AWARD NUMBER: W81XWH-22-1-0002

TITLE: A Spectroscopic Approach to Overcome the Barriers of Early Familial Hypercholesterolemia Diagnosis

PRINCIPAL INVESTIGATOR: Carly S. Filgueira

CONTRACTING ORGANIZATION: Methodist Hospital Research Institute, Houston, TX

REPORT DATE: January 2024

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE January 2024	**2. REPORT TYPE** Annual	**3. DATES COVERED** 01Jan2023-31Dec2023

4. TITLE AND SUBTITLE

A Spectroscopic Approach to Overcome the Barriers of Early Familial Hypercholesterolemia Diagnosis

5a. CONTRACT NUMBER
W81XWH-22-1-0002

5b. GRANT NUMBER
PR211376

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Carly S. Filgueira

E-Mail: csfilgueira@houstonmethodist.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

METHODIST HOSPITAL RESEARCH
INSTITUTE
6670 BERTNER AVE
HOUSTON TX 77030-2602

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In diagnosing and treating familial hypercholesterolemia (FH) knowing both the "good" (HDL) and "bad" (LDL) cholesterol levels is extremely important as it allows a person to reduce the risk of heart disease and stroke. We propose to develop novel optical spectroscopy-based methods to cover the gap in FH diagnosis through use of surface-enhanced Raman spectroscopy (SERS). Our innovation is to use "near infrared" light (just outside the visible spectrum) and gold nanorods to measure LDL cholesterol more accurately and directly. We hypothesize that SERS spectra interpreted with quantum chemistry calculations and combined with the unique surface chemistry of gold nanorods will enable two critical advances in cholesterol detection for FH and other diseases. To test our hypothesis, we will 1) identify experimentally observable Raman vibrational modes for cholesterol detection and 2) test sensitivity of cholesterol detection in serum and in tissue. Major findings include that time dependent density-functional theory (TDDFT) calculations for the Raman vibrations of cholesterol show the presence of spectral bands of cholesterol's tetracyclic rings which provide information on local chemical properties and experimentally, a SERS signal is detectable from surfactant coated nanorods when placed onto skin, which could enable new non-invasive tissue analysis methods for disease diagnosis.

15. SUBJECT TERMS

Familial Hypercholesterolemia, diagnostic tools, screening, spectroscopy, non-invasive, low-density lipoprotein (LDL), high-density lipoprotein (HDL)

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRDC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	22	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

1. INTRODUCTION

The scope of the research is to develop novel optical spectroscopy-based methods to cover the gap in familial hypercholesterolemia (FH) diagnosis through use of surface-enhanced Raman spectroscopy (SERS). FH results in excessively high levels of plasma cholesterol, xanthomas, premature atherosclerosis, and can lead to early death if untreated. Although current prevalence of the heterozygous form of FH is estimated to be around in 1 in 250 individuals in the general population, it is estimated that only 10% of the FH population is diagnosed and adequately treated, leading to a significant gap. Most individuals are unaware of their FH condition, as it often presents as symptomless, such that diagnosis is frequently done late in life or after the first cardiovascular event. We intend to use to Raman spectroscopy to establish the unique, vibrational modes of cholesterol and developed a real-time, non-invasive cholesterol meter that does not require any enzymatic reactions, like a standard lipid panel. We will carry out time-dependent density functional theory (TDDFT) calculations of cholesterol Raman modes and compare with experimental Raman spectra of cholesterol powder. We will then combine cholesterol with synthetic phospholipids to form solution-phase vesicles made up of phospholipid and cholesterol bilayers and record spectra from a more realistic sample. Coupling of these vesicles with gold nanorods will provide strong signal enhancements (ie SERS). Measuring the rate at which cholesterol from HDL and LDL particles transfer to gold nanorods in serum and using the kinetics, we will differentiate between these sources of cholesterol. Finally, a lipid-nanorod formulation will be applied to the surfaces of tissues to provide a new assay of non-bloodstream cholesterol, which could serve as an early indicator of xanthomas associated with FH.

2. KEYWORDS

Familial Hypercholesterolemia, diagnostic tools, screening, spectroscopy, non-invasive, low-density lipoprotein (LDL), high-density lipoprotein (HDL)

3. ACCOMPLISHMENTS

What were the major goals of the project?

Major Task 1 Perform time-dependent density-functional theory (TDDFT) calculations to identify Raman modes specific to the fused ring structure of sterols and prepare Institutional Animal Care and Use Committee (IACUC) protocol. (Months 1-4)
Major task 1 was completed 100%

Major Task 2 Experimentally obtain the Raman spectra of cholesterol powder. (Months 1-2)
Major task 2 was completed 100%

Major Task 3 Experimentally obtain the Raman spectra of cholesterol in synthetic lipid solutions. (Months 2-4)
Major task 3 was completed 100%

Major Task 4 Experimentally obtain the SERS spectra of cholesterol in synthetic lipids on gold nanorods. (Months 4-6)
Major task 4 was completed 100%

Major Task 5 To measure the peak intensity of cholesterol on the phospholipid-coated gold nanorods. (Months 7-10)
Major task 5 was completed 90%

Major Task 6 To determine the rate at which cholesterol from HDL and LDL particles transfer to gold nanorods in serum and use the kinetics to differentiate between these sources of cholesterol. (Months 10-14)
Major task 6 was completed 50%.

Major Task 7 To measure the SERS signal from the interaction of surfactant or lipid-stabilized gold nanorods within tissue. (Months 15-24)
Major task 7 was completed 20%

What was accomplished under these goals?

Specific Aim 1. To identify experimentally observable Raman vibrational modes for cholesterol detection.

Major Task 1 Perform time-dependent density functional theory (TDDFT) calculations to identify modes specific to the fused ring structure of sterols and prepare Institutional Animal Care and Use Committee (IACUC) protocol. (Months 1-4)

The subtasks achieved were: 1) Use the Amsterdam DFT commercial quantum chemistry package to run TDDFT calculations, modify parameters (numerical accuracy, conformer comparison, hydrogen bonding), assign peaks (Figure 1), 2) Initiate IACUC protocol, and 3) Obtain DoD ACURO (Animal Care and Use Review Office) approval.

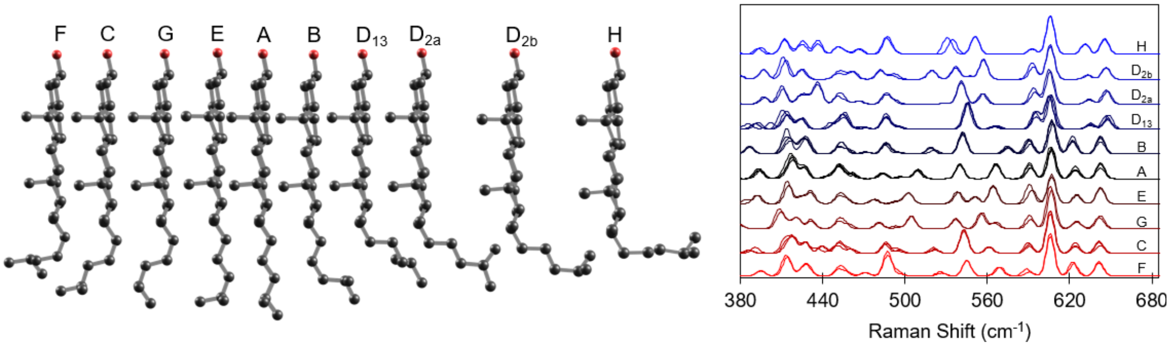


Figure 1. The structure (left) and Raman spectra (right) of the 10 cholesterol structure groups in the low wavenumber band that features vibrations of the steroid rings.

TDDFT calculations were performed on 27 different conformational structures of cholesterol with different dihedral angles in the iso-octyl chain.[1] We found that the conformers fall into 10 structural groups defined by the protrusion angle of the iso-octyl chain from the plane of the steroid rings. Based partially on a precedent from previous reports, we labeled these groups A, B, C, D_{13}, D_{2a}, D_{2b}, E, F, G, and H.[2] Furthermore, the TDDFT calculations showed that cholesterol conformers in the same structure group also have the same spectrum in the low wavenumber region. We found that conformer group A was the most accurate match to the experimental powder spectrum, as expected since group A has an extended chain that matches the crystal structure.[3] The full TDDFT calculated spectrum for group A is displayed in Figure 2 (green), and the corresponding powder spectrum is in black. The numbers above each peak in the spectra are assigned to a vibration of the molecule, indicating that we can make 23 peak assignments. We present the vibration maps of four peaks (10, 13, 16, and 23) below the spectra shown in Figure 2. Spectra and vibration maps of all assigned peaks for four straight-chain conformers are shown in the Appendix.

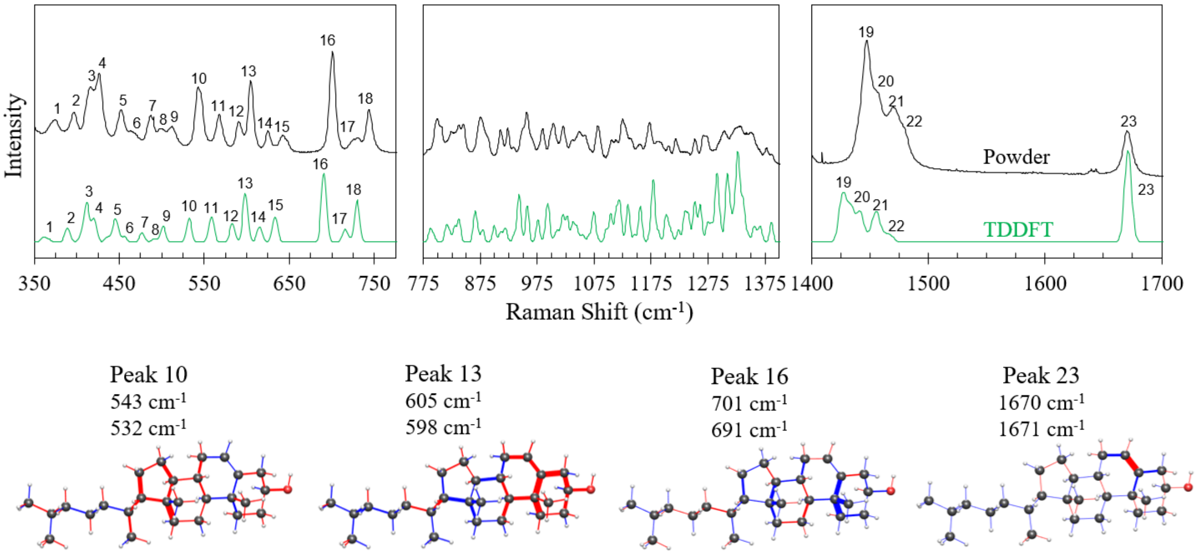


Figure 2. TDDFT calculations and peak assignments to identify modes specific to the fused ring structure of sterols. The calculated (green) and experimental (black) Raman spectra of cholesterol. Individual peaks that match based on position and relative amplitude are labeled. Vibration maps are displayed for four modes with their experimental (top) and calculated (bottom) Raman shifts. In the maps, each bond's thickness indicates its stretching amplitude, and the red/blue color indicates the relative phase.

The IACUC protocol for this study entitled “2021 DoD PRMRP Discovery Familial Hypercholesterol” (ID IS00006547) was approved by the The Methodist Hospital Research Institute IACUC on 11/16/2021; IACUC approval expires 11/15/2024.

DoD ACURO approval was obtained as of 12/16/2021 for the use of mice and pigs and will remain so until modification, expiration or cancellation.

Major Task 2 Experimentally obtain the Raman spectra of cholesterol powder. (Months 1-2)

The subtasks achieved were: 1) Acquire cholesterol power from the commercial vendor (Sigma-Aldrich) and determine acquisition time for reproducible Raman spectra with low background and 2) Identify the peak assignments and vibrational modes.

Raman spectra were recorded from cholesterol powder. We determined that the optimal signal was obtained for acquisition times of 20 minutes for each of the four spectral windows. The powder spectra are plotted in Figure 2 in Major Task 1 (black spectra). The plots were used to complete subtask 2 of Major Task 1, where 23 experimental peaks were assigned to Raman modes calculated by TDDFT.

Major Task 3 Experimentally obtain the Raman spectra of cholesterol in synthetic lipid solutions. (Months 2-4)

The subtasks achieved were: 1) Prepare stable solutions of large multilamellar vesicles made up of synthetic phospholipid and cholesterol bilayers and obtain Raman spectra (Figures 3-5) and 2) Identify the peak assignments and vibrational modes present from the cholesterol and/or lipids solutions (Figure 5).

To identify the spectral peaks in a more biologically relevant sample, solution-phase phospholipid vesicles that contain 25 mol-% cholesterol were created by the following protocol: stable lipid solutions were prepared by first obtaining powdered cholesterol from Sigma-Aldrich (>99% purity). Samples with four different phospholipid component were used, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC), 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), >99% purity, were obtained from Avanti Polar Lipids in chloroform solution. The phospholipid-cholesterol solutions with a 3:1 molar ratio of phospholipids to cholesterol were prepared. The lipids were dried under gentle argon flow for ~45 min, then placed in a turbopumped vacuum chamber for ~2 hours until the pressure dropped below 3 x 10^{-6} Torr. The solutions were then hydrated with deionized water to obtain a 10 mg/mL phospholipid concentration. The resulting solutions were mixed and sonicated for ~20 min. This removes the lipids from the glass container wall forming a milky white multilamellar vesicle (MLV) solution.

Dynamic light scattering (DLS) was carried out on the DOPC MLV sample with and without cholesterol to confirm the lipid vesicle size was not adversely affected by the presence of cholesterol. The results, displayed in Figure 3, show that there is no effect on the size distribution or the appearance of the MLV solution.

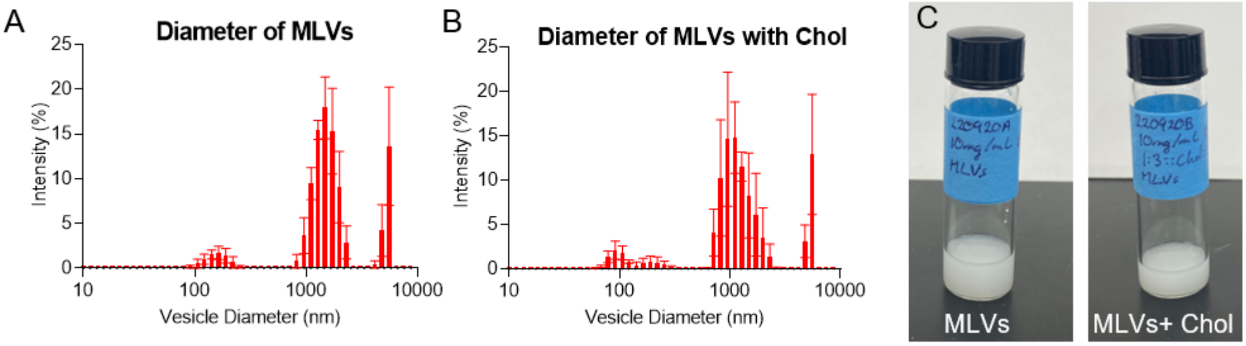


Figure 3. Size distribution histogram for DOPC vesicle diameter of (A) large multilamellar vesicles (MLV) and (B) MLVs with cholesterol obtained from Dynamic Light Scattering (DLS) measurements and (C) photos of the lipid vesicles without and with cholesterol.

Although not included as an initial subtask we also used sonication to transform the MLVs into small unilamellar vesicles (SUVs) turning the solution clearer with a bluish tint, displayed in Figure 4. Aqueous solutions were stored in a refrigerator and used for measurements within 2 days. The advantages of preparing and using stable SUVs over MLVs include: 1) The SUV are optically clearer which reduces the extinction with gives better Raman signal, 2) the SUVs have a more consistent structure resulting in sharper Raman peaks, and 3) the SUVs more efficiently transfer lipid material to the nanorods.

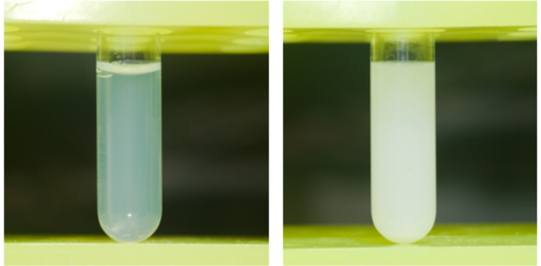


Figure 4. Photographs of solution-phase MLV (left) and SUV (right) lipid vesicles. The less turbid nature of the SUV solution is indicative of the smaller vesicle size.

To identify the peak assignments and vibrational modes present from the cholesterol and/or lipid solutions we loaded the samples in 1x1 mm glass capillaries and actively pumped the sample throughout the measurement to avoid settling and heating. Spectra were acquired for both the pure phospholipid vesicles and the sample that contained cholesterol. Spectra were recorded with 60 mW, 785 nm laser excitation and a 2 hour integration time. For DOPC the entire spectrum was recorded from 350 – 1700 cm^{-1}. For SOPC, POPC, and DPPC only the low spectral window (350 – 650 cm^{-1}) was recorded. The pure phospholipid spectra were subtracted from the cholesterol samples to remove phospholipid peaks and reduce the background. All spectra are presented in Figure 5. From these solution-phase lipid vesicle measurements, 21 of the 23 cholesterol peaks were detected.

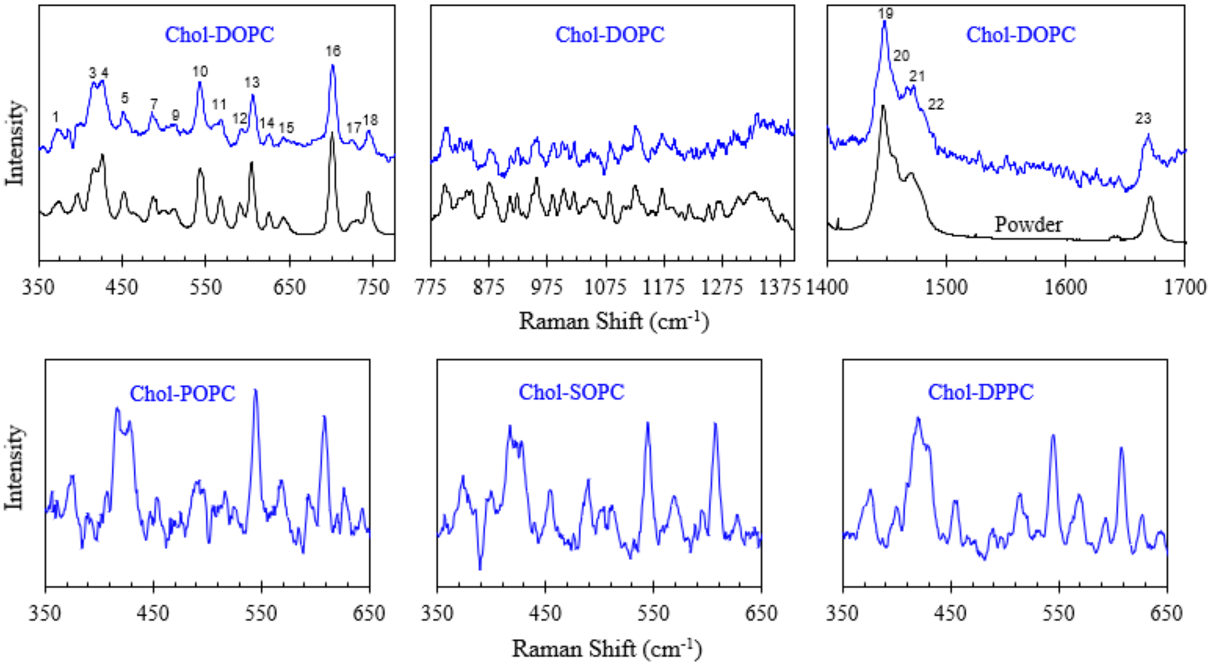


Figure 5. A comparison of the measured Raman spectrum of cholesterol in phospholipid vesicles (blue) and as a microcrystalline powder (black). Peaks in the DOPC vesicle spectrum (top panel) that were identified in Figure 1 are labelled. The low wavenumber band is shown for cholesterol in POPC, SOPC, and DPPC in the lower panel.

Major Task 4 Experimentally obtain the SERS spectra of cholesterol in synthetic lipids on gold nanorods. (Months 4-6)

The subtasks achieved were: 1) Prepare stable gold nanorod solutions with a surface coating of synthetic phospholipid layers containing cholesterol and obtain SERS spectra (See Figure 6A, B) and 2) Identify enhancement and peak assignments and vibrational modes present from the cholesterol and/or lipids solutions on the gold nanorods (Figure 6C, D).

Nanorods were synthesized in cetyltrimethylammonium bromide (CTAB) surfactant.[4] They were pelleted via centrifugation, the CTAB solution was removed, and the nanorods were resuspended in the lipid SUV solution described above. Under these conditions, lipids displace CTAB at the nanorod surface.[5] This process of putting lipids on nanorods was carried out both for pure phospholipids (DOPC) and phospholipids with cholesterol. Figure 6A confirms the rod geometry and Figure 6B displays the resulting SERS spectra. We observe one of the phospholipid peaks (labelled PC) and 5 of the peaks from the powder cholesterol spectra (3, 4, 10, 13, and 16), indicating that cholesterol remains within the lipid bilayer. The signal is enhanced by the nanorods surface (Figure 6C, D) as seen by the increasing signal intensity with increasing nanorod concentration.

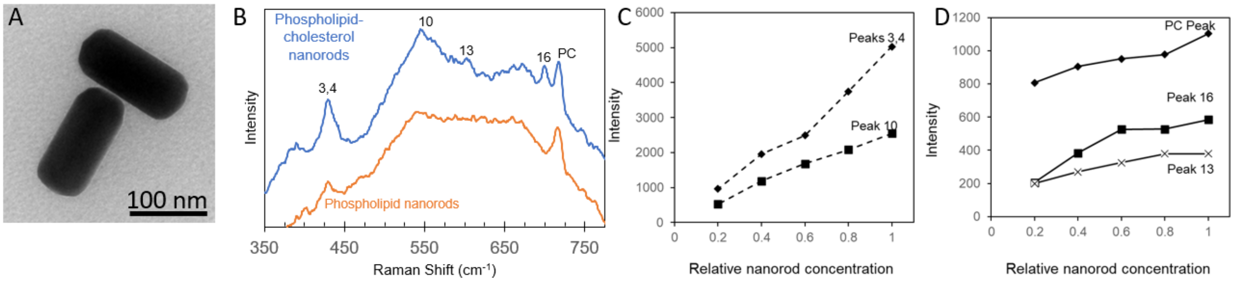


Figure 6. (A) TEM image of gold nanorods encapsulated with lipids, (B) SERS spectrum of nanorods encapsulated with lipids (orange) and nanorods with phospholipids + cholesterol (blue). PC = phosphocholine. Peaks assignments are indicative of cholesterol. (C,D) Peak intensities with varying nanorod concentration indicate the SERS intensity.

Major Task 5 To measure the peak intensity of cholesterol on the phospholipid coated gold nanorods. (Months 7-10)

The subtasks achieved were: 1) Prepare stable gold nanorod solutions with a surface coating of synthetic phospholipid layers containing cholesterol and obtain SERS spectra (See Figure 6 above), 2a) Collect blood samples (n=8 from pigs, 30 ml/animal and n=8 from mice, 1 ml/animal) from other studies upon euthanasia or alternatively purchase blood samples from a commercial source (Animal Technologies, Inc). (**although not treated by ultracentrifugation or spiked with cholesterol in lipid vesicles), and 3) Obtain background Raman spectra and identify strong peaks in spectral region of interest (200 – 1000 cm^{-1}), strength of the Raman background, and fluorescence (See Figure 7).

The subtasks that were not yet achieved were: 2b) Collect blood samples (n=8 from pigs, 30 ml/animal and n=8 from mice, 1 ml/animal) from other studies upon euthanasia or alternatively purchase blood samples from a commercial source (Animal Technologies, Inc). Treat by ultracentrifugation to remove HDL-C and LDL-C particles. Spike with known concentrations of cholesterol in lipid vesicles and 4) Add gold nanorods to treated blood samples and record and analyze SERS measurements.

We first tested serum that still has HDL and LDL particles present with surfactant-coated gold nanorods (rather than lipids) as a control to be able to identify what type of signal and background would be present. The surfactants used were CTAB and sodium dodecyl sulfate (SDS) which in future preparations would be displaced by the phospholipid DOPC. The investigators have already made progress on the use of SDS for nanorod stabilization.[6,7] Figure 7 shows some peaks in the raw serum (black spectrum). Even though the serum samples were clear without evidence of hemolysis, serum bands were detected from 600-650 cm^{-1}, 800-850 cm^{-1}, and 1000-1200 cm^{-1}. We anticipate that these peaks will also appear as background signal in spectra where we add phospholipid nanorods. Unfortunately, no new peaks appear from the nanorod spiked samples indicating the lack of any SERS signal.

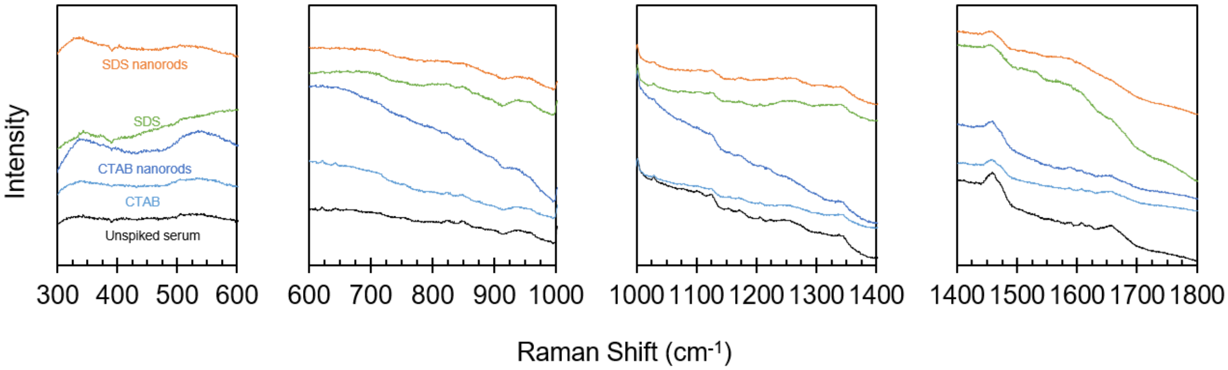


Figure 7. Raman and SERS spectra of unspiked rodent serum (black), serum with CTAB (light blue), serum with CTAB encapsulated nanorods encapsulated (dark blue), serum with SDS (green), and serum with SDS encapsulated nanorods (orange).

Considering this limitation (a lack of SERS signal) we considered using our discovery of the variation of the cholesterol Raman spectrum with conformation to distinguish HDL and LDL particles. We recorded the unenhanced Raman spectra of HDL and LDL particles purified in serum to see if the molecular conformation of cholesterol could distinguish them in their native state. The particles were isolated by adjusting the solution density to 1.063 or 1.21 mg/mL and then ultracentrifuged to float the LDL and HDL, respectively. The isolated lipoproteins were homogeneous according to size exclusion chromatography. Initial spectra of HDL and LDL are presented in Figure 8. A peak related to the phosphocholine headgroup was detected at 720 cm^{-1}, a peak associated with tyrosine in proteins was found near 650 cm^{-1}, and 8 peaks that match the cholesterol spectrum were found. The quality of the signal is sufficient for conformer analysis (compare Figure 8 to Figure 5).

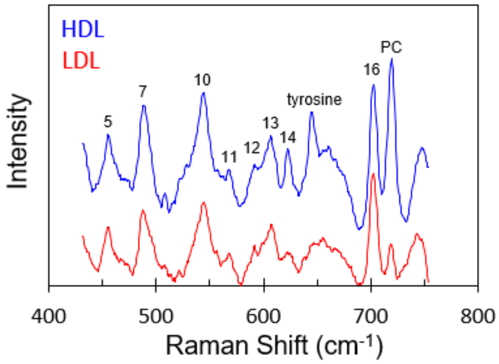


Figure 8. The unenhanced Raman spectrum of HDL and LDL particles, with cholesterol, phosphocholine and tyrosine peaks labelled.

Major Task 6 To determine the rate at which cholesterol from HDL and LDL particles transfer to gold nanorods in serum and use the kinetics to differentiate between these sources of cholesterol. (Months 10-14)

The transfer concept was tested with cholesterol in pure phospholipid vesicles (See Figure 9). As cholesterol transfers from vesicles to the nanorod surface, the cholesterol SERS signal increases with time.

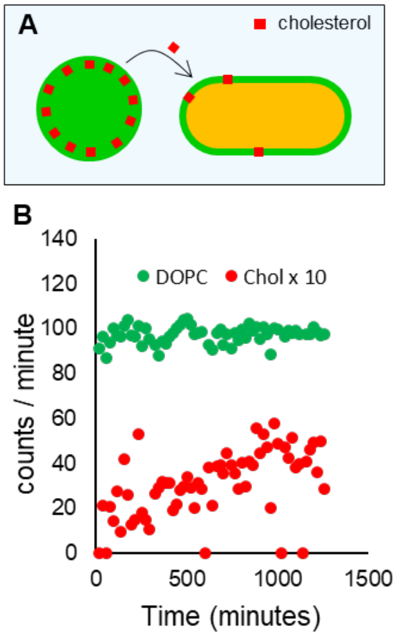


Figure 9. Cholesterol transfer from synthetic vesicles to gold nanorod surfaces. (A) A schematic of the process. (B) Preliminary data based on the time dependence of the intensities of the phospholipid headgroup peak at 718 cm^{-1} and the cholesterol peak at 701 cm^{-1}.

The subtasks that were not yet achieved were: 1) Measure the growth of cholesterol SERS peak over time after mixing lipid coated nanorods with serum. Fit kinetic models to identify and quantify specific rates for LDL-C and HDL-C. 2) Calibrate the LDL-C and HDL-C rate measurements for sensing with spiked serum measurements.

These subtasks were not yet achieved due to the lack of cholesterol SERS peaks identifiable in serum under the conditions tested so far. Increased integration time was not a viable solution since the background signal is strong and contributes significant shot noise. Furthermore, long integration times suffer from cosmic ray streaks that disturb spectral processing.

Rather than cholesterol transfer to nanorods and SERS, moving forward we will investigate *unenhanced* Raman signatures to distinguish HDL and LDL particles. Based on the results presented above (Figure 8), the cholesterol chain structure may be found to depend on lipoprotein particle packing. Also, note the relative level of phosophocholine (720 cm^{-1}) and tyrosine (640 cm^{-1}) in the particles. Both are much higher than the cholesterol peaks in HDL.

Major Task 7 To measure the SERS signal from the interaction of surfactant or lipid-stabilized gold nanorods within tissue. (Months 15-24)

The subtasks achieved were: 1) Collect tissue samples (liver, heart, adipose, dermal – ~1g/sample/mouse and ~50g/sample/pig) from animals (n=8 from pigs and n=8 from mice) on other studies upon euthanasia or alternatively purchase tissues from a commercial source (Animal Technologies, Inc) and apply lipid-nanorod formulations. Monitor the SERS signals.

The subtasks that were not yet achieved were: 2) Statistical analyses, interpretations, report writing, and 3) Prepare manuscript for publication and submit abstract for conference presentation of results.

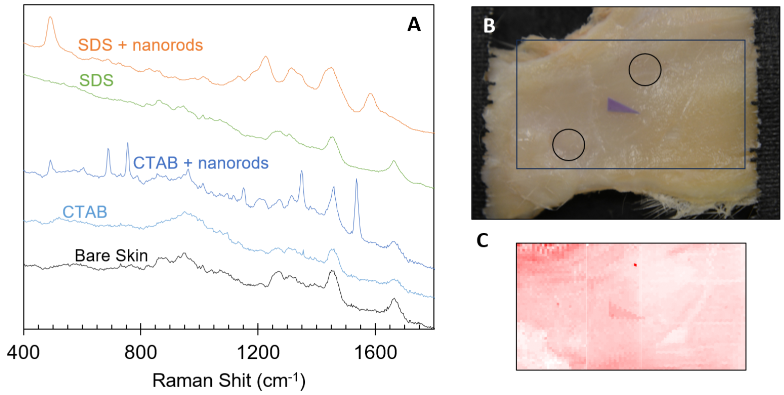


Figure 10. SERS from tisue. (A) Spectra from a skin sample including an untreated region, regions treated with CTAB and SDS surfactant solution, and regions treated with surfactant stabilized nanorods. (B) Photograph of a skin sample that had lipid-encapsulated nanorods deposited in the circled locations. (C) The total Raman signal of the region highlighted region where the triangular reference marker can be seen in the center.

To observe the effects of nanorods we spiked areas of shaved rodent skin with 2 uls of 10 mM surfactant solution (control) and 2 uls of highly concentrated nanorods (~10 OD at the plasmon peak) in 10 mM surfactant or lipid solution solution. The skin was then scanned across its surface with a Raman probe to obtain Raman and SERS spectra. Scanning was performed using a motorized system that maintains a constant probe-sample separation. Each spectrum is recorded with an integration time of 5 s and at a spatial resolution of 0.2 mm.

With surfactant coated nanorods, SERS signals were detected at the nanorod deposited regions with both SDS and CTAB surfactants.[8,9] Representative spectra are displayed in Figure 10A. The source of the strong peaks was not clear. Importantly, peaks appear with the addition of nanorods with both surfactants (see Table 1 for a list of peaks). Some peaks are present with both surfactants (e.g. 492 cm^{-1}), while some peaks are only present for a single surfactant (e.g. 687 cm^{-1}). This suggests we can control what biomolecules we are detecting by changing surfactants. However, none of them were a clear match for cholesterol. Their vibrational assignments will be further investigated.

The above tissue experiments were also carried out with phospholipid coated nanorods. Figure 10B shows a skin sample and the two locations where nanorods were deposited as above. A small triangular reference marker was also placed on the sample to help align the Raman map. The map of total Raman intensity is displayed in Figure 10C. The general tissue structure can be seen, as well as the reference marker. However, there was no enhanced Raman signal from the nanorod regions. It seems that the lipid-coated rods did not recruit molecules to the nanorod surface. Another possibility is that the lipid-coated nanorods did not aggregate into clusters as they may have for the surfactant-stabilized nanorods above. The difference in nanorod behavior in the tissue between surfactant and lipid stabilization will be studied further.

Table 1. SERS peaks that appear on rodent skin spiked with CTAB encapsulated rods and SDS encapsualted rods.

CTAB stabilized	SDS stabilized	Assignment
492 cm^{-1}	492 cm^{-1}	
605 cm^{-1}		
687 cm^{-1}		
755 cm^{-1}		Tryptophan
	1138 cm^{-1}	
1150 cm^{-1}		
	1187 cm^{-1}	
1202 cm^{-1}		
1226 cm^{-1}	1224 cm^{-1}	
1315 cm^{-1}	1313 cm^{-1}	
1349 cm^{-1}	1346 cm^{-1}	Tryptophan
	1447 cm^{-1}	CH_2/CH_3 scissor
1456 cm^{-1}		CH_2/CH_3 bend
1535 cm^{-1}		
	1585 cm^{-1}	

References.

(1) Birkenfeld, K. R.; Gandhi, T.; Simeral, M. L.; Hafner, J. H. The Distribution of Cholesterol Conformers in Lipid Bilayers Detected by Raman Spectroscopy. *In Prep.* **2023**.

(2) Duax, W. L.; Griffin, J. F.; Rohrer, D. C.; Weeks, C. M. Conformational Analysis of Sterols: Comparison of X-Ray Crystallographic Observations with Data from Other Sources. *Lipids* **1980**, *15* (9), 783–792. https://doi.org/10.1007/BF02534032.

(3) Simeral, M. L.; Demers, S. M. E.; Sheth, K.; Hafner, J. H. A Raman Spectral Marker for the Iso-Octyl Chain Structure of Cholesterol. *Appl. Anal. Sci.* **2023**, *under revision*.

(4) Ye, X.; Zheng, C.; Chen, J.; Gao, Y.; Murray, C. B. Using Binary Surfactant Mixtures To Simultaneously Improve the Dimensional Tunability and Monodispersity in the Seeded Growth of Gold Nanorods. *Nano Lett.* **2013**, *13* (2), 765–771. https://doi.org/10.1021/nl304478h.
(5) Matthews, J. R.; Payne, C. M.; Hafner, J. H. Analysis of Phospholipid Bilayers on Gold Nanorods by Plasmon Resonance Sensing and Surface-Enhanced Raman Scattering. *Langmuir* **2015**, *31* (36), 9893–9900. https://doi.org/10.1021/acs.langmuir.5b01203.
(6) Terracciano, R.; Zhang, A.; Simeral, M. L.; Demarchi, D.; Hafner, J. H.; Filgueira, C. S. Improvements in Gold Nanorod Biocompatibility with Sodium Dodecyl Sulfate Stabilization. *J. Nanotheranostics* **2021**, *2* (3), 157–173. https://doi.org/10.3390/jnt2030010.
(7) Terracciano, R.; Zhang, A.; Butler, E. B.; Demarchi, D.; Hafner, J. H.; Grattoni, A.; Filgueira, C. S. Effects of Surface Protein Adsorption on the Distribution and Retention of Intratumorally Administered Gold Nanoparticles. *Pharmaceutics* **2021**, *13* (2), 216. https://doi.org/10.3390/pharmaceutics13020216.
(8) Lee, S.; Anderson, L. J. E.; Payne, C. M.; Hafner, J. H. Structural Transition in the Surfactant Layer That Surrounds Gold Nanorods as Observed by Analytical Surface-Enhanced Raman Spectroscopy. *Langmuir* **2011**, *27* (24), 14748–14756. https://doi.org/10.1021/la202918n.
(9) Matthews, J. R.; Shirazinejad, C. R.; Isakson, G. A.; Demers, S. M. E.; Hafner, J. H. Structural Analysis by Enhanced Raman Scattering. *Nano Lett.* **2017**, *17* (4), 2172–2177. https://doi.org/10.1021/acs.nanolett.6b04509.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

A manuscript is in preparation.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

While studying the calculated Raman vibrations of cholesterol, spectral bands were found among the complex fingerprint vibrations of cholesterol's tetracyclic rings that provide information on local chemical properties. The intensities of peaks 3 and 4 vary with hydrogen bonding at cholesterol's hydroxyl group, and the spacing and intensities of peaks 10-15 are sensitive to two dihedral angles in the iso-octyl chain.[3] This suggests that Raman spectroscopy can contribute much more to biomolecular structure than previously thought, given the use of TDDFT to identify vibrational motions. Also, these peak identifications would help with biomedical detection since cholesterol is dysregulated in a myriad of diseases. This work demonstrates the power of quantum chemical methods if appropriately applied to biomedical problems.

Also importantly, we found that the spectral set up allowed for sensitivity high enough to be able to detect SERS signal from surfactant coated nanorods when placed onto skin tissue. We also have enough throughput to be able to scan across different areas of tissue in rapid succession. Furthermore, we find different SERS peaks with different nanorods surface chemistry suggesting we can selectively detect substances in tissue. This could enable new non-invasive tissue analysis methods for disease diagnosis and progression monitoring.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

The original proposal described applications of gold nanorod SERS detection of cholesterol. The first was to detect cholesterol in serum and to distinguish between cholesterol from HDL and LDL based on the rate of transfer to gold nanorod surfaces. Although initial data was promising, it came from SERS with gold nanorods in pure synthetic lipid vesicle solutions. We have found that we cannot reliably detect SERS from gold nanorods in serum. As described in the Accomplishments, our DFT calculations have revealed that a certain spectral band of cholesterol is sensitive to the cholesterol chain conformation. It is therefore possible that we can distinguish HDL and LDL base on the unenhanced Raman spectrum. In addition to promising initial data (Figure 8), this method could serve as a simpler serum cholesterol assay since it does not require the addition of gold nanorods to the solution.

Actual or anticipated problems or delays and actions or plans to resolve them

Actual: Unknown SERS peaks in tissue spectrum. Solution: search databases, publications on Raman spectra from tissues. Test different tissues (skin from different regions across the animal) to see if the unknown peaks vary.

Actual: Lack of SERS signal in serum. Solution: moving to unenhanced Raman of cholesterol in natural state (HDL/LDL). Our improved understanding of spectral variation with conformation (30 conformers, Fig. 1) for cholesterol and growing knowledge of phospholipid peaks (four types, see Fig. 5) will be applied for spectral interpretation. This will allow us to better identify ratios of phospholipids/cholesterols that could be used in future cholesterol diagnostics.

Anticipated: Cholesterol in natural HDL/LDL particles is 80% esterified to long chains (oleate, linoleate, and palmitate). This additional molecular mass is too large for DFT calculations, and the additional chains could affect the peaks of the fused rings in Fig. X. To account for this, DFT calculations of a model molecule with an esterified methyl group will be run and compared to measurements on cholesterol esters to gauge the impact and our ability to calculate the effect.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS

Publications, conference papers, and presentations

Journal publications.

1. Uyeki, C. M.; Pacheco, C. M.; Simeral, M. L.; Hafner, J. H. The Raman Active Vibrations of Flavone and Quercetin: The Impact of Conformers and Hydrogen Bonding on Fingerprint Modes. *J. Phys. Chem. A* v. 127, n. 6, p. 1387-1394.
2. Simeral, M. L.; Demers, S. M. E.; Sheth, K.; Hafner, J. H. The Fingerprint Raman Modes of Cholesterol: Spectral Markers for Hydrogen Bonding and Iso-Octyl Chain Conformation. *Applied Analytical Sciences,* under revision.
3. Birkenfeld, K. R.; Gandhi, T.; Simeral, M. L.; Hafner, J. H. The Distribution of Cholesterol Conformers in Lipid Bilayers Detected by Raman Spectroscopy. In preparation, 2023.
4. Pacheco, C. M.; Birkenfeld, K. R., Fallon B. C., Filgueira, C. S., Hafner, J. H. The Raman Active Vibrations of Hydroxycholesterols. In preparation 2023.

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers, and presentations.

1. Fallon BC, Carcamo-Bahena Y, Simeral ML, Royal ALR, Hafner JH, Filgueira CS A Spectroscopy-Based Non-Enzymatic Method to Detect Cholesterol for Familial Hypercholesterolemia, HMRI Nanomedicine Department Symposium, Houston, TX December 5th, 2022. Poster Presentation.
2. Carcamo-Bahena Y, Simeral M, Hafner J, Filgueira CS Coupling Raman Spectroscopy with Thin Layer Chromatography to Investigate Lipid Membranes. 2022

ACS Fall Meeting “Sustainability in a Changing World”, August 21-25th, 2022. Poster Presentation.

3. Carcamo-Bahena Y, Simeral M, Hafner J, Filgueira CS A Spectroscopy-based Non-enzymatic Method to Detect Cholesterol for Early Familial Hypercholesterolemia Screening. George and Angelina Kostas Research Center for Cardiovascular Nanomedicine Annual International Meeting, October 24, 2022. Oral/Blitz Talk Presentation. *Third place presentation winner.
4. Fallon BC, Simeral ML, Hafner JH, Filgueira CS Membrane steroid detection using Raman spectroscopy, 2023 ACS Fall Meeting “Harnessing the Power of Data”, San Francisco, CA/Hybrid, August 13-17th, 2023. Poster Presentation.

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Site 1: Houston Methodist Research Institute

Name:	**Carly Filgueira**
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-3246-303X
Nearest person month worked:	1.2
Contribution to Project:	Dr. Filgueira obtained IACUC and ACURO approval, obtained the cholesterol powder, assisted in peak assignment identification of powder cholesterol, cholesterol solutions and lipid solutions, and cholesterol and/or lipids solutions on gold nanorods, collected blood and tissue samples from animals, spiked known concentrations of cholesterol in lipid vesicles, and added gold nanorods to treated blood and tissue samples.

Name:	**Yareli Carcamo-Bahena**

Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	0000-0001-9998-9696
Nearest person month worked:	2.4
Contribution to Project:	Ms. Carcamo assisted with performing dynamic light scattering on the vesicles, collecting blood and tissue samples from animals, performing lipid extractions, and ultracentrifugation.

Site 2: Rice University

Name:	**Jason Hafner**
Project Role:	Co-PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-6943-4232
Nearest person month worked:	0.25
Contribution to Project:	Dr. Hafner performed the time dependent density-functional theory (TDDFT) calculations, identified the peak assignments and vibrational modes of powder cholesterol, cholesterol solutions and lipid solutions, and cholesterol and/or lipids solutions on gold nanorods, prepared the stable gold nanorod solutions with phospholipid layers with and without cholesterol, and measured the growth of cholesterol SERS peak over time after mixing lipid coated nanorods with serum.

Name:	**Mathieu Simeral**
Project Role:	Graduate Student
Nearest person month worked:	6
Contribution to Project:	Mr. Simeral assisted with monitoring Raman background and SERS signals, determined the acquisition time for reproducible Raman spectra of cholesterol with low background, preparing stable solutions of large multilamellar vesicles made up of synthetic phospholipid and cholesterol and preparing stable gold nanorod solutions with synthetic phospholipids.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes, attached are the changes to the active support for Dr. Filgueira and Dr. Hafner.

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

Not applicable

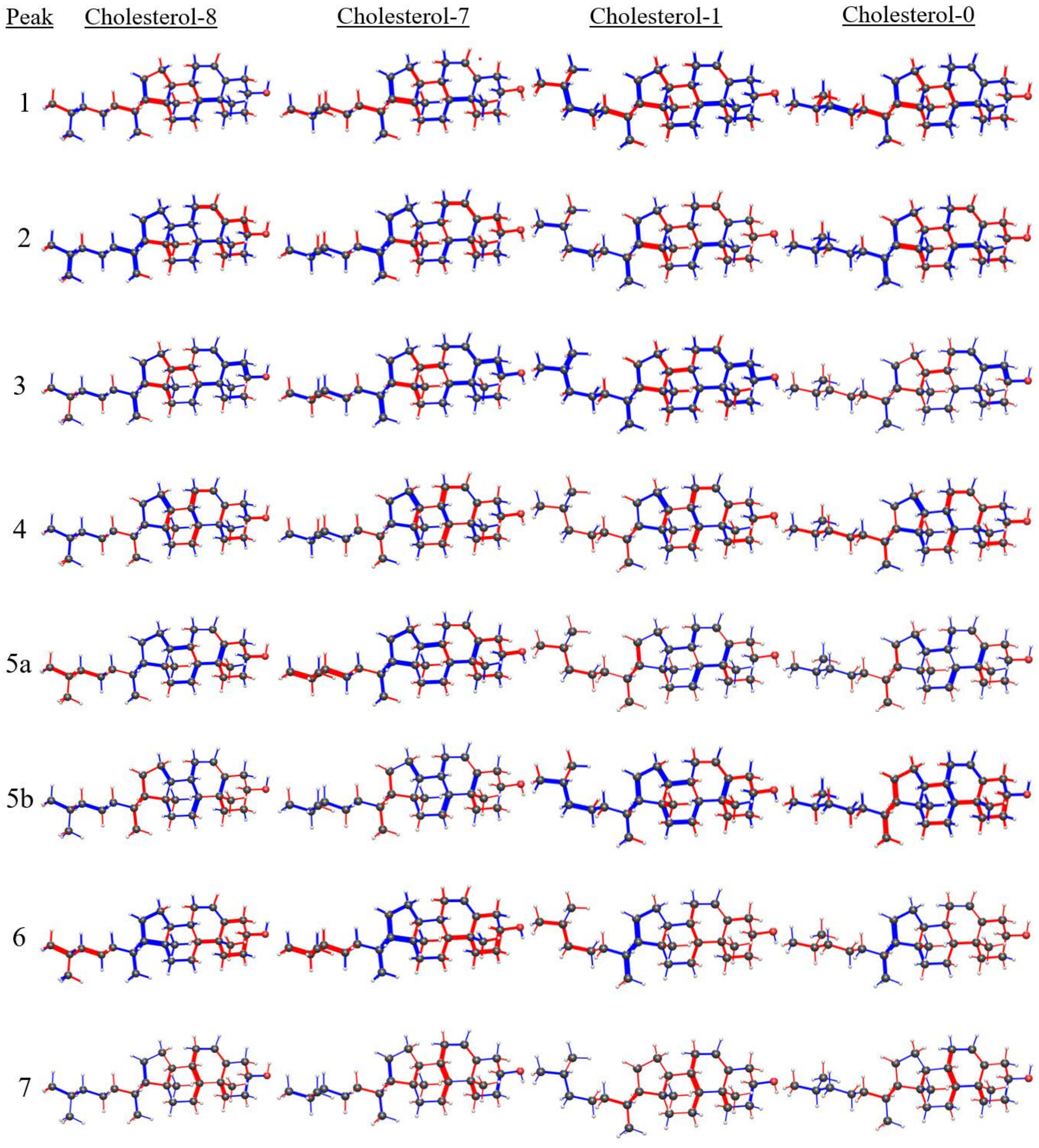
9. APPENDICES

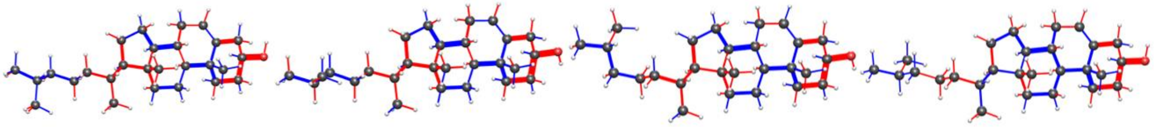
Spectra and Vibration Maps

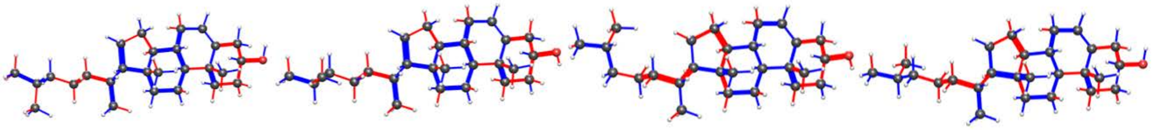
Changes to ongoing research support

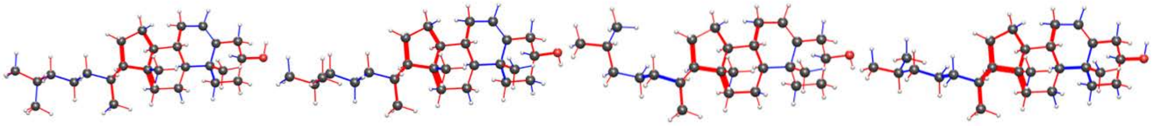
Spectra and Vibration Maps

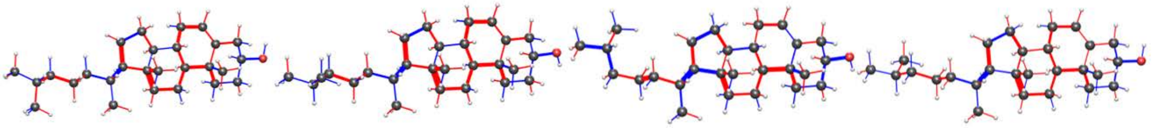
Below are the vibration maps for all identified vibrational modes (1 – 23) for each of the four conformers described (Chol-0, -1, -7, and -8). The widths of the bonds indicate stretching amplitudes and the colors indicate relative phase. Note that each map is normalized to the largest vibration for that map, so comparisons of the vibration amplitude between modes or conformers cannot be made. Also, since these maps show bond stretching, they do not illustrate CH_2 or CH_3 deformations. Peaks 19-22 are therefore not representative of the motion that creates the Raman peaks in the 1400-1500 cm^{-1} window. When two maps are shown (a/b), the calculations found two modes at essentially the same wavenumber shift.

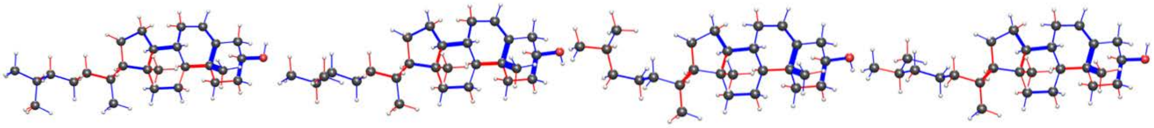


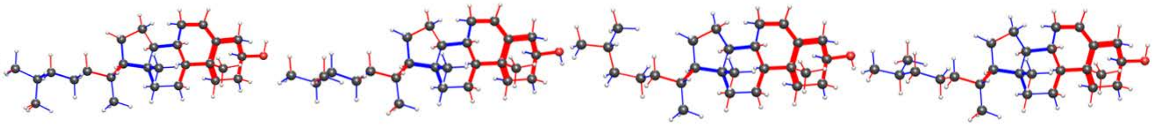
8


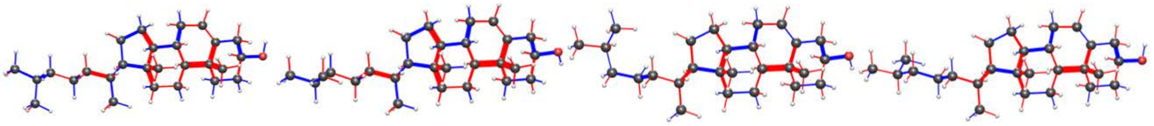
9


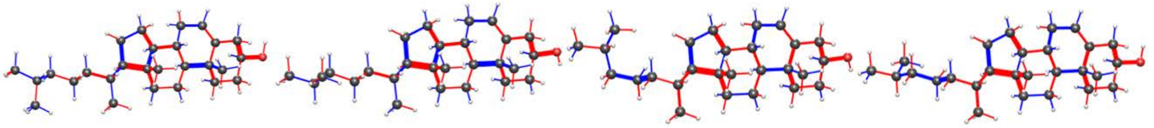
10


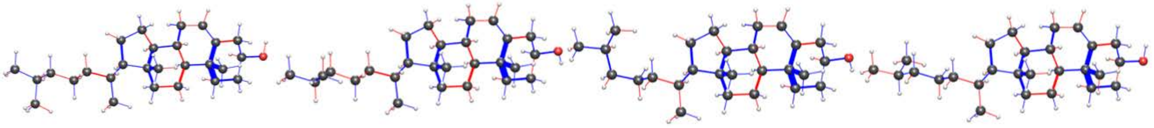
11


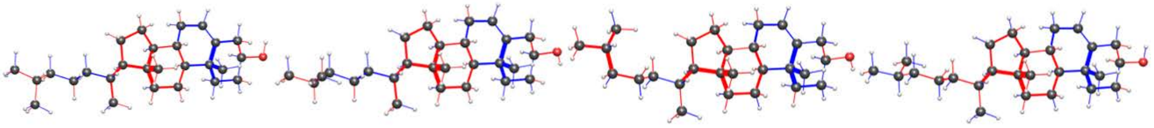
12


13


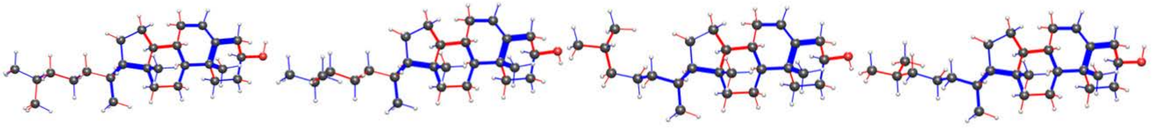
14


15


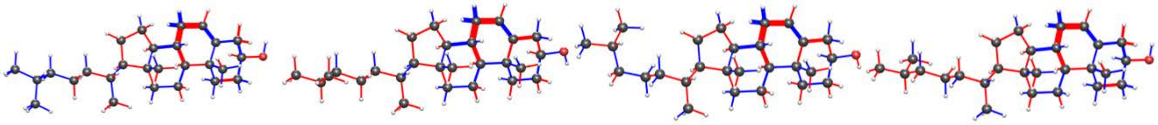
16


17


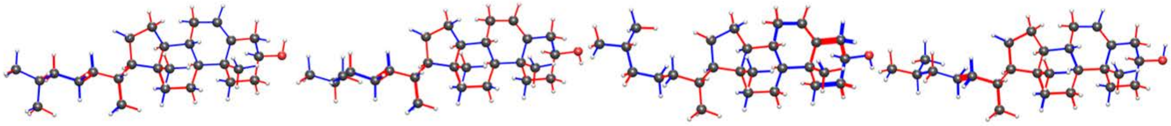
18



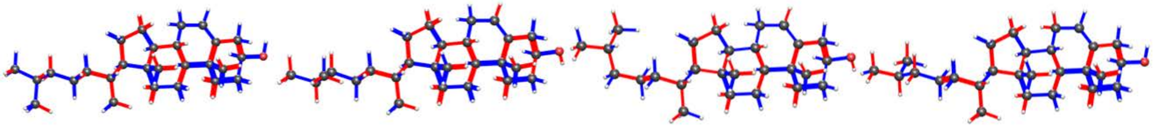
19a



20a

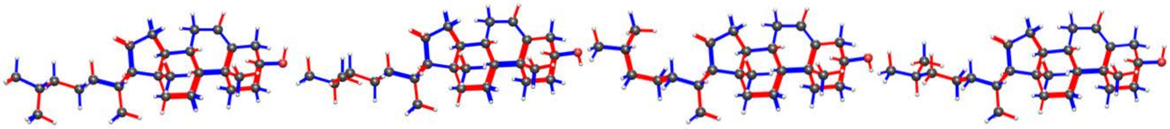


20b

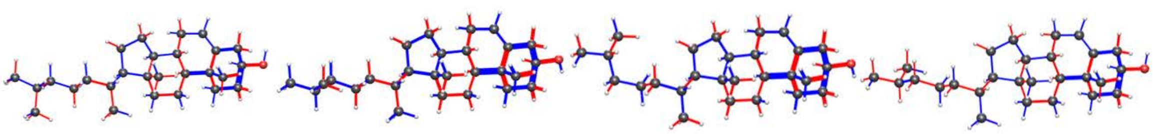


21a

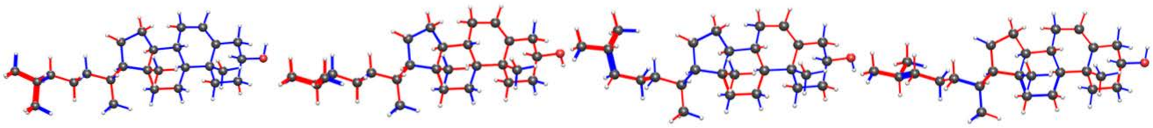
21b



22a



22b



23