Extracellular Synthesis of Gold Nanoparticles by the Fungus *Fusarium oxysporum*

Priyabrata Mukherjee,[b] Satyajyoti Senapati,[b] Deendayal Mandal,[b] Absar Ahmad,[a] M. Islam Khan,*[a] Rajiv Kumar,*[b] and Murali Sastry*[c]

KEYWORDS:

colloids · enzymes · gold · nanostructures · oxidoreductases

The synthesis of nanoparticles of different chemical compositions, sizes, and controlled monodispersity is an important area of research in nanotechnology. As far as the synthesis of metal nanoparticles (and gold in particular) is concerned, a number of chemical methods exist in the literature.[1] Ever-increasing pressure to develop environmentally benign nanoparticle synthesis has lead to a renewed interest in biotransformations as a route to growth of nanoscale structures. While many biotechnological applications, such as remediation of toxic metals, employ microorganisms like bacteria[2] and yeast[3] (the detoxification often occurring through reduction of the metal ions/formation of metal sulfides) it is only recently that microorganisms for use in the synthesis of nanomaterials have been viewed with interest.[4, 5] We have just demonstrated that the fungus *Verticillium* sp., when treated with an aqueous solution of $AuCl_4^-$ or Ag^+ ions, resulted in the in situ reduction and consequent intracellular formation of gold[6] and silver[7] nanoparticles of good monodispersity. However, such biotransformation-based nanoparticle synthesis strategies would have greater commercial viability if the nanoparticles could be synthesised extracellularly directly in the aqueous medium. Towards this objective, we have screened a number of species of fungus belonging to different genera and have observed the extracellular synthesis of gold nanoparticles by treatment of the fungus *Fusarium oxysporum* with aqueous $AuCl_4^-$ ions. The details of this investigation are presented below. Most probably, the reduction of the $AuCl_4^-$ ions occurs due to reductases released by the fungus into solution; this opens up a novel fungal/enzyme-based in vitro approach to nanomaterials.

The inset of Figure 1 shows two conical flasks with the *Fusarium oxysporum* biomass before (A) and after (B) reaction with $AuCl_4^-$ ions for 72 h.[8] The biomass has a pale yellow colour before reaction with the gold ions (A) which changes to dark purple on completion of the reaction (B). The appearance of the purple colour clearly indicates the formation of gold nanoparticles in the reaction mixture.[9] The characteristic pink-purple colour of colloidal gold solutions is due to excitation of surface plasmon vibrations in the nanoparticles and provides a convenient spectroscopic signature of their formation.[9] Upon filtration, it was observed that the biomass was still pale yellow but that the aqueous solution contained the gold nanoparticles. This indicates that the reduction of the $AuCl_4^-$ ions takes place extracellularly, which is an important observation that we will return to subsequently.

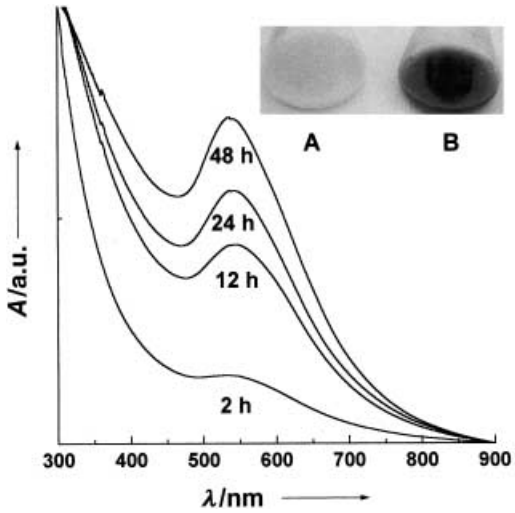

Figure 1. *UV/Vis spectra recorded after addition of 2×10^{-3} M $HAuCl_4$ solution (100 mL) to water (100 mL) in which* Fusarium oxysporum *biomass (10 g) was incubated for 72 h. The curves are recorded after different reaction times. The inset shows conical flasks with* Fusarium oxysporum *before (A) and after (B) exposure to $AuCl_4^-$ ions for 72 h. A colour version of the inset can be seen in the Table of Contents.*

In another experiment, the biomass (10 g) was immersed in water (100 mL) for 72 h. After immersion the solution was filtered and exposed to 10^{-3} M $AuCl_4^-$ ions in the dark. It was observed that the metal ions were slowly reduced in solution as evidenced by a gradual and steady increase in intensity of purple coloration in the solution. Figure 1 shows the UV/Vis spectra recorded from the reaction solution after different reaction times. The strong resonance centred at about 545 nm is clearly seen and increases in intensity with time, after 48 h it stabilises. Quite interestingly, the solution was extremely stable with no evidence of flocculation of the particles even a month after reaction. The resonance is sharp and indicates only little aggregation of the particles in solution.

It is clear from the above that the fungus *Fusarium oxysporum* releases reducing agents into solution that are responsible for

[a] *Dr. M. I. Khan, Dr. A. Ahmad*
Biochemical Sciences Division
National Chemical Laboratory
Pune, 411 008 (India)
Fax: (+91) 20-589-3300
E-mail: mikhan@dalton.ncl.res.in

[b] *Dr. R. Kumar, P. Mukherjee, S. Senapati, D. Mandal*
Catalysis Division
National Chemical Laboratory
Pune, 411 008 (India)
Fax: (+91) 20-589-3761
E-mail: rajiv@cata.ncl.res.in

[c] *Dr. M. Sastry*
Materials Chemistry Division
National Chemical Laboratory
Pune, 411 008 (India)
Fax: (+91) 20-589-3044
E-mail: sastry@ems.ncl.res.in

formation of the gold nanoparticles. UV/Vis and fluorescence measurements of the aqueous solution exposed to *Fusarium oxysporum* for 72 h clearly showed the presence of proteins in solution (data not shown). Preliminary gel electrophoresis investigations indicate that a number of proteins are released by the biomass. The protein extract obtained by suspending the *Fusarium oxysporum* mycelial biomass for 48 h contains a minimum of four proteins of molecular masses between 66 kDa and 10 kDa as seen on gel electrophoresis.[10] This extract was concentrated by ultrafiltration through a YM-3 ultrafiltration membrane and then dialysed against distilled water by using a 3K cut-off dialysis bag. This process removes the low molecular weight components in the extract such as co-factors. The protein mixture obtained after dialysis failed to reduce $AuCl_4^-$ ions. However, on addition of stoichiometric amounts of NADH to the protein extract, the reduction of $AuCl_4^-$ ions occurs quite readily. This clearly indicates the reduction of $AuCl_4^-$ ions by NADH-dependent reductases in the extract. It may be mentioned here that in bacteria, an NADH-dependent iron(III) reductase has been observed in the outer membrane of *Geobacter sulfurreducens*[11] while a periplasmic iron(III) reductase has been isolated from *Magnetospirillum magnetotacticum*.[12] We believe that out of four proteins of this study, one is responsible for the reduction of $AuCl_4^-$ ions and the subsequent formation of gold nanoparticles. This reductase is specific to *Fusarium oxysporum*—prolonged reaction of $AuCl_4^-$ ions with *Fusarium moniliforme* did not result in the formation of gold nanoparticles either intra- or extracellularly. It is well known that proteins could bind to gold nanoparticles through cysteine residues as well as by linkage through amine groups in lysine residues.[13] The long term stability of the gold nanoparticles in solution may be due to stabilisation by the proteins.

A film of the gold nanoparticle solution formed by reaction of gold ions with extracellularly secreted reductases for 72 h was cast on a Si (111) substrate and carbon-coated transmission electron microscopy (TEM) grids and than analysed by Fourier transform infrared (FTIR) spectroscopy and TEM (Figure 2 A and 2 B, respectively).[8] The FTIR spectrum shows the presence of three bands at 1650, 1540 and 1450 cm^{-1}. The bands at 1650 and 1540 cm^{-1} are identified as the amide I and II bands (labelled 1 and 2 in Figure 2A) and are due to carbonyl stretch and –N—H stretch vibrations in the amide linkages of the proteins, respectively.[13, 14] The positions of these bands are close to that reported for native proteins[14] and are in excellent agreement with that observed in gold colloid:pepsin bioconjugates.[13] The FTIR results thus show that the secondary structure of the proteins is not affected as a consequence of reacting with the $AuCl_4^-$ ions or binding with the gold nanoparticles. The band at approximately 1450 cm^{-1} is assigned to methylene scissoring vibrations from the proteins in the solution.

The two TEM pictures recorded from different regions of the gold nanoparticle film are shown in Figure 2 B. In the left picture, well-separated gold particles having spherical and triangular morphology are seen. These particles have a size range of 20 – 40 nm. The right picture shows aggregates of gold nanoparticles. Even though there is large-scale association of the particles, individual, discrete gold nanoparticles can clearly be

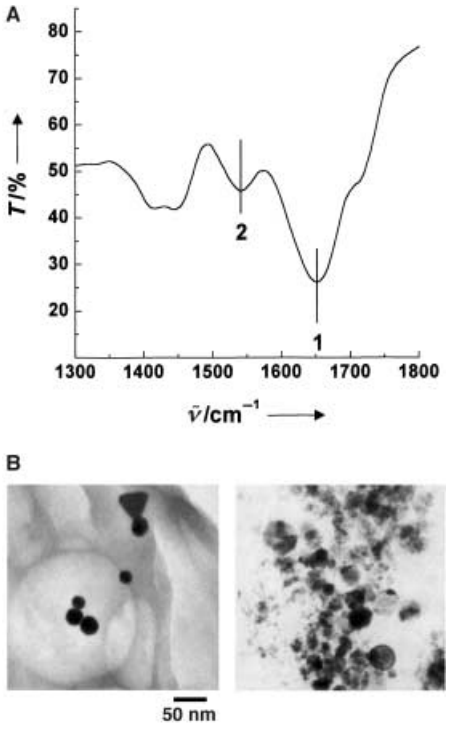

Figure 2. *A) FTIR spectrum recorded from a drop-coated film of an aqueous solution incubated with* Fusarium oxysporum *and treated with $AuCl_4^-$ ions for 72 h. B) TEM micrographs recorded from two different regions of a drop-coated film of an aqueous solution incubated with* Fusarium oxysporum *and treated with $AuCl_4^-$ ions for 72 h.*

discerned in this micrograph. The gold nanoparticles are thus undoubtedly stabilised by the proteins that prevent their sintering, a result in agreement with the UV/Vis spectroscopy measurements which showed the gold solutions to be exceptionally stable. Optical microscopy analysis of this picture yielded nanoparticles of 8 – 40 nm. The TEM results indicate that it is indeed possible to synthesise gold particles of nanoscale dimensions and tolerable monodispersity by using the fungus *Fusarium oxysporum*. The gold nanoparticles synthesised with *Fusarium oxysporum* are much smaller and more monodisperse than those synthesised intracellularly with bacteria.[4, 5]

This, to the best of our knowledge, is the first report on the extracellular synthesis of gold nanoparticles by a eukaryotic system such as fungi. We would like to point out that even though gold/silver nanoparticles have been synthesised by using prokaryotes such as bacteria[4, 5] and with the fungus *Verticillium* sp.[6] the nanoparticles grow intracellularly presumably by reduction of the metal ions by enzymes bound to the cell walls of the organisms. This first report will be followed up with a more detailed study aimed at controlling the nanoparticle size, monodispersity, etc. by varying the gold ion concentration and reaction time. The use of specific enzymes secreted by organisms such as fungi in the extracellular synthesis of nanoparticles is exciting for the following reasons: the synthesis of nanoparticles in solution would be of importance in homogeneous catalysis, while the nanoparticles may be immobilized in different matrices or in thin-film form for optoelec-

  1439-4227/02/03/05 $ 20.00+.50/0 ChemBioChem **2002**, No. 5

tronic applications. Indeed, the bioconjugates of gold nanoparticles with the proteins in solution might show novel nonlinear optical properties. We believe the biggest advantage of this method based on fungal enzymes is the possibility of developing a rational approach for the synthesis of nanoparticles over a range of compositions such as oxides or nitrides, and we are currently working towards this end.

S.S. and D.M. thank the Council of Scientific and Industrial Research (CSIR), Government of India, for research fellowships. The TEM measurements were carried out by Rajesh Gonnade, Materials Chemistry Division, NCL Pune, and are gratefully acknowledged.

[1] D. A. Handley, *Colloidal Gold: Principles, Methods, and Applications, Vol. 1* (Ed.: M. A. Hayat), Academic Press, San Diego, **1989**, chap. 2.
[2] a) J. R. Stephen, S. J. Maenaughton, *Curr. Opin. Biotechnol.* **1999**, *10*, 230; b) T. J. Beveridge, D. J. Doyle, *Metal Ions and Bacteria*, Wiley, New York, **1989**, pp. 31 – 90; T. J. Beveridge, D. J. Doyle, *Metal Ions and Bacteria*, Wiley, New York, **1989**, p. 363; T. J. Beveridge, D. J. Doyle, *Metal Ions and Bacteria*, Wiley, New York, **1989**, p. 374.
[3] M. K. Mehra, D. R. Winge, *J. Cell. Biochem.* **1991**, *45*, 30.
[4] G. Southam, T. J. Beveridge, *Geochim. Cosmochim. Acta* **1996**, *60*, 4369.
[5] T. Klaus-Joerger, R. Joerger, E. Olsson, C. G. Granqvist, *Trends Biotech.* **2001**, *19*, 15.
[6] P. Mukherjee, A. Ahmad, D. Mandal, S. Senapati, S. R. Sainkar, M. I. Khan, R. Ramani, R. Parischa, P. V. Ajayakumar, M. Alam, M. Sastry, R. Kumar, *Angew. Chem.* **2001**, *113*, 3697; *Angew. Chem. Int. Ed.* **2001**, *40*, 3585.
[7] P. Mukherjee, A. Ahmad, D. Mandal, S. Senapati, S. R. Sainkar, M. I. Khan, R. Parischa, P. V. Ajayakumar, M. Alam, R. Kumar, M. Sastry, *Nano Lett.* **2001**, *1*, 515.
[8] *Fusarium oxysporum* biomass (10 g) was taken in a conical flask containing distilled water (100 mL). A carefully weighed quantity of $HAuCl_4$ was added to the conical flask to yield an overall $AuCl_4^-$ ion concentration of 10^{-3} M in the aqueous solution; the reaction was carried out in the dark. UV/Vis spectroscopy measurements in this study were performed on a Shimadzu dual-beam spectrophotometer (model UV-1601PC) operating at a resolution of 1 nm while FTIR studies were carried out on a Shimadzu FTIR-8201PC instrument in the diffuse reflectance mode at a resolution of 4 cm^{-1}. TEM measurements were made on a JEOL 1200EX instrument operating at a voltage of 80 kV.
[9] A. A. Lazarides, G. C. Schatz, *J. Phys. Chem. B* **2000**, *104*, 460.
[10] K. Weber, M. Osborn, *J. Biol. Chem.* **1969**, *244*, 4406.
[11] T. S. Magnuson, A. L. Hodges-Myerson, D. R. Lovley, *FEMS Microbiol. Lett.* **2000**, *185*, 205.
[12] Y. Noguchi, T. Fujiwara, K. Y. Yoshimatsu, K. Fukumori, *J. Bacteriol.* **1999**, *181*, 2142.
[13] A. Gole, C. Dash, V. Ramakrishnan, S. R. Sainkar, A. B. Mandale, M. Rao, M. Sastry, *Langmuir* **2001**, *17*, 1674, and references cited therein.
[14] C. V. Kumar, G. L. McLendon, *Chem. Mater.* **1997**, *9*, 863.

Received: October 24, 2001 [Z310]

OmpA Membrane Domain as a Tight-Binding Anchor for Lipid Bilayers

Philippe Ringler and Georg E. Schulz*[a]

KEYWORDS:

biosensors · liposomes · membrane proteins · proteoliposomes · streptavidin

Liposomes have been used extensively to analyze membrane protein functions because they are realistic membrane substitutes.[1] In many applications liposomes are immobilized on solid supports,[2] which often causes protein malfunctions.[3] This problem was alleviated by suspending the lipid bilayers by long tethers that extend from the solid support to a lipid molecule immersed in the bilayer.[4] Such a lipid, however, cannot grip the bilayer tightly enough to yield high stability. Here we report the large-scale production of an engineered version of the small membrane protein OmpA, which fastens the outer membrane of *Escherichia coli* to the cell wall.[5] The presented OmpA derivative attaches liposomes to streptavidin molecules wherever these are located.

OmpA consists of an integral membrane domain (residues 1 – 171) and a periplasmic peptidoglycan-binding domain (residues 172 – 325). A fourfold mutant (F23L/Q34K/K107Y with an additional methionine residue at the N terminus) of the membrane domain[6] forms an eight-stranded β barrel with an aliphatic waist bordered by two girdles of aromatic side chains (Figure 1). This β barrel is tightly anchored in the membrane. We added the mutation N26C, which introduces a cysteine residue at the tip of one of the four long and mobile extracellular loops. The gene for this cysteine-containing fivefold mutant "OmpAfc" was verified by DNA sequencing. OmpAfc can be mass-produced in *E. coli* inclusion bodies and (re)natured therefrom.[7] Labeling Cys 26 with a tethered biotin[8] permits a tight association of membranes to streptavidin and their release by disulfide reduction.

The accessibility of Cys 26 was checked photometrically by Ellman's reaction,[9] which resulted in 0.7 thiols per OmpAfc. The disulfide exchange reaction with tethered biotin (Scheme 1) yielded 80 % "OmpAfc – biotin" as determined by photometric detection of the leaving pyridine-2-thione. Given the length of the spacer arm and the exposed position of Cys 26 (Figure 1), the maximum distance between the biotin and the membrane center amounts to about 70 Å.

Solutions of OmpAfc – biotin in buffer A (20 mM tris(hydroxymethyl)aminomethane (Tris) – HCl (pH 8.5), 140 mM NaCl, 0.6 % (w/w) octyltetraoxyethylene (C_8E_4)) were tested for streptavidin binding by titration of a fixed amount of OmpAfc – biotin with streptavidin. Streptavidin and OmpAfc – biotin keep their overall

[a] *Prof. Dr. G. E. Schulz, Dr. P. Ringler*
Institut für Organische Chemie und Biochemie
Albertstrasse 21, 79104 Freiburg im Breisgau (Germany)
Fax: (+49) 761-203-6161
E-mail: schulz@bio.chemie.uni-freiburg.de