Master's Thesis

Exploring the antibacterial property of silver nanotriangles synthesized using biocompatible polymers

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Preface

The work presented in this thesis was carried out in collaboration with Jussi Toppari from the Department of Physics and Leona Gilbert from the Division of Cell and Molecular Biology at the University of Jyväskylä.

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Jyväskylä, I've had the best memories here!

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Abstract

Medical devices contaminated with pathogens are the most common source of hospital acquired infections. To prevent the spreading of the infections to other patients and to ensure the safety of the medical devices, hospitals undertake obligatory decontamination procedures. The current decontamination procedures use expensive and hazardous disinfectants and lengthy sterilization protocols. Some bacteria have also developed resistant strains against common disinfectants, as well as against antibiotics of a similar structures through the process of cross resistance. Thus, there is an emerging need for the development of an alternate class of antibacterial agents that can be coated on all categories of medical devices and are active against a broad range of pathogens.

Studies on silver nanoparticles in the past have highlighted their antibacterial nature, but toxicity associated with metallic silver has limited its applicability in biomedical science. With a future aim of developing an antibacterial coating for medical devices that overcomes all the above problems, we synthesized silver nanotriangles using biocompatible polymers like polyethylene glycol (PEG) and poly(sodium) styrene sulphonate (PSSS). The use of biocompatible polymers as a surface coating is hypothesized to reduce the cytotoxicity associated with the nanoparticle. Nanotriangles were of particular interest to us due to their high reactivity with bacterial surfaces that comes from their pointy vertexes and the presence of large number of high atom density facets such as {111}, which are not present in other shapes such as sphere, rods, etc.

Techniques like light spectroscopy and Transmission electron microscopy were used to characterize the nanotriangles. Nanotriangles of PEG were mostly triangular with sharp edges and 35.6 % of them had edge length between 40-50 nm, while the nanotriangles of PSSS were quasi spherical to triangular with blunt edges and 37.5 % of them had edge length between 20-30 nm. The antimicrobial effect of nanotriangles of PEG and PSSS on bacteria were examined using agar plates and liquid cultures of *Escherichia coli* strain (DH5α). At lower dilution ratios (3/5) of PEG and PSSS nanotriangles, a clear antibacterial effect was observed, whereas, higher dilution ratios (1/10) only revealed a reduction in growth of the *E. coli* cells. With these set of results, silver nanotriangles made using biocompatible polymers provides innovative prospects in being used as a future antibacterial coating for medical devices.

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Abbreviation

AA	Ascorbic Acid
AA	Acetic Acid
Ag	Silver
$AgNO_3$	Silver Nitrate
AgNTs	Silver Nanotriangles
AMR	Antimicrobial Resistance
BF-TEM	Bright Field Transmission Electron Microscopy
CDC	Centers for Disease Control and Prevention
ddH_2O	Double Distilled water
DH5α	recombinant strain Douglas Hanahan
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EtO	Ethylene Oxide
FDA	Food and Drug Administration
g	Gram
H_2	Hydrogen
H_2O_2	Hydrogen Peroxide
HAIs	Hospital Acquired Infections
HCl	Hydrochloric acid
HNO_3	Nitric acid
hrs	Hours
IL	Interleukins
kV	kiloVolts
L	Liter
LaB_6	Lanthanum Hexaboride
LB	Luria Bertani
LSPR	Localized Surface Plasmon Resonance
M	Molar
mg/l	Milligram per liter
MIC	Minimum Inhibitory Concentration
min	Minute
ml	Milliliter

ml/min	Milli liter per minute
mM	Milli molar
Mw	Molecular weight
$NaBH_4$	Sodium borohydride
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NIR	Near Infrared region
nm	Nanometer
O.D.	Optical Density
PEG	Polyethylene glycol
PSSS	Poly (sodium) styrene sulphonate
ROS	Reactive Oxygen Species
rpm	Rotations Per Minute
SEM	Scanning Electron Microscopy
SERS	Surface Enhanced Raman Scattering
T	Thickness
TEM	Transmission Electron Microscopy
TSC	Tri Sodium Citrate
UV	Ultra Violet
UV-Vis	Ultraviolet - Visible
WHO	World Health Organization
°C	Degree Celsius
λ_{max}	Lambda max/ absorption maxima
µl	Micro liter
µM	Micro molar
µm	Micro meter
%	Percent
0D	Zero Dimension
1D	One Dimension
2D	Two Dimension

1. Introduction

1.1 Current scenario on sterilization of medical devices

In modern times, surgery has become an inevitable procedure in the management of chronic health conditions, which has led to our growing dependence on it. To support this statement, the latest statistics extracted from the Eurostat's database shows that in Finland for example, there has been a clear increase in the number of surgeries from 467805 in 2000 to 621731 in 2010 (WHO, 2014). All surgical procedures involve the contact of the medical device with the patient's body tissues or fluids (Spach *et al*., 1993). During such procedures, a patient is at high risk of contracting an infection from the medical device if it is not decontaminated properly. To avoid problems related to contamination, such as Hospital Acquired Infections (HAIs), The Centers for Disease Control and Prevention (CDC) has enlisted guidelines for hospitals to clean and disinfect hospital environments, working surfaces, and surgical equipment's (CDC, 1983 and Rutala *et al*., 2008). The lack of compliance with the guidelines established by the CDC, along with the abusive use of antibiotics, has led to a worldwide spread of multi drug resistant bacteria (Knobler *et al*., 2003). According to the latest CDC report, HAI from medical devices alone affects 1.7 million people in the United States, killing nearly 99,000 people every year with 5% to 10% of patients getting infected while in the hospital (Golkar *et al*., 2014 and Klevens *et al*., 2007). Despite undertaking costly precautionary measures to prevent the spread of HAI, it is still emerging to be one of the greatest challenges to the healthcare industry today (Scott, 2009).

1.1.1 Hospital Acquired Infections

HAIs are known as health care acquired infections or more commonly referred to as nosocomial infections in the healthcare industry. HAIs are microbial infections that people get through contact of medical devices while being treated in a healthcare facility, for example, hospitals, long-term care centers, outpatient clinics, dialysis centers, and surgical centers (Stone *et al*., 2002). HAIs can be viral, bacterial or a fungal pathogen by origin. The most common infections are those of *Escherichia coli*, Vancomycin Resistant *Enterococc*us (VRE), *Clostridium difficile*, *Acinetobacter baumannii*, Methicillin Resistant *Staphylococcus aureus* (MRSA) and Norovirus (Dancer, 2008, Martinez *et al*., 2003, Tankovic *et al*., 1994, Green *et al*., 1998 and Kaatz *et al*., 1988). The source of these pathogens is usually patients already infected with HAIs in the hospitals. These patients transmit pathogens to uninfected patients via hospital surfaces, nursing staff, and medical devices which are in common use. Lack of improper disinfection strategies may facilitate easy transfer of HAIs through surgical

equipment's (Haley *et al.*, 1985). HAI are so common, that nowadays, about one in twenty-five hospital patients are diagnosed with a HAI (CDC, 2016).

1.1.2 Antibiotic Resistance

In the early 20th century, infectious diseases were the major cause of mortality worldwide (Cohen, 2000). The gradual decrease in mortality rate around the 1940s was attributed to the introduction of antibiotics. However, the consequence of their overuse in the past few decades has led to the evolution of bacterial resistance (Komolafe, 2003). Literature states that 30% to 60% of the antibiotics prescribed in intensive care units (ICUs) have been found to be unnecessary, inappropriate, or sub optimally used (Luyt *et al.*, 2014). Resistance occurs when the bacteria change in a way (through mutations) that the effectiveness of drugs, chemicals, or other agents, that were otherwise designed to cure or prevent infections, reduces (WHO, 2017). This means that the antibiotics have little or no effect on killing bacteria anymore. Various attempts have been made to overcome the bacterial resistance by discovering new antibiotics or modifying current antibiotics, however, the discovery of new drug molecules is not up to par with the rate at which pathogens are undergoing mutations to develop the resistance against the drug (Huh *et al.*, 2011). HAIs and antibiotic resistance is closely related. More than 60% of the HAIs worldwide are due to the emergence of multi drug resistant bacteria, for example methicillin-resistant *Staphylococcus aureus* (MRSA) and Carbapenem-resistant-Enterobacteriaceae (CRE) (Ventola, 2015).

1.1.3 Traditional Methods of decontaminating medical devices

Cleaning the hospital premises and decontaminating medical devices to prevent transmission of HAIs is of aesthetic necessity. Disinfection and sterilization are two types of decontamination processes. The basic difference between these two processes being that disinfection only destroys harmful organisms whereas sterilization destroys all types of organisms present on the surface or inside a liquid/object. Currently sterilization followed by disinfection is the protocol employed to reduce the bio burden present on medical equipment.

Chemical disinfectants such as quaternary ammonium compounds, aldehydes, alcohols, and halogens are being used to tackle the problem of HAIs (Rutala *et al.*, 2001, Simões *et al.*, 2010, Hota *et al.*, 2004, Russell *et al.*, 1999). As per Talon (1999), a disinfectant is never able to kill 100% of the bacteria present on the equipment because of inefficient cleaning protocols and there are some bacteria that have developed resistance against the active compounds of the disinfectant. Some of the disinfectants used nowadays are known to leave

surface residues. The chemical residues left behind continue to kill the bacteria, meanwhile, a small population of those bacteria undergo mutations under stress and develop a defense mechanism against the active compounds of the disinfectant. These lineages continue to replicate and develop a family of resistant species (McMurry *et al.*, 1998). Studies have also shown that bacteria that develop tolerance against the active compounds in disinfectants have the potential to develop resistance against antibiotics due to the phenomena of cross-resistance (Russell *et al.*, 1998). Triclosan is a common active compound present in most disinfectants. Triclosan has a similar inhibitory target in bacteria as some antibiotics. Mutations in bacteria make them tolerant not only to triclosan but also the antibiotic isoniazid, which is used to treat tuberculosis in patients. Thus, due to similar targets, mutations are able to endow tolerance even against common antibiotics, which is a serious concern (McMurry *et al.*, 1998).

Sterilization is the other decontamination procedure that is used in hospitals to treat surgical devices, as they are capable of destroying all microorganisms including spores (Rutala *et al.*, 2013). Given the range and type of medical devices available in today's hospitals, it is not possible for any one sterilizing agent to be used for all types of medical devices and some of the sterilizing agents even have drawbacks associated with their use. For example, heat labile devices cannot be sterilized using autoclaving; hydrogen peroxide (HP) cannot be used on instruments coated with zinc, brass or nickel (Knox *et al.*, 1961 and Walt *et al.*, 2004). In addition, some of the chemical agents used for sterilization can cause health hazards to the person handling it. For example, ethylene oxide (EtO) is toxic if ingested and it is highly flammable and thus needs careful storage and handling (Rutala *et al.*, 2008). Moreover, the use of a HP gas plasma (HPgp) setup requires a dedicated facility and a specialist to handle it. This makes the process of sterilization more complicated, expensive and time consuming (Rutala *et al.*, 2004).

1.2 Silver: antimicrobial agent

With the emergence of pathogenic strains that are resistant towards disinfectants, antibiotics and to avoid hassles of multiple sterilization procedures, there is a need for an alternate class of disinfection systems that can be easily coated on all types of surgical devices and more importantly benefit to all class of hospitals (Fischbach *et al.*, 2009 and Huh *et al.*, 2011). Oligodynamic metals like silver are to these days one of the most promising candidates to fill this role. A Swiss botanist, Von Ngeli (1893) defined the term oligodynamic ("oligos" is small & "dynamis" is power) for metals that exhibited bactericidal properties at minute

concentrations such as copper, tin, and silver. The use of pure metallic silver and salts of silver in wound has been recorded quite early in history (Chernousova *et al*., 2013). This means that the bactericidal nature of silver has been known for a long time. Building on this fact, scientist in recent times started exploring the properties of nanoparticles of silver (Sondi and Salopek, 2004 and Rai *et al*., 2009). The properties of nanoparticles are different from their bulk counterparts because as the particle size decreases, a greater proportion of atoms are exposed to the surface, and more surface area is available for reaction (Lambert *et al.,* 2010). The advantage of using silver nanoparticles over their bulk counterparts is that they are significantly more toxic to microbes than Ag^+ from metallic silver or salts of silver (Bachelor *et al*., 2014). They are effective at killing even at nano molar concentrations, compared with micro molar levels for bulk Ag^+ (Lok *et al.,* 2006). Moreover, the nanoparticle synthesis route is quite simple and the morphology is easily tunable (Marin *et al*., 2015). One drawback witnessed with the use of silver was the development of resistance against silver in bacteria. However, it was noticed that the mutations for resistance were unstable, difficult to maintain and non-transferable (McHugh *et al*., 1975, Hendry *et al*., 1979, Deshpande *et al*., 1994, Annear *et al*., 1976 and Bridges *et al.,* 1979).

Despite having very good bactericidal properties, toxicity associated with nanoparticles (that come from the chemicals that are used during synthesis) have restricted their use in many biomedical based applications (Stensberg *et al*., 2011). The uptake of silver nanoparticles in eukaryotic cells can induce the production of Reactive Oxygen Species (ROS), leading to oxidative stress and genotoxic effects (Wu *et al*., 2010). ROS is produced as a result of disruption in the flux of ions and electrons across the mitochondrial membrane. Excess ROS production leads to cell death via apoptosis or necrosis and also impairs the energy-dependent DNA repair mechanisms (Asharani *et al*., 2009). Ag^+ released from the silver nanoparticles is also known to directly damage the DNA (Yang *et al.,* 2009). In a study reported by Carlson and colleagues (2008), the exposure of silver nanoparticles to alveolar macrophages stimulated an immune reaction by inducing the production of pro inflammatory cytokines TNF-α, MIP-2 and IL-1β. With this information available on the toxic effects of silver nanoparticles on eukaryotic systems, there is a need to shift our focus towards synthesizing nanoparticles using more natural products like bio extracts from plants or using polymers during synthesis which are already known to be biocompatible. The limitations while using bio extracts for synthesis are that the obtained nanoparticles are polydisperse (Chowdhury *et al*., 2014). Moreover, seasonal variation and different geographic locations of the plants for

the bio extracts restrict the ability to reproduce the same set of nanoparticles (Singh *et al*., 2013). On the other hand, using polymers during synthesis offer rapid reproducibility, prevents aggregation and reduces cytotoxicity associated with the nanoparticle (Larsen *et al*., 2009).

1.3 Biocompatible Polymers

Polymers are defined as macromolecules composed of one or more identical units called monomers that are repeated throughout the chain (Elias, 1997). Polymers can be synthetic or natural. Example of synthetic polymers are Polyethylene, Polypropylene, Polyvinyl chloride, Polystyrene, Nylon, Teflon, Polyurethanes, and natural polymers are DNA, proteins etc. (Davis, 2004). Biocompatibility is one of the most important characteristics associated with a polymeric material if it is intended to be used in a biomedical application (Anderson, 2001). Yu and colleagues (2012) have shown in their study that surface modification of nanoparticles with biocompatible polymers greatly reduces the cytotoxicity associated with the metal nanoparticles. In a similar study carried out by Larsen and coworkers (2009), polymer coated nanoparticles have indicated a reduced phagocytic capture by the immune system which prevented an inflammatory response. Some of the polymers that are being used in recent times for biomedical based applications are polyethylene glycol and poly (sodium) styrene sulphonate (Inada *et al*., 1995).

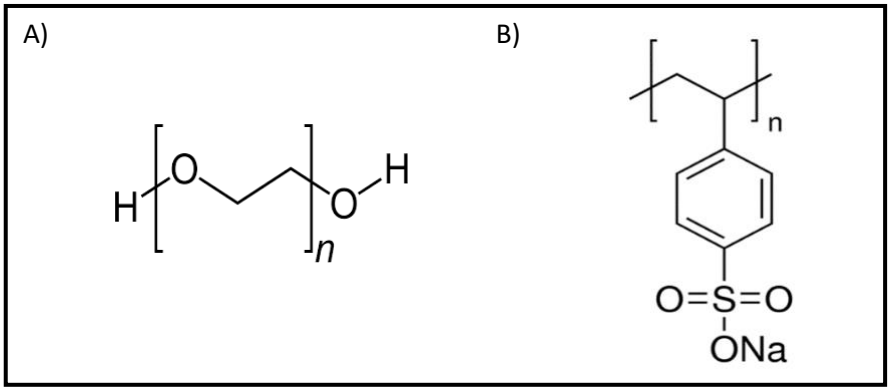

Figure 1: Chemical structure of A) Polyethylene glycol. B) Poly (sodium) styrene sullphonate.

Polyethylene glycol (PEG) is a synthetic polyether available in a range of molecular weight. Polyether's having $M_w \leq 100000$ are commonly referred to as polyethylene glycols. PEG 3350 is nontoxic, approved by the Food and Drug Administration (FDA) in drug formulations, and is commonly used as a strong laxative (Fuertges *et al*., 1990). Experimental evidence suggests that PEG 3350 does not interact with the immune system and is easily cleared from the system unaltered (Working *et al*., 1997).

Poly (sodium) styrene sulphonates (PSSS) are polymers of polystyrene with a modified sulphonate group. They are generally available as sodium or calcium salts with an average molecular weight of M_w ~10,000,000. It is commonly used in the treatment of patients with hyperkalemia; it binds to excess potassium secreted by the kidneys and clears it from the system (Harris *et al*., 1997). It has also been used as an antimicrobial agent in the formulation of a vaginal gel (Herold *et al*., 2000). Cytotoxicity assay performed on human epithelial cells suggested very little toxicity, due to their inert nature (Fahey *et al*., 1998).

1.4 Silver nanotriangles

Nanoparticles of a specific shape in coordination with synthetic polymers like PEG have demonstrated good biocompatibility, while maintaining their anti-microbial property (Debnath *et al*., 2017). Meanwhile the efficacy of the nanoparticles at killing a microbe considerably changes depending on their size and shape. (Lu *et al*., 2013, Pal *et al*., 2007). In a review published by Pal and coworkers (2007), triangular shaped nanoparticles displayed a higher killing rate against both gram positive as well as gram negative bacteria as compared to other common shapes such as spherical and rods. Silver nanotriangles have previously been popular as ultrasensitive tools in Surface Enhanced Raman Scattering (SERS) based detection system. The presence of sharp edges or tips in nanotriangles creates an enhanced electromagnetic hotspot in noble metals, thus making the detection of analytes highly sensitive. Pal and coworkers (2007) in their research have suggested that the pointed edges in nanotriangles along with the presence of large number of high atom density facets such as {111} are responsible for the higher antibacterial activity against bacteria. Thus, there is a need to focus our attention towards exploring this property of silver nanotriangles.

1.5 *Escherichia coli*: a model organism for study

The best ways of assessing the antimicrobial activity of newly discovered disinfectant/sterilizing agent is to test them against the most frequently occurring HAIs which are those of the gram-positive *Staphylococcus aureus* and the gram-negative *Escherichia coli* (Preibe *et al*., 2017, Reybrouck, 1998). Amongst all the known HAIs, *E. coli* is the most common strain found across hospitals worldwide (Peleg *et al*., 2010). *E. coli* are rod shaped, facultative, gram-negative bacteria belonging to the family Enterobacteriaceae. It encompasses a family of serotypes, which have genetic and phenotypic diversity. There are various reasons why *E. coli* is a preferred choice of model organism for a research study (Cooper, 2000). Firstly, the bacterium grows under minimum nutrients with a reproduction time of only 20 mins and thus scaling up is much easier compared to other bacterial species.

Quick reproduction time also allows for easy replication and faster expression of genes. A clonal population of *E. coli* cells, which are derived from the propagation of a single cells can be isolated as a single colony on LB agar plates (Cooper, 2000). The entire genome of strain MG1655 and W3110 was sequenced in 1997 (Blattner *et al.*, 1997). The 4.64 million base pair genome encodes 4,289 genes, whose functions have been known and stored in the EcoCyc database (Keseler *et al.*, 2009) and Coli Genetic Stock Centre (Riley *et al.*, 2005). With this preexisting information, a more detailed understanding of the bacteria and nanoparticles interactions could be made in our study.

2. Aim of Study

The aim of this thesis work was to synthesize nanotriangles of silver and test their antibacterial activity so that they could be used as future antibacterial coatings on surgical instruments. The main objectives were:

- To synthesize silver nanotriangles using biocompatible polymers such as poly ethylene glycol and poly (sodium) styrene sulphonate.
- To characterize their size and shape using techniques like U.V.-Visible Spectroscopy and Transmission Electron Microscopy.
- Test and compare the antibacterial activity of the PEG and PSSS nanotriangles against the gram-negative bacterium *E. coli*, both in liquid cultures and on agar plates.

3. Materials and Methods

3.1. Synthesis of Silver nanotriangles

3.1.1 Using polyethylene glycol (PEG)

The synthesis of Silver nanotriangles using polyethylene glycol was a one pot process. The protocol for synthesis was adapted from the literate published by Zhang and coworkers (2011). The glassware used for synthesis was rinsed in aqua regia once followed by three times washing with ddH_2O. Aqua regia was prepared by mixing 1 ml of HNO_3 to 3 ml of HCl (1:3). The moisture from the glassware was dried out completely before use. During synthesis, a volume of 50 µl of 0.05 M Silver Nitrate ($AgNO_3$) (Fluka), 500 µl of 75 mM Trisodiumcitrate (TSC) (Merck), 100 µl of 17.5 mM PEG (LaySan Bio), 60 µl of 30 % Hydrogen Peroxide (H_2O_2) (Emsure) and 150 µl of 100 mM Sodium Borohydride ($NaBH_4$) (Flucka) were added sequentially to a volumetric flask containing 24.14 ml of ddH_2O. The reaction mix was stirred vigorously at 1500 rpm for 10-15 mins or until any further color change occurred. For every reaction, $NaBH_4$ and $AgNO_3$ were prepared fresh. $NaBH_4$ reacts violently and quickly with water at room temperature. Thus, $NaBH_4$ was prepared with slightly cool ddH_2O (ddH_2O at 4°C for 30 mins). Post synthesis, the prepared silver nanotriangles were stored in dark at +4°C until further use. This synthesis protocol was repeated three times.

3.1.2 Using poly (sodium) styrene sulfonate (PSSS)

The synthesis of silver nanotriangles using PSSS was a two-step process, involving seed production of small spherical silver nanoparticles, followed by growth into larger nanotriangles. The protocol was adapted from Aherne and coworkers (2008). All the experiments were performed using glassware washed with aqua regia or sterile plastic containers under a fume hood.

a. Seed production

For seed production, 250 µl of 500 mg/L of PSSS (Sigma) and 300 µl of 10 mM $NaBH_4$ (Fluka) were added rapidly and sequentially to the flask containing 5 ml of 2.5 mM TSC (Merck). Further, 5 ml of 0.5 mM $AgNO_3$ was added to the flask dropwise using a tubing pump (ISMATEC REGLO ICC Digital Peristaltic Pump, EW-78001-70). The addition was controlled at a flow rate of 2 ml/min, while stirring continuously at 1500 rpm. The color of the solution gradually turned from colorless to light yellow and finally to chartreuse color

over a time span of ~ 3-4 mins. For every synthesis protocol, $NaBH_4$ and $AgNO_3$ were freshly prepared as above.

b. Growth of silver nanotriangles

The silver nanotriangles were grown from the seed solution. A volume of 75 µl of 10 mM Ascorbic Acid (AA) (Sigma) and 80 µl of seed solution were added to a sterile falcon tube containing 5 ml of ddH_2O. The contents in the tube were mixed vigorously using vortexing (Vortex-Genie 2, USA Scientific). To this, 3 ml of 0.5 mM $AgNO_3$ was added dropwise at a rate of 1 ml/min using a syringe pump (ISMATEC REGLO ICC Digital Peristaltic Pump, EW-78001-70). There was a gradual change in the color of the solution from light yellow to orange-vine-red-violet-purple over a time span of 8-10 mins.

c. Stabilizing the silver nanotriangles

Newly formed nanoparticles tend to stabilize themselves by adsorbing molecules on their surface or lower their surface area through agglomeration or coagulation (Kraynov *et al*., 2011). In order to avoid the later and produce uniformly sized nanoparticles, 500 µl of 25 mM TSC (stabilizing agent) was added at the end of the synthesis process. The nanotriangle solution was stored in the dark at +4°C until further use (Aherne *et al*., 2008). This protocol was repeated three times for a total volume of 10 ml of nanotriangles.

3.2. Characterization

Post synthesis, the samples were analyzed using UV-Vis Spectroscopy (Elmer Perkin Lambda 850), Transmission Electron Microscopy (JEOL-JEM 1400 HC) and Scanning Electron Microscopy (Raith E-Line) to study the size, shape and distribution.

3.2.1 UV-Vis Spectroscopy

A sample volume of 1 ml of both the PEG and PSSS AgNT was aliquoted individually into cuvettes (VWR) of path length 1 cm and the extinction spectra was recorded from 899 nm to 300 nm using the Spectrophotometer (Elmer Perkin Lambda 850). The raw data was plotted using the software ORIGIN.

3.2.2 Transmission electron microscopy

The samples for microscopy were prepared by pipetting a clean colloidal solution of silver nanotriangles onto formvar coated copper grid (Electron Microscopy Sciences, Lot no 110526). The nanotriangle solution were cleaned prior to deposition on TEM grids to remove salts from the sample. Crystals of salts hinder the imaging process and prevent uniform distribution of the nanoparticles on the grid. For washing the triangles, in step one, 1.5 ml of

the silver nanotriangles was spun down at a speed of 6000 rpm for 5 mins. In step two, an amount of 500 µl was discarded from the supernatant and the pellet was resuspended with fresh 500 µl of ddH2O and spun down again (5 mins, 6000 rpm). Step number two was repeated two more times. Finally, in step five, a volume of 300 µl of the spun down sample was resuspended with 100 µl of 70 % ethanol in a new Eppendorf tube. The copper grids were made more hydrophilic and decontaminated for the presence of any residual hydrocarbons by treating them with low energy plasma for 15 seconds, using the glow discharge machine (Electron Microscopy Sciences). Amongst all the cleaning processes available today, plasma induced cleaning is the least aggressive methods.

A sample volume of 4 µl was pipetted on the pretreated TEM grids and left to dry overnight in a nitrogen chamber. Normal drying can introduce artifacts that obscure the dispersion of nanoparticles on the grid and hinder image analysis (Michen *et al*., 2015). Keeping the TEM grids in a chamber with a constant flow of dry nitrogen prevents oxidization of sample through moisture (inert environment) and the hygroscopic nature of N_2 gas allows the grids to dry very quickly. In our experiment, the grids were left for long overnight drying to secure fully moisture free grids. The images of the silver nanotriangles were acquired using the JEOL-JEM 1400 HC electron microscope, equipped with a field emission gun, LaB_6 filament, operating at an acceleration voltage of 80 kV, in the BF- TEM imaging mode. Before every imaging session, the microscope was aligned and a blank correction was conducted to prevent noise and to enhance the resolution. A total of 25 images from different sections of each grid were taken. For each batch of nanotriangles that were synthesized, TEM grids were made in duplicates as a backup to damages that happen while handling the grids. Since the synthesis protocol was performed 3 times each for both types of nanotriangles, a total of 6 grids were prepared before imaging. The edge length of 129 PEG AgNTs and 100 PSSS AgNTs was measured using a scale bar provided in the image to plot the histogram.

3.2.3 Scanning electron microscopy

The same nanotriangle sample that was used for Transmission Electron Microscopy was used for Scanning Electron Microscopy. In this case, a volume of 2 µl of the washed silver nanotriangle solution was pipetted onto silicon/silicon dioxide chips and left to dry in a nitrogen chamber. Prior to coating the chips with the sample, the chips were immersed in warm 100 % acetone for 5 mins to remove any contaminants present on its surface and preceded by a quick wash with 70 % isopropanol (IPA) and drying with a nitrogen gun. IPA is used later in the cleaning step as it removes contaminants and any residual acetone stains.

3.3 Antibacterial studies

The silver nanotriangles synthesized using PEG and PSSS were tested against the most commonly found bacterial strain associated with hospital infections, i.e., *E. coli* using agar culture and liquid media culture (Bondi *et al*., 1947, Stamm, 1978).

3.3.1 Bacterial culture and media preparation

3.3.1.1 Preparation of culture media

The Luria-Bertani (LB) broth was prepared to culture *E. coli* cells for the antimicrobial tests. LB is commonly used to culture *E. coli* because it permits fast and good growth yields (Sezonov *et al*., 2007). The LB broth was prepared by combining 10 g of 1 % Bacto - tryptone (Biokar), 5 g of 0.5 % yeast extract (Biokar), 10 g of 85 mM NaCl (VWR) and 1 liter of distilled water. The pH of the media was adjusted to 7.0 with 1 M NaOH. To make LB agar plates, 15 g of 1.5 % Bacto-Agar (Biokar) was supplemented to 1 L of Luria-Bertani broth prior to autoclaving. The media was prepared with deionized distilled water after which it was sterilized by autoclaving at 121°C for 20 min.

3.3.1.2 Preparation of Luria-Bertani agar plates

Sterile media with agar was heated in a microwave, till all the agar dissolved and then cooled roughly to 50-60°C. An amount of 15 ml of LB agar was aliquoted into each petri plate (Sarstedt) such that the bottom of the dish was completely covered. The plates were left standing until the media solidified under sterile conditions. Prepared agar plates were stored upside down at +4°C until further use to prevent condensation droplets from falling on the agar surface which could be potential sources of contamination.

3.3.1.3 Culturing of *E. coli* cells

In order to culture the *E. coli* DH5α cells (Ec) for our antimicrobial tests, the stock solution of *E. coli* that was previously stored at -80°C was thawed on ice. A loop full of *E. coli* cells were scooped form the stock solution with an inoculation loop (Sarsdtedt) and added to a culture tube containing 5 ml of freshly prepared LB. The culture was grown in a shaker incubator (New Brunswick Scientific, C24 incubator shaker) at 37 °C, 225 rpm for 6-7 hrs. The *E. coli* cells were allowed to grow up to an O.D. of 0.6. The growth was measured by recording the optical density of the media at 595 nm using a spectrometer (Multiskan™ FC Microplate Photo). The underlying principle being that the presence of bacteria in the sample scatter the incoming light that is converted into an absorbance value, which we read as O.D.

3.3.2 Testing of silver nanotriangles on *E. coli*

3.3.2.1 Qualitative analysis using the spread plate method

Prior to the test, silver nanotriangle solution that was prepared under nonsterile conditions, was filtered through a 0.2 μm filter (Millipore, Billerica, MA, USA) to prevent contamination during the experiment. An amount of 40 μl of cultured *E. coli* suspension (O.D. 0.6 at 595 nm) was pipetted onto a LB agar plate and spread using a disposable sterile L shaped loop (Sarstedt). To this, 40 μl of PEG and PSSS AgNTs were supplemented individually to different agar plates and left to dry under the fume hood. The plates were incubated upside down at +37°C, overnight. As for the negative control, only *E. coli* was grown on LB agar plates without any addition of nanoparticles and the positive control for this experiment was a LB agar plate streaked with *E. coli* and supplemented with 40 μl of 70% ethanol. This test was performed three times each with PEG and PSSS AgNTs.

3.3.2.2 Bacterial time kill study

Prior to the quantitative experiment study, *E. coli* cells (DH5α) were grown in liquid LB medium at 37°C, 225 rpm. Different dilutions of PEG and PSSS nanotriangles (Table 1) were added to the culture tubes containing the *E. coli* cells and incubated in the shaker incubator at 37 °C, 225 rpm for a total time period of 24 hrs. Sample 1 was the most concentrated with 3 ml of silver nanotriangle solution and 1 ml of media in a total sample volume of 5 ml. Other samples were more diluted for example, sample 2 had 2 ml of nanotriangle solution and 2 ml of media, sample 3 had 1 ml of nanotriangle solution and 3 ml of media and sample 4 had 0.5 ml of nanotriangle solution and 3.5 ml of media.

In all the samples, the amount of *E. coli* cells was kept constant at 1 ml of O.D. 3. Optical density of the culture at 595 nm was recorded using a microplate reader (Multiskan™ GO Microplate Spectrophotometer) at intervals of 1 hour, for the first 9 hours and then at 24 hrs, in order to plot the bacterial cell growth curves (Holowachuk *et al*., 2003). These experiments were performed three times each with PEG and PSSS AgNTs.

Table 1: Different dilutions of Silver nanotriangles to be tested on the growth of *E. coli*.

Sample	Dilution	Amount of Silver nanotriangles suspension (ml)	Amount of Luria Bertani media (ml)	Amount of *E. coli* suspension (ml) O.D. = 0.3	Total volume (ml)
Sample 1	3/5	3	1	1	5
Sample 2	2/5	2	2	1	5
Sample 3	1/5	1	3	1	5
Sample 4	1/10	0.5	3.5	1	5

In order to measure the O.D. of the culture at each time point, a volume of 150 µl was aliquoted from the culture tubes into a 96 well plate (VWR) under the laminar hood to maintain sterility. Bacteria growing in silver free media was used as the negative control and bacterial culture supplemented with 70 % ethanol and 99 % ethanol were used as the positive controls. Since the experiment was performed in triplicates, an average of the optical density at 595 nm versus time in hours was plotted using the ORIGIN software. The optical density of the media and nanoparticles were blanked from the readings and data was plotted. This experiment was performed three times, with all set of dilutions of PEG AgNTs and PSSS AgNTs.

4. Results

With the aim of using silver nanomaterial as a bactericidal coating on surgical instruments, we aimed at exploring the antibacterial property of silver nanotriangles that were synthesized using different biocompatible polymers like polyethylene glycol and poly (sodium) styrene sulphonate. Using biocompatible polymers during synthesis is known to drastically reduce the cytotoxicity while maintaining their antibacterial property (Yu *et al.*, 2012). We wanted to examine if the same effect is perceived in nanotriangles synthesized using polymers, as nanotriangles in general are known to have a better killing effect towards bacteria, as compared to any other shape (Pal *et al.*, 2007). In this section, results from the synthesis of nanotriangles using polyethylene glycol and poly (sodium) styrene sulphonate, their characterization with UV-Vis Spectroscopy, Transmission electron microscopy and lastly, the effect of silver nanotriangles on the growth kinetics of *E. coli* are discussed.

4.1. Silver nanotriangles made with polyethylene glycol

4.1.1 Synthesis

The protocol for the synthesis of silver nanotriangles using poly ethylene glycol was adapted from Zhang and coworkers (2011). The synthesis process was observed as a result of color change from colorless to yellow-orange-red-purple and finally a blue colored solution (Andrew *et al.*, 2010). The blue colored solution marked the end of the synthesis process and an image of the silver nanotriangles can be viewed in figure 2. The change in color occurred due to the one-step reduction of silver ions into nanotriangles with the help of a strong reducing agent. The total reaction time was 15 min and the color change appeared at 4 min.

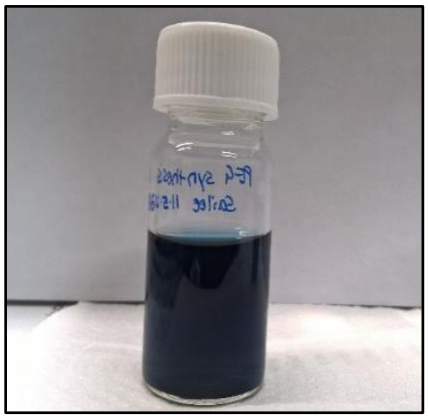

Figure 2: Aqueous colloidal dispersions of silver nanotriangles synthesized using the one pot process with poly ethylene glycol (PEG).

4.1.2 Characterization

The presence of silver nanotriangles in the violet colored solution was initially confirmed by measuring the optical property with a UV-Vis Spectrophotometer (Perkin Elmer Lambda 850). Noble metallic nanoparticles exhibit a strong size dependent optical resonance known as Localized Surface Plasmon Resonance (LSPR). The plasmon couple with the incident light and produce huge enhancement of electromagnetic field around it. The interaction between the incident light and the oscillating electric field results in the scattering and absorption of light which is recorded by the Spectrophotometer and is displayed as the extinction spectrum in figure 3 (Chen *et al.*, 2012). The strong absorption maxima near the infrared region (○) and the shoulder between 350-450 nm (↔) as viewed in figure 3 were indicators of the presence of triangular silver nanoparticles (Wu *et al.*, 2015). The absorption maxima of the in-plane dipole were recorded at 648 nm and quadrupole mode between 350-540 nm. The short narrow peak near the U.V.-vis region of the spectrum around 340 nm could be due to residual silver, as silver ionizes around this energy or it also could be small spherical silver nanoparticles (Wu *et al.*, 2015).

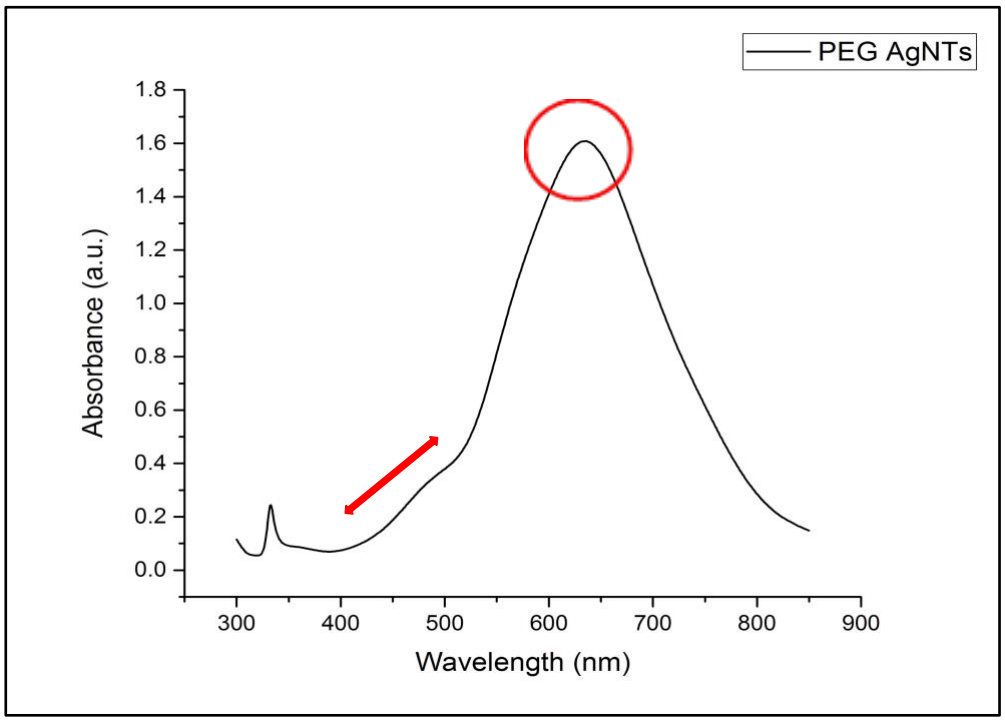

Figure 3: **UV-Vis absorption spectrum of PEG silver nanotriangles.** The silver nanotriangles (AgNTs) are synthesized using poly ethylene glycol (PEG). The main (in-plane dipole) LSPR absorption peaks for PEG AgNTs is at 648 nm (red circle), and the in-plane quadrupole plasmon absorption is between 350-450 nm (double-sided arrows). These figures are a representative image of a triplicate experiment.

Further confirmation on the size and shape of the silver nanoparticles was obtained using the transmission electron microscope. From the TEM image in figure 4a, we can discern that the

silver nanotriangles are polydisperse, quasi spherical to triangular with relatively sharper edges as compared to the particles synthesized using PSSS (data presented in figure 9a).

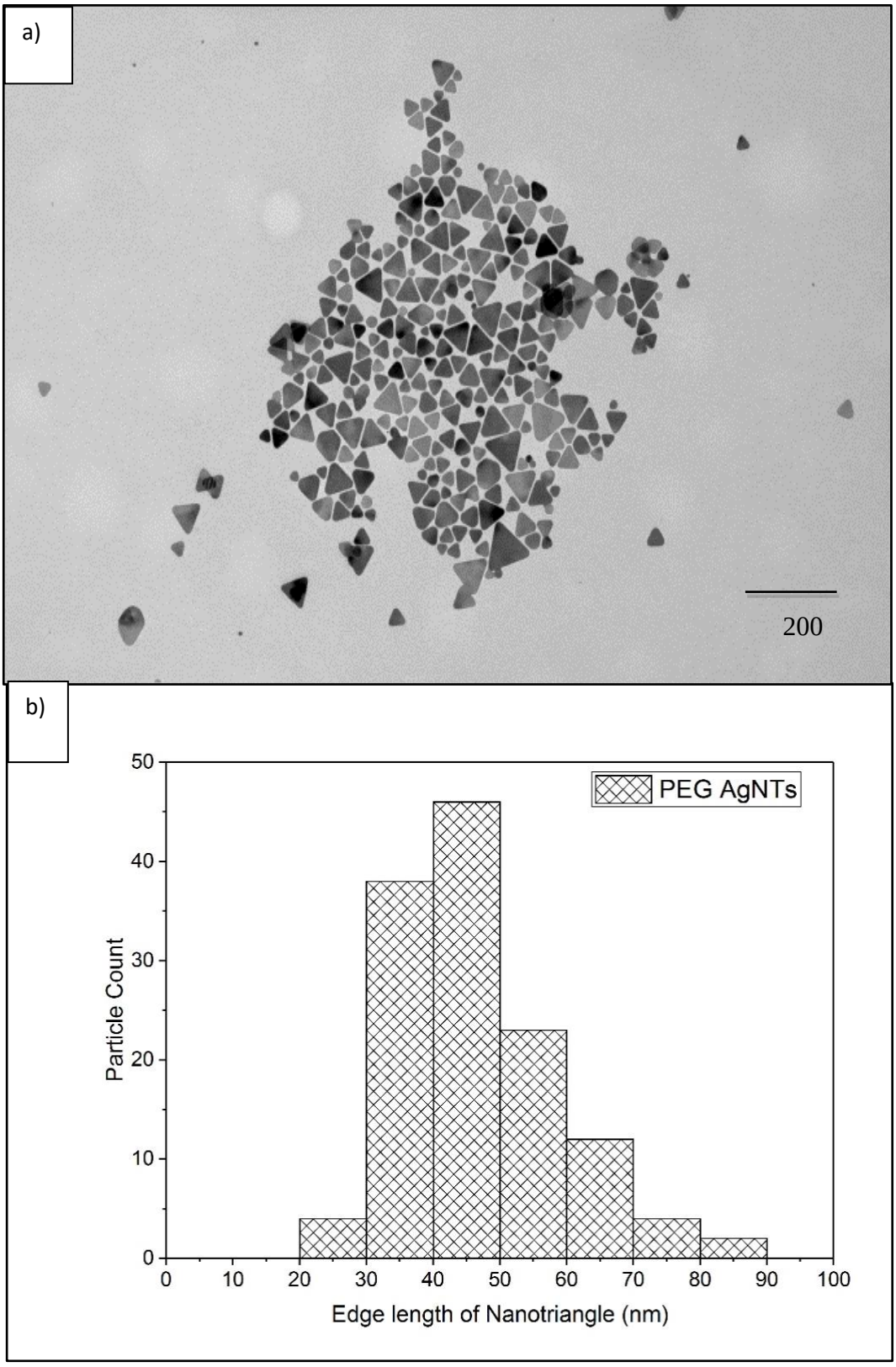

Figure 4: **Visual confirmation and structural morphology of the synthesized PEG silver nanotriangles**. a) Transmission electron microscopy image of the silver nanotriangles. b) The histogram of silver nanotriangle edge lengths indicating a broad (20-90 nm) particle size distribution.

To know the particle size distribution, a histogram of the edge lengths of 129 nanotriangles was plotted using the ORIGIN software. In total 25 TEM images were selected from all the experimental repeats of the PEG and PSSS AgNTs synthesis to list down the edge length of the nanotriangles. The data in the histogram (in figure 4b) indicates a broad particle size distribution ranging from 20-90 nm with 35.6 % of particles having edge length between 40-50 nm.

4.1.3 Antimicrobial testing

The biocidal activity of PEG AgNTs was tested by performing two set of experiments on the laboratory strain of *E. coli* (DH5α). As we can observe in figure 5a, the agar plate having the positive control, i.e., 70% ethanol, does not show the presence of any *E. coli* colonies post 24 hrs incubation. The negative control, figure 5b, had a lawn of *E. coli* cells growing throughout the plate. Whereas the test plate which was supplemented with silver nanotriangles (PEG AgNTs), as observed in figure 5c, had only 103 colonies. These qualitative results suggested that the presence of PEG AgNTs restricts the growth of *E. coli* cells.

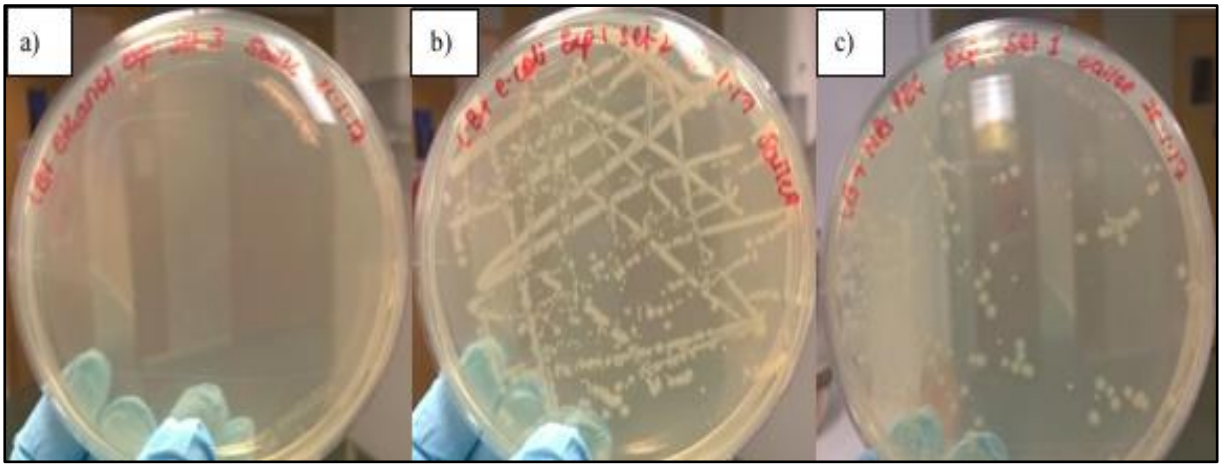

Figure 5: **Inhibitory effects of PEG silver nanotriangles on *E. coli.*** Observation of *E. coli* inoculated on 1.5 % Luria Broth agar plates at 24 h post incubation; a) positive control with 70% ethanol demonstrated no colony growth, b) negative control without any treatment had abundant bacterial colonies and c*) E. coli* treated with PEG silver nanotriangles. The white dots represent the colonies of *E. coli*. The plates supplemented with PEG AgNTs displays lesser colonies as compared to the negative control plate.

A quantitative analysis was made by performing a time dependent study of the effect of different dilutions of silver AgNTs of PEG on the growth of *E. coli*. Optical density of *E. coli* at 595 nm was recorded at different time points starting from 1-12 and 24 hrs. A graph of optical density measurements versus time was plotted using the ORIGIN software which can be observed in figure 6.

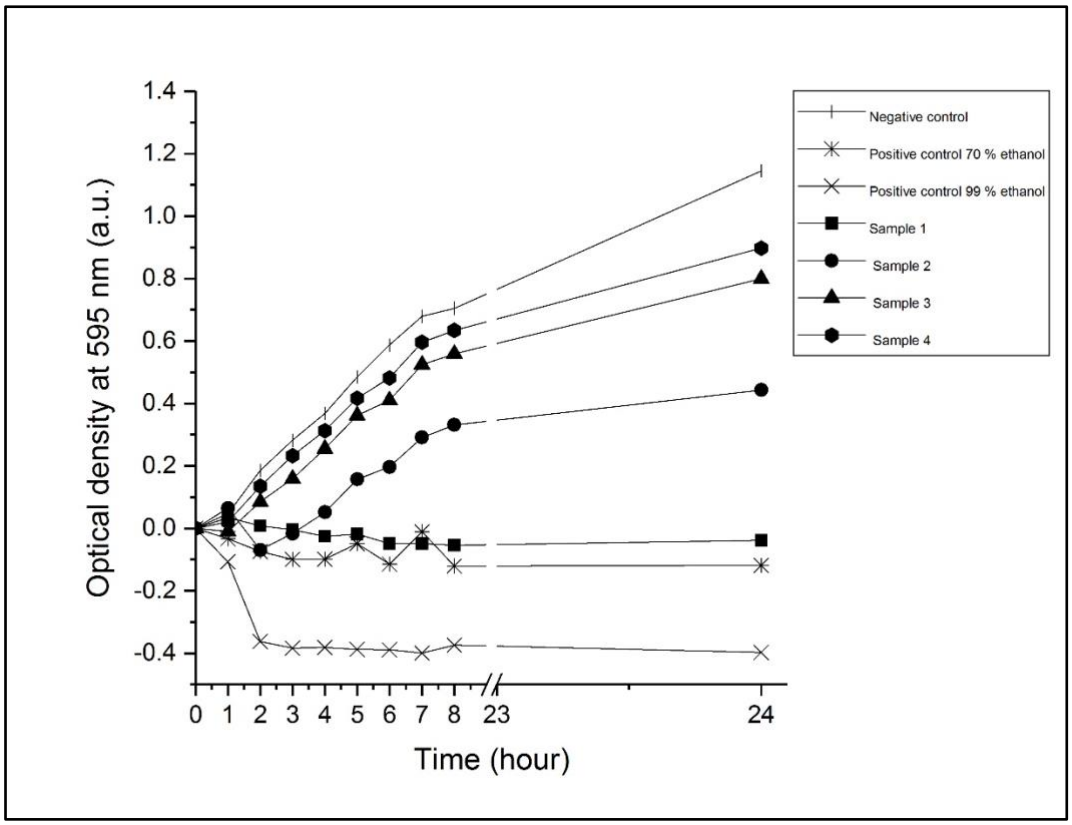

Figure 6: Inhibitory effects of different dilutions of silver nanotriangles on the growth of *E. coli.* Optical density of *E. coli* measured at 595 nm is plotted as a function of time in hours. A concentration dependent inhibition pattern in the growth curve of *E. coli* is observed in the above graph.

In this experiment, bacteria growing in the presence of 70 % and 99 % ethanol were selected as the two positive controls and the negative control was only *E. coli* growing in LB media. As depicted in figure 6, an inhibition of growth was observed in the growth curve of *E. coli* with sample 1 [PEG AgNT dilution of 3/5 (■)]. The growth curve of sample 1 is similar to that of the positive controls, where *E. coli* was grown in the presence of 70 % ethanol (∗). Both these growth curves had no increase in O.D. values over a time span of 24 hrs. The growth curve of *E. coli* with sample 2 [PEG AgNT dilution of 2/5 (●)] had a decrease of 0.1 O.D. in the first 2 hrs; however, O.D. values increased steadily after that. The *E. coli* grown in the presence of sample 3 and 4 PEG AgNTs [dilutions 1/5 (▲) and 1/10 (⬣)] had a growth pattern that clearly depicted a lag phase and exponential phase observed in a typical bacterial growth curve. These results indicated that lower nanoparticles dilutions killed the existing bacteria and inhibited the growth of *E. coli* completely whereas, higher dilutions of nanoparticles could only restrict growth to a certain extent for this given time.

4.2. Silver nanotriangles made with poly (sodium) styrene sulphonate

4.2.1 Synthesis

The protocol for the synthesis of silver nanotriangles using poly (sodium) styrene sulfonate was adapted from Aherne and coworkers (2008). The synthesis was a seed catalyzed two-step protocol, involving the reduction of Ag+ into Ag^0 seeds and then growing these spherical silver seeds into nanotriangles.

The step 1 of the synthesis involved the use of a strong reducing agent like sodium borohydride to reduce the silver salt ($AgNO_3$). The outcome of this reaction was the formation of a chartreuse colored solution of silver spherical seeds as viewed in figure 7.

Figure 7: Aqueous colloidal dispersions of silver nanotriangles synthesized using a seed mediated process using poly sodium styrene sulfonate (PSSS).

Using silver seeds from the step one as the base, additional silver was reduced with a mild reducing agent ascorbic acid. PSSS was found to play a crucial role of shape determination (Aherne *et al.*, 2008). PSSS binds to certain crystal facets which permits growth in one direction, and introduces some defects in the crystal structure of the seeds that predisposed them to nanotriangle formation. This reaction led to the formation of a violet colored solution of silver nanotriangles as shown in figure 7.

4.2.2 Characterization

The presence of silver nanotriangles in the solution was detected by measuring the optical property with a spectrophotometer (300-850 nm). Interaction between the incident light and the localized surface Plasmon generated a typical extinction spectrum as displayed in figure 8. The position of the LSPR band was studied to deduce some information on the shape of the nanoparticle. The extinction spectrum depicted in figure 8 showed the presence of three

dominant peaks due to the different modes of localized surface plasmon excitation. A strong absorption band is noted at the near infrared red region at 595 nm due to the inplane dipole oscillations, a shoulder between 350 - 450 nm could be due to the inplane quadrupole or residual seeds. The dotted curve in figure 8 is the extinction spectrum of the seed solution. The peaks in figure 8 are typical of LSPR excitations of silver nanotriangles and were compared and confirmed with previous literature (Wu *et al*., 2015).

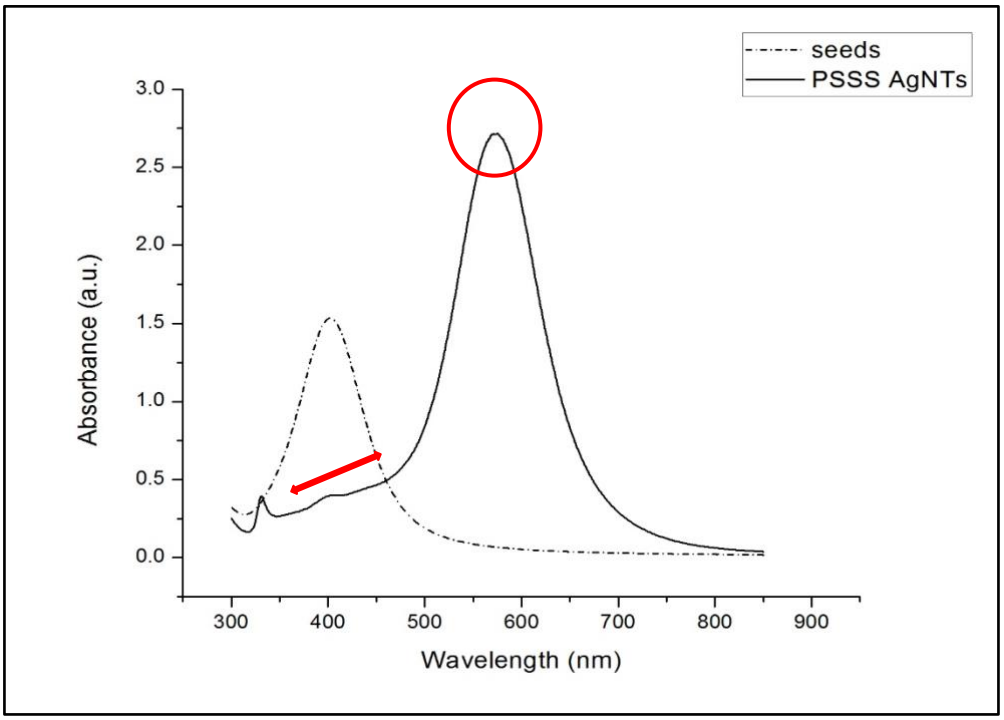

Figure 8: **UV-Vis absorption spectrum of PSSS silver nanotriangles.** The main (in-plane dipole) plasmon absorption peak for PSSS AgNT is at 595 nm (red circle), and the in-plane quadrupole plasmon absorption is between 350-450 nm (double sided arrows). This graph is a representative of nanotriangle synthesis that was reproducible.

The structural morphology of the synthesized particles such as the size, shape, edge length was studied with electron microscopy. The particles were visualized with the JEOL JEM 1400 LaB_6 at 80kV to confirm the shape, as predicted by the UV-Vis data. The edge length could be calculated using either the tools in the RADIUS software or using an image processing software like Fiji. As observed in figure 9a, there is a heterogeneous distribution in the shape of the particles, right from small spherical to large triangular ones. The edges of the PSSS AgNTs are truncated and the particles are stacked one above the other as presented in figure 9a. Using the scale bar, the thickness of the nanotriangles was calculated to be 5 nm. Of the total images were taken from different sections of the copper grid, the edge length of 100 particles was calculated using the Fiji software and a histogram was plotted using the ORIGIN software. The histogram in figure 9b shows quite a narrow size distribution of the

size of the nanoparticles as compared to the particles synthesize using Polyethylene glycol. In majority, 37.5 % of nanotriangles had an edge length between 20-40 nm.

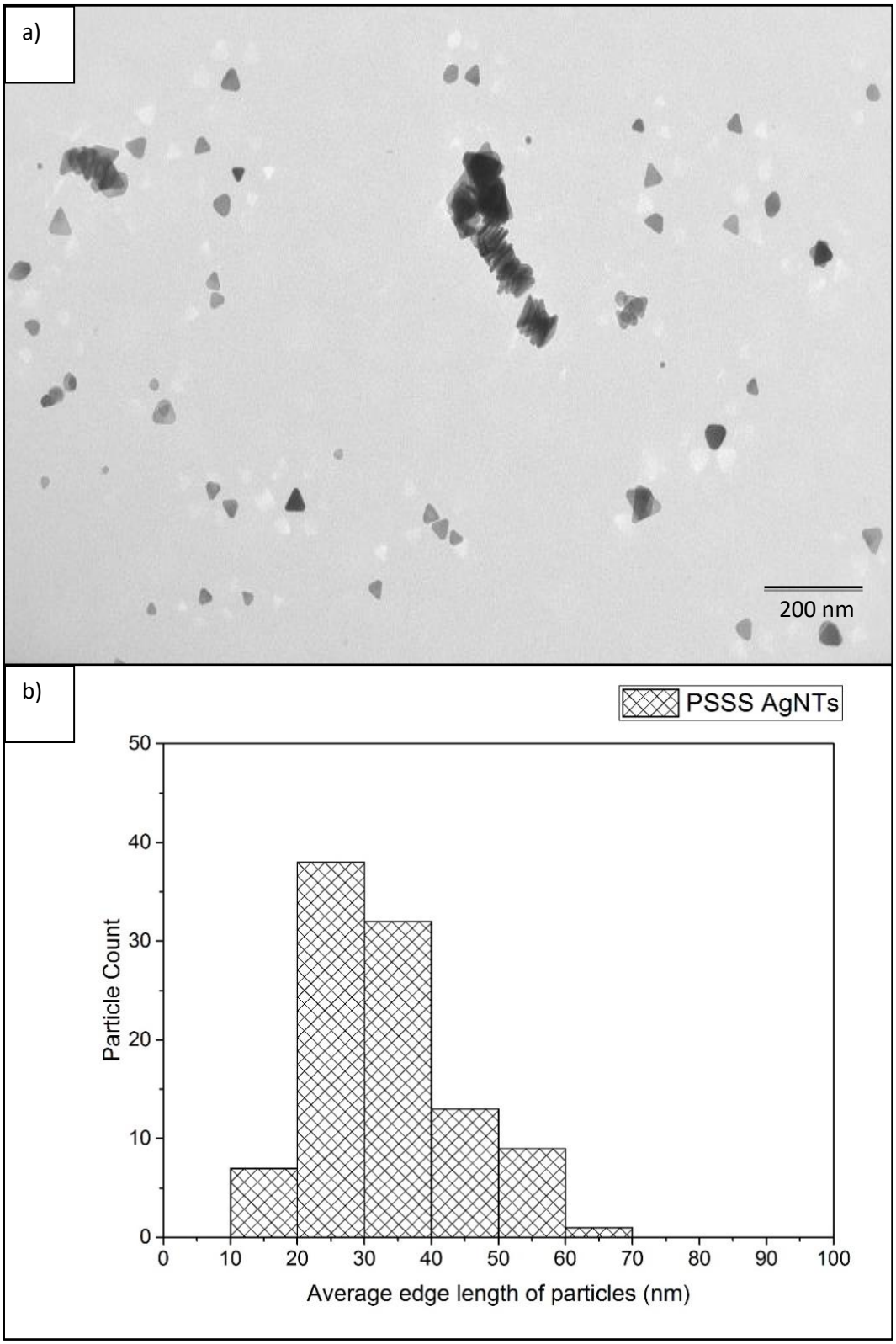

Figure 9: **Visual confirmation and structural morphology of the PSSS silver nanotriangles**. a) Transmission electron microscopy image of Silver nanotriangles. b) Particle size distribution of silver nanotriangles depicting a relatively narrow particle size distribution (10-70 nm).

Additionally, based on the information from the absorption spectrum (λ_{max}) and TEM images (where Thickness = 5 nm), an average edge length of the particles could also be predicted using the following equation.

$$L = T.\ (\frac{\lambda max - 418.8}{33.8})$$

$$L = 26.065$$

When, λ_{max} = 595 nm and T is considered to be 5 nm.

This equation was useful to predict the average edge length of only PSSS AgNTs because; we could estimate the thickness of these particles from the TEM images, whereas for the PEG AgNTs we were not able to because of lack of information on its thickness. Also it was important to note here that the thickness was constant for all the nanotriangles and thus this equation could be applied.

4.2.3 Antimicrobial testing

Like the PEG AgNTs, the PSSS AgNTs were also tested for their antibacterial property by inoculating them on the surface of an agar plate streaked with *E. coli* and allowed to grow at 37°C, overnight in an incubator. Same set of controls were used as that to test the PEG AgNTs. As depicted in figure 10c, there are 134 colonies in the test plate, which are definitely lesser than the lawn of *E. coli* present in the negative control. These results suggest that these particles also have a similar antibacterial effect like the PEG AgNTs.

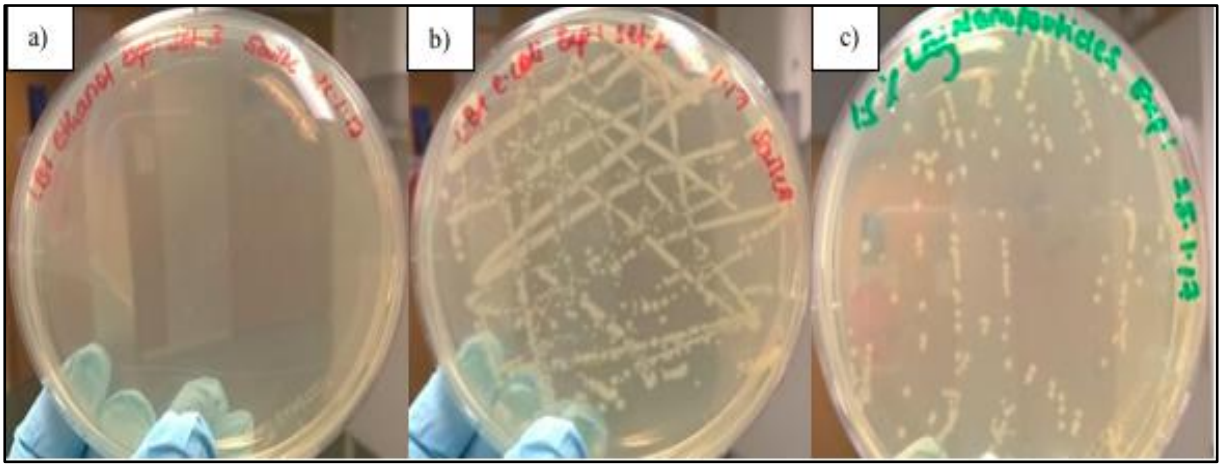

Figure 10: **Growth inhibitory effects of PSSS silver nanotriangles in *E. coli.*** Observation of *E. coli* inoculated on 1.5% Luria Broth agar plates at 24 h post incubation; a) positive control- 70% ethanol with no colonies, b) negative control is only *E. coli*, c*) E. coli* with PSSS silver nanotriangles and. The white dots represent the colonies of *E. coli*. The test plate inoculated with *E. coli* and PSSS AgNTs display lesser colonies as compared to the positive control.

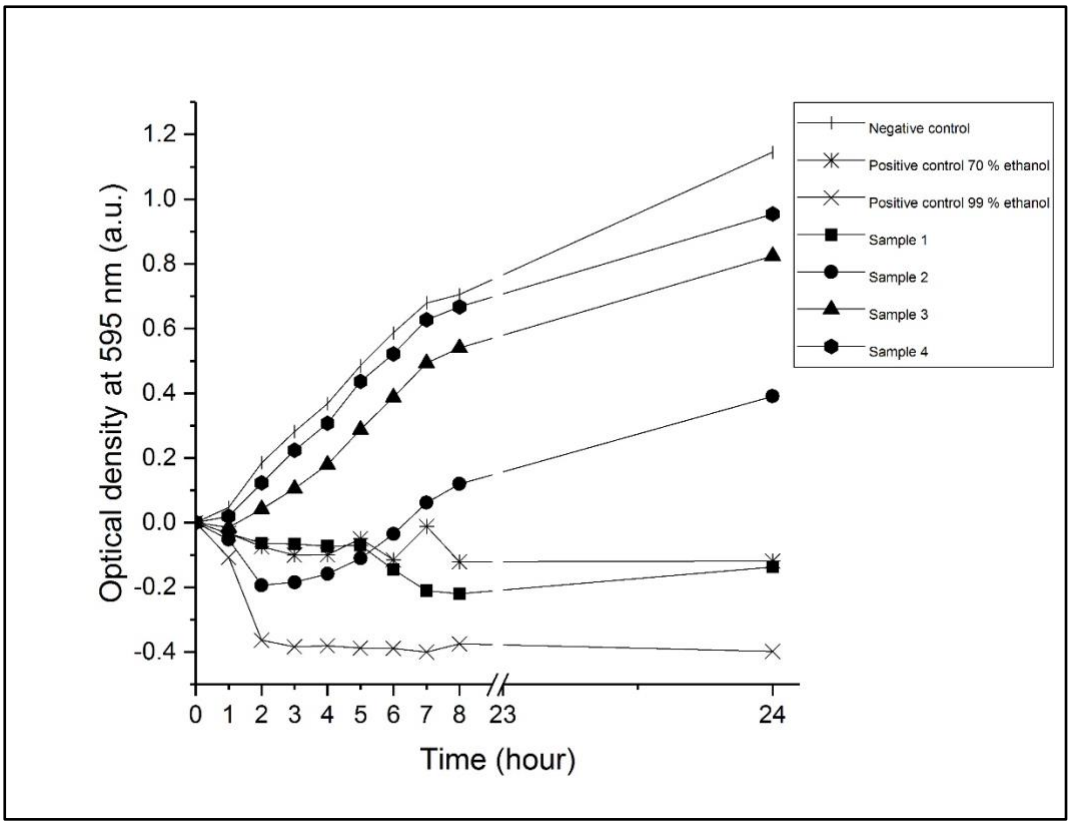

Figure 11: Inhibitory effects of different dilutions of PSSS silver nanotriangles on the growth of *E. coli.* Optical density of *E. coli* measured at 595 nm is plotted as a function of time in hours. Positive controls are 99% and 70% ethanol added to *E. coli* growing in Luria Broth media. Negative control is only *E. coli* growing in Luria Broth media. A concentration dependent inhibition pattern in the growth curve of *E. coli* is observed for Poly (sodium) styrene sulfonate (PSSS) AgNTs. An inhibition of growth was observed in the curve of sample 1 (■) and 2 (●).

The time dependent study of the effect of different dilutions of PSSS AgNTs on the growth curve of *E. coli* elucidated a similar trend in graph as that seen with PEG AgNTs. The highest concentrated sample 1 [PSSS AgNTs dilution 3/5 (■)] showed a clear drop in O.D. values like the positive controls, as seen in figure 11. Interestingly, a slight difference was observed for the sample 2 [PSSS AgNTs dilution 2/5 (●)], where there was a rapid decrease in O.D. value for the first 2 hrs, later it started to rise steadily and rapidly. Unlike the lower dilutions, the sample 3 [PSSS AgNTs dilution 2/5 (▲)] and sample 4 [PSSS AgNTs dilution 1/10 (⬣)] demonstrated a well-defined bacterial growth curve, which was similar to the growth curve of the negative control. This suggests that the higher dilutions have very little inhibitory effect on the growth of *E. coli.*

5. Discussion

Most of the approaches used today for cleaning surgical devices rely on the use of harsh disinfectants or lengthy sterilization procedures (Cowperthwaite *et al*., 2015). In an attempt to find an alternative to these routine procedures, we tried to synthesize nanotriangles of silver using biocompatible polymers like polyethylene glycol and poly (sodium) styrene sulphonte.

The synthesis of silver nanotriangles using poly ethylene glycol was a one-step reduction protocol adapted from Zhang and coworkers (2011). The silver nanotriangles were produced by reducing aqueous $AgNO_3$ with $NaBH_4$ in the presence of H_2O_2, PEG and TSC. For every synthesis reaction, $AgNO_3$ and $NaBH_4$ were prepared fresh because $AgNO_3$ is a silver salt and is photosensitive, it oxidizes quickly if exposed to light. Similarly, $NaBH_4$ quickly oxidizes into H_2 gas and borates when exposed to air. Although the synthesis process was straight forward and the nanotriangles were stable, there was no control over the synthesis process. Reaction conditions like different batches of reagents, ddH_2O, beakers and pipetting hands etc. were implicated as factors directly influencing the reaction product. The advantage of this protocol was the easy scalability to higher volumes.

The protocol for the synthesis of silver nanotriangles using poly (sodium) styrene sulphonate was a two-step seed catalyzed method adapted from Aherne and coworkers (2008). In the first step, silver seeds were produced by a one-step reduction from Ag^+ to Ag° with the help of Sodium borohydride. PSSS played a crucial role of shape determination while growing nanotriangles form the seeds (Aherne *et al.,* 2008). The edge length of the nanotriangles could be easily controlled by adjusting the volume of seed solution in the growth medium. While synthesizing higher volumes of the PSSS AgNTs, the particles aggregated and settled to the bottom. Thus, scaling up to higher volumes was not possible with this protocol. Silver nanotriangles were always stored in dark conditions until use because silver as a metal is susceptible to oxidation by light (Nau *et al*., 2015).

U.V. Vis- Spectroscopy was our first choice for preliminary characterization of the silver nano triangles that we had synthesized because it is sensitive, selective for different types of NPs and only needs a short period of time for measurement (Zhang *et al*., 216). Plasmonic material have unique optical properties which make them strongly interact with certain wavelength of light, producing electric dipoles and higher order pole excitations which are represented by different peaks in a typical extinction spectrum. The excitation of the inplane dipole of a silver nanotriangles with light produced a broad peak near the near infrared region

of the visible spectrum, as noted in figure 3 and 8. The absorption spectra we got for PEG and PSSS AgNTs correlated very well with the dipole approximation made for a triangular nanoparticle from previous literature (Munechika *et al*., 2007). For our set of PEG AgNTs, the peak due to the in-plane dipole oscillation appeared at 648 nm and the shoulder between 450-500 nm was due to the collective in-plane quadrupole oscillations. Likewise, for the PSSS AgNTs, the in-plane dipole oscillation peaks appeared at 595 nm and the in-plane quadrupole oscillation was between 350-450 nm. If we compared our in-plane dipole absorption maxima of PEG AgNTs at 648 nm in figure 3 to the absorption maxima at 800 nm as presented by Zhang and coworkers in their paper, there was a clear shift in peak. The in-plane dipole had blue-shifted because of the decrease in size of the edge length of the nanotriangles from 75 to 40 nm. This blue-shift can be assigned to the decrease in charge separation during plasmon oscillation due to the small size. Also, both the PEG and PSSS AgNTs spectra, in figure 3 and 8, have a short peak at 330 nm. This peak could be due to the presence of atomic silver, as silver ionizes around this energy or could be due to the out-of-plane dipole oscillations or could be small spherical silver nanoparticles that did not grow into triangles (Wu *et al*., 2015).

A preliminary study on the effect of silver nanotriangles on the growth and killing of bacteria was made using a qualitative and a quantitative test. The qualitative test was performed to determine whether the bacteria grow in the presence of silver nanotriangles. From our results of inoculating nanotriangles with *E. coli* on an agar plate, it was clearly distinguishable that there was a reduction in the number of colonies of *E. coli* when compared to the negative control. This meant that both PEG and PSSS AgNTs were capable of preventing growth to an extent. Previously, Raza and coworkers (2016), Tanvir and coworkers (2017) and Pal and coworkers (2007) have tried to study the size and shape dependent effects of silver nanoparticles on gram negative and gram-positive bacteria. Their nanoparticles had completely inhibited the growth of *E. coli* on an agar plate whereas in our case there was only reduction in the number of colonies. Quantitatively it got extremely difficult to compare our results with their work because we weren't successful in determining the concentration of our particles in solution. This was due to the small size of our nanoparticles and volatile nature of the colloidal solvent. Even Mass Spectroscopy analysis failed because the nanotriangles aggregated in methanol. Additionally, they have incorporated results from the Kirby Bauer test which we hadn't performed. The quantitative analysis was made to determine whether the silver nanotriangles can kill pre-existing bacteria in media. Despite not knowing the

concentration of silver nanotriangles, we made a quantitative analysis by making a series of dilutions of nanotriangles to media and inoculating them with a bacterial density of 0.3 O.D. One observation was that the nanoparticles did not precipitate in media which meant that they could be used in biological applications. From the results based on the growth curve of *E. coli* in the presence of PEG and PSSS AgNTs in figure 6 and 11, we saw that the curve of the highest concentration of nanotriangles (sample 1) for both PEG and PSSS AgNTs had a decrease in O.D. over a time span of 24 hrs, which meant that it was not only killing the pre-existing bacteria but also was preventing the growth of new bacteria. It followed the trend exactly similar to the positive control which was 70 % and 99 % ethanol. In the case of the diluted samples 2, 3 and 4 for both PEG and PSSS AgNTs, in figure 6 and 11, there was a slight decrease in O.D. as compared to the negative control, but the curve still represented a well-defined bacterial growth curve, which meant that the lesser concentrated samples of PEG and PSSS AgNTs restricted the growth to a certain extent.

A key observation was made in the graph of PSSS AgNTs with the sample 2 (2/5 dilution) curve in figure 11. There was a sharp decrease in the slope for an initial time period of two hours, however, later it resumed a steady growth. This trend of sharp decrease in O.D. was not spotted in the graph of *E. coli* with PEG AgNTs of the same dilution. What was interesting is that, even a lesser concentrated sample was able to push the growth curve towards the death phase for an initial time period of 2 hrs. The probable explanation could be from the theory proposed by Raffi and coworkers (2008), that smaller nanoparticles are more toxic to bacterial cells (Raffi *et al*., 2008). From the size distribution histogram in figure 4b, we had majority of PEG AgNTs with an average edge length of 40-50 nm, while majority of PSSS AgNTs in the histogram in figure 9b, had an average edge length of 20-30 nm. This explains why PSSS AgNTs were able to kill more bacteria as compared to PEG AgNTs at the same dilution and in the same time. Another possible explanation could be that, the number of nanoparticles present in the sample of 2 (2/5 dilution) of PSSS AgNTs was higher as compared to that of PEG AgNTs of the same dilution. Since the concentration was unknown, it is hard to comment anything about this explanation and compare our results with previous findings.

6. Conclusion and Future prospects

In conclusion, we were successful in reproducing silver nanotriangles using the protocol from Zhang and co-workers (2011) and Aherne and co-workers (2008). However, scaling up to higher volumes of nanotriangles synthesis was only possible for PEG AgNTs. The nanotriangles of PEG were triangular with sharp edges while the PSSS AgNTs had a mix population of quasi-spherical to triangular with blunt edges. From our antibacterial testing results, it is clear that that both the types of nanotriangles are able to kill bacteria at higher concentrations. Due to time restrains, we were not able to test the effect of our nanotriangles of PEG and PSSS AgNTs on cellular and immunological functions. It would also be interesting to test the washed nanoparticles against these bacteria, to know if the killing effect comes from the nanoparticles or the buffer they are in. A lot more could be commented about its applicability as an antibacterial agent had we got some preliminary results from making experiments like the cell viability assay, studying the uptake mechanism of nanoparticles by cells using fluorescence microscopy and flow cytometry, testing the production of pro inflammatory cytokines after exposure to these nanoparticles, effect of nanoparticle on the cellular metabolic activity using the MTT assay and Haemolysis assay.

Another crucial result would to be find a technique to quantify the number of nanotriangles in the aqueous solution, because for any pharmaceutical application it is important to know the MIC of the compound. One possibility would be to use simulations to identify the extinction coefficient and then apply the Beer Lamberts law to find the concentration. The other method would be to use dynamic light scattering to determine the size distribution profile of nanoparticles in the solution. Knowing the diameter of the nanoparticle from this technique one can measure the extinction spectra for quantifying the concentration.

The results presented in this thesis only studies the effect on a lab strain of *E. coli*, thus a hospital strain or a resistant strain would throw mores light and impact on this thesis. Also, nanoparticles behave differently with gram-negative and gram-positive bacteria (Priebe *et al.*, 2017). It would be worthwhile to consider doing these same set of experiments on gram positive bacteria. Another interesting insight to this project would be study properties of silver nanotriangles bound to metallic surface and protein coronas. Binding of nanoparticles to a surface and formation of protein coronas alter the properties of nanoparticles to a great extent. Both these studies would be crucial for this project because nanotriangles would be coated on surgical instruments and since the surgical instruments would come in contact with the body tissues and fluid, it would tend to form protein coronas around the nanoparticles.

Silver nanotriangles seem to be promising candidates as biocidal agents but further investigation of its efficiency in real hospital conditions is needed.

7. References

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