the well numbered 18 as indicated by the light yellow color in the photograph taken of a section of the frame shown in Figure 3.42 (a). The colored spot eventually moved to the well numbered 17 (during 6.2 kV hours of focusing) and stayed in the same well during another 11.3 kV hours of focusing as shown in Figure 3.42 (b). The content of each well was transferred into an Eppendorf tube and illuminated by the 280 nm beam of a UV light. The migration of the pI marker can be seen clearly in the photographs shown in Figure 3.43. The pH values of all wells were measured and the pH profile is plotted in Figure 3.44. The solution in well numbered 17 in which the tested pI marker sample focused has a pH value of 7.45 which agrees very well with the calculated pI value of the marker.
Figure 3.42 The OFFGEL experiment with the pI marker having a calculated pI value of 7.45 (a) loaded into the well numbered 18 and (b) focused into the well numbered 17.

Figure 3.43 Photographs of the fractions collected from the OFFGEL experiment with the pI marker having a calculated pI value of 7.45. The vials were illuminated by the 280 nm beam of a UV light: (a) blank of the focused carrier ampholyte fractions in the absence of a pI marker (background fluorescence, blue); (b) pI marker sample loaded into the well numbered 18 (sample fluorescence green); (c) the collected fractions after 6.2 kV hours of focusing and (d) the collected fractions after 17.5 kV hours of focusing.
There are two problems that plague this pI determination method. The first is that after focusing, the pH of the solution in any well will correspond to the value expected from the position of the well over the IPG strip only when the concentration of the CAs trapped in the well is higher than the minimum concentration needed to achieve a concentration-independent pH value. This concentration varies from well to well and is much higher in the very low and very high pI regions than in the vicinity of pI = 7. These required high concentrations cause the second problem: to reach them, the concentration of the CA mixture initially loaded into the OFFGEL system must also be high. Therefore, any very acidic and very basic minor component or contaminant of the commercial CA mixture will accumulate in the first few wells on the anodic side and the
last few wells on the cathodic side. They will cause high conductivities – and corresponding low electric field strengths - in the first few wells and last few wells. Consequently, electrophoretic transport out of these wells will be very slow. Also, their high buffering capacity may overwhelm the buffering capacity of the IPG strip under the well and locally prevent the IPG strip from acting as an isoelectric trapping membrane.

To illustrate the severity of these problems, an OFFGEL experiment was set up using a 24 cm long $3 < pI < 10$ IPG strip (Agilent) and a 24 compartment high resolution well frame. The wells were initially loaded with 150 $\mu$L aliquots of a 4% w/w solution of a $3 < pI < 10$ CA mixture (Agilent). The initial pH of the CA mixture was 6.85. Focusing was interrupted after 1.27 kVh, the solution from each well was transferred into an Eppendorf vial and their mass and pH were measured. Then the fractions were reloaded into the respective wells and focusing was continued until 2.2 kVh was reached. The mass and pH of the solution in the wells were again measured as before, then reloaded. The process was repeated at 4.8 kVh and 10 kVh. The results are plotted in Figure 3.45. The two teal-colored straight lines indicate the pH limits expected for the solutions in the wells based the positions of the respective wells. The 1.27 kVh curve indicates that focusing moves out the low and high pI CAs first from the 1st and the 24th wells, just as in cIEF, leaving the composition of the CA mixture in the middle mostly unchanged. By 4.8 kVh, the pH in the 1st well and the 24th well is more acidic and basic than expected from the position of the wells over the IPG strip and the nominal pI range of the CA mixture (3 to 10). By 10 kVh, the pH in the first four wells ranges from 1.4 to 1.8, well
below what can be expected from the nominal composition of the $3 < pI < 10$ CA mixture. The most basic components (that set the pH at 4.8 kVh to 10.6 in well 24) have mostly migrated out of the 24th well (into the cathodic electrode pad) by 10 kVh.

![Graph showing pH gradient profile and focusing volt hours](image)

Figure 3.45 The relationship between the pH gradient profile and focusing volt hours obtained in the OFFGEL experiments with a 4% w/w solution of a $3 < pI < 10$ CA mixture (Agilent).

Therefore, in order to avoid the problems caused by the necessarily high initial CA concentration, another type of OFFGEL experiment was run with very low carrier ampholyte concentration and the pI range of the components trapped in a given well
were determined from the position of the well over the IPG strip [87]. 0.1% w/w pre-focused CA fractions were loaded into each of the 24 compartments of the well frame positioned over the pH 3-10 IPG strip and pyrene-based pI markers were loaded in a way that they would migrate toward pH 7 (opposite to the direction of any ITP transport, if present). The pI value of pyrene-based pI markers having calculated pI values of 4.16, 5.73, 7.45 were measured this way and the results are shown in Figures 4.46 to 4.48 and Table 3.8.

Figure 3.46 Photographs of the fractions collected from the OFFGEL experiment with the pI marker having a calculated pI value of 4.16. The vials were illuminated by the 280 nm beam of a UV light: (a) pI marker sample loaded into the well numbered 2; (c) the collected fractions after 44.8kVh of focusing.
Figure 3.47 Photographs of the fractions collected from the OFFGEL experiment with the pI marker having a calculated pI value of 5.73. The vials were illuminated by the 280 nm beam of a UV light: (a) pI marker sample loaded into the well numbered 7; (c) the collected fractions after 24.3kVh of focusing.

Figure 3.48 Photographs of the fractions collected from the OFFGEL experiment with the pI marker having a calculated pI value of 7.45. The vials were illuminated by the 280 nm beam of a UV light: (a) pI marker sample loaded into the well numbered 19; (c) the collected fractions after 11.9kVh of focusing.
Table 3.8 Comparison of calculated and measured pI values of the pyrene-based pI markers.

<table>
<thead>
<tr>
<th>Calculated pI value</th>
<th>pI value measured in the OFFGEL experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.16</td>
<td>3.88-4.14</td>
</tr>
<tr>
<td>5.73</td>
<td>5.98-6.24</td>
</tr>
<tr>
<td>7.45</td>
<td>7.75-7.81</td>
</tr>
</tbody>
</table>

Table 3.8 indicates that the calculated pI values are close to the possible pI range values found in the OFFGEL experiment with the help of the wide pH-range IPG strips. Therefore, in order to narrow the range and get a better estimate for the pI values of the markers, the OFFGEL experiments will have to be repeated in the future once 24 cm long IPG strips are found with sufficiently narrow pH ranges.
Figure 3.49 (a) cIEF separation of 7 pyrene-based pI markers, the mobilization trace was obtained by simultaneously applying both pressure (0.5 psi) and potential (21 kV). The gray trace shown is the corresponding current. (b) The relationship between the calculated pI values of the markers and the mobilization times in the run shown in (a).
The carrier ampholytes have multiple anionic and cationic functional groups, just as the APTS-based pI markers. In a cIEF experiment, the carrier ampholytes are present in a $10^3$-to-$10^4$ times higher concentration than the APTS-based pI markers. Therefore the carrier ampholytes efficiently compete with the pI markers for the surface binding sites and prevent – or at least greatly mitigate – the chromatographic retention of the pI markers. Therefore, in cIEF, the peak shapes of APTS-based pI markers are reasonable.

Seven APTS-based pI markers were analyzed by cIEF under conventional cIEF settings by filling the capillary with a mixture of CAs and blockers. The pI marker samples were prepared in the same mixture of CAs and blockers and introduced into the capillary with pressure from the short end of the capillary. A focusing potential of 30 kV was applied for 9 minutes, then the mobilization trace was obtained using 0.5 psi pressure while potential was maintained at 21 kV. The calculated pI values were plotted as a function of mobilization time in Figure 3.49 and a linear fitting was applied which resulted in $R^2 = 0.9275$. The fitting result served as an indication of how much the calibration curve deviated from the expected linear relationship [88, 89].

In Figure 3.49, the basic end of the calibration curve is steeper than the acidic end.

Though assumptions concerning the cause of the curvature were discussed in the literature [26, 27], a clear explanations is yet to be provided. A number of factors can alter the pH profile in cIEF. Righetti calls it a “chemical dogma” due to the fact that in all synthetic approaches of making CAs, the amount of acidic CAs made is larger than the amount of basic ones, resulting in a nonlinear pH profile [22]. The most significant
cause of the nonlinearity of the pH gradient might be the unequal rate of bi-directional isotachophoresis (ITP) in the capillary that both accompanies and follows IEF. ITP occurs in a discontinuous BGE system, e.g., in a cIEF setting where the BGEs are nonhomogeneous. Since current is conserved along the capillary, the velocity of the analytes in ITP depends on the local field strength (E) and the effective mobility of the analyte ($\mu_{\text{eff}}$). Species with the highest and lowest $\mu_{\text{eff}}$ are called the leading and terminating electrolytes, respectively, i.e., $\mu_{\text{leading}} > \mu_{\text{analyte}} > \mu_{\text{terminating}}$. Take anionic ITP for example: anions move toward anode with different velocities. The leading electrolyte accumulates at the anode and is present in a high concentration where the conductivity is the highest and the field strength is the lowest. The anion with the second fastest velocity follows the leading electrolyte anion and stacks to a certain concentration at the boundary due to the sudden drop of field strength in the leading electrolyte zone. With the same principle, all anion bands become arranged in order of their decreasing effective mobilities. The steady state velocity of any band in a developed ITP train is proportional to the effective mobility of the analyte in the band and the field strength at the position of the band. For monovalent strong electrolytes, the concentration of each adjacent band follows the Kohlrausch regulation function [90]:

$$\sum_{i} \frac{c_i z_i}{\mu_i} = \text{constant}$$

where $c$ is the concentration, $z$ is the charge and $\mu$ is the mobility of the analyte.
In cIEF, both anionic and cationic ITP take place simultaneously, going in opposite
directions, leaving a segment of \( \text{pl 7} \) carrier ampholyte or water in the middle of the
capillary [91]. This unavoidably distorts the shape of the pH gradient. In order to
minimize the pH profile distortion caused by bi-directional ITP in the cIEF experiment,
one should apply potential only as long as necessary to finish the focusing step. To
prove this point, another cIEF experiment was performed using only pressure
mobilization once the focusing was complete as shown in Figure 3.50. Compared to
Figure 3.49, all peaks are much wider when mobilization is done by pressure only,
because there is no electrophoretic process that mitigates Taylor dispersion caused by
the laminar flow. The calibration curve obtained with pressure-only mobilization yields
\( R^2 = 0.9867 \). The better linear fit obtained for the shorter application of the electric field,
12 minutes vs. 100 minutes in this case, implies that the distortion of the pH gradient
caused by bidirectional ITP is less serious.
Figure 3.50 (a) cIEF separation of 7 pyrene-based \( p/l \) markers, the trace was obtained using pressure-only mobilization (1 psi). The gray trace is the corresponding current. (b) The relationship between the calculated \( p/l \) values of the markers and their mobilization time in the run shown in (a).
The cIEF experiments in the previous section were performed with a mixture of CAs, samples and blockers filled into the capillary. Arginine and IDA were used as blockers in the mixture and were mostly in their ionic forms [46, 47]. Therefore, in the beginning of the focusing step, most of the transported charges were carried by the blockers as they went through the capillary. The role of blocker in the cIEF process is to act as a sacrificial reagent to prevent the loss of CAs into the electrode vials. To shorten the focusing time needed, the blockers can be prepositioned by loading them segment-wise into the capillary. In these segments, most of the blocker molecules will already be in their zwitterionic form. The cIEF mobilization trace shown in Figure 3.51(b) was obtained by loading a 6 cm long plug of 200 mM arginine, a 6 cm long plug of 10% CA and sample mixture and an 8 cm long plug of 450 mM acetic acid as three sequential segments before the detection window of the capillary. A focusing potential of 30 kV was applied for 9 minutes, then the mobilization trace was obtained using 1 psi pressure. Three HPTS-based pI markers with pI values of 9.36, 7.83, 6.38 and an APTS-based pI marker with a pI value of 4.16 were used in this test to profile the pH gradient. APTS was injected before the pressure mobilization step to mark the position in the mobilization trace that corresponded to the end of the capillary. As it can be seen in the current trace, at about 7 minutes the current had decreased to a constant level suggesting that the focusing step was complete. The calculated pI values were plotted as a function of mobilization time in Figure 3.51(c) and a linear fitting was applied which resulted in \( R^2 = 0.9906 \). This is by far the best result we have observed in cIEF experiments.
Figure 3.51 (a) In this cIEF test, the anodic blocker, analytes dissolved in the CAs and the cathodic blocker were loaded into the capillary as separate segments. (b) cIEF separation of 4 fluorescent pI markers. The gray trace is the corresponding current. (c) The relationship between the pI values of the markers and their mobilization time in the run shown in (b).
3.3 Ampholytic APTS-based Carbohydrate Tagging Reagent

3.3.1 Background and Objective

Carbohydrate analysis is challenging due to the structural diversity of carbohydrates and their lack of chromophores. Therefore, one common strategy is to derivatize carbohydrates with property-enhancing tags to facilitate their analysis and detection [92]. Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique for the structural analysis of carbohydrates, but cannot be used when the amount (or concentration) of the available sample is too low. Capillary electrophoresis (CE) coupled with laser induced fluorescence (LIF) and/or mass spectrometric (MS) detection provides supreme detection sensitivity which makes it an excellent choice for carbohydrate analysis. Derivatizing reagents that facilitate carbohydrate analysis by CE coupled with LIF and/or MS detection need to be fluorescent and charged. 1-Aminopyrene-3,6,8-trisulfonic acid (APTS) (shown in Figure 3.52(a)) is a commercially available tagging agent and is wildly used in CE-based carbohydrate analysis today. Jackson first used APTS [93] to derivatize oligosaccharide ladders by reductive
amination. His method then had been developed into a validated standard bioanalytical method by Evangelista [94]. With this method, APTS-derivatized sugars can be detected by LIF using the 488 nm argon-ion laser and by MS in negative ion mode.

Here, we propose to exploit a unique property of the newly developed fluorescent pl markers and use them as pyrene-based ampholytic carbohydrate derivatizing reagents. A proof-of-principle example is shown in Figure 3.52(b), which makes the carbohydrate derivatives have higher molar absorbance at 488 nm and make them detectable in positive ion mode of MS for better detection sensitivity compared to their APTS-based counterparts. Moreover, our proposed tag converts the derivatized carbohydrates into ampholytes allowing their manipulation by isoelectric focusing (IEF) and isoelectric trapping (IET). One common, major advantage of both IEF and IET is that they facilitate sample concentration, a major advantage in bioanalysis where samples are often very dilute.

3.3.2 Carbohydrate Derivatization

3.3.2.1 Chemicals and Equipment

The p/l4 derivatizing agent was synthesized as described in 3.1. D-glucose, sodium cyanoborohydride and acetic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO). HPLC analyses, CE analyses and LC-MS analyses were done with the instruments and column described in 2.1.2.1. The cIEF analysis kit was purchased from Beckman-Coulter Inc. (Fullerton, CA).
3.3.2.2 Derivatization of D-glucose with the pI4 Reagent

![Chemical Reaction Scheme]

Figure 3.53 Reaction scheme of derivatizing D-glucose with the pI4 reagent.

3 mg of D-glucose was mixed with 50µg of the pI4 reagent in 42 µL of 15% acetic acid followed by the addition of 17 µL of 1 M sodium cyanoborohydride in tetrahydrofuran with stirring. Maltodextrin ladders were derivatized with the pI4 reagent under the same set of conditions.
Figure 3.54 RP-HPLC analysis of samples of (a) D-glucose and (b) maltodextrin ladder derivatized with the pI4 reagent.

The reaction mixtures were analyzed by RP-HPLC: the results are shown in Figure 3.54. Based on the integration of the peaks at 500 nm in the chromatogram, the derivatization of D-glucose was about 80% complete after 16 hours at 50°C. Under acidic conditions, the glucose derivative was retained in the C-18 column slightly less than the tag itself. The UV absorbance spectra of the pI4 derivatizing agent and its glucose derivative are compared in Figure 3.55. The $\lambda_{\text{max}}^{\text{ex}}$ of the glucose derivative is red-shifted by about 20 nm due to the monoalkylation of the anilinic 8-amino group after reductive amination.
with glucose. A group of peaks less retained than the pI4 derivatizing agent can be seen in the chromatogram of the derivatized maltodextrin ladder (Figure 3.54(b)) and they have the same red-shifted UV absorbance spectra as observed in the UV spectrum of glucose derivatized by the pI4 reagent.

Figure 3.55 Overlaid UV absorbance spectra of the pI4 derivatizing agent and the derivatized D-glucose

Derivatization of D-glucose was confirmed with ESI mass spectrometry. The positive and negative ion mode spectra are shown in Figure 3.56: the signal obtained in the positive ion mode is about 200 times stronger than the signal obtained in the negative ion mode.
Figure 3.56 ESI mass spectra of D-glucose tagged with the pI4 derivatizing agent in positive ion mode (top) and negative ion mode (bottom).

The derivatized carbohydrate samples were also analyzed by CE in free zone mode using a pH 2.2, 20 mM sodium phosphate background electrolyte and a bare fused silica capillary. Electropherograms of the labeling reagent and the labeled D-glucose and maltodextrin ladder samples are shown in Figure 3.57: the pI4 derivatizing agent itself has a higher cationic mobility than its sugar derivatives.
Figure 3.57 CE analyses of the pI4 derivatizing agent and its (a) D-glucose and (b) maltodextrin ladder derivatives.
The pI4 reagent-tagged glucose sample was analyzed by capillary isoelectric focusing using the Beckman cIEF kit in the PA 800. As shown in Figure 3.58, the laser induced fluorescent signals of the pI4 derivatizing agent and its glucose derivative were observed at the same time in the electropherograms, which means that the addition of glucose does not alter the pI of the tag significantly. This was also confirmed by spiking the glucose derivative sample with pI4 derivatizing agent: only one peak could be observed during cIEF analysis.

3.4 Concluding Remarks
In this work, correlations between the structure of an ampholyte and its pI value were discovered and applied to design fluorescent pyrene-based pI markers in a methodical
and systematic way. The generic structure of the designed fluorescent pyrene-based pH markers contains two identical buffering groups as part of the sulfonamido substituents attached at the 1 and 3 positions and a charge-balancing (titrating) group attached as part of an alkoxy substituent at the 6 position of the pyrene core. We successfully made 7 pyrene-based pH markers that represent the highest structural complexity in the designed marker class: they were built using three amino acids (aspartic acid, histidine and lysine) as the buffering groups and amino, alcohol and sulfate functional groups as the charge balancing (titrating) groups proving that the proposed synthetic approach is feasible. Due to hydrophobic and electrostatic interactions between the pyrene-based pH markers and CE capillary walls, the widely used zone electrophoretic measurement-based pH determination method failed. An alternative electrophoretic pH determination method that combined the advantages of the IPG-based OFFGEL instrument and semiprepartive-scale IEF method was designed and tested with the most hydrophobic pyrene-based pH marker (having histidines in the buffering groups and yielding a calculated pH of 7.45). The measured pH value was 7.45 which agrees with the calculated value.

The 7 pyrene-based pH markers were also used to profile the pH gradient in a conventional cIEF method. The calculated pH values were plotted as a function of the mobilization time and a linear fitting was applied which resulted in $R^2 = 0.9275$. The most significant cause of the nonlinearity of the pH gradient might be the unequal rate of bi-directional isotachophoresis (ITP) in the capillary that both accompanies and follows IEF. In order to minimize the pH profile distortions caused by bi-directional ITP in the
cIEF experiment, a cIEF experiment was modified using only pressure mobilization once the focusing was complete, this improved the $R^2$ value to 0.9867. An even better linear fit was obtained for the shorter application of the electric field, 12 minutes vs. 100 minutes in this case, implying that the distortion of the pH gradient caused by bidirectional ITP was rendered less serious. To shorten the focusing time, the blockers were prepositioned around the carrier ampholyte band by loading them segment-wise into the capillary. We optimized the cIEF method by loading a 6 cm long plug of 200 mM arginine, a 6 cm long plug of 10% CA and sample mixture and an 8 cm long plug of 450 mM acetic acid as three sequential segments before the detection window of the capillary. The calculated pI values of the pI markers were plotted as a function of mobilization time and a linear fitting was applied resulting in $R^2 = 0.9906$ which is by far the best result we have observed in cIEF experiments.

Moreover, we exploited a unique property of the newly developed fluorescent pI markers and used them as pyrene-based ampholytic carbohydrate derivatizing reagents. In a proof-of-principle experiment, D-glucose and maltodextrins were derivatized with the pI4 reagent using reductive amination. Derivatization of D-glucose was confirmed with ESI mass spectrometry and the signal obtained in the positive ion mode was found to be about 200 times stronger than the signal obtained in the negative ion mode with APTS as the derivatizing agent. In cIEF, the laser induced fluorescent signals of the pI4 derivatizing agent and its glucose derivative were observed at the same time in the electropherograms, indicating that the addition of the glucose moiety did not alter the pI
of the tag significantly. Compared to the commercially available carbohydrate
derivatizing reagent, APTS, the p/4 reagent yielded carbohydrate conjugates with higher
molar absorbance at 488 nm and rendered them detectable in positive ion mode MS
affording better detection sensitivity.
4. CONCLUSIONS

Two types of fluorescent molecules had been designed and synthesized to improve the analytical infrastructure of capillary-electrophoretic separations.

4.1 Amine-Reactive Fluorescent Labeling Reagent

An ideal fluorescent labeling reagent for capillary electrophoresis should result in conjugates that are hydrophilic, stable and exhibit strong and pH-independent fluorescence emission. The acridine orange-based amine-reactive monocationic fluorescent labeling reagent synthesized in our laboratory two years ago possesses most of the ideal qualities mentioned above, but it is not sufficiently hydrophilic. A more water-soluble version of the permanently cationic acridine-based fluorophore, HEG$_2$Me$_2$-DAA, with a calculated log$P$ of 0.18 was synthesized by connecting oligo(ethylene glycol) groups to the diamino acridine core structure. In the spectral studies of HEG$_2$Me$_2$-DAA, $\lambda_{ex}^{\max}$ was found at 490 nm which remains compatible with the 488 nm line of the argon ion laser, while $\lambda_{em}^{\max}$ was about 5 nm blue-shifted compared to acridine orange. The emission spectra of HEG$_2$Me$_2$-DAA are also pH-independent and the emission intensities are higher than in acridine orange due, presumably, to the presence of methyl-terminated oligo(ethoxy) chains. HEG$_2$Me$_2$-DAA was used in CE with pure aqueous background electrolyte and was found to be free of the majority of peak tailing that was caused by hydrophobic adsorption of the acridine orange-based fluorophore on the fused silica capillary. Bovine serum albumin was
labeled with excess of FL-CA-PFP and analyzed by SDS-CGE with LIF detection. The lowest tested concentration for tagged bovine serum albumin was 15 nM. Chicken ovalbumin was also labeled with FL-CA-PFP and analyzed by cIEF-LIF. The pI of the tagged proteins was shifted in the alkaline direction by about 0.02 compared to the non-tagged protein. A tri-functional probe potentially enabling selective enrichment and selective detection of a variety of molecules (e.g., natural products, pharmaceuticals, inhibitors, etc.) that carry an alkyne anchoring group that minimally perturbs their biological function was also designed and synthetized by combining biotin, FL-CA and an azide group in a “proof-of-principle” level experiment.

4.2 Pyrene-based Fluorescent pI Markers

In cIEF, the profile of the pH gradient can only be determined with the help of pI markers. Up to now, the need for LIF-compatible pI markers remained unsatisfied which limits highly sensitive and accurate cIEF-LIF analysis. A large set of pyrene-based fluorescent pI markers was rationally designed to cover the pI range 3 to 10 and guarantee excellent focusing properties. To prove the feasibility of the proposed synthetic approach, the family with the greatest structural complexity among the designed pI markers was synthesized and characterized. The classical zone electrophoretic pI determination method that relies on the measurement of the effective mobilities of the markers in BGEs of different pH values failed, because hydrophobic and electrostatic interactions between the markers and the coated capillary wall caused strong chromatographic retention even on the best commercially available coated
capillaries. Exploratory work was done to design a new pI value determination method that combines the immobilized pH gradient technology of the OFFGEL instrument and carrier-ampholytes based IEF.

The method aspects of cIEF have also been improved in this work. After evaluating the currently known and used cIEF methods, two alternatives were developed to minimize the uneven stretch of the pH gradient caused by bi-directional ITP. The linearity of the pH gradient was first improved by replacing chemical mobilization of the focused bands by pressure-only mobilization. To shorten the time during which ITP acts on the pH gradient, the focusing time was significantly reduced by loading the anodic and cathodic blockers and and the sample-containing carrier ampholytes as three sequential segments before the detection window. The new segmented loading method yielded a more linear pH gradient than the previously known methods.

To exploit a unique property of the newly developed fluorescent pI markers, we had used them as pyrene-based ampholytic carbohydrate derivatizing reagents. In proof-of-principle experiments, D-glucose and maltodextrins were derivatized by reductive amination. The pI4 carbohydrate derivatization reagent proved advantageous over APTS: the new derivatives have higher molar absorbance at 488 nm and become detectable in positive ion mode of MS affording better detection sensitivity.
cIEF-LIF can be a powerful analytical method for bio-analysis, however, three aspects needs to be further improved. The first aspect is the need to synthesize a large set of closely-spaced ($\Delta pI = 0.5$) fluorescent pI markers with good focusing ability for properly profiling the pH gradient in cIEF. A list of 18 recommended pyrene-based pI markers covering the pH range from 2.7 to 11.3 with a $\Delta pI$ of 0.5 is shown in Table 4.1. The second aspect is to search for a more suitable capillary coating to minimize analyte adsorption onto the capillary wall facilitating the analysis of dilute samples in combination with highly sensitive detectors. The third aspect is the development of cIEF methods that minimize the distortion of the pH gradient and produce reproducible and reliable results.
Table 4.1 Calculated pI values of the eighteen selected pyrene-based pI markers having a ΔpI of approximately 0.5 and a pyrene-based neutral marker.

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[55] Schulze, P., Link, M., Schulze, M., Thuermann, S., Wolfbeis, OS., et al.,


[61] Xie, H. B., Orit; Gu, Li-Qun; Cheley, Stephen; Bayley, Hagan., *Chemistry & Biology* 2005, 12, 109-120.


$^1$H-NMR (500 MHz, D$_2$O) $\delta$ 8.26 (s, 1H), 7.61-7.55 (m, 2H), 7.38 (d, 1H, J= 9.0 Hz), 7.31 (d, 1H, J= 9.0 Hz), 6.04 (s, 1H), 3.91 (broad, 2H), 3.852 (broad, 2H), 3.75-3.73 (m, 4H), 3.70-3.68 (m, 2H), 3.51-3.47 (m, 3H), 3.43-3.40 (m, 1H), 3.26-3.25 (m, 4H), 3.06 (s, 9H), 2.91-2.81 (m, 4H), 2.79 (s, 3H), 2.76 (s, 3H), 2.59-2.41 (m, 4H), 1.98-1.89 (m, 4H); $^{13}$C-NMR (500 MHz, D$_2$O) $\delta$ 176.6, 172.5, 156.6, 149.3, 133.5, 132.8, 128.8, 126.0, 125.8, 125.7, 123.8, 121.1, 120.1, 115.8, 115.0, 110.4, 108.0, 96.5, 70.0, 69.7, 68.9, 67.1, 65.2, 64.3, 59.3, 53.7, 48.7, 47.1, 46.8, 46.5, 43.9, 35.1, 35.0, 33.9, 33.7, 23.9, 23.8, 8.14.
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$^1$H-NMR (D$_2$O)
$^{13}$C-NMR (D$_2$O)
VITA

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