Biogenic Synthesis of Antimicrobial Silver Nanoparticles Capped with L-cysteine

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Abstract

The number of medical applications of silver nanoparticles is constantly expanding due to their high bactericidal properties coupled with low toxicity towards mammalian cells. Because of this expanding use of silver nanoparticles, novel methods of synthesis have been developed in order to achieve nanoparticles preparation through inexpensive and environmentally friendly processes; biogenic synthesis of silver nanoparticles is an approach that meets those requirements.

In this work, *E. coli* cultures centrigugates were used to synthesis L-cysteine capped silver nanoparticles. The ratio between $AgNO_3$ and L-cysteine has been proven to affect the size of the nanoparticles: higher amount of L-cysteine returns smaller nanoparticles, but does not affect the percentage of organic matter in the nanoparticles as determined through TGA.

The silver nanoparticles prepared had antimicrobial activity against *E. coli* and *S. aureus* with Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) independent of the nanoparticles size; for both bacterial species the values of MIC and MBC were the same, 0.0432 mg/ml for *E. coli* and 0.0216 mg/ml for *S. aureus.*

Keywords: silver nanoparticles, *E. coli*, biogenic synthesis, *S. aureus*, green chemistry

Introduction

Nanoparticles offer an intermediate between bulk materials and individual atoms with unique properties. Their chemical properties result from the high percentage of atoms on the surface of the particle. This leads to increased reactivity which depending on the application can give increased surface catalysis, improved loading of the surface or greater release of ions into solution [1].

Nanoparticles have been employed in many fields such as catalysis [2-4], ceramics [5], drug delivery [6,7] and diagnostics and therapies of oncology [8]. The increasing detection of bacterial strains capable of resisting one or more antibiotic has driven the research in the development and application of nanomaterials to inactivate pathogens [9]; this is in virtue of the inability of such agents to induce resistance in bacteria and the constantly decreasing efficacy of common antibiotics. Silver nanoparticles antimicrobial properties have found application in bone cement [10], wound dressing [11] catheters [12,13] and fabrics [14].

It is difficult to differentiate between antibacterial effects of ionic silver and Ag nanoparticles as all nanoparticles release a degree of silver ions [15,16]. The antimicrobial effect of silver is thought to be the combination of several mechanisms of action; for example, silver ions and small silver nanoparticles (Ag-NPs) (less than 10nm) have been shown capable of entering the bacterial cytoplasm and inhibit cell growth [17]. Furthermore, they are able to attach covalently to the surface of cell membrane via phospholipids, inhibit cell wall formation, attach to ribosomal 30s subunit, attach to the thiol groups of enzymes and interact with L-cycteine in bacterial peptides [18]. Silver nanoparticles damage the cell wall and membranes and diffuse into the bacteria where they meditate the release of potassium out of the cell [19] and destroy DNA by intercalating between bases [9,20]. Park et al. [18] found that silver ions (Ag^+) exhibit better bactericidal activity against *E. coli* and *S. aureus* under aerobic conditions than under anaerobic conditions. Silver-mediated reactive oxygen species have synergistic effects with thiol-grouping and the mechanisms are thought to be closely related [19]. Hwang et al. [20] also proposed a combined effect of Ag^+ ions and Ag nanoparticles; once Ag^+ ions have started the creation of reactive oxygen species the membrane becomes damaged and Ag

nanoparticles can enter the cell causing further damage [20]. This was supported by Taglietti et al. [21] who described a 'short distance' interaction mechanism of local physical action of the Ag nanoparticles and 'a long distance' ion release mechanism.

Although metal nanoparticles have been synthesised by a variety of physical and chemical routes, it is the reduction of metal salts that has become the standard route for their synthesis due to the flexibility of the particles produced and its reproducibility. For example, to prepare silver nanoparticles, in general ionic silver (Ag^+) contained in a salt, generally $AgNO_3$, is reduced to metal silver (Ag^0) through a reducing agent such as citrate [22], Al-alkoxide [23], $NaBH_4$ [24], N,N-dimethyl formamide [25] or sugars [26,27]. The nanoparticles can also be stabilized using chelating substances such as: citrates [22,28], oleic acid [26], poly(acrylic acid) [29], starch [30] and glutamic acid [31] or prepared with a capping agent that allows binding of other compounds to the nanoparticles such glutathione [32] or tiopronin [33]. Silver nanoparticles of very different geometrical (size and shape) characteristics can be obtained modifying the reaction conditions. However, many of these synthetic routes employ chemical reagents or physical agents that are expensive and/or harmful; the increasing interest in using low energy processes and safe chemicals (so called "green chemistry") has, therefore, originated novel synthetic routes for the preparation of nanoparticles. Plants extracts [34,35], fungi [36-38] and bacteria [39-41] have been used for synthesising silver nanoparticles, but the faster growth rate of bacteria makes their use more industrially appealing. *E. coli* is a widely used bacteria used in biotechnology and biogenic synthesis of nanomaterials [42-44]. In these works no capping agent was employed, therefore limiting the possibility of further bioconjugation, or size control; moreover the antimicrobial properties of the synthesised nanoparticles was not always determined [43,44].

In this paper, we employed *Escherichia coli* surnatant to prepare silver nanoparticles capped with cysteine; we determined how the ratio between silver salt and cysteine affects the size, composition and antimicrobial properties of the nanoparticles against *E. coli* and *Staphylococcus aureus*.

Materials and Methods

Chemical

All chemicals were purchased from Sigma-Aldrich, UK unless stated

Bacteria

E. coli MG1655 and *S. aureus* NCTC 6571 were stored frozen at -80°C; 10 µl of the frozen stock of cells were streaked on LB Agar and incubated for 24 h at 30°C; the cultures obtained were kept in a fridge at 4°C for no more than a week.

Nanoparticles synthesis

A loopful of *E. coli* cells was employed to inoculate 200 ml of fresh sterile LB broth contained in a 500 ml Erlenmyer flasks; the broth was incubated for 24 hours at 30°C under shaking at 70 rpm.

Following incubation, cultures were poured aseptically into sterile 750 ml polypropylene centrifuge bottles (Beckman Coulter UK Ltd.) centrifuged at 1851g for 10 minutes in a centrifuge (JOUAN 420) fitted with a swinging bucket rotor. After centrifugation, the surnatant was transferred in a clean sterile 500 ml Erlenmyer flask, $AgNO_3$ and L-cysteine were added from stock solutions of 1 M to reach a final concentration of 1 mM or 5 mM. The flasks were then incubated for 24 hours at 30°C under shaking at 70 rpm to allow the synthesis of nanoparticles to proceed.

The nanoparticles were separated through the addition of 50 ml of methanol and centrifuged at 1851g for 10 minutes in a centrifuge (JOUAN 420) fitted with a swinging bucket rotor, washed three times with distilled water and dried at 40°C.

Nanoparticles characterization

TEM – particles size

For transmission electron microscopy (TEM) characterization a 4 µl droplet of nanoparticles suspension was placed on a plain carbon-coated copper TEM grid and allowed to evaporate in air under ambient laboratory conditions for several hours. Bright field TEM images were obtained using

a TEM (Philips CM12, FEI Ltd, UK) operating at 80kV fitted with an X-ray microanalysis detector (EM-400 Detecting Unit, EDAX UK) utilising EDAX's Genesis software. Typical magnification of the images was x 100 000. Images were recorded using a SIS MegaView III digital camera (SIS Analytical, Germany) and analyzed with the computer program ImageJ; the diameters of at least 150 particles for each synthetic condition were determined.

UV-Vis spectra

Absorption spectra of the nanoparticles suspensions were recorded using a spectrophotometer U-3000 (Hitachi High-Technologies Europe GmbH) at wavelength interval of 1 nm.

TGA

Thermogravimetric analysis (TGA) was performed using a Stanton Redcroft, STA-780 series TGA. Data was recorded from 25 to 1000 °C with a constant heating rate of 10 °C minute-1.

FTIR

A Shimadzu 8700 FT-IR spectrometer was employed to collect Infrared spectra (3800 to 500 cm^{-1}) of samples pressed into a KBr plate

Antimicrobial *test*

In the antimicrobial experiment silver nanoparticles were added to sterile Brain-Heart Infusion (BHI) broth at a concentration of 0.0864 mg/ml. The suspension was stored in a refrigerator (5°C) before use. A loopful of *E. coli* cells was employed to inoculate 10 ml of sterile BHI contained in a sterile tube and incubated at 37 °C for 24 hours statically. The same procedure was repeated with *S. aureus*. After incubation of 24 hours, the concentration of both cells was 10^9 CFU/ml as determined through serial plating. The cells suspension were diluted 1:10000 using sterile BHI broth giving an initial cell count of 10^5 CFU/ml. 50 µl of sterile BHI broth added in wells in a row of a 96 wells plate. The silver nanoparticles solution 0.864 mg/ml (50 µl) was added and carefully mixed. Then 50 µl were pipette out of the first well and transferred to next the well on the right and mixed. A plate reader was used to measure the optical density (at 600nm), another reading was taken after the well plate had been

incubated for 24 hours in 37 °C. The Minimum Inhibitory Concentration (MIC) was determined from the plate reader as the lowest concentration of nanoparticles capable of preventing cell growth.

The Minimum Bactericidal Concentration (MBC) was determined after establishing MIC for each bacterium. 25 µl were taken from each well where the concentration of nanoparticles was greater than MIC and either spread on BHI Agar plates or serially diluted into 225 µl of sterile PBS and spread on BHI Agar. Plates were placed in an incubator for 24 hours at 37 °C before reading the results. The MBC is lowest concentration of Ag nanoparticles solution that showed a reduction of number of cells from the initial count of 10^5 CFU/ml.

Results

The UV-vis spectrum of the nanoparticles suspension exhibited a clear absorption maximum at ~430 nm for $AgNO_3$:L-cysteine of 1:1 and 5:5 and ~440 nm when the $AgNO_3$:L-cysteine ratio was1:5 (Figure 1); TEM analysis (Figure 2) revealed that the silver nanoparticles synthesized were rounded regardless of the ratio $AgNO_3$:L-cysteine. The distributions of particles diameters are presented in Figure 3 along with the correspondent Gaussian distribution; when the ratio $AgNO_3$:L-cysteine was 1:1 or 5:5 the particles had a mean diameter of 14.9±2.8 and 13.8±2.8 nm respectively, whilst the nanoparticles obtained with a ratio $AgNO_3$:L-cysteine of 1:5 had a mean diameter of 5.0±1.9 nm. It is evident that the diameters followed a Gaussian distribution (Figure 3). No nanoparticles were synthesised when no L-cysteine was added along with $AgNO_3$ or when both L-cysteine and $AgNO_3$ were added to PBS.

The presence of L-cysteine on the surface of the nanoparticles was investigated through FTIR (Figure 4); in all case, L-cysteine was found on the surface of the nanoparticles as the characteristic peaks of pure L-cysteine were also found when the nanoparticles were analysed.

The effect of the concentration of $AgNO_3$ and L-cysteine on the organic composition of the nanoparticles was investigated through TGA (Figure 5). It appeared that when the ratio $AgNO_3$ to L-cysteine was 1:1 and 5:5 the behaviour was similar up to 300 °C. All silver nanoparticles exhibited a reduction of about 20% at 300 °C, but overall the nanoparticles synthesised with a ratio $AgNO_3$ to cysteine was 1:5 returned the lowest mass reduction.

MIC and MBC were determined to assess the antimicrobial properties of the synthesised silver nanoparticles against two bacterial species: *E. coli* and *S. aureus* (Table 1). The synthetic conditions tested here, and consequently their effect on the nanoparticles characteristics, did not influence the antimicrobial activity against either of the bacterial species. Furthermore, both *E. coli* and *S. aureus* exhibited the same value of MIC and MBC (0.0432 mg/ml and 0.0216 mg/ml respectively).

Discussion

Stabilisation is necessary to obtain nanoparticles in the range below 100 nm, furthermore, nanoparticles stability is also required for practical application. Unwanted overgrowth is suppressed by coating the surface of nanoparticles during synthesis with a capping layer of organic molecules. This capping agent reduces the surface energy of the nanoparticles enhancing their separation and prevents further agglomeration. Depending on the properties of this capping agent, the characteristics (geometry and solubility) of the nanoparticles can be controlled. The choice of capping agent must be directed by the characteristics and application of the intended nanoparticles; for biological applications, polypeptides (gluthatione) [21,45,46], tiopronin [10,33] and aminoacids have been previously employed [31,45]. The common feature of these compounds is the presence of sulphur whose strong affinity for Ag results in coverage of the nanoparticles and provides stability during nanoparticles ripening. Increasing the amount of capping agent, from a ratio $AgNO_3$:L-cysteine of 1:1 to 1:5, resulted in a reduction of the mean particles diameter as the additional cysteine stabilised the particles further, consequently, the nanoparticles reached maturation at smaller size; similar results were found using tiopronin as capping agent [10]. Moreover, the size of the nanoparticles was not affected by the amount of $AgNO_3$ and cysteine but only by the ratio, however it was not possible to test whether a $AgNO_3$:L-cysteine of 5:25 would return the same particles size distribution as a ratio 1:5 as L-cysteine was not soluble at this concentration. The different nanoparticles size caused by varying amounts of $AgNO_3$ and L-cysteine is also responsible for the slight shift in absorption maximum in the UV-vis spectrum. It has been shown that another way of controlling nanoparticles size is through the reaction temperature [38,43], with higher temperatures resulting in smaller particles. $AgNO_3$ concentration and pH have been proven controlling parameters of the nanoparticles size during biogenic synthesis, with the smallest nanoparticles achievable at an optimum value of such parameters [43]. Our approach, through the reagents ratio, seems more energy and environmental friendly as the higher the reaction temperatures the higher the energy cost associate to the synthesis.

The presence of cysteine on the surface of the nanoparticles was confirmed further by FTIR as the spectra of the nanoparticles were similar to pure L-cysteine; furthermore, TGA analysis showed the

presence of organic matter in the nanoparticles as mass reduction was detected at about 200 °C. The different synthetic conditions did not influence the proportion of L-cysteine in the nanoparticles as shown by TGA (Figure 5), however, as different ratios of reagents resulted in nanoparticles with various diameters, the number of L-cysteine molecules per silver atoms varied with changing reagents ratios.

In order to unequivocally prove that the synthesis of nanoparticles is performed by an extracellular compound(s) produced by the growing *E. coli* cells we performed the synthetic step using either fresh medium or PBS, in both cases no nanoparticles were detected. The reducing agent is either an extracellular enzyme or another non enzymatic compound produced by *E. coli* and released during growth [47]. Two enzymes, NADPH-dependant reductase [48] and nitroreductase NfsA [49], have been proven involved in the reduction of Ag^{+} to Ag^{0}. Reducing sugars are thought to be responsible for the non enzymatic reduction; these are more likely to be found in plant extracts [48].

Another green method of synthesising nanoparticles recently gaining a lot of attention employs plants extract to reduce metal ions [50-53]; despite the proven efficacy and flexibility of this process, the use of bacterial cultures sub-products, such as filtrate or centrifugate, is likely to lead to more efficient and cheaper industrial processes as the cost and time required to grow cells are smaller and shorted, respectively, that growing plants and extracting compounds. For the same reason, biogenic synthesis performed using bacteria is a more appealing process than fungi as the growth kinetics of eukaryotic cells are comparably slower than prokaryotic organisms. A variety of bacteria species have been employed to perform biogenic synthesis of nanoparticles, i.e. *Streptococcus thermophilus*, *Pseudomonas putida* and *Bacillus cereus* [42,54], nevertheless the use of non-pathogenic bacteria, such as *E. coli* MG1655 employed here, is ultimately a critical factor in biogenic synthesis of nanoparticles to become an industrially relevant approach.

The antimicrobial activity of the nanoparticles synthesised in this work was tested against Gram-positive *S. aureus* and Gram-Negative bacteria *E. coli* that are some of the most common microorganisms causing infections. The antimicrobial action of a compound can be classified as:

inhibitory, when the concentration of the microorganisms does not increase with time compared to an expected cell growth in absence of the chemical; or bactericidal was the concentration of the microorganisms decrease upon exposure to the compound. Antibiotics generally exhibit inhibitory activity at low concentrations and bactericidal at high doses.

The silver nanoparticles presented in this work exhibited an identical value of MIC and MBC; Panacek et al. [27] also noted the same behaviour with silver colloid nanoparticles in their experiments. These findings suggest that Ag nanoparticles damage the bacteria irreplaceably and the action is more bactericidal than inhibitory, therefore, unlikely to induce the insurgence of resistance.

Ag nanoparticles smaller than 10 nm have been shown to have a high degree of interaction and capacity to enter the cells [55]. However, size is not the only determining factor in assessing nanoparticles antimicrobial activity, for example Prokopovich et al. [10] have shown that silver nanoparticles capped with tiopronin with a diameter of 10 nm were more antimicrobial than nanoparticles with 5 nm diameter, this was attributed to the higher silver content of the larger nanoparticles. Also the shape of the nanoparticles plays a significant role in their antimicrobial activity. Triangular, spherical, rod particles demonstrated antimicrobial activity in decreasing order [9].

The higher resistance of *E. coli* cell to Ag nanoparticles compared to *S. aureus*, demonstrated by the lower MBC values for the latter (43.2 mg/ml and 21.6 mg/ml respectively), is a general trend associated to the structural differences between Gram positive and Gram negative bacterial cell [56].

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Conclusions

The surnatant obtained after centrifuging *E. coli* cultures was employed to synthesis Ag nanoparticles capped with L-cysteine. The higher the ratio $AgNO_3$: L-cysteine the smaller the diameter of the nanoparticles.

The nanoparticles prepared showed antibacterial activity against both Gram-positive *S. aureus* and Gram-Negative bacteria *E. coli*; MIC and MBC were determined to be 21.6 mg/ml and 43.2 mg/ml, respectively. Differences in ratios of $AgNO_3$ and L-cysteine during synthesis did not influence the antibacterial activity of the Ag nanopartilcles. Identical MIC and MBC values indicated Ag nanopartilces having a bactericidal mechanism of action unlikely to induce the insurgence of resistance.

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Table 1. MIC and MBC of biogenically synthesised Ag NP capped with L-cysteine for *E. coli* and *S. aureus*

Bacteria		1:1 mol	1:5 mol	5:5 mol
E. coli	MIC	0.0432 mg/ml	0.0432 mg/ml	0.0432 mg/ml
S. aureus		0.0216 mg/ml	0.0216 mg/ml	0.0216 mg/ml
E. coli	MBC	0.0432 mg/ml	0.0432 mg/ml	0.0432 mg/ml
S. aureus		0.0216 mg/ml	0.0216 mg/ml	0.0216 mg/ml

Figure caption

Figure 1 UV-vis absorption spectra of of Ag NP synthesized with different ratios of $AgNO_3$ and cysteine.

——— 1:1 $AgNO_3$:L-cysteine - - - - - 1:5 $AgNO_3$:L-cysteine ····· 5:5 $AgNO_3$:L-cysteine

Figure 2 TEM images of nanoparticles. Bar equal to 100 nm.

(a) 1:1 $AgNO_3$:L-cysteine (b) 1:5 $AgNO_3$:L-cysteine (c) 5:5 $AgNO_3$:L-cysteine

Figure 3 Size distribution of Ag NP synthesized with different ratios of $AgNO_3$ and cysteine. Lines represent Gaussian fitting and columns experimental data

——— 1:1 $AgNO_3$:L-cysteine - - - - - 1:5 $AgNO_3$:L-cysteine ····· 5:5 $AgNO_3$:L-cysteine

1:1 $AgNO_3$:L-cysteine 1:5 $AgNO_3$:L-cysteine 5:5 $AgNO_3$:L-cysteine

Figure 4 Fourier Transformed Infra Red spectra (FTIR) of the three sets of nanoparticles and pure cysteine.

——— 1:1 $AgNO_3$:L-cysteine - - - - - 1:5 $AgNO_3$:L-cysteine ····· 5:5 $AgNO_3$:L-cysteine

L-cysteine

Figure 5 Thermal Gravimetric Analysis of the three sets of nanoparticles.

——— 1:1 $AgNO_3$:L-cysteine - - - - - 1:5 $AgNO_3$:L-cysteine ····· 5:5 $AgNO_3$:L-cysteine

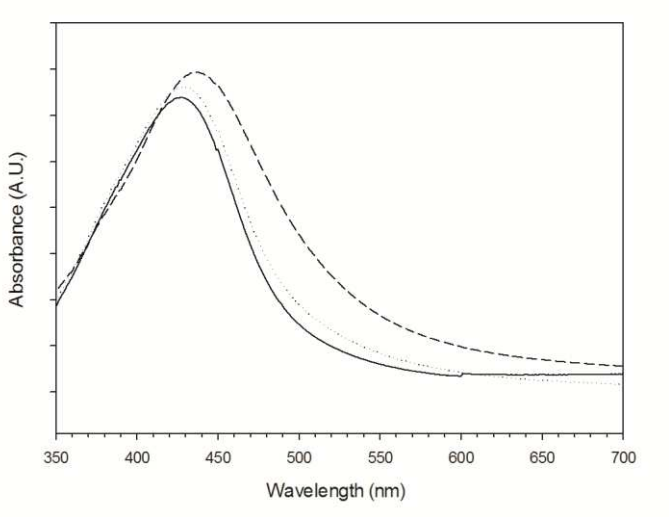

Figure 1

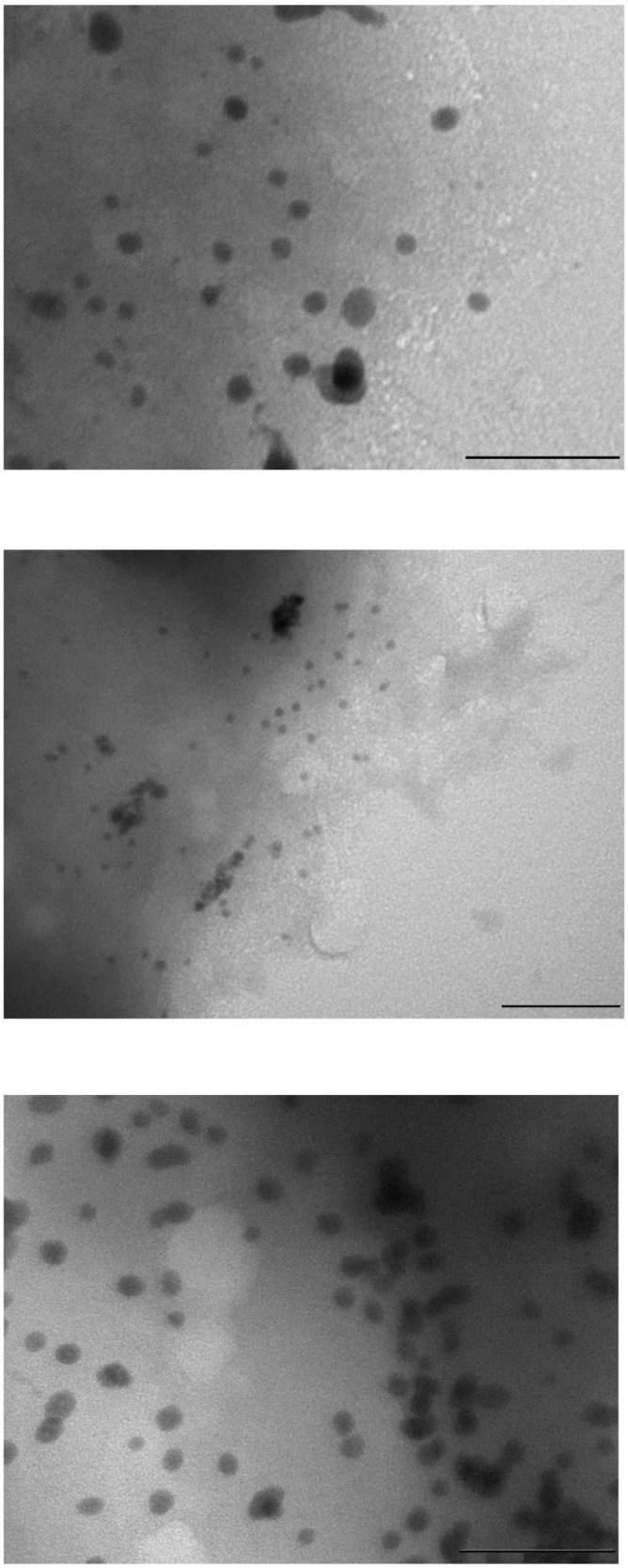

Figure 2

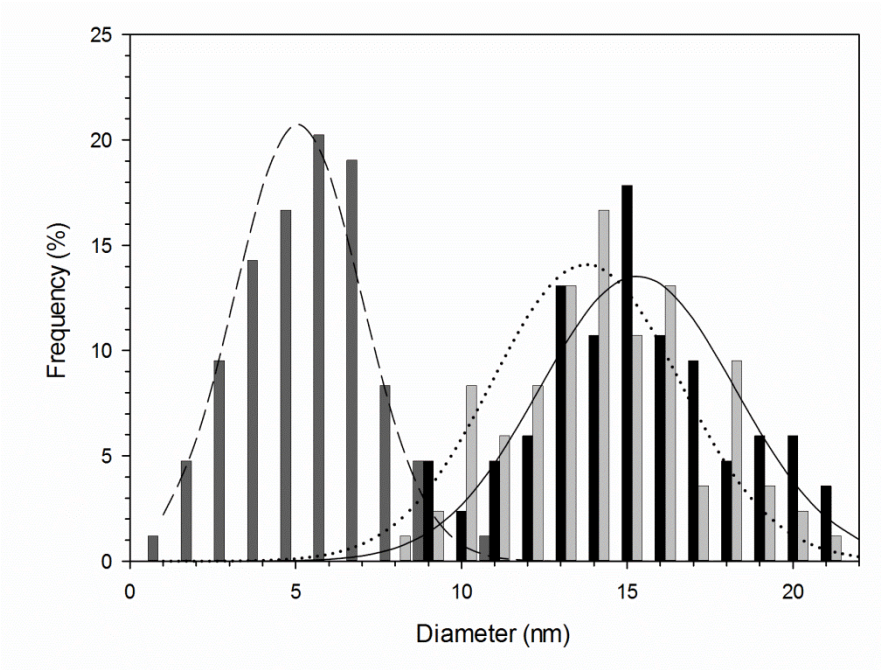

Figure 3

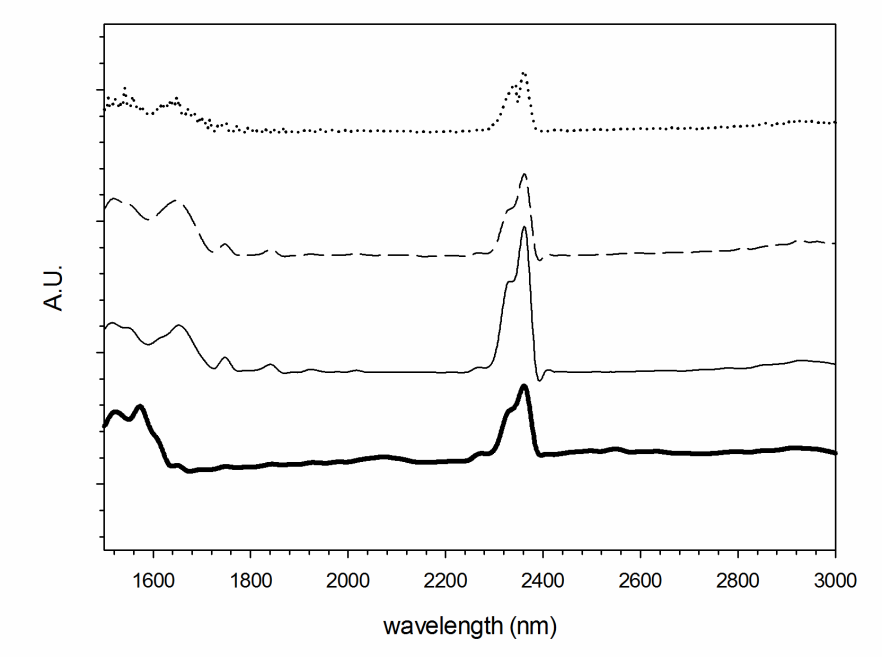

Figure 4

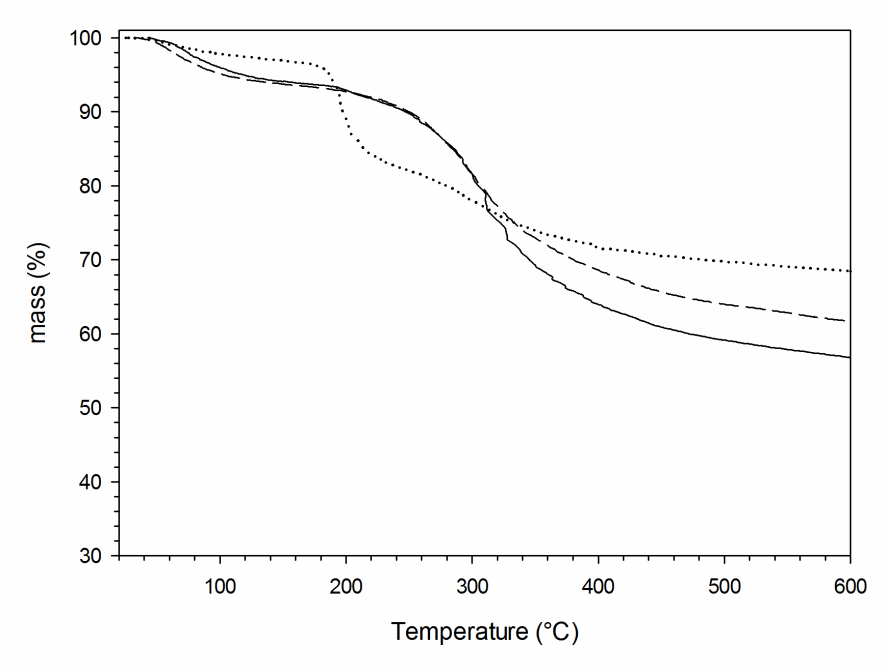
100
90
80
70
60
50
40
30
mass (%)
100
200
300
400
500
600
Temperature (°C)

Figure 5