Short communication

Multi-walled carbon nanotubes accelerate interspecies electron transfer between *Geobacter* cocultures

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**A B S T R A C T**

Carbon nanotubes (CNTs) have been reported to promote symbiotic metabolism in bacteria by accelerating interspecies electron transfer. However, this phenomenon has not been investigated or proven in a cocultures system. In this study, multi-walled CNTs (MWCNTs) were added into *Geobacter* cocultures systems with the ability of direct interspecies electron transfer (DIET). Results showed that addition of MWCNTs accelerated the metabolic rate of the cocultures. Succinate production rate in a test with 1.0 g L⁻¹ MWCNTs was 1.12 mM d⁻¹, 1.67 times higher than without MWCNTs. However, the biotoxicity effect became evident with the addition of much higher levels of MWCNTs addition. This study supports the possibility that carbon nanotubes accelerate interspecies electron transfer and provides a theoretical basis for the MWCNTs application in the process of anaerobic wastewater treatment.

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1. Introduction

The mechanisms whereby microbial interspecies electron transfer (IET) occurs are of interest because they are widely distributed in anaerobic environments [1]. Direct interspecies electron transfer (DIET) is a more effective mechanism for interspecies electron exchange in which electrons transfer through electrically conductive pili, outer cell surface proteins or electrically conductive materials, such as carbon nanotubes (CNTs), graphene and minerals from electron-donating cells to electron-accepting partners [2–4].

Many studies have reported that CNTs enhance symbiotic metabolism in bacteria due to their potentially accelerating IET [5]. In a previous report, single-walled CNTs (SWCNTs) were added into anaerobic sludge granules at a concentration of 1.0 g L⁻¹, which accelerated substrate utilization and methane production rates [6]. In another study, 1.0 g L⁻¹ SWCNTs also promoted start-up of thermophilic anaerobic digestion at high methane production rates [7]. Compared to control groups, conductivity of the sludge with CNT addition was increased by 27-fold, which may form efficient IET channels. In addition to SWCNTs, multi-walled CNTs (MWCNTs), elongated cylindrical nanoobjects (diameter: 3–30 nm; length: several cm) made of sp² carbon [8], were also reported to facilitate DIET process. It was shown that addition of 5 g L⁻¹ MWCNTs enhanced the rate of methane production by 50% due to enhanced DIET [9]. In our recent work, MWCNTs were added to an anaerobic digestion reactor treating beet sugar industrial wastewater [10]. Results showed a marked enhancement of methane production, where MWCNTs acted as electron transfer channels for IET. A MWCNTs hybrid biofilm was also fabricated in our previous work [11] and results showed that MWCNTs may also accelerate the IET, promoting COD degradation in the influent. These results all show the enhancement effect of CNTs in the mixed-culture system.

However, the impact of CNTs on IET, as the electrically conductive material, has never been investigated or proven in a cocultures system with DIET ability [12]. Because the DIET phenomenon had been proven in a cocultures system of *Geobacter metallireducens* GS-15 and *G. sulfurreducens* PCA [13], the effect of CNTs on *Geobacter* cocultures was investigated in this study.

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2. Materials and methods

2.1. Microorganisms, media and growth conditions

Cocultures of *G. metallireducens* GS-15 and *G. sulfurreducens* PCA were established in strictly anaerobic NBF medium with a gas phase of N₂/CO₂ (80:20, v/v) [14] with ethanol (10 mM) as the electron donor and fumarate (40 mM) as the electron acceptor. Before establishment of the cocultures, *G. metallireducens* for inocula was grown in ferric citrate (FC) medium with 20 mM ethanol as the sole electron donor and 55 mM ferric citrate as the electron acceptor. *G. sulfurreducens* for inocula was grown in NBF medium with 20 mM acetate as the sole electron donor and 40 mM fumarate as the electron acceptor. After three transfers of *G. metallireducens* or *G. sulfurreducens*, they were added together into anaerobic NBF medium and the cocultures were dosed with different concentrations of MWCNTs as referenced by previous experiments [12]. The control experiment was just the cocultures without MWCNTs.

Metabolism of ethanol and reduction of fumarate were monitored over time using high-performance liquid chromatography (HPLC) analysis as previously described [15]. Ethanol metabolism rates (Re) and succinate production rates (Rs) were calculated during the linear phase of metabolism in the cocultures.

2.2. Morphology and measurement of the bacteria on the MWCNTs

The morphology of cells on the MWCNTs surface was examined by a scanning electron microscope (SEM). Pretreatment process of all the samples with microorganisms was as follows: samples were first fixed overnight in the fixative containing 2.5% glutaraldehyde at 4 °C and then washed with 0.1 M phosphate buffer solution three times. Next, samples were dehydrated in increasing concentrations of ethanol solution (50, 70, 90 and 100%) and were blown with nitrogen gas and dried using a vacuum freeze-drying method. Samples were then sputter coated with 10 nm of gold and imaged by FEI XL30 Sirion SEM) at an accelerating voltage of 5 kV. Quantitative analysis of the cells was performed based on the protein mass on the surface of the MWCNTs and in the solution [16]. Briefly, after the coculture experiment, samples were soaked in 0.2 M KOH solution and heated for half an hour at 90 °C. Protein concentrations were determined by BCA method according to the manufacturer's protocol (Solarbio, China). Conductivity of the MWCNTs was determined with the four-probe method by a powder resistivity tester (STZ722, Suzhou Jingge, China) [17].

3. Results and discussion

3.1. The influence of MWCNTs on the Geobacter cocultures metabolism

As previously reported, *G. metallireducens* can directly transfer electrons to *G. sulfurreducens* in a defined medium with ethanol as the electron donor and fumarate as the electron acceptor [18]. During this process, fumarate is reduced to succinate, and ethanol is oxidized to CO₂ [19]. According to our calculation, the ratio of succinate production/ethanol degradation was approximately 6. Thus, increases in succinate concentration and decreases in ethanol concentration represent the metabolism of the cocultures and the DIET rate. MWCNTs were added into cocultures to test the possibility of promoting metabolism in the Geobacter co-cultures. As shown in Fig. 1a, MWCNTs addition accelerated the accumulation of succinate. However, without MWCNTs addition, levels of succinate production were markedly lower than the others during the experimental period. This facilitating effect was promoted by MWCNT concentration increasing from 0.1 g L⁻¹ to 1.0 g L⁻¹. Meanwhile, this facilitating effect showed a slight decrease in MWCNT concentration that reached 3.0 g L⁻¹. Corresponding succinate production rates (Rs) during the linear phase of metabolism in cocultures are shown in Fig. 1b. Rs gradually increased from 0.42 ± 0.03 mM d⁻¹ (Control) to 1.12 ± 0.08 mM d⁻¹ (1 g L⁻¹, MWCNTs). When the MWCNT concentration exceeded 1.0 g L⁻¹, Rs decreased from 1.10 ± 0.08 mM d⁻¹ (1.5 g L⁻¹, MWCNTs) to 0.81 ± 0.11 mM d⁻¹ (3 g L⁻¹, MWCNTs). These results indicate that MWCNTs effectively facilitates the syntrophic growth of Geobacter cocultures and this facilitation effect reached a maximum when the MWCNT concentration increased to 1.0 g L⁻¹.

The decrease in ethanol concentration also showed a similar trend as the accumulation of succinate. As shown in Fig. 1c, the decreasing rate of ethanol concentration in the test without MWCNTs was apparently lower than that with the addition of MWCNTs. Corresponding ethanol metabolism rates (Re) during the linear phase of metabolism in the co-cultures were shown in Fig. 1d. The Re of the control test was just 0.066 ± 0.024 mM d⁻¹ and gradually increased to 0.158 ± 0.022 mM d⁻¹ with 0.5 g L⁻¹ MWCNTs addition. For tests with MWCNT concentrations of 0.5, 1.0 and 1.5 g L⁻¹, the ethanol metabolism rates were at a similar level. However, when MWCNT concentration increased to 3.0 g L⁻¹, Re decreased to just 0.074 ± 0.025 mM d⁻¹. As the tubular structure of CNTs is beneficial for electron transfer, the enhanced metabolism of Geobacter cocultures is likely due to the accelerated DIET from *G. metallireducens* to *G. sulfurreducens*.

Pure culture experiments with MWCNTs were also designed to determine whether MWCNTs can accept electrons from *G. metallireducens* or donate electrons to *G. sulfurreducens* in the pure culture system. We measured ethanol consumption and fumarate production performance in this system and results are shown in Fig. S1 and S2. As shown in Fig. S1, ethanol consumption was measured for 12 days and no concentration decrease was observed, indicating that there was no consumption of ethanol in the G.m + MWCNTs system. The concentration difference between G.m, G.m + 1.0MWCNTs, and G.m + 3.0MWCNTs is likely due to the adsorption of MWCNTs. This result clearly demonstrates that MWCNTs cannot accept electrons from *G. metallireducens* to induce ethanol oxidation. Similarly, as shown in Fig. S2, no succinate production was observed, indicating that MWCNTs cannot donate electrons to *G. sulfurreducens* for fumarate reduction. In brief, MWCNTs cannot accept or donate electrons to cells; rather, they merely act as the bridge to transfer electrons from *G. metallireducens* to *G. sulfurreducens*. A reduction in the facilitation effect with greater concentration of MWCNTs may be caused by biological toxicity from MWCNTs [20].

3.2. Geobacter adhesion on the MWCNT aggregates

Direct contact between bacteria with the capacity for extracellular electron transfer and MWCNTs is pre-condition for promoting IET. Cells in the cocultures exist in one of two types: planktonic, freely existing in the solution, and sessile, attached to the surface of MWCNTs [17]. Thus, we compared the total biomass concentration at the end of the incubation to determine protein concentration. Compared to controls (12.32 ± 1.09 mg L⁻¹), total cell protein concentrations with 0.1 g L⁻¹ MWCNTs increased to 13.21 ± 1.28 mg L⁻¹, which is 7.22% higher than the control. In contrast, when MWCNT concentrations were over 0.1 g L⁻¹, total biomass in the cocultures slightly decreased from 13.18 ± 1.44 g L⁻¹ (MWCNT concentration, 0.5 g L⁻¹) to 10.35 ± 0.93 g L⁻¹ (MWCNT concentration, 3 g L⁻¹). MWCNTs addition accelerates IET and enhances metabolism in the cocultures. However, no obvious biomass concentration differences were observed between samples with and without MWCNTs, which is likely due to the inadequate supply of electron acceptors, inhibiting bacteria growth. Thus, these results indicate that the biotoxicity of MWCNTs is likely the primary reason for the slight decrease observed in total biomass.

It was also observed that the ratios of biomass on the MWCNTs aggregates surface to the total cells were at the same (approximately 90%) with MWCNTs dosage increasing from 0.1 g L⁻¹ to 1.0 g L⁻¹ (Fig. 2b). For cocultures with MWCNT concentration of 0.1 g L⁻¹, 87.37 ± 8.06% of the bacteria were attached to the aggregates, while,
Fig. 1. Concentrations of succinate as a function of time in G. metallireducens/G. sulfurreducens cocultures amended with MWCNTs of different concentrations (a). Succinate production rates (Rs) in cocultures with different MWCNTs dosage (b). Concentrations of ethanol as a function of time in G. metallireducens/G. sulfurreducens cocultures amended with MWCNTs of different concentrations (c). Ethanol metabolism rates (Re) in cocultures with different MWCNTs dosage (d). Control experiment had no MWCNTs addition. Black, red, magenta, olive, navy, and purple sphere represent the control, 0.1, 0.5, 1.0, 1.5, and 3.0 g L\(^{-1}\) tests, respectively. Two-tailed Student’s \(t\)-test: \(*p < .05\), \(**p < .01\), and \(***p < .001\).

Fig. 2. Variation in protein concentration of the total cell, as well as cells on the MWCNTs aggregates and in the solution. Dark cyan, red, and dark yellow colour columns represent planktonic, total, and sessile cells, respectively (a); Ratios of cells on the MWCNTs aggregates to the total cells with different MWCNT concentrations (b). Two-tailed Student’s \(t\)-test: \(*p < .05\) and \(**p < .01\).
with a MWCNT concentration of 1.0 g L\(^{-1}\), the ratio was 90.70 \(\pm\) 4.49%. However, for the test with MWCNT concentration of 3.0 g L\(^{-1}\), the ratio decreased to 73.15 \(\pm\) 4.42%, 19.34% lower than the test with MWCNT concentration of 1.0 g L\(^{-1}\), suggesting that additional MWCNTs may reduce the adherence of bacteria.

SEM image of the cocultures with MWCNTs aggregates (Fig. 3, Fig. S3, S4, SS and S6) further revealed that Geobacter cells were distributed on the MWCNTs aggregate surface rather than forming Geobacter aggregates as previously reported [18], yielding similar results to granular activated carbon (GAC), carbon cloth, and biochar added in the cocultures for accelerating DIET [12,17,21,22]. DIET enhancement effect of these materials was due to their excellent conductivity that supports electron transfer. Thus, we measured the conductivity of MWCNTs and compared it to other materials. The conductivity of MWCNTs was 7558.58 S m\(^{-1}\), much higher than that of the GAC (0.30 S m\(^{-1}\)) [12] and biochar (0.41–582.92 S m\(^{-1}\)) [17]. Therefore, we believe that, in addition to the direct contact of the cells, MWCNTs aggregate is the other channel finishing IET. MWCNTs perhaps provide sites for cells to attach for promoting electron transfer via electrical connections associated with DIET. The tight connection between MWCNTs aggregates and cells also support that Geobacter cells directly transfer electrons to and accept electrons from the aggregates, which act as electron transfer channels for IET.

4. Conclusions

In this study, we demonstrate that MWCNTs can facilitate interspecies electron transfer in Geobacter cocultures. With 1.0 g L\(^{-1}\) MWCNTs addition, the succinate production rates increased to approximately 1.67 times higher than in controls. However, higher MWCNTs dosages induced obvious biotoxicity to cell growth. The prevalence of DIET in some anaerobic systems suggests that MWCNTs should be considered for promoting electron transfer via electrical connections associated to have important implications for efficient wastewater treatment. This study not only proves the enhancement effect of MWCNTs on microbial IET process, but also provides a theoretical foundation for the application of MWCNTs in energy recovery and wastewater treatment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2019.107346.

References

[1] A.J.M. Stams, C.M. Plugge, Electron transfer in syntrophic communities of anaerobic bacteria and archaea, Nat. Rev. Microbiol. 7 (2009) 588–577.

[2] D.R. Lovley, Syntrophy goes electric: direct interspecies electron transfer, Annu. Rev. Microbiol. 71 (2017) 643–664.

[3] L. Xiao, F. Liu, J. Liu, J. Li, Y. Zhang, J. Yu, O. Wang, Nano-Fe\(_3\)O\(_4\) particles accelerating electromethanogenesis on an hour-long timescale in wetland soil, Environ. Sci. 5 (2018) 436–445.

[4] O. Wang, S. Zheng, B. Wang, W. Fang, F. Liu, Necessity of electrically conductive pili for methanogenesis with magnetite stimulation, PeerJ 6 (2018), e4541.

[5] G. Martín, A.F. Salvador, L. Pereira, M.M. Alves, Methane production and conductive materials: a critical review, Environ. Sci. Technol. 52 (2018) 10241–10253.

[6] L.-L. Li, Z.-H. Tong, C.-Y. Fang, J. Chu, H.-Q. Yu, Response of anaerobic granular sludge to single-wall carbon nanotube exposure, Water Res. 70 (2015) 1–8.

[7] W.W. Yan, N. Shen, Y.Y. Xiao, Y. Chen, F.Q. Sun, V.K. Tyagi, Y. Zhou, The role of conductive materials in the start-up period of thermophilic anaerobic system, Biosens. Technol. 239 (2017) 336–344.

[8] S. Ravindran, S. Chaudhary, B. Colburn, M. Ozkan, C.S. Ozkan, Covalent coupling of quantum dots to multiwall carbon nanotubes for electronic device applications, Nano Lett. 3 (2003) 447–451.

[9] J.C. Zhang, Y.H. Lu, Conductive Fe\(_3\)O\(_4\) nanoparticles accelerate syntrophic methane production from butyrate oxidation in two different lake sediments, Front. Microbiol. 7 (2016).

[10] J.J. Ambuchi, Z. Zhang, L. Shan, D. Liang, P. Zhang, Y. Feng, Response of anaerobic granular sludge to iron oxide nanoparticles and multi-wall carbon nanotubes during beet sugar industrial wastewater treatment, Water Res. 117 (2017) 87–94.

[11] P. Zhang, J. Liu, Y.P. Qu, J. Zhang, Y.J. Zhong, Y.J. Feng, Enhanced performance of microbial fuel cell with a bacteria/multi-walled carbon nanotube hybrid biofilm, J. Power Sources 361 (2017) 318–325.

[12] F. Liu, A.-E. Rotaru, P.M. Shrestha, N.S. Malvankar, K.P. Nevin, D.R. Lovley, Promoting direct interspecies electron transfer with activated carbon, Energy Environ. Sci. 5 (2012) 8982–8989.

[13] D.R. Lovley, Extracellular electron transfer: wires, capacitors, iron lungs, and more, Geobiology 6 (2008) 225–231.

[14] M.V. Copp, C. Leang, S.J. Sandler, D.R. Lovley, Development of a genetic system for Geobacter sulfurreducens, Appl. Environ. Microbiol. 67 (2001) 3180–3187.

[15] S.L. Zheng, H.X. Zhang, Y. Li, H. Zhang, O.M. Wang, J. Zhang, F.H. Liu, Co-occurrence of Methanosarcina mazei and Geobacteraceae in an iron (III)-reducing enrichment culture, Front. Microbiol. 6 (2015).

[16] X.P. Zhu, M.D. Yates, M.C. Hatzell, H.A. Rohn, P.E. Saikaly, B.E. Logan, Microbial community composition is unaffected by anode potential, Environ. Sci. Technol. 48 (2014) 1352–1358.

[17] P. Zhang, S.L. Zheng, J. Liu, R.C. Wang, F.H. Liu, Y.J. Feng, Surface properties of activated sludge-derived biochar determine the facilitating effects on Geobacter cocultures, Water Res. 142 (2018) 441–451.

[18] H.E.F. Zarath, M. Summers, Ching Leang, Ashley E. Franke, Nikhil S. Malvankar, Derek R. Lovley, Direct exchange of electrons within aggregates of an evolved syntrophic coculture of anaerobic bacteria, Science 330 (2010) 3.

[19] P.M. Shrestha, A.-E. Rotaru, M. Aleksijevic, F. Liu, M. Shrestha, Z.M. Summers, N. Malvankar, D.C. Flores, D.R. Lovley, Syntrophic growth with direct interspecies electron transfer in Geobacter sulfurreducens PC-1 cocultures, Front. Microbiol. 9 (2018) 1–6.

[20] O. Wang, S. Zheng, B. Wang, W. Fang, F. Liu, Necessity of electrically conductive pili for methanogenesis with magnetite stimulation, PeerJ 6 (2018), e4541.

[21] G. Martín, A.F. Salvador, L. Pereira, M.M. Alves, Methane production and conductive materials: a critical review, Environ. Sci. Technol. 52 (2018) 10241–10253.

[22] L.-L. Li, Z.-H. Tong, C.-Y. Fang, J. Chu, H.-Q. Yu, Response of anaerobic granular sludge to single-wall carbon nanotube exposure, Water Res. 70 (2015) 1–8.

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electron transfer as the primary mechanism for energy exchange, Environ. Microbiol. Rep. 5 (2013) 904–910.

[20] M.C. Gutiérrez, Z.Y. García-Carvajal, M.J. Hortigüela, L. Yuste, F. Rojo, M.L. Ferrer, F. del Monte, Biocompatible MWCNT scaffolds for immobilization and proliferation of E. coli, J. Mater. Chem. 17 (2007) 2992–2995.

[21] S. Chen, A.E. Rotaru, P.M. Shrestha, N.S. Malvankar, F. Liu, W. Fan, K.P. Nevin, D.R. Lovley, Promoting interspecies electron transfer with biochar, Sci. Rep. 4 (2014) 5019.

[22] S. Chen, A.E. Rotaru, F. Liu, J. Philips, T.L. Woodard, K.P. Nevin, D.R. Lovley, Carbon cloth stimulates direct interspecies electron transfer in syntrophic co-cultures, Bioresour. Technol. 173 (2014) 82–86.