Nonlinear degenerate four-wave mixing is presented as an ultrasensitive optical absorption-based method for detection and measurement of biological samples. Wave-mixing imaging detection technique can localize and quantify biomolecules in single cells and tissue sections with excellent spatial distribution of light absorbed by a target sample. Cellular components can be label-free or labeled with a chromophore or a fluorophore and imaged by wave mixing using a CCD camera. In a 2-D forward-scattering wave-mixing geometry, two overlapping laser beams form interference gratings and transfer their energy to an absorbing medium, creating thermal gratings followed by changes in the refractive index. The probe beam diffracts off these laser-induced gratings to produce the signal beam, which is detected by a CCD camera or a photodiode. A single bio cell can be placed in a glass slide and as the laser beams probe the labeled cellular component, the CCD camera captures wave-mixing signals corresponding to the absorbing cellular components. This nonlinear imaging technique can be used for both live and fixed cells in real time to obtain information on sequential changes in the number, morphology and distribution of cellular components in a single cell.
Nonlinear laser wave-mixing spectroscopy coupled with capillary electrophoresis provides a novel ultrasensitive method for single-cell protein analysis. This method is used to detect proteins separated within a single cell. Nonlinear wave mixing has many advantages including quadratic dependency on analyte concentration, high spatial resolution and small sample requirements. Furthermore, wave mixing offers excellent detection sensitivity levels even when using very short optical path lengths, and hence, it can be easily interfaced to capillary electrophoresis separation systems. A single cell is injected into a coated capillary, lysed and labeled inside the capillary with a chromophore. Labeled proteins are separated in a sieving matrix under applied voltage through the capillary based on their mass-to-charge ratio differences.

To further improve protein separation, a random amphiphilic copolymer, poly(n-dodecylacrylate-3-sulfopropylmethylacrylate) or C\textsubscript{12}SO\textsubscript{3}H (25/75%), is synthesized and used for protein separation in capillary electrophoresis. Amphiphilic polymers offer many benefits for protein separation. They can be used in both hydrophobic and hydrophilic moieties, they are easy to synthesize, they require less polymer percentage, and they are cost effective. Shorter analysis times have been obtained with this polymer for standard proteins. Amphiphilic polymers can be used for the analysis of hydrophobic proteins to obtain higher separation efficiency and resolution. Nonlinear laser wave mixing coupled with this new polymer can enhance CE separation of proteins.
High throughput CE is performed for the analysis of many samples in a short time scale. Using wave mixing, chromophore-labeled proteins passing through the capillary windows can be detected in an array of capillaries lined up tightly. Signal spots are collected by a photodiode array with an NMOS image sensor. The wave-mixing signal is a collimated coherent laser-like beam, and hence, it can be collected with nearly 100% optical collection efficiency against a dark background. Unique features include short optical path lengths, quadratic dependency on sample absorption coefficient and concentration, and cubic dependency on laser power.

Deep UV 266-nm laser wave-mixing detection is presented as a novel sensitive method for proteins in their native form without using labels. We demonstrate absorption-based wave mixing as an inherently effective detection method for several viable proteins in their native form using capillary electrophoresis. Excellent detection sensitivity levels are obtained using this unusually sensitive absorption-based method.
Protein Analysis at the Single Cell Level by Nonlinear Laser Wave-Mixing Spectroscopy for High Throughput Capillary Electrophoresis Applications

by
Behrokh Bagherifar Sadri

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

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APPROVED BY:

_____________________________  _____________________________
Edmond Bowden     Charles Boss

_____________________________  _____________________________
Kenneth Hanck     William Tong
Co-Chair of Advisory Committee

_____________________________
Morteza Khaledi
Chair of Advisory Committee
DEDICATION

I dedicate my dissertation to my wonderful parents Dr. Jafar Bagherifar (MD) and Nosrat Nasser for the way they raised me and with their love, encouragements and support I became the person I am today.

Furthermore, I dedicate my dissertation to my wonderful, loving and caring husband Dr. Ali Soheil Sadri (PhD), my handsome son Sena Ali Sadri and my beautiful newborn daughter Salena Rojean Sadri for all their love and patience.

I love you all forever!
BIOGRAPHY

Behrokh Bagherifar Sadri was born and raised in Tehran, Iran. She had always been an honored student and highest ranking in her class throughout high school.

She obtained her Bachelor of Science degree in Applied Chemistry from the University of Tehran, Iran in 1993. Behrokh also received a Bachelor of Art degree in English Language Translation, at the same time, from the Azad University of Tehran, Iran.

Following graduation she began working in an analytical instrument company as technical customer service manager. Shortly after her initial job, Behrokh and two other coworkers founded an importing company called Pars Dafineh Tadjhiz.

Behrokh move to the US in 1997 and joined the graduate school at North Carolina State University, chemistry department in 1999. She completed all course works and the qualifier exams for PhD candidacy in 2001.

Behrokh along with her family moved to San Diego, CA in 2002. She continued her research at San Diego State University as a collaborative research with North Carolina State University under Dr. Lifang Sun direction. Due to personal reasons Dr. Lifang Sun departed from the school and Behrokh joined Dr. William Tong research group at SDSU in 2004. Behrokh joined Pfizer Inc in 2004 and continued her efforts to finish her degree for the third time in spite of all the responsibilities she had at work, school and home as a Mom. Dr. Behrokh Sadri graduated in 2008.
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# TABLE OF CONTENTS

LIST OF TABLES ........................................................................................................ xiv

LIST OF FIGURES ..................................................................................................... xv

CHAPTER 1. INTRODUCTION .................................................................................. 1

Motivation .................................................................................................................. 1

Laser Application in Analytical Spectroscopy ....................................................... 2

Laser Medium .......................................................................................................... 2

Laser Types .............................................................................................................. 4

Review of Laser Application in Previous Works .................................................. 5

Non-linear Laser Wave-mixing Spectroscopy ....................................................... 7

Dissertation Outline ............................................................................................... 7

Reference Cited ....................................................................................................... 9

CHAPTER 2. DEGENERATE FOUR-WAVE MIXING THEORY ............................. 11

Degenerate Four Wave Mixing .......................................................................... 11

Formation of Grating ........................................................................................... 13

Interference Pattern ............................................................................................. 13

Diffraction .............................................................................................................. 17

D4WM Optical Geometry ................................................................................... 17

Generation of D4WM Signal .............................................................................. 19

Reference Cited ................................................................................................... 23
CHAPTER 3. EXPERIMENTAL SETUP

Optical Setup

Laser Excitation Sources
  Argon Ion Lasers
  Helium-Neon Laser
  Frequency-quadrupled Nd-YAG Laser

Sample Cells
  Fused Silica Capillaries
  Microchip

Capillary Electrophoresis System
  Capillary Conditioning and Control
  Peristaltic Pump
  XYZ Translational Stage

Signal Detection and Processing
  Photodetector
  Photodiode Array
  CCD Camera
  Microscope

Reference Cited

CHAPTER 4. AMPHIPHILIC POLYMERS: SYNTHESIS, CHARACTERIZATION AND LINEAR SOLVATION ENERGY
Introduction ........................................................................................................ 112

Preventing Protein Absorption on Capillary Wall ........................................... 113

pH Effect .............................................................................................................. 113

Permanent and Dynamic Coating ................................................................... 114

Improving CE Detection Limits ..................................................................... 115

Laser Induced Fluorescent (LIF) ................................................................. 115

Conventional CE Techniques for Protein Analysis ...................................... 117

Experimental: Materials and Methods ....................................................... 119

Reagents ............................................................................................................. 119

Cell Culture ....................................................................................................... 119

Capillary Electrophoresis Setup ........................................................................ 120

Capillary Coating ............................................................................................. 120

Protein Labeling ............................................................................................... 121

Cell Injection .................................................................................................... 124

Apparatus .......................................................................................................... 125

Results and Discussion .................................................................................... 127

Reproducibility and Limit of Detection .......................................................... 127

Molecular Weight Estimation ......................................................................... 139

Conclusion ......................................................................................................... 142

Reference Cited ................................................................................................. 144

CHAPTER 7. AMPHIPHILIC POLYMER-BASED PROTEIN SEPARATION
CHAPTER 9. NATIVE CELLULAR PROTEIN ANALYSIS BY CAPILLARY ELECTROPHORESIS AND NON-LINEAR LASER WAVE-MIXING SPECTROSCOPY

Introduction ................................................................. 209
Experimental ............................................................... 211
Reagents ................................................................. 211
Real-World Samples ....................................................... 211
Mouse Mitochondria Heat Shock Proteins ............................... 211
Leech Ganglia ............................................................. 213
Optical Setup .............................................................. 214
Capillary and Dynamic Coating ........................................... 216
Results and Discussion .................................................... 216
Conclusion ................................................................. 228
Reference Cited ............................................................ 229
## LIST OF TABLES

| Table 3.1 | Laser sources and experimental specifications | 27 |
| Table 3.2 | Capillaries Dimensions | 28 |
| Table 4.1 | Monomer ratios | 42 |
| Table 4.2 | Polymers estimated molecular weight | 48 |
| Table 4.3 | pH and electrophoretic mobility for poly (2-EtHA-AA) | 51 |
| Table 4.4 | Test solute and their descriptor and log k’ values obtained with poly (C₆-AA) | 66 |
| Table 4.5 | LSER model for poly (C₆-AA) | 67 |
| Table 4.6 | Comparison of log k’ values in poly (2-EtHA-AA) and its partitioning in 1-octanol/water | 73 |
| Table 6.1 | Retention time and molecular weight of standard proteins | 141 |
| Table 7.1 | MW estimation of polymer | 154 |
| Table 8.1 | Image size calculation for lens with different focal length | 184 |
| Table 8.2 | Divergence angle measured experimentally in z-axis | 184 |
| Table 8.3 | Divergence angle and beam spot calculation (top view) | 185 |
| Table 8.4 | Image size calculation when signals are not focused Capillary dimension: Tube capillary 100um I.D., 234um O.D, 13um coating | 190 |
| Table 8.5 | Recommended capillary | 193 |
| Table 8.6 | Signal image file size when collected by a CCD camera. Calculation for a 30 minutes run is based on the real data collection for one minute run | 197 |
LIST OF FIGURES

DEGENERATE FOUR-WAVE MIXING THEORY

Figure 2.1   Interference grating of the two pump beams $I_1$ and $I_2$, which creates thermal modulation in absorbing medium ............ 12
Figure 2.2   Interaction of four light beams and their propagation vectors. 14
Figure 2.3   Grating period is proportional to the angle of the pump beams and the wavelength ................................. 16
Figure 2.4   (A) Backward-scattering (B) forward-scattering wave-mixing signal in liquid phase. Only one of the signal beams is collected................................................ 18
Figure 2.5   Exact locations of signal beams are predictable................. 22

EXPERIMENTAL SETUP

Figure 3.1   Optical setup for forward-scattering wave mixing .............. 26
Figure 3.2   Capillary holder......................................................... 30
Figure 3.3   Micro channel dimension (top and side view).................... 31
Figure 3.4   Custom built capillary electrophoresis............................ 33

AMPHIPHILIC POLYMERS: SYNTETHSIS, CHARACTERIZATION AND LINEAR SOLVATON ENERGY RELATIONSHIP (LSER) STUDY

Figure 4.1.a   GPC results for PS standards of 780KD, 100KD, 35KD, 8000D and 4600D with retention times of 5.978, 6.874, 7.143, 7.590 and 7.669 min are shown................................. 46
Figure 4.1.b   GPC results for the set of synthesized polymers with retention times of greater than 7 minutes confirms polymers formations... 47
Figure 4.2   Plot of log (MW) of the standards vs. their retention time $y=-1.6121x + 16.077$ and $R^2=0.9939$)........................................ 49
Figure 4.3   Electrophoretic mobility of poly (2-EtHA-AA) at different pH values................................................................. 52
Figure 4.4   Electropherogram of polymer at different pH levels............. 53
Figure 4.5   Titration curve for poly (2-EtHA-AA)................................. 54
Figure 4.6.a.   Polymer concentration effect on electrophoretic mobility for poly (2-EtHA-AA).................................................. 56
Figure 4.6.b  Polymer concentration effect on electrophoretic mobility for poly \((C_{12}\text{-SO}_3\text{H})\)………………………………………………………………………………… 57

Figure 4.7  Comparison of retention factors, \(k'\), for 0.3% poly (2-EtHA-AA) (bottom) and 0.5% poly (2-EtHA-AA) (top)…………………………………………………………………………………………… 59

Figure 4.8  Temperature variation study and its effect on \(k'\) for a homologue series of alkylbenzoate using poly (2-EtHA-AA) as micelle………………………………………………………………………………… 62

Figure 4.9  Plot of log \(k'\) vs. \(1/T\) for a homologue series of alkylbenzoates using poly (2-EtHA-AA) as micelle…………………………………………………………………………………………… 63

Figure 4.10  Plot of log \(P_{ow}\) vs. log \(k'\) for poly (C\(_6\text{-AA}\))…………………………………………………………………………………………………………………………… 71

Figure 4.11  Plot of log \(k'\) poly (C\(_6\text{-AA}\)) vs. log \(k'\) SDS …………………… 72

Figure 4.12  Compatibility of log \(k'\) for Poly (2-EtHA-AA) with log \((P_{ow}) \)………………………… 74

IMAGING OF DISTRIBUTED ABSORBING SPECIES WITHIN A SINGLE CELL BY NONLINEAR LASER WAVE MIXING SPECTROSCOPY

Figure 5.1  Experimental optical setup: capillary can be replaced with microchannel, glass slide or flow cell ………………………………………………………………………………………………………… 84

Figure 5.2  A single cell flowing inside a capillary ………………………………………… 86

Figure 5.3  Image of two labeled mitochondria within a single cell obtained by confocal microscopy…………………………………………………………………………………………………………………………… 90

Figure 5.4  Wave-mixing signal of a single cell is compared to that of a running buffer. No signal is observed for buffer, but signal is observed for each cell passing through the capillary………………… 91

Figure 5.5  Flow study of single cells. A decreasing number of cells pulling through the capillary yield a smaller number of signal peaks……… 92

Figure 5.6  Single cell detection is shown in a microchannel……………………………………… 94

Figure 5.7  2-D detection of non-homogenous dye and mesh grid by a photodetector (top) and CCD camera (bottom)…………………………………………………………………………………………………………………………… 96

Figure 5.8  Comparison of the wave-mixing image of the dye mesh grid collected (A) under an inverted phase 10X microscope and (B) CCD-based wave-mixing imaging……………………………………………………………………………………………………………………………………… 97

Figure 5.9  Mapping of labeled mitochondria within a single cell ……………… 98

Figure 5.10  Single cell images obtained from (A) fluorescent microscopy and (B) wave-mixing imaging. Cell surface antibodies are directly conjugated to the Cy5 fluorescent probe ………………………………………… 100
SINGLE-CELL PROTEIN ANALYSIS BY CAPILLARY SIEVING ELECTROPHORESIS (CSE) AND LASER WAVE-MIXING SPECTROSCOPY

Figure 6.1 Chemical structure of chromophore QSY35 and UV-Vis spectra of labeled and non-labeled protein 122
Figure 6.2 Optical setup for CSE wave mixing 128
Figure 6.3 Reproducibility for three separate CE runs for 10 µg/mL β-Lactoglobulin 129
Figure 6.4 Concentration variation study for β-lactoglobulin: From bottom to top: 10 mg/mL, 10 ng/mL and 10 fg/mL of β-Lactoglobulin injected 131
Figure 6.5 Electropherogram of β-Lactoglobulin when only 10 fg/ml protein standard is injected A concentration detection limit of 3.58 x 10^-16 M and a mass detection limit of 2.2 ymole determined 132
Figure 6.6 Comparison of electropherograms of β-Lactoglobulin protein within a single cell, protein standard (10 µg/mL) and a mixture of protein standards including β-Lactoglobulin (1 µg/mL) 134
Figure 6.7 Day to day reproducibility for β-Glactosidase (10 µg/mL) 136
Figure 6.8 Comparison of electropherograms of β-Glactosidase protein within a single cell, protein standard (10 µg/mL) and a mixture of protein standards including β-Glactosidase (1 µg/mL) 137
Figure 6.9 β-Glactosidase protein is identified in four single cells injected individually 138
Figure 6.10 Molecular weight estimation plot for a mixture solution containing Orange G, \(\beta\)-lactoglobulin, trypsin inhibitor, carbonic anhydrase, ovalbumin and \(\beta\)-galactosidase ………... 140

Figure 6.11 Comparison of electropherograms obtained for single cell (top) and the standard mixture (bottom)…………………………………… 143

AMPHIPHILIC POLYMER FOR PROTEIN SEPARATION BY CAPILLARY ELECTROPHORESIS AND LASER WAVE-MIXING SPECTROSCOPY

Figure 7.1 Optical D4WM Setup………………………………………………………… 152
Figure 7.2 Electropherogram of protein standards 10 \(\mu\)g/mL \(\beta\)-lactoglobulin and Tripsyn inhibitor (2% polymer, p\(\text{H}\) 8.6, 12 kV). \(\beta\)-lactoglobulin is eluted earlier than trypsin inhibitor due to its smaller MW …………………………………………………… 159
Figure 7.3 Reproducible results (1309.3 +/- 5.5 s) for protein standard 10 \(\mu\)g/mL trypsin inhibitor (2% polymer, p\(\text{H}\) 8.6, 12 kV)………………….. 160
Figure 7.4 Reproducible results (752.7 +/- 1.1s) for protein standard 10 \(\mu\)g/mL \(\beta\)-lactoglobulin (2% polymer, p\(\text{H}\) 8.6, 12 kV)………………….. 161
Figure 7.5 Comparison of retention times for the protein standard \(\beta\)-lactoglobulin using 2% pullulan (bottom) and 2% polymer (top). All the other conditions are the same. A faster elution time is observed when the polymer is used as the running buffer……….. 162
Figure 7.6 A faster elution time is observed at pH 6.54 for \(\beta\)-lactoglobulin (top) as compared to that at pH 8.6 (bottom)………………….. 163
Figure 7.7 Concentration variation study. A faster elution time is obtained when a 1% polymer is used (bottom) as compared to the 2% polymer (top)………………………………………………………… 165
Figure 7.8 Day-to-day comparison for the \(\beta\)-lactoglobulin protein standard using the 1% polymer running buffer. …………………….. 166

HIGH THROUGHPUT PROTEIN SEPARATION BY CAPILLARY ELECTROPHORESIS WITH NON-LINEAR LASER WAVE-MIXING DETECTION

Figure 8.1 (a) Beam shape after a cylindrical lens in the form of a thin line, (b) expanded and focused beams resulting from a cylindrical lens and a convex lens ……………………………………. 179
Figure 8.2  Optical setup for high-throughput CE using a 488-nm argon-ion laser. A cylindrical lens expands the focused beams on the capillary array with 10 square capillaries

Figure 8.3  Array of 8 fused Silica square capillaries (71 um I.D., 360 um O.D., 37 cm total length, 25 cm effective length)

Figure 8.4  Capillaries with different diameters

Figure 8.5  Signal divergence (a) side view: image size is measured experimentally at the 3-foot distance, (b) top view: divergence angles of beams and beam-spot distances at the 3-foot distance in the xy-axis are calculated assuming that $\theta_1 = \theta_2$

Figure 8.6  (a) Focused Gaussian beam at waist radius. (b) Fresnel diffraction from a Gaussian aperture assuming that capillary I.D. = $2W_0$

Figure 8.7  Signal divergence, laser leakage and beam interferences for an array of 10 square capillaries

Figure 8.8  Comparing signal quality when capillary gaps are covered and uncovered. When the gaps are covered, improved signal intensity is obtained (bottom). The probe beam is blocked and unblocked three times to show the signal intensity

Figure 8.9.a  Image of 8 signal spots obtained by a CCD camera for an array of 8 capillaries (left) and images when chopped beam is blocked (right). 1376 x 1024 pixels, 4-mm image size

Figure 8.9.b  Signal spots from all capillaries recorded by a single photodiode. Probe and chopped beam are blocked and unblocked three times and once, respectively, to show the signal and the background

Figure 8.10  A portion of the photodiode array and its pixel dimension (not to scale)

Figure 8.11  Signal comparison for the capillary array of 8 square capillaries when the probe beam is unblocked (top) and blocked (bottom). Each peak corresponds to the signal generated from each capillary in the array. Differences in the number of pixels affected by each signal are due to slight variations between capillaries

Figure 8.12.a  High throughput CE wave-mixing results from an array of 10 square capillaries when 10 fg/mL b-lactoglobulin is injected and separated at 12 kV
Figure 8.12.b  When four-point smoothing is used for capillary 6 (from bottom to top)……………………………………………………………………………... 203

Figure 8.13  Day-to-day reproducibility for Orange G and β-lactoglobulin. First peak is for Orange G (10 kD) at frame number 550 and second peak is for β-lactoglobulin (18.3 kD) at frame number 835 ……………………………………………………………………………… 205

Figure 8.14  Probe volume calculation for a square capillary with 71 um I.D. Laser beam width at the focal pinot is 1070um………………… 206

NATIVE CELLULAR PROTEINS SEPARATION BY CAPILLARY ELECTROPHORESIS AND NON-LINEAR LASER WAVE-MIXING SPECTROSCOPY

Figure 9.1  Experimental capillary electrophoresis wave-mixing setup……… 215

Figure 9.2  Electropherogram of trypsin inhibitor protein standards separated in a 37 cm square capillary (71 um I.D., 360 um O.D., 22 cm effective length, 12 kV applied voltage) …………………… 217

Figure 9.3  Electropherogram of carbonic anhydrous protein standards at 10.3 pg/mL (bottom) and 10.3 fg/mL (top) obtained under the same separation conditions. Results are reproducible at different concentration levels ……………………………………… 218

Figure 9.4  CE run for 10.3 fg/mL carbonic anhydrase protein standard. Limit of detection of 1.3 ymole is obtained …………………… 220

Figure 9.5  Day-to-day reproducibility check for trypsin inhibitor protein standards under the same separation conditions ………………… 221

Figure 9.6  Electropherogram for mouse mitochondria heat proteins (18.8 pg/μL) …………………………………………………………… 222

Figure 9.7  Electropherogram for leech ganglion 5 (vortexed)……………… 224

Figure 9.8  Comparison of electropherograms for leech ganglion 5 supernatant (bottom) and vortexed (top) ……………………… 225

Figure 9.9  Comparison of electropherograms of leech ganglion 5 and ganglion 10………………………………………………… 226

Figure 9.10  Comparison of electropherograms of ganglion 5 from two different leeches …………………………………………… 227
Chapter 1

Introduction

1.1. Motivation

Nonlinear laser wave mixing is a one-color one-laser sensitive spectroscopic tool that has been successfully used for a wide range of applications such as detection of biomolecules. The presence of biomolecules and their functions along with their interaction are important in many areas including diagnosis, prevention and treatment of many diseases. Biomolecules, especially proteins, may be used as biomarkers for early disease detection. Analyzing and detecting these extremely small amounts of biological materials are challenging. However, the outcome may be rewarding and leads to early detection of life threatening diseases. Consequently, there is a growing need for a high resolution, rapid, high throughput, and ultra sensitive detection method. Nonlinear wave mixing is presented as a suitable optical method for studying interactions of biomolecules down to the single-cell level. Wave mixing is one of the most versatile methods for generating a coherent laser-like signal beam. It is a sensitive detection method that utilizes both low-energy pulsed lasers (nJ) and low-power continuous wave (mW) lasers\textsuperscript{1, 2, 3, 4}. Furthermore, high power laser sources have made it possible to observe many novel nonlinear optical phenomena\textsuperscript{5}. 
1.1.1. Laser Application in Analytical Spectroscopy

Theodore H. Maiman at Hughes Research Laboratories in Malibu, California, observed the first successful Light Amplification by Stimulated Emission of Radiation (LASER) on an optically excited ruby rod at the 694 nm wavelength in pulse mode with a high intensity flash lamp in 1960\textsuperscript{6}. An Iranian physicist, Ali Javan, together with William Bennet and Donald Herriot, made the first gas laser using helium and neon later in the same year. Javan later received the Albert Einstein Award in 1993\textsuperscript{7}. Due to unique properties such as high power density, excellent spatial and temporal coherences, directionality, focusability, polarization, tunability, monochromaticity, spectral resolution and brightness, lasers are useful for many applications in science and industry.

These features provide orders of magnitude improvements in detection sensitivity and spectral resolution levels as compared to those of conventional methods including laser-induced fluorescence (LIF), optoacoustic spectroscopy, thermal lens spectroscopy and Raman scattering. Unique laser properties enhance new multi-photon laser spectroscopic methods including laser wave mixing, optical Kerr effect, Stimulated Raman Spectroscopy (SRS) and optical phase conjugation.

1.1.2. Laser Medium

A laser beam is created when an active medium (gain medium in an optical cavity) is energized or excited by an external energy source. An optical cavity contains
a coherent beam of light that travels multiple times between reflective surfaces and passes through the medium. When the number of the excited or higher energy particles exceeds the number of those in the lower energy state, population inversion is formed. When a beam passes through the medium with population inversion, more stimulated emission is produced and the beam is amplified. Any photons traveling across the gain medium will be amplified, but only those that are aligned with the cavity can make multiple traverses across the medium, and hence, amplified. Consequently, the laser beam is either lost to diffraction or absorption of the medium or amplified and emitted from the output aperture.

When amplification in the medium is stronger than the cavity losses, the circulating light power can rise significantly. Since stimulated emission eventually returns a particle from its excited state or higher energy level to the ground state, the capacity of the gain medium is diminished for further amplification. When this reduction increases, the gain is saturated.

When excited/amplified power reaches a balance against gain saturation and cavity losses, an equilibrium is reached. If the power is too small, the gain cannot reach to the adequate level to overcome the resonator losses. Consequently, the emitted laser will have very small power density.

In free space, the beams inside and outside the cavity are usually Gaussian distributed and are highly collimated with very small divergence. The distance over
which the laser beam remains collimated depends on the square of the beam diameter while divergence angle varies inversely with the beam diameter.

1.1.3. Laser Types

Depending on the techniques used in the cavity, such as Q-switching, mode locking or gain-switching, the laser output may be continuous wave (CW) or pulsed. When the waveform is pulsed, higher peak powers are achieved. Dye lasers and vibronic solid-state lasers can generate a wide range of wavelengths that are appropriate for generating extremely short pulses of light ($10^{-15}$ s).

Prior to applying lasers for any experiment, its safety threshold must be carefully considered. For CW lasers, power density (laser power per beam area) must be calculated. For pulsed lasers, energy density levels vary as a function of the square root of the pulse width. For ultra fast pulsed lasers, peak powers are quite high and its electric field may affect the electronic bonds and damage the samples. Depending on the type of the laser in use, optics should be chosen carefully to prevent laser damage. Depending on the wavelength ranges, there are several optical materials commonly used including borosilicate crown glasses (BK7), UV grade fused Silica, CaF$_2$, MgF$_2$, crystal Quartz, Pyrex and Zerodur.
1.2. Review of Laser Application in Previous Works

Since the beginning of the development of laser spectroscopic techniques, scientists have studied the interaction of the radiation and matters with low and high intensity lasers leading them to new linear and nonlinear laser spectroscopic methods. Nonlinear spectroscopy could provide information on the molecular states that are not observed in linear or conventional spectroscopic methods. It owes its progress to the development of nonlinear optics and nonlinear optical effects and tunable lasers in the UV, visible and IR ranges. Also, lasers can be used in a spectral region that most organic molecules are transparent, and hence, allowing interaction of light and material while minimizing surface effects. Using multiple laser beams in nonlinear spectroscopy allows more freedom in choosing polarization of incident fields as compared to conventional spectroscopic methods [5].

Using a laser, one can conduct spectroscopy in the time domain. Hartmann studied coherent transient processes such as free induction decay and photon echoes in the optical domain. These processes had been observed earlier in radio frequency ranges\(^9\).

In addition, Hahn studied the interaction of laser light with optically thick samples for the first time\(^10\). He observed that coherent re-radiation could significantly modify the response of the sample and then affect self-induced transparency in which a normally opaque sample becomes transparent to an intense light pulse. Feld noticed
Dicke super radiance in which atoms are made to undergo emission proportional to the square of the number of radiators\textsuperscript{11}.

Bloembergen developed the formalism of using intense laser light in the field of nonlinear optics\textsuperscript{12}. Franken discovered frequency mixing processes such as second harmonic generation. He used these processes to generate coherent light at new wavelengths deep in the ultra-violet and far infrared ranges\textsuperscript{13}.

Raman discovered Raman scattering which occurs when a light is scattered from a sample that is illuminated with a laser beam. When the excitation photon has higher energy than the output photon, red shift or Stokes shift is observed. Energy levels of the laser photons being shifted up or down provide chemical information. It should be noted that the Raman effect can be induced at many laser frequency ranges\textsuperscript{14}. Hellworth also studied the stimulated Raman effect and other nonlinear optical effects including four-wave mixing induced by high power lasers (large number of photons per mode)\textsuperscript{15}.

Letokhov used multi-photon absorption in atoms to ionize and detect trace quantities. This technique was used in molecules to produce large quantities of highly excited state species\textsuperscript{16}. Dehmelt and Paul developed atom traps and efficient laser cooling that creates highly localized confinement of isolated atoms\textsuperscript{17}. Chu performed dense collections of atoms\textsuperscript{18} while Weimann, Cornell and Ketterle studied Bose-
Einstein condensation. Study of these species arrested in space free of usual external perturbations provides data with extremely high resolution for fundamental studies.

1.3. Nonlinear Laser Wave-Mixing Spectroscopy

Degenerate four-wave mixing (DFWM) is one of the most sensitive nonlinear laser spectroscopic techniques. This absorption-based method uses three laser beams that interact in an absorbing medium and generates a laser-like signal beam (the forth beam). Wave-mixing signal can be created either by stimulated emission or diffraction from a laser-induced grating. Laser-induced gratings may also be generated either by Soret Effect or Dufour Effect. In Soret Effect, a diffusion flux occurs due to a temperature gradient while in Dufour Effect, a heat flux occurs based on a chemical potential gradient. Whether it is Soret or Dufour Effect, the induced thermal grating is the dominating effect used in projects described in this dissertation. The grating diffracts the probe beam by acting on its amplitude or phase. DFWM methods have been used in gas, liquid and condensed phases in a variety of applications [1-4, 21]. In this dissertation, we applied this method for detection of biomolecules, especially proteins in the liquid phase.

1.4. Dissertation Outline

This work demonstrates the use and application of degenerate four-wave
mixing as a sensitive tool to detect and analyze biomolecules, such as proteins in capillaries and microchips. A general overview of lasers, laser types and nonlinear laser spectroscopic methods is explained in this chapter. The theory of DFWM and its signal characteristics are explained in Chapter 2. Experimental conditions, optical setups, laser sources, cells, and capillary electrophoresis setups are explained in detail in Chapter 3. Chapter 4 explains the amphiphilic polymers, their synthesis, characterization and advantages over low molecular surfactant in capillary electrophoresis. It also contains information on amphiphilic polymers solvation properties based on their linear solvation energy relationships. In Chapter 5, single-cell detection in a capillary is discussed. Wave mixing is introduced as a new technique for imaging cells. In Chapter 6, single cell and its proteins are detected and analyzed using wave-mixing spectroscopy and capillary electrophoresis. Wave mixing-based capillary electrophoresis is one of the most powerful separation techniques with high resolving power. Single cells can be detected and their proteins analyzed with excellent detection limits. In Chapter 7, applications of amphiphilic polymers for protein separation and its advantages over other sieving matrix are discussed. Chapter 8 describes a high-throughput nonlinear laser wave- mixing detection system for protein analysis. Lastly, Chapter 9 discusses 266 nm UV laser-based protein separation and wave-mixing detection in their native form.


7 C. A. Townes, How the laser happened, adventures of a scientist, March 1999

8 www. Newport.com


14 www. Wikipedia.org

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20 J. Knittle, Doctoral Dissertation, University of California San Diego and San Diego State University, 2004


2.1. Degenerate Four-Wave Mixing

Degenerate four-wave mixing (DFWM) involves nonlinear mixing of light waves of the same frequency (degenerate since all waves have identical frequencies). Two coherent input light beams (one pump and one probe) or three input beams (two pumps and one probe) interact in an absorbing medium and generate the signal beam. In a typical setup, a laser beam is split into two input beams (pumps). The two excitation pump beams travel in the same direction until focused and mixed with a focusing lens. An interference pattern is formed as the two pump beams recombine and create light and dark bands corresponding to the areas of constructive and destructive interferences. Inside an absorbing medium, the interference patterns cause thermal modulations, which in turn create thermal gratings as shown in Figure 2.1.

Excited molecules take on the periodic spatial distribution of the interference pattern. In the excited state, if the molecules release their energy nonradiatively, thermal gratings cause thermal expansion of the solvent following a change in refractive index. Incoming photons from a probe beam interact with the grating and part of the energy is diffracted, creating the signal beam. The signal beam has the characteristic of a Gaussian beam.
Figure 2.1 Interference grating of the two pump beams $I_1$ and $I_2$, which creates thermal modulation in absorbing medium.
2.2. Formation of Grating

2.2.1. Interference Pattern

When four light waves with frequencies of $\omega_1$, $\omega_2$, $\omega_3$, $\omega_4$ and propagation vectors of $k_1$, $k_2$, $k_3$ and $k_4$ interact in an absorbing medium as illustrated in Figure 2.2, a degenerate wave-mixing signal is generated\(^1\) if:

\begin{align*}
\omega_1 &= \omega_2 = \omega_3 = \omega_4 \quad (2.1) \\
k_1 &= -k_2 \quad (2.2) \\
k_3 &= -k_4 \quad (2.3)
\end{align*}

Next, two laser beams, $I_1$ and $I_2$, interfere with each other with $\theta$ angle and a grating or interference pattern is created that is defined by the intensity modulation expression\(^2\):

\[
I = I_0 \left[ 1 + \frac{m \cos(2\pi x)}{\Lambda} \right] \quad (2.4)
\]

where $I_0$ is the sum of the intensities of the two beams, $m$ represents pattern contrast, $x$ is spatial coordinate and $\Lambda$ is the spatial grating period. Variable $m$ depends on the intensity of the light beams:
Figure 2.2 Interaction of four light beams and their propagation vectors
This interference grating creates spatially periodic light intensity or polarization that in turn changes the optical properties of the absorbing medium.\(^3\)

According to Eichler,\(^4\) the interference pattern or the grating has a vector \(q\) that is the sum of two interfered light vectors \(k_1\) and \(k_2\) with the angle \(\theta\).

\[
q = \pm (k_1 + k_2) \quad (2.6)
\]

The period or spacing between the thermal gratings can be calculated using the following equations:

\[
\Lambda = \frac{2\pi}{q} \quad (2.7)
\]

\[
\Lambda = \frac{\lambda_e}{2\sin\frac{\theta}{2}} \quad (2.8)
\]

where \(\Lambda\) is the grating period, \(\lambda_e\) is the excitation wavelength and \(\theta\) is the angle between the two pump beams.  (Figure 2.3)
Figure 2.3 Grating period is proportional to the angle of the pump beams and the wavelength.
2.3. Diffraction

Generally, amplitude or phase gratings\(^5\) diffract the probe beam and create the signal beam. When the amount of the absorbed light in one part of the grating is different from the other part, the density and refractive index of the solvent and local properties change as well. The thermal grating usually is created when medium absorption is due to optical saturation or medium density changes. However, phase grating is generated when thermal relaxation of the absorbing particles within the medium changes.

In most cases, there is a combination of amplitude and phase gratings (mixed grating)\(^6,7,8\). However in the liquid phase, phase grating (thermally generated density grating) is mostly responsible for the grating diffraction.\(^9,10,11,12,13\)

2.4. D4WM Optical Geometry

Depending on the sample and experiment type, forward or backward-scattering geometry may be used in a wave-mixing optical setup. Generally for gas-phase samples, a backward-scattering geometry is used. Two counter propagating pump beams and a probe beam intersect in a nonlinear medium. Signal beam is generated and retraces the probe beam path as shown in Figure 2.4. (A). In the liquid phase, for ease of optical alignment, forward-scattering geometry with only two beams is used (Figure 2.4. (B)). It should be noted that although only two input pump beams (I\(_1\) and I\(_2\)) are used, a portion of one of the pump beams serves as the probe beam (I\(_3\)).
Figure 2.4 (A) Backward-scattering (B) forward-scattering wave-mixing signal in liquid phase. Only one of the signal beams is collected.
2.5. Generation of D4WM Signal

Depending on the experimental conditions and sample types (gas or liquid), mostly two different types of gratings may be generated that are responsible for the probe beam diffraction and wave-mixing signal generation. Generally in the gas phase, population gratings are formed due to the absorption of the analytes inside the probe volume. After interacting two pump beams in an absorbing medium and formation of the grating, the probe beam diffracts off the grating in a predicted propagation direction, and hence, creating the signal beam. In this case, population gratings are responsible for diffracting the probe beam. The signal intensity in a backward-scattering wave-mixing optical setup can be described as:

\[
I_s = \alpha_0^2 L^2 \frac{4 \left( \frac{I}{I_{sat}} \right)^2}{\left( 1 + 4 \frac{I}{I_{sat}} \right)^3} I_p \quad (2.9)
\]

\[
I_{sat} = \frac{\varepsilon_0 c \eta^2}{2 T_1 T_2 |\mu_{1,2}|^2}
\]

(2.10)

In a forward-scattering wave-mixing setup, the signal can be described as:

\[
I_s \approx I_p \pi \alpha_0^2 L^2 \left[ \frac{I_p}{I_{sat}} \right]
\]

(2.11)
where \( I_s \) and \( I_p \) are the intensity of the signal and probe beams, \( \alpha_0 \) is line center absorption coefficient of the interacting analyte, \( L \) is induced grating length and \( I_{\text{sat}} \) is required intensity for achieving an equal population of analyte particles in the excited and ground states. \( \varepsilon_0 \) is permittivity of vacuum, \( c \) is the speed of light and \( \eta \) is Planck’s constant over \( 2\pi \). Signal is collected over a dark background and one can obtain virtually 100% optical collection efficiency.

If the analyte is undisturbed during the natural lifetime of the excited states, population gratings are mainly responsible for signal generation. This phenomenon happens mostly in low-pressure gas-phase systems. However, when these excited particles deactivate by collision in condensed-phase media or high-pressure gases, thermal gratings are responsible for signal generation.

In such conditions, wave-mixing signal intensity can be summarized and described by the following equation:\(^{14}\)

\[
I_s \approx C \left( \frac{b}{8\pi} \right)^2 I_2^2 I_1 \frac{\lambda_e^2}{\sin^4(\theta/2)} \left( \frac{dn}{dT} \right)^2 \frac{\alpha^2}{\kappa^2}
\]

(2.12)

The two pump beam intensity levels are represented by \( I_1 \) and \( I_2 \). \( C \) is a constant and \( b \) is the optical path length. \( \lambda_e \) is the excitation wavelength, \( \frac{dn}{dT} \) is the change in the index of refraction of the solvent with respect to temperature while \( \kappa \) is the thermal conductivity of the solvent, \( \alpha \) is the absorptivity of the analyte, and \( \theta \) is the angle between the two pump beams.
As shown in the above equation, the signal has a cubic dependence on laser power, allowing efficient use of low laser power levels when analyzing very low concentration levels. The wave-mixing signal intensity has a quadratic dependence on analyte concentration and on $dn/dT$. These nonlinear dependencies are effective for sensitive detection of small changes in analyte properties. In addition, signal intensity can be easily enhanced if a solvent with desired properties (i.e., refractive index, $dn/dT$, thermal conductivity, etc.) is chosen.

It should be noted that if dipole moments or polarizability of the particles within the induced field of the pump beams create a density change on the absorbing medium, an electro-optical gratings may formed. Diffraction from this kind of grating is called Brillouin scattering.\(^{15}\)

Based on the laser power distribution used for the two pump beams, one or two signal beam may be created in a predicted propagation direction and distance from the pump beams. If the two pump beams have the $d$ distance from center to center, signal beams will have exactly the same distance on both sides of the pump beams. This characteristic makes it easy to predict signal beam direction and location, and therefore, the signal can be easily detected by the detector. (Figure 2.5)
Figure 2.5 Exact locations of signal beams are predictable.
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3 S. Berniolles, Doctoral Dissertation, University of California, San Diego, San Diego state University, CA, 1997


11 B.Y. Zel’dovich, N. F. Pillipetsky, V. V. Shkunov, Principles of Phase Conjugation; Springer Series in Optical Conjugation; Springer-Verlag: New York, NY, 1985; Vol 42 pp181-183


14 J. Knittle, Doctoral Dissertation, University of California, San Diego, San Diego
State University, CA, 2004

15 www.scienceworld.wolfram.com
3.1. Optical Setup

Figure 3.1 shows a typical forward-scattering wave-mixing optical setup used for liquid samples. The laser beam is split into two input (pump) beams of different intensities using a 70:30 (T/R) beam splitter. These beams are focused with a focusing lens and mixed inside the sample cell. Interferences of the two pump beams create a thermal grating. This thermal grating diffracts off the probe beam and generates the wave-mixing signal beam. The signal is detected with a photodetector. An optical chopper (Stanford Research System, Model SR541) is used to chop one of the input beams at 200 Hz. The signal is amplified by a lock-in amplifier (Stanford Research System, Model SR810DSP) at 200 Hz and digitized by a personal computer-based data acquisition system (TongLab AIDA program). A small probe volume (nL to pL) resulting from two overlapping input beams allows easy coupling with microfluidic systems.
Figure 3.1 Optical setup for forward-scattering wave mixing
3.2. Laser Excitation Sources

Various laser sources used in our wave-mixing experiments are summarized in Table 3.1.

Table 3.1. Laser sources and experimental specifications

<table>
<thead>
<tr>
<th>Laser Excitation Source</th>
<th>Wavelength (nm)</th>
<th>Power (mW)</th>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argon Ion</td>
<td>514</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Argon Ion</td>
<td>488</td>
<td>10-100</td>
<td>6, 7, 8</td>
</tr>
<tr>
<td>Helium-Neon</td>
<td>633</td>
<td>5</td>
<td>5, 8</td>
</tr>
<tr>
<td>Nd-YAG</td>
<td>266</td>
<td>5</td>
<td>9</td>
</tr>
</tbody>
</table>

3.2.1. Argon-Ion Laser

A 514-nm water-cooled argon-ion laser (Coherent Inc., Palo Alto, CA, Model I90-6) is used for the experiments described in Chapter 5. For experiments in Chapter 6, 7 and 8, a 488-nm argon-ion laser is used. In an argon-ion laser, electrical discharge excites the lasing medium and a few output wavelengths can be selected.

3.2.2. Helium-Neon Laser

A helium-neon laser has a 5:1 He-Ne gas mixture inside the laser cavity. This laser offers low power consumption, cost effectiveness and a good beam quality. A red
He-Ne laser (Uniphase, Model 1125P, 632.8 nm, 5 mW) is used in experiments described in Chapter 5 and 8.

3.2.3. Frequency-Quadrupled Nd:YAG Laser

A frequency-quadrupled Nd:YAG compact UV laser provides the 266 nm excitation line suitable for native protein absorption measurements. This UV laser (Model, NU-10210-100, Teem Photonics, France) also offers low power consumption and a good beam quality.

3.3. Sample Cells

3.3.1. Fused Silica Capillaries

Fused Silica capillaries with different inside diameters and coatings are used as sample cells throughout our experiments. They are obtained from Polymicro Technologies, Inc., and their dimensions and coatings are summarized in Table 3.2.

<table>
<thead>
<tr>
<th>Capillary</th>
<th>I.D. (µm)</th>
<th>O.D. (µm)</th>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fused Silica round</td>
<td>50</td>
<td>365</td>
<td>4</td>
</tr>
<tr>
<td>Fused Silica round</td>
<td>75</td>
<td>365</td>
<td>4, 5, 6, 7</td>
</tr>
<tr>
<td>Fused Silica round</td>
<td>100</td>
<td>150</td>
<td>8, 9</td>
</tr>
<tr>
<td>Fused Silica square</td>
<td>70</td>
<td>360</td>
<td>8, 9</td>
</tr>
</tbody>
</table>
To create an optical window for the input laser beams, a small portion of the polyimide coating (0.5 mm) is removed using a micro torch and then cleaned by methanol. As shown in Figure 3.2, a capillary holder is used to stabilize the capillary cell inside the laser setup. The cell holder also allows easy movement of capillary ends for sample injection and rinsing when connected to a peristaltic pump. Capillaries are mounted and cushioned through plastic tubings in order to reduce pressure from the cell holder.

Square capillaries are used in experiments described in Chapters 8 and 9 instead of round capillaries to minimize background optical noise levels. The square capillaries show less diffraction, and hence, lower optical scattering background levels when interfaced to the input beams. Array capillaries are assembled by simply gluing eight to ten windowed capillaries together and mounted in a cell holder.

3.3.2. Microchip

A microchip is used in the single-cell detection and imaging experiment described in Chapter 5. Microchips (95 mm x 16 mm x 2.2 mm) are obtained from Micralyne, Inc., (Model MC-BF4-TT100) and they are made of Borofloat glass with a T-shaped inner channel (50 µm wide and 20 µm deep) as shown in Figure 3.3.
Figure 3.2 Capillary holder
Figure 3.3 Microchannel dimensions (top and side view)
3.4. Capillary Electrophoresis Systems

A custom-built CE system and a commercial automated P/ACE 2500 (Beckman) CE system are used. Our custom-built CE system is powered by a high-voltage power supply (Glassman, Model PS/MJ30P0400-11 30 KV) using two nickel-coated electrodes. Both ends of the capillary are inserted into glass vials containing running buffer as well as the electrodes as shown in Figure 3.4. This custom-built CE is used for the experiments described in Chapters 5, 6, 7, 8 and 9.

The commercial Beckman P/ACE CE system is fully automated and programmable for capillary rinsing and conditioning, sample injection and CE separation, with a wide range of voltages and injection times. It is supplied with a UV lamp, two platinum electrodes and an auto sampler. It also has a variable wavelength detector (200 – 400 nm). The capillary is held in a cartridge and its temperature is controlled through a coolant running through the cartridge. This CE system is used for the experiments described in Chapter 4.

3.5. Capillary Conditioning and Control

3.5.1. Peristaltic Pump

A peristaltic pump (Rainin Instrument Co., Woburn, MA, Model Rabbit Plus) is used to introduce rinsing solutions and conditioning buffers as well as an alignment dye solution to assist wave-mixing optical alignment. Color-coded pump tubings (orange –blue) with 0.25 mm I.D. (Fisher Scientific) are connected to the capillaries.
Figure 3.4. Custom-built capillary electrophoresis system
3.5.2. XYZ Translational Stage

A three-dimensional (xyz) translational stage (Newport, Irvine, CA, Model 461) is used for positioning the capillaries, the microchip and the photodetector. By adjusting these stages, the capillary or the microchip can be aligned and interfaced properly to the wave-mixing optical setup in order to obtain the best S/N.

3.6. Signal Detection and Processing

3.6.1. Photodetector

A simple Si photodiode (Thorlabs, Inc., Model PDA 55) is used for wave mixing signal collection in the experiments described in Chapters 5, 6, 7 and 8. The photodetector in these experiments has a 13-mm² active area with a spectral range of 400 – 1100 nm and a bandwidth of 10 MHz. A similar photodiode (Thorlabs, Inc., Model DET 210) is used to collect the 266 nm UV signal as described in Chapter 9. This detector has a 0.8 mm² effective area with a spectral range of 200 - 1100 nm. The wave-mixing signal is digitized using a custom-designed software package (TongLab AIDA).

3.6.2. Photodiode Array

For the high-throughput CE system, a wider photodiode array (Hamamatsu, Model HC233-1010) is used. This PDA incorporates a NMOS linear image sensor chip, a driver/amplifier circuit and a temperature controller. The NMOS sensor chip
has 1024 diodes (25 µm x 2500 µm). To minimize temperature drifts and lower dark currents, a temperature controller is used to cool the sensor chip thermoelectrically. The built-in driver/amplifier circuit is interfaced to a computer through a 16-bit I/O board (National Instruments). This board has a pulse generator that provides master clock pulses and master start pulses required by the sensor. The PDA and all the data acquisition operations are controlled by a software package (HC233-1010 code, Hamamatsu, and National Instruments LabView).

Signal images show pixel intensity levels for each frame. Images are collected in a single Microsoft Excel file (250 images at 1-s interval). The collected signal is digitized by the LabView software and analyzed by Microsoft Excel.

3.6.3. CCD Camera

For the single-cell imaging experiment described in Chapter 5, a CCD camera (Sony Model XCD-SX910CR) is used to collect two-dimensional images. The camera is controlled and the images captured by using the custom Sony software. The CCD camera has an array of linked or coupled capacitors that transfer charges to one another. After the image is projected on the capacitor array by a lens, an electric charge is accumulated on each capacitor that is proportional to the light intensity. After exposing the image on each capacitor and transferring it to the neighbors, the final capacitor transfers its charge to an amplifier. The resulting signal is digitized, displayed and stored.¹
Although CCD chips are analog devices, its signal is digitized off the camera in the board installed in a computer. In digital CCD cameras, the digitizers are detached from the CCD to minimize electrical or read-out noise levels and improve signal-to-noise ratios (S/N), and hence, increasing the dynamic range for maximum attainable gray scales. To further reduce the noise levels, Peltier coolers are effectively used in CCD cameras. Otherwise, hot/white pixels begin to cloud images beyond 10 seconds of camera integration. They can be cooled down to –40 °C, and therefore, one can obtain integration times exceeding one hour. Other improvements, such as clocking, sampling and efficient digitizing methods, can further minimize electrical read-out to 4-5 electrons per photodiode or pixel, which in turn would require increasing the number of pixels or surface area. Hence, there should be a balance between large pixels for increased sensitivity and small pixels for maximum resolution.

3.6.4. Microscope

A phase-contrast microscope (10X magnification) is used to illuminate transparent media and capture images of single cells traveling through the capillary window. It simulates the phase and intensity distribution at the focal point by a density variation found inside a cell. An annular aperture controls the intensity of non-diffracted light and provides a quarter-wave phase shift with reference to the diffracted light. The resulting image is an interference of the two phases of the diffracted and
non-diffracted light beams. Jurkat cell images are captured using a digital camera (Minolta, Model 78324438) held at the eyepiece of the microscope.
1 http://en.wikipedia.org

2 Bioimaging systems application note FP-114

3 The Theory of the Microscope by Bausch & Lomb Incorporated 23-24
Chapter 4

Amphiphilic Polymers Synthesis, Characterization and
Linear Solvation Energy Relationship Study

4.1. Introduction

Amphiphilic polymers are linear or branched polymers that are made of hydrophilic and hydrophobic segments. Polymers with a hydrophilic skeleton carrying some hydrophobic groups, either randomly distributed on the chain (grafted or comb like) or fixed at one or two ends (telechelic), are called water-soluble associating polymers.\(^1\) The differences in polarity between the various parts of the polymer molecules, attractive interactions among hydrophobic residues and electrostatic interactions among charged segments, give them special properties in solution and in the solid state.

Using different monomers with different ratios and lengths, various polymers can be made to replace common low molar-mass surfactants. This allows a good control of the hydrophilic/lipophilic balance (HLB) and the possibility of selectively introducing additional functions along the polymer chain.\(^2\) Furthermore, attaining a constant length of a hydrophobic chain is possible by using the initiator method. Controlling the degree of polymerization is also possible by adjusting the head group size of the surfactant. Therefore, the synthesized polymer offers some advantages of
both low molecular-weight surfactants and polymer amphiphiles. However, in previous works, crystallinity with high melting point and poor solubility in water have been reported for some polymeric micelles with an octadecyl group.\(^3\)

These polymeric micelles with different selectivity can expand the range of pseudo stationary phases in MEKC. The main application is the separation of highly hydrophobic solutes where high concentration of organic solvent is needed. These micelle-like amphiphilic polymers have been utilized as hosts for various molecules. They were used in many applications like catalysis, chemical separations and controlled release of drugs or cosmetics\(^4\). Slight variation of the properties of the hydrophobic chain can lead to dramatic changes in the properties of microenvironment of polymer self-assemblies. Therefore, both thermodynamic and kinetic properties of the microenvironment can be altered.

As part of the goal for finding an optimal amphiphilic polymer, we investigate the polymer containing branched hydrophobic side chains, 2-ethylhexyl functional group, in more detail. As the branched molecule is well known for use as plasticizers due to its excellent fluidity, we explore the potential of incorporating it in amphiphilic polymers for various applications. We also investigate functional group effects such as \(-\text{COOH}\) and \(-\text{SO}_3\text{H}\) along the polymer chain. Because micelle formation is a thermodynamic phenomenon, it is important to find the most stable conditions for the polymer. Factors like pH\(^5,6\), temperature\(^7,8\) polymer concentration, chemical structure of solute-polymer, polarity of the solute and location of the solute within the micelle...
affect self-association of polymer and solute association with a micelle. Also, when polymeric micelles are used as carriers for water-insoluble drugs, it is important to understand its stability under various experimental conditions. Since they are relatively unstable in infinitely dilute environments, it is of great importance to detect the best experimental conditions for these polymers. On the other hand, these experimental parameters affect separation resolution in MEKC.

We also investigate solvation properties of these micellar amphiphilic polymers. Due to synthetic flexibility, the monomers composition is varied. As a result, chemical selectivity of the microenvironment of these polymers may further be optimized for a specific separation problem. By changing the structure of polymer self-assemblies, solvation properties of micellar micro domains of polymers can also be altered.

In this study, the monomers listed in Table 4.1 are used to synthesize linear random amphiphilic polymers. Test solutes including alkylbenzoate are used for probing the properties of microenvironment of assemblies due to polymer self-association. Gel permeation chromatography (GPC), pH, temperature and titration studies, and concentration variation study in capillary electrophoresis (CE) are used to characterize these polymers. Their retention factor is also compared with other surfactants like sodium dodecyl sulfate (SDS), which gives a good insight of the solvation properties of the polymeric system. Hence, a wide range of choices is available for selectivity manipulation in a separation process.
### Table 4.1. Monomer ratios

<table>
<thead>
<tr>
<th>Hydrophobic Monomer</th>
<th>Hydrophilic Monomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-dodecylacrylate (25%)</td>
<td>3-sulfopropylmethylacrylate (75%)</td>
</tr>
<tr>
<td>0.6 mmole (0.17ml)</td>
<td>1.8 mmole (0.443g)</td>
</tr>
<tr>
<td>n-dodecylacrylate (25%)</td>
<td>Acrylic acid (75%)</td>
</tr>
<tr>
<td>0.6 mmole (0.17ml)</td>
<td>1.8 mmole (0.125ml)</td>
</tr>
<tr>
<td>2-ethylhexylacrylate (25%)</td>
<td>Acrylic acid (75%)</td>
</tr>
<tr>
<td>0.6 mmole (0.125ml)</td>
<td>1.8 mmole (0.125ml)</td>
</tr>
<tr>
<td>Hexylacrylate (50%)</td>
<td>Acrylic acid (50%)</td>
</tr>
<tr>
<td>1.8 mmole (0.318ml)</td>
<td>1.8 mmole (0.125ml)</td>
</tr>
<tr>
<td>Cyclohexylacrylate (50%)</td>
<td>Acrylic acid (50%)</td>
</tr>
<tr>
<td>1.8 mmole (0.315ml)</td>
<td>1.8 mmole (0.125ml)</td>
</tr>
</tbody>
</table>

### 4.2. Experimental

#### 4.2.1 Chemicals and Reagents

All chemicals are reagent grade or better and obtained from several makers. All solutes are dissolved in methanol (MeOH) and 10 mM phosphate buffer (pH 7) and prepared in Barnstead (18.2 MΩ) water. Buffers are filtered with 0.45 μm plastic filters prior use.
4.2.2. Apparatus and Conditions

All the CE separations are performed using a Beckman P/ACE system (Model 5510) with a UV diode array detector. Capillaries are made of fused Silica (37 cm total length, 30.5 cm effective length, 75 μm I.D., 360 μm O.D.). The CE runs are performed at 20 kV at 25 °C. Capillaries are conditioned by rinsing first with (1) a 50/50% mixture of isopropanol alcohol (IPA)/sodium hydroxide (NaOH) 0.1M, (2) methanol/NaOH 0.1M, (3) Barnstead water, and finally (4) with a running buffer (polymer dissolved in 10 mM phosphate buffer at pH 7) for 10 minutes each. Capillary is rinsed between each run with the same set of solution for 1 minute each. Optical detection is made at 214 nm.

4.2.3. Polymer Synthesis Example

First we measure and prepare 0.6 mmole 2-ethylhexylacrylate (0.125 mL), 1.8 mmole acrylic acid (~0.125 mL) and 0.01 mmole 2-[(cyano-dimethyl-methyl)-azo]-2-methyl-propionitrile (AIBN) (1.642 mg). AIBN is dissolved in 1 mL of N,N-,dimethylformamide (DMF). In a dry test tube, monomers are added to 19 mL of DMF, and then the radical initiator AIBN is added. The test tube is capped with rubber and tightened with a plastic strap and then degassed with vacuum and purged with nitrogen for three times to remove oxygen. The test tube is then placed in an oil bath with the temperature set at 60 °C for 24 hours. The solution is stirred during the reaction. The synthesis is shown in Scheme 4.1.
Scheme 4.1. Polymer synthesis
4.2.4. Polymer Formation and Solubility Test

The purity is confirmed by injecting crude polymers into a GPC column of a HPLC system. First, 0.5 mL of the crude polymers are transferred into small glass vials containing 1 mL mixture (20/80) of mobile phase (acetonitrile) and 10 mM phosphate buffer (pH 7). For those polymers that are still cloudy in this solution, a few drops of tetrahydrofuran (THF) and MeOH are also added to completely dissolve them in the mobile phase solution. After this solubility test, GPC is performed using Agilent 1100 Chemstation with a 300 mm x 7.8 mm Ultrahydrogel Linear GPC column (Waters Corp.) at 45°C and a flow rate of 1 mL /min. A set of standard polymers, polystyrene sodium sulfonat with molecular weights (MW) of 4600, 8000, 35000, and 100000 D are also injected and used for molecular weight estimation of synthesized polymers. Optical detection is made at 210 nm using a diode array detector.

Figure 4.1.a shows GPC results for the MW standards and Figure 4.1.b shows GPC results for the synthesized polymers. The polymer retention times are in the range of those of the MW standards, and hence, they confirm our polymer synthesis.
Figure 4.1.a. GPC results for PS standards of 780, 100, 35, 8 and 4.6KD with retention times of 5.978, 6.874, 7.143, 7.590 and 7.669 min are shown.
Figure 4.1.b. GPC results for the set of synthesized polymers with retention times of greater than 7 minutes confirms polymers formations.
Polymers molecular weights are estimated based on their retention times plotted against log MW of the standards (Figure 4.2). Results are summarized in Table 4.2.

Table 4.2. Polymer estimated molecular weight

<table>
<thead>
<tr>
<th>Polymer</th>
<th>MW (KD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-EtHA-AA</td>
<td>6</td>
</tr>
<tr>
<td>C12-SO3H</td>
<td>50</td>
</tr>
<tr>
<td>Cyc-C6-AA</td>
<td>50</td>
</tr>
<tr>
<td>n-C6-AA</td>
<td>30</td>
</tr>
</tbody>
</table>

All the synthesized polymers are tested for solubility. All of them are soluble except for poly (cyclohexylacrylate- acrylic acid) 50/50%, even after sonication.

4.2.5. Polymer Purification

To purify the polymers the following steps are taken. Polymers with the –COOH functional group are dissolved in tetrahydrofuran (THF) and MeOH, and then transferred into a round bottom flask. Solvent is removed using Rotovap R-3000 under vacuum in a water bath thermostated at 70 °C. Then THF is added to dissolve the polymer and diethyl ether is added to precipitate it. Separated supernatant is centrifuged to make sure that all the polymers are precipitated. This process is repeated three times and the solvent is dried under vacuum. GPC is performed again to
Figure 4.2. Plot of log (MW) of the standards vs. their retention time

\[ y = -1.6121x + 16.077 \text{ and } R^2 = 0.9939 \]
make sure that the polymer is reasonably purified.

For polymers with the -SO$_3$H functional group, a slight different purification process is used. The polymer is first dissolved in H$_2$O and then precipitated in a mixture of 50/50 IPA/ACN. This process is repeated three times and then the same procedure is used to dry the sulfonated polymer.

4.3. Results and Discussion

4.3.1. pH Variation Study

To find the optimum pH for these polymers and its effect on their mobility, capillary zone electrophoresis (CZE) is performed using a running 50/50 buffer mixture of 10 mM phosphate and 10 mM boric acid at different pH levels from 6 to 9.5. This buffer mixture provides the same ionic strength at different pH values. The pH levels of the running buffers are adjusted to the desired value by adding 1M NaOH and monitoring with a Fisher Scientific pH meter (Accumet Research AR15 with Orion glass pH electrode). Poly (2-EtHA-AA) (0.5 %) is dissolved in the same buffer mixture at pH 7 and injected into the capillary. Electrophoretic mobility ($\mu_{ep}$) levels of the polymer at different pH levels are calculated using following equation:

$$\mu_{ep} = \frac{(1d/E)(1/t_{c0} + 1/t_i)}{\mu_{ep}}$$

(4.1)
where \( l_d \) is the effective length of the capillary, \( E \) is the electrical field, \( t_{eo} \) and \( t_r \) are the retention times of unretained (marker) and retained solute. As shown in Figure 4.3, \( \mu_{ep} \) is at the highest level at pH 8. At pH 6, there is no separation. Comparison of poly (2-EtHA-AA) electropherograms at different pH values is shown in Figure 4.4. Similar data are also obtained for poly (C6-AA) (data not shown in figure).

4.3.2. Titration

To verify the results obtained from the pH study, a series of titration is also performed on the poly (2-EtHA-AA) system. Standardized NaOH (0.0179 M) is used as titrant on 7 mL of 0.3% poly (2-EtHA-AA) that contains 0.01 M hydrochloridric acid and 0.05 M sodium chloride. As NaOH is added to the polymer solution, pH is measured and recorded at room temperature. As shown in Figure 4.5, the titration curve increases gradually up to pH 8 followed by a relatively sharp increase. The titration curve confirms that the highest electrophoretic mobility of the polymer is observed at pH 8.

<table>
<thead>
<tr>
<th>pH</th>
<th>6.54</th>
<th>7.04</th>
<th>7.59</th>
<th>8.10</th>
<th>9.00</th>
<th>9.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu_{ep} \times 10^{-3} )</td>
<td>1.943 +/-</td>
<td>1.969 +/-</td>
<td>1.965 +/-</td>
<td>1.992 +/-</td>
<td>1.958 +/-</td>
<td>1.923 +/-</td>
</tr>
<tr>
<td>(95% C.I.)</td>
<td>0.003</td>
<td>0.003</td>
<td>0.010</td>
<td>0.007</td>
<td>0.023</td>
<td>0.021</td>
</tr>
</tbody>
</table>
Figure 4.3. Electrophoretic mobility of poly (2-EtHA-AA) at different pH values
Figure 4.4. Electropherogram of poly (2-EtHA-AA) at different pH levels
Figure 4.5. Titration curve for poly (2-EtHA-AA)
4.3.3. Concentration Variation Study

Different concentrations of 0.1, 0.3, 0.5, 0.7, 1, 1.5 and 2% (w/v) of poly (2-EtHA-AA) and poly (C\textsubscript{12}-SO\textsubscript{3}H) are prepared in a 10 mM phosphate buffer at pH 7 and used as buffers in a MEKC system. Toluene is injected into the capillary and its $\mu_{ep}$ calculated for different polymer concentration levels. Figure 4.6 shows plots of $\mu_{ep}$ vs. polymer concentration percentage for two polymers with different functional groups. For poly (2-EtHA-AA), the highest $\mu_{ep}$ is obtained at 0.3% (w/v) polymer concentration, and for poly (C\textsubscript{12}-SO\textsubscript{3}H), the highest value is obtained at 0.7% (w/v) concentration.

To study the micellar behavior of the polymer, a mixture of alkyl benzoate is injected into the capillary using different concentration levels of poly (2-EtHA-AA). For the 0.1 % polymer, there is no separation for alkylbenzoate, and for concentration levels above 0.5 %, it is not possible to acquire any retention time for the micelle, $t_{mc}$ (data are not shown). Therefore, at these experimental conditions, the concentration range is limited between 0.3 to 0.5% of poly (2-EtHA-AA).
Figure 4.6.a. Polymer concentration effect on electrophoretic mobility for poly (2-EtHA-AA)
Figure 4.6.b. Polymer concentration effect on electrophoretic mobility for poly (C$_{12}$-SO$_3$H)
A plot of retention factor, \(k'\), vs. the number of \(-\text{CH}_2\) chain is also obtained for a homologue series of alkylbenzoate. Retention factor is calculated using the migration time of the unretained solute \((t_{eo})\), the migration time of the polymeric micelle \((t_{mc})\) and the migration time of the retained solute \((t_r)\) using the following equation:

\[
k' = \frac{(t_r - t_{eo})}{t_{eo}(1 - t_r/t_{mc})}
\]  

(4.2)

As shown in Figure 4.7, \(k'\) increases as the number of \(-\text{CH}_2\) chain increases, for both 0.3 and 0.5% poly (2-EtHA-AA). Increasing the concentration of the polymer also yields higher \(k'\) values.

4.3.4. Temperature Variation Study

In previous works, it has been reported that in some polymer systems, phase transition is seen in response to external stimuli such as changes in temperature and pH because of imbalance of hydrophilic and hydrophobic interactions in the system. Depending on the polymer structure and its hydrophilicity, low temperature levels can cause a coil conformation of the polymer chain. In other words, polymer-solvent interactions are stronger than the polymer-polymer interactions. But as the temperature increases, due to the hydrophobic interactions, solvent dissolution ability decreases and the polymer-polymer interactions increase. At some point these interactions are equal.
Figure 4.7. Comparison of retention factors, $k'$, for 0.3% poly (2-EtHA-AA) (bottom) and 0.5% poly (2-EtHA-AA) (top)
Finally at temperatures above the critical temperature, the situation reverses and water is no longer a good solvent for the polymer. Therefore polymer chains form compact “globules”, and hence, phase separation occurs. Any factor affecting the polymer-solvent, polymer-polymer or solvent-solvent interactions will affect the phase transition temperature.\textsuperscript{11} In other words, temperature has a great effect on viscosity, and consequently, electroosmotic flow (EOF). Its effect on selectivity is not significant, however, increase in buffer temperature decreases separation efficiency due to Joule heating. Temperature variation also affects CMC, aggregation number, micellar size and shape, and therefore, the number of theoretical plates, N.

In this study, a mixture of alkylbenzoates is injected into the capillary using 0.3\% poly (2-EtHA-AA) as the running buffer at temperature levels from 288 to 313 °K. Each run is repeated three times. The retention factor is calculated and plotted against temperature. As shown in Figure 4.8, increasing the temperature decreases $t_{\text{me}}$ and solutes elute faster. However, at 298 °K (25°C), the best reproducible results obtained. Therefore, this temperature is chosen for our studies.

At this chosen temperature, the partition coefficient ($K$) of a solute can be predicted simply by checking the difference in free energy of transfer of a functional group from aqueous to the micellar phase ($\Delta\Delta G$) using the following equation:

\[
\Delta\Delta G = -RT \ln K
\]  
(4.3)
where $R$ is the gas constant and $T$ is the temperature (°K). With the $K$ and phase ratio $\Phi$ values, the retention factor of a solute can be determined using:

$$k' = K\Phi \quad (4.4)$$

Replacing $K$ in equation 4.3 gives

$$\Delta\Delta G = -RT \ln \frac{k'}{\Phi} \quad (4.5)$$

Or

$$\Delta\Delta G = -2.3RT \log \frac{k'}{\Phi} \quad (4.6)$$

It shows $\log k'$ is correlated with $1/T$. Therefore, plotting $\log k'$ vs. $1/T$ provides a straight line with a slope for $\Delta\Delta G$. As shown in Figure 4.9, a straight-line plot for a homologue series of alkylbenzoate is obtained, as expected. Also as the $-\text{CH}_2$ chain increases, the slope ($\Delta\Delta G$) also increases. Similar data for methylbenzoate and ethylbenzoate are observed. As illustrated in this figure, their log $k'$ values are not affected by temperature increase as much as those of propyl and butylbenzoates.
Figure 4.8. Temperature variation study and its effect on k’ for a homologue series of alkylbenzoate using poly (2-EtHA-AA) as micelle
Figure 4.9. Plot of log k’ vs. 1/T for a homologue series of alkylbenzoates using poly (2-EtHA-AA) as micelle
4.3.5. LSER study

LSER provides useful information to investigate cogeneric behavior of solutes. It is useful to estimate octanol-water partition coefficients ($P_{ow}$), even for structurally similar solutes.\textsuperscript{12} LSER modeling is done for these polymers using Abraham’s modified version of the LSER equation:

$$
\log k' = vV + eE + sS + aA + bB + c
$$

where retention factor, $k'$ is calculated using equation (4.2).

The terms $V$, $E$, $S$, $A$, $B$ are solute descriptors. $V$ represents the solute characteristic volume in $\text{cm}^3\cdot\text{mol}^{-1}/100$, calculated from its structure by summing the characteristic atomic volumes for each atom and subtracting a fixed amount for each bond. $E$ is solute excess molar refraction divided by 10, calculated from the refractive index and characteristic volume as the difference in molar refraction of the solute and an n-alkaline of identical volume. $S$ is solute dipolarity. In other words, it is the solute that can stabilize the neighboring dipole by virtue of its capacity for orientation and induction interactions. The terms $A$ and $B$ represent hydrogen bond donating (acidity) and hydrogen bond accepting (basicity) ability respectively. The terms $v$, $e$, $s$, $a$ and $b$ are coefficients of these descriptors and are related to the contribution of the micellar phase toward each type of interaction. The coefficient $v$ describes the cohesiveness and dispersive interactions of the micellar phase. It measures the relative ease of cavity
formation and general dispersion interactions of the solutes in micelle and mobile phase. Coefficient $e$ describes the ability of the micelle to interact with the n- or $\pi$-electrons of the solute. The coefficient $s$ represents the dipolarity of the micellar phase, the difference in capacity of the micelles and the mobile phase to take part in dipole-dipole and dipole-induced dipole interactions. Coefficients $a$ and $b$ represents the ability of the micelle to form hydrogen bond interactions with solute molecules. The term $c$ is a regression constant and it increases with increasing phase ratios, however, the other LSER coefficients are not affected.

### 4.3.5.1. Poly (C$_6$-AA) (90/10%)  

A set of 32 solutes is chosen for the LSER study. Initially, MEKC is run for all these test solutes using 0.5% poly (C$_6$-AA) (90/10%) dissolved in 10 m M phosphate buffer at pH 8. Their solvation descriptors,$^{13}$ log $P_{ow}$, values and the calculated log $k'$ values for poly (C$_6$-AA) are all listed in Table 4.4. Performing linear regression in Excel creates the LSER model as shown in Table 4.5.
Table.4.4. Test solute and their descriptor and log k’ values obtained with poly (C$_6$-AA)

<table>
<thead>
<tr>
<th>Solute</th>
<th>log k’</th>
<th>V</th>
<th>S</th>
<th>B</th>
<th>A</th>
<th>E</th>
<th>log P$_{ow}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>-1.59992</td>
<td>0.716</td>
<td>0.52</td>
<td>0.14</td>
<td>0.00</td>
<td>0.610</td>
<td>2.13</td>
</tr>
<tr>
<td>Toluene</td>
<td>-1.58025</td>
<td>0.857</td>
<td>0.52</td>
<td>0.14</td>
<td>0.00</td>
<td>0.601</td>
<td>2.69</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>-1.09301</td>
<td>0.998</td>
<td>0.51</td>
<td>0.15</td>
<td>0.00</td>
<td>0.613</td>
<td>3.15</td>
</tr>
<tr>
<td>Propylbenzene</td>
<td>-1.08281</td>
<td>1.139</td>
<td>0.50</td>
<td>0.15</td>
<td>0.00</td>
<td>0.604</td>
<td>3.68</td>
</tr>
<tr>
<td>p-xylene</td>
<td>-1.53179</td>
<td>0.998</td>
<td>0.52</td>
<td>0.16</td>
<td>0.00</td>
<td>0.613</td>
<td>3.15</td>
</tr>
<tr>
<td>Chlorobenzene</td>
<td>-1.56025</td>
<td>0.839</td>
<td>0.65</td>
<td>0.07</td>
<td>0.00</td>
<td>0.718</td>
<td>2.84</td>
</tr>
<tr>
<td>Bromobenzene</td>
<td>-0.7669</td>
<td>0.891</td>
<td>0.73</td>
<td>0.09</td>
<td>0.00</td>
<td>0.882</td>
<td>2.99</td>
</tr>
<tr>
<td>4-Chlorotoluene</td>
<td>-1.08037</td>
<td>0.980</td>
<td>0.67</td>
<td>0.07</td>
<td>0.00</td>
<td>0.705</td>
<td>3.33</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>-0.55291</td>
<td>1.324</td>
<td>0.99</td>
<td>0.22</td>
<td>0.00</td>
<td>1.360</td>
<td>3.90</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>-0.81141</td>
<td>1.085</td>
<td>0.92</td>
<td>0.20</td>
<td>0.00</td>
<td>1.360</td>
<td>3.35</td>
</tr>
<tr>
<td>1-methylnaphthalene</td>
<td>-0.47904</td>
<td>1.226</td>
<td>0.90</td>
<td>0.20</td>
<td>0.00</td>
<td>1.344</td>
<td>3.87</td>
</tr>
<tr>
<td>Acethophenone</td>
<td>-1.16373</td>
<td>1.014</td>
<td>1.01</td>
<td>0.48</td>
<td>0.00</td>
<td>0.818</td>
<td>1.58</td>
</tr>
<tr>
<td>Benzonitrile</td>
<td>-1.71233</td>
<td>0.871</td>
<td>1.11</td>
<td>0.33</td>
<td>0.00</td>
<td>0.742</td>
<td>1.58</td>
</tr>
<tr>
<td>Nitrobenzene</td>
<td>-1.63298</td>
<td>0.891</td>
<td>1.11</td>
<td>0.28</td>
<td>0.00</td>
<td>0.871</td>
<td>1.85</td>
</tr>
<tr>
<td>Methylbenzoate</td>
<td>-1.67529</td>
<td>1.073</td>
<td>0.85</td>
<td>0.46</td>
<td>0.00</td>
<td>0.733</td>
<td>2.16</td>
</tr>
<tr>
<td>Ethylbenzoate</td>
<td>-1.56564</td>
<td>1.214</td>
<td>0.85</td>
<td>0.46</td>
<td>0.00</td>
<td>0.689</td>
<td>2.64</td>
</tr>
<tr>
<td>4-nitrotoluene</td>
<td>-1.4841</td>
<td>1.032</td>
<td>1.11</td>
<td>0.28</td>
<td>0.00</td>
<td>0.870</td>
<td>2.45</td>
</tr>
<tr>
<td>4-chloroacetophenone</td>
<td>-1.48517</td>
<td>1.136</td>
<td>1.09</td>
<td>0.44</td>
<td>0.00</td>
<td>0.955</td>
<td>2.35</td>
</tr>
<tr>
<td>Methyl 2-methylbenzoate</td>
<td>-1.54127</td>
<td>1.214</td>
<td>0.87</td>
<td>0.43</td>
<td>0.00</td>
<td>0.772</td>
<td>2.75</td>
</tr>
<tr>
<td>Phenyl acetate</td>
<td>-1.97285</td>
<td>1.073</td>
<td>1.13</td>
<td>0.54</td>
<td>0.00</td>
<td>0.661</td>
<td>1.49</td>
</tr>
<tr>
<td>3-methylbenzyl alcohol</td>
<td>-1.68015</td>
<td>1.057</td>
<td>0.90</td>
<td>0.59</td>
<td>0.33</td>
<td>0.815</td>
<td>1.58</td>
</tr>
<tr>
<td>Phenethyl alcohol</td>
<td>-1.73262</td>
<td>1.057</td>
<td>0.83</td>
<td>0.66</td>
<td>0.30</td>
<td>0.078</td>
<td>1.36</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>-1.73937</td>
<td>0.916</td>
<td>0.87</td>
<td>0.56</td>
<td>0.33</td>
<td>0.803</td>
<td>1.08</td>
</tr>
<tr>
<td>Phenol</td>
<td>-1.62058</td>
<td>0.775</td>
<td>0.89</td>
<td>0.30</td>
<td>0.60</td>
<td>0.805</td>
<td>2.58</td>
</tr>
<tr>
<td>4-methyl-phenol</td>
<td>-1.42889</td>
<td>0.916</td>
<td>0.87</td>
<td>0.31</td>
<td>0.57</td>
<td>0.820</td>
<td>1.94</td>
</tr>
<tr>
<td>4-ethylphenol</td>
<td>-1.41398</td>
<td>1.057</td>
<td>0.90</td>
<td>0.36</td>
<td>0.55</td>
<td>0.800</td>
<td>1.49</td>
</tr>
<tr>
<td>4-fluore phenol</td>
<td>-1.54344</td>
<td>0.793</td>
<td>0.97</td>
<td>0.23</td>
<td>0.63</td>
<td>0.670</td>
<td>1.77</td>
</tr>
<tr>
<td>4-chlorophenol</td>
<td>-0.83502</td>
<td>0.898</td>
<td>1.08</td>
<td>0.20</td>
<td>0.67</td>
<td>0.915</td>
<td>2.35</td>
</tr>
<tr>
<td>4-bromophenol</td>
<td>-1.35153</td>
<td>0.950</td>
<td>1.17</td>
<td>0.20</td>
<td>0.67</td>
<td>1.080</td>
<td>2.59</td>
</tr>
<tr>
<td>4-chloroaniline</td>
<td>-1.16979</td>
<td>0.939</td>
<td>1.13</td>
<td>0.31</td>
<td>0.30</td>
<td>1.060</td>
<td>1.83</td>
</tr>
<tr>
<td>3-methylphenol</td>
<td>-1.5712</td>
<td>0.916</td>
<td>0.88</td>
<td>0.34</td>
<td>0.57</td>
<td>0.822</td>
<td>1.96</td>
</tr>
<tr>
<td>3,5-dimethylphenol</td>
<td>-1.47919</td>
<td>1.057</td>
<td>0.84</td>
<td>0.36</td>
<td>0.57</td>
<td>0.820</td>
<td>2.35</td>
</tr>
</tbody>
</table>
As seen in this table, the coefficient $v$ is small and positive compared to other surfactants in other studies [13], indicating that cohesiveness and dispersive interactions of this micellar microenvironment do not play significant roles in solute retention as compared to other surfactants. This small value is not surprising due to the fact that the polymer has a large percentage of hydrophilic parts (90%), and therefore, small hydrophobic interactions in the micellar system is observed.

Dipole-induced dipole (i.e., the $s$ coefficient) is small relative to $v$ and has a close value to other surfactants. Its negative sign indicates that the pseudo stationary phase is more dipolar than water. Based on the LSER results, this polymer has a dipolarity close to those of SDS, SDC and SLSA, and significantly higher than those of SDP and SDCV. This big difference is expected due to larger head groups of SLSA and SDCV.

The polarizability or interaction of a micelle with solute $n$-and $\pi$-electrons is related to the $e$ coefficient. As compared to other surfactants, $e$ is significantly large and its positive value indicates the willingness of the system to interact with $n$- and $\pi$-
electrons of the neighboring solute. This large value could be explained due to the acidic head group that its electrons are less tightly bound. One of the important factors that play a significant role on selectivity is the hydrogen bond interaction between solutes and micelles. The relatively large negative value for b compared to other surfactants [13] indicates a weak proton-donating phase. However, the a value is very small and insignificant, indicating that the hydrogen bond acceptance is similar to those of bulk aqueous phase and has little influence on solute partitioning.

In quantitative structure-activity relationships (QSAR), solute biological activities are related to their structural and/or physico-chemical descriptors. One of these descriptors is the distribution coefficient between 1-octanol and water, $P_{ow}$. According to linear free-energy relationship (LFER), a linear relationship exists between changes in free energies of physico-chemical processes where hydrophobic interactions play a major role. Therefore, a linear relationship between log $k'$ and log $P_{ow}$ can be expected as follows:

$$\log k' = a \log P_{ow} + b \quad (4.8)$$

The relationships between log $k'$ and log $P_{ow}$ have been reported for various surfactants in previous works. As reported, these relationships for the same set of solutes could be different for various micellar phases. This is due to the difference in micellar phase selectivity, suggesting that the hydrophobic interaction is not the major
force affecting retention in MEKC [13]. A plot of log k’ of these solutes vs. log P_{ow} is shown in Figure 4.10. Based on the LSER results obtained for poly (C₆-AA) and when compared to 1-octanol, the big difference is their hydrogen bond donating or accepting capacity. 1-octanol is the strongest hydrogen bond acceptor and the weakest hydrogen bond donor. Therefore, considering the correlation coefficient of the line in Figure 4.10 (R² = 0.5263), it is not surprising that the overall trend in the MEKC retention behavior with poly (C₆-AA) micelles does not correlate well with log P_{ow} values.

It should be noted that although solute retention is due to multiple interactions in the LSER study, the selectivity of micelles could be explained by hydrogen bond interactions. Non-hydrogen bonding solutes should show a straight line with a slope of one while hydrogen bond donating groups would deviate from this line toward the surfactant that has a stronger hydrogen bond acceptor. The same trend should be observed for the hydrogen bond accepting solutes, that is, they must deviate from this line toward the micellar phase with a stronger hydrogen bond donating ability. As shown in this figure, some solutes are very close to this line and some show deviation toward the polymeric micelle.

In this work, the first 12 solutes in Table 4.4 are considered non-hydrogen bond donors, the next 11 solutes are hydrogen bond acceptors and the rest are categorized as hydrogen-bond donors. However, an attempt to recognize a trend in the grouping of these solutes in three lines, according to their hydrogen bond acceptor strengths, is not successful (poor R² values). This again supports the fact that the MEKC retention of
this polymer does not correlate with the log $P_{ow}$ values. We also perform the same comparison with the SDS micellar phase for 15 solutes. A plot of log $k'$ for poly (C$_6$-AA) vs. log $k'$ for SDS is shown in Figure 4.11.

Log $k'$ values for SDS are reported by Abraham et al.$^{15}$ A poor correlation is also observed between these two micellar phases as shown in the figure. We should note that poly (C$_6$-AA) provides very small $k'$ values for our test solutes. MeOH is chosen as our modifier and added at different levels (10, 15, 25, 30 and 45%). As MeOH is increased, the current decreases and it is hard to get the peak for dodecanophenone (used as a marker for $t_{me}$).

Since adding the modifier does not improve separation, increasing the hydrophobic part of the polymer from 10% to 30% seems logical. Therefore, we use poly (C$_6$-AA) (70/30%) instead of poly (C$_6$-AA) (90/10%). However as the hydrophobic part of the polymer is increased, solubility becomes an issue. As a result, 0.5% poly (C$_6$-AA) (70/30%) is insoluble in 10 mM phosphate buffer pH 7. Therefore, less concentrated polymer (0.4% w/v) is prepared. The LSER modeling for different percentage levels of polymers needs to be further investigated.

### 4.3.5.2. Poly (2-EtHA-AA)

The same set of solutes is used for the poly (2-EtHA-AA) LSER study. However, some of these solutes do not show reproducible peaks (poor $R^2$ values). After eliminating these solutes from the test set, only a set of 10 solutes remains that
Figure 4.10. Plot of log $P_{ow}$ vs. log $k'$ for poly (C$_6$-AA)
Figure 4.11. Plot of log k’ poly (C₆-AA) vs. log k’ SDS
show good separation. Hence, a larger set of test solutes is needed for the LSER modeling. Table 4.6 lists the log $k'$ and the log $P_{ow}$ values. The plot of log $k'$ vs. log $P_{ow}$ shows a straight line, indicating that there is a similarity between the selectivity of this polymer and their partitioning in 1-octanol/water solution, as shown in Figure 4.12.

Table 4.6. Comparison of log $k'$ values in poly (2-EtHA-AA) and its partitioning in 1-octanol/water

<table>
<thead>
<tr>
<th>Solute</th>
<th>log $k'$ poly (2-EtHA-AA)</th>
<th>log $P_{ow}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>-1.06</td>
<td>2.13</td>
</tr>
<tr>
<td>Toluene</td>
<td>-0.78</td>
<td>2.73</td>
</tr>
<tr>
<td>Ethyl benzene</td>
<td>-0.47</td>
<td>3.15</td>
</tr>
<tr>
<td>Propylbenzene</td>
<td>0.11</td>
<td>3.72</td>
</tr>
<tr>
<td>4-chlorotoluene</td>
<td>-0.17</td>
<td>3.33</td>
</tr>
<tr>
<td>Ethyl benzoate</td>
<td>-0.79</td>
<td>2.64</td>
</tr>
<tr>
<td>4-nitrotoluene</td>
<td>-0.90</td>
<td>2.37</td>
</tr>
<tr>
<td>4-chloroacetophenone</td>
<td>-0.92</td>
<td>2.32</td>
</tr>
<tr>
<td>Methyl-2-methylbenzoate</td>
<td>-0.78</td>
<td>2.75</td>
</tr>
<tr>
<td>4-ethylphenol</td>
<td>-0.93</td>
<td>2.58</td>
</tr>
</tbody>
</table>
Figure 4.12. Compatibility of log k’ for Poly (2-EtHA-AA) with log (P_{ow})
4.3.5.3. Poly (C\textsubscript{12}-SO\textsubscript{3}H)

The same set of solutes is tested using poly (C\textsubscript{12}-SO\textsubscript{3}H) as micelle in MEKC. All the conditions are the same. Also for this polymer, we do not observe reproducibility and good separation for the whole set of 32 solutes. As with poly (2-EtHA-AA), only ten of them show good MEKC results. To further investigate these polymers, a larger set of test solutes or a mixture of surfactants is needed. Application of this polymer for separation of large molecules (proteins) is presented in Chapter 7 of this dissertation.

4. Conclusion

Amphiphilic polymers are useful as pseudo stationary phases for MEKC. Due to their synthetic flexibility, the composition of hydrophobic and hydrophilic monomers may be varied in the polymer. Therefore, the chemical selectivity of the microenvironment of the polymer can potentially be “tuned” for a specific separation problem. To find the optimum experimental conditions for these polymers as pseudo stationary phases in capillary electrophoresis, these polymers are successfully synthesized and their molecular weights estimated by GPC. Electrophoretic mobility of poly (2-EtHA-AA) is calculated using a pH variation study. Temperature and concentration variation studies also give a good insight for future studies. The best pH (8) and temperature (25°C) are determined for these polymers. Linear solvation energy relationships are also studied for a set of 32 solutes. The results provide a clear view of
how monomer ratios and functional groups along the polymer chain can affect its solvation properties. By choosing suitable monomers, their solvation properties and selectivity can be tuned for specific applications. Some of the real-world applications are described in the following chapters.
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Chapter 5

Single cell Detection and Imaging of Distributed Absorbing Species within a Single Cell by Nonlinear Laser Wave Mixing Spectroscopy

5.1. Introduction

The study of single cell systems is becoming an attractive area of research. With applications in metabolism, signal transduction and physiological processes, single cell studies offer important insights into biological systems.

Reliable optical imaging of chemical species within and on the surface of a single biological cell and sensitive detection of chemical differences and abnormalities can lead to early disease detection, diagnosis, disease state and therapy, and effects of treatment through analyzing the changes of components and contents within the cell.

These optical methods capture images that provide visual representation, characterization and quantification of biological events at the single cell level. Samples are within the real setting of a living cell rather than cell extracts, as used in many conventional methods (e.g., chromatography, Western blot). Current methods do not provide information on the analytes distribution and their changes, in vitro. Single-cell studies offer some advantages including the elimination of averaging features of conventional optical methods and better access to heterogeneity, dynamic fluctuations,
diffusion, reorientation, co-localization and conformational changes at the molecular level.

A number of techniques have been used for single cell imaging. Current methods provide important information, but they offer limited detection sensitivity levels and require significant sample preparation steps. For example, samples analyzed by scanning tunneling microscopy (STM) must be conductive since it is based on probing the density of states of a material (metals and semiconductors) using tunneling current. STM can be a challenging technique, as it requires extremely clean surfaces and sharp tips. Scanning probe microscopy is also generally slower in acquiring images. Also, the maximum image size is generally smaller. Similarly, scanning electron microscopy (SEM) has a relatively low resolution for atomic scale analytes, requires an expensive vacuum, and samples must be treated with heavy metals to obtain contrast within the image. In addition, this method cannot be applied to living cells. In electron microscopy (EM), sample needs to be rapidly frozen and hydrated. Other techniques like scanning near-field optical microscope (SNOM) can only be applied to surface analysis with limited access to soft samples. Atomic force microscopy (AFM) also suffers from slow data collection (hence, not applicable for dynamic phenomena) and tip convolution and shadowing problems.

Surface enhanced Raman scattering (SERS) along with colloidal gold particles (60 nm in size) positioned in cells can be used to map a cell monolayer with 1 µm lateral resolution. Native chemicals inside a cell, such as DNA and phenylalanine, can
be detected, however, it is limited to the non-uniform distribution of the SERS-active nanostructures over the cell.\(^3\)

In other imaging techniques including confocal fluorescence microscopy, laser-induced fluorescence (LIF) and scanning optical microscopy it is necessary to label the analytes within the cell or on cell surfaces, which affects the cell or the cell function. Wave mixing is an optical absorption-based detection method, and provides dynamic holographic images of both stained and non-stained (native) cells.

In this work, nonlinear laser wave mixing is adapted as a cell imaging technique. A single bio cell can be placed on a glass micro slide or in a flow cell and moved with an automatic actuator while the laser beam placement is fixed. As the laser beam passes through the labeled cellular component, a CCD camera records the wave-mixing signals and the corresponding topographic images.

Live and fixed cells can be imaged in real time using nonlinear wave mixing to obtain information on sequential changes in the number, morphology and distribution of cellular components within a single cell. Wave-mixing imaging serves as a highly sensitive detection technique for spatial distribution of light absorbed by a target sample. This sensitive analytical tool can localize and quantify biomolecules in single cells and tissue sections. Cellular components can be labeled with a chromophore or fluorophore and imaged by wave mixing using a CCD camera.

In an unsaturated and optically thin medium thermal gratings created by non-radiative relaxation of absorbing analytes produce the wave-mixing signal with
quadratic dependencies on analyte concentration and the local concentration of the species responsible for the nonlinearity of the medium. Therefore signal beam is a coherent laser-like beam.

Preliminary studies on monitoring single-cell images indicate that scattering of the input laser beams off the cell membrane and its particles has small effect on the wave-mixing signal. In this work, a novel imaging technique for recording the distribution of chemical species within a single cell based on absorption-based laser wave mixing is reported for the first time.

5.2. Experimental
5.2.1. Apparatus
5.2.1.1. Laser Setup

A He-Ne laser operating at 633 nm is used for Cy5-based surface antigen detection, and a 514 nm argon-ion laser (Coherent, Model Innova 90-6) is used for native cytochrome C detection in a separate yet similar experimental setup.

In a 2-D forward-scattering wave-mixing optical geometry, a 70/30-beam splitter splits the laser beam. A convex lens focuses and mixes the two pump beams on a microchannel, capillary or flow cell with a beam overlap angle of approximately one degree. The resulting probe (30 µm) efficiently excites the absorbing medium and creates an interference pattern of constructive and destructive interactions between the pump beams. Input laser beams transfer their energy as heat into the absorbing
medium, and these thermal gratings change the refractive index of the absorbing medium. The probe beam diffracts off these laser-induced gratings to produce the laser-like signal beam, which carries the image of the absorbing analyte in the cell. The signal beam is detected with a photodiode detector and a CCD camera (Sony Corp., Model XCD-SX910CR) in a two dimensional detection. One can profile the concentration variation of cellular components, within and on the surface of a cell and record the dynamic images on a CCD camera.

In order to suppress background incoherent light a focusing lens, an aperture and a collimating lens, are placed immediately in front of the CCD camera. To enhance the S/N, a mechanical chopper set at 200 Hz along with a lock-in amplifier (Stanford Research Systems, Model SR810 DSP) is used to modulate the amplitude of the pump beam. A data acquisition system digitizes and stores the wave-mixing images. An experimental optical setup is illustrated in Figure 5.1.
Figure 5.1. Experimental optical setup: capillary can be replaced with microchannel, glass slide or flow cell.
5.2.1.2. Microscope

A phase-contrast microscope (10x) is used to illuminate our transparent media. It captures an image of a cell traveling through the capillary window using a peristaltic pump at 0.2 ml/min flow rate. Based on density variations found inside a cell, the phase-contrast microscope can observe the phase and intensity distributions at the focal point. An annular aperture controls the illumination of the object. The resulting image is obtained from the interference of the two phases of the diffracted and non-diffracted light beams. Jurkat cell images are captured for the study of density variations using a digital camera (Minolta 78324438) held at the eyepiece on the microscope (Figure 5.2).

A laser scanning confocal microscope (Leica, TCS SP2, Heidelberg, Germany) with 63X objective and a 675/20 PMT is also used for capturing images of the stained single cells. Obtained images are compared with images created by wave mixing imaging technique. Stained cells are placed on a 0.1 % poly-L-lysine (Sigma-Aldrich, St. Louis, MO) coated slide, covered with ProLong mounting media (Molecular Probes), and finally covered with a cover slip.
Figure 5.2. A single cell flowing inside a capillary
5.2.1.3. Capillary and Microchannel

A fused Silica capillary is used initially for the detection of cells. The detection end of the capillary is connected to a peristaltic pump and the injection end is inserted in a cell reservoir. The capillary length is 40 cm with an effective length of 25 cm (50 µm I.D. and 360 µm O.D.).

A CE microchannel is also used for single-cell detection and imaging since it offers good microfluidic controls. The channels are 20 um deep and 50 um wide with a single cross channel design. The microchannel is mounted on a translational stage that is controlled by an actuator (Zaber Technologies, Model T-LA28).

5.2.2. Materials and Methods

5.2.2.1. Reagents

All the chemicals used are reagent grade obtained from Sigma Aldrich. Mito Tracker green is obtained from Molecular Probe. Barnstead (18.2Ω) water is used in all the solutions prepared. All the buffers are filtered with 0.45 µ nylon disposable filters.

5.2.2.2. Single Cell Culture

In our wave-mixing experiments, 25 µm Jurkat cells (American Type Culture Collection, Manassas, VA) are used. They are cultured in medium consisting of 2 mM L-glutamine, 90% RPM1 1640 (Mediatech, Herndon, VA), 10 mM HEPES, and 10%
fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). Before each single-cell injection, cells are checked under the microscope. A 9 mL portion of the cell solution is taken out and centrifuged for 3 minutes at 4 °C. Then the supernatant is aspirated and 1 mL of a PBS buffer at pH 7.4 is added. Approximately $10^6$ cells are washed with PBS three times (i.e., pelleting at 50 g for 5 minutes and re-suspending the pellet in PBS in a microcentrifuge tube). To the remaining cells, another 9 mL fresh portion is added. Cells are incubated at an appropriate confluence in an incubator at 37°C with 5% CO₂.

5.2.2.3. Single Cell Labeling

To label T-cell receptor, cells are incubated with 10 µg/mL anti-CD3e mAb OKT3 hybridoma (American Type Culture Collection) in PBS at 4 °C for 30 minutes. The cells are then washed by centrifuging and re-suspending in PBS. Finally they are incubated with 10 mg/mL Cy5 conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) at 4°C for another 30 minutes.

Mito Tracker Green (Molecular Probes, Eugene, OR) is used to label mitochondria within a cell and labeling is performed as follows. A pre-warmed (37 °C) growth medium containing diluted Mito Tracker (500 nM) is added to the cells and incubated for 30 minutes under growth conditions. The loading solution is replaced with a fresh pre-warmed medium. The cells are observed under a fluorescence
microscope fitted with an appropriate filter set to make sure the cells are sufficiently labeled (Figure 5.3).

For each type of labeling, cells are centrifuged and re-suspended in 3.7% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) in PBS for 30 minutes at 4°C. They are finally washed with PBS for three times.

5.2.2.4. Single-cell Detection

5.2.2.4.1. Capillary Cell

The initial goal is to detect absorbing components of a cell within a capillary. A peristaltic pump is used to pull cells, one or two at a time, through a 50 µm I.D. capillary.

Measurements of single cells and bursts of cells flowing in a stream are made by wave mixing and confirmed by comparing data collected from a buffer that contains no cells. No signal is observed for buffer solution as shown in Figure 5.4. Signal intensity is different for labeled cells due to the cells aggregation, labeling efficiency and their positioning in the probe volume. In addition, optimal flow rate studies are performed for easy confirmation and isolation of cell peaks detected by wave mixing. The wave-mixing signal is generated instantaneously for cells that pass through the probe area. Cell counting experiments (cytometry) may also be performed for biological applications using wave-mixing spectroscopy (Figure 5.5).
Figure 5.3. Image of two labeled mitochondria within a single cell obtained by Confocal microscopy
Figure 5.4. Wave-mixing signal of a single cell is compared to that of a running buffer. No signal is observed for buffer, but signal is observed for each cell passing through the capillary.
Figure 5.5. Flow study of single cells. A decreasing number of cells pulling through the capillary yield a smaller number of signal peaks.
5.2.2.4.2. In a Microchannel

Following visual confirmation and wave-mixing detection, a single cell is docked inside a 50 μm glass microchannel. Small probe volumes and accurate movements provided by an actuator yield a signal of a docked cell. In this work, a simple dilution technique is used to inject a single cell into the microchannel. Unlike other methods, mechanical devices such as capillary mounts, syringes, multi-purpose injectors, and micromanipulators are not necessary to collect the cell into the apparatus. Freshly rinsed cells with PBS are diluted 1:1000 in a pH 7.4 PBS buffer solution. Then 1 μL of diluted cells is placed on the microchannel reservoir. The single cell is visually confirmed under the microscope and pushed through the microchannel by capillary action and the hydrostatic pressure of the system. As a result, there is no need to use a potential (i.e., on and off) to dock the cell inside the microchannel. A single cell is docked inside the microchannel and its location marked with a marker under the microscope. Then the microchannel chip is placed on the actuator and moved through the laser probe area as shown in Figure 5.6.
Figure 5. 6. Single cell detection is shown in a microchannel
5.2.2.4.3. 2D Detection on a Glass Slide

Preliminary results show effectiveness and capabilities of laser wave mixing in detecting absorbing components within an intact cell. Nonlinear laser wave mixing can be used as an optical imaging method. Concentration variations within a medium can be mapped onto the signal beam to produce an image. Wave-mixing imaging is a technique analogous to dynamic holography. It is first aligned and optimized using a mesh grid suspended in a dye solution (1 x 10^-4 M erythrosine B in ethanol) in a 1 mm thick custom-built flow cell. Initial images are obtained with and without a mesh grid placed inside the flow cell. Current images show its ability to produce a hologram or image from optical excitation of a non-homogenous dye and mesh grid (a) by a photodetector and (b) by a CCD camera (Figure 5.7).

Figure 5.8 shows wave-mixing images of the dye mesh grid collected by the CCD camera and an inverted phase 10X microscope. The grid displays apparent distributions of absorbing and non-absorbing (opaque) areas.

After capturing images of a mesh grid, a stained single cell is placed on a glass slide and is covered with a cover slip. The glass slide is moved in 1 um increments by an actuator and the signal beam is collected for two seconds. As a result, a topographic image of the labeled mitochondria all over the cell is obtained by the photodetector. A topographic image of the mapped cell with its labeled mitochondria is shown in Figure 5.9.
Figure 5.7. 2-D detection of non-homogenous dye and mesh grid by a photodetector (top) and CCD camera (bottom)
Figure 5.8. Comparison of the wave-mixing image of the dye mesh grid collected (A) under an inverted phase 10X microscope and (B) CCD-based wave-mixing imaging
Figure 5. 9. Mapping of labeled mitochondria within a single cell
Image of surface antigens on a Jurkat cell is also obtained by wave-mixing imaging system and illustrated in Figure 5.10. An antibody directly conjugated to a fluorescent probe Cy5 is used to stain antigens on the surface of a single cell. Recorded images of the stained cell promise good detection sensitivity for native detection and imaging of the distribution of cytochrome C within a Jurkat cell. One of the key steps for signal transduction in the apoptosis process is the release of cytochrome C. Therefore monitoring small changes of cytochrome C distribution during the apoptosis process provides important information in a wide range of applications in cellular science.12

5.3. Results and Discussion

When two laser input beams with intensities of \( I_1 \) and \( I_2 \) and wavelength \( \lambda_{ex} \) cross at the angle \( \theta \), they excite molecules in an absorbing medium. Energy releases in the form of heat, creating dynamic thermal gratings followed by change of refractive index (dn/dT). A wave mixing coherent laser-like signal beam is then generated and its intensity \( I_s \) can be described as:13, 14, 15, 16, 17, 18, 19:

\[
I_s \approx CI_2^2I_1 \frac{\lambda_e^2}{\sin^4(\theta)} \left( \frac{dn}{dT} \right)^2 \left( \frac{\alpha}{\kappa} \right)^2 b^2 t^2 \quad (5.1)
\]

where C is a constant, \( \alpha \) is the analyte absorptivity, \( \kappa \) is the solvent thermal conductivity, \( b \) is the optical path length, and \( t \) is the thermal quantum yield.
Figure 5. Single cell images obtained from (A) fluorescent microscopy and (B) wave-mixing imaging. Cell surface antibodies are directly conjugated to the Cy5 fluorescent probe.
Wave mixing owes its high detection sensitivity to its nonlinear dependences, like quadratic dependence on analyte concentration, providing more sensitive monitoring of small changes as well as non-destructive detection and non-invasive probing of analytes.

In this study initial optimization of wave-mixing imaging is performed by measuring a $1 \times 10^{-4}$ M erythrosine B solution within the defined regions of a mesh grid. An argon ion laser beams are focused by 1000 mm focusing lens and provided a laser probe diameter of approximately 300 $\mu$m which can excite approximately six squares of the mesh grid in erythrosine B. Figure 5.8 demonstrate comparison of mesh grid images obtained by (a) an inverted phase 10X microscope and (b) wave-mixing imaging technique. As expected, only the absorbing areas within the boundaries of the mesh grid produce thermal gratings, which are then imaged and profiled by the wave-mixing signal beam.

Figure 5.9 shows a topographic image of labeled mitochondria in a single cell on a glass slide that is controlled by an actuator. As the actuator moves, focused laser beams scan over the absorbing medium and a wave-mixing signal is created and detected by a photodetector. This topographic signal provides information on the distribution of mitochondria within the single cell. Comparing the peaks obtained by the scanning laser beam over the single cell to the images obtained by the CCD camera confirms the effectiveness of laser wave mixing as a tool for cell imaging. Intercellular
variations are common in single cell measurements due to the heterogeneity of cell populations.

Images of the distribution of single cell surface antibodies directly conjugated to the Cy5 fluorescent probe is obtained using fluorescent microscopy and wave-mixing imaging and are shown in Figure 5.10 (A) and Figure 5.10 (B) respectively. A 5 mW He-Ne laser at 633 nm excites surface Cy5 probes (250,000 M⁻¹cm⁻¹ extinction coefficient). In order to cover and excite the entire single cell with approximately 25 μm diameter, a 75 mm focusing lens is used to adjust wave-mixing probe beam diameter to approximately 40 μm. Minimum of two grating periods calculated for the stained cells producing thermal gratings, which exhibit a circular shape of light intensity on the order of the cell size. Thermalization, non-instantaneous and non-local solute-solvent energy transfer may contribute to irregular and blurred edges displayed in the wave-mixing image.

To control existing of a true wave mixing signal a photodetector is also placed in our setup in addition to the CCD camera providing a two dimensional detection. The wave-mixing signal generated from the same Cy5 probed Jurkat cell is detected simultaneously. Figure 5.11 shows collected wave-mixing signals and compares it to a “blank” solution containing the PBS buffer only in three signal intervals. A very small background signal is still observed although the blank solution does not contain any Cy5. Concentration detection limit of 66 nM or 0.66 attomole is calculated for Cy5.
Figure 5.11. Wave-mixing signal generated from the same Cy5-stained Jurkat cell compared to that of a blank solution containing the PBS buffer only. Signal is collected by a photodetector.
In an approximately 40 µm excitation probe diameter almost two cells may be accommodated. As observed in figure 5.12 two distinct circular bright patterns are imaged showing the presence of two Cy5 stained Jurkat cells.

Figure 5.13 shows the generated wave-mixing signal by two stained Jurkat cells that are collected by the photodetector. As shown, results were reproducible when the two input laser beams were blocked on and off three times. Also as the amount of Cy5 increases, the wave-mixing signal intensity increases. Signal intensity decreases when only blank solution is used.

We were also able to capture images of cytochrome C that is present at the outer parts of a cell and only in mitochondria. Since cytochrome C does not fluoresce it should be stained with MitoTracker Green or other dyes to be observed in situ by fluorescence microscopy.

Figure 5.14 demonstrates images of both labeled and non-labeled (native) cytochrome C by wave-mixing dynamic holography that records and reconstructs cytochrome C distribution in a single cell.

Its wave-mixing signal collected simultaneously by the photodetector along with the image on the CCD camera is shown in figure 5.15. Reproducibility and signal intensity is also compared to that from a blank solution (PBS buffer) in this figure.
Figure 5.12. Wave-mixing image that captures the presence of almost two Cy5 stained Jurkat cells
Figure 5.13. The wave-mixing signal generated by two stained Jurkat cells collected with a photodetector.
Figure 5.14. Cytochrome C imaging by (A) fluorescence of a Jurkat cell stained with Mito Tracker Green and (B) wave-mixing dynamic imaging of native cytochrome C
Figure 5.15. Wave-mixing signal collected by a photodetector. Signal is reproducible and strong when compared to that of a blank solution (PBS) buffer.
5.4. Conclusion

Non-linear laser wave mixing spectroscopy is introduced as an ultra sensitive imaging technique for imaging of chemical species within biological cells. This technique is an absorption-based optical method, thus can be used for both fluorescing and non-fluorescing analytes. As demonstrated it is an effective and promising tool for imaging trace amounts of analytes in single biological cells in vitro. Similar to the processes in dynamic holography, the coherent laser-like wave-mixing signal beam carries physical/chemical properties of the absorbing analytes encoded within the nonlinear medium. Light absorption outside the nonlinear medium does not contribute in generating the wave-mixing signal. The signal is collected with 100% collection efficiency (unlike fluorescence methods) by a photodetector and a CCD camera. Wave-mixing imaging promises a wide range of potential applications including in situ analysis of intact single cells.
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Chapter 6

Single-Cell Protein Analysis by Capillary Sieving Electrophoresis (CSE) and Laser Wave-Mixing Spectroscopy

6.1. Introduction

Single-cell analyses have attracted a lot of attention due to their importance in studying biological activities. The living cell, with its amazing characteristics in molecular signal amplification, processing and genome-level responses to perturbation, provides the information of their own temporal and spatial activities needed for early disease detection, diagnosis and treatment. Most diseases are due to irregularities in the genetic code of cells. As a result expressions of gene products, RNAs and proteins are altered.

Large portions of mammalian cells are made of proteins (18%) and these proteins are responsible for many crucial cellular functions. For example, proteins catalyze chemical reactions, identify and control other biomolecules and genes, control permeability of cell membranes, and modulate the concentration of metabolites. They can also be used for diagnosis of diseases, drug delivery and development of vaccine. Analysis of expressed cellular proteins can also provide information on the differentiation and development of a single cell and its function in response to irregularities. Some irregularities in cells are due to an infection by a disease. In these
cases only a minor fraction of the total population of cells may be affected, therefore cannot effectively be detected by population-based detection techniques\textsuperscript{6}. For example in conventional single-cell analysis by flow cytometry only a few components are detected. An average measurement is provided that is extrapolated to the entire cell assuming the average measurement is indicative of each individual entity in the population. Therefore, a more reliable method is required to detect and measure individual cell components for the study of irregularities.\textsuperscript{7}

The analysis of cellular proteins requires a highly sensitive detection method with high temporal resolution as well as a separation method that can accommodate extremely small sample volumes. Capillary electrophoresis (CE) coupled to laser wave-mixing spectroscopy is the proposed method for single cell protein detection. Laser wave mixing is presented as an ultra sensitive detection method, which offers a small probe volume, small analyte requirement and high spatial resolution.

Capillary Electrophoresis (CE) offers many advantages as a separation technique including a small sample volume, high selectivity in determining multiple analytes, ease of automation, fast speed and high efficiency.

6.1.1. Preventing Protein Absorption on Capillary Walls

6.1.1.1. pH Effect

One of the strategies to prevent protein absorption is the use of high and low pH buffers. At extreme pH levels, both proteins and capillary walls have the same charge,
preventing protein wall absorption. While the absorption of proteins is diminished, there are some disadvantages to this approach. At low pH levels, electroosmotic flow (EOF) is high and deamidation or peptide bond scission may occur. At very high pH levels (greater than 11), dissolution of the silica (wall material) becomes an issue.

In addition, biological activities of proteins may be very different under these extreme pH conditions. The electrophoretic mobility of proteins tends to be very sensitive to certain pH ranges. Extreme pH levels preclude the study of many native molecular conformation interactions. For example, at the pH extremes, unfolding and/or aggregation of proteins may occur, which could lead to undesirable appearance of multiple and/or broad peaks.

### 6.1.1.2 Permanent and Dynamic Coating

Another strategy to prevent protein absorption on capillary wall is to coat silanol groups in order to suppress EOF permanently or dynamically. Different permanent coatings have been used in capillaries including poly (vinyl alcohol)\(^8\), poly (dimethylacrylamide) (PDMA), anionic poly (acrylate) and cationic acrylate-base polymers.\(^9\) Some other polymers like poly (ethyleneimine) (PEI)\(^10\) or N, N-dimethylacrylamide-ethylpyrrolidine methacrylate (DMA-EpyM)\(^11\) can be physically absorbed and they are good for basic protein separation in CE or CE-MS\(^12\) applications. Polybrene (PB) and poly (vinyl sulfonated) (PVS) can make a non-covalent bilayer coating simply by flushing them through the capillary.\(^13\)
Dynamic coating can be easily performed by using buffer additives. For example, salt or high ionic strength buffers, Zwitterionic salts, divalent cationic amines and non-ionic surfactant may be added to a running buffer. However, these additives are limited to specific applications and some types of proteins.

Cationic starch derivatives\textsuperscript{14}, poly (ethylen Oxide)\textsuperscript{15} and polyDuramide\textsuperscript{16} can be used as dynamic coating additives with some advantages including shorter analysis time and possibility of coating automation and regeneration.\textsuperscript{17} Gue et al. also used a sol-gel method to make a hydrolytically stable amino-silica coating\textsuperscript{18}. Using surfactants like SDS may also modify proteins to enhance separation. SDS binds to proteins in a variety of modes and can break up protein-carbohydrate-lipid complexes. This prevents protein aggregation and reduces conformer formation, giving protein a more uniform charge distribution for easier separation. Therefore, a single symmetrical peak is observed in the electropherogram.\textsuperscript{19}

6.1.2. Improving CE Detection Limits

6.1.2.1. Laser Induced Fluorescence (LIF)

Commercial CE instruments are equipped with UV-Vis absorption detectors. They offer poor concentration detection limits (∼ 10\textsuperscript{-6} M) and require a relatively long sample pathlength (i.e., sample size). To improve detection limits in CE, two strategies have been applied widely. The first one is to use laser-induced fluorescence (LIF) as a detection method. Unless analytes are naturally fluorescent, it is necessary to label
them with a fluorophore before CE separation (pre-capillary labeling). However, labeling at low concentration levels is difficult due to the increase in background interference levels and the decrease in labeling efficiency. Also, multiple sites may be labeled and multiple peaks may be detected for a single protein due to multiple surface charges on a protein. Furthermore, many fluorophores have poor quantum yields and short shelf life. Structural requirements for fluorophore are more difficult to define and quenching must not occur while the molecule is in the excited state. Most biological molecules have an aromatic ring in their structure and electron withdrawing groups tend to produce quenching substituents on the aromatic ring, and hence, they decrease the fluorescence yield. Conversely, electron rich groups inhibit quenching. Wave-mixing spectroscopy is an unusually sensitive absorption-based method that offers reproducible spectra and sensitivity levels comparable or better than those of fluorescence methods.

Dyes such as Rhodamine cyanine, solvatochromic, albumin blue series and phycobiliproteins have been used in many protein analyses based on CE-LIF. Increasing labeling reaction temperature improved limit of detection and reduced analysis time. In some work, cell lysis is performed in a closed volume micro-fabricated device to keep dilution of the single cell content minimized. The amount of analytes is measured either directly or indirectly by measuring the dye concentration before or after cell lysis.
In this work, we use a chromophore, QSY35, instead of a fluorophore. The advantages of using a chromophore include wider availability and better cost effectiveness.

6.1.2.2. Conventional CE Techniques for Protein Analysis

One of the methods widely used to improve protein separation in CE is isoelectric focusing. Yan et al. used a low-voltage electric field perpendicular to the liquid phase streamline in size-exclusion electrochromatography.

Another widely used method to enhance detection in CE is the use of sample pre-concentration techniques. Samples can be stacked by the difference in conductivity in the sample zone while running the buffer. High electric fields in the sample zone cause the analytes to migrate rapidly until they reach the interface of the sample zone and the buffer. Therefore, samples are stacked and the detection sensitivity enhanced. Isotachophoresis (ITP), field amplification or field reversal, isoelectric focusing or chromatographic enrichment, can also be used for pre-concentration. All of these can improve detection limits by up to 1000 times. Buffers can also play an important role in efficiently decreasing wall adsorption of proteins.

However, with all the applied strategies for protein detection at the single cell level, more powerful and ultra sensitive detection systems are still needed. Nonlinear laser wave-mixing spectroscopy with all the inherent and unique properties offers excellent detection sensitivity levels required for protein detection at the single cell
level. By applying this ultrasensitive absorption-based detection technique, the problems associated with the use of fluorophores are minimized. Our custom-built CE system is the capillary version of a SDS-PAGE system, also known as capillary sieving electrophoresis (CSE) or SDS-DALT-CE. This mode of capillary electrophoresis offers some advantages including replaceable and water-soluble buffers.

Considering a single-cell with 25 µm diameter, 8 fL volume and average protein mass of 25 KD, and assuming that 10% of a single-cell is protein, cell protein content will be in fmol range. Therefore a very high-sensitivity-protein analysis is required for single cell protein analysis. Nonlinear laser wave-mixing spectroscopy coupled with capillary sieving electrophoresis provides a novel and ultra sensitive method for single-cell protein analysis. This absorption-based detection method is used to detect proteins separated within a single cell. A single cell is injected into a coated capillary, lysed and labeled inside the capillary with a chromophore. Labeled proteins are separated in a sieving matrix under applied voltage through the capillary based on their mass-to-charge ratio differences.

In this study, we demonstrate that laser wave mixing provides excellent detection sensitivity levels needed to detect cellular components at low concentration levels while using one of the best protein separation methods. Capillary sodium dodecyl sulfate-Dalton electrophoresis (SDS-DALT) is a size-based CE separation method that offers excellent separation resolution. Adding sodium dodecyl sulfate and a low viscose-sieving matrix pullulan to the buffer and capillary, respectively, has
made this an even stronger technique for single-cell analyses. These polymers replaced
gels since gels cannot be used easily in capillaries.\textsuperscript{29} These modifications in separation
mechanism offer on-column lysis chamber for single cell as well as improved
estimation of the molecular weight and proteins elution providing higher peak
resolution.\textsuperscript{30}

6.2. Experimental: Materials and Methods

6.2.1. Reagents

All chemicals required for coating (acrylamide powder, APS, TEMED), sieving
matrix (Aureobasidium pullulan), buffer solution (Tris, 2-(N-cyclohexylamino)
ethanesulphonic acid (CHES)) and protein standards are reagent grade and ordered
from Sigma-Aldrich. Chromophore QSY35 is ordered from Molecular Probes. Water
used in all the solution preparation is Barnstead (18.2Ω). All buffers are filtered with
0.45 μ nylon disposable filters before using in the capillary. All protein standard and
buffers are stored at 8°C however labeled proteins as well as QSY35 were stored in –
20°C and thawed at room temperature before each run.

6.2.2. Cell Culture

Jurkat cells (Cell Culture Bank, Institute of Cytology, Academy of Sciences)
are used in this study. They are cultured in a medium of 0.01% L-glutamine, 90%
RPMI 1640 (Sigma) and 10% fetal bovine serum. Cells are grown under appropriate
confluence in an incubator at 37 °C and 5% CO₂. Approximately 10⁶ cells are prepared. They are washed three times in 1 mL of 1% PBS at pH 7.4, by pelleting and removal of supernatant.

6.2.3. Capillary Electrophoresis Setup

Analysis of proteins produced in the Jurkat cell line is performed on a custom-built CE laser wave-mixing system. A 40-cm bare fused-silica capillary (Polymicro Technologies, Inc., Phoenix, AZ) with 50 μm I.D., 360 μm O.D. and 25 cm effective length is used for separation. The capillary is then mounted on a rigid translational stage, keeping 10 cm of the capillary completely straight and immobile for detection purposes. The capillary is conditioned for several hours with the appropriate solutions for separation.

6.2.4. Capillary Coating

To prevent protein adsorption on the capillary walls, the capillary is coated using the Hjerten method. First, treatment is performed using 0.5 M NaOH for one hour followed by a one-hour rinse (Barnstead water, 18.2Ω). The next step involves a one-hour reaction of MAPS (γ-methacryloxypropyltrimethoxysilane) with silanol groups on the capillary wall. The capillary is then rinsed with water before filling with the polymerizing solution (4% acrylamide solution containing 0.1% APS and 0.1 % TEMED). It takes approximately an hour to completely coat the inner wall. This
additional step minimizes the accessibility of silanol groups and consequently their interaction with proteins. Furthermore, the acrylamide solution increases the capillary surface viscosity and therefore suppresses the EOF. Finally, the capillary is rinsed with water to remove any excess polyacrylamide. With this coating applied, negatively charged proteins at basic pH levels would easily be separated by their mass-to-charge ratio as they move through the capillary under applied high voltage.

6.2.5. Protein Labeling

The chromophore QSY35 is used for labeling the proteins. It is an amine reactive dye that makes a complex with proteins. In general, any QSY35 protein complex has an absorbance maximum at 460 nm. Figure 6.1 shows the absorption spectra of native protein, ovalbumin and the complex comprised of QSY35-ovalbumin obtained by conventional UV-Vis spectrometer. An argon ion laser operating at 488 nm is used to excite the QSY35 protein complex when the single cell proteins are separated by capillary electrophoresis. The wave-mixing signal is strong and the optical signal collection is efficient. Excellent detection sensitivity is obtained even if the laser excitation wavelength is not at the maximum absorption wavelength of the analyte. The QSY35 chromophore with an extinction coefficient of 23,500 cm$^{-1}$M$^{-1}$ does not fluoresce.

To make sure that this dye does not have any emission when excited by the argon-ion laser, we performed fluorescence spectroscopy, and no evidence of emission
Figure 6.1 Chemical structure of chromophore QSY35 and UV-Vis spectra of labeled and non-labeled protein
was observed. Proteins were labeled as described by the protocol supplied by the manufacturer. Specifically, protein standards were weighed and dissolved in the Tris 0.1M and 2% SDS solution (pH 8.6). The basic pH is used due to the fact that proteins are negatively charged and should be repelled from negatively charged capillary wall.

Basic pH buffers minimize protein absorption on the wall, and hence, band broadening. SDS concentration is also below CMC to minimize background and effects on protein mobility. The minimum protein concentration recommended by the protocol was 2 mg/ml. In addition, 5 mg of chromophore QSY35 was dissolved in 0.5 mL DMSO and sonicated using ULTRasonic28X. Them, 50 µL of chromophore solution was added, while vortexing proteins. Labeled proteins were incubated at room temperature for one hour. All the labeled proteins were diluted and aliquots were stored at – 20 °C for future use.

Multi-labeling was not an issue since (a) attaching each protein with many SDS molecules assists shielding the heterogeneity in the charge-to-size ratio of labeled proteins, and (b) bonding of low molecular mass labels to large molecular mass proteins does not change the protein size and consequently its migration velocity when using a size-based separation technique such as SDS-Dalt CE. However, in microchip-based CE of proteins, post separation labeling is recommended because of the extreme short path lengths and to prevent multi-labeling of proteins.

Wave mixing is an absorption-based method and it can detect proteins labeled with fluorophores or chromophores. Working with more labeling schemes, rather than
just fluorophores, affords a large sampling pool and also grants the use of robust and inexpensive lasers to probe proteins in the visible spectral range. The QSY35 structure has aromatic rings that give rise to optical absorption near 488 nm, but due to NO₂ and other electron withdrawing groups, this compound does not fluoresce.

To purify labeled proteins from unreacted dye and lower molecular weight protein fractions, dialysis is performed using dialysis cells (Bel-Art Products) and dialysis membranes with a molecular weight cutoff at 6000 Da.

6.2.6. Cell Injection

Cell lysis is one of the steps that must be as fast as possible to prevent any dilution of the target analytes by the influx of ions from the buffer during the cell membrane disruption. Several methods have been used to lyse the cells including the use of chemicals (surfactants) and laser-induced shock waves in laser-micropipet methods\textsuperscript{35}, and mechanical disruption\textsuperscript{36} or electrical pulses (electroporation). It has been reported that the chemical cell lysis is several folds longer than the high voltage methods\textsuperscript{37}. We have used a combination of chemical lysis (SDS) and DC voltage to obtain rapid cell lysis and to better denature proteins and improve separation.

In our experiment, we placed 1 µL aliquots of SDS buffer, QSY35 and a single Jurkat cell suspension on a micro-slide. Using a standard inverted phase microscope at 10X magnification (Swit Phase Master, Model 765875, Hinkle’s Optical, Inc.) the injection end of the capillary is placed first in SDS buffer and then in QSY35. The cell
was injected by elevating the capillary end followed by injection of QSY35 and SDS buffer again. The single cell injection is visually confirmed under microscope. Each aliquot was injected for three seconds. Using this sandwich method, the cell is lysed and the cellular components of one cell are completely stained. Also, sample handling is efficiently minimized which prevents bias of the data.\textsuperscript{38}

A pressure difference drives the samples into the capillary and the injection duration controls the volume injected. This simple technique eliminates the need for the computer-controlled vacuum pulse to the distal end of the capillary, using a syringe\textsuperscript{39} or etching the injection end of capillary by hydrofluoric acid\textsuperscript{40}. Consequently, shorter analysis time is obtained, as fewer steps need to be done. A height difference of 3 to 4 cm for 3 seconds injects about 6 nL using a 50 µm I.D. capillary.

No incubation is required for this QSY35 chromophore label; thus, the capillary end is placed in the 1.5 mL glass vial filled with CE buffer immediately. Nickel coated cadmium electrodes are mounted and inserted into the CE buffer reservoirs. High voltage is supplied by a 0-30 kV dc power supply (Glassman, Model PS/MJ30P0400-11 30kV). A voltage of 12 kV is applied to the electrodes to move the proteins across the capillary.

6.2.7. Apparatus

An argon ion laser (Coherent Inc., Model Innova 90-6) at 488 nm is used as the excitation source with a small (nL) probe volume. The low-power argon ion laser (2 -
5 mW) provides tight focusing of laser beams on the capillary with no photo degradation or saturation of the analyte. The wave-mixing signal is generated when a laser beam is split in two with a 90-degree angle toward each other by a 70/30 beam splitter. The split beams are refracted off two mirrors set at 45-degree angle and are focused using a convex lens. The focused beams generate a 30 µm spot size, which efficiently excite analytes passing through the capillary. This focused spot size is an approximation governed by the following equation:

\[ 2W_o \approx \frac{4f}{\pi D \lambda} \]  

(6.1)

in which \( W_o \) is the waist radius of the transmitted beam as it is focused at a given distance, \( f \) is the focal length, \( D \) is the diameter of the transmitted beam, and \( \lambda \) is the wavelength. This approximation is suitable because the focus depth of the transmitted beam is longer than the lens focal length. Therefore, it can be assumed that the focused depth is equal to the focal length of the lens. \(^{41}\) The focused spot size is where an interference pattern is formed. Variables like wavelength and the angle that two beams cross, characterize the interference pattern. Analytes that pass through the focused spot absorb the focused beam and are excited in the form of the interference pattern and release their heat energy to surrounding molecules. This heat energy creates a thermal grating followed by a change in density and refractive index of the matrix. Thus can be
read by incoming photons from one of the two pump beams acting as the probe. The diffracted probe beam propagates in a predictable direction away from the bisector of the excitation beams. The signal beam is collimated producing less background noise. It is visible to the naked eye providing easy means of alignment. The generated wave-mixing signal is detected with a photo-detector (Thorlabs PDA55) and is converted to a digital signal with a digital-analog converter to help display the electropherogram on the computer. A chopper controlled by a chopper controller (Stanford research system, model SR540) is set at 200Hz. A lock-in-Amplifier (Stanford research system, model SR830 DSP) is also set to 200Hz and is used to reduce the background noise as much as possible. Figure 6.2 shows the experimental optical setup.

6.3. Results and Discussion

6.3.1. Reproducibility and Limit of Detection

Reproducible signal detection is necessary to confirm the stability and reliability of our custom-built CE and the wave-mixing detection system. Reproducible results for 3-s injections of the protein standard, β-Lactoglobulin, were obtained. The concentration of the protein was 10 µg/mL. The sample was analyzed with an applied voltage of 12 kV. Protein peaks have a migration time of approximately 919 seconds (Figure 6.3). Slight variations in migration times are attributed to human error and the use of manual injections.
Figure 6.2. Optical setup for CSE wave mixing
Figure 6.3. Reproducibility for three separate CE runs for 10 µg/mL β-Lactoglobulin
Assuming 10% of the mass of a mammalian cell are proteins with average weight of about 25 kDa, a typical Jurkat cell (if a sphere) with 25 µm diameter and a volume of about 8 fL would include a total of about 32 fmole of proteins. The average protein is present at the attomole range. Therefore single-cell analyses require a highly sensitive detection method. Wave-Mixing Spectroscopy is an absorption-based method that offers the detection sensitivity levels required for detecting cellular components at the single-cell level.

To demonstrate this point, serial dilutions of a standard protein (β-Lactoglobulin) were prepared from a 10 mg/mL solution (i.e., 10 mg/mL, 10 µg/mL, 10 ng/mL, 10 pg/mL and 10 fg/mL). Since the average expressed protein in a typical cell is in the high attomole range, this wide dynamic range was necessary to prove the validity of the method for single-cell analysis. The detection sensitivity and dynamic range is depicted in Figure 6.4, which shows a concentration variation study of β-Lactoglobulin. As shown in the figure, Gaussian peaks at 919 seconds are observed over a wide concentration range. As expected, decreasing protein concentrations result in decreasing peak intensity.

In conclusion, this nonlinear optical method can be used to identify and detect proteins found at trace concentration levels within a single cell. Figure 6.5 illustrates the ultra-sensitive detection capability of our wave-mixing CE setup. When only 10fg/ml β-Lactoglobulin is injected, a concentration detection limit of $3.58 \times 10^{-16}$ M is determined. With an estimated detection probe volume of 6 nL, a highly sensitive
Figure 6.4. Concentration variation study for β-lactoglobulin: From bottom to top: 10 mg/mL, 10 ng/mL and 10 fg/mL of β-Lactoglobulin injected
Figure 6.5. Electropherogram of β-Lactoglobulin when only 10fg/ml protein standard is injected. A concentration detection limit of $3.58 \times 10^{-16}$ M and a mass detection limit of 2.2 ymole determined.
mass detection limit of 2.2 yocto mole for β-Lactoglobulin labeled with QSY35 is determined. It should be noted that β-Lactoglobulin has multiple QSY35 binding sites per protein (approximately 15 lysine binding sites per molecule) and it is the absorbing QSY35 chromophore, not the protein that generates the wave-mixing signal. We compare the electropherogram of the standard β-Lactoglobulin protein within a single cell with that of β-Lactoglobulin protein standard (10 µg/mL) and a mixture solution consisting of multiple protein standards including β-Lactoglobulin (1 µg/mL) (Figure 6.6). The β-Lactoglobulin protein within the cell elutes at approximately 919 seconds, comparing favorably to that of the protein standard alone and mixture of standards. Peak intensities vary slightly from one electropherogram to another due to variations in protein concentration. It should be noted that other cellular components present in the cell could create higher background levels in the single-cell electropherogram.
Figure 6.6. Comparison of electropherograms of β-Lactoglobulin protein within a single cell, protein standard (10 µg/mL) and a mixture of protein standards including β-Lactoglobulin (1 µg/mL)
The CE and laser wave-mixing detection system is robust and sensitive for proteins. We performed CE for other proteins like β-glactosidase. Figure 6.7 shows day-to-day reproducibility results for β-Glactosidase (10 µg/mL) with a migration time of 1573 seconds and stable background levels. It is clear from the figure that the capillary is still robust and effective for protein separation from day-to-day.

The detection of β-Glactosidase within in a single cell is compared to the standard solutions (Figure 6.8). Migration time is compared favorably to standards at approximately 1577 seconds. As previously mentioned, peak intensities are different because of variation in protein concentration levels. The standard β-Glactosidase concentration is 10ug/mL and its concentration in the mixture is 1 ug/mL. Please note that electropherograms with different peak shapes can be attributed to the number of binding sites available per protein and the overall concentration of proteins after performing dialysis.

We also study the cell content by analyzing different Jurkat cells with four separate injections. Four separate injections of individual cells using the sandwich lysis and labeling method provide detectable wave-mixing peaks eluted at approximately 1577 seconds. As Shown in Figure 6.9, each peak features the individual cell characteristics. Varying growth rate and protein expression can be seen by the different peak intensities and widths. In conclusion, wave mixing can detect and fingerprint proteins within a single cell, and relative protein abundances can be further correlated to diseases.
Figure 6.7. Day to day reproducibility for β-Glucosidase (10 µg/mL)
Figure 6.8. Comparison of electropherograms of β-Glactosidase protein within a single cell, protein standard (10 μg/mL) and a mixture of protein standards including β-Glactosidase (1 μg/mL)
Figure 6.9. β-Glactosidase protein is identified in four single cells injected individually.
6.3.2 Molecular Weight Estimation

Factors affecting injection volumes include viscosity, evaporation, surface tension, sample dissolving solvent viscosity, temperature, sample solution level, voltage ramping/volume expansion, injection vial positioning, injection pressure, and sample carryover. Variations in injection volumes resulting from these factors can be corrected by adding a suitable internal standard.  42

SDS-DALT-CE experiments are performed using a mixture of protein standards. An internal reference marker, Orange G with a molecular weight of 10 kDa is also added to the mixture. There is no interaction between Orange G and proteins and it elutes before the proteins of interest due to its lower molecular weight compared to the proteins used in the mixture.

Figure 6.10 shows the linear correlation between molecular weight (MW) and the migration times of Orange G and proteins (β-Lactoglobulin, tris inhibitor, carbonic anhydrase, ovalbumin and β- Glactosidase). The regression line \(y = 673.98 x -1959\) is obtained with a \(R^2\) value of 0.9911. This linear correlation of the MW and mobility of protein standards can be used to identify and predict the MW of other known or unknown proteins found within the cell. Table 6.1 shows proteins, molecular weights, migration times and relative standard deviations for each of the detected proteins using Orange G as an internal standard. The detectable dynamic range obtained by SDS-DALT electrophoresis and laser wave-mixing detection is wide, and hence, it is
Figure 6.10. Molecular weight estimation plot for a mixture solution containing Orange G, β-lactoglobulin, trypsin inhibitor, carbonic anhydrase, ovalbumin and β-galactosidase
Table 6.1. Retention time and molecular weight of standard proteins.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Molecular Weight (Da)</th>
<th>Migration Time (s)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orange G</td>
<td>10000</td>
<td>746</td>
<td>3.03</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>18400</td>
<td>915</td>
<td>6.31</td>
</tr>
<tr>
<td>Trypsin Inhibitor</td>
<td>20100</td>
<td>970</td>
<td>7.64</td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td>30000</td>
<td>1030</td>
<td>4.93</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>43000</td>
<td>1140</td>
<td>4.29</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>116000</td>
<td>1570</td>
<td>6.91</td>
</tr>
</tbody>
</table>

especially useful when analyzing single cells with a wide range of proteins that are expressed.

Finally, separation of a mixture solution containing β-lactoglobulin, trypsin inhibitor and carbonic anhydrase at 3.3 µg/mL is compared to an electropherogram obtained for a single cell. In the electropherogram obtained for the mixture, β-Lactoglobulin elutes from the column first at approximately 920 s, trypsin inhibitor at 965 s and carbonic anhydrase at 1030 s. All three proteins show matching peaks in the single-cell electropherogram, confirming the identities of these proteins in the Jurkat cell. Some peak height variation is observed due to the varying number of binding
sites found on each protein. The electropherograms obtained for the single cell and the standard mixture are also compared in Figure 6.11.

6.4. Conclusion

In this study, we have demonstrated that laser wave-mixing provides the excellent detection sensitivity needed in order to detect cellular proteins at low concentration levels while using capillary sodium dodecyl sulfate-Dalton electrophoresis (SDS-DALT) as a size-based CE separation method that offers excellent separation resolution.

The SDS-DALT-CE wave-mixing system provided ultra sensitive yoctomole-level detection of proteins using a non-fluorescing label in a small probe volume. Laser wave mixing is an ultrasensitive detection method, which can be used for both fluorescing and non-fluorescing analytes. For this reason, proteins may be labeled with either chromophores or fluorophores. To the best of our knowledge, no one has detected single-cell proteins labeled with a chromophore using an absorption-based optical method. Laser wave mixing and its inherent properties afford sensitivity levels comparable or better than those of other laser methods including laser-induced fluorescence methods.
Figure 6.11. Comparison of electropherograms obtained for single cell (top) and the standard mixture (bottom)
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Chapter 7

Amphiphilic Polymer-Based Protein Separation by Capillary Electrophoresis and Laser Wave-Mixing Detection

7.1 Introduction

The microenvironment of micelle-like polymer aggregates (self-organized assemblies) can increase the range of pseudo-stationary phases with different selectivity in micellar electrokinetic chromatography (MEKC). The main application of these polymers is in the separation of highly hydrophobic solutes where a high concentration of organic solvent is needed. These polymeric micelles compete with other surfactants that cannot be formed in high concentration of organic solutes.\textsuperscript{1,2} It has been suggested that self-organized assemblies can provide functional fine particles that act as hosts for various molecules. They are used in many applications including diagnostic testing, bioseparations, controlled release of drugs, vaccines, antibodies, hormones and other biological agents, gene therapy, catalysis, water-borne coatings, cosmetics, agricultural, pesticides, colloid and interface science, water treatment, enhanced oil recovery, paints, surface modification, chiral separation, artificial receptor, immobilization of biologically active molecules (DNA, proteins, enzymes, peptides, drugs, etc., onto solid supports).\textsuperscript{3,4,5,6,7,8,9,10,11,12,13,14,15}
Polymers are an attractive alternative to gels in capillary electrophoresis due to the difficulties associated with the use of gel in capillaries. Factors such as pH, temperature, chemical structures of solute and polymer, and polarity of the solute and location of the solute within the micelle affect solute association with a micelle. Various polymers can be made and used instead of low molar-mass surfactants. The availability of a wide variety of different monomers allows some user control over the hydrophilic/hydrophobic balance and also allows the ability to selectively introduce additional functional groups along the polymer chain. Thus, the synthesized polymer has characteristics of both low molecular weight surfactants and polymer amphiphiles.

Surfactant micelles have been used in protein separation in micellar electrokinetic chromatography (MEKC). Non-ionic surfactants such as Brij 35, Brij 78 and Tween 20 have been used for protein separation in milk by capillary electrophoresis. Non-ionic surfactant polyoxyethylene alkyl ether was used for protein extraction and purification by using an aqueous micellar solutions two-phase system (AMTPS). The addition of surfactant Brij 35 to the running buffer improved separation of phospholipids and proteins in PDMS-coated capillaries and SDS controlled the electro-osmotic mobility. Linear or slightly branched polymers such as polyethylene oxide (PEO), dextran, polyethylene glycol (PEG) and polyacrylamide have been used as a sieving matrix. Biopolymers (amphibiopols), such as pullulan, have also been used as surfactants for membrane protein solubilization. Pullulan is
hydrophilic natural linear glucan that is not soluble in organic solvents. Therefore, it has a limited number of applications as compared to amphiphilic polymers.

The successful synthesis of the random amphiphilic polymer, poly (n-dodecylacrylate-3-sulfopropylmethylacrylate) or \( \text{C}_{12}\text{SO}_3\text{H} \) (25/75%), allowed for improved separation over previous matrices since it has the desired characteristics necessary for use in CE protein separation methods. Amphiphilic polymers offer many benefits for protein separation. The polymers have the ability to be used in both hydrophobic and hydrophilic moieties. They are easy to synthesize, require lower polymer percentage, and are cost effective. These polymers allow shorter analysis times and provide high efficiency separation of hydrophobic proteins.

Nonlinear laser wave mixing coupled with this new polymer can enhance CE separation with improved peak resolution and detection sensitivity. Chromophore-labeled proteins flow through the capillary and are separated based on their mass-to-charge ratio. The thermal gratings formed by optical absorption produce the laser wave-mixing signal. The signal is a collimated coherent laser-like beam that is easily collected with nearly 100% collection efficiency against a dark background.

7.2. Experimental

7.2.1. Reagents

All chemicals including n-dodecylacrylate, 3-sulfopropylmethylacrylate and 2-[(cyano-dimethyl-methyl)-azo]-2-methyl-propionitrile \( \text{AIBN} \), \( \text{N} \), \( \text{N} \),
dimethylformamide (DMF), polystyrene standards, pullulan and the protein standards β-lactoglobulin and trypsin inhibitor, and chemicals used for coating the capillary including acrylamide, TEMED and MAPS (γ-methacryloyloxypropyltrimethoxysilane) were ordered from Sigma-Aldrich. Molecular Probes provided the chromophore QSY35. All the solutions were prepared using Barnstead (18.2 Ω) water. Buffers were filtered with 0.45 µm nylon disposable filters and stored at 8 °C. The labeled proteins were stored in a -20 °C freezer in aliquots that were thawed at room temperature before each run.

7.2.2. Apparatus

An argon-ion laser (Coherent Inc., Model Innova 90-6) at 488 nm was used as the excitation source (2 to 5 mW). The laser beam is split into two separate beams by a 70/30-beam splitter. These two input beams propagate in the forward direction. They are focused and mixed inside the analytes in the capillary. The molecules that pass through the laser probe volume are excited, forming interference gratings. The heat energy released to surrounding solvent molecules creates thermal gratings, which in turn changes the refractive index of the matrix. Incoming photons from one of the two pump beams (acting as the probe) diffract off the grating and generate the signal beam propagating in a predictable direction. The coherent laser-like signal beam is collimated and visible to the naked eye, allowing easy optical alignments. The generated wave-mixing signal is detected by a simple photodetector (Thorlabs,
PDA55) and digitized and stored on a computer. The weaker input beam is chopped with an optical chopper at 200 Hz (Stanford Research System, Model SR540) and analyzed by a lock-in-amplifier (Stanford Research System, Model SR830 DSP), as shown in Figure 7.1.

### 7.2.3. Polymer Synthesis

To synthesize the polymer, n-dodecylacrylate, 3-sulfopropylmethylacrylate and 2-[(cyano-dimethyl-methyl)-azo]-2-methyl-propionitrile (AIBN) is first dissolved in N, N\textsubscript{-}dimethylformamide (DMF). In a dry test tube, monomers are dissolved in DMF, followed by the radical initiator AIBN. The test tube is then capped, degassed with vacuum, and purged with \textit{N} \textsubscript{2} three times to remove oxygen. After purging, the test tube is stirred and incubated at 60 \textdegree{}C for 24 hours in an oil bath. Scheme 7.1 shows the reaction mechanism.
Figure 7.1. Optical D4WM Setup
Scheme 7.1. Polymer synthesis

3-sulfopropylmethylacrylate (75%)

n-dodecylacrylate (25%)

DMF, N₂, 60°C, 24 hrs

75% / 25%
7.2.4. Gel Permeation Chromatography

After synthesis, the product is assayed for polymer weight. A gel permeation chromatographic (GPC) assay for crude protein is used to determine the weight of the synthesized polymer. A very small amount of the crude polymer (0.5 mL) is mixed with 1 mL of a mixture of acetonitrile (ACN) and phosphate buffer at 10 mM, pH 7 and 20/80% (v/v). This mixture is also used as the mobile phase in a GPC system (Agilent 1100 Chemstation) with a 300 mm x 7.8 mm Ultra Hydrogel Linear GPC column (Waters Corp.) at 45 °C with a flow rate of 1 mL/min. Standard polymers of polystyrene sodium sulfonat (PS) with molecular weights of 4.6, 8, 35 and 100 kDa are used for the determination of polymer weight. Observation of peaks at 210 nm for polymers at retention times between the retention times of the PS standards confirms polymer formation. As shown in Table 7.1, the polymer molecular weight is estimated to be 50 kDa.

Table 1. MW estimation of polymer

<table>
<thead>
<tr>
<th>Polymer</th>
<th>MW (D)</th>
<th>Log MW</th>
<th>tr (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polystyrene</td>
<td>4600</td>
<td>3.66</td>
<td>7.67</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>8000</td>
<td>3.90</td>
<td>7.59</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>35000</td>
<td>4.54</td>
<td>7.14</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>100000</td>
<td>5.00</td>
<td>6.86</td>
</tr>
<tr>
<td>Poly (C_{12}SO_{3}H) (25/25%)</td>
<td>49980</td>
<td>4.70</td>
<td>7.06</td>
</tr>
</tbody>
</table>
7.2.5. Polymer Purification

The polymer is purified using the following procedure. First, the polymer is dissolved in H$_2$O and then precipitated in a mixture of isopropanol alcohol (IPA)/ACN 50/50% (v/v). After transferring to a round bottom flask, the solvent is removed using a Rotovap (R-3000) at 70 $^\circ$C. The remaining liquid is centrifuged and the supernatant removed to make sure that the polymer is precipitated. This process is repeated three times and then the solvent is dried under vacuum. GPC is performed again to make sure that the polymer is reasonably purified.

7.2.6. Sample Preparation and Labeling

The protein standards are dissolved in a Tris/SDS (0.1 M, 2%) solution in which SDS acts as a denaturant to unfold protein standards. Then, the proteins are labeled with the chromophore QSY35. The protein standards are prepared by first weighing and dissolving in Tris 0.1 M and 2% SDS solution at pH 8.6. The basic pH of the solution imparts a negative charge on the proteins. This negative charge minimizes adsorption to the similarly charged capillary wall due to repulsion, and hence, minimizes band broadening. The concentration of SDS is also below CMC, and hence, minimizing background and providing better protein mobility. The chromophore QSY35 (5 mg) is dissolved in DMSO (0.5 mL) and sonicated using ULTRasonic 28X. A 50 µL portion
of the QSY35 solution is added to the protein solutions while vortexing. Then the labeled proteins are incubated at room temperature for one hour. Aliquots of diluted labeled proteins (1000 fold) are made and stored at –20°C for future use. After dialysis of labeled proteins in order to get rid of unreacted dye and low molecular impurities, proteins are ready for use in our capillary electrophoresis system.

7.2.7. Capillary Electrophoresis Setup

Analysis of proteins is performed on a custom-built CE laser wave-mixing system using a 40-cm bare fused-silica capillary (25 cm effective length, 50 µm I.D. and 360 µm O.D.). A small portion of the polyimide capillary coating is removed to allow the beams to pass through the capillary. The capillary is then mounted on a capillary holder on a rigid translational stage, providing a stable platform for detection purposes. The capillary is then conditioned with the water and running buffer before separation.

7.2.8. Coating Capillary

To prevent protein adsorption on the capillary walls, the capillary is coated using the Hjerten method\textsuperscript{32} with a four-step rinsing and treatment process that utilizes the polymerizing solutions for one hour. First, it is treated with 0.5 M NaOH followed
by a one-hour water rinse. The next step involves a one-hour reaction of silanol groups on capillary wall with MAPS (γ-methacryloxypropyltrimethoxysilane). After rinsing the capillary with water, it is filled with the polymerizing 4% acrylamide solution containing 0.1% APS and 0.1 % TEMED. After one hour, the inner wall of the capillary is coated completely. This additional step minimizes the availability of the silanol groups that might interact with proteins and serves to suppress the EOF by increasing the capillary surface viscosity. Finally, the capillary is rinsed with water to remove any excess polyacrylamide. The negatively charged proteins at basic pH levels are easily separated by their mass-to-charge ratios as they move through the capillary under applied high voltage.

7.3. Results and Discussion

This amphiphilic polymer is tested for protein separation. Concentration and pH variation studies are also performed to find the experimental conditions that give the best separation characteristics. The results are compared to those of pullulan sieving matrix commonly used for protein separation.

Initially, protein standards are injected into the capillary filled with a running buffer (2% polymer solution at pH 8.6). An electropherogram (3-s injection at 12 kV) of a standard mixture of 10 µg/mL β-lactoglobulin and trypsin inhibitor is shown in Figure 7.2. Since the separation is good and the baseline clean, our amphiphilic polymer is
acceptable for use in protein separation assays. We obtain reproducible results for protein standards, β-lactoglobulin and trypsin inhibitor, at 10 μg/mL in three electropherograms (all 3-s injection, 12 kV, 2% polymer, pH 8.6) with retention times of 752.7 (+/- 1.1) and (1309.3 +/- 5.5) seconds respectively. Slight variations in migration times and peak heights are attributed to the variable nature of manual injections and changes to the position of the capillary in the probe and sensing volume. As shown in Figures 7.3 and 7.4, we observe signal reproducibility levels necessary for our custom-built CE and the wave-mixing detection system.

A 10 μg/mL β-lactoglobulin sample is also injected into the capillary using 2% pullulan solution (pH 8.6) as the running buffer. The results are compared with the results obtained for the 2% polymer as shown in Figure 7.5. A faster migration time is observed for the 2% polymer as compared to that of the 2% pullulan. Therefore, this polymer provides shorter CE runs for protein separation under similar circumstances.

To further investigate the polymer dynamic pH range, a 2% polymer is prepared in a running buffer Tris/SDS/CHES (0.1 M, 0.1%, 0.1 M). The pH is adjusted to 6.54 and then used as a running buffer. As shown in Figure 7.6, a faster migration time is obtained for β-lactoglobulin at this pH as compared to that at pH 8.6. Therefore, one can further improve and shorten analysis time by adjusting the pH levels.
Figure 7.2. Electropherogram of protein standards 10 μg/mL β-lactoglobulin and Tripsyn inhibitor (2% polymer, pH 8.6, 12 kV). β-lactoglobulin is eluted earlier than trypsin inhibitor due to its smaller MW.
Figure 7.3. Reproducible results (1309.3 +/- 5.5 s) for protein standard 10 ug/mL trypsin inhibitor (2% polymer, pH 8.6, 12 kV).
Figure 7.4. Reproducible results (752.7 +/- 1.1s) for protein standard 10 ug/mL β-lactoglobulin (2% polymer, pH 8.6, 12 kV).
Figure 7.5. Comparison of retention times for the protein standard β-lactoglobulin using 2% pullulan (bottom) and 2% polymer (top). All the other conditions are the same. A faster elution time is observed when the polymer is used as the running buffer.
Figure 7.6. A faster elution time is observed at pH 6.54 for β-lactoglobulin (top) as compared to that at pH 8.6 (bottom).
It is well known that the concentration and the ionic strength of the running buffer in a CE system can play an important role on separation resolution and efficiency. To confirm this, a 1% polymer solution at pH 8.6 is prepared the same way as for the 2% polymer solution. Then, β-lactoglobulin is injected into the capillary, and as expected, a faster migration time is observed for the 1% polymer solution. The migration time for this protein using a 1% w/v polymer is almost half of the 2% polymer as shown in Figure 7.7.

We also check our system for day-to-day reproducibility using the 1% polymer and the standard protein β-lactoglobulin. System robustness is confirmed as shown in Figure 7.8.
Figure 7.7. Concentration variation study. A faster elution time is obtained when a 1% polymer is used (bottom) as compared to the 2% polymer (top).
Figure 7.8. Day-to-day comparison for the β-lactoglobulin protein standard using the 1% polymer running buffer.
4. Conclusion

Amphiphilic polymers offer many advantages over low molecular surfactants and sieving matrixes used for protein separation. They are easy to synthesize and more cost effective. The use of this amphiphilic polymer in both hydrophobic and hydrophilic moieties offers significant advantages including a wider dynamic range and excellent detection sensitivity levels. It also provides faster separation times as compared to those of the sieving matrix pullulan. Shorter separation times can also be obtained using lower pH values and lower concentration levels. Nonlinear laser wave-mixing spectroscopy offers unique inherent properties and one can analyze both fluorescing and non-fluorescing analytes. In this study, proteins are labeled with a chromophore, separated by capillary electrophoresis and detected by laser wave mixing. Using amphiphilic polymers as running buffers offer desirable experimental conditions as compared to other matrices.
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8.1. Introduction

Many researchers have developed a wide range of high throughput methods to characterize samples in different applications including study of combinatorial processes of chemistry and biology. For example, the ultra-thin-layer agarose gel electrophoresis in conjunction with laser-induced fluorescence (LIF) scanning detection (632-nm solid state laser) has been used as a high throughput separation method for individual or multiplexed polymerase chain reaction (PCR) products with sub-nanogram sensitivity by Guttman and Barta.  

Others demonstrated a multi-channel chip array (16 channels) with a replaceable sieving polymer to separate DNA fragments in a size-based electrophoretic separation system with a scanning confocal LIF detector. Chen and Lillard introduced high-throughput analysis of single cells using a flow-based interface for continuous injection of intact cells by electroosmotic flow into a lysis junction. He and Yeung performed a two dimensional mapping of cancer cell extracts using a LC-CE system with a 214 nm UV detector. In this work, fractions obtained by HPLC were collected
in a 96-well microtiter plates. After drying under vacuum and reconstituting with deionized water, they were concentrated on-column by large-volume sample stacking and then separated by capillary electrophoresis using capillary arrays and a photodiode array (PDA)\(^4\). They also performed on-column protein digestion for peptide mapping\(^5\), screening of kinase inhibitors\(^6\) and DNA sequencing\(^7\) using a multiplexed CE system. Gong used a 96-capillary array and a PDA to detect small molecules at 254 nm using MEKC and reported a detection limit of \(1.8 \times 10^{-5}\) M for rhodamine 6G\(^8\).

Tu and Wehmeyer also used a multiplexed CE system with a LIF detector for monitoring conversion of fluorescent-labeled peptide substrates to a phosphorylated fluorescent-labeled peptide product. A CCD image of 96-well plate was obtained and slight variations in migration time and intensity of the internal standard were explained by differences in capillary surface chemistry, vacuum injection pressure, camera digitization errors, capillaries diameters and laser intensity across the capillaries\(^9\).

In a study by Luo and Pang, proteins were separated using commercially available cePro9600, a 96-capillary electrophoresis system with a 214-nm UV detector\(^10\). They obtained a 5 ug/mL detection limit in a SDS-CGE mode for bovine serum albumin (BSA) prepared in 12.5 mM Tris-HCl with dynamic coating of capillaries using 5% dextran in 0.1% SDS in a 50 mM Tris-borate buffer\(^11\). Although they obtained a relatively fast separation time of \(~30\) min., it has a limited sample throughput\(^12\).
The detection methods used so far for high throughput characterization of small molecules include evaporative light scattering (ELS), chemiluminescent nitrogen, mass spectrometry and NMR. However, all of these methods have inherent limitations. For example, ELS provides only relative purity measurements and its response diminishes at lower than 300 MW. Chemiluminescent nitrogen can only be used for compounds that contain nitrogen and not for compounds with functional groups such as azides and tetraazoles that yield N$_2$ on combustion. The response factor of mass spectrometers depends on the compound ionization efficiencies and sample matrixes. The mass resolution for high MW compounds is also limited. Finally, the NMR data analysis is complex, especially in the presence of impurities, and it is difficult to integrate on-line with popular separation systems$^{13}$.

For high throughput CE applications, a variety of detection techniques have been reported including thermo-optical absorption$^{14}$, direct and indirect absorption, photo thermal refraction, direct on-column, post-column and indirect LIF, potentiometric, conductivity and amperometric methods, refractive index detectors, Raman, NMR radioisotope methods, and laser-induced capillary vibration detection. Among these detection techniques, Raman and NMR provide the weakest limit of detection (LOD) (10$^{-3}$ M) and post-column LIF yields the best LOD (10$^{-16}$ M)$^{15}$.

Techniques used for high-throughput application of large molecules (i.e., characterization of peptides, oligonucleotides and catalysis efficiency) include mass spectrometry, gas chromatography (GC), multiplexed CE-UV absorbance detection,
fluorescence emission, photoionization detection, photoacoustic detection, infrared thermography, infrared spectroscopy and colorimetric detection. Among these, only mass spectrometry and infrared spectroscopy provide detailed chemical information, but they cannot be used in high throughput parallel modes. While most of these techniques can analyze samples in 1 minute to 15 minutes per library, CE multiplexed systems can analyze up to 96 samples in 30 minutes\textsuperscript{16}.

To date, almost all commercially available high-throughput capillary electrophoresis systems (multiplexed CE) use laser-induced fluorescence (LIF) detectors. Only one multiplexed CE instrument (Combisep, Model cePRO 9600) is equipped with a UV absorption detector. According to manufacturer specifications, this UV detector can yield a detection sensitivity level of only 5 $\mu g/mL$, a relatively poor sensitivity level for proteins [11, 12].

In this work, we report for the first time, a high-throughput CE system coupled to a nonlinear wave-mixing detector for the analysis of many samples on a short time scale. Using an array of tightly arranged capillaries, proteins can be detected as they pass through the laser probe. Unique features and advantages include excellent detection sensitivity, short optical path lengths and small samples, quadratic dependency on sample absorption coefficient and concentration, cubic dependency on laser power, coherent laser-like signal beams, and 100% optical collection efficiency for signals measured against a dark background. In a typical setup, a cylindrical lens is used to expand the input laser beams on a capillary array. As different proteins with
different molecular weights, and therefore, different migration times, are injected and flowed through, the laser beams are absorbed and the wave-mixing signal is generated. A photodiode array detector collects the signal beams.

8.2. Experimental: Materials and Methods

8.2.1. Reagents

All reagent grade chemicals (Aureobasidium Pullulans, acrylamide powder, APS, TEMED, 2-(N-cyclohexylamino)ethanesulphonic acid (CHES) and β-Lactoglobulin) are obtained from Sigma-Aldrich. QSY35 is ordered from Molecular Probe (Eugene, OR). All solutions are prepared using Barnstead (18.2Ω) water. Buffers are filtered with 0.45 µm nylon disposable filters. All protein standards and buffers are stored at 8 °C and labeled proteins and QSY35 are stored at –20 °C.

8.2.2. Labeling the Proteins

First, the β-lactoglobulin protein standard is weighed (10 mg/mL) and dissolved in a Tris 0.1M and 2% SDS buffer solution at pH 8.4. The basic pH provides negatively charged proteins that can be repelled from negatively charged capillary wall, minimizing protein adsorption on the wall and band broadening while SDS denatures them and provides easier movement of proteins in capillary. Then, 5 mg of chromophore QSY35 is dissolved in 0.5 mL DMSO and sonicated using an ULTRAsonic 28X. A 50 µL portion of QSY is added while vortexing and the β-
lactoglobulin standard is incubated at room temperature for one hour. Labeled protein is placed in cubical dialysis chambers (Bel-Art Products) with a dialysis membrane cutoff of 6000-8000Da to filter out small molecular fragments. Proteins are diluted consecutively down to the 10 fg/mL concentration level. Aliquots of diluted standards are stored at – 20 °C for future use.

8.2.3. Optical Setup

A 488-nm water-cooled argon-ion laser (Coherent, Inc., Palo Alto, CA, Model I90-6) is used as the excitation source. The laser beam is split into two beams with a 70/30 beam splitter. These two input beams are then focused and mixed by a 10-cm focusing lens inside the capillary array. To expand the laser beams along one axis and to focus the light into a thin line on the capillary array, a UV fused Silica cylindrical plano-concave lens (Thorlabs Photonics) is used. The laser beam expands if the source is placed at the focal point with a half angle $\theta$ of $r_0/f$. At distance $z$ after the lens (when $z > f$), a line with thickness $2r_0$ is formed. Its length is calculated by

$$L = 2 \left( \frac{r_0}{f} \right) (z + f) \quad (8.1)$$

If $z >> f$, the expansion ratio is close to $z/f$ as shown in Figure 8.1 (a).

When another lens is placed in the setup as shown in Figure 8.1(b), image lengths $y_2$ and $y_3$ can be calculated using the following equations$^{18}$:
\begin{align*}
\theta_2 &= y_1 / |f_1| \quad (8.2) \\
y_2 &= \theta_1 |f_1| \\
y_3 &= \theta_2 f_2 \\
\theta_3 &= y_2 / f_2 = \theta_1 |f_1| / f_2 \quad (8.5) \\
y_3 / y_1 &= \theta_2 f_2 / \theta_2 |f_1| = f_2 / |f_1| \quad (8.6) \\
2y_3 &= 2 \theta_2 f_2 = 2y_1 f_2 / |f_1| \quad (8.7)
\end{align*}

For our high-throughput setup, a cylindrical lens is necessary. However, expanding the laser beams decreases the laser power at the capillary array, and hence, the signal strength. Placing the cylindrical lens before the focusing lens expands the beams more than is required to cover the capillary array. This excess expansion also decreases the laser power density per capillary. Therefore, the cylindrical lens is placed right after the focusing lens and before the capillary array. After focusing and expanding the laser beams on the capillary array, a laser induced grating is formed, and the probe beam diffracts off the grating to generate the signal beam. Another lens collimates the signal beams created by the capillary array so that they can be conveniently split and collected by a single photodiode and a photodiode array detector. The single photodiode is placed on a translational stage to collect individual wave-mixing signal beams generated by the capillaries. The photodiode array with a C5964 NMOS image sensor (1024 pixels, Hamamatsu, Japan) records the 2-D images and store them in a
Microsoft Excel file at 1-s intervals. To minimize background noise levels, an optical chopper (Stanford Research Systems, Model SR540) modulates the signal at 200 Hz and a lock-in amplifier recovers and amplifies the signal (Stanford Research Systems, Model SR810), as shown in Figure 8.2.

**8.2.4. Capillary Array**

Ten square fused Silica capillaries (71 um I.D., 360 um O.D., 37 cm long, 25 cm effective length) are held tightly together with a holder mounted on a XYZ stage. The polyimide coatings are removed by a small flame to create an array of detection windows. To prevent protein adsorption on the capillary wall, all capillaries are coated with polyacrylamide solution using the Hertjen method. Briefly, the capillaries are rinsed with sodium hydroxide (0.5 M) for an hour followed by rinsing with water. Then, they are reacted with MAPS (γ-methacryloxypropyltrimethoxysilane) followed by a water rinse. The capillaries are filled with the 4% acrylamide polymerizing solution containing 0.1% APS and 0.1% TEMED for one hour. Finally, the capillaries are rinsed again with water to remove any excess acrylamide solution.
Figure 8.1(a). Beam shape after a cylindrical lens in the form of a thin line, (b) expanded and focused beams resulting from a cylindrical lens and a convex lens [18]
Figure 8.2. Optical setup for high-throughput CE using a 488-nm argon-ion laser. A cylindrical lens expands the focused beams on the capillary array with 10 square capillaries.
Before each run, capillaries are rinsed with water and running buffer. The ends of the capillaries are placed in sieving matrix reservoirs containing 8% aueobasidium pullulan, 0.1M Tris, 0.1M CHES and 0.1% SDS (pH 8.4). A capillary array is illustrated in Figure 8.3. The capillary array is assembled using a set of uniform and smooth capillaries.

For an array of eight capillaries, larger net O.D. of each capillary provides larger total capillary surface area per array with larger distance between each capillary probe area. The resulting configuration provides less interference from adjacent capillary and laser leakage. Some examples of capillaries dimensions are shown in Figure 8.4.

In order to optimize the signal and minimize background optical noise, image size from each capillary after removing the coatings as well as its divergence in both xy and z-axis needs to be calculated and measured experimentally.

If each capillary window acts as an optical source and the capillary I.D. is considered the optical source length $p$, then its image length $q$ can be calculated using the following equation:

$$\frac{1}{p} + \frac{1}{q} = \frac{1}{f} \quad (8.8)$$

Table 8.1 shows calculated image size $q$ for a 71 um I.D. capillary when using different focusing lenses.
Figure 8. 3. Array of 8 fused Silica square capillaries (71 um I.D., 360 um O.D., 37 cm total length, 25 cm effective length)
Figure 8.4. Capillaries with different diameters
Table 8.1. Image size calculation for lens with different focal length

<table>
<thead>
<tr>
<th>Focal length of the lens (um)</th>
<th>Capillary I.D. = p (um)</th>
<th>Signal beam Length = q (um)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100000</td>
<td>71</td>
<td>-71.0504</td>
</tr>
<tr>
<td>200000</td>
<td>71</td>
<td>-71.0252</td>
</tr>
<tr>
<td>300000</td>
<td>71</td>
<td>-71.0168</td>
</tr>
<tr>
<td>400000</td>
<td>71</td>
<td>-71.0101</td>
</tr>
<tr>
<td>500000</td>
<td>71</td>
<td>-71.0126</td>
</tr>
</tbody>
</table>

Divergence angle $\theta_3$ of the signal beam is also measured experimentally in the z-axis (i.e., side view) at a 3-foot distance from the detector, as shown in Table 8.2.

Table 8.2. Divergence angle measured experimentally in z-axis

<table>
<thead>
<tr>
<th>Image Size q (um)</th>
<th>q/2 (um)</th>
<th>Distance after capillary (um)</th>
<th>$\tan(\theta_3)/2$</th>
<th>$\theta_3/2$</th>
<th>$\theta_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3000</td>
<td>1500</td>
<td>914400</td>
<td>0.00164</td>
<td>0.094</td>
<td>0.19</td>
</tr>
<tr>
<td>4000</td>
<td>2000</td>
<td>914400</td>
<td>0.002187</td>
<td>0.125</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Divergence angles and beam-spot distances are also calculated for the 3-foot distance from the detector, as shown in Table 8.3. Signal divergence in both axes is shown in Figure 8.5.
Table 8.3. Divergence angle and beam spot calculation (top view)

<table>
<thead>
<tr>
<th>Distance between beams before lens (um)</th>
<th>a/2</th>
<th>Focal point</th>
<th>tg((\Theta_1))/2</th>
<th>(\Theta_1) ((\Theta_1 \sim \Theta_2))</th>
<th>Distance after capillary (um)</th>
<th>Distance between beam spots after capillary (um)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>500</td>
<td>100000</td>
<td>0.29</td>
<td>0.57</td>
<td>914400</td>
<td>262089.17</td>
</tr>
<tr>
<td>2000</td>
<td>1000</td>
<td>100000</td>
<td>0.57</td>
<td>1.15</td>
<td>914400</td>
<td>524178.34</td>
</tr>
<tr>
<td>3000</td>
<td>1500</td>
<td>100000</td>
<td>0.87</td>
<td>1.72</td>
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<td>100000</td>
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<td>2.29</td>
<td>914400</td>
<td>1048356.69</td>
</tr>
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</table>
Figure 8.5. Signal divergence (a) side view: image size is measured experimentally at the 3-foot distance, (b) top view: divergence angles of beams and beam-spot distances at the 3-foot distance in the xy-axis are calculated assuming that $\theta_1 = \theta_2$. 

Distance from capillary to detector
$q = \text{image size } \sim 3000\mu\text{m in 3 feet}$
$\Theta_3 = \text{diverging angle in } z \text{ direction}$
Since our signal beams are considered Gaussian beams, when focused at waist radius, \( W_0 \), Gaussian beam divergence \( \theta_0 \) is calculated by:

\[
\theta_0 = \frac{\lambda}{\pi W_0} \quad (8.9)
\]

where \( \lambda \) is the wavelength of the laser beam. Since the laser beams are focused inside the capillary, the diameter of the capillary equals \( 2W_0 \). If the capillary inner diameter is considered a Gaussian aperture, beam divergence can be calculated based on Fresnel diffraction, as shown in Figure 8.6.

The air gaps between capillaries (after the coating is removed) create some laser leakage and background noise levels. Figure 8.7 shows signal divergence, laser leakage and interference for an array of 10 square capillaries. Image size is calculated for different laser sources and different capillaries under the same condition for a 633-nm He-Ne laser and a 266-nm UV laser in both \( xy \) and \( z \) coordinates, as shown in Table 8.4. When signals are not focused, their image sizes are much greater than what is desired and clearly they will interfere with each other. Covering these capillary gaps can improve the signal quality, however, it is time consuming and somewhat difficult (Figure 8.8). Therefore, a collimating lens is used to prevent too much signal divergence and to minimize optical interference between capillaries. Based on these calculations, 360 um O.D. capillaries are determined to offer minimum optical interference inside our custom-designed capillary array (Table 8.5).
Figure 8.6. (a) Focused Gaussian beam at waist radius. (b) Fresnel diffraction from a Gaussian aperture assuming that capillary I.D. = 2W₀
Distance from lens to capillary  Distance between capillary array and detector

Square capillary array side view after coating removal

Figure 8.7. Signal divergence, laser leakage and beam interferences for an array of 10 square capillaries
Table 8.4. Image size calculation when signals are not focused. Capillary dimension:
Tube capillary 100um I.D., 234um O.D, 13um coating

<table>
<thead>
<tr>
<th>Wave length (nm)</th>
<th>Slit size/capillary (μm)</th>
<th>Slit size/capillary I.D. (nm)</th>
<th>θ₀ (rad)</th>
<th>tg θ₀</th>
<th>Distance (μm)</th>
<th>Image size (μm two triangles)</th>
<th>Total image size considering laser leakage</th>
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<td>633</td>
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**Laser leakage**

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<th>Slit size/capillary I.D. (nm)</th>
<th>θ₀ (rad)</th>
<th>tg θ₀</th>
<th>Distance (μm)</th>
<th>Image size (μm two triangles)</th>
<th>Total image size</th>
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Table 8.4. continued

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<th>$\tan \theta_0$</th>
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<td>0.0000114</td>
<td>304800</td>
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<td>475.25</td>
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</table>

**Laser leakage**

<table>
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<th>Wave length (nm)</th>
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<th>$\theta_0$</th>
<th>$\tan \theta_0$</th>
<th>Distance (um)</th>
<th>Image size (um) (two triangles)</th>
<th>Total image size</th>
<th>Total image size considering laser leakage</th>
</tr>
</thead>
<tbody>
<tr>
<td>266</td>
<td>100000</td>
<td>0.0017</td>
<td>2.96E-05</td>
<td>304800</td>
<td>18.02</td>
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<td>213.31</td>
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<td>0.0000114</td>
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<td>95.30</td>
<td>300.63</td>
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<td>164.59</td>
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<tr>
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<td>1219200</td>
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<td>0.0000114</td>
<td>304800</td>
<td>207.89</td>
<td>233.89</td>
<td>475.25</td>
</tr>
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</table>
Figure 8.8. Comparing signal quality when capillary gaps are covered and uncovered.

When the gaps are covered, improved signal intensity is obtained (bottom). The probe beam is blocked and unblocked three times to show the signal intensity.
Table 8.5. Recommended capillary

<table>
<thead>
<tr>
<th>Wave Length (nm)</th>
<th>Slit size/capillary I.D. (nm)</th>
<th>$\theta_0$</th>
<th>$\tan \theta_0$</th>
<th>Distance (um)</th>
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<th>Total image size considering laser leakage</th>
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<tbody>
<tr>
<td>266</td>
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<td>609600</td>
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*Laser leakage*

<table>
<thead>
<tr>
<th>Wave Length (nm)</th>
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<th>$\theta_0$</th>
<th>$\tan \theta_0$</th>
<th>Distance (um)</th>
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</table>
As compared to round capillaries, square capillaries yield less diffraction. We make arrays of 8 and 10 fused Silica capillaries with 71um I.D. and 360 um O.D. After aligning the optical setup using erythrosine B and obtaining a signal from each of the capillaries using a single photodiode detector, an image of all the eight signal beams is obtained using a CCD camera. As shown in Figure 8.9, signal spots are well separated and can be identified for each capillary.

8.2.5. Choosing the Detector

Before utilizing a photodiode array, a CCD camera is used for initial test runs since it offers good pixel resolution and optical sensitivity. However, due to the large size of the resulting data files, it is not the best detector for a high-throughput CE system with an average run time of 30 minutes. Estimated file sizes easily exceed 2 GB (Tables 8.6) when using the lowest resolution and a slow frame rate (3.75 fps). Furthermore, due to CCD digitization errors, some variations in retention times and signal intensity levels are observed among the capillaries [9].

Hence, we use a C5964-1010 multi-channel array detector with a NMOS photosensitive section for our application. The photosensitive section has P-N junction photodiodes consisting of N-type diffusion layer formed on P-type silicon substrate, which serves as photoelectric converter and stores the obtained charge temporarily. The sensor has 1024 pixels of 25 x 2500 um arrays with an effective area of 25.6 (H) x
Figure 8.9.a. Image of 8 signal spots obtained by a CCD camera for an array of 8 capillaries (left) and images when chopped beam is blocked (right). 1376 x 1024 pixels, 4-mm image size.
Figure 8.9.b. Signal spots from all capillaries recorded by a single photodiode. Probe and chopped beam are blocked and unblocked three times and once, respectively, to show the signal and the background.
2.5 (V) mm, and it provides good sensitivity levels for our system, as shown in Figure 8.10. Its photosensitivity is comparable to that of our photodiode (Thorlabs Photonic DET210) in the UV range.

Table 8.6. Signal image file size when collected by a CCD camera. Calculation for a 30 minutes run is based on the real data collection for one minute run.

<table>
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<th>Output Size</th>
<th>Frame rate</th>
<th>Data file size in 30 min (GB)</th>
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<td>1.90</td>
</tr>
<tr>
<td></td>
<td>3.75</td>
<td>2.06</td>
</tr>
</tbody>
</table>
Figure 8.10. A portion of the photodiode array and its pixel dimension (not to scale).

- \( a \) = pixel pitch
- \( b \) = width of the photodiode diffusion layer
- \( c \) = pixel height
- If \( a = 50 \text{um} \), \( b = 45 \text{um} \)
- If \( a = 25 \text{um} \), \( b = 20 \text{um} \)
8.3. Results and Discussion

After preliminary calculations and optimization steps, the wave-mixing optical setup is aligned using erythrosyn B. The signal is verified for each capillary using a single photodiode that is moved vertically (Figure 8.9, bottom). The 2-D signal images are also tested, aligned and verified on the PDA.

Capillaries are rinsed with water and a sieving matrix solution to clean and condition the capillary array using a multi-channel peristaltic pump. A mixture of 10 fg/mL chromophore-labeled β-lactoglobulin and an Orange G (10 kD) standard is injected for 5 seconds in each capillary. Then, 12 kV is applied to the sieving matrix reservoirs through electrodes. Images of signal peaks for this mixture are captured by the NMOS PDA and saved to an Excel file.

As shown in Figure 8.11, a single signal spot from a capillary occupies about 100 pixels on the PDA detector. Therefore, to analyze the data for each capillary, the signal intensity is averaged over the intensities of the exact number of pixels that are affected by each capillary’s signal. It should be noted that not all the capillaries show the same signal intensity and background levels, as shown in Figure 8.9. It is due to the fact that all the capillaries have some variations in quality, smoothness, thickness, etc. Slight differences in capillary surface chemistry and in-house coatings can cause these signal intensity variations among capillaries.
Figure 8.11. Signal comparison for the capillary array of 8 square capillaries when the probe beam is unblocked (top) and blocked (bottom). Each peak corresponds to the signal generated from each capillary in the array. Differences in the number of pixels affected by each signal are due to slight variations between capillaries.
Data from each capillary are analyzed by an in-house program and Microsoft Excel. As shown in Figure 8.12 (a), capillaries 1 - 4 (from bottom) show a good S/N for 10 fg/mL β-lactoglobulin while capillary 5 yields a hump. Capillary 6 shows a noisy electropherogram, possibly due to air bubbles trapped in the capillary. After smoothing the data for this capillary using four-point averaging, a broader, smaller peak is observed for β-lactoglobulin at the same frame number, as observed by the other capillaries (Figure 12 b). Capillaries 7 - 9 show smaller signals as compared to those from capillaries 1 - 4 with a small difference in retention times. Capillary 10 is also noisier, however, a matching peak is still observed. Extra peaks collected are due to impurities in the standards, flaws in the capillary walls, and other variations found in individual capillaries.

Small variation in retention times are due to slight variations in manually injecting the capillaries by inserting the injection ends inside the solute reservoir for 5 seconds and wiping off the ends in order to prevent cross contamination. All image data are recorded in a single Excel file. However, due to size limitations in Excel, a new file is started after 250 images are recorded.
Figure 8.12 (a) High throughput CE wave-mixing results from an array of 10 square capillaries when 10 fg/mL b-lactoglobulin is injected and separated at 12 kV.
Figure 8.12 (b) When four-point smoothing is used for capillary 6 (from bottom to top).
Image files are reproducible day to day and run to run with a good S/N, indicating the robustness of our setup. As shown in Figure 8.13, the first peak at frame number 550 is from Orange G (10 kD) and the second peak at frame number 835 is from β-lactoglobulin (18.3 kD).

This technique owes its sensitivity and robustness to the unique features of wave mixing including short optical path lengths, coherent laser-like signal, quadratic dependency on sample concentration and absorption coefficient, cubic dependency on laser power, and 100% optical collection efficiency measured against a dark background. A preliminary concentration detection limit of $5.4 \times 10^{-16}$ M and a mass detection limit of 2.9 yoctomole are determined for β-lactoglobulin using a probe volume of 5.4 nL, as shown in Figure 8.14. Hence, our ultrasensitive wave-mixing detector not only yields one of the best detection limits for proteins, it also allows high-throughput parallel optical detection.
Figure 8.13. Day-to-day reproducibility for Orange G and β-lactoglobulin. First peak is for Orange G (10 kD) at frame number 550 and second peak is for β-lactoglobulin (18.3 kD) at frame number 835.
Figure 8.14. Probe volume calculation for a square capillary with 71 um I.D. Laser beam width at the focal pinot is 1070 um.
6 Y. He, E. S. Yeung, Electrophoresis, 2003, 24, 101-108
10 www.combisep.com
12 H. Pang, J. Kenseth, S. Coldiron, DDT, Vol.9, No. 24 December 2004
15 K. Swinney, D. J. Bornhop, Electrophoresis, 2000, 21, 1239-1250

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9.1. Introduction

When using conventional UV-Vis absorption spectrometry, most proteins demonstrate a broad absorption band at 280 nm, corresponding to the strong absorption of amino acids, tyrosine, tryptophan and phenylanalyn.\textsuperscript{1, 2} Wetlaufer et al. showed that the spectra of aqueous O-methyl tyrosine at various pH values (1.08, 5.7, 9.6 and 11.5) all have a strong absorption at 266 nm.\textsuperscript{3} In an ideal situation, it is desirable to prevent dilution of the contents in a single cell or any biological samples by labeling and derivatizing.\textsuperscript{4} However, due to poor sensitivity levels available from conventional UV absorption methods, one must use a fluorophore label in order to use relatively more sensitive laser-induced fluorescence (LIF) detection methods. Most analytes are not easily detectable in their native state and they must be either derivatized or labeled with a fluorophore. Obvious disadvantages of these steps include multiple reaction products due to incomplete labeling of all possible sites, and band broadening as a result of photo bleaching and blinking.\textsuperscript{5}
Different UV sources have been used by researchers including a frequency-doubled argon-ion laser at 257 nm, a frequency quadrupled Nd:YAG pulsed laser at 266 nm, a Xe-Hg lamp at 266 nm, a Cu laser operating at 248.5 nm, and a deuterium lamp at 214 nm.

In our study, we use a compact (butter stick-sized) quadrupled Nd:YAG laser emitting 266 nm radiation at a high pulse frequency. This wavelength is suitable for excitation of natively fluorescent amino acids. The laser wave-mixing signal is generated when two input laser beams form laser-induced gratings, and an input laser beam is diffracted off this grating when proteins absorb the excitation laser light. The wave-mixing signal is a well-collimated laser-like signal beam and can be measured with nearly 100% collection efficiency with excellent signal-to-noise ratios.

For protein separation studies, we use a custom-built capillary electrophoresis system. The capillary is first treated to prevent protein adsorption to capillary walls. UV transparent polymers, such as dextran or poly (ethylene glycol), can be used as a sieving matrix. They are more suitable than polyacrylamide since they offer better UV transmission. Poly (dimethylsiloxane) (PDMS) has been used in other studies to coat capillaries and microchips. Cross-linked poly (vinyl alcohol) has been used as permanent and dynamic coating in capillary electrophoresis systems for studies in the UV region. These PVA-coated capillaries are biocompatible and they offer the highest bio cell survival rate. Barron also used poly-N-hydroxyethylacrylamide as a dynamic coating. Highly hydrophilic-substituted cellulose including hydroxethyl...
cellulose (HEC), hydroxypropyl cellulose (HPC) and hydroxypropylmethyl cellulose (HPMC) have also been used for capillary coating.\textsuperscript{19} In this study, we use a dynamic coating and a sieving matrix solution containing 50 mM TRIS borate, 2.5 mM EDTA, 0.5% methycellulose (high viscosity), 5% Dextran and 0.1% SDS.

9.2. Experimental

9.2.1. Reagents

All reagent grade chemicals including protein standards, protein buffers and sieving matrix buffers are ordered from Sigma-Aldrich. All the solutions are prepared using Barnstead water (18.2 $\Omega$). Protein standards are dissolved in 12.5 mM TRIS-HCl containing 0.5% SDS and 5 mM dithiotheritol (DTT). Protein standards are diluted several times to prepare low concentration analytes (fg/mL). All the protein standards and buffers are stored at 4 °C.

9.2.2. Real-World Samples

9.2.2.1 Mouse Mitochondrial Heat Shock Proteins

Heat shock proteins (Hsps) are a group of proteins important for assembly, folding and translocation of other proteins.\textsuperscript{20} Expressing heat shock proteins is one of the cell defense mechanisms when cells are under stress.\textsuperscript{21} These proteins, when induced by heat shock, protect intercellular proteins from damaging events. Other
inducers include alcohol, UV radiation, oxidants, cell cycle, growth factor, infection, inflammation and recovery from anoxia and glucose starvation.

Mammalian Hsps are categorized into five main families based on their molecular weight\textsuperscript{22} and each family is responsible for a different cell defense mechanism. For example, Hsp 70 is highly antigenic and induces auto antibodies during infection and it has been used as a diagnostic antigen.\textsuperscript{23} Mitochondrial Hsp 70 is involved in protein translocation into mitochondria.\textsuperscript{24} Hsps are also protective against oxidative damages that are involved in a variety of pathological processes including diabetes, arteriosclerosis, Alzheimer’s disease, aging, renal failure, obstructive nephropathy, rhabdomyolysis, hyperlipidemia and glomerular damage to chronic renal failure.\textsuperscript{25}

Because of the important roles Hsps play in cell functions, mitochondria databases have been created for these proteins with useful information on their molecular functions and associated diseases.\textsuperscript{26} According to one of these databases (subcellular localization database), there are 58,128 proteins in mouse and 1,554 proteins in mitochondria, and 369 and 49 proteins are identified in inner and outer mitochondria, respectively.\textsuperscript{27} Genetic mapping of mouse Hsps is also reported elsewhere.\textsuperscript{28} For our study, the mouse mitochondria heat shock proteins (18.8 ug/uL) are provided by Dr. Gottlieb at the BioSience Center (SDSU).
9.2.2.2. Leech Ganglia

For thousands of years, leeches have been used for bleeding and as treatments for a wide range of illnesses. More recently, leeches are used mostly in treatment of impaired venous circulation following surgery, especially in reattachments of ears and fingers. They are also useful in the study of basic principles and phenomena of cellular neurobiology due to their simple yet elegant nervous system. Leech central nervous system consists of dorsal and ventral brain components, and a longitudinal chain of ganglia arranged along a pair of connectives. Each single ganglion contains 400 - 700 neurons. The total count of the neuron is about 15000 - 20000. They are organized in a repetitive pattern and are individually identifiable. For example, mechanosensory neurons in each ganglion are divided into three different cell types: (a) T cells for touch (b) P cells for pressure, and (c) N cells (nonreceptive). The size of a leech neuron is about 10 - 60 um and it can survive for hours outside of the body in bathing solutions.\textsuperscript{29, 30}

In our study, we use leech ganglia 5 and 10. Ganglion 5 is a CNS ganglion that has a specialized function in controlling the sex organs of the animal, and ganglion 10 (located in the mid part of animal’s body) is a non-specialized segmental ganglion. Hence, one expects to see different protein patterns for these two ganglia. Our leech ganglia samples are provided by Dr. Macagno (UCSD).
9.2.3. Optical Setup

A frequency-quadrupled Nd:YAG laser emitting at 266 nm is used to excite amino acids (tyrosine, tryptophan and phenylalanine) in our wave-mixing setup. This UV laser (Teem Photonics, France, Model NU-10210-100) offers low power consumption and a good beam quality. It allows tight focusing on the capillary without photo bleaching or degradation or analyte saturation.

The Nd:YAG output beam is spilt in two by using a 70/30 quartz beam splitter. The two input beams are then focused and mixed inside the capillary by a convex UV lens. The excited molecules in the form of interference patterns release their heat energy to surrounding solvent molecules, creating dynamic thermal gratings, and as a result, refractive index gratings. The incoming photons from the probe beam diffract off the gratings to generate the signal beam. The wave-mixing signal beam is a coherent laser-like beam and it is easily detected by a simple photodetector (Thorlabs Inc., Model DET 210). A chopper (Stanford Research System, Model SR540) is used to modulate the signal at 200 Hz. The signal is amplified by a lock-in amplifier (Stanford Research System, Model SR830 DSP), as shown in Figure 1. The signal is finally digitized by an analog-to-digital converter and the electropherograms are stored on the computer using a custom software package (TongLab AIDA).
Figure 9.1. Experimental capillary electrophoresis wave-mixing setup
9.2.4 Capillary and Dynamic Coating

A 37-cm long square capillary (71 µm I.D., 360 µm O.D., 22 cm effective length) is used in our custom capillary electrophoresis (CE) system. The capillary is mounted on a rigid translational stage, keeping the capillary detection window stabilized and vibration isolated. The capillary column is rinsed with water before CE runs and it is filled up with a dynamic coating and sieving matrix buffer containing 50 mM TRIS borate, 2.5 mM EDTA, 0.5% methycellulose (high viscosity), 5% Dextran and 0.1% SDS. A high voltage power supply (Glassman High Voltage Co., Model PS/MJ30P0400-11 30 kV) and two platinum electrodes power our custom CE system.

9.3. Results and Discussion

After aligning the optical setup with a dye and conditioning the capillary, a trypsin inhibitor standard (21.5 kD) is injected for 5 seconds and a 12 kV potential is applied through the electrodes in the buffer reservoirs. As shown in Figure 9.2, the main peak for this standard is obtained at 611 s. The same conditions are applied to carbonic anhydrase analytes (31 kD) at 10 pg/mL and 10 fg/mL concentration levels. A distinct peak is obtained at 1003 s for both concentration levels. As illustrated in Figure 9.3, the electropherograms are reproducible for different runs using different concentration levels. Small variations in retention times are due to slight differences in manual injections.
Figure 9.2. Electropherogram of trypsin inhibitor protein standards separated in a 37 cm square capillary (71 um I.D., 360 um O.D., 22 cm effective length, 12 kV applied voltage).
Figure 9.3. Electropherogram of carbonic anhydrous protein standards at 10.3 pg/mL (bottom) and 10.3 fg/mL (top) obtained under the same separation conditions. Results are reproducible at different concentration levels.
Our UV laser wave-mixing setup provides ultrasensitive detection sensitivity levels and we can detect 10.3 fg/mL of carbonic anhydrase with a S/N of 20. Preliminary detection limits of 1.3 yoctomole and $3.3 \times 10^{-16}$ M for carbonic anhydrase and 1.9 yoctomole and $4.8 \times 10^{-16}$ M for trypsin inhibitor in an approximately 20um spot size are determined. To our knowledge, these are some of the best detection limits reported for the detection of native proteins using an absorption-based or any other detection method (Figure 9.4).

To check our system robustness and reproducibility, trypsin inhibitor is injected on different days and CE runs performed. As shown in Figure 9.5, the day-to-day reproducibility is obtained for this standard when they are injected individually or in a mixture.

The wave-mixing detection setup is then tested for our cellular proteins in their native form using the mouse mitochondria heat proteins. The original 18.8 ug/uL analyte is diluted several fold down to the pg/uL concentration level in the same buffer. As shown in Figure 9.6, proteins with different masses are detected and matching retention times near those of our standards (611 s and 1003 s) indicate the presence of these proteins or proteins with similar masses.
Figure 9.4. CE run for 10.3 fg/mL carbonic anhydrase protein standard. Limit of detection of 1.3 ymole is obtained.
Figure 9.5. Day-to-day reproducibility check for trypsin inhibitor protein standards under the same separation conditions
Figure 9.6. Electropherogram for mouse mitochondria heat proteins (18.8 pg/uL).
We also observe protein separation and the expected distinctly different patterns of protein peaks from leech ganglia 5 and 10. For ganglion 5, we prepare two sets of samples. We observe several major peaks for vortexed ganglion 5 as shown in Figure 9.7. However, when we use centrifuged supernatant of ganglion 5, we observe some major peaks with smaller peak intensity levels and a relatively noisy baseline, as shown in Figure 9.8. Therefore, we use vortexed samples rather than centrifuged supernatant versions for other studies.

Same experimental conditions are applied to leech ganglion 10. As shown in electropherograms for ganglia 5 and 10 in Figure 9.9, we observe the expected distinctly different protein patterns. The electropherogram for ganglion 10 shows more small peaks and identification of each major peak would require a larger group of protein standards to be injected and retention times compared. In any case, this CE-based pattern recognition system is already promising for a wide range of biomedical applications.

We also compared electropherograms of ganglion 5 from two different leeches. We expect to see similar strong peaks in both ganglia, but not exactly the same patterns since they are from two different animals. As shown in Figure 9.10, there are some peaks at similar retention times, and as expected, there are some major peaks that are missing in ganglion 5 (a). Some of these variations could be also due to possible heterogeneity of the samples and need to be further investigated.
Figure 9.7. Electropherogram for leech ganglion 5 (vortexed)
Figure 9.8. Comparison of electropherograms for leech ganglion 5 supernatant (bottom) and vortexed (top)
Figure 9.9. Comparison of electropherograms of leech ganglion 5 and ganglion 10.
Figure 9. 10. Comparison of electropherograms of ganglion 5 from two different leeches
4. Conclusion

A 266-nm UV laser wave-mixing detection system is presented as a sensitive CE detector for proteins in their native form. Using a compact low-power laser, we observe yoctomole-level detection sensitivity with minimum sample preparation steps and no labeling procedures. Wave mixing offers unusually sensitive absorption measurements since it has a few unique inherent properties including a coherent collimated laser-like signal beam that can be collected with nearly 100% collection efficiency over a dark background. Although one could identify all the protein peaks as in other CE methods, this system is presented as an effective CE-based pattern recognition system that is needed for a wide range of biomedical applications.

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