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Bioreduction of chloroaurate ions by geranium leaves and its endophytic fungus yields gold nanoparticles of different shapes

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Development of biologically inspired experimental processes for the synthesis of nanoparticles is an important branch of nanotechnology. In this paper, we report on the use of geranium leaves (*Pelargonium graveolens*) and its endophytic fungus in the extra-cellular synthesis of gold nanoparticles. Sterilized geranium leaves and an endophytic fungus (*Colletotrichum* sp.) growing in the leaves were separately exposed to aqueous chloroaurate ions. In both cases, rapid reduction of the metal ions was observed resulting in the formation of stable gold nanoparticles of variable size. In the case of gold nanoparticles synthesized using geranium leaves, the reducing and capping agents appear to be terpenoids while they are identified to be polypeptides/enzymes in the *Colletotrichum* sp. case. The biogenic gold nanoparticles synthesized using the fungus were essentially spherical in shape while the particles grown using the leaves exhibited a variety of shapes that included rods, flat sheets and triangles. While the exact reasons for shape variability are not clear at this stage, the possibility of achieving nanoparticle shape control in a host leaf–fungus system is potentially exciting.

Introduction

An important aspect of nanotechnology concerns the development of experimental processes for the synthesis of nanoparticles of different chemical compositions, sizes, shapes and controlled dispersity. Many biological organisms, both unicellular and multicellular, are known to produce inorganic materials either intra- or extra-cellularly[1,2] often of nanoscale dimensions and of exquisite morphology and hierarchical assembly. Some well-known examples of bio-organisms synthesizing inorganic materials include magnetotactic bacteria (which synthesize magnetite nanoparticles),[3–5] diatoms (which synthesize siliceous materials)[6–8] and S-layer bacteria (which produce gypsum and calcium carbonate layers).[9,10] Recognizing the importance of developing eco-friendly nanoparticle synthesis methods, more and more researchers have turned to biological microorganisms for inspiration.

Even though many biotechnological applications such as remediation of toxic metals employ micro-organisms such as bacteria[11] and yeast[12] (the detoxification often occurring *via* reduction of the metal ions/formation of metal sulfides), it is only relatively recently that materials scientists have been viewing with interest such microorganisms as possible eco-friendly nanofactories.[13–18] Beveridge and co-workers have demonstrated that gold particles of nanoscale dimensions may be readily precipitated within bacterial cells by incubation of the cells with Au^{3+} ions.[13–15] Klaus-Joerger and co-workers have shown that the bacterium *Pseudomonas stutzeri* AG259 isolated from a silver mine, when placed 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.[16–18] Nair and Pradeep have synthesized nanocrystals of gold, silver and their alloys by reaction of the corresponding metal ions within cells of lactic acid bacteria present in buttermilk.[19] In a break from tradition, which has hitherto relied on the use of prokaryotes such as bacteria in the intra-cellular synthesis of nanoparticles, we have recently shown that eukaryotic organisms such as fungi may be used to grow nanoparticles of different chemical compositions and sizes. A number of different genera of fungi have been investigated in this effort and it has been shown that fungi are extremely good candidates in the synthesis of gold,[20,21] silver[22,23] and indeed quantum dots of the technologically important CdS by enzymatic processes.[24] We have recently reported that the alkalothermophilic (extremophilic) actinomycete, *Thermomonospora* sp., can synthesize extra-cellularly high concentrations of gold nanoparticles of 8 nm average size with good monodispersity.[25] As can be seen from the above, the use of biological organisms in the deliberate and controlled synthesis of nanoparticles is a relatively new and exciting area of research with considerable potential for development.

Recently, Jose-Yacaman and co-workers have demonstrated the synthesis of gold nanoparticles within live alfalfa plants by gold uptake from solid media.[26] It has been reported that the Au(III) ions are reduced in the solid media to Au(0) by the plant and then the atoms are absorbed into the plant where the nucleation and growth of gold nanoparticles takes place. This method can be very efficient in decontaminating soil polluted with heavy metal ions.[26] Barring the fungus *Fusarium oxysporum*[21,24] and the actinomycete *Thermomonospora* sp.,[25] all other microorganisms and plants investigated thus far when challenged with metal ions have resulted in their reduction and growth of nanoparticles intra-cellularly. Such biosynthetic processes for the synthesis of nanoparticles would be more useful from the application point of view if the nanoparticles can be formed extra-cellularly. For nanotechnology related reasons mentioned above, we have screened a number of plants and their endophytic fungi for the efficient and rapid extra-cellular synthesis of metal nanoparticles and have identified the geranium plant (*Pelargonium graveolens*) and its endophyte as exciting candidates for the synthesis of gold nanoparticles. More specifically, we have studied the reduction of aqueous chloroaurate ions by both the leaves of the geranium plant as well as an endophytic fungus, *Colletotrichum* sp., isolated from the leaves of this plant. In both cases, we have observed rapid extra-cellular growth of gold nanoparticles that are extremely stable in solution. (The search for plant-associated

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fungi producing extracellular gold nanoparticles both from plants and their endophytic fungi is justified by previous examples of plant-associated microbes producing "plant" compounds such as taxol[27] and gibberellins.[28] The pathways of gibberellin biosynthesis in the fungus and higher plants are identical up to GA_{12}.[29] This suggests the possibility of intergeneric genetic exchange between plant and fungus.) Furthermore, the morphology of the leaf and fungus biogenic gold nanoparticles is markedly different. While the nanoparticles synthesized using the fungus, *Colletotrichum* sp., are predominantly spherical in shape and rather polydisperse, interesting nanoparticle shape variation was observed for gold synthesized using geranium leaves. In this case, the particles exhibited a mixture of flat disk and rod-like morphology. It is well known that the shape of nanoparticles plays a crucial role in modulating their optical properties,[30] and therefore the possibility of developing biological processes to achieve such shape control is exciting. Presented below are details of the investigation.

Experimental details

The broth used for reduction of $AuCl_4^-$ ions to Au^0 was prepared by taking 20 g of thoroughly washed and finely cut *Pelargonium graveolens* leaves in a 500 mL Erlenmeyer flask with 100 mL sterile distilled water and then boiling the mixture for 1 min. The process of boiling the leaves leads to rupture of the walls of leaf cells and, thus, release of intra-cellular material into solution. After boiling, the solution was decanted, filtered and 5 mL of this broth was added to 100 mL of 10^{-3} M $HAuCl_4$ aqueous solution. The bioreduction of the $AuCl_4^-$ ions in solution was monitored by periodic sampling of aliquots (2 mL) of the aqueous component and measuring the UV-vis spectrum of the solution. UV-vis spectra of these aliquots were monitored as a function of time of reaction on a Hewlett-Packard diode array spectrophotometer (model HP-8452) operated at a resolution of 2 nm.

An endophytic fungus of the *Pelargonium graveolens* plant, identified to be *Colletotrichum* sp., was isolated from surface disinfected leaves of *Pelargonium graveolens*. The leaves were cut into small pieces and then surface sterilized with 0.01% mercuric chloride solution for 1 min, washed thoroughly with sterile distilled water and placed onto potato dextrose agar (PDA) poured into Petri dishes. The colonies which emerged around the leaves were purified by single spore isolation, and single spore isolates of the fungus *Colletotrichum* sp. were maintained on PDA slants at 25 °C ± 1 °C. Stock cultures were maintained by subculturing at monthly intervals. Subcultures were made on fresh PDA slants from the stock culture and after four days were used as the starting material for nanoparticle biosynthesis experiments. For the synthesis of the gold nanoparticles, *Colletotrichum* sp. was grown in 500 ml Erlenmeyer flasks containing 100 ml MGYP medium which is composed of malt extract (0.3%), glucose (1%), yeast extract (0.3%) and peptone (0.5%). The culture was grown under continuous shaking on a rotary shaker (200 rpm) at 25–27 °C for 96 hours. After 96 hour of fermentation, mycelia were separated from the culture broth by centrifugation (5000 rpm) at 20 °C for 20 minutes and then the mycelia was washed thrice with sterile distilled water under sterile conditions. The harvested mycelial mass (10 gm of wet mycelia) was then resuspended in 100 ml of 10^{-3} M aqueous $AuCl_4$ solution in 500 mL Erlenmeyer flasks. The whole mixture was put into a shaker at 25–27 °C (200 rpm) and maintained in the dark. The bioreduction of the $AuCl_4^-$ ions in solutions was monitored by periodic sampling of aliquots (2 mL) of the aqueous component and measuring the UV-vis spectra of the solution. X-Ray diffraction (XRD) measurements of the two bioreduced chloroauric acid solutions drop-coated onto glass substrates were done on a Philips PW 1830 instrument operating at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation. For Fourier transform infrared (FTIR) spectroscopy measurements, two separate 20 mL chloroauric acid solutions after 48 h of reaction with *Pelargonium graveolens* leaf broth and 4 days of reaction with the *Colletotrichum* sp. biomass respectively were centrifuged at 8000 rpm for 10 min and then redispersed in 20 ml of sterile distilled water to get rid of any free proteins/enzymes that are not capping the gold nanoparticles. This process was repeated three times for each solution to ensure better separation. The above redispersed centrifugate solutions were drop-coated on Si (111) substrates for FTIR measurement which were carried out on a Perkin Elmer Spectrum One instrument in the diffuse reflectance mode at a resolution of 4 cm^{-1}. Samples for transmission electron microscopy (TEM) analysis were prepared on carbon-coated copper TEM grids. The films on the TEM grids were allowed to stand for 2 min following which the extra solution was removed using blotting paper and the grid allowed to dry prior to measurement. TEM measurements were performed on a JEOL model 1200EX instrument operated at an accelerating voltage of 120 kV.

Results and discussion

Reduction of the aqueous chloroaurate ions during exposure to the broth of boiled *Pelargonium graveolens* leaves may be easily followed by UV-vis spectroscopy. It is well known that gold nanoparticles exhibit lovely pink-ruby red colors, these colors arising due to excitation of surface plasmon vibrations in the gold nanoparticles.[30] The gold nanoparticle surface plasmon band occurs in the range 510–560 nm in an aqueous medium. Fig. 1 shows the UV-vis spectra (solid lines) recorded from the aqueous chloroauric acid–geranium leaf broth reaction medium as a function of time of reaction. It is observed that the gold surface plasmon resonance band occurs initially at *ca.* 547 nm after 2 min and steadily increases in intensity as a function of time of reaction. After completion of the reaction, the wavelength of the surface plasmon band stabilizes at 551 nm. The nanoparticle absorption band is slightly asymmetrical with indications of an additional weaker component at *ca.* 610 nm. The presence of this shoulder indicates either

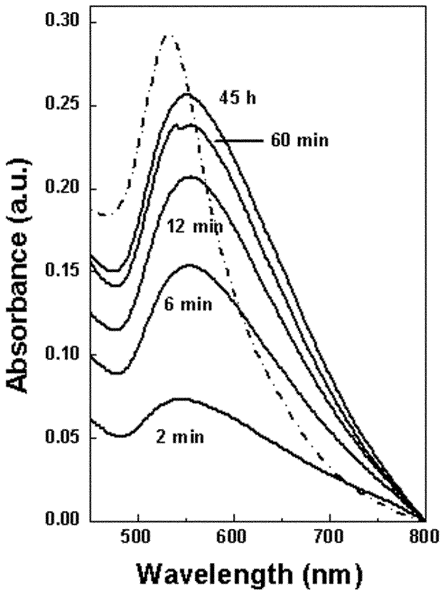

Fig. 1 UV-vis spectra recorded as a function of time of reaction of a 10^{-3} M aqueous solution of $HAuCl_4$ with geranium leaf extract (solid lines). The time of reaction is indicated next to the respective curves. The dash-dotted line represents the UV-vis spectrum of a 10^{-3} M aqueous solution of $HAuCl_4$ after four days of reaction with the endophytic fungus *Colletotrichum* sp. isolated from the geranium leaf.

formation of stable aggregates of the gold nanoparticles in solution or shape anisotropy in the particles. This issue will be addressed subsequently during TEM analysis of the nanoparticle solution. Reduction of the $AuCl_4^-$ ions is nearly complete after 60 min of reaction. In our earlier studies on the use of the fungus *Fusarium oxysporum*[21] and the actinomycete *Thermomonospora* sp.[25] in the extra-cellular synthesis of gold nanoparticles by reduction of aqueous chloroaurate ions, the time for complete reduction of the gold ions was 48 and 120 h respectively (under nearly identical reaction conditions). The significant reduction in the reaction time with the geranium leaf broth is an important result and will enable nanoparticle biosynthesis methods to compete favorably with chemical methods for the formation of gold nanoparticles that are currently much more rapid and reproducible.

After completion of reaction of the chloroaurate ions with the geranium leaf broth (*i.e.*, after 24 h of reaction), the gold nanoparticle solution was tested for stability. It was observed that the nanoparticle solution was stable for more than six months with little sign of aggregation (as determined by UV-vis spectroscopy and TEM measurements) even at the end of this period. To monitor the kinetics of reduction of $AuCl_4^-$ ions by the endophytic fungus *Colletotrichum* sp., UV-vis spectra were measured for this system in parallel. Fig. 1 shows the spectrum recorded from this sample after 4 days of reaction (dash-dotted line). It is observed that the gold surface plasmon resonance band occurs at 534 nm and, compared to the UV-vis spectra obtained from the gold nanoparticles synthesized using *Pelargonium graveolens* leaf broth, is much sharper and more symmetric indicating less polydispersity and shape anisotropy of the gold nanoparticles.

Fig. 2 shows the XRD patterns obtained for films of gold nanoparticles synthesized using the geranium leaf broth (curve 1) and the endophyte *Colletotrichum* sp. (curve 2) on glass substrates. A number of prominent Bragg reflections can be seen in both cases that could be indexed based on the fcc structure of gold. The reflections appear to be broader for the gold nanoparticles synthesized using the endophytic fungus indicating that the particles are of smaller dimensions relative to those synthesized using the geranium leaf broth.

The gold nanoparticles synthesized using the *Pelargonium graveolens* leaf broth and the endophyte *Colletotrichum* sp. were subjected to FTIR analysis to identify (if possible) the biomolecules stabilizing the nanoparticles in solution and also to provide clues as to what the reducing agents might be. The two reaction media after complete reduction of $AuCl_4^-$ ions by the geranium leaf broth and *Colletotrichum* sp. and formation of gold nanoparticles were centrifuged at 8000 rpm for 10 min separately to isolate the gold nanoparticles from free proteins or other compounds present in the solution and the centrifugate. The gold nanoparticle pellets obtained after centrifugation were redispersed in water prior to FTIR analysis. In case of the gold nanoparticles synthesized using *Colletotrichum* sp., strong bands at 1658, 1543 and 1240 cm^{-1} are observed (Fig. 3, curve 1). These bands correspond to the amide I, II and III bands of polypeptides/proteins respectively and agree with those reported in the literature.[31,32] It is possible that the polypeptides capping the gold nanoparticles are glutathiones which are known to be produced by yeast cells.[12] It is well known that proteins can bind to gold nanoparticles either through free amine groups or cysteine residues in the proteins[33] and therefore stabilization of the gold nanoparticles by surface-bound proteins is a possibility in the case of gold nanoparticles synthesized using the endophyte *Colletotrichum* sp. Curve 2 in Fig. 3 represents the FTIR spectrum recorded from gold nanoparticles synthesized using *Pelargonium graveolens* leaf broth. A number of broad bands are observed in the region 1150–1800 cm^{-1} centered at 1716, 1607, 1512, 1444 and 1221 cm^{-1}. *Pelargonium graveolens* leaves have been reported to consist of many terpenoids of which citronellol and geraniol form the major component and linalool a much smaller fraction.[34,35] The peak at 1607 cm^{-1} can be assigned to the vibrational modes of C=C double bonds of these molecules. The peak at 1716 cm^{-1} falls in the region of C=O stretching frequency. It is possible that during reduction of the chloroaurate ions the alcohol groups of the terpenoids are oxidized to carbonyl groups thus resulting in a band at 1716 cm^{-1}. Apart from the presence of terpenoids, the presence of a weak broad band centered at 1221 cm^{-1} characteristic of the amide III band in proteins indicates a small concentration of protein in the gold nanoparticle solution synthesized using geranium leaf extract.[32] The FTIR results thus show that the surface capping of gold nanoparticles synthesized using the fungus is predominantly by proteins while terpenoids are implicated in stabilization of the nanoparticles synthesized using geranium leaf extract. While it is possible that the capping agent also plays the role of a reducing agent, considerably more work involving separation of the different compounds present in the geranium leaf and *Colletotricum* sp. fungal extracts followed by

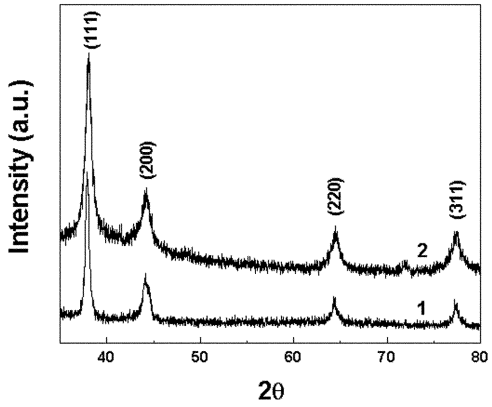

Fig. 2 XRD patterns recorded from drop-coated films of gold nanoparticles synthesized using geranium leaf extract (curve 1) and *Colletotricum* sp. (curve 2) on glass substrates (see text for details). The Bragg reflections are indexed on the basis of the fcc gold structure.

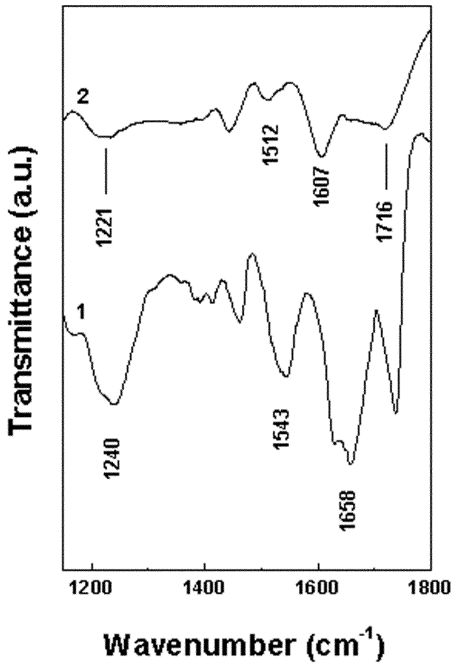

Fig. 3 FTIR spectra of gold nanoparticles synthesized by exposure of aqueous chloroaurate ions to *Colletotrichum* sp. isolated from geranium leaves (curve 1) and gold nanoparticles obtained by reaction of gold ions with *Pelargonium graveolens* leaf extract (curve 2).

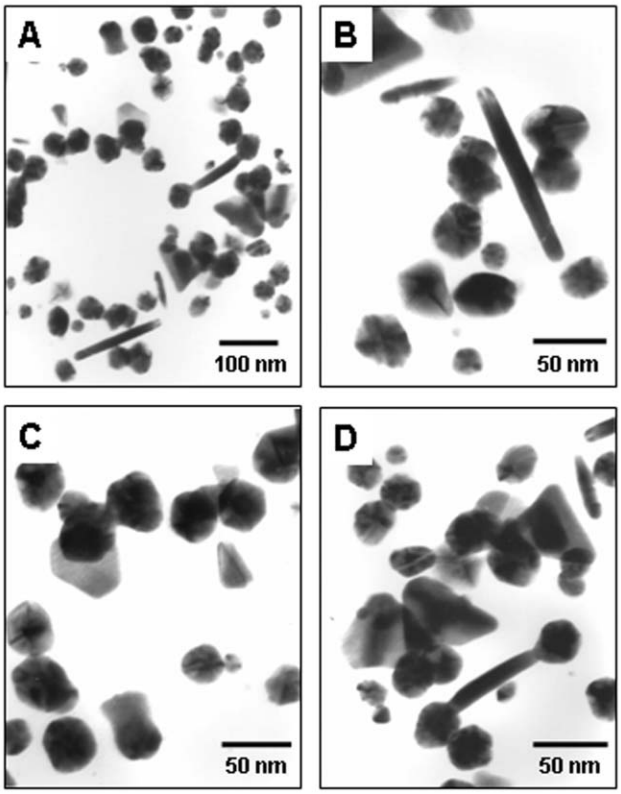

Fig. 4 A–D—Representative TEM images of gold nanoparticles synthesized using geranium leaf extract showing the different shapes of nanoparticles.

assaying each component is required before an unequivocal statement can be made. These efforts are currently in progress.

In an earlier study, we had demonstrated that the fungus *Fusarium oxysporum*, when exposed to aqueous solution of chloroauric acid, resulted in extra-cellular formation of polydisperse gold nanoparticles forming large aggregates in solution.[21] The monodispersity of gold nanoparticles can be improved by reacting gold ions with the extremophilic actinomycete *Thermomonospora* sp. as shown by us very recently.[25] To make nanoparticle biosynthetic procedures a viable alternative to the more sophisticated chemical methods, it would be important to achieve nanoparticle size, polydispersity and if possible, shape control. Figs. 4 and 5 show TEM pictures recorded from drop-coated films of the gold nanoparticles synthesized using *Pelargonium graveolens* leaf broth after reaction with chloroauric acid solution for 48 h. The low magnification TEM image (Fig. 4A) clearly shows a number of gold nanoparticles of a range of sizes and shapes.

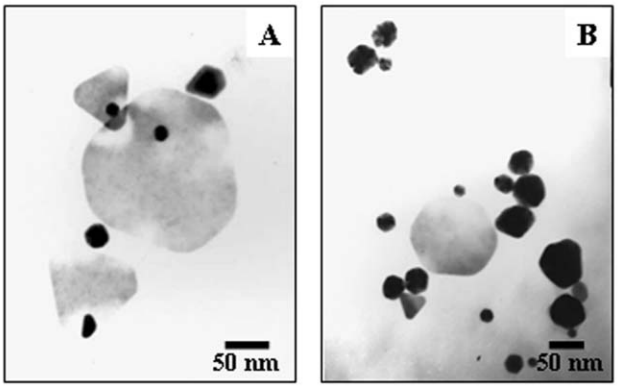

Fig. 5 A and B—TEM images of gold nanoparticles synthesized using geranium leaf extract.

The structural features of the individual gold nanoparticles are more clearly seen in the higher magnification TEM images (Figs. 4B–D). The particles are predominantly decahedral and icosahedral in shape, ranging in size from 20–40 nm, and exhibit contrast that suggests that they are multiply twinned particles (MTPs). What is more interesting is the existence of triangular and rod shaped gold nanoparticles of fairly high density in the ensembles. While triangular nanoparticles of silver and gold have been observed in earlier studies,[16,21] there have been no reports on biogenic gold nanorods. The large nanorod seen in Fig. 4B shows contrast in the terminal regions implicating possible involvement of MTPs in their growth process. Fig. 4D shows a gold nanorod straddled by two MTPs in a dumbbell configuration. Whether this is due to a fortuitous juxtaposition of the nanorod and MTPs or if the nanorod is actually growing outwards from the MTPs is not clear at this stage. Gai and Harmer have recently presented a detailed electron microscopy investigation of gold nanorods synthesized by wet chemical methods involving cetyltrimethylammonium bromide (CTAB) as a templating surfactant.[36] They have shown that gold nanorods grow by diffusion of gold atoms to sites within weaker bonded twinned regions of MTPs. This leads to growth of gold nanorods along the (100) direction, an important prerequisite for this process being stabilization of the highly unstable (110) faces by CTAB molecules.[36] The presence of a large density of MTPs and gold nanorods in the TEM images suggests that a similar templating effect involving MTPs as seeds for growth of the nanorods is operative in the geranium leaf extract experiment of this study.

Seemingly flat gold nanoparticles of predominantly triangular (prismatic) shape were frequently observed in the TEM images (Fig. 4C). These flat nanoparticles are more clearly seen in higher magnification TEM images shown in Figs. 5A and B. The edges of the triangles are truncated in most cases and the inference that they are extremely thin (on a nanoscale) can clearly be made from Fig. 5A, which shows increased contrast in the region of overlap of two triangular particles. In the case of gold nanoparticles synthesized using the endophytic fungus *Colletotrichum* sp. isolated from geranium leaves, the particles were predominantly spherical and aggregated into larger irregular structures with no well-defined morphology (Fig. 6). The smaller spherical particles ranged in size from 8 to 40 nm. It is interesting to note here that even though there is evidence for the presence of surface bound proteins from the FTIR spectra of the gold nanoparticles synthesized using *Colletotrichum* sp. (Fig. 3, curve 1), very little gold nanoparticle shape control appears to be possible using this microorganism.

The ability to grow gold nanoparticles of rod-like and prismatic morphology by biological methods is tremendously

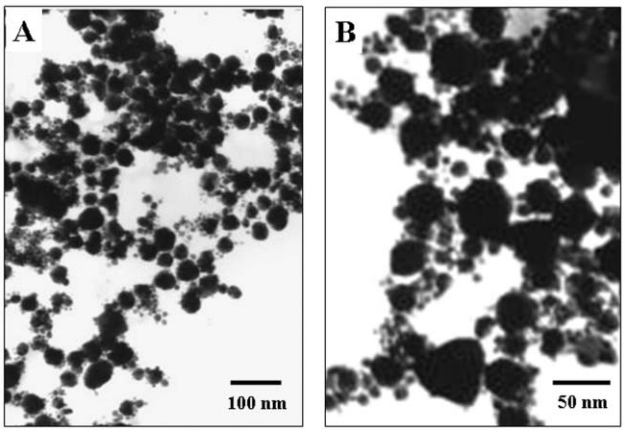

Fig. 6 A and B—TEM images of gold nanoparticles synthesized by reduction of chloroaurate ions with the endophytic fungus isolated from geranium leaves at different magnification.

exciting given that the optical, electronic and catalytic properties of metal nanoparticles are strong functions of not only particle size but shape as well.[37,38] Gold nanoparticles grown using the endophytic fungus *Colletotrichum* sp. show little morphology variation/control and are similar in that respect to gold nanoparticles grown using other fungi and actinomycetes investigated by us earlier.[21,25] In the previous studies, analysis of the bioorganic molecules secreted by the microorganisms indicated that reduction of the gold ions was carried out by enzymes and that the gold nanoparticles were stabilized by surface-bound proteins.[21,25] As mentioned above, proteins stabilize the gold nanoparticles synthesized using *Colletotrichum* sp. and a similar mechanism involving reduction of gold ions by enzymes released by the fungus may be operative here. The gold nanoparticle shape control exhibited by the geranium leaf extract indicates that the process is more involved in this case. In addition to reducing agents (that could be identical (different) to that secreted by the symbiotic endophytic fungus *Colletotrichum* sp.), the geranium leaf extract contains bioorganic molecule(s) capable of stabilizing highly unstable crystallographic faces of gold nanoparticles. Possible stabilizing/reducing molecules could be citronellol and geraniol which are known to be present in large concentrations in *Pelargonium graveolens* leaves.[34,35] A detailed analysis is currently underway to separate the different bioorganic molecules in the geranium leaf extract.

In conclusion, we have shown that reaction of aqueous chloroaurate ions with the extract of geranium leaf and an endophytic fungus, *Colletotrichum* sp., present in the leaves leads to the formation of gold nanoparticles in solution. The reduction is extremely rapid in the case of geranium leaf extract and yields gold nanoparticles of rod-like and thin prismatic shapes. The ability to synthesis gold nanoparticles rapidly with morphology control by eco-friendly biological methods is exciting and represents an important advance in making them viable alternatives to the more popular chemical methods. It is well known that symbiotic biological systems such as the geranium leaf and the endophytic fungus *Colletotrichum* sp. produce secondary metabolites that are different for each organism and advantageous for the symbiotic system as a whole.[39] This is clearly demonstrated by the significant differences in the interaction of aqueous gold ions with the geranium leaf and *Colletotrichum* sp. observed in this study. The possibility of rational use of secondary metabolites of such symbiotic systems in programmed nanomaterials synthesis is an exciting possibility that this study has addressed for the first time.

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