Abstract

Coleman, Donna M. Design and Delivery of Water Soluble Gold Nanoparticles Containing Mixed Monolayers of Thiolated Polyethylene Glycol and Peptides to HeLa Cells (Advised by Professor Daniel L. Feldheim).

Drug delivery has become a major area of research interest today, particularly in cancer research. Many of the current cancer treatments have many undesirable side effects on patients leaving them weak and susceptible to other illness. Targeted drug delivery at the molecular level has many positive applications. First one could target a cancer therapeutic drug directly to the site of infection and reduce the many side effects associated with cancer therapy. Also less medication would be needed also reducing some of the side effects associated with current cancer therapeutics. Many researchers have turned their attention to nanotechnology as a way to address such cancer drug delivery issues. This thesis presents the design of a two-phase type water-soluble gold nanoparticle. The gold nanoparticle is passivated with a peptide sequence that has known cell-penetrating abilities. The thiolated polyethylene glycol imparts stability to the gold nanoparticle enabling it to stay in a stable state and remain functional in high salt concentrations such as inside of a human body. Presented in this thesis is data to confirm the preliminary results of a two-phase type gold nanoparticle passivated with thiolated polyethylene glycol and $Arginine_9$, which could facilitate travel into the cytosol and

nuclear regions of a HeLa cell. This type of delivery system has the potential of a possible future drug delivery vector.

Design and Delivery of Water Soluble Gold Nanoparticles Containing Mixed Monolayers of Thiolated Polyethylene Glycol and Peptides to HeLa Cells

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Dedication

This thesis is dedicated to mom and Dad and my entire family. I would not have been able to do this without your love and support.

Biography

Donna Marie Coleman was born in 1966 in Chicago Illinois. Her parents are Dorothy and Melvin Williams. Donna attended Academy of our Lady high school. Donna was very active in high school. Donna played Basketball, and participated in the marching and jazz bands in high school. Donna also placed in state music competitions. Donna also participated in writing and science competitions in the Afro-Academic Cultural Technological and Scientific Olympics (ACT-SO) competitions in Chicago. After High School Donna enlisted in the United States Army (USA) with duty stations in the United States and Germany. Donna served as a Medical Laboratory Technician for the USA. Donna served during the Gulf war era. Donna trained reserve soldiers to run the laboratory as soldiers were being deployed to the Gulf War combat area. After Donna's time in the military she began her studies at Howard University where she earned a Bachelors of Science degree in Chemistry. After graduation from Howard University she began graduate studies at North Carolina State University in Pursuit of her Masters of science in Chemistry. At North Carolina State University Donna worked with Dr. Daniel Feldheim in Nanotechnolgy and drug delivery applications.

Few things help an individual more than to place responsibility upon him, and to let him know that you trust him.

Booker T. Washington

Acknowledgments

I would first like to give honor and thanks to my lord and Savior Jesus Christ. Without his sacrifice and his fathers Grace I would not be here today. I would then like to thank my parents. My parents came to my games, music competitions and award ceremonies for me no matter how tired they were and were always the one I looked and saw clapping for me. I cherish the days when I use to play basketball for the YMCA and they would come to the games and we would go out as a family and share time together, those are the times I enjoyed the most. It was those times I am most thankful for because I have parents who love me molded and inspired me. I am especially thankful for my mother who made many sacrifices so that I could have the education and opportunities I did at Academy of Our Lady High School.

I would also like to thank all of the teachers in my life that said I could when many others said I could not do something. I am especially thankful to my mentors I have had the pleasure of having here at NCSU. I would like to thank Dr. Wandra Hill who told me to hold on to your dreams no matter what others may say. I would also like to thank Dr. Harold Freeman who motivated me because he believed in me when others who were suppose to believe in me and support me did not. That meant so much to me.

I also can honestly say that the friendships I have developed here at NCSU will last a lifetime. My friends, KeAndra, Sandra, Angela, Damian, Chiamaka, and Eddie have kept me grounded and with their support my time here at NCSU has been a memorable experience. They were the ones who also told me not to give up on my dreams when others said give up on them. I love you all and wish you success in all you do. I would

also like to thank my best friend Adrenia Woodall who has been my friend for almost twenty years. She has been more of a friend to me than I have been to her and she still stands by my side no matter what.

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I would also like to thank my classmates who have been wonderful and supportive. I would also like to thank my group members who have been an asset to me as well. I would also like to thank my advisor Dr. Daniel Feldheim for his financial support during my graduate studies here at NCSU. My time at NCSU has been an enlightening experience for me that will only add to my character and strength.

Philippians 4:13

" For I can do everything through Christ who gives me strength"

Proverbs 3: 5-6

"Trust in the lord with all your heart, and lean not on your own understanding; In all your ways acknowledge him, and he shall direct your paths"

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Design and Delivery of Water Soluble Gold Nanoparticles Containing Mixed Monolayers of Thiolated Polyethylene Glycol and Peptides to HeLa Cells

Chapter One: Introduction and Background

1.1 Introduction

Cancer is one of the most deadly diseases of our time. Cancer accounted for 553,768 deaths in 2001 contributing 22.9% of deaths in America [1]. Cancer is defined as a disease in which abnormal cells divide without control. Cancer cells can invade nearby tissues and spread through the blood stream and lymphatic system to other parts of the body. The current treatments of cancer involve chemotherapy (treatment with various anticancer drugs), surgery (surgical removal of the tumor tissue) and radiation therapy (treatment with high-energy radiation). As a result of these current forms of treatment a variety of side effects can arise. Some of these side effects include destruction of the healthy cells surrounding the cancerous area, as well as healthy cells overall, which can result in hair loss and a reduction of white blood cell counts thereby compromising a person's ability to fight off infections. Patients also suffer from the discomfort of the administering of treatments such as painful intravenous delivery (IV, a needle is placed in the patients arm or neck vein), nausea, fatigue and even depression. Another surging problem on the rise is that of multidrug resistance in cancer treatments (a current drug routine that no longer works to treat the cancer it was prescribed for) [2]. As a result scientists are currently involved in research to find more effective ways to treat and cure cancer. Scientists today are now beginning to look at ways to deliver drugs at the cellular level. The National Cancer Institute recently announced that they would develop and apply nanotechnology to cancer treatment and prevention. Nanotechnology, the development and engineering of devices so small that they are measured on a molecular

scale, has already demonstrated promising results in cancer research and treatment [1]. If scientists can attack and treat cancer at the site of disease, the numerous side effects associated with chemotherapy can be drastically eliminated.

1.2 Background and Significance

One of the major drawbacks of drug delivery at the cellular level is the inability of delivery vectors (the transporting device) to cross the cellular membrane and effectively enter into the nucleus and deliver the desired drug. In order for a drug to work effectively it must reach the nuclear region intact (see Figure 1). One way scientists are addressing the issue of drug delivery in cancer treatments today is by designing nanoscale drug delivery devices that can deliver therapeutic drugs at the cellular level [3]. Being able to deliver drugs at the cellular level will enable the therapeutic drug to have a more pronounced effect and reduce the many side effects associated with the current treatments for cancer. One such design that is being explored in many research groups to include the Feldheim group is bioconjugation of Cell Penetrating Peptides (CPP) and Nuclear Localization Signals (NLS) to nanoparticles. Bioconjugation involves the linking of two or more molecules to form a novel complex having the combined properties of its individual components [4]. Our research currently involves bioconjugation of Cell Penetrating Peptides enhanced with Nuclear localization Signals (NLS). Our current strategy involves the conjugation of peptide sequences to gold nanoparticles (see Figure 2) [5,6]. Bovine Serum Albumin (BSA) serves as a stabilizer, m-Maleimidobenzoly-N-hydroxysuccinimide ester (MBS) as a cross linker and the peptide sequence facilitates cellular entry of our gold nanoparticle once they are conjugated to the gold nanoparticle.

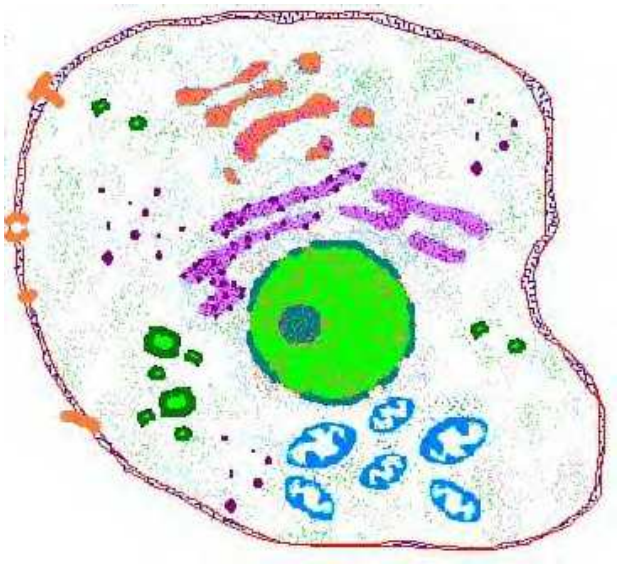

Figure 1: Cell with nucleus colored as green.

Figure 2. 20 nm gold nanoparticles with Nuclear Localization Signal (NLS) peptide attached to the gold surface via a thiol bond.

Cell Penetrating Peptides are short peptide sequences that are able to translocate across plasma membranes in a nonreceptor-mediated fashion. Over the past several years many researchers have been investigating how to utilize these CPP's as transporters of nanoparticles, liposomes and therapeutics into cells. Several families of CPP's are known today. Most are derived from naturally occurring proteins (table 1) [7].

Table 1. Examples of CPP's introduced by various research groups:

1. Penetratin	RQIKIWFQNRRMKWKK [8]
2. Tat fragment (48-50)	GRKKRRQRRRPPQC [9]
3. Signal sequence-based peptides	GALFLGWLGAAGSTMGAWSQPKKKRKV [10]
4. pVEC	LLIILRRRIRKQAHAHSK [11]
5. Transportan	GWTLNSAGYLLKINLKALAALAKKIL [12]
6. Amphiphilic model peptide	KLALKLALKALKAALKLA [13]
7. Arg_9	RRRRRRRRR [14]

Since the discovery that CPP's translocate across the plasma membrane of living cells and permit intracellular transport of cargo such as conjugated peptides, proteins, oligonucleotides and nanoparticles [7], they have opened new possibilities in biomedical research. The subject of this thesis is the use of gold nanoparticles modified with Nuclear Localization Signals (NLS) peptides and CPP's. A Nuclear Localization Signal (NLS) is a short stretch of amino acids that mediates the transport of nuclear proteins into the nucleus. Our research group has been able to successfully show intracellular delivery of gold nanoparticles conjugated to various peptide sequences Figure 3 [5,6] .As shown in

Figure 3, particles conjugated to peptide sequences were able to target the nucleus of a Human Cervical Cancer Cell (HeLa). In order to observe the delivery of gold nanoparticles, a combination of microscopy techniques, including Video-enhanced color and differential interference contrast microscopy (VECDIC) were used.

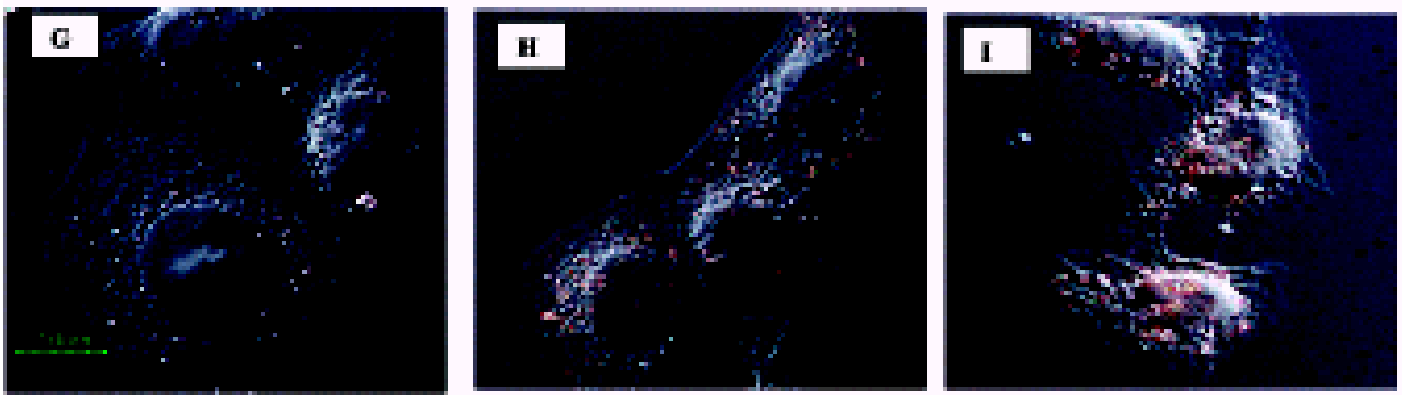

Figure 3. Incubation of HeLa cells with 20nm diameter gold nanoparticle conjugates observed by VECDIC, G-I 0.5, 1.5 and 3 hour incubation with nanoparticles carrying peptide sequence, M4, (CKKKKKKGGRGDMFG).

Through research we found that the stability of our particles needed to be addressed. Once particles are made we immediately deliver them to cells. In some instances our gold nanoparticles aggregate in solution during sample preparations and could not be used. There were also instances in which excess BSA was needed in order to maintain stability of our particles so that they could be used for cell delivery. Having to add excess BSA may hinder the delivery of these conjugates to cells (possibly by

increasing the diameter of our gold nanoparticle and making it difficult for larger size conjugates to penetrate the cell membrane) [15]. Many research groups have also looked into stabilizing gold nanoparticles for biotechnical applications [16,17]. Ensuring the stability of gold nanoparticles enables it to withstand high salt concentrations, for example if it were traveling in the human body. It is therefore necessary to find suitable stabilizers for gold nanoparticles. One useful stabilizer researchers are exploring is Polyethylene glycol (PEG) [4]. This thesis will explore the use of PEG as a method for stabilizing gold nanoparticles.

Delivery to the nuclear region of a cell is a challenge. In order for a cancer therapeutic drug to be effective, nuclear delivery is essential [18].The desired material must first cross the outer cell membrane before it even gets to the nuclear membrane. Cellular membranes are protective membranes that regulate transport in and out of the cell and the subcellular regions within. They are essential for the structure of the cell. They also allow selective permeability so that certain needed materials from the environment can enter and other undesirable or harmful substances cannot enter into the cell. Only compounds within a narrow range of molecular size, net charge and polarity are able to cross effectively into a cell. This is especially true for materials that travel from the cytosol region across the nuclear membrane. One of the mechanisms of internalization is based on Receptor-Mediated Endocytosis (RME) or transporter based uptake [19]. Some viral and bacterial proteins are endowed with properties enabling them to cross the plasma membrane and gain entry into a cell [20,7]. The endocytic machinery of the cell then tags and directs the internalized macromolecule to its desired location within the cell. CPP's

and NLS's are essential for research that utilize peptide sequences coded to have certain functions once introduced into a cell [21].

In order for a cell to keep its proper function, many harmful entities that try to get into cells are denied entry or if they do enter into the cell they are quickly and effectively destroyed by the cell through a process called phagocytosis. This process involves the cell engulfing a particle by wrapping pseudopodia around it and packing it within a membrane-enclosed sac called a vacuole and disposing of the material before it can cause harm to the cell (see Figure 4) [22].

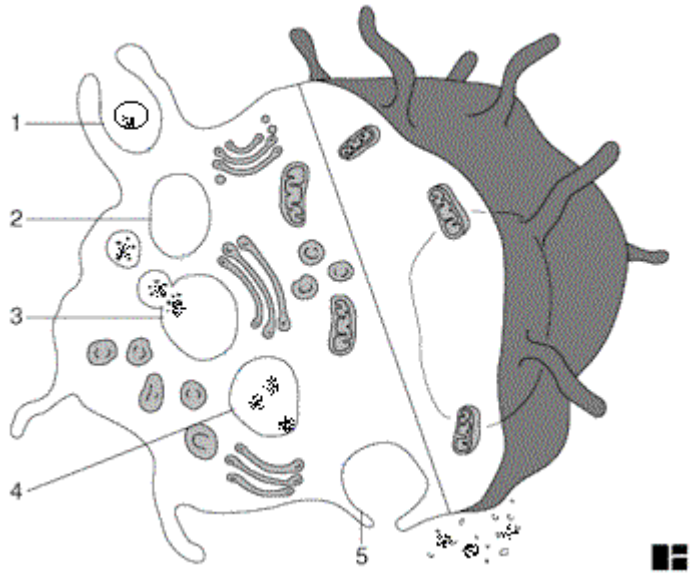

Figure 4: Phagocytosis occurring in a cell. 1) Attachment of particle to pseudopodia. 2) Ingestion of the particle and formation of phagosome. 3) Fusion of the lysome and phagosome releasing lysosomal enzymes into the phagosome. 4) Digestion of the ingested materials. 5) Release of digested products from the cell.

The current design of many drug delivery vectors has attempted to address the issue of avoiding recognition by the cell as being harmful. Many of the designs include the following criteria for an effective drug deliver vector:

1) It must be nontoxic.
2) Able to get into the cell to the desired location without being viewed as harmful to a cell and thereby removed out of the cell.
3) Small enough to cross the cellular membrane.
4) Biocompatible.
5) Particle should be able to remain stable in the body so it can travel through the body to the site of the tumor.
6) Possess a CPP and or NLS for cellular internalization and recognition.

Feldherr and coworkers have already laid the foundation for the use of gold nanoparticles whose surfaces are passivated with nucleoplasmin [23,24,25]. Their delivery mode was via microinjection and they observed how the particles traveled from the cytosol into the nucleus after microinjection of the particle. Microinjection for intracellular studies of cellular activity is useful, however it is not an option for a successful drug delivery of a nanoparticle.

A CCP that has drawn much attention recently in the literature is arginine rich peptides. Arginine is one of the 20 amino acids, (see Figure5). In arginine, the side chain contains a guanidino group separated from the α-carbon by three methylene groups. Arginine rich peptides have been shown to enhance cellular uptake of molecules

to which they were conjugated. Arginine rich peptides are highly basic and these peptides have been found to be of rather low toxicity to cells in culture media and could serve as suitable carrier vectors [26,27,28]. The exact mechanism of internalization however is not yet known.

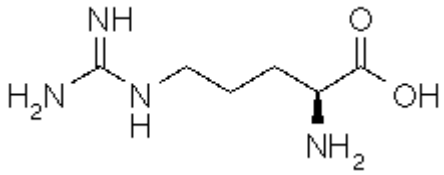

Figure 5: Arginine

1.3 Motivation

In order to overcome some of the drawbacks of current nanoparticles used in cellular delivery applications, this thesis will introduce the design and application of a mixed monolayer of gold nanoparticles for drug delivery purposes. This thesis will address the following:

1) Synthesis of PEG molecules that have different chain lengths and PEG units.

2) Synthesis of water-soluble gold nanoparticles.

3) Gold nanoparticles that are stable at high salt concentrations.

4) Design of a gold nanoparticles passivated with PEG and arginine units.

5) Characterization of designed gold nanoparticles.

6) Delivery of designed gold nanoparticle to HeLa cells.

7) Observation via Transmission Electron Microscopy to determine if these gold nanoparticles will travel to the inside of HeLa cells.

The following hypothesis will be investigated and addressed:

Hypothesis to test:

1) Can gold nanoparticles passivated with PEG undergo ligand exchange reactions with arg_9 peptide?
2) Will gold nanoparticles containing mixed monolayers of PEG and arg_9 be able to cross a cellular membrane, escape the endosomal pathway and deliver the newly designed gold nanoparticles to the internal regions of a HeLa cell?

This will involve the synthesis of water-soluble gold nanoparticles, which utilize PEG as a stabilizer and an arginine rich peptide sequence, CGGWGRRRRRRRRRA, to facilitate cellular internalization of the nanoparticle. The nanoparticle vector should be able to escape the endosomal pathway and get to the cytosol and nuclear regions of a targeted cell.

Chapter two of this thesis will introduce PEG and its many uses to include its use as a stabilizer of gold nanoparticles. Chapter two of the thesis will also discuss the synthesis of Polyethylene glycol thiols and their characterizations. Chapter 3 will discuss the synthesis of water-soluble gold nanoparticles and their characterizations. Chapter 4 will discuss the delivery results of the proposed gold nanoparticle design, delivered to HeLa cells.

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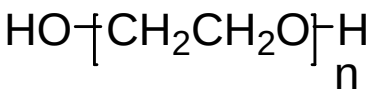
Chapter 2: Synthesis and Characterization of Polyethylene Glycol Thiol Molecules

2.1 Introduction

For many years polyethylene glycol (PEG) has been a molecule of interest to the biotechnical and biomedical research communities. Pioneering researchers such as Zalipsky and Harris have published books and a vast number of papers on PEG's many uses [1,7]. Polyethylene glycol is a linear neutral polymer, available in a variety of molecular weights. It is made from the anionic polymerization of ethylene oxide resulting in the formation of polymer strands of various lengths, depending on the polymerization conditions. These molecules are soluble in water and most organic solvents, (Figure 2.1) [1].

Figure 2.1



The two hydroxy groups located at each end of the polymer allow it to be functionalized for various applications to allow for the coupling of peptides, drugs and other materials. PEG coupled to other molecules can be used for altering the solubility characteristics of the molecules they are coupled with. PEG imparts nonimmunogenicity (will not cause an immune response) to molecules they are functionalized with. This polymer is also nontoxic and does not harm the bioactivity of proteins or cells [1]. PEG has also been used to increase the solubility of formally insoluble drugs and decrease the toxicity of others [8,9]. This is a major benefit for any drug delivery system. PEG has also

been shown to enhance the half-life of substances in vivo, aid in penetrating cell membranes, increase biocompatibility and prevent protein adsorption to surfaces [2].

PEG has also been utilized in many research groups as a stabilizer of nanoparticles including gold nanoparticles [3,4]. When PEG is used unmodified it inhibits colloid aggregation by causing steric repulsion of particles and by shielding particles from high ionic strengths that facilitate flocculation (aggregation of particles)[4]. When one of the OH groups of the PEG molecule is thiolated, the SH group is able to form bonds with an Au surface via covalent bonding [6.] These are just some of the reasons why PEG molecules are useful in biotechnology and will be used in this research project.

2.2 Experimental

2.2.1 Materials

All chemicals were used as received from vendors without further purification except if specifically mentioned. Triethylene glycol, tetraethylene glycol, thioacetic acid, 11-chloro-undecane, 11-bromo-undecane, allyl bromide, hydrochloric acid, azobisisobutyronitrile (AIBN), and sodium hydroxide were purchased from Sigma Aldrich. Octaethylene glycol was purchased from Polypure. Solvents were purchased from Fisher and Sigma Aldrich and used as received unless otherwise stated. Reaction progression and Purification's were monitored by thin layer chromatography (TLC) on silica gel.

2.2.2 Synthesis

2.2.2.1 Octaethylene Thiol

2-(2-{2-[2-(2-{2-[2-(2-undec-10-enyloxy-ethoxy)- ethoxy]- ethoxy}- ethoxy)-ethoxy]-ethoxy}- ethoxy)-ethanol (1) [10]**.** The entire synthetic scheme is shown in Figure 2.2, and was based on Whitesides synthesis of polyethylene glycol molecules [10]. A three-neck 50-ml round bottom flask with 3 g of Octaethylene glycol in place was attached to a condenser and this entire apparatus was purged with argon for 5 minutes. After purging 0.43 ml of 50% aqueous sodium hydroxide was added and the flask was lowered into an oil bath heated to 100 ° C. The sample turned to a clear yellow solution. This reaction was allowed to proceed for 40 minutes. After 40 minutes 0.43 ml of 11-chloroundec-1-ene was added and solution turned a deep yellow color. This reaction was allowed to continue for 24 hours. After 24 hours the reaction mixture was removed form the oil bath and allowed to come to room temperature. The sample was then extracted 6 times with hexane and the organic layers were rotary evaporated down to recover a clear yellow crude material. The crude material was then purified via column chromatography with silica gel. The solvent system used was 100 % ethyl acetate. Purification was followed by thin layer chromatography (Rf=0.35) and product fractions were rotary evaporated down to recover a clear yellow pure product. Sample was then placed on a dry line to remove any remaining solvents. ^{1}HNMR revealed the desired product was obtained.

s-(11-[2-(2-{2-[2-(2-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy}- ethoxy)- ethoxy]-ethoxy}- ethoxy)-ethoxy]-undecyl} ester (Thioacetate) (2). A two-neck 50-ml round bottom flask with stir bar in place and 0.164 grams of AIBN in flask was attached to a photochemical reaction box, and the flask was purged with argon for 5 minutes. After purging flask with argon, 0.4463 grams of product 1 was mixed with 4.2 ml methanol and 0.29 ml of thioacetic acid, mixed well and transferred immediately via a glass syringe to reaction flask. Stirring was initiated, water to condenser turned on and photochemical reaction box was closed and reaction was started. The reaction was run under argon with a 450-W medium-pressure mercury lamp. The reaction was allowed to continue for 6 hours. After 6 hours the photochemical reaction was stopped and the sample was allowed to come to room temperature. The sample was then rotary evaporated down to recover a yellow product and this crude material was then purified via column chromatography with silica gel. The solvent system used was a 3:1 ethyl acetate/methanol system. Purification was followed by thin layer chromatography and product fractions (RF= 0.52) were rotary evaporated down to recover a clear yellow pure product. Sample was then placed on a dry line to remove any remaining solvents. ^{1}HNMR confirmed desired product was obtained.

2-[2-(2-{2-[2-(2-(2-[2-(11-Mercapto-undecyloxy}-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxy}-ethoxy]-ethanol, HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH, (Thiol) PEG (3). A two-neck 25-ml round bottom flask was attached to a condenser and this apparatus was purged with argon for 5 minutes. After purging flask with argon, 0.100 grams of compound 2 was

mixed with 3.2 ml of a 0.1M HCl in methanol solution, mixed well and this material was immediately transferred to reaction flask and the flask was lowered into 80 °C oil bath and this material was refluxed for 5 hours. After 5 hours sample was removed from oil bath and allowed to come to room temperature. The sample was rotary evaporated down to recover a clear yellow material and this crude material was then purified via column chromatography with silica gel. The solvent system used was a 3:1 ethyl acetate/methanol system. Purification was followed by thin layer chromatography (Rf = 0.57) and product fractions were rotary evaporated down to recover a clear yellow pure product. Sample was then placed on dry line to remove any remaining solvents. A typical yield for the thiol is usually 30% of the desired product. ^{13}C NMR and ^{1}HNMR, (300MHz spectrum in $CDCl_{3)}$ data are shown in Figures 2.3, 2.4 respectively. ^{1}HNMR (300 MHz, CDCl3) δ 1.2 (br s, 14H), 1.3 (t, 1H) ,1.5 (m, 4H), 2.5 (q, 2H), 2.6 (s, 2H), 3.4 (t, 2H), 3.55-3.71 (m, 30H); ^{13}CNMR (75MHz, CDCl3) δ 24.85 (t) 26.29 (t), 28.57 (t), 29.26 (t), 29.67 (t), 29.71 (t), 29.75 (t), 29.83 (t), 34.24 (t, CH_2SH), 61.90 (t, CH_2OH), 70.26 (t), 70.48 (t), 70.76 (t), 71.74 (t), 76.94 (t), 77.25 (t), 77.57 (t). High Resolution Exact Mass Measurement-FAB confirmed an elemental composition of $C_{27}H_{56}O_9S$, MW=557.37.

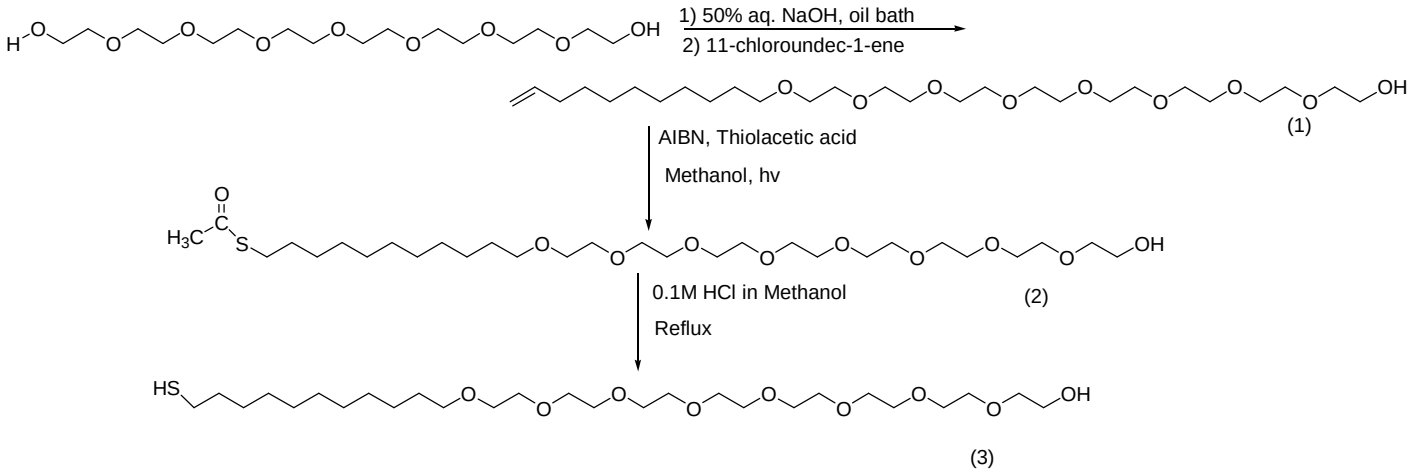


Figure 2.2. Synthetic scheme for molecules 1,2, and 3.

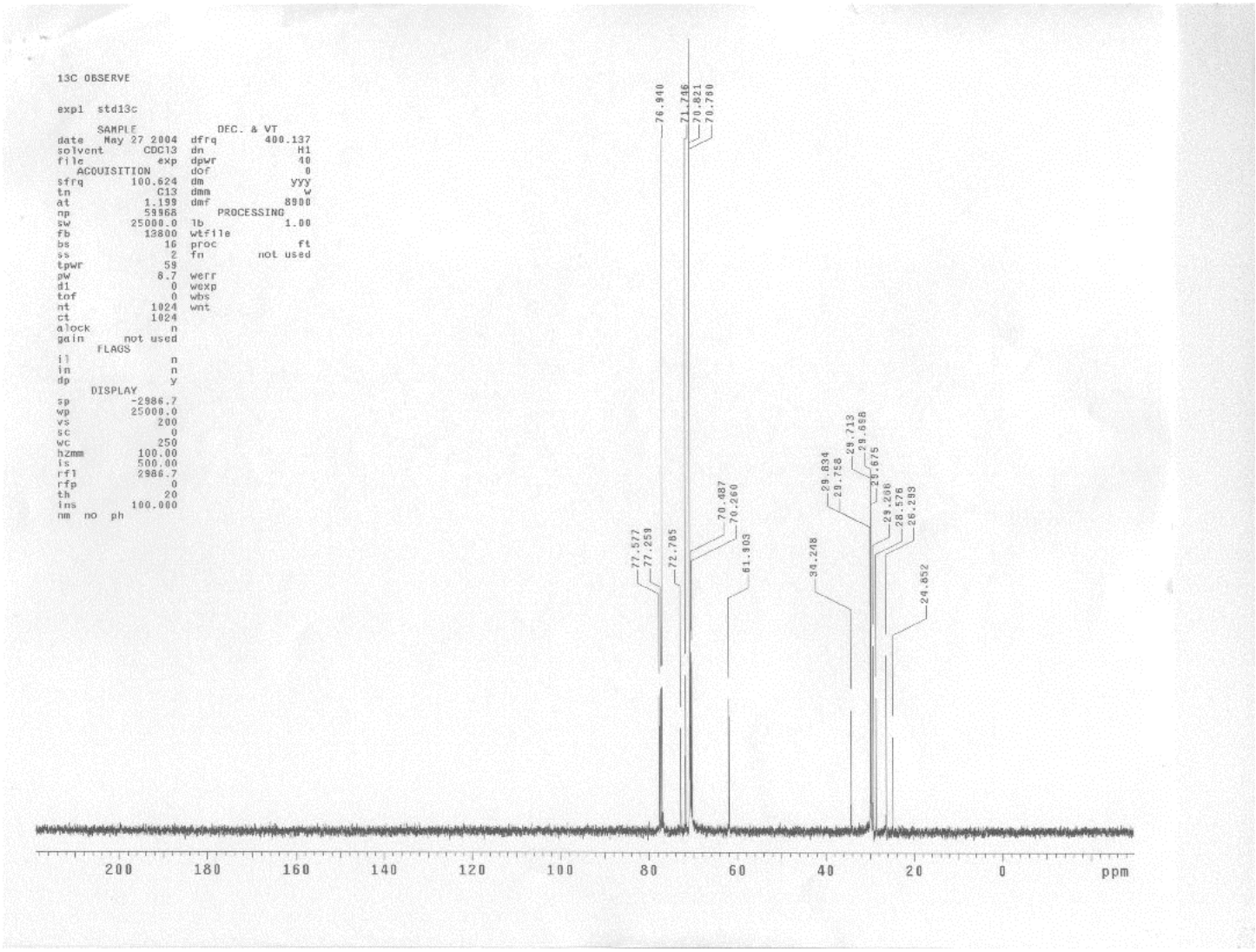


Figure 2.3 ^{13}C NMR spectrum for octaethylene thiol, HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH.

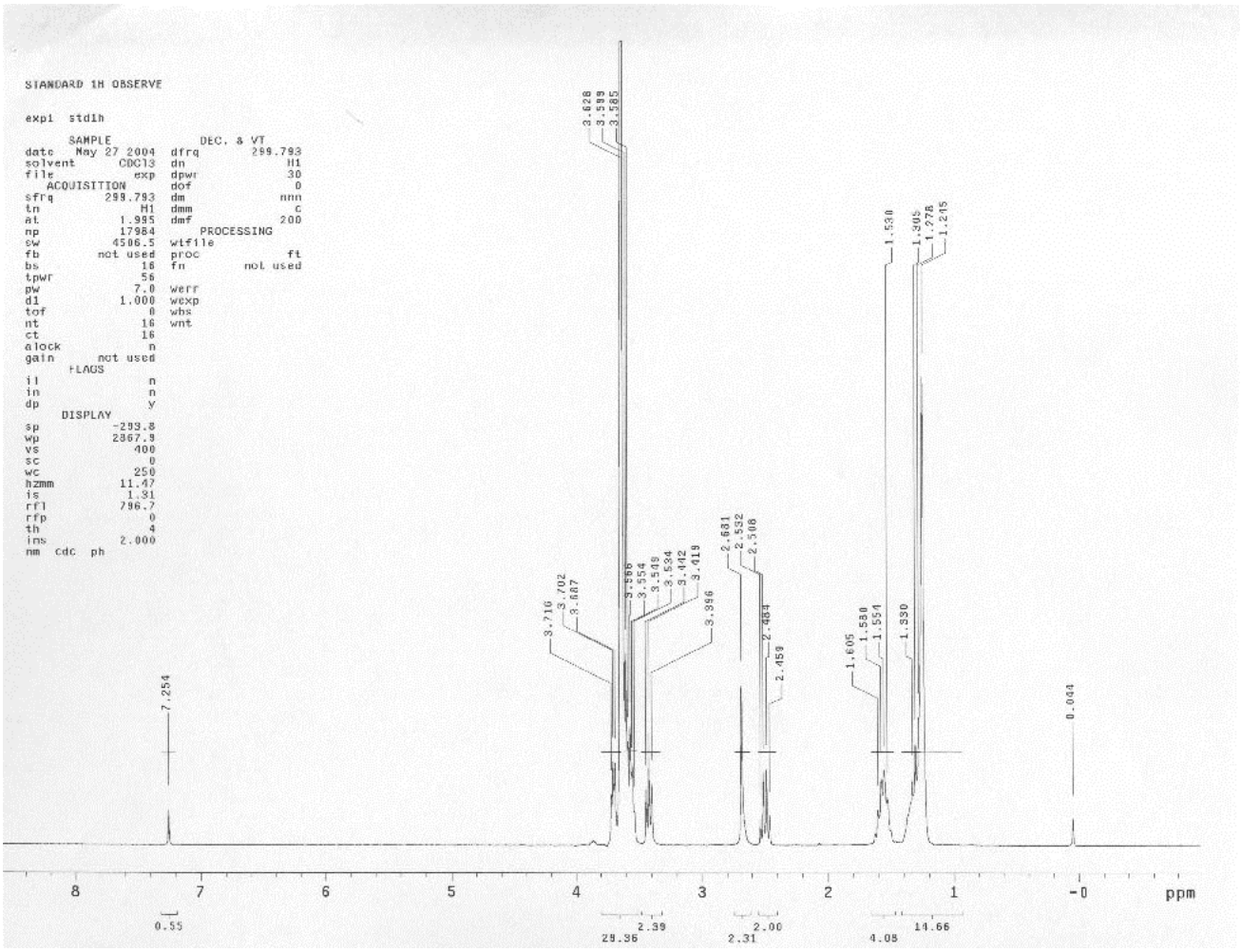


Figure 2.4 ^{1}HNMR spectrum for octaethylene thiol, HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH.

2.2.2.2 Synthesis of Tetraethylene Thiol

2-{2-[2-(2-Undec-10-enyloxy-ethoxy]-ethoxy}-ethanol (1) [10]**.** The entire synthetic scheme is similar to figure 2.2., and was also based on Whitesides synthesis of Polyethylene glycol molecules [10]. A three-neck 100-ml round bottom flask with 20 g of Tetraethylene glycol in place was attached to a condenser and this entire apparatus was purged with argon for 5 minutes. After flask was purged 2 ml of 50% aqueous sodium hydroxide was added and flask was lowered into an oil bath that was 100 °C. The sample turned to a clear yellow solution. This reaction was allowed to proceed for 30 minutes. After 30 minutes 2 ml of 11-chloro-1-undecane was added and solution turned a dark brown color. This reaction was allowed to continue for 24 hours. After 24 hours this reaction mixture was removed form the oil bath and allowed to come to room temperature. The sample was then extracted 6 times with hexane and the organic layers were rotary evaporated down to recover a clear yellow crude material. The crude material was then purified via column chromatography with silica gel. The solvent system used was 100 % ethyl acetate. Purification was followed by thin layer chromatography and product fractions (RF= 0.31) were rotary evaporated down to recover a clear yellow pure product. Sample was then placed on a dry line to remove any remaining solvents. ^{1}H NMR confirms desired product was obtained.

2-{2-[2-(2-Undecyloxy-ethoxy)-ethoxy]-ethoxy}-ethanol, thioacetic acid (2). A two-neck 50-ml bound bottom flask with stir bar in place and 0.1290 grams of AIBN in flask was attached to a photochemical reaction box and the flask was purged with argon for 5

minutes. After purging flask with argon, 1.3256 grams of product 1 was mixed with 20 ml methanol and 0.43 ml of thioacetic acid, mixed well and transferred immediately to flask via a glass syringe to reaction flask. Stirring was initiated, water to condenser turned on carefully and photochemical reaction box was closed and reaction was started. The reaction was run under argon with a 450-W medium-pressure mercury lamp, all of which are located in photochemical box. The reaction was allowed to continue for 11 hours. After 11 hours the photochemical reaction was stopped and sample was allowed to come to room temperature. The sample was then rotary evaporated down to recover a yellow product and this crude material was then purified via column chromatography with silica gel. The solvent system was a 1:1 2-propanol/hexane system. Purification was followed by thin layer chromatography and product fractions (RF= 0.47) were rotary evaporated down to recover a clear yellow pure product. Sample was then placed on a dry line to remove any remaining solvents. ^{1}HNMR confirms desired product was obtained.

2-(2-{2-[2-(11-Mercapto-undecyloxy)-ethoxy]-ethoxy)-ethanol, HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH,Thiol PEG (3). A two-neck 50-ml round bottom flask was attached to a condenser and this apparatus was purged with argon for 5 minutes. After purging flask with argon, 0.5526 grams of compound 2 was mixed with 6.5 ml of a 0.1M HCl in methanol solution, mixed well and this material was immediately transferred to reaction flask and flask was lowered into an 80 ° C oil bath and this material was refluxed for 6 hours. After 6 hours sample was removed from oil bath and allowed to come to room temperature and sample was the rotary evaporated down to recover a clear yellow material and this crude material was then purified via column chromatography with silica

gel. The solvent system used was a 3:1 ethyl acetate/2-propanol system. Purification was followed by thin layer chromatography and product fractions (Rf = .55) were rotary evaporated down to recover a clear yellow pure product. Sample was then placed on dry line to remove any remaining solvents. A typical yield for the thiol is usually 62% of the desired product. ^{1} HNMR, ^{13}CNMR and IR data are shown in Figures 2.5, 2.6, 2.7, respectively. ^{1}HNMR (300 MHz, CDCl3) δ 1.2 (br s, 14H), 1.3 (t, 1H), 1.5 (m, 4H), 2.5 (q, 2H), 2.7 (s, 1H), 3.4 (t, 2H), 3.55-3.71 (m, 16H); ^{13}CNMR (75MHz, CDCl3) δ 25.00 (t), 26.42 (t), 28.71 (t), 29.40 (t), 29.80 (t), 29.90 (t), 29.94 (t), 34.15 (t CH_2SH), 62.03 (t, CH_2OH), 70.32 (t), 70.61 (t), 72.81 (t), 76.90 (t), 77.32 (t), 77.74 (t). IR (neat) 3457, 2929, 2859, 2558, 1640, 1462, 1351, 1295, 1248, 1121, 941, 885 cm-^{1} . High Resolution Exact Mass Measurement-FAB confirmed an elemental composition of $C_{19}H_{40}O_5S$, MW=381.27.

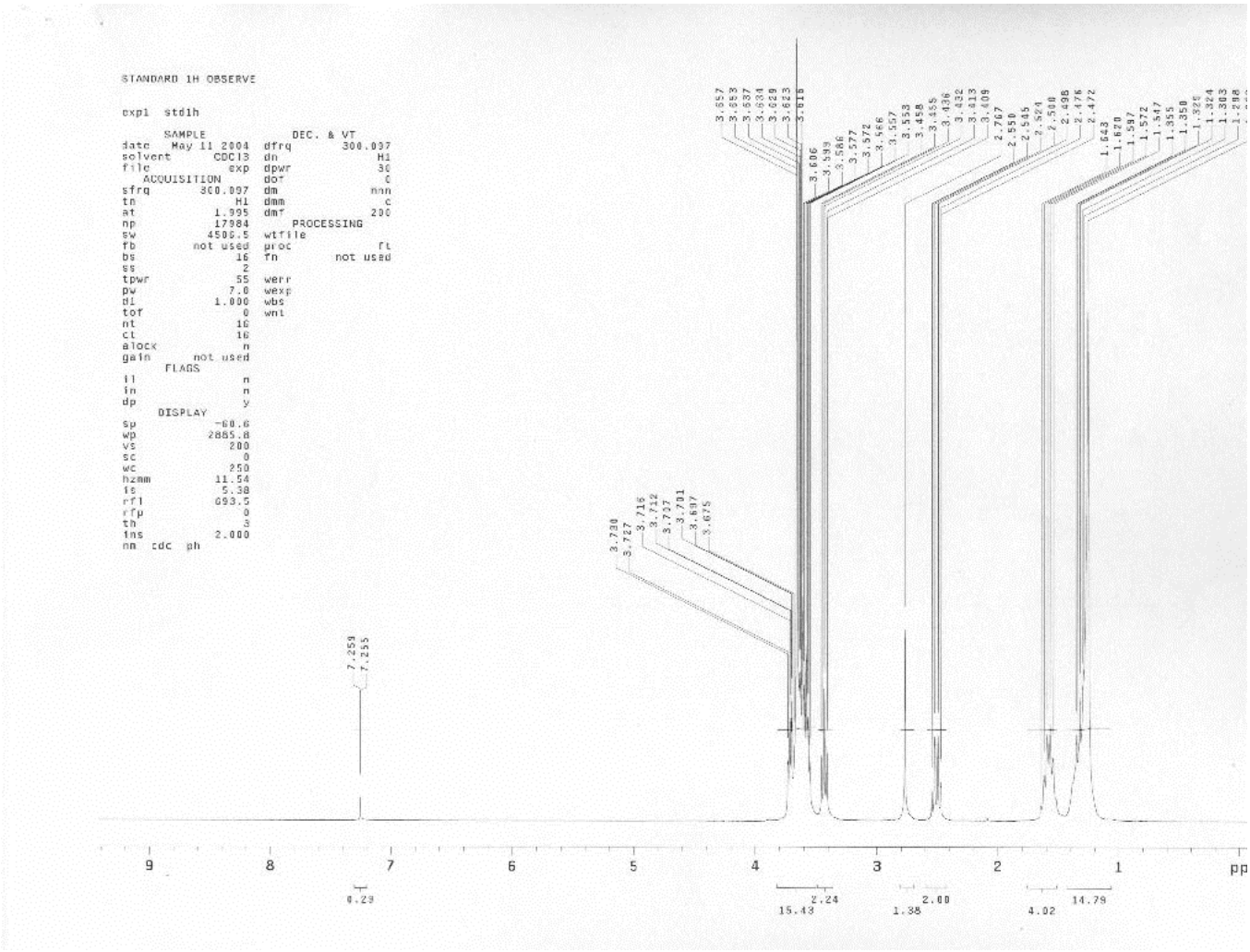


Figure 2.5 ^{1}HNMR spectrum for tetraethylene thiol, HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH.

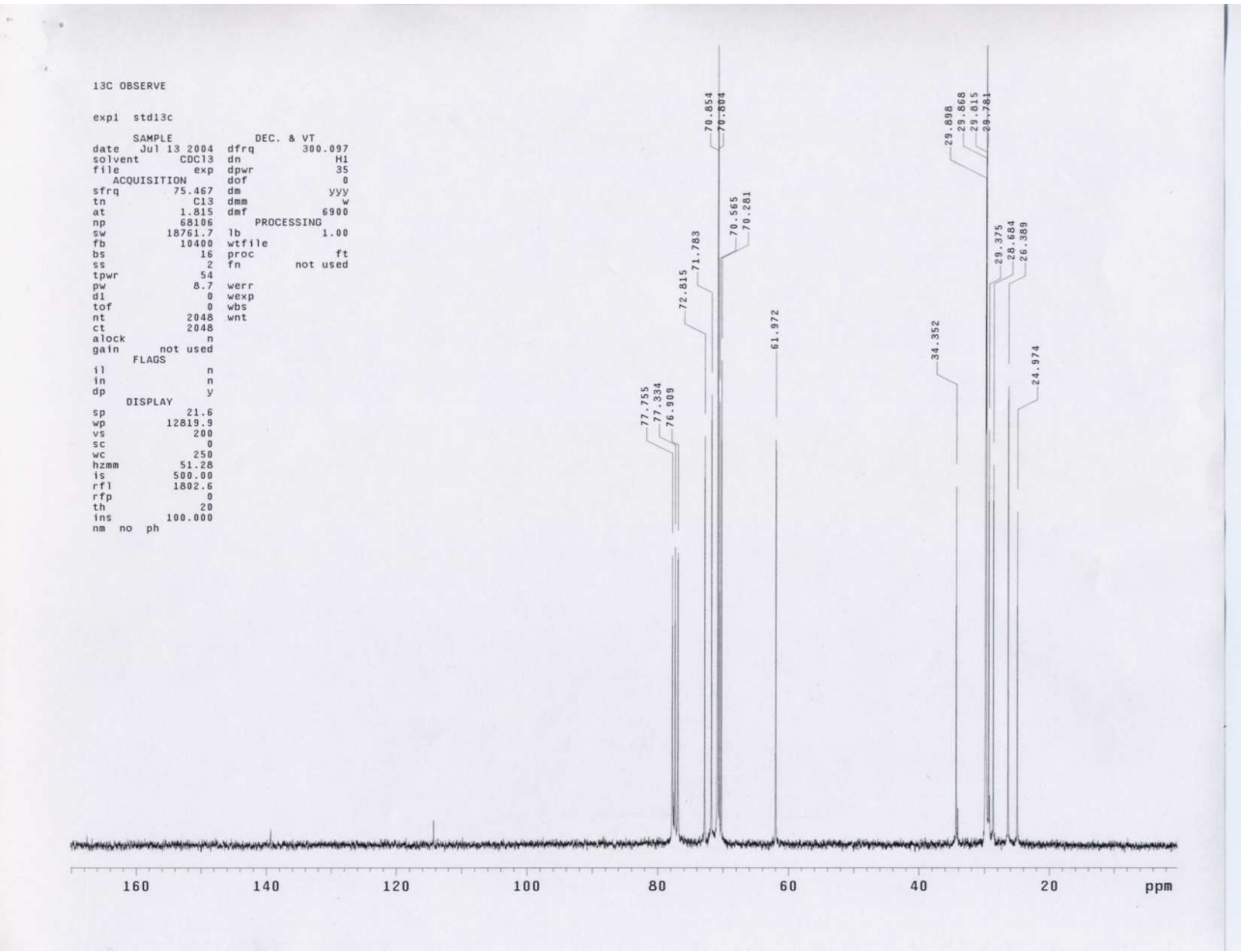


Figure 2.6 ^{13}CNMR spectrum for tetraethylene thiol HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH.

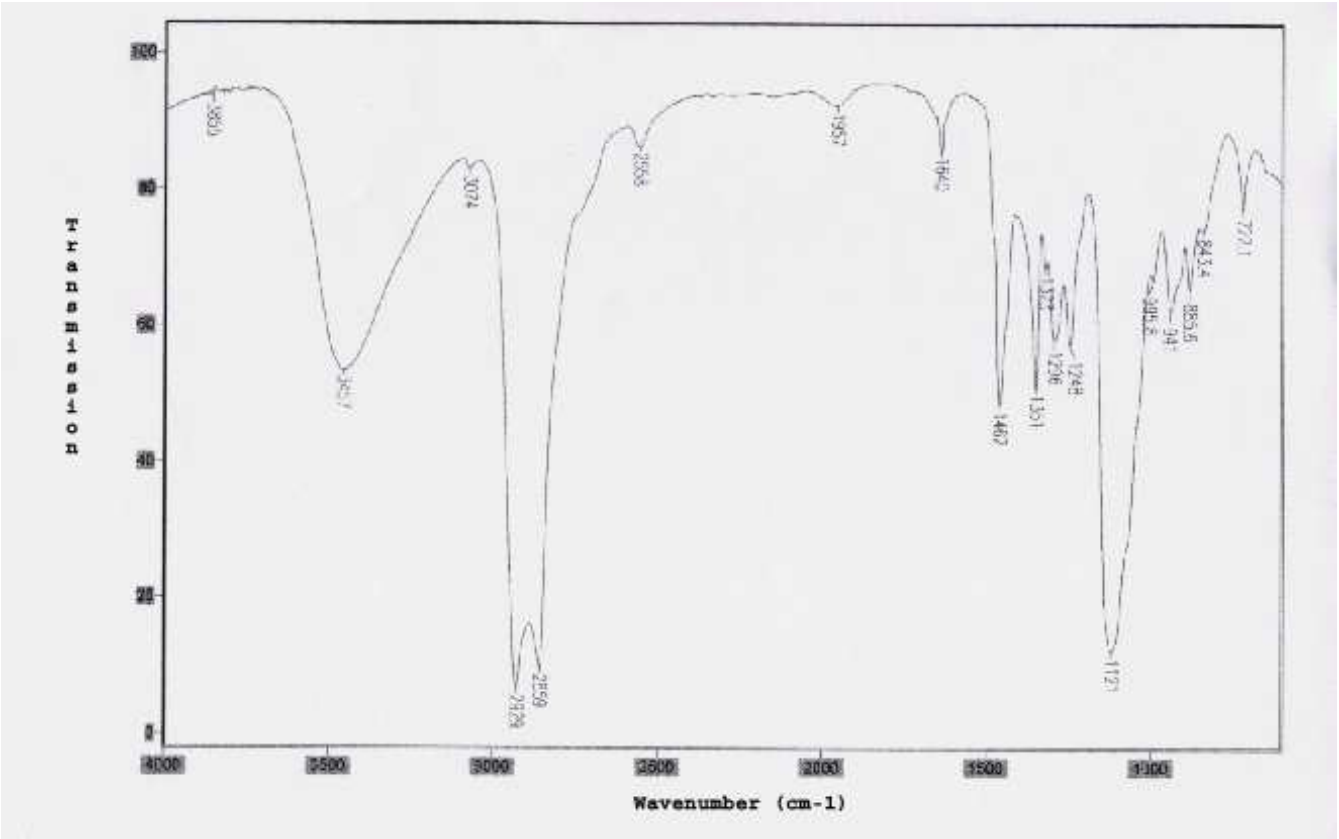


Figure 2.7 Infrared spectrum for tetraethylene thiol, HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH.

2.2.2.3 Synthesis of Tetraethylene thiol with short Alkyl chain

2-(2-{2-[2-(3-Mercapto-propoxy)-ethoxy]-ethoxy}-ethoxy)-ethanol ,thiol HS $(CH_2)_3$ $(OCH_2CH_2)_4$ OH (3) [10]**.** The synthetic scheme for the synthesis of this molecule is similar to figure 2.2. This thiol was synthesized in the same fashion as the other thiols presented. The only change that occurred here was the variation in the alkyl chain (a three carbon is used here and not the 11 carbon previously used). The use of allyl bromide allows the introduction of a shorter carbon chain attached to the PEG. A typical yield for this molecule is usually 49 % of the desired product. ^{1}HNMR and ^{13}CNMR (300MHz spectrum in CDCl3) data are shown in figures 2.9, 2.10 respectively. ^{1}HNMR (300 MHz, CDCl3) δ 1.35 (t , 1H), 1.8 (t 2H), 2.5 (q, 2H), 2.7 (s, 1H), 3.55-3.71 (m, 17H); ^{13}CNMR (75MHz, CDCl3) δ 21.77 (t), 34.03 (t CH_2SH), 62.02 (t, CH_2OH), 69.37(t), 70.48 (t), 70.60 (t), 70.85 (t), 70.80 (t), 77.90 (t) 77.32 (t), 77.74 (t). High Resolution Exact Mass Measurement-FAB confirmed an elemental composition of $C_{11}H_{24}O_5S$, MW=269.14.

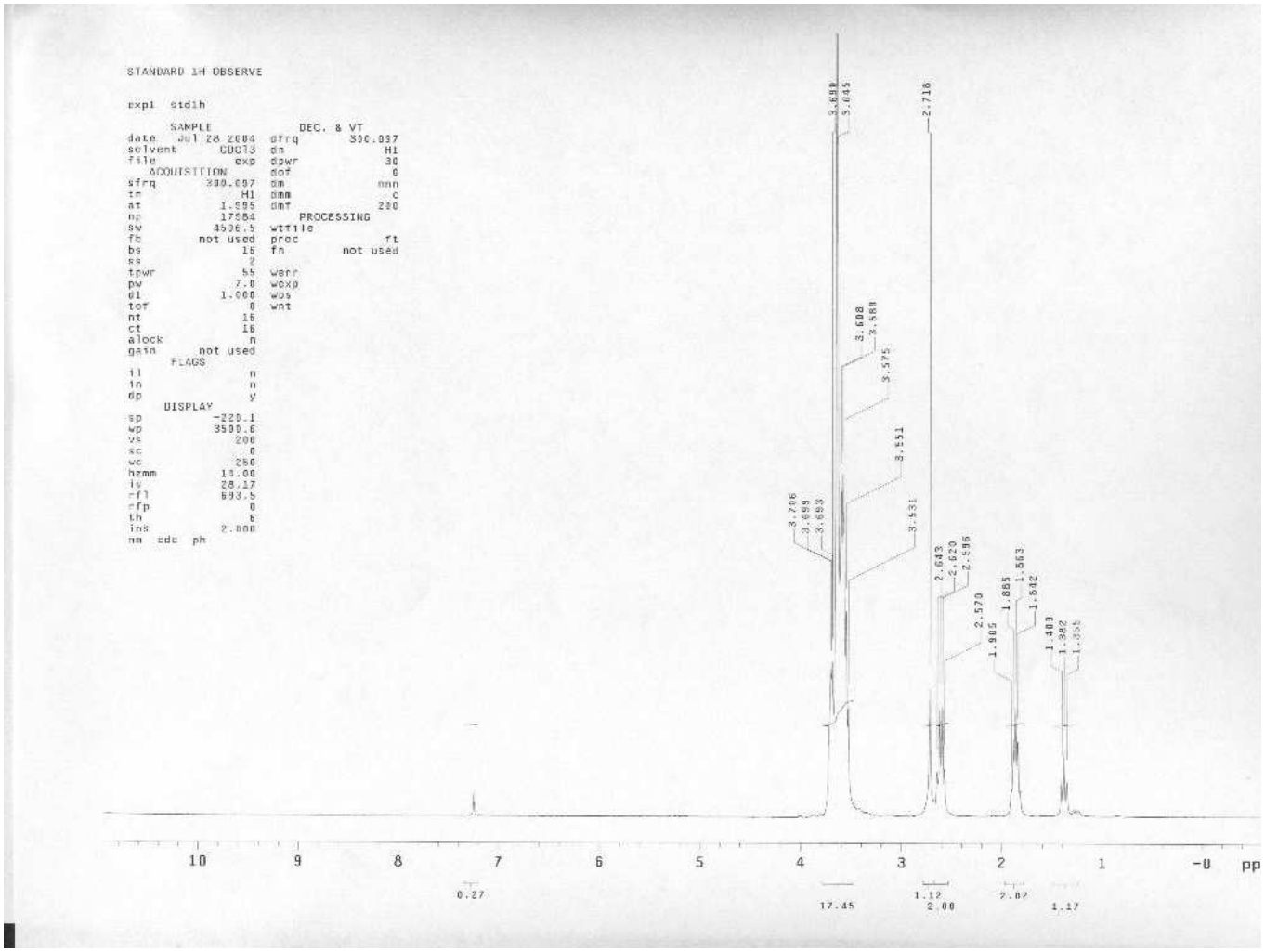


Figure 2.9 ^{1}HNMR spectrum for tetraethylene thiol, HS $(CH_2)_3$ $(OCH_2CH_2)_4$ OH.

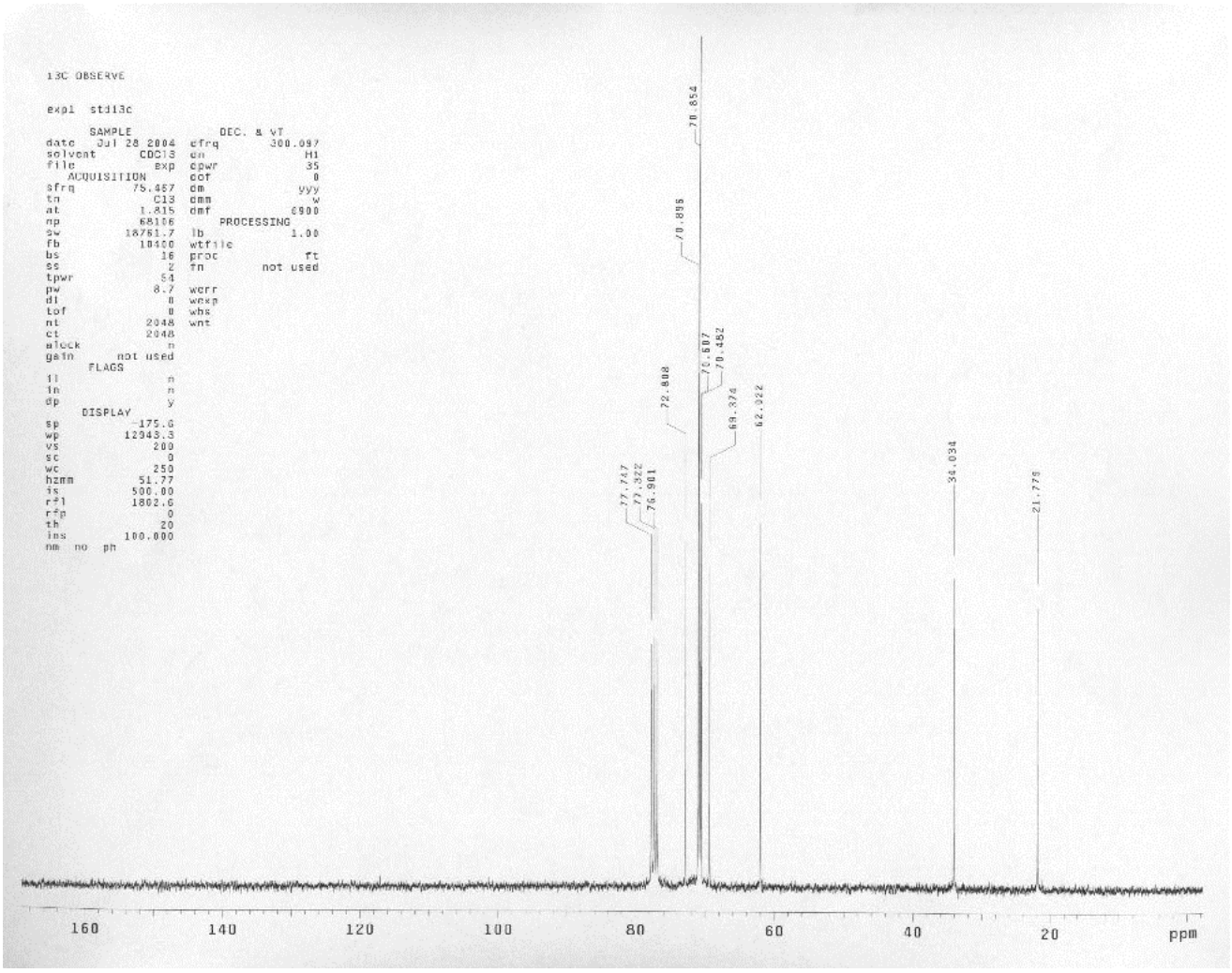


Figure 2.10 ^{13}CNMR spectrum for tetraethylene thiol, HS $(CH_2)_3$ $(OCH_2CH_2)_4$ OH .

2.2.3 Instrumental Setup

Compound identification was confirmed by Proton and Carbon Nuclear Magnetic Resonance (^{13}CNMR, ^{1}HNMR), Infrared Spectrometry (IR), and Mass Spectrometry.

Nuclear Magnetic Resonance. ^{1}H NMR was obtained using Varian Mercury 300 MHZ or 400 MHz Spectrometer.

Infrared Spectrometry. IR data were obtained on a MIDAC M Series IR with an operating system of GRAMS-A1-700 systems. A sodium chloride salt plate was utilized.

Mass Spectrometry. High Resolution Exact Mass Measurement- FAB, of the protonated molecular ion of samples was done by using electric field scanning and fast atom bombardment (FAB) with a JEOL HX110HF mass spectrometer. The mass spectrometry was performed in the chemistry department's mass spectrometry facilities.

2.3 Results and Discussion

The molecules synthesized were chosen because of their known behaviors on gold surfaces. Whitesides reported that long chain thiols adsorb from solution onto flat gold surfaces and form well packed, ordered, and oriented monolayers [11]. Kanaras reported success in stabilizing a gold nanoparticle with HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH [12] . Zheng reports that PEG chains form random coils on a nanoparticle surface Figure 2.11. B [13]. If

long chain PEG is capable of forming random coils on a gold nanoparticle surface, it is believed that this could hinder other ligands being able to attach to the surface. To circumvent that problem Zheng proposed to synthesize densely packed PEG monolayers with well-defined length, Figure 2.11 A. If the spacer is to long then no ligand exchange of arg_9 is expected. The variation in the chain length attached to the PEG on a gold nanoparticle [14] warrants further investigation thus the reasoning behind the variation of the alkyl chain attached to the PEG molecule, and the reasoning why these molecules were synthesized. The desired aim of this research is to design a gold nanoparticle stabilized with PEG, but if the PEG molecule totally covers the entire surface of the gold nanoparticle, there may be little chance of being able to attach other ligands to the surface. Tetraethylene thiol will be abbreviated as, HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH, tetraethylene thiol short chain will be abbreviated as, HS $(CH_2)_3$ $(OCH_2CH_2)_4$ OH, and octaethylene thiol will be abbreviated as, HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH. These abbreviations will follow throughout the remaining portions of this thesis.

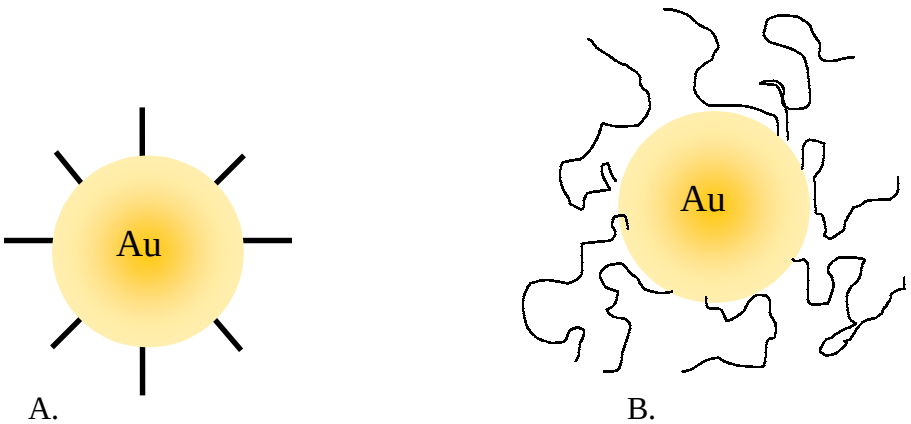


Figure 2.11. Gold nanoparticles coated with (A) a well-defined monolayer of PEG molecules and (B) PEG molecules that can coil around the gold surface.

Characteristic peaks in ^{1}HNMR spectra for tetraethylene glycol were consistent with established literature to determine that the desired products were obtained. Modifications included varying the PEG unit and varying the alkyl chain attached to the PEG, thus the generation of HS $(CH_2)_3$ $(OCH_2CH_2)_4$ OH and HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH molecules. Confirmation of Mass Spectrometry supports the production of this molecule. Examination of IR data shows SH stretching absorption occurring in the 2558 cm^{-1} region of IR spectra. The C-O-C stretch present in PEG shows up at 1121 cm^{-1}. The broad OH region form PEG appears at 3457 cm^{-1} . The CH stretching regions form alkenes appear at 722.1 cm^{-1}. Examination of ^{1}HNMR reveals the characteristic quartet occurring at 2.5 δ for protons on the CH_2SH. The PEG protons occur in the areas of 3.5-3.71 δ. The triplet arising form the proton on HS appears at 1.3 δ. During purification, solvent systems consistently gave retention factor (RF) values consistent with values, which ensures efficient separation of any unreacted material or nonproduct materials [15]. The molecules synthesized in this chapter will be used for the remaining experiments described in chapter 3.

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Chapter 3: Synthesis of Gold Nanoparticles

3.1 Introduction

Gold nanoparticles have been a topic of interest in research for many years. Gold nanoparticles have attracted much attention in part due to their unique optical properties, and excellent compatibility with biomolecules [1]. One of the optical properties of gold nanoparticles is the behavior of their Localized Surface Plasmon Resonance (LSPR) bands [2]. When metal nanoparticles are excited by electromagnetic radiation, they exhibit collective oscillations of their conduction electrons known as Localized Surface Plasmons [15]. The plasmon band reflects the behavior of the gold nanoparticle in relation to the colloids size and stability in strong ionic solutions [3] . This is a valuable tool in studying the behavior of gold in a variety of applications. For example gold nanoparticles in the size range of 20 nm give an absorbance at 525nm.

Gold nanoparticles can be synthesized in a variety of sizes, in the range of 2-100nm [4]. The surface of gold nanoparticles have surface reactivity amenable to a variety of surface reactions such as ligand exchange reactions and other surface modifications, making them excellent colloid materials [5]. Pioneering work from Brust, Schiffrin and coworkers [6,7], Murray [8,9] and Turkevitch [10] have shown the versatility of this colloid in a vast number of scientific studies. Gold nanoparticles can be synthesized in a variety of ways, however the synthetic routes that receive the most attention are one phase, Turkevitch [10] and two phase, Brust and coworkers, [6]. In 1994 Brust introduced the synthesis of thiol derivatised gold nanoparticles in a two-phase liquid-liquid system [6]. With this synthesis gold nanoparticles are created in the range of 1-3 nm. The particle is

protected with an alkenethiol layer thus termed monolayer-protected cluster (MPCs). The particles synthesized in this manner however are not water-soluble. In the synthesis of Turkevitch particles one obtains larger, water soluble particles in the range of 10-100nm. These particles have been cited and used extensively in colloidal science especially biomedical applications [14]. Murray and co-workers adopted the Brust and Schiffrin procedures in an exploration of the synthesis, characterization and functionalization of MPSs as a new class of molecules [11]. With their studies many groups began to explore ways in which to synthesize water-soluble stable gold nanoparticles. In 1995 Brust and coworkers synthesized a class of larger gold nanoparticles in the size of 8 ± 2nm via a modification[12,13] of their standard synthesis. They later passivated the surface of these larger nanoparticles with PEG and were able to design a water-soluble gold nanoparticle. The gold nanoparticles in this research thesis were based upon the synthesis of water-soluble gold nanoparticles [13] .

3.2 Experimental

3.2.1 Materials

All chemicals were used as received from vendors without further purification except if specifically mentioned. Hydrogen tetrachloroaurate ($HAuCl_4$), magnesium sulfate and sodium borohydride were purchased from Sigma Aldrich and used as received. Tetraoctylammonium bromide (ToABr) was purchased from Fulka chemicals. All solvents were purchased from Fisher Scientific and used as received without further purification. Carbon mesh film supported on a copper mesh grid was purchased from Ted Pella. PEG molecules synthesized in chapter two are used to stabilize gold nanoparticles

introduced in this chapter. Arg_9 peptide sequence (CGGWGRRRRRRRRRA), was synthesized at Duke University laboratories. Agarose DNA grade powder and Tris Boric Acid EDTA (TBE) were obtained from Biorad.

3.2.2 Synthesis

Brust-Schiffrin Particles [12]: 0.3091 g $HAuCl_4$ was dissolved in 30 ml deionized water and vigorously mixed. The resulting solution turned a bright yellow color. 2.1912g ToABr was dissolved in 80 ml of toluene, mixed well and was added to the $HAuCl_4$ solution, with vigorous stirring continued. The solution immediately turned to a dark brown color. This solution was allowed to continue stirring for 50 minutes. After 50 minutes 25 ml of a 0.4M solution of $NaBH_4$ was added to the solution slowly via an addition funnel. The resulting solution turned a deep burgundy color. The reaction was allowed to continue vigorous stirring for 2 hours. After 2 hours the solution was placed into a 250-ml seperatory funnel and the clear aqueous layer was separated from the organic burgundy color layer, which contained gold nanoparticles. The toluene phase, which contained the desired particles, were dried over magnesium sulfate. At this stage particles are not water-soluble. A drop of particles was placed on a carbon film supported on a cooper mesh grid, allowed to dry and particles were examined via TEM to establish size, and particle distribution. See Figure 3.1 for Transmission electron microscopy (TEM) of pure brust particles. The concentration of these gold nanoparticles is 3.0 nM.

Tetraethylene and Octaethylene Thiol Particles [13]: 5 mg of thiol HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH, (and or HS $(CH_2)_3$ $(OCH_2CH_2)_4$ OH, HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH.)) was dissolved in 2 ml of 2-propanol. 20 ml of particles was placed in a 50ml round bottom flask and the thiol solution was added to the particles. A slight purple color change was observed as thiol reacted with particles. This solution was allowed to continue gently mixing for 3 hours. After 3 hours the solution was transferred to a 100-ml seperatory funnel and 10 ml of deionized water was added and this solution was vigorously mixed. After mixing two layers were observed a burgundy color aqueous layer and a colorless organic layer. The aqueous layer was transferred to a 100-ml seperatory funnel and this layer was washed three times with diethylether. The particles were then centrifuged at 13,000 rpm and the particles were separated from excess diethylether solution and particles were placed in 20-ml vials. Particles were prepared for TEM analysis as described earlier, for particle size and distribution. The particles were soluble in water, alcohol's, acetone and DMSO. See Figure 3.2 for TEM of octaethylene thiol capped nanoparticles. See Figure3 .3 for UV-Vis of all of the thiol-capped particles.

3.2.3 Ligand Exchange with Arg_9 Peptide

Gold Nanoparticles Particles Capped with a Mixed Monolayer of Octaethylene Thiols and Arg_9 Peptides: Two milliliters of HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH thiol passivated particles were placed in 3 5-ml vials. To each vial a solution of peptides (3.8 mg/ml) was added in the amounts of 50, 150 and 250µl aliquots and this solution was allowed to react for 4 hours. After 4 hours the solutions were centrifuged. The concentrated pellet of particles was washed with deionized water 3 times to remove any

excess or unreacted materials and remaining sample diluted to 500µl. See Figure 3. 4 for a TEM image of HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH/arg_9 particles. See figure 3.6 for IR spectra for pure gold nanoparticles particles, arginine and particles capped with HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH thiol and arg_9. The same experiment was performed for HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH thiol and HS $(CH_2)_3$ $(OCH_2CH_2)_4$ OH thiols.

3.2.4 Stability testing of particles

In order for a gold nanoparticle to be an effective delivery vector for cell delivery experiments, stability must be established. To ensure that particles will be stable, Critical Coagulation Concentrations (CCC) measurements were performed [16]. Critical coagulation concentration testing involves adding a sodium chloride (NaCl) solution to a specified volume of gold nanoparticles until the particles aggregate out of solution [34]. For these testing purposes a 1.7M solution of sodium chloride was added to 500 µl samples of thiol passivated gold particles and mixed monolayers with Arg_9 . The NaCl was added to solution until the plasmon band was no longer detectable. At that point no visible aggregation of particles was observed and the plasmon band did not shift to longer wavelengths (See Figure 3.5 B), thus indicating particles were stable up to a 4.5 ml addition of a 1.7M NaCl solution. The CCC values obtained were reasonable to ensure stability of particles in cell delivery media which has a salt concentration of 150 mM. Particles that were tested are shown in table 2 along with their CCC values. A sample

UV-Vis table is shown in figure 3.5 A for HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH and HS $(CH_2)_3$ $(OCH_2CH_2)_4$ OH particles with sodium chloride (1.7M) additions up to 4.5 ml (1a-2h).

Table 2. CCC data for gold nanoparticle solutions of thiol passivated particles and particles with thiols and Arg_9.

Monolayer on Gold Nanoparticles	**CCC Value (M)**
HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH Au	> 1.5
HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH Au with 50µl Arg_9	> 1.4
HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH Au with 150µl Arg_9	> 1.4
HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH Au with 250µl Arg_9	> 1.4
HS $(CH_2)_3$ $(OCH_2CH_2)_4$ OH Au	> 1.5
HS $(CH_2)_3$ $(OCH_2CH_2)_4$ OH Au with 50µl Arg_9	> 1.4
HS $(CH_2)_3$ $(OCH_2CH_2)_4$ OH Au with 150µl Arg_9	> 1.5
HS $(CH_2)_3$ $(OCH_2CH_2)_4$ OH Au with 250µl Arg_9	> 1.5
HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au	> 1.6
HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au with 50µl Arg_9	> 1.5
HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au with 150µl Arg_9	> 1.6
HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au with 250µl Arg_9	> 1.5

3.2.5 Gold nanoparticle surface studies

In order to establish if PEG is present on the surface of the gold nanoparticles a sample of gold nanoparticles capped with PEG (only) was dissolved in a potassium cyanide (KCN) solution. A 1:3 solution of gold nanoparticle solution and KCN were combined and allowed to mix overnight. After overnight mixing the sample was rotary evaporated and the remaining sample was analyzed via ^{1}HNMR see figure 3.8.

3.2.6 Gel Electrophoresis

Gel electrophoresis was performed to further establish if thiols and peptide are on the gold nanoparticles. If the surface of the gold nanoparticle is covered with PEG and peptide a shift should be observed in the gel [27]. The gel was prepared by dissolving 400mg agarose in 40 ml of TBE. This solution was then heated a standard microwave oven until the solution becomes clear. The solution is removed from the microwave and allowed to come to room temperature. Once gel had come to room temperature the gel is poured into an electrophoresis chamber and allowed to solidify. Once the gel has solidified the gel was loaded with sample mixtures and then the electrodes were attached and the gel was started. The gel was allowed to run for1 hour and 15 minutes. Once the time was over the gel was removed from the chamber and scanned for observation. See Figure 3.9 for gel image.

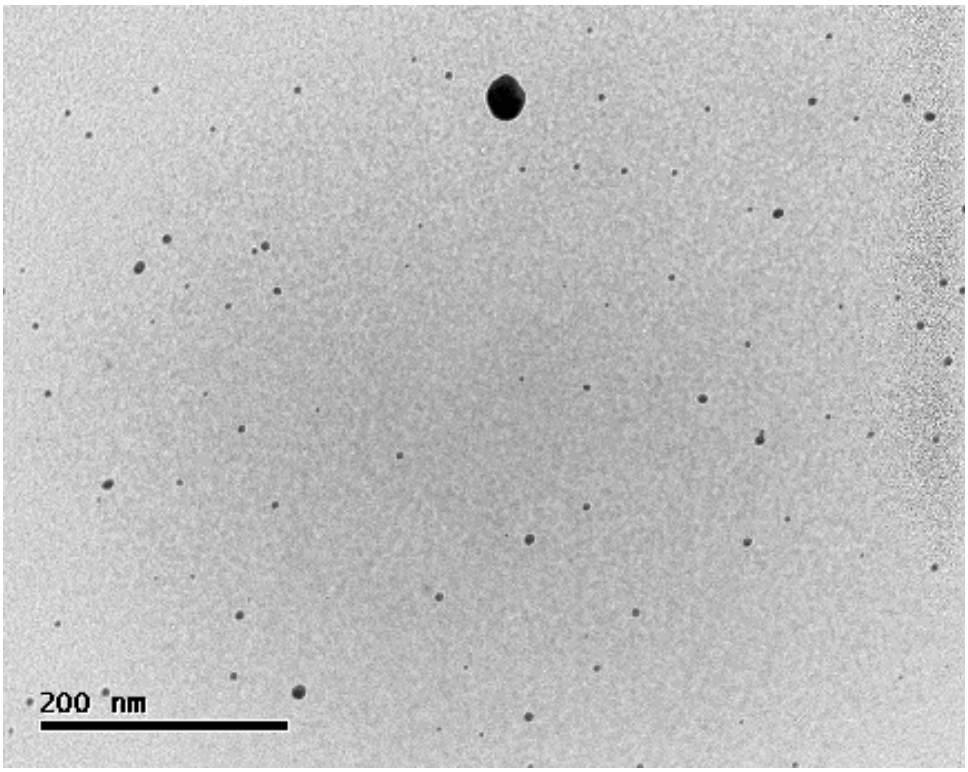


Figure 3.1. TEM image of gold nanoparticles synthesized in section 3.2.2.

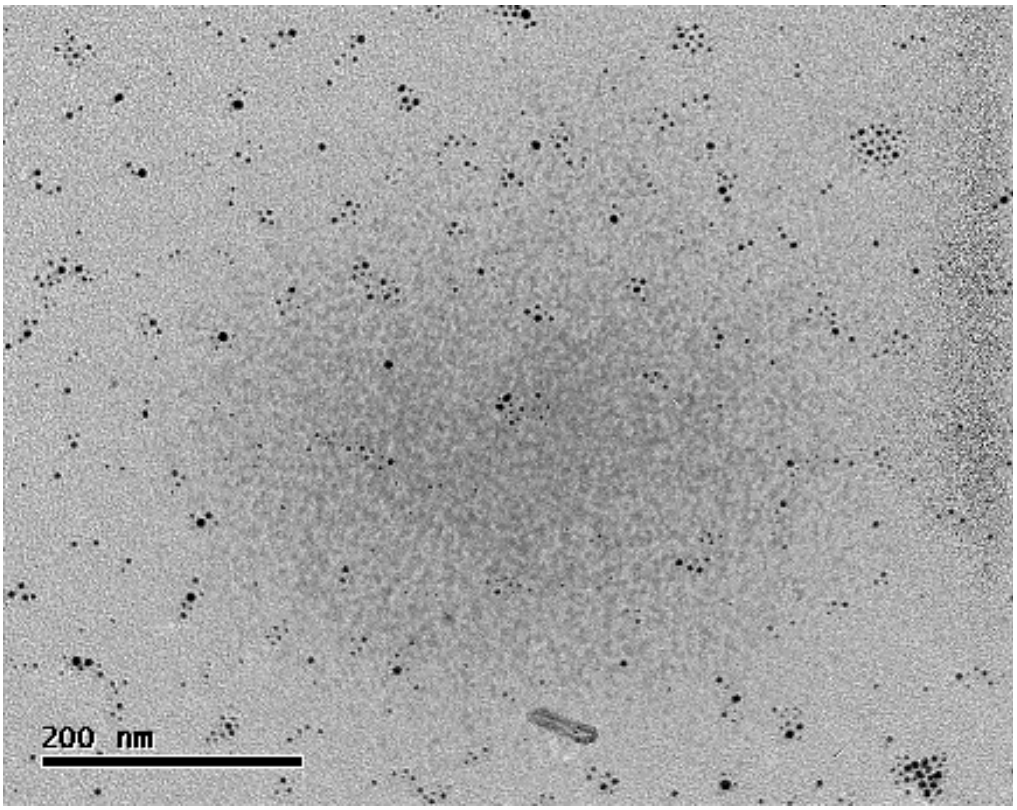


Figure 3.2: TEM image of HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au thiol capped gold nanoparticles synthesized in section 3.2.2.

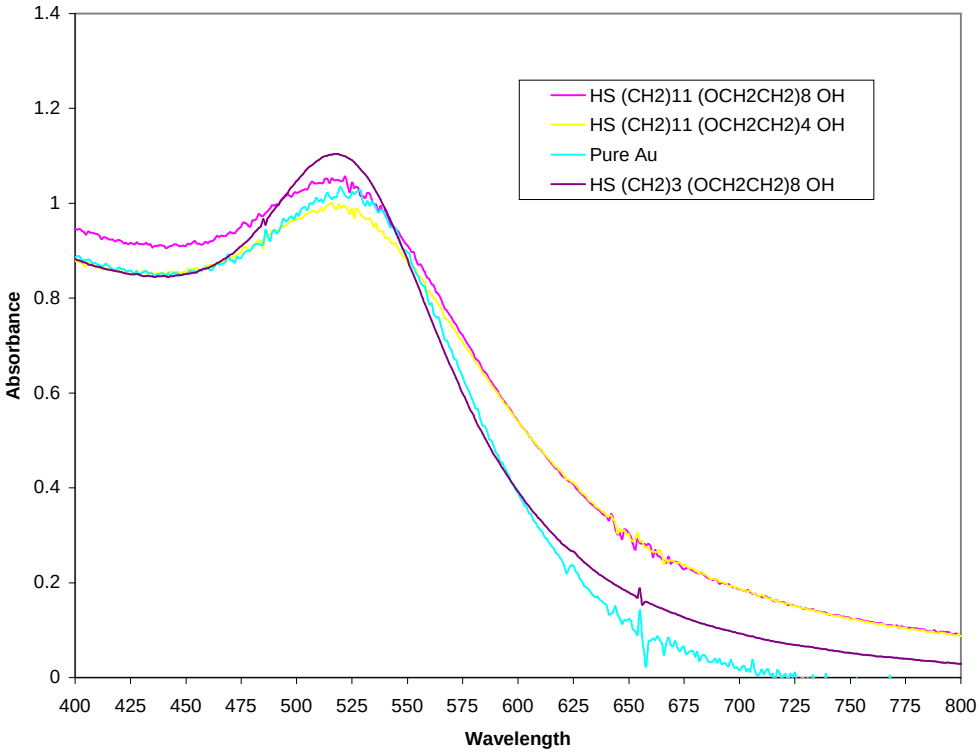


Figure 3.3. Uv-Visible spectra for thiol passivated gold nanoparticles.

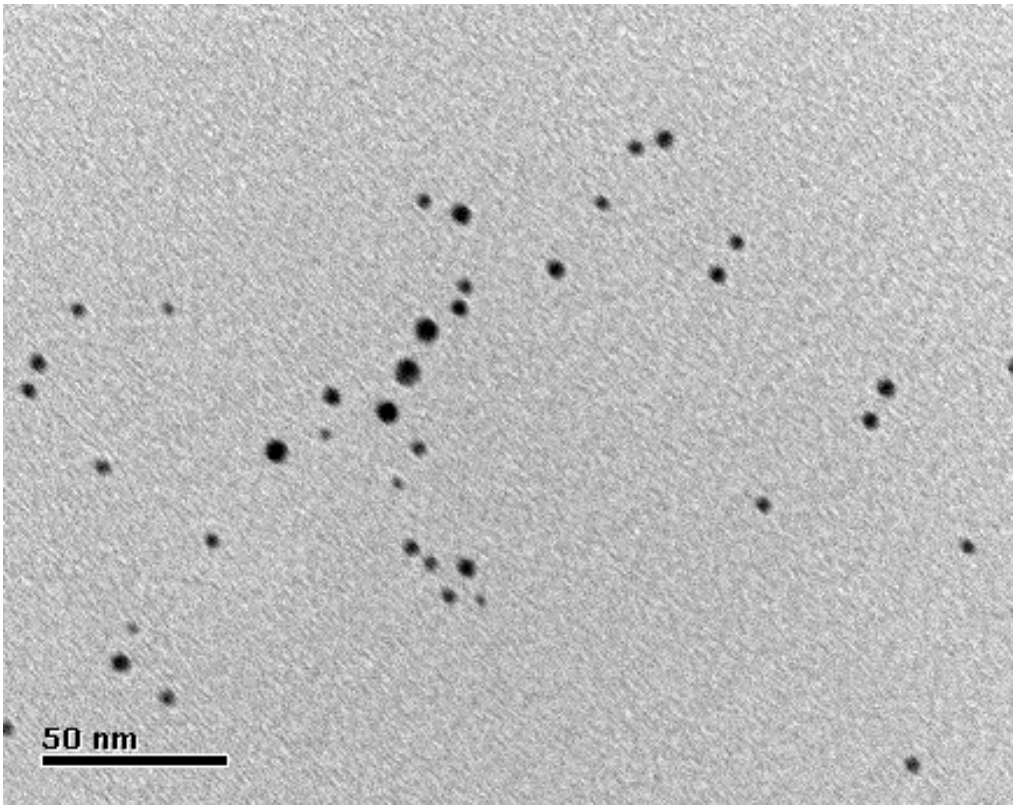
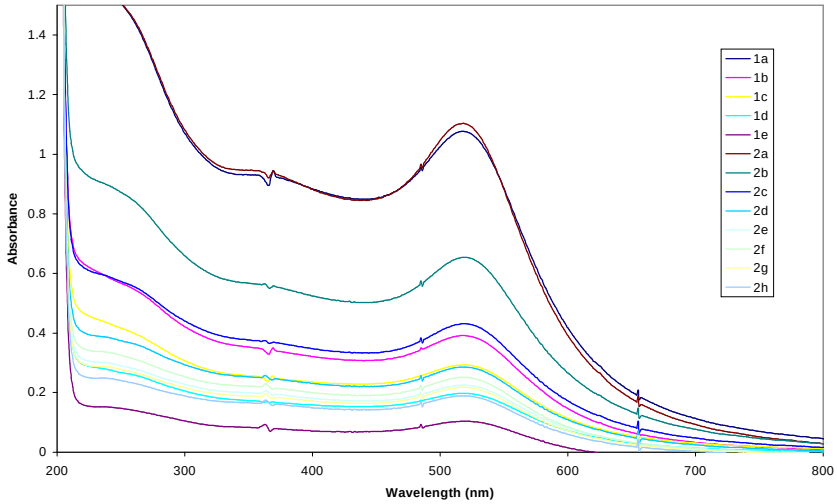
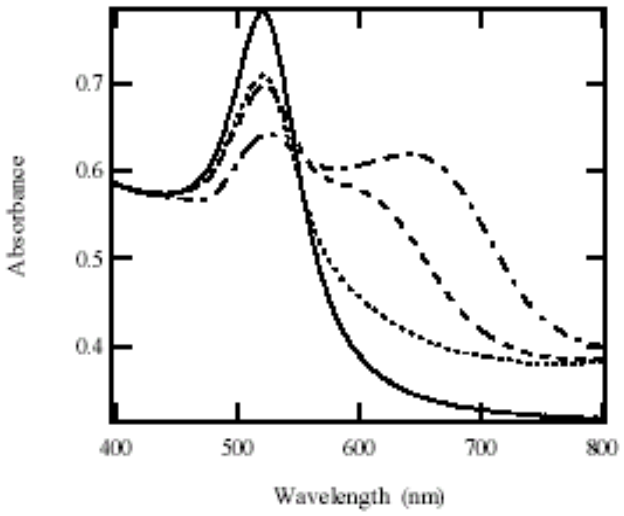


Figure 3.4. TEM image of ligand exchange experiment of thiol passivated gold nanoparticles with $Arg_{9.}$

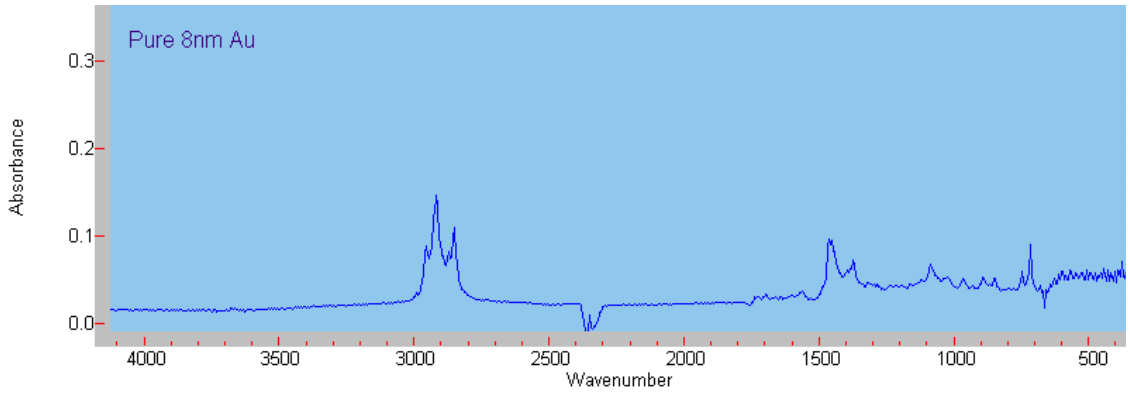


A.

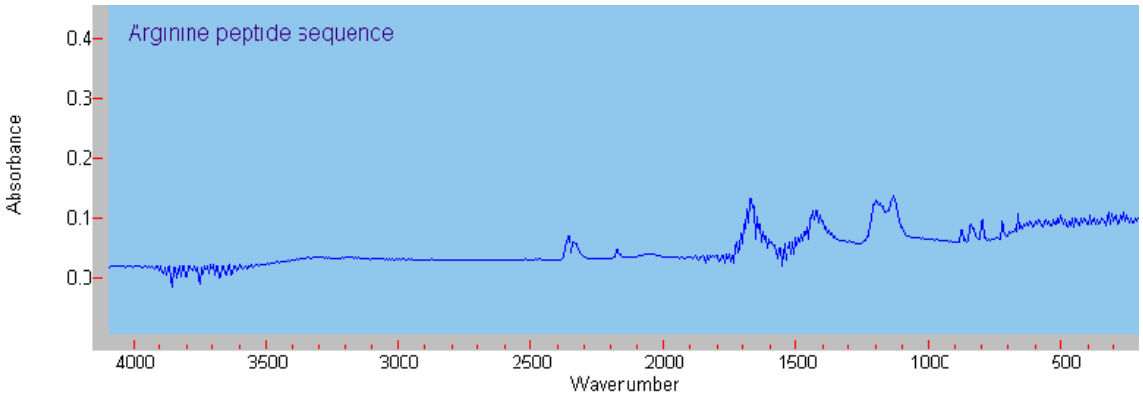
Figure 3.5. A. Uv-Visible spectra of HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH and HS $(CH_2)_3$ $(OCH_2CH_2)_4$ OH molecules. 1a represents a pure sample of HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH. 1b-1e represents the subsequent additions of a 1.7 M NaCl with 1e being the 4.5 ml addition of NaCl. 2a represents a pure sample of HS $(CH_2)_3$ $(OCH_2CH_2)_4$. 2b-2h represents the subsequent additions of a 1.7M NaCl solution, with 2h being the 4.5 ml addition of NaCl. Figure 3.5 B. represents a Uv-Visible spectra of gold nanoparticles that have aggregated as seen by the shift in the plasmon band to longer wavelengths.



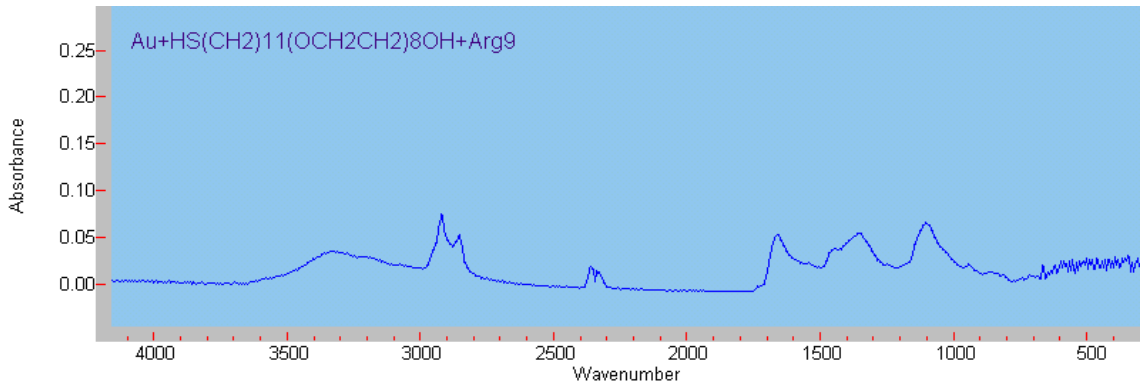
B.



A.



B.



C.

Figure 3.6: Infrared spectra. A) Pure gold nanoparticles. B) Arginine peptide. C) Gold nanoparticles with HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH and Arg_9 peptide.

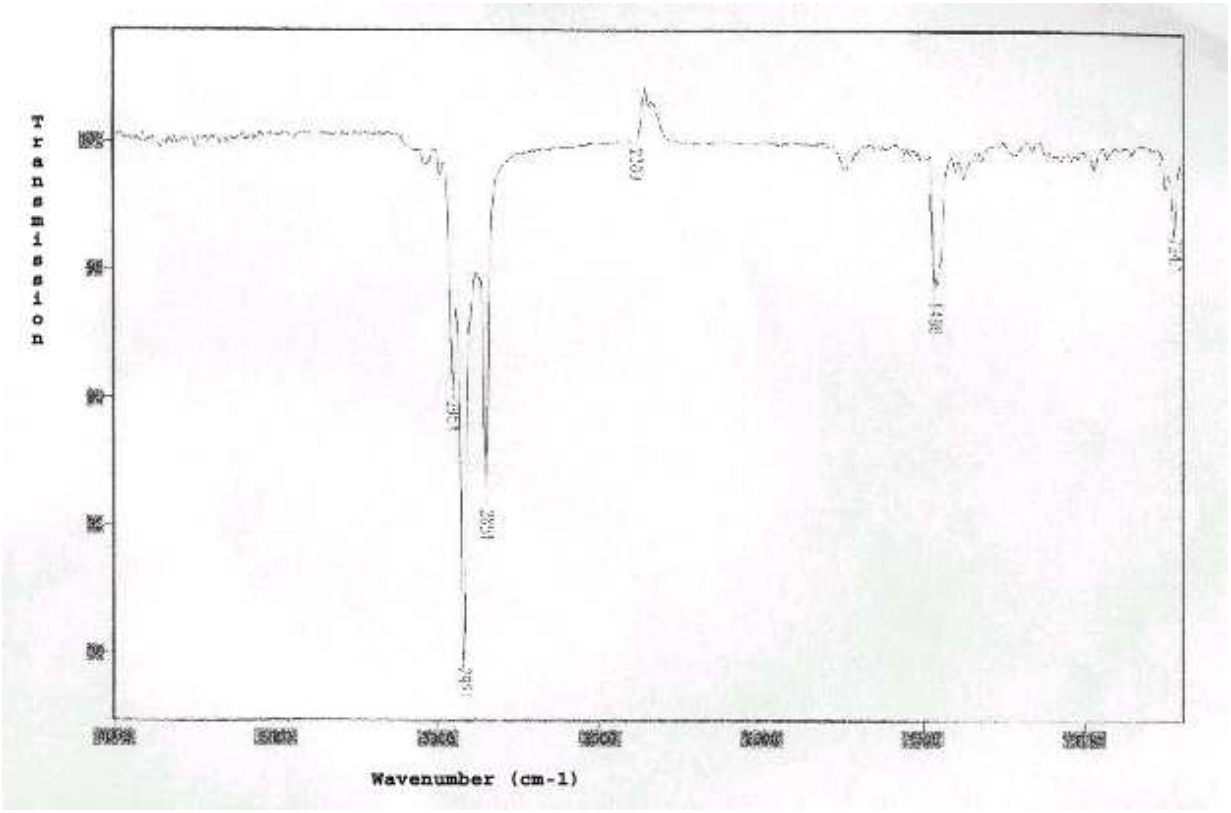


Figure 3.7. Infrared spectrum of a pure sample of Tetraoctylammonium bromide.

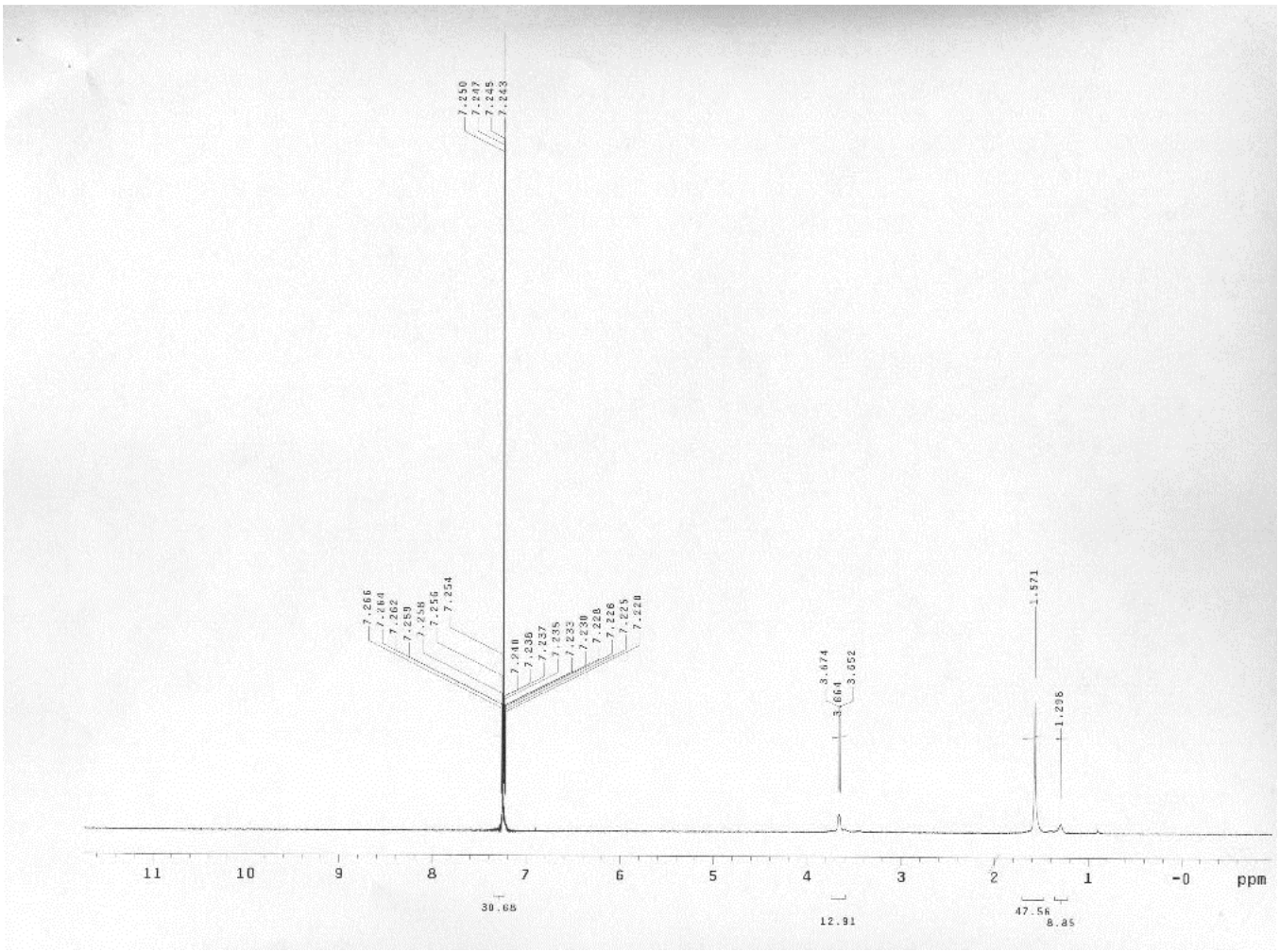
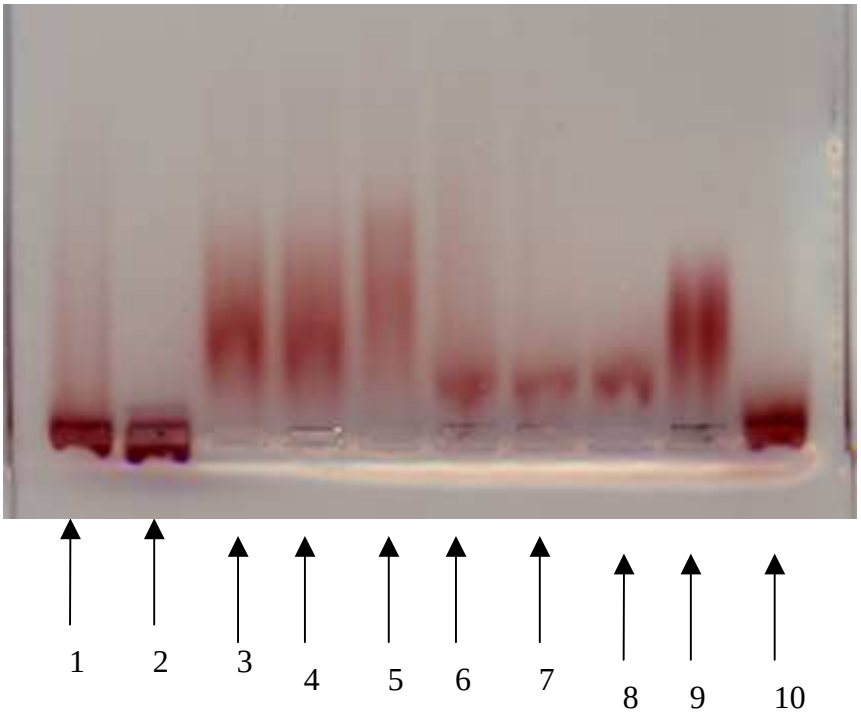


Figure 3.8. [1]HNMR of PEG after oxidatively removing gold nanoparticles.

Figure 3.9. Gel image of gold nanoparticles



Lane	Gold Monolayer	Au:Arg
1	Au-S$(CH_2)_{11}(OCH_2CH_2)_8OH$	1: 0.5
2	Au-S$(CH_2)_{11}(OCH_2CH_2)_8OH$	1: 0
3	Au-S$(CH_2)_3(OCH_2CH_2)_4OH$	1: 0.5
4	Au-S$(CH_2)_3(OCH_2CH_2)_4OH$	1: 1
5	Au-S$(CH_2)_3(OCH_2CH_2)_4OH$	1: 2
6	Au-S$(CH_2)_{11}(OCH_2CH_2)_4OH$	1: 0.5
7	Au-S$(CH_2)_{11}(OCH_2CH_2)_4OH$	1: 1
8	Au-S$(CH_2)_{11}(OCH_2CH_2)_4OH$	1: 2
9	Au-S$(CH_2)_3(OCH_2CH_2)_4OH$	1: 0
10	Au-S$(CH_2)_{11}(OCH_2CH_2)_4OH$	1: 0

Figure 3.9. Gel image of gold nanoparticles. Ten lanes are present and are read from left to right starting at lane1. Lane 1 is HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au plus 150 µl aliquots of $agr_{9.}$ Lane 2 is pure gold nanoparticles passivated with HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH. Lane 3 –5 show gold nanoparticles passivated with HS $(CH_2)_3$ $(OCH_2CH_2)_4$ OH and 50, 150 and 250 µl aliquots of $agr_{9,}$ respectively. Lane 6-8 show gold nanoparticles passivated with HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH and 50, 150 and 250 µl aliquots of arg_9, respectively. Lane 9 shows gold nanoparticles passivated with HS $(CH_2)_3$ $(OCH_2CH_2)_4$ OH, and lane 10 shows gold nanoparticles passivated with HS $(CH_2)_{11}$ $(OCH_2CH_2)_4$ OH.

3.2.7 Instrumental

Transmission Electron Microscopy (TEM). TEM was performed on a Philips CM 12 instrument located at the UNC Dental School lab.

Ultraviolet /Visible Spectroscopy (UV-Vis). UV-Vis was performed on a HP 8453 UV Spectrophotometer.

Infrared Adsorption spectroscopy, (FTIR). FTIR was performed on a FTS 6000 Attached UMA-500 Infrared microscope using a Germanium single pass Attenuated Total Reflectance attachment.

Gel Electrophoresis. Gel electrophoresis was made of 1% agarose. Gel was run at 110mv on a standard gel chamber.

Infrared Spectrometry. IR data were obtained on a MIDAC M Series IR with an operating system of GRAMS-A1-700 systems. A sodium chloride salt plate was utilized.

3.3 Results and Discussion

Examination of synthesized particles via TEM, Figure 3.1, showed that the particle have an average size of 8± 2nm [13]. Once particles were capped with the desired thiol stability tests were performed to establish their usefulness as stable particles for future delivery experiments. Stability has to be established in order to ensure that they will serve as viable delivery vectors for cell delivery experiments. As observed from table 2. all particles synthesized were stable up to at least 1.4M, a CCC value, which is sufficient to show the particles are stable for cell delivery experiments. At this concentration it is not expected that particles will aggregate once introduced to cell culture media (cell culture media has a salt concentration of 150mM). Figure 3.3 shows representative UV-Visible spectra of particles that were capped with thiols from chapter 2. Once particles were stabilized with thiols experiments were performed to see if these particles would undergo ligand exchange reactions and still remain stable. As shown in table 2 the particles remained stable upon the ligand exchange experiment. Only one time point of 4 hours was performed initially to establish if a ligand exchange reaction could occur

and indeed the reaction was confirmed by IR spectra. Also looking at UV-Visible spectra in figure 3.5 the particles remain stable as demonstrated by the plasmon band remaining constant at 525nm with 1.7M solution of sodium chloride up to 4.5 ml generating a CCC value of 1.5M. Once particles underwent ligand exchange reactions TEM analysis were performed to further confirm that particles did not aggregate upon addition of arg_9 peptide to thiol capped particles. As shown from figure 3.4 TEM shows that particles are dispersed and not aggregated. This further confirms stability of HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au /arg_9 capped particles. In a number of cases it has been shown that through careful choice of the passivating ligands and or reaction conditions, one can produce nanoparticles tailored to possess desired properties [17].

FTIR data was obtained on a pure sample of particles, a pure sample of arginine and the final particle construct of gold nanoparticles, PEG thiol, and Arg_9 peptide. Figure 3.6 shows IR spectra on each particle. Utilizing FTIR, functional groups can be identified that may be present on particles. In Figure 3.6 A of the pure gold nanoparticles the characteristic regions of the CH_2 groups are present in the 2850-2900 cm^{-1} regions are from the TOABr present on the particle (see Figure 3.7 for IR of TOABr). The methylene (CH_2) groups appears in the 721 cm^{-1} region are also associated with the presence of TOABr and the methyl groups,(C-H) in the 1400 cm^{-1} regions also arise from the presence of TOABr. These data are expected due to presence of TOABr, which is bound to gold prior to the addition of PEG to the surface. The spectra in figure 3.6 B, represent a pure sample of

arginine. The amide band appears in the 1655-1580 cm^{-1} region. The carboxylate anion (COO^-) appears in the 1400 cm^{-1} regions. The IR spectra in 3.6 C, represents gold nanoparticles with PEG and arg_9. The broad OH band in the 3200-3600 cm^{-1} regions are indicative of the presence of the OH group from the octaethylene thiol PEG. The C-O-C ether absorbs strongly in the 1150-1085cm^{-1} regions. The ether results from the ether regions within the PEG. The amide band appears in the 1655-1580 cm^{-1} region [18] . The CH_2 groups arising from the PEG appears in the 2850-2900 cm^{-1} regions. This confirms the presence of Arg_9 peptide and PEG on the gold nanoparticle.

To further study the surface properties of PEG passivated gold nanoparticles surfface^{1}HNMR studies were performed to evaluate if PEG is still present once gold is dissolved. Once the gold is dissolved PEG should still remain. The ^{1}HNMR shown in figure 3.8 shows weak signals arising from the PEG once present on the surface (see Figure 2.4 for 1HNMR of HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH PEG). A stronger signal was expected however this may be due to the proportions of gold nanoparticles used with KCN. Very weak PEG signals were observed in the 1.2 δ area which are signals arising from the protons present in the alkyl chain attached to the PEG. Protons attached to the actual PEG (OCH_2CH_2) are showing up weakly in the 3.55-3.71 δ regions. Protons associated with the CH2 next to the OCH_2CH_2 PEG units appear in the 1.5 δ regions of the spectra. The data support that PEG is still present and has not been compromised once introduced to the surface of the gold nanoparticle.

Gel electrophoresis was also performed to determine if a visible shift would be observed if a positive species (arginine) were present on the surface of a gold nanoparticle. Once a gold nanoparticle surface is passivated with HS $(CH_2)_{11}$ $(OCH_2CH_2)OH_8$ thiol/Arg_9 the surface should contain the positive charge generated by the presence of the positively charged Arg_9 peptide sequence. Figure 3.9 shows such a gel. As shown in the gel a noticeable shift is observed in lanes which have peptide attached and it is clear that a shift corresponds to the amount of peptide that underwent ligand exchange reactions. The gel shift is also moving in the predicted direction towards the negative electrode. Lanes 2 and 10 show little if any movement occurring, indicating that PEG has covered the gold nanoparticle surface and as predicted in literature should create a neutral surface [27] . Lane 9 shows movement which is not anticipated and this is due to the particles aggregating during the running of the gel.

All of the above data present preliminary evidence of the design of a suitable gold nanoparticle delivery vector for cellular delivery experiments. Synthetic protocols were developed for making mixed monolayers of PEG's and peptides on a brust type water-soluble gold nanoparticle. With this design chapter 4 will show that an HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au/Arg_9 delivery vector will enable a gold nanoparticle to cross the cell membrane and reach the cytosol region of a HeLa cell.

3.4 References

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Chapter 4: Delivery of Newly Designed Gold Nanoparticles to HeLa Cells

4.1 Introduction

Nanoparticles are being investigated by many research groups for cellular delivery applications . Much of the research has involved looking into long circulating particles [1]. The goal of drug delivery is to provide therapeutic levels of medications to the target region. Nanoparticle use can be used to address many of these drug delivery needs. Nanoparticles are one of the only colloids that can be injected intravenously [2]. If particles are unable to escape uptake by the reticuloendoethelial system then the particles will simply be cleared out of the body without even reaching its desired location. Colloid gold nanoparticles represent a completely novel technology in the field of delivery. The use of a colloid surface can provide an excellent platform for imparting multifuctionality to a particle. Multifuctionality can include attaching several molecules to the particle, i.e. a NLS, DNA or other variations. The literature to date has not presented a formulation of the use of brust type (two-phase) water-soluble gold nanoparticles for cellular delivery. This chapter will address that proposed application in relation to the nanoparticle presented in chapter 3 of this thesis.

4.2 Experimental

4.2.1 Materials

All chemicals were used as received from vendors without further purification except if specifically mentioned. Minimum Essential Medium Eagle (EMEM), Dulbecco's phosphate buffered saline, (DPBS), T75 cell culture flasks, Trypsin, glass slides and cell culture well plates were purchased from Fisher Scientific. Original HeLa cell line was obtained from American Type Culture Collection (ATCC) and subsequent cell lines were maintained in our cell lab for our research purposes. Osmium Tetraoxide (OsO_4) was purchased from Electron Microscopy Sciences. LR White Resin, hard grade Acrylic Resin, was purchased from London Resin Company. Concentrated HNO_3 and HCl and Disposable plastic base molds embedding trays were purchased from Fisher Scientific.

SPECIAL NOTE: Cell culture is performed under strict sterile conditions to ensure that infections or harm to cells does not occur. See Appendix for explanation of typical sterile techniques.

All Hela cells used in this delivery experiment were initially grown to 80 % confluency in 3 T75 flasks in an incubator maintained at 37° C and 5% CO_2. Cells were fed and split as needed. Cells were cultured in EMEM media. EMEM media was supplemented with 10% FBS, 2-ml antibiotics, 2 ml L-Glutamine per 500-ml bottle.

Preparation of HeLa Cells: Ten 12 well cell culture dishes were prepared as follows: Well plates were sprayed down with 70% ethanol (while still in packing) and placed into a laminar flow hood, with UV light. Outer wrapper was removed, glass cover slides were dipped in alcohol, flamed and placed into well plates. After coverslips were put into well plates, the contents of 3 T75's with HeLa cells were split into well plates as follows: Each T75 was removed from incubator, and placed into a sterile hood. Media was removed from flask and the cells attached were rinsed with 10 ml of DPBS. DPBS was removed and 1 ml of trypsin was placed in flask and placed back into incubator for 5 minutes. After 5 minutes flask was removed from incubator and 5 ml media were added to flask and this mixture was mixed via a pipette several times to create a concentrated cell suspension. To this cell suspension 54 ml of EMEM media was added and flask was gently mixed. From this mixture 3-12 well plates had 1.5 ml of this suspension delivered to them and plate was gently swirled around to ensure an even distribution of cells. When all well plates were filled they were labeled and placed in incubator. This procedure was repeated until all 10 well plates were seeded with cells. The well plates with HeLa cells were allowed to grow 24 hours at 37°C and 5% CO2.

Delivery of Nanoparticles: After 24 hours cells were checked for confluency. All 10 well plates were at 85-90% confluency, which corresponds to approximately 5.0 X 10^5 cells, and all cells appeared healthy. Conjugates of particles were prepared as follows: Octaethylene thiol/Arg_9/Au construct was prepared as described in chapter 3 and are used for this delivery experiment.

Each well plate was treated in the following manner:

Media was removed from well plate and each well was rinsed three times with DPBS. DPBS was removed and replaced with fresh media. Then to each well plate 50, 100 and 150µl of construct was delivered for experimental delivery times of 30min. 1 hour and 3 hours. Once constructs were added the plate was gently swirled to ensure even distribution and well plates were placed back into incubator. After the appropriate delivery times media was removed and wells were gently rinsed three times with DPBS (at this point well plates were checked to ensure that there were still cells on cover slip and minimum cell loss occurred). After rinsing DPBS was replaced with 1 ml of fixative formula (except wells for Inductively Coupled Plasma, (ICP) analysis). The fixative was allowed to stay on cells for 15 minutes, after 15 minutes fixative was removed and replaced with DPBS. Wells indicated for TEM experiments were placed in refrigerator for later preparation.

ICP preparation: Coverslips with cells were removed from wells and rinsed with deionized water and allowed to dry cell side up. Once coverslips were dry they were placed in new well plates (ICP experiments were set up in duplicate so there are two coverslips of cells per delivery) as follows: one coverslip with cell side facing up was placed into well plate then the duplicate slide was placed on top of that coverslip with cell side facing down. This allowed a coverslip "sandwich" of cells for ICP data collection. Once coverslips were in place 500 µl of aqua regia solution (3:1 HCL, HNO_3) was placed into each well. Plate was swirled every 30 minutes for 2 hours. After 2 hours 400µl of sample was placed into 3.5-ml deionized water (in 15-ml centrifuge tubes). This was

done for each sample. The labeled tubes were then taken over to Gardner hall, soil science lab, for ICP data collection.

TEM Preparation: Well plates for TEM were removed from refrigerator and allowed to come to room temperature. DPBS was removed from wells rinsed with fresh DPBS and replaced with 1% OsO_4 for one hour. Cells were then rinsed with DPBS three times and cells were then dehydrated with ethanol at 50%, 70%, 90% and 100% as follows: DPBS was removed and replaced with 1 ml of 50% ethanol for 15 minutes. 50% ethanol was removed and replaced with 1 ml 70%ethanol for 15 minutes and this process continued until cells were totally dehydrated. After the 100% ethanol dehydration ethanol solution was removed and replaced with 1.5 ml of LR white embedding resin for 30 minutes. The 30-minute exposure to LR white resin continued for three cycles with the last cycle occurring under vacuum. After vacuum the cover slips were removed form well plates and placed on embedding trays. The coverslips on embedding trays were allowed to cure under UV-light overnight. The samples were then taken to UNC for preparation for TEM analysis.

4.2.2 Instrumental

Transmission Emission Microscopy: TEM was performed on a Philips CM 12 instrument located at the UNC Dental school lab.

Inductively coupled argon plasma, ICP: ICP analysis were performed by Dr. Wayne Robarge in the soil science department.

4.3 Results and discussion

The delivery experiment outlined in section 4.2 leads to the ability to view gold nanoparticles via TEM. TEM analysis allows visual examination of conjugates to see if they delivered into a HeLa cell. By combining all of the experiments presented in this thesis a successful cell delivery experiment was performed. The aim of this project was to design a gold nanoparticle vector that will posses the following:

1) Stability in high salt concentrations.
2) Able to cross a cellular membrane.
3) Deliver a water-soluble gold nanoparticle to the inside of a HeLa cell.

ICP data lead to the selection of the cells selected for TEM analysis. Since these particles are so small, microscopy will not allow visual observation of gold nanoparticles in our cells so this is why ICP analysis is used.

Inductively Coupled Plasma (ICP), is an analytical technique used for the detection of trace metals in samples. ICP allows a quantitative determination of the number of gold nanoparticles present in a sample. By observing that number one can determine if gold is

present inside of a cell. For cell delivery projects this approach is used. Once the cell is dissolved during ICP preparation, all that should be left behind are the gold nanoparticles, which can then be measured. The data shown in Figure 4.1 represent such data. By looking at the sample verses a control conclusive results that HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au/arg_9 nanoparticles entered into a HeLa cell are shown. As seen in Figure 4.1 it appears as though there was some delivery of gold nanoparticles. Samples which had 150µl of constructs made with 2ml HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au+150µl arg_9 (sample 11), and 100 µl of construct made with 2ml HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au+50µl arg_9 (sample 2) for one hour seem to give promising results. It appears that all of the cells had some sort of delivery occurring. All of the controls had fewer particles than the actual particles with peptide attached.

TEM analysis of sample number 11 appear in Figure 4.2 and 4.3. Looking at Figure 4.2 a particle can be seen in the nuclear region of a HeLa cell. Figure 4.3 shows three particles in the cytosol region of a HeLa cell. TEM shows promising delivery of the gold nanopaticles. The amount of gold nanoparticles delivered were not as high as hoped for. There are many possibilities for a low delivery. One possibility is that particles that may be stuck to the outside of the cell and are possibly getting counted, thereby giving such a large ICP value. This problem will have to be addressed more thoroughly in future experiments. More TEM analysis are pending of this delivery experiment.

TEM images obtained at UNC show preliminary data to support the aim of this project.

The hypothesis presented in chapter1 have been investigated and addressed:

Hypothesis tested:

1) Can gold nanoparticles passivated with PEG undergo ligand exchange reactions with arg_9 peptide?
2) Will gold nanoparticles containing mixed monolayers of PEG and arg_9 be able to cross a cellular membrane, escape the endosomal pathway and deliver the newly designed gold nanoparticles to the internal regions of a HeLa cell?

Presented in this thesis is preliminary data of the delivery of a brust type gold nanoparticle stabilized with HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au thiol/arg_9, into the cytosol and nuclear regions of HeLa cells. It is believed that this sort of delivery vector could be used in future drug delivery applications.

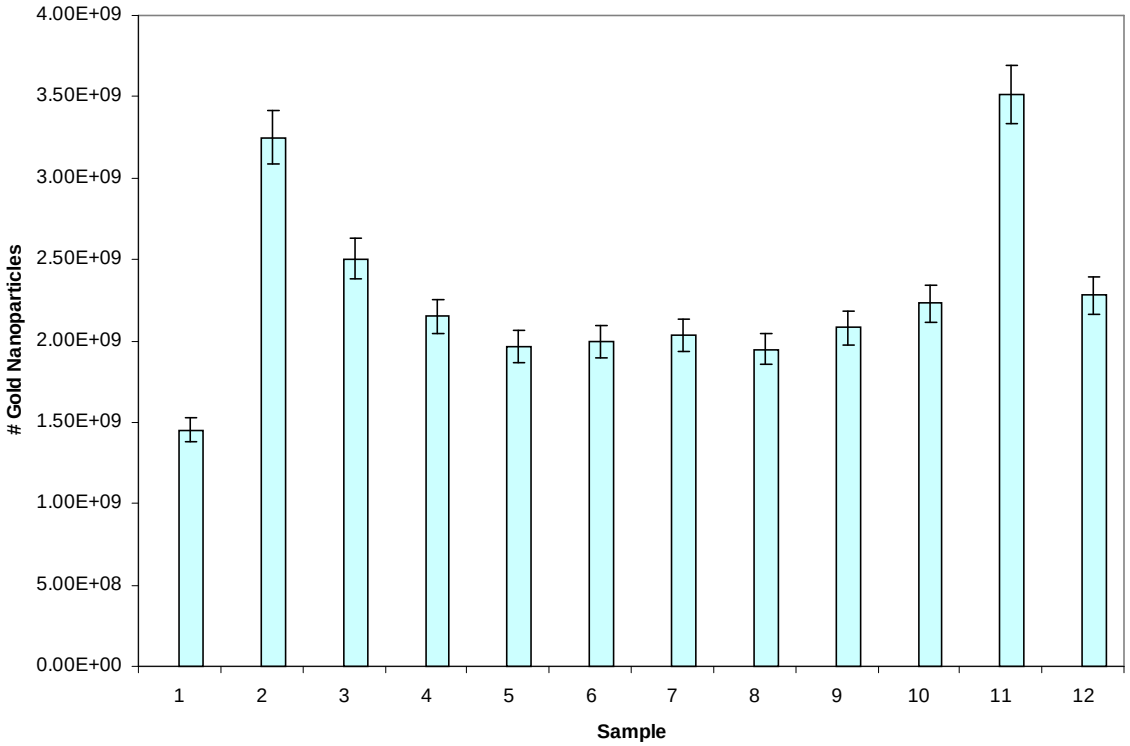
4.4 Future Work

The foundation has been laid for what is believed to be a useful gold nanoparticle drug delivery system. The following experiments are desired to fully study and optimize this system:

1) Thermogravimetric analysis (TGA).
2) X-ray photon spectroscopy (XPS) studies.
3) Experimentation into the design of larger particles. This will allow VEDIC microscopy to be used to see gold nanoparticles.
4) Conjugations of other peptide sequences to particles.

5) Variation in the alkyl chain to PEG to study if delivery of gold nanoparticles will be enhanced in any way.
6) Possible delivery of a multifunctional brust particle, to HepG2 and Breast cancer cells.

Thermogravimetric analysis will allow the determination of the organic composition of monolayer protected gold nanoparticles [17]. X-ray photon spectroscopy is a surface analysis technique, which will allow a more through study of the surface composition of the mixed monolayer gold nanoparticle. Quantitative analysis of the surface will allow the study of the elemental surface composition of this gold nanoparticle [18]. By conjugating other peptide sequences to this gold nanoparticle could delivery be enhanced in any way. By shortening the alkyl chain attached to the PEG in the HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH molecule, a better surface may be created which may allow more peptides to attach to the gold nanoparticle. If one could deliver these gold nanoparticles to other cell lines the delivery activity of these particles could be better understood. For example will delivery be more effective in breast cancer cells or hepatic cancer cells. These are just a few of the future projects that are hoped for in order to more fully understand the behavior of these gold nanoparticles.

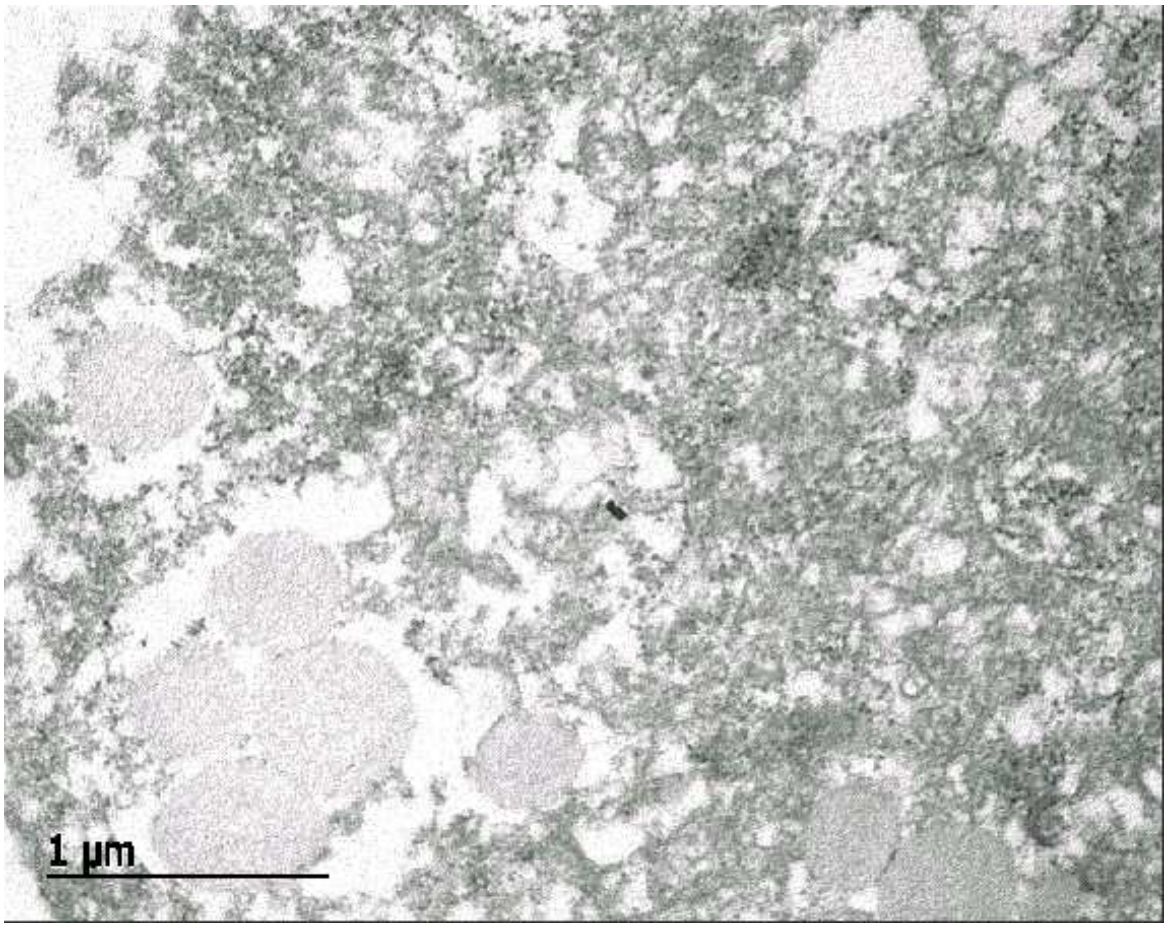


Sample	Gold Monolayer	Au/Arg Ratio (nM)	Time
1	Au-S$(CH_2)_{11}(OCH_2CH_2)_8OH$	1:0	1hr
2	Au-S$(CH_2)_{11}(OCH_2CH_2)_8OH$	1:45	1hr
3	Au-S$(CH_2)_{11}(OCH_2CH_2)_8OH$	1:113	1hr
4	Au-S$(CH_2)_{11}(OCH_2CH_2)_8OH$	1:163	1hr
5	Au-S$(CH_2)_{11}(OCH_2CH_2)_8OH$	1:0	0.5hr
6	Au-S$(CH_2)_{11}(OCH_2CH_2)_8OH$	1:45	0.5hr
7	Au-S$(CH_2)_{11}(OCH_2CH_2)_8OH$	1:113	0.5hr
8	Au-S$(CH_2)_{11}(OCH_2CH_2)_8OH$	1:163	0.5hr
9	Au-S$(CH_2)_{11}(OCH_2CH_2)_8OH$	1:0	1hr
10	Au-S$(CH_2)_{11}(OCH_2CH_2)_8OH$	1:45	1hr
11	Au-S$(CH_2)_{11}(OCH_2CH_2)_8OH$	1:113	1hr
12	Au-S$(CH_2)_{11}(OCH_2CH_2)_8OH$	1:163	1hr

Figure 4.1. ICP data for HeLa cell delivery of gold nanoparticles. Sample 1 is a one-hour control which has only100 µl gold passivated with HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH delivered to HeLa cells. Sample 2 is 1 hr delivery of 100µl Au construct (2ml HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au+50µl arg_9). Sample 3 is 1 hr delivery of 100µl Au construct (2ml

HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au+150µl arg_9). Sample 4 is 1 hr delivery of 100µl Au construct (2ml HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au+ 250 µl arg_9). Sample 5 is a 30-minute control which has only 50µl gold passivated with HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH delivered to HeLa cells. Sample 6 is 30-minute delivery of 50µl Au construct (2ml HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au+50µl arg_9). Sample 7 is 30-minute delivery of 50µl Au construct (2ml HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au+150µl arg_9). Sample 8 is 30-minute delivery of 50µl Au construct (2ml HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au+ 250 µl arg_9). Sample 9 is a 1hr control which has only 150µl gold passivated with HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH delivered to HeLa cells. Sample 10 is 1hr delivery of 150µl Au construct (2ml HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au+50µl arg_9). Sample 11 is 1hr delivery of 150µl Au construct (2ml HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au+150µl arg_9). Sample 12 is 1 hr delivery of 150µl Au construct (2ml HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au+ 250 µl arg_9).

Figure 4.2: Gold particle constructs of HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au thiol/arg_9 conjugate inside the cytosol region of a HeLa cell.



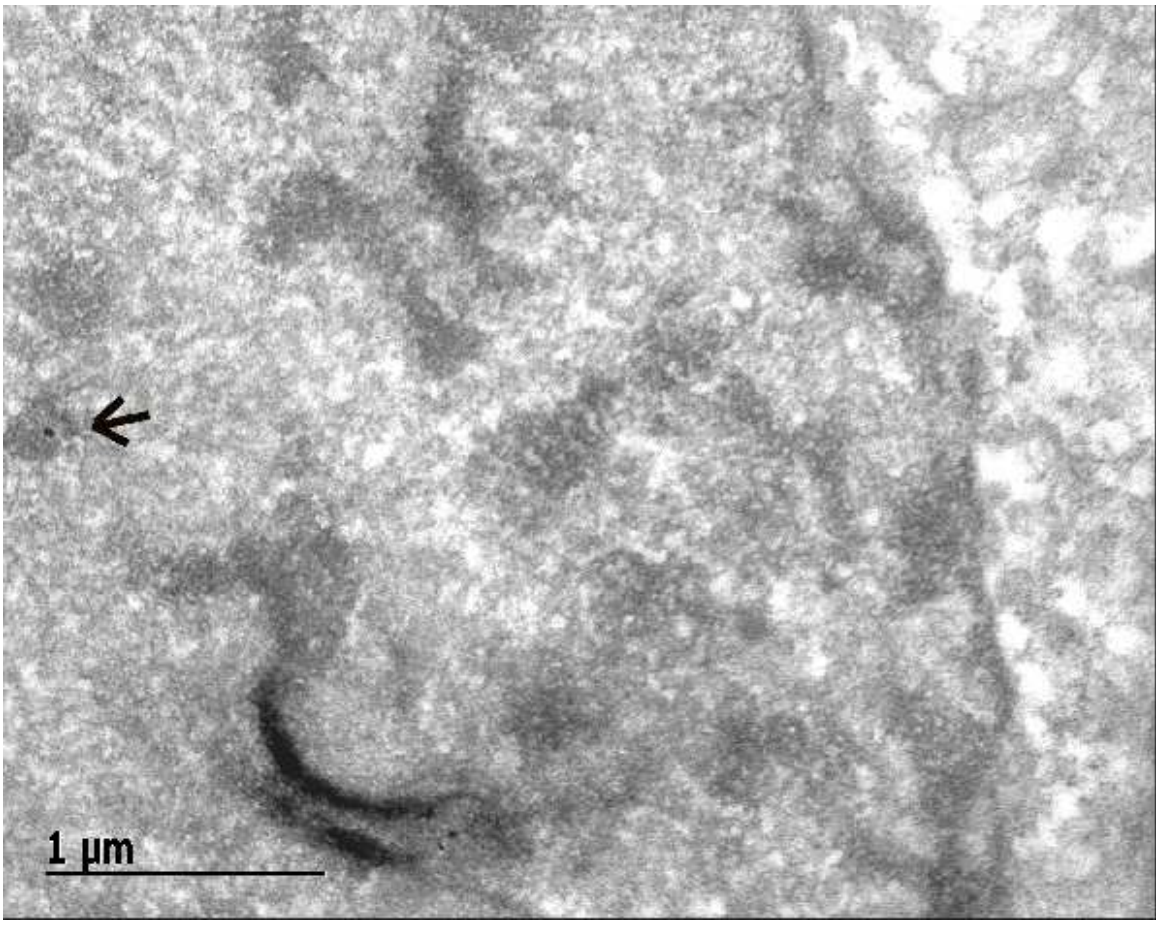


Figure 4.3: Gold particle HS $(CH_2)_{11}$ $(OCH_2CH_2)_8$ OH Au thiol/arg_9 constructs in the nuclear region of a HeLa Cell.

4.4: References

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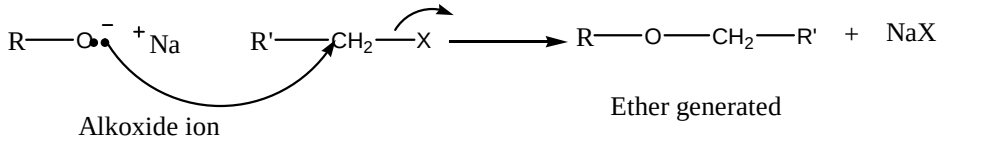
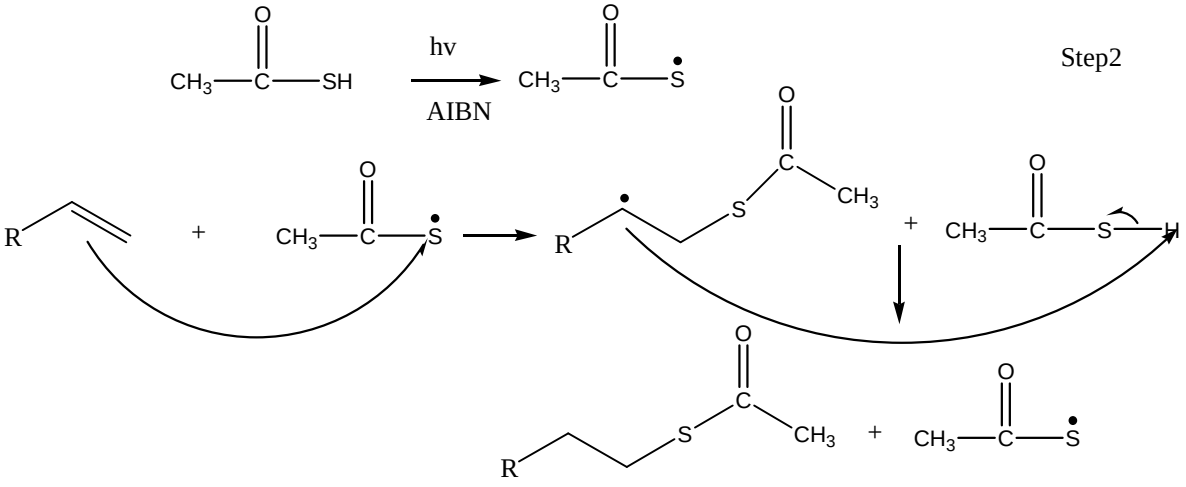
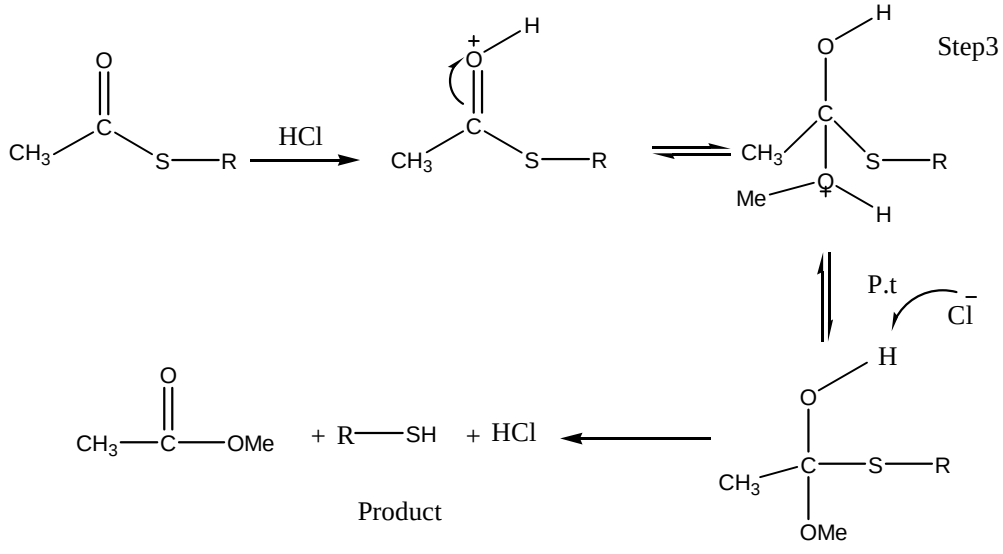
Appendix



Step one



Step2



Step3

Mechanism for the synthesis of PEG's in chapter2.

In step one a Williamson ether synthesis is occurring. In a Williamson ether synthesis a SN_2 displacement occurs. The alkyl halide must be primary so that backside attack is not hindered. The generated alkoxide ion then reacts with the alkly halide.

In step two alkene photochemistry is used. Photochemical addition of thioacetic acid occurs across the double bond to generate the thioacetate.

In step three acidic deprotection occurs to generate the free thiol.

Sterile Cell culture practices:

In order to ensure that cells will not become infected the working area (i.e. hood, bench tops, microscopes, etc.) must be wiped down with alcohol prior to placing cells in the area. It is also essential to have a laminar flow hood with an ultra violet light source. Prior to handling cells, individuals should wash hands and spray them down with alcohol and then put on gloves. Proper laboratory attire must be worn which includes gloves, and a clean lab coat.