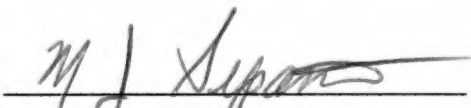
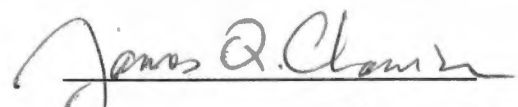
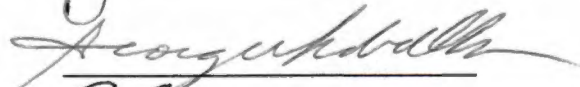
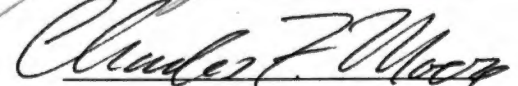


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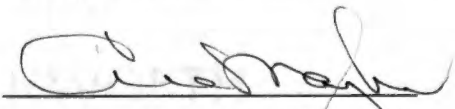
I am submitting herewith a dissertation written by William Franklin Nirode entitled "Innovative Laser-Based Optical Detection Methodologies for Capillary Electrophoresis." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Chemistry.


Michael J. Sepaniak, Major Professor

We have read this dissertation
and recommend its acceptance

Accepted for the Council:


Associate Vice Chancellor and
Dean of The Graduate School

INNOVATIVE LASER-BASED OPTICAL DETECTION METHODOLOGIES FOR CAPILLARY ELECTROPHORESIS

A Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

William Franklin Nirode
August 2000

DEDICATION

This dissertation is dedicated to my parents whose undying love and support was invaluable in the completion of this work.

This dissertation is dedicated to my wife Shannon with all my love for her constant support and love through it all.

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There are many people that I wish to thank for helping me make this work possible. I owe deep gratitude to my family for giving me their endless love and support, which made this all possible. I thank my wife for her support, encouragement, and love giving me the strength and determination to see this project to completion.

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ABSTRACT

Capillary electrophoresis (CE) has emerged as a powerful analytical technique for the separation science and other disciplines. CE has been applied to the separation of a wide variety of analytes ranging from inorganic ions, organics, proteins, DNA, and much more. However, detection methods have always been a primary concern. In fact, from the early reports of CE to the modern day advent of CE, detection methods have been problematic. Many detection methods have been employed with CE ranging from absorbance, electrochemical, conductivity, laser induced fluorescence, thermal lens, and others. The increasing pressure to detect small amounts down to the single molecule level has led to the improvement of the above methods and the implementation of new methods. One new method introduced is the use of sheath flow cells (SFC) for ultrasensitive detection down to the attomole level and approaching the single molecule level. With this development of SFC, has come the implementation of new applications. A post-column derivatization technique based on the SFC arrangement for the study of DNA-protein interaction is explored. The development of SFC as a detection methodology has warranted the need for a rugged device that can be used in a routine laboratory setting. A new device based on this concept is introduced, constructed, and implemented for reduced dimension separation techniques such as CE.

One of the drawbacks to the aforementioned detection methods is the inability to obtain structural information from a CE separation. With the recent growth and explosion of surface enhanced Raman scattering (SERS), detection levels have approached the single molecule level with this method. This work combines the advantages of CE with the structural information inherent to SERS to obtain structural

information from a CE separation. CE-SERS is introduced, implemented and evaluated in this work. The coupling of CE with SERS offers the advantages of an alternative for qualitative analysis, kinetic studies, and others.

ORGANIZATION

Chapter 1 is intended to introduce the reader to the fundamentals of chromatographic techniques such as liquid chromatography and capillary electrophoresis. Emphasis is placed on CE, which governs the work presented. However, effort is made to draw comparisons between the two techniques. Current detection methods are presented and background information is given for the detection strategies developed in this work.

Chapter 2 discusses the use of a sheath flow cell for the study of DNA-protein interactions. The major focus of this chapter is the evaluation of a sheath flow cell as post-column reactor for post-column fluorescence derivatization in CE.

Chapter 3 details the design and construction of a robust sheath flow cell based detection system. Extensive research and development on various experimental designs are examined and evaluated to produce a working model. The design is tested for routine analysis in a laboratory setting.

Chapter 4 entails the development of novel detection method mating SERS with CE. This new means of detection for CE is evaluated with a set of test compounds. A variety of experimental conditions are optimized and presented. Finally, preliminary SERS-CE work is shown to be a useful tool for biochemical analysis in the pharmaceutical field.

Chapter 5 summarizes the work presented in chapters 2-4 and offers some future direction and possible applications of the detection methods developed.

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ABBREVIATIONS AND SYMBOLS

A	absorbance
a'	radius of ion
AP	ammonium persulfate
Ar^+	argon ion laser
α	polarizability
b	detection pathlength
bp	base pairs (of DNA fragment)
BKG	background
c	velocity of light
C	sample concentration
CCD	charged couple device
CE	capillary electrophoresis
CEMSA	capillary electrophoretic mobility shift assay
CGE	capillary gel electrophoresis
CMC	critical micelle concentration
CZE	capillary zone electrophoresis
D	diffusion coefficient
e	charge per unit surface area
E	applied electric field
$\Delta \bar{E}_{\text{vib}}$	vibrational transition energy

E_{in}	electric field of incident light
E_{vib}	vibration energy levels
ϵ	permittivity of free space
	molar absorbtivity
EB	ethidium bromide
EDTA	ethylenediaminetetraceitc acid
EMSA	electrophoretic mobility shift assay
EOF	Electroosmotic flow
F	force
	fluorescence emission
F'	frictional force
f	frictional coefficient
f/l	focal length of one
FWHM	full width half maximum
FITC	fluorescein isothiocynate
GC	gas chromatography
g	gravitational constant
γ MTMS	γ -methacryloxypropyltrimethoxysilane
h	Plank's constant
$h\nu$	energy incident photon
H	plate height
η	viscosity
He-Cd	helium-cadmium laser

He-Ne	helium-neon laser
HPLC	high performance liquid chromatography
HQS	8-hydroxy-quinoline sulfate
HV	high voltage
Δh	height difference
i.d.	inside diameter (of column)
$I_{\max}$	intensity maximum
IR	infrared absorption spectroscopy
I_o	excitation source intensity
I_f	fluorescence intensity
k	Boltzman constant
kV	kilovolt
k'	analyte retention
κ_b	conductivity of running buffer
κ_a	conductivity of sample
k_f	fluorescence rate constant
k_i	intersystem crossing rate constant
k_{ec}	external conversion rate constant
k_{ic}	internal conversion rate constant
k_{pd}	predissociation rate constant
k_d	dissociation rate constant
λ	wavelength
l	effective length (to the detector)

L	length of column
LC	liquid chromatography
LDR	linear dynamic range
LIF	laser induced fluorescence
LOD	limit of detection
m	meter
mW	milliWatt
μA	microamps
mm	millimeter
M	molarity
M	magnification
MC	methyl cellulose
MECC	micellar electrokinetic capillary chromatography
μ	oscillating dipole
$\mu(t)$	magnitude of the dipole moment at time t
μ^0	maximum induced dipole moment
μ_{app}	apparent electrophoretic mobility
μ_{eof}	mobility of electroosmotic flow
μ_{int}	intrinsic electrophoretic mobility
μ_{obs}	observed electrophoretic mobility
$\bar{\mu}$	average mobility
$\Delta\mu$	average mobility

μm	micrometer
nA	nanoamp
nm	nanometer
N	efficiency
OPA	o-phthaldialdehyde
o.d.	outside diameter
PAGE	polyacrylamide gel electrophoresis
PDA	photodiode array
PMT	photomultiplier tube
Φ	fluorescence quantum yield
Φ^*	entanglement threshold
Φ_f	fluorescence quantum efficiency
ΔP	pressure drop
P_o	power of excitation beam
P	power
Phe	L-phenylalanine
P_f	fluorescence signal intensity
q	charge on ion
ρ	density of the sample
Q	sample plug
RB	running buffer
Rb	riboflavin (vitamin B12)
Rh6G	rhodamine6G

R_s	resolution
R_g	radius of gyration
r^2	inner radius
RRS	resonance Raman scattering
S	signal
S/N	signal to noise ratio
SEM	scanning electron microscopy
σ^2	variance
σ_{sc}	cross section
S_0	ground singlet state
S_1	first singlet excited electronic energy level
S_2	second singlet excited electronic energy level
SGE	slab gel electrophoresis
SSCE	size selective capillary electrophoresis
SERS	surface enhanced Raman scattering
SERS	surface enhanced Raman spectroscopy
SFC	sheath flow cell
t	migration time
t_0	void time
t_m	migration time of micelle
T	total time
T_1	first triplet excited electronic energy level
TBE	tris-boric-acid-EDTA

TEMED	N,N,N',N'- tetramethylethylenediamine
Try	L-tryptophan
Tyr	L-tyrosine
UV	ultraviolet
UV-VIS	ultraviolet-visible
V	volts
	volume injected
v	velocity of ion
$\bar{\nu}$	vibration frequency
$\bar{\nu}_s$	frequency of Raman scattered radiation
$\bar{\nu}_o$	frequency of excitation line
V_{eof}	electroosmotic flow
w	width of peak at base
$w_{1/2}$	width of peak at half-height
WD	working distance
z	valence electrons
ζ	zeta potential
ζ'	pore size

CHAPTER 1

INTRODUCTION TO CHROMATOGRAPHY, CAPILLARY ELECTROPHORESIS, AND METHODS OF DETECTION

INTRODUCTION TO CHROMATOGRAPHY

Brief Historical Perspective

In today's world of complex mixtures and matrices there is an intrinsic need to separate, purify, and identify individual components of these mixtures. Consequently, the separation of individual components from a complex mixture is more often than not a vital step in analytical procedures. Without doubt, the most widely used method of performing these separations of individual components is chromatography. Traditionally, classical sample preparation methods (e.g. solvent extraction and solid phase extraction) were used for the separation of mixtures of compounds into individual components. However, instrumental chromatographic techniques have become more popular alternatives to the aforementioned classical approaches as evidenced by their wide use ranging from metal analysis, environmental contaminants, peptides, DNA analysis, various assays, and more [1-10]. Today, the instrumental chromatographic techniques most often used by the analytical chemist are traditional chromatography and electrokinetic based techniques.

Chromatography includes a diverse and significant group of techniques, which allow for the separation of closely related components of complex mixtures. Chromatography can be defined as a separations technique in which the individual components of a mixture are separated based on the rates that they are carried through a

stationary phase by a mobile phase. In all chromatographic techniques, the sample is dissolved in the mobile phase, which can be either a liquid or a gas. The mobile phase is then forced through an immiscible stationary phase, which is fixed in place in a column or on a solid surface. The overall separation process is governed by the partitioning of analytes between the stationary phase and the mobile phase depending on their affinity for either the stationary or mobile phase. For example, components, which are strongly retained by the stationary, migrate slowly with the mobile phase. Conversely, components, which are weakly retained by the stationary phase, move quickly with the flow of the mobile phase. In gas chromatography, the vapor pressure of the component at the column temperature is also a factor.

There are two basic classifications of chromatography: column chromatography and planar chromatography. Column chromatography techniques will be briefly discussed here (a review on planar chromatography can be found in the literature) [11]. In column chromatography, the stationary phase is coated on, chemically bonded to, or packed in, the capillary. The mobile phase is forced through the column through a pressure driven system. There are two general types of column chromatography, which will be discussed: Gas Chromatography (GC) and Liquid Chromatography (LC).

Gas Chromatography

Although chromatographic techniques were first reported in 1903 by Tswett [12], not until 1952 was an instrumental separation method introduced when James and Martin first introduced Gas Chromatography (GC) [13]. GC quickly became the chromatographic technique of choice due to its simplicity and reproducibility. Early

forms of GC used glass or stainless steel tubing as columns (usually $\frac{1}{4}$ inch inside diameter) and were packed with particles coated with a desired stationary phase. A drawback to this first type of GC employed was the poor efficiency that was obtained with these poorly packed columns. In turn, the poor efficiency limited quantitative analysis of complex mixtures. Furthermore, the high pressure drops associated with these packed columns limited lengthening of the column to improve the overall efficiency of the separation.

The greatest improvement in GC was the introduction of open tubular columns. Open tubular columns have a 200-500 μm inner diameter (i.d.) with the stationary phase coated on or chemically attached to the capillary wall. Because of greater permeability, these columns can be made up to 32 times longer than packed columns while maintaining low pressure drops and increasing the efficiency. This evolution in column preparation made it possible to separate and quantitatively analyze moderately complex mixtures and made GC a powerful separations technique. However, a major disadvantage to GC is the necessity that the sample be volatile at relatively low temperatures. This requirement limits the range of samples that can be analyzed by GC to only about 20% of organic compounds. A need for the development of a chromatographic technique for the analysis of liquid samples was warranted.

Liquid Chromatography

Liquid chromatography (LC) was originally introduced by Tswett [12]. His separations were carried out in glass columns with diameters of 1 to 5 cm and lengths of 50 to 500 cm. For reasonable flow rates, the diameters of the particles of the solid

stationary phase were usually in the 150 to 200 μm range. The typical flow rates used were in the range of tenths of an mL per minute that resulted in separation times of several hours. Attempts to increase the flow rates to improve efficiency were unsuccessful. Early in the development of LC it was realized that in order to increase column efficiency there was a need for major changes in the particle size of the packing. Early development in LC involved decreasing the particle size of the packing. However, the development of LC did not take off until the late 1960's when greater emphasis was placed upon developing LC as a complementary technique to GC. Advances in both instrumentation and column packings occurred very rapidly. It was in the late 1960's that the technology for producing particle diameters as small as 3 to 10 μm was developed. It was this column technology, as well as advances in instrumentation, that gave birth to "high-performance liquid chromatography" (HPLC). Today, HPLC is the most widely used of all the analytical separation techniques. The reasons that HPLC has reached tremendous popularity is that HPLC is readily adaptable to accurate quantitative determination, suitable for separating nonvolatile species and has widespread applicability to a variety of materials. The introduction of HPLC expanded the applicability of instrumental chromatographic techniques to 90% of all compounds.

INTRODUCTION TO CAPILLARY ELECTROPHORESIS

Brief Historical Perspective

As liquid chromatography was further developed in the late 1960's so was a technique that would later compete with LC, electrophoresis. This technique may be traced back as far as 1930 to the work of Arne Tiselius who demonstrated the first real

example of electrophoresis by separating proteins based on the electrical charges on their surfaces [14]. In 1948, Arne Tiselius was awarded the Nobel Prize in Chemistry for his work with electrophoresis. However, potentially the most significant advance in the development towards capillary electrophoresis (CE) as an analytical method was the transfer of electrophoresis from a slab format to a column format.

The early years of development of CE saw the technique used for samples to identify one or two major components. Stellan Hjerten reported on the theory of applying an electric field to a sample of solution, which would separate the analytes into distinct zones [15]. Furthermore, Hjerten noticed that under this applied electric field that analytes migrated at different rates forming distinct zones. However, Hjerten observed that with the electric field, the system is heated causing thermal gradients and producing poor resolution. In order to reduce the effects of Joule heating, Hjerten demonstrated electrophoresis in a column format for free solution electrophoretic separations in 1967 [16]. The column format allowed for the application of higher applied fields without the consequences of heating effects. Unfortunately, Hjerten was limited by detection methods for small bore columns; hence, he used 3 mm diameter tubes for his separations. To help counteract the thermal problems associated with this large size tubes, the tubes were slowly rotated about the longitudinal axis.

Further development of column electrophoresis was described by Virtanen where the author used a potentiometric detector for free zone electrophoresis in 200-500 μm diameter Pyrex tubes [17]. Mikkers, Everaerts, and Verheggen utilized 200 μm diameter Teflon tubes for free solution separation [18]. The authors separated a variety of organic and inorganic anions using both ultraviolet (UV) and conductometric detection schemes.

However because of the poor detectability of the technique, large injection volumes were necessary which led to very poor efficiencies in these column electrophoresis techniques.

The works described above were very important in the development of CE. However, it was the pioneering works of Jorgenson and Lukacs that led to modern day CE [19, 20]. These works demonstrated highly efficient (4×10^5 theoretical plates) electrophoretic separations of peptides, amines, and amino acids. The separations were done in 75 μm i.d. fused silica capillaries, which could successfully dissipate the heat generated by the applied electric field of 30 kV. To overcome problems in detection methods as reported by previous authors, Jorgenson and Lukas used fluorescence detection with these narrow-bore capillaries. In addition, the theory of CE was expanded to include the importance of electroosmotic flow in determining the mobility of ions and the affects on both resolution and analysis time. This work led to the explosion and continued growth of modern day CE. Today, CE is used to separate a wide variety of analytes ranging from peptides, proteins, metal ions, DNA, toxins, and much more [21-28]. Almost any detection method can be coupled to CE with several reviews in the literature [29-32]. Concurrently, vast improvements in CE separations have occurred (see review in literature) [33].

Theory of CE

Electrophoretic Migration

Electrophoretic separations are based on the different migration rates of ionic species in an applied electric field. The direction and magnitude of the ion's migration rate is dependent on the magnitude and charge of the ion and the magnitude and direction

of the applied electric field. In an applied electric field, the ion experiences a force (F) that is proportional to the charge of the ion (q) and the electric field (E) given by:

$$F = qE \quad (1.1)$$

The applied electric field (E) can be defined as follows:

$$E = \frac{V}{L} \quad (1.2)$$

where V is the applied voltage and L is the column length (usually in cm). This resultant force causes the ion to migrate towards the oppositely charged electrode. Opposing this directional force of the ion is a counteracting frictional force (F') given by:

$$F' = 6\pi\eta a'v \quad (1.3)$$

where η is the solution viscosity (N s/m²), a' is the radius of the ion (m), and v (m/s) is the velocity of the ion. During electrophoresis a steady state is reached almost immediately and is defined by the balance of F and F' where the forces are equal but opposite as shown in Eq. 1.4:

$$qE = 6\pi\eta a'v \quad (1.4)$$

Solving Eq. 1.4 for velocity yields:

$$v = \frac{q}{6\pi\eta a'} E \quad (1.5)$$

The frictional coefficient ratio ($q/6\pi\eta a'$) is proportional to the charge to size ratio and determines the electrophoretic mobility (μ_e) of a given ion. The μ_e is defined as the velocity per unit field strength and is derived from Eq. 1.5 to yield the following:

$$\frac{q}{6\pi\eta a'} = \mu_e = \frac{v}{E} \quad (1.6)$$

From the above equations it is evident that small, highly charged species will have high electrophoretic mobilities where large, lowly charged species will have small mobilities. Electrophoretic separations are based on the differences in μ_e values.

Electroosmotic Flow

The governing force and a very important phenomenon behind free solution CE is electroosmotic flow (EOF). EOF is the bulk flow of the liquid in the capillary and is a consequence of the surface charge on the capillary wall. EOF results from the electroosmotic pumping action, which originates at the wall of the capillary due to the interaction of solvated cations with the negatively charged capillary surface (discussed in more detail later). When the electric field is applied across the capillary, these solvated cations at the wall-solution interface will migrate towards the cathode due to electrostatic attraction while dragging along with it the entire solution within the capillary. A depiction of EOF is shown in Figure 1.1. The velocity of EOF (V_{EOF}) depends on properties of the solution and the capillary as follows:

$$V_{EOF} = \frac{E\zeta\epsilon}{\eta} \quad (1.7)$$

where ζ is the zeta potential at the capillary wall, ϵ is the dielectric constant of the solvent and η is the solution viscosity. The zeta potential is the potential at the interface between the immobile capillary wall (including any adsorbed ions) and the freely moving buffer. As can be seen from Eq. 1.7 there is a direct relationship between the velocity of EOF and the zeta potential.

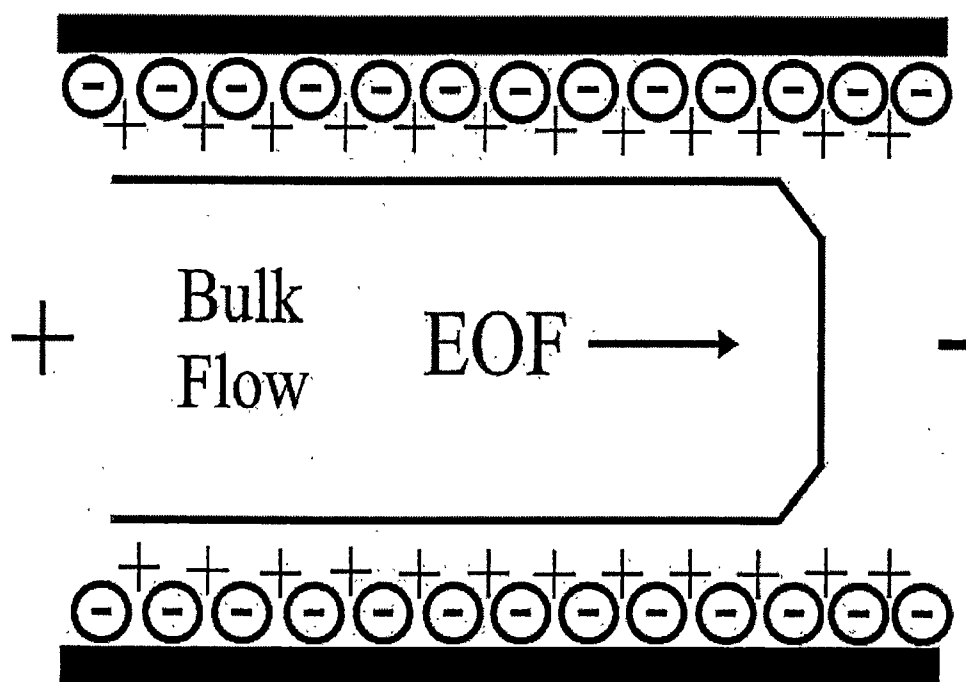


Figure 1.1. Depiction of EOF. The effect of electroosmotic flow (EOF) dragging the bulk flow of the liquid towards the cathode

The walls of the capillary are made up of fused silica and exhibit a negative charge above a pH of 3.0. The solvated cations in solution are attracted to the negatively charged surface, thus they will migrate to the capillary wall. This build up of cations at the capillary-solution interface results in the formation of the charged double layer. When an electric field is applied across the capillary, the solvated cations are attracted towards the cathode end of the capillary. With this migration towards the cathode, the cations drag along with it the bulk flow of the solution. Therefore, the formation of a charged double layer gives rise to the zeta potential and consequently EOF. This formation of the charged double layer and consequent build up of EOF can be seen in Figure 1.2.

Examining Eq. 1.7 and recalling Eq. 1.6, the mobility of EOF can be defined as:

$$\mu_{\text{EOF}} = \frac{\zeta \varepsilon}{\eta} \quad (1.8)$$

Equation 1.8 demonstrates the intrinsic nature of the mobility of EOF because it is independent of the applied field as well as its lack of radial dependence. This lack of radial dependence for EOF results in a flat or plug-like flow profile that is partially responsible for the high efficiencies attained in CE. Since EOF is the driving force of flow and is generated at the walls of the capillary, a uniform distribution of flow across the capillary is observed, as seen in Figure 1.3. Comparing the flow profile observed in CE to that of an ideal case, it is seen that flow profiles are nearly identical. However, in the case of LC methods that use the shear force of pumps to attain flow, a laminar flow profile is observed which generates unequal flow rates for analytes at different positions

Charged Double Layer

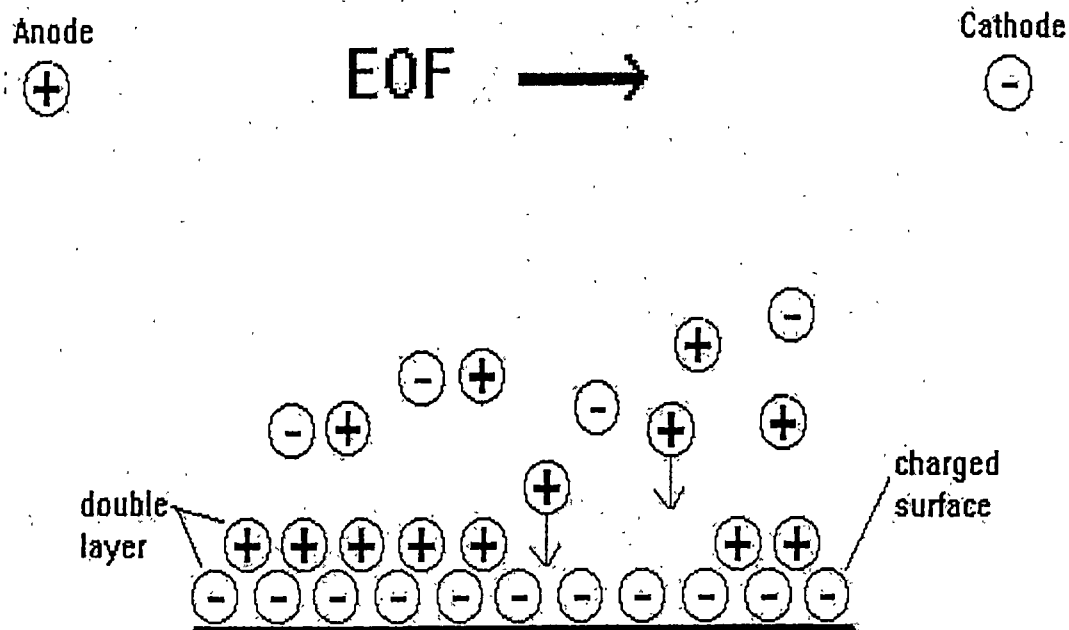


Figure 1.2 Charged double layer. The formation of the charged double layer giving rise to the zeta potential and the phenomenon of electroosmotic flow (EOF).

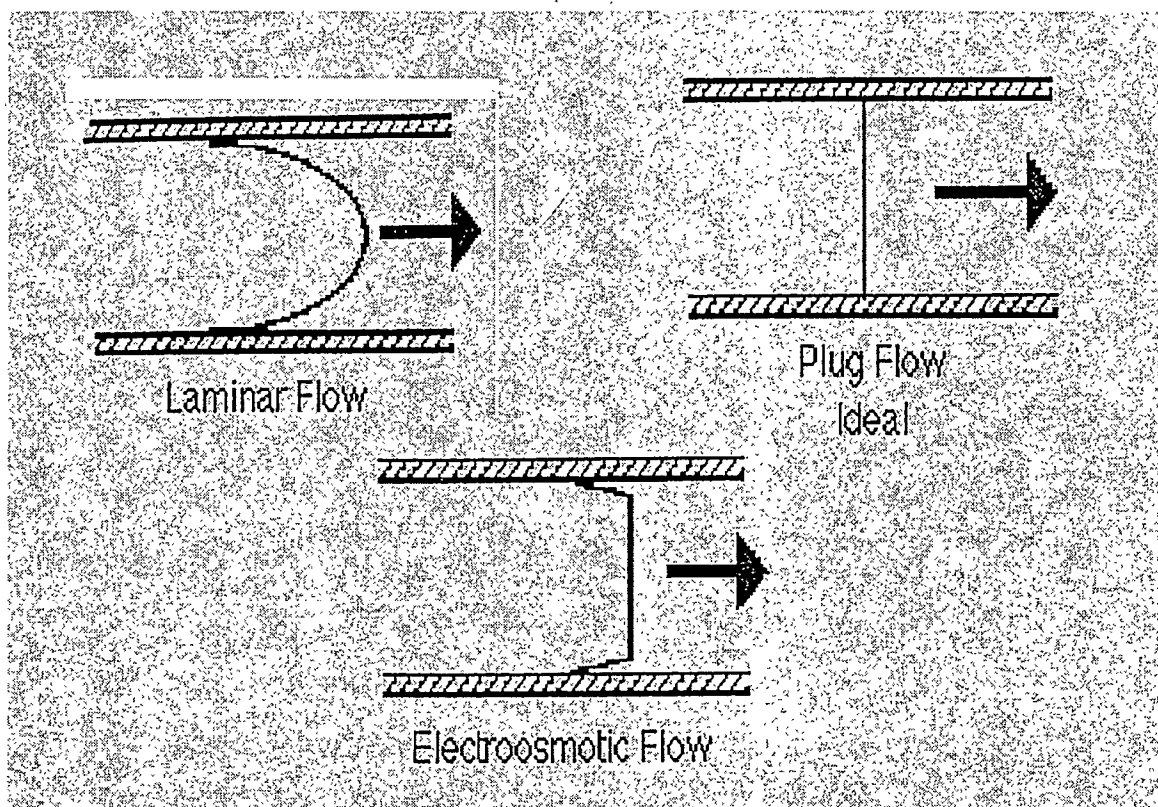


Figure 1.3. Flow profiles. Pictorial depicting of the laminar flow profile of HPLC, the flat flow profile of CE, and the ideal plug flow profile.

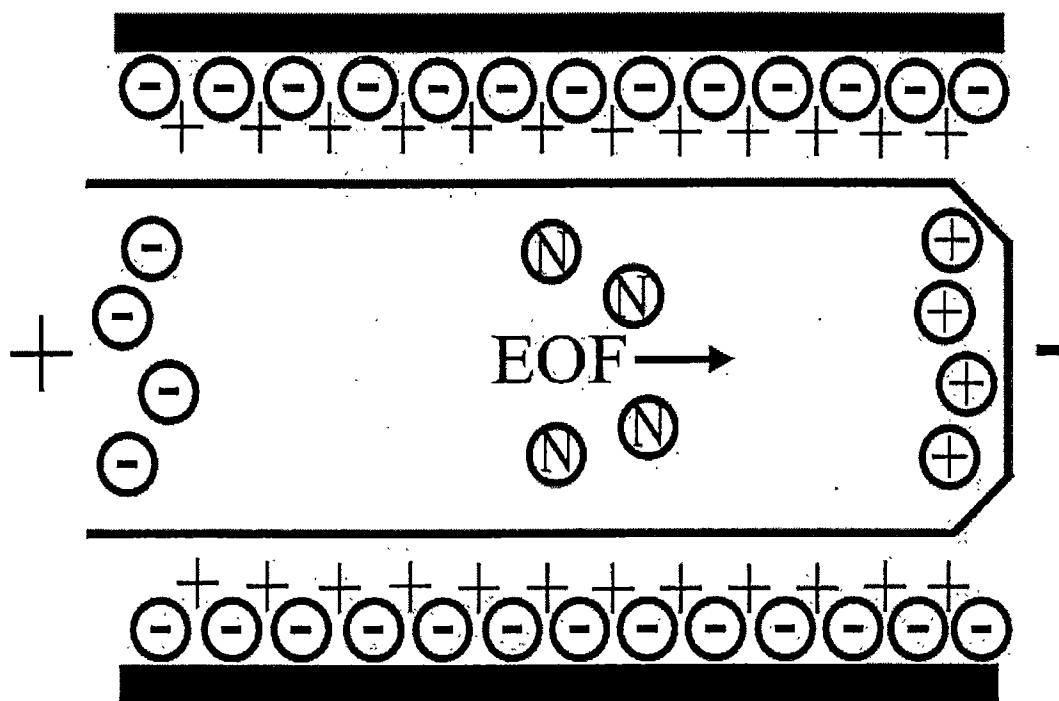
within the column. This laminar flow profile represents a significant source of band dispersion in LC techniques, which are not present in CE.

Observed Electrophoretic Mobility

The phenomenon of EOF allows for the detection of all analytes regardless of charge in a single analysis. This process is true because the magnitude of μ_{EOF} is generally greater than the electrophoretic mobilities of the analyte ions. The observed electrophoretic mobility that any individual ion experiences in the capillary is an additive affect of its intrinsic mobility, μ_e , and the mobility of electroosmotic flow, μ_{EOF} . This observed electrophoretic mobility μ_{obs} , is given by:

$$\mu_{obs} = \mu_e + \mu_{eof} \quad (1.9)$$

Understanding Equation 1.9 is crucial in understanding how a separation occurs in CE. As seen in Figure 1.4 each analyte has its own μ_{obs} but it is the effect of μ_{EOF} that influences the migration rate of the analyte. Cationic analytes have μ_e that is in the same direction as μ_{EOF} ; hence, the mobilities are added producing a μ_{obs} greater than that of μ_{EOF} . Conversely, anionic analytes have an intrinsic mobility, which is towards the anode ($-\mu_e$) the opposite the cathodic direction of EOF. When the $-\mu_e$ and μ_{EOF} are added in this case, the resultant is an observed mobility less than that of μ_{EOF} . Hence, anions will be dragged towards the cathode by the stronger EOF. Neutral analytes do not exhibit an electrophoretic component, thus they are carried with the mobility of EOF. This is shown in Figure 1.4 and summarized in Figure 1.5.



$$\mu_{\text{obs (+)}} = \mu_{\text{EOF}} + \mu_{\text{e(+)}}$$

μ_{EOF} = mobility of neutral species

$$\mu_{\text{obs (-)}} = \mu_{\text{EOF}} + \mu_{\text{e(-)}}$$

Figure 1.4. Mobilities in CE. The observed mobilities for a cationic, neutral, and anionic analytes respectively in CE.

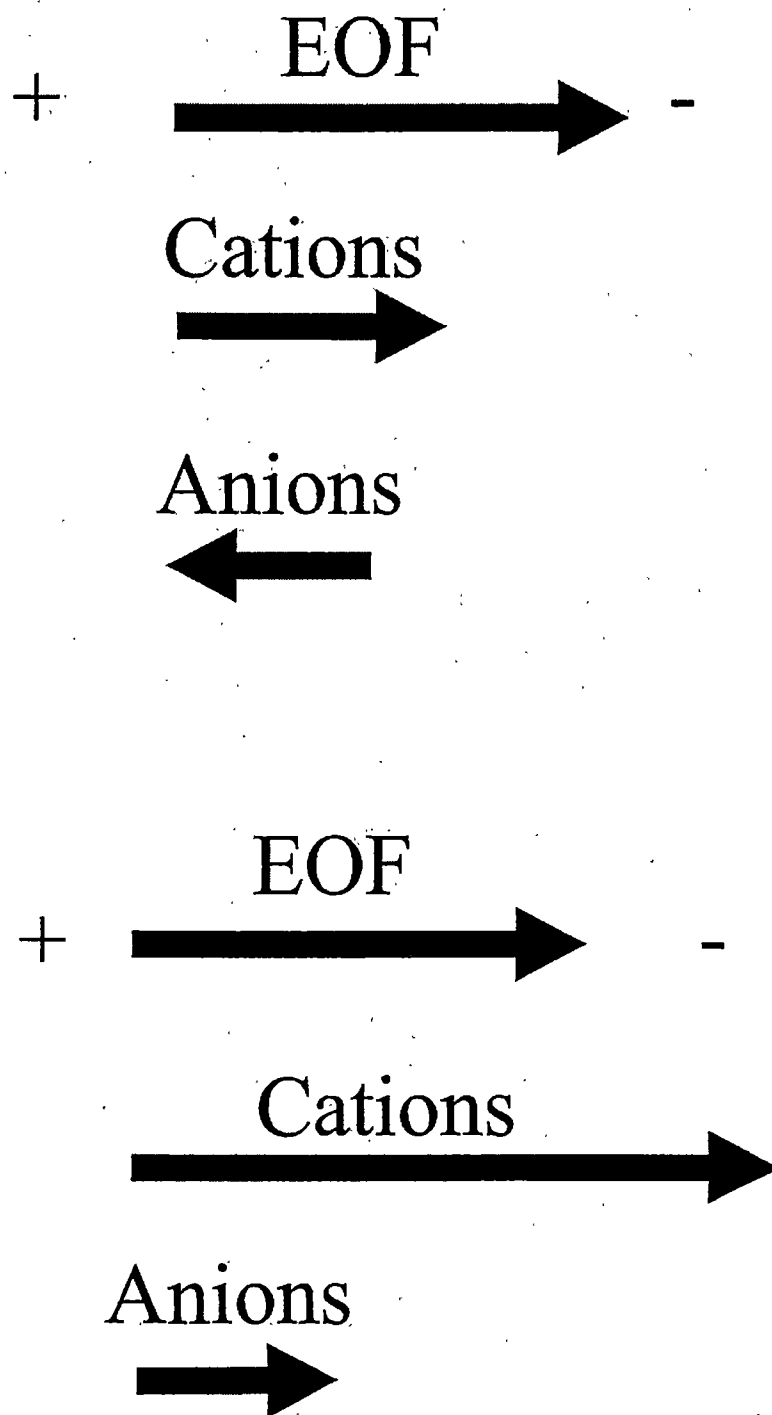


Figure 1.5. Flow Vectors in CE. (*top*) The flow vectors of EOF, cations, and anions in CE. (*bottom*) The resultant flow vector after taking into account the effect of EOF.

The time required for a given analyte to reach the point of the detection along the capillary is called the migration time and given simply by the quotient of distance migrated and velocity it traveled. The migration time of an analyte can be used to determine the observed mobility from the analysis by the following:

$$\mu_{obs} = \frac{l}{tE} = \frac{lL}{tV} \quad (1.10)$$

where l is the length of the capillary to the detector (effective length), L is the total column length, t is the migration time, and V is the applied voltage.

Dispersion and Efficiency

In electrophoresis, the separations are based on the differences in μ_e , which are related to charge ratios (e.g. different mobilities) of the analytes in solution. Dispersion is an important parameter in CE to be controlled because it can widen the zone and the mobility difference needed to achieve a well-resolved separation. To achieve a separation with well-resolved bands, the chromatographic band should remain in a narrow Gaussian distribution as it travels through the column. As the band travels through the capillary, there will be a certain amount of dispersion or broadening of the band. The broadening of a Gaussian solute band is represented by its standard deviation, σ , or variance σ^2 . The width of a Gaussian solute band, w_b , can be defined as:

$$w_b = 4\sigma \quad (1.11)$$

Plate height (H) is usually calculated to relate the amount of variance per unit length (L) of the separation column as given by:

$$H = \frac{\sigma^2}{L} \quad (1.12)$$

Efficiency (N) of a separation is the number of theoretical plates obtained and can be related to H by the following:

$$N = \frac{L}{H} \quad (1.13)$$

There are several factors that can influence dispersion in CE and hence, the efficiency of a separation. In CE, the absence of packed stationary phase and the laminar flow profile seen in LC separations, eliminate two significant sources of dispersion. Therefore, CE separations are generally much more efficient than LC separations. The factors in CE that affect dispersion such as diffusion, injection, detection, thermal effects, and others as given by:

$$\sigma_{total}^2 = \sigma_{diff}^2 + \sigma_{inj}^2 + \sigma_{det}^2 + \sigma_{thermal}^2 + \sigma_{other}^2 \quad (1.14)$$

Other sources of variance can include solute adsorption to the capillary wall and mismatched conductivities of the sample and buffer (electrodispersion). Minimizing dispersion due to adsorption can be accomplished by optimizing buffer content and pH. Proper selection of buffers can help to minimize electrodispersion. The effects of dispersion from injection and detection can be partially overcome by small injection and detection plug lengths. Thermal dispersion effects are minimal for narrow bore capillaries since they dissipate heat much more effectively as previously discussed. Under ideal conditions, it can be assumed that the major source of dispersion is due solely to longitudinal diffusion. Variance from molecular diffusion can be determined from the Einstein equation as follows:

$$\sigma^2 = 2Dt \quad (1.15)$$

where D is the molecular diffusion coefficient (cm^2/s) and t is the time for diffusion to occur. Solving Equation 1.10 for t and substituting into Equation 1.15, efficiency can be related to the molecular diffusion term in chromatography as given by:

$$\sigma^2 = \frac{2DL}{\mu_e V} \quad (1.16)$$

Substituting Equation 1.12 for H in Equation 1.13 yields a fundamental expression for N in CE as given by:

$$N = \frac{L^2}{\sigma^2} = \frac{\mu_e El}{2D} \quad (1.17)$$

From Equation 1.17 it is evident that higher fields results in an increase in efficiency simply because the analyte spends less time in the capillary and has less time to diffuse. Efficiency can be determined directly from an electropherogram from the following equation:

$$N = 5.54 \left(\frac{t}{w_{1/2}} \right)^2 \quad (1.18)$$

where $w_{1/2}$ is the width of a peak at half the height in time units.

Resolution

Resolution of a mixture of components in a sample is a very important parameter in any separations technique. It can be problematic to accurately identify and/or quantitate an individual component if they are poorly resolved. All of the parameters discussed in the previous section, which affect dispersion and efficiency, play an

important role in determining the resolution of a separation. In fact, resolution can be expressed with respect to efficiency as given by:

$$R_s = \frac{\sqrt{N}}{4} \left(\frac{\Delta\mu}{\mu_{\text{avg}}} \right) \quad (1.19)$$

where $\Delta\mu$ is the difference in the mobilities of two given analytes and μ_{avg} is the average of the two mobilities of the two analytes. Substituting Equation 1.18 into Equation 1.19 yields a theoretical expression for resolution given by:

$$R_s = \left(\frac{1}{4\sqrt{2}} \right) (\Delta\mu) \left(\frac{V}{D(\bar{\mu}_e + \mu_{\text{EOF}})} \right)^{1/2} \quad (1.20)$$

It is apparent from Equation 1.20 that an infinite resolution can be reached when $\bar{\mu}$ and μ_{EOF} are equal but opposite each other (e.g. the two analyte migrate in the opposite direction). However, analysis time would approach infinity and only one analyte would be detected. Experimental parameters should be controlled to optimize both resolution and analysis time. Resolution can be measured directly from the electropherogram and can be simply stated by:

$$R_s = \frac{2(t_2 - t_1)}{w_2 + w_1} \quad (1.21)$$

where t_2 and t_1 are the retention times of two respective analytes and w_2 and w_1 are their corresponding baseline peak widths.

Instrumentation

One of the unique features of CE is the inherently simple instrumentation needed (see Figure 1.6). Typically for CE separations, fused silica capillaries of 25 – 75 μm i.d.

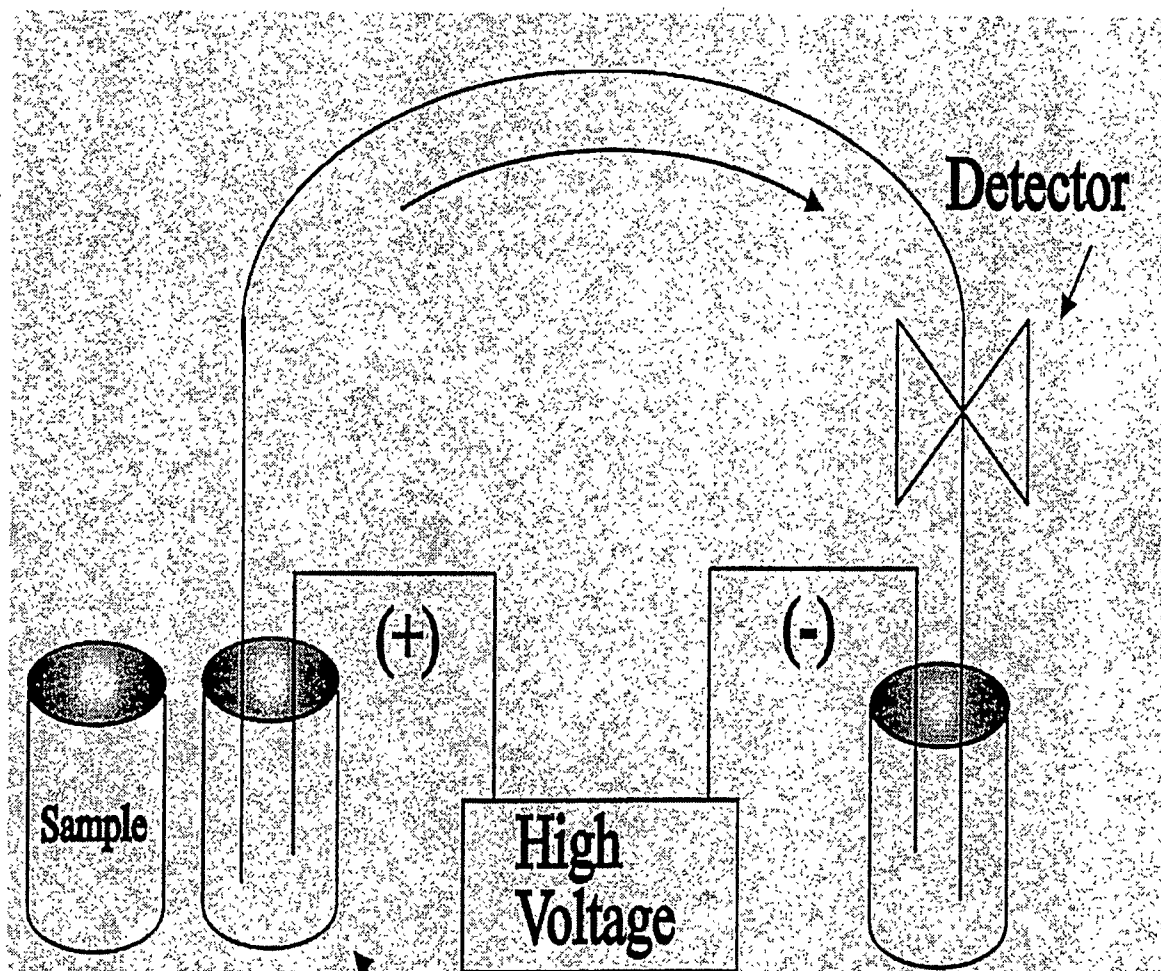


Figure 1.6. Schematic of a typical CE setup.

and 150 – 365 μm o.d. are used. The capillary is placed between two buffer reservoirs whose content is identical to that of the capillary and the reservoirs are placed at equal heights to eliminate any hydrostatic flow in the capillary. Sample introduction is usually achieved by either replacing the inlet end of the column (anode) with a reservoir containing the sample and applying pressure or a small electric field.

Pressure injection or hydrodynamic injection is often accomplished by raising the capillary inlet with respect to the outlet for a given period of time and the sample is introduced by siphoning. The volume injected by hydrodynamic injection can be determined by the Poiseuille equation:

$$V = \frac{\Delta P (\text{i.d.})^4 \pi}{128\eta L} \quad (1.22)$$

where ΔP is the pressure drop defined by:

$$\Delta P = \rho g \Delta h \quad (1.23)$$

where ρ is the density of the sample, g is the gravitational constant, and Δh is the height difference between the sample vial and the outlet vial.

Applying an electric field or electrokinetic injection will introduce a quantity, Q , into the capillary, which can be calculated by:

$$Q = \frac{(\mu_e + \mu_{EOF}) V \pi r^2 C t}{L} \quad (1.24)$$

where r^2 is the inner radius of the capillary, V is the voltage used for electrokinetic injection, C is the sample concentration, t is the length of time for injection, and L is the capillary total length. As can be seen by Equation 1.24, the electrokinetic injection discriminates between solutes based on their mobility.

Once injection of the sample is completed, a high voltage is applied across the capillary usually ranging from 10 – 30 kV producing electric fields ranging from 100 – 1000 V/cm. After application of the electric field, injected solutes will migrate at different velocities from the inlet (anode) to the outlet (cathode) while separating into discrete bands based on their different mobilities. Progression of a typical CE separation occurs from all analytes existing as a narrow (typically 1-2 mm of capillary length) injection zone (see Figure 1.7). Next, the analytes begin to migrate into separate bands based on their different velocities, and finally the analytes will separate into discrete bands, which can be detected individually. Detection in CE is typically accomplished on-column by the removal of a section of the polyimide coating from the capillary. Removal of the polyimide can be accomplished by using a lighter or fuming sulfuric acid. Detection schemes employed are discussed in more detail later in this chapter.

Modes of Capillary Electrophoresis

Capillary Zone Electrophoresis

Capillary zone electrophoresis (CZE) is the most widely used form of column electrophoresis due to its simplicity of operation and versatility. CZE is fundamentally the simplest form of CE because of the simplicity in separation mechanism. Typically the separation occurs because analytes migrate into zones based on their different velocities as previously discussed (see Figure 1.7). As already noted, both anion and cationic analytes are separated by EOF. However, neutral species do not have an intrinsic mobility; hence, they migrate with EOF. To extend the applicability of CE to

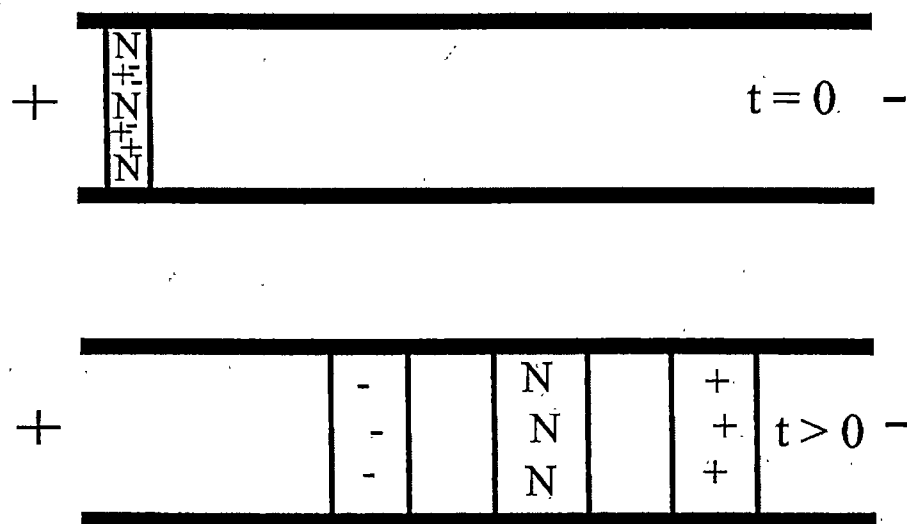


Figure 1.7. The progression of a CE separation.

neutral compounds, another mode of CE is employed, Micellar Electrokinetic Capillary Chromatography (MECC or MEKC).

Micellar Electrokinetic Capillary Chromatography

MECC is a cross between electrophoresis and chromatography and is one of the most widely used modes of CE today. The separation of neutral species is accomplished by addition of surfactants to the running buffer. Once above their critical micelle concentration (CMC), aggregates of surfactant molecules are formed called micelles. Micelles generally consist of long hydrophobic tails, which are oriented towards the center of the micelle to avoid interaction with the hydrophilic running buffer, and charged heads, which are oriented toward the running buffer. Anionic surfactants have little effect on EOF. However, cationic surfactants can reduce and eventually reverse the direction of EOF as depicted in Figure 1.8.

The charged micelle constitutes a secondary pseudostationary phase, which migrates at a velocity different than that of EOF. For example, an anionic surfactant will migrate toward the anode away from EOF. However, since EOF is generally greater than the velocity of the micelle the net movement is in the direction of EOF just as most anionic solutes (see above). During the analysis, the solutes will interact with the micelle both through hydrophobic and electrostatic interactions. For neutral species, it is the partitioning in and out of the micelle that effects the separation. Association of neutral molecules with the micelles is generally in proportion to the hydrophobicity of the solute. When the solute is not interacting with the micelle it is simply carried by EOF and will elute earlier. The separation process in MECC is shown in Figure 1.9.

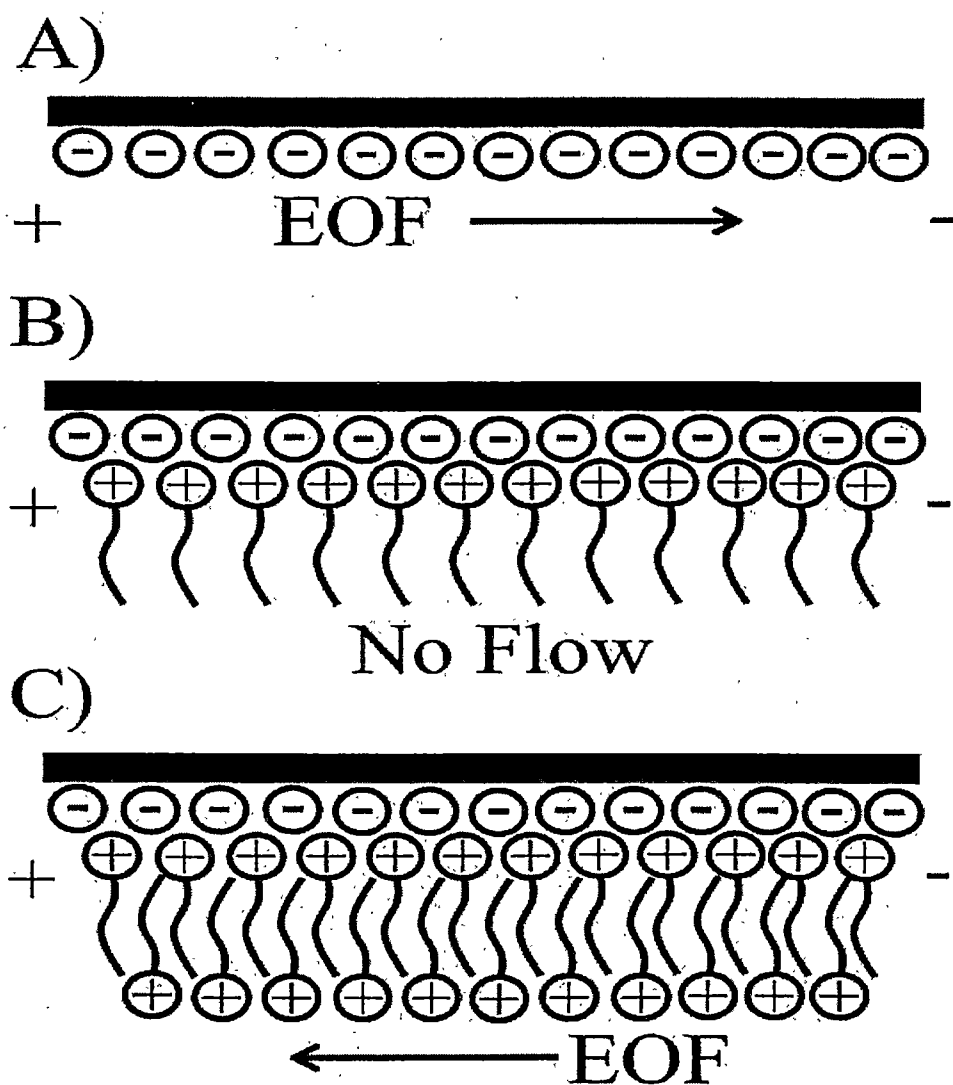
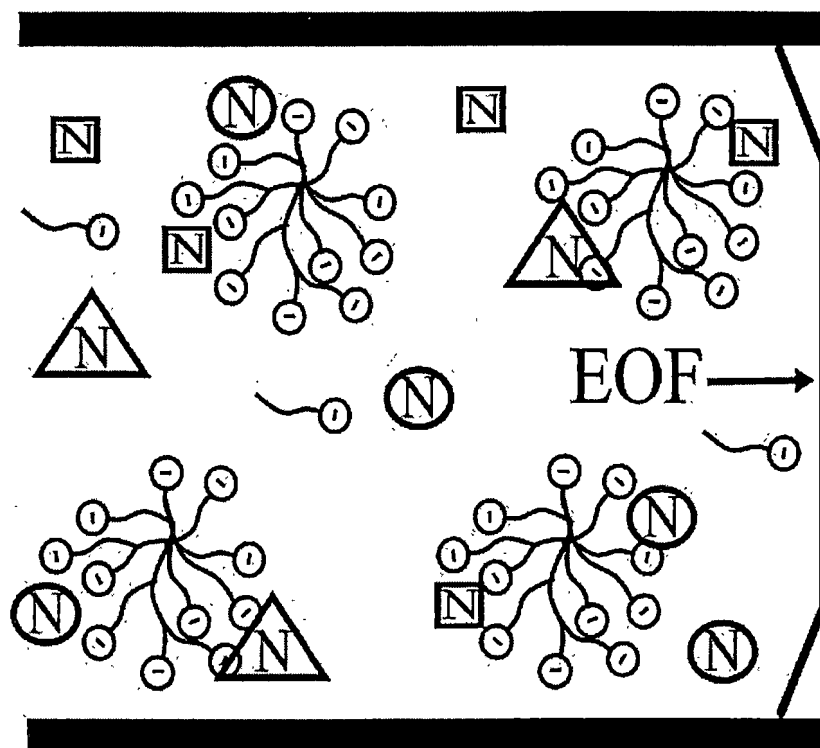


Figure 1.8. Effect of surfactants on EOF in CE. (A) Depiction of the direction of EOF in CE, (B) the elimination of EOF upon addition of micelles to the running buffer, and (C) the subsequent reversal of EOF using a cationic surfactant.



$$\mu_{\text{EOF}} = \longrightarrow$$

$$\mu_{\text{obs (N)}} = \longrightarrow$$

$$\mu_{\text{obs (micelle)}} = \longrightarrow$$

Figure 1.9. Depiction of the separation mechanism for MECC.

MECC is more versatile than traditional CZE since it can separate both charged and neutral species. In addition, separation selectivity can be readily changed by the addition of different micelles or reagents or modifiers to the running buffer. The ability to quickly change the system selectivity by modification of the running buffer is a great advantage of MECC and CE over traditional chromatographic techniques such as LC.

A disadvantage to MECC is that since the secondary phase is not stationary, there is a limited time window during which the solutes may elute. The time window is limited by the void time of the system (t_o) and the effective migration time of the micelle (t_m). This limitation produces some potential drawbacks to the technique. First, the limited time window reduces peak capacity which simplistically is the number of peaks (analytes) that can be separated in a given time window. Another drawback is that as retention of an analyte increases (i.e. capacity factors, k' , increase), resolution will first increase, but then reach a maximum before decreasing as the analyte retention time approaches that of t_m . This results in poor resolution for highly retained (hydrophobic) analytes; therefore, hydrophobic analytes are difficult to separate by MECC.

Capillary Gel Electrophoresis

Before the advent of capillary gel electrophoresis, slab gel electrophoresis was often employed. Traditionally, slab-gel electrophoresis is the technique of choice for the separation of large biomolecules such as nucleic acids. Traditional electrophoresis was combined with a sieving medium, polyacrylamide, and the technique was termed polyacrylamide gel electrophoresis (PAGE). DNA migrates through the fixed pores of a polymeric network driven by an electric field. Small molecules migrate easily because

they do not exhibit resistance from the pores. Large molecules must travel a more exhausting path finding a pore large enough to allow them to pass through before migrating through the polymeric network. Therefore, there is an inverse relationship between a molecule's mobility and its size. Early forms of this technique suffered from poor dissipation of heat necessitating the use of low electric fields and thus analysis times were long. In addition, sample recovery is necessary for additional analyses.

Hjerten modified this approach, which came to be termed capillary gel electrophoresis (CGE) [34]. Unfortunately, this method resulted in poor efficiencies. Hjerten realized that this degradation in efficiency might be due to solute-wall interaction between the biopolymers and the wall of the capillaries. Hjerten then published a benchmark work in CE [35]. He employed a column coating procedure, which successfully coats the charges on the wall of the capillary minimizing the solute-wall interactions. In order to efficiently separate DNA fragments, the capillary must first be modified with a linear polymeric coating, which effectively minimizes DNA-wall adsorption and EOF. Once the capillary is coated, EOF is essentially negligible and for the separation of negatively charged DNA fragments the electric field applied is in the opposite polarity (i.e., detection is at the anodic side) of that of traditional CE. The effect of a coated capillary on EOF for typical DNA analysis is shown in Figure 1.10.

There are two models, which are applied to help describe the migration of DNA through polymer networks in CE. The migration of DNA through a polymer network in a CE column is shown in Figure 1.11. The fundamental basis of these models is that the polymer networks possess a random distribution of pore sizes through which DNA molecules with different radii of gyration migrate. The Ogston model describes a DNA

Coated Column

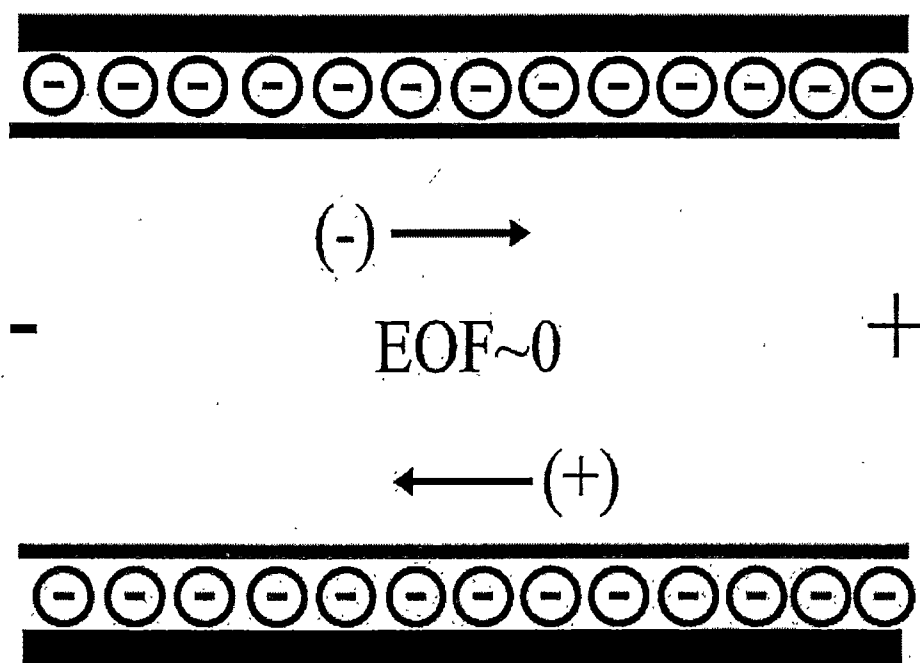


Figure 1.10. Coated column. The effect of coating a column on the EOF and overall flow direction for analysis in CE.

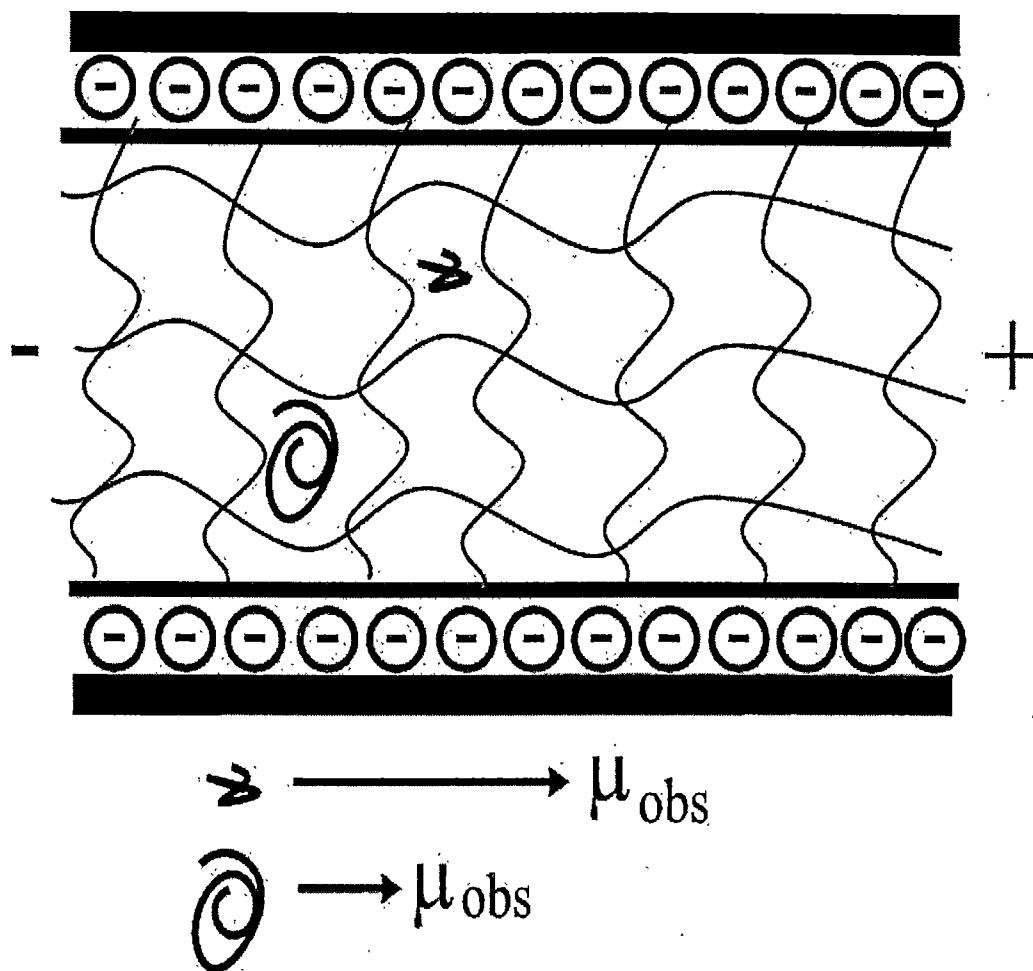


Figure 1.11. A depiction of the mesh network formed with the use of the soluble entangled polymers in CE. Two DNA fragments are shown with their observed mobilities.

molecule as a sphere migrating through the polymer network [36]. The DNA sphere diffuses laterally until it reaches a pore of the appropriate size to pass. The reptation model of migration assumes that if the DNA behaves as a sphere then size sieving matrices that have pore sizes too small for the DNA to pass would trap it [37-40]. Migration would occur because the DNA reptates, head first, through the polymer network by continuously stretching and relaxing as needed. The reptation model stresses the importance of the DNA mobility on field strength, pore size, and other experimental parameters.

Since the net charge on a DNA molecule is directly proportional to its mass, regardless of the number of base pairs (bp) on the DNA, each fragment will migrate at the same rate. Hence, traditional free solution CE is often unsuccessful at separation of DNA materials. Sieving matrices are often employed in capillary gel electrophoresis for the separation of DNA materials. With traditional slab-gel electrophoresis, a sieving matrix is employed to induce additional selectivity necessary for the separation of DNA. Two DNA molecules of different size will have identical electrophoretic mobilities causing them to co-elute; however, the sieving matrix used allows for separation.

Early works using sieving matrices were often chemically bonded inside the column. Problems associated with these matrices include poor reproducibility of the gels and the inability to introduce fresh matrix into the capillary. Today, soluble polymers are used for DNA separations [35, 41-48]. Soluble polymers do not require cross-linking to form the mesh network and fresh polymer can be easily introduced into the capillary. Another advantage of these soluble polymers is that they are a dynamic system without a fixed pore size. Varying concentrations around the entanglement threshold, Φ , where

polymer strands interact with each other to form an entangled mesh network, controls the pore size of the polymer network. The soluble polymer method described for the use of DNA separations are often referred to as size-selective CE (SSCE).

INTRODUCTION TO DETECTION METHODS

Absorbance

As noted previously by earlier works on column electrophoresis, detection in CE can be a great challenge due to the small optical pathlength of the small diameter capillaries employed. There is a wide variety of detection methods employed for CE including absorbance, fluorescence, Raman, conductivity, mass spectrometry, electrochemical, circular dichroism, and others with absorbance and fluorescence the two most common methods of detection. Absorbance of a solute (A) can be defined by Beer's Law as given by:

$$A = \epsilon b C \quad (1.25)$$

where ϵ is molar absorptivity, b is the optical pathlength, and C is concentration. As can be seen from Equation 1.25 there is a direct dependence of absorbance on the pathlength of the cell used. Since the pathlength is small in CE due to the diminutive capillaries used, absorbance is not a very sensitive method of detection in CE. However, absorbance detection is still routinely used since a large number of analytes will absorb UV light.

Fluorescence Spectroscopy

Theory

A brief description on theory fluorescence spectroscopy is warranted, however for a more comprehensive description the reader is referred to Skoog et al. for further reading [49]. Fluorescence emission requires that a molecule absorb photons of light in order to populate excited electronic states. Relaxation from the excited state via fluorescence is a short-lived process ($< 10^{-5}$ s). Fluorescence can occur without a change in the frequency of the fluorescence emission from that of the excitation frequency called resonance fluorescence. However, more often emission is found at wavelengths longer than that of the excitation termed Stokes Shift. In order to understand the basis of fluorescence spectroscopy, it is important to discuss molecular orbitals.

The characteristics of fluorescence can be described by means of simple molecular orbitals for the given compound. Organic compounds absorb radiation when the energy associated with the radiation is appropriate to promote electrons to higher energy molecular orbitals and when certain selection rules are observed [49]. The electrons that will lead to absorption in an organic molecule are those directly involved in bond formation between atoms and the nonbonding or unshared electrons that are localized around such atoms as oxygen, sulfur, nitrogen, and halogens.

The molecular orbitals associated with the single bond formation in organic molecules are termed sigma (σ) orbitals and the corresponding electrons are termed sigma electrons. Double bonds formed in organic compounds contain one pair bonding electrons (σ electrons) and a set of π electrons corresponding to the other pair of electrons. In addition to the electrons mentioned above, many organic compounds have

nonbonding electrons. The unshared electrons in a compound are designated by n . Figure 1.12 depicts an organic compound (formaldehyde) with the three different types of electrons and orbital energy ordering is shown in Figure 1.13.

Fluorescence occurs from the promotion of electrons to a higher energy or excited state due to the absorption of radiation. The four types of transitions are $\sigma \rightarrow \sigma^*$, $n \rightarrow \sigma^*$, $n \rightarrow \pi^*$, and $\pi \rightarrow \pi^*$ [49]. Observation of Figure 1.13 reveals that for $\sigma \rightarrow \sigma^*$ transitions to occur a large amount of energy is needed for this transition; thus absorption maxima for these transitions are seldom observed in the accessible ultraviolet region. Even though $n \rightarrow \sigma^*$ require less energy, they are not very common since the number of functional groups with these $n \rightarrow \sigma^*$ transitions in the readily accessible ultraviolet region is small.

Most applications of absorption spectroscopy are based upon transitions for n or π electrons to the π^* excited state because the energies needed are in an easily accessible region of the spectral region (200 to 700 nm) [49]. Both of these transitions require unsaturated functional groups (chromophores) to provide the π orbitals. There are a wide variety of organic chromophores (see Table 1.1), which will absorb radiation and may produce fluorescence.

Excited States

A molecular electronic state where all the electron spins are paired is termed a singlet state [49]. One of a pair of electrons can be promoted to higher energy orbitals producing either singlet or triplet excited states. In the singlet excited state, the promoted electron is still paired with the electron in the ground state. Conversely, in the triplet

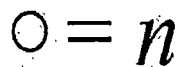
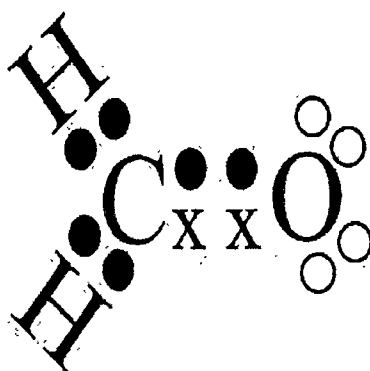


Figure 1.12. Molecular orbitals. A pictorial representation of the three different types of molecular orbitals found in most organic compounds.

Source: Skoog, D.A.; Holler, F.A.; Nieman, T.A *Principles of Instrumental Analysis*, Harcourt Brace & Company, Orlando, Fla, p.331, 5th Edition.

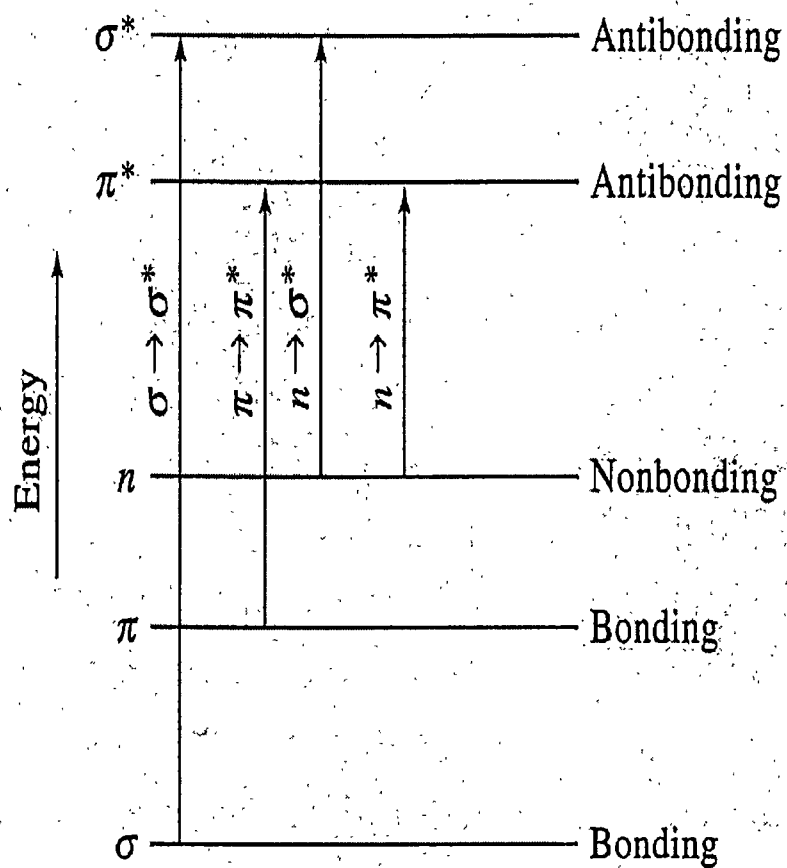


Figure 1.13. Molecular energy levels for electronic transitions.

Source: Skoog, D.A.; Holler, F.A.; Nieman, T.A *Principles of Instrumental Analysis*, Harcourt Brace & Company, Orlando, Fla, p.331, 5th Edition.

Table 1.1: Common Chromophores

Chromophore	λ_{max} (nm)	Type of Transition
Alkene	177	$\pi \rightarrow \pi^*$
Alkyne	178 196 225	$\pi \rightarrow \pi^*$
Carbonyl	186 280 180 293	$n \rightarrow \sigma^*$ $n \rightarrow \pi^*$ $n \rightarrow \sigma^*$ $n \rightarrow \pi^*$
Carboxyl	204	$n \rightarrow \pi^*$
Amido	214	$n \rightarrow \pi^*$
Azo	339	$n \rightarrow \pi^*$
Nitro	280	$n \rightarrow \pi^*$
Nitroso	300 665	$n \rightarrow \pi^*$
Nitrate	270	$n \rightarrow \pi^*$

Source: Skoog, D.A.; Holler, F.A.; Nieman, T.A *Principles of Instrumental Analysis*, Harcourt Brace & Company, Orlando, Fla, p.333, 5th Edition.

excited state the spins of the two electrons are unpaired and exhibit different properties than the excited singlet state. The transition from a singlet to a triplet state or vice versa is much less likely to occur than a transition from the singlet to the singlet excited state.

Figure 1.14 is a partial energy diagram for a photoluminescent molecule. The lowest energy state of a molecule is the ground state labeled as a singlet state (S_0) by the horizontal line. The vertical lines in the figure indicate promotion to an excited energy level indicated by S_1 , S_2 , and T_1 that are the first singlet, second singlet, and first triplet excited electronic energy levels respectively [49]. The energy of the first excited triplet state is lower in energy than the corresponding single state. The horizontal lines in each state show vibrational levels associated with the electronic states. Excitation of the molecule to an excited state is done by absorption of two bands of radiation around either the wavelength corresponding to $S_0 \rightarrow S_1$ transition or the wavelength around the $S_0 \rightarrow S_2$ transition [49]. This excitation process, which occurs in low femtoseconds, results in conversion to any of the several excited vibrational states. Direct excitation to the triplet state is not shown because of the low probability as previously mentioned.

An excited molecule can return to its ground state by a variety of mechanisms. Two of these processes, fluorescence and phosphorescence, involve the emission of a photon of radiation. The other processes are nonradiative. The preferred route back to the ground state is the one that minimizes the lifetime of the excited state [49]. Therefore, if fluorescence is rapid enough with respect to the radiationless processes then emission will occur. Conversely, if any of the nonradiative process has a more favorable rate constant then fluorescence is either less intense or absent. Fluorescence lifetimes are in the range of nanoseconds (10^{-6} - 10^{-9} s).

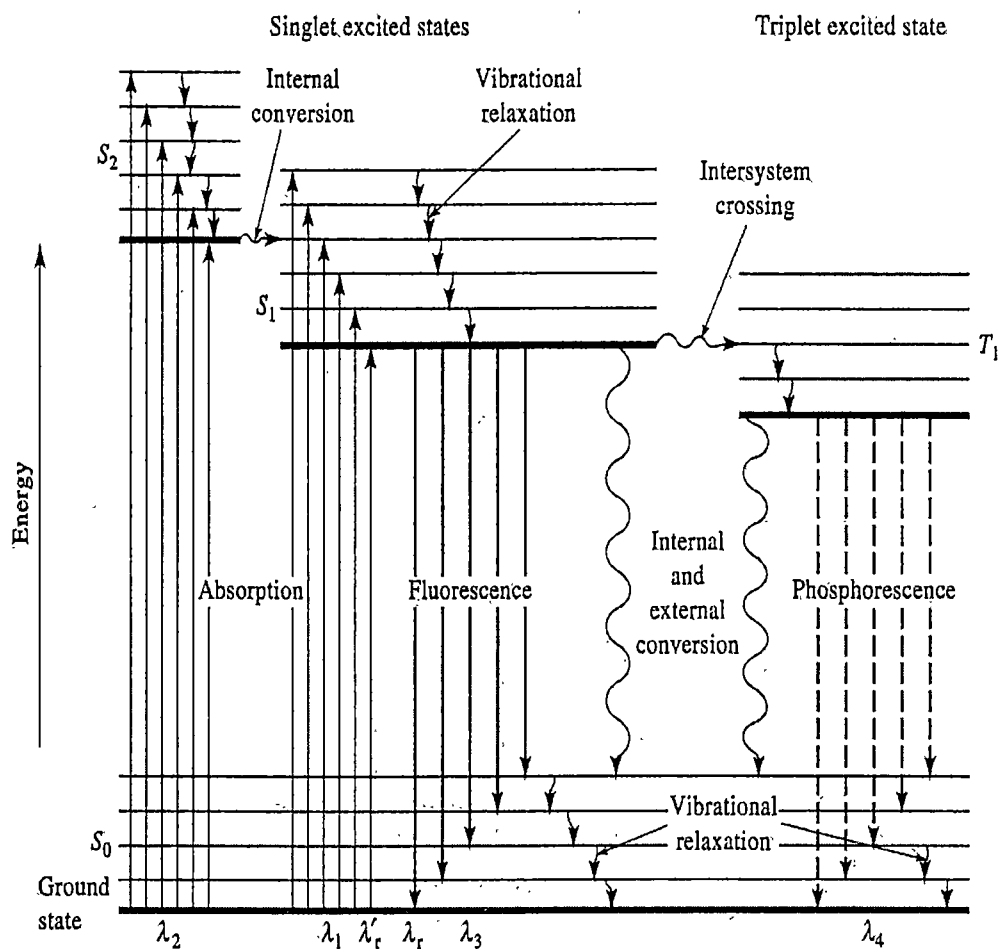


Figure 1.14. Partial Energy Diagram.

Source: Skoog, D.A.; Holler, F.A.; Nieman, T.A. *Principles of Instrumental Analysis*, Harcourt Brace & Company, Orlando, Fla, p.357, 5th Edition.

One of the competing processes from an excited state back to the ground state is vibrational relaxation. A molecule may be promoted to any one of several vibrational levels, but the excess vibrational energy is almost immediately lost due to the collisions between the molecules of the excited species and the solvent [49]. Relaxation to the ground state can occur to any of the vibrational ground state levels and can be followed by subsequent vibrational relaxation. The relaxation process has a lifetime of 10^{-12} seconds or less which is much shorter than the average lifetime of the excited state. Therefore, fluorescence will generally occur from the lowest vibrational level of an excited electronic state, which is usually the lowest excited singlet state. A consequence of the fast vibrational relaxation is that fluorescence band for a given electronic transition occurs at longer wavelengths from the absorption band (Stokes shift) [49].

Internal conversion is another nonradiative process, which occurs when a molecule passes to a lower energy electronic state without emission of radiation. This process seems to be highly efficient since few molecules fluoresce. Another competing process to fluorescence is external conversion in which deactivation of an excited electronic state may involve interaction and energy transfer between the excited molecule and the solvent. The details of external conversion are not well understood.

Intersystem crossing occurs in which the spin of an excited electron is reversed resulting in a change of multiplicity. The probability of this process is enhanced if the vibrational levels of the two states overlap. For example in Figure 1.14, the lowest singlet vibrational state overlaps one of the upper triplet vibrational levels and intersystem crossing is more probable. After intersystem crossing to a triplet state, deactivation can occur by phosphorescence. However, phosphorescence is less probable

since it involves triplet $\rightarrow$ singlet transition (see above) with a lifetime of 10^{-4} to 10 seconds or more.

Factors Affecting Fluorescence

The quantum efficiency for fluorescence can be defined simply as the ratio of the number of molecules that luminescence to the total number of excited molecules. For a highly fluorescent molecule (e.g., fluorescein), quantum efficiencies can approach unity [49]. On the other hand, chemical species that exhibit low to no fluorescence have efficiencies approaching zero.

The fluorescence quantum yield ϕ for a compound can be determined by the following equation:

$$\Phi = \frac{k_f}{k_f + k_i + k_{ec} + k_{ic} + k_{pd} + k_d} \quad (1.26)$$

where k_f , k_i , k_{ec} , k_{ic} , k_{pd} , and k_d are the rate constants for fluorescence, intersystem crossing, external conversion, internal conversion, predissociation, and dissociation respectively. Equation 1.26 shows the interpretation of many structural and environmental factors that influence fluorescence intensity of a molecule. Variables that lead to a high k_f value and low values for the other k terms enhance fluorescence. The magnitude of k_f is mainly dependent upon chemical structure. Many of the competing processes with fluorescence are dependent on the environment (e.g., solvent, polarity, temperature) the excited molecule experiences.

Transitions in Fluorescence

Fluorescence will seldom occur from absorption of ultraviolet radiation from wavelengths below 250 nm because the radiation will cause deactivation of the excited species by either dissociation or predissociation mechanisms [49]. Instead fluorescence emission is usually due to the less energetic $\pi^* \rightarrow n$ and $\pi^* \rightarrow \pi$ transitions. However, fluorescence is more likely to occur in compounds where the lowest energy transition is a $\pi \rightarrow \pi^*$ transition than in compounds which the lowest energy transition is of the $n \rightarrow \pi^*$ transition [49]. This is primarily due to the shorter lifetimes associated with the $\pi \rightarrow \pi^*$ transitions (10^{-7} to 10^{-9} s) compared to the $n \rightarrow \pi^*$ transition lifetime of 10^{-5} to 10^{-7} s and thus, the k_f value in Equation 1.26 for the $\pi \rightarrow \pi^*$ transition is larger than the $n \rightarrow \pi^*$ transition. In addition, the rate constant for intersystem crossing k_i is smaller for the $\pi \rightarrow \pi^*$ excited states because the energy difference between the singlet/triplet states is larger; therefore, it requires more energy to unpair the electrons in the π^* state for intersystem crossing to occur [49]. Consequently, the probability decreases for of intersystem crossing to occur.

Effect of Concentration

The power of fluorescence emission (F) is directly proportional to the power of the excitation beam (P_0) as given by:

$$F = K'(P_0 - P) \quad (1.27)$$

where K' is a proportionality constant and P is the power of the beam after passing through a length b of the medium. The constant K' depends on the quantum efficiency of the fluorescence process. Relating F to concentration, c , rearrange Beer's law as follows:

$$\frac{P}{P_0} = 10^{-\epsilon bc} \quad (1.28)$$

where ϵ is the molar absorptivity of the fluorescing molecules and ϵbc is the absorbance

A. Substituting can obtain:

$$F = K' P_0 (1 - 10^{-\epsilon bc}) \quad (1.29)$$

Provided that $2.303 \epsilon bc = A < 0.05$ then arrangement of the Equation 1.29 gives:

$$F = 2.3K' \epsilon bc P_0 \quad (1.30)$$

or if assuming constant P_0 :

$$F = Kc \quad (1.31)$$

A plot of power versus concentration of the emitting species would be linear at low concentrations. However, when c becomes greater such that the absorbance is larger than 0.05, linearity is lost. In addition, at higher concentrations self-quenching and self-absorption become problematic. Self-quenching is the result of the collisions of excited molecules. Self-absorption occurs when the wavelength of emission overlaps with an absorption peaks and fluorescence is decreased because other fluorescent molecules reabsorb the emission [49]. There are many other parameters that may affect fluorescence including ring substitution, structural rigidity, temperature, solvent effects, and pH.

Fluorescence Detection in Capillary Electrophoresis

Fluorescence detection methods are generally more sensitive than absorbance detection methods. This increase in sensitivity is due primarily because of the linear relationship of increased fluorescence emission (F) for increased excitation power (P_o) seen in Equation 1.27 that is called the "fluorescence advantage". The sensitivity is also related to measurement of an absolute quantity (F) rather than an attenuation of P ($P_o - P$) as observed with absorbance detection. This advantage combined with the selectivity found with fluorescence detection results in superior detectability over absorbance detection. In addition, the attributes of lasers make them well suited for fluorescence detection methods for CE (discussed later in this section).

Even though there are advantages to laser induced fluorescence (LIF) detection methods as mentioned above, it is not a universal detection method. LIF detection requires that the analyte itself fluoresce which can introduce a degree of selectivity, but limits the number of analytes used. Unfortunately, most species exhibit very little fluorescence in their natural state. In order to broaden the scope of CE-LIF to a wider variety of compounds, the compounds must first be modified in some manner to be detected. One method of modification is to derivatize the analyte of interest with a fluorescent label. Many different derivatization agents have been used such as naphthalene-2,3-dicarboxaldehyde (NAD) [50, 51], o-phthalaldehyde (OPA) [52, 53], fluorescein isothiocyanate (FITC) [54, 55], and 8-hydroxy-quinoline sulfate (HQS) [56]. Forms of derivatization of compounds to be analyzed by LIF include either pre-, on-, or post-column derivatization techniques.

Pre-column derivatization is the simplest and most straightforward form of derivatization for use with CE since it requires no changes to the CE system. The derivatization procedure is performed prior to sample injection and separation. Pre-column derivatization being done prior to injection has no time restrictions. However, pre-column derivatization can result in multiple labeling of peaks and can make the analytes of interest more similar in their charge to size ratios; hence, more problematic to separate.

Another derivatization procedure that has been performed with CE is on-column derivatization. With on-column derivatization, the capillary is first filled with the fluorogenic reagent prior to sample injection. After sample injection, the fluorogenic reagent and the analyte reach an equilibrium state as the analyte migrates through the column; however, analytes can still be multiple labeled. The adverse effects of multiple labeling can be mitigated by the fact that this labeling method is dynamic (i.e., covalent attachment of the label to the analyte is not involved).

With the aforementioned problems with on-column and pre-column derivatization, the use of post-column derivatization is warranted. In most instances, the CE capillary and system must be modified in some manner to accommodate post-column derivatization. Modification must allow for the derivatization agent to be added after the separation without significantly diminishing the efficiency of the overall separation. Many different designs have been employed for the introduction of the derivatization agent post-column by co-axially coupled capillaries [53, 57, 58] or cross connectors built into the CE capillary [59]. In addition, EOF has been used to facilitate the derivatization reaction by the way of a free-solution reactor [60] or a fluid gap reactor [61]. However, it

has been determined that the sheath flow design results in minimal band dispersion and superior detection capabilities [62].

However, post-column derivatization is not without drawbacks. For instance, post-column derivatization schemes require fast kinetics between the derivatization agent and the analyte in order to minimize band broadening of the separation. This can severely limit the number of fluorescent tags that can be used with post-column derivatization. In addition, a source of dead volume may be introduced with the connections involved between the capillary and flow cell.

Forms of derivatization such as pre-, on-, or post-column derivatization can overcome this lack of native fluorescence of analytes. However, derivatization schemes can be problematic as mentioned. Each of these derivatization techniques is alluded to again some of the subsequent chapters.

Instrumentation

Basic fluorescence instrumentation normally consists of the following components: excitation source, filters, detector, cell, and miscellaneous excitation and emission optics to collect and direct the radiation. A basic CE-LIF apparatus is shown in Figure 1.15. The system consists of a laser for the excitation source, a focusing lens, CE capillary for the detection cell, collection lens, appropriate filters to isolate the radiation desired, and a photomultiplier tube (PMT) for detection. LIF-CE is done on-column through an optically transparent window created by removal of a short section of the polyimide protective coating from the capillary. A laser beam is tightly focused through this window and analytes are detected as they migrate pass this window. Fluorescence is

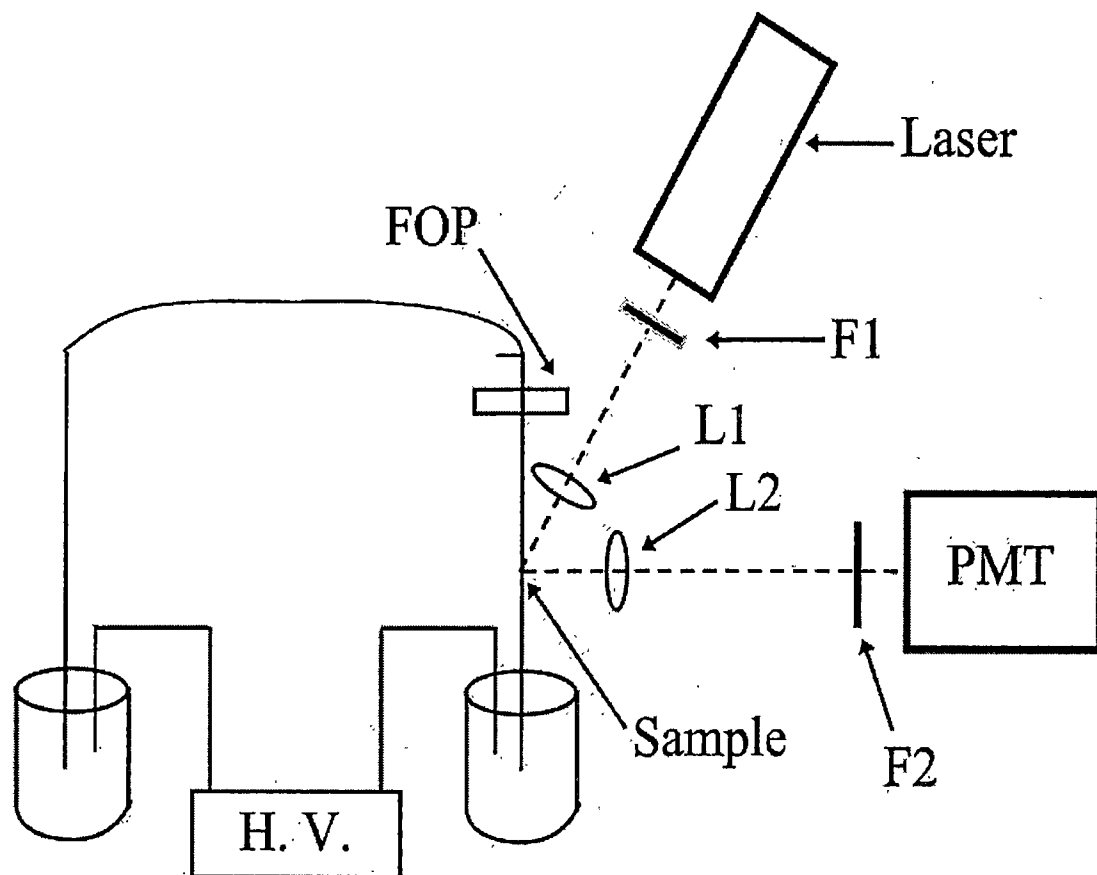


Figure 1.15 Pictorial representation of a CE-LIF apparatus. F1 is an appropriate laser line filter, L1 is focusing lens, L2 is collection lens, FOP is a fiber optic positioner, F2 is a bandpass filter, and PMT is a photomultiplier tube.

collected at a right angle to the excitation source, spectrally filtered, and detected by a PMT. As mentioned previously in the discussion of fluorescence, only a small class of compounds will natively fluoresce. Most analytes that do indeed fluoresce will require excitation from a laser, which emits radiation in the UV spectral region. UV lasers are often expensive and can be problematic because special optics is required for work in the UV to eliminate fluorescence emanating from optical surfaces.

To achieve high sensitive detection, lasers are preferred over conventional incoherent light sources. Lasers have many properties that make them ideal as sources for CE-LIF including the following: power, collimation, monochromaticity, temporal, and polarization. Each of the five properties offers their own advantages as sources for CE-LIF. Due to the fluorescence advantage mentioned previously, the higher the power the higher the fluorescence emission. Lasers are very capable of providing very intense light from a few mW to several watts. Collimation of a laser beam is an important property because of the small size of capillaries used in CE. The ability to focus a laser beam through a narrow-bore CE capillary is advantageous offering low focusing volumes. When using pulsed lasers, temporal rejection of unwanted signals is possible. In addition, polarization properties are helpful in rejecting certain unwanted contributions to background signals.

In general, on-column fluorescence detection methods suffer from a large amount of light scatter off of the capillary producing a large amount of background signal due to reflection and refraction. Ideally, fluorescence detection should occur off-column in a flow chamber made of good quality and flat windows. It would seem logical to use a square capillary for electrophoresis; however, it is difficult to make long segments of

small dimension capillary with flat surface. Therefore, the use of a post-column fluorescence detection chamber to achieve high sensitivity is desirable.

Great care must go into the design of a post-column detector to minimize band broadening due to dead volume in the transfer of the sample from the capillary to the flow cell. Dovichi has developed a post-column fluorescence detector based on the sheath flow cuvette [62]. With this design the sample is transferred from the capillary to the flow cell with little loss of separation efficiency and very high detection sensitivity is achieved. The sheath flow cell (SFC) is made from optically flat quartz with 2 mm thick windows and has 200 μm square i.d. The outlet end of the capillary is inserted a desired distance into the SFC. Sheathing fluid, which is identical to the makeup of the CE running buffer, is introduced into the cuvette at a flow rate of low microliters per hour usually via a syringe pump. Completion of the electrical circuit for CE requires the stainless steel plumbing associated with the sheath stream be held at ground potential. Under the very low flow rates the sample stream is approximately 10 μm in diameter through the center of the SFC. Fluorescence is collected by a microscope objective at a right angle to the sample stream. Filters reject scattered laser light spectrally and a 200 μm pinhole is used to isolate the field of the PMT to the illuminated sample stream. The sheath flow cuvette has produced very low detection limits approaching single molecule detection.

Evaluation, optimization, and implementation of various fluorescence methods will be discussed in subsequent chapters. In addition, a detailed discussion and demonstration of SFCs will be given.

Raman Spectroscopy

Brief History

In 1928, C.V. Raman discovered that the visible wavelength of a small fraction of radiation scattered differs from the incident light and the shifts in wavelength are dependent upon the chemical structure of the molecule causing the scatter [63-66]. Raman was awarded the Nobel Prize in Physics in 1931 for his discovery; hence this phenomenon bears his name.

Basic Theory

Raman scattering results from the same type of vibrational changes associated with infrared absorption spectroscopy (IR) [49]. However, there are differences in the classes of compounds that can be analyzed between the two techniques, which make the techniques complementary. The biggest advantage Raman has over IR is the fact that water does not cause interference, thus aqueous solution can be analyzed. In addition, Raman spectra can be acquired in glass or quartz a cell, which eliminates working with unstable window materials or even handling the compound at all. Raman spectroscopy did not become widely used until the advent of lasers in the 1960s. Furthermore, fluorescence is usually an interfering process in Raman spectroscopy unless near-infrared lasers are employed.

In general, molecules that have polar functional groups and low symmetry are weakly Raman active while molecules with polarizable functional groups and high

symmetry are strongly Raman active. The Raman effect is an emission phenomenon and not limited by the laws of absorption. The intensity of the signal produced is a linear function of the contribution of the Raman scattering center and the intensity of the incident light source. Raman scattering intensity varies as a function $1/\lambda^4$ where λ is the incident wavelength.

Raman spectra are acquired by using a powerful laser to irradiate a sample and the scattered radiation is measured at some angle with a spectrometer. The intensities of the Raman lines are very weak compared to the intensity of the source (usually 0.001%); therefore, detection can be difficult. Monochromatic incident light can induce an oscillating dipole, μ , in certain molecules and emit radiation at the frequency of the dipole oscillation (ν_{sc}) as given by:

$$\mu(t) = \mu^o \cos(2\pi\nu_{sc}t) \quad (1.32)$$

where $\mu(t)$ is the magnitude of the dipole moment at time, t , and μ^o is the maximum induced dipole moment which can be approximated by:

$$\mu(t) = \alpha E_{in}(t) \quad (1.33)$$

where α is the polarizability of the molecule with units of $C^2 m^2 J^{-1}$ which is a measure of the degree with which an electric field of incident light (E_{in}) can distort molecular orbitals. The induced dipole may be compromised of a variety of harmonic frequencies. The scattered radiation, which is at the same frequency as the incident radiation, is Rayleigh scattering ($\nu_{sc} = \nu_{in}$). Additional components, which are not at the same frequency as that of the incident radiation give rise to Raman scattering as two types: Stokes scattering and anti-Stokes scattering.

The vibrational energy levels (E_{vib}) termed the Raman effect can be defined as:

$$\bar{\nu}_s = \bar{\nu}_0 \pm \frac{\Delta E_{vib}}{hc} \quad (1.34)$$

where $\bar{\nu}_s$ is the frequency of Raman scattered radiation, $\bar{\nu}_0$ is the frequency of the excitation line, h is Plank's constant, and c is the speed of light. The Raman effect produces two sets of lines one for Stokes given by:

$$\bar{\nu}_s = \bar{\nu}_0 - \frac{\Delta E_{vib}}{hc} \quad (1.35)$$

and anti-Stokes lines given by:

$$\bar{\nu}_s = \bar{\nu}_0 + \frac{\Delta E_{vib}}{hc} \quad (1.36)$$

The abundance of the anti-Stokes lines is a function on how many molecules are in the first vibration state compared to the ground state. The ratio is given by a Boltzmann distribution:

$$\frac{n_{v=1}}{n_{v=0}} = \exp \frac{\Delta \bar{E}_{vib}}{kT} \quad (1.37)$$

where $\Delta \bar{E}_{vib}$ is the vibrational transition energy in joules per molecule, k is the Boltzmann's constant, and T is temperature in Kelvin. Rayleigh scatter is more intense than the Stokes and anti-Stokes scattering.

A typical Raman spectrum of carbon tetrachloride (CCl_4) demonstrating the three types of scattering is shown in Figure 1.16. The abscissa in Figure 1.16 is the wavenumber shift, which is defined as the difference in wavenumbers (cm^{-1}) between the observed radiation and the incident radiation of the source. The Raman peaks found are on either side exhibiting identical Δcm^{-1} with the Stokes lines found at wavenumbers

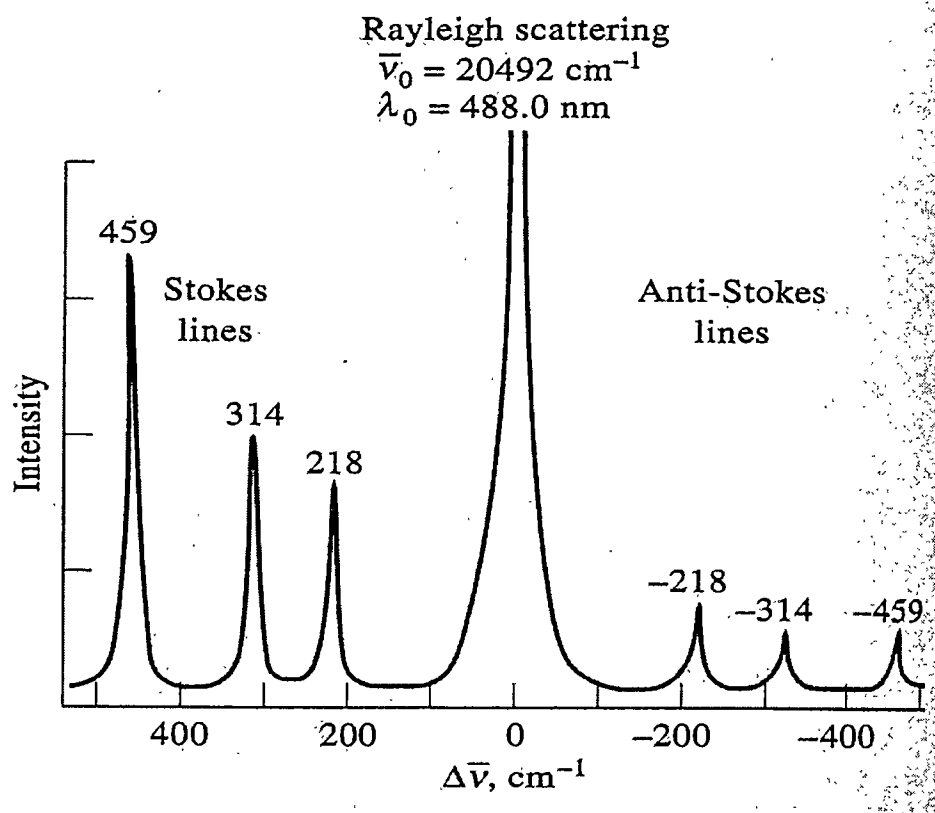


Figure 1.16. A Raman spectrum of CCl_4 .

Source: Skoog, D.A.; Holler, F.A.; Nieman, T.A *Principles of Instrumental Analysis*, Harcourt Brace & Company, Orlando, Fla, p.430, 5th Edition.

smaller than the Rayleigh peak and anti-Stokes lines found at wavenumbers greater than the Rayleigh peak. However, anti-Stokes lines are less intense than Stokes lines and thus, the Stokes portion of the spectrum is generally reported. It is important to note that the magnitudes of Raman shifts are independent of the wavelength of excitation. Thus, the same spectral bands would be obtained for CCl_4 no matter what the excitation wavelength used.

Spectral excitation is normally done by radiation having a wavelength that is away from any adsorption peaks of the analyte. An energy diagram is shown in Figure 1.17 showing a picture of the sources of Raman and Rayleigh scatter. The far left vertical arrow indicates the energy change in the molecule when it interacts with a photon from the excitation source. The energy change is equal to the energy of the incident photon $h\nu$.

The second arrow indicates the type of change that would be associated with the molecule if it encountered a photon that happened to be in the first vibrational level of the electronic state. The probability of the fraction of the molecules to be in this state is small as indicated by narrowness of the arrow in the diagram. The middle set of arrows indicates the change in energy that produces Rayleigh scatter. It is important to note that no energy is lost in Rayleigh scattering; hence, the collisions between the photon and the molecule are termed elastic collisions. The arrows to the left indicate the Stokes and anti-Stokes emission. The two emissions differ from the Rayleigh radiation by the frequencies corresponding to $\pm \Delta E$, the energy of the first vibrational level of the ground state. The relative populations of the two upper energy states are that the Stokes emission is more favorable than the anti-Stokes emission.

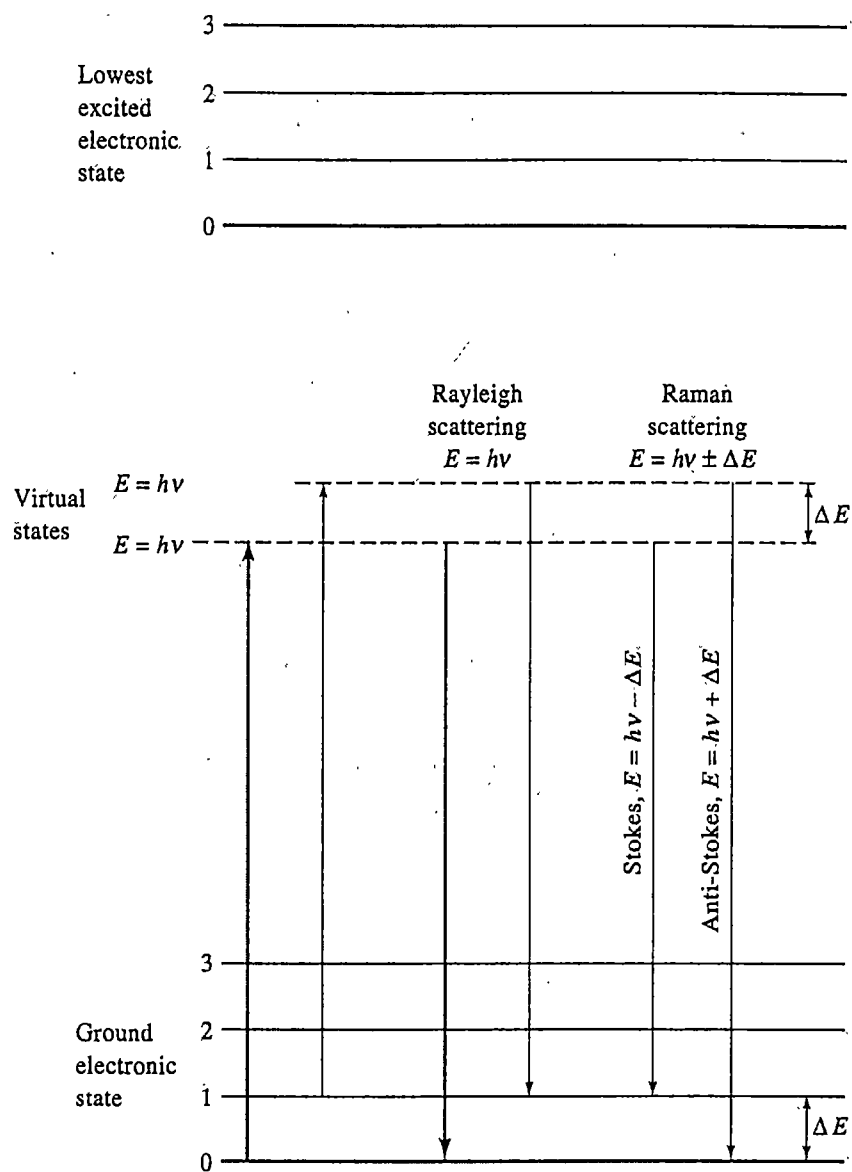


Figure 1.17. Energy diagram for Raman and Rayleigh scatter.

Source: Skoog, D.A.; Holler, F.A.; Nieman, T.A *Principles of Instrumental Analysis*, Harcourt Brace & Company, Orlando, Fla, p.431, 5th Edition.

Applications

Raman scattering spectroscopy is a powerful tool in structural elucidation. Raman spectra have very sharp spectral features offering the potential for spectral selectivity for complex mixtures. Coupling of Raman to specialized forms of CE can allow for the identification of analytes. There have been some reports of Raman scattering coupled to specialized forms of CE [67-72]. Unfortunately, Raman scattering has a low sensitivity, which has hindered its acceptance as a routine detection method for CE. Even though Raman scattering has inherently low cross-sections, phenomena such as resonance Raman scattering (RRS) and surface enhanced Raman scattering (SERS) greatly enhance observed Raman signals. The sensitivity issue can be overcome by using one of these other forms of Raman spectroscopy (see discussions below).

Resonance Raman Spectroscopy

Resonance Raman scattering (RRS) occurs when the Raman line intensities are enhanced by the excitation of wavelengths that closely approach that of an electronic absorption peak of an analyte. The excited electronic state of the molecule contributes to the virtual state, which enhances the probability that the transition will result in Raman scatter. The magnitudes of the Raman peaks associated with the most symmetric vibrations are enhanced by a factor of 10^2 to 10^6 . RRS is much more sensitive than Raman spectroscopy offering detection at concentrations as low 10^{-7} M compared to 0.1 M with Raman spectroscopy.

For RRS to occur, the electron is promoted into an excited state followed by an immediate relaxation to a vibrational level of the electronic ground state. RRS differs

from that of fluorescence in that the relaxation to the ground state is not preceded by prior relaxation to the lowest vibrational level of the excited state. A major limitation to RRS is the interference of fluorescence by the analyte or other species present in the sample. For a more detailed description and theories of RRS see the literature [73-76].

Surface Enhanced Raman Scattering

Brief Historical Perspective

SERS is an alternative Raman enhancement phenomenon with greater applicability than RRS. The first report of SERS was in 1974 by Fleischmann who observed a strong enhancement for a pyridine molecule adsorbed onto a silver electrode [77]. Later reports of this observation by Jeanmaire and Van Duyne [78] and then Albrecht and Creighton [79] speculated that this enhancement was due to complex surface enhancement processes. There have been many reports of SERS in the literature following these observations [80-84]. In fact, today there are reports of even single molecule detection work with SERS [85, 86].

Theory of SERS

SERS produces Raman signals from molecules adsorbed on or very near to metal surfaces. A fully accepted theory on the basis of SERS has not yet been reported. However, two important models as part of the overall theory have been proposed, electromagnetic and chemical models. Controversies still exist over the relative contributions of these effects and the details of their description. A full discussion of

these models is beyond the scope of this work; however a brief explanation is warranted. For a full explanation, many detailed reviews can be found in the literature [83, 87-90].

To help understand the basis of the proposed models for the SERS effect, recall the relationship:

$$\mu = \alpha E_{in} \quad (1.33)$$

where μ is the electric dipole and it is proportional to the polarizability of the molecule, α , and the magnitude of the incident electric field, E_{in} . Therefore, if either α or E_{in} are increased, then the Raman scattering intensity is enhanced. The electromagnetic model has been proposed to account for the increase in E_{in} . On the other hand, the chemical model has been proposed to account for the increase in α . At this point in time, the chemical model is the least understood of the two models.

Electromagnetic Model

Molecules on or near metal surfaces will experience enhanced electric fields with respect to the incident light. Enhancements of 10^6 to 10^7 have been reported for roughened metallic surfaces. This enhancement can be attributed to surface plasmons [91]. When an incident field irradiates a metallic surface, conduction electrons are displaced into an oscillation of equal frequency to the incident light. Once these electrons become confined about a region in space, normally around metallic spheres or roughened metallic surface, a plasmon frequency is produced. With the plasmon frequency, there is a resonant response of the collective oscillation to the initial field producing intense electric fields localized around a molecule near the metal surface. Important to note is that Raman scatter from an adsorbed molecule can stimulate surface

plasmons in the metallic substrate, called the image effect, which can provide an additional enhancement [82]. Other enhancement effects include the Fresnel reflection effect and the “lightning rod” effect. The Fresnel effect arises from the oscillating dipole of vibrating molecule interacting with surface electrons to produce Raman shifted reflection of light [82]. The “lightning rod” effect occurs when electric field lines are concentrated to provide field enhancements at high curvature points of a metallic feature.

It is also important to note that the enhanced field decreases with the cube of the distance, r , from the center of the metallic surface. In addition, the intensity of the Raman scatter produced increases with the square of the field applied to the molecule, E_{in} . To maximize the electromagnetic field, the surface plasmon effect must first be optimized. Various parameters are adjusted, to match the plasmon frequency to the frequency of the intended excitation source, which include the metal used, and the size and shape of the metallic surface. The best metals to be used for SERS in the visible region are silver, gold, and copper with silver providing the best enhancement. Other metals have been investigated and are reported in the literature [90, 92]. The overall size of the metallic surface has been shown to be a very important factor in the plasmon frequency. For simplicity, a spherical shape is considered. For silver spheres, a maximum enhancement of 10^6 would occur for a 5 nm radius particle with 382 nm excitation [93]. With increasing particle size, the plasmon frequency bandwidth increases and the enhancement decreases.

Chemical Model

The chemical effect is more specific than the electromagnetic effect and is less understood. The basis of the chemical effect seems to be from the overlap of metal and adsorbate electronic wavefunction. The overlap promotes light-induced charge transfer processes that increase the apparent value of the molecule polarizability and the intensity of the Raman scattering [90, 92]. This effect seems to be a much shorter effect than the electromagnetic effect occurring from 0.1 to 0.5 nm. The overall chemical effect is dependent on the atomic-scale features of the metallic surface, the geometry of the bonding between the analyte and the metallic surface, and the energy levels of the analyte adsorbed onto the metallic surface. The overall extent of the chemical effect varies for a range of analytes, but it is quite specific for adsorbate-surface systems producing enhancements from 10 to 1000 [94]. Chemical effects are still considered minor contributors to the overall enhancement seen with SERS. At this time there are two proposed models, the charge transfer model and the adatom model.

The charge transfer model proposes that stimulation by an incident photon causes an electron from the metal surface to move into a charge-transfer excited state of the adsorbed analyte. This process results in an ionized molecule with geometry different than the initial geometry of the neutral molecule, thus a change in the polarizability of the molecule. Next, the molecule relaxes with the return of the electron to the metal surface. It is this relaxation that produces a vibrationally excited neutral adsorbed molecule which emits a Raman shifted photon.

With the adatom model, there are interactions between the adsorbed molecules and the atomic scale specific active sites in the metallic surface. When the charge

transfer process occurs, there can be additional chemical enhancement from very specific molecules [92]. It is hypothesized that the enhanced Raman scattering could be attributed to the additional momentum available for scattering of the electron-hole pairs at atomic scale roughness or by relaxation of the momentum by the presence of the adatom site that the adsorbed molecule is bound.

Instrumentation

The instrumentation required for Raman and SERS include three basic components (1) a monochromatic excitation source, (2) a high-resolution dispersive element, such as a monochromator or a polychromator, and (3) an appropriate detector. The most common and versatile excitation sources used today for Raman scattering are lasers because of the high power output provided which is needed to produce Raman scattering of sufficient intensity to be measured. Most sources are continuous wave (cw) laser, but a few pulsed lasers have been used. The most common lasers used are the argon ion (Ar^+ at 488nm or 514.5nm), krypton ion (Kr^+ at 647.1 nm) and the helium-neon (HeNe at 632.8nm). However, near-infrared lasers such as a diode laser or the Nd-doped yttrium-aluminum-garnet (Nd:YAG) laser are used because they can be operated at higher powers and are not energetic enough to populate a significant number of fluorescence-producing excited electronic energy states in most molecules. Consequently, fluorescence is less problematic with these lasers as excitation sources.

The problems associated in the detection of a Raman signal are how to measure such a weak signal in the presence of the intense excitation source, how to effectively remove interference from the dominant signal, and if possible how to enhance the weaker

signal. Early instruments were based on a triple monochromator, which made these instruments complex and limited the optical throughput of the instrument (usually down to 5% or less overall efficiency). A variant to the triple monochromator design was the use of a double monochromator with a fixed grating spectrograph. A schematic of a single-channel instrument can be seen in Figure 1.18. With the advent of holographic notch filters the monochromator design was simplified. In fact, the need for the pre-monochromators for Rayleigh rejection was eliminated which significantly reducing the size of the instrumentation. This allowed for the use of array detectors.

Early instruments almost exclusively used a PMT as the detection source. PMTs are generally used in the ultraviolet and visible regions from about 400 nm to 800 nm; however, extended wavelength tubes can be used which allow for detection beyond 900 nm. A major improvement in Raman performance from dispersive instruments has been the use of multichannel detectors such as diode arrays and charge couple devices (CCDs). These multichannel detector based instruments exploit the multiplex advantage. Another advantage of using an array detector is that the grating can remain static, which eliminates the need for moving parts within the spectrograph resulting a very stable and reproducible optical setup. An important factor with an array detector based system is that there is a fixed spectral range and resolution, which is limited by the grating, the slit, and the array size of the detector.

A typical Raman dispersing spectrometer with a CCD detector an Ar^+ laser as the source is shown in Figure 1.19. The laser beam is directed and sent to a microscope probe where the laser beam is focused onto the sample. The emission is collected and sent to a slit of monochromator where it passes through a filter that rejects the Rayleigh

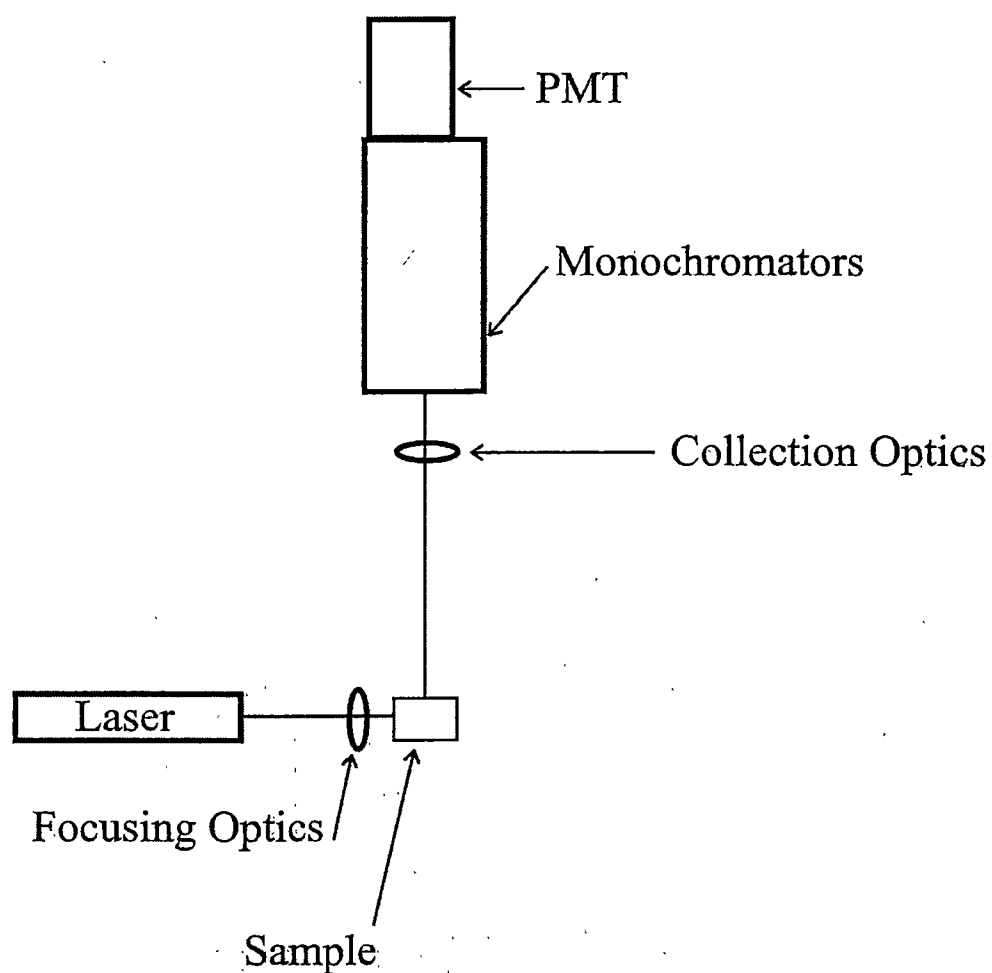


Figure 1.18. Single-channel Raman instrument.

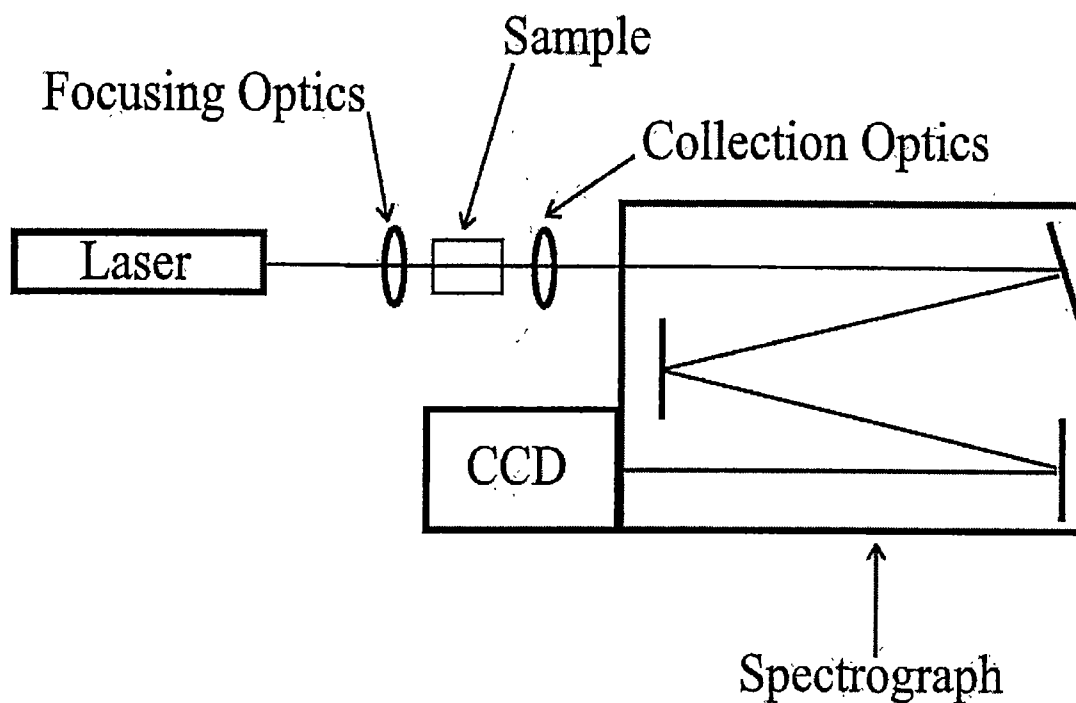


Figure 1.19 Multichannel Raman instrument.

Source: Skoog, D.A.; Holler, F.A.; Nieman, T.A *Principles of Instrumental Analysis*, Harcourt Brace & Company, Orlando, Fla, p.440, 5th Edition.

scatter. Then a diffraction grating is used to disperse the radiation and reflect it upon a CCD. The ruling density or the number of grooves per mm defines the dispersion by the grating. The greater the number of grooves per mm, the greater the resolution, but the narrower the spectral range covered by the grating. Typically, gratings between 1000 and 2000 grooves/mm are used. Sometimes more than one grating is on a rotatable turret, which allows for easy changing of the grating in the instrument.

A CCD is a metal-insulator semiconductor that can store charge in localized potential wells. A CCD array can be related to picturing the potential wells as a row of buckets (pixels) filled with charge. To obtain readout from a CCD array, the charge is transferred from the end pixel (bucket) to the readout electronics. The pixel that is adjacent to the end pixel then transfers its data to the end pixel that in turn transfers it to the readout electronics. The transfer of charge continues sequential from pixel to pixel until all pixels have been transferred to the electronic readout. There are two main disadvantages in using CCDs. One is the limitation to sequential readout of the pixels, which causes these arrays to be slower than the photodiode array (PDA) detectors (see discussion below). A second limitation is the affect of blooming. Blooming occurs when a charge overfills a pixel and spills into the adjacent pixel.

Dispersive instruments may also use a PDA detector for acquiring spectra. A PDA is simple and consists of an array of photodetectors. A PDA is operated either by responding to light and generating an electrical current proportional to the number of photons that strike the surface termed, photovoltaically or its electrical resistance is reduced proportionally to the number of photons that strike its surface, termed

photoconductor. A PDA is normally used with an image intensifier to increase the weak Raman signal.

There are important parameters to investigate in the detector used in Raman spectroscopy. First, the detector noise and dark current are important. Second, are the detector range and the response profile. Selection of the appropriate detector can depend on the complexity of the optics used to collect the light, the speed of detection, and the sensitivity required. For instance, a PDA requires optics that allows the appropriate portion of radiation to strike only the desired photodetector. With a CCD this is less problematic because of the larger area available to direct the signal. In terms of speed of light detection, a CCD is the slowest and the PDA has the potential to be the fastest. A PDA is generally more sensitive than a CCD because of the associated charge-transfer efficiency. The efficiencies are high, but are still less than 1, which makes CCD less sensitive than a PDA.

STATEMENT OF PROBLEM

Even though CE offers many advantages such as speed, efficiency, and small sample requirements outlined earlier in Chapter 1, CE still suffers from some inherent problems due to injection, separation, and detection. Due to the fact that CE is relatively young in age compared to competing techniques such as LC, there is still a lack of understanding regarding the fundamental processes of CE. Although significant advances in detection methods for CE have been reported, a need for further advancement and improvement is warranted. The purpose of the research presented is to utilize the advantages of CE while improving, refining, and developing detection

methodologies for CE. The work presented herein entails the development of a post-column derivatization technique for the study of DNA-protein interactions, the improvement in the design of SFCs, and the first ever coupling of SERS detection with CE.

CHAPTER 2

EVALUATION OF A SHEATH FLOW CUVETTE FOR POST-COLUMN FLUORESCENCE DERIVATIZATION OF DNA FRAGMENTS SEPARATED BY CAPILLARY ELECTROPHORESIS

INTRODUCTION

Analytical methods centered on studying large, complex biomolecules have shown success in physical and life sciences. The study of these complex molecules is demonstrated in such fields as chemistry, biochemistry, biology, and others. CE has had success for the analysis of DNA. In fact, CE is rapidly becoming a routine instrumental technique in many laboratories replacing traditional techniques such as slab-gel electrophoresis for DNA analysis [95-98]. As techniques have improved for preparing capillaries containing size sieving media, the emergence of CE as a powerful new separation tool for DNA analysis has turned into reality [25, 98, 99].

Analytical methods for other biomolecules such as proteins are just as prevalent. Size separations of proteins by CE with gel filled capillaries were first demonstrated by Hjerten [34]. The implementation of CE for protein-digestion, peptide derivatization [100, 101], and peptide mapping [102, 103] has been investigated. The success CE has shown in the study of DNA and proteins makes it suitable for the study of DNA-protein interactions.

The study of DNA-protein interactions is an active area of biochemical research. Jacob and Monod first proposed the model of genetic regulation for protein synthesis by DNA-binding repressor proteins in 1961 [104]. Research has shown that genetic

regulation arises as the consequence of DNA-binding repressors and activators. To gain an understanding of genetic regulation at the molecular level, it is imperative to study the interaction between nucleic acids and regulatory proteins. In fact, with the very recent sequencing of the human genome makes this study of DNA-protein interactions a significant contribution. The binding of a given protein to a very specific binding site on a DNA molecule results in the activation or repression of genetic signals and consequences. The method of choice for the study of DNA-protein interaction is the gel retardation assay or more specifically the electrophoretic mobility shift assay (EMSA) [105, 106]. EMSA is used for a wide variety of applications in nucleic acid research [105-111].

The general mechanism of EMSA is based on that of a gel retardation scheme. A solution comprising of a mixture of DNA-protein complexes and unbound protein is loaded into the gel. Subsequently, free DNA will introduce into the gel as a discrete band when placed under electrophoresis. This occurs because the mobility of the DNA-protein complex can differ from that of the free DNA. The proteins that are DNA-bound are electrophoretically retarded with respect to that of the unbound DNA. The free DNA is generally unaffected by dissociation/association processes; hence, the assay procedure can provide an accurate value for the quantity of uncomplexed DNA. Since the concentration of input DNA and uncomplexed DNA is known, the amount of DNA that exists in the complexed form can be determined.

EMSA does suffer from some limitations such as long analysis time, inaccurate quantitation, and trouble with determining dissociation constants [112]. CE is well established as a replacement for the traditional EMSA method. CE offers the advantages

over traditional slab-gel and gel chromatography techniques as previously discussed in Chapter 1. The current method is termed capillary electrophoretic mobility shift assay (CEMSA). CEMSA will combine the advantages of both EMSA and CE while minimizing the limitations suffered in EMSA. CEMSA has been developed in regards to both a quantitative and qualitative aspect of the technique.

The development of CEMSA was characterized with a well know system, the interaction between the *trp* repressor (protein) of *Escherichia coli* (*E. coli*) and the *trp* operator (DNA) by Stebbins et al. [113]. The *trp* repressor protein is known to control transcription in the *trp*, *arch*, and *trpR* operons [114]. The transcription process is when enzymes synthesize an RNA copy of one strand of a DNA sequence. Consequently, the RNA then controls the synthesis of a cell's proteins, termed translation. An operon can be defined as a cluster of genes, an operator, and its structural genes. An operon's transcription is blocked by the repressor proteins and initiated by stimulatory proteins. Consequently, the binding of the repressor to the operator inhibits transcription of structural genes from occurring.

This resultant competition between the repressor and the RNA for binding sites in the promoter/operator region is termed repression. The DNA binding in the *trp* repressor/*trp* operator system is an allosteric controlled process. The *trp* repressor regulates the production of L-tryptophan in *E. coli* and is completed by a negative feedback loop [115]. If the concentration of L-tryptophan is low, the *trp* repressor is inactive and the *trp* operon is expressed which results in the synthesis of L-tryptophan. This process is due to the fact that the inactive repressor is not able to bind with the operon due to steric hindrance. This is depicted in Figure 2.1 (A). However, at high

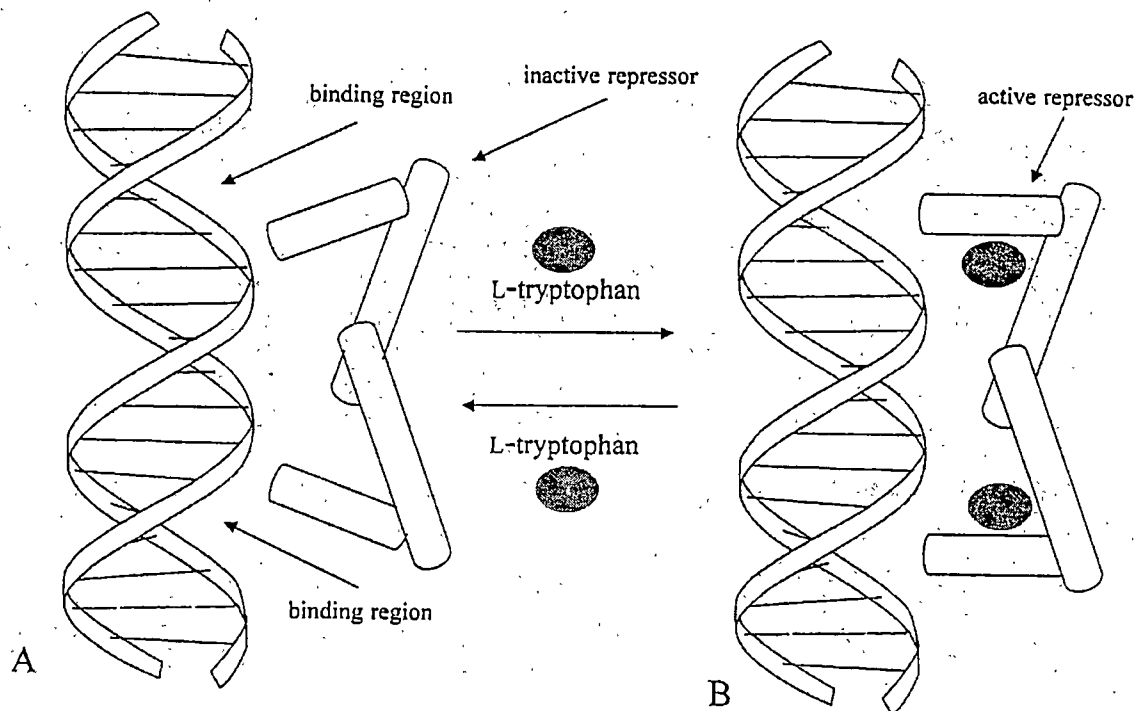


Figure 2.1. The interaction between the *trp* repressor and the *trp* operator. (A) the inactive repressor cannot bind to the operator with high affinity, (B) the binding of L-tryptophan creates a conformational change in the repressor (protein) that changes the orientation of the repressor, which allows the active repressor to bind into two successive major grooves of the operator DNA.

concentrations of L-tryptophan the active repressor is formed. Concurrently, the operator is bound which inhibits transcription activation in the *trp* operon and represses the synthesis of L-tryptophan. Consequently, the binding of the L-tryptophan forces a conformational change in the *trp* repressor that changes the conformation of the recognition helices allowing a fit into the two successive grooves of the operator DNA. This is shown in Figure 2.1 (B) [115].

The investigation of DNA-protein interactions by CE with LIF detection was performed by Stebbins et al.[113]. The specific interaction between the *trp* repressor and the *trp* operator was used to evaluate CEMSA. In CEMSA and in traditional assays, the mobility of a DNA-protein complex differs from the mobility of the free DNA. Many studies were done to characterize and develop CEMSA as a tool for the study of DNA-protein interactions [113]. Running buffer conditions in terms of pH and composition were evaluated in order to optimize complex formation and resolution of free DNA from the complex. Quantitative studies were done to show the reaction of the repressor with operator resulting in a decrease of the operator signal and an increase in the repressor-operator peak. Nonspecific interactions between the operator and complex protein that demonstrate the specificity of the operator for the repressor were shown. Studies demonstrated that this specificity of the operator for the repressor could be employed to separate the repressor from a complex sample, which contains a non-specific protein. The above studies were implemented to show CEMSA's ability to selectively distinguish between bioaffinity specific and nonspecific interactions. In addition, experiments done to characterize CEMSA for the study of DNA-protein interactions included experimental evidence of *trpR*:DNA complex, the dependence of complex formation on L-tryptophan,

the effect of buffer composition (methyl cellulose) on the efficiency and resolution of the separation, the effect of concentration of *trpR* on the free DNA and the complex, and the effect of non-DNA binding proteins on the selectivity of the repressor/operator interaction.

However, improvements in the technique are warranted which would allow for the determination of a range of parameters important to DNA-protein interactions. Efficient labeling of the DNA would allow for an increase in the number of DNA molecules that could be detected; hence, lower concentrations of analyte can be used. In order to use lower concentrations, an improvement in the overall detectability of the previously used fluorescence detection system is needed. Alternative systems can be implemented such as lens-based collection of fluorescence, spatial rejection of specular scatter, and the use of extended light path capillaries to improve the detectability. However, for the ultimate in detection capabilities the use of SFCs is warranted.

Detection schemes in CE are usually performed on-column where the detection zone is limited to the cross section over a short segment (< 1 mm) of the capillary (see Chapter 1 for a more detailed discussion). When coupled with the sheath flow cell, laser induced fluorescence (LIF) detection has achieved ultra-sensitive limits of detection [116-119]. However, since most analytes separated by CE are not natively fluorescent, chemical derivatization is employed to extend the use of LIF to non-fluorescent analytes through the use of fluorescent tags. Techniques exist for pre-column, on-column, and post-column derivatization of many classes of non-fluorescent analytes as alluded to in Chapter 1. The discussion below applies each of the three different derivatization techniques for the DNA-protein analysis.

Pre-column derivatization is performed prior to sample injection and separation. In the investigation of DNA-protein interactions, DNA fragments must first be tagged with a fluorogenic reagent before analysis can be performed. Most commonly a fluorescein compound is covalently bonded to the DNA fragments. However, this technique has the shortcomings of poor labeling efficiency and multiple labeling of analytes, making the acquisition of any quantitative information difficult.

Another derivatization procedure that has been performed with CE is on-column derivatization where the capillary is first filled with the fluorogenic reagent prior to sample injection. After sample injection, the fluorogenic reagent and the analyte reach an equilibrium state as the analyte migrates through the column; however, analytes can still be multiply labeled. The adverse effects of multiple labeling can be mitigated by the fact that this labeling method is dynamic (i.e., covalent attachment of the label to the analyte is not involved). In terms of DNA-protein interactions, on-column derivatizing agents can bind with the DNA fragment, which contains the specific binding region for the protein; thereby, hindering the interaction of the protein with that particular DNA fragment.

With the aforementioned difficulties in pre-column and on-column derivatization techniques for the study of DNA-protein interactions, investigation of post-column derivatization techniques is warranted. Several reactor designs have been demonstrated for post-column derivatization work; [53, 59-61] however, it has been determined that the sheath flow design results in minimal band dispersion and superior detection capabilities in the chemiluminescence detection of iso-luminol thiocarbamyl-amino acids [62].

The investigation and evaluation of the SFC as a reaction chamber to post-column fluorescently derivatize DNA fragments separated by CE is described herein. Use of the SFC arrangement facilitates the mixing of the intercalating dye, ethidium bromide (EB), and the effluent from the separation capillary by diffusion without a high degree of band dispersion. Theoretical plate counts of $> 1 \times 10^6$ are reported with the post-column derivatization technique and resolution of all of the fragments in a ϕ x-174-*Hae*III digest is achieved. Optimization of experimental parameters such as flow rate, position of the detection zone, and EB concentration are examined. Analytical figures of merit such as limit of detection (LOD) and linear dynamic range (LDR) is reported for a sample of ϕ x-174-*Hae*III digest. Evaluation of post-column derivatization for the investigation of DNA-protein interactions is demonstrated. The integrity of a DNA- *trp*-repressor protein interaction is maintained with the post-column approach, but comprised when EB is added to the running buffer. This arrangement is evaluated for the study of DNA-protein interactions where other derivatization techniques are unsuitable.

EXPERIMENTAL

Materials

Tris(hydroxymethyl)aminomethane, boric acid, ethylenediamine tetracetic acid (EDTA), methyl cellulose (MC) polymer (M_r 100000; 4000 M_r for 2% w/v), ethidium bromide (EB), and L-tryptophan were obtained from Sigma (St. Louis, MO, USA) and were used to prepare running buffers. Samples of ϕ x-174 -*Hae*III digests (480 μ g/mL) were purchased from Sigma. Acrylamide, γ -methacryloxypropyltrimethoxysilane (γ -MTMS), N,N,N',N'-tetramethylethylenediamine (TEMED) and ammonium persulfate

(AP) were obtained from Sigma and used to prepare column coatings. Samples used for the DNA-protein binding study (*trp* repressor and pBKH26 digest) were generous gifts of B.K. Hurlburt (University of Arkansas for Medical Sciences, Little Rock, AR, USA). Fused-silica capillaries (50 μm i.d. x 365 μm o.d.) were purchased from Polymicro Technologies (Phoenix, AZ, USA).

Column Preparation

The fused-silica capillaries were cut to a total length of 40 cm. The outlet end of the capillary was tapered down with a lathe to an approximate o.d. of 150 μm to help minimize the formation of eddy currents. Columns were coated with linear polyacrylamide using a modified version of Hjerten's method [35, 120]. The columns were rinsed with 0.1 M NaOH for 1 hour, rinsed with deionized water for 10 minutes, rinsed with 0.1 M HCl for 1 hour, and then rinsed again with deionized water for 10 minutes. An acidic solution of γ -MTMS was prepared by the mixing 20 mL water with 50 μL 6 M acetic acid and 80 μL of γ -MTMS and was then flushed through the column for 18 hours followed by water for 10 minutes. A 4.0% solution (0.40 g) of acrylamide was prepared in 9 mL of phosphate buffer (9 mL water + 0.087 g K_2HPO_4) along with a solution of 0.15 g AP in 10 mL of water and was allowed to degass by bubbling helium for 30 minutes. Polymerization reaction was started by adding 1 mL of AP with 7.5 μL of TEMED to the acrylamide solution followed by subsequent introduction of the solution into the capillary to allow for in-situ polymerization for approximately 12 hours. The column coating reaction is shown in Figure 2.2. The capillary was then flushed with

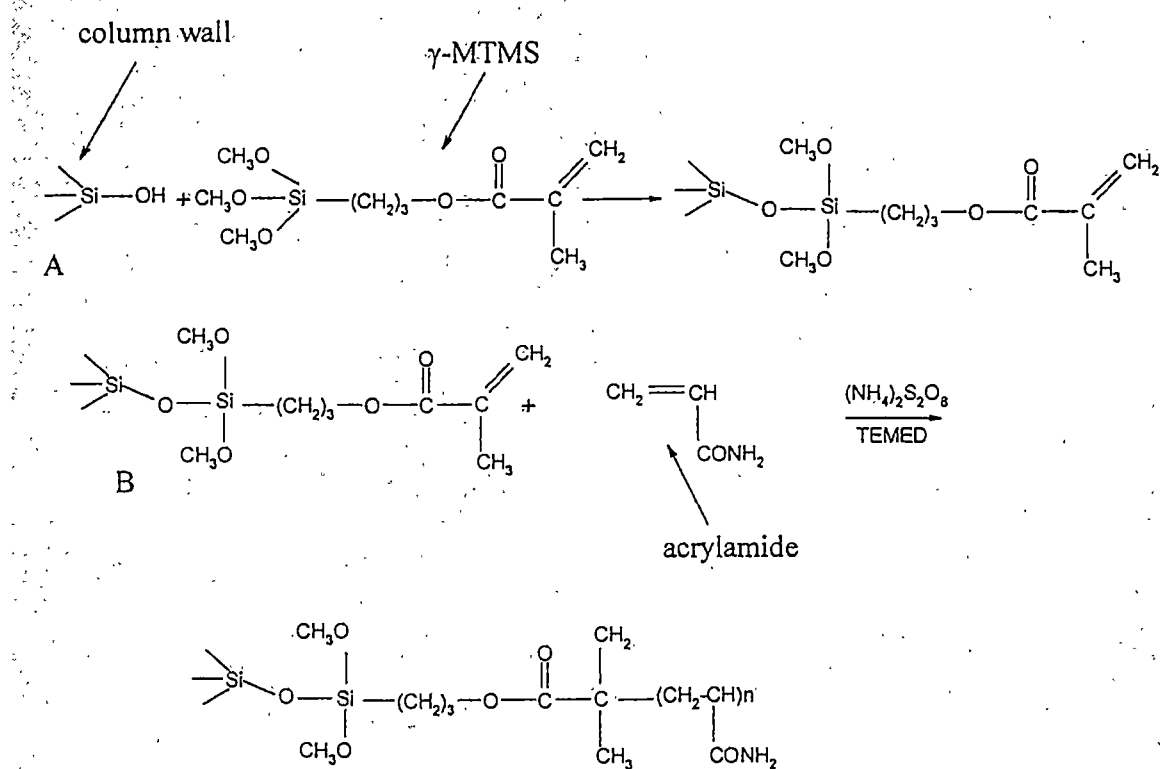


Figure 2.2. Linear polyacrylamide coating reaction.

10 mM H_3PO_4 to remove any excess polyacrylamide. Columns were stored under 10 mM H_3PO_4 .

Running Buffer and Sample Preparation

Stock solutions of tris-borate EDTA (TBE) were prepared by weighing out an appropriate amount of solid and diluting with deionized water to final concentrations of 0.45 M Tris, 0.45 M boric acid, and 0.01 M EDTA. A 10-fold dilution of the stock solution resulted in a running buffer containing 45 mM Tris, 45 mM boric acid, and 1 mM EDTA and referred to as 45 mM TBE. Methyl cellulose stock solutions were prepared by weighing out the appropriate amount of solid MC (100,000 MW) into 80-90 °C 45 mM TBE. Once the MC was dissolved, the solution was cooled on ice with stirring until a clear viscous consistency.

The stock MC solutions were used to prepare fresh running buffers daily by dilution with 45 mM TBE to a final concentration 0.5 % MC. LIF detection of the DNA was accomplished by adding the appropriate amount of concentrated EB stock (1 mg mL^{-1}) to 10 mL aliquots of MC running buffers giving final EB concentrations ranging from 1 to 5 μM . The running buffers were then degassed under vacuum to remove any bubbles that may have been generated.

DNA samples of $\phi\text{x-174}$ -*Hae*III digests were prepared by diluting an aliquot from the stock with 45 mM TBE to a desired final concentration. Samples of purified *trp* repressor were prepared and handled as described in Czernik et al [109]. The DNA plasmid pBKH26 was digested with the restriction enzyme *Hinf*I (Boehringer Mannheim,

Indianapolis, IN, USA) and the resulting fragments were fluorescein labeled with the FluoroAmp T4 Kinase Green Oligonucleotide Labeling System (Promega, Madison, WI, USA) on the 5' end and was prepared by B.K. Hurlburt (University of Arkansas for Medical Sciences, Little Rock, AR, USA) as described [121].

Intercalation with Ethidium Bromide

As discussed in more detail in Chapter 1, detection schemes vary in CE. In terms of DNA separations, the main detection schemes used are electrochemical, absorbance and fluorescence. Absorbance is a simpler technique but is less sensitive than fluorescence. At very low pHs and using UV lasers, DNA will natively fluoresce. However, for more routine analysis there is a need for some form of derivatization of the sample. One method of detection for DNA analysis involves using LIF via the addition of an intercalating dye into the running buffer that labels double stranded DNA fragments.

Ethidium bromide has been used in DNA analysis [122]. The spectral properties of EB make it a very useful intercalation dye for LIF detection of DNA by CE. When EB intercalates into the hydrophobic interior of the double helix, the quantum yield increases 20 fold [122]. EB has maximum intercalation stoichiometry when one ethidium cation per four base pairs (bp) [122] as shown in Figure 2.3a. After intercalation, EB has a spectral shift shown in Figure 2.3b. A helium neon "greenie" (He-Ne, 543.5 nm) laser is often used for DNA analysis with EB. EB offers other advantages in the separation of DNA fragments by CE. In fact EB can increase the resolution in CE separations [123]. Note that one ethidium will intercalate into four bp resulting in a reduction μ_{obs} of the

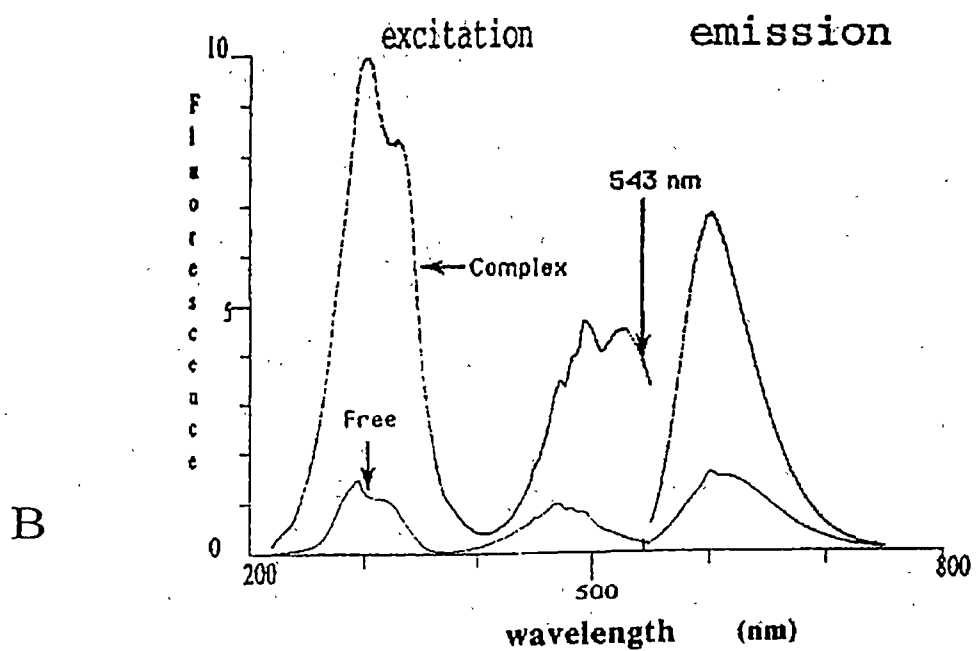
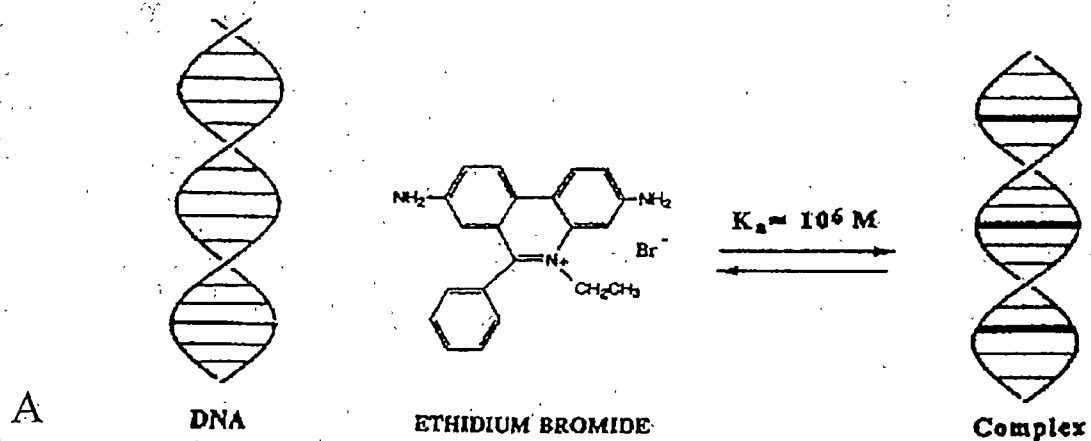


Figure 2.3. Ethidium bromide. The effect of ethidium bromide intercalation on DNA (A) and the subsequent spectroscopy (B).

labeled DNA fragments is observed. The change in the intrinsic mobility results in a greater difference in the migration times between fragments and increases resolution (see Equation 1.19 page 18 in Chapter 1).

Apparatus

The electrophoresis, fluorescence, and sheath flow cell apparatus was assembled in-house. The pictorial representation of a sheath flow cell is shown in Figure 2.4. The sheath flow cell was made in house using epoxy to affix a 500 μm i.d. x 700 μm o.d. x 5 cm piece of round quartz tubing (VitroCom Inc., Mountain Lakes, NJ, USA) into a 5 mm x 0.030" i.d. piece of PEEK tubing which was subsequently fastened into a Swagelock tee. The separation capillary was fastened into the Swagelock tee via a graphite ferrule and was centered inside the sheathing capillary with the aid of a video microscope. The sheath flow was introduced via a piece of 0.02" i.d. PEEK tubing fastened into the side port of the Swagelock tee. The inlet end of the PEEK tubing was placed in a reservoir of the sheathing fluid at a height of 8 - 12 cm above the outlet end of the sheathing capillary. The Swagelock tee was then secured into a fiber optic positioner. The actual SFC apparatus used for experiments is shown in Figure 2.5.

The CE-laser fluorimetry instrumentation consisted of a PMS Electro-Optic helium-neon greenie laser (1.5 mW at 543.5 nm) used as the excitation source (Edmund Scientific, Barrington, NJ, USA). The laser beam was focused through the sheathing capillary with a 25 mm quartz $f/1$ lens (focused spot size estimated to be 20-30 μm) and fluorescence emission was collected at 90° to the flow cell with a 25 mm quartz $f/1$ lens

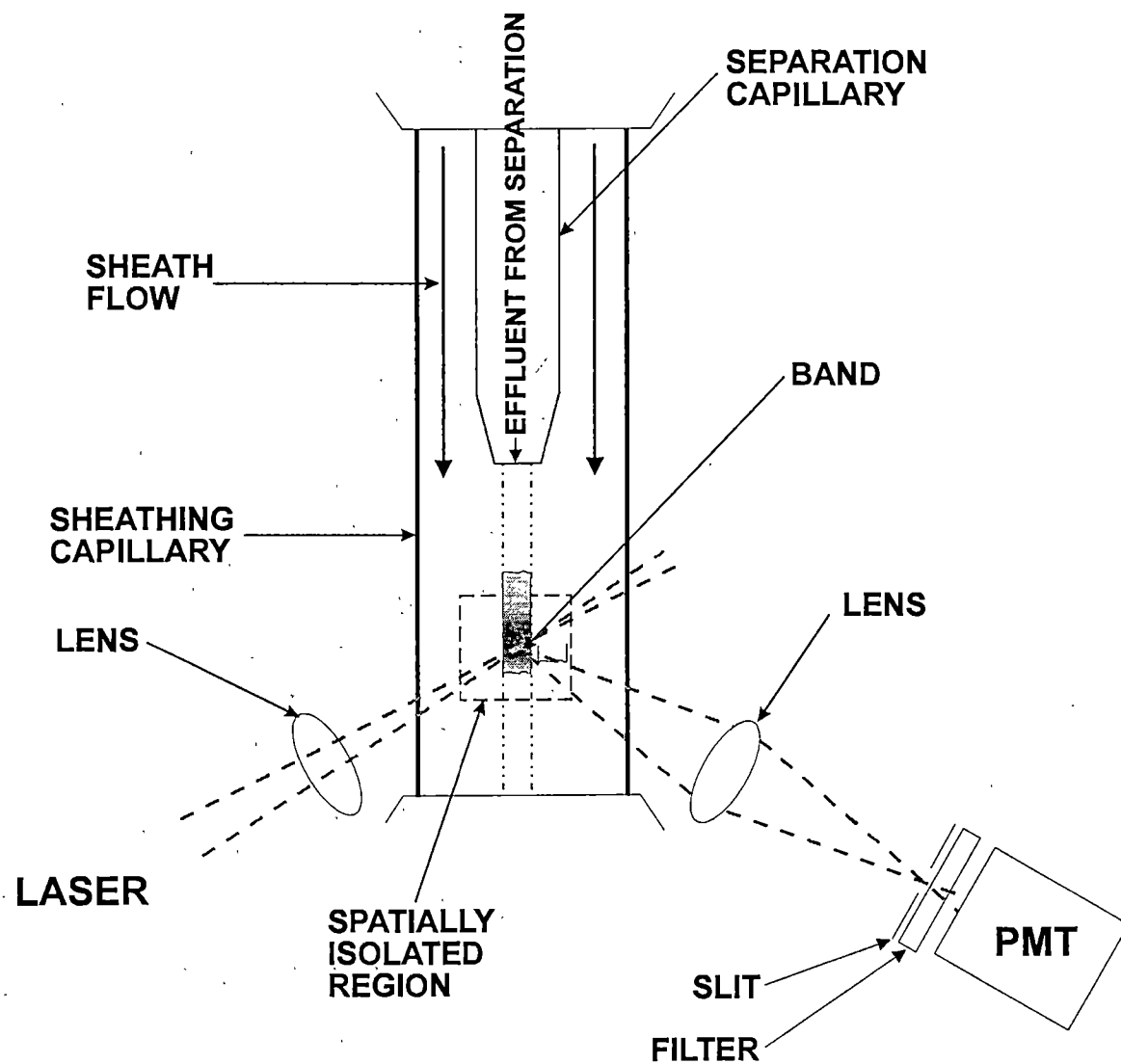


Figure 2.4. Pictorial representation of a sheath flow cell used for EB post-column DNA intercalation.

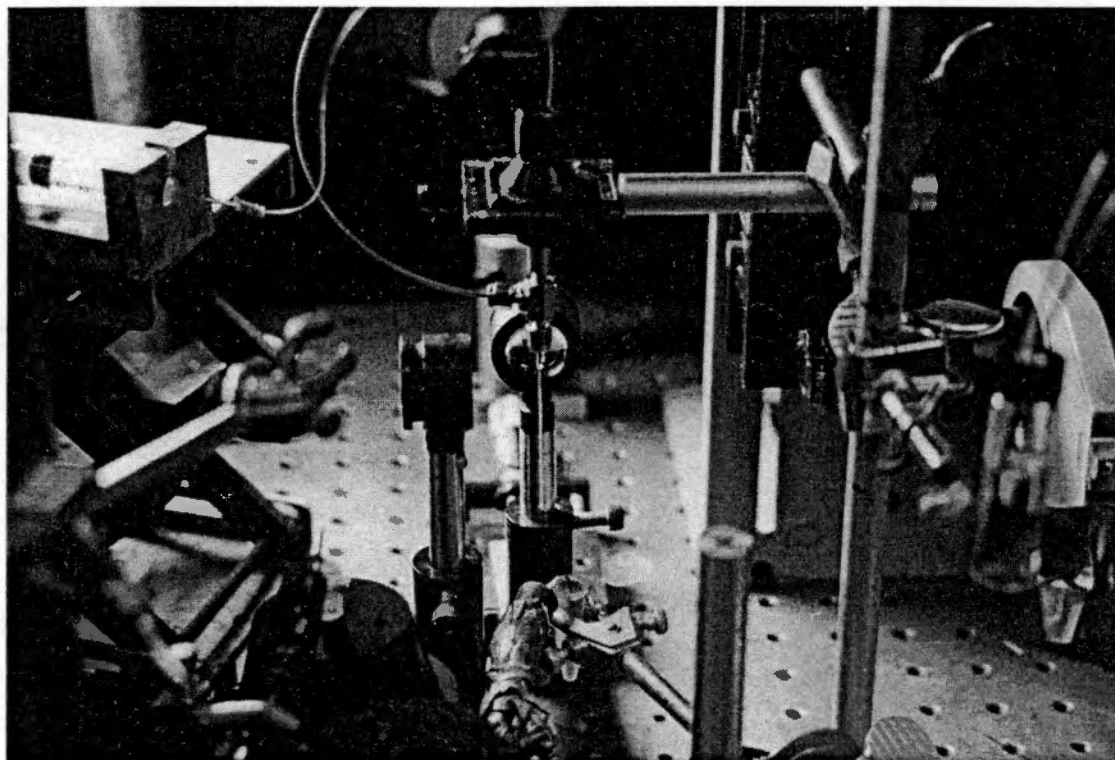


Figure 2.5. Picture of the SFC apparatus with video microscope used for the study of DNA-protein interaction.

(Edmund Scientific, Barrington, NJ, USA). The fluorescent image was spatially isolated using an adjustable optical slit (Edmund Scientific, Barrington, NJ, USA). The isolated radiation was passed through a 570 nm cut-on filter and allowed to impinge on a RP-1 type photomultiplier tube (Hamamatsu Corp., Bridgewater, NJ, USA). The PMT photocurrent was monitored by a Pacific Precision Instruments (Concord, CA, USA) Model 126 photometer. The photometer was operated with a 0.1 s output time constant. Electropherograms were recorded with a program written in-house using a PS/2 model 50 IBM computer equipped with a uCDAS-16G data collection board (Keithley Metrabyte, Taunton, MA).

A Hipotronics (Brewster, NY, USA) Model 840A high-voltage power supply was used to apply electrophoretic fields. Injections were performed electrokinetically (for running buffers incorporating MC) by placing the inlet of the capillary into the sample vial and applying -600 V for 30 s unless otherwise noted. Following injection, the capillary inlet was replaced into the running buffer vial and a running potential of -10 kV was applied across the 40 cm capillary.

Video images of EB-DNA fluorescent band profiles were recorded via a SONY zoom CCD Color Video Camera (Edmund Scientific, Barrington, NJ, USA) interfaced with a Trinitron Color Video Monitor (Edmund Scientific, Barrington, NJ, USA) and a SONY VHS recorder. The corresponding images of the band profiles shown herein were digitally enhanced with Corel Draw/Corel Photo-Paint using a Pentium 133 MHz PC compatible computer.

Safety Considerations

EB is a toxin, irritant, and mutagen and may cause heritable genetic damage. Extreme care should be exercised when handling EB and EB solutions. Suitable protective clothing, gloves, and eye protection should be worn at all times when handling EB. Solutions of EB should be filtered by charcoal filtration or chemically deactivated before disposal. A commercially available filter funnel kit that uses a packaged charcoal disk can be used to filter the EB through the charcoal filter. The filtrate can go down the drain and the charcoal filter should be placed in a sealed bag and placed in the biohazardous waste box for incineration. EB solutions can be chemically deactivated and neutralized by the Lunn and Sansone method [124]. The Lunn and Sansone method entails adding 5% hypophosphorous acid, 12 mL of 0.5 M sodium nitrate, stir briefly, let stand for 20 hours, adjust pH to 4.9 using sodium hydroxide, and disposed. Spills of EB solutions should be wiped up using paper towels soaked in the freshly prepared decontamination solution (an aqueous solution of sodium nitrite and hypophosphorous acid) as described by the Lunn and Sansone method [124]. Used towels should then be neutralized with sodium bicarbonate and can be discarded in the trash.

RESULTS AND DISCUSSION

Characterization

In an attempt to characterize the flow cell, studies on the effects of flow rate, detector position, and concentration of EB were performed. Figure 2.6 depicts the effect of the separation flow velocity (effluent) on signal intensity ($I_{\max}$) versus position of the

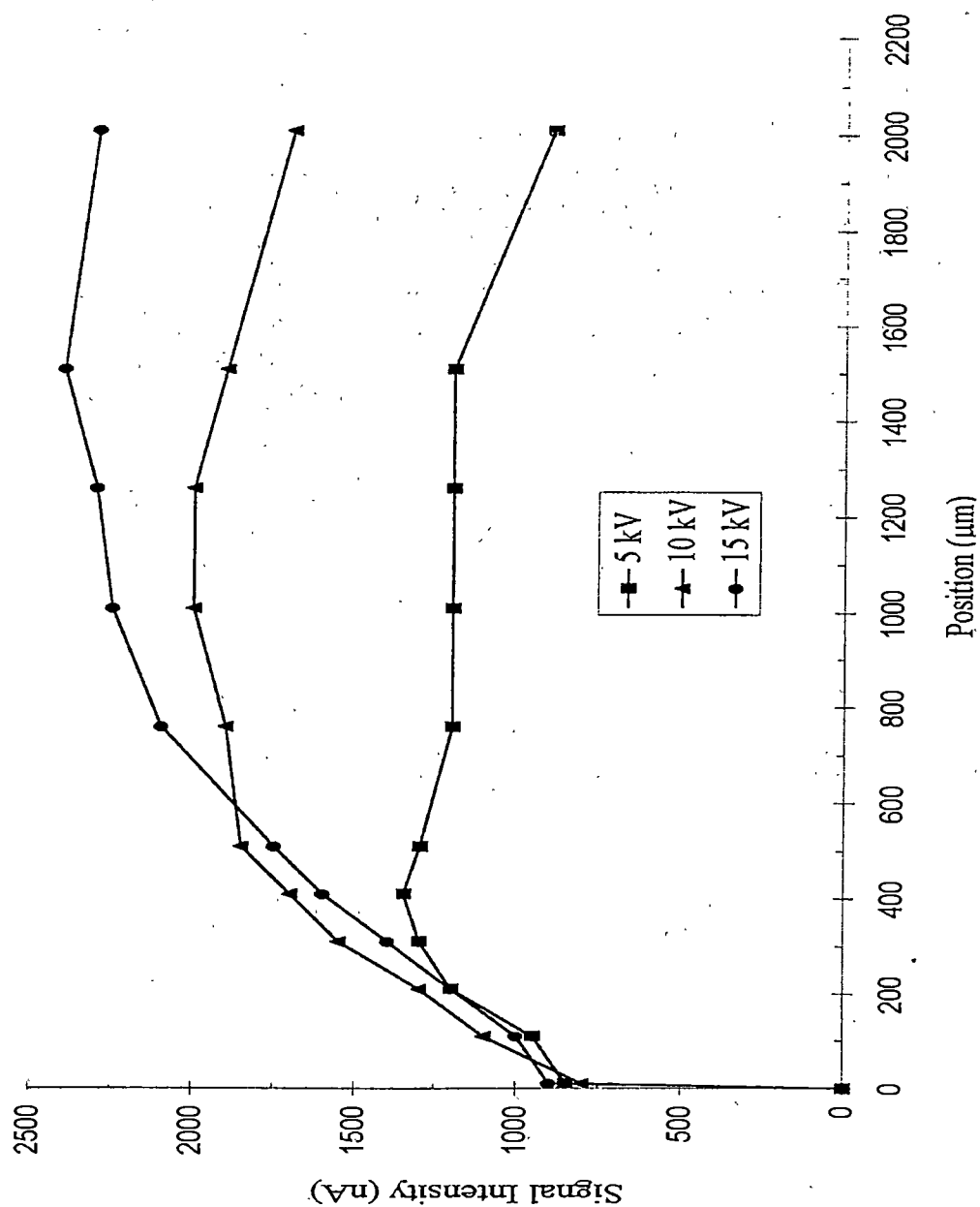


Figure 2.6. Effect of separation flow velocity on signal intensity versus position. Detector position in μm down from outlet end of separation capillary and performed via continuous injection of $\phi\text{x-174}$ *Hae*III digest in 45 mM TBE with a sheathing fluid of 2.5 μM EB. Flow velocities were determined to be matched at 10 kV.

separation flow velocity was varied by adjusting the applied voltage. As DNA is continuously exiting the capillary due to electrophoretic migration, it is entrained in the sheathing fluid and travels down the sheathing capillary. Concurrently, the EB diffuses into the DNA flow stream. The diffusion of the high molecular weight DNA is expected to be very slow. At some point in time the EB will completely diffuse into the DNA stream and signals should reach a maximum. This is seen in Figure 2.6. The faster the separation flow velocity (in this case the higher the applied voltage) the further down the sheathing capillary this would be expected to occur; again as seen in the figure. The slight decrease in signal at large distances from the separation capillary may be due to diffusion of DNA out of the optical imaging field or simply misalignment. At relatively low and high flow rates the DNA stream may be expected to hydrodynamically constrict and expand, respectively. Constriction may increase the effective DNA concentration in the focused laser beam but will decrease the effective pathlength. Expansion should have the opposite effect. Since the expansion (high voltage) case has the greater signal in Figure 2.6, the pathlength effect seems to dominate. In reality, the flow patterns are somewhat more complicated, particularly very near the separation capillary (see below).

Figure 2.7 shows video images of band profiles at 3 arbitrary positions (adjacent to separation capillary, 210 μm , and 1200 μm) at each of the flow velocities investigated. The i.d. of the separation capillary (50 μm) is drawn to scale, but the three detector positions are not drawn to scale. The focused laser beam is estimated to have a spot size of approximately 50 μm through the center of the flow cell. The fluorescent images are shown in dark black and the laser scatter off of the flow cell are shown in gray. Figure 2.7b reveals the band profiles when the flow velocities are matched (10 kV) between the

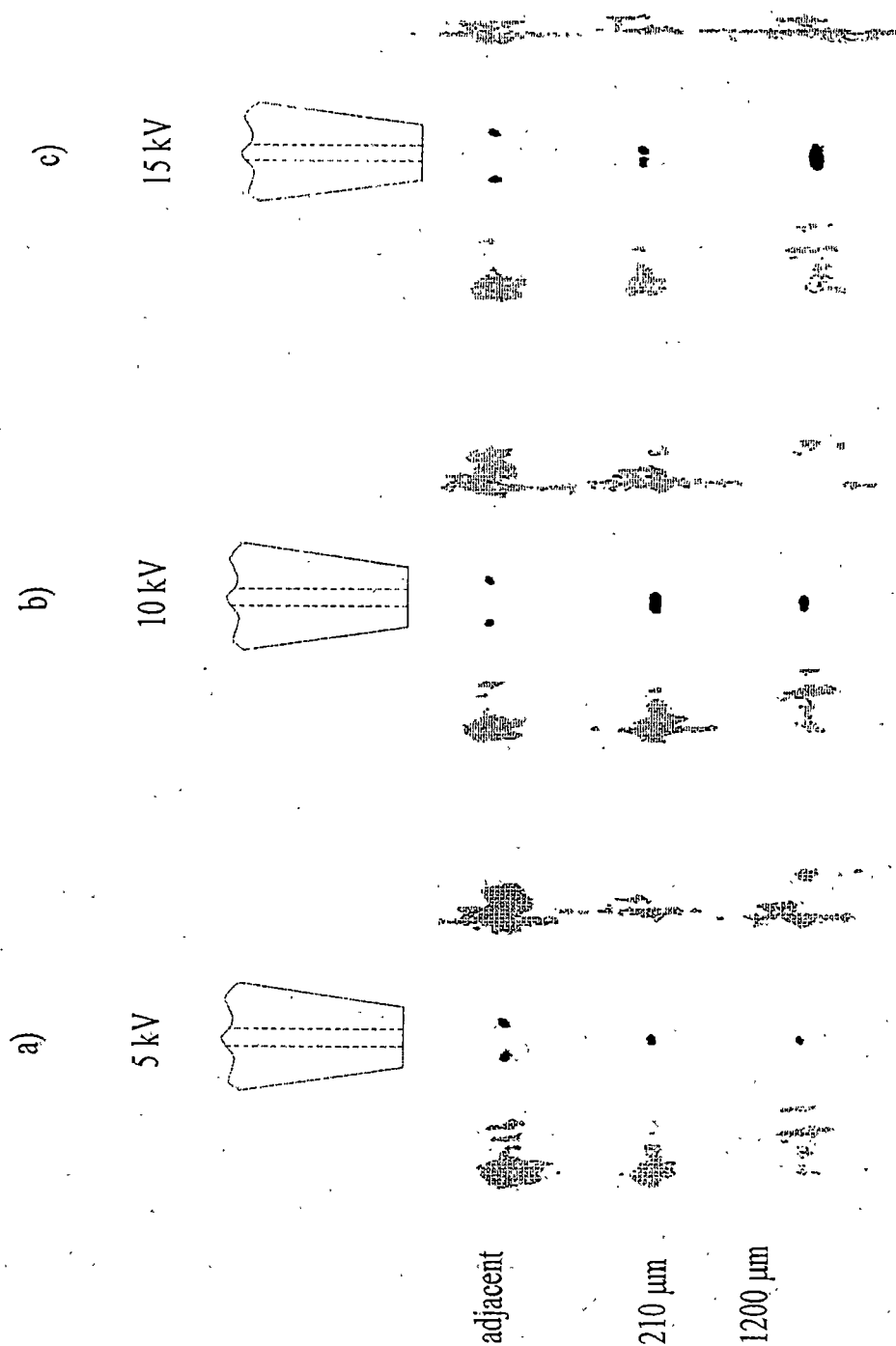


Figure 2.7. Video images of DNA-EB fluorescence band profiles at three separation flow velocities at three different focused laser beam positions (in μm from outlet end of separation capillary) down the sheath flow cell. The adjacent images are estimated to be 10-20 μm from the separation capillary. There is no significance to the vertical spacing of these images.

separation and sheathing fluids (notice that the image of the focused laser beam remains approximately constant). In each flow velocity investigated, there is evidence of eddy currents around the outside of the capillary tip by the image being slanted (adjacent images) which indicates that the EB has not yet diffused into the effluent channel. This occurs despite the very low Reynolds number for this system. The slower flow rates (5 and 10 kV) at 210 μm show that the EB has almost completely diffused into the effluent channel. On the other hand, Figure 2.7c shows the opposite effect when the effluent flow is faster (15 kV) than the sheathing flow. In this case the EB has not completely diffused into the effluent channel by the 210 μm position (distinguished by the separation of the fluorescent dots in the image). This separation of the fluorescent dots also indicates that the migration of positively charged EB into the outlet (negative polarity at the inlet) of the separation capillary is not appreciable. Figures 2.7a and 2.7c (1200 μm image) show the hydrodynamic constriction and expansion, respectively, that occurs due to the mismatched flow rates (slower in the 5 kV case and faster in the 15 kV case). The effects of the eddy currents on signal and separation performance were not directly studied. Future work will include tapering the separation capillary to a thinner wall thickness with the goal of reducing these eddy currents.

Next the effects of EB concentration in the sheathing fluid on I_{max} were evaluated. The experiment was carried out at the potential of 15 kV with an EB concentration of 1.0 μM , 2.5 μM , and 5.0 μM which all gave different I_{max} at 410 μm , 310 μm , and 110 μm respectively. This demonstrates the effect of concentration on the diffusional mass transport of the EB into the effluent channel. As expected, the lower concentrations of

EB (1.0 and 2.5 μM) require more time to achieve DNA-EB equilibrium; thus, I_{max} occurs further down the sheathing capillary.

Evaluation of Post-Column Derivatization

As with any post-column derivatization detector, resolution and efficiency are governing factors in determining the suitability of the approach. Figure 2.8b is an electropherogram of $\phi\text{x-174}$ *Hae*III digest with complete resolution of all 11 fragments obtained using the sheath flow cell and post-column derivatization. A resolution of 2.4 for the 271/281 bp fragments and an efficiency of 2.2×10^6 plates/meter based on the 603 bp fragment are demonstrated which compares well to the on-column detection of $\phi\text{x-174}$ *Hae*III (Figure 2.8a) with a resolution of 2.8 for 271/281 bp fragments and efficiency of 2.4×10^6 plates/meter based on the 603 bp fragment. Figure 2.8b was obtained with the focused laser beam within 50 μm of the separation capillary. At a position of 250 μm from the outlet of the separation capillary the efficiency of the separation degrades to 2.1×10^5 plates/meter and the resolution decreases to 0.8 for 271/281 bp fragments (not shown). Comparing figures 2.8a and 2.8b, the minimal loss of efficiency between on-column and post-column derivatization is approximately 10% with a decrease in resolution of the 271/281 bp fragments of 15%. Some of the difference in resolution may be due to the effect of the EB on the separation [125]. This effectively demonstrates the suitability of this post-column derivatization approach for the detection of DNA fragments. However, modification, such as more effective tapering of the separation capillary, is needed to allow greater freedom in positioning the detection zone without excessive band dispersion. To determine a limit of detection (LOD) for $\phi\text{x-174}$ *Hae*III, a

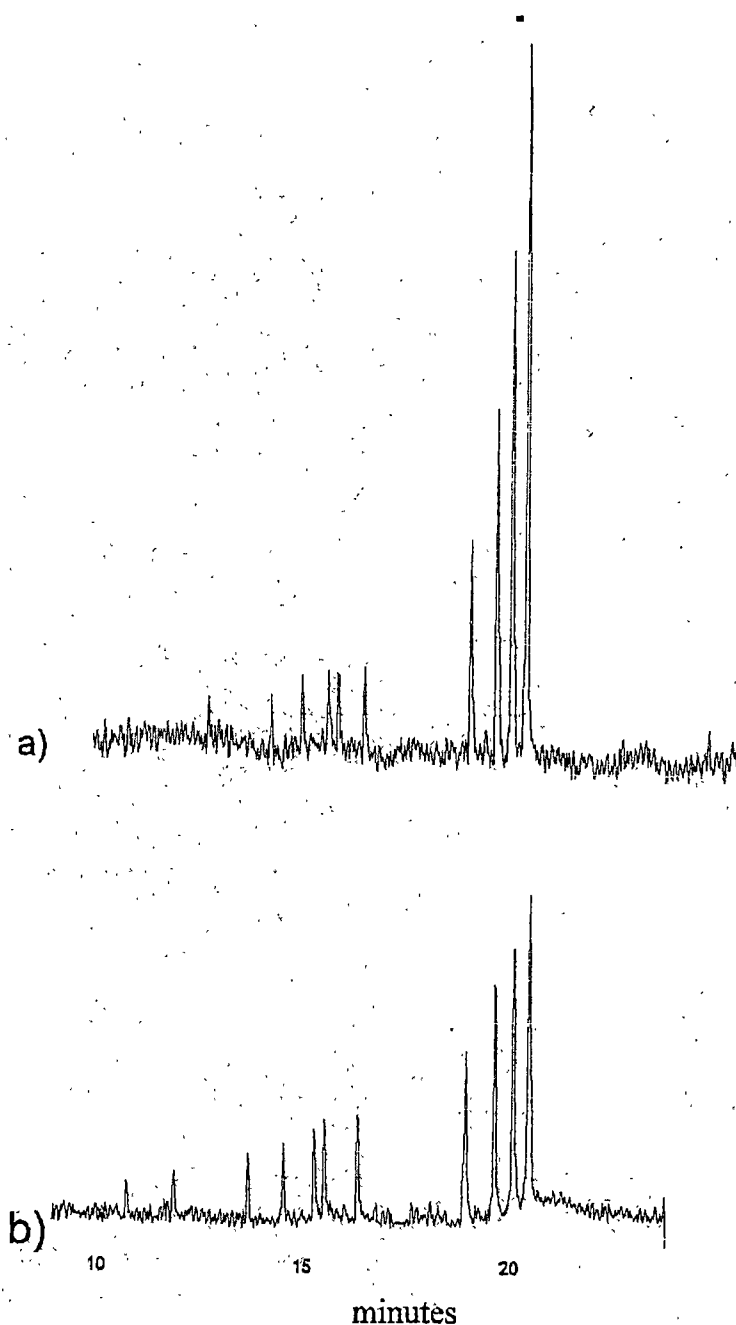


Figure 2.8. On-column versus post-column separation. a) On-column detection of 20 $\mu\text{g/mL}$ of $\phi\text{x-174}$ *Hae*III digest. Running buffer used was 0.5% MC in 45 mM TBE with 2.5 μM EB. b) Post-column derivatization detection of $\phi\text{x-174}$ *Hae*III digest adjacent to separation capillary. Running buffer consisted of 0.5% MC in 45 mM TBE with a sheathing fluid of 2.5 μM EB. Fragment sizes in order of elution are 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078, and 1353 base pairs.

continuous injection was performed and an LOD of 1.6 ng/mL of DNA for the raw data and 0.71 ng/mL with 5 point Savitsky-Golay smooth was obtained with a linear dynamic range (LDR) spanning 3 orders of magnitude was determined. This LOD was nearly a 5-fold improvement over the 3.3 ng/mL for on-column detection (with EB in the running buffer) based on the 310 bp fragment considering chromatographic dilution previously reported by our group [126].

Investigation of DNA-Protein Interactions

Figure 2.9a demonstrates the post-column derivatization separation of the DNA plasmid pBKH26. Important to note is the 264 bp fragment (*trp* operator), which contains the active binding site for the *trp* repressor protein. Upon addition of the protein (*trp* repressor) to the sample, a significant decrease in the peak intensity of the 264 bp fragment occurs (Figure 2.9b) due to the large binding constant between the *trp* repressor and *trp* operator. Addition of EB to the running buffer prior to sample injection improves the resolution of certain fragments (see peak(s) at about 19 minutes). However, EB will intercalate into the DNA fragments; consequently, interfering with the active binding site on the 264 bp fragment and hindering specific DNA-protein interactions. This is illustrated in Figure 2.9c. The 264 bp fragment does exhibit a slight decrease in peak height, which can be attributed to non-specific interactions between the DNA and the protein; however, the decrease is obviously not as significant as in Figure 2.9b. Figure 2.9 effectively demonstrates the need for the addition of EB post-column instead of on-column in order to preserve the integrity of the DNA-protein interaction.

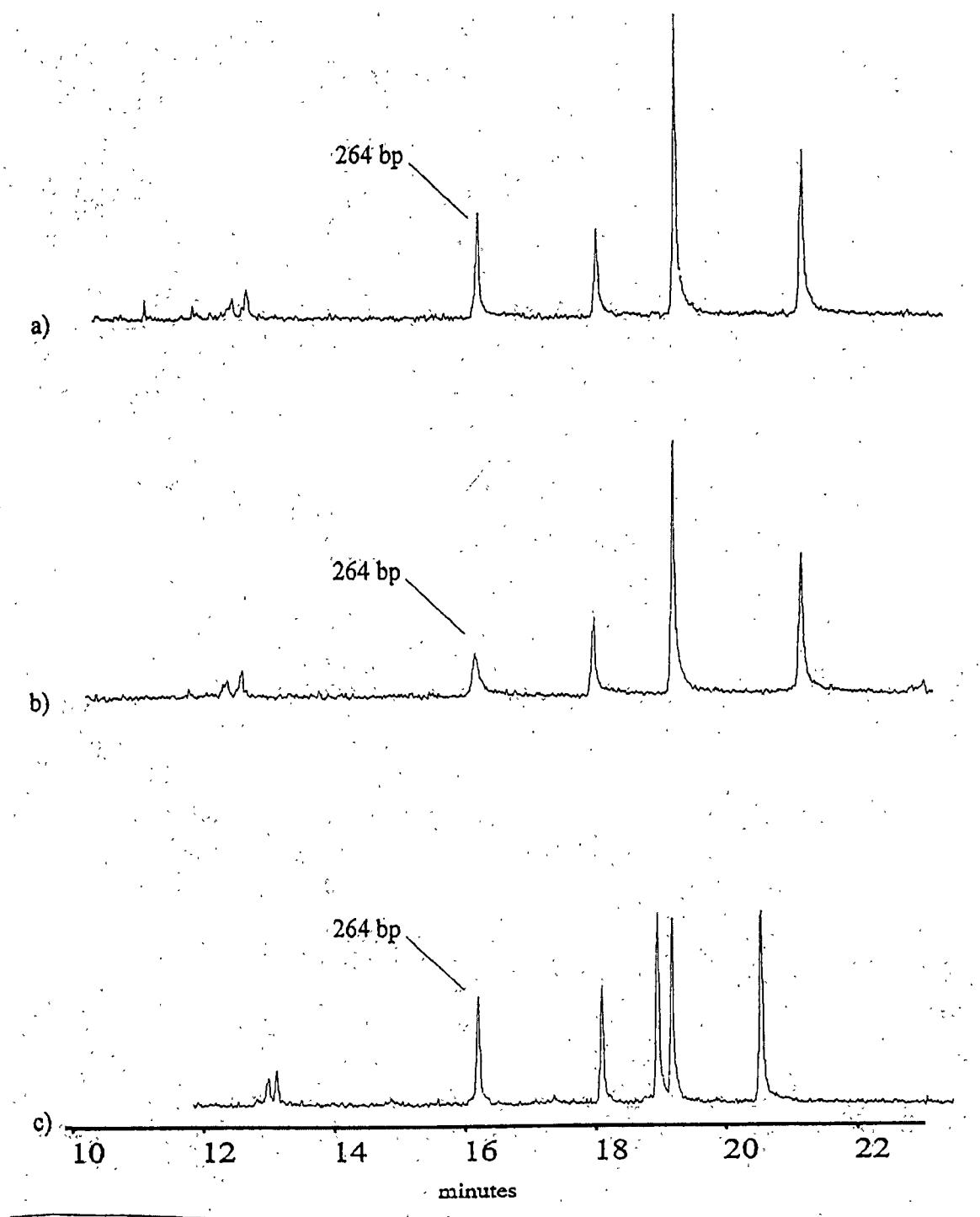


Figure 2.9. Electropherograms depicting the effect of adding EB on-column compared to post-column for the study of DNA-protein interactions. a) Free DNA. b) DNA-protein complex. c) DNA-protein complex but with addition EB on-column (in the running buffer). Running buffer conditions were 0.5% MC in 45 mM TBE with 0.5 mM L-tryptophan, pH 8 with a sheathing fluid of 2.5 μ M EB except in (c) as indicated. Position of detection zone was adjacent to the separation capillary.

CONCLUSIONS

The SFC arrangement is readily adaptable for post-column derivatization. The addition of EB pre-column or on-column affects the integrity of the DNA-protein interaction. However, the SFC facilitates the ease of mixing of EB post-column to derivatize DNA fragments while maintaining the integrity of the DNA-protein interaction. SFCs offer sensitive detection capabilities that allow the use of in lower DNA concentrations. Consequently, this will expedite the acquisition of quantitative information regarding the DNA-protein interaction including the equilibrium dissociation and the association rate constants. However, improvements in the sheath flow cell design are needed. A design, which is more rugged and easier to use, is warranted.

CHAPTER 3

A SHEATH FLOW CELL-BASED LASER INDUCED FLUORESCENCE DETECTION SYSTEM WITH PRE-IMAGING SPATIAL FILTERING FOR REDUCED DIMENSION SEPARATION TECHNIQUES

INTRODUCTION

LIF detection has been employed in high sensitivity separation applications since the late seventies [127]. This approach to detection combines the classical advantages of conventional fluorescence with the added laser-based features of high excitation beam intensity and excellent focusing characteristics. These characteristics allow ultra trace analysis of complex mixtures with the absolute detection limits at the attomole level [128] and below [129]; [119]. As a result, LIF has been extensively utilized for both LC [130-132] and CE applications [55, 113, 125, 133].

Two primary bottlenecks are responsible for the slow evolution of LIF development for practical routine purposes in everyday laboratory settings. First, practical application of this technique requires excitation in the UV spectrum to allow quantitation of a reasonable number of analytes without some form of derivatization [50, 134-136]. With derivatization drawbacks can occur such as multiple labeling, poor labeling efficiency (pre-column and on-column derivatization); band broadening, and slow reaction kinetics (on-column and post-column derivatization), which was described in more detail in Chapter 1. Second, a key challenge in the development of LIF instrumentation suitable for routine analysis is the design of a rugged procedure for the optical alignment of the detection flow cell with the focusing and collection optics.

Application of this technology to ultra sensitive analysis in a routine laboratory setting will require instrumentation which is rugged, simple, easy to use, highly precise in terms of inter-day performance, small size, and cost effective. Thus, improvements in a LIF flow cell design are desirable.

A critical component in the development of LIF detection schemes is the design of the flow cell. In terms of detectability, the key objectives in developing a flow cell are threefold: (1) Maximizing pathlength (signal levels increase with pathlength), (2) efficiently coupling the laser radiation into the flow cell (without damage to the flow cell), and (3) efficiently collecting fluorescence emission while rejecting unwanted background. In LIF detection, rejection of unwanted background can be accomplished by spatial, spectral, or temporal means. Background can consist of the following: (1) specular scatter off of the optical interfaces of the flow cell (spatial, spectral, and temporal rejection possible); (2) Rayleigh scatter from the mobile phase (spectral and temporal rejection possible); (3) Raman scatter from the mobile phase (temporal and to some degree spectral rejection possible); and (4) flow cell impurity fluorescence. Spatial and spectral rejection can be accomplished easily; however, temporal rejection can be more problematic.

Spatial rejection is usually accomplished via a far-field mode of spatial filtering (see Figure 3.1). Traditionally, an illuminated image is collected with a lens. The lens will collect the desired fluorescence from the analyte as well as the contributions from the four sources of background described above (specular scatter, Rayleigh scatter, Raman scatter, and fluorescence). However, spatial rejection is most effective in minimizing the

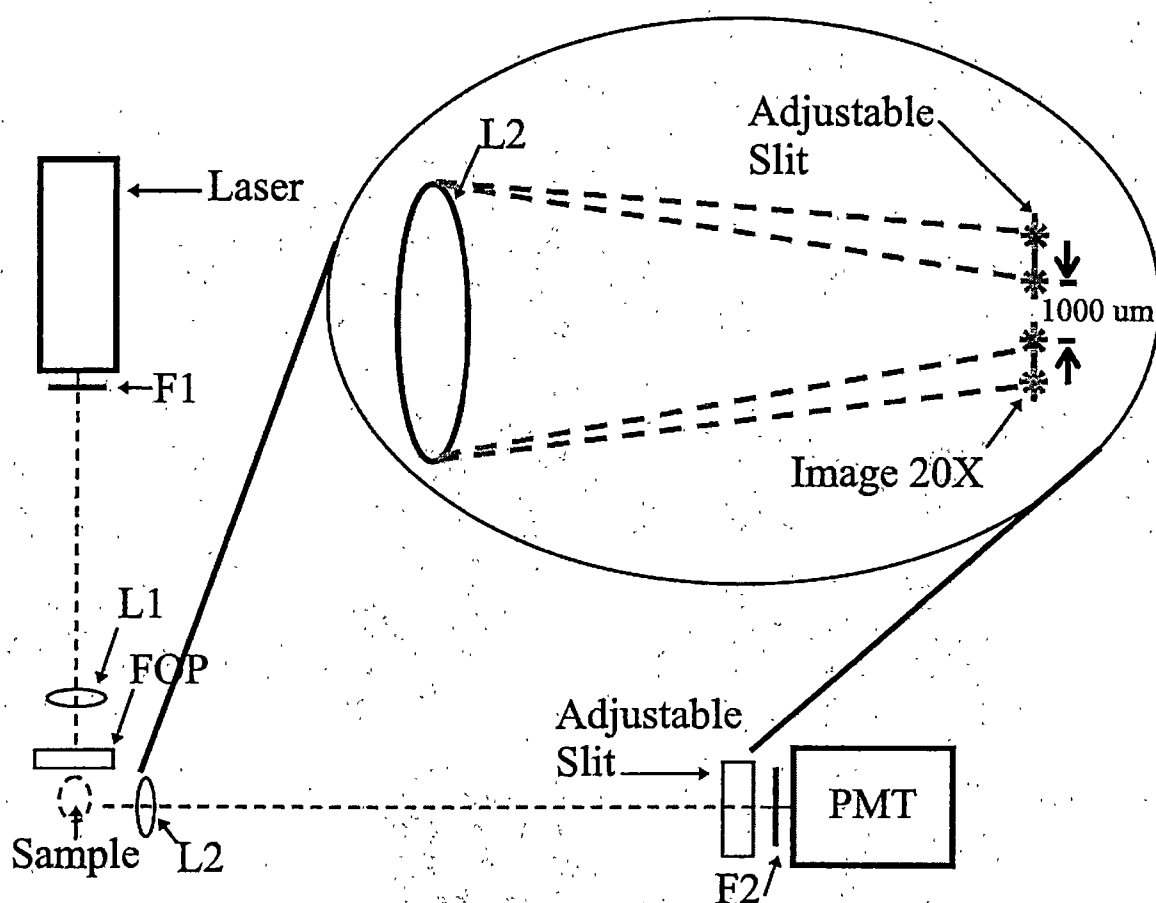


Figure 3.1. A depiction of far-field spatial imaging with a traditional LIF setup. Components are as follows: F1 a laser line filter, F2 bandpass filter, L1 focusing lens, L2 collection lens, FOP fiber optic positioner, and PMT photomultiplier tube. The corresponding illuminated image is 20 X (corresponding to a magnification of 20).

contribution from specular scatter off of the capillary of flow cell walls. All of this will be collected by the lens and magnified to some desired degree in the far field of the optical system. The magnified image is then spatially isolated using a slit or other device such as a pinhole or iris. In this case for comparison purposes, an adjustable slit will be described.

Taking a 50 μm i.d. x 365 μm o.d. capillary and a one inch lens with a f number of 1 as an example, image sizes (S_i) and magnification (M) can be determined from the following equations:

$$\frac{1}{f} = \frac{1}{S_i} + \frac{1}{S_o} \quad (3.1)$$

where f is the focal length of the lens and S_o is object distance (e.g. the CE capillary in the presented example). M can be determined as follows:

$$M = \frac{S_i}{S_o} \quad (3.2)$$

Applying Equation 3.1 to the example, if the capillary is placed a focal length away (1 inch), S_o would equal one then S_i would be infinite meaning the image is collimated over a infinite distance. However, if the lens is moved to a distance of two focal lengths from the capillary, S_o would equal $2f$ (2 inches), then S_i would also equal $2f$. Using Equation 3.2, the corresponding M would be equal to one. Consequently, if an M of 20X is desired with an $f/1$ lens, S_o set to one inch (f), then S_i would equal 20 inches. Thus, the adjustable slit and detector apparatus for spatial filtering would be placed at the distance corresponding to S_i . Taking the 50 μm x 365 μm capillary case with a desired magnification of 20X, then the size of the image of the i.d. of the capillary will be 1000

μm and $7300\ \mu\text{m}$ for the o.d. of the capillary. Therefore, for effective minimization of specular scatter a desired slit setting of less than $1000\ \mu\text{m}$ is needed.

Care must be done with adjustment of the slit to the appropriate size to help from decreasing signal from the fluorescence of the analyte. Optimization of the desired slit size is normally done to maximize the signal to background ratio (S/BKG) of the optical system. Once this is accomplished, the far field mode of spatial imaging is complete and the optical system is "aligned". Spatial imaging can also be done as in the above but with slits with pre-determined widths. However, these slits suffer from a degree of freedom offered by the adjustable slit. An iris or aperture can be used which is adjustable allowing for convenience and increased optimization. In addition, microscope objectives are sometimes used to collect the desired fluorescence and the image is focused through a pinhole corresponding to the size of the fluorescent image. The pinhole serves as a means of spatial filtering as specular scatter will be blocked by the pinhole. Microscope objectives are faster optics and can effectively collect a smaller isolated image than traditional lenses used. Traditionally, these forms of far-field spatial imaging are employed with CE-LIF; however, they can be time consuming procedures.

Spectral rejection quite simply employs the use of filters (or other wavelength selectors) to effectively isolate the desired radiation. Narrow band pass, wide band pass, cut-on, and holographic notch can be used. To help reduce specular scatter from and Rayleigh scatter from the mobile phase, a holographic notch filter can be used. Notch filters are narrow band pass filters usually 10 or 20 nm at full-width-half-maximum (FWHM) that can effectively reduce the Rayleigh and specular scatter to very low levels. These types of filters are limited to lasers in the visible portion of the electromagnetic

spectrum; hence, UV lasers are particularly problematic in minimizing Rayleigh scatter. The other types of filters mentioned are commonly used to help reduce to some degree the effects of specular scatter, Raman scatter, and unwanted fluorescence. Obviously, the narrower a band pass filter is at FWHM, the better the minimization of the above effects. However, often this is not the case due to a mixture of analytes with a wide range of emission wavelengths. Consequently, a narrow band pass filter is not effective in this case because it will decrease the amount of signal collected for the mixture of analytes. A more appropriate choice in this scenario is a wide band pass filter (usually over a range of 50-100 nm) or a cut-on filter. A cut-on filter will block all radiation up to a desired wavelength and then transmit all radiation above a desired wavelength (i.e. it will "cut-on" at a desired wavelength). Even though filters are effective in spectral rejection, they can weakly fluoresce themselves contributing to background levels.

Temporal rejection is commonly employed when using pulsed lasers, instead of continuous wave lasers, as the excitation source. Pulsed laser systems suffer from high peak powers that can degrade analytes of interest and the flow cell, pulse to pulse power reproducibility is poor, and signal recovery is complicated. Therefore, pulsed lasers temporal rejection are seldom used with CE.

The fluorescence of the flow cell is particularly problematic in capillary scale separations using fused silica capillaries (such as CE) when UV excitation is employed. Some flow cells can be employed for post-column detection with CE. Properly designed flow cells can permit spatial rejection of much of this fluorescence from the capillary. However, it has been shown for the ultimate in background rejection, the flow cell wall must be effectively removed, such as with SFCs [137]. SFCs allow for the walls to be

removed from the collection angle (i.e. view) of the appropriate optic whether it is a lens or microscope objective. Therefore, fluorescence of the flow cell walls is less problematic than with the fused silica capillaries used in CE [137].

Many SFC designs have been employed for post-column detection schemes in reduced dimension separation techniques such as CE. Some of these SFCs have been used as reaction chambers for post-column labeling [135] while others have been used as a flow cell solely for detection purposes [129, 137]. It has been shown that SFCs are superior in sensitivity and lowering limits of detection (LOD)[62]. Furthermore, SFCs preserve the integrity of a chromatographic separation, without a significant decrease in key chromatographic parameters such as resolution and efficiency [62, 129, 135]. However, many of the current designs have several drawbacks including: somewhat cumbersome to use, difficulty in optically aligning the system, a potential requirement for a large amount of space, fragile, expensive, and generally a low degree of ruggedness [62, 129, 135].

In order to overcome these drawbacks, a new design is warranted. The construction, performance, and practical implementation of a rugged device for LIF detection in CE and LC techniques are described. This device is based on a SFC arrangement with attributes that include ruggedness, simplicity, ease of use, low cost, intra-day reproducibility, and small size. With this design, a pre-imaging mode of spatial filtering is employed to effectively reduce the contributions of both wall scatter and wall fluorescence to the background signal levels. A comparison of the pre-imaging spatial filtering and traditional methods of far-field spatial filtering is performed. This SFC system has been designed specifically for use in the UV spectral region, which can be

very advantageous since it broadens the scope of the possible applications of LIF; however, fluorescence of the flow cell walls is particularly problematic in the UV region. This flow cell design can be used with an UV laser, which presents the capabilities for the use of this system for a routine, practical analysis in the everyday laboratory. The current design will offer advantages over other designs by facilitating an easier alignment procedure, better compatibility for both CE and LC techniques, lower cost, reduction in the overall size of the detection system, and a higher degree of ruggedness. In addition, the dimensions of the SFC allow for the coupling of different scaled separation techniques without changing the SFC to accommodate differences in flow rates and peak volumes. The effect of flow cell geometry, slit widths, and detector position on fluorescent background levels are shown. The device has been applied to the fluorescence detection of three dansyl amino acids for both CE and capillary LC techniques. Efficiencies on the order of $>10^5$ plates/meter and 10^4 plates/meter are shown for CE and capillary LC techniques respectively. A comparison is made with CE between on-column and post-column detection demonstrating the latter preserves the integrity of a chromatographic separation.

EXPERIMENTAL

Chemicals and Materials

Sodium phosphate (monobasic) and sodium phosphate (dibasic) were purchased from Sigma (St. Louis, MO, USA) and were used to prepare running buffers (RB). Acetonitrile UV and water (all HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA) and used to prepare mobile phases. Alignment dyes used were sodium

fluorescein (Sigma) and kiton red purchased from Exciton (Dayton, OH, USA) and serial dilutions were made in RB or mobile phase. Dansyl amino acids L-tyrosine (Tyr), L-tryptophan (Try), and L-phenylalanine (Phe) were purchased from Sigma and diluted in RB or mobile phase. All sizes of fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). A capillary scale LC column (500 μm i.d. x 15 cm) hypersil C18 BDS, 5 μm packed column was purchased from Keystone Scientific (Bellefonte, PA, USA). All sizes of PEEK tubing used were from UpChurch Scientific (Oak Harbor, WA, USA). Square and rectangular borosilicate and fused quartz tubing used for the sheath flow cells were purchased from Wilmad Glass (Buena, NJ, USA) and cut to desired length using a diamond glass cutter.

Instrumentation

The instrumental requirements for a separation based LIF detection system is similar to that previously described in Chapter 1. The fluorometry apparatus used in this work consisted of one of three lasers a 488 nm line from an argon ion laser (Ar^+) (Uniphase Model 2201-20SL, San Jose, CA, USA), a 325 nm line from a helium-cadmium (HeCd) laser (Omnichrome Model 2074-20M, Chino, CA, USA), and a 257 nm frequency doubled Ar^+ laser (Lexel Laser, Waterford, CT, USA). A laser line filter, 488 nm and 325 nm respectively, for each laser was placed in the path of beam to remove any plasma lines from the laser emission. The beam was focused onto the sheathing capillary using a 12.5 mm diameter ($f/1$) quartz lens. Emission was collected at a 90° angle with respect to the laser beam with a 12.5 mm diameter ($f/1$) lens. The isolated image was

passed through a bandpass filter and the isolated radiation was allowed to impinge on an RP-1 type photomultiplier tube (Hamamatsu Corp., Bridgewater, NJ, USA). The PMT photocurrent was monitored by a Pacific Precision Instruments (Concord, CA, USA) Model 126 photometer. The photometer was operated with a 1.0 s output time constant. Electropherograms and chromatograms were recorded with a program written in house using LabView and a 486 DX4 100 MHz computer equipped with an uCDAS-16G data collection board (Keithley Metrabyte, Taunton, MA). A Hipotronics (Brewster, NY, USA) Model 840A high-voltage power supply was used to apply electrophoretic fields. A hydrostatic injections were made by raising the inlet end of the capillary 10 cm above the outlet end for 10 sec. Following injection, the capillary inlet was replaced into the running buffer vial and a running potential of + 15 kV was applied across the capillary. Mobile phases were delivered via an Hewlett Packard Model 1050 HPLC system. Injections were made with a Rheodyne Model 7520 500 nL fixed volume sample injector (Rheodyne, Cotati, CA, USA). Sheathing fluids were delivered with a single-syringe infusion pump (Fisher Scientific, Fair Lawn, NJ, USA). Video images of fluorescent band profiles were recorded via a SONY zoom CCD Color Video Camera (Edmund Scientific, Barrington, NJ, USA) interfaced with a Trinitron Color Video Monitor (Edmund Scientific, Barrington, NJ, USA) and recorded with a SONY VHS recorder.

Capillary and Column Preparation

Fused silica capillaries used for CE separation capillaries (50 μm i.d. x 365 μm o.d.) were cut to a total length of 40-60 cm using a capillary cutter. The outlet end of the capillary was tapered down to an approximate o.d. of 100 μm to help minimize turbulent

flow. This was achieved in house with a metal lathe, sanding stone, and a piece of fine sandpaper. The capillary was securing fastened into the lathe and allowed to rotate at a high speed. A sanding stone was placed into the lathe at a desired angle to produce the taper of the capillary. While rotating and applying gentle pressure, the capillary was slowly moved across the sanding stone until the desire taper was produced. This was done for all capillaries to help minimize turbulent flow. A detection window (for on-column comparison) was made by removing a section of the polyimide coating near the outlet end of the capillary with fuming concentrated sulfuric acid. For all CE experiments, the capillaries were treated with 1 M NaOH for 1 hr. followed by 30 minute water rinse. The capillaries were then filled with RB before use and allowed to equilibrate under the running voltage for 10 minutes. Capillaries were stored in water overnight. Transfer capillaries for LC separations were mated to the corresponding peak volumes for the desired LC column dimension to help minimize band broadening while maximizing pathlength. A 25 μm i.d. x 150 μm o.d. fused-silica capillary was nested inside and secured with epoxy to a 250 μm i.d. x 365 μm o.d. fused silica capillary, which was used as the transfer capillary between the column and the sheathing capillary. LC columns were rinsed with the desired mobile phase and allowed to equilibrate for 30 minutes at the working flow rate.

Sheath Flow Cell Fabrication Procedure

For comparison purposes, a previous design for the sheath flow cell arrangement was described in detail in Chapter 2 (See Figure 2.5, page 82). The prior design suffers from the drawbacks of lack of ruggedness, need for additional equipment to align the

flow cell (squared with respect to the laser beam), cumbersome to use, poor reproducibility, requires a far-field mode of spatial filtering, a somewhat more difficult alignment procedure, takes up more space, and is not easily adaptable for routine use in both an academic and an industrial setting.

In the design described herein, the sheathing capillary was either a piece of square borosilicate tubing (0.400 mm i.d. x 0.800 mm o.d. x 5 cm for CE scale; 2 mm i.d. x 2.8 mm o.d. x 5 cm for μ -bore LC scale) or a rectangular piece of borosilicate tubing (0.400 mm i.d. x 1.4 mm o.d. x 5 cm used for both scales) that were cut and placed into the corresponding fabricated cell holder (discussed below).

The SFC design was machined in house and it consisted of the following: cell holder, alignment block, translation plate, solvent tee, detector body, and lens holders (see Figure 3.2). The cell holder was machined in half with a center slot machined into each half to match the half of corresponding o.d. for the sheathing capillary to be used (see Figure 3.3). When placed together and held secured by two screws, the center groove keeps the sheathing capillary square and secured in the appropriate cell holder (see Figures 3.4). Furthermore, into each cell holder was both an excitation slit (200 μ m) and an emission slit (300 μ m) at 90° with respect to one another (see Figure 3.5). The emission slit was mated to f/1 imaging optics and was machined with a 45° bevel. The cell holder (with the appropriate sheathing capillary secured) was fastened into the underside of an alignment block as shown in Figure 3.6. The alignment block served a dual purpose. First, it provided a means of connecting the cell holder to the translation plate and solvent tee. Second, placed inside the alignment block was a metal disk, which

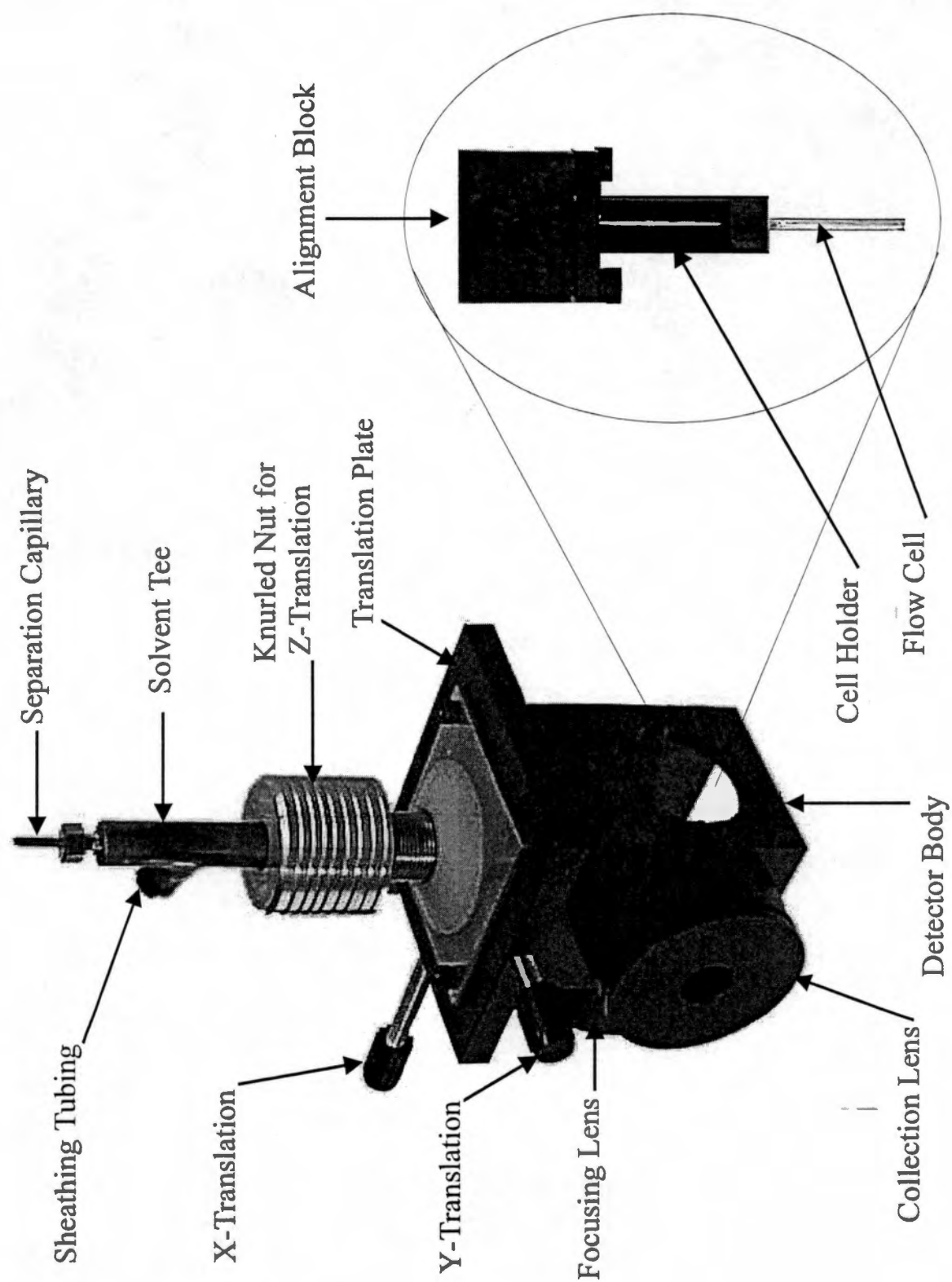


Figure 3.2. SFC Design.

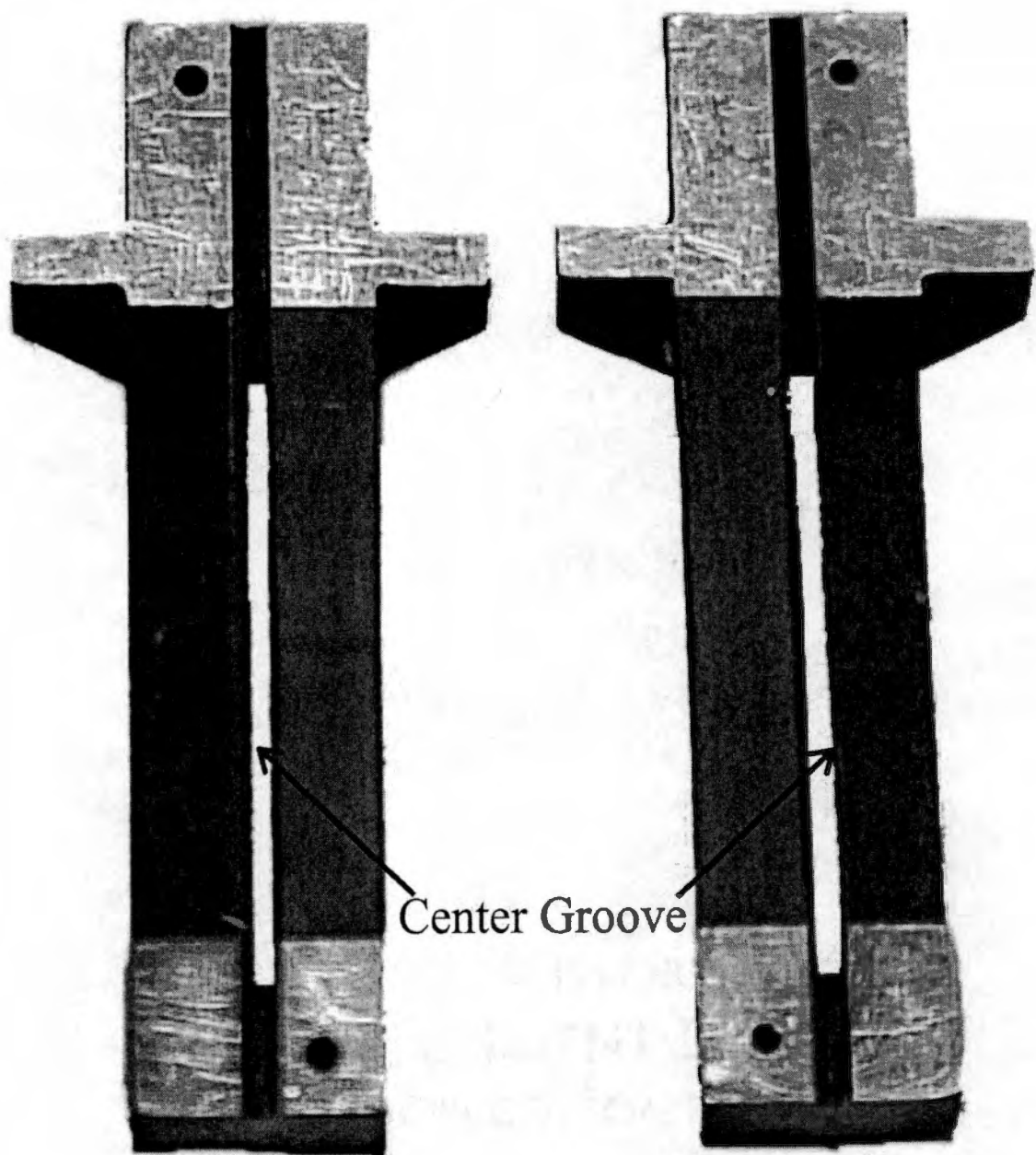


Figure 3.3. Cell holder. Picture of the two halves of cell holder with the center groove for attaching the flow cell to the cell holder.

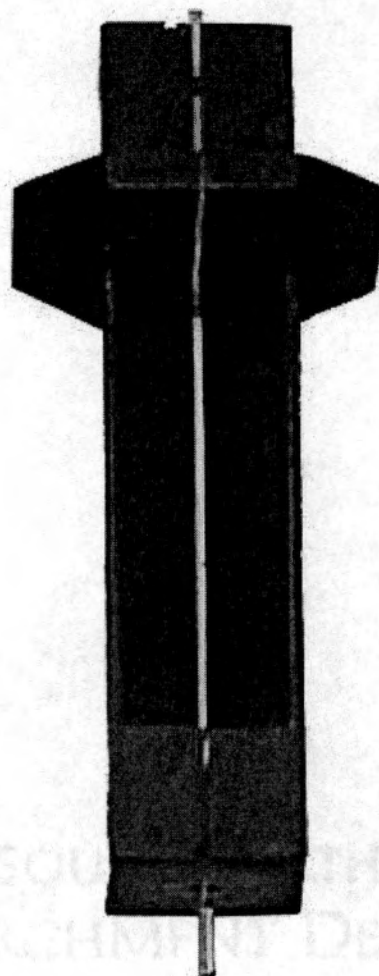


Figure 3.4. Cell holder after attachment of sheath flow cell and fastening of the two halves.

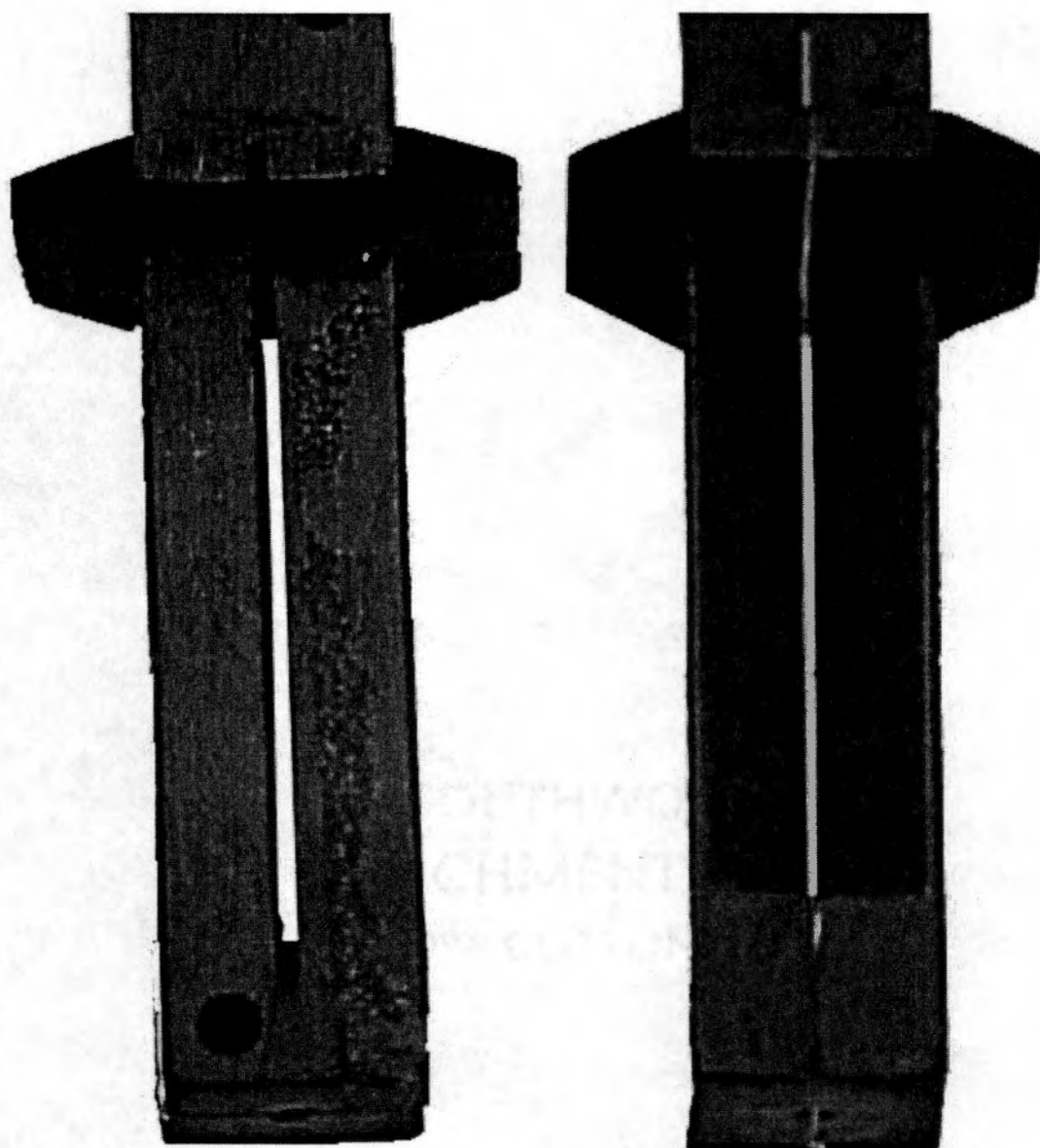


Figure 3.5. Picture of the slits mated into the cell holder. (Right) excitation slit and (left) the emission slit.

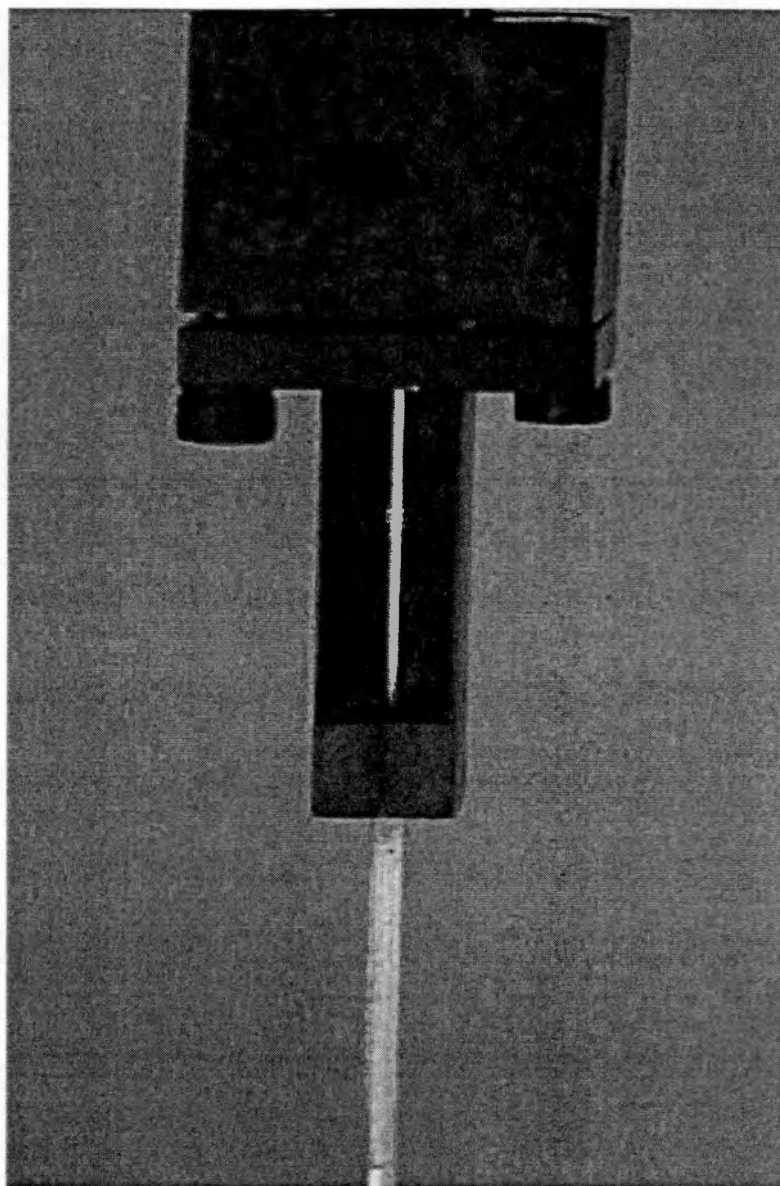


Figure 3.6. Depiction of the alignment block with the cell holder already attached to the underside of the alignment block.

provided a means for a liquid tight seal around the sheathing capillary and between the solvent tee and the sheathing capillary (see Figure 3.7). This liquid seal was accomplished by placing an o-ring around the sheathing capillary and another o-ring around the end of the solvent tee. The cell holder was fastened to the underside of the alignment with a metal plate, which slipped around the cell holder. Four screws secured the plate to alignment block and held the cell holder in place. The fastening of cell holder to alignment compressed the o-ring around the sheathing capillary forming a liquid tight seal as shown in Figure 3.8. The solvent tee was connected to the translation plate with a knurled fitting that provided translation in the z-direction (see Figure 3.9). To fabricate the entire design, the alignment block was secured to the translation plate by screwing the solvent tee into the topside of the alignment block (Figure 3.10). This translation plate allowed for translation about a x, y, and z-axis much in the same way as a commercially available fiber optic positioner. This entire fabrication was done only once. The only assembly, which may have to be done more than once, was the attachment of the cell holder to the underside of the alignment block when a new sheathing capillary was needed. The separation capillary was held in place via a piece of PEEK tubing which was secured into the solvent tee with a stainless steel Valco ferrule and fitting (see Figure 3.11). The sheathing fluid was introduced via a piece of 0.02" i.d. PEEK tubing fastened into the sidearm of the solvent tee (see Figure 3.11). Once the cell holder, solvent tee, alignment block, and translation plate were all securely fastened, a detector body fitted over the cell holder and mounted directly to the underside of the translation plate. The detector body houses both the focusing and collection lenses. Figure 3.12 shows the two homemade lens holders that secured the focusing and collection lenses. Once assembled

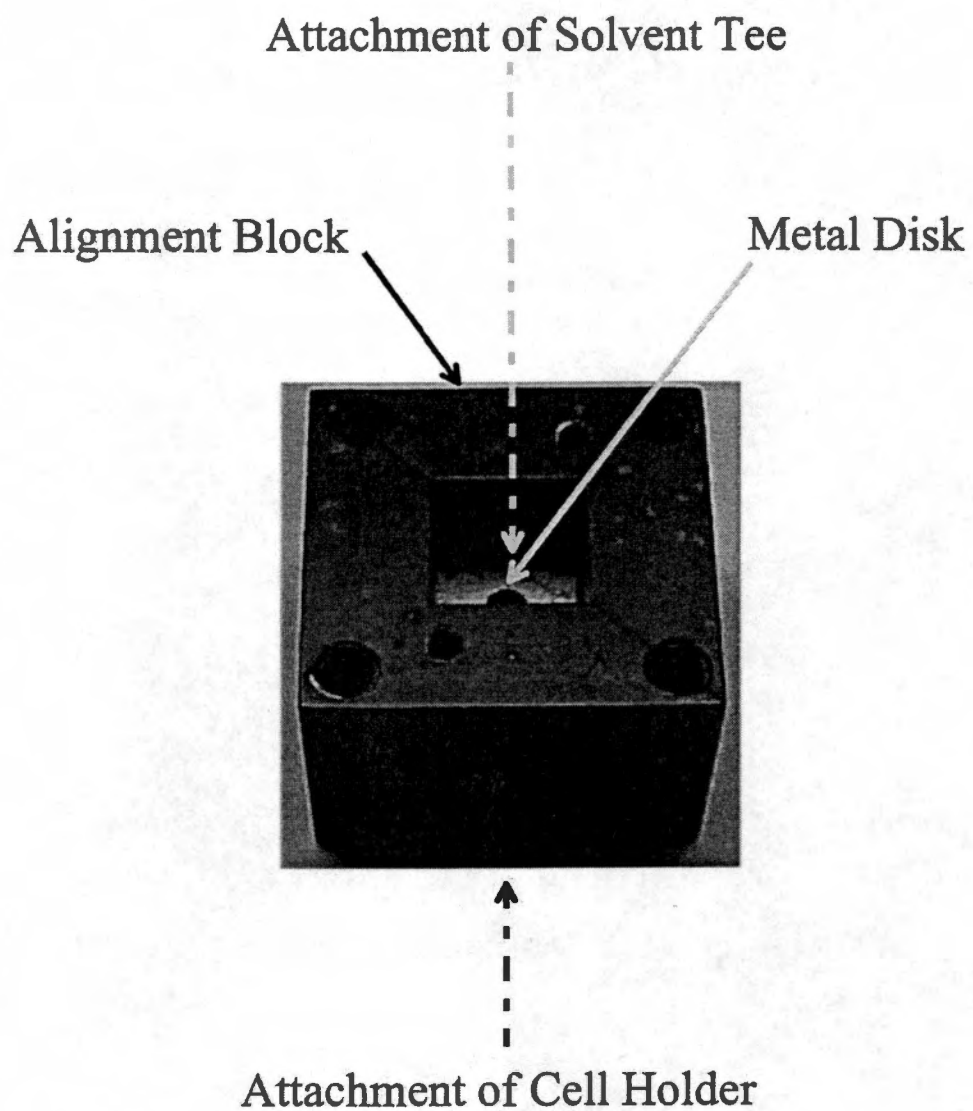


Figure 3.7. Picture of the alignment with metal disk used for liquid tight assembly of solvent tee and cell holder.

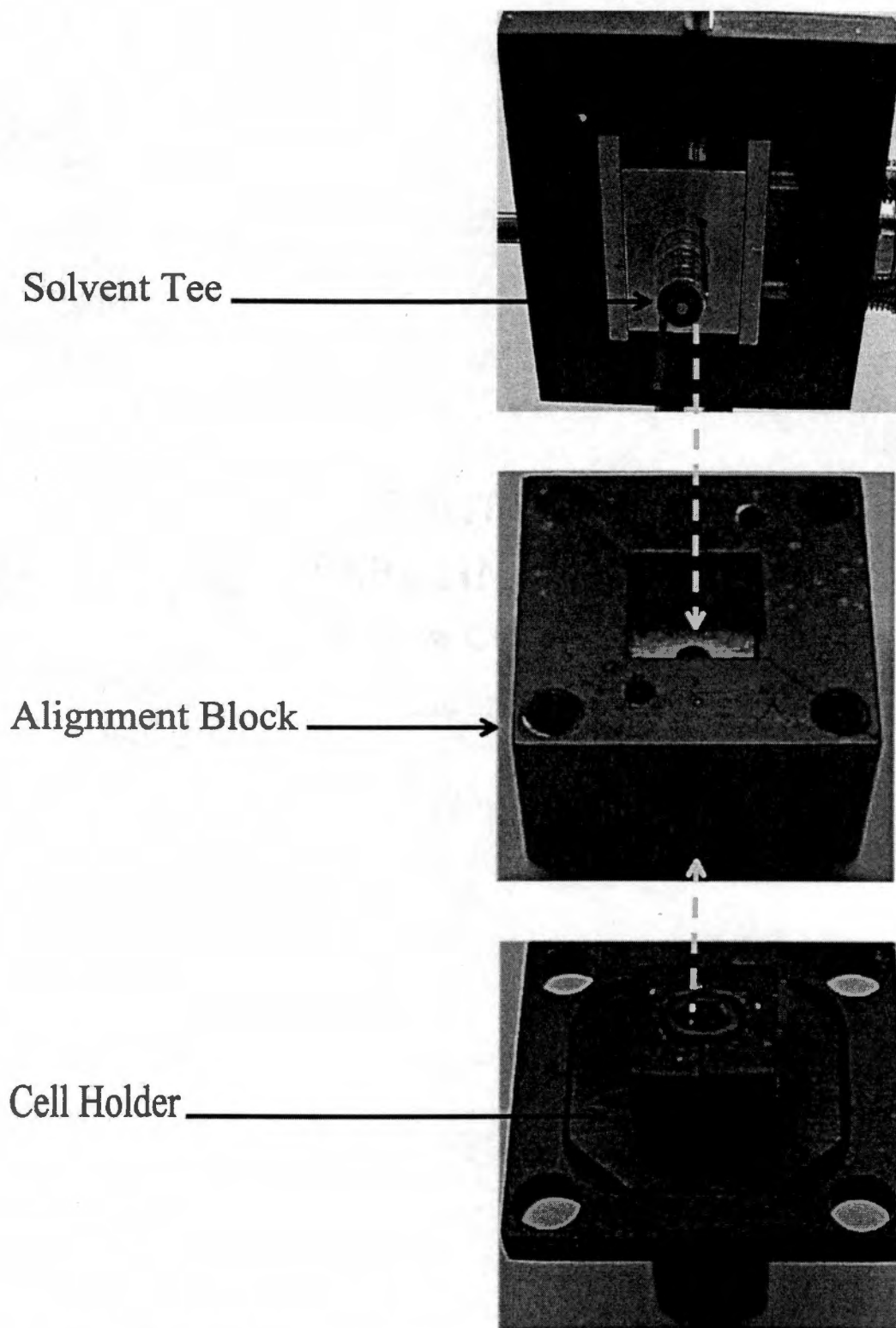


Figure 3.8. Depiction of the liquid tight seal formed by the alignment between both the solvent tee and the cell holder.

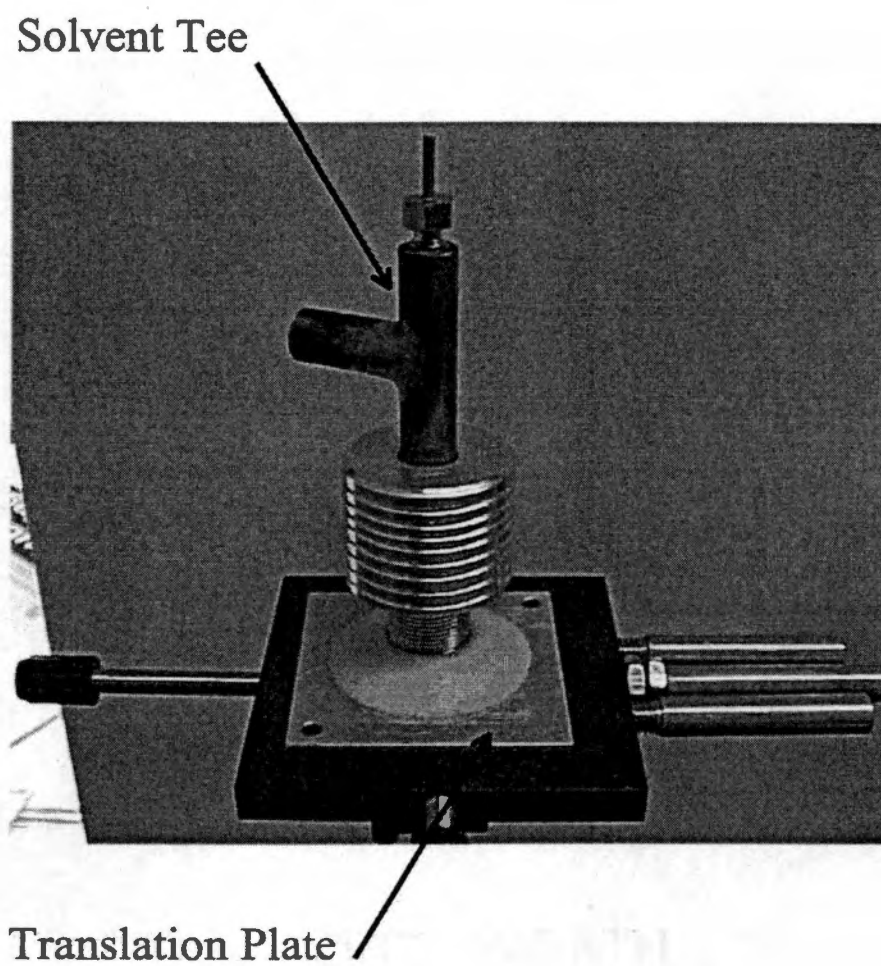


Figure 3.9. Solvent tee and translation plate.

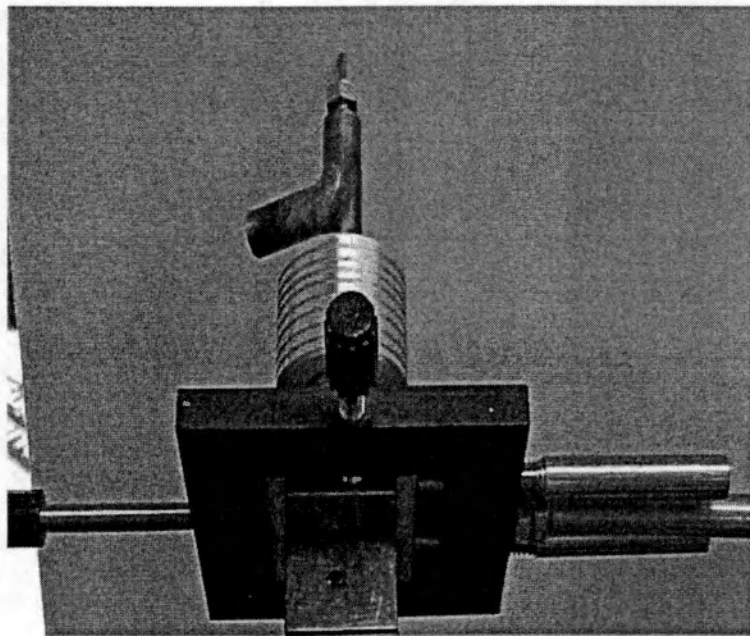
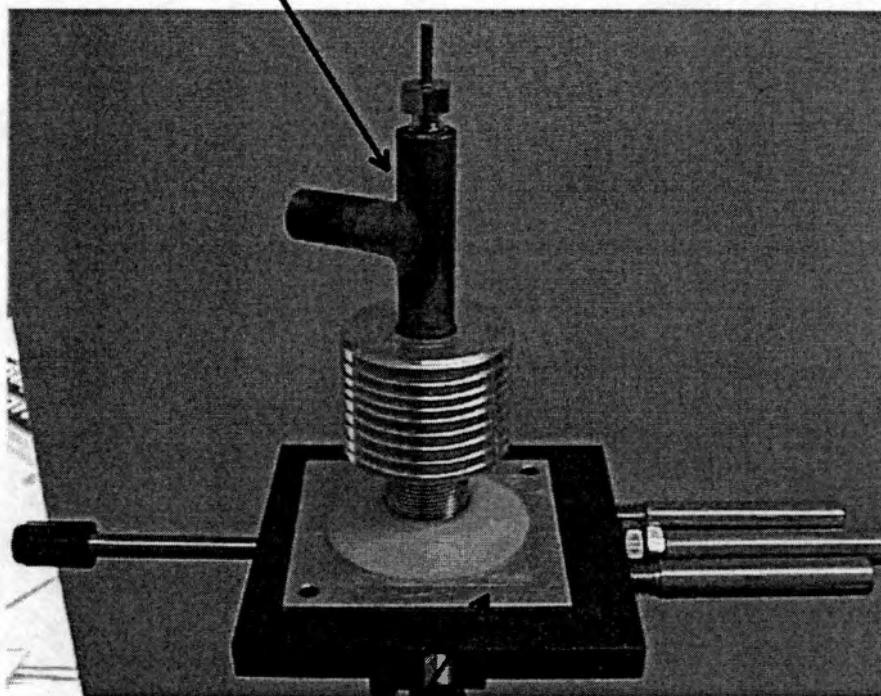


Figure 3.10. The alignment block, solvent tee, and translation plate after assembly of solvent to alignment block by way of the translation plate.

Solvent Tee



Translation Plate

Figure 3.11. Picture of the top portion of the solvent tee. The separation capillary connected via the top and the sheathing fluid delivered via PEEK tubing connected from the sidearm.

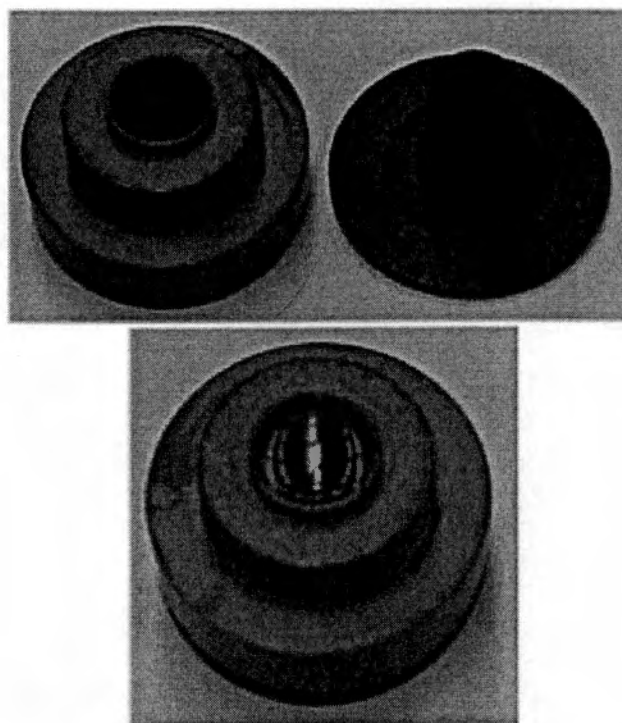


Figure 3.12. Picture of lens holder. (top) Disassembled and (bottom) assembled with lens in place.

the two lens holders screwed into the detector body at 90° to each other as shown in Figure 3.13. In addition, the detector body allows for adjustment of the focusing and collection lenses by simply screwing the lens holders in either direction (see Figure 3.13). For detailed construction plans, see Appendix. In the back of the detection body was a 1/16" pinhole, which was used to properly align the laser beam with respect to the rest of the system (see discussion below). The additional equipment needed for CE-laser and HPLC-laser fluorometry setup was as described (see Instrumentation section).

Alignment Procedure

To better demonstrate the simplicity of the overall ease of alignment with the proposed design, a comparison to the previously used design is warranted. The optical alignment procedure for the old SFC design consisted of the following steps. First, the laser beam was centered through a 25.4 mm f/1 lens and a spot was marked to indicate the path of the laser beam. Second, the laser beam was focused onto the sheathing capillary using this f/1 lens and the beam was determined to be going through the center of the sheathing capillary when the image was on the same spot marked previously. To "square" the sheathing capillary so that the front face was at no appreciable angle with respect to the laser beam, a video microscope was used. This procedure was important to help minimize wall scatter due to the laser beam hitting the sheathing capillary at any angle and causing the fluorescent image to be skewed in the sheathing capillary with respect to the collection lens. It was very difficult to know if the sheathing capillary was perfectly square even with the use of the video microscope because of the crudeness of the design. A collection lens (25.4 mm, f/1) was placed at 90° with respect to the laser

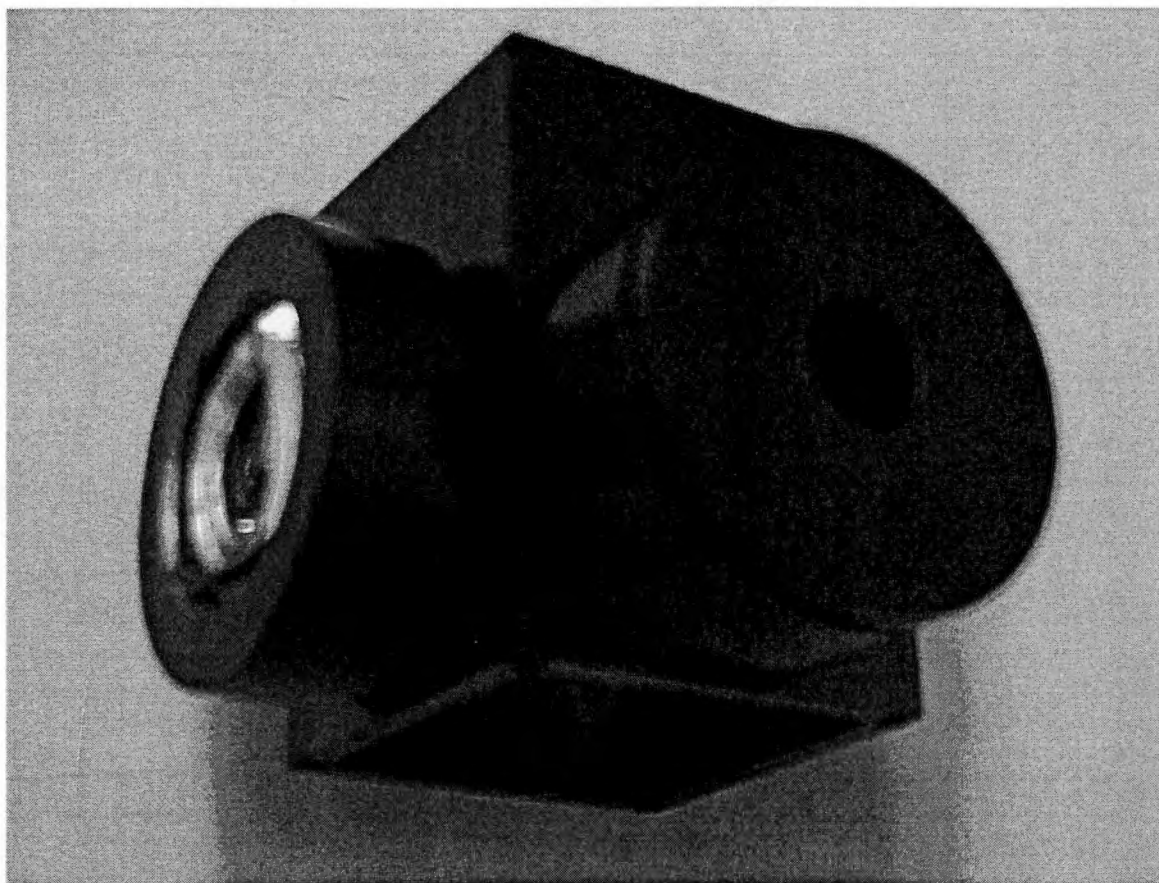


Figure 3.13. Detector body after the insertion of the two lens holders.

beam and the corresponding wall scatter and fluorescence was collected and magnified in the far field to some degree (15X in most instances). This magnified image was spatially isolated in the far field using an external adjustable slit as described previously. The fluorescent image was passed through a bandpass filter and then allowed to impinge on a PMT.

To optimize the alignment of the system, a laser dye was continuously injected through the separation capillary into the sheathing capillary. The sheathing flow was matched to the separation flow with the aid of a video microscope and was optimized when the fluorescent image eluting from the separation capillary was the same diameter as the flow channel of the separation capillary (i.e. 75 μm for a 75 μm i.d. capillary). This was accomplished by varying the flow rate of the sheathing flow with the syringe pump. Final optimization of alignment included translating the PMT horizontally and vertically across the fluorescent image, focusing the collection lens, and adjusting the external slit while obtaining the best signal/background (S/BKG) possible.

In contrast, using the pre-imaging spatial filtering technique proposed here, the alignment procedure was much simpler, quicker and reproducible. First, the laser beam is centered in much the same way as described above with the marking of the spot the laser hits before and after it passes through the focusing lens. To center the laser beam through the sheathing capillary, the sheathing capillary is simply translated in the x-direction until the laser beam exits the pinhole in the back of the detector body and is at the same position as previously marked. The filtering of wall scatter and fluorescence is accomplished prior to the collection of the fluorescent image by the collection lens. This is accomplished due to the coupling of the sheathing capillary geometry with the

appropriate slit width in this cell holder (discussed in more detail later). Consequently, the fluorescence is collected by the lens, passed through a bandpass filter, and imaged onto the PMT positioned within a focal length away of the collection lens. There is no need for any far field imaging or additional spatial filtering with this design unlike that of the previous design. The final optimization of the system is accomplished as above with the exception that varying the external slit is not needed. This procedure is less time consuming and difficult from day-to-day operation. This design exhibits a more rugged, reproducible alignment procedure that can be used daily in a practical laboratory setting.

RESULTS AND DISCUSSION

Characterization of Sheath Flow Cell

In an attempt to characterize the new SFC design, studies on the effects of flow rate and detector position on the overall signal intensity of a laser dye were performed. In this case, the effect of the separation flow velocity (effluent) on the signal intensity ($I_{\max}$) versus position of the detection zone was studied. In this experiment, the sheathing flow velocity was held constant and changing the applied voltage varied the separation flow velocity. As laser dye was continuously exiting the capillary due to electrophoretic migration, it was entrained into the sheathing fluid and travels down the sheathing capillary. As seen in Figure 3.14, the faster the separation flow velocity (in this case the higher the applied voltage) the higher $I_{\max}$ will be at every position down the sheathing capillary. For instance, $I_{\max}$ was approximately 2800, 1900, and 1200 nA for 15, 10, and 5 kV respectively at a position adjacent to the separation capillary. Translating down the SFC away from the separation capillary decreases the signal. In the 15 kV case, the

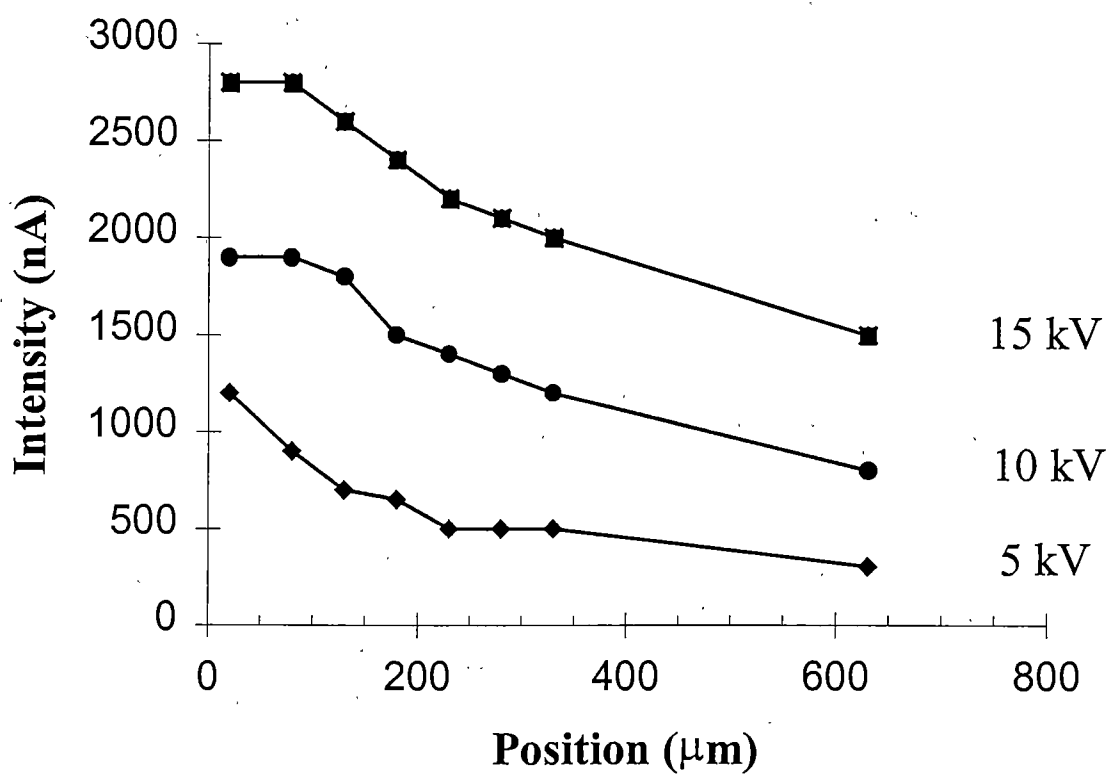


Figure 3.14. Effect of flow rate and detector position on signal. Sheathing flow was held constant while adjusting the applied voltage varied the separation flow. A solution of 1×10^{-7} sodium fluorescein was continually eluting from the separation capillary. Flow rates were matched in the 10 kV case.

signal decreases from 2800 nA at a position of 100 μm to 2300 nA at a position of 250 μm . Consequently, the signal gradually levels at a position of 650 μm to a signal of 2000 nA. This general trend is also observed in the 5 and 10 kV cases. Expansion may decrease the effective dye concentration (due to diffusion) in the focused laser beam, but it will increase the effective pathlength. Since the expansion (15 kV) case has the greatest signal, this pathlength effect dominates in influencing I_{max} .

To determine if an improvement in detectability was feasible with the proposed design compared to the previous design, a set of experiments was performed. A series of concentrations of sodium fluorescein were injected. Important analytical information pertinent to a LIF setup such as signal, BKG, noise, and S/N were recorded for both designs and summarized in Table 3.1. Noise levels for both systems remained constant at approximately 0.20 nA. Even though a slight increase in background (1.7 nA) was noted in the new design (probably due to a slight misalignment of the flow cell), a 4-fold increase in signal/noise (S/N) was observed. This improvement in S/N gave a good indication that a pre-imaging mode of spatial filtering was achieved by the SFC holder and that an overall improvement in detectability was possible.

A LOD for sodium fluorescein was determined for both the CE and μ -bore LC systems. For each separation technique a different sheathing capillary was used to accommodate the differences in peak volumes. Calibration plots of concentration of sodium fluorescein injected versus S/N were made for both CE and LC (not shown). For the CE technique, a LOD in the low picomolar range (7.5×10^{-12} M) was observed (using $\text{LOD} = \text{S/N of } 2$). This corresponds to approximately 0.42 attomoles injected. A LOD of

Table 3.1: Fluorescence Data Comparing Previous Design to Proposed Design

Old Design	1×10^{-10} (M)	1×10^{-11} (M)	5×10^{-12} (M)
BKG (nA)	4.5	4.5	4.5
Signal (nA)	1.2	.65	.30
Noise (nA)	.15	.15	.15
S/N	8.0	4.3	2.0
New Design			
BKG (nA)	6.2	6.2	6.2
Signal (nA)	5.7	1.9	0.5
Noise (nA)	0.2	0.2	0.2
S/N	28	8.5	2.5

2.2×10^{-11} M was observed for μ -bore LC (using LOD= S/N of 2 and correcting for chromatographic dilution).

However, one drawback with this above approach was the need to modify the SFC to accommodate both CE and LC separation techniques. In the above, a separate sheathing capillary was used for each technique. This can be problematic and time consuming to have to change the sheathing capillary to toggle between CE and LC techniques. Hence, a better sheathing capillary geometry is needed which would accommodate both CE and reduced dimension LC techniques without having to use two different sheathing capillaries. In order to overcome this, a rectangular sheathing capillary was implemented. This unique geometry offers two advantages over those traditional flow cells used. First, coupled with the appropriate slit width matched to $f/1$ imaging optics, this arrangement will more efficiently block laser wall scatter and wall fluorescence using a pre-imaging mode of spatial filtering. Figure 3.15 depicts the emission image (fluorescent image and wall scatter) seen with the square and rectangular flow cell arrangements coupled to $f/1$ imaging optics. The top figure shows a depiction of a square sheathing capillary used with a LC mode of operation. In this mode of operation, signal is collected with a $f/1$ collection lens with 2.8% efficiency (solid angle defined by - - -) in Figure 3.15. A slit is made for the sheathing capillary, which matches the corresponding collection optics. With this approach specular wall scatter is still collected at 1.2 % efficiency (solid angle defined by · · ·) in Figure 3.15. Conversely, with the rectangular sheathing capillary geometry mated to the same $f/1$ collection optics, the collection efficiency is 6.2 % and specular wall scatter is collected at 0.0 % efficiency. Based on the above observation, the rectangular sheathing capillary coupled

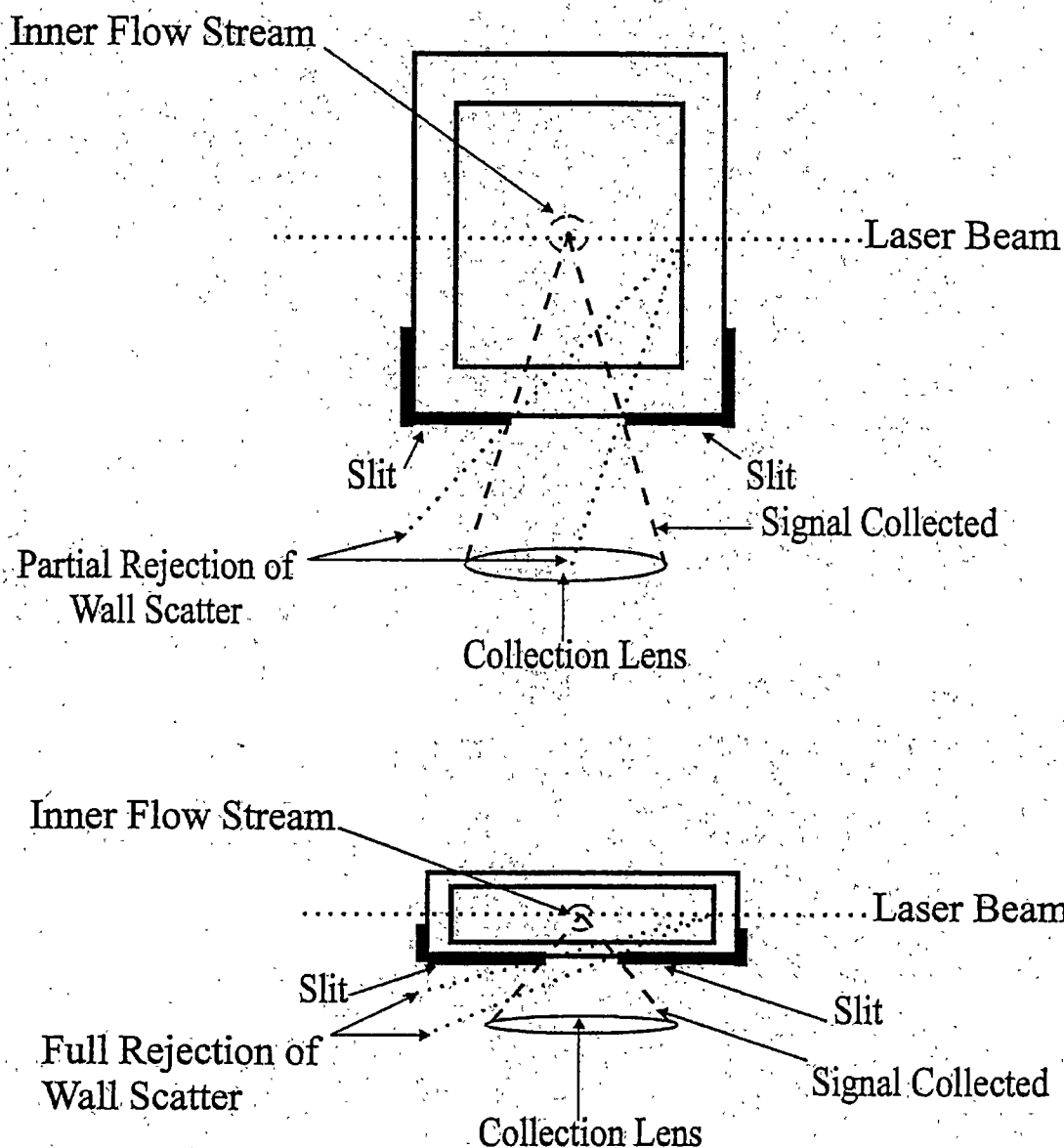


Figure 3.15. Comparison of square and rectangle sheathing capillary geometry for pre-imaging spatial filtering. (Top) Depiction of square sheathing capillary used with μ -LC. Signal collection is based on $f/1.5$ collection lens with a 2.8% efficiency (dark dash line). Specular wall scatter is collected at only 1.2% (light dash line). (Bottom) Depiction of rectangular sheathing capillary which can be used with both CE and LC. The signal is collected at $f/1.0$ at 6.2% efficiency (dark dash line). Specular wall scatter is collected at 0.0% efficiency. The rectangle sheathing capillary demonstrates the superiority in near-field rejection of wall scatter without compromising a loss in signal collection.

with the appropriate slit was superior in spatial rejection of wall scatter compared to the commonly used square sheathing capillaries. Second, the dimensions of the sheathing capillary allow for both CE and LC scales to be used without having to change sheathing capillaries to accommodate the different flow rates and peak volumes used with the different separation techniques. It is feasible to for both CE and LC to be done with the square sheathing capillary; however, keeping the separation capillary centered and secured in CE is problematic.

Evaluation of Rectangular Flow Cell Design

To evaluate the feasibility of the rectangular sheathing capillary geometry, a series of experiments was done to demonstrate the capabilities of pre-imaging spatial filtering compared to a far-field mode of spatial filtering. For these experiments three different lasers were used, a visible Ar^+ at 488 nm, a near UV HeCd at 325 nm, and a UV frequency doubled Ar^+ at 257 nm. With each of these lasers the effects of collection lens position, detector position, and slit widths on fluorescent background contributed by both wall scatter and wall fluorescence. To determine the effectiveness of the cell holder with the machined slit mated for pre-imaging spatial filtering, a comparison was made to an identical cell holder with no pre-imaging spatial filtering slit. Essentially, this cell holder only served as a means of securing the sheathing capillary into place. The emission side had an opening, which was bigger than the sheathing capillary itself, which would allow all scatter to pass through and be collected by the collection lens (see Figure 3.16).

A visible Ar^+ laser was chosen as a source because it could be used with an available holographic notch filter that could determine the spatial filtering ability of the

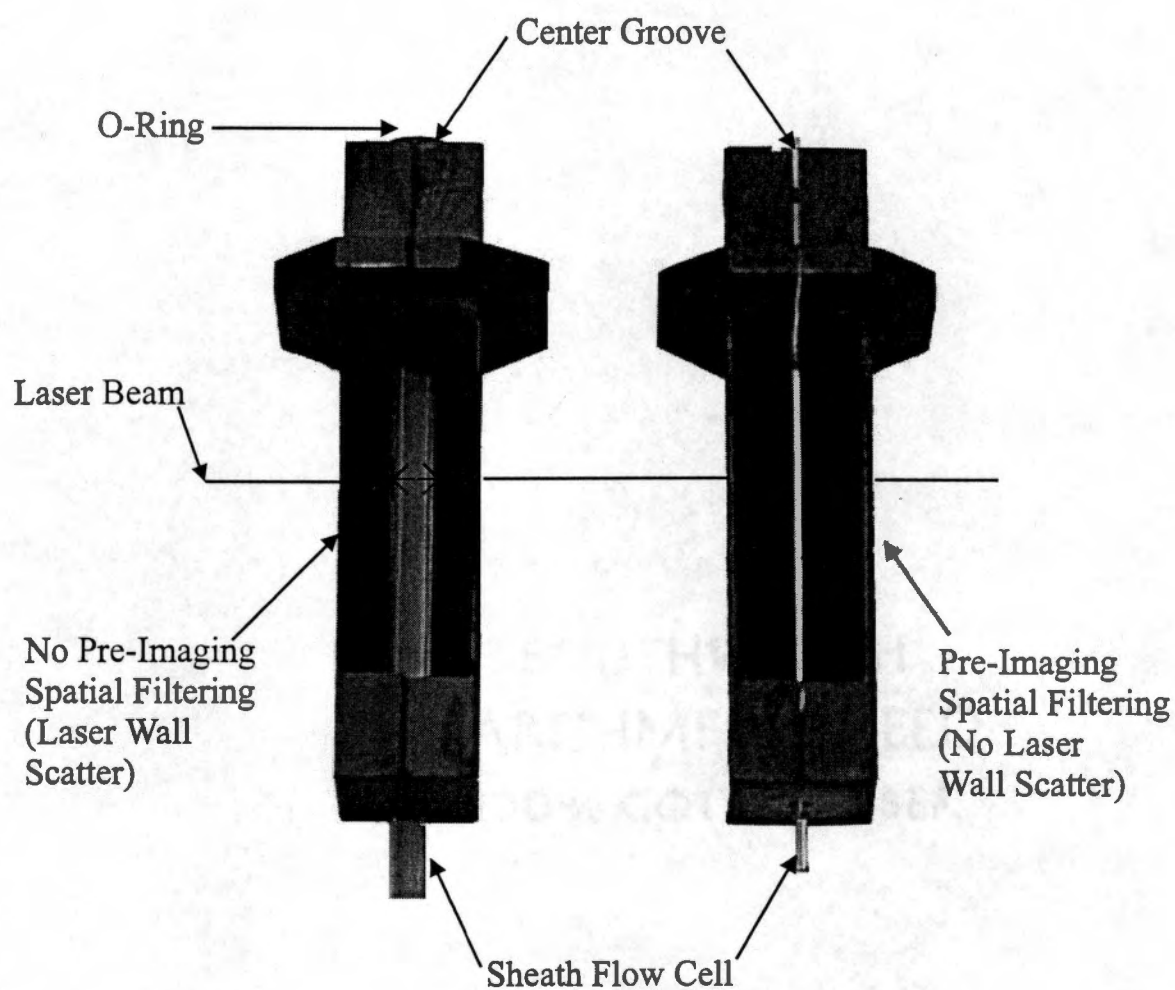


Figure 3.16. Comparison of two cell holders. (Right) Cell holder with pre-imaging spatial filtering and (Left) cell holder without pre-imaging spatial filtering. Notice the laser wall scatter depicted in the left figure while this is absent in the right figure. Demonstration of the pre-imaging spatial filtering employed is seen on the right.

pre-imaging technique by comparing the scatter blocked with the pre-imaging technique to that blocked by the holographic notch filter. By placing the notch filter in the path of the detector, all laser scatter is effectively blocked; henceforth, a low signal (background) is achieved. Furthermore, there should not be much wall fluorescence because of the higher wavelength used (488 nm in this case) so the biggest contribution to BKG in this case would be specular wall scatter.

Tables 3.2 and 3.3 compare the cell holder with the pre-imaging spatial filtering to the cell holder without the pre-imaging capabilities. Table 3.2 summarizes positioning arrangement 1 where the collection lens is placed at a distance of approximately 1.07 focal lengths from the sheathing capillary and the PMT is approximately 1 focal length beyond the collection lens. The table contains data for different adjustable, external slit (placed directly in front of PMT) widths. With positioning arrangement 1, the scatter and fluorescence is convergent upon the detector, but the detector is not placed at a distance that corresponds to the image being focused. The cell holder with the slit efficiently blocks laser scatter better off of the rectangular sheathing capillary better than the cell holder without the slit at all external slit widths as is seen in Table 3.2. Placing the notch filter in the path of the detector reinforces this conclusion because the signal is reduced to 1.8 nA in the cell holder without a slit.

Table 3.3 shows data from positioning arrangement 2 where in the detector is placed at a distance from the lens, which corresponds to 15X magnification of the focused scatter and fluorescent image. With this arrangement most of the scatter off of the sheathing capillary is blocked; however there is a small amount of scatter off the slit interface. The scatter is much wider than the external, adjustable slit used and is outside

Table 3.2: Positioning Arrangement 1, Ar⁺ Laser at 488 nm

	Cell Holder-No Slit	Cell Holder-Slit
External Slit (μm)	Signal (nA)	Signal (nA)
250	120	1.0
500	280	1.4
1000	800	1.8
2000	1800	2.9
3000	2600	3.8
4000	3600	4.3
5000	4100	5.0
6000	4800	5.0
6500	5000	5.0

Table 3.3: Positioning Arrangement 2, Ar⁺ Laser at 488 nm

	Cell Holder-No Slit	Cell Holder-Slit
External Slit (μm)	Signal (nA)	Signal (nA)
250	2.0	1.2
500	4.0	1.2
1000	4.0	1.2
2000	8.2	1.2
3000	13	1.2
4000	17	1.2
5000	23	1.2
6000	27	1.2
6500	27	1.2

the detection range of the PMT. Consequently, there is a very low signal (1.2 nA) for this detector position and the signal is constant for every width of the adjustable slit. Furthermore, by using a far field mode of spatial filtering with the cell holder with no slit (as is traditionally done), the majority of the background is blocked which gives small signal values.

For a further evaluation of this pre-imaging spatial filtering technique, a HeCd laser was used as a laser source closer to the UV spectral region where fluorescence of the flow cell walls introduces another significant source of background. In order to determine the contribution of wall fluorescence over wall scatter to the overall signal, a 350 nm cut-on filter and 600 nm cut-on filter were used. If there were a contribution of wall fluorescence, the signal would be greater for the 350 nm filter than the 600 nm filter because the 600 nm would more effectively block the wall fluorescence. Table 3.4 summarizes this effect at positioning arrangement 1 with both cell holders used. With the pre-imaging spatial filtering, most of the scatter and fluorescence was blocked using the 350 nm filter (21 nA) at the largest external slit opening; however, by comparison with the 600 nm filter the signal is reduced to 5.0 nA at the same external slit width. This reduction in signal shows that there must be a contribution of wall fluorescence that is not efficiently blocked with the 350 nm filter, but is with the 600 nm. In addition, the cell holder without the slit shows this effect as well, but with a more dramatic decrease in signal from 21,000 nA to 2800 nA at the widest external slit width (6500 μm).

Table 3.5 depicts positioning arrangement 2 with a traditional far-field mode of spatial filtering. Demonstration of a far-field mode of spatial filtering was shown with the cell holder without pre-imaging capabilities by the reduction of the background

Table 3.4: Positioning Arrangement 1, HeCd Laser at 325 nm

	Cell Holder- No Slit	Cell Holder- No Slit	Cell Holder- Slit	Cell Holder- Slit
External Slit (μm)	Signal (nA) w/ 350 nm filter	Signal (nA) w/ 600 nm filter	Signal (nA) w/ 350 nm filter	Signal (nA) w/ 600 nm filter
250	450	70	1.2	1.0
500	1200	200	1.8	1.2
1000	3800	550	3.4	1.8
2000	7800	1200	6.8	2.8
3000	12000	1700	9.2	3.4
4000	16000	2100	13.0	4.0
5000	19000	2600	17.0	4.4
6000	20000	2800	20.0	4.8
6500	21000	2800	21.0	5.0

Table 3.5: Positioning Arrangement 2, HeCd Laser at 325 nm

	Cell Holder- No Slit	Cell Holder- No Slit	Cell Holder- Slit	Cell Holder- Slit
External Slit (μm)	Signal (nA) w/ 350 nm filter	Signal (nA) w/ 600 nm filter	Signal (nA) w/ 350 nm filter	Signal (nA) w/ 600 nm filter
250	1.2	0.8	1.0	0.8
500	2.0	1.0	1.0	1.0
1000	4.2	1.2	1.8	1.0
2000	9.0	2.0	2.6	1.2
3000	13.0	2.4	3.2	1.8
4000	18.0	3.2	3.8	2.0
5000	21.0	4.4	4.2	2.0
6000	25.0	5.0	5.0	2.0
6500	25.0	5.0	5.0	2.0

(lower signal) at all external slit widths compared to the signal values observed in Table 3.4. The further decrease of signal with the cell holder with pre-imaging spatial filtering demonstrates the effect of the scatter image (from the slit interface) magnified to a degree outside the detection zone of the PMT.

The design of this pre-imaging spatial filtering technique ultimately was developed to be used in the UV spectral region where many analytes natively fluoresce. However, wall fluorescence becomes a very significant source of background, which can create very high background levels compromising the sensitivity of the technique. It was hypothesized that with the proposed pre-imaging spatial filtering technique that a significant portion of wall fluorescence would be blocked and the background would be decreased. Examination of Tables 3.6 and 3.7 summarize data collected with an UV frequency doubled Ar^+ laser at 257 nm using a 320 nm cut on filter or a 600 nm cut on filter with the two different cell holder holders. At positioning arrangement 1 (Table 3.6), the cell holder without the pre-imaging filtering shows a very high fluorescent signal of 40,000 nA at the widest external slit width with the 320 nm filter, but reduced to only 15,000 nA with the 600 nm filter. Conversely, the cell holder with the pre-imaging spatial filtering the signal is only 60 nA with the 320 nm filter and dropped to 38 nA with the 600 nm filter with no external slit (wide open at 6500 μm). Furthermore, with 15X magnification and using the cell holder without pre-imaging spatial filtering, signal levels for fluorescent background were much lower; (100 nA for the 320 nm filter and 40 nA for the 600 nm filter). The same reduction in signal was observed with the 15X magnification for the cell holder with the pre-imaging as with the two previous lasers.

Table 3.6: Positioning Arrangement 1, Ar⁺ Laser at 257 nm

	Cell Holder- No Slit	Cell Holder- No Slit	Cell Holder- Slit	Cell Holder- Slit
External Slit (μm)	Signal (nA) w/ 320 nm filter	Signal (nA) w/ 600 nm filter	Signal (nA) w/ 320 nm filter	Signal (nA) w/ 600 nm filter
250	240	90	2.0	2.0
500	580	220	2.8	2.4
1000	1700	640	5.8	4.2
2000	3900	1600	12.0	7.6
3000	5800	2550	19.0	12.0
4000	7800	3400	26.0	16.8
5000	12000	4800	34.0	20.8
6000	28000	10500	50.0	32.0
6500	40000	15000	60.0	38.0

Table 3.7: Positioning Arrangement 2, Ar⁺ Laser at 257 nm

	Cell Holder- No Slit	Cell Holder- No Slit	Cell Holder- Slit	Cell Holder- Slit
External Slit (μm)	Signal (nA) w/ 320 nm filter	Signal (nA) w/ 600 nm filter	Signal (nA) w/ 320 nm filter	Signal (nA) w/ 600 nm filter
250	3.0	2.0	1.8	1.4
500	6.0	3.0	1.8	1.4
1000	15	6.4	2.4	2.0
2000	34	14	4.0	2.8
3000	52	22	5.8	3.8
4000	68	29	7.0	4.6
5000	80	34	9.2	5.4
6000	90	40	10.0	6.2
6500	100	40	10.2	6.2

Further characterization of the rectangular cell holder with the pre-imaging spatial filtering was done by continuously injecting a flowing stream of 1×10^{-7} sodium fluorescein through the separation capillary while maintaining a sheathing flow matched to the separation flow. The matching of two flow rates was accomplished by continuously eluting a stream of laser dye from the separation capillary (in this case sodium fluorescein). Concurrently, the sheathing flow was adjusted with the syringe pump until the fluorescent image eluting from the separation capillary was approximately the same size as the i.d. of the separation capillary (50 μm in this case). The matching of the flows was accomplished with the aid of a video microscope and the fluorescent image was measured on the video screen. Once the flow rates were matched, signal and background levels were recorded at a series of external slit widths by adjusting the external slit from 250 μm to 6500 μm wide. A plot of the Signal/Background (S/BKG) ratio vs. slit width demonstrated the effect of using an external slit for spatial rejection. A steady increase in S/BKG is observed with an optimization around a slit width of 2500 μm before decreasing to 130 at a 4500 μm slit width. As the slit is gradually widened from 250 μm to 2500 μm , background levels are increased slightly but the signal obtained increases greater, hence a maximum level is reached at 2500 μm slit width. As the external slit is increased wider from 2500 μm to 4500 μm slightly more signal is detected, but more background is also detected. Hence, the S/BKG levels begin to decrease.

The S/BKG level was calculated to reach a maximum level at approximately 1500 μm external slit width. However, it was observed at an external slit width of 2500 μm that the maximum S/BKG was reached. This is due to a slight hydrodynamic expansion

of the fluorescent image, the fluorescent image was not centered on the external slit, and the image was unfocused at the external slit interface. Because of these aforementioned difficulties with a far-field mode of spatial filtering with an external slit, it is more practical and easier to use the pre-imaging spatial filtering which does not require an external slit.

The data presented shows that the pre-imaging spatial filtering technique efficiently blocks both wall scatter and wall fluorescence even in the UV spectral region where wall fluorescence is particularly problematic. However, for any post-column detection arrangement to be feasible, the integrity of a chromatographic separation must be studied. Therefore, an examination of the rectangular sheathing capillary geometry for use in a chromatographic separation is warranted.

Separation of Amino Acids

To evaluate the rectangular sheathing capillary geometry as a suitable SFC for the preservation of the integrity of a chromatographic separation, a comparison between on-column and post-column detection was made using a three component mixture of dansyl amino acids. Figures 3.17a and 3.17b show post-column detection of three dansyl amino acids (Tyr, Trp, and Phe) using CE and the HeCd laser (a) and CE and the Ar⁺ UV laser (b). Figure 3.17c shows post-column detection of three free form amino acids (Tyr, Trp, and Phe) using capillary LC and the Ar⁺ UV laser (c). The efficiencies for on-column detection with the HeCd laser were 6×10^5 , 12×10^5 , and 8.4×10^5 plates/meter for Tyr, Trp, and Phe respectively. However, for the post-column detection the efficiencies were 5×10^5 , 10×10^5 , and 7.6×10^5 plates/meter for Tyr, Trp, and Phe respectively. This

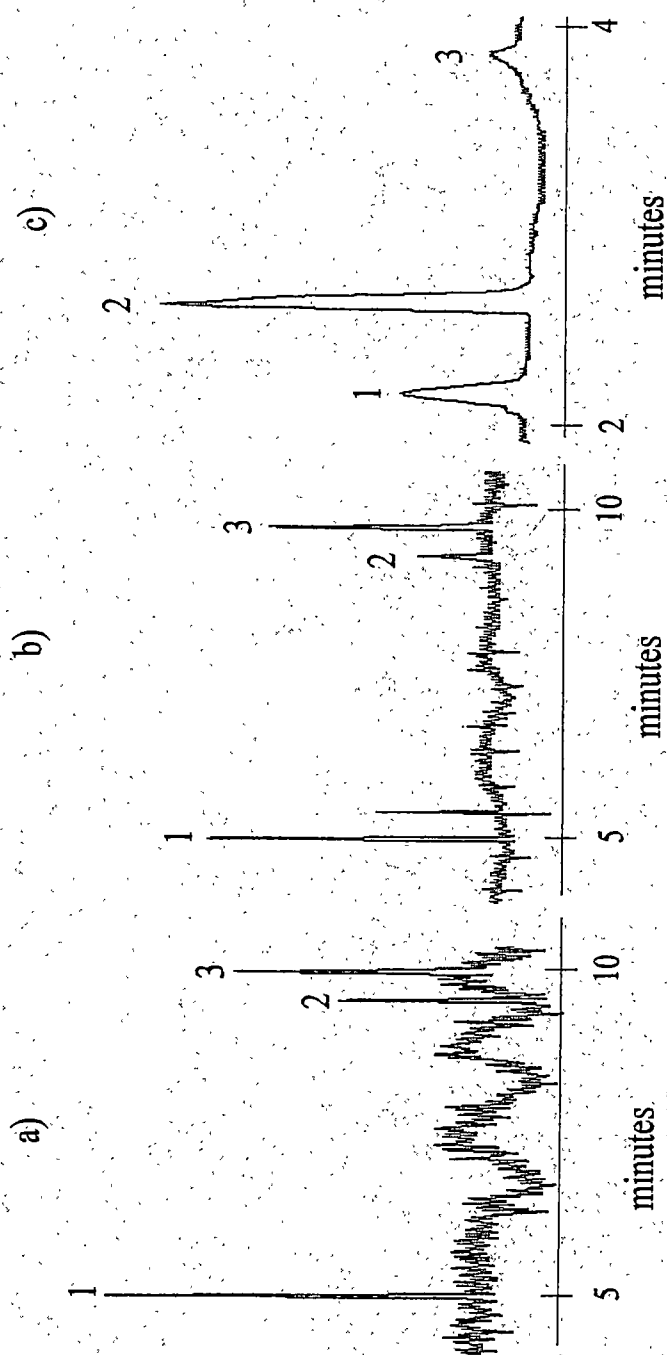


Figure 3.17. CE separation of three dansyl amino acids. (a) HeCd laser and (b) Ar^+ laser at 257 nm with post-column detection adjacent to separation capillary. Separation conditions are 50 mM phosphate running buffer, 15 kV, sheathing flow 0.5 mL/hr. Elution order is (1) Tyr, (2) Try, and (3) Phe. (c) Capillary LC separation of free form amino acids with Ar^+ laser at 257 nm with post-column detection adjacent to separation capillary. Separation conditions: 500 μm x 15-cm column, .020 $\mu\text{L}/\text{min}$ flow rate, 50/50 ACN/ H_2O mobile phase. Elution order was (1) Tyr, (2) Try, and (3) Phe.

corresponded to a decrease in efficiency of 17% for Tyr, 17% for Trp, and only 10% for Phe. Similar decreases in efficiencies were observed with the UV laser. On-column detection efficiency for Tyr was 3.4×10^5 plates/meter, 6.2×10^5 for Trp, and 5.0×10^5 plates/meter for Phe. The decreases in efficiencies were 18% for Tyr (2.8×10^5 plates/meter), 15% for Trp (5.3×10^5 plates/meter), and 12 % for Phe (4.4×10^5). This degradation of efficiency for post-column detection with a SFC is minimal and comparable to that reported previously with a more traditional sheathing capillary geometry [62, 135].

An integral reason of choosing rectangular sheathing capillary geometry was the ability to use different dimension separation techniques such as CE and LC without having to change the sheathing capillary. A capillary LC separation of the three native aromatic amino acids (Tyr, Trp, and Phe) was done with the UV Ar^+ laser. Figure 3.17c shows a rapid, efficient separation for capillary LC with all three peaks eluting in less than 5.5 minutes (Elution order was Tyr, Try, and Phe). The efficiencies reported were 1.8×10^4 plates/meter for Tyr, 4×10^4 plates/meter for Try, and 8×10^4 plates/meter for Phe.

CONCLUSIONS

The SFC design and pre-imaging mode of spatial filtering described represented a significant advancement in the development of a routine, practical LIF detection system. The overall design itself is rugged, simple, small, and easy to use LIF detection system. A pre-imaging mode of spatial filtering was shown to be very comparable to a more traditional far field mode of spatial filtering, but inherently easier and simpler. The

sheathing capillary geometry employed in this design contributes very minimally to band dispersion and degradation of separation efficiency. Furthermore, this sheathing capillary geometry was compatible with both CE and LC techniques. In addition, the capillary geometry used with this SFC arrangement maintains the integrity of a separation demonstrated with a mixture of amino acids. Compatibility with an UV laser presents the applicability for a routine, practical system for LIF detection with reduced dimension separation techniques.

CHAPTER 4

ON-COLUMN SURFACE ENHANCED RAMAN SPECTROSCOPY DETECTION IN CAPILLARY ELECTROPHORESIS USING RUNNING BUFFERS CONTAINING SILVER COLLOIDAL SOLUTION

INTRODUCTION

Despite very low absolute detectability, ultimately reaching the single molecule detection level in certain LIF experiments, [117, 138, 139] detection in CE generally suffers from modest-to-poor concentration detectability. Moreover, CE detection has not matured relative to other separation methods with regards to implementing strategies that provide spectroscopic information for high selectivity or solute identification purposes. Thus, it is not surprising that many reports of CE development work focus on the important issue of detection [33]. Raman spectroscopy has the potential to provide high information content spectra for solutes dissolved in the aqueous media employed most often in CE. Moreover, the inherent inefficiency of normal Raman scattering has been overcome with remarkable success using resonance and surface enhanced techniques. In some instances extremely low levels and even the probing of single molecules has been accomplished by surface enhanced Raman spectroscopy (SERS) [140-144]. SERS is performed most often by employing silver colloidal solutions as the enhancing medium, although the preparation of effective colloidal solution remains somewhat an art form. Other substrates that can be reproducibly produced have also been shown to provide large Raman enhancements [142, 144]. There is a modest history of employing SERS in planar [143, 145] or liquid [68, 72, 146-149] chromatography. For example, Fateley and

co workers added silver colloidal solutions post column and detected organic dyes using a 1 mm i.d. flow cell [146]. Spectra were obtained in a stop flow mode for injections of 200 ng of the dyes. Cotton and co workers detected purine bases, in some cases at low nanomole injected quantities, using HPLC with post separation addition of silver colloidal solutions [147]. Laserna and coworkers found it advantageous to employ a windowless flow cell for SERS detection of banned drugs [148, 149]. SERS has not been employed in CE. However, Morris and co workers have published several reports of normal Raman spectroscopy detection in conjunction with capillary electrokinetic separations [67-72]. They made very effective use of the concentrating effects of capillary isotachopheresis to perform normal Raman spectroscopy on-column at analytically significant solute concentration levels. In a recent report, these researchers performed this mode of separation and detection on a microchip and alluded to the potential for employing a SERS active surface on the chip [69].

Reported in this chapter is the implementation of SERS detection for conventional CE employing a zonal mode of separation. Direct on-column surface enhance Raman spectroscopy (SERS) detection is demonstrated in capillary electrophoresis (CE). Riboflavin and the very SERS active compound Rhodamine 6G are used as test solutes. Distinctive SERS spectra of two test compounds, riboflavin and Rhodamine 6G, are obtained in 100 μ m i.d. fused silica capillaries under CE conditions using running buffers that contain silver colloidal solution. A common method is employed to prepare silver colloidal solutions, which are used directly as running buffer or added to conventional CE running buffers. It has been shown that optimization of the size of the silver particles, relative to the laser wavelength employed, is very important in order to realize extremely

large enhancements [150]. Colloidal solution optimization was not performed in this work. Incorporation of the colloidal solution into running buffer is shown to have little effect on the separation of the test compounds as monitored using a laser induced fluorescence instrumental scheme. However, SERS spectra degrade if the capillary is not rinsed between experiments. The effects of numerous operating conditions on the quality of SERS spectra obtained under flowing conditions in 100 μm i.d. CE capillaries are evaluated. The effects of laser power, wavelength, spectra acquisition time, silver colloidal concentration, and applied voltage (i.e., flow rate) on the quality of SERS spectra are evaluated.

Detection is performed using an unmodified commercial Raman spectrometer in a confocal microscope mode of operation. A commercial Raman instrument is used without modification for on-column detection. Rapid sequential acquisition of spectra, as is necessary to produce 3-dimensional (migration time versus signal versus wavenumber) plots, is not possible with this spectrometer. However, spectra could be obtained on-the-fly with spectral acquisition times that maintain the integrity of CE bands (1-second in this work). Using laser powers of 17 mW (at the sample) at 515 nm and employing 1-second spectral acquisition times, spectra with bands exhibiting signal-to-noise ratios greater than 10 could be obtained for 1.0×10^{-6} M riboflavin and very low nM concentrations of Rhodamine 6G. In this manner, injections of low pg and low fg quantities of riboflavin and Rhodamine 6G, respectively, yielded distinctive spectra. In the case of the latter solute, the injected quantity was approximately 90 attomoles.

EXPERIMENTAL

Materials

Sodium phosphate (monobasic) and sodium phosphate (dibasic) were purchased from Sigma (St. Louis, MO, USA) and were used to prepare running buffers. Riboflavin (vitamin B2) (Rb) and Rhodamine 6G (Rh6G) were purchased from Sigma (St. Louis, MO, USA) and used as is to prepare 1×10^{-3} M stock solutions and diluted as test compounds. Silver nitrate (ACS grade) and sodium citrate were used in the silver sol preparation and purchased from Sigma (St. Louis, MO, USA). Fused-silica capillaries (100 μm i.d. x 365 μm o.d.) were purchased from Polymicro Technologies (Phoenix, AZ, USA).

Silver Colloidal Solution Preparation

Silver colloidal solutions were prepared according to a modified Lee and Miesel procedure [151]. Briefly, 90 mg of AgNO_3 was dissolved in 500 mL of H_2O and brought to a boil. A 1% solution of sodium citrate (10 mL) was added drop wise over a period of 10 minutes. The solution was kept boiling for approximately 1 hr. The final product was a greenish yellow color and had an absorption maximum at 430 nm. The silver colloidal solutions were used directly at pH 6 and diluted as running buffer with 25 mM phosphate at pH 6.0 for CE experiments.

Capillary Preparation

Fused silica capillaries used for CE-SERS and CE-LIF separations (100 μm i.d. x 365 μm o.d.) were cut to a total length of 36-40 cm using a capillary cutter. An on-

column detection window was made by removing a section of the polyimide coating at 15 cm from the inlet end of the capillary with fuming concentrated sulfuric acid. For all CE experiments, the capillaries were pre-treated with 1 M NaOH for 1 hr. followed by 30 minute water rinse and an 1 hr. acid rinse with 1 M HCl. Capillaries were rinsed with 1.0 M HCl for 10 minutes between individual experiments and stored in dilute acid (0.1 M HCl) overnight.

CE-SERS Apparatus

SERS spectra were collected using a Dilor Model OMARS 89-Z24 microprobe spectrometer operated in a confocal mode with an excitation wavelength of 515 nm or 488 nm provided by a Lexel argon ion laser (Lexel Laser, Waterford, CT, USA). A charged-coupled device detector is employed. A microscope objective (80 X) is used to focus the laser-radiation and to collect the 180° Raman scattering. The CE capillary was placed horizontally into a plexiglass stage machined in house [152]. A depiction of this plexiglass stage is shown in Figure 4.1. This plexiglass assembly allowed for the CE capillary to be placed and secured horizontally to allow for CE experiments. The plexiglass assembly was then secured onto the microscope stage of the Raman spectrometer that allowed for x, y, and z translation of the capillary for focusing the laser beam through the capillary. A picture of the CE-SERS apparatus is shown in Figure 4.2.

Samples were analyzed in a 100 μm i.d. fused silica capillary. Capillaries were filled with the test analyte with a syringe for static studies and electrokinetically pumped by a high voltage power supply (Spellman, Brewster, NY, USA) for dynamic studies. All scans of either 1, 10, or 100 s acquisition were with a grating centered at 1200 cm^{-1} and a

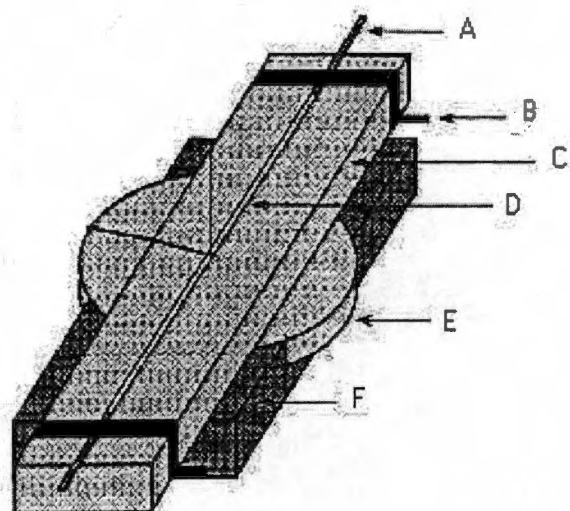


Figure 4.1. Plexiglass stage for CE-SERS. (A) needle electrode, (B) high voltage lead, (C) plexiglass stage, (D) capillary, (E) translation stage, and (F) aluminum plate.

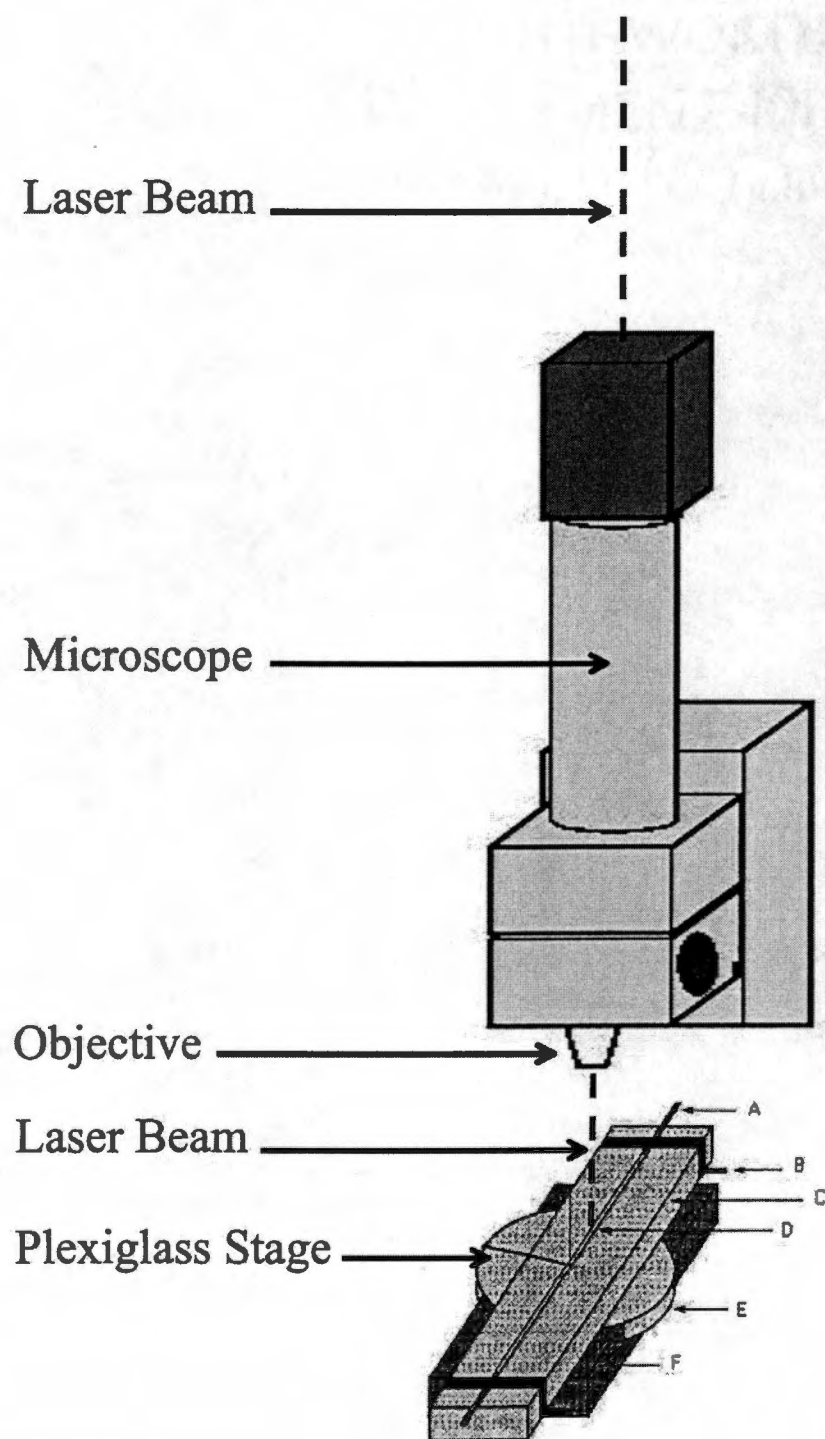


Figure 4.2. CE-SERS apparatus.

spectrograph and foremonochromator at 1450 cm^{-1} giving a spectral range of 1024- 1874 cm^{-1} with a spectral slit width of 4.4 cm^{-1} . All spectra presented were treated using a Sovitsky-Golay nine point smooth.

CE-LIF Apparatus

The CE-laser fluorimetry instrumentation consisted of a 257 nm frequency doubled Ar^+ laser (Lexel Laser, Waterford, CT, USA) used as the excitation source. The laser beam was focused through the capillary with a 25 mm diameter quartz $f/1$ lens and fluorescence emission was collected at 90° to the flow cell with a 25 mm diameter quartz $f/1$ lens (Edmund Scientific, Barrington, NJ, USA). The fluorescent image was spatially isolated using an adjustable optical slit (Edmund Scientific, Barrington, NJ, USA). The isolated radiation was passed through a 380 nm long pass filter (Edmund Scientific) and allowed to impinge on a RP-1 type photomultiplier tube (Hamamatsu Corp., Bridgewater, NJ, USA). The PMT photocurrent was monitored by a Pacific Precision Instruments (Concord, CA, USA) Model 126 photometer. The photometer was operated with a 0.1 s output time constant. Electropherograms were recorded with a program written in house using LabView and a Pentium II computer equipped with an uCDAS-16G data collection board (Keithley Metrabyte, Taunton, MA). A Spellman (Brewster, NY, USA) Model 840A high-voltage power supply was used to apply electrophoretic fields.

Injectons for both fluorimetric and SERS detection experiments were performed electrokinetically by placing the inlet of the capillary into the sample vial and applying +1 kV for 10 s unless otherwise noted. Following injection, the capillary inlet was

replaced into the running buffer vial and a running potential of +2.5 kV was applied across the capillary.

SEM Apparatus

A Hitachi S-3200N Scanning Electron Microscope (Hitachi Scientific Instruments, San Jose, CA, USA) was used for all experiments. Operating conditions included a beam voltage of 20 kV, pressure of 20 Pa, and a working distance of 13.1 mm. The images recorded were backscattered electron images and were recorded and analyzed with Scion Image Shareware.

RESULTS AND DISCUSSION

Evaluation of Parameters

Different parameters were evaluated to determine their effects on the quality of SERS spectra in CE applications. Figure 4.3 depicts the effect of laser power and acquisition time on the SERS spectrum of Rb obtained with a stagnant 1.0 μ M Rb in the stock silver colloidal solution in the capillary. With a low laser power (4 mW at the sample) and a long acquisition time of 100 seconds, the integrity of the SERS spectrum is preserved with 12 distinct bands for Rb (not labeled in this case). Conversely, an increase of laser power to 17 mW with the same acquisition time results in a substantial degradation of the Rb spectrum. The spectrum appears to suffer from a broadening of the Raman bands and an increase in background fluorescence. It is not clear at this point if this is due to degradation of the Rb itself or if it is related to a change in the condition of the silver colloidal solution or the capillary wall. Of course it could be a combination of

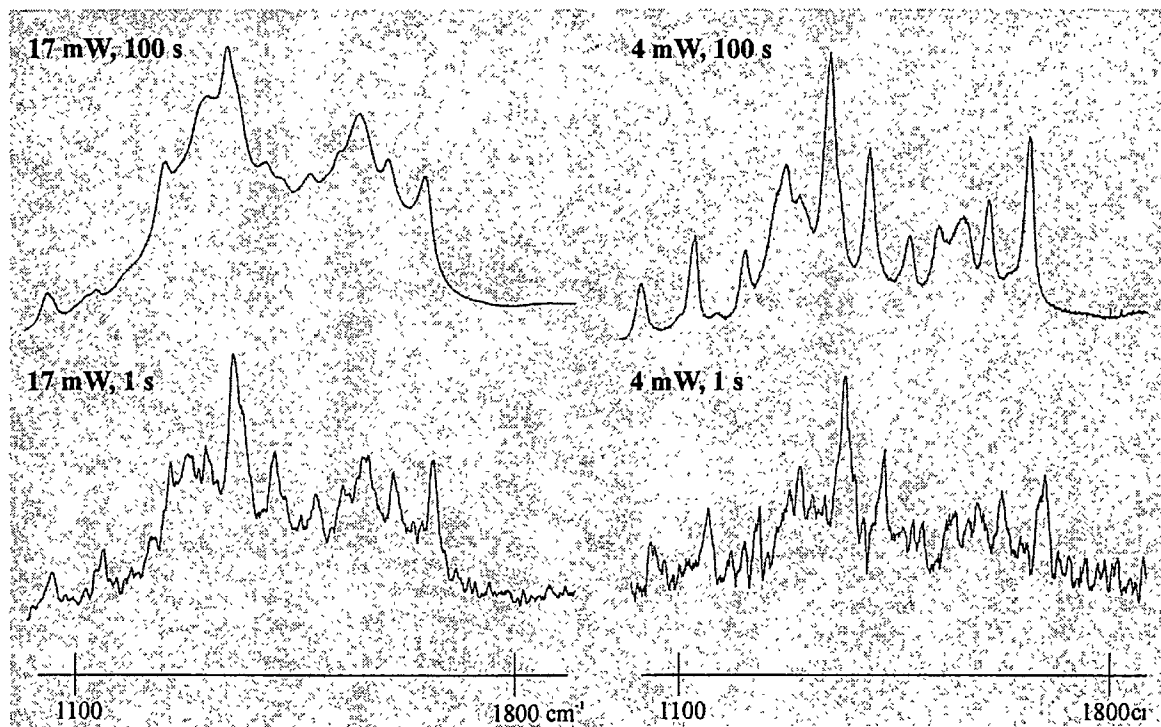


Figure 4.3. Effect of laser power and acquisition time on the integrity of a SERS spectrum. Acquisition conditions were 1×10^{-6} M Rb in a 1% w/v silver colloidal solution under static conditions in 100 μm i.d. capillary. Laser wavelength was 514.5 nm with an estimated spot size of 25 μm .

these effects. It is not likely that the degradation is due to the detector being saturated under these conditions. Since the spectra were acquired in this study under static conditions, the higher laser powers would seem to cause the degradation previously mentioned. Fortunately, if a lower acquisition time is used (1-second) with a laser power of 17 mW, a good quality spectrum is obtained with a higher signal/noise (S/N) than that obtained using a lower laser power of 4 mW and 1-second acquisition time. A spectral acquisition time that is 10% of the temporal peak width should produce negligible band broadening. Thus, a 1-second acquisition time (τ) would be compatible with non-detection CE plate counts (N) of 40,000 for a solute with a 500-second migration time (t) [$N = 16 (t / 10\tau)^2$]. Decreasing τ to 0.5-seconds would increase this plate count to 160,000, which would be acceptable in most CE applications. Therefore, a 0.5-1-second acquisition time with a laser power of 17 mW should be suitable for performing on-the-fly SERS detection in CE separations.

Experiments shown in Figure 4.3 were done under static conditions. However, in order to perform on-the-fly SERS detection with CE, the analyte of interest will be in a flowing system (i.e. electrokinetic flow under an applied voltage). Table 4.1 summarizes the results found when the applied voltage was varied over the range of 5 to 15 kV and compared to that of static conditions. Signal/noise ratios of three prominent bands in the Rb spectrum are shown. Noise levels in the SERS spectra were quantified using Grams 32 Spectral Notebase Version 4.01 by measuring the height of the noise from peak to valley. As seen in the table, there is an improvement in S/N from static conditions to dynamic conditions. However, there is little difference between the three different

Table 4.1. Parameters Affecting S/N of SERS Spectrum

Parameter (S/N)	1155 cm^{-1}	1347 cm^{-1}	1629 cm^{-1}
Static (S/N)	5.08	15.6	12.4
5 kV (S/N)	6.67	21.3	16.0
10 kV (S/N)	6.72	22.0	17.1
15 kV(S/N)	7.02	20.7	15.4
1% w/v (S/N)	20.7	72.6	53.2
0.6 % w/v (S/N)	5.74	15.8	9.41
0.3 % w/v (S/N)	2.06	5.09	3.71

CONDITIONS

- Static study was done by filling the capillary with a 1 μM Rb solution diluted with silver colloidal solution
- Dynamic studies were done by a continuous electrokinetic injection of 1 μM Rb solution diluted with silver colloidal solution
- Silver concentration study was done by diluting the silver with water and preparing 1 μM Rb solutions with each % silver colloidal solution

applied voltages. This indicates that even mild electrokinetic flow will replenish the detection zone with fresh Rb-silver colloidal solution leading to improved SERS signals.

Quantification of silver particles was done by weighing a known volume of silver colloidal solution and subtracting the weight of a same volume of water and calculating a weight percent taking in account the density of silver to be 10 g/mL. The stock silver colloidal solution was determined to be 1% w/v. This solution has a pH of approximately 6 and no effort was made to adjust the pH to a good buffering region for citrate-citric acid buffer. A study of the effect of the concentration of the silver colloidal solution on the quality of the SERS spectrum of Rb was also performed. Table 4.1 shows S/N for the stock solutions and two lower concentration solutions resulting from dilution with a pH 6.0, 25 mM phosphate running buffer. Three bands for Rb were chosen based on their location in the SERS spectrum and their reproducibility. Signal-to-noise ratios were calculated from a single SERS spectrum. As the concentration of colloidal solution is decreased SERS signals diminished dramatically as seen in the table. Concurrently, the quality of the SERS spectra degrades to a point where the three bands of interest are barely detectable. As shown in Table 4.1, the stock silver colloidal solution produced the best SERS spectra for Rb. Therefore, the 1% w/v stock silver colloidal solution was used as running buffer for all subsequent experiments.

With the experiments performed to create the data in Table 4.1, it was determined that acid rinsing was needed after a period of time to preserve the integrity of SERS spectra. It is assumed that a gradual build up of silver particle on the walls of the capillary occurs with constant exposure to the flowing colloidal solution. The spectra for Rb shown in Figure 4.4 illustrate this effect. All spectra in Figure 4.4 were acquired with

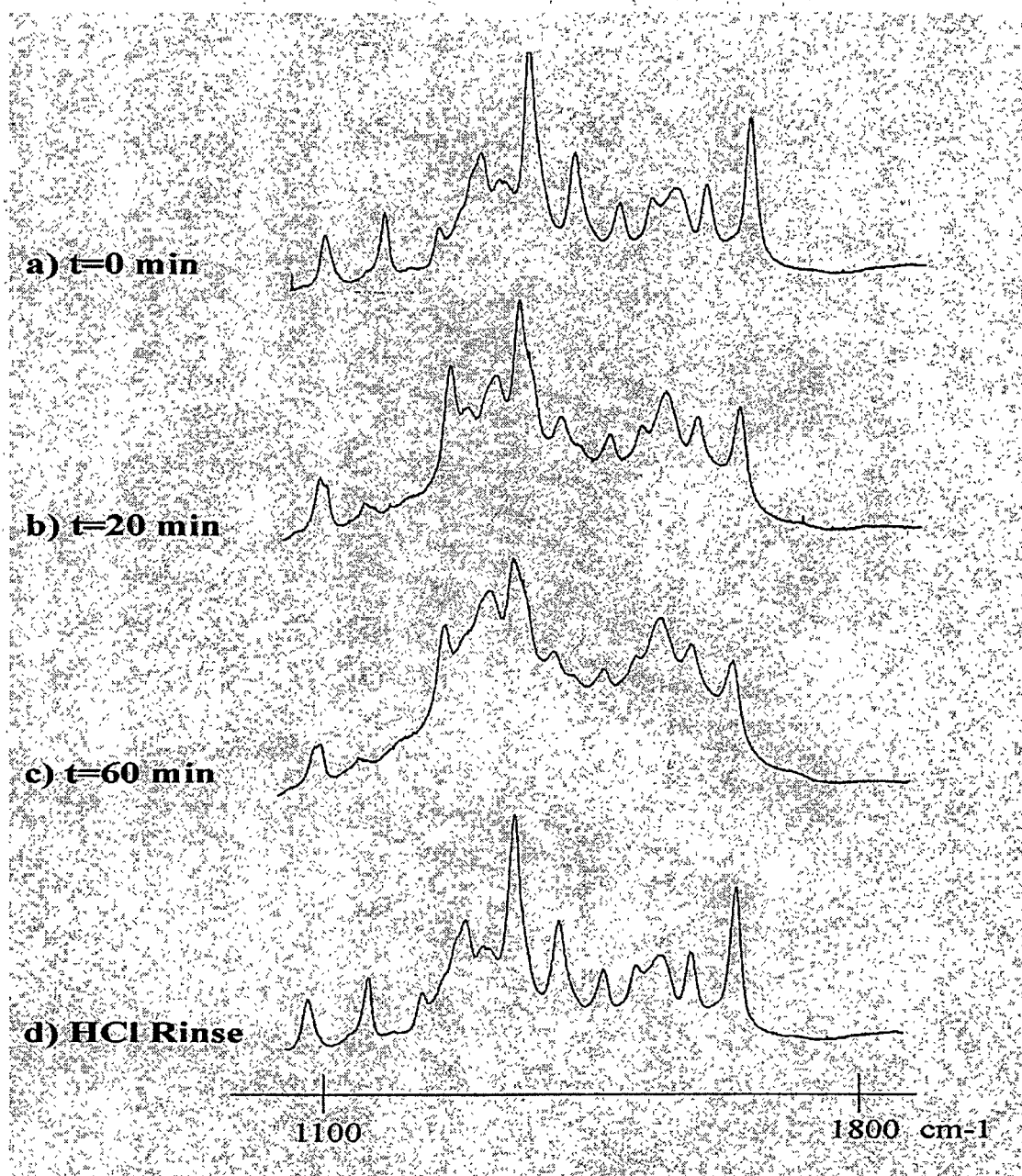


Figure 4.4. Effect of rinsing the CE capillary between runs on the integrity of the SERS spectrum. Analyte used was 1×10^{-6} M Rb. SERS conditions were +10 kV running voltage, 514.5 nm, 100 mW power, and 1 s acquisition time.

a 10 second time constant. Figure 4.4a depicts the initial SERS spectrum of 1×10^{-6} Rb with a newly prepared capillary. Without rinsing the capillary after the initial run, the SERS spectrum degrades in less than 20 minutes (see Figure 4.4b) and further degrades in 60 minutes (see Figure 4.4c) with very poor resolution and an apparent increase in fluorescence background. However, rinsing the CE capillary with 1.0 M HCl rinse for approximately 10 min (see Figure 4.4d) the integrity of the SERS spectrum is preserved and is reproducible to the initial spectrum shown in Figure 4.4a. Therefore, it was deemed necessary to rinse the CE capillary after each run with 1.0 M HCl for 10 minutes to obtain high quality, reproducible SERS spectra.

Matching excitation wavelength to silver particle size has been shown to be a critical parameter in producing large SERS enhancements [86]. We performed a comparison of the argon ion laser wavelengths of 488-nm and 515-nm. The effect on Rb SERS spectra was minimal but the effect on Rh6G was significant. The S/N of three arbitrary bands were observed to be about 5-to-6-fold better for the 515-nm laser line. Moreover, the 488-nm line resulted in more Rh6G fluorescence background. This fluorescent background masked the SERS effect in the 488 nm case; hence, smaller S/N ratios were observed for each of the three bands. This increase in fluorescence background for 488 nm may be attributed to a mismatch between particle size and laser wavelength or a result of poor rinsing of the capillary with 1.0 M HCl solution.

The presence of a narrow size range of “hot” particles that produce large ($\geq 10^{14}$) enhancements has been documented. Nie and co workers used atomic force microscopy to determine that the “hot” particles for 488-nm, 568-nm, and 647-nm excitation had narrow size-ranges centered around 70-nm, 140-nm, and 195-nm, respectively [150]. We

have made no effort to optimize the size distribution of particles in our colloidal solutions, nor have we sieved these particles to produce narrow ranges that could be matched to the excitation wavelength. Thus, we did not expect a dramatic wavelength effect in this study.

A scanning electron micrograph of the silver particles used in these studies was obtained and demonstrated a wide particle size distribution with many large particles or particle aggregates ≥ 200 nm. Figure 4.5 shows a scanning electron micrograph of the silver particles used in these studies. There is clearly a wide range of particle sizes and even evidence of agglomerations of particles. While this is not a straight forward project, the presence of a greater number of properly sized particles and the absence of poorly sized particles that dilute the effect of "hot" particles should greatly improve signals in future SERS-CE work and represents strong motivation.

CE-LIF Detection

Before attempting an on-the-fly CE separation with a SERS mode of detection, separation conditions were tested and evaluated in a more traditional LIF mode of detection to determine the effect, if any, the silver colloidal solution may have on the integrity of the CE separation of Rh6G and Rb. Figure 4.6 shows electropherograms for Rb and Rh6G separations using normal phosphate running buffer and the stock colloidal solution as running buffer (both at roughly pH 6). The points labeled A, B, and C in Figure 4.6 corresponds to the three different one second acquisition points, which would be used for the on-the-fly CE-SERS case (see next section). The peak shaped observed in Figure 4.6b is somewhat poorer than often observed in CE. This is possibly due to some

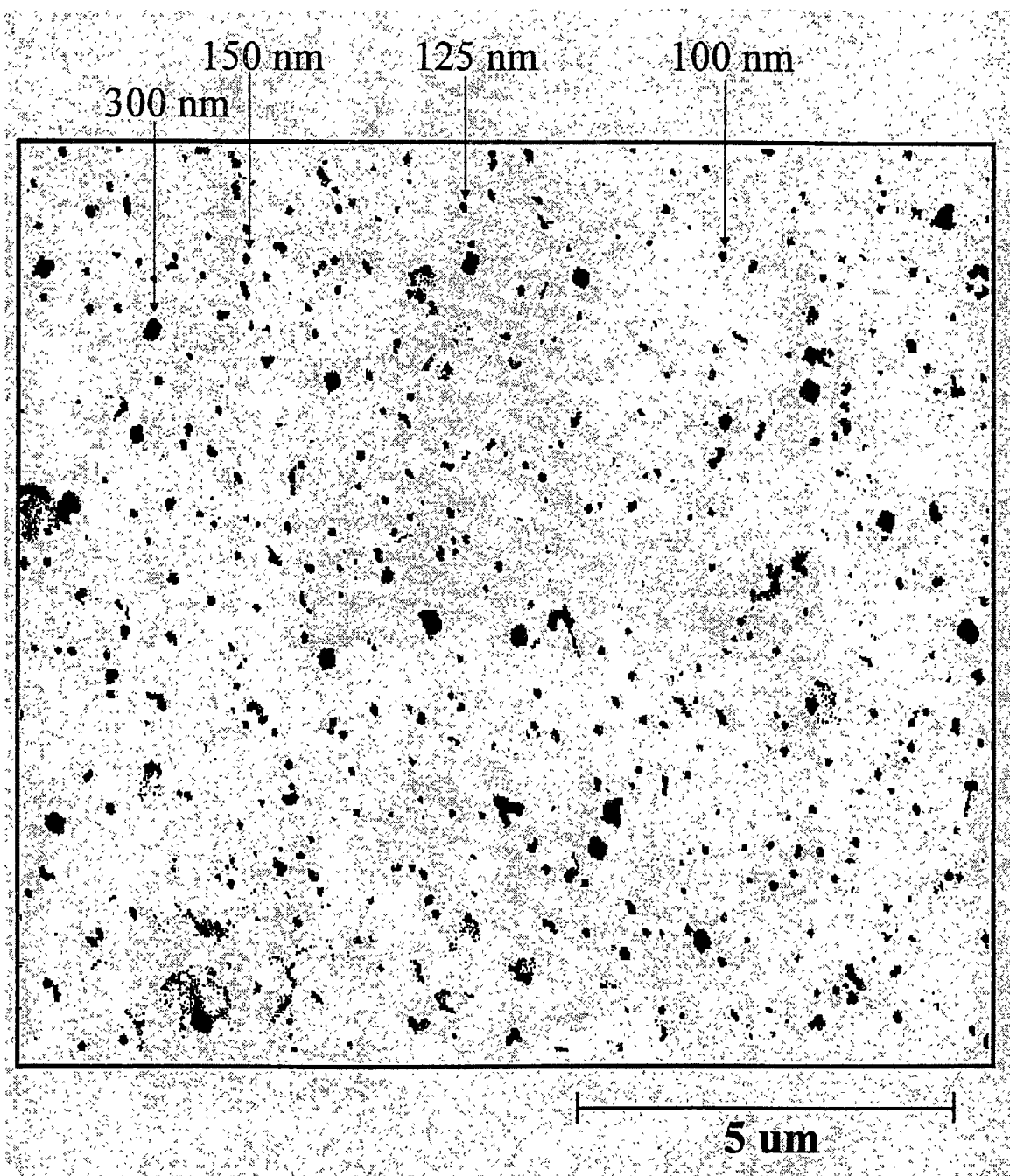


Figure 4.5. SEM micrograph of 1% w/v stock silver colloidal solution. A drop of solution was deposited on a silicon wafer and allowed to dry prior to obtaining the image.

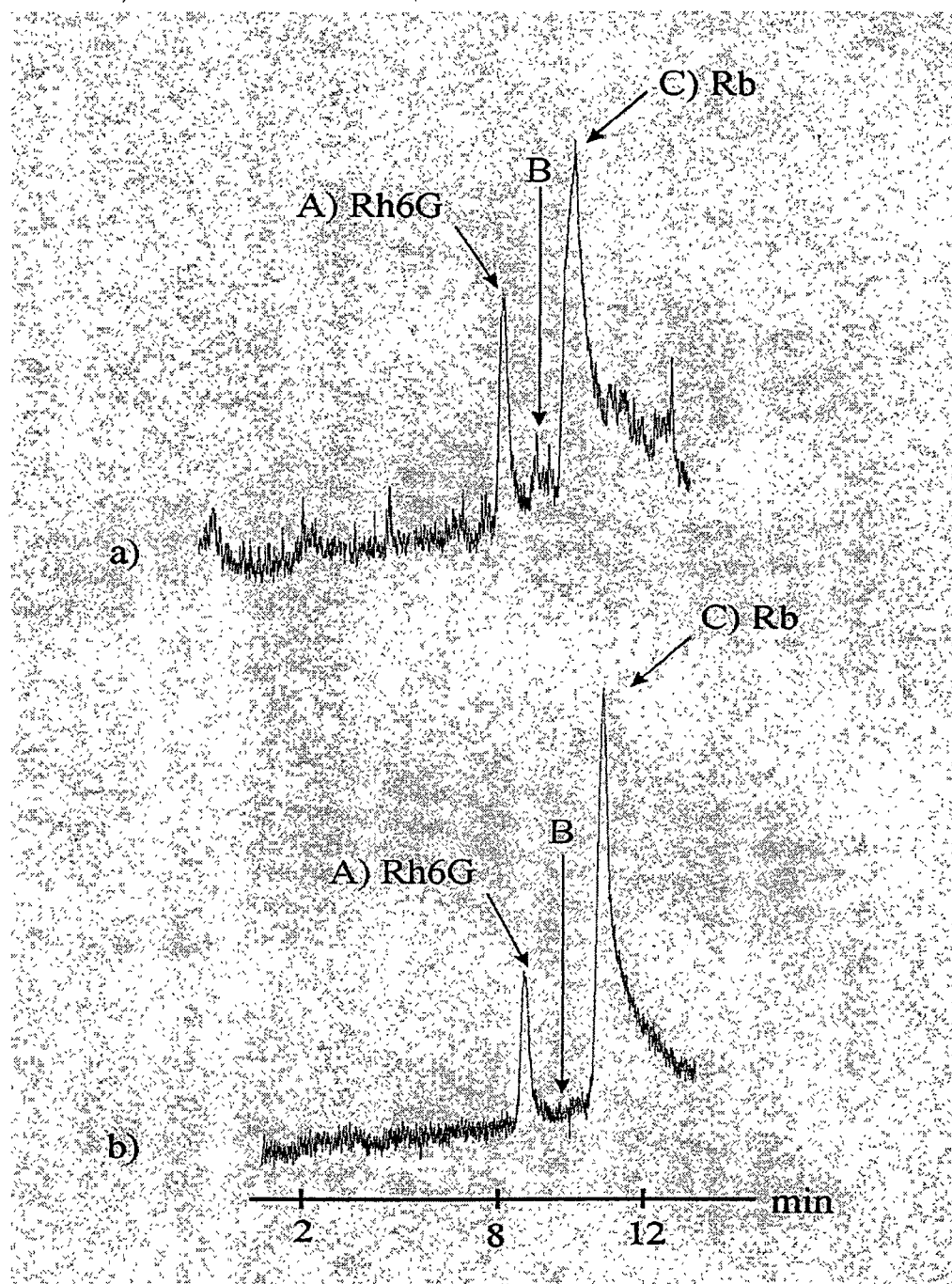


Figure 4.6. Effect of the addition of silver colloidal solution to the RB on a CE-LIF separation. (a) A 1% w/v silver colloidal solution as running buffer; (b) Normal running buffer. Separation conditions were 1×10^{-6} M Rb and 1×10^{-8} M Rh6G electrokinetically injected at 1 kV for 10 s and a running voltage of 2.5 kV. Length of the column to the detection window was 15 cm. A 257 nm frequency doubled Ar^+ laser was used at 60 mW at the source for excitation. Positions A, B, and C correspond to SERS spectra taken under CE-SERS conditions (see Figure 4.7).

wall adsorption and the fact that the capillary i.d. is at the upper range of those employed in CE. The small additional peaks in Figure 4.6a may be due to scatter by large colloidal aggregates or simply laser fluctuations. Although an LIF instrumental arrangement was used to obtain the electropherograms, it is largely the broad envelop of Raman bands that are isolated by the LIF emission filter in the case with colloidal particles. The differences in relative peak heights of the test compounds for the two running buffers is likely due to differences in fluorescence quantum yields and Raman enhancements for these compounds. Important LIF detection parameters such as background and noise were monitored and little change was observed with the use of the colloidal solution, although electrophoretic current was 5 – 10 μ A higher. The RSD's for migration times in the presence of the colloid were 5% and 3% respectively for Rh6G and Rb. Low running voltages (2.5 kV) were used to allow for the SERS detection of CE bands on-the-fly (see below).

It is well known that large SERS enhancements occur for solutes adsorbed onto the substrate. It is interesting to note that the migration times do not change substantially (< 10%) comparing Figure 4.6a and 4.6b. If the large SERS effects we observe are due to solutes irreversibly adsorbed onto these large (relative to solutes) colloidal particles in the running buffer then one would expect substantial changes in migration times and/or peak shape. Most likely there is a bound/unbound equilibrium with fairly rapid exchange kinetics. The bound solutes provide the largest SERS enhancements but the equilibrium appears to favors the unbound state because of the minor influence on migration rate. The situation may be similar to other forms of on-column labeling in CE such as the use of running buffers containing intercalating dyes to measure DNA or chelating ligands to

measure metal ions [56, 153]. If one looks closely at the peaks it appears there is some minor broadening in Figure 4.6a relative to 4.6b, which may be a consequence of the bound/unbound exchange kinetics. If kinetics are rapid then mass transfer between solute migrating as free species or as particle bound should not limit efficiency [56, 153]. This appears to be mostly the case in these studies.

CE-SERS Detection

As alluded to previously, the data acquisition capabilities of the spectrometer used in these studies did not permit the acquisition of automated repetitive scans. Thus, it was not possible to produce intensity, wavenumber, and migration time as an appropriate three-dimensional plot. It was possible, however, to use spectral acquisition times that maintain the integrity of CE separations, observe spectra at numerous points along the course of a separation, and save relevant observed spectra. To simplify catching a spectrum near band center the applied voltage was fairly low in this work (2.5kV). Nevertheless, the separation was accomplished in just over 10 minutes. We feel that experiments performed in this manner fully demonstrate the capability of acquiring SERS spectra on-the-fly under realistic CE conditions.

The spectra shown in Figure 4.7 were obtained on-the-fly for a separation at points that correspond to A (Rh6G band), B (baseline between bands), and C (Rb band) shown in Figure 4.6. These spectra were acquired by using a 1-second acquisition time and a laser power of 17 mW at the sample. We believe this is the first such report for on-the-fly CE-SERS detection. The corresponding wave number assignments are made for both Rh6G and Rb and correspond well to those previously reported in the literature

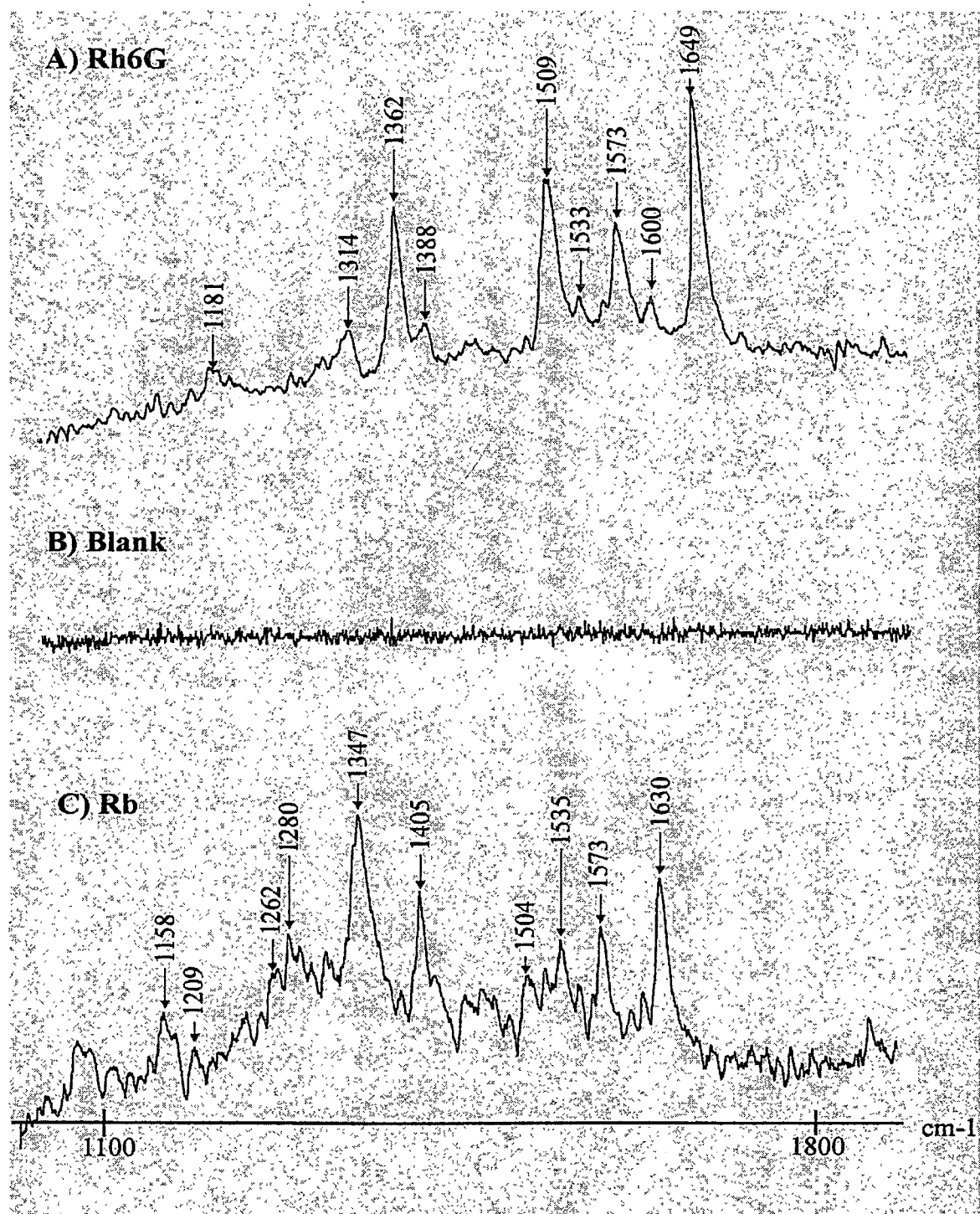


Figure 4.7. CE-SERS spectra. The spectra corresponding to points A, B, and C in Figure 4.6 are (A) SERS spectrum of 1×10^{-8} M Rh6G injected with an elution time of 9 minutes (B) Blank spectrum between the two analytes of interest at a time of 11 minutes (C) SERS spectrum of 5×10^{-6} M Rb injected with an elution time of 13 minutes. CE conditions were same as in Figure 4.6. SERS conditions were 514.5 nm, 17 mW, and 1 s acquisition time.

[154, 155]. It is shown that on-the-fly CE-SERS detection is feasible upon the addition of non-optimized silver colloidal solution as running buffer for a separation of two test compounds. The amount of Rh6G injected is 90 attomoles (9.0 nL at 1.0×10^{-8} M). Assuming a Gaussian band profile, the concentration of Rh6G at peak center is 2.7×10^{-9} M. Thus, spectra have been acquired (S/N for most bands > 10) on-the-fly at concentrations of approximately 10^{-9} M (see Figure 4.7). With optimization of the silver colloidal solution and other parameters we expect improved detectability. Improvements in spectrometer design are also desirable both in terms of increasing signals via greater optical throughput and increasing spectra collection frequency (see below).

A preliminary evaluation of improvements in the spectrometer design was done. The Dilor instrument was upgraded with a new CCD detector and software which would allow for continuous, sequential spectral acquisition allowing for higher CE flow rates to be used. To test the capabilities of the instrumental improvements, a four component mixture containing Rh6G, Rb, sodium fluorescein (Fl), and dibenzylfluorescein (DBF) was used. Figure 4.8 is a CE-LIF separation of the test mixture with a RB containing Ag colloidal solution, 2 mM sodium borate, and 10 mM sodium chloride (NaCl). Using a higher CE flow rate (8 kV) than previously used, all four components are separated in under six minutes. Figure 4.9 is a three dimensional (3D) plot of the same four component separation shown in Figure 4.8, but with SERS detection demonstrating the feasibility of the instrumental improvements. On-the-fly spectra were acquired for one second every two seconds in a continuous format for a period of seven minutes.

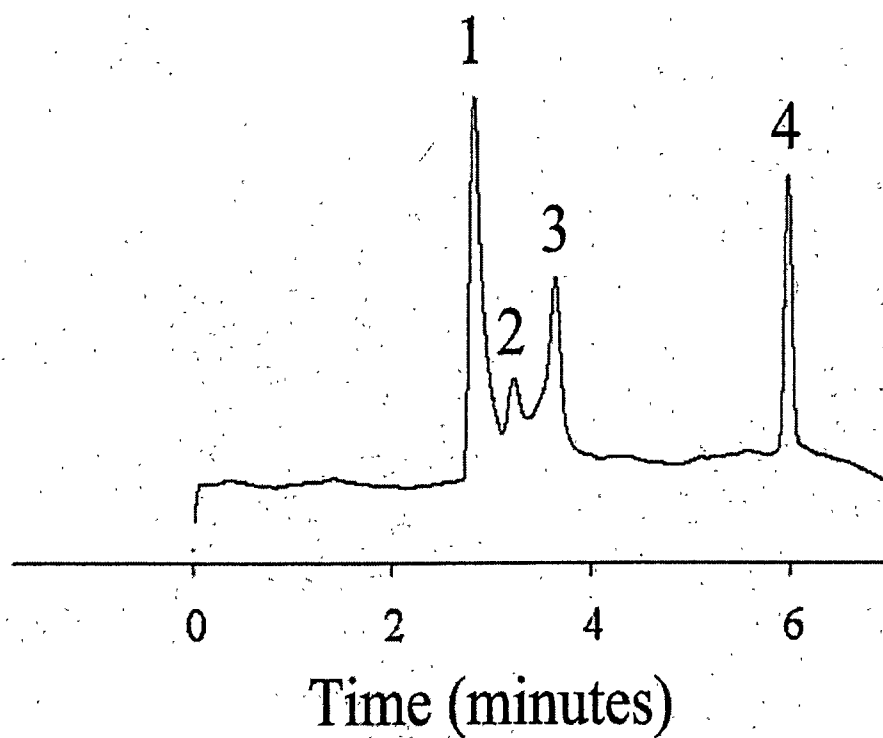


Figure 4.8. CE-LIF separation of four component mixture. Elution order is (1) 1×10^{-6} M Rh6G, (2) 1×10^{-5} M Rb, (3) 1×10^{-5} M DBF, and (4) 1×10^{-6} M FI. RB contained silver colloidal solution in 2 mM sodium borate and 10 mM NaCl. The CE running voltage was 8 kV.

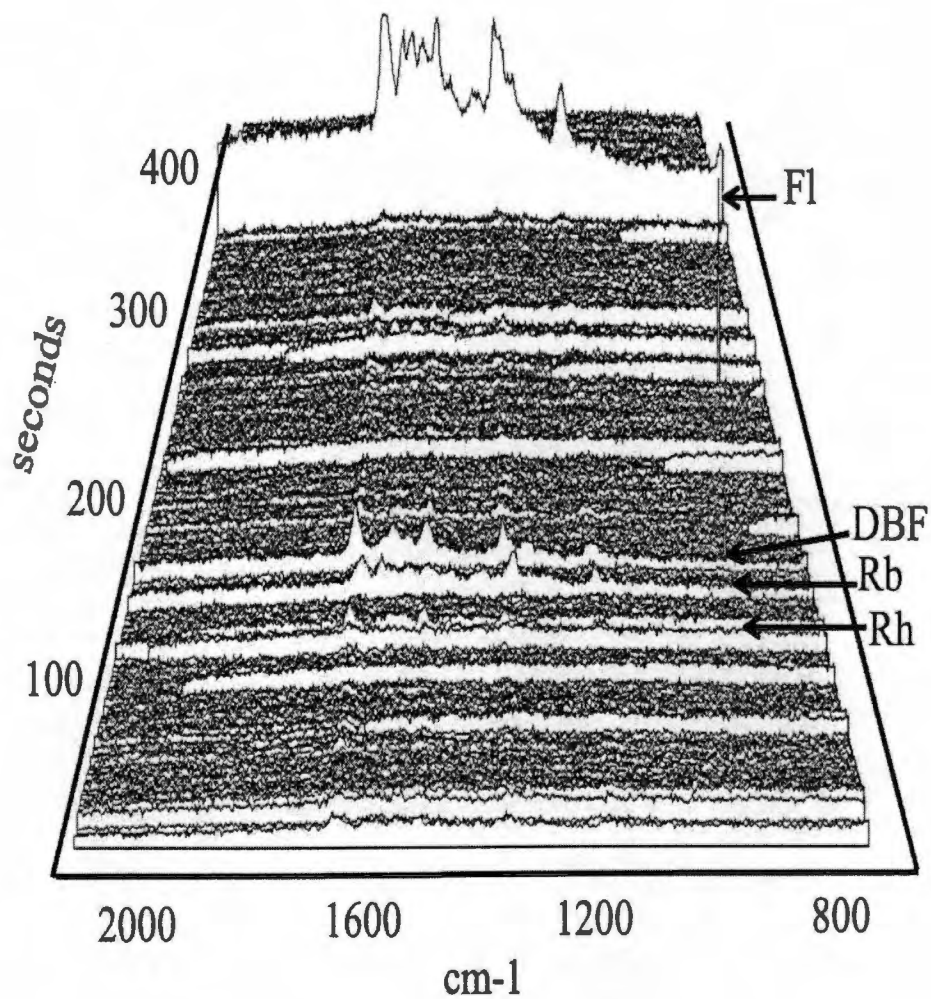


Figure 4.9. Three-dimensional plot of test mixture. Experimental conditions were the same as in Figure 4.8. Concentrations injected were 1×10^{-8} M Rh6G and 1×10^{-6} M Rb, DBF and F1. Spectra was acquired 1 second every 2 seconds.

CONCLUSIONS

The successful first coupling of CE with SERS detection was demonstrated. A test separation of two test compounds, Rh6G and Rb, showed was shown. Even though optimal CE conditions were not used (i.e. high electrokinetic flow rates and smaller i.d. capillaries) for this test separation the integrity of the separation was maintained. Little effect on the integrity of the CE test separation was shown when incorporating silver colloidal solutions as a buffer additive in CE. Parameters that affect SERS spectra such as laser power, acquisition time, laser wavelength, and rinsing of the CE capillary was shown. In addition, preliminary work, which allowed for the acquisition of spectra in a continuous format, was done generating useful 3D plots of wavenumbers vs. time vs. intensity.

CHAPTER 5

CONCLUDING REMARKS

Overall, the development of detection methodologies for CE was a testing and gratifying experience. The successful advancement and implementation of these detection methodologies is described. Although conclusions were drawn in each of the preceding data chapters, an overall conclusion, with some remarks regarding the future of the described detection methodologies, is provided here.

The previous development of the CEMSA technique for the study of DNA-protein interactions combined the advantages of CE with those of a mobility shift assay. A well-characterized system between the *trp* repressor of *E. coli* and the *trp* operator served as a test assay for this new technique. Different methods of detection served to be problematic for CEMSA. One method employed was to fluorescently label the DNA, which allowed for LIF detection instead of radiolabeled detection. However, this pre-column derivatization approach suffered from poor labeling efficiencies. In addition, another method tried was the use of an intercalation dye such as EB to help improve detection sensitivity of the system; however, problems arose. For instance, upon the addition of EB pre- or on- column, the DNA-protein interaction was disrupted. In order to preserve the integrity of the DNA-protein interaction and allow for lower detection limits a new design was warranted. Combining the advantages of superior detection sensitivity and the use as a post-column reactor cell, SFCs were employed. The SFC arrangement allowed for a post-column derivatization procedure where the EB was added after the separation; therefore, the DNA-protein interaction was maintained.

Furthermore, the SFC approach improved LIF detection limits for a sample of Φ X-174 DNA digested with *HaeIII*.

While this implementation of the SFC arrangement as a post-column derivatization cell for DNA-protein interaction was successful, further improvements can be made. Alternative intercalating dyes or more efficient fluorescent labeling procedure could be used to increase the detectability of the DNA-protein interaction. In addition, increased sensitivity could be accomplished by an improvement in the SFC design that would facilitate an easier more reproducible optical alignment procedure. With these improvements in the SFC design, additional studies on the *trp* repressor/*trp* operator can be done that would include the determination of a variety of parameters inherent to DNA-protein interactions.

Many SFC designs used today are based on the same geometry of flow cell and optical setup. There are drawbacks to these designs including difficulty in alignment, intra day reproducibility, size and cost. Most traditional LIF setups whether based on a SFC or other cell arrangement, use far-field imaging to spatially filter any background signal. A refinement in these approaches was warranted.

A new pre-imaging mode of spatial filtering was introduced successfully reducing sources of background from Rayleigh scatter, spectral scatter, and fluorescence. The pre-imaging mode offered the added advantage over far-field imaging in that it occurs right at or very near the optical interface between the excitation source and the flow cell eliminating additional optical components, thus dramatically reducing the size of the optical apparatus. Furthermore, with this design the optical alignment became easier and more reproducible. However, in order to use the pre-imaging mode of spatial filtering a

non-traditional rectangular flow cell geometry was employed offering the same advantages as previously mentioned SFCs. In addition, the proposed SFC design can be effectively used with an UV laser as an excitation source which expands the range of analytes that can be used without some form of derivatization. With the previously mentioned features, this new rugged, compact design can be used for routine practical analyses.

Qualitative analysis in CE is often accomplished by peak identification from migration times, which can be difficult due to poor run-to-run reproducibility from varying flow rates or column degradation. If it were possible to obtain structural information from an actual CE separation, then the affects of these sources of poor reproducibility in CE would be minimal. With the first ever coupling of SERS detection to CE, obtaining structural information from a CE separation was realized. With the recent introduction of CE-SERS, many cursory studies important to the development and future of this detection methodology were necessary. The effects of included laser power, acquisition time, laser wavelength, silver particle concentration, and running buffer composition on the integrity of SERS spectra were done. The applicability of this method was demonstrated with an on-the-fly spectrum obtained for a two component mixture. In fact, in the case of Rh6G an injected quantity was approximately 90 attomoles. However, there is room for improvement. Preliminary work on instrumental improvement yielded a 3D plot for the separation of a four component mixture by CE.

Future studies may include the optimization of the silver colloidal solution preparation in terms of reproducibility and particle size. It may be important to match particle size with excitation laser wavelength. The use of smaller i.d. capillaries (75 μm)

with higher CE flow rates (applied voltage) will allow for a more complex test separation to be used. Improved instrumentation capable of acquiring data in a repetitive mode in faster times would allow a more "realistic" CE mode of operation and use the data to generate 3D plots (intensity vs. wavenumber vs. time). In addition, a real world sample is needed to demonstrate SERS as a viable detection method for not only CE but also for the scientific community. Because CE-SERS has just recently been introduced, much is still being learned and many possible applications exist for this exciting detection methodology.

In conclusion, it has been shown that these innovative detection methodologies further promote CE as a powerful analytical tool. In the preceding chapters, each demonstrating an individual detection method that set the stage for the further development and implementation of CE into the general scientific community. While some of these detection methodologies need refinement, they will become more robust and significantly more important as they continue to grow and mature.

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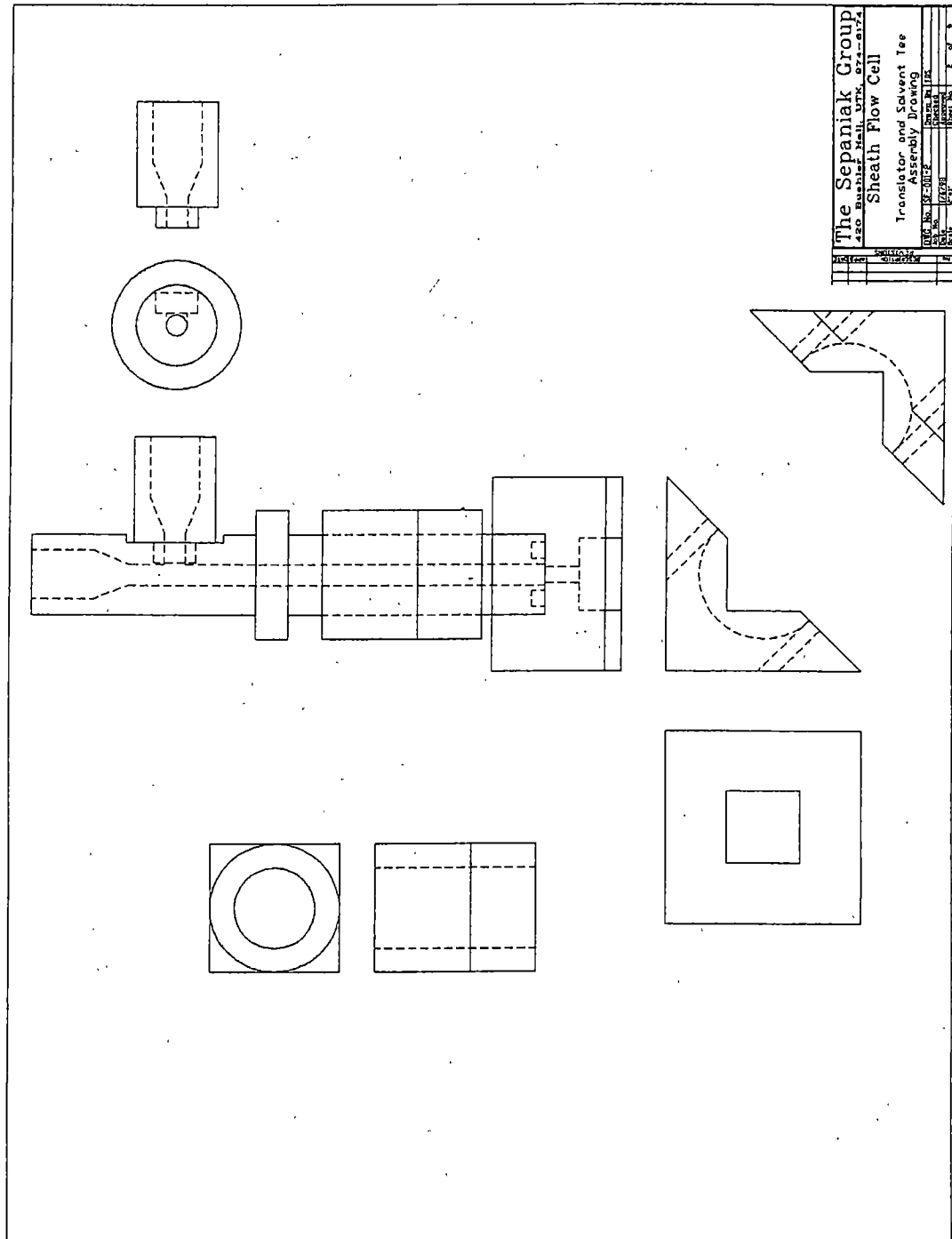
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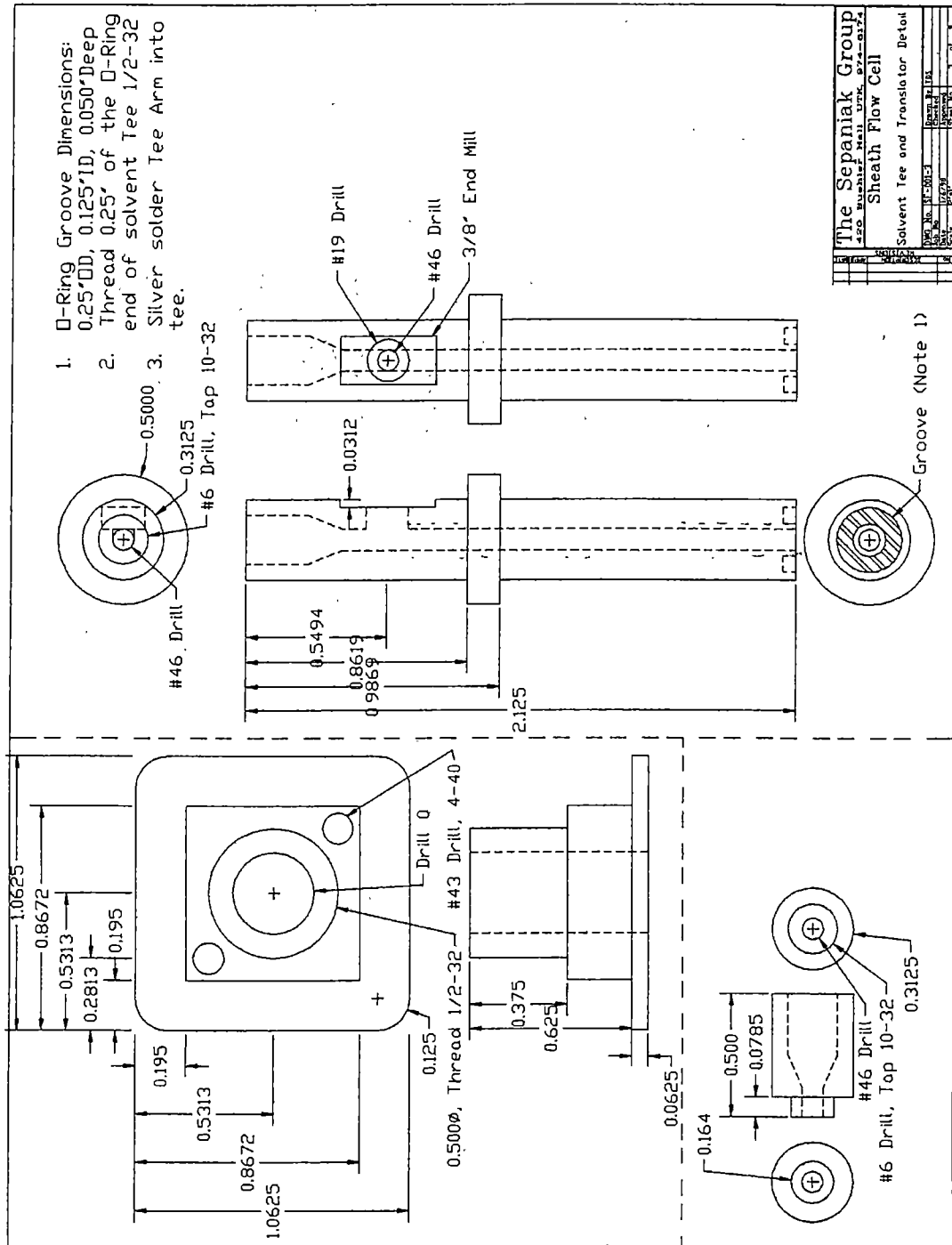
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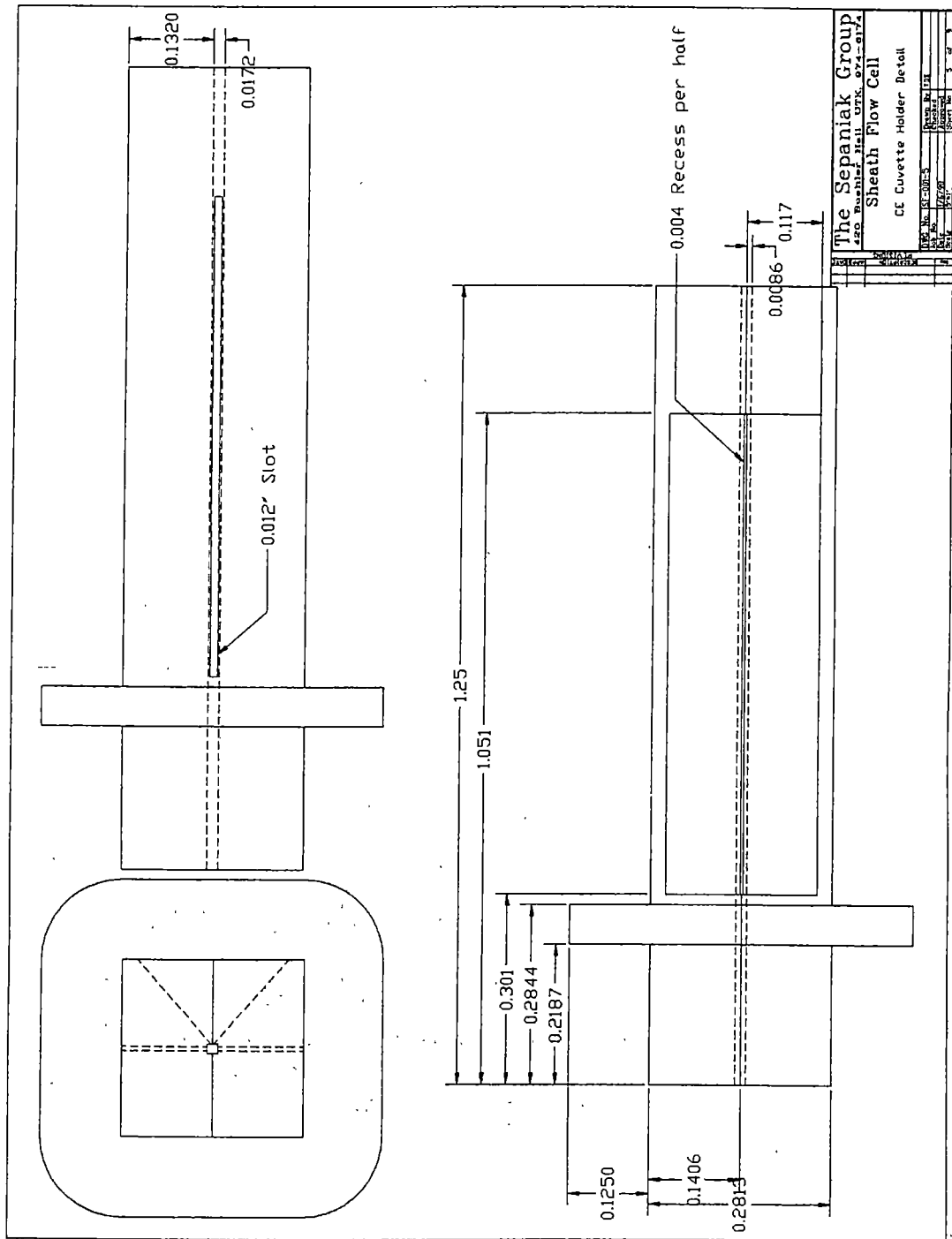
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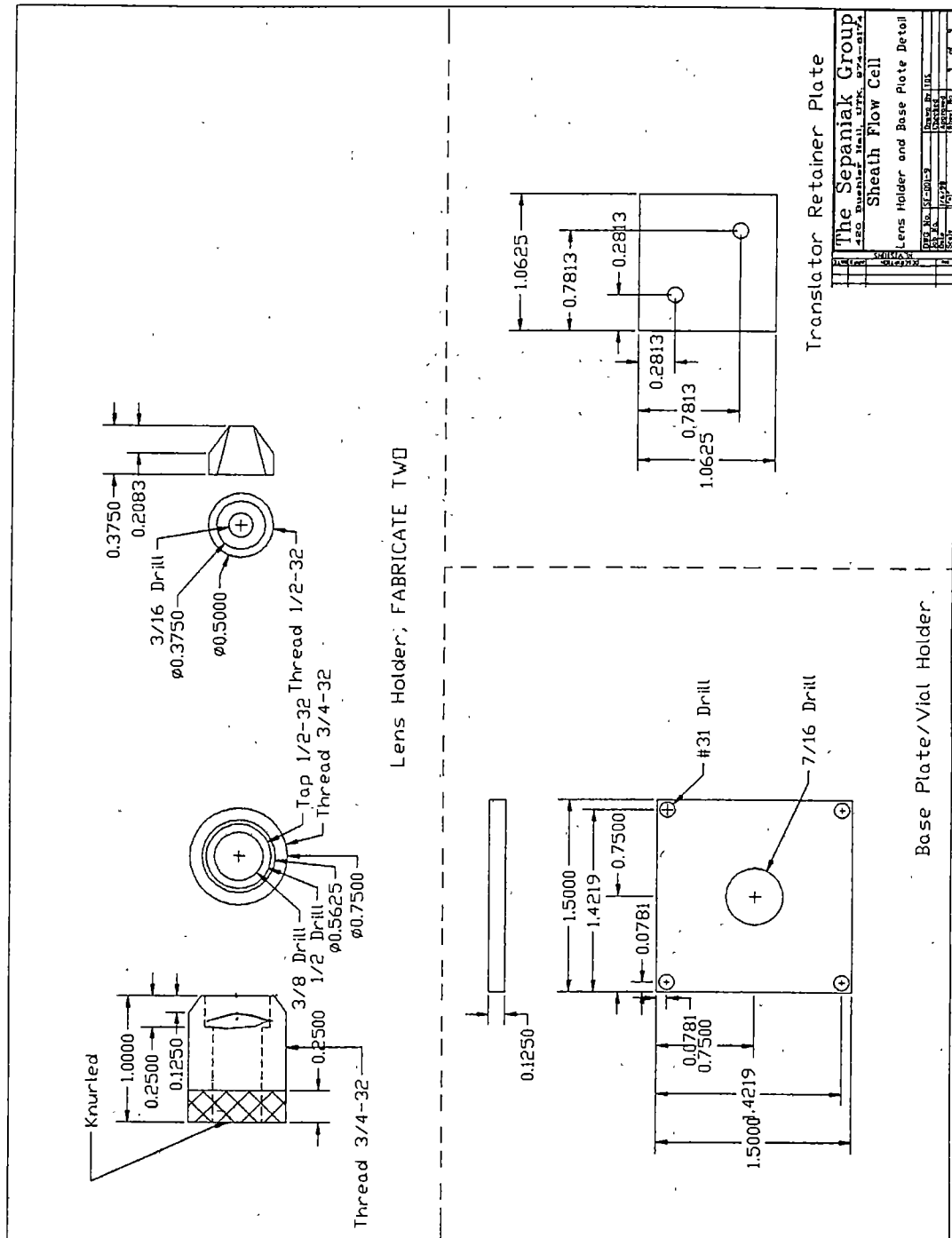
APPENDIX

APPENDIX









VITA

William Franklin Nirode was born in a small town outside of Cleveland, Ohio in 1973. He was raised in this same small town and graduated from high school in 1991. After graduation he entered Baldwin-Wallace College in Berea, Ohio a short distance from Cleveland, Ohio where he graduated in 1995 with a Bachelor of Science degree in Chemistry. Upon graduation, he entered graduate school at The University of Tennessee, Knoxville undertaking research in analytical chemistry. He earned his Ph.D. in Chemistry in July 2000 and will be employed at Hofstra University in Hempstead, New York as an assistant professor of analytical chemistry.