WORLD BANK STEP-B SHESTCO-AUST-PRINCETON PROGRAMME ON MATERIALS SCIENCE

STAKEHOLDERS MEETING, WORKSHOP AND PAN AFRICAN SCHOOL OF MATERIALS RESEARCH, TRAINING AND EDUCATION

PASMAT Biosynthesis lab session.

INTRODUCTION

Nanotechnology is the creation, exploitation and synthesis of material at a scale smaller than 1um. The concept was given by Physicist Professor **Richard Feynman** in **1959.** Nanoparticles are 1-100nm and they exhibit different shapes like spherical, triangular, rod etc. This field has opened up new worlds of possibility with distinctive approaches such as the making nanoscale structures by machining and etching techniques, or the molecular nanotechnology approach which refers to building organic and inorganic structures atom-by-atom, or molecule-by-molecule.

This field is multidisciplinary, whereby R&D at nanoscale is fused by the skills or knowledge on tools and techniques from different arms of science, including information on the physics affecting atomic and molecular interactions. This includes the collaboration between Materials scientists, engineers, medical researchers are forming teams with biologists, physicists and chemists.

The synthesis of nanoparticles is a cornerstone of nanotechnology. New methods to study synthesis of nanoparticles are an area of active research. The methods currently being used encompass chemical routes. A big impediment to encouragement of these methods is that the by-products associated with metal production have become a great concern with respect to environmental pollution, additionally; some of these processes are expensive. In addition, the synthetic procedures involve conditions such as high temperature, pressure and environmental inertness, which are cost intensive.

A common application of nanoparticles is cancer research. In the United States, breast cancer is the second leading cause of cancer-related death among women. The National Cancer Institute estimates that, in 2010, there were over 207,000 new cases, and nearly 40,000 deaths, from breast cancer among women in the United States [1]. There is, therefore, a need for advancements in the early detection or cancer. Such advancement could increase survival rates and improve other clinical outcomes [2]. In addition, localized treatments are needed to overcome the limitations of current systemic therapies [3].

Recently, there has been interest in targeting cancer cells with gold nanoparticles (AuNPs) [4-6]. One motivation is that gold nanoparticles display a strong plasmon resonance in the near infrared (NIR) region. This property is extremely sensitive to the size, shape and aggregation state of the nanoparticles [7]. The NIR region is where tissue transmissivity is highest, due to low scattering and absorption from intrinsic

chromophores [8]. Therefore, this property can be exploited for imaging, as well as photothermal therapy [9, 10].

Gold nanorods and nanoshells are also being developed as photothermal therapy agents. Nanoparticle-based techniques have the potential to offer many advantages over more conventional forms of cancer treatment. At present, the most common cancer treatment methods are based on chemotherapy, radiation therapy, or surgery, all of which can be successful, but have substantial disadvantages. Chemotherapy often induces severe side effects and can cause damage to healthy cells, and radiation is only useful on localized, well-defined tumours. Surgical removal also requires that the tumour be well localized, and is often impossible if the tumour is surrounded by sensitive tissues such as the brain.

Hyperthermic treatment has been tried in many forms, but conventional techniques tend to cause substantial damage to surrounding tissues. Modern nanotechnology, though, offers the possibility of materials that selectively bind to particular types of cancer cells, sensitizing them to light without affecting surrounding healthy tissues.

As a diagnostic tool, these nanoparticle techniques can be used to greatly enhance image contrast when studying a tumour. As a treatment tool, they may take several forms: photodynamic therapy if the cancer is destroyed chemically by the light-activated sensitizer particles, or photothermal therapy if it is destroyed by heating the nanoparticles with an external energy source (often an infrared laser).

For example, Loo et al [9] conjugated nanoshells to antibodies specific to the human epidermal growth factor receptor 2, (HER2). After incubating the cancer cells with over-expressing HER2 receptors, the cells were irradiated with near-IR light at a frequency resonant with the surface plasmon resonance of the nanoshells. The light absorption led to heating, and thereby resulted in cell death.

AuNP–antibody conjugates are being developed that combine photothermal therapy with near-IR imaging capabilities for cancer cells [9, 12].
AuNPs can also scatter visible light. Reflectance confocal microscopy [13] and optical coherence tomography [14, 15], are microscopy techniques that image reflected light, and provide detailed, three-dimensional images of tissue, without the need for physical sectioning. These techniques provide spatial resolutions of ~1-10 μm and a penetration depth, ranging from ~ 300 μm to 1-2 mm [16]. Sokolov et al. [16] have demonstrated the use of gold bioconjugates for such types of reflective imaging.

The potential advantages of using gold nanoparticles in cancer therapies have resulted in increased interest in their synthesis [17-20]. However, most of the common methods that are used to synthesize gold nanoparticles are cumbersome and involve the use of toxic chemicals [21-24], high temperatures [25, 26] and high pressures [27, 28]. In addition, many issues have been raised related to the stability of the resulting nanoparticles in aqueous solutions [29], toxicity [30] and control over their size and shape [31].

There are different methods for the synthesis of nanoparticle which include chemical, physical, hybrid and biological methods. The later method proffers a non toxic and cost effective route of synthesis. Furthermore, this method does not need the use of high

temperature, pressure, energy and toxic chemicals, it is easy to scale-up, does not cause inflammation. It also does not need elaborate process of maintaining cell culture.

In contrast, the biosynthesis of AuNPs provides a novel approach to the development of non-toxic, environmentally benign methods that have the potential to replace conventional chemical and physical synthesis techniques that are normally used to synthesize AuNPs. Furthermore, the biosynthesis of AuNPs can be achieved through the use of environmentally benign bacteria, fungi, and plants. These provide useful new pathways to the synthesis of particles with a range of shapes and sizes.

Prior work on the bacterial synthesis of AuNPs has shown that the particles may be formed intracellularly or extracellularly. The shapes and sizes of the resulting AuNPs have also been shown to vary with pH and the type of bacteria. In an earlier study, *Bacillus subtilis* 168 [32] was used to reduce Au^{3+} ions to AuNPs with sizes between 5 and 25 nm. Other microbes are also known to have the ability to synthesize AuNPs. *Shewanella algae* can reduce Au^{3+} ions to AuNPs [13], while *actinomycete Thermospora* [16] and photosynthetic bacterium *Rhodococcus sp.* [17] can synthesize AuNPs intracellularly and extracellularly.

Alternative metal recovery/removal methods are being considered which are based on metal sequestering and or metal uptake from solution by biological systems. Nature however, is no stranger to nanotechnology; living organisms from bacteria to beetles rely on nano-sized protein-based machines that do everything from whipping flagella to flexing muscles. The molecular machinery of nature out performs anything that mankind knows how to construct with conventional manufacturing technology by many orders of magnitude [33].

Biological systems have a unique ability to control the structure, phase, orientation and nano-structural topography of inorganic crystals. It is well known that inactivated biological systems interact with metal ions; the connection between the two is more in depth. As is well known that many elements in trace concentration are essential to plant growth and propagation; however these very elements become toxic to the plants at higher concentrations [34]. It has been shown that many plants as well as bacteria can actively uptake and reduce metal ions from soil and solutions. A well-known example of reduction and production of nanoparticles is the magneto-tactic bacteria that can synthesize magnetic nanoparticles which have an enormous number of applications.

Another example is the production of gold nanoparticles using inactivated Alfalfa biomass. The possibility of using Lactic acid bacteria in the whey of buttermilk has shown the production of gold-silver composite materials when challenged to a mixture the ions of the two metals. These are some examples that show the biotechnological solutions to material-science. Although many biotechnological applications such as remediation of toxic metals employ microorganisms such as bacteria and yeast, it is only relatively recently that material science has been viewing these as possible eco friendly nano-factories.
Microbes affect the redistribution of metal by oxidation, reduction or biosorption. Microbes may solublize the metals as in the case of uranium, or reduce them, as in the case of iron and manganese. Microbial biomass can retain relatively high quantities of metal by biosorption (passive mode) or by bioaccumulation (actively by viable cells). It has been recently shown that several types of inactivated biomasses and living organisms have the ability to remove high concentrations of Au^{3+} from solution by converting it to Au^{0}.

The remediation of toxic metals and other contaminants by microorganisms and plants have been suggested. Using parts of, or the whole plants for metal nanoparticle synthesis could be advantageous over the microbial approach whereby it is more cost effective, proffering an ease of scale and does not require maintenance of cell cultures as demonstrated in the synthesis of gold and silver nanoparticles with *alfalfa* plants.

Bioactive compounds said to be present in Medicinal substances found in plants are referred to as secondary metabolites or biologically active compounds. The bioreduction of gold and silver nanoparticles from silver and chloroaurate ions has been achieved using plants such as *Azardirachta indica and Zingiber officinale*.

Moringa Olifera has a wide range of uses and is known for its medicinal values and has been used to treat skin diseases, colorectal cancer, arthritis, heart condition etc It has also been reported for its antibacterial properties. Medicinal plants such as *Moringa Oliefera* are said to be effective in metal biosorption from aqueous effluents. Reports suggest that metals such as arsenic, cadmium, lead, copper and silver contaminants can easily be removed using the seeds of *moringa olifera*. 4 α L-rhamnosyloxy-benzyl isothiocyanate had been identified as an antimicrobial agent in the seeds. During the course of this school, this plant would be used in the phytosynthesis of gold, and Iron oxide (paramagnetic) nanoparticles for the first time!

However, the basic mechanisms of biologically-assisted nanoparticle formation are not fully understood. Furthermore, the potential for the scale-up of the Au biosynthesis is yet to be demonstrated. There is, therefore, the need to explore the potential of scalable biologically-based methods for the synthesis of gold nanoparticles. There is also a need to establish the basic mechanisms of gold nanoparticle formation during biosynthesis. Such understanding could guide the future scale-up and applications in niche areas, such as nano-medicine. Furthermore, since nano-medicine requires the specific targeting of diseased cells, there is, therefore, a need to develop a basic understanding of the adhesion of nanoparticles to diseased cells, such as cancer cells. Also, since basic studies of adhesion have not been conducted on biosynthesized gold nanoparticles or antibody-conjugated gold nanoparticles, there is a need to study the adhesion of biosynthesized AuNPs and specific antibody-coated AuNPs with the potential for future applications in breast cancer detection and treatment.

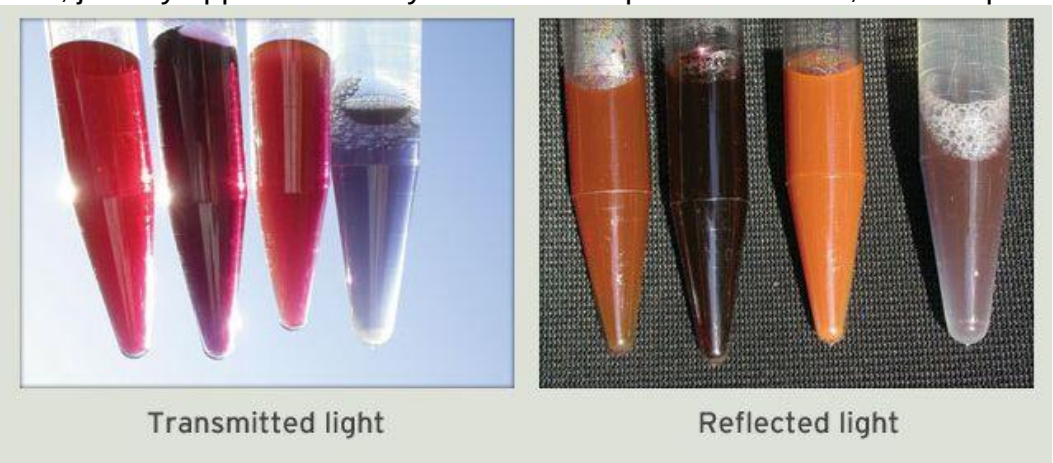
This laboratory session explores the mechanisms by which AuNPs are formed by bacterial reduction of chloroauric acid ($HAuCl_4$) with *Bacillus megaterium, Bacillus subtilis, Serretia marcescens,* along with a yet to be identified microbe, all common, non-pathogenic soil bacteria. 4 plants indicated above are also used in the phytosynthesis of gold, magnetite, and silver nanoparticles. Characterization via UV/VIS spectroscopy, SEM/EDS, XRD as well as visual observations would be conducted in the laboratory, providing each candidate with the opportunity to use these array of equipment. Further characterization would be conducted at the Princeton University USA. These would include Atomic Force Microscopy, Transmission Electron Microscopy etc.

The color of nanoparticles

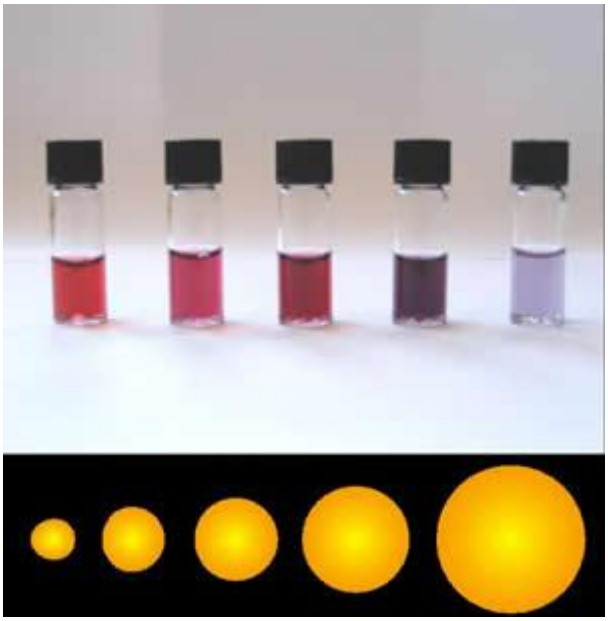
The color known as "Purple of Cassius" in glass and glass enamel is created by incorporating a colloidal suspension of gold nanoparticles, a technology in use since

ancient times. Colloidal silver is yellow, and alloys of gold and silver create shades of purple-red and pink.

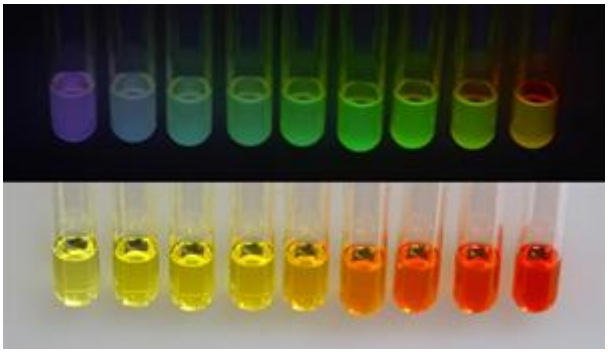
Nanoshells are a recent product from the field of nanotechnology. A dielectric core is coated with metal, and a plasmon resonance mechanism creates color, the wavelength depending on the ratio of coating thickness to core size. For gold, a purple color gives way to greens and blues as the coating shell is made thinner. In the future, jewelry applications may include other precious metals, such as platinum.



Dispersions of discrete gold nanoparticles in transparent media provide a fascinating range of colors, only recently exploited in the manufacture of paints and coatings. The shape of the particles and the viewing conditions determine the color we see. The gold particles in the test tubes on the left are shown in transmitted light, while the image on the right shows the same gold nanoparticles viewed in reflected light.



The diameter of gold nanoparticles determines the wavelengths of light absorbed. The colors in this diagram illustrate this effect.



Different sized quantum dot nanoparticles are shown above, first in ultraviolet light and then in ambient light. The length of the synthesis reaction determines particle size for CdSe, increasing from left to right. In colloidal suspension, this semiconductor behaves in the same way as a metal.

EXPERIMENTAL PROCEDURES

Chemicals used for synthesis of gold and silver nanoparticles were chloroauric acid ($HAuCl_4$) and silver nitrate ($AgNO_3$) (Sigma-Aldrich). Fresh seeds of *Moringa Olifera* as well as leaves of *Terminalia catappa, Calotropis spp* and *Nauclea latifolia* were collected from the staff quarters of the research complex of the SHEDA SCIENCE & TECHNOLOGY COMPLEX (SHESTCO) were used as a plant source for green nanoparticle synthesis.
Equipments including an Innova®44 incubator shaker and a PerkinElmer spectrophotometer were used initially for nanoparticle synthesis.

Biosynthesis of Gold nanoparticles

Bacillus megaterium, Bacillus subtilis, Serretia marcescens, etc bacterial cells were cultured in enriched nutrient broth (ENB). After growth for 48 hours at 30°C, the bacterial cells were harvested by centrifugation. The cells were washed five times with sterile distilled water. Both the washed bacterial cells, and the nutrient broth obtained from the 48 hr growth period (termed conditioned media, or CM), were used in nanoparticle synthesis reactions. One gram (wet weight) of washed bacterial cells was suspended in 10 mL of a sterile aqueous solution of 1 mM HAuCl4 at pH 4 and 7.

To determine whether or not cells were required for the formation of the nanoparticles, the CM was aseptically filtered using filters with pore sizes of 0.22 µm to remove any bacterial cells. Solutions of 1 mM HAuCl4 in 10mL of filtered CM were then prepared at pH of 4 and 7. In addition, the following combinations served as controls: 1 gm (wet weight) of bacteria in water at pH 4 and 7; 1 mM HAuCl4 at pH 4 and 7; CM at pH 4 and 7; and ENB and 1 mM HAuCl4 at pH 4 and 7. The pH of each starting solution was adjusted using 0.1M NaOH and HCl.

Seeds of *Moringa Olifera* and *Prosopsis africana* were soaked in tap water for 2 hours. 5g of the wet seeds were carefully added to 5ml of 1 mM aqueous $HAuCl_4$ and $AgNO_3$ solution in 20ml pyrex test tube used for the bioreduction process. Fresh leaves of *Calotropis spp* were washed and dried at room temperature (for 1 week) after which they were ground and placed in 20ml pyrex tubes. Add 5ml of 1 mM aqueous $HAuCl_4$ and $AgNO_3$ solution into the respective test tubes. The tubes containing the mixture were incubated in a shaker at 130 rpm in dark conditions.

Viability of biomaterial to chloroauric acid exposure

On subjecting the four test microbes to 1mM concentration of $AuCl_4$ - ions, visual observations of the biotransformation indicated the formation of nanoparticles either extracellularly or intracellularly, which resulted in the solution or biomass turning pink-purple in colour. This colour was very distinct as compared to the control, which was golden yellow in colour.

Characterization of Au

The UV–Visible spectra of the synthesized gold nanoparticles were measured on a PerkinElmer spectrophotometer operated at a resolution of 0.5 nm with either quartz (UV–vis) or disposable polystyrene (visible only) reduced volume cells.

- Centrifuge the samples for 10mins @ 3000rpm and collect the supernatant (Dry the biomass for further experiments)
- Repeat this step 3x to ensure no biomass material
- Dilute small aliquots of each sample with distilled H_2O then run the samples with the UV-Vis spectrometer using H_2O as the blank.

The samples indicating a reduction of gold could also be analyzed with methods based on a metallized optical element illuminated by laser beam at the surface of which deeply sub-micron (nanoscale) particles in suspension can be directly visualized, counted and analyzed in real time using optical microscope.
The amount of light scattered by a particle varies strongly as a function of its size (radius). Although large particles scatter significantly more light than small particles, the later move rapidly under Brownian motion appearing to 'jump' distances significantly larger than their apparent 'size'. This is particularly characteristic of particles below 100nm and increases in smaller particles. Larger particles move much more slowly and accordingly can be easily distinguished from smaller ones through their slower Brownian motion and brighter appearance.

Further characterization via SEM and EDX is as follows;

- Thin layer of the dried plant leaf samples were prepared and placed on a carbon coated tape.
- Back Scatter mode was selected for contrast imaging also viewing the dispersive nature of the NPs embedded with the leaves.

RESULTS AND DISCUSSION

PASMAT Biosynthesis of Gold Nanoparticles

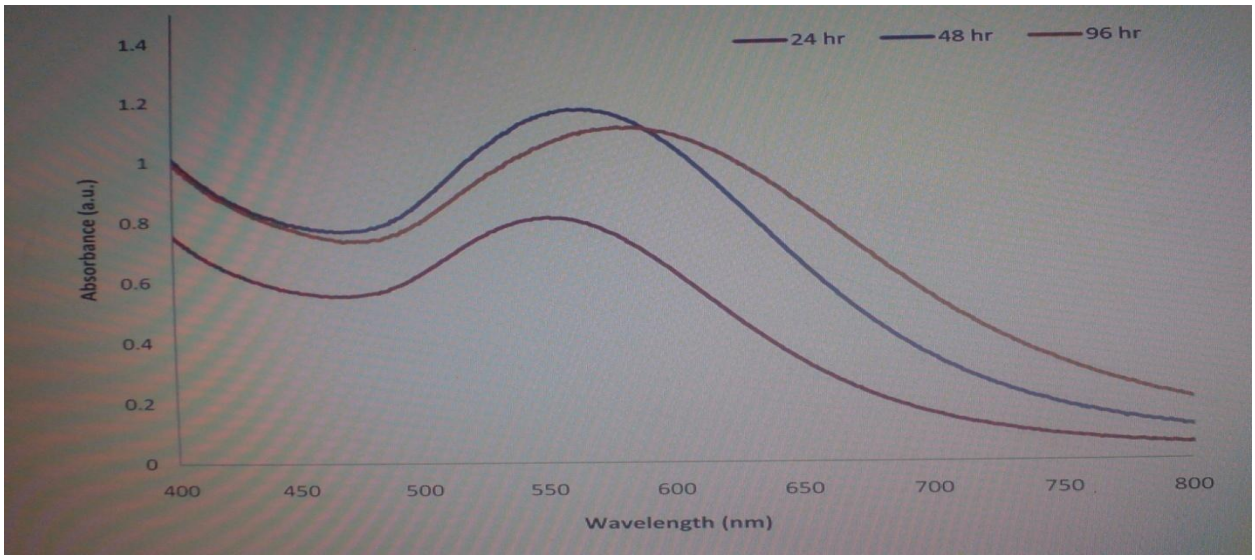
Chloroaurate ions were reduced during exposure to the respective microbes. The colour of the reaction solutions at pH 4 and 7 turned from pale yellow to purple, within 24 hours, which indicated the formation of gold nanoparticles in the presence of bacteria.
To determine whether the reaction occurred extracellularly, the colour of the conditioned media with aqueous chloroaurate ions was monitored. In these reactions, a colour change was noticed at both pH 4 and 7, with the most notable colour change at pH 4. Similarly, for these conditions, the reaction solutions changed from a pale yellow to deep purple, indicating the formation of gold nanoparticles through the possible involvement of extracellular proteins. Control reactions exhibited no change in colour, indicating the requirement of extracellular proteins in the biosynthesis of AuNPs.

The extracellular reaction was also monitored using UV-visible spectroscopy at time intervals up to 96 hours. The UV-visible absorption spectra recorded from the reaction solutions in CM after just 24-96 hours of reaction. The results indicate that the reaction solution of CM with 1 mM $HAuCl_4$ at pH 4 had an absorption maximum at 553, 562, and 581 nm, after 24, 48 and 96 hours, respectively. These absorption maxima can be attributed to the surface plasmon resonance band (SPR) of the gold nanoparticles [37].

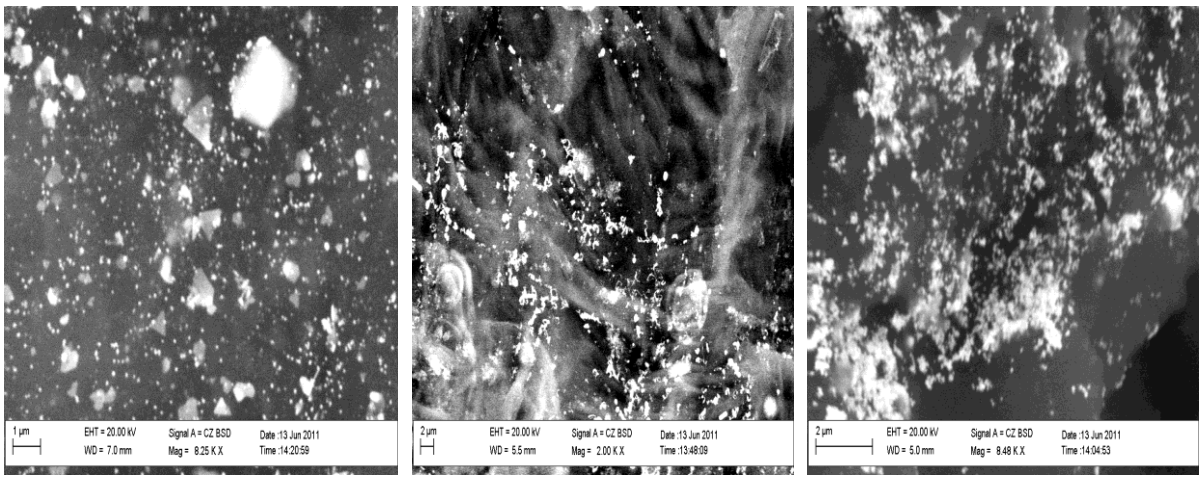
The red shift with reaction time can be attributed to increases in particle size. The shift in the wavelength at a maximum absorbance (λ_{max}) agrees with earlier work by Njoki et al., who showed that a change in the absorbance or wavelength of the surface plasmon (SP) resonance band is dependent on the particle size. Njoki et al. [38] also reported that the red shift is also accompanied by a small broadening of the SP band; which is in agreement with the present results. Absorbance peaks were not apparent at these wavelengths in any of the controls, and, therefore, are not shown.

The redox biotransformation of the aqueous $HAuCl_4$ and Silver Nitrate with the plant plant materials mixture revealed the synthesis of gold and silver nanoparticles respectively within 24 hours in the dark through a visible change in colour of the solution from pale yellow to pinkish-purple for gold and from a clear to dark brown colour for silver. These are indicative of the production of gold and silver nanoparticles. These colour changes are as a result of the excitation due to surface plasmon resonance with the gold and silver nanoparticles [39, 40].
This colour change is thought to be as a result of interaction between the plant proteins and sugars within the leaves seed coats of the respective plants.

Figures below show the absorbance peaks for gold and silver from the respective 24hour reaction mixtures. Gold nanoparticles from the reduction of aqueous $AuCl_4^-$ ions as an absorbance reading of 541nm for *Calotropis Spp.*, 542nm for *Moringa Olifera* and 538nm for *Nauclea latifolia* were identified. The results obtained are in accordance with reports obtained from bioreduction of gold and silver nanoparticles using *Papaya* fruit extract, biomass of *Avena Sativa, Sesbania* seedlings and *Terminalia Catappa* which reported that the SPR band for gold and silver respectively occurred between 520-560nm and 420–450 nm [41, 42, 43].
The monodispersive nature of the synthesised nanoparticles within the plant materials were analysed using back-scattering SEM. The contrast in imaging as depicted in figures is indicative of the presence of the reduced gold and silver partly absorbed by the plant materials.



UV–Vis absorption spectra of gold nanoparticles. UV-Vis Absorption Spectra of Au NPs after Reaction of $HAuCl_4$ with cell-free extract at pH 4 for bacterial samples



The synthesis of gold and silver nanoparticles using the leaves and seeds of *Calotrophis Spp, Prosopis africana* and *Moringa Olifera* has been demonstrated. This could be as a result of secondary metabolites present in the plant material. The synthesis of gold and silver nanoparticles using the 3 plant materials has been demonstrated. The bio-reduction of the metal ions and the stabilization of the gold and silver nanoparticles proved to be stable. The antimicrobial and possible anticancer properties of these plant materials could be exploited in the use of these nanoparticles in cancer therapy, biosensor development, other bio-detection approaches and the development of antimicrobial consumables.

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