Functionalization of hemoglobin-specific ligand on gold nanostructured substrate fabricated by pulsed laser deposition for surface enhanced Raman scattering applications

CHEM 3410U

Supervisors: Nisha R. Agarwal, Olena V. Zenkina

By: Ryan Kwok

Submitted: April 13th, 2020

This report was completed in fulfillment of the report component of the course CHEM 3410U - Chemistry Laboratory Techniques instructed by Dr. Nisha R. Agarwal and Dr. Olena V. Zenkina in the winter 2020 semester at Ontario Tech University.©

Abstract

Hemoglobin disorders are characterized by the genetic alteration of the protein's structural configuration. A standard diagnosis typically involves acquiring vials of patients blood and sifting through it to separate and detect harmful variants of hemoglobin. These tests are often conducted in a laboratory away from the location of care and can delay a diagnosis up to a few days.

This report proposes the usage of surface enhanced Raman spectroscopy accompanied by a nanobiosensor to detect hemoglobin and as future work lay the foundation for differentiating among different hemoglobin disorders. The nanobiosensor is comprised of a gold-nanoparticle (Au-NP) substrate and a novel ligand designed to attach to hemoglobin. The nanobiosensor also serves as a platform on which SERS spectra of hemoglobin can be acquired. Since SERS has the ability to determine the vibrational 'fingerprint' of a molecule at ultra-low concentrations, it is possible to assign the unique spectra of a hemoglobin variant to its corresponding disorder. This allows for the fast classification and differentiation of hemoglobin disorders in a point-of-care setting, similar to a glucose-meter.

This paper reports on the functionalization of the novel ligand to the Au-NP substrate. The success of the functionalization is based on the shifting of the surface plasmon resonant peak seen in absorption plasmon resonance data of the substrate. A distinctive blueshift is seen in one of the functionalized substrates and is hypothesized to be a sign of the successful adsorption of the ligand to the Au-NP surface.

Keywords: Hemoglobin, SERS, Ligand, Au-NP, Nanobiosensor

Introduction

Hemoglobin is a complex protein comprised of two α and two β globin subunits attached to a central heme group. Hemoglobin related disorders are caused by inherited mutant genotypes that alter the process of protein synthesis. The specific mutation that the globin gene undergoes will determine the variation of disorder that occurs. Hemoglobin disorders stem from the quantitative and qualitative characteristics concerning globin subunits in the protein [1].

Issues stemming from quantitative characteristics refer to the partial or complete absence of globin subunits while qualitative characteristics refer to structural defects in globin subunits [1]. Both involve some change in the structure of hemoglobin which, in turn, may also negatively impact hemoglobin's ability to function. These impaired hemoglobin limit the number of properly functioning red blood cells (RBC) which can lead to a variety of medically relevant problems.

An example of an infamous disorder stemming from structural defects in hemoglobin is sickle cell anemia. This disorder is caused by a mutation in hemoglobin that results in 'sickling', the reversible process of changing the shape of a RBC from a disc to a crescent. This repeated process can lead to early RBC breakdown, decreased efficiency in oxygen transport due to clogged blood vessels and accumulated organ damage [1, 2].

Significant portions of the human population suffer from blood related illnesses. Those that carry the significant gene variant required for a hemoglobin disorder comprise 5.2% of the world's population [2]. In developed countries, most children with hemoglobin disorders receive appropriate treatment and are able to live well into adulthood with a chronic disorder. However, children born in developing countries are less fortunate, as most have been found to succumb to the disorder before the age of five [2].

The World Health Organization recommends a variety of measures to aid prevention of hemoglobin disorders around the world. These preventive measures are based around the idea of screening for the disease, early if possible, and informing those that are affected. Diagnoses of children with a disorder will allow parents to receive genetic counselling and make informed decisions regarding family size [2, 3]. Screening may also help prospective couples make informed decisions when it comes to choosing a partner [3]. Early screenings provided for the entire population would be the ideal scenario for prevention. However, realizing this goal will require an improvement regarding the accessibility of these tests. One way to achieve this is to find a quick and convenient method to test for hemoglobin disorders.

Hemoglobin disorders tend to display a wide variety of symptoms in patients as a result of the specific variant that is present. For this reason, it is necessary to quickly identify the disorder so that the patient may receive appropriate care. In an emergency situation, it is imperative that the patient receives a timely diagnosis as a delay in treatment may severely worsen symptoms and increase the possibility of death [4].

Diagnoses for hemoglobin disorders can take anywhere from a few hours up to a few days depending on whether or not blood samples need to be shipped to a lab [5]. There currently exist over one thousand unique variants of hemoglobin disorders with numerous options available to test for them in a laboratory setting [3]. Standard electrophoresis tests are often used to separate common variants of hemoglobin. Additional lab tests with other electrophoretic techniques may be required to definitively distinguish between other variants that are difficult to detect [6].

This project seeks to harness the power of a technique known as Surface Enhanced Raman Spectroscopy (SERS) for the quick detection of hemoglobin in blood. SERS is used to determine the vibrational modes of a molecule, which are unique to each molecular configuration. For this reason, SERS spectra acts as a 'fingerprint' that can be used to classify and differentiate different types of molecules [7].

The concept of traditional Raman spectroscopy lies in obtaining information from light scattered off of a sample molecule. Most light scatters off the molecule at the same wavelength as the incident beam, which is known as Rayleigh scattering. However, a small percentage of light is scattered off at a wavelength that is not the same as the incident beam and contains useful information pertaining to the vibrational modes of the molecule; this is known as Raman scattering [7]. The resulting spectra will plot intensity by wave number and will include a variety of characteristic peaks. These peaks correspond to unique vibrational modes that are all occurring simultaneously in the molecule.

Vibrational modes in a molecule result in either a change in dipole moment, a change in polarizability, or both. Raman detects modes with a change in polarizability, but is not sensitive to other mode types. In order to observe modes that result in a change in dipole moment, it is necessary to use IR spectroscopy. Both techniques are often used in conjunction, as they are complimentary and provide a complete picture for the vibrational modes in a molecule [7].

SERS uses a roughened metallic nanoparticle surface to enhance the traditional Raman signal. This is achieved through a phenomenon known as localized surface plasmon resonance (LSPR) which is the rapid oscillation of groups of electrons in the conduction band of the metal surface [7]. LSPR occurs when particles comparable in size to the wavelength of light are excited by an incident beam. The electric field component of the incident light will cause the the electrons of these particles to shift back and forth repeatedly. This results in an enhanced electric field and Raman signal for pi-conjugated molecules placed upon this metallic surface [7]. Noble metals such as gold, silver or copper are selected for SERS applications as they have plasmon frequencies close to that of the visible or near-infrared spectrum. For this reason, even greater signal enhancements can be achieved using an incident beam of the appropriate plasmon resonant frequency, as it will maximize the absorbance of light by the metal nanoparticles [7].

In addition to the ability to classify and differentiate molecules, SERS has the advantage of doing so label-free at ultra-low concentrations [8]. This project attempts to create a nanobiosensor by functionalizing a solid gold-nanoparticle (Au-NP) substrate with a novel ligand. The lipoic acid end of the ligand will graft to the surface of the substrate via Au-S interactions as seen in figure 1. The nitrogen-containing aromatic rings on the other end of the ligand will attach to the heme group in hemoglobin as shown in figure 2.

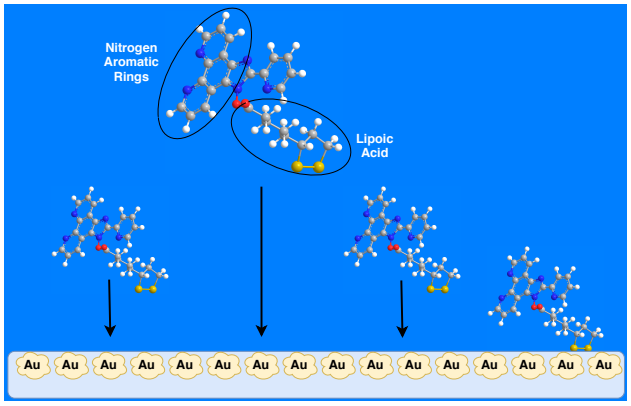


Figure 1: Ligand functionalizing onto the Au-NP substrate.

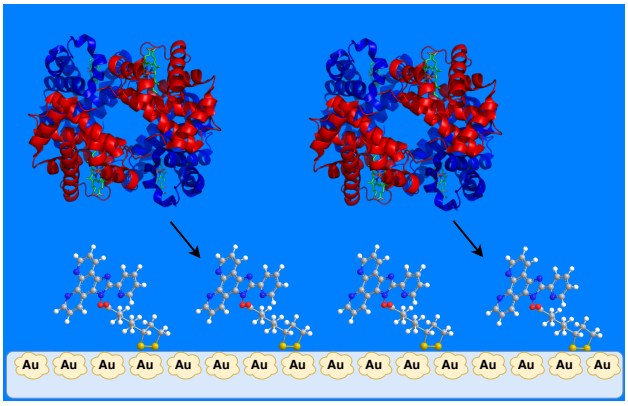


Figure 2: Hemoglobin attaching onto ligand of functionalized substrate.

Successful functionalization of the novel ligand to a colloidal solution of Au-NPs was reported in the 2018 paper by Egan et al [9]. The ligand functionalized Au-NPs were able to bind to hemoglobin in solution. This paper attempts to functionalize a solid Au-NP substrate using the same ligand to acquire reproducible SERS spectra of hemoglobin. A similar goal of protein detection was accomplished by Agarwal et al, in which SERS spectra of lysozyme proteins binded directly to a Au-NP substrate were acquired [8]. Hemoglobin, being a more complex molecule with a higher molecular weight may not effectively bind to a Au-NP surface via Au-thiol interactions alone. The novel ligand receptor will provide a stable support for hemoglobin to securely anchor itself to the rigid Au-NP surface.

A colloidal nanoparticle solution is a potential candidate for SERS of hemoglobin, but may pose some problems. Colloidal solutions allow hemoglobin to probe Au-NPs from all different directions, which may not be very consistent and in some cases may fold and denature the protein [8]. A solid substrate will create more order and allow hemoglobin to probe the sensor from one side only. This allows hemoglobin to secure itself in the same orientation across the substrate, which is hypothesized to generate more reproducible SERS results [8].

SERS of hemoglobin in colloidal solutions is already well known in academia, but the proposed nanobiosensor presents the ability to differentiate between different hemoglobin disorders in a timely manner. A structural defect in hemoglobin will be reflective in the corresponding SERS spectra. Acquiring numerous spectra of a particular hemoglobin variant and assigning those spectra to that particular disorder will make it possible to build a database of these classifications. Minute differences in spectra compared to that of standard hemoglobin may not be apparent to the naked eye, but a machine learning algorithm may be able to make the distinction.

Experimental Methods

Materials

1,4 Dioxane was purchased from Tokyo Chemical Industry (TCI). Hemoglobin from bovine blood was purchased from Sigma Aldrich. The novel ligand 2-(pyridin-2-yl)-1H-imidazo[4,5-f][1,10]phenanthrolin-1-yl-5-(1,2-dithiolan-3-yl)pentanoate was synthesized using the procedure detailed in the Experimental section of 'Hemoglobin-driven iron-directed assembly of gold nanoparticles' [9].

Gold nanoparticle substrates were synthesized using the top-down approach of pulsed laser deposition (PLD). A KrF laser was used with wavelength of 248 nm, repetition rate of 10 Hz and pulse width of 25 ns. Laser shots were fixed at 10000 laser shots with a laser fluence of 2 J cm^{-2}. Deposition took place in a vacuum chamber with Ar gas pressure of 70 Pa. The Au-NP were deposited on a 7059 Corning glass substrate. The complete procedure for the production of these substrates can be found in 'Protein-Metal Interactions Probed by SERS: Lysozyme on Nanostructured Gold Surface' [8].

Instrumentation

UV-Visible spectra were acquired using the Perkin Elmer Lambda 750S UV/Vis Spectrometer. Substrates were sandwiched between two polyacrylic holders held together by a binder clip as depicted in figure 3. Raman spectra were acquired using the Renishaw Raman imaging microscope System 2000.

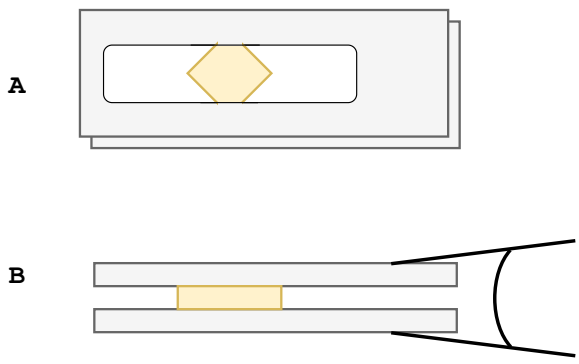


Figure 3: Diagram of the polyacrylic holders used to hold the Au-NP substrates from (A) top-down view and (B) side view.

Preparation of Ligand Solution

25.08 mg of the novel ligand was dissolved in 100 mL of 1,4 Dioxane, creating a stock solution with a concentration of 0.5 mM. The solution was stirred on a stir plate for 30 minutes to ensure complete solubility. The solution was poured into a dark nontransparent bottle and was stored in the refrigerator.

Preparation of Hemoglobin Solution

3386 mg of hemoglobin was dissolved in 169.3 mL of type I deionized water creating a final solution concentration of $3.1 \times 10^{-4} M$. The solution was stirred on a stir plate for 30 minutes to ensure complete solubility. The solution was poured into two dark nontransparent bottles and was stored in the refrigerator.

Au-NP Substrate Functionalization

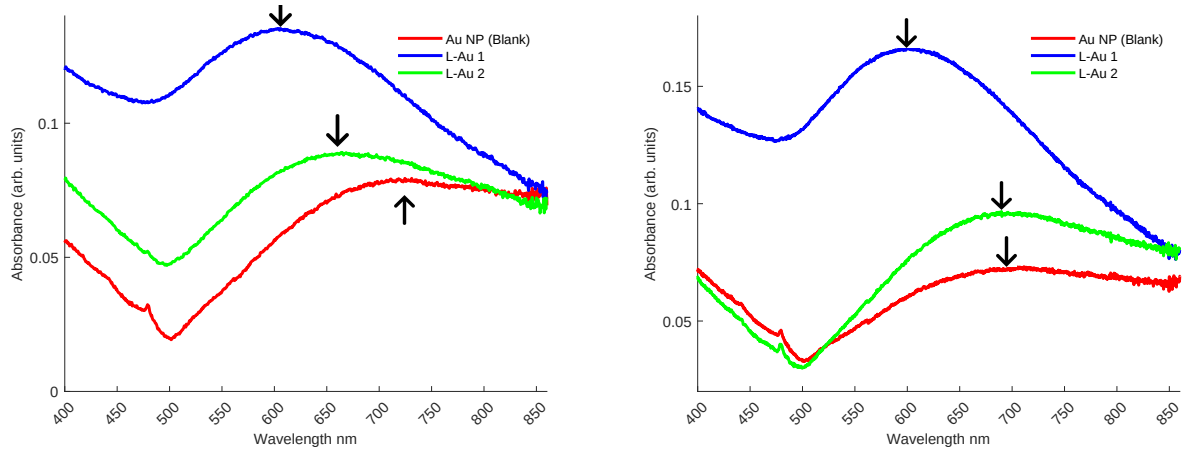
Au-NP substrates were picked up by the corner with Teflon-wrapped tweezers and dried with an air-valve under the fume hood. Substrates were then washed with ethanol, air-dried again and individually deposited into small vials with the Au-NP surface facing upwards. An aliquot of the ligand solution was pipetted into each vial such that the substrates were completely submerged in solution. The substrates were left in solution for 8 hours to allow functionalization to occur, after which they were pulled out of their respective vials, air-dried and stored in a Petri dish.

Results and Discussion

Two Au-NP substrates were functionalized with the novel ligand. The UV visible absorbance spectrum was acquired for both functionalized substrates along with a nonfunctionalized substrate for reference. The spectra was acquired once immediately after the 8 hour functionalization period and again five days later.

A broad surface plasmon resonance (SPR) peak in figures 4a and 4b can be seen in the nonfunctionalized Au-NP substrate around 700 nm. The functionalized substrate represented by the blue line (L-Au 1) in figures 4a and 4b display a shift in SPR peak of about 100 nm relative to the nonfunctionalized substrate. The magnitude of the shift appears to be the same

across both dates of collection. The SPR peak of the substrate represented by the green line (L-Au 2) shifts approximately 50 nm in figure 4a but does not appear to shift at all in figure 4b.



(a) (b)

Figure 4: UV visible absorption spectra for two ligand functionalized Au-NP substrates (blue & green) and one nonfunctionalized substrate (red) acquired on (a) March 4th with a data collection interval of 0.5 nm, (b) March 9th with a data collection interval of 0.25 nm.

The blueshifts seen in these figures suggest that the ligand has adsorbed onto the surface of the Au-NP substrate, albeit to varying degrees. Since the shift in SPR peak among figures 4a and 4b is more pronounced and consistent in L-Au 1 than compared to L-Au 2, it is likely that more of the ligand has successfully functionalized on to L-Au 1. This variance in shifting/successful functionalization between substrates could be attributed to a variety of reasons; these include the amount of the Au-NP monolayer present, the age of the substrate and potential unknown contaminants on the surface of the substrate.

Egan et al reported redshifting of the SPR peaks in their UV visible data from 519 nm to 536 nm upon the functionalization of the novel ligand to the citrate Au-NPs [9]. The direction that the shift occurs in depends on the initial SPR peak of the Au-NPs as well as the SPR peak of the ligand by itself. Here, a SPR peak blueshift is observed in L-Au 1 of 700 nm to 600 nm upon functionalization of the same ligand used by Egan et al. Since the blueshift exhibited in this paper is more drastic than the redshift exhibited by Egan et al, the SPR peak of the ligand likely lies in the upper 500 nm mark.

Although some blueshifting is observed for these substrates, it should be noted that these results are not conclusive. UV visible data of more functionalization trials with more substrates must be acquired to confirm surface attachment of the ligand. In addition, this project could also benefit from other methods of surface characterization to further support the claims made off of the UV visible data.

Once ligand functionalization on the substrate is confirmed, SERS testing may commence. Going forward, SERS spectra of functionalized substrates submerged in a hemoglobin solution can be acquired. Different concentrations of hemoglobin in solution will affect the spectra, so it is important to find a balance so as to not overcrowd the substrate or have too few hemoglobin present. Results of SERS spectra will determine whether or not the nanobiosensor is effective for hemoglobin detection and future disorder differentiation.

Conclusions

The preliminary steps of verifying the functionalization of a Au-NP substrate with a novel ligand have been completed. Of the two Au-NP substrates that have been functionalized, one has shown a significant blueshift away from the initial SPR peak. It is hypothesized that the blueshift of the SPR peak is indicative of the successful adsorption of the ligand onto the substrate. The shift is consistent with a previous paper that uses the same ligand to functionalize a colloidal solution of Au-NPs. The mentioned paper reports a redshift from a lower initial SPR peak while this paper reports a blueshift from a higher SPR peak.

Future Works

Additional trials are required to confirm these findings as the results of this paper are based on limited data. Further research involving SERS of the substrate in hemoglobin solution can be completed. SERS testing will verify the eligibility of the functionalized Au-NP substrate as a nanobiosensor for hemoglobin disorder differentiation.

Glossary

Dipole Moment: Separation of positive and negative charges in a molecule

Instantaneous Dipole: Sudden change in the distribution of electrons, causing a charge difference (temporary dipole moment)

Polarizability: A vibrational mode's ability to produce an instantaneous dipole when subject to an electric field

Plasmon: Quantization of electron oscillations

References

[1] Bernard G Forget and H Franklin Bunn. Classification of the disorders of hemoglobin. *Cold Spring Harbor perspectives in medicine*, 3(2):a011684, 2013.

[2] Bernadette Modell and Matthew Darlison. Global epidemiology of haemoglobin disorders and derived service indicators. *Bulletin of the World Health Organization*, 86:480–487, 2008.

[3] Maria D P Aguinaga, Ming Chan, Dorley M C Davis, Tim C, Dy Bonifacio, Marie Earley, Althea M Grant, Mary Hulihan, Suzanne Karabin, Joanne Mei, Christine Moore, Laxmi Nayak, and Kwaku Ohene-Frempong. Hemoglobinopathies: Current practices for screening, confirmation and follow-up. 2015.

[4] Rebecca Hampton, Vinod Balasa, and Sheryl E Allen Bracey. Emergencies in patients with inherited hemoglobin disorders—an emergency department perspective. *Clinical Pediatric Emergency Medicine*, 6(3):138–148, 2005.

[5] Cynthia Cobb. How long does it take to get blood test results? Retrieved from: https://www.healthline.com/health/how-long-for-blood-test-results, 2018.

[6] Daniel E. Sabath. Molecular Diagnosis of Thalassemias and Hemoglobinopathies: An ACLPS Critical Review. *American Journal of Clinical Pathology*, 148(1):6–15, 06 2017.

[7] Peter Larkin. *Infrared and Raman spectroscopy: principles and spectral interpretation*. Elsevier, 2017.

[8] NR Agarwal, M Tommasini, E Ciusani, A Lucotti, S Trusso, and PM Ossi. Protein-metal interactions probed by sers: lysozyme on nanostructured gold surface. *Plasmonics*, 13(6):2117–2124, 2018.

[9] Jacquelyn G Egan, Nicole Drossis, Iraklii I Ebralidze, Holly M Fruehwald, Nadia O Laschuk, Jade Poisson, Hendrick W de Haan, and Olena V Zenkina. Hemoglobin-driven iron-directed assembly of gold nanoparticles. *RSC advances*, 8(28):15675–15686, 2018.

Appendices

Procedure for Preparation of Ligand Solution

1. Wash 1 dark bottle, a 20 mL graduated cylinder and 250 mL beaker with type I DI water.
2. Measure 25.08115 mg of ligand powder on a scale and 100 mL of 1,4 Dioxane solvent in a graduated cylinder.
3. Pour the PHEN ligand powder into a beaker and add enough 1,4 Dioxane solvent such that there is 100 mL of the solution (note the amount of solvent left in the graduated cylinder). This 100 mL solution will have a concentration of 0.5 mM and will be used for several experiments throughout the project.
4. Place a clean stir bar into the beaker and leave on a stir-plate (no heat) for 30 minutes. Dispose of remaining solvent in the nonhalogen waste container.
5. Pour the solution into a dark bottle and wrap with parafilm. Store the solution in the refrigerator.

Procedure for Preparation of Hemoglobin Solution

1. Wash 2 dark bottles, a graduated cylinder and 250 mL beaker with type I DI water.
2. Measure 3.386 g of hemoglobin on a scale.
3. Pour the hemoglobin into a 250 mL beaker and dissolve in 169.3 mL of type I DI water. This hemoglobin stock solution will have a concentration of $3.1 \times 10^{-4} M$.
4. Place a clean stir bar into the beaker and leave on a stir-plate for 30 minutes.
5. Pour the solution into dark bottles and wrap with parafilm. Store the solution in the refrigerator.

Procedure for Au-NP Substrate Functionalization

1. Wash 2 vials, 1 small beaker and a Petri dish with type I DI water.
2. Thaw out and aliquot of the ligand solution.
3. Take two substrates and air dry them under the fume hood.
4. Prepare ethanol in a small beaker. Using Teflon wrapped tweezers, pick up the substrate by its marked corner and dip it into the ethanol. Air dry once again.
5. Place the dried substrate into a labelled vial. Repeat step 4 for the other substrate.
6. Pipette an appropriate amount of ligand solution into the vials such that the substrates are covered completely. Store in a dark location and wait 8 hours for functionalization to occur.
7. Air dry and store substrates in a labelled Petri dish.