Production of gold nanoparticles by electrode-respiring *Geobacter sulfurreducens* biofilms

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Abstract

The goal of this work was to synthesize gold nanoparticles (AuNPs) using electrode-respiring *Geobacter sulfurreducens* biofilms. We found that AuNPs are generated in the extracellular matrix of *Geobacter* biofilms and have an average particle size of 20 nm. The formation of AuNPs was verified using TEM, FTIR and EDX. We also found that the extracellular substances extracted from electrode-respiring *G. sulfurreducens* biofilms reduce Au^{3+} to AuNPs. From FTIR spectra, it appears that reduced sugars were involved in the bioreduction and synthesis of AuNPs and that amine groups acted as the major biomolecules involved in binding.

Keywords: Gold nanoparticles, *Geobacter sulfurreducens*, Extracellular substances, Biofilms.

INTRODUCTION

The growing demand for and effectiveness of metal nanoparticle (NP) applications in a wide range of fields—drug delivery, electronic devices, subversive environments, etc.—have brought an increased scrutiny to the various methods of NP synthesis. A number of chemical and biological approaches are available. Chemical methods include citrate reduction [1], ligand-stabilized direct synthesis [2], and electrochemical methods [3]. All of these chemical methods are energy-intensive and generate toxic waste. In contrast to the chemical methods, biological methods with metal-reducing microorganisms use little energy because they operate at lower temperatures and generate no chemical toxic waste, which represents an environment-friendly way to produce metal NP [4, 5].

Dissimilatory metal-reducing microorganisms, such as *Shewanella oneidensis* MR-1 and *Geobacter metallireducens*, possess the unique ability to transfer electrons extracellularly [6, 7]. These microorganisms use *c*-type cytochrome-based pathways to transfer electrons from the microbial cytoplasmic membrane, through the entire cell envelope to the microbial cell surface, where they directly reduce metal ions [8, 9]. For this reason, *Shewanella* spp. and *Geobacter* spp. can produce nanoparticles such as Fe(II), palladium [Pd(0)] or selenium [Se(0)] on cell surfaces through the extracellular reduction of Fe(III), Pd(II) or Se(IV/VI), respectively [10-12]. The biogenic metal NPs can be used for the remediation of contaminants, catalysis, data storage, drug delivery and cancer treatment [13-15]. The extracellular formation of metal NPs also makes separation easier because the step of rupturing the cells [11] can be skipped. Furthermore, *Geobacter*, when grown on a surface as biofilms with promising catalytic activity and controlled electrochemical reactions, can be advantageous for rapid NP synthesis [16]. When *G. sulfurreducens* is grown on electrodes, it reduces cytochromes in the extracellular matrix, which transfers electrons from cells to the solid electron acceptor as the respiration process [17-19]. In some cases the entire biofilm matrix can become reduced [20]. A reduced biofilm matrix on an electrode, with its high availability of electrons, can be used to reduce gold ions (Au^{3+}) to gold nanoparticles (AuNPs). Further, the biomolecules in this matrix can effectively bind metal ions and serve as sites for the nucleation and stabilization of AuNPs [21, 22]. Although AuNP formation on the surface of cells and in EPS through Au^{3+} reduction by *G. sulfurreducens* cultures is suggested in the literature [11], it has not previously been investigated.

The goal of this work is to investigate the reduction of Au^{3+} and formation of AuNPs by electrode-respiring *Geobacter sulfurreducens* biofilms and the involvement of extracellular polymeric substances (EPS) in the reduction and stabilization of AuNPs. *G. sulfurreducens* biofilms were grown on the working electrode in a three-electrode system. Au^{3+} was introduced into *G. sulfurreducens* biofilm. The generation of AuNPs was verified and quantified using HRTEM, FTIR and EDX. EDX. HRTEM was also used to measure the size of the NPs. Since most of the AuNPs were found to be generated in the extracellular matrix, we extracted EPS from the *G. sulfurreducens* biofilms and tested whether EPS can directly reduce Au^{3+} to AuNPs.

MATERIALS AND METHODS

Media and growth conditions. The growth medium for *G. sulfurreducens* (ATCC 51573) biofilms contained: 0.38 gL^{-1} KCl, 0.2 gL^{-1} NH_4Cl , 0.069 gL^{-1} $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.04 gL^{-1}

CaCl₂·H₂O, 0.2 gL⁻¹ MgSO₄·7H₂O, 2 gL⁻¹ NaHCO₃, 10 mL Wolfe's vitamin solution and 10 mL modified Wolfe's minerals. Acetate (20 mM) was provided as the electron donor. For inoculum preparation in vials, fumarate (40 mM) was added as electron acceptor. No fumarate or other soluble electron acceptor was added to the medium for biofilm growth. The pH of the medium was maintained at 6.8. The medium was then sterilized at 121°C for 20 minutes and cooled to room temperature. The medium was sparged with a gas mixture of N₂/CO₂ (80%/20%) for 24 h to remove oxygen and used for *G. sulfurreducens* biofilm growth in a bioelectrochemical system following our published procedure [20].

***G. sulfurreducens* biofilm growth on electrodes.** We used a three-electrode bioelectrochemical system. The *G. sulfurreducens* biofilms were grown on a graphite anode as the electron acceptor [20] in fumarate-free growth medium. We used a graphite cathode and a standard Ag/AgCl reference electrode [23]. In order to keep the reactor environment anaerobic, it was bubbled continuously with a N₂/CO₂ (80%/20%) mixture. The experiments were carried out under potentiostatic control by polarizing the biofilm electrode at +300 mV_{Ag/AgCl} using a potentiostat built in-house [24]. Before the system was inoculated, cyclic voltammetry was applied to check the operability of the system and test background current. A Gamry Reference 600™ potentiostat (Gamry Instruments, Warminster, PA) was used for cyclic voltammetry. The biofilm reactor was inoculated with *G. sulfurreducens* inoculum (10% v/v). The current began to increase within 24 h, and the biofilm was allowed to grow continuously. All experiments were conducted at 30 °C and pH 6.8.

Synthesis of AuNPs by *G. sulfurreducens* biofilms

Within 8 days, the biofilm was fully developed on the electrode and reached a maximum steady current of ~3 mA. At this stage, HAuCl₄ solution was added in order to initiate the synthesis of AuNPs by the biofilm. One ml of a HAuCl₄·3H₂O stock solution (50 mM) was added to the bioelectrochemical system for 1 h to reach a final concentration of 250 μM Au³⁺ inside the reactor. During this time, Au³⁺ was reduced and the color changed to the typical purple that is associated with the formation of AuNPs [25, 26]. Whereas, in the absence of *G. sulfurreducens* biofilms, no such change in color was observed indicating no applicable generation of AuNP.

Spectroscopic and microscopic analysis

Eight hours after the AuNPs were synthesized, a small aliquot of the suspension was collected to measure the UV-VIS absorbance spectrum using a Hewlett-Packard Model 8453 UV-VIS spectrophotometer to characterize the surface plasmon resonance (SPR) of the gold. A second spectrum was collected eight hours later. A FEI Technai G2 20 Twin model transmission electron microscope (TEM) equipped with a LaB6 electron source (FEI, USA), operated at 200 kV, was used to investigate the morphological structure of the NPs in the biofilm. Samples for TEM measurements were prepared by drop casting on a formvar-lined TEM grid. The microtomy was done on plastic-embedded samples. Biofilm from the electrode with HAuCl₄ solution was collected by carefully scraping the biomass into the centrifuge tube in the anaerobic chamber. Cells were fixed overnight at 4 °C in 2.5% glutaraldehyde/2% paraformaldehyde. Samples were fixed with 1% osmium tetroxide overnight at 4 °C. Samples were then dehydrated in an ascending series of ethanol concentrations (10 min each) until three rinses of 100% ethanol and one rinse of propylene oxide were performed. A sample infiltration in SPURRS resin (EMS) was cured overnight at 60 °C. The blocks were sectioned to ~70 nm with a Leica Reichert

Ultracut R ultramicrotome. The thin sections were mounted on a formvar-lined TEM grid and viewed under TEM. These TEM images were analyzed using public domain software, imageJ 1.49v (NIH; <http://rsb.info.nih.gov/ij>). The elemental analysis of the ultrathin cross section NP sample was performed using energy-dispersive X-ray spectroscopy (Oxford Instruments) with INCA analytical software.

Synthesis of AuNPs using extracellular polymeric substances extracted from *G. sulfurreducens* biofilms

EPS extraction and characterization. Samples of *G. sulfurreducens* biofilms, including cells and EPS, were harvested from multiple graphite rod anodes inside an anaerobic chamber. The harvested cells from each electrode were resuspended in 1 ml of sterile, deoxygenated 0.9% NaCl in 1.5-ml micro-centrifuge tubes. EPS were extracted from the suspensions, following protocols published in the literature [27, 28], with slight modification. Each biofilm suspension was centrifuged ($4,180 \times g$) for 10 min to form a pellet. Each centrifuged pellets was resuspended in 1 ml of 0.9% NaCl and centrifuged at $5,000 \times g$ for 6 min, and the resulting cell pellets were collected for bound EPS extraction. For simplicity we use “EPS” to refer to bound EPS in this manuscript. The cell pellets were resuspended in 0.9% NaCl, and the suspension was incubated at 80 °C for 10 min followed by centrifuging for 15 min at $15,000 \times g$ and 4 °C. The supernatant was filtered twice through a 0.45- μ m filter to remove bacteria and retained as EPS. EPS were stored at -20 °C for later chemical analyses.

Reduction of Au^{3+} and formation of AuNPs in EPS. Bound EPS are tightly associated with the cell surface [27] and used for Au^{3+} reduction because of their high numbers of total potential binding sites, high protein content and evidence of significant contribution to both metal reduction and sorption [29]. Aqueous EPS (500 μ l) were kept in an anaerobic chamber ($\text{N}_2:\text{CO}_2:\text{H}_2$ (75%:20%:5%)) overnight to make the EPS completely anaerobic. $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ solution was added to the EPS solution to achieve a final concentration of 250 μM Au^{3+} and left in the anaerobic chamber at 30 °C for 24 h. The AuNP formation in EPS solution was identified visually from its light purple color. The formation of NPs was characterized using transmission electron microscopy (TEM) and Fourier transform infrared (FTIR) spectroscopy analysis.

Transmission electron microscopy and Fourier transform infrared spectroscopy of EPS.

Samples for TEM analysis were prepared on the formvar-coated grids. Five- μ L samples of EPS only and the solution of EPS with NPs were carefully placed on the grids and air-dried prior to imaging. The grids were viewed on the FEI Tecnai TEM (FEI, USA) at 200 KV. FTIR spectra were collected on a ThermoFisher Nicolet iS10. EPS alone and EPS with NPs were freeze-dried. Each freeze-dried sample was separately combined with KBr (mass ratio of 1:500), and pellets were prepared using a hand press. Absorbance spectra at a resolution of 8 cm^{-1} with 128 scans were collected and analyzed with OMNIC spectra software.

RESULTS AND DISCUSSION

Biofilm growth on electrodes and Au^{3+} reduction. Figure 1 shows a typical current response from electrode-respiring *G. sulfurreducens* biofilm. Cells attached to the electrode surface and current started increasing as inoculum was added to the reactor. This response consists of a lag

phase, an exponential phase and a stationary phase, similar to previous observations [30]. Figure 1 shows that the current response fluctuated with Au^{3+} addition over 1 h and then steadily decreased. However, within ~5 h, the current production recovered, suggesting that the reduction of Au^{3+} to AuNP did not inhibit the anodic current.

Figure 1. Typical electrode-respiring *G. sulfurreducens* biofilm growth on the graphite electrode. The inset shows the decrease in current for 1 h after $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ solution was added to the reactor. The dashed arrow indicates the inset, which is a magnification of the area inside the rectangle, and the solid arrow inside the inset shows the current after the addition of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ to the reactor.

UV-VIS spectroscopy confirmed the uniformity of the synthesized AuNPs. Figure 2 shows typical UV-VIS spectra for the suspension at 8 and 16 h after the addition of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$. The surface plasmon resonance band (SPRB) is a quick indicator of the presence of small AuNPs whose redshifts indicate larger NPs, and broadening typically indicates polydispersity in NPs [31-34]. After 8 h of incubation, the SPRB absorption peak was observed at ~550 nm, which indicates the formation of AuNPs (Figure 2). The presence of this absorption peak indicates the presence of relatively small nanoparticles [34, 35]. The appearance of a single but comparatively strong band absorption peak also is an indication that these nanoparticles are uniform in shape (isotropic) but not uniform in size [25]. The absence of the SPRB after 16 h of incubation suggests uncontrolled growth of the existing AuNPs into bulk particulates. These phenomena lead to the aggregation of AuNPs after 16 h of incubation.

Figure 2. UV-VIS absorption spectra for AuNPs in *G. sulfurreducens* suspensions after a) 8 h of incubation and b) 16 h of incubation. The surface plasmon resonance band absorption peak was observed only for the 8 h incubation, indicating an isotropic shape and uniformly sized particles in this condition.

Transmission electron microscopy showed extracellular synthesis of AuNPs. TEM revealed the synthesis to be mostly extracellular, spherical in shape, occurring just outside the bacterial cells (Figure 3A and B), although Figure 3C suggests the existence of some intracellular particles that are more monodispersed than those outside the cell walls. A similar observation was reported in the literature and attributed to the penetration of smaller particles or clusters through the cell wall without disturbing its integrity, due to nonspecific interactions between positively charged extracellular AuNPs [36] and negatively charged biomolecules inside the cell [37, 38]. The monodispersity or aggregation behavior of synthesized NPs may differ from one bacterium to another for the same type of NPs. HRTEM showed the crystalline nature of the nanoparticles (Figure 3D). Particle diameter distribution calculated from TEM images shown in Figure 3E indicates particle sizes were varied between 10 and 90 nm with an average particle diameter of 20 nm. Forty percent of total synthesized particles had size smaller than 15 nm. The difference between the largest and the smallest size of the nanoparticles was 75 nm which indicates the narrow distribution [39].

Figure 3. TEM images of extracellular synthesis of AuNPs in *G. sulfurreducens*. (A, B, C) In accordance with the UV-VIS data, TEM also confirms the formation of nanoclusters along the

outer membrane as a result of aggregation. (D) The individual newly formed AuNPs show a nanocrystalline character. (E) Particle diameter distribution calculated from TEM images for AuNP formed in biofilms and on *G. sulfurreducens* cell surface.

Energy-dispersive X-ray spectroscopy. EDX provided elemental information about the particles formed, as each element has a characteristic spectrometry at its atomic level. EDX data recorded from an ultrathin section of the *G. sulfurreducens* biofilm sample showed the peaks corresponding to Au atoms. The Ni peak corresponds to the Ni TEM grid that was used for the thin section support.

Figure 4. EDX spectrum of the biofilm sample (area highlighted in Figure 3, panel B), confirming Au signal.

Formation of AuNPs through Au^{3+} reduction by EPS. Figure 5 shows TEM images of *G. sulfurreducens* EPS exposed to Au^{3+} (250 μM) for 24 h. These images show that spherically shaped AuNPs were also directly produced from Au^{3+} in aqueous EPS. These AuNPs have an identical pattern to that of metallic gold crystal [40]. From the analysis with imageJ software, the average diameter of these AuNPs was found to be 26 nm. A visual and quantitative comparison of Figure 5A and Figure 3C revealed that the average size of an AuNP directly formed in EPS was larger (by 6 nm) than that of one produced on the cell surface through extracellular synthesis by the biofilm. The AuNPs were observed to be more dispersed in the EPS than on the cell surface of biofilm cells. Particle diameter distribution given in Figure 5C shows a particle size distribution range between 15 and 45 nm. The difference between the largest and the smallest nanoparticles was 30 nm. Twenty nine percent of these particles had an average size of 17 nm. Extracellularly synthesized AuNPs by the biofilm had relatively smaller size than that synthesized directly by EPS. Considering smaller sizes of AuNPs generated by biofilms or EPS it is possible to use them for drug delivery or as catalysts.

Figure 5. (A) TEM images of extracellular polymeric substances (EPS) extracted from *G. sulfurreducens* biofilms with newly formed AuNPs. (B) The particles alone. (C) Particle diameter distribution calculated from TEM images for AuNP formed in the EPS extracted from *G. sulfurreducens* biofilms.

Fourier transform infrared spectroscopy identified EPS involved in AuNP binding. There is limited information on the involvement of functional groups and biomolecules in Au^{3+} reduction by *G. sulfurreducens* biofilm EPS. In this study, when comparing the FTIR spectra of EPS before and after they reacted with Au^{3+} (Figure 6), we observed the possible involvement of reducing sugar components in EPS in Au^{3+} reduction. Functional groups arising from proteins ($1650\text{--}1500\text{ cm}^{-1}$), polysaccharides and nucleic acids ($1200\text{--}800\text{ cm}^{-1}$) were observed for the EPS (Figure 6) [27, 41]. The band at 1650 cm^{-1} is attributed to C=O stretching, whereas the band at 1550 cm^{-1} is attributed to N-H bending and C-N stretching, thus confirming the presence of amines; this is similar to observations by others [4, 35, 42]. Thus, Figure 6 shows the involvement of amine groups in Au^{3+} reduction and the binding of AuNPs [35, 43, 44].

Figure 6. Comparison of FTIR spectra of EPS before and after they reacted with Au^{3+} . The arrows indicate shifts in peaks due to C=O stretching (1650 cm^{-1}) and N-H bending with C-N stretching (1550 cm^{-1}).

Proposed mechanism

Two types of mechanisms are possible for NP synthesis by biofilms: intracellular and extracellular metal ion reduction. *G. sulfurreducens* is a metal-reducing bacterium which can use metal ions outside their cell walls as a terminal electron acceptor for respiration [18, 29]. When Au^{3+} comes near the cell walls of *G. sulfurreducens*, c-type cytochromes supply electrons to the ions all the way from the periplasm to the outer membrane, where the electron exchange occurs. Outer membrane cytochromes OmcB and OmcZ are reported to transfer electrons to Au^{3+} [45]. After the reduction, the reduced atoms start to nucleate and grow into AuNPs. Heterogeneous nucleation on the surface of the membrane would provide a lower activation energy barrier to overcome than reduction in the bulk solution [46]. The above observation on AuNPs formation by electrode-respiring *G. sulfurreducens* biofilms fits all these hypothesized mechanisms.

On the other hand, as Figure 3C shows, most of the AuNPs remain outside the cell envelope, which suggests the EPS as a potential reducing agent. *G. sulfurreducens* biofilm matrix has been reported to have an open circuit potential of $-400\text{ mV}_{\text{Ag}/\text{AgCl}}$ when grown on an anode [20], whereas the reduction of Au^{3+} to Au^0 has a standard reduction potential of $+1.20\text{ V}_{\text{Ag}/\text{AgCl}}$ [47]. Therefore, EPS biomolecules with a reduction potential of less than $+1.20\text{ V}_{\text{Ag}/\text{AgCl}}$ can reduce Au^{3+} under standard conditions. EPS consist of proteins, polysaccharides and other constituents that control the redox processes and pathways [4, 48, 49]. The presence of considerable reducing constituents that cause the formation of AgNPs has been identified in the EPS of *E. coli* [4]. Here, we observed that similar behavior is achievable for EPS from electrode-respiring *G. sulfurreducens* biofilms.

A few NPs also formed inside the cell envelope (Figure 3, A-C). Whether Au³⁺ slip through the membrane and are directly reduced intracellularly or externally formed Au clusters actually penetrate the membrane is not clear. However, it is proposed in the literature that NPs stabilized by amphiphilic ligands are able to slip through the periplasm without any disruption [38, 50, 51]. In the thermodynamic point of view, binding interactions such as hydrophobic and coulombic forces must balance out the resistive forces (for example, the elasticity of the cell membrane) in order for NPs to penetrate the cell membrane [38].

As for the stabilization process, FTIR spectra analysis suggests the involvement of amine groups in the EPS in binding AuNPs. Some researchers have studied the interactions between biomolecules and NPs in detail. Their results suggest the possible binding agents: proteins and other polymeric substances in the EPS [4, 38]. Proteins and polysaccharides can form electrostatic bonds with charged particles [48]. Proteins are reported to be electrostatically stronger than polysaccharides for binding particles [37]. Electrostatic and steric forces can act upon particles with a synergistic effect, which might cause the NPs to be stabilized. Also, as our UV-VIS results suggest, the electrostatic forces may be less dominant, causing the particles to be aggregated within 16 h. Further studies are required to identify the exact mechanisms, compounds involved in NP formation and stabilization mechanisms.

CONCLUSIONS

This paper reports AuNP synthesis by an electrode-respiring *G. sulfurreducens* biofilm in a three-electrode bioelectrochemical reactor. The extracellular synthesis of AuNPs was quantified using TEM, EDX and UV-VIS, which indicated possible aggregation of the particles within 8 h of their formation. We also found that the extracellular substances extracted from electrode-respiring *G. sulfurreducens* biofilms reduced Au³⁺ to AuNPs. From FTIR spectra, it appears that reduced sugars were involved in the bioreduction and synthesis of AuNPs and that amine groups acted as the major biomolecules involved in binding.

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