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Superoxide-Mediated Extracellular Biosynthesis of Silver Nanoparticles by the Fungus *Fusarium oxysporum*

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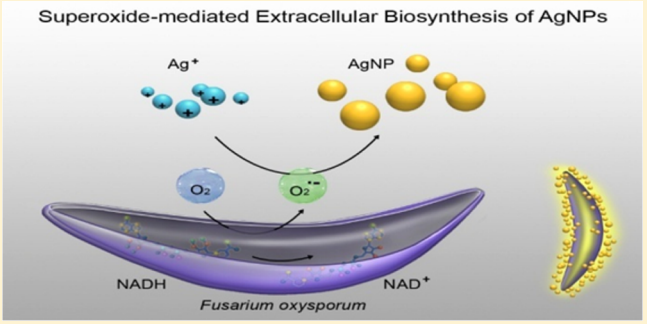
Supporting Information

ABSTRACT: The biosynthesis of silver nanoparticles (AgNPs) by microorganisms has become a hot topic in recent years, although its mechanism is still not well understood. Here we report the extracellular biosynthesis of AgNPs by the fungus *Fusarium oxysporum* through a superoxide-dependent mechanism. Reduction of Ag^+ to AgNPs in the extracellular region of *F. oxysporum* was verified by transmission electron microscopy, while the superoxide produced extracellularly by *F. oxysporum* was evidenced by chemiluminescence. We further demonstrated that the biosynthesis of AgNPs was inhibited by a superoxide scavenger or the inhibitor of NADH oxidases, and the addition of NADH significantly improved the formation of AgNPs. These results demonstrated that, for the first time, the fungus *F. oxysporum* can mediate the synthesis of AgNPs through the enzymatic generation of extracellular superoxide, which is helpful in understanding the biosynthesis of AgNPs and the biomineralization and transformation of silver and other metals or metalloids.



INTRODUCTION

Because of the wide application of silver nanoparticles (AgNPs),[1−4] there is an ever-growing need to develop environmentally friendly processes for their synthesis. As inspired by the bioreduction of silver ion (Ag^+) by *Pseudomonas stutzeri*,[5] the biosynthesis of AgNPs with microorganisms has become a hot topic, which was suggested as a green approach to synthesizing AgNPs.[6] These AgNPs are usually localized extracellularly,[6] which would simplify the downstream processing.[7,8] Although the biosynthesis of AgNPs has been demonstrated with a variety of bacteria, fungi, and actinomycetes,[6] the mechanism of this process is yet to be elucidated, which will be helpful for the enhancement of AgNP synthesis[9] as well as our understanding of the biomineralization of silver and the formation of naturally occurring AgNPs.[10] Several studies suggested that nitrate reductase (NR) was involved in this bioreduction process.[11−14] It was observed that the fungus *Fusarium oxysporum* can mediate AgNP synthesis,[15] and NR purified from *F. oxysporum* can reduce Ag^+ into AgNPs.[9] In addition, NR inhibitor piperitone can partially inhibit the biological reduction of Ag^+ to AgNPs by enterobacteria.[16] Infrared spectrometry and ^{13}C nuclear magnetic resonance suggested that aldehyde groups of extracellular polysaccharides may also play a crucial role in the reduction.[17,18] These results suggested that multimechanisms may present for this bioreduction. However, the pathway and electron donor involved in this process are still not well understood.

Recently, it has been demonstrated that bacteria and fungi can produce superoxide,[19,20] which can further mediate the oxidation of Mn(II)[20,21] and I^-,[22] and the reduction of Fe(III).[23,24] As demonstrated previously, superoxide produced from KO_2[25,26] or photoirradiated natural organic matter[27] can also reduce Ag^+ into AgNPs. Therefore, it is reasonable that superoxide bioproduced by microorganisms could reduce Ag^+ into AgNPs.

The main objective of this study was to probe the role of bioproduced superoxide in the microorganism-mediated biosynthesis of AgNPs, by using the fungus *F. oxysporum* as a model. We hypothesized *F. oxysporum* produces extracellular superoxide, which can subsequently reduce Ag^+ into extracellular AgNPs. To test this hypothesis, the extracellular formation of superoxide and AgNPs was demonstrated by continuous flow chemiluminescence (CL) and transmission electron microscopy (TEM), respectively. Then the role of superoxide in the biosynthesis of AgNPs was symmetrically

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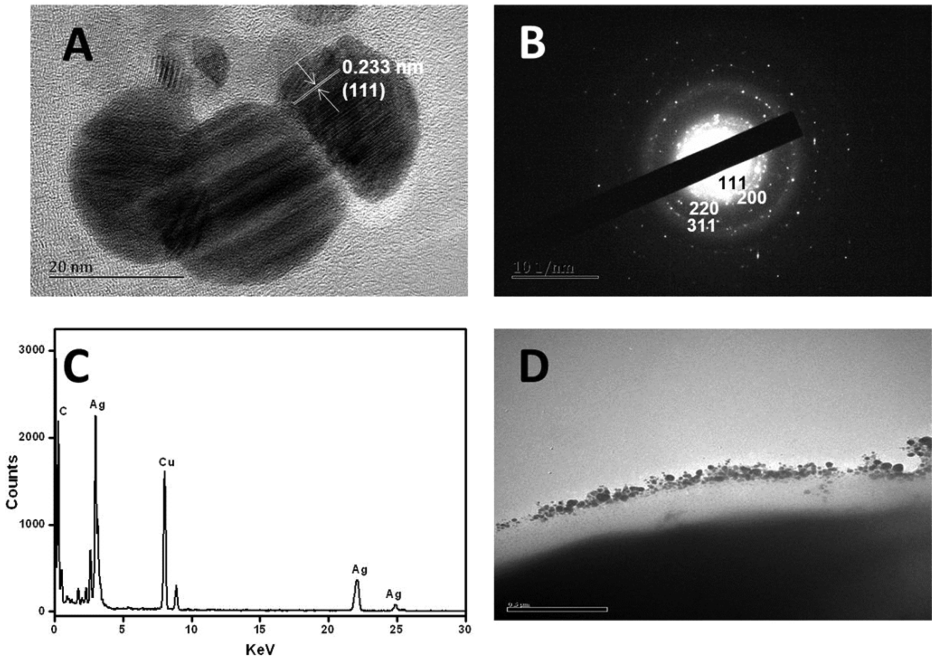

Figure 1. Extracellular synthesis of AgNPs by fungus *F. oxysporum* as demonstrated by (A) high-resolution TEM, (B) SAED, (C) EDS, and (D) TEM. The scale bars in panels A and D are 20 nm and 0.5 μm, respectively.

examined by using heat sterilization and the addition of a superoxide scavenger, reduced nicotinamide adenine dinucleotide (NADH), or a NADH enzyme inhibitor.

MATERIALS AND METHODS

Materials. *F. oxysporum*, separated from soybean root soil, was purchased from China General Microbiological Culture Collection Center (CGMCC, catalog no. 3.6787). Potato dextrose broth (PDB) and potato dextrose agar (PDA) were from Aobox Biotechnology (Beijing, China). Analytical grade $AgNO_3$, glucose, $Zn(NO_3)_2$, and $Cu(NO_3)_2$ were purchased from Sinopharm Chemicals (Shanghai, China). Superoxide dismutase (SOD) from bovine erythrocytes, diphenyleneiodonium chloride (DPI), NADH, and NAD^+ were from Sigma-Aldrich (St. Louis, MO). Other chemicals were from Beijing Chemicals (Beijing, China). Ultrapure water (18.3 MΩ) from a Milli-Q gradient system (Millipore, Bedford, MA) was used throughout the experiments.

Cell Culture of *F. oxysporum*. PDA medium is more favorable for the growth of *F. oxysporum*, while the PDB liquid medium can facilitate the separation of *F. oxysporum* from the matrix. Thus, first the frozen-dried *F. oxysporum* was suspended in 0.3−0.5 mL of PDB medium and transferred onto PDA slant culture medium to active the mycelia at 25 °C. Then the *F. oxysporum* was inoculated into 100 mL of PDB medium and cultured at 25 °C while being shaken at 180 rpm for 4 days.

Treatment of *F. oxysporum* and the Biosynthesis of AgNPs. To prevent the interference from the culture medium for biosynthesis of AgNPs, *F. oxysporum* in its fast-growing period was first washed three times with sterilized water via centrifugation (2000*g* for 20 min at 10 °C) and then diluted with sterilized water to a final volume of 20 mL. The obtained *F. oxysporum* solution was then filtered through sterilized monolayered gauze (pore size of 1 mm × 1 mm) for further experiments.

Into a sterile glass tube (15 mm × 100 mm) were sequentially added 5 mL of a filtered *F. oxysporum* solution, 2% (m/v) glucose (as an energy source to maintain the growth of fugus), and $AgNO_3$ (1 mmol L^{-1}), and the tube was sealed with a cotton plug. The glass tube was kept at 25 °C while being shaken at 150 rpm. The solution sampled at different times was filtered through 0.2 μm glass fiber filter for further characterization.

To probe the possible role of superoxide and oxidoreductase in the biosynthesis of AgNPs, the superoxide scavenger [SOD and $Cu(NO_3)_2$] [$Zn(NO_3)_2$ as a control], NADH, NAD^+, and oxidoreductase inhibitor (DPI) were also added to investigate their effects on the biosynthesis of AgNPs.

Characterization of AgNPs. The UV−visible (vis) spectra were recorded by using a UV−vis−NIR spectrometer (UV-3600, Shimadzu). TEM, coupled with an energy dispersive spectrometer (EDS) and selected area electron diffraction (SAED), was conducted with a H-7500 (Hitachi) or JEM 2100F (JEOL) instrument.

Detection of Superoxide by CL. A continuous flow CL apparatus[28] was used for online detection of superoxide from *F. oxysporum*. The apparatus includes a CL analyzer (Institute of Biophysics, Beijing, China) and two peristaltic pumps (Longer Precision Pump Co., Hebei, China). It should be noted that the photoreactor in a previous study[28] was omitted in this study. Methyl cypridina luciferin analogue (MCLA, 0.1 mmol L^{-1}, pH 11.6) was mixed with *F. oxysporum* through Tygon pump tubing (inside diameter of 1 mm), and the CL intensity is measured with a photomultiplier tube at the spiral detection cell in the CL analyzer.

RESULTS AND DISCUSSION

Extracellular Synthesis of AgNPs by *F. oxysporum*. After incubation of $AgNO_3$ with *F. oxysporum* for 12 h, the formation of AgNPs was demonstrated comprehensively by TEM, SAED, and EDS (Figure 1). Spherical particles with an average diameter of 20.7 nm (Figure S1) were observed. The lattice planes observed in the TEM image (Figure 1A) and SAED patterns (Figure 1B) can be indexed to face-centered

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Environ. Sci. Technol. Lett. XXXX, XXX, XXX−XXX

cubic silver metal. EDS (Figure 1C) further confirmed these nanoparticles are silver. Other signals of C and Cu are from the carbon-coated copper grid. The TEM image in Figure 1D revealed that these AgNPs are located extracellularly, which is consistent with the finding of Ahmad et al.[15] These combined results demonstrated that the live fungus *F. oxysporum* can extracellularly synthesize AgNPs.

The evolution of AgNPs with incubation time was further probed by UV−vis spectrometry (Figure S2). The UV−vis spectrum of the blank (2% glucose and $AgNO_3$ incubated at 25 °C for 48 h) did not show any surface plasmon resonance (SPR) absorption from AgNPs, demonstrating that glucose in the matrix should not produce any artifact of AgNPs during the incubation procedure. Figure S2 indicates the SPR intensity from AgNPs at ∼450 nm increased with incubation time, suggesting the increased AgNPs concentration accordingly.[27]

Production of Superoxide by *F. oxysporum*. It was reported that superoxide can be produced by bacteria and fungi.[19−21,23,24] Here, for the first time, we demonstrated the production of superoxide by *F. oxysporum* by using continuous flow CL (Figure 2). No CL signal was observed when only *F. oxysporum* was present (in the absence of CL reagent, MCLA), indicating the *F. oxysporum* would not interfere with the CL determination. In the absence of *F. oxysporum*, MCLA can produce a weak CL signal; however, a continuous enhanced CL signal was observed when *F. oxysporum* was mixed with MCLA, suggesting a continuous production of superoxide by fungi at an approximately constant rate. However, the magnitude of the CL signal decreased significantly in the presence of a specific superoxide scavenger, SOD, which further confirmed that the CL signal was from superoxide. In addition, after the fungus was heat-deactivated, the magnitude of the CL signal decreased significantly, suggesting the superoxide was produced by live *F. oxysporum*. Superoxide, as a charged radical species, is unlikely to be able to diffuse through the cell membrane,[29] and exogenous macromolecular SOD also cannot enter the cell from the extracellular space because of the molecular-sieving function of the cell wall.[21,30] Therefore, these results clearly demonstrated that the live fungus *F. oxysporum* can produce superoxide extracellularly.

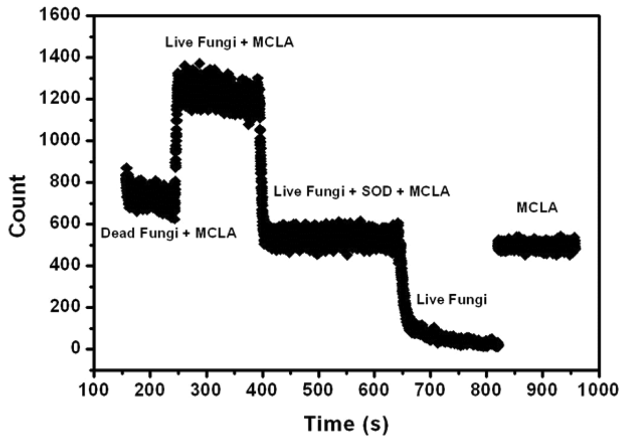

Figure 2. Online detection of superoxide produced by the fungus *F. oxysporum* by using continuous flow chemiluminescence. The concentration of SOD is 600 units mL^{-1}.

Superoxide-Mediated Extracellular Biosynthesis of Silver Nanoparticles. *Superoxide Scavenger.* Cu^{2+} is an effective and rapid superoxide scavenger, while chemically similar Zn^{2+} cannot catalyze the dismutation of superoxide.[20,21,31] The effect of Cu^{2+} (as a superoxide scavenger) and Zn^{2+} (as a control) on the formation of AgNPs by *F. oxysporum* was therefore investigated (Figure 3A). The addition of Cu^{2+} can induce a concentration-dependent inhibition of AgNP formation, suggesting a critical role of superoxide as the reductant of Ag^+. However, addition of Zn^{2+} can only slightly red-shift the UV−vis spectrum. This red-shift was also observed for AgNPs in the presence of Cu^{2+}, which should be ascribed to the divalent metal ion-induced aggregation and fusion of AgNPs.[32,33]

Then, SOD, a specific superoxide scavenger,[34,35] was added to the growing cultures to further validate the role of superoxide in AgNP formation. Figure 3B shows that SOD can partially abolish the formation of AgNPs by *F. oxysporum*, demonstrating the critical role of superoxide in the reduction of silver. It should also be noted that at 600 units mL^{-1} SOD, the formation of AgNPs can still be observed. As Figure 2 demonstrates that SOD at this concentration can scavenge the fungally produced superoxide, therefore, other possible reductive processes besides superoxide should exist.

It should be also noted that in panels A and B of Figure 3, the formed AgNPs in the absence of Cu^{2+} or SOD showed slightly different peak shapes, although the culture of fungus and biosynthesis of AgNPs are performed under the same conditions but in different batches. The different peak shape of spectra indicated the different sizes or aggregation states of AgNPs,[33] which may be associated with the experimental batch-dependent composition and concentration of extracellular polymeric substances that play an important role in stabilizing AgNPs.[36]

Activity of Enzymes Responsible for Superoxide Production. A variety of oxidoreductases can produce superoxide by engaging one-electron transfer from substrate to O_2, including NAD(P)H oxidases,[37,38] multicopper oxidases,[39] and NR.[40] Thus, the role of the primary enzymes, NAD(P)H oxidases, responsible for superoxide production was investigated in the formation of AgNPs by *F. oxysporum*.

DPI, an inhibitor of transmembrane oxidoreductases and other NAD(P)H binding enzymes,[41] results in the partial inhibition of AgNP formation at 12 and 24 h (Figure 3C,D). In addition, a concentration-dependent inhibition of DPI on AgNPs was observed, which further supports the possibility that superoxide-mediated AgNP formation is associated with the activity of NAD(P)H oxidase enzymes.

NAD(P)H oxidases, located within the plasma membrane, can utilize cytosolic NADH as the substrate to reduce O_2 to extracellular superoxide.[19] Therefore, the role of the oxidase substrate, i.e., NADH, was studied in AgNP formation. As shown in Figure S3, NADH significantly enhanced the formation of AgNPs by *F. oxysporum*. However, the addition of the oxidized form NAD^+ did not increase the level of formation of AgNPs. In addition, compared with that of NADH spiking, the simultaneous addition of NADH and SOD significantly inhibits the formation of AgNPs, suggesting the NADH-enhanced enzymatic superoxide production and the subsequent reductive formation of AgNPs by superoxide.

Live and Heat-Deactivated F. oxysporum. As demonstrated above, a superoxide scavenger can only partially inhibit the production of AgNPs by *F. oxysporum*, indicating the presence of another AgNP synthesis pathway besides superoxide. We then compared the heat-deactivated and live *F.*

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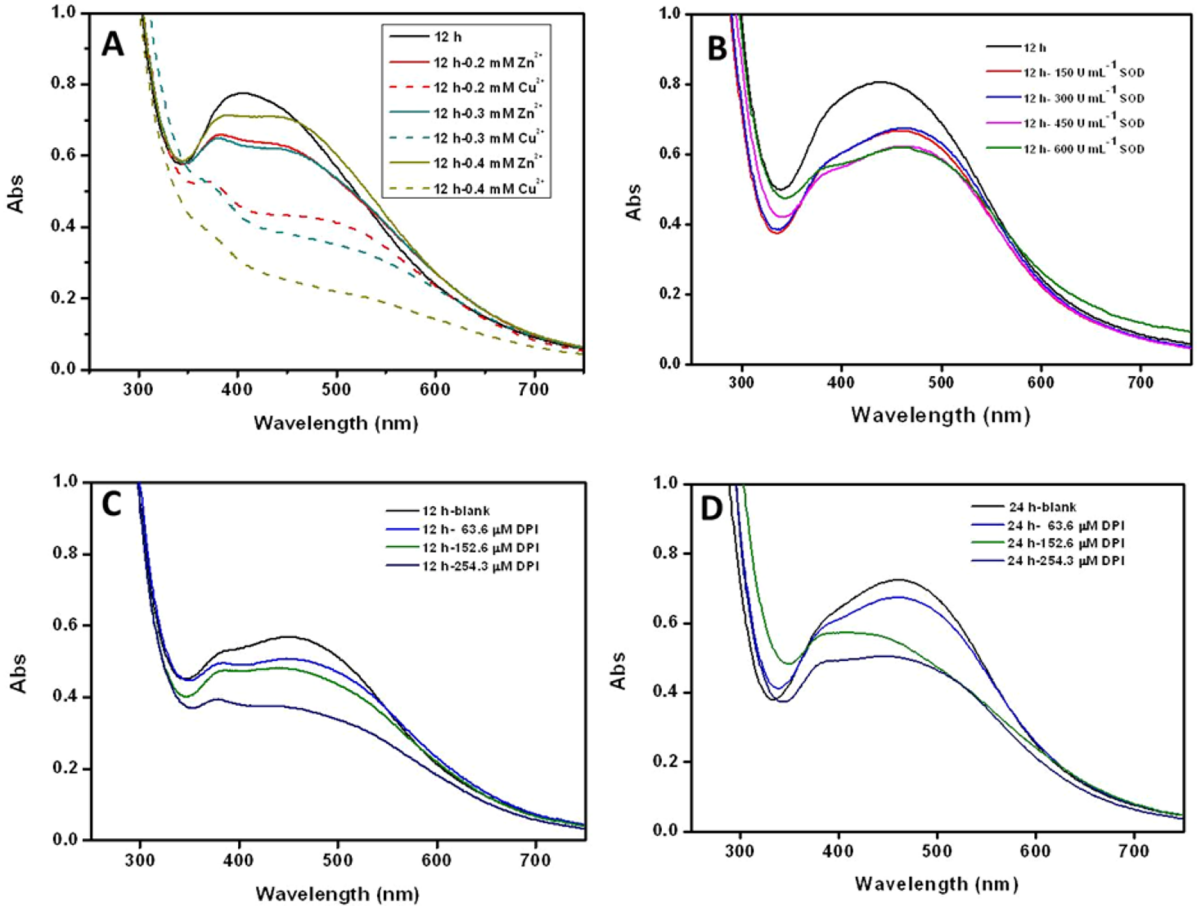

Figure 3. UV−vis absorption spectrum of AgNPs formed by *F. oxysporum* in the presence of (A) Cu^{2+} or Zn^{2+}, (B) SOD, and (C and D) DPI after incubation for 12 or 24 h.

oxysporum for their AgNP synthesis capability. As shown in Figure S4, although the heat deactivation of *F. oxysporum* significantly decreased the level of AgNP formation, the SPR absorption suggested there is still AgNP formation even in the presence of heat-deactivated *F. oxysporum*. As only live *F. oxysporum* can produce superoxide (Figure 2), the synthesis of AgNPs by heat-deactivated *F. oxysporum* indicated that other AgNP formation processes besides superoxide may also present for *F. oxysporum*. The formation of AgNPs by dead *F. oxysporum* could possibly be ascribed to the reductive aldehyde groups of extracellular polysaccharides.[18] Figure S4 also shows that the difference in AgNP synthesis capability of dead and live *F. oxysporum* decreases with incubation time, possibly due to the toxicity of Ag^+ and thus the inhibition of cell viability and reproduction to *F. oxysporum*.[42]

It should be noted that several studies also suggested that NR plays a crucial role in AgNP biosynthesis,[9,11−14] which is not contradictory with our proposed superoxide pathway. As NR from various sources can also reduce O_2 to superoxide,[40,43−45] it is reasonable that the previously proposed NR pathway also involved superoxide as a reductant.

Environmental Implications. Living bacteria, fungi, algae, and plant species can reduce Ag^+ into AgNPs[6] and potentially serve as "nano-factories" for AgNP synthesis.[46,47] Here we discovered superoxide plays a crucial role in the extracellular synthesis of AgNPs by the fungus *F. oxysporum*. As superoxide is widely produced in bacteria,[19] fungi,[20] algae,[48] and plants,[49] this study is helpful for understanding the mechanism of AgNP biosynthesis, which will have great implications for the enhancement of AgNP synthesis in the future. In addition, this study also improves our understanding of biomineralization of silver and the source of naturally occurring AgNPs. As the antibacterial activities of AgNPs are much lower than that of Ag^+,[50] this bioreduction is also a possible natural antidote to mitigate the toxicity of silver to organisms. Considering the key roles of superoxide in the redox cycles of Fe,[23,51] Mn,[20,21] Cu,[32,52,53] Cr,[54,55] As,[56,57] and I,[22] it is expected that the superoxide-produced organisms should also have great impacts on the biogeochemical cycles of these elements.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.6b00066.

TEM image and size distribution of AgNPs and and the UV−vis spectrum of AgNPs under different conditions (PDF)

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Notes

The authors declare no competing financial interest.

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Environ. Sci. Technol. Lett. XXXX, XXX, XXX−XXX

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Environ. Sci. Technol. Lett. XXXX, XXX, XXX−XXX