Fungus-mediated biosynthesis of silica and titania particles†

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The synthesis of inorganic materials by biological systems is characterized by processes that occur at close to ambient temperatures, pressures and neutral pH. This is exemplified by biosilicification in marine organisms such as diatoms while laboratory-based synthesis of silica involves extreme temperature and pH conditions. We show here that silica and titania particles may be produced by challenging the fungus *Fusarium oxysporum* with aqueous anionic complexes SiF_6^{2-} and TiF_6^{2-} respectively. Extra-cellular protein-mediated hydrolysis of the anionic complexes results in the facile room temperature synthesis of crystalline titania particles while calcination at 300 °C is required for crystallization of silica.

Introduction

Biology is replete with examples of exquisite hierarchically assembled inorganic structures synthesized under mild pH, pressure and temperature conditions. Some well-known examples of microorganisms synthesizing inorganic materials include magnetotactic bacteria (magnetite nanoparticles),[1,2] diatoms (siliceous materials)[3–8] and S-layer bacteria (gypsum and calcium carbonate layers).[9,10] Silica formation in diatoms/sponges proceeds by hydrolysis of silicic acid by proteins such as silicatein[4,5] and polycationic peptides termed silaffins[6,7] while laboratory-based synthesis of silica involves chemical transformation of silicate precursors under extreme temperature and pH conditions. We show here that silica and titania particles may be produced by challenging the fungus *Fusarium oxysporum* with aqueous anionic complexes SiF_6^{2-} and TiF_6^{2-} respectively. Extracellular hydrolysis of the anionic complexes by low molecular weight cationic proteins secreted by the fungus results in the room temperature synthesis of crystalline silica and titania particles. The use of non-mineral forming microorganisms in advanced materials synthesis opens up exciting possibilities for commercially viable biological synthesis of technologically important oxides and semiconductors.

Biosilicification in diatoms has been studied in much detail both from materials point of view[3,7] and for understanding the link between global silicon and carbon cycles.[8] Elucidation of the biological synthesis of silica through silicatein and silaffin has resulted in the design of synthetic cationic polypeptides that mimic the behavior of silicatein.[11,12] While purely biological and bioinspired methods for the synthesis of silica provide environmentally benign and energy-conserving processes, they have not been extended to the formation of other technologically important oxides such as titania and zirconia. Nor have microorganisms other than diatoms been investigated in the formation of silica by hydrolysis of silicon precursors. In a preliminary report, some of us have shown recently that hexafluorozirconate ions can be reacted with the fungus *Fusarium oxysporum* to yield zirconia nanoparticles of average size 7–8 nm.[13] Herein, we have extended our findings through a detailed study of the reaction of the fungus *Fusarium oxysporum* with aqueous anionic complexes of SiF_6^{2-} and TiF_6^{2-} resulting in their protein-mediated hydrolysis and room temperature formation of silica and titania particles. We observe that exposure of the fungus to the silicon and titanium complexes induce the secretion of proteins of molecular weights 21 and 24 kDa. These proteins have been tested for hydrolytic activity and indeed transform the metal complexes to silica and titania. The molecular weights of these proteins are quite close to those observed in the earlier study on zirconia formation with the same fungus implying that they are the same proteins or their post-translationally modified variants.[13] *Fusarium oxysporum* is a plant pathogenic fungus and during its life cycle is not exposed to such ions. That this fungus should secrete proteins capable of hydrolyzing SiF_6^{2-} and TiF_6^{2-} complexes is unexpected but with significant potential for development. Titania is a wide-bandgap semiconductor with important applications in optoelectronics, photocatalysis and as pigments in paints and cosmetics[14] while silica plays an important role in silica-based materials such as resins, molecular sieves and catalysts.[15] Chemical methods such as the sol–gel method for the synthesis of these oxides proceed by hydrolysis of suitable metal complexes under extreme pH and temperature conditions.[16] Biological methods for synthesis of titania and silica particles would thus help mitigate the shortcomings of chemical methods.

Experimental

Synthesis of biogenic silica and titania

The plant pathogenic fungus *Fusarium oxysporum* was cultured as described elsewhere.[17] The fungal mycelia was inoculated in a 500 ml Erlenmeyer flask containing 100 ml of MGYP medium and incubated for 72 hours under shaking conditions

† Electronic supplementary information (ESI) available: Fig. S1: EDAX spectra recorded from biogenic silica (curve 1) and titania (curve 2) particles synthesized using the fungus *Fusarium oxysporum*. See http://www.rsc.org/suppdata/jm/b5/b503008k/

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(200 rpm) at 27 °C. After incubation, the fungal mycelia was harvested and washed thoroughly under sterile conditions. 20 g (wet weight) of the fungal biomass was then resuspended in 100 ml aqueous solutions of 10^{-3} M K_2SiF_6 (pH 3.1) and K_2TiF_6 (pH 3.5) separately in 500 ml Erlenmeyer flasks and kept on a shaker (200 rpm) at 27 °C. The reaction between the fungal biomass and the SiF_6^{2-} and TiF_6^{2-} ions was carried out for a period of 24 hours. The bio-transformed products were collected under sterile conditions at various time intervals during the reaction by separating the fungal mycelia from the aqueous extract through filtration.

Control experiments

In control experiments, the fungal biomass was resuspended in deionized water in the absence of hexafluoro derivatives and the filtrate obtained thereafter was characterized by TEM and FTIR. This reaction did not result in the formation of silica and titania nanoparticles. In another control experiment, the hydrolysis of hexafluoro derivatives in deionized water in the absence of fungal biomass at pH 3.1 (K_2SiF_6) and 3.5 (K_2TiF_6) was studied by TEM and FTIR. This control experiment also was negative and no silica or titania nanoparticles could be detected.

Sample characterization

The samples for transmission electron microscopy (TEM) were prepared by drop coating on to a carbon-coated copper grid. Selected area electron diffraction (SAED) analysis was also carried out for the above prepared TEM samples. FTIR spectroscopy kinetics of the purified and dried biogenic silica and titania powders taken in KBr pellets at various time intervals were recorded on a Perkin-Elmer Spectrum One instrument at a resolution of 2 cm^{-1}. For FTIR kinetics, the biotransformed products present in the filtrate were dried to powders and monitored at different time intervals of reaction. XRD measurements of drop-coated films of the biogenic silica and titania on glass substrates were carried out on a Phillips PW 1830 instrument operated at a voltage of 40 kV and a current of 30 mA with Cu K_α radiation.

Energy dispersive analysis of X-rays (EDAX) measurements were performed on a Leica Stereoscan-440 instrument equipped with a Phoenix EDAX attachment. The EDAX instrument was calibrated automatically.[18] For calibration, one Al sample stub containing Cu is kept inside the microscopic chamber. With the voltage at 20–25 kV, the specimen stub is moved until the field of view contains 25% Al and 75% Cu. The instrument is then calibrated until the two peaks (1.486 keV for Al and 8.040 keV for Cu) are equal. Spectrum acquisition ceases once 8000 counts are reached. The spectrometer was set to these values automatically and used them to carry out further iterations of the calibration process until the peaks were within 1 eV of the default reference value. Thereafter, EDAX measurements were made by casting the silica and titania nanoparticle films on etched Cu substrates.

X-Ray photoemission spectroscopy (XPS) measurements of films of biogenic SiO_2 and TiO_2 nanoparticles cast on to Cu substrates were carried out on a VG MicroTech ESCA 3000 instrument at a pressure better than 1×10^{-9} Torr. The general scan and C 1s, Si 2p and F 2p core level spectra were recorded with un-monochromatized Mg K_α radiation (photon energy = 1253.6 eV) at a pass energy of 50 eV and electron takeoff angle (angle between electron emission direction and surface plane) of 60°. The overall resolution was ~1 eV for the XPS measurements. The core level spectra were background corrected using the Shirley algorithm[19] and the chemically distinct species were resolved using a nonlinear least squares fitting procedure. The core level binding energies (BE) were aligned with the adventitious carbon binding energy of 285 eV.

Thermogravimetric analysis (TGA) of powders of dried biogenic silica and titania nanoparticles was performed on a TGA-7 Perkin Elmer instrument at a scan rate of 10 °C min^{-1}. 20 g (wet weight) of the fungal biomass was suspended in 100 ml aqueous solutions of 10^{-3} M K_2SiF_6 (22.3 mg) and K_2TiF_6 (24.0 mg) separately for a period of 24 hours at 27 °C under shaking conditions. The filtrates obtained thereafter were lyophilized to powders and TGA measurements were carried out on carefully weighed amounts of the powders.

Protein separation

To identify the fungal protein(s) responsible for hydrolysis of the aqueous anionic complex of SiF_6^{2-} into silica, 20 g of the fungal biomass was suspended in 100 ml of sterile distilled water and aqueous 10^{-3} M K_2SiF_6 solution respectively for a period of 24 h at 27 °C under shaking conditions. The filtrate containing the extracellular proteins secreted by the fungus in the absence of K_2SiF_6 was separated from the mycelia by filtration and reacted with 10^{-3} M K_2SiF_6 under ambient conditions for 24 h. The reaction product formed was analyzed by FTIR and TEM. The extracellular proteins secreted by the fungus in the filtrate in the absence of SiF_6^{2-} anions were salted out overnight at 4 °C using ammonium sulfate precipitation followed by centrifugation. The proteins obtained thereafter were dissolved in the minimal volume of 20 mM phosphate buffer (pH 7.2) and dialyzed (using a 12 kDa cutoff dialysis membrane). The dialyzed protein fraction containing a mixture of proteins was applied on a DEAE-Sephadex column. The unbound protein fraction showing the activity was dialyzed against 20 mM sodium acetate buffer (pH 4.5), concentrated by lyophilization and then loaded on a CM-Sephadex column equilibrated with 20 mM sodium acetate buffer (pH 4.5). The bound proteins were eluted using a step-gradient of NaCl in 20 mM sodium acetate buffer (pH 4.5). The various fractions obtained were checked for their activity. Out of various fractions obtained, only one fraction showed the activity to convert complex SiF_6^{2-} and TiF_6^{2-} ions into silica and titania particles respectively under appropriate reaction conditions. The activity of the purified fraction thus obtained was further checked by TEM and FTIR. In a separate experiment, the extracellular proteins secreted by the fungus in the filtrate in the presence of SiF_6^{2-} anions were salted out overnight at 4 °C using ammonium sulfate precipitation followed by centrifugation. The proteins obtained thereafter were dissolved in the minimal volume of deionized water and dialyzed (using a 12 kDa cutoff dialysis membrane). The salt-precipitated fungal protein obtained in the presence of SiF_6^{2-} ions and purified protein fraction obtained by ion exchange columns were

analyzed by 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) carried out at pH 8.2 and run along with the protein standard molecular weight marker according to the procedure published by Laemmli.[20] In a separate experiment, the column purified fraction of protein was analyzed through native (non-denaturing) PAGE. The polyacrylamide gels were stained using silver staining.

Results and discussion

Representative transmission electron microscopy (TEM) images from the fungus–K_2SiF_6 reaction medium after 24 h of reaction are shown in Fig. 1A. The particles are irregular in shape presenting an overall quasi-spherical morphology. The particle size histogram of the silica particles (Fig. 1C) shows that the particles range in size from 5 to 15 nm and possess an average size of 9.8 $\pm$ 0.2 nm. Fourier transform infrared (FTIR) analysis of particles from the fungus–SiF_6^{2-} reaction medium after 24 hours of reaction showed the presence of a prominent resonance at *ca.* 1086 cm^{-1} (curve 2, Fig. 2A). The strong 1086 cm^{-1} band is attributed to excitation of the antisymmetric Si–O–Si stretching mode of vibration[21] and is absent from the spectrum of pure K_2SiF_6 (curve 1, Fig. 2A). The presence of the absorption band at *ca.* 750 cm^{-1} indicates the presence of some unreacted SiF_6^{2-} ions in the particles. Two absorption bands centered at *ca.* 1650 and 1540 cm^{-1} (amide I and II bands respectively; curve 2, inset of Fig. 2A) attest to the presence of proteins in the quasi-spherical particles that are clearly missing in the K_2SiF_6 sample (curve 1, inset of Fig. 2A).

Energy dispersive analysis of X-rays (EDAX) measurements of drop-coated films of the silica particles from the fungus–SiF_6^{2-} reaction medium after 24 hours of reaction clearly

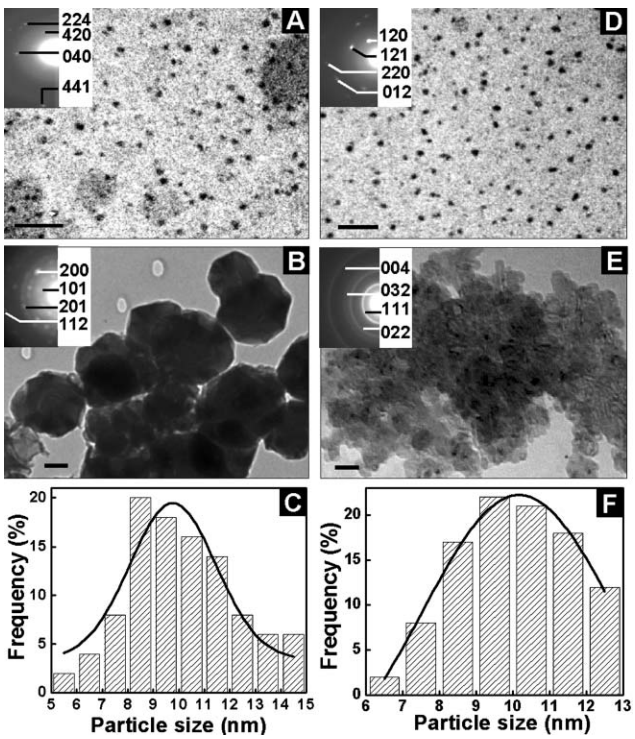

Fig. 1 TEM micrographs of silica particles synthesized using *Fusarium oxysporum* before (A) and after calcination at 300 °C for 3 h (B). The insets in A and B are SAED patterns recorded from representative silica particles. TEM micrographs of titania particles synthesized using *Fusarium oxysporum* before (D) and after calcination at 300 °C for 3 h (E). The insets in D and E are SAED patterns recorded from representative titania particles. The scale bars in all the images (A–D) correspond to 50 nm. Particle size histograms of the silica and titania particles shown in images A and D are shown in (C) and (F) respectively.

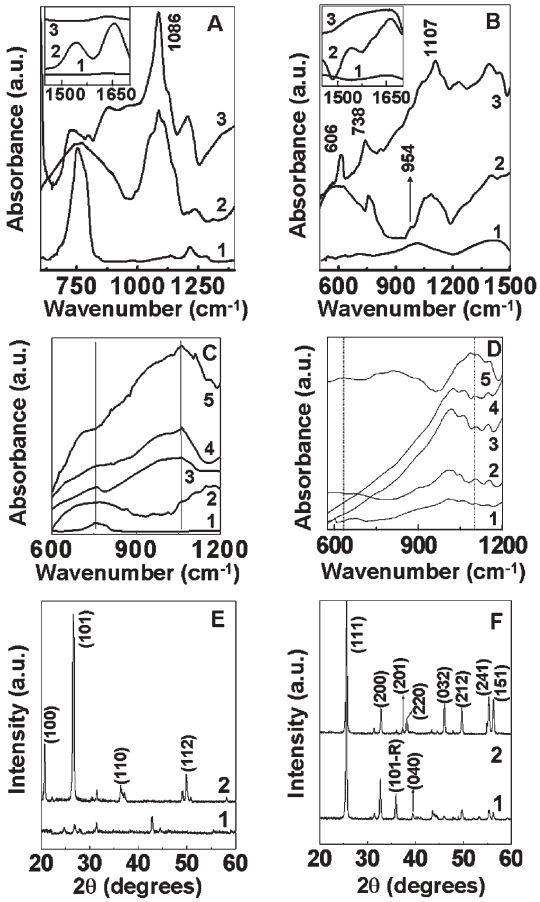

Fig. 2 A) FTIR spectra recorded from powders of K_2SiF_6 (curve 1); silica particles synthesized using *Fusarium oxysporum* before (curve 2) and after calcination at 300 °C for 3 h (curve 3). The inset shows an expanded view of these spectra in the region of protein amide bands. B) FTIR spectra recorded from powders of K_2TiF_6 (curve 1); titania particles synthesized using *Fusarium oxysporum* before (curve 2) and after calcination at 300 °C for 3 h (curve 3). The inset shows an expanded view of these spectra in the region of protein amide bands. FTIR kinetics recorded from silica (C) and titania (D) particles synthesized by the reaction of appropriate precursors with *Fusarium oxysporum*. In both figures, curves 1–5 correspond to patterns recorded at reaction intervals of 1, 6, 12, 18 and 24 h respectively. XRD patterns recorded from silica (E) and titania (F) particles synthesized by the reaction of appropriate precursors with *Fusarium oxysporum*. In both figures, curves 1 and 2 correspond to patterns recorded from the as-prepared and calcined samples respectively: silica [4.26 Å (100); 3.36 Å (101); 2.46 Å (110); 1.85 Å (112)]; titania [3.46 Å (111); 2.71 Å (200); 2.48 Å (101); 2.40 Å (201); 2.34 Å (220); 2.29 Å (040); 1.96 Å (032); 1.83 Å (212); 1.66 Å (241); 1.64 Å (151)]. The peak marked with 'R' corresponds to the rutile phase of TiO_2.

show the presence of Si and O with an atomic ratio of 1 : 1.83. This is in reasonable agreement with the expected stoichiometry of silica (ESI†Fig. S1, curve 1). Signatures of K and F were also observed in the EDAX spectrum indicating the presence of some unreacted K_2SiF_6. A more refined chemical analysis of the biogenic silica nanoparticles was performed by X-ray photoemission spectroscopy (XPS), which is known to be a highly surface sensitive technique (Fig. 3). In Fig. 3A, curves 1, 2 and 3 correspond to the chemically distinct C 1s core levels originating from the hydrocarbon chains, α-carbon and –COOH groups present in the proteins in the protein–silica composite with binding energies (BEs) of 285, 286.6 and 289 eV respectively. In Fig. 3B, the Si 2p spectrum could be resolved into two spin–orbit pairs (spin–orbit splitting ~ 0.6 eV) with $2p_{3/2}$ binding energies (BEs) of 102.7 eV[22] (curve 1 in Fig. 3B) and 106.2 eV[23] (curve 2 in Fig. 3B) respectively. The low BE component at 102.7 eV agrees excellently with values reported for SiO_2,[22] while the high BE component at 106.2 eV is assigned to unhydrolyzed SiF_6^{2-} ions present on the surface of the silica nanoparticles.[23] From the peak intensities of curve 1 (SiO_2) and curve 2 (SiF_6^{2-}), it is observed that the amount of unhydrolyzed SiF_6^{2-} ions is extremely small indicating that most of the SiF_6^{2-} ions have been transformed into SiO_2 nanoparticles. In addition to the Si 2p spectrum, a F 2p signal was also observed in the sample (Fig. 3C) supporting the presence of some amount of unhydrolyzed SiF_6^{2-} ions in the nanoparticle samples (Fig. 3B).

Selected area electron diffraction (SAED) analysis of the silica particles clearly shows the crystalline nature of the silica particles (inset, Fig. 1A). The diffraction spots could be indexed based on the tridynite polymorph of SiO_2 wherein the *d* values obtained (3.24 Å, 3.00 Å, 2.50 Å and 2.09 Å) match well with the standard *d* values (3.24 Å, 2.98 Å, 2.48 Å and 2.07 Å) for the (420), (224), (040) and (441) planes of this polymorph of SiO_2.[24] We did not observe diffraction spots from potential impurities such as unreacted K_2SiF_6. Drop-coated films of the as-prepared silica particles (curve 1, Fig. 2E) failed to show well-defined Bragg reflections indicating that the particles were X-ray amorphous. While the reasons for the silica particles being X-ray amorphous but crystalline under electron diffraction analysis are not clear at this point, we speculate that the particles undergo mild heating during electron irradiation in the TEM that facilitates their crystallization. In order to monitor the kinetics of hydrolysis of SiF_6^{2-} ions into SiO_2, FTIR spectroscopy measurements of the fungus–K_2SiF_6 reaction medium were carried out after 1 h, 6 h, 12 h, 18 h and 24 h of reaction (curves 1–5 respectively, Fig. 2C). It is observed that there is a gradual and monotonic increase in absorbance at *ca.* 1086 cm^{-1} that is accompanied by a decrease in intensity of the resonance at 750 cm^{-1} with time clearly indicating the hydrolysis of SiF_6^{2-} ions into SiO_2.

Fig. 1D show a representative TEM image obtained from the fungus–K_2TiF_6 reaction medium after 24 h of reaction. A number of spherical particles ranging in size from 6 to 13 nm with an average size of 10.2 ± 0.1 nm were seen for which the particle size histogram is shown in Fig. 1F. EDAX measurements of drop-coated films of the biogenic titania particles obtained by reaction of the fungus with TiF_6^{2-} ions after 24 hours of reaction clearly show the presence of Ti and O at an atomic ratio of 1 : 1.92. This is in good agreement with the expected stoichiometric ratio (ESI† Fig. S1, curve 2). K and F signals were also observed in the EDAX spectrum indicating the presence of some unreacted K_2TiF_6. XPS analysis of the biogenic titania nanoparticles indicated the almost complete hydrolysis of TiF_6^{2-} ions to TiO_2; these data are not shown for brevity.

Selected area electron diffraction (SAED) analysis of the as-prepared TiO_2 particles indicated that they were crystalline (inset, Fig. 1D). The diffraction spots could be indexed based on the brookite structure of TiO_2 wherein the *d* values obtained (3.53 Å, 2.90 Å, 2.47 Å and 2.34 Å) match well with the standard *d* values of the brookite polymorph of TiO_2 [3.51 Å (120), 2.90 Å (121), 2.47 Å (012) and 2.34 Å (220)].[24] The XRD pattern obtained from solution-cast films of these TiO_2 particles also shows a number of Bragg reflections characteristic of the brookite phase of TiO_2 with one peak arising from the rutile phase (curve 1, Fig. 2F).[24,25] The FTIR spectrum recorded from the TiO_2 particles taken in a KBr pellet showed the presence of broad absorption bands at *ca.* 600 cm^{-1} and 1100 cm^{-1} (curve 2, Fig. 2B) that are missing in the K_2TiF_6 sample (curve 1, Fig. 2B). These peaks correspond to excitation of Ti–O–Ti vibrational modes in the particles.[26,27] A weak band at 954 cm^{-1} corresponds to excitation of the Ti–O antisymmetric stretching of Ti–O–Ti bonds.[28] As in the case of biogenic silica particles, the presence of proteins in the titania particles is indicated by the amide I and II bands in the FTIR spectrum (curve 2, inset, Fig. 2B), these bands being absent in the K_2TiF_6 powder (curve 1, inset, Fig. 2B). The kinetics of titania formation in the fungus–TiF_6^{2-} reaction medium was followed by FTIR spectroscopy after 1 h, 6 h, 12 h, 18 h and 24 h of reaction (curves 1–5 respectively,

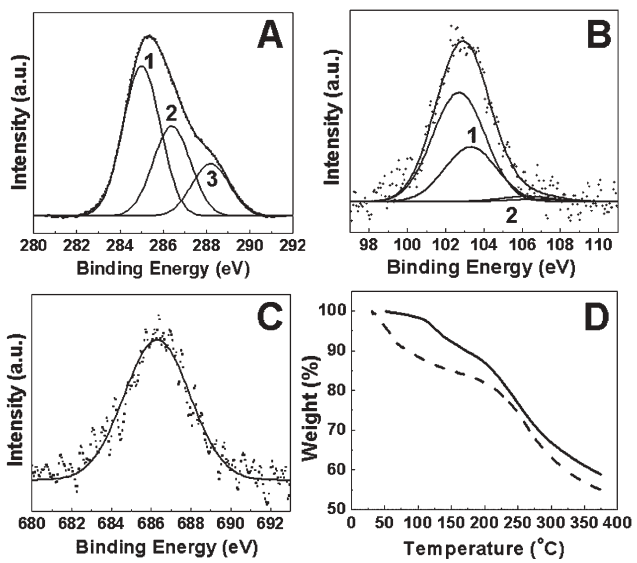

Fig. 3 (A–C) XPS data showing the C 1s (A), Si 2p (B) and F 2p (C) core level spectra recorded from a biologically synthesized silica nanoparticle film cast onto a Cu substrate. The chemically resolved components are shown as solid lines and are discussed in the text. (D) TGA data obtained from dried biogenic silica (solid line) and titania (dashed line) nanoparticle samples.

Fig. 2D). It is observed that there is a steady increase in intensity of the resonance at *ca.* 1100 cm^{-1} with time (the Ti–O–Ti vibrational modes) indicating hydrolysis of the TiF_6^{2-} ions into TiO_2.

FTIR, SAED and XRD analysis of the products of the reaction between K_2SiF_6 and K_2TiF_6 with *Fusarium oxysporum* indicate the formation of crystalline SiO_2 and TiO_2 particles with a fair degree of proteins occluded into their structures. The SiO_2 and TiO_2 powders were then calcined in air at 300 °C for 3 h to remove the occluded proteins and promote further crystallization of the oxides. The TEM images of SiO_2 (Fig. 1B) and TiO_2 (Fig. 1E) particles after calcination show that these particles undergo a significant change in particle morphology and form large aggregates after calcination. The SAED analysis of the silica particles after calcination suggests that the particles have become more crystalline with a number of well-defined Bragg reflections (101, 200, 201 and 112) appearing in the SAED pattern (inset, Fig. 1B), wherein the *d* values obtained (3.35 Å, 2.13 Å, 1.98 Å and 1.82 Å) match well with the standard *d* values (3.36 Å, 2.13 Å, 1.98 Å and 1.82 Å) for the tridynite polymorph of SiO_2.[23] This is mirrored in the XRD pattern of the calcined silica sample that shows intense Bragg reflections (curve 2, Fig. 2E) characteristic of crystalline silica.[24] A sharp SAED ring pattern is observed in the calcined TiO_2 particles (inset, Fig. 1E) that could be indexed based on the brookite structure for titania, wherein the *d* values obtained (3.47 Å, 2.24 Å, 1.97 Å and 1.28 Å) match reasonably well with the standard *d* values (3.46 Å, 2.24 Å, 1.97 Å and 1.28 Å) for the 111, 022, 032 and 004 planes respectively of the brookite polymorph of TiO_2.[24,25] This is in agreement with XRD analysis of the titania powder that shows an increase in intensity of all the brookite peaks after calcination (curve 2, Fig. 2F). FTIR analysis of the silica and titania powders after calcination (curves 3 in Fig. 2A and B respectively) shows a sharpening of the Si–O–Si and Ti–O–Ti vibrational bands that is accompanied by a disappearance of the protein amide I and II bands from the particles (insets of Fig. 2A and B, curve 3 in both cases). Thus, removal of the occluded proteins by calcination improves the crystallinity of the biogenic silica and titania particles.

To identify the fungal protein(s) responsible for hydrolysis of the aqueous anionic metal complexes, 20 g of the fungal biomass was suspended in 100 ml of sterile water for a period of 24 h at 27 °C under shaking conditions. The filtrate containing the extracellular proteins secreted by the fungus in the absence of the metal complexes was separated from the mycelia by filtration and reacted with 10^{-3} M K_2SiF_6 under ambient conditions for 24 h. The reaction product formed was analyzed by different techniques and hydrolysis of the SiF_6^{2-} ions was clearly established (FTIR: curve 2, Fig. 4B; TEM: Fig. 4C). However, the percentage hydrolysis of the SiF_6^{2-} ions was considerably lower than that observed when the reaction of the metal complexes was carried out directly in the presence of the fungus indicating that the hexafluorosilicate ions stimulate the secretion of the hydrolyzing proteins by the fungus.

The extracellular proteins secreted by the fungus in the filtrate obtained in the aforesaid experiment in the absence of SiF_6^{2-} anions were salted out overnight at 4 °C using ammonium sulfate precipitation followed by centrifugation. The proteins obtained thereafter were dissolved in the minimal volume of deionized water and dialyzed (using a 12 kDa cutoff dialysis membrane). The dialyzed protein fraction containing a mixture of proteins was further purified by ion exchange columns. The various fractions obtained from the CM-Sephadex column were checked for their activity. Of the various fractions obtained, only one fraction when reacted with SiF_6^{2-} and TiF_6^{2-} ions, was capable of hydrolysis of these complexes to silica and titania respectively. The reaction product formation was monitored by different techniques and hydrolysis of the SiF_6^{2-} and TiF_6^{2} ions by column-purified fraction was clearly established (FTIR: curve 3, Fig. 4B; TEM: Fig. 4D). For brevity, the results pertaining to silica synthesis using this fraction alone are described.

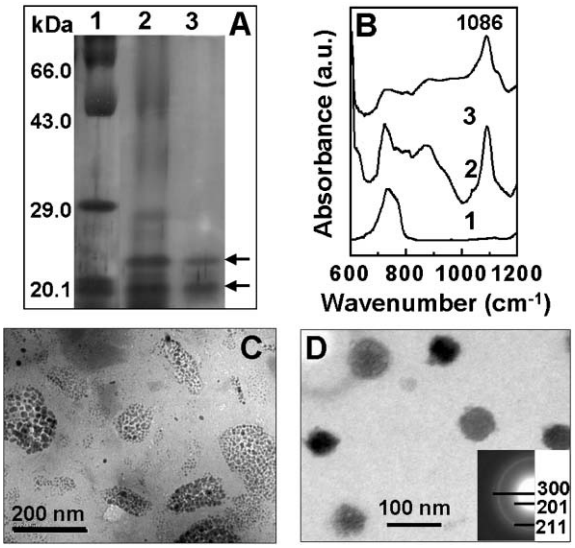

Fig. 4 SDS-PAGE data (A) showing the extracellular protein profile of *Fusarium oxysporum.* Lane 1 shows standard protein molecular weight markers with the bands showing corresponding to molecular weights in kDa; lane 2 shows extracellular fungal proteins obtained by 80% ammonium sulfate precipitation of filtrate in the presence of SiF_6^{2-} and lane 3 shows protein fraction purified using ion exchange columns. The arrows highlighting the bands indicate the proteins responsible for hydrolysis of the metal complexes into silica particles. FTIR spectra (B) recorded from pure K_2SiF_6 (curve 1), powders of silica synthesized using fungal crude extract (curve 2) and silica formed using column purified protein fraction (curve 3) respectively. TEM micrographs of silica particles synthesized using the fungus extract (C) and the column purified protein fraction (D). The inset in D corresponds to the SAED pattern recorded from the particles shown in the main figure [1.98 Å (201); 1.58 Å (211); 1.42 Å (300)].

In a separate experiment, 20 g of the fungal biomass was suspended in 100 ml of sterile water containing 10^{-3} M K_2SiF_6 for a period of 24 h at 27 °C under shaking conditions and the extracellular proteins secreted by the fungus in the filtrate in the presence of SiF_6^{2-} anions were salted out overnight at 4 °C using 80% ammonium sulfate followed by centrifugation. The proteins obtained were thereafter dissolved in the minimal volume of deionized water and dialyzed (using a 12 kDa cutoff dialysis membrane). The activity of this dialyzed fraction of protein could not be checked due to the presence of silica

particles in the protein fraction itself, since these proteins were extracellularly secreted by the fungus in the presence of SiF_6^{2-} ions and even repeated washing and centrifugation failed to completely remove the silica particles from the protein fraction.

The protein standard molecular weight markers (lane 1, Fig. 4A), protein fraction obtained in the presence of SiF_6^{2-} ions by ammonium sulfate precipitation (lane 2, Fig. 4A) and the purified protein fraction obtained by ion exchange columns that tested positive for hydrolysis of SiF_6^{2-} ions (lane 3, Fig. 4A) were analyzed by 10% SDS-PAGE carried out at pH 8.2. The protein fraction obtained by ammonium sulfate precipitation (lane 2, Fig. 4A) shows few proteins in the form of well-separated bands along with two intense bands of *ca.* 21 and 24 kDa. Moreover, the active purified protein fraction obtained by ion exchange columns (lane 3, Fig. 4A) also showed only those two bands, which appear at the same level as that of the intense bands in lane 2.

In a separate experiment, the column purified fraction of protein when analyzed by native (nondenaturing) PAGE again showed two bands corresponding to the two most intense bands in the ammonium sulfate precipitated fraction (data not shown) clearly suggesting that these proteins are not the subunits of a single protein but are two separate proteins. Moreover, the cationic nature of these proteins was also confirmed using native PAGE. This suggests that either one or both of these cationic proteins must be responsible for the hydrolytic conversion of hexafluorosilicate and hexafluorotitanate ions into silica and titania particles respectively. In the previous study on the formation of zirconia using *Fusarium oxysporum*, we have found that 24 and 28 kDa proteins were responsible for the hydrolytic conversion of hexafluorozirconate complexes into zirconia nanoparticles.[13] This slight difference in molecular weights of the proteins involved in the two cases suggests the involvement of similar proteins in the hydrolysis process and this difference might be articulated to various levels of post-translational modification of proteins involved in the above two cases.[13] Further investigations are in progress to understand the individual role of these two proteins in silica/titania formation both in terms of the amino acid sequence of these proteins and the nature of their interaction with the silica and titania particles.

The hydrolysis of other silicon precursors such as tetraethoxysilane (TEOS) by the fungus proceeds equally well yielding large amounts of silica particles. These metal anions are not toxic to the fungus, which can be extracted from the metal ion–fungus reaction medium and grown further in culture media. A number of other genera of fungi were tested for hydrolytic activity (*Curvularia lunata*, *Colletotrichum gloeosporioides*, *Phomopsis* sp., *Aspergillus niger*) but did not yield positive results.

20 g (wet weight) of the fungal biomass when reacted with 100 ml aqueous solution of 10^{-3} M K_2SiF_6 yielded 34.2 mg of silica nanoparticles when dried to a powder. As mentioned earlier, the nanoparticles are capped with proteins/biomolecules that stabilize them against aggregation. To remove the biomolecules on the surface of the biogenic silica particles, thermogravimetric analysis (TGA) of the silica powder was performed up to 400 °C and 55% loss in weight was observed (Fig. 3D, solid curve). Simple arithmetic shows that 0.77 mg of silica nanoparticles are formed per gram of wet fungal biomass. From the TGA analysis of biogenic titania nanoparticles (Fig. 3D, dashed curve) and the weight of the titania nanoparticles formed after reaction of 20 g (wet weight) of the fungal biomass with 100 ml aqueous solution of 10^{-3} M K_2TiF_6, the yield of pure titania nanoparticles per gram of wet biomass was estimated to be 0.765 mg.

Even though biotechnological applications such as remediation of toxic metals routinely employ micro-organisms such as bacteria and yeast,[29] it is only recently that bacteria[30–33] and fungi[21,34,35] have been investigated as possible eco-friendly inorganic nanofactories. The report on extra-cellular synthesis of CdS nanoparticles by reaction of aqueous $CdSO_4$ solution with the fungus *Fusarium oxysporum* highlighted the fact that specific fungal enzymes (such as sulfate reductases)[35] once identified, may be used to synthesize inorganic materials over a range of chemical compositions enzymatically. We have shown here that the fungus *Fusarium oxysporum* secretes proteins capable of hydrolyzing SiF_6^{2-} and TiF_6^{2-} extracellularly to form silica and titania at room temperature. It is also possible that the non-proteinaceous component of the fungal exudates such as gums and gels could hydrolyze the metal complexes to yield SiO_2 and TiO_2 particles. This possibility was tested by precipitating out the proteins in the fungal extract and then reacting the remaining non-proteinaceous component with aqueous SiF_6^{2-} and TiF_6^{2} ions. We did not observe any hydrolysis of the metal complexes in this reaction thus clearly underlining the crucial role played by only the proteins in the formation of silica and titania nanoparticles. Particularly gratifying is the fact that the fungus is capable of hydrolyzing tough metal halide precursors under acidic conditions. While the hydrolytic proteins secreted by *Fusarium oxysporum* are yet to be sequenced and studied for their role in the fungus metabolic pathways, our studies indicate that they are cationic proteins of molecular weight centered around 21 and 24 kDa. The regenerative capability of biological systems coupled with the discovery that fungi such as *Fusarium oxysporum* are capable of hydrolyzing metal complexes that they never encounter during their growth cycle shows enormous promise for development, particularly the large-scale synthesis of metal oxide semiconductor materials. The eco-friendly and energy-conserving nature of the fungus-based biological process for metal oxide synthesis in comparison with chemical processes such as the sol–gel method cannot be overemphasized.

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