

Silver Nanoparticles

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Formation of Silver Nanoparticles by Electron Transfer in Peptides and c-Cytochromes

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Abstract: The reduction of Ag^+ ions to Ag^0 atoms is a highly endergonic reaction step, only the aggregation to Ag_n clusters leads to an exergonic process. These elementary chemical reactions play a decisive role if Ag nanoparticles (AgNPs) are generated by electron transfer (ET) reactions to Ag^+ ions. We studied the formation of AgNPs in peptides by photoinduced ET, and in c-cytochromes by ET from their Fe^{2+} /hemes. Our earlier photoinduced experiments in peptides had demonstrated that histidine prevents AgNP formation. We have now observed that AgNPs can be easily synthesized with less-efficient Ag^+ -binding amino acids, and the rate increases in the order lysine < asparagine < aspartate < serine. The ability of Fe^{2+} /hemes of c-cytochromes to reduce Ag^+ to AgNPs was studied in an enzymatic experiment and with living bacteria *Geobacter sulfurreducens* (Gs).

Microbial respiration processes in bacteria are gaining important relevance for the synthesis of metal nanoparticles from metal salts.^[1] Electrons, needed for the metal-ion reduction, are generated by respiration processes in the inner cell membrane, and c-type cytochromes (Mac, Ppc, and Omc) have been proposed to transport these electrons by their Fe^{2+} /heme cofactors to the outer cell membrane, where subsequent electron transfer (ET) from the Fe^{2+} /hemes through the peptide of the c-cytochromes reduces the attached metal ions to metal nanoparticles (Figure 1).^[2,3] This mechanism might also be an efficient way to protect the bacteria from toxic heavy-metal ions. For example, Lloyd et al. have demonstrated that *Geobacter sulfurreducens* (Gs) can even withstand generally toxic concentrations of Ag^+ ions by reducing them to Ag nanoparticles (AgNPs).^[4] Herein we present experiments at the molecular level that shine light on our observations of the bacterial biomineralization processes of silver with Gs.

Recently, some of us have observed that the formation of AgNPs by (photoinduced) ET through peptides is not

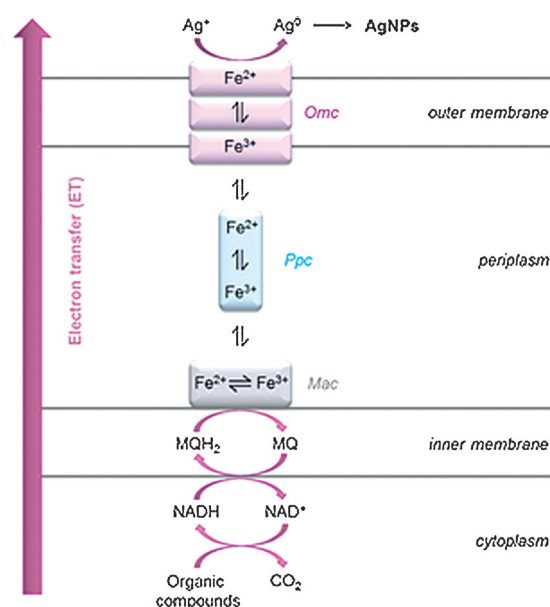
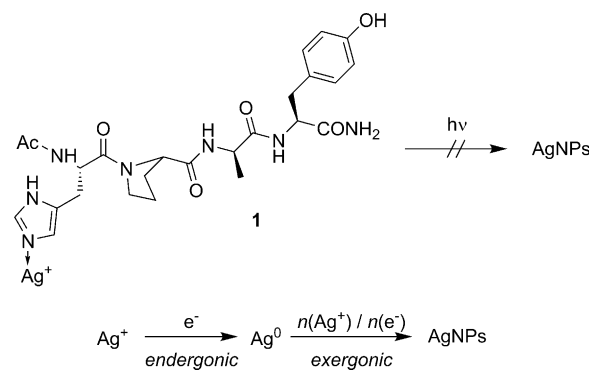


Figure 1. Schematic representation of the electron transfer from NADH to extracellular metal ions (Ag^+ ions as an example) in *Geobacter sulfurreducens*.

a simple process, as ET through Ag^+ /peptide **1** did not yield AgNPs (Scheme 1).^[5] Although irradiation of the C-terminal tyrosine of tetrapeptide **1** generated tyrosyl radicals and electrons, a reduction of Ag^+ ions, bound to the N-terminal histidine, did not occur. The reason for this is the endergonic first reduction step of Ag^+ ions to Ag^0 atoms ($E^0 = -1.8$ V), hence only the aggregation to Ag_n clusters ($n > 10$) leads to stable AgNPs ($E^0 = 0.8$ V).^[6] We have now studied the



Scheme 1. Inhibited formation of Ag nanoparticles (AgNPs) by photo-induced electron transfer (ET) in Ag^+ /peptide **1**.

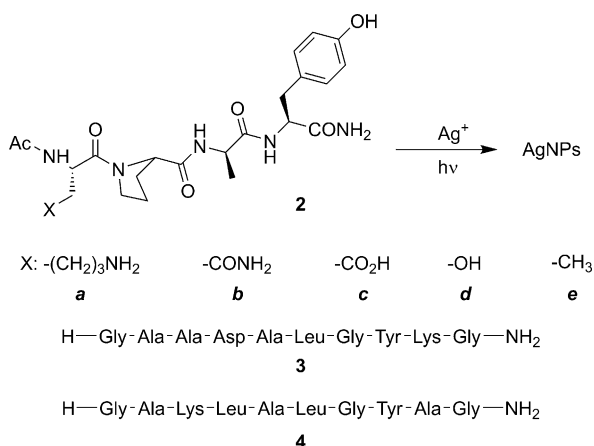
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synthesis of AgNPs by photoinduced ET to Ag^+ bound peptides, as well as ET to Ag^+ in enzymatic reductions in vitro and compare these to microbial reductions by Fe^{2+} /hemes in living Gs.

In a previous experiment, we assumed that the failure of AgNP formation by photoinduced ET in Ag^+ /peptide **1** was caused by the high Ag^+ binding energy of the imidazole side chain of histidine preventing the aggregation of Ag^+ ions with Ag atoms (Scheme 1).^[5] As a consequence of this hypothesis, amino acids with weaker Ag^+ -binding side chains, such as aliphatic amines, amides, acids, alcohols, or alkyl groups should allow AgNP formation by photoinduced ET. Therefore, we exchanged the N-terminal histidine of tetrapeptide **1** by lysine, asparagine, aspartate, serine, as well as alanine, to yield the tetrapeptides **2a–e**, respectively. After addition of AgNO_3 and generation of electrons by irradiation of the C-terminal tyrosine, AgNPs were generated in all cases (Scheme 2), however with different speed (Figure S1 in the Supporting Information).^[7]



Scheme 2. AgNP formation by photoinduced ET with Ag^+ /peptides **2–4**.

As an example, Figure 2 illustrates for **2c** the time dependence of the AgNP formation determined by UV/Vis spectroscopy (plasmon resonance), the transmission electron micrographs (TEMs), and the size distribution of the AgNPs. Under identical conditions, the formation rate for the AgNPs increases in the order **2a** < **2b** < **2c** < **2d** < **2e**. This rate acceleration of the AgNP formation from lysine via asparagine and aspartate to serine and alanine corresponds to the decrease of the calculated binding energies of Ag^+ ions with these amino acids.^[8] This is in accord with our assumption that the strong Ag^+ binding energy of histidine prevents the aggregation to AgNPs in peptide **1**, while weaker binding amino acids facilitate the aggregation of silver atoms to AgNPs. Hence, with less strongly binding side chains the synthesis of AgNPs from Ag^+ /peptides by ET is not inhibited.

This finding was confirmed with even larger, histidine-free decapeptides such as **3** and **4** which also generated AgNPs by photoinduced ET. In contrast to the tetrapeptides **2**, these AgNPs underwent rapid Ostwald aggregation, as shown by the shift of the plasmon resonances to longer wavelengths

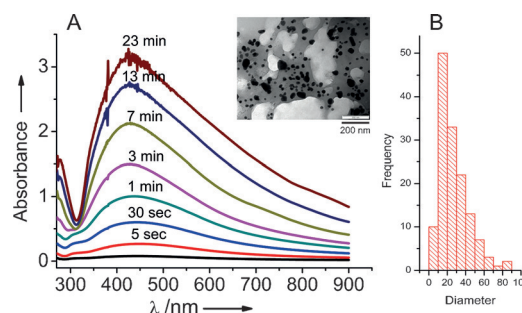


Figure 2. Formation of AgNPs by photoinduced ET in Ag^+ /peptide **2c**. The increase of the AgNP formation with time is measured by the UV/Vis spectra of the plasmon resonances (A). The sizes of the AgNPs are shown by the TEMs of a representative probe (B).

during their formation (Figure 3). Clearly, the peptide coronas of **3** and **4** stimulate the Ostwald ripening of the AgNPs.^[9]

Recently, we had also shown that peptide-bound AgCl crystals can be easily reduced to AgNPs even in the presence of the strongly Ag^+ -binding histidine (of peptide **1**).^[5] We explained this by the short distances between Ag^+ ions in the AgCl crystals so that the aggregation to Ag_n clusters is not hindered by histidine. Interestingly, this reaction occurred in two steps. In a first fast reaction, large Ag@AgCl composites were formed, which were then slowly cleaved reductively by ET to small AgNPs (Figure 4A). Both reaction steps were first order demonstrating that the processes occurred intramolecularly in the AgCl crystals as well as in the Ag@AgCl composites. We have now carried out experiments with AgCl complexes of peptides **2a–e**.^[10] Again, in all cases AgNPs were formed by photoinduced ET (Figure 4B). The AgNP formation was always of first order, and occurred 5–10 times faster than in the Cl^- free solutions using Ag^+ complexes of

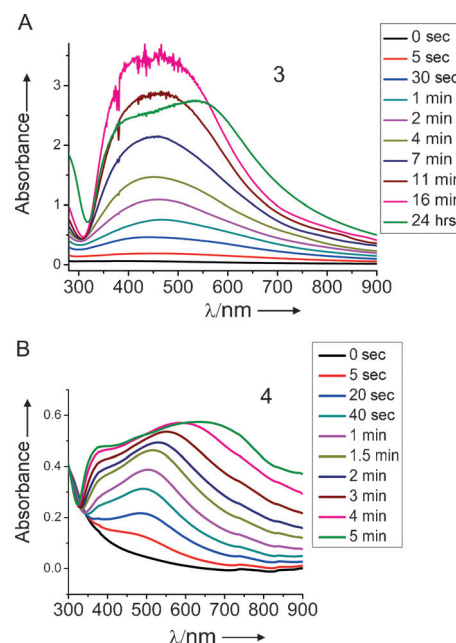


Figure 3. Formation of AgNPs by photoinduced ET with Ag^+ /peptides **3** and **4**. The shift of the UV/Vis spectra demonstrates the Ostwald ripening of these AgNPs during or after formation.

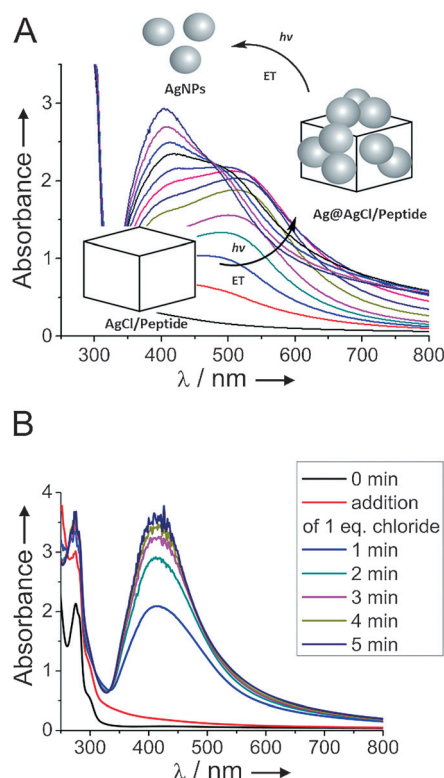


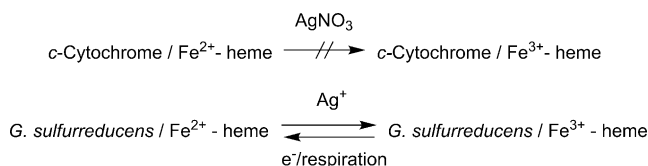
Figure 4. Formation of AgNPs by photoinduced ET in AgCl/peptides using peptides **1** (A) or **2c** (B). (A) shows the intermediate formation of Ag@AgCl composites (see Ref. [5]). Such composites are absent in (B).

2a–e described above (Figure S2). Interestingly, the formation of Ag@AgCl composites could not be observed.^[11] This can also be explained by the different silver-binding ability of the amino acid side chains. The strong Ag⁺-binding amino acid histidine slows down the burst of the Ag@AgCl composites to small AgNPs, which increases the lifetime of the Ag@AgCl composites. With weaker Ag⁺ binding amino acids the Ag@AgCl composites are too short-lived to be detected during the ET reaction.^[11] Thus, we could observe and characterize the intermediate Ag@AgCl composites only because we had used histidine as Ag⁺ binding amino acid in our earlier experiments.^[5]

In our experiments with peptides **1–4** the ET occurs by light-generated electrons. On the other hand, bacteria use Fe²⁺/heme cofactors of c-cytochromes as sources for electrons that reduce Ag⁺ ions bound at the peptide surface of the enzyme. This ET through the peptides depends upon the potential differences of the redox couples. As the reduction of Ag⁺ ions to Ag atoms is strongly endergonic ($E^0 = -1.8$ V), a reduction of Ag⁺ to Ag atoms by the Fe²⁺ ions of an isolated monoheme cytochrome ($E^0 = 0.26$ V) should not occur.^[12] We verified this by adding a tenfold excess of AgNO₃ to the monoheme horse heart c-cytochrome and confirmed that under anaerobic conditions the Fe²⁺/heme cofactor was not oxidized to the Fe³⁺/heme within two hours (Figure S3).^[13]

The exergonic AgNP formation did not occur with the monoheme c-cytochrome, as the Fe²⁺ ions, which are needed for the reduction of further Ag⁺ ions to AgNPs, were not

regenerated in the enzymatic experiment *in vitro*. We then compared these results at the molecular scale with what we observed with living bacteria. Indeed, the situation changes in going to living bacteria, such as *Geobacter sulfurreducens* (Gs) where multiheme cytochromes occur, providing a reservoir of electrons, and where the respiration processes in the inner cell membrane produces a flux of electrons, which can regenerate Fe²⁺/hemes quickly after their oxidation by Ag⁺ to Fe³⁺/hemes (Scheme 3 and Figure 1). To verify this mecha-



Scheme 3. Reaction of the enzyme c-cytochrome or the living *Geobacter sulfurreducens* with Ag⁺ ions.

nism, we carried out experiments with 0.7 pM Gs in 2.5 mL aqueous solutions. The bacteria were initially in the resting state and contained their cytochrome cofactors as Fe²⁺/hemes (as verified by UV/Vis).^[14] Under strictly anaerobic conditions 0.075 mM AgNO₃ was added and the oxidation states of the iron/hemes were analyzed by their Soret and Q bands.^[15] Figure 5 A,B show that AgNO₃ addition oxidized Fe²⁺/hemes to Fe³⁺/hemes. The peak at 419 nm (Soret band for Fe²⁺/hemes) showed that the experiments started with Gs, carrying the cofactors of their c-cytochromes as Fe²⁺/hemes. Under strictly anaerobic conditions a shoulder at 409 nm appeared

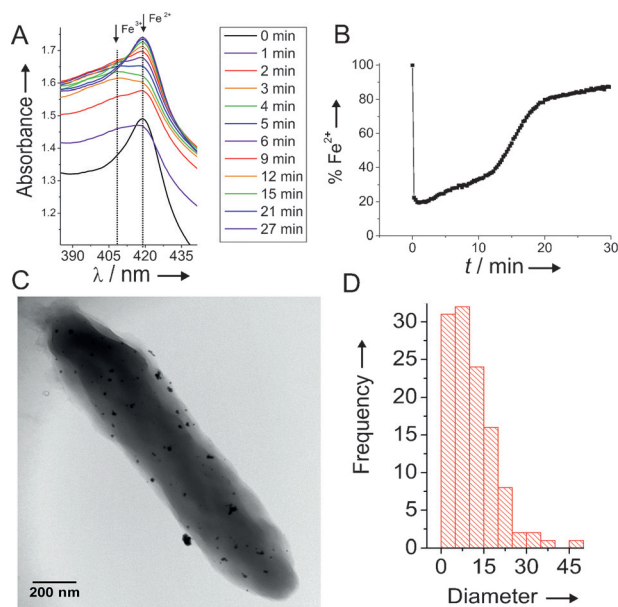


Figure 5. Formation of AgNPs through extracellular electron transfer by Gs. A) UV/Vis spectra of the Soret band obtained before (0 min) and after contact with 75 μM AgNO₃ at different times. B) Q band analysis of the percentage of Fe²⁺/heme as function of time using the absorbance at 554 nm. C) Representative TEM image of a Gs cell decorated with AgNPs.^[16] D) Size distribution of the AgNPs formed in (C).

immediately after AgNO_3 addition (Figure 5). This corresponds to the absorption of the Soret band for Fe^{3+} /hemes, demonstrating the oxidation of Fe^{2+} to Fe^{3+} /hemes. A maximum value of 80 % Fe^{3+} /hemes: 20 % Fe^{2+} /hemes was reached after about 2 min. At this point, the oxidation of Fe^{2+} /hemes by Ag^+ ions at the outer cell membrane, and the reduction of the Fe^{3+} /hemes by the electron flow from the respiration at the inner cell membrane occurred at the same rate. From here on the reduction to Fe^{2+} /hemes dominated. Figure 5B shows the time dependence of Fe^{2+} /heme concentrations using the absorption change of their Q band at 553 nm (Figure S5).^[15] After a fast decrease of the Fe^{2+} oxidation state (2 min) the increase to the Fe^{2+} /hemes occurred in a reaction sequence that reminds of a redox titration curve with its maximum change at around the 1:1 $\text{Fe}^{3+}/\text{Fe}^{2+}$ ratio. The electrons needed for this reduction are delivered by the respiration process at the inner cell membrane (Figure 1).

The microbial formation of AgNPs, which causes the increase of the intensity of the UV/Vis spectra in Figure 5A, is demonstrated in Figure 5C. The medium average size of the AgNPs is about 5–10 nm, that is, slightly smaller than those with the model peptides (Figure 2).

Conclusion: The reduction of Ag^+ ions to Ag^0 by ET is an endergonic reaction. Thus, a critical requirement for successful synthesis of AgNPs is the rapid, exergonic aggregation to Ag_n clusters. In ET experiments with Ag^+ /peptides this is possible using amino acids, which are weaker Ag^+ binders than histidine (Figure 2). Another condition favoring AgNP formation is a steady electron flux, which can be achieved by *Geobacter sulfurreducens*. Respiration at the inner cell membrane generates electrons that are transported by Fe^{2+} /hemes of c-cytochromes (Ppc) through the periplasm and reduces Fe^{3+} /hemes of the outer cell-membrane cytochromes (Omc). Subsequent ET through the protein shell of Omc to the attached Ag^+ ions or Ag_n^+ clusters then produces AgNPs oxidizing the Omc cofactors to Fe^{3+} /hemes (Figure 1).^[17] Ongoing respiration regenerates Fe^{2+} /hemes (Figure 5B), thus c-cytochromes of *Geobacter sulfurreducens* act as biocatalysts.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: electron transfer · *geobacter sulfurreducens* · mineral respiration · silver nanoparticles · silver peptides

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- [8] J. Jover, R. Bosque, J. Sales, *Dalton Trans.* **2008**, 6441. In peptide **2e** the N-terminal amino acid is alanine, whose methyl group does not bind Ag^+ ions. Thus, **2e** can be used as a standard. The exchange of alanine by aspartate in peptide **2c** leads to a 6-fold decrease of the AgNP formation rate (Figure S1), demonstrating the binding ability of the carboxylic side chain, see also K. M. Fromm, *Chimia* **2013**, *67*, 851. In going to **2b** and **2a** the rate further decreases as shown in Figure S1.
- [9] R. Anderson, R. Buscall, R. Eldridge, P. Mulvaney, P. J. Scales, *Colloids Surf. A* **2014**, *459*, 58.
- [10] The experiments were carried out as described in Ref. [5]. A pH of 8.5 was used.
- [11] Only with lysine as an amino acid in **2a**, a broad or two overlapping peaks could be observed in the beginning of the reaction (Figure S2). As it is typical for $\text{Ag}@\text{AgCl}$ composites, the long-wavelength peak disappears during the reaction; see Ref. [5].
- [12] R. Margalit, A. Schejter, *Eur. J. Biochem.* **1973**, *32*, 492.
- [13] The Fe^{3+} /hemes of 10 μM c-cytochrome were reduced by vitamin C to Fe^{2+} /hemes and 10 equivalents of AgNO_3 or $\text{Fe}(\text{NO}_3)_3$ were added. The redox states of the, iron/hemes were analyzed by their Soret and Q bands.
- [14] *Gs* was anaerobically cultured in sealed serum bottles in 50 mL sterile growing media containing 30 mM NaHCO_3 , 9.4 mM NH_4OAc , 1.3 mM KNO_3 , 2 μM KCl , 2.6 mM NaH_2PO_4 , 20 mM sodium acetate as electron donor and 40 mM sodium fumarate as electron acceptor and trace minerals and vitamins. Fumarate was growth limiting and depleted when the experiments were conducted leaving the hemes in the reduced state. A silicone cap-sealed, O_2 -tight macro quartz cuvette (HELLMA) was used for the experiments. The clean quartz cuvette was degassed with argon to remove O_2 and sealed with a silicone rubber. 2.5 mL of the *Gs* solution ($\text{OD}_{600}=0.55$, 4.4×10^8 cells mL^{-1} or about 0.7 μM) was injected through a needle into the cuvette. A blank spectrum was measured before 75 μL of a O_2 -free 2.5 mM AgNO_3 solution was added, giving rise to 75 μM Ag^+ , by a needle through the silicone cap. The experiments were conducted in a Varian Cary 5E UV/Vis-NIR spectrometer. Ultra-pure water (Millipore, 18 M Ω cm, $\text{TOC}<5$) was used in the reference cuvette.

- [15] The Q band of the reduced hemes of the Omc has a maximum around 553 nm. The oxidized hemes have no spectral feature in this region. The Fe²⁺/heme percentage was determined by subtracting a straight baseline between 543–568 nm and normalizing the obtained absorption at 553 nm to 0 and 1, where 0 corresponds to 100% reduced and 1 to 100% oxidized hemes.
- [16] After Fe²⁺/hemes reappeared, the sample was centrifuged at 4400 rpm for 4 min. The supernatant solution was discarded and the pellet re-suspended in MilliQ water to remove residual Ag⁺-ion species. The solution was again centrifuged at 4400 rpm for 4 min, the supernatant solution discarded and the pellet re-suspended in 2% glutaraldehyde solution to fix the bacteria for several hours. For TEM investigations, several drops of this solution were dropcast on a carbon-coated copper TEM grid (Alfa Asear). Microscopy was performed on a FEI Tecnai Spirit transmission electron microscope, operated at 80 kV.
- [17] In recent publications also the role of polysaccharides during the microbial formation of AgNPs were discussed: a) F. Kang, P. J. Alvarez, D. Zhu, *Environ. Sci. Technol.* **2014**, 48, 316; b) S.-W. Li, X. Zhang, G.-P. Sheng, *Environ. Sci. Pollut. Res.* **2016**, 23, 8627. In these publications the formation of AgNPs by bacterial polysaccharides took several hours and days. Our experiments with *Geobacter sulfurreducens* (*Gs*) were finished in less than one hour (Figure 5A). In addition, if we added AgNO₃ to a solution from which *Gs* had been removed no AgNPs were formed within two hours. One referee asked whether saccharides, bound to the outer cell membrane can compete with redox reactions catalyzed by c-cytochromes. This question remains to be studied.

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