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Bioreduction of $AuCl_4^-$ Ions by the Fungus, *Verticillium sp.* and Surface Trapping of the Gold Nanoparticles Formed**

Priyabrata Mukherjee, Absar Ahmad, Deendayal Mandal, Satyajyoti Senapati, Sudhakar R. Sainkar, Mohammad I. Khan, R. Ramani, Renu Parischa, P. V. Ajayakumar, Mansoor Alam, Murali Sastry,* and Rajiv Kumar*

The area of nanotechnology, which spans the synthesis of nanoscale matter, understanding/utilizing their exotic physicochemical and optoelectronic properties, and organization of nanoscale structures into predefined superstructures, promises to play an increasingly important role in many key technologies of the new millennium.[1] As far as the synthesis of nanoparticles is concerned, there is an ever-growing need to develop clean, non-toxic, and environmentally friendly ("green chemistry") synthetic procedures. Consequently, researchers in the field of nanoparticle preparation have been looking at biological systems for inspiration. The above factors, combined with academic curiosity, has lead to the development of biomimetic approaches for the growth of advanced materials. Many organisms, both unicellular and multicellular, are known to produce inorganic materials either intra- or extracellularly,[2] examples include magnetotactic bacteria (which synthesize magnetite nanoparticles),[3] diatoms (which synthesize siliceous materials),[4] and S-layer bacteria (which produce gypsum and calcium carbonate layers).[5]

Even though microbes have been used with considerable success in biotechnological applications, such as remediation of toxic metals,[6] reports on their use in the synthesis of nanomaterials are extremely limited. Beveridge and coworkers have demonstrated that nanoscale gold particles

[*] Dr. M. Sastry, Dr. S. R. Sainkar, Dr. R. Parischa
Materials Chemistry Division
National Chemical Laboratory
Pune, 411 008 (India)
Fax: (+91) 20-5893952/5893044
E-mail: sastry@ems.ncl.res.in

Dr. R. Kumar, Dr. P. Mukherjee, D. Mandal, S. Senapati
Catalysis Division
National Chemical Laboratory
Pune, 411 008 (India)
Fax: (+91) 20-5893761
E-mail: rajiv@cata.ncl.res.in

Dr. A. Ahmad, Dr. M. I. Khan
Biochemical Sciences Division
National Chemical Laboratory, Pune, 411 008 (India)

R. Ramani
Department of Pathology, Armed Forces Medical College, Pune, 411 001 (India)

P. V. Ajayakumar, Dr. M. Alam
Department of Microbiology and Plant Pathology
Central Institute of Medicinal and Aromatic Plants, P.O. CIMAP, Lucknow, 226 015 (India)

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readily precipitate in bacterial cells on incubation of the cells with Au^{3+} ions.[7] A study of the growth of Au nanoparticles by using *Bacillus subtilis* 168 revealed the precipitation of non-crystalline Au particles which subsequently transformed into crystalline octahedral gold containing sulfur and phosphorus.[7b] More recently, Klaus-Joerger, and co-workers have demonstrated that placing the bacteria *Pseudomonas stutzeri* AG259 (isolated from a silver mine) in a concentrated aqueous solution of $AgNO_3$, resulted in the reduction of the Ag^+ ions and formation of silver nanoparticles of well-defined size and distinct morphology within the periplasmic space of the bacteria.[8] Furthermore, they have shown that biocomposites of nanocrystalline silver and the bacteria, when thermally treated, yield a carbonaceous material with interesting optical properties for potential application in functional thin-film coatings.[9] Herein, we demonstrate that exposure of the fungus, *Verticillium sp*. (AAT-TS-4), to aqueous $AuCl_4^-$ ions results in reduction of the metal ions and formation of gold nanoparticles of around 20 nm diameter. The gold nanoparticles are formed on both the surface and within the fungal cells (on the cytoplasmic membrane) with negligible reduction of the metal ions in solution. To our knowledge, this is the first demonstration of the use of eukaryotic organisms in the biological synthesis of noble metal nanoparticles such as gold.

Figure 1 A shows the *Verticillium sp.* fungal cells after removal from the culture medium and before immersion in $HAuCl_4$ solution, the pale yellow color of the fungal cells can clearly be seen. After exposure to aqueous solution of $HAuCl_4$ the fungal cells are vivid purple which indicates the formation of Au nanoparticles in the cells (Figure 1 B); the aqueous $HAuCl_4$ medium is colorless, which indicates that extracellular reduction of $AuCl_4^-$ ions has not occurred. Gold nanoparticles absorb radiation in the visible region of the electromagnetic spectrum (ca. 520 nm) because of excitation of surface plasmon vibrations giving gold nanoparticles striking colors in various media.[10] The UV/Vis spectra show no evidence of absorption in the range 400 – 800 nm for the as-harvested fungal cells, the fungal cells exposed to $AuCl_4^-$ ions show a distinct absorption at around 540 nm (Figure 1 C). This absorption is close to that observed for thin films of gold nanoparticles grown by different techniques.[10, 11] Just as for the as-harvested fungal cells the UV/Vis spectrum of the aqueous $HAuCl_4$ solution after immersion of the fungal cells shows no discernible absorption in the 540 nm region. This result supports the visual evidence (Figure 1 B) that no extracellular reduction of the $AuCl_4^-$ ions occurred. Further evidence for the intracellular formation of gold nanoparticles is provided by X-ray diffraction (XRD) analysis of the Au nano-*Verticillium* biofilm deposited on a Si substrate (Figure 1 D). The presence of intense peaks corresponding to the (111), (200), and (220) Bragg reflections of gold (identified in the diffraction pattern) agree with those reported for gold nanocrystals.[12] An estimate of the mean size of the gold nanoparticles formed in the cells was made by using the Debye – Scherrer equation by determining the width of the (111) Bragg reflection.[13] The size of the gold nanoparticles was thus determined to be about 25 nm.

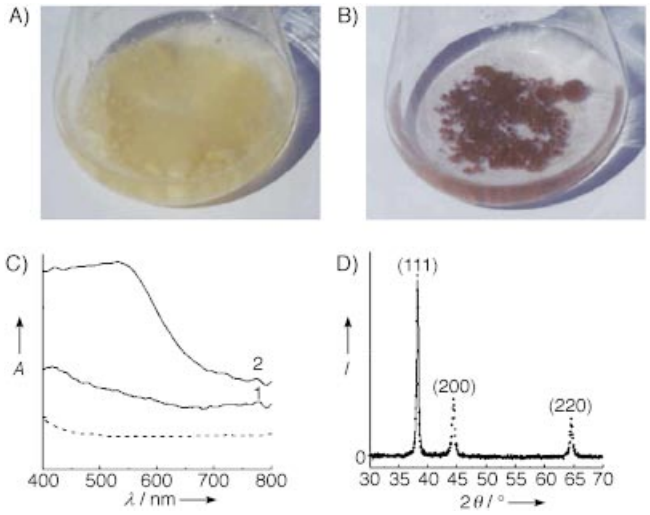

Figure 1. A) *Verticillium* fungal cells after removal from the culture medium. B) *Verticillium* fungal cells after exposure to 10^{-4} M aqueous solution of $HAuCl_4$ for 72 h. C) UV/Vis spectra recorded from biofilms of the *Verticillium* fungal cells before (curve 1) and after exposure to 10^{-4} M aqueous $HAuCl_4$ solution for 72 h (curve 2). The spectrum recorded from the $HAuCl_4$ solution after immersion of the fungal cells for 72 h is shown for comparison (dashed line). D) XRD pattern recorded from an Au nano-*Verticillium* biofilm formed on a Si(111) wafer. The principal Bragg reflections are identified.

The presence of mycelia covered with well-dispersed gold nanoparticles can be observed by scanning electron microscopy (SEM; Figure 2 A). The gold nanoparticles appear to be on the surface of the mycelia. A spot-profile energy-dispersive analysis of X-rays (EDX) of one of the gold nanoparticles shows the presence of strong signals from the gold atoms together with weaker signals from C, O, and Cl atoms (Figure 2 b). The C and O signals arise from X-ray emission from proteins/enzymes either directly bound to the gold nanoparticle or in the vicinity of the particle, while the presence of a weak Cl signal indicates the presence of a small fraction of $AuCl_4^-$ ions in the region being investigated.

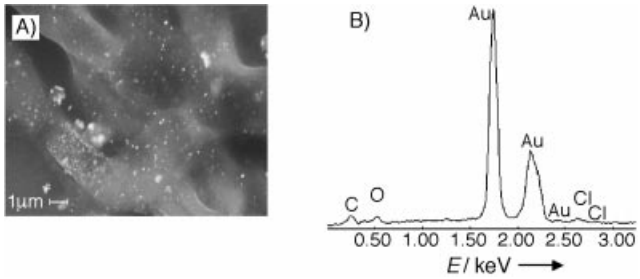

Figure 2. A) SEM image of a biofilm of the *Verticillium* fungal cells deposited on an Si(111) wafer after immersion of the cells in aqueous 10^{-4} M $HAuCl_4$ solution for 72 h. B) Spot-profile EDX spectrum recorded from one of the gold nanoparticles shown in Figure 2 A.

To understand, albeit tentatively, the mechanism of formation of the gold nanoparticles in the biofilm, knowing the location of the gold nanoparticles relative to the fungal cells is important. This is conveniently done by transmition electron microscopy (TEM) analysis of thin sections of the Au nano-*Verticillium* cells (Figure 3). At lower magnification, a number

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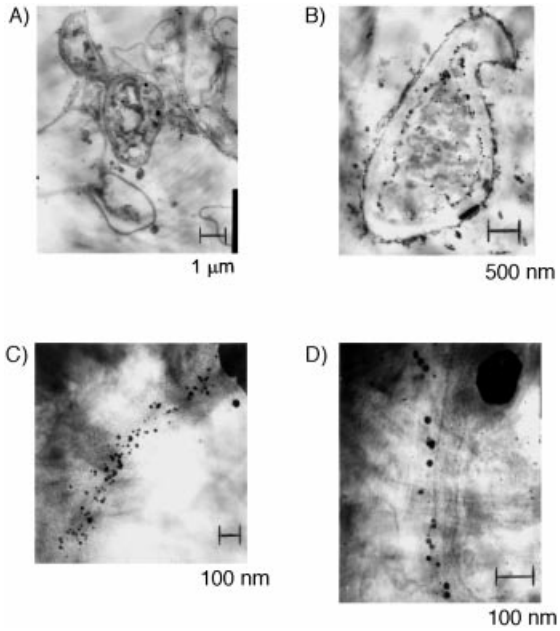

Figure 3. TEM images at different magnifications (A – D) of thin sections of stained *Verticillium* cells after reaction with $AuCl_4^-$ ions for 72 h.

of *Verticillium* cells can be seen (Figure 3A). Careful inspection of the picture shows extremely small particles of gold organized on the walls of the cells. Larger particles of gold are seen within the cells and are considerably smaller in number. Figure 3B shows a TEM image of a single cell in which the gold nanoparticles are formed both on the cell wall (outer boundary) as well as on the cytoplasmic membrane (inner boundary). The number of gold nanoparticles is clearly higher on the cytoplasmic membrane than on the cell wall. As observed in Figure 3A, a very small percentage of larger gold particles is observed within the cytoplasm. Selected area diffraction analysis of a single gold particle (data not shown) revealed diffuse rings with lattice spacings in excellent agreement with those expected for gold. From many TEM images of single *Verticillium* cells a statistical analysis of the size of gold nanoparticles formed both on the cell wall and on the cytoplasmic membrane gave an average gold nanoparticle size of 20 ± 8 nm. Note that in this analysis, the bigger gold particles (often larger than 100 nm, Figure 3C and D) have been excluded.

Higher magnification TEM images of one of the cells show the highly organized assembly of gold nanoparticles on the membrane (Figure 3C and D). The large number of the gold nanoparticles on the membrane surface is striking—this marks a significant departure from bacterial cells, where often not more than 10 – 15 nanoparticles are observed in one cell.[8] Of note is that the size distribution of gold nanoparticles produced using *Verticillium sp.* is much narrower than that observed for silver particles produced in bacteria.[8] The higher magnification TEM thin-section image (Figure 3D) shows the gold nanoparticles are mostly spherical but that there are a few triangular and hexagonal particles. The cytoplasmic membrane is clearly seen in Figure 3D and a large, quasi-hexagonal gold particle is observed within the cytoplasm.

The full potential for using micro-organisms for the synthesis of nanoscale matter may be realized if the ability of the cells to multiply (and thus cover large surface areas with nanomaterials) is not compromised by the presence of metal ions. Consequently, a small number of the Au nano-*Verticillium* cells were removed from the biofilm and placed in the culture medium on an agar plate. Within a week, the seed fungal cells had multiplied to cover the surface of the agar plate of diameter 8 cm (picture shown in the Supporting Information) thus clearly demonstrating that the $AuCl_4^-$ ions and Au nanoparticles do not lead to cell death.

While the exact mechanism of the formation of gold nanoparticles by reaction with the fungal cells is not understood, based on our and other results we speculate as follows: Esumi et al. have shown that sugar persubstituted (amidoamine)dendrimers spontaneously reduce $AuCl_4^-$ ions, which leads to the formation of gold nanoparticles bound to the dendrimers.[14] We have demonstrated the reduction of $AuCl_4^-$ ions by silanol groups and entrapment of the gold nanoparticles within the pores of mesoporous MCM-41 silicate matrices.[15] To rule out the reduction of $AuCl_4^-$ ions by sugars in the cell wall, reaction of the metal ions with different genera of fungi, such as *Fusarium oxysporum*, *Colletotrichum gloeosporioides*, and *Curvularia lunata* were carried out in a manner identical to that for *Verticillium sp.* No intracellular reduction of the $AuCl_4^-$ ions was observed in these cases, thus ruling out a nonspecific mechanism for the growth of the nanoparticles. Indeed, that most of the gold nanoparticles are observed to form on the cytoplasmic membrane of *Verticillium* cells (Figure 3) rules out the role of cell-wall sugars in the reduction of the $AuCl_4^-$ ions. Since the nanoparticles are formed on the surface of the fungal cells and not in solution, we believe the first step involves trapping of the $AuCl_4^-$ ions on the surface of the fungal cells. This could occur by electrostatic interaction with positively charged groups (such as, lysine residues) in enzymes present in the cell wall of the mycelia. Thereafter, the gold ions are reduced by enzymes within the cell wall leading to the aggregation of the metal atoms and formation of gold nanoparticles. TEM analysis of thin sections also shows the presence of gold nanoparticles on the cytoplasmic membrane (Figure 3B, D), which indicates that some of the $AuCl_4^-$ ions/small gold particles diffuse across the cell wall and are localized on the cytoplasmic membrane. It is possible that enzymes present in the cytoplasmic membrane also participate in the reduction of the gold ions. A detailed structural analysis of single *Verticillium* cells is currently underway as part of efforts to elucidate the mechanism of formation of gold nanoparticles by reduction of $AuCl_4^-$ ions.

In conclusion, the bioreduction of aqueous $AuCl_4^-$ ions by the fungus *Verticillium sp*. has been demonstrated. The reduction of the noble metal ions occurs on the surface of the mycelia as well as on the cytoplasmic membrane leading to the formation of gold nanoparticles of fairly well-defined dimensions and good monodispersity. The nanoparticles are bound to the surface of the fungal cells and may be used for other applications including catalysis and as precursors for synthesis of coatings for electronic applications. This fungal-mediated green chemistry approach towards the synthesis of

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gold nanoparticles has many advantages, such as the ease with which the process can be scaled up, economic viability, possibility of covering large surface areas by suitable growth of the mycelia etc. The shift from bacteria to fungi as a means of developing natural "nano-factories" has the added advantage that processing and handling of the biomass would be much simpler. Further, compared to bacteria, fungi are known to secrete much higher amounts of proteins, thereby significantly increasing the productivity of this biosynthetic approach. Application of such bio-nanocomposites in catalysis and other electronic applications are currently being pursued.

Experimental Section

The acidophillic fungus, *Verticillium sp.*, was isolated from the Taxus plant and maintained on potato-dextrose agar slants at 25 °C. The fungus was grown in 500 mL Erlenmeyer flasks each containing MGYP media (100 mL), composed of *m*alt extract (0.3 %), *g*lucose (1.0 %), *y*east extract (0.3 %), and *p*eptone (0.5 %) at 25 – -28 °C under shaking (200 rpm) for 96 h. After 96 h of fermentation, mycelia were separated from the culture broth by centrifugation (5000 rpm) at 10 °C for 20 min and the settled mycelia were washed three times with sterile distilled water. Some of the harvested mycelial mass (10 g) was re-suspended in sterile distilled water (100 mL) in 500 mL conical flasks at pH = 5.5 – 6.0 and to this suspension an aqueous solution of chloroauric acid ($HAuCl_4$, 100 mL, 10^{-4} M) was added. The whole mixture was put into a shaker at 28 °C (200 rpm) and left for 72 h. The bio-transformation was monitored by visual inspection of the biomass as well as measurement of the UV/Vis spectra from the fungal cells. Films of the fungal cells (both before and after exposure to $AuCl_4^-$ ions for 72 h) for UV/Vis spectroscopy, X-ray diffraction, and SEM studies were prepared by solution casting the fungal cells onto quartz and Si(111) substrates and thoroughly drying the films in a flow of N_2. UV/Vis spectroscopy measurements of the films on quartz were made on a Shimadzu dual-beam spectrophotometer (model UV-1601PC) operating in the reflection mode at a resolution of 2 nm. Since the films of the bio-nanocomposite were rough, the results are not quantitative and have been used merely to detect the presence of gold nanoparticles in the biomaterial. UV/Vis spectra of the aqueous $HAuCl_4$ solution after reaction with the *Verticillium* cells for 72 h were also recorded in the transmission mode on the same instrument. SEM and EDX measurements of the fungal cells after formation of the gold nanoparticles were carried out on a Leica Stereoscan-440 scanning electron microscope equipped with a Phoenix EDX attachment. Since the images obtained for the as-prepared Au nano-*Verticillium* biofilms were good, no sputtering of either gold or carbon was required. EDX spectra were recorded in the spot-profile mode by focusing the electron beam onto one of the gold nanoparticles present on the surface of the mycelial cells. XRD studies of the Au nano-fungus biofilm on Si(111) substrates were carried out in the transmission mode on a Philips PW 1830 instrument operating at 40 kV voltage and a current of 30 mA with $Cu_{K\alpha}$ radiation.

Thin sections of the *Verticillium* cells, after reaction with $AuCl_4^-$ ions in the manner described above, were prepared for TEM analysis as follows: approximately 1 mm^3 aliquots of the Au nano-*Verticillium* biomass were taken and fixed in 2.5 % gluteraldehyde in distilled water for 2 h at room temperature. After fixation, the cells were sedimented (1500 rpm, 10 min) and washed three times with distilled water. Without postfixation, the pellet was subjected to dehydration with 30, 50, 70, and 90 % ethanol for 15 min at each concentration followed by two changes in absolute ethanol. Since ethanol is poorly miscible with epoxy resins, propylene oxide was used as a linking agent. The dehydrated pellet was kept in propylene oxide for 15 min following which the infiltration of the resin was carried out by placing the pellet in a 1:1 mixture of propylene oxide and Epon 812 overnight at room temperature. Embedding was carried out by using a mixture of the resin (Epon 812) and hardeners (DDSA: dodecynyl succinic anhydride and MNA: methyl nadic anhydride) in the ratio 1:1.5. To this, two drops of tridimethylaminomethyl phenol (DMP30) was added to accelerate the polymerization process. Polymerization was carried out using this mixture at 60 °C for 3 days. Ultrathin sections were cut using an ultramicrotome (Leica Ultracut UCT) and were taken on copper TEM grids (40 μm × 40 μm mesh size). The sections were slightly stained with uranyl acetate and lead citrate prior to TEM analysis. TEM measurements were carried out on a JEOL Model 1200EX instrument operating at an accelerating voltage of 60 kV. A low operating voltage was used to minimize damage to the thin sections by electron beam heating.

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