American Journal of
Nanoscience and Nanotechnology Research
http://www.ivyunion.org/index.php/ajnnr

Research Article

Biosynthesis, Characterization and Antimicrobial Activity of Silver Nanoparticles Using Cell Free Lysate of Bacillus Subtilis: A Biotechnology Approach

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Abstract: Present study was aimed at the bio-synthesis and characterization of silver nanoparticles (AgNPs) using bacterial cell free lysate of *Bacillus* specie isolated from iron-rust contaminated soil sample from the Niger Delta region of Nigeria. This method was cost effective, eco-friendly and an alternative to chemical synthesis which is hazardous and requiring tedious synthetic manipulation. This study also evaluated the antimicrobial effect of the synthesized silver nanoparticles. Silver nanoparticles produced by reacting cell-free lysate of *Bacillus subtilis* and 1 mM of aqueous silver nitrate solution were characterized by Ultraviolet-visible spectroscopy, Transmission electron microscopy (TEM) and Photon correlation microscopy (Zeta sizer). The UV-Visible spectrophotometric result revealed an absorption maxima corresponding to peaks near 428 nm, depicting reduction of ionic silver (Ag^{+}) to silver atom (Ag^{0}). It has already been reported that nitrate reductase enzymes are implicated in metal ion reduction reactions. The Transmission electron microscopy analysis revealed that the AgNPs size ranged between 58.24 ± 1.04 nm and 72.20 ± 2.10 nm complementing the result obtained from Photon correlation microscopy (76.86 ± 1.14 nm). The antibacterial activity of AgNPs gave highest inhibition zone diameter of 26 mm on *Pseudomonas aeruginosa* at the dose of 0.10 mg/ml and 14 mm on tested *Candida albicans*. The synthesized silver nanoparticles were found to produce a dose dependent antimicrobial inhibitory effect while surface adsorption and lysis were implicated as the mode of action.

Keywords: Bacillus subtilis; silver nanoparticles; antimicrobial; photon correlation microscopy
Received: February 11, 2018; **Accepted**: March 3, 2018; P**ublished**: April 30, 2018
Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Synthesis and application of nanometal particles have rapidly gained interest in recent years. Areas of nano materials application especially the silver nanoparticles (AgNPs) include biomolecular detection, antimicrobials [1], antivirals [2] and catalysis [3]. Green synthesis of nanometal particles is cost effective, eco-friendly and has a profound advantage over chemical and physical synthesis since it can take place at room temperature [4]. Oftentimes, the morphology of nanoparticles can also be controlled during synthesis. There are postulations that microbial synthesis of metal nanoparticles can take place either intracellularly or extracellularly [5-8]. While intracellular synthesis may require ultrasonification [9], extracellular synthesis which is cost effective requires simple or one-pot synthesis affording great advantage in large-scale or industrial synthesis, hence, increased research in silver nanoparticles and their applications. When the bacteria cell-free lysate of *Bacillus subtilis* was treated with aqueous solution of silver nitrate (Ag^{+}) ions, silver nanoparticles were formed and this is a reduction reaction (Ag^{+} +e • Ag^{0}). Result has shown that other variable microorganisms like *Pseudomonas*, *Arthrobacter* and *Staphylococcos* species have the potential of extracellular nanometal formation [10].

In the current study, soil microorganisms from soil contaminated with rusted iron were screened for their potential to produce silver nanoparticles, the selected specie was characterized to be *Bacillus subtilis*. The stability of the nanoparticles were due to presence of capping agents and the potency of the nanoparticles against pathogens were attributed to their particle size and stability [11].

Experimental Section

Materials

Silver nitrate, nutrient media, and other reagents used were of analytical grade obtained from Merck, Germany and Hampshire, UK. *Bacillus subtilis* was isolated from rusty metal contaminated soil sample collected from Niger Delta region of Nigeria (Port Harcourt).

Instrumentation

The mean particle size and morphology of the AgNPs were characterized by transmission electron microscopy (TEM) (VEGAimuGmbH,Germany). Surface resonance Plasmon absorption was determined using UV-Visible spectrophotometer (Perkin Elmer Lambda 35). The polydispersity Index and Zeta Potential was determined using Photon correlation microscope (Mavern Nano ZS, ZS290, and UK). Infrared Spectroscopy (FTIR) was performed using the (FTIR)(Shimadzu-8400).

Bacterial isolation and purification

Bacterial isolation and handling were carried out in the Department of Microbiology and Biotechnology of the University of Port Harcourt, Nigeria under aseptic condition using an inoculation hood. In this research, bacterial isolate from metal rust contaminated soil was considered as source of Nicotinamide Adenine Dinucleotide (Enzyme), which is implicated in the biosynthesis of metal nanoparticles. Following soil sample pre-treatment by heating at 80^{oC} for 45 min using a thermostatic hot plate, sufficient quantity of biomass was obtained from bacterial culture aerobically grown in a nutrient agar and was latter sub-cultured in a nutrient broth (500 ml). The organism was harvested by centrifugation at 12000 rpm for 15 min at ambient temperature. The isolate was biochemically and

morphologically characterized following standard protocol [12]. The pure isolate gave a positive reaction on gram staining and was morphologically seen as rods under light microscope.

Preparation of cell-free lysate

The biomass was washed severally with double-distilled and UV-treated water via centrifugation. It was resuspended in 20 ml phosphate buffer (pH 7.4) and ground with sterile (autoclaved) white sharp sand, recentrifuged at 12000 rpm to get the cell-free lysate, which was stored at -20^{oC} until use.

Biosynthesis of silver nanoparticles (AgNPs)

Silver nanoparticle synthesis was carried out using 30 ml of cell-free lysate and 70 ml of an aqueous solution of silver nitrate (1 mM $AgNO_3$) in a 250 ml capacity conical flask. The content were kept under dark at a regulated temperature of 37^{oC} for 48 hr. Organoleptic result shows a colour change from creamy yellow to silvery black. While the negative control showed no colour change (Aqueous silver nitrate).

Characterization of Synthesized silver nanoparticles

The absorption spectra of the silver nanoparticle sample were taken at 190 to 610 nm using a UV-Visible spectrophotometer (Perkin Elmer Lambda 35) to determine the maximum point of production of silver nanoparticles. Double distilled water was used as blank.

The morphology and size of silver nanoparticles were determined by Transmission Electron Microscopy (TEM). Sample preparation for TEM analysis involve depositing a drop of aqueous silver nanoparticle suspension on a carbon- coated copper grid and allowed to dry at room temperature, the Transmission Electron Micrographs were produced and studied for particle size and morphology.

The average particle size, size distribution by intensity as well as polydispersity index were determined by injecting 1:20 dilution of aqueous silver nanoparticle solution into the U-shaped glass cuvette of the Photon Correlation Microscope. FTIR studies were carried out on the cell-free lysate by employing KBr pellet technique this was done to investigate the active principle involved in the reduction of silver ion (Ag^+) to atom (Ag^0). The FTIR spectra were generated at a resolution of 4 $^{cm-1}$ in a transmission mode (4000-400 $^{cm-1}$).

Antimicrobial activity of synthesized silver nanoparticles

Microorganisms

Bacterial and fungal isolates were procured from the Department of Microbiology of the University of Port Harcourt Teaching Hospital, Nigeria. The antimicrobial activity determinations were carried out under high asceptic condition**.**

This research employed the disc diffusion method ~~method of agar well diffusion assay~~ to investigate the antimicrobial activity of silver nanoparticles [13]. The tested micro-organisms were seeded in the nutrient agar plates, and then six 4-mm diameter paper discs were saturated with 0.05 mg/ml, 0.1 mg/ml AgNPs aqueous solution, Chloramphenicol (0.2 mg/ml), ketoconazole (0.5 mg/ml), 0.05 silver nitrate (AgNO3) aqueous solution and double distilled water (control) respectively. The paper disc was placed on the solidified agar plates and was allowed to incubate at 37^{oC} for 24 hr and 48 hr for bacteria

and yeast cultures respectively. The inhibition zone diameter was measured.

Results and Discussion

Biochemical reaction between silver nitrate aqueous solution and bacterial-cell free lysate produced a colour change from creamy yellow to silvery black due to the excitation of surface Plasmon vibration [14-18] , which clearly shows the presence of AgNPs. The above reaction yielded silver nanoparticles which were analyzed by UV-Visible spectroscopy.

Characterization of silver nanoparticles by UV-visible spectroscopy

Synthesized silver nanoparticle solution showed an absorption surface Plasmon peak at the region of 428 nm after 48 hr (see fig:1). The absorption peak intensity between 402-460 nm suggests the presence of surface Plasmon resonance which is characteristic for presence of silver nanoparticles [19].

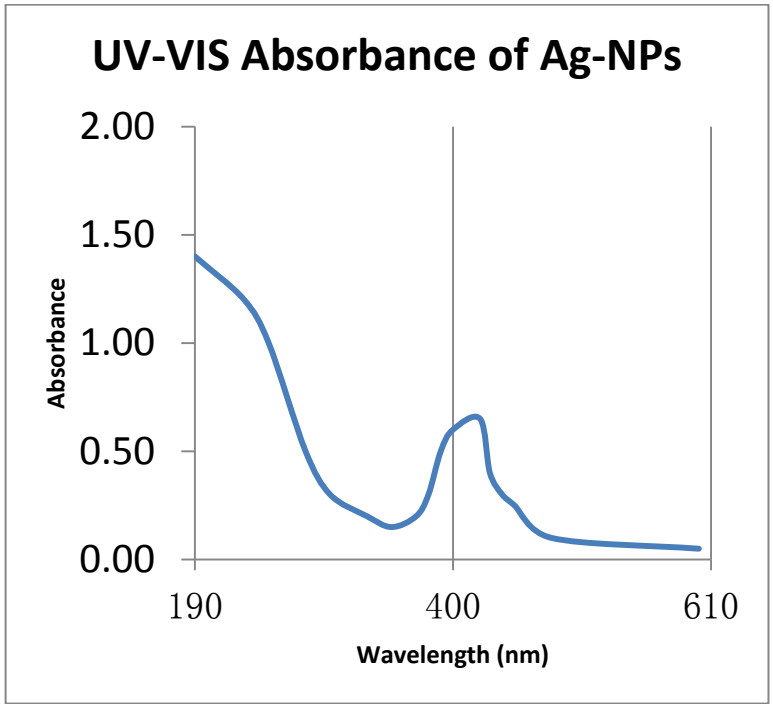

Fig 1 UV-VIS absorption band of synthesized silver nanoparticles.

Characterization of silver nanoparticles by Photon correlation microscopy

The mean particle size, zeta potential and polydispersity index of the synthesized silver nanoparticles were determined using Photon correlation microscope [20]. Result of Photon correlation microscopy analysis gave the zeta potential of the synthesized silver nano particles as -31.10 ± 0.42 mV. The polydispersity index (PI) was shown to be 0.24 ± 0.02 while the average size was observed to be 76.86 ± 1.14 nm. (See Fig: 2a**). Particle size range were found to be between 45 and 120 nm.**

PHOTON CORRELATION MICROSCOPY RESULT FOR Ag-NPs

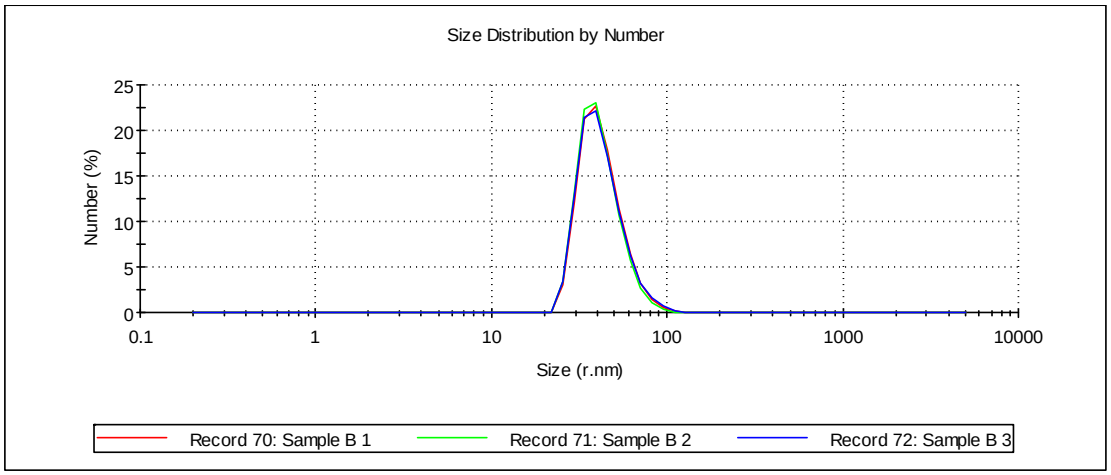

Fig 2a Zeta size distribution by number for synthesized silver nanoparticles.

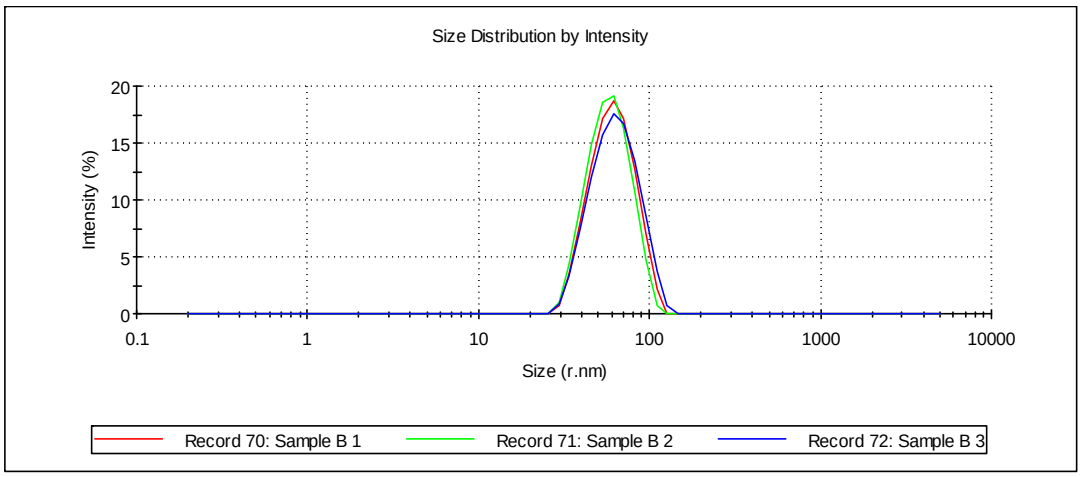

Fig 2b Zeta size distribution by intensity for synthesized silver nanoparticles.

Characterization of silver nanoparticles by TEM

The morphology of the synthesized silver nanoparticles was observed to be discreet and spherical with average particle size of 56 nm. (Fig. 3) The observed average particle size is in perfect correlation with Zeta sizer result.The silver nanoparticles produced were quite characteristic, distinct and polydispersed in their distribution.

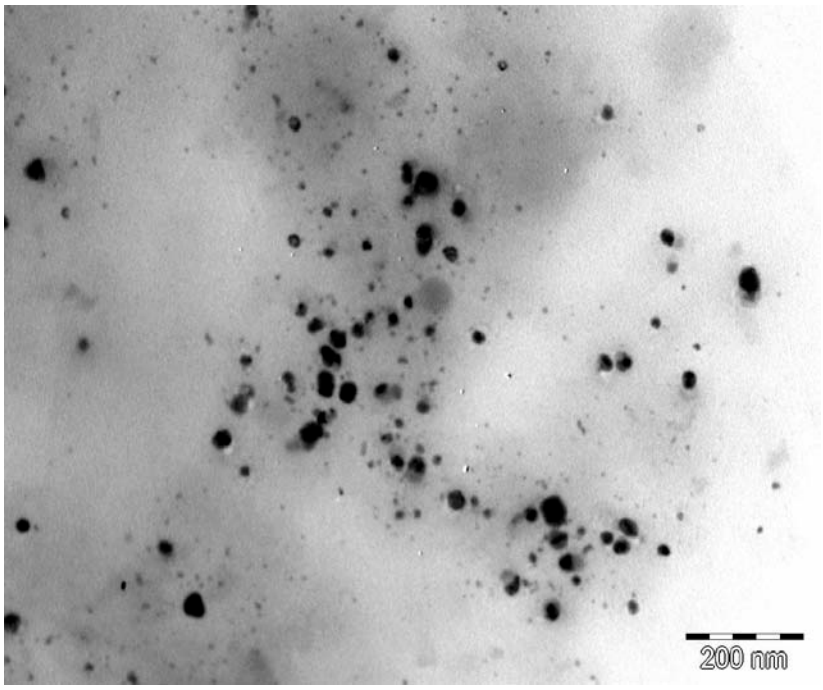

Fig. 3 TEM micrograph showing synthesized silver nanoparticles.

Characterization of silver nanoparticles by FTIR

Possible biochemical interaction which could occur between Ag^{+} and protein molecules was studied using FTIR to account for the reduction of silver ions to atom. Intercalations of protein molecules in the presence of silver ions are possible postulations. The spectra revealed three prominent peaks at the region of 1750.32 cm^{-1}, 3618.56 cm^{-1}, and 2160.36 cm^{-1} for aqueous silver nanoparticles. The hydrogen bonding of lipids and presence of protein (enzymes) give a well known signature in the infrared region of the electromagnetic spectrum. The bands at 1750.32 cm^{-1} are attributed to C-N stretching vibration. The bands at 3618.56 cm^{-1} and 2160.36 cm^{-1} are possibly due to amide stretching vibration of enzyme proteins arising from C=O stretching and C-N bending vibration. The overall observation hence confirmed the presence of protein in the cell-free lysate supporting the previous work of Jain et al.2011 . Proteins have been implicated in the binding, reduction of Ag+ to Ag^{0} (atom) and source of enzymes involved in the overall reaction. (Fig:4). Other components include aromatics, lipids and amino acids. It has been reported in previous work that protein binds with nanoparticles through cysteine residues or free amino acids and negatively charged carbonyl moiety in proteins can also bind with the nanoparticle through electrostatic interactions. Membrane associated cytochromes (enzymes) and redox proteins are the main components of cell free lysate which are involved in direct electron transfer and hence nanoparticle formation.

Possible mechanism of nano silver synthesis

Nicotinamide adeninine dinucleotides (NAD+) are component of enzymes and these are present in the

bacterial cell-free lysate. They are known to carry electrons from one reaction to another. The ability of NAD to initiate a redox reaction could have caused the transformation of Ag^+ to Ag^0.

$AgNO_3$ • $Ag^+ + NO_3^-$

$NAD^+ + e^-$ • NAD

$NAD + H^+$ • $NADH + e^-$

$e^- + Ag^+$ • Ag^0 .

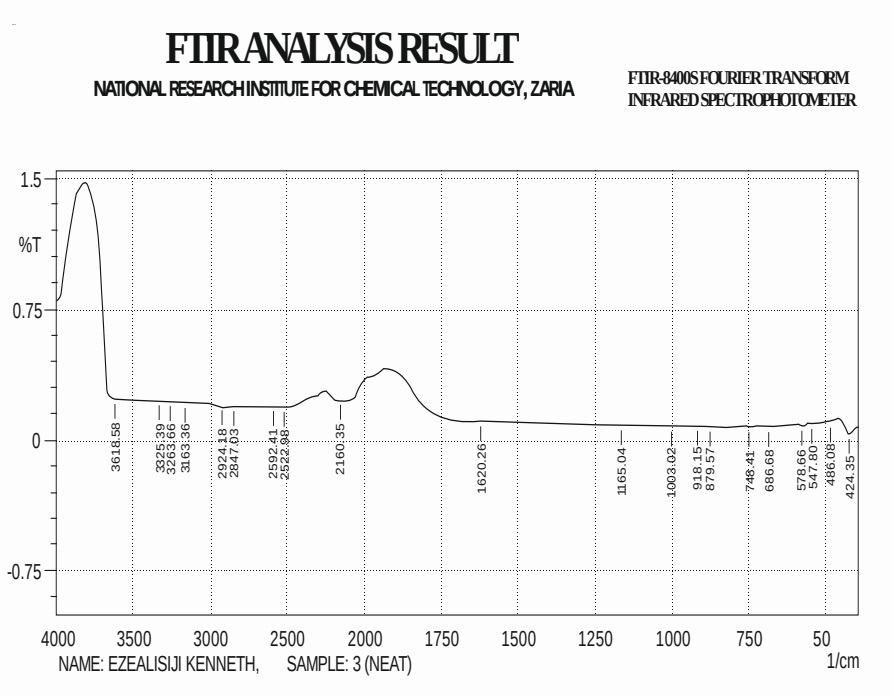

Fig.4 FTIR spectrum of bacteria cell free lysate.

Antimicrobial activity of synthesized AgNPs

The antimicrobial efficacy of synthesized silver nanoparticles was investigated against selected pathogenic organisms such as *B. subtilis, pneumonia*, *S. aureus*, *P. aeruginosa*, *E. coli*, and *C. albicans* using agar well diffusion method. The inhibition zone diameter (mm) were recorded against a positive control (chloramphenicol). At the dose of 0.05 mg/ml and 0.10 mg/ml respectively, the silver nanoparticles were found to possess a remarkable antimicrobial activity against all the tested pathogenic organisms in a dose-dependent manner. All the tested organisms, except *B. subtilis,* were highly susceptible. *B. subtilis* was found to be less susceptible. The antibacterial activities of the synthesized silver nanoparticles were found to be more efficacious when compared with the standard drug chloramphenicol (0.2 mg/ml). The anti-fungal activities of the AgNPs were also remarkable though slightly lower than those of ketoconazole (0.5 mg/ml) (Fig.5). Bacterial cell lysis and death could be due to ~~adsorption~~ penetration of silver nanoparticles through bacterial protein cell membrane

as well as their reaction with biological components of the cells such as sulphur and phosphorus–containing amino acid component of DNA (plasmid bodies) leading to protein denaturation and cell death.

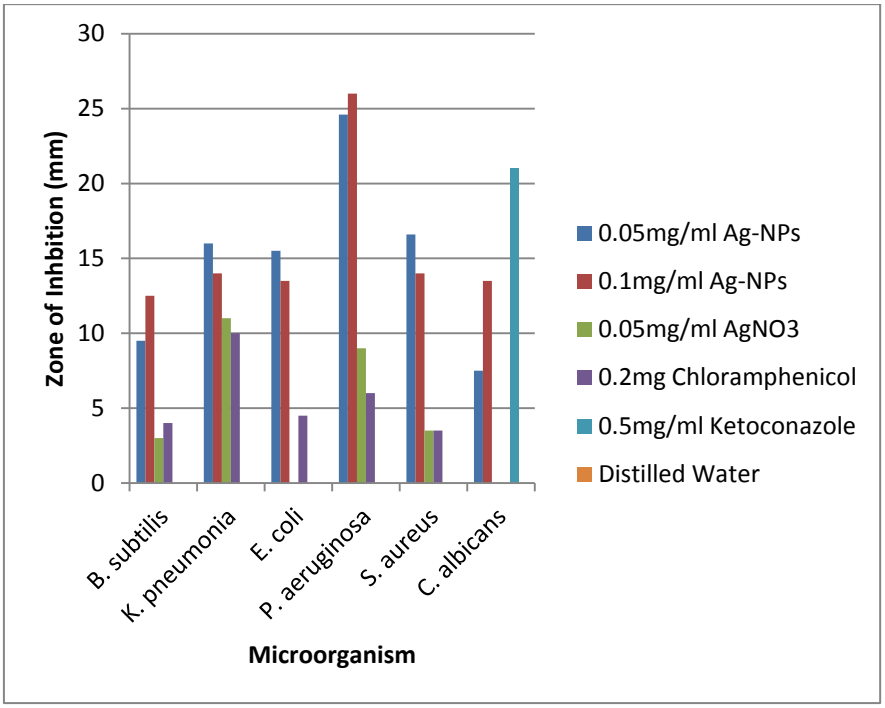

Fig. 5 Inhibitory activities of synthesized silver nanoparticles.

Conclusion

In this study, silver nanoparticles were successfully synthesized from highly virulent *Bacillus subtilis* isolated from iron rust contaminated soil sample from the Niger Delta region of Nigeria. The efficacies of the synthesized silver nanoparticles against pathogenic microorganisms were established. Synthesis and formation of silver nanoparticles were confirmed through highly sensitive analytical tools which include UV-vis spectrophotometer, Photon correlation microscopy, TEM and FTIR. Thus, bio-synthesized silver nanoparticles could be included as a component of transdermal patches intended to be used for healing of wounds since *P. aeruginosa* is implicated in wound infections.

Acknowledgement

The authors are grateful to management of Department of Chemistry, Rhodes University, Grahamstown, South Africa for granting the research team access to their state-of-the-art Nanotechnology centre.

Conflict of Interest

The authors report no conflicts of interest.

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