Deep Ultraviolet Resonance Raman Spectroscopy as a Tool for Biomolecule Analysis

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List of Abbreviations

CD	Circular dichromism
DLPC	1,2-didodecanoyl- <i>sn</i> -glycero-3-phosphocholine
DMPC	1,2-Dimyristoyl- <i>sn</i> -Glycero-3-Phosphocholine
DOPG	1,2-dioleoyl- <i>sn</i> -glycero-3-phospho-(1'- <i>rac</i> -glycerol)
DPH	Diphenylhexatriene
DTT	Dithiothreitol
dUVRR	Deep-ultraviolet resonance Raman spectroscopy
FPLC	Fast protein liquid chromatography
HDL	High-density lipoprotein
IDL	Intermediate-density lipoprotein
IR	Infrared
LDL	Low-density lipoprotein
MCR-ALS	Multivariate curve resolution-alternating least squares
NMR	Nuclear magnetic resonance
OCD	Oriented CD
PARAFAC	Parallel factor analysis
PLS	Partial least squares
SEC	Size exclusion chromatography
VLDL	Very low-density lipoprotein

Abstract

Cholesterol is a major component of membranes, reducing their fluidity at higher concentrations. The UV Raman vibrational mode of the carbon-carbon double bond in cholesterol occurs at 1675 cm^{-1} is strongly resonance enhanced in deep ultraviolet resonance Raman (dUVRR) spectra of natural and model membranes at an excitation wavelength of 197 nm. DUVRR is a vibrational spectroscopic technique that is used to gather information on protein secondary structure and in this work is extended to study systems in the presence of the membrane component cholesterol and the cholesterol analog 5α -cholestan- 3β -ol, which lacks the carbon-carbon double bond.

DUVRR studies of melittin indicated that little loss of helical structure occurred with increasing temperature as was suggested by circular dichroism (CD), another structurally sensitive spectroscopic technique. A cholesterol analog was introduced to decrease the fluidity of membrane in a similar manner to cholesterol, while minimizing spectroscopic interference from the resonant enhanced carbon-carbon double bond vibration. With the introduction of the cholesterol analog a loss in helical structure of the melittin was seen in both the dUVRR and CD.

Lipoproteins transport cholesterol through the blood stream. Studies have shown that an individual's lipoprotein levels are correlated with their risk of cardiovascular disease. The strongly resonantly enhanced dUVRR feature of the carbon-carbon double bond was used to quantify cholesterol in lipoprotein particles isolated from whole blood. Like cholesterol, unsaturated lipids give rise to a strongly enhanced vibrational mode in dUVRR spectra, which is slightly downshifted from that of cholesterol. The vibrational

mode of unsaturated lipids occurs in the region of 1655-1661 cm^{-1} , depending on the type of lipid. The feature arising from the unsaturated lipids was found to be sufficiently resolved from cholesterol's feature to allow quantitation of cholesterol in model membranes and lipoproteins.

Isolation and characterization of lipoproteins is an emerging field as studies show that cardiovascular disease risk is a complex function of not just cholesterol and lipoprotein levels but also their size distribution. Spectroscopic methods are readily coupled with chromatographic techniques. Size exclusion chromatography (SEC) was employed to separate lipoproteins based on their size from a series of blood serum samples. Each volume fraction was spectroscopically characterized via dUVRR spectroscopy producing a two-way data matrix for each sample, which could then be analyzed using higher order multivariate methods. Parallel factor (PARAFAC) analysis and multivariate curve resolution-alternating least squares (MCR-ALS) were employed to resolve the underlying spectroscopic and chromatographic profiles. The resolved profiles were attributed to lipid/cholesterol, protein and buffer components.

Introduction

Deep Ultraviolet Resonance Raman (dUVRR) spectroscopy has traditionally been applied to secondary structure determination in aqueous proteins. Work with this technique in membrane systems is a relatively new endeavor¹⁻⁴. Membrane proteins are a vital part of our cellular biology but are not as well studied as their aqueous soluble counterparts due to the experimental challenges of working with lipids for traditional techniques like nuclear magnetic resonance (NMR) or X-ray crystallography. Saturated lipids are nearly invisible in dUVRR spectra of lipid-solubilized proteins, leaving only the structurally sensitive modes from peptide bond of the protein itself resonantly enhanced. Thus, dUVRR spectra can be collected with very little sample preparation and with no required modifications to the membrane protein. Additionally, the amide I vibrational mode is a built-in marker for lipid solvation³⁻⁴.

One of the greatest health risks we currently face is heart disease⁵. Heart disease has been linked to blood cholesterol levels and its carrier molecules, lipoproteins⁶⁻¹². Current clinical methods for the analysis of blood cholesterol levels do not measure cholesterol or the lipoproteins directly but instead rely on enzymatic reactions, associated protein quantitation, and calculations. These indirect methods can lead to inaccurate results which could imply an improper course of treatment. The vibrational mode arising from the carbon-carbon double bond found in biological molecules like fatty acids, unsaturated lipids, and cholesterol is resonant enhanced in dUVRR spectra. The ability to quantitate cholesterol while also monitoring the protein content associated with lipoprotein particles

makes dUVRR a unique tool in this field. Coupled with a separation technique like size exclusion chromatography (SEC) dUVRR can play a vital role in a new direct approach to blood cholesterol analysis.

Chapter 1

Eagleburger, M.K.; Cooley, J.W.; JiJi, R.D., Effects of Fluidity on the Ensemble

Structure of a Membrane Embedded α -helical Peptide. Biopolymers **2014**, 101(8), 895-902.

Introduction

Membrane proteins are often termed the gatekeepers of the cell and are involved in cellular trafficking, environmental sensing, and energy metabolism. Membrane proteins are associated with a number of different diseases and thus make up over 50% of current drug targets¹³⁻¹⁴. While the role of membrane proteins in cellular processes is quite important, less is known about membrane proteins than their soluble counterparts¹⁵⁻¹⁶. It is thought that 20-30% of the genome codes for membrane proteins¹⁷⁻¹⁸. However, there are currently only 387 unique 3D structures of membrane proteins, less than 1% of all known 3D protein structures¹⁹.

Traditional techniques for soluble protein structure determination such as X-ray crystallography and solution-state nuclear magnetic resonance (NMR)²⁰ are not amenable to structural analysis in intact bilayers. This means that analysis of membrane proteins and their dynamics in their native environments has not traditionally been feasible. As a result, infrared (IR) and circular dichroism (CD) spectroscopies have been relied upon for structure determination²¹⁻²². These techniques report on the ensemble secondary structure content of a protein.

Deep-ultraviolet resonance Raman spectroscopy (dUVRR) is an emerging vibrational method that has been used to estimate the secondary structural content of soluble proteins. As the dUVRR spectra, like IR, represent the sum of all of a protein's secondary structural elements, the spectra can therefore be deconvoluted into the pure secondary structure content components²³⁻²⁷. Only recently has dUVRR spectroscopy been applied to the field of membrane proteins^{3-4, 28-30} revealing that selective excitation of the amide backbone results in a strong protein signal with little interference from the lipid and buffer components. Furthermore, the dUVRR spectra of lipid desolvated proteins have an enhanced amide I mode³⁻⁴. Yet, despite its promise as a complimentary tool for the study of membrane proteins, further work is needed to determine the effects of the membrane environment on the dUVRR spectra of proteins.

Melittin is a small 26-residue amphiphilic protein, and is the main hemolytic component of honeybee venom. Melittin is readily soluble in water where it adopts a disordered structure³¹⁻³², but in the presence of a suitable membrane environment melittin will spontaneously insert into the membrane and adopt an α -helical conformation³³⁻³⁵. Once associated with a membrane system the structure and function of melittin are dependent upon the type and phase of the lipid membrane³⁶⁻³⁹. Cholesterol is also known to influence the ability of melittin to bind to lipid membranes and induce cell lysis⁴⁰⁻⁴¹. A great deal of the melittin literature is devoted to studying this lytic ability, with the focus on the changes effected by melittin on the membrane systems. Studies aimed at observing the secondary structure of melittin in different membrane conditions or with cholesterol are few^{36, 40, 42}. Anderson *et. al.* showed a small reduction in the α -helical character of melittin in bicelles and in cholesterol doped bicelles with an increase in temperature by

CD. Wessman *et. al.* studied the interaction of melittin with DOPC liposomes doped with cholesterol by CD and also saw a decrease in the α -helical content of melittin in the presence of cholesterol. Here, we further elucidate the structure of melittin upon interaction with 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC) liposomes with differing fluidities by CD and dUVRR spectroscopy, and reveal a unique sensitivity of dUVRR spectroscopy for resolving subtle structural fluctuations associated with changing membrane environment.

Materials and Methods

Cholesterol, monosodium phosphate monohydrate, disodium phosphate heptahydrate, sodium chloride, and chloroform were purchased from Fisher Scientific (Pittsburgh, PA). 5 α -cholestan-3 β -ol and diphenylhexatriene (DPH) were purchased from Sigma-Aldrich (St. Louis, MO). 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC) was purchased from Avanti Polar Lipids Incorporated (Alabaster, AL). Melittin was purchased from Pi Proteomics (Huntsville, AL).

Lipid Vesicle Preparation for Anisotropy Measurements

DMPC was dissolved in a 65:35:8 mixture of chloroform, methanol, and water, respectively. DPH, cholesterol, and 5 α -cholestan-3 β -ol were dissolved in chloroform. Cholesterol and 5 α -cholestan-3 β -ol were added to the DMPC at 0, 10, and 25 molar percent. For anisotropy measurements DPH was also added to the solution. Each solution was then dried under a stream of argon until a thin film was formed on the bottom of the container. The mixture was then further dried in a vacuum desiccator for more than one

hour. The mixture was rehydrated in 10 mM phosphate buffer (pH 7.4), and sonicated in a heated water bath for one hour and extruded 21 times through an Avestin LiposoFast (Ottawa, ON) extruder with a 100 nm filter to form monodisperse liposome vesicles with a hydrodynamic radius of 31 nm. These vesicles were then diluted in phosphate buffer to give a final lipid/cholesterol/5 α -cholestan-3 β -ol concentration of 50 μ M and a final DPH concentration of 0.5 μ M for anisotropy measurements. Melittin containing vesicles were prepared as above with Melittin being dissolved in water and added to the vesicle solution at a concentration of 2 molar percent.

Measuring membrane fluidity with fluorescence anisotropy

Fluorescence anisotropy (r) of DPH was measured with a Cary Eclipse fluorescence spectrometer with a manual polarizer. The following equations were used to determine r ⁴³:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad G = \frac{I_{HV}}{I_{HH}}$$

I_{VV} is the fluorescence intensity with the excitation and emission polarizers in the vertical position. I_{VH} is the fluorescence intensity with the excitation polarizer in the vertical position, and the emission polarizer in the horizontal position. I_{HV} and I_{HH} are the fluorescence intensities with the excitation polarizer in the horizontal position, and the emission polarizer in the vertical, and horizontal positions, respectively. G is a correction factor for the sensitivity of the instrument to vertically and horizontally polarized light. The fluorescence anisotropy of the DMPC vesicles was observe at 37 °C. The temperature in the fluorescence spectrometer was controlled using a Neslab RTE-111

temperature control unit and samples were kept in a constant temperature water bath when not being measured.

Deep Ultraviolet Resonance Raman (dUVRR)

The dUVRR instrument setup has been described previously⁴⁴. In short, the fourth harmonic of a tunable Ti:Sapphire Coherent laser (Santa Clara, CA) was focused on a flowing sample. The sample chamber contained two nitinol wires approximately 1 mm apart, between which the sample flowed. The sample chamber was purged with nitrogen gas to remove ambient oxygen. The samples were excited with 197 nm light with an average power of 0.5 mW to minimize the risk of sample degradation. Samples were kept at a constant temperature using a custom-made water-jacketed cell (Mid Rivers Glassblowing, St. Charles, MO) and a Fischer Scientific (Pittsburgh, PA) Isotemp 3016D circulating water bath. Melittin samples were made as above, 2 molar percent melittin in DMPC liposomes, with 20 mM phosphate buffer (pH 7.4, 5 mM NaCl) and without the addition of DPH. Melittin/DMPC liposomes were diluted to a final melittin concentration of 0.5 mg/mL. Sodium perchlorate (50mM) was added to the samples as an internal intensity standard.

Circular Dichroism

Circular dichroism measurements were taken on a Jasco (Easton, MD) J-710 Spectrophotometer with a 1 mm path length cuvette. Samples were scanned from 250 nm to 190 nm with a data pitch of 0.1 nm, a band width of 1 nm, a scanning speed of 50 nm/min, a response time of 4 seconds, and with 5 total accumulations. Temperatures for the CD spectra were controlled by a Julabo (Allentown, PA) AWC100 temperature

control unit. Samples were prepared in a similar manner as the above dUVRR samples. All spectra were buffer and DMPC corrected and converted to mean residue ellipticity (θ_{MRE}). Multiple trials were collected and only a single representative spectrum from each experiment is shown. Oriented CD (OCD) measurements were obtained using the Jasco J-710 Spectrophotometer fitted with an OCD attachment manufactured by Jasco. OCD samples were prepared as the above dUVRR samples, with a small volume being placed on a quartz plate. The plated sample was then dried in a desiccator containing an 80% zinc chloride solution. The plate was then placed in the OCD sample cell and rehydrated with 1 mL of water. OCD spectra were taken at six positions, averaged, and DMPC corrected.

Results

Figure 1-1: Fluorescence anisotropy of DPH in DMPC liposomes containing cholesterol (squares), 5 α -cholestan-3 β -ol (triangles), and 5 α -cholestan-3 β -ol plus two molar percent melittin (circles). Error bars indicate the deviation of the fluorescence anisotropy between three experimental collections.

The fluorescence anisotropy of DPH in DMPC membranes was measured to compare the effect of cholesterol, which is known to decrease membrane fluidity, with 5 α -cholestan-3 β -ol, a cholesterol analog. The use of 5 α -cholestan-3 β -ol is necessitated by spectral interference from the double bond in cholesterol in both CD and dUVR spectra. An increase in anisotropy with increasing concentration was observed for both cholesterol and 5 α -cholestan-3 β -ol (Figure 1-1). An increase in anisotropy is correlated with a decrease in the fluidity of the DMPC bilayer. The addition of melittin to the DMPC liposomes with 5 α -cholestan-3 β -ol did not significantly change the DPH anisotropy. This

suggests that 5 α -cholestan-3 β -ol decreases membrane fluidity in a similar manner as cholesterol and that melittin itself does not alter the fluidity of the DMPC liposomes.

Previous studies have shown that melittin adopts a disordered structure in an aqueous environment³¹⁻³², and α -helical structure in a membrane or membrane-mimicking environment³³⁻³⁵. The CD spectrum of melittin in an aqueous environment has a single minimum at 198 nm consistent with disordered structure (Figure 1-2)⁴⁵⁻⁴⁶. Introduction of DMPC liposomes induces folding of melittin⁴⁷⁻⁴⁸ and results in an α -helical CD spectrum with two minima at 209 nm and 222 nm, and a single maximum at 192 nm (Figure 1-2).

Figure 1-2: DUVRR (top) and CD (bottom) spectra of melittin in water (grey) and in DMPC liposomes (black).

DUVRR spectra of proteins are dependent on their secondary structure composition.

Recent studies have shown that dUVRR spectroscopy is amenable to structural studies of membrane-solubilized proteins^{3-4, 30, 49}. There are four observable amide modes, the amide I, II, III and S modes. The amide I is a carbonyl stretching mode and is typically quite weak in α -helical and disordered conformations. Hydrogen bonding between the backbone carbonyl and the solvent greatly diminishes the intensity of the amide I band^{28, 50-51}. The amide II is a coupled N-H bending/C-H stretching mode. Although the position and intensity of the amide II mode is sensitive to secondary structure, the observed shifts are much smaller than those observed for the amide III band. The amide III mode is a C α -H bending/C-N stretching mode and is coupled with the amide S mode, a C α -H bending/N-H bending mode, in disordered and β -sheet conformations⁵². The amide S mode is not resonance enhanced in α -helical conformations^{24, 53}.

Similar to the CD spectra, the UVRR spectra indicate a major structural difference in melittin between the aqueous and membrane environments. The maximum of the amide III mode for aqueous melittin occurs at 1251 cm⁻¹ and the amide S maximum occurs at 1391 cm⁻¹. The position and intensity of the amide III and S modes, as well as the amide II maximum at 1553 cm⁻¹ and amide I maximum at 1665 cm⁻¹ are consistent with disordered structure^{24, 54-55}. For melittin in DMPC liposomes the amide III maximum shifts up to 1285 cm⁻¹ and the intensity of the amide S mode decreases dramatically, both indications of folding into an α -helix^{25, 56}. While the position of the amide I band (1652

cm^{-1}) is consistent with α -helical structure, the intensity of the amide I maximum is much stronger than typically observed for globular α -helical proteins. The strong intensity of the amide I band indicates that the backbone of melittin is desolvated in the presence of DMPC bilayers consistent with previous dUVR studies of lipid solvated proteins³⁻⁴.

Figure 1-3: CD spectra of melittin in DMPC from 7°C to 37°C.

Fluorescence anisotropy of DPH in DMPC bilayers is approximately 0.35, 0.3 and 0.1 at 7°C, 23°C, and 37°C, respectively, indicating that the DMPC bilayer is in the gel state at room temperature and below. CD spectra of melittin (Figure 1-3) show a slight increase in the intensity of the maximum in the 192-194 nm region and the minimum at 222 nm with increasing temperature up to 22°C, indicating a slight increase in helical content from 7° to 22°C. Above 22°C, the maximum and minimum at approximately 193 and 222 nm decrease in intensity to a much larger extent, suggesting a significant loss of helical

content at physiological temperature (37°C). A red shift (192 nm to 193.5 nm) in the maxima around 193 nm and the minima around 208 nm can be seen from 7°C to 37°C.

Figure 1-4: DUVRR temperature profile of melittin in DMPC liposomes. Spectra were collected from 7°C to 37°C in intervals of 5°C.

To determine if melittin partially unfolds at physiological temperatures, dUVRR spectra were collected from 7°C to 37°C (Figure 1-4). The amide S mode (1370-1400 cm^{-1}) is a

marker for non-helical structure in dUVRR spectra of proteins^{53, 57}. In the dUVRR temperature profile of melittin, the amide S mode at 1383 cm^{-1} in all the spectra is very weak and does not change significantly. This indicates that melittin is adopting a highly helical structure in DMPC liposomes at all observed temperatures. However, an increase in the amide III intensity at 1240 cm^{-1} can be observed with increasing temperature in the spectra above 22°C . Given that there is little change in the amide S intensity, this indicates that the helical structure of the melittin remains intact⁵⁸.

Figure 1-5: Plot of dUVRR amide III intensity at 1240 cm^{-1} and CD intensity at 222 nm versus temperature of the dUVRR temperature profile (solid squares) and CD temperature profile of melittin (dashed circles).

The tryptophan modes in the dUVRR spectra are weak and seen as shoulders on the amide I and amide II modes. The dUVRR data coupled to the CD data suggests that

melittin is not unfolding at higher temperatures but is sampling a greater range of dihedral angles, as the lipid environment changes from a gel to liquid state. The intensity of the amide III band at 1240 cm^{-1} increases concurrently with a decrease in the negative ellipticity at 222 nm (Figure 1-5). The position and intensity of the lowest frequency amide III band has been shown to be dependent on the ψ dihedral angle⁵⁸⁻⁶¹. The appearance of a band at 1240 cm^{-1} is consistent with formation of a small amount of 3_{10} -helical structure, possibly at the termini of the α -helix⁶⁰. It has been suggested the 3_{10} -helical structure is an intermediate between α -helical and disordered structure⁶².

The temperature dependence of the CD and dUVRR suggest that in a gel like lipid environment the peptide adopts a more uniform helical structure. Alternatively, cholesterol is known to decrease lipid fluidity as does 5α -cholestan- 3β -ol (Figure 1-1). In order to study the effect of decreasing lipid fluidity on the structure of melittin at physiological temperature 5α -cholestan- 3β -ol was added to DMPC liposomes at 10 and 25 molar percent.

The CD spectra of melittin with 5α -cholestan- 3β -ol shows a slight increase in the maxima in the 192-194 nm region and a slight decrease in the minima at 208 nm and 222 nm between 0 and 10 molar percent 5α -cholestan- 3β -ol (Figure 1-6). Between 10 and 25 molar percent 5α -cholestan- 3β -ol, a large decrease in intensity across the spectra can be observed, which may be interpreted as a loss of helical structure (Figure 1-6). The OCD spectra show an increase in the ratio of 214 nm intensity/228 nm intensity from 0.669 at 0 molar percent 5α -cholestan- 3β -ol to 0.784 at 10 molar percent 5α -cholestan- 3β -ol. The ratio increases again to 0.834 between 10 and 25 molar percent 5α -cholestan- 3β -ol. The

increase in the relative intensity of the 214 nm minimum suggests an increase in the tilt angle of the melittin helix with respect to the bilayer normal⁶³⁻⁶⁵.

The CD spectra of melittin with increasing molar percentages of 5 α -cholestan-3 β -ol suggest a loss of helical structure at 25 molar percent 5 α -cholestan-3 β -ol. A small increase in the amide S mode at 1389 cm⁻¹ is observed in the dUVRR spectrum of melittin at 25 molar percent 5 α -cholestan-3 β -ol (Figure 1-7). This increase in the amide S mode indicates a small loss of helical content in the peptide. Concurrently, the amide III band at 1240 cm⁻¹ increases. The position of the amide II (1542 cm⁻¹) and amide I (1651 cm⁻¹) bands are still consistent with those of an α -helical peptide, but with the amide II position being slightly downshifted from that observed for soluble helical peptides⁶⁶⁻⁶⁷. Although there is a small loss of helical structure, the dUVRR spectra indicate that the structure of melittin remains largely intact at the highest molar percentage of 5 α -cholestan-3 β -ol, which is further evidenced by the lack of change in the intensity of the amide I and amide II modes.

Discussion

Melittin is a highly soluble peptide that spontaneously folds in a membrane or membrane-mimicking environment. While dUVRR spectroscopy has been employed to study the structure and dynamics of a variety of globular proteins, its application to membrane proteins has been limited³⁻⁴. Of particular note in these previous studies was the upshifted and extremely intense amide I mode of membrane proteins versus their

soluble counterparts. The dUVRR spectrum of melittin, a much simpler protein, in a lipid environment, also has a more intense amide I band versus soluble α -helical proteins. However, the position of the amide I band at 1651 cm^{-1} is consistent with other soluble α -helical proteins. It can be concluded from the dUVRR spectra of melittin that desolvation by bilayer insertion does result in an increase in the intensity of the amide I mode, presumably from the loss of hydrogen bonding between water molecules and the carbonyl of the amide backbone. However, the upshift and extreme intensity of the amide I band in previous studies may derive from additional factors other than the solvation environment of the peptide backbone as these were not observed for lipid solvated melittin.

Figure 1-6: CD (top) and OCD (bottom) spectra of melittin in DMPC liposomes at 37°C with 0 molar percent 5 α -cholestan-3 β -ol (black), 10 molar percent 5 α -cholestan-3 β -ol (grey), and 25 molar percent 5 α -cholestan-3 β -ol (dashed).

CD spectroscopy has many advantages and is often used for rapid assessment of protein structure. However, studies on smaller non-globular proteins show that the CD and dUVRR spectra can suggest dramatically different secondary structure distributions^{44, 54, 58}. Similar discrepancies were observed between the CD and dUVRR spectra of melittin in DMPC liposomes at 37°C and with 5 α -cholestan-3 β -ol, where a large loss of helical structure could be inferred from the CD spectra (Figure 1-3 and Figure 1-6), but the dUVRR spectra indicated that the helical structure of melittin remains largely intact. The loss of intensity in the CD spectra may be due to a loss of coupling along the axis of the helix as the motion of the peptide increases at higher temperatures and becomes more elongated at lower fluidities. The ability of 5 α -cholestan-3 β -ol to disrupt the transmembrane orientation of melittin and force the melittin toward a more parallel orientation nearer the surface of the membrane could account for the small loss of helical structure indicated by dUVRR and the additional loss of CD spectral intensity.

Figure 1-7: DUVRR spectra of melittin in DMPC liposomes at 37°C with 5 α -cholestan-3 β -ol (molar percentages as indicated).

The temperature profile of melittin shows a decrease in the intensity of the CD spectrum with increasing temperature, as was seen previously³⁶. However, the results obtained here in lipid vesicles show a much larger change in the CD spectrum over a much narrower temperature range than those in bicelles. The large loss in CD intensity is not coupled with an increase in the amide S mode of the dUVRR spectra, as would be expected if the melittin were losing α -helical character, but rather an increase in the amide III mode. This suggests a more fluid membrane environment enables melittin a greater freedom of movement resulting in sampling of 3₁₀-helical structure but conservation of the peptides overall helical structure (Figure 1-8).

Figure 1-8: Plot of dUVRR amide III position versus w-angle.

The introduction of 5 α -cholestan-3 β -ol to the DMPC vesicles at 10 molar percent induced little structural change in melittin as indicated by the CD and dUVRR spectra. The addition of 25 molar percent 5 α -cholestan-3 β -ol shows both a dramatic decrease in the intensity of the CD spectrum and an increase in the amide S and amide III dUVRR modes. These spectral changes indicate a decrease in the α -helical character of melittin with increasing 5 α -cholestan-3 β -ol concentration as is expected from previous studies (Figure 1-8)^{40, 42}. The OCD spectra show melittin being mostly transmembrane without 5 α -cholestan-3 β -ol. Melittin then becomes less transmembrane with higher concentrations of 5 α -cholestan-3 β -ol. This indicates that the melittin is being forced out of the transmembrane orientation by 5 α -cholestan-3 β -ol. This change in tilt angle for melittin could contribute to the loss of α -helical character seen in the CD and dUVRR spectra.

In conclusion, lipid induced desolvation results in an increase in the intensity of the amide I band in the dUVRR spectra of melittin in a membrane bilayer. The amide II, III, and S modes appear unaffected by the polarity of the environment of the protein. In combination, the amide bands can be used to monitor both a protein's environment (amide I) and secondary structure (amide II, III, and S). In all, by combining CD, OCD and dUVRR the subtle structural fluctuations of melittin in changing fluidities were elucidated in a way not experimentally achieved previously.

Chapter 2

Introduction

Heart disease is currently the leading cause of death in the United States⁵. High blood cholesterol levels can be an indication of an increased risk of heart disease⁶⁸⁻⁷⁰.

Cholesterol is naturally produced and metabolized by the liver and transported through the bloodstream by particles called lipoproteins⁶. Lipoproteins are disk- or spherical-shaped complexes with a hydrophilic exterior made of lipids and proteins that carry cholesterol in their hydrophobic core. There are five major classes of lipoproteins that are distinguished by the size and density of the particle. The classes, from least dense to most dense, are chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoprotein and high-density lipoproteins (HDL). For the purposes of this study only VLDL, LDL, and HDL will be discussed. Higher HDL blood levels have been linked to a reduction in the risk for heart disease,⁹⁻¹⁰ as these particles can transfer cholesterol that has built up on arterial walls back to the liver where it can be broken down and removed from the body. The opposite is true of LDL. Higher LDL blood levels increase the chance of heart disease^{7-8, 11-12} as LDL can promote transport of cholesterol to the arterial walls causing the arteries to harden and be restricted.

The most widely used clinical testing methods for cholesterol, LDL, and HDL do not directly measure either LDL or HDL⁷¹⁻⁷². Instead these tests measure total cholesterol in the blood, triacylglycerides and the protein associated with HDL. LDL levels are then calculated using the Friedewald⁷³ equation. This manner of lipoprotein testing can yield

widely varied results depending on how patients prepare for the test. Considering the link between LDL, HDL, and heart disease a more direct approach is needed for lipoprotein characterization in blood.

Deep Ultraviolet Resonance Raman (DUVRR) Spectroscopy is a vibrational technique that has been used to monitor both soluble and membrane-associated proteins^{2, 24, 28, 52, 58, 66}. The utility of dUVRR spectroscopy in protein studies is derived from the resonance enhancement of the structurally informative peptide backbone modes over side chain or background solvent modes, which can dominate the spectra with traditional Raman techniques. This signal enhancement can also be applied to the double-bonded structure found in many types of biological molecules like fatty acids, lipids, and cholesterol. The ability to simultaneously probe protein, lipid, and cholesterol modes in a sample makes dUVRR a unique tool for lipoprotein analysis.

Materials and Methods

Cholesterol, monosodium phosphate monohydrate, disodium phosphate heptahydrate, sodium chloride, sucrose, oleic acid, linoleic acid, vaccenic acid, arachidonic acid, dithiothreitol (DTT) and chloroform were purchased from Fisher Scientific (Pittsburgh, PA). 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DOPG) and 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine (DLPC) were purchased from Avanti Polar Lipids (Alabaster, AL). The Amplex Red Cholesterol Assay Kit was purchased from Thermo Fisher Scientific (Waltham, MA). Commercial low-density lipoprotein (LDL) and high-

density lipoprotein (HDL) were purchased from Feldan (Quebec, Canada). Frozen cow brain was purchased from a local supermarket.

Synaptosome isolation was performed per the method of Hui et. al⁷⁴ using frozen cow brain. The frozen cow brain was homogenized in a 10% sucrose solution and 1 mM DTT with a glass homogenizer. The homogenized brain was centrifuged at 700 rcf for 20 minutes. The pellet was then homogenized in 10% sucrose and centrifuged at 700 rcf for 20 minutes. The supernatants from the previous centrifugations were combined and centrifuged at 10,000 rcf for 30 minutes. The resulting pellet was then re-suspended in 10% sucrose, layered on 27%/41% sucrose levels, and centrifuged at 100,000 rcf for 2 hours. The interface of the 27%/41% sucrose layers was collected and centrifuged at 100,000 rcf for 1 hour. The pellet was then re-suspended in 10% sucrose and the solution was centrifuged at 100,000 rcf for 1 hour. The pellet was then incubated in 5 mM Tris-HCl pH 8.1 for 35 minutes at 0°C and then centrifuged at 100,000 rcf for 30 minutes.

Synaptosomes were re-suspended in 20 mM phosphate buffer.

Standard lipid vesicles with cholesterol were formed by first individually dissolving DLPC, DOPG, and cholesterol in chloroform. The dissolved lipids/cholesterol were then combined to form solutions of 15, 10, and 5 percent cholesterol and 30, 25, and 20 percent DOPG by weight, with DLPC making up the remaining mass in a glass culture tube. The chloroform was then evaporated under a stream of argon to leave a thin film on the bottom of the tube. The tubes were further dried overnight in a vacuum desiccator. The dried solutions were then rehydrated using a 20 mM phosphate buffer (pH 7.4) and sonicated in a bath sonicator until the solutions changed from cloudy to opaque. The vesicles formed from this preparation had an average diameter of 25.8 ± 1.5 nm.

Whole blood samples were obtained from a local clinical testing laboratory. Blood serum was isolated from 10 separate whole blood samples by centrifugation at 10,000 rcf for 10 minutes⁷⁵. VLDL, LDL and HDL were separated from each of the 10 samples using a gradient of 48% w/w sucrose, and 0.67 M NaCl. The blood serum was adjusted to 4 M NaCl then layered on the gradient and centrifuged for 2 hours at 180,000 rcf. VLDL was collected from the top of the 0.67 M NaCl layer. LDL was isolated from the interface between the 0.67 M NaCl and the 4 M NaCl fractions. HDL was collected from the interface of the 4 M NaCl and 48% w/w sucrose fractions.

Independent cholesterol concentrations were determined using the Amplex Red Cholesterol Assay which detects the hydrogen peroxide production of an enzymatic reaction with cholesterol⁷⁶. Fluorescence measurements for the assay were taken on a Biotek SynergyMX fluorescence microplate reader.

The deep ultraviolet resonance Raman (dUVRR) instrument has been described previously⁴⁴. Briefly, the fourth harmonic of a tunable Ti:Sapphire Coherent (Santa Clara, CA) laser was focused on a sample flowing between two thin nitinol wires in a nitrogen purged sample chamber. The excitation wavelength used was 197 nm with power attenuated at the sample to 0.5 mW. Data processing and partial least squares (PLS) analysis were done using the MatLab (Natick, MA) environment.

DUVRR spectra of three sets of standard lipid vesicles were obtained as the calibration set for the PLS analysis of LDL isolated from whole blood yielding 27 standard lipid vesicle spectra. PLS was performed using the plsregress function in MatLab and the root mean square error of calibration (RMSEC) for the prediction of the standard lipid vesicle concentrations was calculated.

Results

Figure 2-1. DUVRR spectra ($\lambda_{\text{ex}} = 197 \text{ nm}$) of synaptosomes (left) and commercially available HDL and LDL (right).

DUVRR protein spectra contain four discernable amide modes. The amide I (carbonyl stretch), amide II (coupled N-H bend/C-H stretch), amide III ($\text{C}_\alpha\text{-H}$ bend/C-N stretch), and in certain protein structures amide S (coupled to the amide III, $\text{C}_\alpha\text{-H}$ bend/N-H bend, not resonantly enhanced in α -helical conformations). These amide modes provide insight into protein secondary structure and have proven particularly useful in the analysis of lipid-solubilized proteins^{1-4, 28, 77}. Previous studies have shown that the amide I intensity is strongly enhanced in the dUVRR spectrum of α -helical transmembrane proteins³⁻⁴.

Synaptosomes were isolated to obtain a dUVRR spectrum of a biologically relevant membrane system. The dUVRR spectra of synaptosomes (Figure 2-1) reveals a strong feature in the amide III region at 1263 cm^{-1} , an amide II band at 1545 cm^{-1} , a weak amide S mode at 1383 cm^{-1} , and a very intense feature in the amide I region at 1662 cm^{-1} . The position of the amide II band (1545 cm^{-1}) is indicative of α -helical structure, though the presence of a weak amide S band (1383 cm^{-1}) is consistent with at least a small amount of disordered structure⁷⁸. Fully disordered proteins typically have an amide III band of relatively equal intensity to that of the amide S band⁷⁸. Thus, the strong feature in the amide III region at 1263 cm^{-1} is more likely the tyrosine ring-O mode⁷⁹.

DUVRR spectra of commercially isolated HDL and LDL have intense amide I bands at 1664 cm^{-1} ; however, the intensity of this peak in the LDL spectrum is much greater than that in the HDL spectrum suggesting the intensity derives from a source other than transmembrane α -helices. The aromatic modes at 1211 cm^{-1} , 1261 cm^{-1} , and 1605 cm^{-1} are less intense in the LDL dUVRR spectrum, likely indicating a higher protein content within the HDL particles.

Figure 2-2. DUVRR spectra of fatty acids (left), the unsaturated lipid, DOPG (right, black), and cholesterol (right, gray).

Many biological membrane components like fatty acids, unsaturated lipids, and

cholesterol contain C-C double-bonded structures. Previous studies have shown that saturated lipids do not exhibit a strong dUVRR feature in this region^{2, 28}. DUVRR spectra of isolated fatty acids, the unsaturated lipid DOPG, and cholesterol have intense features in the 1655-1670 cm^{-1} region (Figure 2-2).

Both cis (arachidonic acid, linoleic acid, and oleic acid) and trans (vaccenic acid) fatty acids were evaluated for this study. All cis fatty acid dUVRR spectra contained one main spectral feature at 1661 cm^{-1} , which was slightly red shifted in dUVRR spectrum of the trans fatty acid to 1655 cm^{-1} . Both vaccenic acid and oleic acid contain a single double bond, but the intensity of the dUVRR spectrum for the trans vaccenic acid is less than that of the cis oleic acid. The intensity of the dUVRR special feature at 1661 cm^{-1} for the

cis fatty acids also increases with increasing number of double bonds. Oleic acid has a single double bond and is the least intense, linoleic contains two double bonds and is second most intense, and arachidonic acid has four double bonds and is the most intense. Cholesterol and lipids are major components in animal cell membranes. DOPG was chosen as a representative unsaturated lipid species and was analyzed in conjunction with cholesterol. DOPG contains two cis C-C double bonds with a main dUVRR spectral feature at 1660 cm^{-1} . Cholesterol has a single double bond but it is contained on the inside of a ring structure. The dUVRR feature for cholesterol can be seen at 1668 cm^{-1} .

Figure 2-3. Representative DUVRR spectra of VLDL (top), LDL (middle), and HDL (bottom) and the underlying lipid (lipid peak) and cholesterol (cholesterol peak) components.

The three major features in the dUVRR spectra of VLDL, LDL, and HDL separated from blood serum (Figure 2-3) are the tyrosine/amide III feature (1265 cm^{-1}), a lipid feature

around 1660 cm^{-1} , and a cholesterol feature around 1675 cm^{-1} . The tyrosine/amide III band is used as an indicator of protein content in the lipoprotein particles.

Figure 2-4. Correlation plots for the ten VLDL (left), LDL (center), and HDL (right) samples of the integrated tyrosine/amide III peak area versus Amplex Red cholesterol assay concentration (top) and the integrated cholesterol peak area versus Amplex Red cholesterol assay concentration (bottom).

The dUVRR tyrosine/amide III peak area increases with increasing cholesterol concentration, as measured by the Amplex Red cholesterol assay (Figure 2-4, top). These data suggest that the measured cholesterol concentration is correlated with the total amount of lipoprotein present in the blood serum samples. Comparison of the peak area of the fitted band at $\sim 1675\text{ cm}^{-1}$ versus the measured cholesterol concentration shows that there is no linear relationship between the total cholesterol content and the 1670 cm^{-1} peak intensity excepting the VLDL samples (Figure 2-4, bottom). For the HDL samples, it is likely that the intensity of the amide I band interferes with estimation of the

cholesterol peak intensity. However, the dUVRR spectra of LDL samples were largely devoid of protein bands suggesting that the lack of correlation may not be solely an interference issue. It is possible that dUVRR is sensitive to individual variations in the amount of cholesterol with the lipoproteins themselves. For example, a sample that is low in total LDL will have a lower measured cholesterol value via the Amplex Red assay but could have a higher percentage of cholesterol within the LDL that is present in the sample.

Figure 2-5. Representative dUVRR spectra of the standard lipid vesicles (left) and a table representing the standard vesicle compositions (right). Each of the nine concentration levels was prepared in triplicate for a total of 27 standard lipid vesicles. DUVRR spectra are colored by cholesterol content: 6 mM (black), 4 mM (dark gray), 2 mM (light gray).

Standard lipid/cholesterol vesicles were made from saturated lipid (DLPC), unsaturated lipid (DOPG), and cholesterol. The dUVRR spectra of the standard lipid/cholesterol

vesicles have a unsaturated lipid feature at 1661 cm^{-1} and a cholesterol feature at 1675 cm^{-1} (Figure 2-5), that are dependent on the percentage of cholesterol. As the cholesterol content increases, the raw intensity of the unsaturated lipid peak decreases, shifting the ratio of the two peaks. This effect may be due to increased self-absorption of the Raman scattering as the content of cholesterol increases.

Normalization of this feature to unit length in both the standard lipid/cholesterol vesicles and the LDL samples corrects for any intensity differences between the two data sets and puts them on the same scale. The standard lipid/cholesterol vesicles can then be used to create a calibration curve to predict the percent cholesterol present in LDL samples using PLS (Figure 2-6).

Figure 2-6. Plot of the cholesterol concentration and predicted cholesterol concentration for the nine samples of replicate 1 (black squares), nine samples of replicate 2 (dark grey triangles), and nine samples of replicate 3 (light grey circles) each of the standard lipid vesicles using PLS.

Figure 2-7. Predicted cholesterol concentration for LDL based on PLS results using standard lipid vesicles (left), dUVRR spectra of LDL sample three (grey) and sample 4 (black) normalized to unit area (center, top), raw dUVRR spectra of LDL sample three (grey) and sample 4 (black) (center, bottom), and cholesterol assay concentrations for the ten LDL samples.

The PLS model was applied to the LDL samples only as they are the closest match in composition to the standard lipid/cholesterol vesicles. The HDL samples have a much higher protein content and will therefore have the largest underlying amide I protein contribution in the 1661 cm^{-1} and 1675 cm^{-1} region of the dUVRR spectrum, which would make cholesterol determination with a model devoid of protein difficult. Further studies are needed to identify a suitable standard model for direct quantification of cholesterol in HDL. VLDL, has very little cholesterol. Consequently, the dUVRR spectra of the VLDL samples have a strong feature, mostly derived from the unsaturated lipid feature, at 1675 cm^{-1} . PLS of the LDL samples returns a narrow concentration range between 13.3% and 15.6% by weight cholesterol (Figure 2-7, left). When the normalized dUVRR spectra used for the PLS analysis of the highest and lowest predicted cholesterol

concentration samples (Figure 2-7, top, center) are compared, a difference in the ratio between the lipid peak and the cholesterol peak is seen. In Sample 3, the lipid peak has a lower intensity than that of the cholesterol peak leading to a higher predicted cholesterol concentration. In Sample 4 the opposite is true. The cholesterol peak is much lower than the lipid peak and leads to the lower predicted cholesterol concentration for that sample.

When compared to the cholesterol concentration determined by the Amplex Red Assay (Figure 2-7, right) we see that Sample 3 has a much lower cholesterol concentration than that of Sample 4. The raw dUVRR spectra of the two samples shows a less intense spectrum for Sample 3 when compared with Sample 4. Taken together with the results of the PLS analysis on the same two samples, an interesting trend emerges. The Amplex Red cholesterol assay reports on total cholesterol in solution and is consequently reporting on the total amount of lipoprotein in solution. This total amount of lipoprotein in solution is also being reported by the raw dUVRR spectrum by the intensity of the combined lipid/cholesterol peaks. Further analysis of the dUVRR spectrum reveals that the ratio between the lipid and cholesterol peaks is reporting on the amount of cholesterol contained within the lipoprotein particles themselves. Thus, dUVRR can simultaneously report on both the global amount of lipoprotein in solution and yield insights on the composition of the lipoproteins.

Discussion

DUVRR spectra of biological molecules (Figures 2-1 and 2-2) reveal a new utility for the technique. The double bond contained within these molecules shows a large resonance

enhancement allowing them to be easily detected at low relative concentrations. DUVRR not only allows for the detection of these molecules but can even discern between the type of double bond in the biomolecule. The trans double bond found in vaccenic acid (Figure 2-2), the cis double bond in the lipid DOPG, and the ring double bond of cholesterol all occur at different frequencies in dUVRR spectra. The sensitivity and selectivity of the double-bond character of these lipid molecules will allow dUVRR to be used in complex systems. Combined with multivariate analysis or fitting, spectral features have the potential to increase the utility of dUVRR in the analysis of lipids and lipid soluble compounds.

Lipoprotein analysis by dUVRR yields a wealth of information as the technique can simultaneously observe the protein, lipid, and cholesterol contained within the lipoprotein molecules. DUVRR and Amplex Red cholesterol assay analysis of a set of 10 VLDL, LDL, and HDL samples separated from blood serum reveals that dUVRR can not only report on total cholesterol but the percent composition of cholesterol within each lipoprotein particle as well. The Amplex Red cholesterol assay measures total cholesterol in solution by a series of enzymatic reactions. The actual analyte of the process is not cholesterol but a hydrogen peroxide product of one of the enzymatic reactions. The Amplex Red assay has no ability to distinguish between a sample with a few lipoprotein particles containing a high percentage of cholesterol and many lipoprotein particles with a low percentage of cholesterol.

DUVRR spectroscopy of lipoproteins has the potential to be useful in the simultaneous monitoring of both total lipoprotein concentration in solution and the percentage of

cholesterol contained within those lipoproteins. The dUVRR tyrosine/amide III band is a good monitor of protein content and scales with the Amplex Red cholesterol assay total cholesterol determination. The percentage of cholesterol within the lipoprotein molecules may be determined using a set of standard calibration samples and PLS regression, though other multivariate calibration methods may perform equally well. Combination of multivariate regression and dUVRR spectral analysis yields reasonable estimates of the percentage of cholesterol within the lipoprotein molecules. More research on the importance of cholesterol content within lipoproteins is needed. Additionally, dUVRR may be an effective detection method for in-line separation and measurement of lipoprotein content and size distributions, which have been shown to have relevance to cardiovascular disease⁸⁰⁻⁸¹.

While one of the most traditional applications of dUVRR spectroscopy has been in the field of protein secondary structure determination, a new utility of dUVRR to study lipids and cholesterol opens the door to a host of new applications for the technique.

Chapter 3

Introduction

Heart disease is currently the leading killer in the United States today and affects over a third of the population⁵. There are many risk factors for heart disease but one of the most talked about is the link to blood cholesterol levels⁶⁸⁻⁷⁰. Cholesterol itself is not soluble in the blood stream as it is too hydrophobic. Instead cholesterol is encapsulated and transported through the body by molecules known as lipoproteins. Lipoproteins have a hydrophobic core made of lipids that hold cholesterol; the hydrophobic core is wrapped in proteins that help solubilize the lipoproteins in the blood stream. Lipoproteins are usually disk or spherically shaped and are defined by their densities. There are five major categories of lipoproteins which include chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL). As protein content increases, lipoproteins become smaller and denser. Chylomicrons have virtually no protein, the largest radius and are the least dense. HDL has the largest protein content, the smallest radius and is the densest type of lipoprotein. As in the previous chapter of this work only VLDL, LDL, and HDL were included in the analysis.

In the clinical setting total blood cholesterol, LDL, and HDL levels are what is most commonly measured. Increased levels of LDL have been linked to an increased risk of heart disease^{7, 11-12}, whereas increased HDL levels have been linked to a decreased risk for heart disease⁹⁻¹⁰. The most common methods used in the clinic do not measure LDL

or HDL levels directly⁷¹⁻⁷². Instead whole blood cholesterol, triglycerides, and the protein associated with HDL is measured. LDL levels are then calculated using the Friedewald equation. More direct methods for measuring blood HDL and LDL levels such as nuclear magnetic resonance⁸²⁻⁸⁵, ultracentrifugation⁸⁶⁻⁸⁹, and electrophoresis⁹⁰⁻⁹³ exist but require more time, money, or data analysis than the current clinical methods.

While lipoproteins are characterized by their densities, a wide distribution of sizes exists within the particle categories. This size distribution allows for the use of size exclusion chromatography to help separate lipoprotein species from one another in a blood serum sample. Size exclusion chromatography (SEC) is a separation technique that works by passing particles through a stationary phase with a certain pore size⁹⁴⁻⁹⁵. If the particles are smaller than the pore size they can enter the matrix of the stationary phase and will be retained on the column. If they are larger than the matrix they will be excluded and elute off the column. Ideally, with a size based separation there will be no interaction between the stationary phase and the analytes as there is in most other separation techniques.

Instead the analytes will be separated by the differences in size alone. There are several advantages to using SEC in the analysis of lipoproteins. The major advantage is that very little sample prep is required. Blood samples can be collected, the serum or plasma isolated, and placed directly into the chromatography system.

Deep Ultraviolet Resonance Raman (dUVRR) Spectroscopy is a vibrational technique that has been used previously in the determination of secondary structure for both soluble and membrane-associated proteins^{2, 77, 96-97}. Resonance enhancement of the peptide backbone vibrational modes provides rapid structural insights with little sample

preparation. DUVRR is also sensitive to the carbon-carbon double bonds found in biological molecules like fatty acids, unsaturated lipids, and cholesterol. Resonant enhancement of this characteristic double bond allows for a new method of cholesterol quantitation.

There have been many studies that employ multivariate calibration and higher order statistical techniques to the dUVRR analysis of proteins^{27, 78, 98-99}. Analyses like multivariate curve resolution-alternating least squares (MCR-ALS) and parallel factor analysis (PARAFAC) can analyze complex data sets and reveal underlying spectral and chromatographic components when the composition of the analyte is not known.

Here we present a new multifaceted approach for measurement of lipoprotein profiles by combining SEC, dUVRR, and multivariate analysis. The vast range in lipoprotein size allows for good separation of the lipoprotein types by SEC. These SEC fractions can then in turn be analyzed by dUVRR for identification and cholesterol content. Finally, the dUVRR data can be processed using multivariate calibration techniques. This workflow allows for a single continuous analysis that yields not only a lipoprotein profile, but potentially critical information about the composition of each lipoprotein fraction.

Materials and Methods

Monosodium phosphate monohydrate, disodium phosphate heptahydrate, and sodium sulfate were purchased from Fisher Scientific (Pittsburgh, PA). Blood collection tubes and needles were purchased from Becton, Dickinson, and Company (Franklin Lakes, NJ).

Five blood serum samples were isolated from whole blood, donated by graduate students, by centrifugation at 10,000 rcf for 10 minutes. The serum was then separated using fast protein liquid chromatography (FPLC) on a Bio-Rad system using a GE (Pittsburg, PA) Superose 6 size exclusion column (5-5000 kDa separation range for globular proteins). A mobile phase of 20 mM phosphate and 250 mM sodium sulfate, injection volume of 500 μ L, and a flow rate of 0.5 ml/min were used. Fractions of 1 mL were collected for the entirety of the FPLC run for each sample.

Independent cholesterol concentrations were determined using the Amplex Red Cholesterol Assay which detects the hydrogen peroxide production of an enzymatic reaction with cholesterol⁷⁶. Fluorescence measurements for the assay were taken on a fluorescence microplate spectrometer.

The deep ultraviolet resonance Raman (dUVRR) setup has been described previously⁴⁴. Briefly, the fourth harmonic of a tunable Ti:Sapphire Coherent (Santa Clara, CA) laser was focused on a sample flowing between two thin nitinol wires in a nitrogen purged sample chamber. The excitation wavelength used was 197 nm with power attenuated at the sample to 0.5 mW. All data processing and analysis were carried out in Matlab (Mathworks, Natick, MA).

Parallel factor analysis is typically used to deconstruct a multi-way data array to aid in identification and quantification of underlying components in samples with unknown concentrations¹⁰⁰⁻¹⁰⁴. The PARAFAC analysis done here is based on a trilinear model¹⁰⁰ as shown in equation 1.

$$r_{ijk} = \sum_{n=1}^N \hat{x}_{i,n} \hat{y}_{j,n} \hat{z}_{k,n} + e_{i,j,k} \quad (1)$$

The trilinear data set for this work was created by combining dUVRR spectra of 17 SEC fractions from 5 different blood serum samples creating a r_{ijk} from equation 1 measuring the i th wavenumber of the j th SEC fraction from the k th blood serum sample. The PARAFAC algorithm has been described previously¹⁰⁵. A core consistency diagnostic¹⁰⁶ was used to determine the PARAFAC model parameters. This type of diagnostic on a similar data set has been described previously⁹⁹. A three-factor model with non-negativity restraints was chosen because this model yielded the best core consistency (34.3%). The addition of a third factor results in a drop in the core consistency from 100% with two-factors but was greater than that of a three-factor model without non-negativity restraints (24.4%), and much greater than a four-factor model (2.4%) and a four-factor model with non-negativity constraints (3.8%).

MCR-ALS analysis was done on the same data set as the PARAFAC analysis using an algorithm originally developed by Bro and Sidiropoulos¹⁰⁷. The MCR-ALS analysis was initialized using the outputs of an evolving factor analysis and was also constrained to nonnegative values. MCR-ALS will resolve a concentration set and pure spectral values from the original data matrix. The concentration set will be comprised of the amount of the underlying factors from the data set.

Results

Figure 3-1. Size exclusion chromatograms (SEC) for five blood serum samples.

Figure 3-2. Representative set of dUVRR spectra from the fractions collected during a SEC analysis of blood serum.

Chromatographic analysis of lipoproteins

Lipoproteins are a composite of lipids, cholesterol and proteins. Tryptophan, which absorbs strongly at 280 nm is a major component of proteins. Thus, monitoring the absorption at 280 nm will enable detection of any component with a significant amount of protein associated with it. The size exclusion chromatograms of blood serum samples with UV detection at 280 nm are very similar and have several interesting features.

(Figure 3-1).

The first chromatographic feature occurs from 8-10 mL and represents the largest particles in the blood serum samples. These large particles have been completely

excluded from the matrix of the SEC column. This early chromatographic band is likely a combination of chylomicrons and VLDL. The relatively low intensity of the absorption at 280 nm can be attributed to the small amount of protein in these particles. From 12-15 mL, a small shoulder appears on the larger band at 15-19 mL. These two chromatographic components represent biomolecules with lower and higher protein absorption, possibly LDL and HDL, respectively. The last chromatographic feature from 19-21 mL is close to the total volume of the column and represents smaller compounds, potentially free protein.

The ability to conduct qualitative analysis of chromatographic data with single wavelength absorption detection is limited. A series of dUVRR spectra were collected from sequential fractions of five SEC separations of blood serum. Typical protein dUVRR spectra contain four distinct amide modes. These amide modes consist of a carbonyl stretch (amide I), a coupled N-H bend/C-H stretch (amide II), a C_{α} -H bend/C-N stretch (amide III), and in certain proteins structures a C_{α} -H bend/C-N bend (amide S) that is coupled to the amide III but is not resonantly enhanced in α -helical conformations^{24-25, 55, 108-109}. The amide modes of the dUVRR spectra report on the secondary structure of proteins and have been utilized effectively to study lipid-solubilized proteins,^{1-4, 28, 77} with a particularly strongly enhanced amide I mode in transmembrane α -helices³⁻⁴. There are also three main spectral regions of interest when studying lipoproteins by dUVRR as seen in Chapter 2 of this work. First is the tyrosine/amide III feature at 1265 cm^{-1} which reports on protein content in the lipoprotein particles. The second and third regions are an overlapped lipid band (1660 cm^{-1}) and

cholesterol band (1675 cm^{-1}). These bands simultaneously carry information on the amount of lipoprotein in solution, which correlated with the magnitude of the combined lipid and cholesterol peak, and the amount of cholesterol within the lipoprotein particle itself, which is related the intensity ratio between the lipid peak and the cholesterol peak.

SEC-dUVRR spectra have characteristic features that can be attributed to the sulfate ion at 978 cm^{-1} and the broad water band at 1640 cm^{-1} in nearly pure buffer at 8 mL (Figure 3-2). After the 8 mL fraction, there is a rapid rise in the lipid/cholesterol feature at 1660 cm^{-1} through the 10 mL fraction, correlating with the earliest feature in the size exclusion chromatograms. As the intensity of the lipid/cholesterol band drops, the amide III/tyrosine feature at 1265 cm^{-1} increases in intensity, indicating particles with higher combined protein and cholesterol contents. Towards the end of the separation, the lipid/cholesterol feature disappears while the protein modes remains, indicating free protein is being eluted from the column.

Figure 3-3. PARAFAC analysis of SEC-dUVRR samples with non-negativity restraints. The three components from the PARAFAC analysis are buffer (light grey), lipid/cholesterol (dark grey), and protein (black). The predicted spectral results (left), amount of component per SEC fraction (middle), and amount of each factor per sample (right) are shown.

Multivariate analysis of SEC-dUVR spectra of blood serum

Combined chromatographic and spectral analysis of several blood serum samples results in a trilinear data array that can be rapidly analyzed using multi-way methods such as PARAFAC. A three-factor PARAFAC model with non-negativity constraints was determined to be the optimal model. Three-factors were resolved that correspond spectroscopically to a buffer component (light grey), a protein component (black), and a lipid/cholesterol component (dark grey) (Figure 3-3, left).

Evaluation of the SEC profiles, indicates that the buffer component is dominant initially and drops through the 22 mL fraction, at which time it increases again (Figure 3-3, middle). Conversely, the protein component is absent initially and increases slowly through the 22 mL fraction, where the protein component begins to taper off.

Interestingly, the protein component increases from the 10-12 mL fractions and then levels off between the 12-14 mL fractions and then increases again through the 16 mL fraction suggesting multiple underlying chromatographic components. There are two maxima in the chromatographic profile of the lipid/cholesterol component. The first peak occurs at the 10 mL fraction, after which the intensity falls sharply, with an asymmetrical shoulder centered around the 14 mL fraction. Comparison of the relative amounts of each resolved component in the serum samples indicates that samples 1 and 3 are more similar

with a greater lipid/cholesterol to protein ratio, whereas samples 2, 4 and 5 have greater protein to lipid/cholesterol ratios (Figure 3-3, right).

There is insufficient resolution in the separation to enable clear differentiation of the lipoprotein particles. However, PARAFAC analysis resolves three distinct spectral and chromatographic features that can be used to differentiate the lipoproteins in the fractions based on their relative proportion of the lipid/cholesterol and protein features. Given that each individual's amount and size distribution of lipoproteins is different, the chromatographic profiles may not be identical for all samples. In such a case, the trilinearity constraint is not entirely fulfilled.

Figure 3-4. Resolved dUVRR spectral components for the MRC-ALS analysis of the SEC fractions.

If it is assumed that the underlying spectral profiles are the same for each sample, but the chromatographic profiles may vary, MCR-ALS can be used to resolve the global spectral and individual chromatographic profiles. The resolved dUVR spectra from the MCR-ALS analysis with non-negativity constraints of the data set (Figure 3-4) is similar to those estimated using the PARAFAC model. Again, there is a buffer component (light grey), a protein component (black), and a lipid/cholesterol component (dark grey).

Figure 3-5. Plot of intensity of the three resolved MCR-ALS factors versus SEC fraction for the MCR-ALS analysis of the dUVR spectra of SEC fractions. The three factors are lipid/cholesterol (left), protein (middle), and buffer (right).

The individual resolved chromatographic profiles from MCR-ALS are again similar to the global chromatographic profiles estimated with PARAFAC analysis, except that sample-to-sample differences become apparent. The lipid/cholesterol and protein profiles are less intense for sample 3 (Figure 3-5, left and middle, respectively), whereas the buffer profile (Figure 3-5, right) is more intense suggesting a lower overall level of lipoproteins. The largest difference in the lipid/cholesterol chromatographic profiles (Figure 3-5, left) is at 11 mL, where chylomicrons and VLDL particles are eluting. The

greatest sample variance is in the protein chromatographic profiles, especially between volume fractions 12-14 mL and 15 to 20 mL (Figure 3-5, middle).

Discussion

SEC separation of blood serum enables separation of various sized lipoprotein particles including chylomicrons/VLDL, LDL and HDL. SEC combined with dUVRR provides insight into the complex distribution of an individual's lipoproteins. Both PARAFAC and MCR-ALS analysis resolved a lipid/cholesterol, protein and buffer spectral profiles. MCR-ALS, enabled evaluation of individual chromatographic profiles. Inclusion of more samples combined with selective analysis of the lipid/cholesterol region may enable resolution of the lipid and cholesterol features in the future.

Conclusion

Here the utility of dUVRR as both a powerful tool for analysis of not only membrane protein structure and solvation state but also as a new means for cholesterol analysis is demonstrated. DUVRR was able to distinguish between melittin losing α -helical structure with an increase in cholesterol in the membrane system and melittin gaining more freedom in a more fluid lipid environment with increasing temperature, a change that could not be distinguished in the CD.

A unique resonance enhancement of the carbon-carbon double bond found in biomolecules like fatty acids, unsaturated lipids, and cholesterol with resolvable frequency shifts between cis, trans, and internal ring carbon-carbon double bonds has been discovered. The intensity of this new feature also scales with increasing number of double bonds and concentration allowing dUVRR to be applied to the field of cholesterol quantitation. When comparing LDL particles to standard unsaturated lipid/cholesterol containing vesicles it was found that the intensity of the lipid/cholesterol feature scales with the total amount of lipoprotein in solution, while the ratio between the lipid and cholesterol features within the dUVRR spectra is correlated to the amount of cholesterol contained within the lipoprotein particles themselves.

When paired with SEC and multivariate analysis methods dUVRR could distinguish between lipoprotein types in the separation chromatogram and determine lipoprotein particle and cholesterol concentrations within those particles as previously discussed.

Overall, dUVRR has been shown to be a powerful technique with applications in not only

the traditional field of protein structural analysis but also in the field of lipoprotein and cholesterol analysis.

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Vita

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