A Dissertation

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Investigation of Energy Transfer, Quantification, and Localization of Peptides and Proteins by Fluorescence Spectroscopy and Mass Spectrometry

By

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for

The Doctor of Philosophy Degree in Chemistry

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The University of Toledo
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An Abstract of

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Peptides and proteins are integral biomolecules found in all organisms, and are responsible for movement, molecular transport, catalysis, and energy transfer. This dissertation is aimed on the analysis of energy transfer involving fluorescent proteins and development of methodologies for quantitative and imaging analyses of proteins. Such studies are important because energy transfer plays a significant role in numerous chemical, physical, and biological processes, while quantity and location of peptides and proteins are often closely associated with their functions.

In the initial study, energy transfer from fluorescent phycobiliproteins to noble metal nanoparticles was analyzed. Solutions of highly fluorescent phycobiliprotein B-phycoerythrin (B-PE) were mixed with colloidal Au and Ag nanoparticles and were characterized by steady-state and time-resolved fluorescence spectroscopy to determine the magnitude and mechanism of the energy transfer. It was found that the protein fluorescence was quenched after the addition of metal nanoparticles. Electron microscopy and absorption spectroscopy confirmed that B-PE was adsorbed onto the nanoparticles, creating a favorable geometry for quenching. Time-resolved fluorescence spectroscopy
showed that B-PE fluorescence lifetimes decreased from 2.2 ns to 0.5 and 0.6 ns upon adsorption onto Au and Ag nanoparticles, respectively, corresponding to energy transfer efficiencies of >70%. Our results, which include lifetimes, efficiencies, and energy transfer distances, show that energy was transferred via the surface energy transfer (SET) mechanism, rather than Förster resonance energy transfer (FRET). The results also imply that efficient energy transfer between proteins and metal nanoparticles may be possible regardless of whether or not resonance conditions between emission spectra of the proteins and absorption spectra of nanoparticles are achieved.

The second project dealt with quantitative analysis of proteins by a combination of fluorescence spectroscopy and electrospray ionization (ESI) mass spectrometry (MS). While MS is a very sensitive and versatile detection technique, quantification of compounds by MS is challenging due to relatively low reproducibility of ionization. We investigated the native fluorescence of phenylalanine, tryptophan, and tyrosine as a tool that can improve quantification of peptides and proteins by LC-ESI-MS. Natively fluorescent amino acids as well as peptides and proteins containing them were successfully separated by HPLC, and quantified with a spectrofluorimetric detector and a mass spectrometer. Two detectors connected in series enabled sequential measurements of fluorescence intensities and ion signals as well as structural characterization of separated polypeptides. Fluorescence detector provided better linearity and repeatability of quantification than mass spectrometer, and similar sensitivity for most of biomolecules analyzed. The fluorescence signal was linear over 3-4 orders of magnitude with limit of detection in the low picomole or high femtomole range. Hence, spectrofluorimetric detection of native fluorescence can be used as a reliable method that can facilitate
quantification of peptides and proteins by LC-ESI-MS.

In addition to analyzing native fluorescence of peptides and proteins, the effect of fluorescence dye labeling on ESI efficiency and charging of polypeptides was studied. Peptides and proteins were labeled with fluorescein isothiocyanate (FITC) and separated from the dye by size exclusion chromatography and HPLC. The ESI-MS and LC-ESI-MS analyses of mixtures of labeled and unlabeled polypeptides confirmed that labeling was successful, leading to slight increase in the charging of the most abundant ions in the mass spectra. Further experiments will confirm how derivatization of the peptides and proteins by FITC affects their ionization efficiencies.

In the last project, distribution and localization of biomolecules within the mouse tissue sections was studied by matrix-assisted laser desorption/ionization (MALDI)-imaging mass spectrometry (IMS). Peptides and proteins were imaged in brain and spinal cord tissue sections originating from non-transgenic mice and transgenic mice containing the gene for amyotrophic lateral sclerosis (ALS). Tissue sections from apparently healthy and diseased (ALS) mice were coated with sinapinic acid (SA) or α-cyano-4-hydroxycinnamic acid (CHCA) matrixes and imaged using a MALDI-MS instrument. These imaging experiments enabled determination of distribution and localization of peptides and proteins in the spinal cord and brain tissue sections.
I would like to dedicate my dissertation to my late father Shri. Om Prakash Saraswat and pray for his peaceful stay in heaven.
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List of Abbreviations

2D DIGE…… 2 Dimensional difference in gel electrophoresis
ALS………. Amyotrophic lateral sclerosis
APC………… Allophycocyanin
B-PE……… B-phycoerythrin
CHCA…….. α-Cyano-4-hydroxycinnamic acid
CID………… Collision induced dissociation
CNS………. Central nervous system
CRP………. C-reactive protein
CSF………. Cerebrospinal fluid
DESI.......... Desorption electrospray ionization
ESI-MS……. Electrospray ionization-mass spectrometry
FITC……….. Fluorescein isothiocyanate
FRET………. Fluorescence resonance energy transfer
GFP…………. Green fluorescent protein
ICAT………. Isotope coded affinity tag
IMS………. Imaging mass spectrometry
iTRAQ…….. Isobaric tag for relative and absolute quantification
LC-MS…….. Liquid chromatography-mass spectrometry
LID………… Laser induced dissociation
MALDI…….. Matrix-assisted laser desorption/ionization
MS…………. Mass spectrometry
MS/MS…….. Tandem mass spectrometry
MSI………… Mass spectrometry imaging
NHS………… N-Hydroxysuccinimide
PBS…………Phosphate buffered saline
PC…………Phycocyanin
PCB…………Phycocyanobilin
PEB…………Phyceroerythrobilin
PUB…………Phycourobilin
PXB…………Phycobiliviolin

Q-TOF…….Quadrupole-time-of-flight

R-PE…….R-phycoerythrin
RP-HPLC……Reverse phase-high performance liquid chromatography

SA…………..Sinapinic acid
SELDI……..Surface enhanced laser desorption/ionization
SET………..Surface energy transfer
SILAC……..Stable isotope labeling of amino acids in cell culture
SIMS………..Secondary ion mass spectrometry
SOD…………Superoxide dismutase

TOF-MS…….Time-of-flight mass spectrometry
TOF/TOF……Time-of-flight/time-of-flight

UV-VIS……..Ultraviolet-visible
Chapter 1

Introduction

1.1 Structure and functions of proteins

Peptides and proteins are an essential part of life considering their role in almost every biological process. Proteins are multipurpose and highly abundant macromolecules, which serve as catalysts, transporters, structural components of cells and tissues, etc. (1). Therefore, it is very important to study these biomolecules. Proteomics is the branch of biochemistry, which studies peptides and proteins.

Peptides and proteins consist of twenty common amino acids. These amino acids are bonded together by peptide bonds to make polypeptides or proteins. These molecules in linear form contain two ends: N-terminal and C-terminal (1).

1.2 Analysis of proteins by fluorescence spectroscopy

Fluorescence spectroscopy is a technique which measures fluorescence from a sample. In this technique, the molecules are excited from their ground state to the excited state by absorption of light. After excitation, these molecules can come back to the ground state in number of ways as explained by the Jablonski diagram (Figure 1-1) (2). Excited molecules can come back from the excited singlet state $S_1$ to the ground state $S_0$. 


by emission of light and this phenomenon is known as fluorescence. Fluorescence of molecules can be measured as a function of their concentration at characteristic excitation and emission wavelengths for qualitative and quantitative analyses. Excited molecule can also relax by other means such as: internal conversion, vibrational relaxation, non-radiative relaxation, intersystem crossing, phosphorescence, and Förster resonance energy transfer (FRET). In internal conversion, a molecule relaxes from higher excited electronic state to lower excited electronic state. In vibrational relaxation, a molecule relaxes from higher vibrational state to lower vibrational state of a particular electronic state. Molecules can relax from excited state to ground state without emission of light through a process known as non-radiative relaxation. Molecules can also go from excited singlet state ($S_1$) to excited triplet state ($T_1$) in a process known as intersystem crossing. Once in the excited triplet state, excited molecules can come back to the ground state by emission of light and this phenomenon is known as phosphorescence. Sometimes, energy emitted by one molecule (donor) can be absorbed by another molecule (acceptor), resulting in excitation of the second molecule (Figure 1-1). This phenomenon is called FRET and depends on the distance between the donor and acceptor (3). The acceptor molecule can come back to the ground state by any of the processes discussed previously. If energy transfer process originates from a fluorescent molecule (donor) to an acceptor molecule, the fluorescence of donor molecules undergoes quenching.
Figure 1-1. Jablonski diagram for transitions between electronic states. Excitation of molecule from the ground state to the excited state is shown by black arrow. Yellow squiggly arrow represents vibrational relaxation from higher vibrational levels to the lowest vibrational level within an electronic state and internal conversion from higher excited state to lower excited state. Non-radiative decay from the excited state to the ground state is represented by black squiggly arrow. Red straight arrow represents fluorescence. Phosphorescence is represented by blue straight arrow. Intersystem crossing and energy transfer from donor to acceptor is represented by black curved arrow (2).

Of the 20 common amino acids, only 3 amino acids are natively fluorescent. Natively fluorescent amino acids are: phenylalanine, tyrosine, and tryptophan. Tryptophan is highly fluorescent and has a quantum yield of 0.14. It excites at 280 nm.
with a maximum wavelength for emission at 348 nm. Tyrosine is not very far from tryptophan with a quantum yield of 0.12, and excites at 274 nm with a wavelength of maximum emission at 324 nm. Phenylalanine has a quantum yield of 0.02 and has excitation and emission wavelengths of 260 nm and 295 nm, respectively (3, 4). Peptides and proteins containing these amino acids also exhibit native fluorescence. Examples of several peptides and proteins that exhibit native fluorescence are MRFA, neurotensin, angiotensin, cytochrome C, myoglobin, and trypsinogen. Fluorescence of these amino acids can be used for quantification and identification of peptides and proteins (5-7).

But, quantum yields of natively fluorescent amino acids are not as good as those of fluorescent dyes. Peptides and proteins can be labeled with dyes which have much higher quantum yields than amino acids. For example, fluorescein isothiocyanate (FITC) has a quantum yield of 0.95 and has excitation and emission wavelengths of 490 nm and 548 nm, respectively (3). FITC is a fluorophore that selectively binds to primary amino groups, for example the protein’s N-terminus and lysine’s side chain amino groups, leading to the formation of highly fluorescent derivatives.

1.3 Energy transfer involving fluorescent proteins

Fluorescent protein’s main function in their respective host organisms is the absorption of light energy, and its efficient transfer to other proteins or emission to the environment (8). For example, green fluorescent protein (GFP) from jellyfish *Aequorea victoria* (9) absorbs the energy transferred from the blue luminescent protein aequorin and emits green light (10). Phycobilisomes, photosynthetic pigment-protein complexes, absorb sunlight and transfer energy via FRET to chlorophyll a (11-14).
Cyanobacteria and eukaryotic algae (red algae, glaucophytes, and criptomonads) contain fluorescent phycobiliproteins. They are composed of two or more subunits containing open-chain tetrapyrrole prosthetic groups. These proteins are divided in three groups: allophycocyanins, phycocyanins and phycoerythrins (15). Prosthetic groups found in phycobiliproteins include: phycoerythrobilin (PEB), phycocyanobilin (PCB), phycobiliviolin (PXB), and phycourobilin (PUB). This family of proteins is highly suitable for several applications, such as energy transfer, due to the exceptional characteristics of phycobiliproteins. These properties can be summarized as but are not limited to: very high absorption coefficients, high fluorescence quantum yields, persistent fluorescence over broad pH range, large Stokes shifts (~ 81 nm), low fluorescence background noise, strong and wide emission bands in the red region of the spectrum, and high solubility in water (11-14,16-19).

R-Phycoerythrin (R-PE) and B-Phycoerythrin (B-PE) are phycobiliproteins found in cyanobacteria and red algae as part of phycobilisomes. They efficiently absorb light in deep water and transfer energy through FRET to other phycobiliproteins phycocyanin (PC) and allophycocyanin (APC), which further transfer the energy to chlorophyll a (20). Holzwarth et al. showed that the pathway of energy transfer in phycobilisomes (PBS) is from PE to PC to APC to APC B to photosynthetic reaction center (21). R-PE and B-PE are 240-kDa autofluorescent proteins consisting of 3 subunits and more than 30 chromophores (13). They have very high extinction coefficients and large Stokes shifts (22). Phycobiliproteins, such as R-PE and B-PE are 10-100 folds brighter than organic fluorophores.

Due to the presence of prosthetic groups, which are covalently bound to
apoproteins, these proteins are highly fluorescent and absorb and emit at wavelengths specific to phycobilin groups (23). For example, B-PE protein has absorption peaks at two wavelengths 545 nm and 565 nm and one shoulder at 498 nm (24). The emission wavelength of B-PE is 570 nm.

Energy transfer related research at the molecular level could provide very useful information about energy transfer between fluorescent molecules and other molecules. Since the efficiency of light transfer in phycobilisomes is 95% (25), it would be beneficial to study the process of energy transfer from these proteins to particles and surfaces in detail. Moreover, mimicking this process on surfaces or particles coated with fluorescent proteins can be an attractive model system for studying protein-particle and protein-surface phenomena that may lead to efficient energy transfer. Fluorescent molecules can transfer energy to the surfaces or nanoparticles through surface energy transfer (SET) (26) or FRET (8). Because of the complexity of the fluorescent proteins, it is hard to predict if the mechanism of energy transfer between fluorescent protein and nanoparticles and/or surfaces will be FRET or SET. Fluorescent proteins on surfaces or in contact with nanoparticles may exhibit a wide range of changes in their fluorescent and structural properties. Fluorescent molecules at surfaces or in suspension with nanoparticles could have quenched or enhanced fluorescence intensities as well as show changes in their fluorescence properties such as fluorescence rate, quantum yields, and fluorescence lifetimes.

FRET is a distance dependent resonant process first described by Förster et al. in late 1940’s (27). Due to distance dependence, it has been used to investigate molecular level distance and fluorescence emission rates of energy transfer and has applications in
biomedical, protein folding, and DNA/RNA energy transfer processes (28-32). During FRET, a molecule called the donor undergoes relaxation by transfer of its excited energy to an acceptor molecule by dipole-dipole coupling between the donor and acceptor (27). The physical phenomenon of FRET cannot happen unless there is adequate overlap between the emission and absorption bands of the donor and acceptor, respectively, and two molecules are close enough. FRET’s dependence on distance is inverse on the sixth power and this dependence gives the opportunity to investigate distance between the two molecules involved in FRET. FRET can occur successfully over distance ranging between 1 nm and 8 nm. FRET efficiency is also affected by the relative orientation of the donor and acceptor transition dipoles. However, the efficiency of energy transfer between donor and acceptor is unaffected by the nature of solvent and other molecules present due to distance dependence nature of energy transfer (27,28).

Unlike FRET, SET does not require a resonant electronic transition, and it is due to interaction of the electromagnetic field of the donor dipole with the free conduction electrons of the acceptor. SET energy transfer rate is also distance dependent phenomenon and depends on $d^{-4}$ (18,19).

The experimental evidence for elucidation of FRET and SET comes from the measurement of distance between donor and acceptor molecules (r and d, respectively), with these distances ranging from several nanometers up to 8 nm in the case of FRET and above 8 nm in the case of SET (18,19,26). The rate of energy transfer in FRET ($k_T(r)$) is based on the Förster equation:

$$k_T(r) = \frac{1}{\tau_D} \left(\frac{R_0}{r}\right)^6$$

(eq. 1-1)

where $\tau_D$ is the lifetime of the donor in the absence of energy transfer, and $R_0$ is the
Förster distance (the distance at which the energy transfer rate of the donor is equal to the decay rate of the donor in the absence of the acceptor) \((8,18,19,26)\). Regarding SET, dependence of energy transfer rate on the distance between the donor and acceptor \((d)\) is given by the equation:

\[
k_{\text{SET}} = \frac{1}{\tau_D} \left(\frac{d_0}{d}\right)^4 \tag{eq. 1-2}
\]

where \(d_0\) is the distance at which a fluorophore will display equal probabilities for energy transfer and spontaneous emission, and can be calculated using Persson’s and Lang’s model \((18,19,26)\).

It has been shown that fluorescence of rhodamine 6G gets quenched by gold nanoparticles because energy is transferred through SET from rhodamine molecules to nanoparticles \((18,19)\). Bringing together semiconductor nanocrystals and organic dyes, Becker \textit{et al.} demonstrated FRET in single nanoparticle-dye complex, and electrically controlled the emission from the dye \((33)\). Fluorescent proteins were investigated by fluorescence spectroscopy in numerous studies, but there are limited reports about their behavior on surfaces and nanoparticles. Recently, using single-molecule fluorescence spectroscopy, Ray \textit{et al.} have shown that R-phycoerythrin fluorescence intensity is enhanced on the surface of silver \((34)\). It was shown in similar studies that the fluorescence intensity of fluorescent molecule is enhanced in the presence of metal nanoparticles via metal enhanced fluorescence (MEF) mechanism \((32,35)\).

\textbf{1.4 Analysis of proteins by mass spectrometry}

There has been a keen interest in knowing how protein expression and function change qualitatively and quantitatively through environmental alterations or challenges.
Since the success of the Human Genome Project in combination with advances in biotechnology and in bioinformatics, proteomic projects are the driving forces for the entire scientific community such as biochemists, bioanalytical chemists, etc. But, the proteome project is much more complex than the genome project due to the diversity in the protein count in cells. Proteomic projects involve examination of all proteins that can be present in a cell, a tissue, or an organism at a given time in a given condition. Therefore, cells, tissues, and body fluids such as plasma can be used as sources of proteins. A single cell produces \( \sim 10^4 \) proteins (36). Also, posttranslational modifications add to the overall complexity of the proteome. On the other hand, tissues composed of different types of cells are an extremely complex heterogeneous system and the number of proteins is much higher than in each individual cell.

Therefore, investigation of complex systems in the proteome is a challenging task and requires efficient analytical methods. Mass spectrometry (MS), which measures the mass-to-charge ratios, has become a principal tool for comprehensive and high-throughput analysis of molecules. Mass spectrometry has emerged for the analysis of peptides, proteins, lipids, disease markers (37), glycopeptides (38) and oligosaccharides (39). Therefore, MS is the most commonly used analytical tool for qualitative (amino acid composition and structure elucidation) analysis of the proteome.

There is a huge interest in the MS analysis of peptides and proteins present in biological fluids and disease-related tissues. Mass spectrometric analysis provides the molecular weights and sequences of peptides and proteins present in the sample, which can allow determination of compositions of these molecules as they are comprised of 20 amino acids. Sample preparation for detection of peptides and proteins is essential to
enhance sensitivity and reproducibility of the analysis using mass spectrometry.

Bottom-up and top-down MS technologies have been used for proteomic analyses in the last 15-20 years (40). In the bottom-up approach, proteins, or protein mixtures are subjected to digestion. LC is then used for separation of the peptide mixture and MS is used for identification and characterization of peptides and proteins. In the top-down approach, protein samples are separated using LC and then individual proteins are characterized and identified directly by means of MS/MS. The top-down approach is still looking for wide use due to practical problems in the separation of intact protein mixtures. Technical problems in protein separations are: solubility in mobile phase, insufficient separation, and slow mass transfer (40). Proteins possess complex structure and closely related physical and chemical properties, which makes separation and identification a laborious task. However, successful separations of proteins have been reported using liquid chromatography (41,42).

1.4.1 ESI-MS and MALDI-MS

Anatomy of a mass spectrometer is shown in Figure 1-2 (43). A mass spectrometer consists of 3 major segments: an ionization source, a mass analyzer, and a detector. An ionization source transforms analyte molecules into ions. A mass analyzer separates the ions formed in ionization sources according to their masses. A detector measures the relative amounts of all separated ions. Electron ionization (EI), chemical ionization (CI), fast atom bombardment (FAB), electrospray ionization (ESI), and matrix assisted laser desorption/ionization (MALDI) are several examples of ionization sources used in mass spectrometry. Most common mass spectrometers used to analyze protein
samples include ESI and MALDI mass spectrometers. They use ESI and MALDI as ionization sources because these are soft ionization techniques. The ESI and MALDI sources yield less fragmentation than EI and FAB. In ESI-MS, peptide and protein solution is dispersed by electrospray and sampled into the mass spectrometer through a capillary as charged jets with assistance of additional nebulization and heating (44). In MALDI-MS, analyte-matrix co-crystals are irradiated by a laser and the matrix absorbs laser energy. A hot matrix plume contains ionized matrix ions where protonation of the analyze molecules takes place (45). Sinapinic acid (SA) and α-cyano-4-hydroxy cinnamic acid (CHCA) are the most common matrixes used for the analysis of proteins and peptides by MALDI-MS, respectively. In ESI-MS, ions are mostly multiply charged, while in MALDI-MS, singly charged ions are generally observed. Once ions are formed in ionization sources, they can be separated and analyzed by: quadrupole (Q), ion trap, magnetic sector, fourier transform ion cyclotron resonance (FT-ICR), and time-of-flight (TOF) analyzer. TOF analyzer is well-matched for pulsed ionization sources due to its requirement for ions to be created in bundles. In TOF analyzer (46), ions are potentially (V_s) accelerated and depending on the time (t) taken by the ions to fly a distance d, mass-to-charge ratios (m/z) are determined by the following equation:

\[ m/z = \frac{t^2(2V_se)}{d^2} \]  

(eq. 1-3)

where e is the charge on the electron.

Advantages of ESI and MALDI coupled to a time-of-flight (TOF) mass analyzer to analyze proteins include: no upper limit on mass range in theory, high sensitivity (100 attomoles), high-resolution, and high mass accuracy (46,47). In addition, with advancement of ESI and MALDI, mass spectrometers can accurately measure masses of
hundreds of peptides in a mass spectrum.

Figure 1-2. Schematic of a typical mass spectrometer (43).

Mass spectrometry is implemented for the analysis of both underivatized and derivatized biomolecules. Some peptides and proteins ionize well during MS analysis, while others do not. Therefore, there is sometimes a need to derivatize peptides and proteins with organic molecules or dyes to improve ionization efficiency and stability of peptides. Previous studies have shown that characterization at the nanogram levels of peptides and proteins is possible after labeling with dyes such as monobromobimane and coumarine (48,49). Peptides labeled with coumarin dyes show 5-15 fold increase in intensity of matrix assisted laser desorption/ionization-mass spectrometry (MALDI-MS) signal over unlabeled peptides. Major requirements for derivatization reagents are: the labeling reaction should be fast and the labeling yield should be as close as possible to 100%; derivatives should be stable during sample preparation; and should not affect physical and chemical properties of peptides (49). Therefore, both native (label free)
fluorescence and fluorescence labeling can be used along with mass spectrometry for characterization of proteomic samples.

However, structural analysis is difficult based on MS data alone. Therefore, tandem mass spectrometry (MS/MS) is often used for the structural characterization and determination of composition of peptides and proteins. It also helps to resolve the presence of isomers by tandem MS.

1.4.2 Structural analysis and sequencing of peptides

For structural analysis of peptides and proteins, MS/MS is an essential technique (43). The structure of each compound can be identified by the measurement of mass-to-charge ratios (m/z) of parent ion and its fragment ions. MS/MS is advantageous for heterogeneous samples where the structure of each component can be identified and characterized. In the MS/MS experiments, a particular parent ion to be identified is selected in the first mass analyzer. Collision induced dissociation (CID) and laser induced dissociation (LID) are well-established fragmentation methods for the fragmentation of peptides and proteins. In CID, the selected parent ion collides in a collision cell with an inert gas such as argon to produce fragment ions and the resulting ions are transmitted to a second mass analyzer. Using tandem mass spectrometry-based peptide sequencing by collision induced dissociation or laser induced dissociation approaches, mass of any peptide can be measured with high accuracy.

1.4.3 Quantification of peptides and proteins

Biological systems are highly complex and comprehensive and directed protein analyses can provide powerful perception to unfold the difficulties of biological systems.
For comprehensive, high-throughput identification of peptides and proteins, MS has become a principal tool. To comprehend the dynamics and functionality of the human proteome and multifaceted biological systems, quantification of peptides and proteins is essential.

There are a number of separation techniques to circumvent the problem of high complexity of proteomic samples such as liquid chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 2D-PAGE, etc. Of the different separation techniques available, liquid chromatography is most useful for peptide and protein separation. Reverse phase, affinity, gel permeation, and ligand exchange are different chromatographic separation methods, which have been used in past for proteomic research. Out of these methods, reverse-phase high performance liquid chromatography (RP-HPLC) is the one which is most widely used, due to its wide range of application. It is the method of choice due to the number of stationary and mobile phase alternatives available and its ease of coupling with different detection techniques such as UV-Vis, fluorescence, electrochemical detection, and especially mass spectrometry. There are a number of reports of the use of RP-HPLC-MS in proteomic studies such as the study of proteome of transfected HeLa cell lines, proteomic analysis of two maurine macrophages cell lines, etc. (50,51). Therefore, LC-MS is a well-developed and highly sensitive detection technique and is widely used for protein identification, sequencing, and characterization.

Peak intensities of chemically different peptides and proteins in mass spectrum do not associate well with their relative abundances (52). Thus, it is generally not possible to perform relative quantification of each peptide, or, by inference, the parental protein,
within the same spectrum or between MS spectra. However, for chemically identical peptides, peak intensities in mass spectrum do associate with the relative abundance of those peptides. Therefore, current MS based strategies to simultaneously and quantitatively measure proteins from different samples are based on either labeling the proteins or protease-derived peptides (in such a way that the proteins or peptides are chemically identical and differ only in isotopic mass), or are based on a label-free approach. The label-free approach is based on reliable identification and alignment of unlabeled chemically identical peptides within different spectra from multiple samples.

To quantitatively measure peptides and proteins in complex mixtures using labeling approaches, the following biodiscovery methods are most common: 2D difference in gel electrophoresis (2D-DIGE) (53), isotope-coded affinity tag (ICAT) (54), isobaric tag for relative and absolute quantification (iTRAQ) (55), and stable isotope labeling of amino acids in cell culture (SILAC) (56).

For quantification using 2D-DIGE, fluorescent cyanine dyes Cy 3 and Cy 5 are used to label representative samples of proteins originating from two different sources. After mixing the covalently labeled samples in equal proportion, proteins are separated on the same 2D isoelectric focusing (IEF)/sodium dodecyl sulfate (SDS) gel. The representative protein spots on the 2D IEF/SDS gel can be detected immediately by the fluorescence imaging of the gel at wavelengths suitable for cyanine dyes. An evaluation of images after fluorescence excitation of each spot on the gel allows quantification of protein changes in the same gel (53).

ICAT approach for protein quantification requires presence of three components: a thiol-specific reactive group, an isotopic linker, and a biotin affinity tag (54). Two
representative samples of proteins from different sources to be quantified are labeled with ICAT reagents (tags). After mixing in equal proportion (1:1), labeled samples are purified by affinity chromatography and identified by MS. However, the ICAT method has limitations such as nonspecific interaction between tag and streptavidin affinity matrix, multiple subsequent reactions, limited application in the detection of acidic proteins, and limited detection of proteins lacking cysteine residues (56,57).

On the other hand, SILAC uses assimilation of isotopically labeled amino acids into proteins during synthesis by cells. One sample of cells is labeled with light amino acid whereas another sample of cells is labeled with heavy counterpart and after some divisions, both samples of cells are collected, and mixed in equal proportion. Mixture may be than exposed to digest using trypsin and tryptic peptides are separated by LC and analyzed by MS and MS/MS (56).

iTRAQ uses a tag which consists of N-Hydroxysuccinimide (NHS) reactive groups coupled to a bipartite tag (55). These tags have constant total molecular weights and bind to primary amines such as the N-terminal amine of a peptide and the side chain of lysine amino acid. Once labeled, peptide samples are mixed in equal proportion, separated, and analyzed by MS. The peptide of interest is exposed to tandem MS, liberating the ions with unique mass. Intensity of these ions symbolizes the quantity of the peptides from which they were released.

ICAT and SILAC - use mass difference as the basis of quantification by measurement of relative peak areas in mass spectra. Due to mass difference labeling approach, these techniques are limited to a binary set of reagents and make quantification of multiple states difficult to undertake. However, iTRAQ techniques can be used for
quantification of multiple states. This method of quantification uses a multiplexed group of reagents that lay isobaric mass tags on peptides in a digest mixture. After derivatization, all peptides are isobaric and chromatographically indistinguishable. But, these peptides when subjected to MS/MS produce signature ions (reporter ions) which can be used for the identification and quantification of individual members of a multiplex set (55). Therefore, isotopic labeling may be performed on intact proteins with specific different isotopically labeled tags after protein purification, or at peptides derived from proteins after purification, or proteins may be metabolically labeled with light and heavy amino acids. For example, isotopically labeled cysteines can be used for detection, identification, and quantification of proteins based on the doublets present in the mass spectra (58). The doublet corresponds to “heavy” isotope and “light” isotope in the mass spectrum. One or more members of the doublets are then subjected to CID- or LID-based MS/MS to identify the peak, and by inference, the protein.

Aforesaid approaches suffer from the requirement of isotopic labeling or Cy dye labeling, limited capacity of multiplexing, and also require sample manipulation (59). Some of these issues can be circumvented using label-free approaches. As mentioned earlier, peak intensity of chemically different peptides and proteins in mass spectrum do not correlate with their relative abundances but they do correlate for chemically identical molecules (52). Therefore, quantities of chemically identical peptides could probably be equated in any number of analyses by keeping the analyses parameters as fixed as possible. Amon et al. and Duan et al. showed the use of a label free approach for quantification of peptides and proteins following an LC-MS method (60,61). Native fluorescence of peptides and proteins during LC-MS analysis can be used for
quantification by utilizing fluorescence detection prior to MS detection (62). Fluorescence detection prior to MS will allow solution-phase measurements as well as gas-phase measurements of peptide and protein molecules.

1.4.4 Imaging Mass Spectrometry

Imaging Mass Spectrometry (IMS) also known as mass spectrometry imaging (MSI) is a mass spectrometric technique used to visualize the spatial distribution and localization of biomolecules, e.g., peptides, proteins, lipids, metabolites, and most importantly biomarkers by measuring their molecular masses. Most developed techniques in field of IMS are secondary ion mass spectrometry (SIMS) imaging, desorption electrospray ionization (DESI) imaging, and MALDI Imaging.

SIMS collects and analyzes secondary ions ejected by sputtering the solid surfaces and thin films with a primary ion beam (such as gold ions) with high-energy (typically 5 to 20 keV) (63). SIMS imaging provides very high spatial resolution down to order of 2-3 \( \mu \text{m} \) for small molecules (<1000 Da) such as lipids (64). Tissue sections were pulsed with \( \text{Au}^+ \) ion beam and secondary ions (positive and negatively charged ions) were collected to prepare ion images. Imaging of small biological molecules in tissue sections using SIMS has been reported in literature (65). SIMS imaging has limited application for large molecules due to fragmentation during ionization and also suffers with reduced ionization efficiency.

DESI is an ionization technique that empowers desorption/ionization of analytes (e.g., peptides) of interest from surfaces (e.g., tissues) under ambient conditions (66). DESI is a hybrid technique which is an amalgamation of electrospray (ESI) and
desorption (DI) ionization methods and hence the name DESI. Ionization takes place by
targeting an electrospray jets (charged droplets) to the insulated surface without a matrix
which is at a reasonable distance away (67). Generated gaseous ions pass through the
ambient interface that is coupled to mass spectrometer which is under vacuum system.
According to Takats and co-workers, numerous fundamental parameters can affect the
ionization efficiency and the collection efficiency of DESI mass spectrometry analysis
such as: electrospray, chemical, geometric, and surface effects. Optimization of these
parameters also depends on the type of molecule, e.g., protein, under investigation (67).
DESI has been used in imaging of molecules in tissue sections (68,69). Tissue section (10
μm thick) was sprayed with electrically charged solvent (acetonitrile / N,N-
dimethylformamide) and MS analysis was performed in negative ion mode. Identification
and characterization of lipids were achieved using DESI in combination with tandem
mass spectrometry (68). Advantage of DESI imaging is in its ambient condition for
imaging biological samples and no sample preparation. However, DESI imaging suffers
from modest resolution and is less sensitive than MALDI imaging.

MALDI imaging uses matrix-assisted laser desorption/ionization technique as a
source of ionization in which a laser of suitable wavelength irradiates the sample, often a
thin tissue section, which is coated with appropriate matrix (a organic compound that
absorbs and transfers energy of the laser to the analyte) (70). Applied matrix co-
crystallizes with analytes in the sample and these crystals absorb the UV radiation from
the laser pulse causing matrix and analyte molecules to desorb from the sample surface.
Gas-phase ionization occurs in the desorbed MALDI plume and these ablated ions
typically fly through the time-of-flight (TOF) mass spectrometer under high vacuum
conditions where they are analyzed. The laser irradiates an area of typically 30-100 micron in diameter and hundreds of compounds ablated from the sample surface after irradiations are represented in the resulting mass spectrum signals. The MALDI imaging technique is universal and can be used to ionize all sorts of molecules with theoretically no upper limit on the molecular mass of the analyzed molecule (46). Molecules analyzed MALDI-IMS range from small molecules (drugs, metabolites) to large molecules (proteins). MALDI-IMS can allow visualization of the spatial distribution within 10-15 µm-thick tissue section of an animal or plant.

A flow chart for identification, characterization, and imaging of peptides and proteins from tissue sections using MALDI-IMS is shown in Figure 1-3 (71). For MALDI imaging of tissue sections, tissue sections were sliced to 10-15 µm in thickness and thaw and mount on a glass slide. Tissue sections were washed with different proportion of isopropanol to remove lipids and other salt molecules. Tissue sections on the glass slide were coated with sinapinic acid (SA) matrix for the mass spectrometric analysis of proteins. In case of peptide analysis, large proteins on tissue sections were digested with trypsin. Tissue sections with tryptic peptides were coated with α-cyano-4-hydroxycinnamic acid (CHCA) matrix. The glass slide was inserted into a slide holder especially designed for MALDI imaging. The slide holder with the glass slide was inserted into the mass spectrometer that will record the spatial distribution of the molecular species such as proteins, and peptides. The mass spectrometer was operated in linear mode for protein analysis and in reflectron mode for peptide analysis. The mass spectrometric data were processed with software for reconstruction of ion images. MALDI imaging provides the widest mass range for molecules, which can be analyzed
and imaged simultaneously in a thin section of tissues. The resulting data set obtained from imaging experiments include x, y coordinates (pixel) and corresponding spectra (70). Extracting the experimental intensities and scheming on a color scale can construct 2-D ion images for ions. Each pixel in 2-D ion images of the tissue section has its own mass spectrum and contains thousands of diverse molecular species and allied relative abundances. For peptide and protein identification, experimental m/z values were compared with literature values. Identification and characterization can be achieved using tandem mass spectrometry.

Schwamborn and co-workers assigned the tumor biomarkers for prostate cancer using MALDI-IMS (72). Eleven tissue section from the diseased group and 11 from the control group were subjected to imaging and 2 proteins were found to be upregulated in disease tissue section and identified as prostate cancer biomarker. However, 3-D reconstruction is more demanding due to tissue tearing and deformation problems, which can occur due to the requirement of the specimen to be sliced into thin slices in MALDI-IMS imaging. Recently, the 3-D MALDI-IMS representation of protein profiles in mouse brain was demonstrated by Crecelius et al. (73). MALDI images and optical microscopy images were registered and superimposed to generate the 3-D images of brain tissue section. IMS has been used to map different anatomic substructures of a mouse brain, and spinal cord tissue section and obtained information on the local peptide and protein composition across a brain and spinal cord tissue section (63,70).
Figure 1-3. Workflow for the distribution, localization, ion imaging, identification, and characterization of peptides and proteins using MALDI-IMS.
Chapter 2

Energy Transfer from Fluorescent Proteins to Metal Nanoparticles

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Abstract

Energy transfer plays a significant role in numerous chemical, physical, and biological processes. While the use of fluorescent proteins in Förster resonance energy transfer (FRET) studies of biomolecules is common, energy transfer between fluorescent proteins and inorganic nanoparticles has not been explored in detail. In this study, energy transfer from fluorescent phycobiliproteins to noble metal nanoparticles was analyzed. Solutions of B-phycoerythrin (B-PE) were mixed with colloidal Au and Ag nanoparticles and were characterized by steady-state and time-resolved fluorescence spectroscopy to determine the magnitude and mechanism of the energy transfer. It was found that the protein fluorescence was quenched after the addition of metal nanoparticles. Electron microscopy and absorption spectroscopy confirmed that B-PE was adsorbed onto the nanoparticles, creating a favorable geometry for quenching. Time-resolved fluorescence spectroscopy showed that B-PE fluorescence lifetimes decreased from 2.2 ns to 0.5 and

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0.6 ns upon adsorption onto Au and Ag nanoparticles, respectively, corresponding to energy transfer efficiencies of >70%. Our results, which include lifetimes, efficiencies, and energy transfer distances, show that energy was transferred via the surface energy transfer (SET) mechanism, rather than FRET.

### 2.1 Introduction

Many useful energy transfer concepts originate from nature. For example, fluorescent proteins have evolved to perform energy transfer with extraordinary efficiency. While fluorescent proteins are commonly used as probes of protein localization in biological cells, their native functions in the host organisms are light absorption, energy transfer to other proteins, or light emission to the environment (8). For example, green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* absorbs energy transferred from blue luminescent protein (aequorin) and then emits green light (8,9).

Cyanobacteria and eukaryotic algae (red algae, glaucophytes, and criptomonads) contain three classes of fluorescent phycobiliproteins, phycoerythrins, allophycocyanins, and phycocyanins, that are assembled into photosynthetic protein complexes called phycobilisomes (10,15). The phycobiliproteins B-phycoerythrin (B-PE) and R-phycoerythrin (R-PE) each contain more than 30 phycoerythrobilin (PEB) and phycourobilin (PUB) chromophores that are assembled in a 240-kDa multimeric complex (αβ)6γ. Hence, phycobilin chromophores, which are linear tetapyrole compounds bound to polypeptide chains by thioether bonds, are responsible for the excellent spectroscopic properties of phycobiliproteins as well as energy transfer involving phycobiliproteins (15).
Phycobilisomes absorb sunlight and then transfer energy via Förster resonance energy transfer (FRET) to chlorophyll a (15). Phycobiliproteins possess unique characteristics that make them suitable for fluorescence detection and energy transfer, which include extremely high absorption coefficients over a broad part of the visible spectrum, high fluorescence quantum yields, and large Stokes shifts (8,11,12). For example, both B-PE and R-PE absorb light from ~ 450 nm to ~570 nm and emit orange fluorescence that peaks at 576 nm with quantum yields of 0.98 and 0.84, respectively (12).

Previously, it was found that light is absorbed in phycoerythrins by phycourobilin and phycoerythrobilin chromophores (12). Energy is then transferred to phycoerythrobilin chromophores by FRET, with subsequent emission from this chromophore at 576 nm (13). In phycobilisomes, FRET flows from the phycobilin chromophores of phycoerythrin, located at the outer rods of the phycobilisome complex, toward the phycocyanobilin chromophores of phycocyanins and allophycocyanins (15). The latter phycobiliproteins are located in the core of the phycobilisome complex, and energy is further transferred toward chlorophyll a. It is important to note that the energy transfer efficiency among phycobiliproteins in a phycobilisome complex is close to 100% (15). This extremely high efficiency of energy transfer in these protein systems motivates the careful study of the energy transfer process from phycobiliproteins to technologically relevant inorganic nanomaterials, such as metal nanoparticles.

Fluorescent molecules in close proximity to metal nanoparticles may exhibit changes in their spectroscopic properties. Previous studies have shown that fluorescent molecules mixed with metal nanoparticles and on metal surfaces may exhibit...
fluorescence intensities, quantum yields, and fluorescence lifetimes that are different than for the fluorophores alone (8,14,16). Energy transfer between fluorescent molecules and nanoparticles can involve mechanisms such as surface energy transfer (SET) and electron transfer (17-20,22,25). In addition, fluorescence intensities can decrease as a result of dynamic or static collisional quenching, thermal deactivation, charge buildup, or reactions of fluorescent molecules with impurities (8).

Since FRET is a resonant process, it requires the emission spectrum of a donor fluorophore to overlap with the absorption spectrum of a nearby acceptor molecule. In contrast, SET does not require a resonant electronic transition, since it involves the interaction of the dipole field of the donor with the free conduction electrons of the metal.

Förster theory predicts the rate of the energy transfer, $k_{\text{FRET}}(r)$, in FRET to be:

$$k_{\text{FRET}}(r) = \left(\frac{1}{\tau_D}\right)\left(\frac{R_0}{r}\right)^6 \quad \text{(eq. 2-1)}$$

where $\tau_D$ is the lifetime of the donor in the absence of energy transfer, and $r$ is the distance between donor and acceptor molecules (8). The Förster distance, $R_0$, is the distance where the non-radiative energy transfer rate equals the radiative decay rate of the donor in the absence of the acceptor (8).

For SET, the energy transfer rate is given by the equation:

$$k_{\text{SET}} = \left(\frac{1}{\tau_D}\right)\left(\frac{d_0}{d}\right)^4 \quad \text{(eq. 2-2)}$$

where $d$ is the distance between donor and acceptor molecules and $d_0$ is the distance at which a fluorophore has an equal probability of non-radiative energy transfer and radiative emission, which can be calculated using Persson’s and Lang’s model (17-20,25).
The distance dependence of FRET and SET are such that FRET tends to be operative at short distances while SET can still be effective at long distances. Experimental observations of FRET are generally limited to distances of up to ~8 nm, whereas SET is effective for much larger distances (17-20,25).

These energy transfer processes between fluorescent donor molecules and acceptors, such as metal nanoparticles, will depend on the physical and chemical characteristics of the molecules and particles involved. For example, it has been shown that the fluorescence of rhodamine 6G is quenched by gold nanoparticles of various size and shape due to energy transfer from rhodamine to the metal nanoparticles by the SET mechanism (18,19). SET has been used to monitor conformation changes of protein bovine serum albumin adsorbed on gold nanoparticles (20). It was also shown to be a dominant mechanism of energy transfer from fluorescent dyes that were separated from gold nanoparticles by 2 nm to 15 nm using DNA linkers (25). In contrast, many recent studies have shown that fluorescence can increase in the presence of metal nanoparticles, via a mechanism known as metal enhanced fluorescence (MEF) (26,33). For example, it was shown recently that the fluorescence intensity of R-phycoerythrin is enhanced on the surface of silver (34,35).

In this study, we investigate energy transfer between the fluorescent phycobiliprotein B-PE, which acts as a donor, and gold and silver nanoparticles, which act as acceptors. The different spectral characteristics of the Au and Ag nanoparticles enable the differentiation of FRET and SET, since the Au nanoparticle plasmon absorption is resonant with the B-PE emission spectrum while that for Ag is not. Lysozyme was also used in control experiments, since its native fluorescence is resonant
with the plasmon absorption for Ag nanoparticles but not Au nanoparticles. Spectra of these proteins and nanoparticles are shown in Figure 2-1.

Solutions of proteins were mixed with colloidal metal nanoparticles and characterized by steady state and time-resolved fluorescence spectroscopy to elucidate the nature of the energy transfer. Electron microscopy imaging and absorption spectroscopy showed that the proteins were adsorbed to the metal nanoparticles. Time-resolved fluorescence spectroscopy measurements provided quantitative evidence for energy transfer. Excited-state lifetime measurements for the protein in the presence and in the absence of the metal nanoparticles provided a measure of energy transfer efficiency and enabled the mechanism to be identified as SET.

2.2 Experimental

2.2.1 Materials

Solutions of B-phycoerythrin (B-PE) at concentrations of 4 mg/mL were purchased from Invitrogen Molecular Probes (Eugene, OR). Protein lysozyme and other chemicals were purchased from Sigma (Saint Luis, MO). HPLC grade water was purchased from Fisher for protein solutions. Purified water from a Synergy ultrapure water system from Millipore (Billerica, MA) was used for nanoparticle synthesis and substrate processing. Osmium tetroxide was purchased from Structure Probe, Inc (West Chester, PA).

2.2.2 Synthesis of metal nanoparticles

All reactions were carried out in water. Gold and silver nanoparticles were synthesized as described in detail elsewhere (74,75). To synthesize gold nanoparticles, 40 mL of 1 mM tetrachloroaurate solution was heated to boiling on a hot plate. To this solution, 4 mL of 38 mM trisodium citrate solution was added all at once to the boiling
solution with constant stirring (~800 rpm). A deep red solution indicated that Au nanoparticles had formed. To synthesize silver nanoparticles, 30 mL of 2 mM sodium borohydride and 30 drops of 38 mM trisodium citrate were first mixed together. The solution was then cooled for 15 minutes in an ice water bath. To this solution, 10 ml of 1 mM silver nitrate was added dropwise with constant stirring (~800 rpm). A bright yellow solution indicated the formation of Ag nanoparticles.

2.2.3 Nanoparticle size distributions

Scanning transmission electron microscopy (STEM) images of nanoparticles were acquired on 200 mesh copper TEM grids. Specimens were prepared for imaging by immersing the TEM grids in gold and silver nanoparticle solutions for 6 hours. After that time, grids were taken out from the solution, rinsed with water, and dried with argon. Samples were imaged with a Hitachi HD-2300A 200 kV scanning transmission electron microscope (STEM). ImageJ was used to process the images and extract particle areas. Diameters were calculated assuming circular particle images, and the resulting histogram was fit with a Gaussian function. The center and half width of the Gaussian were reported as the average size and polydispersity, respectively.

2.2.4 Imaging of protein adsorption onto nanoparticles

Samples were imaged with a JEOL JSM -7500F 30 kV SEM using a STEM detector. TEM grids were immersed in the protein-nanoparticle solution for 6 hours. The grids were then rinsed with water, air dried, and immersed in 2% osmium tetraoxide aqueous solution for 30 min. After incubation in OsO₄, the samples were rinsed with water, dried under flowing N₂, and then imaged.
2.2.5 Nanoparticle absorption spectra and absorption coefficients

Absorption spectra of the Ag and Au nanoparticles were measured in quartz cuvettes from 200 nm to 820 nm using the Nicolet Evolution 300 spectrophotometer from Thermo Electron Corporation (Madison, Wisconsin). Absorption coefficients for the nanoparticles were calculated using Beer’s law, using an estimated nanoparticle concentration. The number of nanoparticles in a solution was determined from the total mass of the metal divided by the mass of spherical metal nanoparticles whose average radius was determined by STEM, as described above.

2.2.6 Protein fluorescence and fluorescence quenching

Emission spectra of proteins in the presence and absence of metal nanoparticles were measured using the LS 50B luminescence spectrometer from Perkin Elmer (Beaconsfield, Buckinghamshire, England). The emission fluorescence spectrum of B-PE was measured between 520 nm and 700 nm using an excitation wavelength of 490 nm and 2 mL of a 20 nM protein solution. The emission fluorescence spectrum of lysozyme was measured between 320 nm and 500 nm using an excitation wavelength of 280 nm and 2 mL of a 1.2 μM protein solution. 10 μL-aliquots of Au and 20 μL-aliquots of Ag nanoparticles were added to the protein solutions and fluorescence spectra were measured. Fluorescence of protein-NP solutions was also measured after serial dilution in order to find a nanoparticle concentration range where the inner filter effect was negligible. The fluorescence intensity of the protein-nanoparticle solutions was linearly proportional to the protein concentration for the NP concentration range used, indicating that the inner filter effect was negligible. Corrected fluorescence emission spectra of phycoerythrin were measured on an Aminco Bowman II luminescence spectrometer.
Control experiments were performed to determine the effect of residual reagents from the nanoparticle synthesis on fluorescence. Residuals were added to the protein solutions by removing the NPs by precipitation and adding the supernatant. NPs were precipitated by adding 0.2 g of NaCl to a 2 ml solution of nanoparticles and then were separated from the supernatant by centrifugation at \( \sim 15,000 \) rpm for 5 minutes. Next, 1 ml of the supernatant was added to 2 ml of a 40 nM B-PE solution and the fluorescence spectrum was measured and compared to fluorescence spectrum of the free protein. NaCl was also added directly to the B-PE solutions to ensure that protein aggregation or precipitation did not occur.

\[ 2.2.7 \text{ Time-resolved fluorescence} \]

The detailed experimental setup for time-resolved fluorescence measurements has been described elsewhere (76). Briefly, the fluorescence lifetimes were recorded by a time-correlated single photon counting (TCSPC) system (SPC-830, Becker & Hickl GmbH) in a single mode. The optical path for the measurement was based on the Axiovert 200M inverted scanning confocal microscope. A pulsed laser was used to excite the fluorescence with a central wavelength of 532 nm, a pulse duration of \( \sim 300 \) fs, and a repetition rate of 76 MHz. Both the polarization of excitation and emission were controlled by Glan Taylor polarizers. The polarized fluorescence at magic angle within 0.3 numerical aperture (NA) of the objective was collected by a single photon counting avalanche photodiode detector (MPD, module: PDM50ct). The counting rate of the signal was controlled at 500 kHz for all measurements through adjusting the intensity of the excitation laser. The lifetimes were calculated by fitting the decay curves with the
convolution of a Gaussian function and exponential or biexponential decays.

2.3 Results and Discussion

2.3.1 Properties of nanoparticles and fluorescent proteins

The size and shape of the synthesized nanoparticles were determined by scanning transmission electron microscopy. It was found that gold and silver nanoparticles were predominantly spherical with diameters of 10.1±0.8 nm and 10.3±1.8 nm, respectively (Figure 2-2). The nanoparticles were further characterized by absorption spectroscopy. Plasmon absorption bands for silver and gold nanoparticles were observed at ~390 and ~525 nm, respectively (Figure 2-1).

![Graph](image)

**Figure 2-1.** Absorption and emission spectra of fluorescent proteins (B-PE and lysozyme) with absorption spectra of Au and Ag colloidal nanoparticles.
Steady-state fluorescence measurements show that the metal nanoparticles are not fluorescent, while B-PE has a fluorescence maximum at 576 nm. It is important to note again that the absorption spectrum of the gold nanoparticles overlaps well with the emission fluorescence spectrum of B-PE, while the absorption spectrum of Ag nanoparticles does not (Figure 2-1). This suggests the possibility for resonant energy transfer in the case of Au but not in the case of Ag.

Figure 2-2. Scanning transmission electron microscopy image of colloidal metal nanoparticles. (a) Au nanoparticles consisted primarily of spheroids with a diameter of 10.1±0.8 nm, with <5% triangular particles. (b) Ag nanoparticles were almost exclusively spheroids with a diameter of 10.3±1.8 nm.

Lysozyme’s native fluorescence emission peaks at 360 nm, upon excitation at 280 nm, which is resonant with the Ag plasmon but not the Au plasmon (Figure 2-1).
Lysozyme was therefore used as a donor, complementary to B-PE, to monitor the influence on quenching of the overlap between the emission band of the protein and the absorption band of the nanoparticles.

### 2.3.2 Adsorption of fluorescent proteins on metal nanoparticles

When nanoparticles were added to the B-PE solution, the proteins were found to adsorb onto the metal nanoparticles. The proteins were stained using osmium tetroxide, enabling their visualization under electron microscope imaging. The stained proteins were clearly visible on the surface of the nanoparticles, as shown in the insets of Figure 2-3. Absorption spectra also showed red shifts for the Au and Ag nanoparticle plasmons upon addition of B-PE (Figure 2-3), further confirming protein adsorption (77).

Phycobiliproteins and organic fluorophores are well known to adsorb to metal and fused silica surfaces (14,34,35,78,79). The Au and Ag nanoparticles were protected only by citrate anions adsorbed onto their surfaces, which should not greatly impair protein adsorption. In fact, the nanoparticles appear to displace adsorbed proteins as they approach one another, leading to interparticle spacings that were much smaller than the thickness of two protein layers. Although this implies that that the proteins are physisorbed, the mechanism of adsorption is not clear. The pI of B-phycoerythrin is ~4.4 (80), so the protein is expected to be negatively charged in pH 7 aqueous solutions. The particles are stabilized with citrate anions, however, so the adsorption of B-PE onto the metal nanoparticles is probably not electrostatic. Hydrophobic and van der Waals interactions are more likely to be involved, since these drive the self assembly of the phycobilisome complex (81). For lysozyme, however, the pI is 11.5 (82) so it is possible for the proteins to be electrostatically bound to the nanoparticles, although hydrophobic
and van der Waals interactions could also be at play. Additionally, B-PE contains multiple cysteine residues (83) that could play a role in chemisorption, although observations of protein mobility suggest that this mechanism does not contribute to binding.

**Figure 2-3.** Absorption spectra of protein-nanoparticle suspensions (dashed curves) compared to absorption spectra of NPs (solid lines). The spectra of B-PE adsorbed on gold and silver NPs show red shift. Insets show STEM images of B-PE adsorbed on Au and Ag NPs.

Direct imaging also facilitates estimation of the number of protein molecules adsorbed onto each individual nanoparticle. From electron microscopy, the protein layer
was observed to be ~5 nm thick. Considering that B-PE is disc shaped with a 10.1 nm diameter and 5.4 nm thickness (84), this implies that B-PE binds to the nanoparticles oriented with its base covering the nanoparticle surface. Based on this binding geometry and the nanoparticle surface area, the number of B-PE molecules bound to NPs is estimated to be ~4 both for gold and silver. Steady-state fluorescence was also used to estimate the number of protein molecules adsorbed onto each individual nanoparticle. By comparing the fluorescence of free protein before nanoparticle addition to the fluorescence of the supernatant after nanoparticle addition and precipitation, the number of proteins per nanoparticle was estimated to be ~3 for Ag and ~4 for Au.
Figure 2-4. Semi-logarithmic plots showing fluorescence intensities of (a) B-PE and (c) lysozyme as a function of Au (blue circles) and Ag (red squares) nanoparticle concentration. Fluorescence spectra of (b) B-PE with Ag nanoparticles and (d) lysozyme with Au nanoparticles.

2.3.3 Characterization of phycobiliprotein-metal nanoparticle mixtures by steady-state fluorescence spectroscopy

Fluorescence spectra were recorded for proteins in aqueous solution mixed with aqueous colloidal metal nanoparticles. Control experiments were done to find a nanoparticle concentration range to measure fluorescence quenching with negligible
influence of the inner filter effect. Steady-state fluorescence from B-PE was quenched after mixing with both Au and Ag nanoparticles (Figure 2-4a). Quenching was found to increase with nanoparticle concentration, but the fluorescence intensity of each mixture did not change in time. This indicated that the incubation time was shorter than the time between mixing and the first measurement, which was approximately 1 minute. Similar quenching results were obtained with lysozyme in the presence of Au and Ag nanoparticles (Figure 2-4c). For both B-PE and lysozyme, there was no shift in the emission wavelength nor were any additional emission peaks observed (Figures 2-4b and 4d), indicating that FRET was probably not present. Control experiments showed that residual materials from the nanoparticle synthesis had no effect on the fluorescence intensities.

The steady-state fluorescence decayed exponentially with increasing nanoparticle concentration (Figures 2-4a and 4c), due to fluorescence quenching upon adsorption of the protein on the nanoparticles. It is interesting to note that fluorescence quenching did not depend on the overlap between the fluorescence spectrum of the protein and the absorption spectrum of the Au and Ag nanoparticles. Once adsorbed on the metal particles the fluorophores within the proteins appeared to be stable, consistent with previous single-molecule studies (34,35). No measurable changes in the fluorescence and absorption maxima of the proteins were observed, indicating that B-PE did not dissociate into its subunits. The native fluorescence of lysozyme was also quenched by the metal nanoparticles with no changes in the fluorescence spectrum, again indicating adsorption without disruption of the native protein fluorophores. These steady-state fluorescence results therefore indicate that neither FRET nor the dissociation of proteins were likely
mechanisms of fluorescence quenching.

2.3.4 Energy transfer between B-PE and metal nanoparticles

To determine the origin and efficiency of the energy transfer between B-PE and metal nanoparticles, excited-state lifetimes of B-PE and B-PE-nanoparticle mixtures were measured by time-resolved fluorescence spectroscopy. Measurements were performed for free B-PE and then upon addition of nanoparticles to the protein solution.

The fluorescence lifetime of B-PE was found to have decreased in the presence of gold and silver nanoparticles (Figure 2-5). While the intensity decay curves for pure phycobiliproteins were best fitted as monoexponential decays, the decay curves for phycobiliprotein-nanoparticle mixtures were best fitted as the sum of two exponential curves. The following biexponential decay equation was used to fit the experimental fluorescence intensity (I):

\[ I(t) = y_0 + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \]  

(eq. 2-3)

where \( t \) is time, \( \tau_1 \) and \( \tau_2 \) are decay time constants, \( A_1 \) and \( A_2 \) are the amplitudes of the two components at \( t = 0 \) s, and \( y_0 \) is an offset. Here, \( \tau_1 \) and \( \tau_2 \) correspond to fluorescence lifetimes of phycoerythrin in two different physical states: in solution and on a nanoparticle.

Monoexponential fits for pure B-PE solutions gave a lifetime of 2.24 ns, which is close to previously determined values for phycobiliproteins (85). For the protein-nanoparticle mixtures, unconstrained biexponential fits were initially used to extract decay components that corresponded to both the free and adsorbed proteins. While the lifetimes of the first decay components closely matched that of the unbound protein, the second
components were shortened. Once this was established, the $\tau_1$ component was fixed using the free protein lifetime and the $\tau_2$ component for the adsorbed protein was found to be 0.5 ns and 0.6 ns for Au and Ag, respectively, as shown in Table 2-1. This was presumably due to energy transfer from the adsorbed protein to the metal nanoparticle. Note that because $A_1/A_2$ was higher in the Au solution than the Ag solution, its decay curve appears to be slower than that of Ag despite the decay constant for Au-bound protein being shorter.

**Table 2-1.** Time-resolved fluorescence lifetimes and energy transfer efficiencies of free B-PE and B-PE adsorbed on metal-nanoparticle surfaces.

<table>
<thead>
<tr>
<th>System</th>
<th>$\tau_1$ (ns)$^*$</th>
<th>$\tau_2$ (ns)$^**$</th>
<th>ET efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free BPE</td>
<td>2.24 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPE Au</td>
<td>2.24 ± 0.01</td>
<td>0.52 ± 0.07</td>
<td>76.8</td>
</tr>
<tr>
<td>BPE Ag</td>
<td>2.24 ± 0.01</td>
<td>0.64 ± 0.01</td>
<td>71.4</td>
</tr>
</tbody>
</table>

$^*$ lifetime of free protein $^**$ lifetime of adsorbed protein

The energy transfer efficiency ($\phi_{ET}$), which is the fraction of photons absorbed by the donor that are transferred to acceptor, can be determined from the fluorescence lifetimes according to the following equation:

$$\phi_{ET} = 1 - \frac{\tau_2}{\tau_1}$$  \hspace{1cm} (eq. 2-4)

As shown in Table 1, energy transfer efficiencies of >70 % were calculated for the mixtures of B-PE with Au and Ag nanoparticles. The similar energy transfer efficiencies for Au and Ag are contrary to what might be expected for FRET, since Ag is far from resonant with the B-PE emission band while Au is not. If FRET were operative in this case, a significant difference in the energy transfer efficiencies for the two metals would
be expected. This again argues against FRET being the energy transfer mechanism.

Figure 2-5. Time-resolved fluorescence of (a) pure B-PE, (b) B-PE incubated with Au nanoparticles, and (c) B-PE incubated with silver nanoparticles.

2.3.5 Distances between fluorescent proteins and nanoparticles

The efficiency of energy transfer is distance dependent so it is important to calculate the distances between the fluorescent protein donors and nanoparticle acceptors. These distances can further indicate if energy is transferred through FRET or SET.

In the case of FRET, the Förster distance \( R_0 \) (in Å) can be calculated using the equation:
\[ R_0 = 0.211[\kappa^2 n^4 Q_{\text{protein}} J(\lambda)]^{1/6} \quad (\text{eq. 2-5}) \]

where \( \kappa^2 \) is the orientation factor (2/3 in solution), \( n \) is the refraction index of the medium (1.4), \( Q_{\text{protein}} \) is the quantum yield of the protein, and \( J(\lambda) \) is the overlap integral between the emission peak of the protein donor and absorption peak of the nanoparticle acceptor (8). The overlap integral is calculated using the following equation:

\[ J(\lambda) = \int F_{\text{protein}}(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda \quad (\text{eq. 2-6}) \]

In this equation, \( F \) is the corrected fluorescence intensity of the protein, \( \varepsilon_A \) is the absorption coefficient of the nanoparticle acceptor and \( \lambda \) is the wavelength in nm (8).

To evaluate the overlap integral, the corrected fluorescence spectrum of the phycobiliprotein was measured and the molar absorptivities of the nanoparticles between 520 and 700 nm were determined. Based on this analysis, the Förster distances \( (R_0) \) calculated for B-PE in the presence of gold and silver nanoparticles were 16.8 and 11.7 nm, respectively. Although these distances are large, they are consistent with previous studies (19,20) once differences in \( \varepsilon_A(\lambda) \) are accounted for. In particular, the nanoparticles used in the present study are significantly larger and therefore have larger \( \varepsilon_A(\lambda) \), which scales with the number of atoms in the nanoparticle (86).

The distances \( (r) \) between fluorescent protein donors and nanoparticle acceptors were further calculated from experimentally determined values of energy transfer efficiencies, using the following equation:

\[ \phi_{\text{FRET}} = 1/ (1+ (r/R_0)^6) \quad (\text{eq. 2-7}) \]

This analysis yielded distances between protein and metal nanoparticles of \( \geq 10 \) nm (see
Table 2-2).

To check if SET is the mechanism of energy transfer, energy transfer distances can be calculated. First, $d_0$ was calculated using the equation:

$$d_0 = (0.225c^3 Q_{\text{protein}}/\omega_{\text{protein}}^2 \omega F \kappa_F)^{1/4} \quad (\text{eq. 2-8})$$

where $c$ is the speed of light ($3 \times 10^{10}$ cm s$^{-1}$), $\omega$ is the angular frequency of the donor’s electronic transition ($3.27 \times 10^{15}$ s$^{-1}$), $\omega_F$ is the angular Fermi frequency ($8.4 \times 10^{15}$ s$^{-1}$ for gold and $8.3 \times 10^{15}$ s$^{-1}$ for silver), and $\kappa_F$ is the Fermi wavevector ($1.2 \times 10^8$ cm$^{-1}$ for both gold and silver) (19,87). From this analysis, the calculated value of $d_0$ between B-PE and metal nanoparticles was 8.6 nm (Table 2-2).

Next, the distances ($d$) between donor and acceptor were calculated from the experimental energy transfer efficiencies, using the following equation:

$$\phi_{\text{SET}} = 1/ (1 + (d/d_0)^4) \quad (\text{eq. 2-9})$$

Distances of 6.4 nm and 6.9 nm were obtained from this analysis for Au and Ag, respectively (Table 2), which are consistent with the physical dimensions of the protein-nanoparticle complex. For example, B-phycoerythrin is a disk-shaped protein with a diameter of 10.1 nm and a height of 5.4 nm (84), while the nanoparticles have diameters of 10.2 nm. If the distance between donor and acceptor were considered to be the distance between the center of the spherical particle and center of the B-PE chromophores, then the donor-acceptor pair distance would be ~7.8 nm. This estimate of the pair distance is consistent with the above calculation of SET distances.
Table 2-2. Calculated energy transfer parameters of phycobiliprotein-nanoparticle mixtures.

<table>
<thead>
<tr>
<th>system</th>
<th>(\lambda_{em}) (nm)</th>
<th>(\frac{J(\lambda)}{\text{M}^{-1}\text{cm}^{-1}\text{nm}^{4}})</th>
<th>(Q_{0})</th>
<th>(R_{0}) (nm)</th>
<th>(r) (nm)</th>
<th>(d_{0}) (nm)</th>
<th>(d) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPE</td>
<td>576</td>
<td></td>
<td>0.98</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPE Au</td>
<td>576</td>
<td>(1.49 \times 10^{18})</td>
<td>0.98</td>
<td>16.8</td>
<td>13.8</td>
<td>8.6</td>
<td>6.4</td>
</tr>
<tr>
<td>BPE Ag</td>
<td>576</td>
<td>(1.72 \times 10^{17})</td>
<td>0.98</td>
<td>11.7</td>
<td>10.0</td>
<td>8.6</td>
<td>6.9</td>
</tr>
</tbody>
</table>

Although the agreement is satisfactory, the slightly longer distance estimate for Ag could be due to the slightly larger average particle size and the broader size distribution. Interestingly, these distances are not consistent with the stacking of B-PE on the nanoparticles, which may be due to the disruption of the stacking geometry upon adsorption onto the nanoparticles.

The same analysis of the geometry using the FRET distance estimates are unphysical. In particular, the distance estimate for Au of 13.8 nm would put the fluorophore beyond the physical size of the protein. Further, the physical position of the fluorophore should be approximately the same with respect to the center of the nanoparticle for both Ag and Au, since the adsorption geometry should be independent of the identity of the metal. Therefore very similar FRET distances would be expected for each metal. Estimates of the FRET distances for Au and Ag differed significantly, however, again indicating that FRET is not the operative energy transfer mechanism.

While >70% of the energy was transferred to the nanoparticles, it is interesting to account for the remaining energy. A significant fraction of the remaining energy was emitted as light, which was observed in steady-state and time-resolved fluorescence measurements. Quantification is difficult, however, as it was neither possible to separate
the free and bound proteins nor to evaluate the quantum yield of the bound proteins. Other possibilities include an intersystem crossing to a triplet state, which could lead to radiative decay on a timescale beyond that of our measurements, or non-radiative mechanisms such as internal conversion and intermolecular exciton-exciton annihilation. Whatever the fate of the remaining energy, it should be still possible to increase the energy transfer efficiency toward 100% by engineering the protein-nanoparticle couple.

2.4 Conclusion

When B-PE was mixed with Au and Ag nanoparticles the proteins were found to adsorb to the metal particles, which led to fluorescence quenching. Scanning electron microscopy and absorption spectroscopy confirmed that B-PE was adsorbed on the metal nanoparticles. Time-resolved fluorescence spectroscopy showed that the energy transfer from B-PE to the metal nanoparticles occurred with > 70% efficiency. The fact that energy transfer efficiencies were very similar for Au and Ag strongly suggests that FRET was not operative, since the resonance conditions were very different. Further, the SET distances between protein donors and nanoparticle acceptors were estimated to be 6.4 nm and 6.9 nm for Au and Ag, respectively. These distances were in agreement with estimates based on the physical size of the constituents. Estimates of FRET distances were not physically reasonable, however. These results indicate that energy was transferred from protein to nanoparticle through surface energy transfer (SET), rather than FRET. This implies that efficient energy transfer between proteins and metal nanoparticles may be possible regardless of whether or not resonance conditions are satisfied.
Chapter 3

Quantification of HPLC-separated Peptides and Proteins by Spectrofluorimetric Detection of Native Fluorescence and Mass Spectrometry

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Abstract

Due to relatively low reproducibility of the ionization and differences when using buffers as mobile phases, the quantitative analysis by electrospray ionization mass spectrometry (ESI-MS) can be often challenging. In the present study, the native fluorescence of phenylalanine, tyrosine, and tryptophan was investigated as an improvement tool for the analytical quantification of peptides and proteins by HPLC-ESI-MS. Natively fluorescent amino acids as well as peptides, proteins, and protein digests were successfully separated by HPLC, and quantified with a spectrofluorimetric detector and ESI-MS. The two detectors were connected in series and enabled the sequential measurements of the fluorescence intensities as well as the measurements of the ion signals and mass spectral characterization of separated polypeptides. Fluorescence detector provided better linearity and repeatability of quantification than mass spectrometer, and similar limits of detection for most of biomolecules analyzed.
The fluorescence signal was linear over 3 to 4 orders of magnitude with limits of
detection in picomole or high femtomole range, depending on nature and number of
natively-fluorescent amino acid residues present in the analyzed polypeptides. Hence,
native fluorescence of phenylalanine, tyrosine, and tryptophan can be used as a label-free
methodology to facilitate quantification of peptides and proteins by LC-ESI-MS.

3.1 Introduction

Mass spectrometry is widely applied for identification and structural
countentization of proteins and their post-translational modifications. Most of protein
MS analyses are conducted by ESI and matrix-assisted laser desorption/ionization
(MALDI) mass spectrometry (44,88). To analyze complex proteomic samples, bottom-up
and top-down MS techniques were developed. In the bottom-up approach, a protein
mixture is subjected to enzymatic digestion, and HPLC-MS is then used for separation of
digest peptides and protein identification (89,90). Reversed-phase HPLC (RP-HPLC) in
combination with ESI-MS is most commonly used in such applications. In top-down
approach, a proteomic sample is separated and individual proteins are investigated
directly by MS/MS (89,90).

In addition to qualitative structural analysis, LC-ESI-MS can be used for
quantification of proteins using labeling and label-free techniques. Isotopic labeling is
often used in the case of mass spectrometric quantification of peptides and proteins
(53,54,91). Quantitative analysis can be done using isotopic labeling by amino acids in
cell culture (SILAC) (91), isotope-coded affinity tags (ICAT) (53), and isobaric tags for
relative and absolute quantification (iTRAQ) (54). For example, cysteines that are
isotopically labeled by ICAT reagents can be used for quantification of proteins based on
the presence of doublets in the mass spectra corresponding to “heavy” and “light” isotopes (53). These procedures require expensive isotopic labels and extensive sample preparation protocols. In addition, label-free methodologies have also been reported for protein quantification in biological samples (92,93). In all of these quantification experiments, the mass spectrometers can operate in single stage acquisition mode (94) and single ion recording (SIR) mode (95), or in multiple stage acquisition modes such as low-energy collision-induced dissociation tandem mass spectrometry (CID-MS/MS) and multiple reactions monitoring (MRM) (96-98). Commonly, HPLC enables separation while an ESI mass spectrometer is used for structural characterization and quantification of polypeptides.

However, LC-ESI-MS has its own quantification drawbacks such as ionization suppression and irreproducible ionization especially when different buffers are used as mobile phases (48). Differences among MS instruments (i.e., variability of ion sources and mass analyzers) also complicate comparative quantification. All these factors can affect accuracy and reproducibility of the MS quantification. The addition of another independent detection method could be useful to facilitate detection and quantification of peptides and proteins by LC-MS. The examples of detection of biomolecules using HPLC-MS in combination with fluorescence detection were reported in the literature (48,49,99-103). These studies showed that the analyses of fluorescent biomolecules by LS-ESI-MS and LC-MALDI-MS are feasible. Fluorescent labeling may change ionization efficiency and MS/MS fragmentation patterns of analyzed biomolecules improving their identification (48,49). However, although it may be useful for quantification of peptides and proteins, fluorescent labeling often requires extensive
procedures for tagging, purification, and separation of proteomic samples.

Native fluorescence of tryptophan, tyrosine, and phenyl alanine was used as a label-free methodology for detection of amino acids, peptides, and proteins separated by HPLC (5,7,104) and for quantification of proteins separated by capillary and gel electrophoresis (104,105). Recently, Russell et al. demonstrated quantification of peptides and proteins separated by nano-LC using parallel detection of intrinsic fluorescence of tryptophan and ESI mass spectrometry (62). They constructed an on-capillary fluorescence detection system employing UV-LED as excitation source, and appropriate optics and a multiplier for fluorescence detection. In combination with ESI, this setup permitted parallel fluorescence and MS quantification of tryptophan-containing polypeptides.

In the present study, we have explored how the spectrofluorimetric detection of all three natively fluorescent amino acids (tryptophan, tyrosine, and phenylalanine) can improve LC-ESI-MS quantification of peptides and proteins. To perform these experiments, an HPLC instrument containing a spectrofluorimetric detector was coupled to an ESI-mass spectrometer for the quantification of separated biomolecules. Using model peptides, proteins, and protein digests (Table 3-1), we evaluated this native fluorescence-mass spectrometry methodology for quantitative analyses. Additionally, we compared two detection techniques in terms of sensitivity, limit of detection, repeatability, and dynamic range for quantification of peptides and proteins.
3.2 Experimental

3.2.1 Chemicals

Phenylalanine (>99% purity), tyrosine, tryptophan (>99% purity), MRFA (>90% purity), bradykinin acetate (>98% purity), angiotensin II human (>93% purity), leucine enkephaline, neurotensin (>99% purity), cytochrome C (>95% purity), myoglobin (>90% purity), trypsinogen, sequence grade trypsin, iodoacetamide (IAM), DL-dithiothreitol (DTT) (>95% purity), and high-purity (>95%) formic acid were purchased from Sigma (St. Louis, MO, USA). Neurotensin (8-13), Glu-fibrinopeptide, and MAGE-3 were purchased from AnaSpec (Fremont, CA, USA). HPLC-grade water and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

3.2.2 Sample preparation

Stock solutions of phenylalanine, tyrosine, and tryptophan were prepared in HPLC-grade water at concentrations of 3 mg/ml (18.16 mM), 0.5 mg/ml (2.76 mM), and 1 mg/mL (4.90 mM), respectively. To evaluate the linearity of quantification of these amino acids, the stock solutions were further diluted with water to obtain 10-μl injection aliquots containing from: 7.3 pmole to 73.0 n mole of phenylalanine, 1.1 pmole to 11.0 n mole of tyrosine, and 1.2 pmole to 10.0 n mole of tryptophan.

For peptide analysis, detection limit and linearity check were performed after preparation of the following aqueous stock solutions: 10 mg/mL (19.1 mM) of MRFA, 10 mg/mL (12.3 mM) of neurotensin fragment (8-13), 3 mg/mL (2.8 mM) of bradykinin, 3 mg/mL (2.9 mM) of angiotensin II, 3 mg/mL (1.9 mM) of Glu-fib, 3 mg/mL (5.4 mM) of leucine enkephalin, 10 mg/mL (5.9 mM) of neurotensin, and 1 mg/mL (9.4 mM) of MAGE-3. The standard solutions were further diluted with water to obtain injection...
aliquots containing from: 0.0318 nmole to 19.097 nmole of MRFA, 0.7 pmole to 699.42 pmole of neurotensin fragment, 7.68 pmole to 1.536 nmole of bradykinin, 1.39 pmole to 6.964 nmole of angiotensin, 0.82 pmole to 4.093 nmole of Glu-fib, 1.23 pmole to 6.171 nmole of leucine enkephalin, 1.708 pmole to 1.708 nmole of neurotensin, and 0.240 pmol to 0.472 nmol of MAGE-3.

To determine detection limit and linearity of protein quantification, aqueous stock solutions of cytochrome C (80.8 µM), myoglobin (58.9 µM), and trypsinogen (41.6 µM) were prepared at concentration of 1 mg/mL each. These stock solutions were mixed and further diluted with water to obtain injection aliquots containing from: 0.808 pmole to 266.75 pmole of cytochrome c, 0.416 pmole to 137.38 pmole of trypsinogen, and 0.589 pmole to 194.489 pmole of myoglobin.

For analysis of a protein digest, a stock solution of BSA was prepared at concentration of 5 mg/mL (75.7 µM) and digested using trypsin. Cysteins in the tryptic digest were reduced and alkylated using DTT and IAM, respectively. LOD and linearity of BSA peptide separation is obtained by further diluting BSA digest with water to obtain injection aliquots containing from: 13.5 pmol to 6.7 nmol of peptide with m/z 740.6, 13.8 pmol to 6.9 nmol of peptide with m/z 720.6, and 21.5 pmol to 10.8 nmole of peptide with m/z 927.9.

For quantitative analysis of a complex peptide mixture, solutions of cytochrome C, myoglobin and trypsinogen were prepared at concentration of 1 mg/mL and digested using trypsin. Cysteins in the tryptic digests were reduced and alkylated using DTT and IAM, respectively. Digests of the three proteins were mixed in equal amounts to prepare
a stock solution. LOD and linearity of tryptic peptide separation is obtained by further
diluting the stock solution with water to obtain injection aliquots containing from: 34.0
pmole to 3.4 nmole of peptide with \( m/z \) 736.0, 77.9 pmol to 5.85 nmol of peptide with
\( m/z \) 751.9, and 47.7 pmole to 4.77 nmole of peptide with \( m/z \) 454.7.

### 3.2.3 Instrumentation and data acquisition

All experiments were performed by HPLC-ESI-MS. The HPLC system
(Shimadzu Technologies, Addison, IL, USA) consisted of LC-20AD binary pump, DGU-
20A3 vacuum degasser, SIL-20A auto sampler, RF-10AXL fluorescence detector, and
SCL-10A VP system controller. The flow rate was 0.2 mL/min and injection volume was
10 µL. HPLC data were acquired using LC Solution software (Shimadzu). The
fluorescence signal was sampled at a rate of 3.33 Hz. Fluorescence detector allowed
adjustments of the excitation and emission wavelengths during the run while excitation
and emission slits had fixed bandwidths of 15 nm.

HPLC was connected to a quadrupole time-of-flight (Q-TOF) mass
spectrometer (Q-TOF Micro, Waters, Milford, MA, USA). MS data were acquired using
MassLynx software, version 4.1 (Waters). MassLynx was also used for integration of
baseline-separated chromatographic peaks in total ion cromatograms (TICs) of amino
acids, peptides, and proteins, and for extraction and integration of tryptic peptide peaks in
extracted ion chromatograms (EICs). ESI-MS experiments were performed in positive
ion mode. The MS scan time was 1.0 s and the interscan time was set to 0.1 s.
Desolvation temperature was 350ºC and desolvation gas flow rate was 650 L/hr. The
MCP detector voltage was set at 2.35 kV. All HPLC-MS quantitative analyses were done
in triplicates, while repeatability experiments were repeated ten times.
3.2.4 LC-fluorescence-MS analysis of amino acids

Separation of amino acids was performed on an analytical scale C18 column (Everest™ 250 × 2.1 mm, Vydac, Deerfield, IL, USA). The mobile phase A was HPLC-grade water containing 1% formic acid and mobile phase B was acetonitrile containing 0.85% formic acid. Elution gradient was: 0-6.5 min 5% of B, 6.5-11 min 18% of B, 11-12 min 90% of B, 12-14 min 90% of B, 14-15 min 5% of B, and the run was stopped at 28.1 minutes. Excitation and emission wavelengths used for fluorescence detection were respectively 274 nm and 304 nm for 5.15 minutes, 260 nm and 295 nm from 5.15 to 7.50 minutes, and 280 nm and 348 nm from 7.50 to 15 minutes. Excitation and emission wavelengths were switched back to 274 nm and 304 nm during the rest of the run. The gain and sensitivity of fluorescence detector were set to 1X and low, respectively.

ESI-MS was performed in the m/z range from 50 to 350. The spray voltage was set at 3.0 kV and sample cone voltage was 30 V. The source temperature was 120°C and collision energy was set at 4 V.

3.2.5 LC-fluorescence-MS analysis of peptides

Separation of peptides was also performed on an analytical scale C18 column (Everest™ 250 × 2.1 mm, Vydac). The mobile phase A was HPLC-grade water containing 0.8% formic acid and mobile phase B was acetonitrile containing 0.7% formic acid. Elution gradient was: 0-2 min 12.5% of B, 2-20 min 13.5% of B, 20-28 min 15.5% of B, 28-36 min 35% of B, 36-40 min 90% of B, 40-42 min 90% of B, 42-45 min 5% of B, and the run was stopped at 58.2 minutes. Excitation and emission wavelengths used for fluorescence detection were respectively 274 nm and 304 nm for 7.0 minutes, 260 nm and 290 nm for 5 minutes, 274 nm and 304 nm for 3.3 minutes, 260 nm and 290 nm for
15.90 minutes, and 274 nm and 304 nm for the rest of the run. For separation of MAGE-3, elution gradient was: 0-2 min 25% of B, 2-6 min 45% of B, 6-10 min 49% of B, 10-13 min 90% of B, 13-15 min 90% of B, 15-17 min 5% of B, and run was stopped at 27.2 minutes. Excitation and emission wavelengths used for fluorescence detection of MAGE-3 were 280 nm and 348 nm. The gain and sensitivity of fluorescence detection were set to 1X and low, respectively.

ESI-MS analysis of peptides was performed in the \( m/z \) range from 50 to 1800. The spray voltage was set at 3.1 kV and sample cone voltage was 44 V. The source temperature was 120\(^\circ\)C and collision energy was set at 10 V.

### 3.2.6 LC-fluorescence-MS analysis of proteins

Separation of proteins was performed on a C4 column (Jupiter 250 × 2.0 mm, Phenomenex, Torrance, CA, USA). The mobile phase A was HPLC-grade water containing 3.7% formic acid and mobile phase B was acetonitrile containing 3.15% formic acid. Elution gradient was: 0-3 min 5% of B, 3-5 min 27% of B, 5-26 min 37% of B, 26-45 min 41% of B, 45-49 min 45% of B, 49-50 min 90% of B, 50-51 min 90% of B, 51-52 min 5% of B, and run was stopped at 65.1 minutes. Excitation and emission wavelengths used for fluorescence detection were 274 nm and 304 nm, respectively. Fluorescence detector was set at 1 X gain and at medium sensitivity.

ESI-MS was performed in the \( m/z \) range from 800 to 2200. The spray voltage was set at 3.0 kV and sample cone voltage was 30 V. The source temperature was 120\(^\circ\)C and collision energy was 4 V.
3.2.7 LC-fluorescence-MS analysis of BSA digest

Separation of peptides from BSA digest was performed on a C18 column, which was used previously for separations of amino acids and peptides (subsections 2.4 and 2.5). The mobile phase A was HPLC-grade water containing 3.7% formic acid and mobile phase B was acetonitrile containing 3.15% formic acid. Elution gradient was: 0-5 min 5% of B, 5-104 min 35% of B, 104-110 min 90% of B, 110-118 min 5% of B, and run was stopped at 130.5 minutes. Excitation and emission wavelengths used for fluorescence detection were 274 nm and 304 nm, respectively. Fluorescence detector was set at 4X gain and medium sensitivity.

ESI-MS was performed in the m/z range from 300 to 1800. The spray voltage was set at 3.0 kV and sample cone voltage was 30 V. The source temperature was 120ºC and collision energy was 4 V.

3.2.8 LC-fluorescence-MS analysis of cytochrome C, myoglobin and trypsinogen digests

Separation and detection of cytochrome C, myoglobin and trypsinogen digests were performed under the same conditions as described for the analysis of BSA digest (subsection 2.7).
3.3 Results and discussion

3.3.1 General considerations

Tryptophan, tyrosine, and phenylalanine exhibit ultraviolet light-induced fluorescence (UV-IF), but have different absorption coefficients and fluorescence quantum yields (8). Tryptophan has an absorption coefficient of $5.7 \times 10^3$ M$^{-1}$cm$^{-1}$ and quantum yield of 0.14. It is commonly excited at 280 nm and its emission maximum is at 348 nm. Tyrosine has an absorption coefficient of $1.6 \times 10^3$ M$^{-1}$cm$^{-1}$ and quantum yield of 0.13. Its excitation maximum is at 274 nm and its emission maximum is at 324 nm. Phenylalanine has an absorption coefficient of $0.4 \times 10^3$ M$^{-1}$cm$^{-1}$ and quantum yield of 0.02, while its excitation and emission maxima are at 260 nm and 295 nm, respectively. The fluorescence properties of tryptophan enable the detection and quantification of peptide and proteins containing this amino acid upon LC separation (7,62). Since tryptophan is more fluorescent, it is possible to achieve a better response of its fluorescence detection than for tyrosine and phenylalanine. However, tryptophan is a rare amino acid in a proteome (62) and it would be beneficial to detect and quantify peptides and proteins containing all natively fluorescent amino acids. A spectrofluorimetric detector enables selective detection and quantification of fluorescence originating from all three natively fluorescent amino acids as well as from peptides and proteins containing them. In present study, a spectrofluorimetric detector was coupled to an ESI mass spectrometer and used for quantification of HPLC-separated biomolecules whose amino acid compositions and molecular weights are shown in Table 3-1.
Table 3-1. Amino acid sequences\(^a\) and molecular weights\(^b\) of analyzed biomolecules.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Amino acid sequence</th>
<th>(M_r) (exp)</th>
<th>(M_r) (theor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>Trp (W)</td>
<td>204.9</td>
<td>204.1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr (Y)</td>
<td>181.8</td>
<td>181.1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe (F)</td>
<td>165.8</td>
<td>165.1</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>Asp-Arg-Val-Tyr-Ile-His-Pro-Phe</td>
<td>1045.6</td>
<td>1046.18</td>
</tr>
<tr>
<td></td>
<td>(DRVYIHPF)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bradykinin Acetate</td>
<td>Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg</td>
<td>1060.7</td>
<td>1060.21</td>
</tr>
<tr>
<td></td>
<td>(RPPGFSPFR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine enkephalin</td>
<td>Tyr-Gly-Gly-Phe-Leu (YGGFL)</td>
<td>554.93</td>
<td>555.62</td>
</tr>
<tr>
<td>[Glu]-Fibrinopeptide B</td>
<td>Glu-Gly-Asp-Asn-Glu-Glu-Gly-Phe-Ser-Ala-Arg (EGVNDNEEGFFSAR)</td>
<td>1569.7</td>
<td>1570.6</td>
</tr>
<tr>
<td>MRFA</td>
<td>Met-Arg-Phe-Ala (MRFA)</td>
<td>522.73</td>
<td>523.65</td>
</tr>
<tr>
<td>Neurotensin (8-13)</td>
<td>Arg-Arg-Pro-Tyr-Ile-Leu (RRPYIL)</td>
<td>816.54</td>
<td>816.99</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Pro-Tyr-Ile-Leu (ELYENKPRRPYIL)</td>
<td>1671.97</td>
<td>1672.92</td>
</tr>
<tr>
<td>MAGE-3</td>
<td>Phe-Leu-Trp-Gly-Pro-Arg-Ala-Leu-Val (FLWGPRLV)</td>
<td>1058.09</td>
<td>1059.09</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>F (4), Y (4), W (1)</td>
<td>12388(^c)</td>
<td>12384(^c)</td>
</tr>
<tr>
<td>Trpsinogen</td>
<td>F (5), Y (12), W (5)</td>
<td>23951(^c)</td>
<td>23981(^c)</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>F (4), Y (1), W (2)</td>
<td>16970(^c)</td>
<td>16934(^c)</td>
</tr>
<tr>
<td>BSA Peptide (927.9)</td>
<td>Tyr-Leu-Tyr-Glu-Ile-Ala-Arg (YLVEIAR)</td>
<td>926.95</td>
<td>928.08</td>
</tr>
<tr>
<td>BSA Peptide (720.6)</td>
<td>Arg-His-Pro-Glu-Tyr-Ala-Val-Ser-Val-Leu-Arg (RHEYAVSVLLR)</td>
<td>1439.3</td>
<td>1440.7</td>
</tr>
<tr>
<td>BSA Peptide (740.6)</td>
<td>Leu-Gly-Glu-Tyr-Gly-Phe-Gin-Ala-Leu-Ile-Val-Arg (LGEYGQNALIYR)</td>
<td>1479.3</td>
<td>1480.7</td>
</tr>
<tr>
<td>Tryp (454.7)</td>
<td>Asn-Lys-Pro-Gly-Val-Tyr-Thr-Lys (NKPGVYTK)</td>
<td>907.4</td>
<td>905.5</td>
</tr>
<tr>
<td>Myo (751.9)</td>
<td>Hys-Pro-Gly-Asp-Phe-Gly-Ala-Asp-Ala-Gln-Gly-Ala-Met-Thr-Lys (HPGDFGADAQGAMTK)</td>
<td>1501.8</td>
<td>1501.7</td>
</tr>
<tr>
<td>Cyt C (736.0)</td>
<td>Thr-Gly-Gln-Ala-Pro-Gly-Phe-Thr-Tyr-Thr-Asp-Ala-Asn-Lys (TGQAPGFTYTDANK)</td>
<td>1470.0</td>
<td>1469.7</td>
</tr>
</tbody>
</table>

\(^a\) Amino acid sequences were either obtained from manufacturers or using UniProt database available at [www.expasy.org](http://www.expasy.org).

\(^b\) Experimental and theoretical molecular weights correspond to monoisotopic masses.

\(^c\) Experimental and theoretical molecular weights correspond to average masses.
Figure 3-1. HPLC separation of amino acids. Amino acids were detected by (A) a fluorescence detector at respective excitation and emission wavelengths for each amino acid and (B) ESI-MS.
3.3.2 Quantification of natively fluorescent amino acids by LC-fluorescence-MS

Initial LC-fluorescence-MS experiments involved separation and quantification of natively fluorescent amino acids. Tyrosine, phenylalanine, and tryptophan were separated from their mixture by HPLC with fluorescence-detected retention times of 4.3, 5.9, and 10.2 min, respectively (Figure 3-1). Retention times and peak widths recorded by mass spectrometer were similar to those recorded by fluorescence detector, i.e., no significant retention time increase and band broadening were observed in MS-detected chromatograms. Singly-charged protonated ions corresponding to natively fluorescent amino acids were detected by ESI-MS and used to determine their molecular weights (Table 3-1). For quantification, calibration curves were plotted using 9 standard solutions of different concentration, and linear regression analyses of peak areas corresponding to native fluorescence (Figure 3-2A) and MS (Figure 3-2B) signals of amino acids were performed. Since amino-acids were baseline separated and underwent fragmentation during ESI, TICs were used to measure areas of peaks in MS chromatograms. A good linear relationship was found for both fluorescence and MS detection in the range between ~1 pmole and 73 nmole, as indicated by correlation coefficients ($R^2>0.99$) for all calibration curves (Figure 3-2 and Table 3-2).
Figure 3-2. Calibration curves prepared upon analysis of standard solutions of amino acids by (A) a fluorescence detector and (B) ESI-MS.

Calibration curves were also used to determine the limits of fluorescence and MS detection, which represent chromatographic peaks with signal-to-noise ratios of ~3.
Concurrently, lower limit of quantification, which is the lowest concentration of analyte that produces a chromatographic signal distinguishable from background noise with a minimum ratio of 10:1, can be determined. LOD values for both, fluorescence and MS quantification of amino acids are given in Table 2. LOD is one order of magnitude lower for fluorescence quantification of tyrosine and tryptophan in comparison to their quantification by MS. Better limit of fluorescence detection for tyrosine and tryptophan was expected because these amino acids are more fluorescent (i.e., have higher molar absorptivities and quantum yields) than phenylalanine. However, MS shows one order of magnitude better LOD than fluorescence for quantification of phenylalanine. This is due to higher ionization efficiency of phenylalanine in comparison to tyrosine and tryptophan.

Linear regression equations for calibration curves obtained using HPLC with fluorescence and ESI-MS detection are represented in table A-1.

The repeatability of fluorescence (Figure 3-3A) and MS (Figure 3-3B) measurements was also determined. For all amino acids, a total of 10 HPLC runs were replicated on a sample under optimal separation conditions described in subsection 3.2.4. Repeatability of the amino acid quantification was greater for fluorescence than for MS detection as represented by relative standard deviation values shown in Table 3-2. Additionally, dynamic range of UV-IF was linear over 4 orders of magnitude for tryptophan and over 3 orders of magnitude for tyrosine and phenyl alanine. Linear dynamic range of MS detection was three orders of magnitude. Above results indicate that fluorescence detector in series with MS can be successfully used for quantitative analysis of amino acids. Calibration curves shown in Figure 3-2 can be used for determination of unknown concentration of a natively fluorescent amino acid that are
separated using RP-HPLC gradient described in subsection 3.2.4.

Figure 3-3. The repeatability of (A) fluorescence and (B) ESI-MS detection of HPLC-separated amino acids measured for 10 replicates.
3.3.3 Quantification of peptides by LC-fluorescence-MS

To further evaluate LC-native fluorescence-MS quantification methodology, a mixture of 7 peptides containing phenylalanine and tyrosine was separated by RP-HPLC (Figure 3-4). Retention times of MRFA (1 Phe), neurotensin (8-13) (1 Tyr), bradykinin (2 Phe), angiotensin II (1 Phe and 1 Tyr), Glu-fib (2 Phe), leucine enkphalin (1Phe and 1 Tyr), and neurotensin (2 Tyr) were 10.4, 13.7, 16.8, 26.1, 30.6, 34.1, and 37.7 min, respectively. Peptides were detected by MS as singly- and doubly-charged ions, and their measured molecular weights were close to theoretical values (Table 3-1). Calibration curves were plotted using 9 standard solutions of different concentrations. Linear regression analysis was performed of peak areas corresponding to fluorescence signal (Figure 3-5A) and total ion count (Figure 3-5B) versus peptide concentration. A good linear relationship was found over the investigated concentration range, as indicated by correlation coefficients ($R^2>0.995$) for all standard peptides (Table 3-2). UV-IF dynamic range was linear over 3-4 orders of magnitude with high femtomole LOD for tyrosine containing peptides. Limit of MS detection is either lower or similar to limit of fluorescence detection for all peptides but neurotensin, which showed lower LOD for fluorescence (Table 3-2). Linear regression equations for calibration curves obtained using HPLC with fluorescence and ESI-MS detection are represented in table A-1.
Figure 3-4. HPLC separation of a peptide mixture. Peptides were detected by: (A) a fluorescence detector at specific excitation and emission wavelengths for each peptide and (B) ESI-MS.
Hence, the highest sensitivity of fluorescence detection (slope of the calibration curve in Figure 3-5) was achieved for neurotensin, which contains two tyrosine residues. Neurotensin fragment (8-13), which contains single tyrosine residue, showed higher sensitivity than peptides that contain one phenyl alanine and one tyrosine residue (leucine enkephalin and angiotensin). This can be due to earlier elution of the former peptide and decreased quenching of its fluorescence by the increase of acetonitrile amount in the mobile phase (62). While they were close (within factor of ~1.6 times), calibration curves for leucine enkephalin and angiotensin did not overlap. However, bradykinin and Glu-fib, which contain two phenylalanine residues, showed overlapping calibration curves. Fluorescence detection of MRFA was the least sensitive because this peptide contains only one phenylalanine. As pointed out previously (6,62), fluorescence signal of the peptides may be affected by primary sequence of the peptides as well as composition of HPLC mobile phase.

After fluorescence and MS quantification of peptides containing tyrosine and phenylalanine, the quantification of a tryptophan-containing peptide MAGE-3 was performed. Retention time for MAGE-3 peptide was 10.8 min. Due to the presence of tryptophan, LOD of MAGE-3 (0.24 pmol) was lower than LOD of neurotensin (0.30 pmol), although latter peptide contains 2 tyrosine residues. Again, the peptide sequence and mobile phase composition may affect comparison of native fluorescence of different peptides. However, it is beneficial to detect and quantify peptides containing all natively fluorescent amino acids.
Figure 3-5. Calibration curves prepared upon analysis of peptide standard solutions by (A) a fluorescence detector and (B) ESI-MS.

As in the case of amino acid separation, the repeatability of peptide detection was
better for fluorescence detection (Figure 3-6A) than for MS detection (Figure 3-6B) as represented by relative standard deviations values (Table 3-2). In general, separations of studied peptides are characterized by slightly lower LOD for MS detection and better repeatability and linearity of fluorescence detection. UV-IF linear dynamic range of 3 orders of magnitude was found for all peptides except for neurotensin, for which linear dynamic range was 4 orders of magnitude. Linear dynamic range of MS quantification was 3 orders of magnitude.
Figure 3-6. The repeatability of (A) fluorescence and (B) ESI-MS detection of HPLC-separated peptides measured for 10 replicates.
3.3.4 Quantification of proteins by LC-fluorescence-MS

To monitor UV-IF of HPLC-separated proteins, a mixture of three proteins was investigated. Fluorescence- and MS-detected chromatograms for separation of cytochrome c, myoglobin, and trypsinogen are shown in Figure 3-7, and retention times for these proteins were 17.7, 24.2, and 37.1 min, respectively. Molecular weights of these proteins (Table 3-1) were determined from the series of multiply-charged ESI-MS ions. Linearity was checked upon the linear regression analysis of peak areas versus proteins quantities (Figure 3-8). A good linear relationship was found over the investigated concentration range for UV-IF, as indicated by correlation coefficients ($R^2>0.99$) for all calibration curves (Table 3-2). LOD values for both fluorescence and MS detection of proteins are presented in Table 3-2. LODs were very similar for MS and fluorescence detection, while a better repeatability is observed for UV-IF than for MS (Figure 3-9) as represented by standard deviation values for 10 replicates (Table 3-2). Linear dynamic ranges were similar for UV-IF and MS detection (~3 orders of magnitude). These results confirm that UV-IF is a promising technique for the quantification of intact proteins by LC-ESI-MS (62). Linear regression equations for calibration curves obtained using HPLC with fluorescence and ESI-MS detection are represented in table A-1.
Figure 3-7. HPLC separation of proteins. Proteins were detected by (A) a fluorescence detector using excitation wavelength of 274 nm and emission wavelength of 304 nm and (B) ESI-MS.
Figure 3-8. Calibration curves for quantification of HPLC-separated proteins prepared upon analysis of standard protein solutions by (A) a fluorescence detector and (B) ESI-MS.
Figure 3-9. The repeatability of (A) fluorescence and (B) ESI-MS detection of HPLC-separated proteins measured for 10 replicates.

3.3.5 LC-fluorescence-MS quantification of BSA digest

To quantify peptides in a biologically relevant sample, protein BSA, which
contains varying number of phenylalanine (28), tyrosine (21), and tryptophan (3) residues, was digested by trypsin and BSA peptide digest was analyzed by LC-fluorescence-MS. Three peptides with retention times of 40.2, 53.1, and 65.3 min (Figure 3-10) were chosen for further LOD, linearity, and repeatability studies. Peptide ion with $m/z$ 927.9 ($t_R$=40.2 min) contained 2 tyrosine residues, peptide ion with $m/z$ 720.6 ($t_R$=53.1 min) contained 1 tyrosine, and peptide ion with $m/z$ 740.6 ($t_R$=65.3 min) contained 1 phenylalanine and 1 tyrosine. These peptides were chosen for two main reasons: 1) they were separated from all other peptides (Figure 3-10) and 2) they contained the same number of phenylalanine and tyrosine residues as 3 peptide standards analyzed previously (Table 3-1 and subsection 3.3.3).

Linearity was determined with the linear regression analysis of peak areas versus protein amounts (Figure 3-11). A good linear relationship was found over the investigated concentration range for all three calibration curves (Table 3-2). UV-IF $R^2$ values exceeded 0.999, with similar responses for all three peptides selected. Nonetheless, $R^2$ values were comparable in the case of MS approach. The UV-IF linear dynamic range was also comparable to that of MS in the studied concentration range (~3 orders of magnitude). Linear regression equations for calibration curves obtained using HPLC with fluorescence and ESI-MS detection are represented in table A-1. LOD values for both, fluorescence and MS detection of tryptic peptides are given in Table 3-2. LOD is slightly lower for fluorescence than for MS. Also, the repeatability of fluorescence detection superseded MS detection for 10 replicates as shown in Figure 3-12 and represented by relative standard deviations values shown in Table 3-2.
Figure 3-10. HPLC separation of BSA tryptic digest. Peptides were detected by (A) a fluorescence detector using excitation wavelength of 274 nm and emission wavelength of 304 nm and (B) ESI-MS.
Figure 3-11. Calibration curves for quantification of three peptides from BSA tryptic digest. Peptides were separated by HPLC and detected by (A) native fluorescence and (B) MS.
Figure 3-12. The repeatability of (A) fluorescence and (B) ESI-MS detection of HPLC-separated BSA peptides measured for 10 replicates.
Quantitative LC-fluorescence-MS analyses of pure peptides and peptide digests (Figures 3-5 and 3-11, respectively) indicate that linear dynamic ranges are similar but sensitivities of peptide detection are higher for pure peptides. The differences in sensitivities are mainly because of different separation and ionization conditions used, and partially due to miscleavages present in tryptic digests, which decrease effective concentrations of peptides. Digest peptides containing the same number of natively fluorescent amino acids as pure peptides were separated in a mobile phase that had a lower pH than mobile phase used for separations of pure peptides (as described in subsections 3.2.7. and 3.2.5.). Hence, detection sensitivity is decreased for digest peptides because their fluorescence is quenched more than fluorescence of pure peptides during the separation. Nevertheless, in-series fluorescence and MS detection will be suitable for quantitative analyses of proteins from complex tryptic digests if calibration curves are constructed using digests of individual pure proteins and HPLC analyses are performed under similar separation conditions.

3.3.6 LC-fluorescence-MS quantification of a complex protein digest

To quantify proteins in a more complex mixture, cytochrome C, myoglobin and trypsinogen were digested with trypsin, and resulting peptides were analyzed by LC-fluorescence-ESI-MS. Three peptides with retention times of 12.6, 25.1, and 28.6 min (Figure 3-13) were chosen for further LOD, linearity, and repeatability studies. Doubly-charged peptide ion with \( m/z 454.7 \) (\( t_R=12.6 \) min) originated from trypsinogen and contained 1 tyrosine, while doubly-charged peptide ion with \( m/z 751.9 \) (\( t_R=25.1 \) min) originated from myoglobin and contained 1 phenylalanine. Doubly-charged peptide ion with \( m/z 736.0 \) (\( t_R=28.6 \) min) originated from cytochrome C and contained 1 tyrosine and
1 phenylalanine residue.

**Figure 3-13.** HPLC separation of tryptic digest of cytochrome C, myoglobin, and trypsinogen. Peptides were detected by (A) a fluorescence detector using excitation wavelength of 274 nm and emission wavelength of 304 nm and (B) ESI-MS.
Figure 3-14. Calibration curves for quantification of three peptides from tryptic digest of cytochrome C, myoglobin and trypsinogen. Peptides were separated by HPLC and detected by (A) native fluorescence and (B) MS.
Figure 3-15. The repeatability of (A) fluorescence and (B) ESI-MS detection of three peptides from tryptic digest of cytochrome C, myoglobin and trypsinogen measured for 10 replicates.
Linearity of quantification was determined with the linear regression analysis of peak areas versus protein amounts (Figure 3-14). The highest detection sensitivity was achieved for peptide originating from myoglobin, which contains 1 tyrosine and 1 phenylalanine residue. A good linear relationship was found over the investigated concentration range for all three calibration curves (Table 3-2). UV-IF $R^2$ values exceeded 0.99 and were slightly better than $R^2$ values obtained from MS calibration curves. The UV-IF linear dynamic range was also comparable to that of MS in the studied concentration range (~3 orders of magnitude). LOD values of MS detection were similar to LODs of fluorescence detection (Table 3-2). But, the repeatability of fluorescence detection superseded MS detection for 10 replicates as shown in Figure 3-15 and represented by relative standard deviations values shown in Table 3-2.

It is important to note that the accuracy of quantification of proteins from the complex peptide mixtures by detection of native fluorescence depends on the resolution of HPLC separation. As shown in Figure 3-16 and Table 3-3, many natively fluorescent peptides were detected, but some of them were not separated with resolution that will allow accurate quantitative analysis. For example, peptides 9 and 10 in Table 3-3 were not separated by LC but were distinguished based on MS data and retention times in extracted ion chromatograms. However, spectrofluorimetric detector can selectively detect peptides containing any of the three natively fluorescent amino acids, and, in this way, simplify HPLC chromatograms, and enable more specific quantification of proteins. Such selectivity complements well to the ability of LC-ESI-MS to quantify peptides and proteins using extracted ion chromatograms.
Table 3-2. Limits of detection, correlation coefficients of linearity, and standard deviations of repeatability determined for native fluorescence and MS quantification experiments.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LOD</th>
<th>LOD</th>
<th>R²</th>
<th>R²</th>
<th>Repeatability</th>
<th>Repeatability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(MS)</td>
<td>(Fluo.)</td>
<td>(MS)</td>
<td>(Fluo.)</td>
<td>(MS)</td>
<td>(Fluo.)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>11.0</td>
<td>2.20</td>
<td>0.997</td>
<td>0.999</td>
<td>10.1%</td>
<td>1.52%</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>7.30</td>
<td>73.0</td>
<td>0.995</td>
<td>0.999</td>
<td>4.79%</td>
<td>3.56%</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>12.0</td>
<td>1.20</td>
<td>0.996</td>
<td>0.999</td>
<td>11.8%</td>
<td>0.81%</td>
</tr>
<tr>
<td>Bradykinin acetate</td>
<td>1.50</td>
<td>13.0</td>
<td>0.995</td>
<td>0.997</td>
<td>3.29%</td>
<td>1.69%</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>1.40</td>
<td>12.0</td>
<td>0.998</td>
<td>0.999</td>
<td>3.11%</td>
<td>0.65%</td>
</tr>
<tr>
<td>[Glu]-Fibrinopeptide</td>
<td>0.80</td>
<td>14.0</td>
<td>0.998</td>
<td>0.998</td>
<td>3.36%</td>
<td>3.26%</td>
</tr>
<tr>
<td>Leucine enkephalin</td>
<td>1.20</td>
<td>1.20</td>
<td>0.999</td>
<td>0.999</td>
<td>2.98%</td>
<td>0.44%</td>
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<tr>
<td>MRFA</td>
<td>3.80</td>
<td>31.8</td>
<td>0.995</td>
<td>0.999</td>
<td>2.16%</td>
<td>2.22%</td>
</tr>
<tr>
<td>Neurotensin (8-13)</td>
<td>0.70</td>
<td>0.70</td>
<td>0.996</td>
<td>0.999</td>
<td>3.29%</td>
<td>0.81%</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>1.70</td>
<td>0.30</td>
<td>0.999</td>
<td>0.999</td>
<td>2.40%</td>
<td>2.91%</td>
</tr>
<tr>
<td>MAGE-3 (Trp)</td>
<td>0.47</td>
<td>0.24</td>
<td>0.997</td>
<td>0.998</td>
<td>4.73%</td>
<td>0.16%</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>0.24</td>
<td>0.40</td>
<td>0.999</td>
<td>0.999</td>
<td>2.76%</td>
<td>1.16%</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>0.60</td>
<td>0.71</td>
<td>0.999</td>
<td>0.999</td>
<td>4.20%</td>
<td>2.43%</td>
</tr>
<tr>
<td>Trypsinogen</td>
<td>0.21</td>
<td>0.40</td>
<td>0.998</td>
<td>0.999</td>
<td>6.62%</td>
<td>5.04%</td>
</tr>
<tr>
<td>BSA Pep (927.9 m/z)</td>
<td>107.7</td>
<td>21.5</td>
<td>0.997</td>
<td>0.999</td>
<td>4.51%</td>
<td>0.41%</td>
</tr>
<tr>
<td>BSA Pep (720.6 m/z)</td>
<td>69.4</td>
<td>69.4</td>
<td>0.995</td>
<td>0.999</td>
<td>5.32%</td>
<td>1.99%</td>
</tr>
<tr>
<td>BSA Pep (740.6 m/z)</td>
<td>67.6</td>
<td>27.0</td>
<td>0.999</td>
<td>0.999</td>
<td>5.46%</td>
<td>0.85%</td>
</tr>
<tr>
<td>Cyto C (m/z 736.0)</td>
<td>34.0</td>
<td>45.4</td>
<td>0.993</td>
<td>0.997</td>
<td>9.91%</td>
<td>5.10%</td>
</tr>
<tr>
<td>Myo (m/z 751.9)</td>
<td>266.3</td>
<td>133.2</td>
<td>0.991</td>
<td>0.998</td>
<td>12.7%</td>
<td>8.59%</td>
</tr>
<tr>
<td>Tryp (m/z 454.7)</td>
<td>147.4</td>
<td>110.5</td>
<td>0.997</td>
<td>0.997</td>
<td>5.24%</td>
<td>4.67%</td>
</tr>
</tbody>
</table>
Figure 3-16. HPLC separation of tryptic digest of cytochrome C, myoglobin, and trypsinogen. Peptides were detected by (A) a fluorescence detector using excitation wavelength of 274 nm and emission wavelength of 304 nm and (B) ESI-MS. Amino acid sequences of numbered natively fluorescent peptides are shown in Table 3-3.
Table 3-3. $m/z$ values, retention times and amino acid sequences of natively fluorescent peptides separated by HPLC (Figure 3-16) from the digest mixture of cytochrome C, myoglobin and trypsinogen. Retention times correspond to those detected by fluorescence detector.

<table>
<thead>
<tr>
<th>Peak #</th>
<th>$m/z$ (charge)</th>
<th>$t_R$(min)</th>
<th>Protein</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>454.7 (+2)</td>
<td>12.59</td>
<td>Trypsinogen</td>
<td>NKPGVYTK</td>
</tr>
<tr>
<td>2</td>
<td>800.0 (+2)</td>
<td>22.90</td>
<td>Cytochrome C</td>
<td>KTGQAPGFTYTDANK</td>
</tr>
<tr>
<td>3</td>
<td>751.9 (+2)</td>
<td>25.08</td>
<td>Myoglobin</td>
<td>HPGDFGADAQGAMTK</td>
</tr>
<tr>
<td>4</td>
<td>736.0 (+2)</td>
<td>28.62</td>
<td>Cytochrome C</td>
<td>TQAPGFTYTDANK</td>
</tr>
<tr>
<td>5</td>
<td>472.2 (+2)</td>
<td>33.88</td>
<td>Myoglobin</td>
<td>YKELGFQG</td>
</tr>
<tr>
<td>6</td>
<td>577.7 (+2)</td>
<td>35.60</td>
<td>Trypsinogen</td>
<td>SSGTSYPDVLK</td>
</tr>
<tr>
<td>7</td>
<td>748.6 (+1)</td>
<td>51.01</td>
<td>Myoglobin</td>
<td>ALELFR</td>
</tr>
<tr>
<td>8</td>
<td>585.2 (+2)</td>
<td>53.21</td>
<td>Cytochrome C</td>
<td>TGPNLHGLFGR</td>
</tr>
<tr>
<td>9</td>
<td>758.9 (+3)</td>
<td>76.11*</td>
<td>Trypsinogen</td>
<td>SIVHPSYNSTNLNDIMLIK</td>
</tr>
<tr>
<td>10</td>
<td>812.6 (+2)</td>
<td>76.11*</td>
<td>Cytochrome C</td>
<td>EETLMYELENPKK</td>
</tr>
<tr>
<td>11</td>
<td>748.5 (+2)</td>
<td>86.40</td>
<td>Cytochrome C</td>
<td>EETLMYELENPK</td>
</tr>
<tr>
<td>12</td>
<td>694.9 (+3)</td>
<td>92.86</td>
<td>Cytochrome C</td>
<td>GITWKEETLMYELENPK</td>
</tr>
<tr>
<td>13</td>
<td>629.3 (+3)</td>
<td>99.27</td>
<td>Myoglobin</td>
<td>YLEFISDAIIHVLHSK</td>
</tr>
</tbody>
</table>

*Peptides were not separated by LC but were distinguished based on MS data and retention times in extracted ion chromatograms.
3.4 Conclusions

In this study, a hybrid methodology was developed for sequential spectrofluorimetric and ESI-mass spectrometric quantification of HPLC-separated peptides and proteins that contain any of the three natively fluorescent amino acids (tryptophan, tyrosine, and phenylalanine). A spectrofluorimetric detector is used to selectively detect and quantify natively fluorescent polypeptides, while ESI-MS must be used to confirm their structural identities. Native fluorescence detection exhibited more linear and reproducible quantification of polypeptides than MS, while LODs and linear dynamic ranges for analyzed biomolecules were similar as in ESI-MS. Therefore, UV-IF can facilitate more accurate quantification of peptides and proteins by LC-ESI-MS.

Present methodology complements well to a nano-LC-fluorescence-ESI-MS quantification technique described previously (62). Although the ease of use and wider availability are advantages of standard HPLC, capillary and nano-LC improve LC-MS sensitivity and often yield better separation efficiencies than analytical HPLC. Spectrofluorimetric detection approach presented in this study can be adopted to capillary and nano-LC-MS analyses and used with LC-MS/MS quantitative methodologies. Future experiments will be aimed toward native fluorescence-MS analysis of more complex mixtures of peptides and proteins.
Chapter 4

Effect of Dye Labeling on Ionization Efficiency and Charging of Peptides and Proteins analyzed by ESI-MS

4.1 Introduction

The use of mass spectrometry with or without liquid chromatography is now routine for mass profiling and identification and characterization of peptide and protein mixtures. However, wide variation in signal intensities of peptides in peptide mixtures influences the sequence coverage in identification of proteins. Derivatization-based strategies have been developed for proteomic analysis due to large variations in peptide signal intensities in mass spectrometry. It has been shown that derivatized peptides and proteins produce increased MS signals in MALDI (106,107), as well as enhanced fragmentation in MS/MS (108). This could be due to stability of the protonated complex in the gas phase (109). Additionally, it has recently been shown that labeling of peptides and proteins with coumarin tags increases MALDI-MS signal intensities and helps in the identification and characterization of peptides (49,110). Several examples of detection and quantification using HPLC with fluorescence detection were reported in the literature (111-114). However, LC with fluorescence detection may not provide sufficient information about the structural characterization of molecules. A combined fluorescent
and mass spectrometric analysis procedure for detection and characterization of analytes has also been described in the literature (48,49).

Here, the effect of dye labeling on electrospray ionization efficiency and charge of peptides and proteins was investigated. The peptides and proteins were labeled with fluorescein isothiocyanate (FITC) due to its high quantum yield (0.95). FITC is a fluorophore that selectively binds to primary amino groups, for example the protein’s N-terminus and lysine’s side chain amino group, leading to the formation of highly fluorescent derivatives. The labeled and unlabeled peptides and proteins were analyzed using LC-ESI-Q-TOF-MS and ESI-ion trap-MS.

4.2 Materials and Methods

4.2.1 Materials

1X PBS, methanol, chloroform, ethanol, HPLC grade water and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ). FITC, acetic acid, and high purity (>95%) formic acid were purchased from Sigma (St. Louis, MO). Bradykinin acetate (>98% purity), angiotensin II human (>93% purity), leucine enkephaline, and Glu-[Fib] were also purchased from Sigma-Aldrich (St. Louis, MO) and were reconstituted in water for immediate use. Quartz cuvettes were purchased from World Precision Instruments, Inc. (Sarasota, FL). C18-Sep-Pak solid phase extraction cartridges were purchased from Waters (Franklin, MA).
4.2.2 Methods

4.2.2.1 Development of a LC-fluorescence-MS method for analysis of labeled peptides

4.2.2.1.1 Conjugation of FITC to peptides (115)

Two mg of each peptide was dissolved in 1 mL of freshly prepared 0.1 M NaHCO₃ (pH 9.0). One mg of FITC was dissolved in 1 mL of anhydrous DMSO. Fifty µL of freshly prepared FITC solution was added very slowly in aliquots of 5 µL to the peptide solution with continuous stirring. The reaction mixture was incubated at 4 °C in the dark for 8 h. To the reaction mixture, 55.3 µL of 1 M NH₄Cl was added and incubated further at 4 °C in the dark for 2 hours. FITC-conjugated peptide was desalted by solid phase extraction.

4.2.2.1.2 Solid phase extraction (SPE) of FITC-conjugated peptides

A C18 Sep-Pak cartridge was washed with 1 mL of ethanol 3 times. The cartridge was further washed 3 times with 1 mL of water. The reaction mixture was then applied 5 times onto a cartridge. The cartridge was washed with 1 mL of water. FITC-conjugated peptide was eluted with 1 mL of acetonitrile. FITC-conjugated peptide was dried using a vacuum evaporator. The dried sample was stored at -20 °C for future use. The dried sample was dissolved in 50:50 ACN:H₂O containing 3.7% FA (i.e., in a solvent mimicking HPLC mobile phases) and analyzed by LC-MS.

4.2.2.1.3 HPLC-ESI-Q-TOF-MS analysis of FITC-conjugated peptides

All experiments were performed by HPLC-ESI-Q-TOF-MS. The HPLC system (Shimadzu Technologies, Addison, IL, USA) consisted of an LC-20AD binary pump, DGU-20A3 vacuum degasser, SIL-20A auto sampler, Rf-10AXL fluorescence detector,
and SCL-10A VP system controller. HPLC data were acquired using LC solution software (Shimadzu). The fluorescence signal was sampled at a rate of 3.33 Hz. HPLC was connected to a quadrupole time-of-flight (Q-TOF) mass spectrometer (Q-TOF Micro, Waters Inc., Milford, MA, USA). The MS data were acquired using MassLynx software version 4.1 (Waters).

Separation of labeled and unlabeled peptides was performed on an analytical scale C18 column (Everest™ 250 × 2.1 mm, Vydac, Deerfield, IL, USA). Mobile phase A was HPLC grade water containing 3.7% formic acid and mobile phase B was acetonitrile containing 3.7% formic acid. The flow rate was 0.2 mL/min and the injection volume was 5 μL. An elution gradient was applied (0-5 min 5% of B, 5-15 min 20% of B, 15-45 min 35% of B, 45-55 min 45% of B, 55-65 min 95% of B, and 65-75 min 5% of B) and the run was stopped at 80 minutes. Excitation and emission wavelengths used for fluorescence detection were 492 nm and 518 nm, respectively. The described conditions above were optimized and used for separation of labeled and unlabeled peptides. The gain and sensitivity of fluorescence detection were set to 1X and low, respectively.

ESI-Q-TOF-MS was performed in positive ion mode in the m/z range from 200 to 1800. The spray voltage was set at 3.0 kV and the sample cone voltage was 30 V. The desolvation temperature was 350 °C and the desolvation gas (nitrogen) flow rate was 500 L/hr. The source temperature was 90 °C and collision energy was set at 4.0 V. The MCP detector voltage was set at 2.35 kV. The scan time was 1.0 s and the interscan time was set to 0.1 s.

**4.2.2.1.4 Excitation and emission spectra of FITC**

Excitation and emission spectra of FITC were measured using the luminescence
spectrometer LS 50B from Perkin Elmer (Beaconsfield, Buckinghamshire, England). To measure the excitation spectrum of FITC, the excitation wavelength was set in the range from 350 nm to 500 nm and the emission wavelength was set at 520 nm. Also, to measure the emission spectrum of FITC, the emission wavelength used was in the range from 460 nm to 700 nm while the excitation wavelength was kept constant at 443 nm.

4.2.2.2. Development of LC-fluorescence-MS method for analysis of labeled proteins

4.2.2.2.1 Conjugation of FITC to proteins (115)

Two mg of lysozyme was dissolved in 1 mL of freshly prepared 0.1 M NaHCO₃ (pH 9.0). One mg of FITC was dissolved in 1 mL of anhydrous DMSO. Fifty µL of freshly prepared FITC solution was added very slowly in aliquots of 5 µL to the protein solution with continuous stirring. The reaction mixture was incubated at 4 ºC in the dark for 8 h. To this reaction mixture, 55.3 µL of 1 M NH₄Cl was added and incubated further at 4 ºC in the dark for 2 hours. FITC-conjugated lysozyme were desalted and purified by size exclusion chromatography as discussed in subsection 4.2.2.1.2.

4.2.2.2.2 Preparation of sepharose gel

Sepharose gel was prepared by soaking 8 grams of sepharose 6B gel in 1X PBS buffer followed by washing with buffer for 2 minutes before use. A column was loaded with sepharose gel to a bed volume of ~15 mL. After loading, the column was stored at room temperature until use making sure there is enough buffer in the column to prevent drying of the gel.

4.2.2.2.3 Size exclusion chromatography for purification of FITC-conjugated protein

The sepharose 6B column was equilibrated with 1X PBS for 5 minutes. The
column was washed with 10 mL of 1X PBS. The reaction mixture was applied to the equilibrated sepharose 6B column. The FITC bound and unbound proteins were eluted with 1X PBS buffer in 5 mL fractions and fractions were collected.

4.2.2.4 Measurement of absorption spectra of the fractions collected from column chromatography

Absorption spectra of the fractions collected during purification were measured in quartz cuvettes at 200 nm to 820 nm using a Nicolet Evolution 300 Spectrophotometer from Thermo Electron Corporation (Madison, Wisconsin). The blank used for fraction analysis was the elution buffer. The absorption spectra for all of the above samples were measured using a spectrometer with a PDA detector.

4.2.2.5 Measurement of excitation and emission spectra of the fractions collected from size exclusion chromatography

Emission spectra of fractions collected during purification were measured in quartz cuvettes using a luminescence spectrometer (LS 50B from Perkin Elmer). To measure the emission spectrum, the excitation wavelength was set at 270 nm and the emission wavelength range used was 290 nm to 500 nm. Also, to measure the excitation spectrum, the emission wavelength was set at 370 nm and the excitation wavelength range used was 200 nm to 340 nm.

4.2.2.6 HPLC-ESI-Q-TOF mass spectrometric analysis of FITC-conjugated lysozyme

All experiments were performed by HPLC-ESI-Q-TOF-MS. The HPLC and ESI-Q-TOF-MS systems were used as described in section 4.2.2.1.3.

Separation of labeled and unlabeled lysozyme was performed on an analytical
scale C4 column (Everest™ 250 × 2.1 mm, Vydac, Deerfield, IL, USA). Mobile phase A was HPLC grade water containing 3.7% formic acid and mobile phase B was acetonitrile containing 3.7% formic acid. The flow rate was 0.2 mL/min and the injection volume was 20 µL. An elution gradient was applied (0-5 min 20% of B, 5-50 min 36% of B, 50-52 min 95% of B, 52-55 min 95% of B, and 55-57 min 10% of B), and the run was stopped at 60 minutes. Excitation and emission wavelengths used for fluorescence detection were 492 nm and 518 nm, respectively. The described conditions above were optimized conditions used for separation of labeled and unlabeled peptides. The gain and sensitivity of fluorescence detection were set to 1X and low, respectively.

ESI-Q-TOF-MS was performed in positive ion mode in the m/z range from 1000 to 2000. The spray voltage was set at 3.0 kV and the sample cone voltage was 30 V. The desolvation temperature was 350 °C and the desolvation gas (nitrogen) flow rate was 550 L/hr. The source temperature was 90 °C and the collision energy was set at 3.0 V. The MCP detector voltage was set at 2.35 kV. The scan time was 1.0 s and the interscan time was set to 0.1 s.

Direct infusion of the reaction mixture was performed on ESI-ion trap mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The MS data were acquired using Esquire control software (Bruker) in positive ion mode in the m/z range from 1000 to 1800. The drying temperature was 305 °C and the dry gas flow rate was 8 L/min. The nebulizer gas pressure was 10.0 psi.
4.3 Results and discussion

4.3.1 Development of LC-fluorescence-MS method for analysis of labeled and unlabeled bradykinin

4.3.1.1 Excitation and emission spectra of FITC and Bradykinin-FITC

Emission spectra of FITC and SPE purified peptide-FITC conjugate were obtained using an emission wavelength range from 510 to 580 nm while keeping the excitation wavelength at 494 nm. After comparison of the two emission spectra, a broad emission band was observed for the FITC-conjugated bradykinin with an emission maximum at 518 nm which was similar to emission band for the FITC alone. It was concluded that peptide was labeled successfully with FITC (data not shown).

4.3.1.2 HPLC-ESI-Q-TOF mass spectrometric analysis of FITC-conjugated peptide

The conjugated peptide was separated from free FITC and unlabeled peptide using high-performance liquid chromatography (HPLC). The separated analytes were detected and characterized using fluorescence and MS detectors (Figure 4-1). Successful separation of the FITC-conjugated peptide was achieved. Retention times of 9.7 minutes, and 18.1 minutes were measured for free bradykinin and conjugated bradykinin-FITC, respectively (Figure 4-1). The mass spectra of bradykinin and FITC-conjugated bradykinin are shown in Figure 4-2 and Figure 4-3, respectively. As can be seen from the insets, labeling of bradykinin with FITC increases the charge of bradykinin as only singly and doubly charged ions are observed for unlabeled bradykinin, but additional triply-charged ion is also observed with FITC-labeled bradykinin (m/z of [M+3H]$^{3+}$ = 483.4288), which was not present in unlabeled peptide. This could be due to extra proton accepted by conjugated dye. Similar results were obtained with angiotensin, leucine
enkephalin, and Glu-fib peptides after labeling with FITC (date not shown).

**Figure 4-1.** HPLC separation of FITC-conjugated bradykinin reaction mixture. Labeled and unlabeled bradykinin were detected by (A) a fluorescence detector using excitation wavelength of 492 nm and emission wavelength of 518 nm and (B) ESI-MS.
Figure 4-2. ESI-Q-TOF mass spectrum of unlabeled bradykinin. Insets represent the isotopic distribution for +1 and +2 charge states of bradykinin.
Figure 4-3. ESI-Q-TOF mass spectrum of FITC-conjugated bradykinin. Insets represent the isotopic distribution for +2 and +3 charge states of FITC labeled bradykinin.
4.3.2 Development of an LC-fluorescence-MS method for analysis of labeled and unlabeled lysozyme

4.3.2.1 Measurement of absorption spectrum of the fraction collected from column chromatography

Fractions collected from sepharose column were tested for the presence of characteristic absorption peaks for the protein in absorption spectroscopy. Characteristic peaks at 214 nm (peptide bond), 256 nm and 280 nm (aromatic amino acids) were observed. This shows the presence of protein in the collected fractions (data not shown).

4.3.2.2 Measurement of excitation and emission fluorescence spectra of the fractions collected from column chromatography

Collected fractions were also tested for the presence of protein using the native fluorescence of tryptophan. The emission spectrum was recorded from 300 nm to 500 nm while the excitation wavelength was kept constant at 274 nm. Fluorescence signals were detected in collected fractions also showing that protein was present in fractions.

4.3.2.3 MS analysis of lysozyme in collected fraction

Lysozyme fractions obtained from the sepharose column were analyzed for the presence of protein by MS analysis. Collected fractions were injected directly into an ESI-ion trap-MS instrument. Mass spectrum of labeled and unlabeled lysozyme is shown in Figure 4-4.
4.3.2.5 HPLC-ESI-Q-TOF mass spectrometric analysis of FITC-conjugated lysozyme

FITC-labeled and unlabeled lysozyme were separated successfully by HPLC with retention times of 37.6 minutes and 40.5 minutes, respectively (Figure 4-5). Extracted ion chromatograms (EIC) for fluorescently labeled and unlabeled lysozyme were prepared (Figure 4-6 a). EICs were integrated for one minute time interval to prepare mass spectrum for the labeled and unlabeled lysozyme (Figure 4-6 b). Lysozyme (MW = 14.3 kDa) showed peaks at \( m/z \) values of 1192.9, 1301.3, 1431.3, 1590.3, and 1788.9 with respective charges of +13, +12, +11, +10, and +9. Similarly charged ions were also observed for FITC labeled lysozyme (\( m/z \) values of 1225.5, 1336.8, 1470.2, 1633.5, and
Mass spectrum of unlabeled lysozyme showed the most intense peak at +10 charge state. On the other hand, most intense peak for labeled lysozyme was +11. It can be concluded that the labeling of lysozyme with FITC increases the average charge on the protein.

Figure 4-5. HPLC separation of FITC-conjugated lysozyme reaction mixture. Labeled and unlabeled lysozyme were detected by (A) a fluorescence detector using excitation wavelength of 492 nm and emission wavelength of 518 nm and (B) ESI-MS.
Figure 4-6. (A) Extracted ion chromatogram for unlabeled (red trace) and labeled lysozyme (blue trace). (B) Extracted ion chromatograms were integrated for a 1 minute interval to obtain corresponding mass spectra of labeled (blue trace) and unlabeled lysozyme (red trace).
4.4 Conclusion

Fluorescence detection and ESI-mass spectrometry were used in a tandem fashion allowing the analysis of FITC-labeled peptides and proteins with two different detection modes. These detection modes provide complementary data for the analysis of FITC-conjugated polypeptides. Labeled and unlabeled peptides and proteins were baseline separated using liquid chromatography and identified and characterized using mass spectrometry. It was found that labeling of peptides and proteins increases the average charge of peptides and proteins. This increased charging upon dye labeling can be useful for ESI-MS analysis of large biomolecules and difficult to ionize biomolecules. Future experiments will be aimed on the measurements of the influence of dye labeling on ionization efficiency.
Chapter 5

MALDI-Imaging of peptides and proteins in brain and spinal cord tissue sections

5.1 Introduction

Imaging mass spectrometry (IMS) is useful in gaining information about the distribution and localization of molecules in a target organ or tissue. IMS also allows for the correspondence between target compound tissue distribution and its effects. Chaurand et al. and Yanagisawa et al. used MALDI-MS to directly analyze tissue sections and showed different protein patterns for normal and tumor tissues. They also accurately distinguished between normal organ and carcinoma tissues (116,117) Chaurand and co-workers identified 3 colon cancer specific biomarker based on amino acids sequence from mouse colon tissue sections. Protein profile of normal and cancerous lung tissues analyzed by Yanagisawa and co-workers resulted in 15 distinct peaks which can be used to isolate healthy from diseased tissue.

Eberlin et al. reported the use of MALDI-IMS for the detection of proteins in mouse brain tissue section tissue (68). A tissue section was first analyzed for the analysis of lipids and same tissue section was subjected to MALDI imaging for the analysis of proteins followed by optical microscopy imaging. Human brain cancer tissue section was
also analyzed and oleic acid (m/z 281.4) and the protein calcyclin (m/z 10085) was observed to be up-regulated. Monroe et al. imaged the multiple peptides and proteins in rat spinal cord tissue section using MALDI-IMS and confirmed identification of analytes by follow-up experiments with tissues extracts using LC-MS/MS (63). Recently, Reyzer et al. took the MALDI imaging technique to the next level by imaging proteins directly from multiple tissue morphologies present in whole-body animal tissue sections (118).

IMS can be useful in biomarker discovery for diseases which involve specific enzymes, for example, amyotrophic lateral sclerosis (ALS) disease which involves superoxide dismutase (SOD). Superoxide dismutase is responsible for catalysis of the dismutation of superoxide into oxygen and hydrogen peroxide (119). There are three major families of superoxide dismutases (120): 1) Cu/Zn-SOD: this enzyme binds both Cu and Zn as cofactor, 2) Fe or Mn-SOD: this type of enzyme binds with either Fe or Mn as cofactor, 3) Ni-SOD: this type of enzyme only binds with nickel as a cofactor. In humans, three forms of SOD (SOD1 in cytoplasm, SOD2 in mitochondria, and SOD3 in extracellular fluids) are present. SOD1 is a dimer while SOD2 and SOD3 are tetramers. The SOD1 gene is responsible for encoding SOD1 enzyme. Mutations in this gene result in amyotrophic lateral sclerosis (ALS) disease (119). A4V and H46R are the most common mutations in this gene. However, G93A is a comparatively rare mutation that has been heavily studied in ALS mouse models (121,122). So far, a number of diverse mutations (greater than 150) have been found associated with SOD1 gene (123). ALS is an invariably chronic, progressive, fatal motor neuron degenerative disease with concurrent muscle weakness, wasting, and spasticity, which may be sporadic (SALS) or
familial (FALS). Out of the known cases of FALS, 20-30 % of cases are triggered by gain-of-function mutations in the SOD1 gene (124,125). Therefore, development of ALS biomarkers could contribute in more prompt diagnosis of the disease.

The current investigation for new and consistent biomarkers of ALS is underlined by the fact that people have been involved with ALS diagnosis worldwide. Early diagnosis could be enhanced by discovery of novel biomarkers so that patients could be diagnosed and treated in early stages of the disease. This would increase ALS survival rates and lower down treatment costs. Cerebrospinal fluid (CSF) may best reveal the pathogenic events in the central nervous system (CNS) as it contains proteins that are secreted or released by injured/dying cells (126). Recently, Ryberg et al. used surface-enhanced laser desorption/ionization-time-of-flight mass spectrometry (SELDI-TOF-MS) to identify an ALS biomarker. A biomarker that can differentiate between the diseased and healthy persons was identified by comparing the protein profile of CSF from ALS and healthy sample set obtained using mass spectrometric analysis (127). C-reactive protein (CRP) as an alleged CSF biomarker for ALS disease was recognized during these experiments. ELISA also confirmed the presence of CRP in CSF.

However, the large number (~1000 to 50,000) of lysed cells are required for a single MS spectrum depending on the method of use and, this hampers proteomics studies due to the limited availability of the cells of interest isolated from heterogeneous tissue (72,128,129). These samples are obtained by tedious microdissection techniques. One frozen tissue section is adequate for highly responsive IMS proteomic analysis and hence do not suffer from biological sample availability problems. It is also of interest to the scientific community to determine the distribution and localization of ALS related
biomarkers and other related peptides.

Therefore, we studied the brain and spinal cord tissue sections from non-transgenic and transgenic mice expressing a mutant human SOD1 gene G93A. Male transgenic B6SJL-Tg(SOD1*G93A)1Gur/J mice on a mixed B6/SJL genetic background (stock No 002726) were obtained from The Jackson Laboratory. This hybrid strain was maintained at the DLAR at The University of Toledo Health Science campus (UTHSC) by breeding hemizygous carrier males to B6SJL, F1 hybrid females (stock No. 100012). Littermates were genotyped at 10 days of age (genotyping protocol 002726 by The Jackson Laboratory) and both male and female transgenic mice were used in the study. After perfusion, brain and spinal cord were isolated from the mice and isolated organs were subjected to tissue slicing. Tissue slices were coated with MALDI matrix for MS analysis. 2D-ion images were reconstructed using FlexImaging software to visualize the distribution and localization of peptides and proteins of interest by applying mass filter of appropriate mass for the analyte of interest. Ion images for myelin basic protein and its fragments, neuropeptides such as angiotensin and bradykinin, and many other peptides of interest were successfully reconstructed.

5.2 Materials and Methods

5.2.1 Materials

HPLC grade water, acetonitrile, hydrochloric acid, methanol, and glacial acetic acid were purchased from Fisher. Trifluoroacetic acid (TFA), Trypsin, ammonium bicarbonate, sinapinic acid (SA) and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma and used without further purification. The protein and peptide calibration standards were purchased from Bruker Daltonics. Gelatin type A mounting
medium was purchased from Vector.

5.2.2 Methods

5.2.2.1 Instrumentation and software

Tissue sections were cut on a cryo-cut Microtome (American Optical Corporation, Southbridge, MA) machine. Tissue sections were mounted onto conductive indium tin oxide (ITO)-coated glass slides (25 mm × 75 mm × 1.1 mm) (Bruker Daltonics, Bremen, Germany). Matrix (sinapinic acid for proteins and CHCA for peptides) were coated on tissue sections using an ImagePrep device (Bruker Daltonics). A MALDI-TOF/TOF-MS (Bruker Daltonics, Germany) equipped with a Smartbeam laser (UltrafleXtreme, Bruker Daltonics, Germany) was used for mass spectrometric analyses. This instrument is also equipped with LIFT technology to increase potential energy (130). In this technology, potential of the ions get raised from 0 to 19 kV in stage one followed by velocity focusing in stage 2 and subsequent post-acceleration by reducing the potential to 2-3 kV in stage 3. This technology facilitates high resolution and sensitivity. Mass spectrometric analysis in this study was performed in positive ion mode with linear mode for protein analysis and reflectron mode for peptide analysis. Five hundred laser shots were accumulated from each spot on the tissue section to collect the peptide and protein profile. Laser repetition frequency of 500 Hz was used and the laser intensity was set at 80% for mass spectrometric analysis. The mass detection range was set between 400 Da to 3000 Da for imaging of peptides and 1000 Da to 20000 Da for imaging of proteins. FlexControl (Version 2.3, Bruker) and FlexImaging (Version 2.1, Bruker) software were used to setup appropriate mass range, laser intensity, raster, image registration, automatic data acquisition, and mass filter for imaging analysis.
5.2.2.2 Tissue sample preparation

Non-transgenic and transgenic mice expressing a mutant human SOD1 gene G93A were used for the present study. Male transgenic B6SJL-Tg(SOD1*G93A)1Gur/J mice on a mixed B6/SJL genetic background (stock No 002726) were obtained from The Jackson Laboratory. This hybrid strain was maintained at the DLAR at UTHSC by breeding hemizygous carrier males to B6SJL, F1 hybridfemales (stock No. 100012). Littermates were genotyped at 10 days of age (genotyping protocol 002726 by The Jackson Laboratory) and both male and female transgenic mice were used in the study. Spinal cord and brain tissue were isolated from symptomatic SOD1G93A-trangetic mice at 90-125 days of age. The animals were euthanized by transcardial perfusion of phosphate buffered saline (pH 7.4) performed under deep isoflurane anesthesia. The tissue was exposed by laminectomy, carefully isolated, placed in a mold and snap-frozen in -75°C isopentane. Frozen tissue was stored sealed at -80°C. These tissues were kept at -20°C for 10 minutes to narrow down the temperature gradient between frozen tissue and mounting media. While waiting for the tissue samples to come to room temperature, gelatin type A was prepared in distilled water at a concentration of 0.1 mg/mL and was heated to 60°C. This gel type solution was used as supporting media to support tissues in vinyl Tissue-Tek cryomolds (Sakura Finetek USA Inc., Torrance, CA). Tissue sections were molded and kept at -80°C for 15-20 minutes to allow for freezing the gel and tissue sections. After 20 minutes, these molds were placed in a cryosectioning machine that was set at -30°C overnight to prevent tissue sections from peeling.
5.2.2.3 Tissue sectioning

Tissue sections of brain and spinal cord from a transgenic and non-transgenic mouse were prepared by sectioning at 10 µm thickness in cryostate. Many tissue sections were prepared for both brain and spinal cord tissues. These sections were mounted on indium-tin oxide coated glass slides and placed in the cryostat to cool down. Sectioning was performed to the point that the bottom-part of the sample tissue is not in contact with the embedding medium and mold. The tissue sections were positioned onto the conductive side of ITO coated glass slides with fingertip warming from the other side. Tissue sections are sensitive to the environmental conditions and can undergo protein degradation and, therefore, should be immediately kept in a vacuum desiccator for 30 minutes. Glass slides containing the tissue sections were transported on dry ice and stored at -80 °C for future use to prevent degradation of peptides and proteins. For MS analysis, glass slides were immediately transferred from -80 °C to desiccators for drying to prevent movement of molecules in the presence of moisture.

5.2.2.4 Tissue washing

After drying and thawing at -20 °C for 10 minutes, followed by 10 minutes at 4 °C, and at last 10 minutes at room temperature in a vacuum desiccator, glass slides with tissue sections were washed with 70% isopropyl alcohol (IPA) for 30s, followed by 95% IPA for 30s, followed by washing with 100% IPA for 30s. Washing is very important for removing lipids and salts from the tissue surface which can affect mass spectrometric analysis. After washing, slides were again dried in desiccators for 20-30 minutes.
5.2.2.5 On-tissue trypsin digestion

For peptide analysis, on-tissue tryptic digestion was performed. On-tissue proteins were reduced and alkylated by spraying DTT and IAM solutions onto the prepared tissue samples, respectively. DTT was prepared at a concentration of 2 mg/mL in 60:40 ACN:H₂O (0.2% Formic acid). IAM was prepared at a concentration of 2 mg/mL in 60:40 ACN:H₂O (0.2% Formic acid). Trypsin solution was prepared at concentration of 1 mg/mL in 40 mM freshly prepared ammonium bicarbonate. Tissue sections were sprayed in the following sequence: DTT, followed by IAM and trypsin using ImagePrep. Tissue sections were incubated overnight at 37 °C for enzymatic digestion of proteins.

5.2.2.6 Teach point and image scanning

After drying, positions of the tissue sections on a glass slide were marked using three teach points with white marker. These teach points helped in locating the position of the tissue section during mass spectrometry imaging experiments. The glass slide with marked tissue section was imaged using a photo scanner and scanned image was registered in FlexImaging software. This registration allowed aligns the position of the tissue section on the glass slide with x, y coordinates of the standard MALDI target plate.

5.2.2.7 Matrix coating using ImagePrep

The glass slide with marked tissue section was layered with sinapinic acid (SA) matrix for proteins or with α-cyano-4-hydroxycinnamic acid (CHCA) for peptides using Imageprep. Sinapinic acid matrix was prepared at a concentration of 20 mg/mL in 60:40 ACN:H₂O with 0.2% trifluoroacetic acid. CHCA matrix was prepared at a concentration
of 10 mg/mL in 50:50 ACN:H₂O with 0.2% trifluoroacetic acid. After coating with matrix, the tissue sections on glass slides were analyzed in linear MS mode for proteins and reflectron MS mode for peptides. Proteins and peptides were irradiated from the surface of the tissues with laser emitting light with an intensity of 80% of the maximum intensity, and 500 shots were accumulated from each spot. For spinal cord tissue sections 75 μm raster was used but 100 μm raster resolution was used for brain tissue sections. On every tissue section, regions of interest were defined, resembling representative regions of predominantly diseased and non-diseased tissue. Analyzed mass spectrum was used in FlexImaging software to reconstruct 2-D ion images for peptides and proteins in the tissue section.
5.3 Results and Discussion

5.3.1 Imaging of peptides and proteins in brain and spinal cord tissue sections

Figures 5-1 (a) and 5-1 (b) show the tissue sections from brain and spinal cord, respectively. These tissue sections after matrix deposition were used for imaging of proteins MALDI-IMS. Figure 5-1 (a) represents region of interest (shaded area) selected for mass spectrometry analysis of brain tissue section. The region of interest (ROI) shown in Figure 5-1 (a) was irradiated by the laser at raster distance of 100 µm and corresponding mass spectra from each laser shot were collected. Figure 5-1 (b) represents spinal cord tissue section on the glass slide before selecting area of interest for imaging experiments.

Figures 5-1. (a) Brain tissue section selected for imaging mass spectrometry analysis (shaded area), and (b) spinal cord tissue section on the glass slide before selecting area of
interest for imaging experiments.

The overall spectra from brain and spinal cord tissue sections are illustrated in Figure 5-2 (a) and (b) respectively, showing peptide and protein profiles from ~1.5 - 20 kDa. Spectra of every spot obtained upon laser irradiation and its position were automatically saved by the software for further analysis. Within this mass range a number of differentially expressed proteins were detected such as myelin basic protein (m/z 14221), and thymosin β-4 (m/z 4962.6). Peptides such as little SAAS (m/z 1785.3), PEN (m/z 2301.4), and synapsin I (1-13) (m/z 1713.9) fragment were also detected (63,73). The most abundant proteins (e.g., myelin basic protein) and proteins that ionize well in tissues gave higher relative abundances compared to other proteins. As shown by Crecelius et. al., myelin basic protein is localized in corpus callosum and anterior commissure (73). Figure 5-3 shows the mass spectrum of a brain tissue sections in the lower mass range and several peaks were labeled according to literature values (63). For example, angiotensin (m/z 1045.85), bradykinin (m/z 1059.93), neurokinin A (m/z 1131.34), myelin basic protein S (119-128) (m/z 1102.72), myelin basic protein S (182-195) (m/z 1432.76) were observed from brain tissue section.
Figure 5-2. Overall mass spectrum of (A) a brain tissue section and (B) a spinal cord tissue section originating from a healthy mouse. The spectra were measured by MALDI-IMS in the positive ion mode in the m/z range between 1500 and 19500.

2-D ion images for proteins of interest can be prepared using reconstruction software (FlexImaging) by selecting peaks from averaged and single spectra and distribution of the peaks can be seen with single- or multi-colored gradient views. 2-D
ion images (distribution maps) prepared for peptides and proteins from brain tissue are shown in Figure 5-4. These reconstructed ion images help in understanding the distribution and localization of peptides and proteins.

**Figure 5-3.** Overall mass spectrum of a brain tissue section originating from a healthy mouse. The spectrum was measured by MALDI-IMS in the positive ion mode in the m/z range between 600 and 2500 with several peaks labeled according to their literature masses (63).
Brain and spinal cord tissue sections from a transgenic mouse were also subjected to tissue imaging studies. Figure 5-5 (a) shows the mass spectrum from non-transgenic mice brain. Figure 5-5 (b) shows the overall mass spectrum for brain tissue sections from transgenic mice. By comparing two types (transgenic vs. non-transgenic) of tissue sections, several up-regulated and down-regulated proteins were detected while some proteins were present in similar abundance between diseased and normal tissue. For example, proteins with m/z 4808.09 and 4959.69 were down-regulated in transgenic mice. Protein (m/z 15859.3) was appeared in transgenic mice. Substantial differences were observed in peptide and protein profile of tissue sections with m/z below 10 kDa. By comparing the two mass spectra, it can be concluded that more proteins were detected from transgenic mice than from non-transgenic mice, which could be due to appearance of new proteins with disease or due to differences between locations of the tissue sections. Therefore, IMS seems to be feasible for classification of ALS tissue samples by comparison of the overall mass spectra and by looking up-regulated or down-regulated peptides and proteins. Although differential protein expression between normal and ALS tissues was noticed, additional experiments should confirm that these differences originate from the same parts of the brain.
Figure 5-4. Ion density map of several peptides and proteins at 100 μm resolution obtained after MALDI-MS imaging of a mice brain tissue section. Peptides and proteins were identified by comparison of experimental $m/z$ values with literature $m/z$ values (63,73).

5.3.2 On-tissue trypsin digest and peptide distribution map

IMS has limited applications for imaging of proteins over the size of 20 kDa and these proteins have not been observed from brain and spinal cord tissue sections during our experiments. However, proteins with $m/z$ greater than 20 kDa can be analyzed by
using bottom-up approach. During bottom-up approach, proteins are digested using enzyme trypsin and tryptic peptides are analyzed. Therefore, proteins were digested using on-tissue enzymatic digestion. Figure 5-6 shows the overall mass spectrum for peptides from the spinal cord after on-tissue trypsin digestion. 2-D ion images for peptides were constructed. These reconstructed ion images provide localization and distribution of peptides of interest within the tissue section. Further experiments need to be performed in order to identify these peptides, and hence, proteins. A tandem mass spectrometry analysis, which requires sufficient signal intensity in imaging mass spectrometry experiments will allow identification, characterization, and sequencing of peptides and hence, parental proteins. Thus, IMS is a promising technique for peptide and protein profiling, for distribution and localization, and for characterization of proteins from a single ALS tissue sections with high spatial resolution (20-100 μm). Another major advantage of IMS is in its requirement of a very small amount of sample, i.e., a single 10-μm thick ALS tissue section was needed to perform these experiments.
Figure 5-5. Mass spectra of the brain tissue section from (A) a non-transgenic mice and (B) transgenic mice in the m/z range between 4000 and 16000.
Figure 5-6. Overall mass spectrum of spinal cord tissue section originating from healthy mice after on-tissue tryptic digest in the $m/z$ range between 400 and 3600.
5.4 Conclusion

MALDI imaging mass spectrometry was used to obtain information about the relative abundance, distribution, and localization of peptides and proteins in brain and spinal cord tissue sections. We were able to identify successfully a few peptides and proteins in mass spectrum such as myelin basic protein fragments, thymosin β-4, angiotensin, bradykinin, little SAAS, PEN, synapsin I (1-13) fragment. By comparison of healthy and diseased (ALS) tissue sections some difference in the protein profiles and relative intensities of the proteins were observed. These differences in protein profiles can potentially be useful to search for biomarkers of ALS.
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Appendix A:

Table A.1. Linear regression equations for calibration curves obtained using HPLC with fluorescence and ESI-MS detection.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Linear Regression equation (MS)</th>
<th>Linear Regression equation (LC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>$y = 1.2806 \times 10^3 x + 338.48$</td>
<td>$y = 1.9369 \times 10^3 x + 1944.9$</td>
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<tr>
<td>Phenylalanine</td>
<td>$y = 2.5116 \times 10^3 x + 105.04$</td>
<td>$y = 1.5259 \times 10^4 x + 2553.3$</td>
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<tr>
<td>Tryptophan</td>
<td>$y = 1.2098 \times 10^3 x + 348.46$</td>
<td>$y = 1.0000 \times 10^6 x + 17994$</td>
</tr>
<tr>
<td>Bradykinin acetate</td>
<td>$y = 1.3912 \times 10^3 x + 3183.1$</td>
<td>$y = 2.0000 \times 10^6 x + 30225$</td>
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<tr>
<td>Angiotensin II</td>
<td>$y = 9.9545 \times 10^4 x + 1431.7$</td>
<td>$y = 7.0000 \times 10^6 x + 1824.7$</td>
</tr>
<tr>
<td>[Glu]-Fibrinopeptide</td>
<td>$y = 1.7503 \times 10^5 x + 1586.5$</td>
<td>$y = 1.0000 \times 10^6 x + 36803$</td>
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<tr>
<td>Leucine enkephalin</td>
<td>$y = 6.9817 \times 10^4 x - 265.13$</td>
<td>$y = 1.0000 \times 10^7 x - 25217$</td>
</tr>
<tr>
<td>MRFA</td>
<td>$y = 5.9138 \times 10^4 x + 346.13$</td>
<td>$y = 2.3823 \times 10^5 x + 2286.5$</td>
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<tr>
<td>Neurotensin (8-13)</td>
<td>$y = 8.1411 \times 10^4 x + 849.22$</td>
<td>$y = 2.0000 \times 10^7 x - 47686$</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>$y = 5.0772 \times 10^3 x + 525.11$</td>
<td>$y = 6.0000 \times 10^6 x + 409030$</td>
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<tr>
<td>Cytochrome C</td>
<td>$y = 1.7611 \times 10^4 x - 2384.6$</td>
<td>$y = 1.0499 \times 10^5 x - 412886$</td>
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<tr>
<td>Myoglobin</td>
<td>$y = 6.0366 \times 10^3 x - 6920.7$</td>
<td>$y = 2.3759 \times 10^5 x - 906950$</td>
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<tr>
<td>Trypsinogen</td>
<td>$y = 7.7043 \times 10^2 x + 371.78$</td>
<td>$y = 1.6887 \times 10^5 x - 415338$</td>
</tr>
<tr>
<td>BSA Pep (927.9 m/z)</td>
<td>$y = 1.2321 \times 10^1 x - 14.315$</td>
<td>$y = 4.2391 \times 10^3 x - 188570$</td>
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<tr>
<td>BSA Pep (720.6 m/z)</td>
<td>$y = 4.3923 \times 10^1 x - 34.143$</td>
<td>$y = 3.3128 \times 10^3 x - 188644$</td>
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<tr>
<td>BSA Pep (740.6 m/z)</td>
<td>$y = 4.3322 \times 10^1 x - 31.133$</td>
<td>$y = 4.6694 \times 10^3 x - 168010$</td>
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<td>Cyto C (m/z 736.0)</td>
<td>$y = 9.9502 \times 10^2 x - 90.123$</td>
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<td>Myo (m/z 751.9)</td>
<td>$y = 4.1788 \times 10^1 x - 13.303$</td>
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<tr>
<td>Tryp (m/z 454.7)</td>
<td>$y = 4.4105 \times 10^1 x + 0.683$</td>
<td>$y = 8.8380 \times 10^2 x + 90415$</td>
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