Carbon Nanotubes Alter the Electron Flow Route and Enhance Nitrobenzene Reduction by Shewanella oneidensis MR-1

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ABSTRACT: Dissimilatory metal-reducing bacteria play an important role in environmental bioremediation, and their extracellular electron transfer (EET) and pollutant reduction process can be affected by various redox-active substances. While it is generally thought that these substances usually only accelerate the EET rate, here we discover that the electron flow route within Shewanella oneidensis MR-1 cells can also be altered by the introduction of carbon nanotubes (CNTs). Addition of 0.5% (w/v) CNTs in the cell-immobilized alginate beads led to a shift of intracellular nitrobenzene (NB) reduction to extracellular reaction and a 74% improvement in NB reduction efficiency. This work provides the first evidence that the electron flow route of microorganisms can be altered by CNTs. It broadens our view about the possible environmental consequence of CNTs from a microbial extracellular respiration perspective and may lead to an improved understanding of microbial respiration and improve the practical application of bioremediation processes.

INTRODUCTION

Shewanella oneidensis MR-1 is a typical and widely distributed dissimilatory metal-reducing bacterium with broad anaerobic respiratory capacity.1,2 This bacterium plays an important role in biogeochemical cycling of metals3−5 carbon elements, etc., and has great potential for environmental remediation applications.6−10 The powerful reducing capability of S. oneidensis MR-1 relies heavily on its flexible respiration pathways and its extracellular electron transfer (EET) ability. It possesses a great number of c-type cytochromes that are capable of transferring electrons to diverse terminal electron acceptors. In particular, some c-type cytochromes at the cell surface, such as OmcA and MtrC, are critically involved in microbial EET and extracellular reduction reactions.11,12

The EET and bioreduction processes of S. oneidensis MR-1 can be substantially affected by environmental conditions, especially the presence of redox-active compounds and materials. Carbon nanotubes (CNTs) are a typical redox-active material13 and are usually used as a support for a biocatalyst at an electrode because of their good biocompatibility and superior conductivity.14,15 CNTs have been found to promote the redox transition of house heart c-type cytochrome16 and to increase the rate of electricity generation by S. oneidensis MR-1 in a microbial fuel cell.17 All these studies suggest that CNTs can accelerate the microbial EET rate. However, there is no evidence to show whether CNTs can also affect the microbial electron transfer pathway.

S. oneidensis MR-1 is known to possess diverse respiration pathways and can reduce a wide range of pollutants intracellularly or extracellularly. Previous studies have proposed that reduction of nitrobenzene (NB) by S. oneidensis MR-1 is mainly an intracellular process.1 It is generally thought that these substances usually only accelerate the EET rate, but here we discover that CNTs are capable of channeling electrons outside the cells and turning NB reduction into an extracellular process and greatly accelerating the reduction process. In this study, we confirm this phenomenon and elucidate how the flow of electrons from S. oneidensis MR-1 cells to NB was changed by CNTs. The NB bioreduction experiment was conducted under environment-mimicking motility-restricted conditions by immobilizing S. oneidensis MR-1 cells in alginate beads. This study might help to broaden our view of the environmental impact of CNTs not just from a toxicity point of view but also from a microbial extracellular respiration perspective. Moreover, in light of the increasing levels of production, use, and release of CNTs to the environment, our study may lead to a better understanding of and improvement of the practical bioremediation processes.

MATERIALS AND METHODS

Preparation of Cell- and CNT-Immobilized Alginate Beads. S. oneidensis MR-1 (ATCC 700550) wild type and its mutant strain deficient in OmcA and MtrC (ΔomcA/ΔmtrC) as precultured and raw multiwalled CNTs were prepared as described in the Supporting Information. The strains and prepared CNTs were then immobilized individually or together in Ca-alginate (AL) beads.
A total of 0.05 g of CNTs was dispersed in 10 mL of sterile distilled water under ultrasonication for 30 min to yield a stable suspension. Then, 0.25 g of sodium alginate was added and the mixture heated in an 80 °C water bath to form a homogeneous sol. After being cooled at room temperature for 24 h and subjected to removal of air bubbles, the sol was mixed with the collected S. oneidensis MR-1 cells, which had been preadjusted to a concentration with an OD600 of 3.0. The mixture was stirred for 10 min before being extruded dropwise into a sterilized 2% (w/v) CaCl2 solution while being stirred using a 1 mL syringe from a height of ~40 cm. After ionic immersion and gelation for 2 h, cell-immobilized alginate beads were obtained. Alginate beads with and without MWNTs were also prepared and used as the controls.

**NB Reduction in Serum Vials and Sample Analysis.** The NB reduction experiments were conducted in the modified BMM medium with 20 mM lactate as the electron donor. The as-prepared beads or free cells were added to 25 mL of modified BMM medium (with a final concentration of OD600 = 0.1) in 50 mL serum vials with butyl rubber stoppers and purged with nitrogen gas to ensure an anaerobic environment. The cultures were incubated at 30 °C while being shaken at 150 rpm. All the experiments were conducted in triplicate. The initial NB concentration was 100 mg/L. In addition, electrochemical reduction tests were also conducted to improve our understanding of the roles of CNTs in EET as described in the Supporting Information.

Samples were taken at given time intervals and centrifuged immediately at 10000g for 10 min. The supernatant was used for concentration measurement. The NB concentration was measured by high-performance liquid chromatography (1200 Infinity, Agilent Co.) following the method reported in the literature. The NB reduction efficiency was defined as the percentage of reduction by the end of the experiment and was calculated on the basis of the initial and residual NB concentrations.

**Morphology Analysis.** The photographs of beads were taken with a digital camera (Power Shot SX200IS, Canon Co.). To reveal the three-dimensional structure and morphology of the beads, the immobilized bacterium MR-1 was replaced with the green fluorescent protein (GFP)-labeled AS93 strain of S. oneidensis MR-1. The AS93-immobilized beads were cut into small pieces and observed with a confocal laser scanning microscope (CLSM) (RX81, Olympus Co.).

**RESULTS**

**Observation of Cell-Embedded Alginate Beads.** The cell-immobilized beads showed light flesh color and became dark after the CNTs had been added. All freshly prepared beads were spherical, with a diameter of 2.324 ± 0.035 mm for the cell-embedded beads and 2.470 ± 0.063 mm for the cells and CNT-embedded beads. The diameter slightly increased after the reduction experiment (Figure 1A,B) because of the adsorption of water to the internal voids of the beads. The distribution of cells in beads was examined by the CLSM using a GFP-labeled AS93 strain derived from S. oneidensis MR-1. The cells (green fluorescence) were uniformly distributed in the bead, and the bright fluorescence indicated that a high activity of imbedded cells was retained (Figure 1C).

**Effect of CNTs on the NB Reduction Efficiency by Immobilized S. oneidensis.** Figure 2A shows that NB was considerably reduced by both the free and immobilized cells, with a similar level of reduction efficiency. Product analysis confirmed that NB was reduced to aniline (Figure S1 of the Supporting Information). The introduction of CNTs led to significantly faster reduction for both the free and immobilized cells. In particular, the reduction efficiency of the AL/CNT/ MR-1 sample after 174 h reached 94.5% (vs 54.4% in the AL/ MR-1 sample), indicating an ~74% increase in the presence of CNTs. Interestingly, better performance was achieved with the
AL/CNT/MR-1 sample than with the AL/MR-1 sample, suggesting that the CNT promotion effects were favored by AL, possibly because of enhanced physical adsorption, mitigation of the biotoxicity of CNTs, or both. On the other hand, the fact that AL enhanced the performance of MR-1 only when CNTs were present also implies there was a synergy between CNT and AL. Considering the comparable performance of the MR-1 and AL/MR-1 samples, the adsorption by AL seemed to be negligible. Thus, the synergy was most likely attributed to the lower mobility of CNTs and cells in the AL beads, which mitigated the biotoxicity.

Impacts of CNTs on the Microbial Electron Flow Route. To elucidate how the NB bioreduction was accelerated by CNTs, we investigated the impacts of CNTs on the microbial electron transfer route by comparing the NB reduction by MR-1 and its ΔomcA/ΔmtrC mutant. OmcA and MtrC are two critical components in the EET chain of S. oneidensis MR-1. In the absence of CNTs, immobilized MR-1 and ΔomcA/ΔmtrC showed similar NB reduction efficiencies, indicating that OmcA and MtrC were not involved in the reduction under such conditions (Figure 2B). This result is consistent with previous studies. Surprisingly, with the addition of CNTs, MR-1 showed markedly faster reduction than ΔomcA/ΔmtrC. In contrast, the performance of the ΔomcA/ΔmtrC mutant was unaffected by CNTs (Figure 2B). These results clearly indicate that OmcA and MtrC were involved in the CNT-accelerated NB reduction. In other words, the promotion effect of CNTs was closely associated with OmcA and MtrC and the EET pathway. Notably, the reduction efficiency of the AL/CNT/MR-1 sample was slightly higher in Figure 2A (94.5%) than in Figure 2B (82.11%) after a 174 h reduction, which might be due to the different beads with slightly different cell activities used in these two experiments.

Electrochemical Reduction Enhancement by CNTs. The effect of CNTs in accelerating electron transfer and NB reduction was further demonstrated by the electrochemical reduction test. As illustrated in Figure S2 of the Supporting Information, the application of a −0.3 V potential (vs the standard hydrogen electrode) led to a rapid reduction of NB compared with the nonelectrochemical controls. The CNT-modified carbon paper electrode reduced NB much faster (61%) than the control carbon paper electrode (37%), strongly confirming a positive role of CNTs in EET and bioreduction.

DISCUSSION

In this study, S. oneidensis cells are immobilized in alginate beads, which could resemble the situation of cells and CNTs confronted in sediments and soils with a restricted mobility in the natural environment. The NB reduction efficiencies were similar for the free cells and immobilized cells, suggesting that the mass transfer was not a rate-limiting step for such immobilized systems. A dose of CNTs added to the MR-1 beads significantly increased the NB reduction efficiency. The CNTs in the alginate sol were mixed with cells and solidified immediately; therefore, most of CNTs were localized outside cells. Thus, the enhanced reduction should be ascribed to an accelerated EET mediated by CNTs.

OmcA and MtrC are terminal reductases anchored at the extracellular side of the outer membrane and are critical gatekeepers in the EET chain of S. oneidensis MR-1. The results of this work and a previous report all suggest that these two c-type cytochromes are not involved in the reduction of NB by S. oneidensis MR-1. Surprisingly, a dose of CNTs profoundly accelerated the reduction of NB by the immobilized cells (Figure 2A). Because CNTs are mainly responsible for the EET as discussed above, this result suggests that CNTs alter the electron flow route and build a new pathway for the extracellular reduction of NB, in which OmcA and MtrC might be essential components (Figure 3). A further analysis convinced us that, after the addition of CNTs, OmcA and MtrC became critically involved in the electron transfer for NB reduction (Figure 2B). The accelerated EET from OmcA and MtrC to NB molecules and promoted NB reduction by CNTs were further validated by the electrochemical reduction tests in which a point potential similar to the redox potential that OmcA and MtrC can provide was applied (Figure S2 of the Supporting Information).

Notably, Figure 2B shows that the ΔomcA/ΔmtrC mutant exhibited a slightly lowered NB reduction efficiency when CNTs were present. This might be due to a toxicity of CNTs as reported in previous studies. In our system, the CNTs might act like a double-edged sword: accelerating electron transfer and NB extracellular reduction on one hand and impairing cell activity on the other. Thus, for the ΔomcA/ΔmtrC mutant, the positive effects of CNT were disabled because of the block of the pathway of OmcA and MtrC, while the negative effects became predominant, which led to an impaired cell activity and a lower NB reduction efficiency. However, for the wide type, the positive effects outweighed the negative; thus, NB bioreduction was improved. The NB reduction rate in our study was comparable to those of other studies with Shewanella sp. [ranging from 23.6 to 55.1 g L−1 h−1 (g of cells)−1 (Table S1 of the Supporting Information)], implying a high potential of this AL/CNT/MR-1 sample for practical bioremediation implementation with improved environmental resistance.

The promotion effects of CNTs should be associated with the unique physicochemical properties of CNTs. First, CNTs possess abundant redox-active sites and consequently favor a high electrical conductivity and therefore accelerate EET. Second, CNTs can absorb diverse organic compounds, thus allowing greater mass diffusion and increased reaction kinetics.
In this study, the CNT-containing alginate beads showed a slightly higher level of NB absorption than the beads without CNTs (Figure 2A), which also might have contributed to the higher NB reduction efficiency. Third, the extracellular reduction could lower the local concentration of NB entering the cells and alleviate the cellular toxicity of NB; thus, a high microbial activity could be maintained. Taken together, the dose of CNTs builds a positive feedback loop for the reduction of NB by S. oneidensis MR-1 through microbial respiration. It is reasonable to expect that other conductive materials might also have similar effects. For example, a graphene scaffold has been found to significantly increase the number of exoelectrogens involved in EET and accelerate the EET at the microbe–electrode interface. 30,31 Thus, a further exploration of the possible effect of other conductive materials on EET shall be needed in future studies.

In summary, CNTs were found to introduce a new electron flow route in S. oneidensis MR-1 to improve NB reduction. This work implies that the redox-active compounds in the environment, for example, humic substances, might have impacts on the microbial respiration and bioremediation processes. Thus, we might need to reconsider the definition of the extracellular electron acceptors, because some inherently intracellular reactions might be shifted to extracellular ones. In addition, the findings in this study suggest that CNTs could be used as a useful material to promote extracellular reduction reactions and hence offer implications for practical bioremediation applications.

**ASSOCIATED CONTENT**

**Supporting Information**

One table, two figures, and detailed experimental methods used in this work. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We thank the Natural Science Foundation of China (21107105 and 51278479) and the Program for Changjiang Scholars and Innovative Research Team in University, China, for partial support of this study.

**REFERENCES**


