Sensitive Nonlinear Laser-Based Spectroscopic Studies of Chemical and Biological Agents for Biomedical and Security Applications

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Chemistry

by

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2015
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Chair

University of California, San Diego

2015
DEDICATION

To my family and friends who have supported me throughout my education
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LIST OF ABBREVIATIONS

ATP adenosine triphosphate
BSA bovine serum albumin
CARS coherent anti-Stokes Raman Scattering
CE capillary electrophoresis
CGE capillary gel electrophoresis
CRDS cavity ring-down laser absorption spectroscopy
CW continuous-wave
DBS deep-brain stimulation
DFWM degenerate four-wave mixing
DMSO dimethyl sulfoxide
DNA deoxyribonucleic acid
EC-QCL external cavity quantum cascade laser
ELISA enzyme-linked immunosorbent assay
EOF electroosmotic flow
EPA US Environmental Protection Agency
FASS field-amplified sample stacking
FDA US Food and Drug Administration
F-DFWM forward-scattering degenerate four-wave mixing
FITC fluorescein isothiocyanate
HIV human immunodeficiency virus
HPLC high-performance liquid chromatography
IMS ion mobility spectrometry
IR infrared
LIF laser-induced fluorescence
LIFE lung imaging fluorescence
MALDI matrix-assisted laser desorption ionization
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>MEKC</td>
<td>micellar electrokinetic chromatography</td>
</tr>
<tr>
<td>NLO</td>
<td>nonlinear optics</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>OPC</td>
<td>optical phase conjugation</td>
</tr>
<tr>
<td>OPS</td>
<td>optically pumped semiconductor</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>R:T</td>
<td>reflection:transmission</td>
</tr>
<tr>
<td>RSD</td>
<td>relative standard deviation</td>
</tr>
<tr>
<td>S/N</td>
<td>signal-to-noise</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TATP</td>
<td>triacetone triperoxide</td>
</tr>
<tr>
<td>TEM</td>
<td>transverse electromagnetic</td>
</tr>
<tr>
<td>TNT</td>
<td>2,4,6-trinitrotoluene</td>
</tr>
<tr>
<td>TTL</td>
<td>transistor-transistor-logic</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WINCS</td>
<td>wireless instantaneous neurotransmitter concentration system</td>
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ABSTRACT OF THE DISSERTATION

Sensitive Nonlinear Laser-Based Spectroscopic Studies of Chemical and Biological Agents for Biomedical and Security Applications

by

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Doctor of Philosophy in Chemistry

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Professor William G. Tong, Chair

Nonlinear laser wave-mixing spectroscopy is presented as an ultrasensitive detection method for chemical and biological agents in thin-film and liquid-phase samples. Wave mixing is an unusually sensitive absorption-based detection method that offers inherent advantages including excellent sensitivity, small sample requirements, short optical path length, high spatial resolution and excellent standoff detection capability. Wave mixing offers excellent optical absorption detection sensitivity even when using thin
samples (<0.1 mm), and hence, it is inherently suitable for interfacing to microarrays, microfluidics, and capillary electrophoresis. Laser excitation wavelengths can be tuned to detect multiple chem/bio agents in their native form. Since the wave-mixing signal is a coherent laser-like beam with its own propagation direction, it offers excellent S/N and allows remote standoff detection capability.

Laser wave mixing is presented as a way to natively detect small biomolecules and nitroaromatic explosives at zeptomole mass detection limits. The absorbance of select neurotransmitters and nitroaromatic explosives at ultraviolet wavelengths make their detection possible using a compact pulsed UV-laser. The detection of these analytes is successfully accomplished both on UV-transparent surfaces and by using capillary electrophoresis separation.

Wave mixing is used for the ultrasensitive detection of cellular proteins and antibodies using fluorescent and non-fluorescent labels. Using sodium dodecyl sulfate capillary gel electrophoresis, proteins can be separated by their size using an appropriate sieving matrix. Newly developed methods employ wave-mixing and capillary electrophoresis for the zeptomole mass detection of cellular proteins and antibodies without the need for time consuming capillary preparation steps.

The HIV-1 p24 capsid protein has been detected using laser wave-mixing spectroscopy and capillary electrophoresis using chromophore and fluorophore labels. Size-based capillary electrophoresis separation has also been used to analyze the products of a p24 antibody-antigen reaction. These studies show the potential of wave mixing to be used to create field-deployable and relatively inexpensive HIV viral load screens in resource-limited settings.
CHAPTER 1
INTRODUCTION

1.1 LASER-BASED ANALYTICAL CHEMISTRY

In less than a century since their inception, lasers are now widely used in a variety of applications from data recording and processing to the development of novel scientific instrumentation. Lasers now play a key role in experimental design in all areas of science, and the fundamental link between lasers and chemistry has led it to become an essential tool in chemistry. Chemistry, the study of matter and its possible state changes, requires specialized tools that can both transform states of matter and simultaneously measure those changes. Laser radiation offers special properties for transforming atomic and molecular states and is much more suited for spectroscopic measurements than ordinary light. The following sections illustrate the properties of lasers and how they can be used in novel spectroscopic measurements.

1.1.1 THE BASICS OF LASERS

The term “laser” comes from an acronym of “light amplification by stimulated emission of electromagnetic radiation.” In the most basic two-energy level system, there are three processes that should be considered when a system is exposed to photons (or energy) in resonance with the transition frequency: absorption, stimulated emission and spontaneous emission. In a simplistic rate equation model, the probability of a photon transition to a higher energy level is higher than the probability for a downward transition.
In this system, no amplification can be observed. Even under optimal conditions for a two-level system, where the photon beam flux and temperature of the medium are optimized, the best one could achieve is equal populations between the two states.

There are two major requirements to make a given medium suitable for light amplification through stimulated emission. The first requirement is that there must be a metastable excited state that exists long enough to participate in stimulated emission. The second requirement is a population inversion where the population in the upper energy state is greater than that of the lower energy state. As stated previously, population inversion is not possible in a two-level photon-matter interaction. Population inversion can only be achieved where there are additional energy levels to serve as detours in the excitation and holding states for population accumulation. These are possible in both three and four-level systems as shown in Figure 1.1.

In the simple three-level system, illustrated in Figure 1.1B, the lasing process takes place between the upper-level excitation-state 2 and the ground-state level 1. The pump radiation elevates a fraction ($\eta$) of pump photons ($h\nu$) from the ground-state to level 3. This leaves the probability of photons pumping to level 2 at $W_p = \eta W_{13}$. It is necessary that the rate-of-decay $A_{32}$ dominate over $A_{21}$ and the rate of spontaneous emission for photons to accumulate preferentially to level 2. In this type of three-level laser system, population inversion is possible if more than half the number of particles in the ground state could be pumped to higher energy states.

If another level is added to create a four-level system, as shown in Figure 1.1C, population inversion could be achieved between level 3 and level 2. It also should be noted that level 2 should be high enough in energy so it is not thermally excited and it should
Figure 1.1 Excitation and emission processes for a two-level (A), three-level (B) and four-level (C) system.
relax back to level 1 at a relatively high rate. This makes population inversion optimal between levels 3 and 2 because the population in level 3 only must exceed the thermal population in level 2. This illustrates that, in laser systems, population inversion takes place immediately after beginning the initial pumping process.

The lasing action of a laser is not only dependent on energy states of a lasing medium; the design of the laser resonator must be optimized to produce useful laser radiation. The basic design of a laser resonator is illustrated in Figure 1.2. Light amplification increases in a laser by multiple passes of light waves through an active medium. After pumping to the upper excited state level, photons resulting from stimulated emission propagate along the optical axis where they are bounced between two resonator mirrors. Each round trip in the resonator lets the beam pass though the active medium twice and leads to amplification as long as population inversion is taking place. Losses can result from many factors including mirror misalignment, contamination of optical surfaces, diffraction losses and reflection losses. Optical resonators are never perfect and one must consider losses and gains in the two-mirror system. If the overall gains exceed the overall losses, laser output will be maintained.

Laser media come in many varieties and are commonly found in solid, liquid and gaseous states. The properties of laser light, including the output wavelength, depend on the energy states of the medium. The most common commercial lasers designed for laboratory use include the helium-neon laser, the argon-ion laser, the fixed-wavelength solid-state laser (e.g., Nd:YAG) and semiconductor diode lasers. These lasers offer many advantages compared to ordinary light sources. They are brighter, monochromatic, highly
Figure 1.2 The basic design of a laser resonator
directional, allow for polarization control, and are temporally and spatially coherent, and some are also tunable. Lasers can be tuned finely to scan and resolve isotopes and hyperfine lines in atomic spectroscopy.

The helium-neon (He-Ne) gas laser can be found in many scientific laboratories as it is relatively small, inexpensive and yields a nice beam profile at 632.8 nm. As the name suggests, the lasing medium comprises a mixture of helium and neon at about a 10:1 ratio. The gas chamber holds both the gases and two high-voltage DC electrodes used to excite the discharge. The He-Ne laser is a four-level laser system. The excitation and de-excitation scheme incorporates collisional energy transfer between the two gas components in the laser medium (Figure 1.3). Energetic electrons elevate the He atom to its two excited metastable states He*\(^{(2^1S_0)}\) and He*\(^{(2^3S_0)}\) which serve as a pumping reservoir. In these excited metastable states, collisions occur with the Ne atom and energy is exchanged to produce unexcited He atoms with Ne atoms in the excited states Ne*\(^{(3s^2)}\) and Ne*\(^{(2s^2)}\). This collisional energy transfer can occur with high probability because of the resonant energy states involved with neon and helium. The Ne excited states mentioned above constitute the long-lived upper level 3 states, and it can decay to the Ne*\(^{(2p^4)}\) level 2 state that consists of ten sublevels. The transition with the highest probability yields the characteristic red color we observe with He-Ne laser radiation with a wavelength equal to 632.8 nm. Although other transitions are possible, they are not optimized in a conventional He-Ne laser.

The basic design and concepts behind the argon-ion laser are similar to that of the He-Ne laser, except that the energy level transitions that contribute to laser action come from argon ions rather than neutral atoms. Rare gas atoms, such as argon, have a filled-
Figure 1.3 Energy level diagram for the helium-neon (He-Ne) laser. Relevant collisional and optical energy transitions are noted.
shell ground-state electronic configuration and require high-energy sources for ionization. The plasma tube filled with argon gas normally has a cooling mechanism as it is connected to a high voltage and high current ionization source. Ground-state and excited-state rare gas ions are formed in the discharge via collisional energy. The excited states responsible for laser action are formed by further collisions between excited state and ground state ions with discharge electrons. The schematic energy level diagram for an argon-ion laser is shown in Figure 1.4. The argon-ion laser can be adjusted to emit up to nine laser lines in the range from 454.6 – 528.7 nm. The strongest lines occur at 488.0 nm and 514.5 nm and the individual lines can be selected with an intra-cavity tuning prism.

Gas lasers are very useful in scientific applications but they are also inefficient in converting energy into lasing radiation. Solid-state lasers, especially semiconductor diode systems, are gaining popularity as they optimize efficiency, portability and cost. Nd:YAG is a widely used material in lasers. Nd:YAG is an abbreviation for neodymium:yttrium aluminum garnet, which consists of neodymium atoms doped into a crystal host (Y₃Al₅O₁₂). The implanted neodymium ions are in the triply ionized form, i.e., Nd³⁺, which serves as the active laser medium. The \( ^4F_{5/2} \) energy levels could be populated by pumping ground state ions with 808 nm light either derived from Xe flash lamps or powerful GeAlAs diode lasers (Figure 1.5). The Nd³⁺ ions relax to the upper laser level \( ^4F_{3/2} \) by coupling to the crystal matrix. The main laser transition occurs between the upper state and the \( ^4I_{11/2} \) corresponding to the 1064.14 nm wavelength. Because diode-based lasers are highly efficient, the overall electrical power to light conversion seen in diode-based Nd:YAG lasers can exceed 15% compared to the argon-ion at 0.1%. The 1064 nm laser radiation from a Nd:YAG laser can be frequency doubled, tripled and quadrupled to yield
Figure 1.4 Energy level diagram for the argon-ion gas laser. Relevant energy transitions are noted.
Figure 1.5 Energy level diagram for the Nd:YAG solid-state laser. Relevant energy transitions are noted.
wavelengths at 532 nm (second harmonic), 355 nm (third harmonic) and 266 nm (fourth-harmonic).

1.1.2 SENSITIVE LASER-BASED ANALYTICAL METHODS

Many sensitive techniques and detection methods used in analytical chemistry rely on lasers to serve as an optimal light source for chemical modification and detection of analytes. The impact that lasers have made over time in analytical chemistry is best illustrated by the number of published papers relating to the topic over the past 20 years. In 1982, *Analytical Chemistry* published 24 papers where “laser” appeared either in the title, abstract or keyword listing for each article. This made up just under 3% of the total number of papers published in the journal that year.¹ The rate of published papers in *Analytical Chemistry* involving lasers jumped in the 1990s and by 2005 over 12% of the total articles published contained “laser” in the context mentioned above. Presently, chemical detection and characterization involving lasers spans a wide variety of applications from chemical desorption/ionization commonly coupled with mass spectrometry, e.g., MALDI, to laser-excited fluorescence, widely used for sensitive detection of chemical separation systems as well as imaging and fluorescence microscopy. These techniques often lend themselves to one universal theme: to alter a chemical system using controlled laser light to observe a wide-range of effects.

*Raman Spectroscopy*

Raman spectroscopy benefits significantly from the inherit properties of lasers. Coherent, monochromatic, powerful and tunable laser sources serve well in a Raman setup. Raman spectroscopy provides information about molecular vibrations that is
complementary to infrared spectroscopy, e.g., homonuclear diatomic molecules. More complex molecules exhibit unique Raman scattering and infrared absorption data that can be combined to provide essential information about molecular vibrations.

Raman spectroscopy exhibits advantages over other spectroscopic techniques used in analytical chemistry. Compared to infrared absorption spectroscopy where it is necessary to scan a vibrational spectrum for a period of time, Raman spectroscopy can collect an entire vibrational spectra in a single laser pulse. Another advantage is that Raman spectroscopy can be used to study vibrational transitions of molecules in water-based solution, an important feature for studying biological molecules in aqueous media.

The main drawback of Raman spectroscopy is the very low cross section of spontaneous inelastic scattering of photons in a given sample. A Raman spectrometer utilizes a relatively powerful source, a bulky monochromator and very sensitive photodetectors for detecting incoherent scattered photons. Because of these requirements, a sensitive Raman system was impractical until lasers became commercially available.

Resonance Raman scattering is performed when the frequency of the excitation radiation is tuned to the absorption band of the analyte. The excitation increases the Raman scattering probability of vibrational modes spatially and, therefore, increases the intensity of the Raman signal significantly as compared to conventional Raman scattering. This enhanced signal is only present when the excitation wavelength is specifically tuned to the analyte and adds a level of specificity for sample analysis. This is important for studying complex biological samples and probing the active site of enzymes by tuning to the absorption peaks of the active sites. Specific spectra have been obtained by measuring the resonance Raman scattering from vibrational modes localized in oxy- and
deoxyhemoglobin.\textsuperscript{2} UV-resonance Raman spectroscopy has been used to specifically detect many types of biological samples from bacteria to whole pollen.\textsuperscript{3,4} It has also been shown to provide criminological information related to the Raman spectra of common narcotics and drugs such as cocaine, heroin, and ecstasy.\textsuperscript{5,6}

Coherent anti-Stokes Raman scattering (CARS) is the most commonly used nonlinear Raman technique and is performed by crossing two or more sources of monochromatic radiation to form a strong and highly directional Raman signal with a small divergence. The CARS signal is produced when two overlapping frequencies, $\omega_1$ and $\omega_2$ produce a new frequency $\omega_s$ when the difference between $\omega_1$ and $\omega_2$ is the molecular frequency, $\omega_M$, produced in normal Raman scattering for a given analyte. The unusually small excitation volume makes it possible to probe biological samples such as stem cells and DNA.\textsuperscript{7}

Raman spectroscopy not only provides excellent sensitivity but also provides a chemical “fingerprint” of chemicals which adds a high level of specificity when compared to other conventional spectroscopic techniques. Adaptations of Raman, such as surface- and tip-enhanced Raman spectroscopy, have generated new molecular sensors that can be used for biomedical environmental and security applications. The non-destructive character of the analysis makes it well-suited for a variety of volatile and sensitive systems.

\textit{Cavity Ring-Down Spectroscopy}

Many absorption-based techniques involve the use of a laser for chemical analysis including laser absorption spectroscopy, frequency-modulated absorption spectroscopy, and multi-pass sample cell laser spectroscopy. Although useful for simple sample analysis,
these techniques often are not able to measure samples at very low concentration levels because they all involve detection of a very small change of total transmitted energy measured against noisy background levels. Techniques that can overcome high background noise, such as intracavity methods, can be useful but are often hard to design and implement. Cavity ring-down laser absorption spectroscopy (CRDS) addresses these issues with a simple and quantitatively accurate technique that measures samples by observing the decay of a light pulse rather than total transmitted light.

The technique was developed for use with pulsed lasers in the 1980s and is now widely used for measuring electronic and vibrational absorption spectra for trace gas-phase analytes. Measurements using CRDS are simple as the technique can make direct absorption measurements on micro-second time scales. These advantages have made it especially useful for monitoring environmental contaminants such as nitrogen oxides, volatile mercury compounds, hydrocarbons, and particulate matter in environmental aerosols. The sensitivities of these measurements reach up to low parts-per-trillion (ppt) levels especially for mercury compounds because of the strong absorbance of atomic mercury at 253.6 nm.

The optical setup for a typical CRDS experiment, shown in Figure 1.6, consists of a laser source, a ring-down cavity containing two highly-reflective optical mirrors (R ≤ 0.999), and a highly sensitive photodetector to capture leakage of light out of the cavity. Light is pulsed on a time scale shorter than the cavity round-trip time and is directed to the input mirror of the cavity. As expected, most of the light is reflected by the input mirror but a small percentage (< 0.1%) enters the cavity and gets reflected back-and-fourth
Figure 1.6 Schematic diagram of the essential features of a cavity ring-down spectroscopic setup.
between the intercavity mirrors. The intensity of the leaked light slowly decays and this
decrease in light is monitored by each pass in the cavity by a sensitive photodetector. The
detector sees an exponential decay of light as it is lost inside the cavity. The exponential
rate of decay for an empty cavity is given by:

$$\frac{dI}{dt} = -\left(\frac{1-R}{t_r/2}\right) \equiv -\frac{1}{\tau_0} I$$

(1)

where $R$ is the reflectivity of the mirrors, $t_r$ is the optical round-trip time, and $\tau_0$ is the cavity
ring-down time. Upon integration, $I(t) = \exp(-t/\tau_0)$, the exponential decay term. The
attenuation constant plays into the decay if an absorbing analyte is introduced into the
cavity. The absorption, which is proportional to the attenuation constant $a$, adds to the
light decay seen with each pass through the cavity and leads to even shorter decay times
given by

$$I(t) = \exp[-\left(\tau_0^{-1} + ac\right)t]$$

(2)

In cases where the laser source can be tuned, on-resonance frequencies can be plotted
against the decay time to produce an absorption spectrum that yields quantitative
information. The fact that the signal depends on decay rates rather than absolute intensity
means that power fluctuations in some laser systems are not an issue for recording
quantitative CRDS measurements. The advantages of high sensitivity and simple setup
make CRDS useful in both laboratory and mobile field applications for quantitative
measurements.

**Laser-Induced Fluorescence**

Laser-induced fluorescence (LIF) is the process by which atoms or molecules that
have been excited by laser radiation spontaneously emit light. The process, illustrated in
Figure 1.7a, occurs as an analyte resonantly absorbs photons from a laser beam and then spontaneously emits photons on return to a lower energy level. The emission spectrum is collected at a 90-degree angle from the input laser beam while an absorption spectrum can be simultaneously obtained as the incident light is used to excite molecules. This provides several advantages for chemical measurement including quantitative measurements, enhanced sensitivity and selectivity over absorption methods, and excellent spatial resolution.

The LIF setup is similar to those for absorption techniques with additional components added to measure emitted photons. The emission is collected at a 90-degree angle from the input beam to reduce background noise from the input beam. The signal is filtered and collected by a detector. Assuming that the excitation laser does not cause saturation, the total LIF intensity is given by:

\[ I_{\text{LIF}} \propto I_{\text{laser}} N_1 B_{12} \Phi \]  

where \( B_{12} \) is the Einstein coefficient for absorption and \( \Phi \) is the fluorescence quantum yield of the upper excited state, and \( N_1 \) is proportional to the number density of particles undergoing excitation from the initial state.

In principle, LIF is a zero-background technique and provides excellent signal-to-noise ratios at very low concentrations. If the molecule of interest does not absorb light, then no fluorescence is observed. Although the interaction region can include sample-cell components such as gas discharge tubes, flames and vacuum cells, we focus primarily on
Figure 1.7 (A) Schematic energy-level diagram of fluorescence. (B) Experimental optical setup for laser-induced fluorescence.
experiments performed under ambient conditions using capillary cells and microchips for sensitive analyses of small molecules and proteins.

In a capillary electrophoresis system with a laser-induced fluorescence (CE-LIF) detector, the sample is injected at one end of the capillary and separated according to size and charge. The details behind capillary electrophoresis will be discussed in Chapter 3. Separations on a microchip can also be accomplished and coupled to a laser-induced fluorescence detector.

CE-LIF has become essential in high-throughput DNA sequencing and for the analysis of PCR products. The sensitivity of the method makes it ideal for measuring low-concentrations in biological samples. The work in this area extends to protein fingerprinting, proteomics, and environmental studies involving the detection of natively fluorescing contaminants in ground water such as phenoxy acid herbicides. The relevance of CE-LIF to current biomedical technology will be discussed in later sections of this chapter.

**Infrared Laser Spectroscopy**

Infrared laser spectroscopy has made huge progress in the past couple of decades with the development of tunable external cavity quantum cascade lasers (EC-QCLs) first introduced by the Bell Labs in 1994. These lasers offer useful mid-IR wavelengths (3.5 – 13 µm) for analytical chemistry applications since many chemical species of interest have strong fundamental absorptions at these wavelength ranges. Most gaseous chemical substances also possess strong absorptions in the mid-IR spectral region (fundamental ro-vibrational transitions). Diode lasers producing mid-IR wavelengths were limited to lead-
salt lasers that incorporated nonlinear crystals to reach appropriate wavelengths. These types of lasers become unattractive for laboratory and field use because of their poor thermal conductivity and poor mechanical stability. Lithium neobate material is now incorporated to produce tunable mid-IR QCLs. These systems are capable of reaching high continuous-wave power and can tune over a range of $800 \text{ cm}^{-1}$ at room temperature.

EC-QCLs are useful in a wide range of biological and environmental applications. Wörle et al. have used EC-QCLs to measure the absolute isotope ratio of $^{12}\text{CO}_2$ and $^{13}\text{CO}_2$ in exhaled breath from mice. These measurements can monitor disease patterns and the ratios can be used to diagnose *Helicobacter pylori* infections and glucose metabolism disfunction. Methane, an important chemical for monitoring global warming and stratospheric chemistry, has been detected by a QCL operating at 8.1 µm. Tunable EC-QCLs offer many advantages for chemical fingerprinting in the IR range, especially for detection of potentially hazardous chemicals such as triacetone triperoxide (TATP), which possesses prominent spectral features in the mid-IR range. This new technology presents an opportunity to explore new and more specific methods for the detection of chemical contaminants, explosives and environmental hazards.

### 1.1.3 Laser Systems for Security Applications

The uses of lasers in the military fall into a few categories including those used directly as a weapon, those used for designating targets and those as sensors. Classical laser designs have not produced output levels capable of neutralizing large military targets; however, lasers have been designed to output sufficient power levels for destroying missile targets. *Northrop Grumman* has developed an electric laser capable of producing 100 kW
of laser power that could potentially be mounted on an aircraft, ship or vehicle to destroy weapons in the battlefield.\textsuperscript{26} The chemical oxygen-iodine laser is a megawatt laser developed to emit a 1.315 \( \mu \text{m} \) beam while attached to an aircraft. The laser system, named YAL-1A by Boeing, has been shown to produce megawatt pulses multiple times while being carried by a 747 aircraft.\textsuperscript{27} The advantage of using laser systems, such as the ones mentioned above, is demonstrated by its ultrafast and fine-precision capabilities in neutralizing targets in the battlefield while minimizing collateral damage.

\subsection*{1.1.4 Laser Systems in Biomedical Technology}

The introduction of the laser to medicine has provided new and more efficient diagnostic and treatment methodologies for treating patients. As a surgical tool, a laser can cauterize as it cuts, reducing trauma that is typical of knife-based procedures. Lasers can also be used to vaporize a tissue surface with high precision. Other important applications include treatment of corneal disorders\textsuperscript{28}, kidney stones\textsuperscript{29} and clogged arteries\textsuperscript{30} (angioplasty).

Many diagnostic techniques in medicine have been developed using lasers. Lung imaging fluorescence endoscopy (LIFE) is used for the early detection of cancer and can distinguish healthy tissue from cancerous/pre-cancerous tissue while laser light probes the lungs of a patient.\textsuperscript{31} Confocal microscopy is another diagnostic tool routinely used for real-time imaging of tissues without the need for fixing or staining. The field of diagnostics is constantly evolving and sensitive methods are being explored using flow cytometry and laser-induced fluorescence for the discovery of new biomarkers. The enhanced specificity
and sensitivity levels of new laser-based methods make them attractive for use in the medical field.

1.2 NONLINEAR LASER SPECTROSCOPY

Single-frequency mode-hop-free lasers offer narrow linewidths that are suitable for sub-Doppler nonlinear laser spectroscopic measurements. These techniques are attractive because they offer higher resolution and sensitivity levels as compared to conventional laser techniques. Although there are several popular nonlinear spectroscopic techniques described in the literature, all of them are similar and only vary by the interaction of the optical field with matter, and the optical field can be characterized by several different parameters. To understand the development of many popular techniques, including coherent anti-stokes Raman spectroscopy and four-wave mixing (FWM), one should explore the development of nonlinear optics (NLO) where the dielectric polarization responds non-linearly, in a given medium, to the electric field of the laser light.

Optical phase conjugation has been a major research area over the past 30 years in the field of nonlinear optics. The generation of “phase-conjugated” optical waves was achieved by Boris Zel’dovich and colleagues through nonlinear optical processes. This work was expanded by Yariv et al. who showed that phase conjugation by degenerate four-wave mixing could be observed if two counter propagating pump waves were each in the same single transverse mode. This geometry results in a medium that is illuminated with a third order nonlinear susceptibility, $\chi^{(3)}$. In 1979, Martin and Hellwarth mixed and phase-matched three waves to generate a fourth phased-matched wave in liquid samples of carbon disulfide. This research has set the foundation for optical phase conjugation applied to
areas of holography, interferometry, high resolution spectroscopy, and select areas of analytical chemistry.

1.3 **Degenerate Four-Wave Mixing**

Degenerate four-wave mixing (DFWM) is a laser-based nonlinear spectroscopic technique that exhibits excellent sensitivity for gas- and liquid-phase analytes under ambient conditions. The setup for a forward-scattering wave-mixing setup is relatively simple; two beams of resonant energy are mixed inside an absorbing analyte to produce two signal beams. The signal produced exhibits a quadratic dependence on analyte concentration and a cubic dependence on laser power. Unlike most conventional light absorbing techniques, DFWM can measure extremely small changes in analyte concentration in real-time because of its nonlinear dependence on analyte concentrations. Even when using low laser power levels (2 – 50 mW), wave-mixing detection sensitivity can exceed that of other laser-based techniques that employ more powerful lasers. Typically, biological samples must be labeled by fluorescent tags to reach sensitivity levels sufficient to monitor cellular processes. DFWM can reach these sensitivity levels by optical absorption alone without labels, and even if a label is used, one can use either chromophores or fluorophores for ultrasensitive detection. The signal produced by wave-mixing detection is a coherent laser-like beam that can be directed and collected against a dark background. Laser wave mixing has been demonstrated to provide excellent sensitivity and high spectral resolution in a wide variety of applications.

DFWM has been used for the sensitive detection of gas-phase analytes and has provided hyperfine spectral resolution capable of measuring isotopes in atomic species.
The ability to measure isotopes is important for a broad scope of applications including the detection of environmental pollutants, geological dating, and applications in homeland security. Gas-phase samples are atomized for detection by various sources including graphite furnace, flames, dc-discharge plasmas, and inductively-coupled plasmas (ICPs).

Previous studies have focused on measuring atomic species in the gas-phase with a variety of laser types. The results from these studies, summarized in Table 1.1, illustrate some of the advantages of wave mixing for isotope analysis including excellent sensitivity and high spectral resolution.\textsuperscript{35–41} Wave mixing can measure hyperfine structures, which are spectroscopic “fingerprints” of isotopes.\textsuperscript{37}

The work presented on laser wave mixing in the following chapters focuses solely on liquid-phase analytes. The broader absorption profile of analytes in the liquid-phase makes wave mixing compatible with a wider variety of laser excitation sources.\textsuperscript{42–53} Wave-mixing detection in liquid-phase samples is typically achieved in a forward-scattering arrangement where two input beams are mixed using a lens. This produces a very small detection probe volume (\textasciitilde pL) and is compatible with sample cells of varying sizes from larger cuvettes and liquid cells to smaller microchips, capillary fibers and microarrays. The small probe volume enables analysis with high spatial resolution. Previous work involving liquid-phase analyte detection by DFWM is summarized in Table 1.2, with mass detection limits ranging from nanomol to yoctomole levels. Although much of this early work involves bulky and expensive laser equipment (e.g., argon-ion), newer studies utilize low-power and compact diode lasers capable of producing wavelengths and power levels similar to the argon-ion laser.
Table 1.1 Summary of analytical results obtained for gas-phase samples using laser wave-mixing spectroscopy with different lasers and atomizers.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DC Cathode Plasma</td>
<td>CW Ar⁺ Laser Pumped Ring Dye Laser (mW)</td>
<td>Na</td>
<td>3s²S₁/₂-3p²P₃/₂</td>
<td>Doppler-Free Spectroscopy</td>
<td>1 µg</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>CW Ar⁺ Laser Pumped Ring Dye Laser (mW)</td>
<td>Na</td>
<td>3s²S₁/₂-3p²P₃/₂</td>
<td>Doppler-Free Spectroscopy</td>
<td>60 ng</td>
<td>36</td>
</tr>
<tr>
<td>DC Cathode Plasma</td>
<td>Pulsed Excimer Laser Pumped Dye Laser (nJ)</td>
<td>Ca</td>
<td>4s²¹S₀-4s4p¹P₁</td>
<td>Doppler-Free Spectroscopy</td>
<td>320 ng</td>
<td>37</td>
</tr>
<tr>
<td>Air-Acetylene Flame</td>
<td>CW Ar⁺ Laser Pumped Ring Dye Laser (mW)</td>
<td>Na</td>
<td>3s²S₁/₂-3p²P₃/₂</td>
<td>Doppler-Free Spectroscopy</td>
<td>5 ng/mL</td>
<td>38</td>
</tr>
<tr>
<td>Air-Acetylene Flame</td>
<td>CW Ar⁺ Laser Pumped Ring Dye Laser (mW)</td>
<td>Na</td>
<td>3s²S₁/₂-3p²P₃/₂</td>
<td>Doppler-Free Spectroscopy</td>
<td>0.2 ng/mL</td>
<td>39</td>
</tr>
<tr>
<td>Air-Acetylene Flame</td>
<td>CW Ar⁺ Laser Pumped Ring Dye Laser (mW)</td>
<td>Ba</td>
<td>6s²¹S₀-6s6p¹P₁</td>
<td>Doppler-Free Spectroscopy</td>
<td>5 µg/mL</td>
<td>40</td>
</tr>
<tr>
<td>Air-Acetylene Flame</td>
<td>Pulsed Excimer Laser Pumped Dye Laser (nJ)</td>
<td>Li</td>
<td>2s²S₁/₂-2p²P₁/₂</td>
<td>Doppler-Free Spectroscopy, Isotope Analysis</td>
<td>2.5 ng/mL</td>
<td>41</td>
</tr>
</tbody>
</table>
Table 1.2 Summary of wave-mixing results obtained for liquid-phase samples.

<table>
<thead>
<tr>
<th>Laser</th>
<th>Sample Cell</th>
<th>Sample</th>
<th>Concentration Detection Limit (M, AU, or pg/mL)</th>
<th>Mass Detection Limit (g or mol)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argon-Ion</td>
<td>Liquid Cell</td>
<td>Eosin B</td>
<td>$2.1 \times 10^{-11}$ M</td>
<td>$2.9 \times 10^{-18}$ mol</td>
<td>42</td>
</tr>
<tr>
<td>Argon-Ion</td>
<td>Capillary</td>
<td>Cobalt (II)</td>
<td>$2.2 \times 10^{-3}$ AU</td>
<td>$4.4 \times 10^{-12}$ mol</td>
<td>43</td>
</tr>
<tr>
<td>Argon-Ion</td>
<td>Liquid Cell</td>
<td>Eosin B, Iodine</td>
<td>$7.1 \times 10^{-9}$ M, $4.6 \times 10^{-7}$ M</td>
<td>$7.0 \times 10^{-19}$ mol, $4.5 \times 10^{-17}$ mol</td>
<td>44</td>
</tr>
<tr>
<td>Argon-Ion</td>
<td>Liquid Cell/CD</td>
<td>(+)Co(en)$_3^{3+}$</td>
<td></td>
<td>$6.8 \times 10^{-13}$ g</td>
<td>45</td>
</tr>
<tr>
<td>Diode Laser</td>
<td>Liquid Cell</td>
<td>Rhodamine 800</td>
<td>$7.7 \times 10^{-8}$ M</td>
<td>$1.2 \times 10^{-17}$ mol</td>
<td>46</td>
</tr>
<tr>
<td>Argon-Ion</td>
<td>CE System</td>
<td>Dabsyl-Glycine</td>
<td>$8.5 \times 10^{-8}$ M</td>
<td>$1.3 \times 10^{-17}$ mol</td>
<td>47</td>
</tr>
<tr>
<td>Argon-Ion</td>
<td>HPLC System</td>
<td>Glycine</td>
<td></td>
<td>$7.8 \times 10^{-13}$ mol</td>
<td>48</td>
</tr>
<tr>
<td>He-Ne</td>
<td>Capillary</td>
<td>BSA, HPV-Ab</td>
<td>$3.4 \times 10^{-19}$ M, $6.4 \times 10^{-14}$ M</td>
<td>$1.7 \times 10^{-22}$ mol, $2.6 \times 10^{-17}$ mol</td>
<td>49</td>
</tr>
<tr>
<td>Argon-Ion</td>
<td>Sol-gel Sensor</td>
<td>Fe(II), Cr(VI)</td>
<td>$1.7 \times 10^{-14}$ M, $1.15 \times 10^{-9}$ M</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Argon-Ion</td>
<td>DNA Microarray</td>
<td>DNA</td>
<td>$8.7 \times 10^{-16}$ M</td>
<td>$1.0 \times 10^{-23}$ mol</td>
<td>50</td>
</tr>
<tr>
<td>Argon-Ion</td>
<td>Protein Microarray</td>
<td>Cytokines</td>
<td>$6.4 \times 10^{-13}$ M (0.01 pg/mL)</td>
<td>$6.0 \times 10^{-21}$ mol</td>
<td>50</td>
</tr>
<tr>
<td>Argon-Ion</td>
<td>CE System</td>
<td>Cellular Proteins</td>
<td>$3.5 \times 10^{-16}$ M (6.6 fg/mL)</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Argon-Ion</td>
<td>CE Microchip</td>
<td>Ni(II), Cu(II)</td>
<td>$2.6 \times 10^{-9}$ M, $8.5 \times 10^{-8}$ M</td>
<td>$5.0 \times 10^{-20}$ mol, $1.6 \times 10^{-18}$ mol</td>
<td>51</td>
</tr>
<tr>
<td>Argon-Ion</td>
<td>CE System</td>
<td>Cellular Proteins</td>
<td>$3.6 \times 10^{-16}$ M</td>
<td>$2.2 \times 10^{-23}$ mol</td>
<td>52</td>
</tr>
<tr>
<td>Pulsed UV</td>
<td>CE System</td>
<td>Cellular Proteins</td>
<td>$3.3 \times 10^{-16}$ M</td>
<td>$1.3 \times 10^{-23}$ mol</td>
<td>52</td>
</tr>
<tr>
<td>Argon-Ion</td>
<td>Capillary</td>
<td>TNT-complex</td>
<td>$2.3 \times 10^{-12}$ M</td>
<td>$2.1 \times 10^{-18}$ mol</td>
<td>53</td>
</tr>
<tr>
<td>Diode Laser</td>
<td>Capillary</td>
<td>TATP-complex</td>
<td>$3.7$ pg/mL</td>
<td>$7.8 \times 10^{-18}$ mol</td>
<td>53</td>
</tr>
<tr>
<td>Pulsed UV</td>
<td>CE System/Surface</td>
<td>Nitroaromatic Explosives</td>
<td>$4.4 \times 10^{-13}$ M</td>
<td>$2.4 \times 10^{-23}$ mol</td>
<td>This Work</td>
</tr>
<tr>
<td>Pulsed UV</td>
<td>CE System</td>
<td>Neurotransmitters</td>
<td>$3.7 \times 10^{-13}$ M</td>
<td>$2.0 \times 10^{-23}$ mol</td>
<td>This Work</td>
</tr>
<tr>
<td>Diode Laser</td>
<td>CE System</td>
<td>Cellular Proteins</td>
<td>$3.0 \times 10^{-10}$ M</td>
<td>$2.4 \times 10^{-20}$ mol</td>
<td>This Work</td>
</tr>
<tr>
<td>Diode Laser</td>
<td>CE System</td>
<td>HIV Protein (p24)</td>
<td>$4.2 \times 10^{-10}$ M</td>
<td>$3.13 \times 10^{-20}$ mol</td>
<td>This Work</td>
</tr>
</tbody>
</table>
1.4 DISSERTATION OUTLINE

Chapters 3 - 6 of this dissertation present wave mixing as a way to detect both small molecules and proteins for biomedical and security applications. The results presented here are obtained from optical and electronic systems that are built to be compact and robust to show versatility for laboratory, clinical, and in-field applications. Chapter 2 introduces the basic theory and concepts behind DFWM and illustrates its key advantages over conventional spectroscopic methods. Chapter 3 covers the experimental parameters for developing new methods for detecting small molecules and proteins in a wave-mixing setup. Chapter 3 also discusses the alignment of optics and key parameters that are necessary to produce a setup capable of performing ultrasensitive detection of analytes. Chapter 4 presents laser wave mixing as a sensitive detection method for nitroaromatic explosives and neurotransmitters with data acquired from a compact passively q-switched pulsed ultraviolet-laser that can detect nitroaromatic species, neurotransmitters and other conjugated molecules in their native form. Chapter 5 presents wave mixing as a sensitive detector for cellular proteins. Coupled to capillary electrophoresis, DFWM is a powerful tool since it can separate and detect proteins at very low concentration levels without the use of fluorescent tags. Chapter 6 presents results for the ultrasensitive detection of p24 antigen, a capsid protein that could be used to develop cost and time efficient HIV-viral load measurements for patients. Chapter 7 presents conclusions and future directions for this work.
1.5 References


CHAPTER 2
THEORY

2.1 NONLINEAR LASER WAVE-MIXING SPECTROSCOPY

Nonlinear laser wave mixing is one of the most sensitive nonlinear laser methods. Four-wave mixing refers to the interaction of laser light by way of the third-order nonlinear polarization. In a degenerate four-wave mixing setup, all the input waves have the same frequency. Nonlinear wave-mixing spectroscopy involves the mixing of two input beams inside an absorbing analyte. When two beams are mixed, they produce a pattern of constructive and destructive interferences. Absorbing analytes interact with this interference pattern to produce laser-induced gratings. Signal from DFWM is produced from photons that diffract off the laser-induced gratings at defined angles from the input beams. In a dual beam forward-scattering setup, input beams serve as both pump and probe beams, and hence, producing two signal beams. These wave-mixing signals can be isolated and directed away from incoherent noise levels since they are coherent laser-like beams. In addition, the wave-mixing signal is detected against a dark background, and hence, yield excellent signal-to-noise ratios. It is important to understand the nature of laser-induced gratings to understand the mechanisms behind wave-mixing signal optimization in an experimental context.
2.1.1 INTERFERENCE PATTERN

The formation of laser-induced gratings begins when two optical waves, $E_1$ and $E_2$, with vector waves $k_1$ and $k_2$, intersect at an angle $\theta$ inside of an absorbing medium, as shown in Figure 2.1, to create an interference pattern described by the light intensity modulation expression:\(^1,2\):

$$I(x) = I_0 \left[ 1 + \beta \cos \left( \frac{2\pi x}{\Lambda} \right) \right]$$  \hspace{1cm} (1)

where $x$ is a spatial coordinate and $\Lambda$ is the spatial period of the interference pattern. The two coherent beams cross to form an interference pattern resulting from constructive and destructive interferences. The pattern contrast, also called the fringe modulation level, is represented by $\beta$ and depends on the intensities of the two input beams:

$$\beta = \frac{2 \sqrt{I_1 I_2}}{I_0}$$  \hspace{1cm} (2)

where $I_0$ is the total incident laser intensity ($I_0 = I_1 + I_2$). The resulting fringe spacing, $\Lambda$, is expressed by the following Bragg equation:

$$\Lambda = \frac{\lambda}{2 \sin \left( \frac{\theta}{2} \right)}$$  \hspace{1cm} (3)

As shown at the top of Figure 2.1, a grating-like modulation of the refractive index, $\Delta n$, is produced in the absorbing medium following the absorption of light by the analyte species. This occurs because the absorbing medium placed inside the interference pattern mimics its spatial modulation, thus, generating laser-induced gratings.
Figure 2.1 Interference pattern formed by the mixing of two input laser waves, $E_1$ and $E_2$, inside a nonlinear medium and the resulting refractive-index grating modulation, $\Delta n$. $\lambda$, excitation wavelength; $x$, spatial coordinate; $\Lambda$, spatial grating period.
2.2 LASER-INDUCED GRATING FORMATION

The refractive index modulation, $\Delta n$, acts as a stationary phase grating that diffracts incident laser light, when an appropriate absorbing analyte is present in the light wave interference pattern, to produce the DFWM signal. It is not the interference pattern alone that causes the diffraction of incident probe beams; it is the absorbing analyte, in response to the illuminated spatial fringes formed from the interference patterns that produces the signal beams. There are a few mechanisms in action that give rise to laser-induced gratings. For atomized vapors and other gas-phase samples, the grating depends on a redistribution of atoms in either the ground- or excited-state that gives rise to spatial modulation of the refractive index. This is called a population grating. The other type of grating important for producing signal in DFWM is the thermal grating, the grating that contributes most significantly when the absorbing analyte is detected in liquids. In this section, these two types of gratings will be discussed in more detail as it is important to understand how these gratings arise and how they generate the DFWM signal. Although some of the basic phenomena describing laser-induced gratings will be discussed here, a comprehensive review is beyond the scope of this dissertation. Transient laser-induced gratings, including those that are not key factors in generating the wave-mixing signal, can be found in Eichler et al.4

2.2.1 POPULATION GRATINGS

Population gratings are observed for atomized and gas-phase absorbing species. They are generated when analytes within a given laser probe are raised to an excited state. The intensity modulation produced in the absorbing medium is spatially coherent with the
constructive and destructive interference patterns present when two laser beams are mixed. It has been shown that population grating effects are maximized for the DFWM signal when the laser intensity is close to the analyte concentration saturation level. The population grating has been shown to dominate with DFWM experiments performed on saturable xanthene-dye-doped polymer films.

2.2.2 Induced Thermal Gratings

Population gratings dominate where absorbing analytes are allowed to reach the population density to induce a spatially coherent pattern of excited- and ground-state particles. Where analyte particles are subject to constant collisional deactivation, these conditions are difficult to obtain. Another type of laser-induced grating dominates when collisional deactivation is present, as with liquid-phase samples, where absorbing analytes are deactivated and release energy to the surrounding medium. This, in itself, generates a diffraction grating called a thermal grating. For experiments in the liquid-phase, molecules excited by incident radiation do not stay excited for long in the “amplitude grating” phase and quickly decay via non-radiative relaxation to produce a thermal grating that is spatially modulated to the laser mixing interference pattern. This means that the same fringe spacing observed by intersecting two laser beams (Figure 2.2) will determine the spacing in the thermal grating as well. Thermal gratings make it possible to generate the DFWM signal in liquid samples, and hence, wave mixing is useful for detecting biological samples in aqueous buffers or in their natural environments. The wave-mixing signal is generated when the input laser beams interact inside a liquid sample to create refractive index modulation, Δn, which follows the spatially modulated absorption and
Figure 2.2 Interference pattern produced by two intersecting laser beams
relaxation of sample within confined interference fringes to produce a thermal grating.
Using small angles between the two input beams can help extend the lifetime of a grating
since it will make the grating period wider and increase the time it takes for thermal
relaxation to occur.\textsuperscript{3}

2.2.3 Degenerate Four-Wave Mixing Signal

There are two primary optical geometries for DFWM: backward-scattering DFWM
and forward-scattering DFWM. The latter is the only configuration used in the
experiments presented in this dissertation. In the backward-scattering arrangement, three
laser beams are involved: two counter-propagating input beams serving as the forward-
pump ($E_f$) and backward-pump ($E_b$) beams and one additional probe beam ($E_p$). The signal
beam ($E_s$) is produced when an absorbing medium is introduced into the laser probe volume
and retraces the probe beam path. The signal is a phase-conjugate “time-reversed” replica
of the incident probe beam. This configuration has been used to study gas-phase analytes
with sub-Doppler spectral resolution.\textsuperscript{9}

For the detection of liquid-phase analytes, the high resolution afforded by the back
backward-scattering DFWM geometry is unnecessary because of the boarder absorption
profiles of condensed-phase samples. A simpler, two-beam forward-scattering geometry
is used for condensed-phase studies. In this arrangement as illustrated in Figure 2.3, the
two input beams work under a self-diffraction mode, i.e., the two beams serve as both pump
and probe beams. Although only two beams are needed in this arrangement, this is still a
four-wave mixing process as four photons are involved in the wave-mixing signal
generation. The process in the forward-scattering wave-mixing arrangement involves two
Figure 2.3 Description of the forward-scattering self-diffraction and the vector diagrams for the formation of the signal beams.
beams, $E_1$ and $E_2$ (with respective wave vectors $k_1$ and $k_2$), intersecting at the cell where an absorbing analyte is present at a small angle $\theta$. The wave-mixing process inside the absorbing medium generates a thermal grating. The input beams, $E_1$ and $E_2$, also act as the probe beam in the forward-scattering arrangement and diffract a portion of its photons to yield signal beams $E_3$ and $E_4$ with respective wave vectors $k_3$ and $k_4$. Depending on the intensity ratio used on the two input beams, one could either generate a single signal beam (the one with more diffracted photons) or two first-order signal beams that would be produced simultaneously with two input beams of equal intensity. Most applied DFWM experiments require only one signal beam so the signal energy is concentrated in one direction (usually $I_2:I_3$ of 7:3).

The directionality of these first-order signal beams are defined under Bragg conditions where both energy and momentum conservation are satisfied. The conservation of energy requires that:

$$\omega_3 = \omega_1 + \omega_1 - \omega_2$$

where $\omega$ is the frequency followed by the subscripts where 1 is both the pump and probe beam, 2 is the second pump beam, and 3 is the signal beam. Since all laser beams have the same frequency, the generated signal beam will also have the same frequency. Momentum is also conserved by:

$$k_3 = k_1 + k_1 - k_2$$

where $k$ is the wave vector followed by the subscripts where 1 is both the pump and probe beam, 2 is the second pump beam, and 3 is the signal beam. This acts as a guide for ideal alignment where input beams can be systematically pre-aligned with templates to fully capture diffracted signal beams with predictable directionality. Mismatches in phase ($\Delta k$)
will lead to quenching in the wave-mixing output signal.\textsuperscript{10} It is important to use forward-scattering arrangements with small angles to increase the interaction length inside the analyte to avoid phase mismatches.\textsuperscript{11}

### 2.2.4 **Forward-Scattering Four-Wave Mixing Signals in Liquids**

In liquid-phase samples, thermally induced nonlinear effects dominate over other types of nonlinearities in the formation of the wave-mixing signal. Light absorption in an absorbing liquid creates a periodic temperature distribution $T(x)$ that can be described as a solution of the following differential equation\textsuperscript{1}:

\[ \rho C_p \frac{\delta T}{\delta t} - K \nabla^2 T = \alpha I \]  

(6)

where $\rho$ is the volumic mass, $C_p$ is the heat capacitance at constant pressure, $K$ is the thermal conductivity, and $\alpha$ is the absorption coefficient of the analyte solution. In the steady-state condition, $\delta T/\delta t = 0$, the temperature modulation, $\Delta T$, can be described as\textsuperscript{12}:

\[ \Delta T = \frac{\alpha I_0}{K} \left[ \frac{\Lambda}{2\pi} \right]^2 \cos \left( \frac{2\pi x}{\Lambda} \right) \]  

(7)

For DFWM, refractive index modulation, $\Delta n$, is then produced within the absorbing sample. The spatial modulation of the refractive index with wave vector $q$ is expressed as\textsuperscript{1,4}:

\[ \Delta n = \frac{\partial n}{\partial T} \frac{\alpha I_0 \tau_{th}}{\rho C_p} \cos \left( \frac{2\pi x}{\Lambda} \right) = \Delta n_0 \cos \left( \frac{2\pi x}{\Lambda} \right) \]  

(8)

where $\partial n/\partial T$ is the temperature gradient of the refractive index at constant pressure and $\tau_{th}$ is the thermal diffraction grating decay time given as:
The amplitude $A_3$ of the first-order diffracted beam is given by the equation:

$$A_3 = \frac{A_1}{\Lambda} \int_0^\Lambda t(x) e^{2\pi i x/\Lambda} \, dx$$

(10)

t(x) is the amplitude of the thermal phase grating:

$$t(x) = e^{i\phi \cos(2\pi x/\Lambda)}$$

(11)

where $\phi$ is the maximum phase variation impressed on the wave front by the index grating defined as:

$$\phi = \frac{2\pi b \Delta n_0}{\lambda}$$

(12)

where $b$ is the sample thickness. The self-diffraction efficiency, $\eta$, of the first-order diffraction signal beam is:

$$\eta = \left| \frac{A_3}{A_1} \right|^2$$

(13)

and can be derived as:

$$\eta = \frac{I_3}{I_1} = |J_1^2(\phi)|$$

(14)

with $J_1$ defined as the first-order Bessel function defined as:

$$J_1(\phi) = \frac{1}{2\pi} \int_0^{2\pi} e^{i\phi \cos(t)} \cos(t) \, dt$$

(15)

when $\phi \ll 1$, $J_1 \approx \phi/2$, and the expression for diffraction efficiency can be simplified as:

$$\eta = \left| \frac{\pi b \Delta n_0}{\lambda} \right|^2$$

(16)

with these expressions, the signal beam intensity, $I_3$, can be described as:
\[ I_3 = \left( \frac{b}{16\pi} \right)^2 I_1 I_0^2 \frac{\lambda^2}{\sin^4 \left( \frac{\theta}{2} \right)} \left( \frac{\delta n}{\delta T} \right)^2 \frac{\beta^2 \alpha^2}{K^2} \]  

Substituting in the expression for the fringe modulation level from section 2.1.1, we get:

\[ I_3 = \left( \frac{b}{8\pi} \right)^2 I_1^2 I_2 \frac{\lambda^2}{\sin^4 \left( \frac{\theta}{2} \right)} \left( \frac{\delta n}{\delta T} \right)^2 \frac{\alpha^2}{K^2} \]  

This final equation is very important as it lays the groundwork for some of the most important principles and advantages of DFWM. The equation shows that the signal intensity exhibits a cubic dependence on input laser power and a quadratic dependence on analyte concentration. This is important because, even at modest laser intensity levels, wave mixing can yield impressive sensitivity levels for the detection of gas- and liquid-phase analytes. The quadratic dependence on sample concentration is a unique feature for chemical detection (unlike most absorption-based techniques that follow Beer’s law on a simple linear dependence). This is advantageous for measuring small changes in chemical content as slight change in concentration will yield dramatic changes in signal intensity.
2.3 REFERENCES


CHAPTER 3
EXPERIMENTAL SECTION

3.1 **FORWARD-SCATTERING DEGENERATE FOUR-WAVE MIXING OPTICAL GEOMETRY**

Forward-scattering degenerate four-wave mixing (F-DFWM) creates a simple configuration for the experiments performed in the studies presented in this dissertation. This optical arrangement is most commonly used for liquid-phase analytes as the broad optical absorption profiles of condensed-phase species negate the need for sub-Doppler spectral resolution available from backward-scattering wave-mixing setups. The typical forward-scattering optical arrangement is illustrated in Figure 3.1. F-DFWM needs only a single laser source that is split to create two parallel input beams. Closer spacing between the two parallel input beams creates smaller mixing angles ideal for increasing the grating period, and thus, optimizing the intensity of the wave-mixing signal. The laser source is split by a 70:30 (R:T) beam splitter and directed by mirrors in an “optical box” that allows the beams to be directed to the sample cell in a parallel configuration. The mirrors are coated with a UV-enhanced aluminum coating that yields an average reflectivity of 90% from 250 nm to 600 nm (Newport Corp., Irvine, CA, 10D20AL.2). This makes it ideal for use with multiple laser sources including UV lasers (266 nm) and blue lasers (447 nm, 473 nm, 488 nm). The laser beams are focused and mixed with a mixing lens at the sample cell. Irises and beam blockers are placed throughout the setup to minimize reflections and optical noise before the beams enter the sample cell. Absorbing analytes are excited to
Figure 3.1 Simplified experimental arrangement for forward-scattering degenerate four-wave mixing. BS, beam splitter; M, mirror; L, lens.
generate laser-induced thermal gratings, which then create an index of refraction change that produces the DFWM signal. The strong wave mixing signal is collected optically against a dark background. The input beams are blocked after they pass the sample cell. The signal is collected by a collimating lens that directs all the signal on a photodiode detector (Thorlabs, Newton, NJ, PDA25K or PDA36A depending on the laser used). An optical chopper (Stanford Research Systems, Sunnyvale, CA, SR540) modulates the intensity of the weaker pump input beam at 200 Hz. The chopper is interfaced to a lock-in amplifier (Stanford Research Systems, Sunnyvale, CA, SR810 DSP) that processes the signal and sends it to the data acquisition system (Measurement Computing, Norton, MA, 1608FS) controlled by a custom-built DAQ software (AIDA). The footprint of this experimental arrangement is already small (< one square meter) and it can be further miniaturized by using smaller optics and mounts.

The F-DFWM optical alignment is relatively simple because the signal beam emerges in a predictable direction from the input beams as shown in Figure 3.2. The wave-mixing signal emerges from the analyte cell as a coherent laser-like beam that can be collected with nearly 100% efficiency through the use of directional mirrors and a collimating lens. This ability to direct a bright coherent signal beam over a dark background gives wave mixing an edge over other optical techniques in terms of sensitivity and S/N levels one could achieve. The optical alignment is further simplified because a bright signal spot visible to the naked eye is produced using alignment solutions.

The focal length of the focusing lens defines the wave-mixing laser probe volume. The focused laser spot size at the sample is typically in the micrometer range. Using small capillary fibers and microfluidic chips (20 - 100 μm i.d. channels), the probe volume is
Figure 3.2 Signal beams diffract off thermal gratings in predictable propagation directions that are spatially distinguished from the two input beams. The center-to-center distance (d) is equidistant between the two input beams and from a single input beam to signal beam as shown in A. Actual photo of the DFWM signal is shown in B.
calculated to be in the picoliter (pL) range. Even though these probe volumes are small, the photon density in the wave-mixing probe is high. Because adequate signal can be produced even with small probe volumes, DFWM is ideal for interfacing to miniaturized devices such as capillary fibers and microfluidic chips.

3.2 LASER SOURCES

Several types of pulsed and continuous-wave (CW) lasers have been used in wave mixing optical setups. No matter which type of laser is used, the general principle behind their core design remains the same: a lasing material is pumped optically, chemically or by an electrical discharge to create population inversion and generate a laser beam. One inherent necessity for performing wave mixing with lasers is that they provide a tunable and monochromatic source. The high power density of the laser is necessary to perform and observe multiphoton processes. The wide range of inexpensive, low-power and compact laser sources can be interfaced to a wave-mixing setup to design a portable detector that is suitable for field use.

3.2.1 PASSIVELY Q-SWITCHED ULTRAVIOLET LASER

Many of the experiments described in Chapters 4 and 5 utilize a compact solid-state low-noise Q-switched 266 nm pulsed laser (CNI, Changchun, China) as an excitation source for small molecules and proteins. Since many highly conjugated systems, such as aromatic amino acids and small molecules, absorb in the UV wavelength range without the use of labels, we use this type of laser for native label-free detection of analytes. The laser outputs 266 nm radiation with an average power of 20 mW (0.1 – 4 µJ, 1.3 ns, 7 kHz
repetition rate). The output beam yields a near TEM$_{00}$ mode in an elliptical beam spot (0.5 mm diameter). UV lasers of this type work well with liquid-phase analytes for native detection; although, thermal stability issues and walk-off effects in nonlinear crystals pose some challenges for routine use.

### 3.2.2 VISIBLE-SPECTRUM DIODE LASER SYSTEMS

Different solid-state visible diode lasers are used in experiments described Chapters 4-7. These compact, efficient lasers are suitable for ultrasensitive wave-mixing detection of chromophore- and fluorophore-labeled analytes. Since liquid analytes exhibit a broad optical absorption profile, fixed wavelength diode lasers are suitable for detection of these analytes. These laser systems are rugged, low-cost, and can be tuned to lower power for alignment via transistor-transistor-logic (TTL) modulation. All laser systems were made available by CNI (Changchun, China). The laser wavelengths used were 447 nm, 473 nm, and 635 nm and they reached maximum continuous-wave (CW) output power levels of 20 mW, 50 mW, and 100 mW, respectively. Beams from these lasers yield a near TEM$_{00}$ mode. Unlike many other CW laser sources such as the argon-ion laser, these systems do not require external cooling or special power conditioning.

### 3.2.3 OPTICALLY PUMPED SEMICONDUCTOR LASER

The laser used to detect labeled neurotransmitter and protein samples in Chapters 5-7 is an optically pumped semiconductor (OPS) laser with a 50 mW laser output at 488 nm (Coherent, Inc., Santa Clara, CA, Sapphire). In OPS lasers, the pump light from a direct-coupled single emitter is re-imaged to the front surface of an OPS chip which
absorbs pump radiation and leads to population inversion. The quantum-well structure of the OPS chip ultimately determines the wavelength and other layers of the chip act as a low-loss distributed Bragg reflector that is optimized for specific output wavelengths. This type of laser offers several advantages as compared to Nd:Yag and Nd:YVO₄ lasers including minimum thermal lensing and green-noise effects due to intracavity frequency doubling. It also acts as an alternative to ion lasers as it can produce ultra-narrow linewidths and high-quality beams in a compact design with low power requirements.

3.2.4 Laser Systems for Laser Wave Mixing

Prior to the advent of small and portable solid-state diode lasers, DFWM was performed with more traditional laser sources for analyte detection. The argon-ion laser (Coherent, Inc., Santa Clara, CA, Innova 90-6) has been used for the detection of small molecules, metals and proteins using DFWM.¹ The laser has several different output wavelengths with the two strongest laser lines centered at 514.5 nm and 488 nm. The laser output can be varied and it exhibits a TEM₀₀ mode. These lasers offer high-quality beams, but they are bulky and require a circulating cooling water system.

Another gas laser with a much smaller footprint is the helium-neon laser (Uniphase, Milpitas, CA, 1125-P). This laser produces a modest amount of power (5 mW) at 632.8 nm and it has been used for ultrasensitive detection of Coomassie Brilliant Blue-stained proteins and antibodies.²
3.3 Sample Cells

Sample cells are placed on a three-dimensional translational stage (Newport, Irvine, CA, TSX-ID) placed at the focal point (10 cm) of the mixing lens for precise XYZ adjustments. Accurate placement of the sample cell is critical in creating the sharpest dynamic gratings, and hence, the maximum wave-mixing signal intensity. Placement of an iris before the sample cell can drastically reduce reflections and minimize noise in the optical setup. Unlike most conventional absorption techniques, long path lengths are not necessary to detect analytes at low concentrations (< 1 mm instead of 10 mm). The small probe volume in DFWM detectors are inherently suitable for interfacing to many types of capillary cells and microfluidics.

3.3.1 Capillary Fibers

Flexible polyimide-coated fused-silica capillary fibers (Figure 3.3A) were used to examine many proteins and small molecules in our continuous-flow and capillary electrophoresis-based analyses. The outer diameter (o.d.) of the capillary used for all the experiments described in the following chapters was 360 µm. The inner diameter (i.d.) varied, depending on the experiment, from 50 µm to 100 µm. Although the wave-mixing signal can be generated from all these capillary types, the 75 µm i.d. capillary offers the best S/N and the lowest Joule heating during CE separation. In the simple continuous-flow detection mode, a peristaltic pump (Rainin, Inc., Oakland, CA, Rabbit Plus) was used to introduce analytes into the capillary.
Figure 3.3 Sample cells used for DFWM absorption experiments. A, capillary fiber; B, “liquid-sandwich” Quartz cell.
3.3.2 “LIQUID-SANDWICH” QUARTZ CELL

Sample cells were constructed from Quartz components to detect a thin film of liquid analytes on a surface. The “liquid-sandwich” sample cells were constructed by stacking a Quartz disc (AdValue Tech, Tuscon, AZ, FQ-D-1N-N1/16) on top of a Quartz microscope slide (AdValue Tech, Tuscon, AZ, FQ-S-003) as shown in Figure 3.3 B. The disc overlay minimized optical lensing effects that can occur from liquid samples placed on surfaces due to surface tension induced curved surfaces.

3.3.3 CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) was used to enhance S/N and chemical specificity levels in our analyses of small molecules and proteins. The custom-built CE system was positioned in the wave-mixing setup so that the mixed laser probe sits on the capillary window. The capillaries used were described in Section 3.3.1. In a typical experiment with no special CE coating or reagents, analytes were introduced at the cathode end and flowed through the capillary based on their charge-to-mass ratios. Uncharged molecules migrated with the electroosmotic flow. Special conditions were employed throughout this dissertation to optimize the CE parameters for each type of analyte. The illustration for a basic capillary electrophoresis setup is shown in Figure 3.4. The power supply (Glassman High Voltage, Inc., High Bridge, NJ, MJ30P400) outputs 5-30 kV with a maximum current output of 400 mA. Platinum electrodes (0.5 mm diameter) were attached to the positive and ground end of the CE power supply and placed into appropriate buffer reservoirs. Small capillary fibers require very small sample amounts. The custom-built CE setup
Figure 3.4 Schematic diagram of a capillary electrophoresis setup. The DFWM detector probes the capillary directly.
allowed easy control of effective capillary lengths, probe position, and voltage and current levels. It also provided better access to components that would otherwise be difficult to access in a commercial capillary electrophoresis instrument. Capillary cartridges from commercial vendors were relatively expensive whereas capillaries purchased in spools and placed in our custom-built CE setup were very inexpensive. Much of this work employed simple electrokinetic injections to introduce the sample into the capillary by applying voltage for a period of time causing ions to flow with the electroosmotic flow. Other sample types (proteins) require pressure injections, performed by applying negative pressure to the opposite end of the capillary by a syringe pump (KD Scientific, Holliston, MA, Legato 111). DFWM makes it possible to perform capillary electrophoresis separation without needing to increase the path length by way of custom capillaries or special flow cells. The technique has been demonstrated to provide excellent detection sensitivity in extremely small detection probe volumes in capillaries.$^3$
3.4 REFERENCES


CHAPTER 4

LABEL-FREE DETECTION OF SMALL MOLECULES IN MICROFLUIDICS AND ON SURFACES USING UV LASER WAVE MIXING FOR SECURITY AND BIOMEDICAL APPLICATIONS

4.1 ABSTRACT

Degenerate four-wave mixing (DFWM) yields excellent detection sensitivity levels for a wide range of biomedical\textsuperscript{1–3} and environmental\textsuperscript{4} analytes. In this chapter, DFWM is presented as an effective method for ultrasensitive detection and high-resolution separation of several nitroaromatic explosive compounds and neurotransmitters. These aromatic small-molecule species can be detected by direct optical absorption in their native form using ultraviolet (UV) lasers. When using UV lasers, high-resolution chemical separation and specificity levels are achieved by coupling the laser wave-mixing detector to a capillary electrophoresis (CE) system. Many studies have reported detection limits for these compounds in the low ppb range for the detection of explosives\textsuperscript{5} and the nano-molar range for the detection of neurotransmitters.\textsuperscript{6} These studies report much better detection sensitivity levels for the analysis of label-free explosive compounds and neurotransmitters using a passively Q-switched pulsed UV laser. Laser wave mixing offers both enhanced sensitivity levels and an effective interface to CE microfluidics due to its inherently small laser probe volume, which is defined as the overlap volume of the two input laser beams. This chapter reports low femtomole detection levels for TNT samples on a Quartz slide.
and zeptomole detection levels in capillaries. Even with a mixture of several structurally similar nitroaromatic compounds, these methods can separate and distinguish individual compounds in the mixture using micellar electrokinetic chromatography capillary (MEKC) electrophoresis. The neurotransmitters dopamine and adenosine were detected at attomole and zeptomole mass sensitivity levels, respectively. The detection of neurotransmitters at very low concentration levels could open the door to better treatment options and diagnostic methods for neurodegenerative diseases.

4.2 INTRODUCTION

Security enforcement agencies throughout the world rely on analytical methods such as mass spectrometry and high-performance liquid chromatography (HPLC) to detect explosive compounds for the prevention and investigation of terrorist attacks. Ideally, detectors for potentially harmful compounds should be sensitive, fast and field deployable at ambient pressures and temperatures with minimum false positives since devices capable of quick identification of these compounds allow law enforcement agencies to make pointed decisions for evacuating and decontaminating suspect sites. However, currently available detection methods for specific identification of explosive compounds are bulky, expensive, not portable, and are too slow for “real-time” analysis and determination. For example, the official U.S. EPA protocol for monitoring explosives in soil and ground water using dual-column HPLC requires analysis times of 60 minutes or longer using bulky instruments. Nitroaromatics could be detected by UV-visible absorption spectrometers; however, conventional UV-visible spectrometers require
absorption cells with a long 10-mm path length and their detection limits are too poor to be effective. Current separation methods, such as HPLC, could be used to separate explosive compounds; however, they yield relatively poor separation resolution for structurally similar compounds. Ion mobility spectrometry (IMS) is widely used for explosive detection at airports; however, they all lack important features including speed, size requirement, cost effectiveness and ruggedness.\(^6\) Capillary electrophoresis is an effective method for resolving structurally similar compounds, such as isomers, and it has been used to separate several nitroaromatic explosives with analysis times under ten minutes.\(^7\)-\(^10\) Even shorter analysis times (~30 s) can be accomplished by adapting separation protocols to a microchip electrophoresis format.\(^11\) Although capillary- and microchip-based electrophoresis systems can resolve structurally similar explosives, their current detectors are often cumbersome and require the use of either florescent labels\(^12\) or costly amperometric\(^13\) techniques for optimum sensitivity.

New technology providing label-free sensitive detection of analytes would not only be useful for the detection of explosives, but it could also applied to the detection of neurotransmitters for biomedical purposes. The dysregulation of neurotransmitters in the body is related to several neurological disorders including Alzheimer’s and Parkinson’s diseases. The concentration of specific neurotransmitters present at localized points in the human body is closely related to these diseases. Parkinson’s disease is characterized by a lack of dopamine that results from a progressive degeneration of dopamine-producing cells in the substantia nigra.\(^7\) Adenosine has also been shown to play an important role in
modulating neural activity. Positron emission tomography (PET) molecular imaging has shown involvement of serotonin in the development of motor and non-motor symptoms and complications in Parkinson’s disease. The sensitive and rapid detection of neurotransmitters shows great potential for the development technologies where neurotransmitters could be used as clinically relevant biomarkers capable of diagnosing specific diseases, and therefore, providing access to more effective treatment regimens. Ultrasensitive techniques capable of measuring neurotransmitters in the bloodstream or in vivo would allow health care workers to monitor patient health and response to treatment more effectively.

In order to effectively measure neurotransmitters in the body without specialized pre-concentration techniques, an ultrasensitive method is needed as the basal levels of these chemicals is relatively low (nano-molar). Various analytical techniques, such as electro-analytical methods, laser-induced fluorescence, confocal Raman mapping spectroscopy, NMR and mass spectrometry have been employed to design highly sensitive and selective methods for monitoring neurotransmitters. Although these methods can reach the basal levels for many neurotransmitter species, they often require bulky equipment or expensive functionalized electrodes.

One of the few FDA approved treatments for Parkinson’s disease is deep-brain stimulation (DBS). In this treatment, electrodes are implanted in the brain to deliver controlled electrical pulses to help mitigate Parkinson’s disease symptoms. Although the mechanism remains unknown, studies have found that DBS treatment stimulates the production of neurotransmitters at localized points in
the brain.\textsuperscript{17} For example, Bekar \textit{et al.} have shown that DBS treatment at the thalamus yields a significant increase of ATP and adenosine.\textsuperscript{18} These studies have led to the development of new devices capable of monitoring the production of neurotransmitters \textit{in vivo} to better understand the mechanisms of DBS treatments and to help develop a chemically guided DBS electrode to improve placements in patients and to improve the overall success rate of these treatments.\textsuperscript{19}

Currently available systems for the detection and measurement of neurotransmitters \textit{in vivo} use a wireless instantaneous neurotransmitter concentration system (WINCS), which employs fast-scan cyclic voltammetry. Although this technique provides a great option for \textit{in vivo} measurements, it is only effective for electroactive analytes.\textsuperscript{20} Detection of non-electroactive analytes requires the use of enzyme-linked electrodes.\textsuperscript{21,22} Optically based techniques have been employed to detect both neurotransmitter targets and amino acids, but these techniques are limited to chemical species that are either native fluorophores or those that can be labeled to fluoresce.\textsuperscript{23} Liquid chromatography coupled to mass spectrometry is a sensitive and versatile detection method for neurotransmitters but the total analysis time and expensive bulky equipment limit its potential use for clinical and \textit{in vivo} applications.\textsuperscript{24}

The wave-mixing signal is orders of magnitude stronger than those of conventional optical absorption techniques because the signal is a coherent laser-like beam that has its own propagation direction (high S/N), and it has a quadratic dependence on analyte concentration (easier detection of small chemical changes)
and a cubic dependence on laser input power (effective use of low laser power levels). The wave-mixing signal intensity can be described as:

\[ I_s \approx I_1^2 I_2 \left( \frac{\lambda^2}{\sin^4 \left( \frac{\theta}{2} \right)} \right) Q \left( \frac{\alpha^2}{\kappa^2} \right) \]  

(1)

where \( I_1 \) and \( I_2 \) represent the intensity of the probe and pump laser beams used to generate the signal beam (\( I_1 \) is lower than \( I_2 \)), \( Q \) is the solvent parameter and is equal to the square derivative of the refractive index change with respect to temperature change \([Q = (dn/dT)^2]\), \( \lambda \) represents the wavelength of the laser, \( \alpha \) is the absorption coefficient of the analyte, and \( \kappa \) is thermal conductivity. These nonlinear dependencies offer more effective monitoring of smaller changes in analyte concentration, making wave mixing an effective chemical sensor.

Taking advantage of the cubic dependence of the wave-mixing signal on input laser power, these studies demonstrate effective use of low-power portable UV lasers (< 30 mW) to generate signal intensities that yield zeptomole-level mass detection limits. This chapter illustrates that wave mixing is an effective and reliable tool for detecting nitroaromatic compounds and neurotransmitters when interfaced to CE-based microfluidics. It overcomes limitations of conventional absorption detection methods because wave mixing offers enhanced sensitivity levels and inherently small probe volumes (pL). The use of compact and portable fiber-based CE systems allows detection of explosives quickly in the field with high sensitivity and specificity levels. Potential integration of wave-mixing detection with real-time chip-based electrophoresis\(^{35} \) offers promise for a new generation of ultrasensitive detection methods for security and biomedical applications. DFWM
is a novel nonlinear absorption-based optical technique that exhibits a quadratic dependence on sample concentration making it possible to detect large changes in signal intensity for small changes in analyte composition. This makes DFWM ideal for monitoring extracellular neurotransmitters.

4.3 EXPERIMENTAL

4.3.1 WAVE-MIXING DETECTOR FOR CAPILLARY ELECTROPHORESIS

As previously illustrated in Chapter 3 (Figure 3.1), a compact low-noise Q-switched pulsed UV laser (CNI, Changchun, China) operating at 266 nm at 7 kHz repetition rate and 20 mW average power served as the excitation light source for explosive and neurotransmitter detection in the laser wave-mixing optical setup. The output of the laser beam is split by a 30:70 (R:T) beam splitter to form two input beams. The transmitted beam from the beam splitter is twice as strong as the reflected beam and serves as both the pump and the probe beam. The reflected beam serves only as the pump beam. An optical chopper (Stanford Research Systems, Sunnyvale, CA, SR540) modulates the intensity of the pump input beam at 200 Hz. The chopper is interfaced to a lock-in amplifier (Stanford Research Systems, Sunnyvale, CA, SR810 DSP), which is connected to a simple photodetector (Thorlabs, Newton, NJ, PDA25K or PDA36A depending on the laser used). The two input beams are arranged in the box configuration to travel equal distances prior to being mixed at the capillary cell or the microfluidics. The wave-mixing signal propagates in the forward direction and behaves as a coherent laser-like beam, and
it is conveniently collected by the photodetector with virtually 100% collection efficiency. The diameter of the laser beam measures 1.1 mm before the mixing lens and corresponds to a calculated probe volume of 55 pL when using a 75 µm inner diameter capillary. This probe volume is calculated using the focused spot size equation where the spot is directly proportional to the focal length of the lens and the laser wavelength. CE separation is driven by a 30 kV adjustable power supply (Glassman High Voltage Inc., High Bridge, NJ, MJ30P400). The signal is processed by the lock-in amplifier and digitized by a data acquisition board (Measurement Computing, Norton, MA, 1608FS) controlled by custom-built DAQ software (AIDA). The wave-mixing detector cell is a simple 75 µm i.d. 360 µm o.d. fused silica capillary (Molex, Lisle, IL) connected to a peristaltic pump (Rainin Instruments, Oakland, CA). The polyamide coating on the outer surface of the capillary is removed using a butane torch to create an optical window for wave-mixing detection. A methyl red (in methanol) solution is used to pre-align and optimize the wave-mixing optical setup using a 266 nm laser.

All optical absorption spectra were collected using a UV-visible spectrometer (Agilent, Santa Clara, CA, 8453) and a 1-cm Quartz cuvette. Absorption spectra were blanked using the appropriate solvent system for each analyte.

4.3.2 CHEMICALS AND REAGENTS

The small molecules targeted for label-free detection using ultraviolet wavelength laser wave mixing are shown in Figure 4.1. Methyl red, methanol, acetone, and acetonitrile were purchased from Thermo Fisher Scientific (Waltham,
Figure 4.1 Nitroaromatic explosives (A) and neurotransmitters (B) targeted for detection.
Analytical reference standards for nitroaromatic explosives were purchased from AccuStandard (New Haven, CT). Adenosine and serotonin were purchased from Acros Organics (Morris Plains, NJ) and dopamine hydrochloride was from Alfa Aesar (Ward Hill, MA). Hydrochloric acid, sodium hydroxide, borax and sodium dodecyl sulfate were obtained from Sigma Aldrich (St. Louis, MO).

Standard dilutions of nitroaromatic compounds were prepared from prediluted (100 μg/mL) analytical reference standards (AccuStandard, New Haven, CT) in acetonitrile. Reference standards 1,3-dinitrobenzene, nitrobenzene, 1,3,5-trinitrobenzene, 2,4-dinitrotoluene and TNT (Figure 4.2) were diluted in 20 mM borate buffer at pH 9.3 and stored at 4 °C prior to analysis. Dilutions were performed using borate buffer (20 mM, pH 9.3).

Solutions used to perform capillary electrophoresis were prepared using distilled water from a compact water distillation system (4000, Waterwise, Leesburg, FL). Sodium borate buffer was prepared at appropriate concentrations using solid sodium tetraborate decahydrate (Borax, Sigma) dissolved in water and adjusted to an appropriate pH level either using sodium hydroxide or hydrochloric acid. High purity electrophoresis-grade sodium dodecyl sulfate (Sigma) was added to borate buffer to create micelle solutions. Native dopamine and adenosine solutions were prepared by dissolving appropriate amounts in borate buffer adjusted to pH 9.
4.4 RESULTS AND DISCUSSION

4.4.1 DETECTION OF NITROAROMATIC COMPOUNDS IN A CAPILLARY

Nitroaromatic explosives were first analyzed by a UV-visible spectrometer to determine absorbance values at the 266 nm laser excitation wavelength (Figure 4.2). Strong optical absorption at 266 nm by these explosive compounds allows direct label-free ultrasensitive measurement of micrometer-thin samples by laser wave mixing. TNT has the highest absorption coefficient at 266 nm among the explosives analyzed here and a concentration detection limit of $4.4 \times 10^{-10}$ M or a mass detection limit of $2.4 \times 10^{-20}$ mol for TNT was determined using the continuous-flow detection mode. Figure 4.3 shows a representative confirmation of wave-mixing signals for nitroaromatic compounds in a capillary cell. Signal is confirmed by blocking the chopped beam, i.e., the pump beam, for a short time then unblocked to measure the net wave-mixing signal level. The probe beam is then blocked to measure the ambient background noise levels. The wave-mixing signal is measured only when both the pump and the probe beams are allowed to excite the sample in the capillary. Fluctuations in signal intensity are mostly due to sinusoidal noise resulting from typical flow fluctuations in the peristaltic pump. When the probe beam is blocked, residual noise that is phase-matched to the lock-in amplifier can be detected, and much of this noise results from imperfections on mirror surfaces and diffraction off the optical arrangement. This noise is reduced drastically by effective use of spatial filters (apertures) to filter the coherent signal beam and block the incoherent background light. The detector is placed 60 cm from the sample to
Figure 4.2 UV-visible absorption spectra of EPA Method 8330A nitroaromatic compounds in borate buffer: (1) nitrobenzene, (2) 2,4-dinitrotoluene, (3) 1,3-dinitrobenzene, (4) 1,3,5-trinitrobenzene, (5) TNT at 1.62 x 10^-4 M, 9.00 x 10^-5 M, 1.16 x 10^-4 M, 9.39 x 10^-5 M, and 4.40 x 10^-5 M respectively. TNT spectrum on secondary scale (right)
Figure 4.3 Detection of TNT solution in a continuous-flow capillary cell at a flow rate of 2.1 μL/min. TNT is diluted in methanol to $4.4 \times 10^{-10}$ M. Pump and probe beams are allowed to excite the sample at time $T_1$. At $T_2$, the probe beam is blocked and the difference between the baselines at $T_1$ and $T_2$ represents residual optical noise from the pump beam. Solvent blank is obtained by flowing methanol only.
collect the coherent laser-like signal while letting incoherent light noise decay before reaching the detector. A small amount of signal may be observed when the solvent alone is pumped through the capillary due to residual absorption from previous sample runs, and rinsing the channel with low-concentration acids and bases can significantly reduce this chemical contamination background noise.

Even at trace-concentration levels, the laser wave-mixing signal is visible to the naked eye and yields excellent signal-to-noise ratios when using low laser power levels (mW). The coherent laser-like nature of the signal beam allows virtually 100% optical collection efficiency. The diameter of the laser probe was calculated to be about 30 μm and, as such, was well suited to probe the 75 μm inner diameter of the fused-silica capillary. The laser probe volume was calculated at 55 pL, and hence, laser wave mixing is inherently suitable for probing small spots on surfaces with high spatial resolution and for interfacing to microfluidics and microchannels to further enhance chemical specificity levels.

Wave-mixing detection of nitroaromatics in a continuous-flow capillary mode allows simple and rapid (within seconds) detection of samples as the first line of rapid inspection. By coupling to a CE-based microfluidics or capillary, one can detect explosives quickly (within minutes) using a single laser, and CE also allows rapid separation of explosive compounds and confirmation of a specific explosive against many potential interferents. One could also use labels, antibodies or color complexing agents that are uniquely suitable for a target analyte, and wave mixing allows the use of widely popular fluorescence labels and tags since the wave-mixing signal is strong for both fluorescing and non-fluorescing samples.
Capillary electrophoresis is a widely used separation technique for small molecules and proteins and previous studies have demonstrated CE-based wave-mixing detection for biomolecules.\textsuperscript{1,2} Figure 4.4 shows an electropherogram obtained using micellar electrokinetic capillary electrophoresis (MEKC), a technique well established for separating nitroaromatics.\textsuperscript{26-29} The surfactant, SDS, is present at a concentration above its critical micelle concentration, forming charged micelles. Each nitroaromatic component with neutral charge partitions migrates with the micelles at different rates along the electroosmotic flow. The results show that several nitroaromatic species can be detected simultaneously in less than 2 minutes using MEKC. These results for the migration times of the individual peaks are highly reproducible with calculated relative standard deviations (RSDs) from 1.6% to 2.5% Previous studies\textsuperscript{16-19} reported concentration detection limits in the micro-molar range. As shown in Figure 4.5, the detection limits are orders of magnitude better in the CE-separated detection mode since we determine a concentration detection limit of 4.4 x 10\textsuperscript{-13} M and a mass detection limit of 24 yoctomole for TNT (based on the laser probe volume used). Low concentration detection of TNT runs were performed in triplicate with a calculated RSD value of 1.5% for migration times. The mass detection limit in the yoctomole range represents fewer than 20 molecules of TNT inside the laser probe volume when the wave-mixing signal is collected.
Figure 4.4  Electropherogram showing separation of five nitroaromatic explosives at 10 µg/mL: (A) 1,3,5-trinitrobenzene, (B) 1,3-dinitrobenzene, (C) nitrobenzene, (D) 2,4,6-trinitrotoluene, and (E) 2,4-dinitrotoluene. CE conditions: fused silica 75 µm i.d. capillary (15 cm effective capillary length, 30 cm total), 13.3 kV (positive), 20 mM Borate - 45 mM SDS buffer, electrokinetic injection (5 s), 8-minute rinse procedure between CE runs. Detector: 266 nm laser. Low attomole amounts of explosives detected inside the 55 pL laser probe volume.
Figure 4.5 Capillary zone electrophoresis separation and detection of zeptomole TNT. CE conditions: fused silica 100 µm i.d. capillary (15 cm effective capillary length, 30 cm total), 12 kV (positive), electrokinetic injection (10 s), 20 mM borate buffer, and 2-minute rinse procedure between CE runs. 266 nm laser.
4.4.2 Detection of Neurotransmitters in a Capillary

Adenosine, dopamine and serotonin are neurotransmitter species that absorb radiation in the ultraviolet range (~200-300 nm) as shown in Figure 4.6. Prior to analysis by capillary electrophoresis, analytes are moved through the capillary by a peristaltic pump at 2.1 µL/min. Signal is verified and measured by creating a “finger-plot” where pump and probe beams are systematically blocked and unblocked to measure the resulting signal-to-noise. At the beginning of the plot, the weaker beam is blocked for a period of time before unblocking and allowing signal to be generated. The wave-mixing signal is measured only when both pump and probe beams are allowed to pass and excite the sample in the capillary. Fluctuations in signal intensity are most likely due to fluctuations in flow from the peristaltic pump. Flow experiments are performed at various concentrations and at various laser powers to verify that the signal exhibits a quadratic dependence on concentration and a cubic dependence on laser power. A typical “finger-plot” of neurotransmitter analyte is shown in Figure 4.7. Although these plots provide information on the suitability and preliminary detection limit of neurotransmitters, capillary electrophoresis is better suited to achieve enhanced sensitivity and specificity levels.

Unlabeled adenosine and dopamine samples were separated and detected by DFWM using a 266 nm laser and capillary electrophoresis. Figure 4.8 shows representative reproducible electropherograms for dopamine obtained using micellar electrokinetic chromatography capillary electrophoresis. Samples can be
Figure 4.6 UV-visible absorption spectra of target neurotransmitters in borate buffer:
(A) adenosine, (B) serotonin, and (c) dopamine at $6.4 \times 10^{-5}$ M, $1.3 \times 10^{-4}$ M, and $2.2 \times 10^{-4}$ M, respectively.
Figure 4.7 Detection of label-free adenosine in borate buffer at $3.7 \times 10^{-4}$ M flowed through a 75 µm i.d. capillary by a peristaltic pump. Wave mixing performed with 266 nm laser. Pump and probe beams are allowed to excite the sample at time $T_1$. At $T_2$, the probe beam is blocked and the difference between the baselines at $T_1$ and $T_2$ represents residual optical noise from the pump beam.
Figure 4.8 Reproducible electropherograms of dopamine using capillary electrophoresis with DFWM detection on a 266 nm laser (20 mW). Sample for both trails is dopamine at $2.1 \times 10^{-4}$ M in sodium borate buffer at pH 9. Run buffer is the same as that for sample with 40 mM SDS. CE parameters: 75 µm i.d., 30 cm, 15 cm effective, 8.1 kV, and 2 s injection.
separated and identified in 2 minutes using this method. The surfactant used in these experiments was sodium dodecyl sulfate (SDS) and was present at a concentration above its critical micelle concentration to form charged micelles. Neurotransmitters partition into the micelles at different rates along with the electroosmotic flow. Sodium borate buffer was used as the run buffer in these experiments to minimize the current at high voltages, thus minimizing Joule heating. Label-free separation and detection of neurotransmitters using wave mixing eliminate the need for chromophore and fluorophore conjugation normally used to reach the sensitivities necessary for neurotransmitter analysis.

Sensitive and highly reproducible results were obtained for the detection of neurotransmitters using wave mixing coupled to capillary electrophoresis. In a typical capillary electrophoresis experiment, standard samples are used to measure the elution times of individual neurotransmitters before running a mixture of samples. It is important to make sure the capillary is rinsed and conditioned consistently for migration time reproducibility. Peak heights also remain reproducible from run to run. Signal-to-noise ratios may vary depending on the cleanliness of the capillary. The capillary remains renewable for hundreds of experiments with proper cleaning and care.

The detection of native dopamine and adenosine by DFWM and micellar electrokinetic chromatography capillary electrophoresis is shown in Figure 4.9. Native detection of neurotransmitters reduces the number of steps needed to work up a biological sample where accurate measurements are important. Both dopamine and adenosine are electroactive molecules. The common label-free detection
Figure 4.9 Electropherograms of native adenosine, dopamine and a mixture of both analytes. For individual samples, the concentration of dopamine and adenosine are $6.5 \times 10^{-4}$ M and $3.7 \times 10^{-4}$ M, respectively. In the mixture, the concentration of dopamine and adenosine are $6.5 \times 10^{-4}$ M and $3.4 \times 10^{-4}$ M, respectively. The run buffer is 20 mM sodium borate buffer at pH 8.9 with [SDS] 60 mM. CE parameters: 75 µm i.d., 30 cm, 15 cm effective, 12 kV, 10 s electrokinetic injection, 266 nm excitation laser at 20 mW.
method used to detect dopamine and adenosine is electrochemical detection.\textsuperscript{30} Capillary electrophoresis yields efficient separation for small molecules and is suitable for analyzing biological samples due to its small sample size requirements (fL - pL). Capillary electrophoresis instruments are typically coupled to a fluorescence detector to reach ultrasensitive levels and only analytes with native fluorescence or those tagged with a suitable fluorophore can be detected at low concentration levels. Wave mixing results show both sensitive detection and good reproducibility for dopamine and adenosine. Our reproducible retention times for dopamine and adenosine promise reliable separation of more complex mixtures of molecules. The separation and identification of neurotransmitters in standard samples and in mixtures were accomplished in about 2 minutes.

Using micellar electrokinetic capillary electrophoresis for adenosine and dopamine, reproducible electrophorograms were obtained at very low concentration levels as shown in Figure 4.10. Samples are initially run at higher concentration levels to verify retention times and reproducibility and are then diluted to lower concentrations for detection limit studies. Native adenosine exhibits the strongest optical absorption at 266 nm as compared to all the neurotransmitter analytes presented here. It yields the best concentration detection limit of $3.7 \times 10^{-13}$ M and a mass detection limit of $2.0 \times 10^{-23}$ mol (i.e., approximately 12 molecules inside the probe volume). The electropherogram for adenosine yields a S/N of 26.3. Dopamine, as expected with its lower absorption at 266 nm, yields a concentration detection limit of $2.1 \times 10^{-9}$ M (sufficient to reach the nM basal levels of dopamine present in the brain\textsuperscript{31} and a mass detection limit of $1.2 \times 10^{-19}$ mol (S/N of 6.1).
Figure 4.10 Electropherograms for target neurotransmitters, pH 9, 60 mM SDS, 12 kV, 5 s injection. (A) Serotonin $4.7 \times 10^{-6}$ M, 25 mM sodium borate run buffer, pH 9, 15 kV, 10 s injection. (B) Dopamine $2.1 \times 10^{-9}$ M, 25 mM sodium borate run buffer, pH 9, 40 mM SDS, 8.1 kV, 2 s injection. (C) Adenosine $3.7 \times 10^{-13}$ M, 20 mM sodium borate run buffer. CE: 75 µm i.d. capillary, 30 cm, 15 cm effective, 266 nm excitation laser.
4.4.3 DETECTION OF TNT SAMPLES ON SURFACES

Laser wave-mixing detection of micrometer-thin liquid TNT samples on surfaces was demonstrated by using a thin layer of 25-mm diameter spot of TNT held between Quartz plates. Methyl red is used as the wave-mixing alignment solution in order to pre-align the detector and to verify the wave-mixing signal based on quadratic dependence on concentration using samples ranging from 50 μg/mL to 0.2 mg/mL. Quadratic dependence on analyte concentration was determined over a range of concentrations, when using micrometer-thin samples on a surface.

After the wave-mixing optical setup was aligned using methyl red (Figure 4.11) and the signal confirmed, trace amounts of micrometer-thin TNT were placed between Quartz plates. Figure 4.12 shows that micrometer-thin layers of TNT can be detected over buffer background signal at a low concentration level of $4.40 \times 10^{-5}$ M. The absolute amount of mass inside the laser probe volume, assuming equal distribution of the molecules across the Quartz surface area, was determined to be 0.75 pg of TNT. The Quartz plate and the buffer both yield similar background noise levels, indicating that the buffer alone yields a minimum amount of background noise. The use of two Quartz plates to hold a thin layer of TNT sample decreases the laser power available for the sample and increases the optical background noise levels. Nevertheless, the wave-mixing detection sensitivity for TNT thin films was better than any other absorption-based label-free optical detection methods using such micrometer-thin samples. Wave-mixing detection
Figure 4.11 Relative average signal vs. analyte concentration for methyl red spots on a “liquid-sandwich” surface sample. Signal exhibits a quadratic dependence on concentration (inset). Concentrations of methyl red: (A) 0.2 mg/mL, (B) 0.15 mg/mL, (C) 0.10 mg/mL, (D) 0.08 mg/mL, (E) 0.05 mg/mL. DMSO used as solvent blank.
Figure 4.12 Laser wave-mixing signals across TNT spots on a Quartz plate. Small 4 μL spots of the sample are isolated between two round 25 mm Quartz plates. (A) 4.4 x 10^{-4} M TNT in sodium borate buffer, (B) 4.4 x 10^{-5} M TNT in sodium borate buffer, and 20 mM sodium borate buffer at pH 9.
limits could be further enhanced by using a single Quartz plate on which a spot of TNT or nitroaromatics is positioned.

Figure 4.13 shows wave-mixing signals collected from a TNT sample deposited on a Quartz plate and allowed to settle to form spots. Similar experiments were done using 1,3-dinitrobenzene and 2,4-dinitrotoluene. Using a known amount of explosive solution at the micromolar concentration range, trace amounts of explosives are deposited on the Quartz plate including 0.2 ng of TNT, 20 ng of 1,3-dinitrobenzene and 20 ng of 2,4-dinitrotoluene. Assuming an even distribution of molecules on the surface of the deposited spot and based on the radius of the laser wave-mixing probe on the Quartz plate ($r_{266} = 3 \times 10^{-5}$ m$^2$), the total absolute amount or mass detected inside the laser probe is determined to be 7.54 femtogram for TNT and 754 femtogram for 1,3-dinitrobenzene and 2,4-dinitrotoluene.

At higher concentration levels, the signal-to-noise ratio is much better and residual optical noise is much lower, as expected. The concentration detection limits for dry explosive spots are generally an order of magnitude better than those determined for thin films of explosives held between Quartz plates. Mass detection sensitivity levels for dry explosive spots are excellent (femtogram range) even when using very low laser power levels (a few mW) to avoid photo bleaching of trace amounts of samples, especially since the samples in the dry spots are static unlike those in the flowing capillary cells. Although dust and other contaminants on surfaces could also create some background scatter light, wave mixing offers unique advantages over conventional optical methods in effectively filtering incoherent
Figure 4.13 Confirmation of laser wave-mixing signals from explosive spots scanned across a Quartz plate: (A) $5.95 \times 10^{-5}$ M 1,3-dinitrobenzene, (B) $5.49 \times 10^{-5}$ M 2,4-dinitrotoluene, and (C) $4.40 \times 10^{-5}$ M TNT. Sample spots are created on the Quartz plate by depositing 1 μL of sample solution dissolved in borate buffer to form 5 mm spots. Both pump and probe input beams are allowed to excite the sample at time $T_1$, and the probe beam is blocked at time $T_2$. 
optical noise levels since the signal beam is a coherent laser-like beam.

These studies have demonstrated ultrasensitive detection of explosives in flowing liquids, surface thin films, and dry spots on surfaces with zeptomole or femtogram detection sensitivity levels. Although the samples were placed at a relatively short standoff distance in these preliminary studies (100 cm), we have demonstrated much longer standoff detection distances (meters) in other studies. Wave mixing offers much longer standoff detection capabilities as compared to conventional optical methods since the wave-mixing signal is a coherent laser-like collimated beam. In addition, the signal beam is a phase conjugate beam of the probe beam in a backward-scattering wave-mixing optical setup, i.e., the signal beam is a time reversed replica of the probe beam, and hence, the signal is more and more collimated as it travels, allowing for much longer standoff detection capabilities without sacrificing optical collection efficiency.

Although, fixed-wavelength lasers are used in the studies reported here, we have demonstrated the use of tunable lasers in wave-mixing analysis of isotope and hyperfine profiles that yield high-resolution chemical fingerprinting. Tunable external cavity diode lasers and tunable quantum cascade mid-IR lasers allow collection of complete spectra over a wavelength range that allows profiling of atomic or molecular spectra and identification and confirmation of chemical species while taking advantage of unique wave-mixing features including ultrasensitive detection levels and long standoff detection capability.
4.5 CONCLUSIONS

Laser wave mixing is demonstrated to be an ultrasensitive absorption-based optical method for detecting nitroaromatic explosives and neurotransmitters in their native states. Coupled to capillary electrophoresis-based microfluidics, wave mixing can separate and distinguish nitroaromatic and neurotransmitter species at zepto-mole detection sensitivity levels (Table 4.1). Hence, wave mixing is orders of magnitude more sensitive than conventional absorption techniques while using micrometer-thin samples (instead of a conventional 1-cm cuvette). Wave mixing offers unique and significant advantages including coherent laser-like signals, virtually 100% optical signal collection efficiency, zeptomole detection sensitivity, compact and portable detection setup, low laser power requirements, the ability to detect both fluorescing and non-fluorescing molecules, and standoff detection capabilities. Wave mixing also allows detection of liquid explosive samples in flowing capillary cells, liquid explosive thin films on surfaces and dry explosive spots on surfaces with better detection sensitivity levels than many other methods that require bulky instruments. These new tools demonstrate the utility of UV laser wave mixing to perform ultrasensitive label-free analysis of small-molecules for biomedical and security applications.
Table 4.1 Detection limits for explosives (top) and neurotransmitters (bottom).

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<th>Mass LOD</th>
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4.6 ACKNOWLEDGEMENTS

Chapter 4, in part, is currently being prepared for submission for publication of the material. Hetu, M. M.; Iwabuchi, M. F.; Maxwell, E. J. The dissertation author was the primary investigator and author of this material.

4.7 REFERENCES


CHAPTER 5
SIZE-BASED SEPARATION AND DETECTION OF CELLULAR PROTEINS BY LASER WAVE MIXING SPECTROSCOPY AND CAPILLARY ELECTROPHORESIS

5.1 ABSTRACT

Degenerate four-wave mixing (DFWM) is presented in this chapter as a sensitive optical absorption-based detection method for cellular proteins and antibodies. Wave mixing is coupled to size-based capillary electrophoresis (CE) to separate and detect native and labeled proteins. Size-based separations are accomplished using sodium dodecyl sulfate capillary gel electrophoresis (SDS-CGE). Protein separations have been optimized for wave mixing by selecting appropriate sieving matrices, developing new dynamic coating methodologies, and changing run buffer conditions. Several proteins from a standard protein ladder are used to optimize the separation conditions. Proteins and antibodies are detected at zeptomole mass detection limits using laser wave mixing and SDS-CGE.

5.2 INTRODUCTION

Polyacrylamide gel electrophoresis (PAGE) is commonly used in biomedical research for the detection of large biomolecules. Under standard conditions, these biomolecules travel at different rates according to their electrophoretic mobilities.
The mobility of these macromolecules, usually proteins or nucleic acids, depends on their conformation, length and overall effective charge. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used for size-based separations of proteins using a large slab gel.\textsuperscript{1,2} The procedure for separating proteins in the gel matrix involves a multi-step procedure in which the gel is assembled, proteins are heated in the presence of SDS to denature the sample before loading onto the gel, and then separated by electrophoresis and stained, so that protein weights and concentrations can be quantified. In the presence of SDS, protein complexes all have similar charge densities, and therefore, they are separated solely based on their mobilities, i.e., smaller proteins have greater mobilities. The mobility decreases linearly with the logarithm of the protein molecular mass. Although widely used, SDS-PAGE is labor intensive and time consuming. Many of these steps are performed by hand, which is often attributed to non-reproducible results.

SDS-capillary gel electrophoresis (SDS-CGE) is based on the same principles for size-based separations as SDS-PAGE except the separation takes place inside a small fused-silica capillary and is not limited to acrylamide polymers as a separation matrix. Some of the first SDS-CGE papers published over two decades ago, reported the use of the same sieving matrices found in a SDS-PAGE gel (cross-linked polyacrylamide), prepared directly inside the capillary fiber.\textsuperscript{3} Later experiments replaced cross-linked polyacrylamide with linear polyacrylamide that increased the lifetime of the capillary columns. Cross-linked polymers often exhibit short collapse times attributed to their rigid dynamic equilibrium whereas
the non-cross-linked polymers offer a more dynamic equilibrium with more flexibility in the overall matrix, and hence, less bubble and crack formations in the gel.4 Current techniques employ water-soluble linear or slightly branched polymers that can be used as replaceable sieving matrices in capillary electrophoresis. Many types of these polymers have been successfully employed for size-based separations including linear polyacrylamide5,6, polyethylene glycol4, polyethylene oxide7, dextran8,9, and pullulan.10–12

The inner walls of the capillary are typically coated to reduce protein-wall interactions and to suppress the electroosmotic flow (EOF). An uncoated capillary interacts with proteins through electrostatic charges (since the silanol groups on the capillary wall are negatively charged) and through hydrophobic interactions. These interactions often lead to problematic separations and can even deteriorate capillary columns by clogging the capillary. The interaction of proteins with the capillary wall is greatly reduced when they are in SDS-protein complexes that are negatively charged. For this reason, capillary coatings in SDS-CGE are primarily used to suppress EOF. Many studies employ a covalently-bound cross-linked polyacrylamide coating first used by Hjertén et al. to suppress EOF in capillary columns.13 This method involves several steps including pretreatment of the capillary (etching, leaching, dehydration and silylation) to obtain adequate coverage of the silylating agent on the capillary wall, and a long coating procedure to form a uniform layer of polyacrylamide on the surface of the capillary wall. Poor performance is attributed to poor silylation yields during the etching and dehydration steps.14 Attempts have been made to optimize the Hjertén reaction, but they require
time-consuming steps, e.g., two overnight reactions.\textsuperscript{15} Even under optimal conditions, some have noted that poor pH stability and lack of hydrolytic stability of the permanent coating make long-term use of the polyacrylamide coating undesirable.\textsuperscript{16}

Dynamic capillary-wall coatings have been explored for their use in protein separations because they can easily be used and flushed out of capillaries for multiples uses. Dynamic coatings are produced by creating a run buffer with appropriate additives that can create dense polymer layers on the capillary wall through hydrogen bonding, electrostatic interactions and hydrophobic forces. These solutions, normally containing polymers at low concentrations, allow capillaries to be coated by simply flowing the solution through the capillary before a separation run. The molecular weight of the polymer used in the coating has been shown to have an effect on coating stability since larger polymers create stronger interactions with the capillary wall.\textsuperscript{17} Polydimethylacrylamide and poly(-hydroxyethylacrylamide) are just a few examples of polymers used to create dynamic coatings. Various surfactants have also been used in buffers to improve separation resolution by modifying the capillary wall such as dimethyldioctadecylammonium bromide (DODAB) with polyoxyethylene (POE) 40 stearate\textsuperscript{18} and hexadecyltrimethylammonium bromide (HTAB).\textsuperscript{19} Both additives and surfactants work towards the same goal of modifying the capillary wall to prevent interaction of proteins with the wall and to suppress electroosmotic flow.

Due to the complexity of the sample media, analyzing proteins in biological fluids is often challenging. Innovative methods that reduce the number of steps for
biological sample analyses are needed for various biomedical applications. Size-based protein separations by CGE offer a powerful tool for new methods in automated protein analysis, cell proteomics, diagnostics and biomarker discovery. Successful detection of erythrocyte membrane proteins has been demonstrated using CGE and these methods serve as new diagnostic tools for hereditary spherocytosis analogous to SDS-PAGE. Two-dimensional CGE arrangements have been developed for the proteomic fingerprinting of breast cancer cells. Single-cell proteomic measurements have been performed on a HT29 human colon adenocarcinoma cell line with excellent sensitivity and resolution. The examples mentioned above represent only a small segment of biomedical applications that is moving forward involving biomolecule separations by SDS-CGE.

Many types of detectors have been reported for CGE systems and UV absorption is the most commonly used detector in commercially available capillary electrophoresis systems. Absorbance detectors with ultraviolet light sources can probe the peptide bonds between amino acids as well as aromatic side-chain groups in protein molecules since they absorb light around 200-220 nm and 280 nm, respectively. Conventional absorption-based detectors yield poor detection sensitivity levels, especially when probing narrow path lengths of capillary fibers. To enhance sensitivity, many groups have opted to first derivatize proteins with a fluorescent dye and detect them by relatively more sensitive laser-induced fluorescence (LIF) methods. Using fluorophores such as Chromeo P540, mass detection limits have been reported in the zeptomole (10^-21 mol) range for labeled
proteins. LIF has been the most commonly utilized sensitive detection method in commercial capillary electrophoresis systems.

Degenerate four-wave mixing has been previously presented as an ultrasensitive absorption-based detector for proteins in flow systems as well as in SDS-CGE using pullulan as a sieving matrix. Yoctomole mass detection limits were reported after successfully separating proteins in single Jurkat cells. Unlike fluorescence-based detectors, wave-mixing detectors allow the use of both fluorophore and chromophore labels, or without using a label at all for label-free native analyte detection. Native label-free proteins have been detected by laser wave mixing using an ultraviolet laser to probe aromatic amino acids in proteins. Although useful for ultrasensitive detection, these studies utilize the Hjertén method and require time-consuming capillary conditioning and preparation steps. Optimization of laser wave mixing and capillary electrophoresis parameters are necessary for efficient detection and separation of protein analytes.

Wave mixing has been demonstrated to detect cellular proteins at low concentration levels (10^{-14} M) using Coomassie Brilliant Blue staining and a helium-neon laser source. The wave-mixing signal is orders of magnitude stronger than those of conventional optical absorption techniques because the signal is a coherent laser-like beam that has its own propagation direction (high S/N) and it has a quadratic dependence on analyte concentration (easier detection of small chemical changes) and a cubic dependence on laser input power (effective use of low laser power levels). The wave-mixing signal intensity can be described as:
$I_s \approx I_1^2 I_2 \left( \frac{\lambda^2}{\sin^4 \left( \frac{\theta}{2} \right)} \right) Q \left( \frac{\alpha^2}{\kappa^2} \right)$

where $I_1$ and $I_2$ represent the intensity of the probe and pump laser beams used to generate the signal beam ($I_1$ is lower than $I_2$), $Q$ is the solvent parameter and is equal to the square derivative of the refractive index change with respect to temperature change [$Q = (dn/dT)^2$], $\lambda$ represents the laser wavelength used, $\alpha$ is the absorption coefficient of the analyte, and $\kappa$ is thermal conductivity. This equation illustrates the quadratic dependence of the signal on analyte concentration, i.e., small changes in protein content can be detected in biological systems with large changes in signal.

In addition to excellent sensitivity, DFWM offers several other advantages over conventional absorption techniques including small sample requirements, shorter analysis times, and a broader range of available functional derivitization agents such as fluorophores and non-fluorescing chromophores. Coupled to capillary electrophoresis, ultrasensitive detection of proteins and other biomolecules of interest can be performed with high specificity and selectivity.

The studies presented in this chapter demonstrate sensitive detection of cellular proteins, antibodies and biomarkers using capillary electrophoresis and laser wave mixing. The experimental arrangement for detecting proteins using laser wave mixing coupled to capillary electrophoresis was shown previously in Figure 3.1. SDS-CGE was used to determine the approximate molecular weight of proteins. For relatively pure samples, the sieving matrix was removed from the run buffer to yield quicker analysis times and promote a greater degree of optical cleanliness during detection. These experiments were optimized to reduce capillary rinsing times while
maximizing reproducibility for peaks in the electropherogram. Fluorescein isothiocyanate was used to label proteins for DFWM analysis at 473 nm and 488 nm. Although native detection can be accomplished by employing an ultraviolet laser for wave-mixing detector, the low extinction coefficient of proteins in the UV makes it challenging to produce strong signals. This chapter demonstrates the detection of proteins and antibodies at sub-zeptomole mass detection limits.

5.3 EXPERIMENTAL

5.3.1 WAVE-MIXING DETECTOR FOR PROTEIN CAPILLARY ELECTROPHORESIS

Many proteins contain amino acids that natively absorb ultraviolet light making their detection possible by wave mixing using a 266 nm UV laser. The amino acid most responsible for native absorbance in proteins is tryptophan. The molar extinction coefficient is relatively low for tryptophan at 5,579 cm\(^{-1}\)/M as compared to commonly used chromophores and fluorophores, and hence, conventional absorption-based methods yield poor detection sensitivity. Therefore, proteins and biomolecules are commonly labeled and probed by visible lasers for sensitive detection. One can use a wide range of visible lasers including a continuous wave (CW) optically pumped semiconductor blue 488 nm laser with adjustable power (Coherent, Santa Clara, CA, Sapphire), and a 473 nm diode laser (CNI, Changchun, China). In a wave-mixing setup, the output of the laser is split by a 30:70 R:T beam splitter to create two input beams. The transmitted beam from the beam splitter is about twice as intense as the reflected beam and serves as both the pump and the probe beam. The reflected beam serves only as the pump beam.
An optical chopper (Stanford Research Systems, Sunnyvale, CA, SR540) modulates the pump input beam at 200 Hz. The chopper is interfaced to a lock-in amplifier (Stanford Research Systems, Sunnyvale, CA, SR810 DSP), which is connected to a simple photodetector (Thorlabs, Newton, NJ, PDA25K). The two input beams travel equal distances to the capillary analyte cell, where they are focused and mixed. The wave-mixing signal is a collimated coherent laser-like beam and propagates in the forward direction and it is conveniently collected by the photodetector with virtually 100% collection efficiency using a lens and a mirror. CE separation is driven by a 30 kV adjustable power supply (Glassman High Voltage, Inc., High Bridge, NJ, MJ30P400). The signal is processed by the lock-in amplifier and digitized by a data acquisition board (Measurement Computing, Norton, MA, 1608FS) controlled by a custom-built DAQ software package (AIDA). The analyte cell is either a microfluidics or a simple 75 μm i.d., 360 μm o.d. fused-silica capillary (Molex, Lisle, IL) that is connected to a syringe pump or a peristaltic pump (Rainin Instruments, Oakland, CA). A methyl red (in methanol) solution is used to pre-align and optimize the wave-mixing optical setup.

All optical absorption spectra were collected using a UV-visible spectrometer (Agilent, Santa Clara, CA, 8453) and a 1-cm Quartz cuvette. Absorption spectra were blanked using the appropriate buffer for each analyte.

5.3.2 CHEMICALS AND REAGENTS

Solutions used to perform capillary electrophoresis on proteins were prepared using distilled water from a compact water distillation system (Waterwise,
Leesburg, FL, 4000). Borax, Tris base, sodium dodecyl sulfate (SDS), acrylamide, PEG 10,000, β-lactoglobulin, and bovine serum albumin (FITC-labeled) were all purchased from Sigma-Aldrich (St. Louis, MO). NHS-rhodamine, FITC, CHES, hydrochloric acid, unstained protein ladder, dialysis tubing (MW\text{CUTOFF} = 12-14 kDa) and dye removal columns are purchased from Thermo Fisher Scientific (Waltham, MA). Sodium borate buffer (100 mM) was prepared by dissolving solid sodium tetraborate decahydrate (Borax) in water and adjusting the pH to 8.6 using 1 M hydrochloric acid. Tris-CHES buffers were prepared at various concentration levels by adding solid Tris base (Trizma base) and CHES, and diluted with water. High purity electrophoresis-grade sodium dodecyl sulfate (SDS) was added to CE run buffers at 0.1%. The sieving matrices, pullulan (TCI America, Portland, OR) and polyethylene glycol (Fluka PEG 10000), were added to the run buffer at various concentration levels. Fluorescein isothiocyanate (FITC), 5-(and 6)-carboxytetramethylrhodamine, succinimidyl ester (NHS-Rhodamine) and succinimidyl 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoate (NBD-X, SE, Marker Gene Technologies, Eugene, OR) are fluorophores used to label proteins. Labels were freshly prepared in solution at 10 mg/mL by dissolving in DMF. Ultratrol LN (Target Discovery, Palo Alto, CA) was used to coat capillaries in order to decrease the electroosmotic flow in CE runs and move proteins in reverse polarity. All solutions used for capillary electrophoresis were filtered through a 0.45 μm membrane on a syringe filter.
5.3.3 **Protein Labeling**

Proteins were prepared for labeling by dissolving them in conjugation buffer (100 mM sodium borate, pH 8.6) at 1-2 mg/mL in a 1.5 mL centrifuge tube. Dye solution was added in molar excess (10-20 fold) and the mixture was allowed to react for 1 hour in the dark. Excess dye was removed using two different methods: (1) centrifuge-driven dye removal columns or (2) dialysis. Labeled proteins were dialyzed into run buffers. These methods were applied to label the cancer antigen 15-3 (CA15-3) monoclonal antibody and standard proteins.

The unstained protein ladder contains seven proteins: beta-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp98I (25.0 kDa), beta-lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa). These proteins are present in the solution at various concentration levels (0.1 – 0.2 mg/mL) and they serve as molecular weight markers for SDS-PAGE gels. The protocol for labeling these proteins involves using a fixed higher concentration of each component (2.0 mg/mL) to ensure that excess FITC is present in the reaction mixture. Aliquots of 150 µL were taken from the stock solution and dialyzed into conjugation buffer. The dialyzed protein mixture was reacted with a 15-fold molar excess of FITC for 1 hour in the dark. Excess dye was removed by dialysis into run buffer.
5.4 RESULTS AND DISCUSSION

5.4.1 PROTEIN LABELING

Protein labeling was performed prior to denaturing so that the sample may be used for experiments where SDS is excluded from the run buffer. This allows for denaturing after the sample has been labeled. In other studies, proteins were also labeled during or after denaturation with SDS since pre-labeled proteins could not effectively bind SDS, and hence, could lead to poor separation due to non-uniform charge states. These studies show that pre-labeling does not drastically affect resolution and allows for multiple types of experiments to be performed.

Although wave mixing allows the use of both chromophores and fluorophores as labels, many fluorophores are widely available and proven as good labels for proteins. Figure 5.1 shows UV-visible absorption spectra of native and labeled-β-lactoglobulin using NBD-X, FITC and NHS-Rhodamine. The spectra show that labeling will change both the degree of absorption and the $\lambda_{\text{max}}$ of the absorbing protein. Although useful for quickly separating labeled protein from excess dye, the use of dye removal columns could lead to poor protein recovery rates. For this reason, dialysis is used instead of the centrifuge-driven columns to remove the excess dye. FITC is chosen since it has the highest absorbance around 473 nm and 488 nm, the excitation wavelengths of the lasers used. It is still possible to use labels or dyes that do not have the $\lambda_{\text{max}}$ exactly tuned to the excitation wavelength since the wave-mixing signal is still strong even when the excitation wavelength is more than 30 nm away from the analyte $\lambda_{\text{max}}$.33,34
Figure 5.1 UV-visible spectra of both native and fluorophore-labeled β-lactogloblin. Protein concentration is 1 mg/mL for β-lactogloblin. Product recovered from derivitization reactions vary by overall recovery from the fluorescent dye removal columns (Thermo, Part 22858). Reactions are 10:1 (protein:dye) in 100 mM sodium borate buffer (pH 8.6). (A) native β-lactogloblin, (B) NBD-X-labeled β-lactogloblin, (C) FITC-labeled β-lactogloblin, (D) NHS-rhodamine labeled β-lactogloblin. Spectra of native protein and NBD-X are plotted on the secondary (right) axis.
5.4.2 Separation Conditions

Optimal experimental conditions for protein separation should yield a simple and reproducible analysis for standard proteins. The proteins should either be labeled completely and uniformly to produce a few peaks (or ideally a single peak) or the experimental conditions should be optimized to produce a small and uniform number of peaks for a given analyte. Because it is difficult to control the degree of labeling, these studies focus on optimizing size-based protein separation. Electrophoresis depends on charge-to-size ratios, and hence, it is necessary to achieve a near-uniform charge for the proteins so that size is the dominant variable in the separation process.

Figure 5.2 illustrates one of the challenges for protein separation. It shows that non-uniform charge states can complicate the identification of proteins in an electropherogram. Even for a single protein, in this case β-lactogloblin, a simple zone electrophoresis run performed in the absence of a sieving matrix produces multiple peaks in both normal and reversed-polarity CE runs. In the reversed-polarity CE run, Ultratrol LN is used to coat the capillary wall in order to prevent absorption of protein on the capillary wall, while still allowing natural electroosmotic flow to move the proteins towards the anode.

Figure 5.2 (A) shows that SDS can be added to the run buffer and bind to the labeled protein in order to yield a uniform charge state. Not only does the addition of SDS reduce the overall number of peaks for a single protein in a given electropherogram, it also increases the net wave-mixing signal for the protein. The peak migration is not drastically affected by the degree of labeling when SDS is
Figure 5.2 Electropherograms of β-lactoglobulin collected by a 473 nm based laser wave-mixing setup. Capillary: 75 µm i.d., 30 cm (15 cm effective); 100 mM Tris-CHES, pH 9.0. (A) Ultratrol LN-coated wall, -18 kV, 80 µA, 2 s injection (scaled to left y-axis) with 0.1% SDS added to the run buffer; (B) Ultratrol LN-coated wall, -18 kV, 24 µA, 2 s injection; (C) Bare-capillary wall, +18 kV, 24 µA, 2 s injection.
present since the number of small molecular weight labels does not dramatically change the size of the large protein molecule.

A suitable sieving matrix must be selected to yield reproducible size-based separations as well as an optically clean beam profile for the wave-mixing signal. The sieving matrix must also be adaptable to a number of wave-mixing experiments including UV laser wave mixing-based detection of label-free native proteins. Figure 5.3 shows UV-visible absorption spectra of four different separation matrices suitable for separation of cellular proteins. Linear polyacrylamide has some advantages over cross-linked polymers since they can handle high electric fields without collapsing as the matrix is formed through physical interactions such as van der Waals forces and hydrogen bonds rather than covalent bonds. Linear polyacrylamide is not an ideal sieving matrix when using 266 nm UV lasers since it absorbs strongly in the UV region. When tested on a capillary, this high UV absorbance causes flashing and current drop-outs in a UV laser wave-mixing CE setup (20 mW). When used in a blue laser wave-mixing setup, linear polyacrylamide distorts the input beam profile and decreases the signal collection efficiency (Figure 5.4c). Hence, the linear polyacrylamide matrix performs poorly in laser wave-mixing setups.

Pullulan is a commonly used sieving matrix for SDS-CGE size-based separations. Pullulan has a low absorbance around 266 nm, making it more adaptable to experiments using UV excitation sources. In a typical SDS-CGE experiment with cellular proteins, pullulan is present at 7% or higher to achieve
Figure 5.3 UV-visible absorption spectra of sieving matrices. (→) 100 mM Tris-CHES buffer, (----) 3% PEG in buffer, (····) 6% pullulan in buffer, (- - -) 4% linear polyacrylamide in buffer.
Figure 5.4 Images of the strong input beam profile after passing through the capillary with different sieving matrices. (A) 100 mM Tris-CHES buffer, (B) 3% PEG in buffer, (C) 4% linear polyacrylamide in buffer, (D) 6% pullulan in buffer. Laser: 473 nm, 50 mW.
adequate separation resolution.\textsuperscript{10–12} The use of pullulan results in a viscous and sticky solution that diminishes the integrity of the capillary window and column over time. The optical integrity is not as important for CE-LIF experiments where the output is a diffuse incoherent fluorescence light. The wave-mixing output beam profile shown in Figure 5.4d confirms that the pullulan matrix affects the integrity of the wave-mixing signal, and hence, the signal collection efficiency. In addition, a high concentration of pullulan in the run buffer often leads to clogging and poor CE reproducibility. The high viscosity of the matrix also requires the use of a syringe to introduce analytes into the capillary. As expected for size-based separations with sieving matrices, the migration times for proteins increase linearly with the log of the molecular weight when using pullulan. The inherent challenges described above made pullulan a secondary choice for size-based separations when used with wave-mixing setups.

Polyethylene glycol (PEG), a less commonly used sieving matrix, has similar UV transparency as pullulan, and therefore, can be adapted to both UV and visible laser wave-mixing experiments. PEG is chosen as the preferred separation matrix in wave-mixing setups for three primary reasons: (1) it has low optical absorption in the UV range, (2) it can be used at low concentration levels and still achieve good resolution, and (3) it does not affect the optical integrity of the capillary window even after multiple runs.

Figure 5.3 shows the UV-visible absorption spectrum of a 3\% PEG solution and closely matches that of pullulan and run buffer spectra. Much like pullulan, PEG is a linear, stable and flexible matrix and it allows size-based separations at
much lower concentration levels as compared to pullulan. The use of lower concentration sieving matrix also allows the use of a simple peristaltic pump to introduce analytes into the capillary and to rinse and recoat the capillary. PEG also yields a clean beam profile as shown in Figure 5.4b.

Figure 5.5 shows that FITC-labeled proteins from a commercially available molecular weight ladder can be separated and detected using a 3% PEG matrix in the run buffer solution. Relative standard deviations for the retention times of each peak in multiple trials were determined to be lower than 0.2%. The linear relationship ($r^2 = 0.968$) observed between the logarithm of the molecular weight and the migration time confirms the effectiveness of PEG in size-based separation. This is useful in molecular weight determination of proteins in a variety of applications including biomarker discovery and cellular proteomics.

5.4.3 OPTIMIZATION OF PROTEIN SEPARATION

In this section, four experimental parameters affecting separation are explored: (1) concentration of the separation matrix, (2) separation potential, (3) run buffer concentration, and (4) the capillary length. These parameters are explored in order to optimize detection sensitivity, analysis time and separation resolution.

Figure 5.6 presents the effect of PEG concentration on resolution, peak height, and analysis time for the analysis of molecular weight ladder proteins. It is clear that a lower PEG concentration leads to a decrease in resolution since there is not enough matrix present to achieve adequate size-based separation, i.e., it does not allow the proteins to spend enough residence time in the capillary to move apart. As
Figure 5.5 (A) Reproducible electropherograms of FITC-labeled protein ladder using a 473 nm laser based wave-mixing setup and 3% PEG. Capillary: 75 µm i.d., 50 cm (30 cm effective); 100 mM Tris-CHES, pH 9.0, Ultratrol LN-coated wall, -15 kV, 12 µA, 2 s injection. Peaks: 1, lysozyme; 2, β-lactoglobulin; 3, REase Bsp98I; 4, lactate dehydrogenase; 5, ovalbumin; 6, bovine serum albumin; and 7, β-galactosidase. (B) Linear correlation between the logarithms of the molecular weights vs. retention time.
Figure 5.6 Electropherogram of FITC-labeled protein ladder using a 473 nm laser based wave-mixing setup with varying amounts of PEG (1%, 3%, 5%). Capillary: 75 µm i.d., 50 cm (30 cm effective); 100 mM Tris-CHES, pH 9.0, Ultratrol LN-coated wall, -15 kV, 12 µA, 2 s injection. Peaks: 1, lysozyme; 2, β-lactoglobulin; 3, REase Bsp98I; 4, lactate dehydrogenase; 5, ovalbumin; 6, bovine serum albumin; and 7, β-galactosidase.
the PEG concentration is increased to 3%, it is clear that there is a marked increase in peak height and separation resolution. Although 5% PEG provides the best resolution, it takes nearly 13 minutes to collect the electropherogram. Longer times between runs can lead to various changes in the capillary including coating deterioration, clogging, current drop-outs, and lower run-to-run reproducibility. It was determined that 3% PEG provides the best balance between separation resolution and signal strength while still allowing relatively fast separations (< 10 min).

Figure 5.7 shows the dependence of protein migration time on CE voltage potential. Increasing the potential does not drastically change the resolution of the protein peaks. It merely shifts them to an earlier migration time as the voltage is increased, as expected. Using 14 kV – 19 kV of separation potential, all separations take place within 3-5 minutes. As with most types of electrophoresis, increasing the voltage and subsequent current can cause other problems including Joule heating, boiling, and electrical arcing. These problems can be avoided by simply using lower CE potentials at the cost of slightly longer retention times (e.g., 2 more minutes).

There is a significant change in peak heights as the concentration of the run buffer is increased from 50 to 100 mM as shown in Figure 5.8. Labeled-proteins were dialyzed into 25 mM buffer to remove excess dye from the reaction and to maintain a high-field strength during an electrokinetic injection. Increasing the concentration of Tris-CHES buffer also increases the size of the CE peak for FITC-labeled BSA. This is most likely due to field-amplified sample stacking (FASS). By increasing the difference in run buffer concentrations between the sample vials
Figure 5.7 Plots of migration time vs. logarithm of the molecular weight for ladder proteins at various CE voltages. Capillary: 75 µm i.d., 30 cm (15 cm effective); 100 mM Tris-CHES, pH 9.0, Ultratrol LN-coated wall, 2 s injection. 488 nm laser source. Voltage: A, 14 kV; B, 15 kV; C, 16 kV; D, 17 kV; E, 18 kV; and F, 19 kV. Filled and unfilled markers are alternated in the plot for clarity. $R^2 > 0.95$ for all trendlines.
Figure 5.8 Electropherograms of FITC-labeled bovine serum albumin using a 488 nm laser based wave-mixing setup with different concentration levels of run buffer. Capillary: 75 µm i.d., 30 cm (15 cm effective); Tris-CHES buffer, pH 9.0, Ultratrol LN-coated wall, -15 kV, 12 µA, 2 s injection. Run buffer concentrations: 50 mM; 75 mM; 85 mM; and 100 mM.
and the capillary, the sample region becomes a high electrical resistance zone. Ions drift faster in the sample region before “stacking” on the low drift velocity region in the capillary. This effect is maximized around 100 mM in the run buffer. Larger peak areas and peak heights offer better detection sensitivity. In order to optimize the signal, samples are typically run with a 1:4 ratio of sample buffer concentration to run buffer concentration. Changing the capillary length does not drastically affect resolution or sensitivity for CE separations of ladder proteins. Figure 5.9A shows reproducible electropherograms collected using a 30 cm capillary with a 15 cm effective separation length. The electropherograms for the protein ladder presented in Figure 5.5A utilized a 50 cm capillary with a 30 cm effective length. The shorter analysis time and ample resolution make shorter capillary lengths more attractive.

The use of a suitable capillary coating is important in size-based CE separation of proteins. One of the challenges in protein separations involves the change in EOF due to the change in capillary wall conditions. Many studies have opted to employ the coating method proposed by Hjerten\textsuperscript{13} where the wall was reacted with 3-(trimethoxysilyl)propyl methacrylate, leaving free acrylic groups exposed on the wall surface. These groups were reacted with linear polyacrylamide to form the coating. Linear polyacrylamide cannot completely cover the entire wall and these exposed surfaces absorb proteins and create EOF. Polyacrylamide coatings bonded to the surface of the capillary wall through Si-O-Si linkages suffer from a lack of long-term stability because the siloxane linkages are prone to hydrolysis under basic conditions. The use of cross-linked linear polyacrylamide yielded poor reproducibility as described above. Ultratrol LN, a commercially
Figure 5.9 (A) Reproducible electropherograms of FITC-labeled protein ladder using a 488 nm laser-based wave-mixing setup with 3% PEG. Capillary: 75 µm i.d., 30 cm (15 cm effective); 100 mM Tris-CHES, pH 9.0, Ultratrol LN-coated wall, -17 kV, 25 µA, 2 s injection. Peaks: 1, lysozyme; 2, β-lactoglobulin; 3, REase Bsp98I; 4, lactate dehydrogenase; 5, ovalbumin; 6, bovine serum albumin; and 7, β-galactosidase. (B) Linear correlation between the logarithm of the molecular weights vs. retention time.
available dynamic coating, offers relatively fast rinsing cycles and more efficient separations.

Ulтратrol LN is a proprietary formulation shipped as a ready to use mixture and has a working pH range from pH 3 to 9.\textsuperscript{35} Ulтратrol LN coating is stable for only a single 10 min run and recoating is necessary for another run. Figure 5.10 shows three sequential electrophoresis runs for FITC-labeled BSA and β-lactoglobulin. It is clear that the coating must be reapplied after each run by rinsing with 0.1 M sodium hydroxide for 2 min and rinsing with Ulтратrol LN solution. Reproducible electropherograms were easily obtained using this modified protocol. All runs used capillaries freshly coated with Ulтратrol LN.

5.4.4 Wave-Mixing Signal and Detection Limits

DFWM offers excellent sensitivity levels even when using thin analytes present in capillaries, microarrays and lab-on-a-chip devices. This section shows characterization of the wave-mixing signal and the detection sensitivity for a FITC-labeled BSA protein, protein standards, neurodegenerative disease biomarkers and antibodies.

Figure 5.11a shows three consecutive electropherograms of low to high concentration FITC-labeled bovine serum albumin. The peak height increases as the concentration of the sample increases. The linear relationship between the log of the peak height vs. the log of the concentration shows that the signal produced exhibits a quadratic dependence on sample concentration. The peaks in this
Figure 5.10 Electropherograms of consecutive CE separation runs where the coating and sieving matrix are not refreshed after each run. Sample is a protein mixture of FITC-labeled bovine serum albumin and β-lactoglobulin (1 mg/mL). Capillary: 75 µm i.d., 50 cm (30 cm effective); 100 mM Tris-CHES, pH 8.9, 3% PEG, Ultratrol LN-coated wall, -15 kV, 12 µA, 5 s injection. Detection by 473 nm laser-based wave-mixing setup.
Figure 5.11  (A) Electropherograms of FITC-labeled bovine serum albumin at different concentration levels using a 473 nm laser-based wave-mixing setup. Concentrations: A, 1 mg/mL; B, 1.3 mg/mL; and C, 1.6 mg/mL Capillary: 75 µm i.d., 50 cm (30 cm effective); 100 mM Tris-CHES, pH 9.0, 3% PEG, Ultratrol LN-coated wall, -15 kV, 12 µA, 2 s injection. (B) Quadratic dependence of peak height on protein concentration.
electropherogram are broad because the proteins are labeled prior to denaturing with SDS.

A separation matrix is not required when the analyte is relatively pure. Figure 5.12 and Figure 5.13 show detection of FITC-labeled BSA and the breast cancer CA15-3 antibody. A concentration detection limit of 0.30 nM was determined for FITC-labeled BSA, which corresponds to a mass detection limit of 24 zeptomole for BSA based on the wave-mixing probe volume of 79 pL. Similarly, a concentration detection limit of 0.6 nM was determined for CA15-3 monoclonal antibody, and it corresponds to a mass detection limit of 47 zeptomole. These results demonstrate ultrasensitive detection limits of laser wave mixing for biomolecules and biomarkers. The 488 nm laser yields better sensitivity levels as compared to the 473 nm laser at the same laser power levels, partly due to the superior laser quality of the optically-pumped semiconductor laser compared to the lower quality diode laser.

5.5 CONCLUSIONS

Wave mixing is presented here as a sensitive and reliable absorption-based detection method for the analysis of labeled-proteins using capillary electrophoresis separation. Although both chromophores and fluorophores can be used to label proteins in wave mixing; readily available and cost effective fluorophores are used in these studies. Conventional capillary zone electrophoresis is not suitable for protein separations, so SDS-CGE is used to carry out size-based separations. The wave-mixing signals from CE runs are strong enough to yield zeptomole mass detection limits. The signal is a coherent laser-like beam and it can be efficiently
Figure 5.12 Electropherograms of FITC-labeled bovine serum albumin using a 488 nm laser-based wave-mixing setup. Capillary: 75 µm i.d., 30 cm (15 cm effective); 100 mM Tris-CHES buffer, 0.1% SDS, pH 9.0, Ultratrol LN-coated wall, -15 kV, 12 µA, 2 s injection. Analyte concentration levels: 3.0 µM, 30 nM, and 0.30 nM.
Figure 5.13 Electropherograms of FITC-labeled CA15-3 monoclonal antibody using a 488 nm laser-based wave-mixing setup. Capillary: 75 µm i.d., 30 cm (15 cm effective); 100 mM Tris-CHES buffer, 0.1% SDS, pH 9.0, Ultratrol LN-coated wall, -15 kV, 12 µA, 2 s injection. Analyte concentration levels: 6.6 µM, 66 nM and 0.6 nM.
collected against a dark background with virtually 100% collection efficiency. The use of small diode-based lasers in these detection systems gives them potential to be field deployable. The methods demonstrated here are also adaptable to UV laser wave-mixing setups. Wave mixing is a sensitive absorption-based detection method, and hence, it allows sensitive detection of label-free native molecules and other macromolecules resistant to labeling. The methods demonstrated here can also be used to determine the sizes and concentration levels of proteins in biological samples.
5.6 REFERENCES


CHAPTER 6
DETECTION OF HIV-ASSOCIATED PROTEINS
AND ANTIBODIES BY DEGERNATE FOUR-
WAVE MIXING

6.1 ABSTRACT

Laser wave mixing is presented as a sensitive detection method for the HIV-1 p24 capsid protein that can overcome current sensitivity limitations of enzyme-linked immunoassay-based methods. Wave mixing, coupled to capillary electrophoresis, provides fast size-based separation and sensitive detection of p24. The wave-mixing signal is a coherent laser-like beam that can be directed against a dark background, and hence, optical collection is efficient and the S/N is high. Low zeptomole-level mass detection limits are determined for FITC- and QSY-labeled p24 in these studies. Size-based CE separations offer enhanced chemical specificity levels for the products of p24 antibody-antigen reaction. Ultrasensitive detection sensitivity and high chemical specificity levels demonstrated here promise wave mixing as a compact field-deployable inexpensive detector for viral load screening in resource-limited settings.

6.2 INTRODUCTION

Although important progress has been made in the past three decades for diagnosing and treating HIV infections, the number of people living with HIV continues
to increase and AIDS-related illnesses remain a leading cause of premature mortality on a global scale.\textsuperscript{1} Sub-Saharan Africa is the most heavily affected region and accounted for 71\% of all new infections in 2008. In that year, it is estimated that there were about 34 million people living with HIV globally. Since there is currently no cure or primary prevention measures for HIV, such as an effective vaccine, health officials have opted for a “test-and-treat” strategy in which high-risk populations are tested and those found to be infected are immediately placed on antiretroviral therapy to reduce transmission rates.\textsuperscript{2} Currently, the detection of reactive antibodies to HIV is one of the most commonly used diagnostic methods for HIV infection. Screening for viral antibodies is accomplished through the use of enzyme-linked immunosorbent assays (ELISAs) and then confirmed by supplemental antibody tests such as Western blot or immunofluorescence.\textsuperscript{3,4} These tests are suboptimal for detecting recent infections as they target antibodies that can take three to six months to appear in a recently infected individual.\textsuperscript{5} At the initial stages of infection, HIV-1 antibody tests are nonreactive and the diagnosis of primary HIV-1 infection is made by either the p24 core antigen test or by PCR-based nucleic acid testing (viral load).

Accurate diagnostics for primary HIV-1 infections are critical for interrupting HIV transmission as persons in the early stages of infection can become sources of new infections.\textsuperscript{6,7} Primary HIV infection is characterized by high levels of viremia, often exceeding a million counts per microliter.\textsuperscript{8} In undiagnosed primary infections, patients might not adopt preventative sexual behaviors to reduce the likelihood of transmission, whereas, many who are diagnosed will actively take steps to reduce the risk.\textsuperscript{9} In addition, patients benefit from an early diagnosis (during primary infection) so they can be monitored for immune function deterioration and initiate appropriate antiretroviral therapy.
Nucleic acid testing is the gold standard for accurately diagnosing primary infections but it is too costly to be routinely implemented in resource poor settings where it is most needed. New tools and methods are needed for sensitive and cost-efficient diagnosis of primary HIV infections.

HIV rapid tests are formatted as a lateral flow assay, similar in design to a home pregnancy kit, and are routinely used in resource poor settings. Various types of these tests have been used in combination with risk-factor assessment data to generate algorithms that could predict acute infections without the need for viral load testing. Another target for the detection of acute HIV infection is the HIV-1 capsid protein, p24 antigen.

Viral proteins are present at the start of infection and each virus will contain approximately 2,000 copies of p24 capsid protein making it readily detectable even at the earliest stages of infection. Nucleic acid amplification methods used in blood screenings are capable of analytical sensitivities reaching 30 viruses/mL. For the detection of 30 viruses/mL the calculated mass detection limit for p24 antigen is approximately 100 zeptomole, which corresponds to approximately 60,000 molecules. Current ELISA techniques for the quantification of p24 have been optimized (>2 pg/mL) using heat to denature the samples and amplify signal but are only prognostically useful at the threshold cutoff for viral quantification where antiretroviral therapy is initiated. In order to monitor patient response to antiretroviral therapy by an approximate viral load measurement, the sensitivity of a new analytical method for the detection of p24 must offer zeptomole-level mass detection limits.

New approaches have been designed to improve p24 detection sensitivity as compared to those of ELISA-based techniques. Nanotechnology-based techniques, such
as the biobarcode amplification assay (BCA)\textsuperscript{16}, have been developed using p24 antibody reactions with biobarcode DNAs that are used for signal amplification, and they exhibit a slight sensitivity improvement over enhanced ELISA methods (0.1 pg/mL). Gold-nanoparticles have also been used to create capacitive immunosensors coupled to p24 monoclonal antibodies for the immobilization and ultrasensitive detection of p24 on surfaces.\textsuperscript{17} Other research has aimed efforts at developing nano-arrays containing antibodies for p24 using dip-pen nanolithography to selectively capture the antigen in order to enhance sensitivity.\textsuperscript{18} Although these new methods exhibit improved detection limits over ELISA-based methods, they require specialized methods and expensive materials, making them too difficult to implement for widespread screening and diagnostic efforts.

Table 6.1 shows a comparison of several commercially available and newly developed methods for the quantification of viral loads for the prognostic monitoring of HIV-positive patients. Nucleic acid-based techniques typically exhibit the highest sensitivity but are costly and inefficient because of the time it takes to prepare and analyze a sample. Even the lower cost NASBA test from bioMérieux requires a larger up-front cost of approximately $140,000 for the analyzer and equipment to prepare samples.\textsuperscript{19} The commercially available p24 ELISA from Perkin Elmer is much cheaper compared to the nucleic acid methods but it requires time-consuming steps and it is not sensitive enough for reliable viral load measurements. New tests are needed to overcome the limitations of commercially available tests in many areas including cost, speed and sensitivity.

Laser wave mixing has the potential to offer an ideal solution that can overcome limitations of current methods for p24 assays. The wave-mixing signal is orders of magnitude stronger than those of conventional optical absorption
Table 6.1 Comparison of selected methods for measuring HIV viral loads (adapted from ref. 19)

<table>
<thead>
<tr>
<th>Company/Lab</th>
<th>Abbott</th>
<th>Rosche</th>
<th>Bayer</th>
<th>bioMérieux</th>
<th>Perkin Elmer</th>
<th>Tang et. al.</th>
<th>Lee et. al.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of Assay</td>
<td>RT-PCR</td>
<td>RT-PCR</td>
<td>bDNA</td>
<td>NASBA</td>
<td>Enzyme Immunoassay p24 Antigen</td>
<td>Nanoparticle Immunoassay p24 Antigen</td>
<td>Dip-Pen Nanolithography Array Assay</td>
</tr>
<tr>
<td><strong>Dynamic Range</strong></td>
<td>HIV virions unless otherwise stated</td>
<td>50 - 1,000,000 (copies/mL)</td>
<td>50 - 750,000 (copies/mL)</td>
<td>75 - 500,000 (copies/mL)</td>
<td>50 - 3,000,000 (copies/mL)</td>
<td>400 (copies/mL)</td>
<td>&gt;0.1 pg/mL p24 antigen; &gt;0.025 pg/mL p24 antigen</td>
</tr>
<tr>
<td><strong>Specimen Volume</strong></td>
<td>200 µL</td>
<td>100 - 500 µL</td>
<td>1,000 - 2,000 µL</td>
<td>10 - 2,000 µL</td>
<td>100 µL</td>
<td>100 µL</td>
<td>1 µL</td>
</tr>
<tr>
<td><strong>Time for Result</strong></td>
<td>5 hours</td>
<td>6-7 hours</td>
<td>22 hours</td>
<td>2 hours</td>
<td>2.30 hours</td>
<td>&gt;2 hours</td>
<td>&lt;6 hours</td>
</tr>
<tr>
<td><strong>Cost per Test</strong></td>
<td>$40</td>
<td>$119</td>
<td>$125</td>
<td>$60</td>
<td>$10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Reference</strong></td>
<td>19</td>
<td>16</td>
<td>18</td>
<td>16</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
</tbody>
</table>
propagation direction (high S/N) and it has a quadratic dependence on analyte concentration (easier detection of small chemical changes) and a cubic dependence on laser input power (effective use of low laser power levels). The wave-mixing signal intensity can be described as:

\[ I_s \approx I_1^2 I_2 \left( \frac{\lambda^2}{\sin^4 \left( \frac{\theta}{2} \right)} \right) Q \left( \frac{\alpha^2}{\kappa^2} \right) \]  

where \( I_1 \) and \( I_2 \) represent the intensity of the probe and pump laser beams used to generate the signal beam (\( I_1 \) is lower than \( I_2 \)), \( Q \) is the solvent parameter and is equal to the square derivative of the refractive index change with respect to temperature change \( [Q = (dn/dT)^2] \), \( \lambda \) is the laser wavelength, \( \alpha \) is the absorption coefficient of the analyte, and \( \kappa \) is thermal conductivity. This equation illustrates the quadratic dependence of the signal on analyte concentration. Small changes in protein content in biological systems can produce large changes in the wave-mixing signal. In addition to excellent sensitivity, wave mixing offers other inherent advantages over conventional absorption techniques including small sample requirements, fast analysis, and a broader range of available derivitization agents such as fluorophores and non-fluorescing chromophores. Coupled to a suitable separation method, such as capillary gel electrophoresis, wave mixing offers sensitive and efficient detection of p24, allowing monitoring of antiviral drug therapy using protein analysis rather than nucleic acid amplification.

The following sections demonstrate sized-based separation and sensitive detection of p24 capsid protein. This chapter also describes wave-mixing detection of ELISA p24 antigen-antibody products and the analysis of monoclonal and
polyclonal antibodies bound to p24. The experimental arrangement used to separate and detect these proteins and antibodies, as shown in Figure 3.1, is similar to the ones described in previous chapters. Analytes are labeled with either chromophore or fluorophore dyes and detected by a blue laser in the wave-mixing setup. Wave mixing only requires the analyte to absorb incident radiation, and hence, fluorescence is not necessary. The methods developed in this chapter report a concentration detection limit of 0.24 nM and a mass detection limit at 31.3 zeptomole for p24. Assuming 2,000 molecules of p24 capsid per virus particle, fewer than 10 copies of HIV can be detected within the detection probe volume of our wave-mixing setup.

6.3 EXPERIMENTAL

6.3.1 WAVE-MIXING DETECTOR AND CAPILLARY ELECTROPHORESIS FOR HIV CAPSID AND ANTIBODY DETECTION

The HIV capsid protein p24, like other proteins, absorbs radiation in the ultraviolet wavelength range; however, conventional UV absorption methods offer poor detection sensitivity. Laser wave mixing offers much better detection sensitivity levels; however, low extinction coefficients of proteins in the UV range necessitates the use of amine reactive labels to enhance detection sensitivity and to allow the use of relatively inexpensive, compact and convenient visible lasers in a wave-mixing setup. In this study, both a UV laser and a blue laser were used in the wave-mixing setups. A compact passively Q-switched pulsed 266 nm 20 mW laser (CNI, Changchun, China) was used to probe label-free analytes. A continuous-wave
(CW) optically pumped semiconductor 488 nm 50 mW laser with adjustable power (Coherent, Santa Clara, CA), a 473 nm 50 mW diode laser (CNI, Changchun, China) or a 447 nm 20 mW diode laser (CNI, Changchun, China) was used to probe labeled analytes.

As shown previously in Figure 3.1, the laser beam is first split by a 30:70 R:T beam splitter to create two input beams. The transmitted beam is about twice as intense as the reflected beam and serves as both the pump and the probe beam. The reflected beam serves only as the pump beam. An optical chopper (Stanford Research Systems, Sunnyvale, CA, SR540) modulates the pump beam at 200 Hz. The chopper is interfaced to a lock-in amplifier (Stanford Research Systems, Sunnyvale, CA, SR810 DSP), which is connected to a simple photodetector (Thorlabs, Newton, NJ, PDA25K). The two input beams are arranged to travel equal distances and then focused and mixed at the capillary cell. The wave-mixing signal propagates in the forward direction and since it is a coherent laser-like beam, it is conveniently collected by the photodetector with virtually 100% collection efficiency. CE separation is driven by a 30 kV adjustable power supply (Glassman High Voltage, Inc., High Bridge, NJ, MJ30P400). The wave-mixing signal is processed by the lock-in amplifier and digitized by a data acquisition board (Measurement Computing, Norton, MA, 1608FS) controlled by a custom-built DAQ software (AIDA). The wave-mixing detector cell is a simple 75 μm i.d., 360 μm o.d. fused-silica capillary (Molex, Lisle, IL) connected to a syringe pump or a peristaltic pump (Rainin Instruments, Oakland, CA). A methyl red (in methanol) solution is used to pre-align and optimize the wave-mixing optical setup.
All absorption spectra were collected by a UV-visible spectrometer (Agilent, Santa Clara, CA, 8453) and a 1-cm Quartz cuvette. Absorption spectra were blanked using the appropriate buffer for each analyte.

### 6.3.2 Chemicals and Reagents

All solutions used in the capillary electrophoresis system were prepared using distilled water from a compact water distillation system (Waterwise, Leesburg, FL, 4000). Borax, Tris base, sodium dodecyl sulfate (SDS) and PEG 10,000 were all purchased from Sigma-Aldrich. FITC, CHES, hydrochloric acid, unstained protein ladder, dialysis tubing (MWCO 12-14 kDa) and dye removal columns were purchased from Thermo Fisher Scientific. QSY® 35 acetic acid, succinimidyl ester (QSY) is purchased from Life Technologies. Recombinant p24 full-length protein was purchased from Abcam (Cambridge, MA, ab43037). The HIV-1 p24 antigen capture assay was purchased from Advanced Bioscience Laboratories (Rockville, MD, Part #5421). The monoclonal antibody for HIV-1 p24 was purchased from MP Biomedicals (Santa Ana, CA, 0856980) and the FITC-labeled polyclonal antibody was purchased from Thermo Fisher Scientific. HIV-1 gag p24, a recombinant protein containing a.a. 77 to 436 of the HIV-1 gag region including p24 antigen, was purchased from Virogen (Watertown, MA, 00111-V). The ELISA assay was prepared using the method contained in the product insert and the p24 standard solution contained in the kit. Sodium borate buffer (100 mM) was prepared by dissolving solid sodium tetraborate decahydrate (Borax) in water and adjusting the pH to 8.6 using 1 M hydrochloric acid. Tris-CHES buffers were prepared at various
concentrations by adding solid Tris base (Trizma base) and CHES and diluting with water. High purity electrophoresis-grade sodium dodecyl sulfate (SDS) was added to electrophoresis run buffers at 0.1%. The sieving matrix, polyethylene glycol (Fluka PEG 10000), was added to the run buffer at 3%. Fluorescein isothiocyanate (FITC) was used as the fluorophore label for proteins, and QSY is used as the chromophore protein label. Both FITC and QSY were freshly prepared in solutions of DMF at 10 mg/mL and 20 mg/mL, respectively. Ultratrol LN (Target Discovery, Palo Alto, CA) was used to coat the capillary wall in order to decrease the electroosmotic flow during CE runs and to allow proteins to move in reverse polarity experiments. All solutions used for capillary electrophoresis were filtered through a 0.45 µm membrane on a syringe filter.

6.3.3 Protein Labeling

HIV antigens were prepared for labeling by dissolving them in conjugation buffer (100 mM sodium borate, pH 8.6) at ~1 mg/mL in a microcentrifuge tube. The dye solution was added in molar excess (20 fold) and the mixture was allowed to react for 1 hour in the dark. Excess dye was removed by dialysis into run buffers.

The unstained protein ladder contains seven proteins: beta-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp98I (25.0 kDa), beta-lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa). These proteins are present in solution at different concentration levels (0.1 – 0.2 mg/mL) and used as molecular weight markers for SDS-PAGE gels. The protocol for labeling these proteins assumes a fixed higher
concentration of each component (2.0 mg/mL) to ensure that excess FITC is present in the reaction mixture. Aliquots of 150 µL were taken from the stock solution and dialyzed into conjugation buffer. The dialyzed protein mixture was reacted with a 15-fold molar excess of FITC for 1 hour in the dark. Excess dye was removed by dialysis into run buffer.

6.4 RESULTS AND DISCUSSION

6.4.1 DETECTION OF ELISA PRODUCTS BY LASER WAVE MIXING

The product of an enzyme-substrate reaction in a commercially available ELISA kit is typically a product that produces a distinct color change that can be read by a plate reader. For the Advanced Biosciences Laboratories HIV-1 p24 antigen capture assay, the conjugate solution contains human anti-p24 polyclonal antibodies coupled to horseradish peroxidase. The resulting product has a yellow color and a maximum absorbance at 450 nm. This product formation is measured by laser wave mixing using a 447 nm laser.

Laser wave mixing exhibits a quadratic dependence on concentration and a cubic dependence on laser power, making it easy to observe small changes in analyte concentration. Sensitivity limitations of current ELISA methods could be overcome by using wave mixing as a detector instead of a plate reader.

Figure 6.1 shows the formation of the yellow enzyme-substrate product in the p24 antigen-capture ELISA plate. The resulting products yield stronger yellow colors with
Figure 6.1 Results obtained from HIV-1 p24 antigen capture assay (ELISA, Advanced Bioscience Laboratories). UV-visible spectra recorded after the formation of the color-forming complex (left). Concentration of p24 captured (bottom to top): None (media control), 6.25 pg/mL, 12.5 pg/mL, 25.0 pg/mL, 50.0 pg/mL, and 100.0 pg/mL. Photo on the right shows solutions with their respective concentrations (bottom to top) listed above.
increasing concentrations of p24, and the 447 nm laser is chosen to probe this analyte in a wave-mixing setup. However, this does not overcome the sensitivity limitations of ELISA assays since solutions containing no antigen still absorb the excitation light. The absorbance of the solutions containing no p24 are close to those at the lower end of the dynamic range of the assay (3.1 pg/mL). Nevertheless, wave mixing can still detect the lower limit of detection of the ELISA assay. Figure 6.2 shows the wave-mixing signal from the resulting solution that contains 3.1 pg/mL of p24 antigen.

\textbf{6.4.2 ANTIGEN LABELING USING CHROMOPHORE AND FLUOROPHORE PROTEIN TAGS}

The recombinant HIV-1 capsid protein p24 was labeled by first dialyzing the stock solution into the appropriate conjugation buffer at basic pH. In order to demonstrate that wave mixing can detect analytes labeled with a chromophore or a fluorophore, both a chromophore (QSY) and a fluorophore (FITC) were tested as labels for the protein. Figure 6.3A shows the absorption spectra of proteins labeled with QSY and FITC. Native proteins only absorb in the ultraviolet range and QSY- and FITC-labeled proteins absorb in the blue wavelength range (473 nm and 488 nm excitation lasers). The samples were reacted with either FITC or QSY for 1 hour and then dialyzed into run buffer. Although the $\lambda_{\text{max}}$ varies in the blue region, wave-mixing detection is excellent even when the excitation wavelength is more than 30 nm from the $\lambda_{\text{max}}$.\textsuperscript{20,21} The sequence for the monomeric unit of p24 antigen shows ten available lysines for labeling (Figure 6.3B) with an amine reactive dye.\textsuperscript{22} To ensure complete labeling, a dye:protein ratio of 20:1 was used for the reaction.
Figure 6.2 Detection of p24 antigen capture assay product at 3.1 pg/mL flowed through a 75 µm i.d. capillary by a peristaltic pump. Wave mixing performed using a 447 nm 20 mW laser. Pump and probe beams are allowed to excite the sample at time $T_1$. At $T_2$, the probe beam is blocked and the difference between the baselines at $T_1$ and $T_2$ represents residual optical noise from the pump beam. Two cycles are shown.
Figure 6.3 (A) UV-visible spectra of QSY and FITC in buffer. Concentrations: 48 µM and 25 µM. (B) Sequence of the full-length p24 capsid protein. Lysines are highlighted in the sequence.
6.4.3 SIZE-BASED SEPARATION AND LIMIT OF DETECTION FOR HIV-1 p24 CAPSID USING FITC

Sized-based separations for p24 were performed using sodium dodecyl sulfate capillary gel electrophoresis (SDS-CGE) with a poly (ethylene glycol) (PEG) sieving matrix. Capillary electrophoresis migration times for proteins depend on their charge-to-size ratios. SDS binds to proteins creating uniform charge states that allow them to travel based on their size. The negatively charged SDS-protein complexes migrate towards the positive electrode in a reverse-polarity capillary electrophoresis mode where analytes travel in the opposite direction of the electroosmotic flow. Ultratrol LN, a commercially available dynamic capillary coating, is used to suppress the electroosmotic flow and to allow proteins to travel unhindered towards the positive electrode. In addition, it prevents protein adsorption on the capillary wall. The capillary is prepared for separation by flowing solutions of sodium hydroxide, water and Ultratrol coating before adding the buffer containing 3% PEG. This preparation process takes less than 10 minutes and it is much more efficient compared to the Hjertén method for coating capillaries in order to suppress electroosmotic flow.\textsuperscript{23}

Figure 6.4A shows an electropherogram that allows approximate molecular weight determination for FITC-labeled p24 antigen. The electropherogram shows a major peak at 34.2 kDa that is almost 10 kDa higher than the actual molecular weight of the protein. Multiple runs of FITC-labeled p24 yielded an average approximate molecular weight of 34.0 kDa with a relative standard deviation of 2.6%. Although improvements can be made to improve accuracy, this method provides qualitative information about the approximate and relative sizes of protein analytes in a given separation. Sizing is accomplished through
Figure 6.4 (A) Size-based electropherogram of FITC-labeled p24 protein using capillary electrophoresis with wave-mixing detection using a 473 nm 50 mW laser and 3% PEG. Capillary: 75 µm i.d., 50 cm (30 cm effective); 100 mM Tris-CHES, pH 9.0, Ultratrol LN-coated wall, -15 kV, 12 µA, 5 s injection. (B) Linear correlation between the logarithm of the molecular weights vs. retention time. Proteins from migration times from low to high: lysozyme, β-lactoglobulin, REase Bsp98I, lactate dehydrogenase, ovalbumin, bovine serum albumin, and β-galactosidase.
calibration of the capillary using a FITC-labeled protein ladder containing 7 proteins ranging in molecular weights from 14.4 kDa to 116 kDa. The methods for labeling this molecular weight ladder are described in Chapter 5. The resulting calibration curve, shown in Figure 6.4B, gave a linear fit where the calculated line predicts values slightly larger than the actual proteins that are below 25 kDa and predicts slightly lower values for proteins over 35 kDa. Hence, the calculated molecular weight for FITC-labeled p24 was higher than the actual value. Although multiple runs of the protein ladder can be averaged to create a calibration for molecular weight determination, using migration times for the proteins ladder separation closest to the analyte separation minimizes systematic error caused by the shifting migration times as the capillary is used for more runs. The accuracy of size determination improves as the $R^2$ value increases in the protein ladder calibration.

Figure 6.5 shows wave-mixing detection sensitivity for FITC-labeled p24. No sieving matrix is added to the run buffer to decrease analysis times and noise levels due to prolonged Joule heating. Three consecutive runs are shown for p24 injected at a concentration of 4.2 nM, and each run shows the p24 peak centered around 70 seconds with increasing noise for each consecutive run. This noise can be minimized by replacing the capillary or by rinsing with 0.1 M sodium hydroxide for 1 hour. Using a wave-mixing probe volume of 79 pL, a mass detection limit of 313 zeptomole was determined for FITC-labeled p24. These results demonstrate that p24 can be efficiently labeled and detected using a relatively inexpensive fluorophore label and a compact wave-mixing detector.
Figure 6.5 Electropherograms of 4.2 nM FITC-labeled p24 antigen detected by laser wave mixing using a 488 nm 50 mW laser. Capillary: 75 µm i.d., 30 cm (15 cm effective); 100 mM Tris-CHES buffer, 0.1% SDS, pH 9.0, Ultratrol LN-coated wall, -15 kV, 12 µA, 2 s injection.
6.4.4 SIZE-BASED SEPARATION AND LIMIT OF DETECTION FOR HIV-1 p24 CAPSID USING QSY 35

Similar to FITC-labeled p24 detection, size-based separations of QSY-labeled p24 were performed using PEG as a sieving matrix for capillary electrophoresis. Figure 6.6A shows an electropherogram for the size-based separation of QSY-labeled p24 with a peak centered at 29.7 kDa, about 6 kDa away from the expected value of 24 kDa, but still closer to the actual value as compared to that obtained for the FITC-labeled analyte. Multiple runs of QSY-labeled p24 yielded an average molecular weight of 28.7 kDa with a relative standard deviation of 4.5%. These variations are attributed to the protein ladder calibration (Figure 6.6B) and the slight variability in migration times for any given separation of this type. Small changes in migration time produce large changes in molecular weight, and therefore, these types of separations should only be used to make approximate determinations of molecular weight or to determine relative sizes of protein analytes in a given separation run.

Figure 6.7 shows wave-mixing detection sensitivity for QSY-labeled p24. Two consecutive runs are shown with minimal noise increases when compared to FITC-labeled p24 electropherograms. FITC contains functional groups (alcohol) that may interact with charged ions in the buffer or capillary wall, causing it to increase random noise after consecutive runs. QSY-labeling produced a single isolated sharp peak and also maintained clean capillary conditions even after high concentration CE runs. The concentration used for the detection limit electropherograms is 0.42 nM, which corresponds to a mass detection limit of 31.3 zeptomole. This limit of detection is around the concentration needed to match nucleic acid amplification sensitivities for monitoring antiviral treatment.
Figure 6.6 (A) Size-based electropherogram of QSY-labeled p24 protein detected by laser wave mixing using a 473 nm 50 mW laser and 3% PEG. Capillary: 75 μm i.d., 50 cm (30 cm effective); 100 mM Tris-CHES, pH 9.0, Ultratrol LN-coated wall, -15 kV, 12 μA, 5 s injection. (B) Linear correlation between the logarithm of the molecular weights vs. retention time. Proteins with low-to-high migration times: lysozyme, β-lactoglobulin, REase Bsp98I, lactate dehydrogenase, ovalbumin, bovine serum albumin, β-galactosidase.
Figure 6.7 Electropherograms of 0.42 nM QSY-labeled p24 antigen detected by laser wave mixing using a 488 nm 50 mW laser. Capillary: 75 µm i.d., 30 cm (15 cm effective); 100 mM Tris-CHES buffer, 0.1% SDS, pH 9.0, Ultratrol LN-coated wall, -15 kV, 12 µA, 2 s injection.
for HIV-1. These results confirm effective use of a chromophore (not fluorophore) label in sensitive wave-mixing detection of p24.

### 6.4.5 Nonlinear Dependence of Wave-Mixing Signal in p24 Separations

Unlike conventional absorption-based detection methods, laser wave mixing exhibits a quadratic dependence on analyte concentration and a cubic dependence on laser power. Figure 6.8A shows electropherograms of FITC-labeled p24 with increasing concentration levels. When the logarithms of the relative peak heights are plotted to the logarithm of the analyte concentration, the resulting slope of the trendline is 1.5 with a linear fit ($R^2 = 0.999$). In an ideal case, the slope of this plot would be 2.0 showing a quadratic dependence on the peak height with analyte concentration; however, background noise levels usually keep the slope under 2.0. The capillary is susceptible to vibrations in the room and thermal stress resulting from hours of multiple CE runs, rinse cycles and re-coating procedures. Chapter 5 describes other examples yielding logarithm plots with slopes closer to 2.0.

### 6.4.6 Reaction of p24 with Monoclonal and Polyclonal Antibodies Monitored by Laser Wave Mixing

Monoclonal and polyclonal antibodies were reacted with FITC-labeled p24 antigen to observe the shift in approximate molecular weight by size-based capillary electrophoresis separations. Previous studies have shown that antibody-antigen binding complexes can be observed even in the presence of SDS up to 70 mM. Mammalian antibodies have an average molecular size of 150 kDa, so a large shift is expected for the
Figure 6.8 (A) Electropherograms of FITC-labeled p24 at different concentration levels detected by laser wave mixing using a 473 nm 50 mW laser. Peak retention times are shifted for clarity. Concentration (bottom to top): 42 µM, 33 µM, 25 µM, and 16 µM; Capillary: 75 µm i.d., 50 cm (30 cm effective); 100 mM Tris-CHES, pH 9.0, 3% PEG, Ultratrol LN-coated wall, -15 kV, 12 µA, 5 s injection. (B) Nonlinear dependence of peak height on p24 concentration.
p24 protein with a size of only 24 kDa. Antibody conjugation provides a greater level of specificity to capillary electrophoresis separation and wave-mixing detection.

Figure 6.9 shows results from fresh samples of FITC-labeled p24 before and after reaction with a monoclonal antibody. The reaction was carried out with a 1:1 molar ratio of antibody to antigen and then allowed to react for 1 hour at room temperature. The resulting electropherogram shows a large peak centered at an approximate molecular weight of 150 kDa. The absence of peaks present in the pre-reaction electropherogram may be an indication that those peaks are fragments of the p24 antigen containing the immune-reactive sequence. These results show that antibodies can be used to increase the specificity of analyte detection by wave mixing coupled to capillary electrophoresis.

Figure 6.10A shows the electropherogram of a FITC-labeled p24 polyclonal antibody solution with a major components centered around 150 kDa, a typical size of a mammalian antibody. The antigen used is a larger protein (HIV-1 gag, a.a. 77 - 436) containing the immunoreactive p24 sequence. This reaction was performed using a 2:1 ratio for antibody to antigen at room temperature for 60 min. Polyclonal and monoclonal antibodies bind to the same antigen, but are dissimilar in their binding because polyclonal antibodies have the ability to bind multiple epitopes on an antigen target whereas a monoclonal antibody only binds to a single epitope.25 Hence, polyclonal antibodies tend to form an extended network of antibody-antigen interactions (Figure 6.10D) and can be used for immunoprecipitation reactions. As expected, the reaction of p24 antigen with the polyclonal antibody solution produced an electropherogram, shown in Figure 6.10B, containing no peaks as the large complex does not allow the sample to inject electrokinetically.
Figure 6.9 Electropherograms showing separation of FITC-labeled p24 before (bottom) and after (top) reaction with monoclonal antibody detected by laser wave mixing using a 473 nm 50 mW laser. Reaction conditions: 42 pmol each of p24 and monoclonal antibody in 16 µL of buffer (25 mM Tris-CHES, pH 9.0, 0.1% SDS); Capillary: 75 µm i.d., 50 cm (30 cm effective); 100 mM Tris-CHES, pH 9.0, 3% PEG, Ultratrol LN-coated wall, -15 kV, 12 µA, 5 s injection.
Figure 6.10 (A) Electropherogram showing separation of FITC-labeled p24 polyclonal antibody. (B) Electropherogram of p24 antigen after reacting with the polyclonal antibody and detected by laser wave mixing using a 473 nm 50 mW laser. Reaction conditions: 53 nmol polyclonal antibody and 27 nmol HIV-1 gag (p24 antigen) in 27 µL of buffer (25 mM Tris-CHES, pH 9.0, 0.1% SDS); Capillary: 75 µm i.d., 50 cm (30 cm effective); 100 mM Tris-CHES, pH 9.0, 3% PEG, Ultratrol LN-coated wall, -15 kV, 12 µA, 5 s injection. (C) Illustration of polyclonal antibodies in solution. (D) Illustration of polyclonal antibodies creating a network of antigen-antibody complexes.
6.4.7 **NATIVE P24 DETECTION BY UV LASER WAVE MIXING**

Figure 6.11 shows label-free native detection of p24 antigen. Optical absorption of native proteins relies on the absorption of aromatic amino acids in the protein sequence, and hence, the overall extinction coefficients of most proteins are relatively low as compared to those of chromophore- or fluorophore-labeled proteins. Hence, detection limits of labeled-proteins are much better using wave-mixing detection compared to UV laser wave-mixing detection of native proteins. The extinction coefficients of labeled-proteins are especially high in the ultraviolet wavelength range. Hence, UV laser wave-mixing setups could detect native label-free proteins (µM to nM) and labeled proteins (nM to pM).

6.5 **CONCLUSIONS**

Laser wave mixing coupled to capillary gel electrophoresis offers quick separation and sensitive detection of HIV-associated proteins and antibodies. The use of ELISA assays for the detection of p24 presents some challenges and limitations as a prognostic tool for monitoring HIV viral loads including poor sensitivity and high background absorption levels. Laser wave mixing allows zeptomole-level detection of p24, i.e., only a few thousand p24 molecules present in each HIV virus, and it is comparable or better than the current sensitivity limits of nucleic acid-based methods and yet wave-mixing detection is fast, simple and portable. The results here also show that the detection of p24 by wave mixing is not limited to using only fluorescent protein labels. Chromophore labels, such as QSY, afford similar or better sensitivity levels as compared to those from fluorophore
Figure 6.11 Electropherogram of native p24 (42 µM) detected by UV laser wave mixing using a 266 nm UV laser. Capillary: 75 µm i.d., 30 cm (15 cm effective); 100 mM Tris-CHES, pH 9.0, 3% PEG, Ultratrol LN-coated wall, -15 kV, 12 µA, 5 s injection.
labels. Laser wave mixing offers several advantages over current methods for monitoring viral loads including small sample size requirements, compact setups, low power requirements, and detection sensitivity without using time-consuming amplification steps, making it possible to use portable tools for viral load screening in resource limited settings.
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Chapter 6, in part, is currently being prepared for submission for publication of the material. Hetu, M. M.; Iwabuchi, M. F. The dissertation author was the primary investigator and author of this material.

6.7 REFERENCES


(2) Dieffenbach CW; Fauci AS. JAMA 2009, 301, 2380.

(3) Carlson, J. R. JAMA 1988, 260, 674.


(19) Sans Frontières, M.; UNICEF. In *Sources and prices of selected medicines and diagnostics for people living with HIV/AIDS*; OMS, 2005.


CHAPTER 7

CONCLUSIONS

Nonlinear laser wave mixing offers many advantages over conventional absorption-based detection methods including the generation of coherent laser-like signal beams, small probe volumes and sample size requirements, short optical absorption path lengths, high spatial resolution, and sensitivity levels that meet or exceed those of sensitive fluorescence-based methods. The coherent laser-like signal can be collected with nearly 100% collection efficiency against a dark background. The signal has a quadratic dependence on analyte concentration, and hence, it yields large changes for small changes in analyte concentration. The signal also exhibits a cubic dependence on laser power making it possible to use low-power (mW) lasers as an excitation source. These characteristics make laser wave mixing useful for the analysis of hazardous chemical agents and small biomolecules, proteins and antibodies.

By using compact portable low-power lasers effectively in a wave-mixing system, one could design field deployable detection systems for a wide range of real-world biomedical and security applications. UV laser wave mixing allows sensitive detection of nitroaromatic explosives both on surfaces and in capillaries and fast and convenient detection of neurotransmitters such as dopamine, serotonin and adenosine in their native form at low concentration levels.

Laser wave mixing coupled to capillary electrophoresis offers powerful new ways for the analysis of cellular proteins and antibodies with enhanced sensitivity and specificity.
levels. By using commercially available dynamic capillary coatings and a polyethylene glycol sieving matrix, one can reduce the capillary preparation time of 1-3 days (for the Hjertén method) to a mere 10 minutes. New methods are presented (Chapter 5) to determine approximate molecular weights of proteins, and one could eventually run cellular lysates for the discovery of new biomarkers. Chapter 6 presents new laser wave-mixing methods for the detection of p24 antigen, a capsid protein that can potentially be used to monitor antiviral therapy for HIV. This new method yields zeptomole-level sensitivity that is comparable or better than those of nucleic acid amplification screens for HIV viral loads without using complex procedures and bulky instruments.