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Biosynthesis of gold nanoparticles using diatoms – silica-gold and EPS-gold bionanocomposite formation

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ABSTRACT Novel synthesis of gold nanoparticles, EPS-gold and silica-gold bionanocomposites by biologically driven processes employing two diatom strains (*Navicula atomus, Diadesmis gallica*) is described. Transmission electron microscopy (TEM) and electron diffraction analysis (SAED) revealed a presence of gold nanoparticles in the experimental solutions of the diatom culture mixed with tetrachloroaureate. Nature of the gold nanoparticles was confirmed by X-ray diffraction studies. Scanning electron microscopy (SEM) and TEM showed that the nanoparticles were associated with the diatom frustules and extracellular polysaccharides (EPS) excreted by the diatom cells. Due to its accessibility, simplicity and effectiveness, this method of nanocomposites preparation has great importance for possible future applications.

KEYWORDS: composite, *Diadesmis gallica*, diatoms, EPS, frustule, gold, nanoparticles, *Navicula atomus*, silica.

Introduction

Development of reliable and eco-friendly procedures of metallic nanoparticles and nanocomposites synthesis is crucial for success of increasingly advancing nanotechnology applications. In present years, increasing attention is paid to a synthesis of composites that either directly appears from or is inspired by the nature (Bellezza et al. 2009). Some relevant ways using "natural factories" of various organisms as well as possible applications of free nanoparticles were previously widely discussed and reviewed by e.g. Mohanpuria et al. 2008, or Bhattacharya and Gupta 2005. Biosynthesis of metallic nanoparticles by photoautotrophic organisms (Brayner et al. 2007, Lengke et al. 2006a, Lengke et al. 2006b, Lengke et al. 2007a) or their components (Brayner et al. 2009, Krpetic et al. 2009) were also tested and described recently.

Gold nanoparticles (AuNPs) exhibit high catalytic activity although bulk gold is typically catalytically ineffective (Dotzauer et al. 2006, Sardar et al. 2009). Catalytic activity of some artificially prepared AuNPs was previously examined (Carregal-Romero et al. 2010, Saha et al. 2010). However, despite of high reactivity and efficiency of metallic colloids as homogenous catalysts, their application to large-scale processes is limited due to the reduction of catalytic activity by particle aggregation. Therefore, for practical utilization (e.g. for medical purposes), nanoparticles are usually immobilized on solid matrices such as silica, aluminum, and metal oxides (Budroni et al. 2006, Bus et al. 2005, Mallick et al. 2004, Kim et al. 2008).

Diatoms are unicellular photosynthesizing microorganisms belonging into the group of brown algae (division *Chromophyta*, class *Bacillariophyceae*) encased in siliceous cell walls – frustules. Frustule of a diatom is always formed by two valves (epitheca and hypotheca) connected together by circular pieces of silica called girdle bands. The construction material of a frustule is mainly nanostructured amorphous polymerized silicic acid (van den Hoek et al. 1995). Surface of the frustule is finely structured with extensions, perforations, thickenings, or thin areas in the wall, and the final pattern together with a frustule shape is characteristic for each species. The ability of reproduction of such precise forms results

from unique mechanism of silica acquirement and processing, which has been previously described in detail (Volcani 1981, Hildebrand 2003). New valves are produced after cell division and cytokinesis of the mother protoplast. The final silica polymerization and its deposition onto the forming diatom wall occur in flattened vesicles called silica deposit vesicles (SDVs). It is likely, that the precise formation of the valve pattern is facilitated by organic macromolecules of the vesicle matrix. When the siliceous wall is completely formed, exocytosis occurs and two daughter cells each containing maternal epitheca and newly formed hypotheca are separated. The inner membrane of the SDVs becomes the new plasmalemma, whereas the outer membrane now forms primary coatings around the silica[18]. The proteins associated with the mature diatom cell wall contain highly conserved repeated building block and have been denoted as frustulins (Kröger et al. 1996, van de Poll et al. 1999). The mechanism of diatom frustule formation is further investigated as a model for biomimetic synthesis of silica nanostructures (El Rassy et al. 2005, Crawford et al. 2009, Hildebrand 2005).

Although biosynthesis of nanoparticles through phototrophic organisms such as cyanobacteria, algae (Chakraborty et al. 2009, Mata et al. 2009) or higher plants (Krpetic et al. 2009) was noted previously, this work describes the very first detailed experiments carried out using diatoms, or organisms with silica based shells respectively. Formation of nanoparticles in presence of siliceous frustules likely provides occasions for novel bionanocomposite use. We present experiments leading to a protocol for synthesis of AuNPs, EPS-gold and silica-gold bionanocomposites by biologically driven processes in diatoms.

Materials and Methods

Diatom strains and cultures

Diatom cultures (*Navicula atomus* CCALA 383 – NA; *Diadesmis gallica* CCALA 766 – DG) were obtained from the Culture Collection of the Centre of Algology in Třebon, Biology Centre of the AS CR, Institute of Hydrobiology, Czech Republic. Strains were kept in 1 L Erlenmayer flasks with cotton

plugs containing WC Medium (Guillard and Lorenzen 1972), into which water glass ($Na_2SiO_3.5H_2O$) was added to obtain final concentration of 500 mg of silica per liter of media, ensuring no silica limitation for the diatom growth. The conditions at the growth chamber (KBW-240, Binder, Germany) were controlled to 21°C and 16h/8h light/dark cycle (5 x 36 W/m² Osram Lumilux Cool Daylight fluorescent lamp). Prior the beginning of the experiment, the cultures were transferred into fresh medium (20% (v/v) of inoculum), and were grown for approximately four weeks to reach a stationary "growth" phase.

Diatoms Biosynthesis Experiments

Experiments were conducted to examine the role of diatoms in the synthesis of AuNPs from aqueous solutions of Au salts. To initiate the experiments, 10 mL of gold solution tetrachloroaureate ($HAuCl_4$; Sigma-Aldrich) (≈ 50 mg/mL Au) was added to 10 mL of 4-weeks old diatom culture in liquid WC Medium (≈ 20 mg dry weight) in 50 mL Falcon flasks. The solutions in flasks were then let to incubate in laboratory conditions (moderate light and 23°C) for 12 hours. Experiments were organized as triplicates. Abiotic control was performed in the same conditions using liquid WC Medium without the diatom cultures.

UV-VIS Analyses and Light Microscopy (LM)

Noble metal nanoparticles exhibit a strong UV-visible (UV-VIS) absorption band that is not present in the spectrum of the bulk metal (surface plasmon resonance – SPR). An amount of 1,5 mL of each sample suspension was centrifuged (2 minutes, 8.000 x g, EBA 21, Hettich, Germany). The optical absorption spectra of the samples were measured by UV-VIS spectrophotometer Cintra 303 (GBC Scientific Equipment, Illinois, USA).

The diatom cultures alone (reference control) as well as their suspension with tetrachloroaureate were examined using an OLYMPUS BX 51 brightfield microscope with high resolution Nomarski DIC optics, equipped with a DC 71 digital camera, under an immersion oil lens at 1000x magnification.

Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM)

Unstained whole sample mounts of diatoms and AuNPs from the experiments were examined with a Jeol 1200 EX TEM microscope (JEOL Ltd., Tokyo, Japan) operating at 120 kV and field emission SEM microscope JSM-7401F (JEOL Ltd., Tokyo, Japan) with cryo system for high-resolution SEM Gatan ALTO 2500 (Gatan Inc., USA). The whole mounts were prepared by floating carbon-coated grids on a drop of culture for several minutes to allow the cells and any fine-grained particles to attach to the grid. Nature of observed crystalline structures was confirmed by the Selected Area Electron Diffraction (SAED) method.

Image analysis

Image analysis tool (JMicrovision: Image Analysis Toolbox, www.jmicrovision.com) was used for size and shape analysis of the nanoparticles in the experimental samples. Two TEM micrographs from each sample underwent the image analyses.

Grazing incidence X-ray diffraction (XRD)

X-ray diffraction patterns were obtained using Bruker AXS D8 Discover diffractometer (Bruker, USA) with CuKα radiation and 12 kW rotating anode in grazing incidence (GI) geometry. The diffraction patterns were taken from dried specimens, incidence angle was 4 degrees.

Results

Approximately 2 hours after experiment initiation, color of all of the experimental solutions started to turn dark reddish. The samples were let to incubate for total amount of 12 hours and subsequently centrifuged. Although the color of the liquid was still red, measurement of UV-VIS spectra did not prove any characteristic AuNPs surface plasmon resonance peak. This indicated presence of AuNPs only in the pellet of the cells.

This presumption was confirmed by LM observations (see Fig. 1). Compared to the reference control (Fig. 1 a),c)), diatom cells in the experimental samples and their proximate surroundings changed their color to deep red, signaling the presence of AuNPs fixed to the living substance (Fig. 1 b),d)). LM

micrograph also showed spilt living substance, which indicated that certain part of diatom cells have died during the bioreduction process.

Photographs from the TEM clearly show presence of spherical AuNPs in experimental samples for both diatom strains (see Fig. 2, ESM Supporting Fig. 1), the Au nature of these crystalline nanoparticles was confirmed by SAED patterns (Fig. 2 b),d)). It was observed that the AuNPs remain captured in EPS net in the extracellular space (e.g. Fig. 2 a)), or are directly associated with the diatom frustule structures (e.g. Fig. 2 c)).

Both detailed TEM pictures (Fig. 2 b),d)) and image analysis (JMicrovision programme) confirmed mostly spherical shape of the nanoparticles. Size of the biosynthesized nanoparticles differed in each strain (see Fig. 3). Whereas DG samples showed larger mean particle size (around 22 nm) and wider range of the size distribution, AuNPs synthesized by NA strain had smaller mean particle size (9 nm) and higher homogeneity in size.

The spatial distribution of the nanoparticles in the sample was examined using SEM with a cryochamber, which allowed avoiding changes caused by dehydration procedures in standard SEM preparation methods. Using the backscatter secondary electron detector, the attachment of the nanoparticles to the diatom mass was observed. The SEM photographs (Fig. 4, see also ESM Supporting Fig. 2, 3) clearly show shape of the diatom frustules, as well as the structures of the intercellular matter (EPS) with attached nanoparticles. In accordance with the light microscopy observations, SEM depicted the nanoparticles distributed in more loosened and thicker layer of the EPS structures around the cells and directly on the frustule surface of the DG strain (Fig. 4 a),b)), whereas dense fibrous net of the EPS close to the frustule surface with entrapped nanoparticles can be seen in pictures of the NA strain (Fig. 4 b),c)).

On the other hand, abiotic control experiments without diatom cultures did not prove presence of AuNPs in the solution neither by the color change nor by means of any microscopy technique (data not shown).

X-ray diffraction explicitly confirmed presence of crystalline gold in all of the experimental suspensions and therefore is in compliance with other observations. As an illustration, diffractogram of DG is shown in Fig. 5. All peaks of the diffractogram are in good agreement with theoretical presumptions for cubical gold. We observed similar diffractogram in case of the NA strain (not shown), which differed in additional peaks indicating minute presence of other ingredients originating most likely from the WC medium salt precipitates or unexpended tetrachloroaureate.

Discussion

Concerning the speed of diatom nanoparticle biosynthesis, the duration of the bioreduction of gold tested in this experiment was in order of hours. Such time is shorter comparing to studies of NPs biosynthesis in cyanobacteria, in which the bioreduction took place in order of days (Lengke et al. 2006a, 2007a) and conversely longer or similar to studies on bacteria or fungi (Lengke and Southam 2006, Vijayakumar and Prasad 2009). However, all of the experiments indeed strongly depend on mass of biomass used, pH of bioreduction, salt concentration etc. (Brayner et al. 2007, Lengke et al. 2007b) and the cultivation have not yet been optimized.

Results obtained in combination of LM, EM, and UV-VIS spectroscopy indicate that the biosynthesized AuNPs are bound to the cellular structures and intercellular EPS. In previous studies of nanoparticle biosynthesis (e.g. Lengke et al. 2007a), presence of free NPs directly in the solution of the cell culture and metallic salt was confirmed through SPR spectra. However, direct measurements of the UV-VIS spectra were not possible for the treated suspension of the diatom culture, as the siliceous frustules cause significant dispersion of the light beam and wavelength changes. Instead, we used a supernatant of the experimental solution centrifuged at 8.000 x g. This speed assured sedimentation of larger particles such as diatom frustules, whereas it was not sufficient to separate particles of the size of AuNPs (these would settle down at the speed of approximately 15.000 x g or more). SPR spectra did not confirm presence of free AuNPs produced by diatoms, suggesting their exclusive fixation onto the cell

structures. This fact has considerable significance for future utilization of the nanoparticles. It is expected that EPS with imbedded AuNPs as well as the siliceous frustules with adsorbed AuNPs could be isolated from the solution and used for further applications (e.g. in heterogeneous catalysis).

TEM micrographs well documented sizes and shapes of the AuNPs. Different size was observed for each of the tested strains. Moreover, JMicrovision image analysis revealed notable difference between the size of the AuNPs embedded in the EPS structures and the AuNPs adsorbed to frustule surface. When compared, particles in the EPS are roughly 50% smaller than the ones on the silica surface. This phenomenon is likely caused either by the nature of the microstructure of the silica surface or by the constitution of the EPS net. Yet, for the potential practical application of the nanoparticles synthesized by diatoms, possibility of certain regulation of the size and the size distribution by using alternative tetrachloroaureate concentration, temperature or other parameters is expected (Lengke et al. 2007b).

Although we did not perform any viability studies during the biosynthesis process, LM showed remarkable changes in the cells. Based on this fact and previous studies (Lengke et al. 2006a, Brayner et al. 2007) we can assume that reduction of $HAuCl_4$ occurs in the presence of both living and lifeless biomass. However, we cannot distinguish contribution of vital and dead diatom cells to the bioreduction process.

The observations from the LM and EM suggest slightly different way of the AuNPs deposition in each diatom strain. DG cells were strongly surrounded by veil of EPS in which the AuNPs were captured and distributed (Fig. 1 c)), whereas in NA cells the AuNPs were attached to thin layer of the EPS in the frustule direct proximity (Fig. 4 c)). Besides, both strains had nanoparticles directly adhered to the surface of the siliceous frustule (Fig. 4 b), 2 c)). Yet, the conclusions on the EPS abundance and extent are uncertain, as we also noticed remarkable disintegration of the living matter due to the unfavorable growth conditions caused by the tetrachloroaureate added into the medium (especially in the DG strain). The cell content discharged off some of the frustules might have mixed with the EPS. On the other hand, observed embedding of the nanoparticles in the organic matter seemed to be very

stable; the very same picture was obtained more than 30 days after the experiment performance.

Significance of the EPS structures for the NPs stabilization was previously discussed in case of cyanobacteria (Brayner et al. 2007). Function of the EPS is basically the same for both cyanobacteria and diatoms. Primarily, they form a mechanical and chemical protective biofilm around the cells; other functions are e.g. formation of interspecies communication network in symbioses etc. (Paerl and Pinckney 1996, Ben-Ari 1999, Christensen 1999, Allison et al. 2000, Flemming et al. 2000, Wimpenny 2000, Flemming and Windenger 2001). EPS are negatively charged and possess metal-binding capabilities (Sutherland 2001a, Sutherland 2001b), so that after intracellular synthesis, gold nanoparticles are released to the culture medium (as observed in cyanobacteria e.g. by Bhattacharia and Gupta (2005), Lengke et al. (2006a)) and can be directly attached to the EPS. Embedding of released nanoparticles into a polysaccharide network of the diatom EPS is clearly visible in both TEM and SEM micrographs attained in this study (Fig. 2, 4). Moreover, our observations indicate a stabilization function of the diatom EPS against NPs aggregation.

It is likely that important role in AuNPs biosynthesis and transport off the cell in diatoms is played by the SDVs, as the formations of corresponding proportions were frequently seen on the TEM micrographs of the experimental samples (see ESM Supporting Fig. 4). However, the mechanism of nanoparticles formation by the living cells (or with their contribution) is still largely unclear, despite increasing number of new studies concerned with this question. According to the accessible sources, reduction in phototrophic organisms occurs through interaction of the metallic salt with cellular organic compounds such as carbohydrates or proteins. Previous studies with heavy metal recovery in brown algae (Mata et al. 2009, Kuyucak and Volesky 1989) indicated that reduction of Au^{3+} to Au^0 occurred through oxidation of hydroxyl groups (abundant in polysaccharides of the algal cell wall) to carbonyl groups. Also algal pigments rich in hydroxyl groups (e.g. fucoxanthins – Kuyucak and Volesky (1989)), or other highly reactive functional groups such as sulfhydryl present in the polysaccharides of the cell wall (responsible for its brown color – fucoidans (Kuyucak and Volesky 1989)), could be involved in

the reduction processes. Greene et al. (1986) determined the importance of these groups in experiments with the green alga *Chlorella vulgaris* (their chemical modification reduced the gold uptake). Last but not least, the role of silaffin polypeptides in the nanoparticles formation should be mentioned. Sillafins are a class of heavily posttranslationally modified proteins responsible for mediating silica deposition at ambient temperature and pressure (Davis et al. 1986, Kroger et al. 1999). Within the native peptides, lysine residues are modified to long-chain polyamines and serine residues phosphorylated Foo et al. 2004, Kroger et al. 2002). Native silaffin polypeptides isolated from a diatom *Cylindrotheca fusiformis* can catalyze the silica precipitation in vitro from a silica precursor under slightly acidic conditions (Kroger et al. 2001)). This process is caused by a self-assembly of the silica due to the silaffins activity resulting into the silica nanoparticle formation (Nam et al. 2009). We expect that the silaffins might play role also in synthesis of other types of nanoparticles such as AuNPs; however additional research beyond the scope of this study would be necessary to verify this hypothesis.

It is well known that gold is an excellent catalyst for many organic oxidation reactions. In fact, current research is focused on the development of gold nanocatalysers for the chemical industry (Hughes et al. 2005). Obtained bionanocomposite appears to be suitable adept for applications in catalysis or further modifications e.g. cell modification by ferrofluids Mosinoewicz-Szablewska et al. 2010).

Conclusions

Biosynthesis of gold nanoparticles has been successfully conducted using two strains of diatoms mixed with aqueous $HAuCl_4$ ($\approx$ 500 mg/L Au) at laboratory conditions. The interaction of diatoms with aqueous salt promoted the precipitation of gold nanoparticles. Shapes and sizes, chemical composition and interaction with siliceous frustules and EPS of the diatoms were described by the methods of light and electron microscopy and X-rays diffraction techniques.

Presented method of tetrachloroaurate reduction by diatoms appears to be worthwhile, effective and low-cost method of binonacomposites preparation. Besides, performance of the described method is

very simple (uses organisms commonly living in streams and ponds worldwide, can be performed at room temperature and in physiologic pH) and environmentally friendly compared to other chemical methods that use toxic reagents. Due to their remarkable properties, we also expect that silica-gold and EPS-gold bionanocomposites have potentially a great value for various applications and should be further studied.

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Electronic Supporting Material Available: TEM micrograph of *Navicula atomus* cells after tetrachloroaureate addition (Supporting Fig. 1); SEM overview micrograph of *Navicula atomus* cells after tetrachloroaureate addition (Supporting Fig. 2); SEM micrograph of *Diadesmis gallica* cells after tetrachloroaureate addition. Association of gold nanoparticles with EPS structures between two DG frustules. (Supporting Fig. 3); TEM micrograph of *Navicula atomus* cells after tetrachloroaureate addition. Detail of silica deposit vesicles (marked with arrow) (Supporting Fig. 4).

Figure captions

Figure 1. Light microscope photographs of the diatom cells before (left) and 12 hours after (right) tetrachloroaureate addition for (a,c) *Diadesmis gallica,* and (b,d) *Navicula atomus.*

Figure 2. TEM micrographs of (a,c) *Diadesmis gallica,* and (b,d) *Navicula atomus* cells after tetrachloroaureate addition. Gold nanoparticles captured in the EPS net of the intercellular space of DG (a). Detail of association of gold nanoparticles with the frustule surface in the raphe region of NA (c). Detail micrographs of AuNPs and SAED patterns for DG (b), and NA strains (d).

Figure 3. Histogram of size distribution of gold nanoparticles synthesized by (a) *Diadesmis gallica,* and

(b) *Navicula atomus* after tetrachloroaureate addition.

Figure 4. SEM micrographs of (a,c) *Diadesmis gallica,* and (b,d) *Navicula atomus* cells after tetrachloroaureate addition. Detail of gold nanoparticles deposition on the silica frustule surface of the DG cells (b). Association of gold nanoparticles with the EPS structures of the NA cell (c). Detail micrograph of gold nanoparticles embedded into the EPS chain of the NA cell (d).

Figure 5. Theta-2theta diffraction pattern of gold nanoparticles synthesized by *Diadesmis gallica* after tetrachloroaureate addition.

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