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SILVER NANOPARTICLES POSSESS ANTI-BIOFILM ACTIVITY AGAINST MULTIDRUG-RESISTANT *STAPHYLOCOCCUS SAPROPHYTICUS* ATCC 15305

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ABSTRACT: An example of the human microbiota is *Staphylococcus saprophyticus*, a gram-positive, non-hemolytic pathogenic bacterium. It's a prevalent culprit behind simple infections associated with the urinary tract, especially among sexually active young women. It has a high colonization rate in the perineum, rectum, urethra, cervix, and gastrointestinal system and is also quite lethal. Because of their ability to form a biofilm, these bacteria are highly resistant to antibiotics. Thus, unconventional methods, such as the use of silver nanoparticles, have been devised to counteract this type of biofilm. The silver nanoparticles are produced in an environmentally friendly way by utilizing an extract of water from banana peels. UV/Vis, FTIR, SEM, and EDAX are the four methods used to characterize the NPs and validate their production. Using the Screening method and SEM analysis, we show that silver nanoparticles inhibit the growth of *Staphylococcus saprophyticus* ATCC 15305, the most prevalent bacteria responsible for urinary tract infections.

INTRODUCTION: Worldwide, healthcare-associated infections have increased due to the spread of methicillin-resistant *Staphylococcus* germs. There are several types of *Staphylococci* that are both beneficial and harmful to human health ¹. *S. saprophyticus*, the second most common cause of simple urinary tract infections, is most common in young, sexually active females (42% of all infections) ^{2, 3}. When cells of one microbe adhere to cells of another species and, in many cases, to a surface, the resulting layer is called a biofilm. These attached cells embed themselves in a polymeric matrix they generate *in-vitro* ⁴.

The attachment of free-floating bacteria initiates the biofilm development process. Weak Vander Waals forces and hydrophobic factors initially cause the biofilm to adhere ⁵. They may securely fasten themselves in place with the help of cell adhesion structures called pili. Biofilms release polysaccharides that trap quorum-sensing autoinducers and ensure bacterial survival. Therefore, quorum sensing and other forms of cell-to-cell communication have played a role in biofilm development ^{6, 7}.

Similar to how nanotechnology offers a fresh strategy for combating and ultimately eliminating such biofilms, it also provides an alternative method of doing so. It's a field of study that draws from many other fields to examine the many uses and applications of nanoparticles (NP). There are many various types of nanoparticles based on their characteristics, shapes, and sizes, with diameters ranging from 1 nm to 100 nm.

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Because of their unique nanoscale sizes and architectures, they exhibit unique chemical and physical characteristics. Because of their absorption in the visible spectrum, they also have optical qualities⁸. NPs have three distinct layers: Surface layers can be modified in several ways, including adding tiny molecules, metal ions, surfactants, and polymers. Second, the shell's outermost layer has a distinct chemical composition. Thirdly, the NPs themselves are the core^{9,10}. There is a wide variety of NPs, each with its own unique size, shape, chemistry, and other characteristics¹¹. Pure metal nanoparticles exhibit a wide absorption band in the visible region of an ultraviolet-visible spectrophotometer and have a localized surface plasmon resonance characteristic. Their unique optoelectrical properties make them applicable to various scientific fields. Also, NPs of noble metals like Ag may be generated using a variety of chemical and physical processes.

On the other hand, AgNPs may be synthesized in a green way that is both economical and kind to the environment. Reduction of Ag salts is a function of several plant components¹². Every year, industries generate a million tonnes of peel trash. This has led to a rise in interest in the use of biological waste in the synthesis of AgNPs among researchers. Peels include a variety of flavonoids, phytochemicals, polyphenols, and alkaloids that have both reducing and stabilizing effects. These compounds can be utilized as an alternative and in large-scale manufacturing of AgNPs since they decrease Ag salt¹³⁻¹⁵.

MATERIALS AND METHODS: The chemical used during the experiment is AgNO₃ which was provided by our Department of Microbiology, Guru Nanak Institute of Pharmaceutical Sciences and Technology, Sodpur, Kolkata, West Bengal, India.

Preparation of Banana Peel Extract: 25g of *M. paradisiaca* peels were washed thoroughly and boiled in distilled water at 70°C for 15 minutes. The solution is filtered through a cheesecloth to remove insoluble fractions and impurities. This extract is stored in the refrigerator at 4°C, which is used as a reducing as well as capping agent.

Synthesis of AgNPs using Banana Peel Extract (BPE): AgNO₃ is used as a source of Ag. 10mM of

50mL AgNO₃ solution is prepared in a conical flask. The BPE is poured into the burette and added dropwise very slowly until the solution turns dark brown. The reaction is carried out under dark conditions.

Characterization of Silver Nanoparticles: Characterizing silver nanoparticles is extremely necessary to understand the behavior, bio-distribution, safety, and efficacy of the formed nanoparticles. UV Visible Spectroscopy is a simple and easier technique used to primarily characterize synthesized nanoparticles. De-ionized water is used as a blank. Fourier transforms infrared spectroscopy (FTIR) is used to determine the primary functional groups in biomolecules that act as reducers and caps for the bio-reduced silver nanoparticles.

The sample was ground into a powder and then combined with KBr powder to produce a pellet. With a resolution of 4cm⁻¹, all measurements were taken between 400 cm⁻¹ and 4000cm⁻¹. SEM is used to study the surface morphology of the NPs. In order to perform this experiment, the powdered sample was coated with gold and palladium, in which a 10kV current is passed. Also, the energy dispersive spectrum of the synthesized nanoparticles gives the quantitative information of the biosynthesized NPs suggesting the presence of Ag as the ingredient element showing absorption at the range between 2.5 keV- 4 keV due to surface plasmon resonance¹⁶.

Preparation of Biofilm of *S. saprophyticus*: 100 ml of biofilm media is prepared by dissolving 0.1g of beef extract, 0.2g of yeast extract, 0.05g of peptone, and 0.05g of NaCl in 100 ml of distilled water. The constituents are heated until they dissolve completely. The media is sterilized by autoclaving at 121°C, 15 Psi pressure for 25 minutes. The media is cooled to 40°C and poured into a test tube. Previously isolated and identified multidrug-resistant *Staphylococcus saprophyticus* ATCC 15305 is inoculated into the medium¹⁷. The test tubes are then incubated at 37°C for 24 hours under sterile conditions. Similarly, biofilms are also grown on five different coverslips. After an incubation period, the test tube contents are poured into a small beaker, and the biofilm formed on the walls of the test tube and the coverslips are stained

with crystal violet (0.4%) for 35 minutes and air dried. Air drying is done after two washes each with double distilled water and PBS to remove any remaining planktonic cells^{18,19}.

Screening of Anti-biofilm Activity of AgNP: The antibiofilm activity of AgNPs is screened by adding AgNP in the concentration of 20 μ l, 40 μ l, 60 μ l, and 80 μ l each to each test tube and cover slip containing biofilm of *S. saprophyticus* ATCC 15305. The change in the presence of biofilm on the walls of the test tube is noted to screen the antibiofilm activity of AgNP. The degree to which

biofilm on the test tube walls has diminished is noted to screen the antibiofilm activity of AGNPs. Furthermore, SEM is used to examine the morphological alteration of the biofilms generated on the coverslips after treatment with AgNPs.

RESULT:

Synthesis of Silver Nanoparticles: On pouring the BPE dropwise slowly from the burette into the AgNO₃ solution, the solution turns brown. The formation of the brown color of the solution (as in **Fig. 1**) is a visible indicator of the formation of silver nanoparticles.



FIG. 1: FORMATION OF SILVER NANOPARTICLES

Characterization of Silver Nanoparticles: The characterization of AgNPs is important to understand their behavior, bio-distribution, safety, and efficacy. Careful characterization of AgNPs is an important step which is done by applying the following techniques:

UV Vis Spectroscopy: Primarily, silver nanoparticles have been characterized using

UV/Vis spectroscopy, which is both a simple and effective method. The absorption spectra of the AgNPs were found at 400nm.

Peak resembles characteristics of surface plasmon resonance of silver NP on plotting obtained absorbance versus wavelength graph **Fig. 2**²⁰. De-ionized sterile water is used as blank.

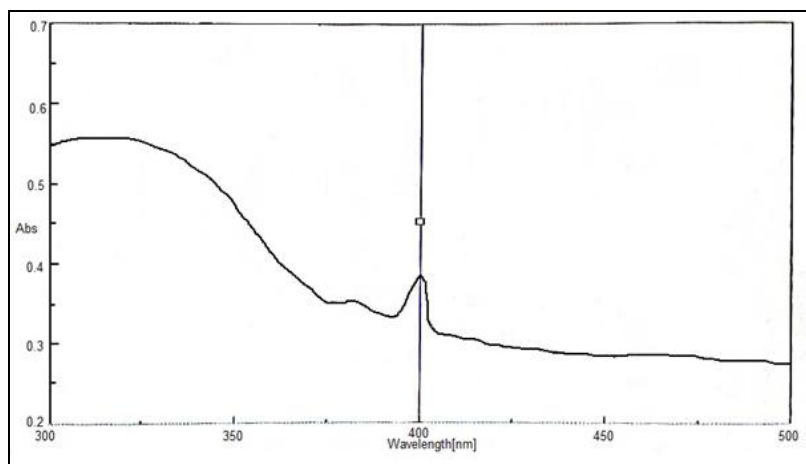


FIG. 2: UV VISIBLE SPECTRA OF SILVER NANOPARTICLES

FTIR Analysis of Silver Nanoparticles: The bio-reduced silver nanoparticles are characterized by their FTIR spectrum to determine the key functional groups in the biomolecules responsible for the reduction and capping processes. FTIR Spectrum shows absorption bands at 3429 cm^{-1} , 2935 cm^{-1} , 1631 cm^{-1} , 1385 cm^{-1} , 1056 cm^{-1} and 557.4 cm^{-1} indicating the presence of reducing as well as capping agents in nanoparticles **Fig. 3**. Furthermore, comparing the results from the previous researches conducted and tallying with our findings, the peaks indicate O-H stretching alcohol group with strong vibration between the

bonds of the atoms (3429 cm^{-1}), C-H stretching alkane with the medium appearance of vibration between the bonds (2935 cm^{-1}), C=C stretching conjugated alkene with medium vibration between the bonds (1631 cm^{-1}), S=O stretching sulfate with the strong appearance of vibration between the bonds (1385 cm^{-1}), C-O stretching primary alcohol group with the strong appearance of vibration between the bonds of the atoms (1056 cm^{-1}), C-I stretching halogen compound with strong vibration between the bonds of the atoms in the nanoparticles (557.4 cm^{-1}), on the surface of BPE is responsible in the process of NPs synthesis²¹⁻²⁴.

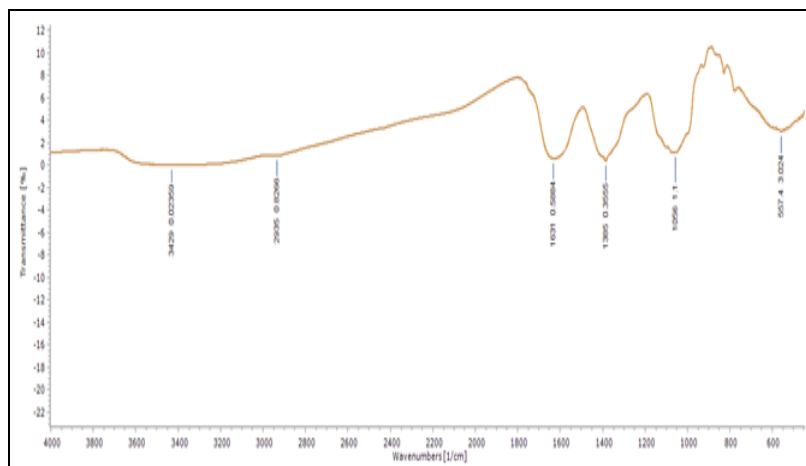


FIG. 3: FTIR ANALYSIS OF SILVER NANOPARTICLES

Analysis of Silver Nanoparticles using SEM: The NP's surface morphology was examined using SEM. It creates a three-dimensional picture of the material by using electrons that have been backscattered and secondary electrons. Magnifications of 2000x and 9000x were used for the scanning electron microscopy study **Fig. 4** and **Fig. 5**. Analyzing the nanoparticles at 2000x

validates the development of silver nanoparticles **Fig. 4** with measured sizes ranging between 30nm-65nm along with triangular, tetragonal, pentagonal, hexagonal structural orientation while examination at 9000x reveals that the NPs were distinct and the shape of the NPs is more or less spherical and quasi-spherical **Fig. 5**.

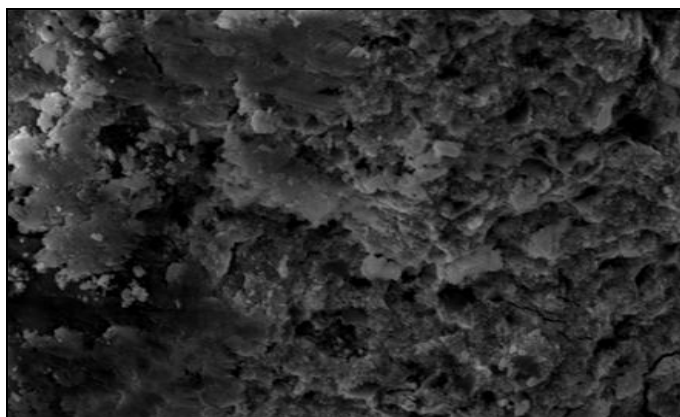


FIG. 4: SEM ANALYSIS OF SILVER NANOPARTICLES (2000X MAGNIFICATION)

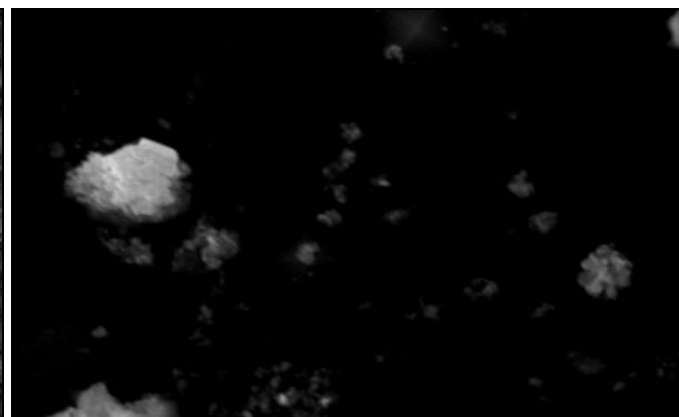


FIG. 5: SEM ANALYSIS OF SILVER NANOPARTICLES (9000X MAGNIFICATION)

Analysis of the Nanoparticles using EDAX: Quantitative information on biosynthesized NPs may be gleaned from their energy-dispersive X-Ray spectra. Absorption between 2.5 keV and 4 keV, as seen in **Fig. 6**, is evidence of surface

plasmon resonance and, by extension, the existence of Ag as the constituent element²⁵. **Fig. 6** further displays the presence of many elements including Ag, O, K, Cl, Ca, P, Na, as well as Mg.

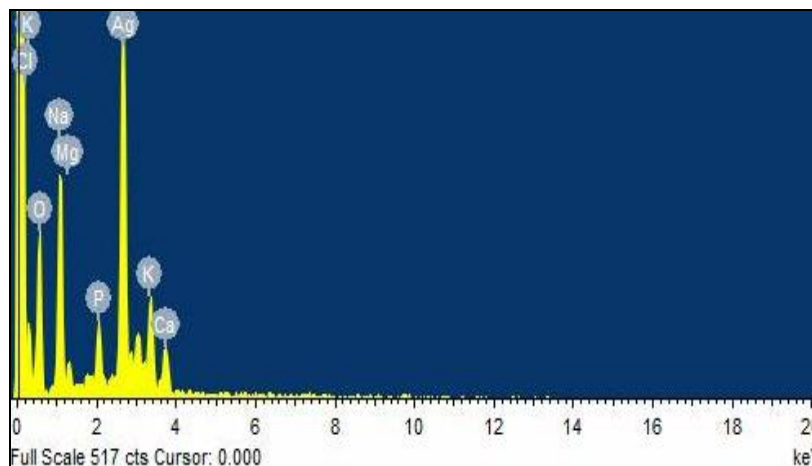


FIG. 6: EDAX SPECTRUM OF SILVER NANOPARTICLES

Biofilm Formation on Test Tube: The inoculated bacterial strain is incubated in the test tube at 37°C for 24 hours **Fig. 7** and forms a biofilm that has been confirmed by staining them using crystal violet.

The crystal violet staining test is a widely used technique for determining the level of microbial biofilm development in a variety of settings^{19, 26}. Air drying the stained test tube indicates the occurrence of visible films along the inner liner of the walls of the test tube **Fig. 8**.



FIG. 7: AFTER INOCULATING THE BACTERIAL STRAINS IN TEST TUBE AND INCUBATION AFTER 24 HOURS



FIG. 8: AFTER DISCHARGING THE PLANKTONIC CELLS AND STAINING THE BIOFILM WITH CRYSTAL VIOLET

Screening of Anti-biofilm Activity of Silver Nanoparticles: Silver nanoparticles are added in four different test tubes at a concentration of 20 µg/ml, 40 µg/ml, 60 µg/ml, and 80 µg/ml. The capacity of AgNPs to suppress *S. saprophyticus* ATCC 15305 biofilm activity was measured as a function of concentration. Adding 20µg/ml and 40 µg/ml of AgNP, show that the amount of biofilm has decreased somewhat. However, biofilm is nearly completely inhibited as AgNP concentration is increased from 60 µg/ml and 80 µg/ml.

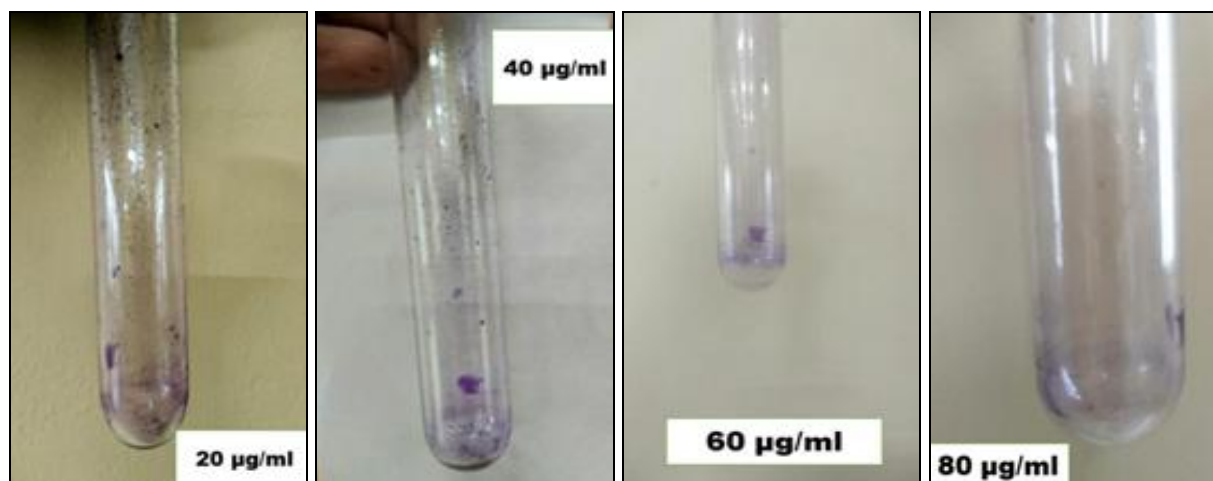


FIG. 9: SCREENING OF ANTI-BIOFILM ACTIVITY OF SILVER NANOPARTICLES

Antibiofilm Activity of Silver Nanoparticles using SEM: SEM was used to characterize the morphology of biofilms treated with AgNPs²⁷. After 48 hours of growth on glass coverslips, *S. saprophyticus* biofilms consisted of EPS threads and clumped, aggregated bacterial cells **Fig. 10**. The growth of biofilm clusters and the formation of

EPS matrices were virtually suppressed upon the addition of AgNPs at two distinct doses, namely at 60 µg/ml **Fig. 11** and 80 µg/mL **Fig. 12**. Therefore, the antibiofilm action of AgNPs against *S. saprophyticus* ATCC 15305 was shown to be concentration dependent.

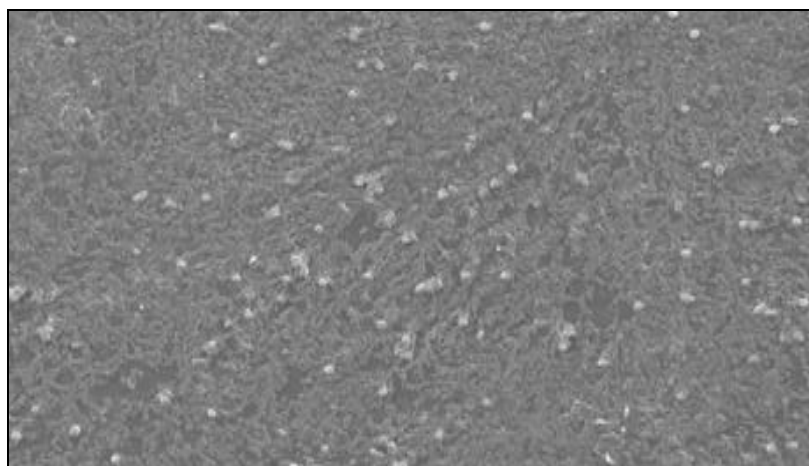


FIG. 10: CONTROL WITHOUT ADDITION OF SILVER NANOPARTICLES (2000X MAGNIFICATION)

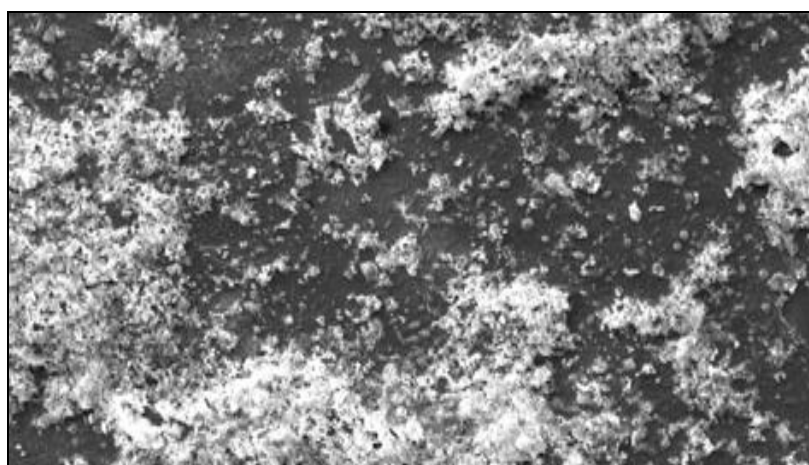


FIG. 11: SUPPRESSION OF BIOFILMS AFTER ADDITION OF SILVER NANOPARTICLES AT 60 µG/ML (2000X MAGNIFICATION)

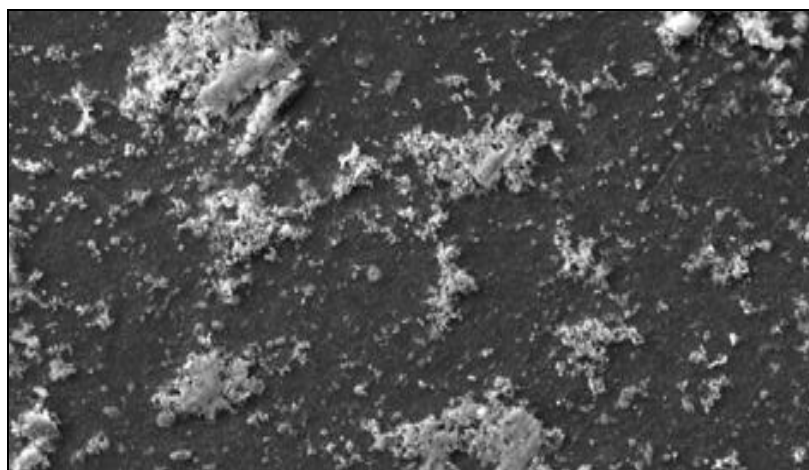


FIG. 12: SUPPRESSION OF BIOFILMS AFTER ADDITION OF SILVER NANOPARTICLES AT 60 µG/ML (2000X MAGNIFICATION)

DISCUSSION: The AgNPs' anti-biofilm efficacy varies with species, and so does sensitivity to their effects. Evidence suggests that AgNPs can kill off a wide variety of *Staphylococci* sp. It is hypothesized that the mechanism is analogous to that of silver ions and that it involves the adhesion and lysis of the bacterial membrane or cell wall, biomolecular interaction and disruption (nucleic acid, enzymes), and the synthesis of reactive oxygen species (ROS) and free radicle species that cause cellular oxidative damage.

When applied to bacterial cells and biofilms, AgNPs efficiently inhibit the formation of biofilms and kill bacteria living inside them. Our work has established the antibiofilm activity of silver nanoparticles against the strain of *S. saprophyticus* ATCC 15305. On addition of silver nanoparticles at a concentration of 20µg/ml and 40 µg/ml, showed minimal antibiofilm activity. Further, increasing the concentration of silver nanoparticles to 60 µg/ml and then to 80 µg/ml, inhibits the formation of biofilms nearly or completely. SEM analysis of the anti-biofilm activity of silver nanoparticles was further confirmed at 2000x magnification, which also depicted the large-scale anti-biofilm activity of AgNP at a concentration of 60 µg/ml and 80 µg/ml. Furthermore, when diagnosing cancer or determining how healthy cells interact with their surroundings, NPs are just as useful as any other diagnostic instrument. Measuring nanoscale forces exerted by proteins on cells may provide new insight into several diseases, including osteoarthritis, cancer, and malaria, due to changes in cells' elasticity and adhesion.

This is made possible by developing nanoceramics, a technology that allows researchers to examine the fundamental behavior of living cells and biomolecules²⁸. Performing even one of these studies can help researchers better understand the molecular basis of disease, aid in the creation of more precise diagnostic tests, and inspire new ways to treat patients. Inorganic nanostructures have several potential uses as biomarkers, including in genomics, proteomics, molecular diagnostics, and high-throughput screening, each offering exciting new possibilities and access to powerful new tools. The development of nanoscale probes has made it possible to track and analyze cellular processes in great detail²⁸. Silver nanoparticles had a bactericidal rather than bacteriostatic impact on the examined bacteria, as evidenced by “the average ratio of the minimum bactericidal concentration to the minimal inhibitory level”²⁹. Theoretically, a bactericidal agent is favored in clinical settings since killing bacteria should hasten the end of an illness, boost clinical outcomes, and lessen the chances of drug resistance and further infection spreading. Microbes are less likely to develop antibiotic resistance mutations if they are eradicated rather than suppressed³⁰.

CONCLUSION: This article has reached the preliminary conclusion that, similar to the effects of pharmaceuticals, different NPs can have both desirable and unintended outcomes in humans³¹. Numerous uses, including those in catalysis, sensing, photovoltaic energy, the environment, and medicine, have been investigated for NPs based on metals^{32,33}.

However, there may be secondary consequences for humans from the growing dangers posed by NPs. Since our understanding of how NPs evolve is still in its infancy, more focus is required to steer these NPs in the right direction. Processing NPs for life science requires extensive research to properly understand their production, characterization and probable toxicity. At now, the pharmaceutical industry is the primary target of NP commercialization. Despite NPs' promising biological uses, it's important to understand how NPs interact with cellular components and mechanisms. Accordingly, the advancement of nanobiotechnology requires attention to the NPs' *in-vivo* biomedical applications.

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