Department of Chemical Engineering

PhD Comprehensive Examination

High-throughput method for the mechanistic understanding of anti-bacterial properties of silver nanoparticles

Candidate:

Amir Kazemi

Examining Committee:

Dr. David Latulippe

Dr. Carlos Filipe

Dr. Younggy Kim

Committee Chair:

Dr. Li Xi

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1. Introduction

Biocides are commonly used for their anti-microbial properties to achieve lower growth rates of bacteria and other microorganisms. The anti-microbial function can be achieved using either organic chemicals (e.g. chlorinated phenols and quaternary amines) or inorganic compounds such as silver[1]. Anti-microbial properties of silver were known since a long time ago when people used silver containers to preserve food and beverages[2]. In the 19th century silver nitrate containing eye drops were used as a child care product[3]. In early 20th century, colloidal silver was used as medicine[2], however, their use was halted due to the progresses in antibiotic production.

During the recent decades, with the development of nanotechnology, colloidal silvers with the size range of 1-100 nm, known as nano-silver or silver nanoparticles (SNPs), have gained great attention among the researchers considering their wide applications. Despite the general consensus, there are reports[1] which claim that SNPs have been used for more than 100 years and have been registered in the United States since 1954. Regardless, different applications including but not limited to alternative medicines, drug delivery, personal care products, bioactive sensors, water purification, anti-bacterial ceramics, and textile industry products have been studied during the recent years. However, there are still numerous unsolved challenges related to SNPs such as development of facile and effective synthesis methods, toxicity to different organisms, mechanism of action, environmental and health effects, and statistical optimization of synthesis and operating conditions affecting the bactericidal activity.

2. Literature review

2.1. Silver nanoparticles (SNPs)

As mentioned above, silver can be engineered into colloidal nanoparticles typically with a size range of 1-100 nm. SNPs, like other nanoparticles, show enhanced physicochemical properties as well as biological-related activities due to their larger available surface area. SNPs are produced in various shapes and sizes which significantly affect the activity of the nanoparticles[4–6]. Fig. 1 shows transmission electron microscopy (TEM) and scanning electron microscopy (SEM) images of a number of sizes and shapes reported for SNPs. In general the smaller the size of the particle, the more bioactive surface area is available. Furthermore, triangle nanoparticles are reported as being more efficient than other common shapes such as spheres[5], although the effect of surface area of the SNP was ignored in that report when comparing shape effect on anti-bacterial properties.

2.2. Use of SNPs

In most of the applications relevant to SNPs, the anti-microbial properties of the nanoparticle is of great importance. For example, SNPs are being used in clothes, personal care and household products as an anti-microbial agent[7]. Some of the common applications of SNPs are briefly described in the next sections.

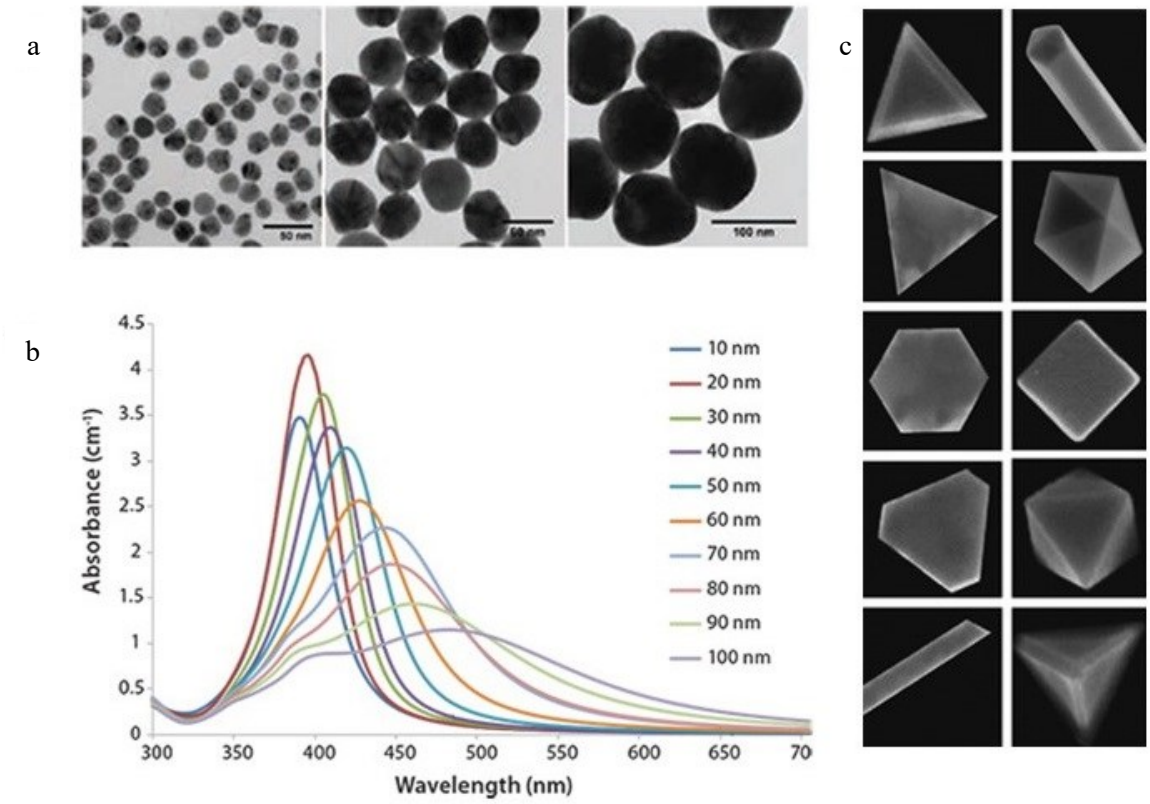


Figure 1. a. TEM image of 20, 60 and 100 nm spherical SNPs (credit: Sigma). b. UV-Vis spectra of 10-100 nm (credit: Sigma). c. SEM image of non-spherical SNPs (adapted from Walters et al.[8]).

2.2.1. Medical applications

Enhanced wound healing using SNPs has been reported in several publications[9]. Wright et al.[10] have studied the potential of SNPs to reduce the number of viable antibiotic resistant bacteria. Tian et al.[11] have investigated the wound healing potential of SNPs and found that healing speed depends on SNP dose. They have also reported inflammation reduction and modulation of fibrogenic cytokines due to the anti-microbial properties of SNPs. Skin drug delivery is another area in which SNPs are being widely used[12].

SNPs can be used for diagnosis, imaging and biosensing as well. Bio-sensors based on SNPs can be used for sensing a variety of proteins that are not detectable with normal biosensors[13]. This feature allows for detection of various diseases. For example, for cancer diagnosis, rod-shape Au–Ag nanoparticles were used as a platform for enhanced multivalent binding by multiple aptamers. The produced nanorod-aptamer conjugates were found to be potentially useful in particular cell detection and targeting in cancer diagnosis and therapy[14]. In the field of imaging and detection the development of surface-enhanced Raman spectroscopy (SERS), as a strong analytical tool, is influenced by application of SNPs in order to prepare the surface. Silver nanorods were recently used in the structure of a SERS which was employed to achieve very sensitive detection of the respiratory syncytial virus[15].

For years, polymers such as polymethylmetacrylate and ultra-high molecular weight polyethylene have been used in orthopaedics field for replacing the artificial joints. However, these

materials may cause infection in the body. Recent studies have used SNPs containing polymers to reduce the risk of infection[16,17]. SNPs have been reported to be suitable as an anti-infectious agent in terms of central venous catheters and ventricular drainage catheters, which are widely used in hospitals but are prone to infection-related incidences and are mostly treated with antibiotics[18,19], as well. Wong and Liu[9] suggested that the future trend will be in development of anti-inflammatory agents, antiviral drugs, and anti-platelet agents using SNPs as well as studying the effect of SNPs on proliferation of the stem cells. For example, in one of the recent studies, Das et al.[20] synthesized silver nanopartciles using $AgNO_3$ and *Ocimum gratissimum* leaf extracts to destroy multidrug resistant bacteria.

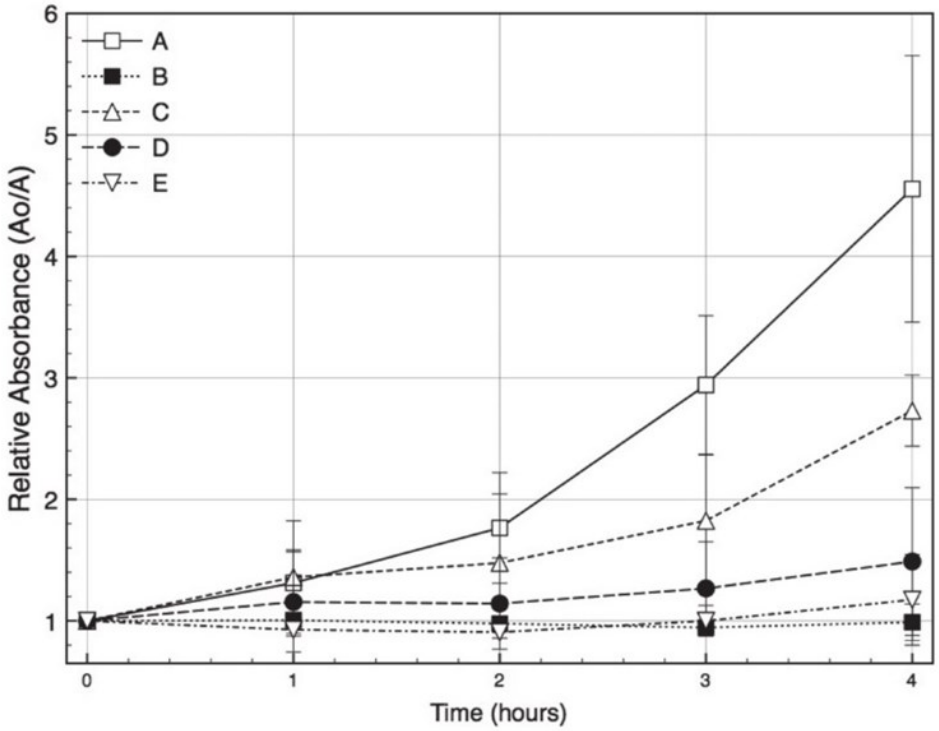
2.2.2. Water treatment

During the recent years nanotechnology has been employed in several studies related to water and wastewater treatment[21]. Due to their strong anti-microbial properties, SNPs have been considered as a potential water disinfection and treatment technology for a long time. Because of the uncertainties about the toxicity and health effects of SNPs and to eliminate an extra separation step, most studies have considered the modification or coating of different solid structures such as papers[22–24], polymeric and ceramic membranes[25–32], and beads[33,34]. Jain and Pradeep[22] impregnated polyurethane (PU) foam with SNPs and used it as an anti-bacterial water filter. They have performed experiments in batch and continuous modes and reported undetectable concentration of *Escherichia coli* ATCC 25922 and *E. coli* MTCC 1302 species in the treated water when using feed waters with 10^3-10^6 colony-forming units per milliliter (CFU/mL).

One of the famous studies in this area was performed at McGill University[23] which was later referred to as 'the drinkable book' in which Dankovich and Gray[23] coated absorbent blotting papers with SNPs by using $NaBH_4/AgNO_3$ in-situ[1] nanoparticle formation. The presence of SNPs on the paper was shown using the reflectance spectra of the papers with UV-Vis reflectance spectroscopy and the concentration of the nanoparticles on the paper was quantified by acid digestion of the paper followed by an inductively coupled plasma atomic emission spectroscopy. In each test 10^8 CFU/mL of a gram-negative bacteria (a strain of *E. coli*) and a gram-positive bacteria (a strain of *Enterococci faecalis*) was passed through the coated paper. The bacterial counts in the effluents were tested using two methods. In one method, the effluent bacteria was grown using a fresh nutrient after centrifugation and separation of the silver and absorbance values at 600 nm were measured every hour to monitor the growth kinetics. The other method was based on counting of bacterial colonies incubated for 24 h at 37 ºC on Agar plates. The SNPs content was determined for both bacteria rich portion and supernatant after centrifugation using graphite furnace atomic absorption. The SNPs loss from the papers was determined using the same absorption technique and inductively coupled plasma atomic emission spectroscopy (ICP-AES). TEM images were used for measuring the average diameter of the nanoparticles. The results indicated that effective bactericidal activity for *E. coli* bacteria suspensions can be obtained with 10 min percolation time. Furthermore, very low contents of SNPs were observed in the permeate water. Recently Dankovich have successfully used another technique based microwave radiation for the coating of SNPs on paper[24]. Fig. 2 is adapted from both mentioned studies[23,24] and shows the inhibitory effect of the paper coated with SNPs and successful coating of the papers using the microwave-assisted synthesis technique.

[1] In-situ formation is generally defined as the formation of nanoparticle on a support such as polymers and ceramics when the material is present in the solution when the reaction is occurring.

I



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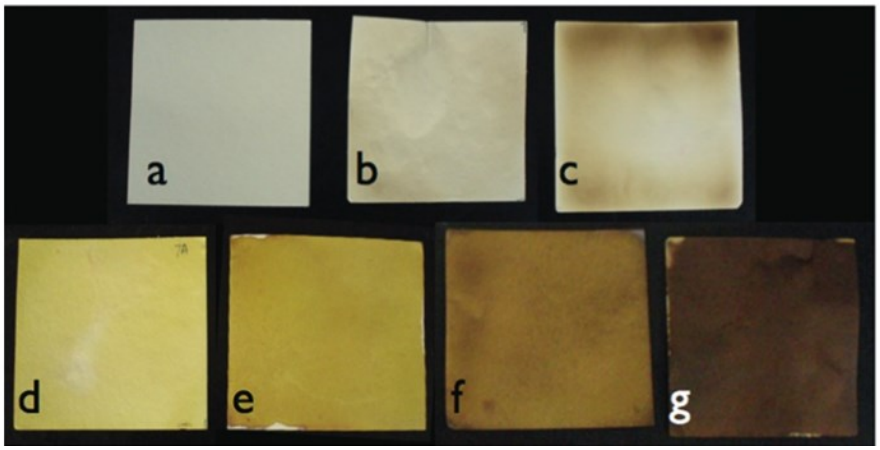

Figure 2. Panel I: *E. coli* regrowth kinetic after filtering through SNP containing paper: A. Positive control (Blotting paper). B. Negative Control (no bacteria). C. 1.6 mg SNP/g paper. D. 2.3 mg SNP/g paper. E. 5.7 mg SNP/g paper. Panel II. Micro-wave-assisted SNP impregnation on paper: a. Blotting paper. b. Microwaved paper. c. Microwaved paper in 1 M glucose. d. Paper coated with 1 mM $AgNO_3$. e. Paper coated with 10 mM $AgNO_3$. f. Paper coated with 25 mM $AgNO_3$. g. Paper coated with 100 mM $AgNO_3$. 0.1 M glucose and 4-6 min heating with microwave was used to form SNPs. (reproduced[23,24])

2.2.3. Other applications

Kumar et al.[35] reported a green approach to synthesize SNPs containing paint with anti-microbial properties. SNPs have been used in food preservation industry as well[36,37]. Fernandez et al.[36] successfully produced a SNP containing cellulose paper by in-situ reduction of silver ions and used it for preservation of melon slices. Results showed strong anti-bacterial activities when using the silver nanoparticle containing papers. Gottesman et al.[37] demonstrated a new ultrasonication

technique for SNP containing paper production. According to their observations, it was suggested that the paper can be used as an anti-microbial agent for food preservation since it is efficient against both gram positive and gram negative bacteria. SNPs are also being used in personal care products. Human skin is populated with different apathogenic bacteria depending on multiple factors. To reduce this microflora, SNPs have been incorporated in deodorants, food powders, socks, and fibers used for clothes and bedding products. SNPs can be used in child products[38], nanofiber and nanocomposites with applications in textile industry[39] and pet-related products[40].

2.2.4. Synthesis of SNPs

SNP synthesis is generally based on the formation of silver colloids dispersed in water or organic solvents. Colloidal silver has a larger surface area with higher conductivity and activity. In order to form colloidal silver, chemical reduction is normally used along with a silver salt (e.g. $AgNO_3$) and a stabilizing agent. Some of the common chemical reductants in this regard are sodium borohydride ($NaBH_4$)[5,6,24,41–43], trisodium citrate (TSC)[6,42] and elemental hydrogen[44,45]. Silver atoms are formed based on silver ion reduction ($Ag^+ \rightarrow Ag^0$) after which agglomeration causes colloidal nanoparticles formation. Employment of strong reductants results in formation of small particles. However, formation of large particles with slow reductants is difficult since generally a wide size distribution is obtained. Large particle formation can be performed using a bottom-up approach[46] which is based on a two-step method. First, a strong reducing agent is used in order to form small colloidal particles[6,45]. Next, these particles are enlarged by further reduction using a weaker agent like TSC. Furthermore, it is reported that the size of the silver nanoparticles depends on the concentration of the silver salt (i.e. $AgNO_3$) and the concentration of reducing and stabilizing agents. Silver colloidal nanoparticles have a yellow color solution and a spectral peak between 390-420 nm[24,43,45].

Green synthesis of SNPs, which is based on selecting nature friendly reducing agents as well as nontoxic solvents and stabilizers, is reported in a number of recent studies[20,45]. For example, monosaccharides and polysaccharides can be used as strong reducing agents for SNP production[47]. Heparin, and Cellulose, are among the natural polysaccharides with potential for silver ion reduction and nanoparticle production. In most cases, reduction using polysaccharides should be performed at temperatures higher than room temperature and the particle size highly depends on the reaction time and temperature[45]. A number of chemical and green reducing agents are shown in Fig. 3.

Another method for reduction of silver ions and nanoparticle generation is irradiation[45]. Laser irradiation was used by Abid et al.[48] in the presence of the sodium dodecyl sulfate (SDS) as a surfactant to form SNPs in a well-defined shape and size distribution. Microwave irradiation was successfully used in the presence of glucose and $AgNO_3$ by Danakovich[24] for in-situ formation of SNPs on a blotting paper for water disinfection. Among the other irradiation methods, ionizing radiation and radiolysis techniques have been successfully employed for SNP production[45].

Use of Tollens' reagent which involves reduction of $Ag(NH_3)_2^+$ is another common method for SNP synthesis. This reagent has a short shelf life and should be produced freshly in the lab. Normally, Tollens' reagent is reduced by an aldehyde according to reaction (1):

$$Ag(NH_3)_2^+{}_{(aq)} + RCHO + 2OH^- \rightarrow Ag_{(s)} + RCOOH_{(aq)} + 2NH_{3(aq)} + H_2O \quad (1)$$

Furthermore, a modified version of Tollens process can be performed using saccharides as reducing agent in the presence of ammonia. The size and morphology of SNPs strongly depend on

the concentration of ammonia and reducing agent as well as the nature of the reducing agent which is used for the synthesis.

Some of the biological organisms or their extracts (such as proteins, enzymes, amino acids, and vitamins) can play the role of reducing agent for nanoparticle production. Protein extracts from *Chlorella vulgaris* can be used as a reducing and size controlling agent in formation of SNPs[49]. The reducing mechanism was attributed to the presence of Tyr residues. *O. gratissimum* leaf extract was used as a reducing agent at pH 10 for the formation of SNPs with 20-30 nm diameters which were used for destroying multi-drug resistant bacteria[20]. Several other studies have reported Ag^+ reduction and SNP formation by usage of biological systems such as fungus[50–52], yeast[53], plant extracts[54] and bacteria[55,56] based on different extracellular and intracellular mechanisms which generally involves enzyme/protein oxidation. Fig. 4 shows an example of green synthesis of SNPs using *O. Sanctum* extract and sunlight[54]**.**

One of the important topics related to synthesis of SNPs is the application of different capping (i.e. stabilizing) agents, such as surfactants, to adjust the particles size and morphology by changing their stability. Low stability of the nanoparticles is a major concern which occurs when the surface charge density is not high enough. This phenomena can result in aggregation and formation of larger particles with altered characteristics and anti-microbial properties[57–59]. Some of the common capping agents for SNPs are cetyltrimethylammonium bromide (CTAB), TX-100, Tween 80, SDS, polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), polysaccharides, and citrates. It is worth mentioning that some of the green reducing agents such as polysaccharides and some of the chemicals such as citrates can play the role of reducing and capping agents simultaneously.

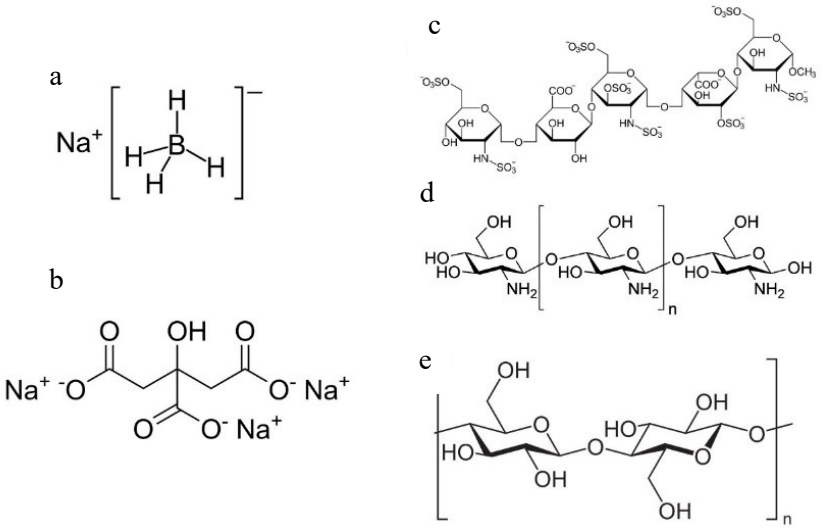

Figure 3. Examples of reducing agents used for SNPs synthesis. Chemical reducing agents: a. $NaBH_4$ (strong reducing agent). b. TSC (weak reducing agent). Green reducing agents: c. Heparin. d. Chitosan. e. Cellulose (credit: Wikimedia).

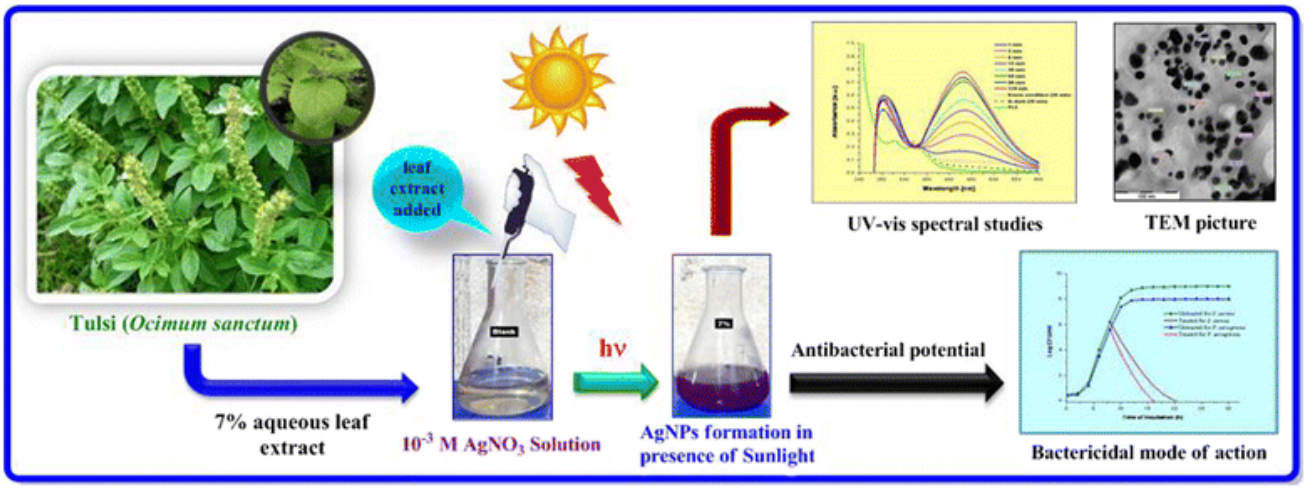

Figure 4. Green synthesis, characterization and analyses of SNP using *Ocimum sanctum* and sunlight with $AgNO_3$ (adapted from Brahmachari et al.[54]).

2.2.5. Characterization and anti-bacterial performance of SNPs

One of the important steps in performing experiments with SNPs is characterization. Characterization can be used in order to determine factors such as size, morphology and stability of the SNPs. The methods reported through different works related to SNPs are generally among the following techniques.

UV-Vis spectroscopy is commonly used for showing the presence of SNPs in solutions by obtaining a peak in 390-420 nm region in UV-Vis spectrum. Furthermore, absorbance readings at wavelengths near the peak can be used for determining the concentration of SNPs. Other methods such as Atomic absorption spectroscopy (AAS)[23,60,61] and Inductively Coupled Plasma Mass Spectrometry (ICP-MS)[5,62,63] can be used for the concentration measurement of SNPs in the solution. ICP-MS integrates high temperature of the plasma with mass spectroscopy. ICP has a greater speed and sensitivity in comparison with other techniques for the detection of the elements such as AAS.

TEM, SEM and atomic force microscopy (AFM) are among the most common techniques related to SNPs characterization. They can be used with several purposes such as determining the size and size distribution of the nanoparticles as well as monitoring their morphology and anti-microbial activity. According to previous studies[64], the advantage of AFM is the measurement of 3D images which is helpful for understanding the morphology of the nanoparticles. X-ray diffraction (XRD) is used for analyzing the size and the crystal structure of SNPs since it can identify individual crystals[65]. Additionally, Fourier transform infrared (FTIR) spectroscopy can be used for qualitative analysis and detection of different functional groups[66].

Dynamic light scattering (DLS) is a characterization method which is used for measuring the hydrodynamic radius (R_h) of SNPs based on Brownian motion. R_h refers to the radius of a sphere with the same diffusion coefficient as the particle in the solution and it is calculated using Stokes-Einstein equation[2] and thus it depends on solution condition. In the interfacial double layer, zeta potential (ζ) is the potential at the slipping plane location relative to the bulk fluid (Fig. 5). Zeta

[2] Stokes-Einstein equation: $R_h = \frac{k_b T}{6\pi\eta D}$ where k_b is the Boltzman-constant (J/K), η is the viscosity (cP), T is the temperature (K), and D is the diffusivity (m^2/s).

potential is widely used as a measure of particle stability in solutions containing SNPs. The majority of zeta potential analyzers which are commercially available work based on electrophoretic mobility, however, ζ can be measured using instruments that work based on electrokinetic and electroacoustic phenomena as well.

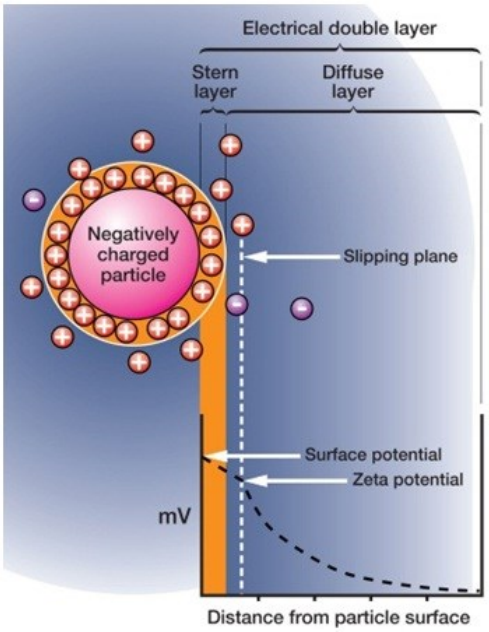

Figure 5. Schematic demonstration of zeta potential for a negatively charged particle (credit: pharmainfo.net).

Anti-bacterial properties of silver nanoparticles can be studied using different methods. Bacterial counting can be performed either manually or using automated cell counters. Alternatively, indirect methods such as measuring the optical density or fluorescence measurement as a result of bacterial activity (such as fluorescent protein production) can be employed. Indirect methods are more facile and appropriate for automation. Furthermore, microscopy and different bioassays can be used for studying the mechanism of action of SNPs which will be further explained in section 3.3.7.

2.2.6. Mechanism of action

Mechanisms of anti-bacterial activities of SNPs are not fully understood. However, there are three major pathways which are suggested in previous studies[67] as the anti-bacterial mechanisms (Fig. 6). First, although some studies suggested SNPs are not toxic by themselves, silver ions may be produced as a result of different paths in the presence of SNPs. Reaction 2 shows one of the most common pathways reported by earlier researchers [68,69] for Ag^+ generation:

$$4Ag_{(s)} + O_2 + 2H_2O \rightarrow 4Ag^+ + 4OH^- \quad (2)$$

Note that presence of O_2 is the main factor which promotes Ag^+ generation.

Next, SNPs have the potential to act as a catalyst and to generate reactive oxygen species (ROS). ROS is naturally present as a result of metabolism of some types of organisms and is controlled by the antioxidant defenses. However, the elevated concentration of ROS results in membrane damage, and similarly, mitochondrial function loss in eukaryotic cells. Third, it is proposed that SNPs can cause direct damage to the cell membrane which may change the proton gradient, increase membrane permeability and cell death[67].

To be more specific concerning the potential mechanisms, SNPs and ions produced from SNPs interact with enzymes of the respiratory chain and prevent ATP synthesis. The metabolic activity of the bacterial cell may be affected as well since SNPs have been reported to affect the mitochondrial function in eukaryotic cells and similarly, cell membrane in prokaryotic cells[67,68]. Furthermore, the ROS generation can cause severe DNA damage due to oxidative stress which results in generation of strand breaks and increasing generation of abasic (AP) sites[70]. Moreover, the interaction of silver ions with the thiol groups and disulfide bonds formation can damage bacterial proteins and result in cell death[67,71]. Additionally, a few of previous toxicity studies have suggested lipid peroxidation as a mechanism of anti-microbial activity[72,73]. In this mechanism, free radicals take an electron from lipids in the cell membrane which results in formation of highly reactive and unstable compounds. This mechanism affects cell membrane fluidity and permeability. Lipid peroxidation is more likely to occur for unsaturated lipids. Lastly, interfering with the cell proliferation process is another activity observed by silver ions in the previous studies[31,67,74].

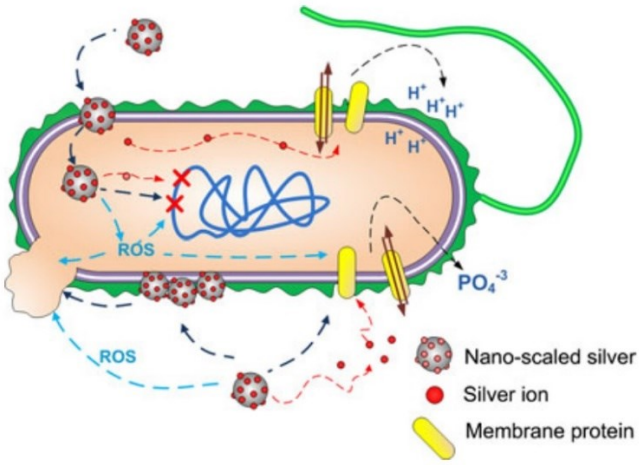

Figure 6. A number of proposed routes for anti-bacterial properties of SNPs (reproduced from Marambio-Jones et al.[67]).

2.2.7. Environmental toxicity and health effects

Although the anti-microbial toxicity of silver (including SNPs) is reasonably valuable, there are numerous contradictory reports on toxicity of silver ions and SNPs for the environment[72,75–80] and the higher cell lines such as zebra fish, *Drosophila melanogaster* (fruit fly)[72], *Caenorhabditis elegans* (a type of roundworm)[81], rats[82,83], and humans[68,84]. The environmental state and toxicity of SNPs is not only related to their concentration and morphology but it also depends on the capping agents used for stabilization, the pH, and the concentration of the other cations and anions in the aquatic environment.

The major paths of human exposure to SNPs are skin (e.g. from different products and cloths containing SNPs), respiratory system (e.g. at the manufacturing site), or ingestion (e.g. through water or food preservation packages). According to Ahamed et al.[75], SNPs pose serious potential risks in long term. For example, a number of studies have reported effects on brain, lung, reproductive organs, vascular system and liver. Furthermore, it has been reported that SNPs may result in cell leakage and may have an adverse effect on mitochondrial function[67,75,84]. US National Center for Complementary and Integrative Health and US Food and Drug Administration have issued warnings related to consumption of over-the-counter drugs containing silver and SNPs due

to health and safety concerns[85]. As a results, toxicity of SNPs is an important research area which requires further studies in the future.

3. Proposal

3.1.Objectives

The anti-bacterial properties of SNPs are reported in several studies with different applications in biotechnology[19,86], textile[87] , water[32], and food industries[36]. However, these anti-bacterial activities depend on numerous factors such as synthesis method, shape, size, chemical composition, solution condition (i.e. concentration of SNPs, pH, ionic strength, and type of ions present in the solution), and type of bacteria. Furthermore, synthesis of novel structures containing SNPs is not only reported to have a significant effect on nanoparticle's morphology and applications, but it has also affected the anti-bacterial activity and mechanism of action.

SNPs are being used for different applications. Hence, it is important to understand their anti-bacterial properties under different operating conditions in each application. To elaborate, understanding of anti-bacterial properties of dispersed SNPs can be used for *in vitro* toxicity tests in drug discovery field[88]. Besides, development of different materials which contain SNPs has raised questions about their safety and anti-bacterial efficiency. Therefore, high-throughput (HT) anti-bacterial testing of materials containing SNPs will be a key. In water treatment field, the disinfection step can be conducted by stabilizing SNPs on different structures (e.g. porous structures). As a result, development of a HT testing module is of a great importance in this field in order to understand the water disinfection processes and to perform more efficient processes. The proposed research aims to solve these challenges and to facilitate the study of anti-bacterial properties of SNPs in different applications in three HT format *phases*:

- *Phase I.* High-throughput screening of bacterial toxicity (BT) of SNPs.
- *Phase II.* High-throughput screening of anti-bacterial activities using a microscale magnetic material containing SNPs (MSNPs).
- *Phase III.* Understanding the water disinfection process using SNPs by design and development of high-throughput water disinfection (HTWD) module.

Process optimization is the important long term goal of most processes in different fields. One of the main goals of designing high-throughput screening (HTS) systems is to implement a strategy to reach the optimum in a rapid, efficient and inexpensive manner. Hence, it is vitally important to use an appropriate statistical methodology for the method and to design the HT experiments based on that particular methodology.

In addition to HTS methods, which are mostly based on performing different chemical and biological reactions, separation processes and assays by means of microplate readers in different modes (i.e. absorbance, fluorescence and luminescence), high-content screening (HCS) has been offered as a unique technique to investigate the effect of nanoparticles on biological systems such as bacteria[89–91]. HCS is an automated screening tool which integrates the efficiency of HTS techniques with microscope imaging. By employing HCS, instead of obtaining a single readout (e.g. microplate readers in HTS) numerous images are captured and analyzed from each well during different steps of cell activity or assaying. Although HCS is beyond the scope of this proposal , this concept is widely used in drug discovery field and has a great potential to be employed in investigation of nanoparticles interaction with bacteria and higher order organisms[91,92] and it can be considered as an eventual long-term objective after completion of the HTS objectives.

Based on the information provided above, the short and long-term objectives of this project are summarized as follows:

3.1.1. Short term objectives

- To prepare an initial experimental design platform based on statistical methods for the proposed phases (section 3.3.1).
- Synthesis and characterization of the proposed SNPs for BT studies (section 3.3.2).
- Synthesis and characterization of MSNPs (section 3.3.3).
- Design and development of a high-throughput water disinfection (HTWD) module (section 3.3.4).
- To study the anti-bacterial mechanism of action of SNPs by using HT biological assays (section 3.3.7) in different phases.

3.1.2. Long term objectives

- To analyze the results obtained from different phases and to perform further experiments under more operating conditions if required.
- To employ a proper optimization and modelling approach for understanding the anti-bacterial properties of SNPs for each application.
- Design and development of future experiments which will be based on employing HCS as a novel tool for understanding the anti-bacterial activities of SNPs.

3.2. Literature pertinent to the proposal

3.2.1. High-throughput screening

High-throughput screening (HTS) is the process of testing a large number of different materials or experimental conditions while using a small amount of sample. Regularly, microplates, small containers which have multiple small wells, are the key tools in HTS techniques. HTS is successfully being employed in biotechnology and pharmaceutical industry to accelerate the discovery of new biomolecules. Numerous HT techniques for different processes such as chromatography [93,94], membrane filtration [95,96], bio-reactions[97,98], adsorption[99], aqueous two phase systems[100,101] and flocculation[102] have been developed during the past years to speed-up and to reduce the cost of process optimization as well. Mainly, miniaturized modules are designed and developed to be able to perform parallel experiments with small amounts of sample. In this way, it is possible to screen and monitor the effect of different operating conditions on the process. HTS techniques can also be integrated with robotic, liquid handling and real time measurement instruments.

3.2.2. Anti-bacterial properties of SNPs

As previously mentioned, the anti-bacterial activity of SNPs depend on several factors. As proposed by Damoiseaux et al.[89], to study the effect of nanomaterials on biosystems, the first step is to characterize the nanomaterial itself. For example, size, size distribution, shape, state of dispersal/aggregation, stability, and the zeta potential of the SNP have to be determined using the characterization techniques described in section 2.2.5. Several studies reported that bacterial death using SNPs is dependent on size and shape of nanoparticle[74]. Pal et al.[5] studied the bactericidal activities of SNPs of different shapes and observed that the shape affects the bactericidal properties. They synthesized and characterized spherical, rod-type and truncated triangular nanoparticles and cultured *E. coli* (ATCC 10536) on agar plates with different concentrations of SNPs. The inhibition of bacterial growth due to the truncated triangular nanoparticles was observed at a lower concentration compared to the other nanoparticles. However, the effect of surface area was ignored by the authors. The effect of size of SNPs on anti-bacterial efficiency was investigated by Agnihotri

et al.[6]. They synthesized SNPs with 10 different sizes (5-100 nm) by a two-step reduction of $AgNO_3$ using $NaBH_4$ and TSC. They observed that the anti-bacterial activity of SNP is inversely proportional with size of the nanoparticle most likely due to the available surface area.

Furthermore, the solution condition affects the interaction between SNPs and bacteria. El Badawy et al.[44] showed that environmental conditions such as pH, ionic strength, and electrolyte type as well as the method of synthesis have a significant effect on SNPs aggregation and their surface charge in aquatic environments. Jin et al.[79] studied the effect of different ions at constant ionic strength on SNPs and concluded that alkalinity and hardness of the aquatic environment play an important role in toxicity of SNPs against gram-negative (*Pseudomonas putida*) and gram-positive (*Bacillus subtilis*) bacterial species. The authors reported that gram-negative bacteria was more resistant. Presence of humic substances in the environment can play a significant role in bactericidal activity of the SNPs as well. Fabrega et al.[63] reported mitigated bactericidal action due to presence of Suwannee River humic acids caused by the sorption of humic acids on the surface of SNPs and consequent changes in the surface properties of SNPs.

After characterization of the nanoparticles, it is important to find the appropriate measures to study and monitor the anti-bacterial properties of SNPs [89]. In conventional studies, this part is heavily based on microscopy such as using fluorescence microscopy, SEM, and TEM. For example, Feng and colleagues[103] studied the anti-bacterial effect of silver ions on *E. coli* and *staphylococcus aureus* by means of TEM and X-ray microanalysis and reported that silver ion affects the replication ability of DNA and it interacts with thiol groups in proteins which results in inactivation of bacteria. Sondi and Salopek-Sondi[104] investigated the anti-bacterial effect of SNPs on *E. coli* as a gram-negative bacteria. Results from SEM and TEM images showed cell wall pitting and accumulation of SNPs on the cell membrane which causes high cell membrane permeability and cell death. Morones et al.[105] were the first to propose three inactivation mechanisms for gram-negative bacteria when being exposed to SNPs (cell membrane damage, DNA damage, and release of silver ions) by analyzing Scanning transmission electron microscopy (STEM) images. Flow cytometry is another method which is widely employed for determining the mechanism of action of different substances on cells and cell viability[106]. Furthermore, other conventional biological techniques such as counting of colony forming on cell culture plates have been performed to study the anti-bacterial properties of SNPs[23,62,107]. However, considering the large number of operating conditions affecting the anti-bacterial properties of SNPs, employing such techniques is both laborious and costly. Therefore, there is a considerable need for research in design and development of HT techniques which facilitate the understanding of anti-bacterial mechanism of silver nanoparticles under different operating conditions in different applications such as anti-bacterial materials, water disinfection and SNPs toxicity studies.

3.3. Methods and proposed approach

3.3.1. Experimental design

As emphasized earlier, there are several factors affecting the anti-bacterial properties of SNPs including but not limited to the method of synthesis, size, and presence of different ions, bacterial growth medium, pH, ionic strength, SNP concentration and biomass concentration. Hence, one of the most important steps for understanding the anti-bacterial properties of silver nanoparticles is to perform an experimental design based on statistical methods. For example, if we design our experiments based on 5 factors with 4 levels and another 5 factors with 2 levels, we will need to perform $4^5 \times 2^5 = 32768$ experiments. This will require around 340 96-well plates to be conducted without considering the negative-controls, positive-controls and replicate experiments which are all

necessary in order to get acceptable results. Therefore, one strategy is to select a sub-set of the required experiments using design of experiments methods such as factorial design or Taguchi method. Factorial design is generally more appropriate when each independent variable (i.e. factor) has two or three levels. On the other hand, Taguchi method can be performed when more levels are involved by selecting the appropriate orthogonal array and performing the experiments based on that particular array. Table 1 shows a summary of the proposed statistical method for this project based on factorial design.

Table 1. Summary of the factorial design proposed for the understanding of the anti-bacterial properties of SNPs.

Phase I			*Phase II*			*Phase III*		
BT	**Level**	**Coded level**	**MSNP**	**Level**	**Coded level**	**HTWD**	**Level**	**Coded level**
Synthesis method	SNP1	−1	Synthesis method	Immersed	−1	Synthesis method	Microwave	−1
	SNP2	+1		In-situ	+1		$NaBH_4$	+1
Bacteria	*E. coli*	−1	Bacteria	*E. coli*	−1	Bacteria	*E. coli*	−1
	S. aureus	+1		*S. aureus*	+1		*S. aureus*	+1
C_{SNP} µg/mL	20	−1	C_{AgNO_3} mM	1	−1	C_{AgNO_3} mM	1	−1
	50	+1		4	+1		10	+1
SNP size (nm)	~ 5	−1	CFU/mL	10^6	−1	CFU/mL	10^6	−1
	~ 30	+1		10^8	+1		10^8	+1
CFU/mL	10^6	−1	Media	Water	−1	Filter paper	Grade 2	−1
	10^8	+1		LB	+1		Grade 1	+1
Media	Water	−1	Effect of magnetic field	Off	−1	Mode of operation	Gravity	−1
	LB	+1		On	+1		Partial Vacuum	+1
$CaCl_2$ (mg/L)	0	−1	$CaCl_2$ (mg/L)	0	−1	NaCl (mg/L)	0	−1
	50	+1		50	+1		30	+1
$MgCl_2$ (mg/L)	0	−1	$MgCl_2$ (mg/L)	0	−1	$MgCl_2$ (mg/L)	0	−1
	50	+1		50	+1		50	+1
NaCl (mg/L)	0	−1	NaCl (mg/L)	0	−1	pH	6.5	−1
	30	+1		30	+1		8	+1
Aeration	Anaerobic	−1	SMPU size (mm^3)	8	−1	$CaCl_2$ (mg/L)	0	−1
	Aerobic	+1		64	+1		50	+1
			Aeration	Anaerobic	−1	Fulvic acid (mg/L)	0	−1
				Aerobic	+1		10	+1
						Filter Paper Pre-treatment	No	−1
							NaOH	+1

Note that this table is only to highlight the importance of using a proper statistical approach prior and during the HT experiments for understanding the anti-bacterial properties of SNPs and may be altered according to the importance of the factors, the interaction among the factors, and the results obtained during the experiments. The factors listed here are mostly based on the literature. It is suggested that synthesis method[41,42,45], bacteria type[108], concentration of the bacteria (based on CFU)[67,109], presence of oxygen[110,111] (listed in phases I and II), concentration of SNP (or silver salt used for synthesis)[6,23,24], SNP size[6], and the solution condition[57,63,79,112] including the present ions (e.g. Mg^{2+}, Ca^{2+} and Cl^-), presence of organic molecules and pH affect the anti-bacterial properties of SNPs. Moreover, moving MSNPs with a magnetic field, type of the filter paper, mode

of operation and presence of humic substances are considered as additional factors in phases II and III. Based on this table 2^{10}, 2^{11} and 2^{12} experiments have to be completed for HT testing of BT, MSNPs and HTWD phases respectively. However, with a factorial design we only need to perform 32 experiments (i.e. 2^{10-5}, 2^{11-6}, and 10^{12-7} for HT testing of BT, MSNPs and HTWD respectively) in each case which is achievable in triplicates in a single 96-well plate in case of the BT or MSNP experiments (3×32=96) and in the case of HTWD it can be completed in triplicates by running the setup 4 times (4×24=96). The experiments can be continued by analyzing the effect of factors and their interactions on the response variables (e.g. killing efficiency, cytotoxicity, cell viability, and log reduction value) if required. Further experiments may be performed by using optimization strategies such as response surface methodology (RSM) to obtain a better understanding of the process in a wider operating range of variables. Different factors for each phase mentioned in Table 1, such as synthesis method and microorganisms, are explained in detail through the next sections of this report.

3.3.2. High-throughput screening of bacterial toxicity (BT) of SNPs

In this phase, two different methods will be employed in order to synthesize SNPs with two different sizes (approximately 4.5 nm and 31 nm). In the first method[6] (SNP1), briefly, $NaBH_4$ (strong reducing agent) and TSC (weak reducing agent and stabilizer), will be heated (60 ºC) and mixed for 30 mins. Next the $AgNO_3$ solution will be added drop-wise (using a syringe pump) and the temperature will be raised to 90 ºC along with pH adjustment with NaOH (pH 10.5). According to Agnihotri et al., nanoparticle size will depend on the concentration of $AgNO_3$ and reducing and stabilizing agents. In this project the protocols for 5 (±0.7) nm and 30 (±5.1) nm nanoparticles will be followed. In the second method (SNP2), microwave-assisted synthesis will be performed using $AgNO_3$ and a solution of TX-100, 2,7-DHN, and NaOH under microwave irradiation[113]. According to the method, nanoparticle size will depend on the concentration of $AgNO_3$ and stabilizing and reducing agents. In this project the protocol for 4 (±0.6) nm and 32 (±3.5) nm nanoparticles will be followed. SNP1 and SNP2 synthesis methods are summarized in Table 2.

Table 2. SNP Synthesis methods for high-throughput understanding of anti-microbial activities in a batch process.

Name	Synthesis Method	Reducing Agent	Stabilizing Agent	Expected size (nm) and shape	Reference
SNP1	Two-step chemical reduction	NaBH4, TSC	TSC	5 (±0.7) nm 30 (±5.1) nm Spherical	[6]
SNP2	Microwave-assisted reduction	Irradiation 2,7-DHN	TX-100	4 (±0.6) 32 (±3.5) Spherical	[113]

The anti-bacterial experiments will be performed according to the experimental design. Since sample volumes greater than a single well of a 96-well plate may be needed for future analysis and bioassays, deep well 96-well plates (well volume ~ 2 mL) will be used in order to achieve different experimental conditions by addition of the appropriate type and concentrations of biomass, SNP and ions. As explained in section 2.2.6, oxygen presence is the key promoter for Ag^+ generation. Hence, a subset of experiments will be performed under anaerobic condition to understand the specific toxicity of SNPs in the absence of Ag^+. Briefly, first, the synthesized SNPs will be washed with 1% HNO_3 and will be deoxygenated by nitrogen purging followed by further purification through a 3 kDa UF membrane in stirred cell or in dialysis tubing format[111]. The tests under anaerobic condition will be performed using a simple anaerobic 96-well plate approach developed

earlier by Eini et al.[114] using AnaeroGen™ atmosphere generation system (from Oxoid), sealing material and BBL GasPak disposable anaerobic indicator (from BD). Alternatively, the experiments can be performed in an anaerobic chamber or incubator.

3.3.3. High-throughput testing of anti-bacterial activities using a microscale magnetic material containing SNPs (MSNPs)

For the second phase of this project, microscale magnetic material containing SNPs will be prepared. Jain and Pradeep[22] have discovered the potential of using PU as a support for SNP impregnation. They observed high anti-bacterial activities and proposed this system as a potential water disinfection technique. It was reported that the binding is due to interaction between nitrogen of the -N(H- of PU and SNPs. On the other hand, Zhou et al.[115] have synthesized a novel PU foam containing magnetic Fe_3O_4 particles. In this phase, a combination of these two approaches will be followed by synthesizing magnetic PU foams impregnated with SNPs.

The proposed method is summarized in Fig. 7. Briefly, first, the PU foam will be prepared by the method proposed by Zhou et al.[115]. Next, the foams will be cut in 2×2×2 mm and 4×4×4 mm pieces (appropriate dimensions for a 96 well plate). SNPs will be synthesized according to method used for SNP1. The pieces of magnetic PU will be immersed in a SNP solution (described elsewhere[22]) for 12 h followed by washing and characterization. Furthermore, in-situ synthesis of SNPs on magnetic PU foam will be performed. The same strategy described above will be followed in the presence of magnetic PU foam portions.

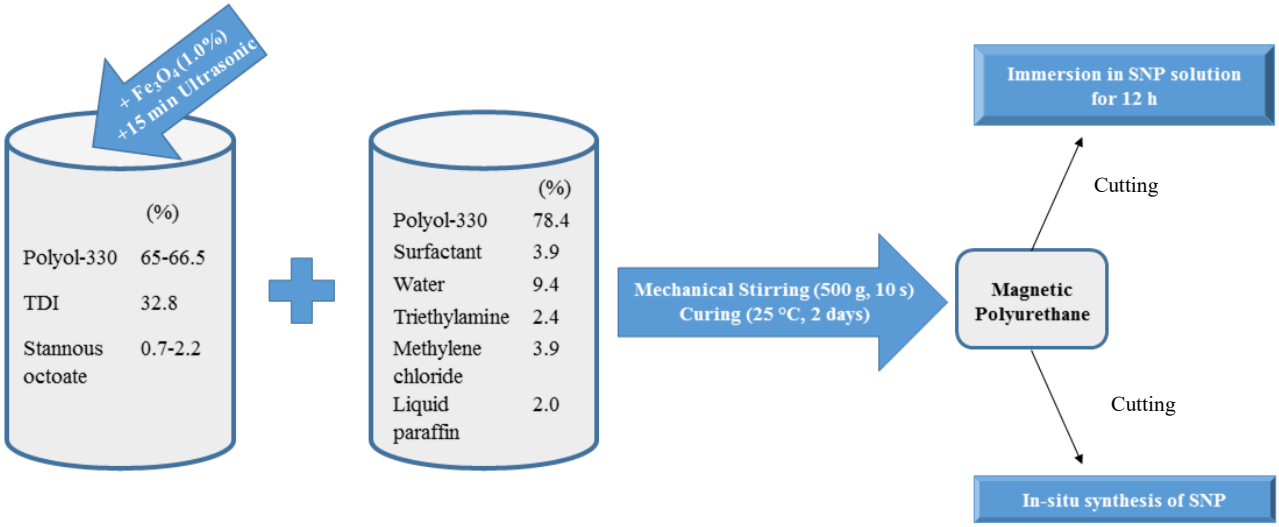

Figure 7. Summary of MSNPs synthesis process

In addition to practical applications of having a magnetic polymeric structure containing silver nanoparticles, one of the major benefits of this design is having the ability to remove the MSNPs from the microplate. This will be done using a 'robotic magnetic pin tool' (from VP scientific) which is designed particularly for microplates. In this way, SNPs will not interfere with the bioassays which will be conducted to understand the effect of SNPs on bacterial cells. Deep well 96-well plates will be used for running the experiments designed for this phase as well. Performing appropriate control experiments will be essential in this phase. For example, it is important to ensure that the potential decrease in biomass concentration is due to the presence of SNPs and not the magnetic PU foam. Control experiments with untreated magnetic PU will be helpful to determine whether the anti-microbial activity is due to the presence of silver nanoparticles or not. Results from

characterization and bactericidal effectiveness will be compared using the experimental design. Furthermore, the anti-bacterial properties will be compared with phase I to see the difference between anti-bacterial activities of the dispersed SNPs and the MSNPs. The anaerobic experiments will be performed using the strategy described in phase I.

3.3.4. Understanding of water disinfection process using SNPs by design and development of high-throughput water disinfection (HTWD) module

For phase III of this project, a HT technique will be developed to understand the bactericidal properties of SNPs using cellulose filter papers impregnated with SNPs. Whatman cellulose filter papers (grades 1 and 2 with max particle retentions of 11 and 8 μm respectively) will be used. Different methods and chemistries can be used for the synthesis and impregnation of SNPs on cellulose. In this report, two methods[23,116,117] will be briefly explained based on the literature. First, for experiments with pretreated papers, pre-treatment will be performed using NaOH described in previous studies[118]. Next, the filter papers (both treated and untreated) will be immersed in 100 mL of $AgNO_{3(aq)}$ solutions of 1 and 10 mM for 24 h. The solutions will be placed in an autoclave for 10 min at 121 ºC and atmospheric pressure. Based on Li et al.[116] cellulose plays the role of both reducing and stabilizing agents during the treatment at this temperature. However, other reports[24,118] suggest that the presence of glucose and NaOH will enhance the stability of SNPs impregnated on the papers. As a result, glucose and NaOH addition steps will be performed if the synthesized nanoparticles are not stable enough[24]. In the second method[23,117], the papers will be immersed in $AgNO_3$ solutions of the same concentrations which were used through the previous method for 30 minutes. After washing with ethanol, the filter papers will be placed in $NaBH_4$ solution for 15 min followed by a drying step in a vacuum dryer.

The high-throughput water disinfection (HTWD) module shown in Fig. 8 includes the following components. This design is inspired by simple laboratory filtration systems where, for example, a Buchner funnel is used as a filter paper holder:

- A mesh-well plate (with 24 individual wells) with 500 μm openings (from electron microscopy science). This component can alternatively be replaced by a custom block made in a machine shop with the same dimensions.
- Filter papers impregnated with SNPs and cut in diameter ≥ 15.6 mm (24-well plate standard well bottom diameter).
- Silicon o-rings (diameter ~15.6) to ensure sealing (from McMaster Carr).
- A 24 well plate insert with 420 μm openings (Prep-Eze™ from PELCO) as a flow director and holder for the filter papers and the o-rings.
- 24 well rack (Piro Tube Rack 24 Well SBS from LTF Labortechnik) and 24 centrifuge tubes (15 mL) as a sample collection platform to collect up-to 15 mL of treated water sample from each well. This sample collection design allows for the separate weighing of the permeate samples if required.
- A custom-designed vacuum manifold which is connected to a pressure regulator and a vacuum pump to control vacuum if required for the experiment (see Table 1).
- A multi-rack syringe pump which will be connected to flow distributors. In Fig. 8 it is assumed the experiment is being run using two different filter papers in triplicates for each operating condition. Therefore, four syringes ($24 \div 2 \div 3 = 4$) and four flow distributors with six outlets are shown in this figure. The number of syringes and flow distributors may vary depending on the experimental design used for each run. The

syringe pump flow rate will be adjusted to achieve a relatively constant hydrostatic pressure.

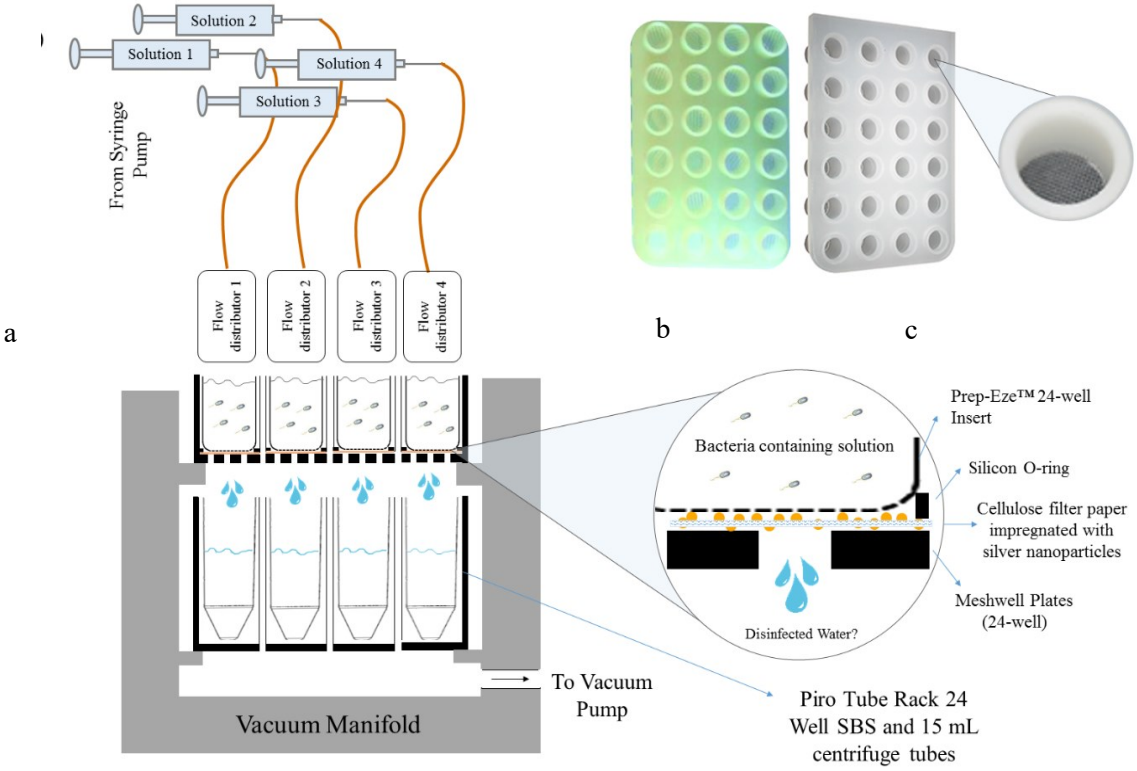

Figure 8. a. Schematic demonstration of the high-throughput water disinfection (HTWD) module for understanding the anti-bacterial mechanism of SNPs. b. Meshwell plate (24-well, electron microscopy science). c. Prep-Eze™ 24-well insert (PELCO).

3.3.5. Characterization methods

Dynamic light scattering (DLS)

Dynamic light scattering will be performed to measure the hydrodynamic diameter of the nanoparticles under different solution conditions (Table 1) using DynaPro Plate Reader II (Wyatt technology), a HT microplate reader which measures the dynamic light scattering based on Brownian motion.

Zeta potential measurement

Zeta potential of the nanoparticles in different solution conditions will be measured using Möbiuζ (Wyatt technology). This zeta potential analyzer is an automated instrument for zeta potential measurement based on electrophoretic mobility measurement. This system can be used with different sample handling systems and auto-samplers and requires only between 65-170 µL of sample for zeta potential measurement.

SNPs concentration

UV-Vis spectra will be recorded between 300-800 nm for nanoparticle samples to detect the peak related to SNPs between 390-420 nm (concentration and size dependent) using a microplate reader (Tecan M1000). The concentration of SNPs in the solutions will be determined using inductively coupled plasma mass spectrometry (ICP-MS). The concentration of SNPs will be measured in permeate water in HTWD setup and a subset of solutions treated with MSNPs using

ICP-MS to learn if the nanoparticles are released into the solution or not. For the detection of SNPs on paper and magnetic PU, UV-Vis reflectance spectra will be recorded between 300-800 nm as described elsewhere[22,23]. Additionally, the concentration of SNPs on papers and magnetic PU will be measured using ICP-MS after acid digestion (method will be adapted from[23]). It is worth mentioning that the ICP-MS system can be used with a compatible HT auto-sampler (e.g. SPS 4 Auto-sampler from Agilent) if required.

Electron microscopy

TEM will be used to obtain a better understanding about size and morphology of the nanoparticles. SEM will be used for surface characterization of MSNPs and filter paper samples in phases II and III. Unfortunately there is no HT method available for this part. However, given the limited number of samples it is not expected that these analyses will affect the HT nature of this study.

3.3.6. Microorganisms

According to previous studies, the interaction between SNP and bacteria depends on the nature of the bacteria[7,119] (Fig. 9). As a result, one gram-negative and one gram-positive species which are occasionally found in environmental systems or are of great importance to larger organisms such as human were selected for this study. *E. coli* (gram-negative), and *S. aureus* (gram-positive) are the microorganisms which will be used to understand the bactericidal properties of SNPs. For simplicity, the bacteria LB broth (Bacterial Media - GIBCO®, Thermo) will be used as the culture media. The bacteria will be added to autoclaved LB broth and will be cultured at 37 °C overnight at 160 rpm[23]. The bacteria will be harvested where the optical density measurements at 600 nm (OD_{600}) measurement is approximately 1.0 absorbance unit which according to previous studies approximately corresponds to 10^8-10^9 CFU/mL[5,23,24]. For the first experiments, pictures will be captured and analyzed with OpenCFU software to determine the exact CFU/mL of each species. Afterwards, the identical incubation-harvesting procedure will be followed and dilutions will be made if necessary to achieve the required biomass concentration according to Table 1. For each experiment, the cells will be centrifuged and re-suspended in appropriate media defined in Table 1.

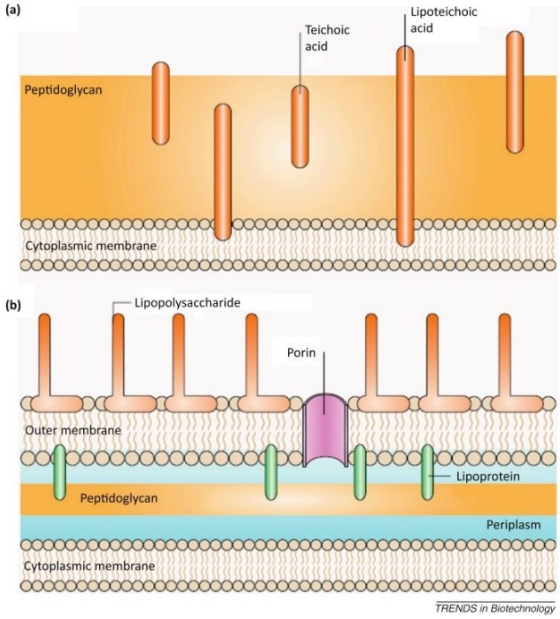

Figure 9. Schematic demonstration of differences between the cell wall structure of gram-positive (a) and gram-negative (b) bacterial cell wall structure. (Reproduced from Hajipour et al.[119])

3.3.7. Bioassays

The following assays will be performed for mechanistic understanding of bactericidal activities of SNPs.

Growth kinetic assay

The growth/death kinetic of each bacteria under the relevant operating condition will be monitored by collecting the samples at different time intervals and performing OD_{600} measurement followed by plotting of OD_{600} vs time. Because of the limited amount of sample available for OD measurements as well as the other bioassays, the OD measurements will be performed immediately

after each sample collection step using a UV-Vis microplate reader for 12 h and the analyzed samples will be returned to the plate containing SNPs. Since a number of HTWD experiments will be performed under partial vacuum condition and there is no access to the samples until the end of the experiment, the log reduction value (LRV) will be calculated for those samples:

$$\text{LRV} = \log\left(\frac{\text{Biomass concentration of initial sample}}{\text{Biomass concentration of treated sample}}\right) \quad (3)$$

The bacteria regrowth will be monitored by centrifugation and addition of new LB broth to the treated bacteria obtained from each experiment. OD_{600} measurements will be performed for 5 h to monitor the regrowth kinetic using a UV-Vis microplate reader.

Live/dead assay

While using OD_{600} is a common practice in biomass concentration measurement, this assay does not measure the dead or alive bacteria population. This type of information can be obtained using assays that measure the viability instead of measuring total biomass concentration. The Live/Dead assay kit (Invitrogen) distinguishes the live and dead bacteria by using two nucleic acid dyes. SYTO 9 green fluorescent dye penetrates cell membranes and labels live and dead bacteria. On the other hand, propidium iodide red fluorescent dye only permeates the membrane of non-viable bacteria. Measurements will be performed at 485/530 nm (excitation/emission) and 485/630 (excitation emission) after addition of SYTO 9 and propidium iodide respectively. The bacterial viability index is calculated as follows which can be converted to percentage of live bacteria using a proper calibration curve:

$$\text{Bacterial viability index} = \frac{\text{Green fluorescence intensity}}{\text{Red fluorescence intensity}} \quad (4)$$

Measurement of reactive oxygen species (ROS)

As indicated earlier, ROS generation is among the major proposed mechanisms for anti-bacterial activity of SNPs. ROS generation will be measured by incubation of the 50 µL of SNP-treated sample with 100 µL 2,7-dichlorofluorosceindiacetate (OxiSelect™ Intracellular ROS Assay Kit, Cell Biolabs, Inc.) followed by fluorescent intensity measurement at 480/530 nm (excitation/emission) using a microplate reader.

MTT assay

It was mentioned that SNPs can affect the metabolic activity of the cell, cause mitochondrial dysfunction in eukaryotic cells and inhibit the cell proliferation process. MTT assay is a standard assay which is commonly used to approximately determine cell proliferation and viability. However, since this assay is based on the metabolic activity, the results obtained here will be attributed to the effect of SNPs on the metabolic activity of bacterial cells in this project. In brief, bacteria treated with SNPs under each experimental condition are incubated with MTT solution (Cayman) at 37 ^{o}C for 4 h periods after which centrifugation is performed for 10 min at 10000 rpm. Centrifuged cells are dissolved in formazan buffer and the supernatant absorbance is measured at 590 nm using UV-Vis spectroscopy in a microplate reader. Decrease in metabolic activity[60] will be calculated as:

$$\text{Decrease in metabolic activity } (\%) = \frac{\text{OD}_{570nm}\text{ (untreated)} - \text{OD}_{570nm}\text{(treated)}}{\text{OD}_{570nm}\text{ (untrearted)} - \text{OD}_{570nm}\text{ (media)}} \times 100 \quad (5)$$

Thiol and Glutathione detection assays

Thiols are efficient antioxidants which are able to protect lipids, proteins, and nucleic acids in the cell against peroxidative damage. They have the ability to react with free radicals due to their

reductive nature. Since the interaction of silver ions with thiol groups is proposed as one of the anti-bacterial mechanisms of SNPs, it is important to perform an assay to detect the toxicity effect of SNP on thiols. Glutathione (GSH) is considered as one of the most important thiols. Hence, GSH measurement can be performed as a measure of thiol interactions with SNPs. The assay will be performed according to the instruction proposed by the manufacturer of the assay kit (Cayman). Briefly, after preparing the appropriate buffers and centrifugation and washing of the cells treated with SNPs, 50 µL of each sample is mixed with150 µL of assay cocktail (mainly contains 5 5'-dithiobis(2-nitrobenzoic acid) (dtnb)) followed by absorbance measurement at 412 nm using a UV-Vis microplate reader. Although GSH measurement is a common measure for thiols interaction quantification, most of the gram positive bacteria including *S. aureus* lack Glutathione. Hence, thiol assay will be performed by using thiol detection assay kit (Cayman). The assay will be performed according to the instruction proposed by the manufacturer. Briefly, after preparing the buffers and centrifugation and washing of the treated cell, 50 µL of sample will be mixed with 50 µL of thiol fluorometric detector followed by incubation for five minutes (room temperature). The fluorescence intensity will be measured at 385 nm (excitation) / 515 nm (emission) wavelengths using a microplate reader.

Respiratory chain enzyme inhibition

The inhibition of respiratory chain enzyme will be determined using lactate dehydrogenase (LDH) assay (Pierce LDH Cytotoxicity Assay Kit). Briefly, 50 µL of each sample will be transferred to a 96 well plate and will be incubated with 50 µL of the reaction mixture for 30 minutes at room temperature. 50 µL of stop solution is added followed by absorbance reading at 490 nm and 680 nm. LDH activity is calculated as:

$$\text{LDH activity} = (\text{Absorbance intensity at 490 nm}) - (\text{Absorbance intensity at 680 nm})$$

Same method will be performed for negative control (water) and positive control (Cell Lysis Buffer 10X) provided in the assay kit and cytotoxicity will be calculated using the following equation:

$$\text{Cytotoxicity} = \frac{\text{Sample LDH activity} - \text{Negative control}}{\text{Positive control} - \text{Negative control}} \quad (6)$$

DNA damage assay

A number of authors[67,72,120] have suggested a SNP anti-bacterial mechanism based on DNA damage. Base damage and AP sites formation in DNA is one of the most common DNA damages generated by oxidative stress. A relatively simple DNA damage detection assay will be used in this project to detect AP sites by using aldehyde reactive probe (ARP). Detailed instruction including cell fixing, labeling, DNA denaturation, blocking and staining steps will be performed as described by the manufacturer (Cayman). The analysis will be performed immediately after performing all the steps using a fluorescence microplate reader at excitation/emission of 485/535 nm.

Lipid peroxidation

As underlined before, a few of previous studies suggested lipid peroxidation as a mechanism of anti-microbial activity[72,73] In this mechanism, free radicals take an electron from lipids in the cell membrane which results in formation of highly reactive and unstable compounds. Lipid hydroperoxide (LPO) assay kit (Cayman) is a sensitive assay in which hydroperoxides are measured by directly reacting with Fe^{2+} and generating Fe^{3+}. In this assay, hydroperoxides are extracted from the samples by using chloroform to prevent the potential interferences. The assay will be performed according to the instruction proposed by the manufacturer. Briefly, the samples will be extracted using saturated methanol and cold chloroform followed by centrifugation. The final sample will be

prepared by mixing 500 µL sample extracted, 450 µL of chloroform-methanol mixture, and 50 µL of chromogen in a deep well plate. The absorbance reading will be performed at 500 nm using 300 µL of the solutions prepared in the previous step.

Experimental considerations

Each HT assay must be run along with negative control and positive control samples as assay quality control measures to ensure the reliability of the assay. The assays are proposed considering the potential anti-bacterial mechanisms of SNPs mentioned earlier. Furthermore, it is important to note that all of the proposed assays are based on HTS and using microplate readers; however, other techniques such as fluorescence microscopy, flow cytometry, and HCS (as briefly described in section 3.1) have the potential to provide more detailed analyses about the anti-bacterial mechanism of action of SNPs. Explaining such techniques is beyond the scope of this project since they are not classified among the HTS methods. Such analyses can be considered among the future objectives of this project. A summary of mechanisms proposed in previous sections is shown in Fig. 10.

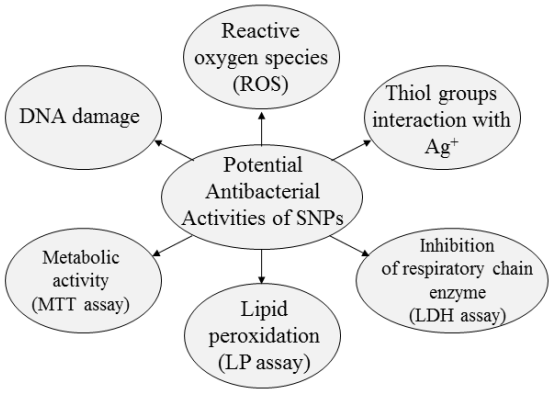

Figure 10. Matrix of different assays proposed in this study for the mechanistic understanding of anti-bacterial properties of silver nanoparticles.

3.4. Challenges

Despite all efforts to design this proposal in a precise manner and to make it as detailed as possible, the following potential challenges still exist:

- The presence of metal nanoparticles such as SNPs strongly interferes with some of the bioassays. Although it is proposed that each assay is performed after centrifugation and washing steps, normally this is not enough to remove all the nanoparticles in the samples. While further washing steps can be performed, the second phase of this proposal is intended to solve this challenge by introducing the removable MSNPs.
- One of the major challenges will be related to the stability of the SNPs. For example, the stability of SNPs highly depends on pH and solution composition. Although there are a few studies providing such information (e.g. stability of SNPs through a wide range of pH when using TSC[57]), they do not include all the different surfactants and care must be taken during the characterization steps when changing the solution condition. Appropriate measures (e.g. modifying the synthesis procedure, changing the surfactant, and modifying the experimental plan) may be needed if very large aggregates of SNP form.
- One of the important challenges is to validate the assays related to HTS. For each assay positive and negative control samples must be prepared. One way to validate the assay is to calculate the 'z-factor' which was proposed by Zhang et al.[121].

3.5. Milestones for specific objectives and tasks

Table 3 shows the milestones for this project based on the approach proposed in previous sections. The short term objectives and tasks will be conducted by a PhD student in 4 years. The long term objectives (mentioned in section 3.1.2) and future enhancements can be conducted by another PhD student or postdoctoral fellow(s) after reaching and analyzing the short term objectives.

Table 3. HTS for the mechanistic understanding of anti-bacterial properties of SNPs – project timeline.

Date	Duration (days)	Task
01-Sep-2016	150	Training, literature review and preparing the experimental design for *phase I*.
01-Feb-2017	90	*Phase I*. Synthesis and characterization of the proposed SNPs for BT studies.
01-May-2017	90	Treating selected microorganisms with SNPs and performing bioassays.
01-Aug-2017	60	Analysis, modification of the operating conditions and conduction of more experiments if required.
01-Oct-2017	90	Training, literature review and preparing the experimental design for *phase II*.
01-Jan-2018	90	*Phase II*. Synthesis and characterization of MSNPs.
01-Apr-2018	90	Treating selected microorganisms with MSNPs and performing bioassays.
01-Jul-2018	90	Analysis (comparing results with *phase I*), modification of the operating conditions and conduction of more experiments if required.
01-Oct-2018	90	Training, literature review and preparing the experimental design for *phase III*.
01-Jan-2019	120	*Phase III*. Design and testing of a high-throughput water disinfection (HTWD) module.
01-May-2019	90	In-situ synthesis of SNPs on filter papers and characterization.
01-Aug-2019	120	Performing HTWD experiments under the operating conditions decided in the experimental design.
01-Dec-2019	60	Performing silver content measurement and bioassays.
01-Feb-2020	90	Analysis (comparing results with *phases I and II*), modifying the operating conditions and designing experiments for future studies.
01-May-2020	140	Final data analysis, thesis writing and oral defence.

3.6. Anticipated significance of the work

The successful completion of this project will result in progress in different fields relevant to anti-bacterial properties of silver nanoparticles such as biotechnology, water, polymer, food, and toxicology research by reducing the cost and speeding up the understanding of the anti-bacterial properties of the SNPs. In this way, the anti-bacterial products containing silver nanoparticles will be synthesized and operated in an optimized condition. Furthermore, by knowing the anti-bacterial mechanism, it will be possible to solve many of the safety concerns regarding the usage of silver nanoparticles related to human health and the environment. Last but not least, the results from this study will pave the way for future research in this field. Student(s) who complete this project will

be able to work at different sectors such as pharmaceutical, biotechnology, food and bioprocessing, nanotechnology, polymer and water industries.

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