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I, April N. Milarcik , hereby submit this original work as part of the requirements for the degree of Master of Science in Chemistry.

It is entitled:

β-cyclodextrin Modified Metal Nanoparticles for the Detection of Cholesterol using SERS

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Committee chair: Peng Zhang, Ph.D.

Committee member: Joseph Caruso, Ph.D.

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β-cyclodextrin Modified Metal Nanoparticles for the Detection of Cholesterol using SERS

A thesis submitted to the

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Master of Science

In the Department of Chemistry

of the College of Arts and Sciences

by

April Milarcik

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Committee Chair: Peng Zhang, Ph.D.

Abstract

β-cyclodextrin modified metal nanoparticles were investigated for the detection of cholesterol using Surface Enhanced Raman Spectroscopy (SERS). Gold and silver nanoparticles were reduced and capped with β-cyclodextrin or modified by polyelectrolyte assembly using PAH and β-cyclodextrin modified PAA polymers. The Raman spectrum of cholesterol should be enhanced due to formation of an inclusion complex with β-cyclodextrin and therefore location close to the metal surface. Suitable detection limit and repeatability for this application was not obtained through successive experiments to improve both the nanoparticles and the measurement parameters. Further improvements of β-cyclodextrin modified metal nanoparticles could provide a detection method for cholesterol using SERS with suitable repeatability and detection limit.

Acknowledgements

I would like to thank my research advisor, Dr. Peng Zhang, for his guidance. I would also like to thank Dr. Changwon Lee for his guidance and instruction. I would like to thank my committee members, Dr. Joseph Caruso and Dr. William Heineman, for their suggestions and review. I would like to thank my family, boyfriend, friends, and colleagues who offered their encouragement, advice, and support. I remember my father with love and gratitude. Finally, I would like to thank God for continuing to work in my life.

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Chapter 1. Introduction

1.1. Cholesterol and its detection

Cholesterol is a lipid that is an essential component in cell membranes and is used in the formation of bile acids and steroid hormones. Cholesterol is carried in the blood in the form of lipoproteins of varied density. Elevated low density lipoprotein (LDL) has been linked to the advancement of coronary heart disease (CHD), while the amount of high density lipoprotein (HDL) is inversely related to the risk of CHD. Total plasma cholesterol <5.2 mM (200 mg/dL) is desirable while >6.2 mM (240 mg/dL) is high and cause for concern.[1-2]

LDL is typically estimated as part of total lipoprotein analysis including total cholesterol, HDL, LDL, and triglycerides. Blood serum or plasma is collected after the patient has fasted for a specified time, and cholesterol and triglyceride levels are measured. Normal concentration of cholesterol in serum is 1.3-2.6 mg/ml (3.36-6.72 mM). About 30% (about 1-2 mM) is present as free cholesterol and 70% as cholesterol esters.[3] Cholesterol esters must be converted to free cholesterol and fatty acids in order to measure total cholesterol. According to Robinet et al., "The most commonly used assays to quantify cholesterol levels can be separated into two groups: 1) analytical methods such as gas-liquid chromatography or liquid chromatography coupled with

1. *Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Final Report*. National Cholesterol Education Program. National Heart, Lung, and Blood Institute. National Institutes of Health. NIH Publication No. 02-5215, September 2002.
2. Wisitsoraat A.; Karuwan C.; Wong-ek K.; Phokharatkul D.;, Sritongkham P.; Tuantranont A. High Sensitivity Electrochemical Cholesterol Sensor Utilizing a Vertically Aligned Carbon Nanotube Electrode with Electropolymerized Enzyme Immobilization. *Sensor* **2009**, *9*, 8658-8668.
3. Ansari A.A.; Kaushik A.; Solanki P.R.; Malhotra B.D. Electrochemical Cholesterol Sensor Based on Tin Oxide-Chitosan Nanobiocomposite Film. *Electroanalysis* **2009**, *21*, 965 – 972.

flame ionization or mass spectrometry detection and quantification; and 2) enzymatic assays, which can be colorimetric or fluorometric". [4] The first methods are time intensive, requiring reaction and extraction steps to prepare samples, and require expensive equipment. Comparatively, enzymatic assays are simpler and faster. Still, possible problems are reagent instability, misleading results, and poor specificity. [2]

A sensor for cholesterol which is inexpensive, reproducible, stable, and fast would be useful to estimate cholesterol levels. Electrochemical enzymatic sensors having good sensitivity have been demonstrated. [2-3] Non-enzymatic sensors have been reported as well. [4-5] In the research reported herein, β-cyclodextrin modified metal nanoparticles have been prepared for use in detection of cholesterol using Surface Enhanced Raman Spectroscopy (SERS).

1.2. Surface Enhanced Raman Spectroscopy

Raman spectroscopy is one method of evaluating the structure of molecules. When a molecule is irradiated with a light source, the photons in the light are absorbed and then emitted. The photons change the energy of the molecule and induce molecular rotations or vibrations. Functional groups in the molecule have different vibrational frequencies.

2. Wisitsoraat A.; Karuwan C.; Wong-ek K.; Phokharatkul D.;, Sritongkham P.; Tuantranont A. High Sensitivity Electrochemical Cholesterol Sensor Utilizing a Vertically Aligned Carbon Nanotube Electrode with Electropolymerized Enzyme Immobilization. *Sensor* **2009**, *9*, 8658-8668.
3. Ansari A.A.; Kaushik A.; Solanki P.R.; Malhotra B.D. Electrochemical Cholesterol Sensor Based on Tin Oxide-Chitosan Nanobiocomposite Film. *Electroanalysis* **2009**, *21*, 965 – 972.
4. Robinet P.; Wang Z.; Hazen S.L.; Smith J.D. A simple and sensitive enzymatic method for cholesterol quantification in macrophages and foam cells. *J. Lipid Res.* **2010**, *51*, 3364-3369.
5. Zhang N.; Liu Y.; Tong L.; Xu K.; Zhuo L.; Tang, B. A novel assembly of Au NPs–b-CDs–FL for the fluorescent probing of cholesterol and its application in blood serum. *Analyst* **2008**, *133*, 1176–1181.

Emitted photons may have the same or different energy when compared to the incident light. In Rayleigh scattering, which is elastic scattering, the frequency of the emitted photon matches that of the incident photon. Raman scattering is inelastic scattering, with a change in energy between the incident photons and the emitted photons. The frequency, and wavelength or wavenumber, of the emitted light is shifted up or down from the original, monochromatic source. In Stokes scattering, part of the incident frequency is transferred to the Raman active mode of the molecule, and the frequency of the emitted photon is lower. In anti-Stokes scattering, the photon is absorbed by a molecule already in an excited state, and the emitted photon has higher frequency. Anti-Stokes scattering is useful for Raman spectroscopy. The Raman signal is typically weak, with only about 0.001% of incident light producing anti-Stokes scattering. [6-7]

The intensity of Raman scattering is proportional to the polarizability of the molecule. The polarizability is a measure of the distortion of a molecule in an electric field. The induced dipole moment of the molecule is described as $P = \alpha E$, where E is the electric field and α is the polarizability of the molecule. Aromatic functional groups have more intense scattering than aliphatic groups.[6-8]

6. Haynes C.L.; McFarland A.D.; Van Duyne R.P. Surface-Enhanced Raman Spectroscopy. *Anal. Chem.* **2005***, 77*, 338A-346A.
7. Raman Spectroscopy Basics. Princeton Instruments online library. http://content.piacton.com/Uploads/Princeton/Documents/Library/UpdatedLibrary/Raman_Spectroscopy_Basics.pdf
8. Atkins, P.W. *Physical Chemistry*, 6th ed.; W.H. Freeman and Company: New York, 1998; pp 453-491.

Raman scattering provides weak signals compared to other spectroscopy methods, such as infrared (IR) and fluorescent. Different techniques are employed to enhance the Raman signal. Surface enhanced Raman spectroscopy (SERS) is among these methods. In SERS, an increase in signal strength is observed when the molecule is placed near a roughened noble metal substrate. Two mechanisms of enhancement, one chemical and one electromagnetic, have been proposed. In the charge transfer model, the polarizability of the molecule is enhanced through electron transfer between the metal surface and the molecule. The signal enhancement factor is reported to be up to 100.[6]

In electromagnetic enhancement, the electric field of the incident radiation is enhanced by localized surface Plasmon resonance. The laser strikes surface irregularities and excites conduction electrons of metal particles. When the electric field is increased, the magnitude of the dipole moment, and therefore Raman scattering, is increased. Electromagnetic enhancement depends on the size, shape, and material of substrate, which determines the resonant frequencies of the metal electrons. Electromagnetic enhancement factors greater than 10,000 have been reported.[6] Metals which are SERS active include gold, silver, and copper. Some common substrates are electrochemically etched silver electrodes, and silver or gold colloids.

In SERS, the molecule of interest must be on or near the metal surface. Targets are typically held in place by chemisorption, physisorption, and partitioning by self assembled monolayer. Signal

6. Haynes C.L.; McFarland A.D.; Van Duyne R.P. Surface-Enhanced Raman Spectroscopy. *Anal. Chem.* **2005***, 77*, 338A-346A.

enhancement is affected by the size and structure of the metal surface features, dielectric environment, and radiation source. It is more difficult to analyze molecules which do not adsorb on the metal surface.

Advantages of Raman spectroscopy include high spectral resolution, non-destructive analysis, and small sample size. Raman spectroscopy can be used to study samples in the solid, liquid, and gas phase. Water is a weak Raman scatterer, and aqueous samples can be measured.

Disadvantages of SERS include difficulty in making reproducible substrates and characterizing substrates.[6] Other disadvantages include difficulties in spectra interpretation. Peaks that are weak in Raman can appear in SERS, and vice versa. Contaminants can interact with surfaces and give misleading peaks.[6-7]

1.3. Cyclodextrin modified metal nanoparticles for use in Surface Enhanced Raman Spectroscopy

To characterize a molecule using SERS it must be close to or on the substrate to experience the enhanced electric field. Many analytes of interest, including cholesterol, do not chemically or physically adsorb on the metal. The metal may be modified to interact with the target and concentrate molecules near the metal surface. Cyclodextrins, which form inclusion complexes with some molecules, have been used to modify metal nanoparticles for use in SERS.

6. Haynes C.L.; McFarland A.D.; Van Duyne R.P. Surface-Enhanced Raman Spectroscopy. *Anal. Chem.* **2005***, 77*, 338A-346A.
7. Raman Spectroscopy Basics. Princeton Instruments online library. http://content.piacton.com/Uploads/Princeton/Documents/Library/UpdatedLibrary/Raman_Spectroscopy_Basics.pdf

Cyclodextrins are cyclic oligosaccharides which consist of glucopyranose units joined by α-1,4-linkages, resulting in a cone shaped structure that is open at both ends. The structure and shape of β-cyclodextrin are shown in Figure 1. The most common cyclodextrins are α-, β-, and γ-Cyclodextrin with 6, 7, and 8 saccharide units, respectively. Cyclodextrins have a hydrophilic outer surface which enables solubility in aqueous systems. The inner cavity is more hydrophobic and interacts with hydrophobic molecules or portions of molecules which match the cavity diameter.[9-10] The end with the smaller diameter is the primary face, with primary hydroxyl groups which may be easily modified through reactions. The wider end has secondary hydroxyl groups and is the secondary face.[10] Table 1 shows some properties of the common cyclodextrins.

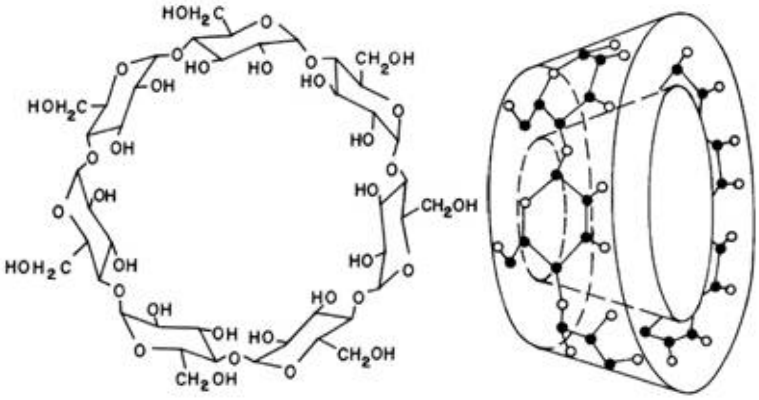


Figure 1. Structure and shape of β-cyclodextrin.[11]

9. López C.A.; de Vries A.H.; Marrink S.J. Molecular Mechanism of Cyclodextrin Mediated Cholesterol Extraction. *PLoS Comp. Biol.* **2011**, *7*, 1-11.
10. Ogoshi T. and Harada A. Chemical Sensors Based on Cyclodextrin Derivatives. *Sensors* **2008**, *8*, 4961-4982.
11. The European Cyclodextrin Society. http://www.eurocdsoc.com/

	α-CD	β-CD	γ-CD
Number of glucose units	6	7	8
Molecular Weight	972.86	1135.01	1297.15
Water Solubility (g/L)	145	18.5	232
Internal Diameter	4.7-5.2	6.0-6.4	7.5-8.3
Depth	6.7	7.0	7.0

Table 1. Comparison of common cyclodextrins.[10]

Cholesterol is also able to fit inside the cavity of cyclodextrin. The diameter of β-CD in particular is a good match for cholesterol.[9] β-CD is commonly used to extract cholesterol from cell membranes and to remove cholesterol from foods. From López et al., "The degree of cholesterol depletion is a function of the cyclodextrin derivative used, its concentration, incubation time, temperature and cell type".[9]

In the research reported herein, gold and silver nanoparticles were directly modified with β-cyclodextrin in an effort to prepare a fast, simple detection method for cholesterol using SERS. Other researchers have reported cyclodextrin modified nanoparticles for use in SERS. Xie et al. used silver nanoparticles (AgNPs) coated with thiol modified β-cyclodextrin to capture polycyclic aromatic hydrocarbons (PAH). They demonstrated enhancement of the Raman spectra

9. López C.A.; de Vries A.H.; Marrink S.J. Molecular Mechanism of Cyclodextrin Mediated Cholesterol Extraction. *PLoS Comp. Biol.* **2011**, *7*, 1-11.
10. Ogoshi T. and Harada A. Chemical Sensors Based on Cyclodextrin Derivatives. *Sensors* **2008**, *8*, 4961-4982.

for pyrene and anthracene, and were able to quantitatively identify them in a mixture.[12-13] Wang et al. prepared one-dimensional gold nanorods decorated with thiol modified β-CD for detection of the insecticide methyl parathion. They reported excellent selectivity and sensitivity using SERS.[14] Huang et al. used α-CD to synthesize AuNP suspensions with controllable particle size and good stability for use as a SERS substrate. α-CD acted as both a reducing and capping agent in the formation of AuNPs.[15]

In the current research, metal nanoparticles were also modified with polyelectrolytes appended with β-CD. Layer by layer assembly of polyelectrolytes has been widely used to modify particles and surfaces.[16-18] Polyelectrolytes are polymers bearing functional groups which may be either positively or negatively charged when ionized. The polymers are sequentially adsorbed and assembled through electrostatic interactions. The polyelectrolytes used in this study were poly(acrylic acid), negatively charged with deprotonated carboxyl groups, and poly(allylamine hydrochloride), positively charged with amine groups. Poly(acrylic acid) was modified with β-CD to interact with cholesterol.

12. Xie Y.; Wang X.; Han X.; Xue X.; Ji W.; Qi Z.; Liu J.; Zhao B.; Ozaki Y. Sensing of polycyclic aromatic hydrocarbons with cyclodextrin inclusion complexes on silver nanoparticles by surface-enhanced Raman scattering. *Analyst* **2010**, *135*, 1389–1394.
13. Xie Y.; Wang X.; Han X.; Song W.; Ruan W.; Liu J.; Zhao B.; Ozaki Y. Selective SERS detection of each polycyclic aromatic hydrocarbon (PAH) in a mixture of five kinds of PAHs. *J. Raman Spectrosc.* **2011**, *42*, 945–950.
14. Wang J.; Kong L.; Guo Z.; Xua J.; Liu J. Synthesis of novel decorated one-dimensional gold nanoparticle and its application in ultrasensitive detection of insecticide. *J. Mater. Chem.*, **2010**, *20*, 5271–5279.
15. Huang T.; Meng F.; Qi L. Facile Synthesis and One-Dimensional Assembly of Cyclodextrin-Capped Gold Nanoparticles and Their Applications in Catalysis and Surface-Enhanced Raman Scattering. *J. Phys. Chem. C* **2009***, 113*, 13636–13642.
16. Zhou Y.; Lee C.; Zhang J.; Zhang P. Engineering versatile SERS-active nanoparticles by embedding reporters between Au-core/Ag-shell through layer-by-layer deposited polyelectrolytes. *J. Mater. Chem. C*, **2013**, *1*, 3695–3699.
17. Chapel J.-P.; Berret J.-F. Versatile electrostatic assembly of nanoparticles and polyelectrolytes: Coating, clustering and layer-by-layer processes. *Curr. Opin. Colloid Interface Sci.* **2012**, *17*, 97–105.
18. Cranford S.W.; Ortiz C.; Buehler M.J. Mechanomutable properties of a PAA/PAH polyelectrolyte complex: rate dependence and ionization effects on tunable adhesion strength. *Soft Matter*, **2010**, *6*, 4175–4188.

Chapter 2. Experimental methods

2.1. Materials

Deionized water was used for all experiments. β-cyclodextrin (β-CD), hydrogen tetrachloroaurate ($HAuCl_4$), poly(acrylic acid sodium salt) (PAA), 4-(Dimethylamino) pyridine (DMAP), and poly(allylamine hydrochloride) (PAH) were purchased from Sigma-Aldrich. Cholesterol, p-aminothiophenol, and pyrene were also obtained from Sigma Aldrich. Sodium hydroxide (NaOH), silver nitrate ($AgNO_3$), and sodium citrate were from Fisher Scientific. N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Thermo Scientific. All materials were used as obtained without further purification.

2.2. Synthesis of β-cyclodextrin capped gold nanoparticles

β-cyclodextrin capped gold nanoparticles (AuNPs) were prepared using a process similar to that reported by Huang et al.[15] Typically 2 ml of 10 mM β-CD in DI water, 3.84 ml of water, and 100 μL of 10 mM $HAuCl_4$ were mixed together. Then 60 μL of 1 M NaOH was added, and the solution was heated at a specified time and temperature to give a suspension of β-CD capped gold nanoparticles. In this solution, final concentrations of β-CD, $HAuCl_4$, and NaOH were 3.33 mM, 0.17 mM, and 10 mM, respectively. The molar ratio of β-CD: $HAuCl_4$ was 20:1, with 0.02 mmol β-CD and 0.001 mmol $HAuCl_4$. The reagent amounts were scaled as necessary to prepare larger batches, or different reactant ratios. Reaction solutions were in glass vials in a heated water bath.

15. Huang T.; Meng F.; Qi L. Facile Synthesis and One-Dimensional Assembly of Cyclodextrin-Capped Gold Nanoparticles and Their Applications in Catalysis and Surface-Enhanced Raman Scattering. *J. Phys. Chem. C* **2009**, *113*, 13636–13642.

AuNPs were purified by centrifugation. The suspension was transferred to a microcentrifuge tube and centrifuged at 12000 rpm for 10-15 minutes. The clear supernatant was removed and replaced with water. The suspension was mixed briefly by sonication or use of a vortex mixer, and centrifuged again. The supernatant was removed and the sample diluted to obtain the desired volume of suspension. Typically particle size and UV-vis absorption measurements were conducted on the final suspension of AuNPs after purification.

2.3. Synthesis of β-cyclodextrin capped silver nanoparticles

Silver nanoparticles (AgNPs) directly reduced with β-cyclodextrin were prepared in a similar manner. For a 40:1 molar ratio of β-CD: $AgNO_3$, 220 μL of 20 mM $AgNO_3$ was added to 17.6 ml of 10 mM β-CD in a vial. This solution was heated to boiling for ten minutes, and then 12 μL of 1 M NaOH was added. The solution was heated another ten minutes as nanoparticles formed and the color changed from clear to yellow. For a 20:1 molar ratio of β-CD: $AgNO_3$, 8.8 ml of 10 mM β-CD and 8.8 ml water was used instead. AgNPs were purified by centrifugation at 15000 rpm for 20 minutes, and characterized with particle size and UV-vis absorption spectroscopy.

2.4. Synthesis of β-cyclodextrin modified PAA

For preparation of β-cyclodextrin modified poly(acrylic acid sodium salt) (PAA), 10 ml of PAA solution (3.5 g/L, 48 mM using the MW of the monomer unit, 72 g/mol) was placed in a vial. 26.4 μL of 0.2 M N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), a water soluble coupling reagent used in the formation of amide and ester bonds, was added while stirring. After about five minutes, 2.64 μL of 0.2 M 4-(Dimethylamino)pyridine (DMAP), a

catalyst for the formation of ester bonds, was added to the solution while stirring.[16, 19] Immediately 480 μL of 10 mM β-cyclodextrin was added. The C6 hydroxyl group on the primary face of β-CD should react with the carboxylic acid group of PAA to form an ester bond, giving a polymer with pendant β-CD.[10, 20]

Final molar amounts of each reagent were 0.48 mmol PAA, 5.28 x 10^{-3} mmol EDC (1.1 equivalent β-CD), 5.28 x 10^{-4} mmol DMAP (0.1 equiv EDC), and 4.8 x 10^{-3} mmol β-CD (1% mol PAA). β-cyclodextrin modified PAA was also prepared with β-CD at 10% mol PAA and 0.1% mol PAA, with other reagent amounts adjusted accordingly.

The reaction was continued overnight. The β-CD modified PAA was purified by three dialysis cycles in deionized water using Spectra/Por membranes having 6000-8000 MWCO.

2.5. Synthesis of PAH-PAA coated nanoparticles

Gold and silver nanoparticles were prepared, and then coated with PAH. Finally, they were coated with β-cyclodextrin modified PAA as an outer layer.[16] Gold nanoparticles were prepared by citrate reduction.[21] 50 ml of DI water was placed in a 100 ml flask to which 625 μL of 20 mM

10. Ogoshi T. and Harada A. Chemical Sensors Based on Cyclodextrin Derivatives. *Sensors* **2008**, *8*, 4961-4982.
16. Zhou Y.; Lee C.; Zhang J.; Zhang P. Engineering versatile SERS-active nanoparticles by embedding reporters between Au-core/Ag-shell through layer-by-layer deposited polyelectrolytes. *J. Mater. Chem. C*, **2013**, *1*, 3695–3699.
19. Neises B.; Steglich W. Simple Method for the Esterification of Carboxylic Acids *Angew. Chem. Int. Ed.*, **1978**, *17*, 522-524.
20. Murakami S.; Aoki N. Bio-Based Hydrogels Prepared by Cross-Linking of Microbial Poly(*γ*-glutamic acid) with Various Saccharides. *Biomacromolecules* **2006**, *7*, 2122-2127.
21. Frens G. Controlled Nucleation for the Regulation of the Particle Size in Monodisperse Gold Suspensions. *Nat. Phys. Sci*. **1973**, *241*, 20-22.

$HAuCl_4$ was added. The solution was heated to boiling in a water bath, and 300 μL of 1% wt sodium citrate in water was added. The solution was heated for another 15 minutes with a color change to pink or red observed.

Silver nanoparticles were prepared in a similar manner. 625 μL of 20 mM $AgNO_3$ was added to 50 ml of DI water in a flask. The solution was heated to boiling in a water bath, and 1 ml of 1% wt sodium citrate in water was added. The solution was heated for about 1 hour until a color change was observed. The final color was yellow-brown.

To prepare PAH-PAA coated nanoparticles, 6 ml of prepared AuNPs were added dropwise to 10 ml Poly(allylamine hydrochloride) (PAH) at 3.5 g/L and allowed to react overnight. The AuNP-PAH particles obtained were purified by two centrifugation cycles at 6250 rpm for 10 minutes. The AuNP-PAH suspension was concentrated by a factor of two, and then 1.5 ml was added dropwise to 3 ml of the prepared PAA-CD solution and allowed to react overnight. The product was purified by two centrifugation cycles at 6250 rpm for 10 minutes and resuspended in water.

Silver nanoparticles were coated with PAH-PAA using the same procedure. 10 ml prepared AgNPs were added dropwise to 15 ml PAH solution and allowed to react overnight. AgNP-PAH particles were purified by two centrifugation cycles at 10000 rpm for 10 minutes, and concentrated by a factor of 5. Newly prepared solutions of PAA-CD were reacted with AgNP-PAH at a ratio of 3:2. The product was purified by two centrifugation cycles at 10000 rpm for 10 minutes and resuspended in water.

2.6. Instruments

Particle size was measured by dynamic light scattering using a Microtrac Zetatrac. Samples were placed in the built in cuvette of the instrument and measured at room temperature. UV-vis absorption was measured with an Ocean Optics USB 4000 Spectrometer with attached USB-ISS-UV-VIS sampling system using a 1 cm path length cuvette.

Samples were prepared for SERS measurement in different ways. Samples were measured as solid by applying several 10 μL drops to a glass coverslip or aluminum substrate (aluminum foil or aluminized tape attached to a 1x3 inch glass microscope slide), drying between drops. Alternately samples were measured as liquid by placing a small amount in a glass capillary tube with approximate 1 mm inner diameter. For a normal Raman spectrum of an analyte, a few crystals of the analyte were fixed on a slide by adding a drop of acetone.

Raman spectroscopy was completed using a Renishaw InVia Raman Microscope. The excitation source was a 514 nm argon ion laser or 785 nm diode laser. Typically measurements were one scan with 10 s exposure time. Magnification power and laser power were varied.

Chapter 3. Results and discussion

3.1. β-cyclodextrin capped gold nanoparticles

Reaction conditions were first investigated for the preparation of β-CD capped AuNPs. Different temperatures, reaction times, and molar ratios of β-CD: $HAuCl_4$ (denoted β-CD:Au) were evaluated. Initial preparation at 70°C resulted in a purple colored suspension with a UV-vis absorption at 551 nm, red shifted from the gold plasmon band at 520 nm, indicating aggregated

particles. Reactions were carried out at room temperature (RT) and 40°C for up to four hours. AuNPs prepared at 40°C had the desired UV-vis peak absorbance at 520 nm and narrower particle size distribution compared to those prepared at RT. For subsequent samples, the reaction was carried out for two hours at 40°C.

AuNPs were prepared with molar ratios of β-CD:Au set at 15:1, 20:1, and 25:1. Ratios of 15:1 and 20:1 provided better repeatability, as indicated by particle size and UV-vis absorption. Results are shown in Figure 2.

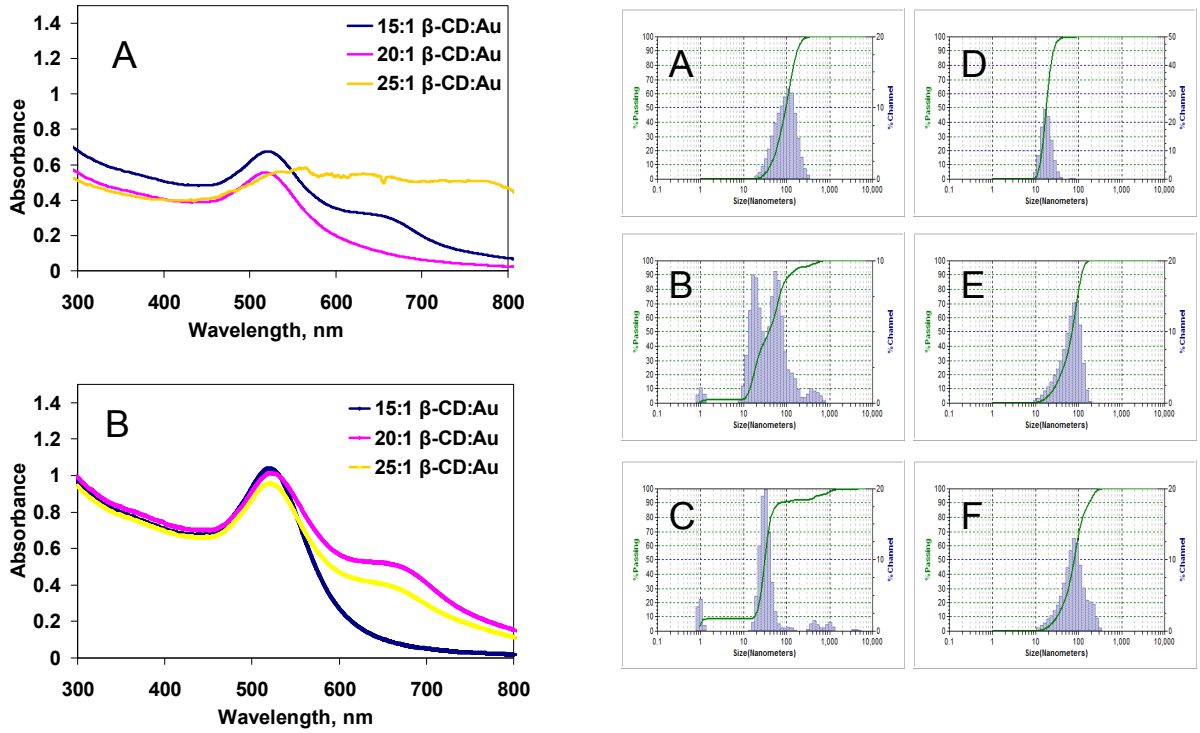


Figure 2. Left: UV-vis absorption spectra for A) AuNPs prepared at RT for 4 hours with different molar ratios of β-CD to Au. B) AuNPs prepared at 40°C for 2 hours with different molar ratios of β-CD to Au.
Right: Particle size for A) 15:1 ratio prepared at RT for 4 hours. B) 20:1 ratio prepared at RT for 4 hours. C) 25:1 ratio prepared at RT for 4 hours. D) 15:1 ratio prepared at 40°C for 2 hours. E) 20:1 ratio prepared at 40°C for 2 hours. F) 25:1 ratio prepared at 40°C for 2 hours.

Settling was observed for some AuNPs. Visual observation indicated the initial suspension settled less than the purified suspension. Samples rinsed and purified using a 10 mM NaOH solution rather than water showed less settling over a two week time span, similar to a control sample which was not purified by centrifugation. pH for the initial suspension, a sample purified using water, and a sample purified using 10 mM NaOH is shown in Table 2.

Rinse/resuspend	**pH**
Control-none	7.48
DI water/ DI water	4.5
10 mM NaOH/ 10 mM NaOH	11.68

Table 2. pH for AuNPs

The effect of pH on AuNP stability was investigated further. AuNPs with 15:1 ratio β-CD: Au were prepared. They were purified by centrifugation using 10 mM NaOH to rinse and dilute, with a resulting pH of 11.76. To this suspension, 1 or 0.1 M HCl was added until the AuNPs became unstable. At pH 5.53 the suspension was purple-pink in appearance, and the peak absorbance shifted from 520 nm to about 570 nm. 100 mM NaOH was then added to the suspension until pH 10.36 was reached. The suspension became faint pink, almost clear, and the peak absorbance remained shifted to the longer wavelength, indicating the aggregration induced by decreasing the pH was not reversible, as shown in Figure 3.

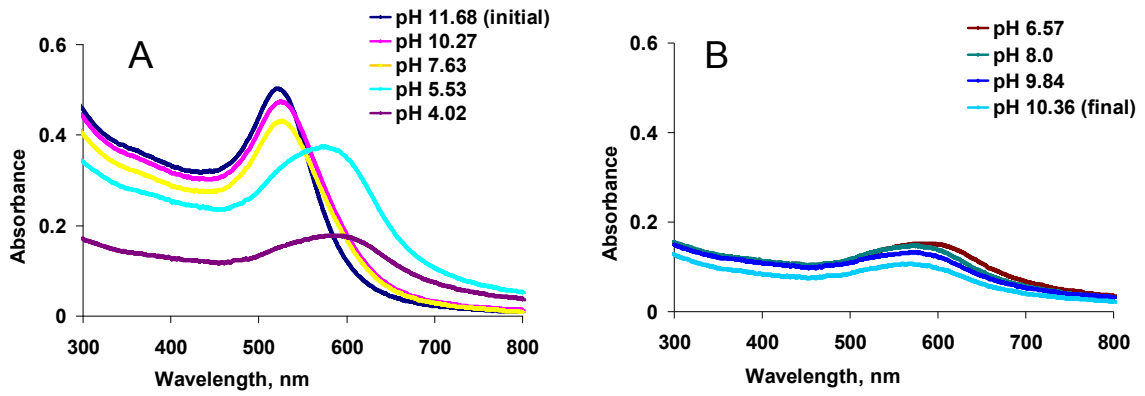


Figure 3. A) UV-vis absorption spectra for AuNPs with addition of HCl to decrease pH B) UV-vis absorption spectra for same AuNPs with addition of NaOH to increase pH

β-CD capped AuNPs were prepared and purified using 1 mM NaOH (pH 10.3). Different amounts of a stock solution of 1 mM cholesterol in ethanol were added to samples for 0.01, 0.05, and 0.09 mM final concentration. The samples were sonicated for two hours after addition of cholesterol, and then centrifuged at 12,000 rpm for 15 minutes to concentrate. Samples were prepared for Raman spectroscopy by dropping 10 µL aliquots on Al foil and allowing sample to dry between drops. Raman spectroscopy was conducted using lasers of 514 nm and 785 nm wavelength. Results are shown in Figure 4a. With the 514 nm laser no peaks were observed. With the 785 nm laser some peaks were observed but none which could be positively identified as cholesterol. The Raman spectrum for cholesterol is shown in Figure 4b.

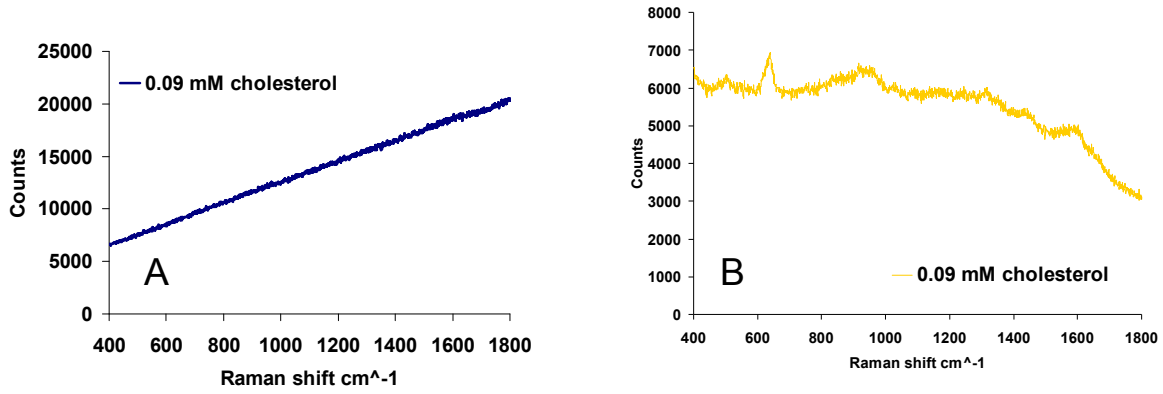


Figure 4a. Raman spectra for A) β-CD capped AuNPs with 0.09 mM cholesterol using 514 nm laser. B) β-CD capped AuNPs with 0.09 mM cholesterol using 785 nm laser.

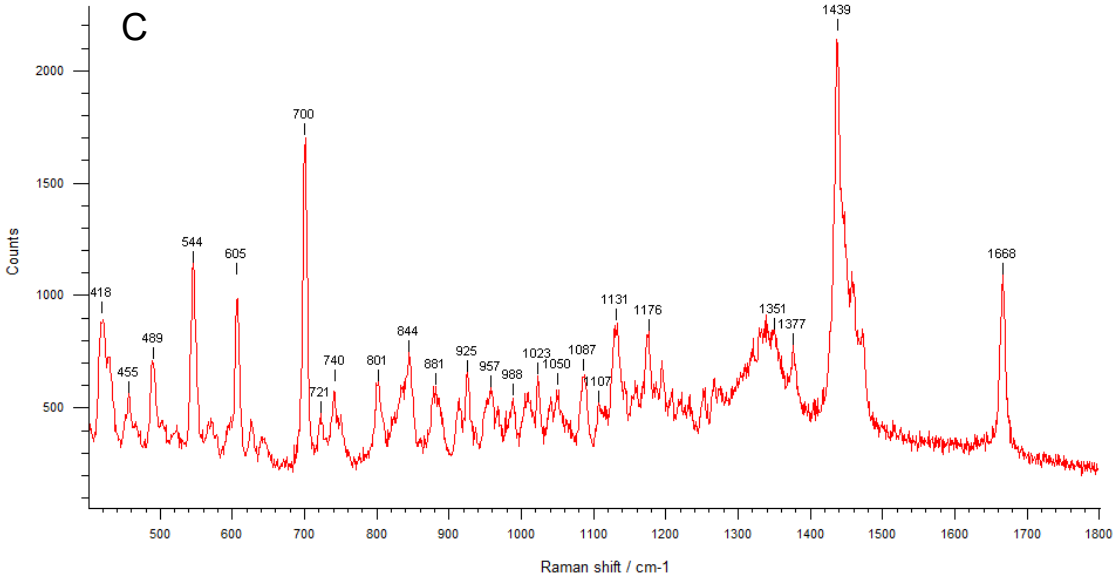


Figure 4b. Raman spectrum for C) Cholesterol measured as solid using 785 nm laser.

It was found in a literature reference that the authors observed lower peak intensity for samples centrifuged at speeds higher than 1500 rpm; they speculated that high speeds separated the guest molecule from the β-CD cavity.[13] To investigate this as a possible explanation for the lack of

13. Xie Y.; Wang X.; Han X.; Song W.; Ruan W.; Liu J.; Zhao B.; Ozaki Y. Selective SERS detection of each polycyclic aromatic hydrocarbon (PAH) in a mixture of five kinds of PAHs. *J. Raman Spectrosc.* **2011**, *42*, 945–950.

peaks observed, another sample rinsed and resuspended in 1 mM NaOH was centrifuged at different speeds until the particles sedimented as shown in Table 3. It was found that a lower speed could be used, but not as low as that given in the literature reference. This could be due to the difference in preparation protocol and stability of the β-CD modified AuNPs.

Time (min)	RPM	Result
10	1500	No separation
10	2500	No separation
10	5000	Slight separation
10	7500	NP separation, faint pink super
10	10000	NP separation, slight pink super

Table 3. Study of centrifuge speed for β-CD capped AuNPs with 0.09 mM cholesterol

After this centrifugation study, one sample containing cholesterol was redispersed by sonication and another was centrifuged to concentrate. A 10 μL drop of each was placed on Al foil for SERS analysis as a liquid droplet. Neither showed any peaks using the 514 nm or 785 nm laser, as shown in Figure 5.

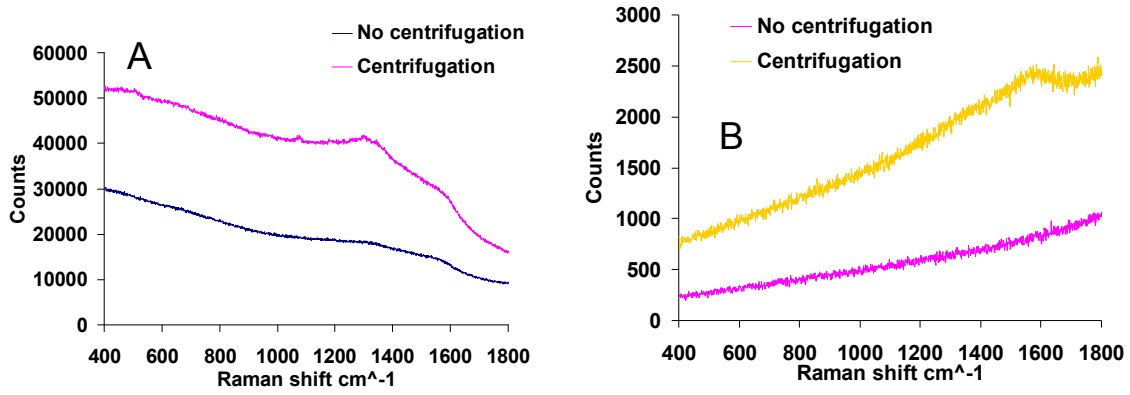


Figure 5. Raman spectra for A) β-CD capped AuNPs with 0.09 mM cholesterol with and without centrifugation using 514 nm laser. B) β-CD capped AuNPs with 0.09 mM cholesterol with and without centrifugation using 785 nm laser.

3.2 Nanoparticles coated with PAH and β-cyclodextrin modified PAA

As an alternate approach, gold or silver nanoparticles were coated with PAH and β-CD modified PAA (denoted as Au PAH-PAA or Ag PAH-PAA) using layer by layer assembly of polyelectrolytes. All particles prepared consisted of one layer of PAH coated onto the citrate stabilized metal nanoparticle, and then one layer of β-CD modified PAA. PAH-PAAs with β-CD at 1% and 10% mol PAA were prepared and evaluated for SERS detection of cholesterol. Another molecule often used to evaluate SERS activity, p-aminothiophenol (PATP) [15], was used to confirm the PAH-PAAs function as a SERS substrate. PATP can also form an inclusion complex with B-CD.[22] Cholesterol and PATP solutions in ethanol were added to suspensions of PAH-PAAs for a final concentration of 0.09 mM. Samples were sonicated for two hours, and then purified by two centrifugation cycles at 6250 rpm for 10 minutes. Finally the particles were redispersed in 100 μL water.

Samples were prepared for Raman spectroscopy as dried material on a silicon wafer or the liquid sample was placed in a capillary tube and measured directly. For both gold and silver particles, no peaks were observed for cholesterol. No clearly identifiable peaks characteristic of cholesterol or PATP were observed. For gold particles, peaks observed ~1072, 1140, and 1585 cm^{-1} could be

15. Huang T.; Meng F.; Qi L. Facile Synthesis and One-Dimensional Assembly of Cyclodextrin-Capped Gold Nanoparticles and Their Applications in Catalysis and Surface-Enhanced Raman Scattering. *J. Phys. Chem. C* **2009***, 113*, 13636–13642.
22. Cappadona T.A.; Daniels L.M.; Siddiquee T.A. Host–Guest Complex of β-Cyclodextrin and Disulfide Form of 4-Aminothiophenol. *Appl. Sci.* 2012, *2*, 773-779.

due to PATP.[15] However, the Au particles with cholesterol also displayed peaks around 1070 and 1579 cm^{-1}. For silver particles, prominent peaks were observed which could be attributed to PATP around 1141, 1389, and 1433 cm^{-1}. No peaks were observed for cholesterol as seen in Figure 6.

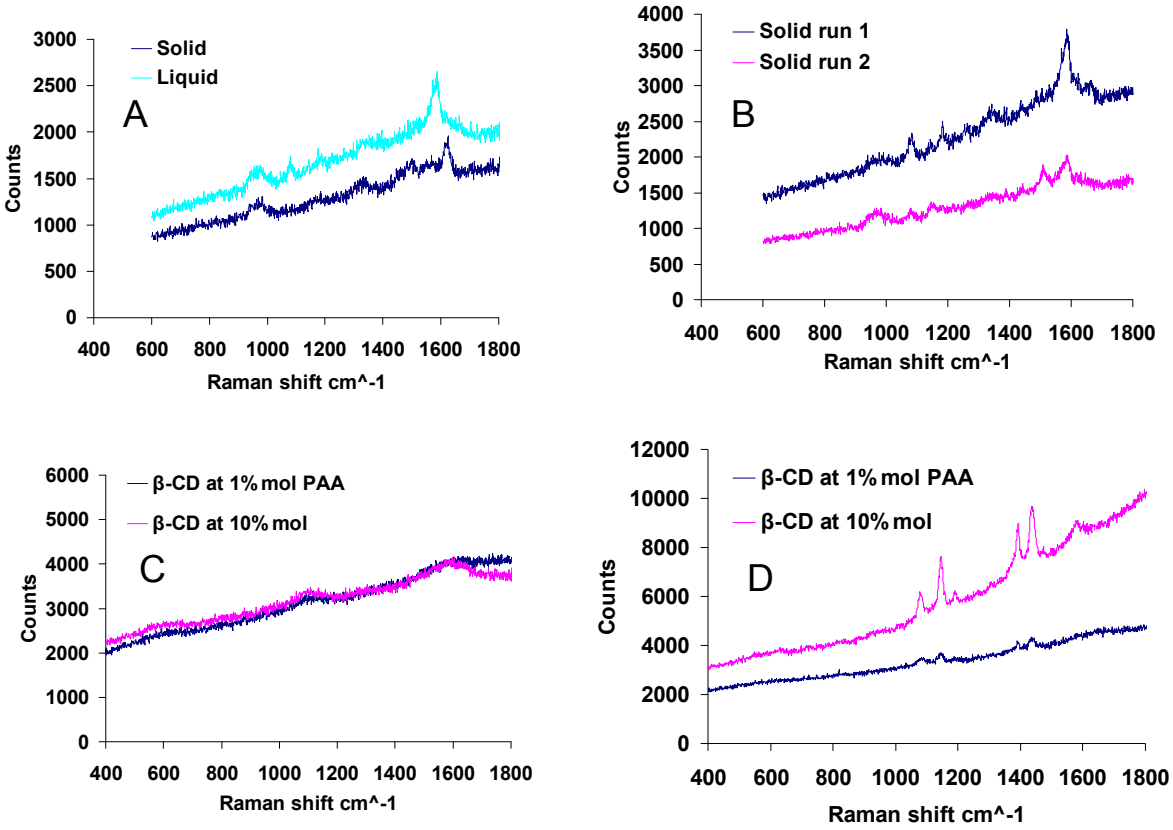


Figure 6. Raman spectra for A) Au PAH-PAA with CD at 1% mol PAA and 0.09 mM cholesterol. B) AU PAH-PAA with CD at 1% mol PAA and 0.09 mM PATP C) Ag PAH-PAA with 0.09 mM cholesterol D) Ag PAH-PAA with 0.09 mM PATP. All samples measured with 514 nm laser.

15. Huang T.; Meng F.; Qi L. Facile Synthesis and One-Dimensional Assembly of Cyclodextrin-Capped Gold Nanoparticles and Their Applications in Catalysis and Surface-Enhanced Raman Scattering. *J. Phys. Chem. C* **2009**, *113*, 13636–13642.

A newly prepared suspension of silver nanoparticles and the previously prepared AgNPs and AuNPs were used to prepare PAH-PAA particles. β-CD was set to 1% and 0.1% mol PAA, and reagent amounts were adjusted accordingly. Pyrene, demonstrated to form an inclusion complex with β-CD resulting in SERS enhancement, was used as a comparison to cholesterol.[12-13] Cholesterol and pyrene solutions in ethanol were added to samples. Samples were sonicated for an hour, and then centrifuged to concentrate. Raman spectroscopy measurements were made on samples as liquid.

The Ag PAH-PAAs prepared with the freshly made AgNPs had the strongest signal intensity, and results are shown in Figure 7. For the Ag PAH-PAA particles with β-CD at 1% mol PAA, peaks characteristic of pyrene were observed at 1238, 1405, and 1628 cm^{-1}.[12] Peaks indicative of pyrene were observed for both silver NPs with β-CD at 1% mol PAA, but not for the gold NPs. This could be due to the age of the suspension or to the better performance of silver as a substrate. No peaks were observed for cholesterol. For all samples, peaks were observed for samples with analyte which were not observed for the control around 876, 1038, 1076, and 1450 cm^{-1}. These are likely indicative of β-CD.[23] This appears to be supported by the fact that the samples with a lower amount of β-CD also show these peaks, while peaks for pyrene are not observed. The reason they are not observed in the control sample is unclear but could be due to a change in conformation of the β-CD molecule with a guest included in the hydrophobic cavity.

12. Xie Y.; Wang X.; Han X.; Xue X.; Ji W.; Qi Z.; Liu J.; Zhao B.; Ozaki Y. Sensing of polycyclic aromatic hydrocarbons with cyclodextrin inclusion complexes on silver nanoparticles by surface-enhanced Raman scattering. *Analyst* **2010**, *135*, 1389–1394.
13. Xie Y.; Wang X.; Han X.; Song W.; Ruan W.; Liu J.; Zhao B.; Ozaki Y. Selective SERS detection of each polycyclic aromatic hydrocarbon (PAH) in a mixture of five kinds of PAHs. *J. Raman Spectrosc.* **2011**, *42*, 945–950.
23. Egyed O. Spectroscopic studies on B-cyclodextrin. *Vib. Spectrosc.* **1990**, *1,* 225-221

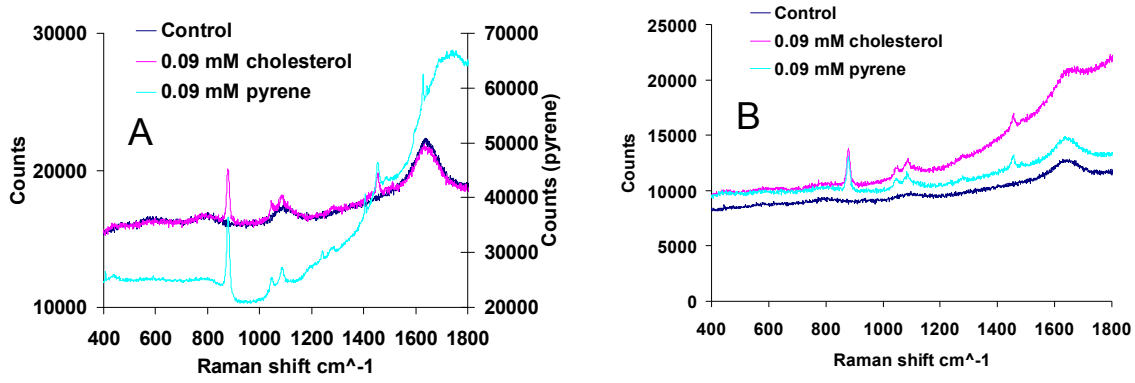


Figure 7. Raman spectra of A) Ag PAH-PAA with CD at 1% mol PAA B) Ag PAH-PAA with CD at 0.1% mol PAA. Samples measured as liquid using the 514 nm laser at 100% power.

3.3. β-cyclodextrin capped silver nanoparticles

The experiments with PAH-PAA coated particles gave better results for silver compared to gold. Silver nanoparticles (AgNPs) were prepared by direct reduction with β-cyclodextrin to determine whether silver would provide SERS detection of cholesterol. AgNPs were prepared with molar ratios of β-CD:Ag at 40:1 and 20:1. AgNPs were characterized by particle size analysis and UV-vis absorption spectroscopy, showing particle size less than 100 μm and peak absorbance around 418 nm. Results are shown in Figure 8.

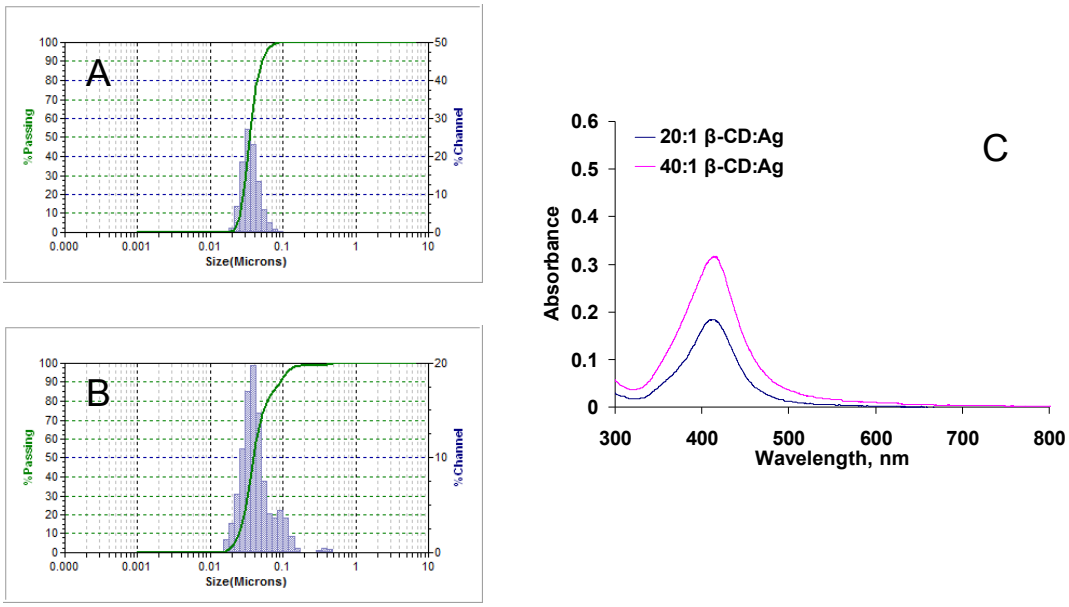


Figure 8. A) Particle size of 20:1 β-CD:Ag particles. B) Particle size of 40:1 β-CD:Ag particles. C) UV-vis absorption spectra of β-CD:Ag particles.

Concentration of AgNPs was varied to observe the effect on SERS detection. Concentration remained as prepared for one sample, and a second was concentrated by a factor of three. PATP and cholesterol were added to each sample. After one week, samples containing PATP were dark blue, a sign of aggregation. This most likely occurred due to the thiol group of PATP having a stronger affinity for the silver surface and replacing the β-CD molecule.

Samples with cholesterol were centrifuged at 10000 rpm for 10 minutes to further concentrate the AgNPs for measurement. A lower centrifuge speed did not result in particle separation. Samples were measured as liquid in a capillary tube. The more concentrated suspension resulted in higher signal intensity for the 40:1 β-CD:Ag sample, but did not appear to provide greater sensitivity. Two large, broad peaks were observed for all samples around 1408 and 1619 cm^{-1} as

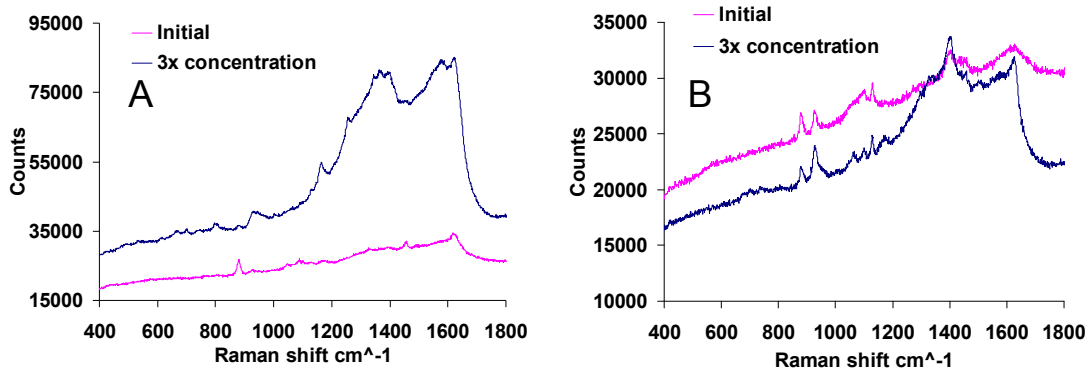


Figure 9. Raman spectra of A) 40:1 β-CD:Ag with 0.09 mM cholesterol. B) 20:1 β-CD:Ag with 0.09 mM cholesterol. Both were measured as liquid with 10 scans at 100% power using the 514 nm laser.

20:1 β-CD:Ag Initial	20:1 β-CD:Ag 3x	Cholesterol (514 nm)	β-CD (514 nm)	β-CD (23)
878	877	420	**480**	**480***
925	926	490	578	580
1089	1058	**546**	709	**850**
1128	1095	**607**	**854**	**950**
1408	1127	**701***	**950**	1010
1619	1396	723	1045	1050
	1448	751	**1130**	1080
	1619	801	1263	**1130***
		845	**1335**	**1160 B***
		882	1390	1205
		926	1451	1250
		1089	**2908***	1340
		1132	**2970**	**1350**
		1177		**1390 B***
		1335 B		1415
		1380		1455
		1440*		
		1669*		
		~2900 (24)		

Table 4. SERS peaks observed for 20:1 β-CD:Ag samples, with corresponding peaks for cholesterol and β-CD shaded. Cholesterol and β-CD labeled 514 nm were measured as solid for this study. Also see references 23, 24. **Bold** type indicates a moderately strong peak, while **bold*** indicates a strong peak.

23. Egyed O. Spectroscopic studies on B-cyclodextrin. *Vib. Spectrosc.* **1990**, *1,* 225-221
24. Nie B.; Masyuko, R.N.; Bohn, P.W. Correlation of surface-enhanced Raman spectroscopy and laser desorption-ionization mass spectrometry acquired from silver nanoparticle substrates. *Analyst* 2012, *137*, 1421-1427.

seen in Figure 9. Sharp peaks were observed, particularly for the 20:1 β-CD:Ag sample, around 878, 926, and 1128 cm^{-1}. These could be due to either cholesterol or β-CD. A comparison of peaks observed for 20:1 β-CD:Ag samples, cholesterol, and β-CD are shown in Table 4.

Many additional SERS measurements were completed for AgNPs directly capped with β-CD. There is an art to successful SERS measurements. Scans should initially be made using lasers with different wavelengths in order to select one which gives the best signal for the sample. Magnification level should also be considered. Higher magnification normally provides signals with higher intensity, and can provide good resolution. However, higher magnification gives a smaller irradiated sample area, and is often less repeatable than lower magnification. Typically, samples measured in liquid state provide more reproducible results than those measured in solid state. They are likely more homogeneous and possibly less aggregated. Multiple measurements, in different locations, should be made for a solid sample. All of these things were not done consistently for samples measured herein.

In addition, many samples studied herein were centrifuged after the addition of analyte to further concentrate the suspension in an effort to increase SERS signal intensity. Early in this research, it was found that Xie et al. achieved better signal resolution for SERS with β-CD modified AuNPs by optimizing the centrifuge speed for samples.[13] Zhang et al. simply incubated the β-CD modified AuNPs with cholesterol.[5]

5. Zhang N.; Liu Y.; Tong L.; Xu K.; Zhuo L.; Tang, B. A novel assembly of Au NPs–b-CDs–FL for the fluorescent probing of cholesterol and its application in blood serum. *Analyst* **2008**, *133*, 1176–1181.
13. Xie Y.; Wang X.; Han X.; Song W.; Ruan W.; Liu J.; Zhao B.; Ozaki Y. Selective SERS detection of each polycyclic aromatic hydrocarbon (PAH) in a mixture of five kinds of PAHs. *J. Raman Spectrosc.* **2011**, *42*, 945–950.

Sample centrifugation was revisited as a possible explanation for lack of clearly identifiable peaks for cholesterol. A control of β-CD capped particles without analyte was included. Pyrene, demonstrated to form an inclusion complex with β-CD resulting in SERS enhancement, was used as a comparison.[12-13] AgNPs were prepared with and without centrifugation after the addition of cholesterol and pyrene solutions in ethanol. Samples were measured as liquid in a capillary tube. For cholesterol, the sample without centrifugation showed a clear peak around 1440 cm^{-1}, likely indicative of cholesterol, that was not observed in the centrifuged sample. For pyrene, the sample without centrifugation had a stronger peak indicative of pyrene around 1240 cm^{-1} compared to the sample which was centrifuged. See Figure 10 for results; B denotes without centrifugation.

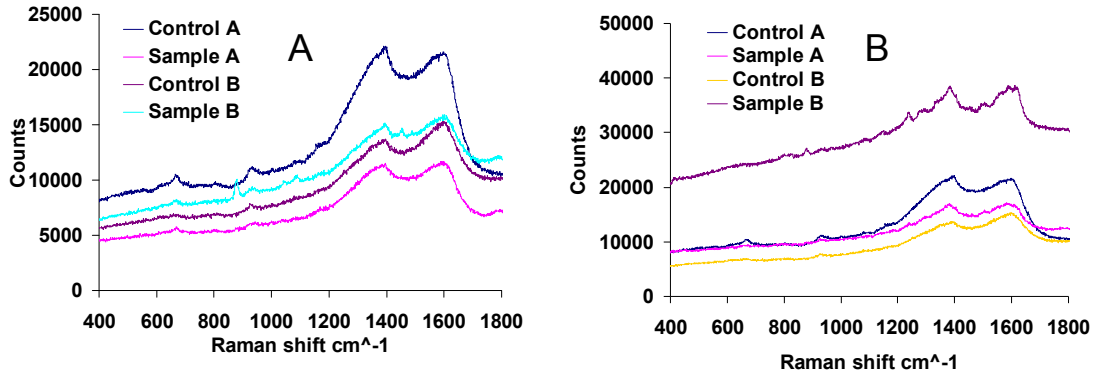
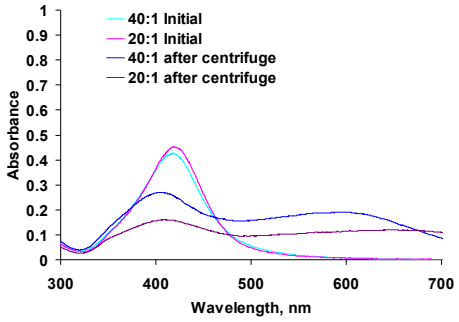


Figure 10. Raman spectra of A) 40:1 β-CD:Ag with 0.09 mM cholesterol where A denotes centrifugation step and B without centrifugation. B) 40:1 β-CD:Ag with 0.09 mM pyrene where A denotes centrifugation step and B without centrifugation. Samples were measured as liquid with 514 nm laser at 100% power, 10x magnification.

12. Xie Y.; Wang X.; Han X.; Xue X.; Ji W.; Qi Z.; Liu J.; Zhao B.; Ozaki Y. Sensing of polycyclic aromatic hydrocarbons with cyclodextrin inclusion complexes on silver nanoparticles by surface-enhanced Raman scattering. *Analyst* **2010**, *135*, 1389–1394.
13. Xie Y.; Wang X.; Han X.; Song W.; Ruan W.; Liu J.; Zhao B.; Ozaki Y. Selective SERS detection of each polycyclic aromatic hydrocarbon (PAH) in a mixture of five kinds of PAHs. *J. Raman Spectrosc.* **2011**, *42*, 945–950.

β-CD capped AgNPs were prepared with different concentrations of cholesterol and pyrene. Freshly prepared AgNPs with 40:1 and 20:1 β-CD:Ag ratios were characterized by particle size and UV-vis spectroscopy. A color change from yellow to red was observed in the typical purification step to remove any unreacted β-CD. UV-vis and particle size results in Figure 11 indicated aggregation of the particles.



Sample	Mi, nm	D50, nm	SD, nm
40:1 initial	38.2	37	9.82
40:1 after centrifuge	112.9	108.1	32.5
20:1 initial	86	59.5	52.3
20:1 after centrifuge	149.6	143.3	49

Figure 11. UV-vis absorption spectra and particle size results for AgNPs

AgNPs with a 40:1 β-CD:Ag ratio showed slightly less aggregation, and were used for SERS measurements. Samples were not centrifuged after addition of analyte, and they were measured as liquid. For samples with cholesterol reported in Figure 12, a peak attributed to cholesterol was observed around 1450 cm^{-1}. Overall, the height of this peak increased with cholesterol concentration, as shown in Figure 13. A peak at 876 cm^{-1} was noted for samples with cholesterol, but not attributed to cholesterol since the same peak was seen for samples with pyrene. All samples, including the control sample without cholesterol, had a peak around 728 cm^{-1}, and broad peaks around 1353 and 1580 cm^{-1}.

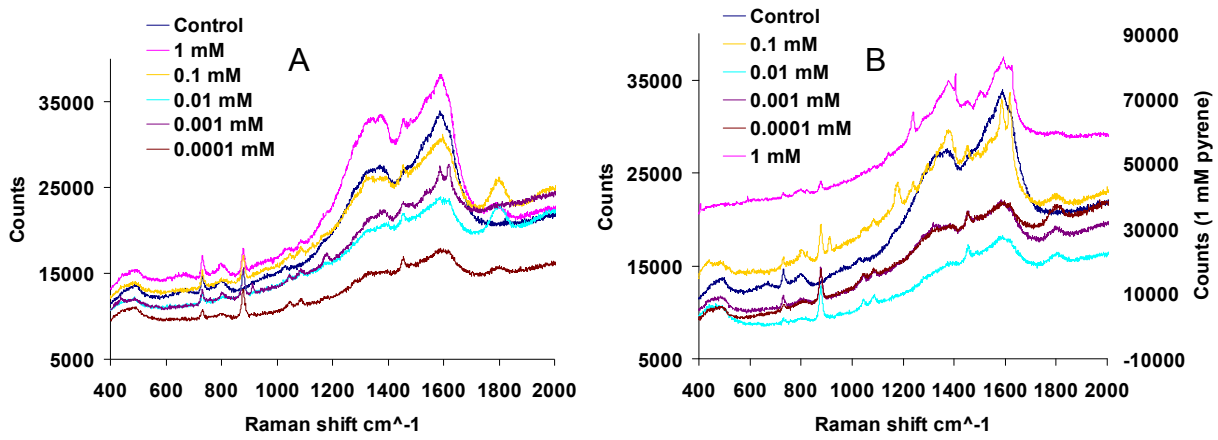


Figure 12. Raman spectra of A) 40:1 β-CD:Ag with different concentrations of cholesterol B) 40:1 β-CD:Ag with pyrene added. Samples were measured as liquid with 514 nm laser at 100% power, 3 scans, 10x magnification.

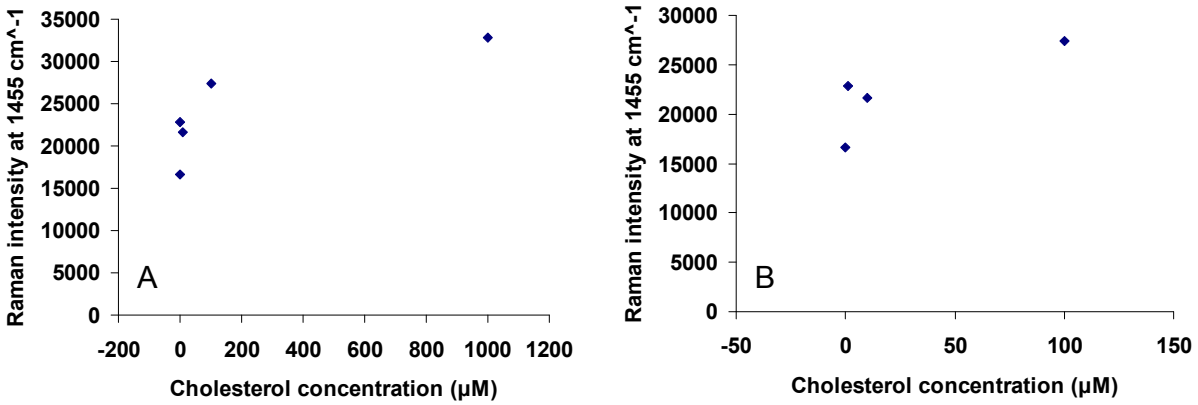


Figure 13. Figure 13. A) Change in Raman intensity at 1455 cm^{-1} with different cholesterol concentrations. B) Expanded view.

For samples with pyrene, a peak characteristic of pyrene was observed around 1236 cm^{-1} for samples with 1 mM and 0.01 mM pyrene, as shown in Figure 12. Samples with lower concentrations of pyrene did not have this peak. Peak height increased with concentration for those samples which had a peak at 1236 cm^{-1}. Samples with pyrene also showed a peak around 1450 cm^{-1}. This is problematic, and could indicate that the peak is due to cyclodextrin rather than

cholesterol. Peaks were also observed at 729 and 875 cm^{-1} for all samples with analyte, and broad peaks were observed around 1353 and 1580 cm^{-1} as for other samples.

3.4. Future work

Finally, peaks for cholesterol were observed using β-CD capped AgNPs. Further study is needed to determine the detection limit for cholesterol using the prepared nanoparticles. Repeatability for both preparation of nanoparticles and detection of cholesterol should be established.

Further investigation of peaks present in the SERS spectra and not attributed to cholesterol is needed. Two broad, strong peaks around 1350 and 1580 cm^{-1} were observed for both control samples and samples with added cholesterol. These peaks are in the same region as the strong peak around 1440 cm^{-1} characteristic of cholesterol and may obscure signals due to cholesterol. The peaks could be due to overlapping signal peaks, or to carbon contaminants.[25] Improvements to the SERS measurement method could include a background subtraction approach for the spectra, possibly subtracting the observed broad peaks.

25. Fang C.; Bandaru N.M.; Ellis A.V.; Voelcker N.H. Beta-cyclodextrin decorated nanostructured SERS substrates facilitate selective detection of endocrine disruptor chemicals. *Biosensors and Bioelectronics* **2013,** *42*, 632–639.

Poor stability and aggregation of nanoparticles could result in lower signal intensity. According to Huang et al., cyclodextrins demonstrate relatively weak capping for metals, and they found that some cyclodextrin left the AuNP surface when toluene filled the cyclodextrin cavity.[15] Similarly, addition of cholesterol could induce cyclodextrin removal from the surface of the nanoparticle. An alternate approach may be to modify nanoparticles with thiol functionalized β-CD, which may remain more strongly adsorbed on the metal surface.[5, 12]

Nanoparticles may need to be stabilized in a buffer solution to improve colloid stability and to achieve higher loading of cholesterol. Settling was observed for many solutions which were purified and then suspended in water. Samples with pH 7 or higher were shown to have better stability. However, this rinsing protocol was not used for all sets of samples. In this study, precipitation of cholesterol was observed for samples with added cholesterol concentration higher than 1 mM. Other researchers used a PBS buffer solution with surfactant to solubilize cholesterol[2] or a Tris-HCl buffer which maintained the NP suspension at suitable pH.[5]

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3.5. Conclusion

In conclusion, gold and silver nanoparticles reduced and capped with β-cyclodextrin were tested for the detection of cholesterol using Surface Enhanced Raman Spectroscopy. Metal nanoparticles modified with PAH and β-cyclodextrin modified PAA were also tested for the detection of cholesterol. Suitable repeatability and detection limit for this application was not obtained through successive experiments to improve both the nanoparticles and the measurement parameters. Further improvements of β-cyclodextrin modified metal nanoparticles could result in a detection method for cholesterol using SERS with suitable repeatability and detection limit.

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25. Fang C.; Bandaru N.M.; Ellis A.V.; Voelcker N.H. Beta-cyclodextrin decorated nanostructured SERS substrates facilitate selective detection of endocrine disruptor chemicals. *Biosensors and Bioelectronics* **2013,** *42*, 632–639.