Microbially-reduced graphene scaffolds to facilitate extracellular electron transfer in microbial fuel cells

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A R T I C L E   I N F O

Article history:
Received 25 January 2012
Received in revised form 30 March 2012
Accepted 30 March 2012
Available online 6 April 2012

Keywords:
Microbially-reduced graphene
Extracellular electron transfer
Scaffolds
Microbial fuel cell

A B S T R A C T

A one-pot method is exploited by adding graphene oxide (GO) and acetate into an microbial fuel cell (MFC) in which GO is microbially reduced, leading to in situ construction of a bacteria/graphene network in the anode. The obtained microbially reduced graphene (MRG) exhibits comparable conductivity and physical characteristics to the chemically reduced graphene. Electrochemical measurements reveal that the number of exoelectrogens involved in extracellular electron transfer (EET) to the solid electrode, increases due to the presence of graphene scaffolds, and the EET is facilitated in terms of electron transfer kinetics. As a result, the maximum power density of the MFC is enhanced by 32% (from 1440 to 1905 mW m−2) and the coulombic efficiency is improved by 80% (from 30 to 54%). The results demonstrate that the construction of the bacteria/graphene network is an effective alternative to improve the MFC performance.

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1. Introduction

Microbial fuel cells (MFCs) are energy-producing devices in which electrical energy can be directly generated from oxidation of organic matter through the catalytic activity of microorganisms. Since MFCs are capable of utilizing various organic matters as a fuel in an environmentally-friendly manner, they have attracted a good deal of research attention during the past decades (Logan et al., 2006; Song et al., 2012). Although being a promising biotechnology, the application of MFCs in the real field is currently limited by its small-scale power output, which is mainly resulted from the lack of efficient means for transferring electrons extracellularly from microbes to solid electrode (Peng et al., 2010).

A few microorganisms, such as Shewanella and Geobacter species, have been identified to be capable of transferring electrons to extracellular electron acceptors in the absence of exogenous mediators (Kim et al., 1999; Bond et al., 2002). Three principal extracellular electron transfer (EET) mechanisms have been proposed, including direct electron transfer via bacterial surface redox proteins (Kim et al., 1999), electrically conductive pili (also known as “nanowires”) (Reguera et al., 2005) and indirect electron transfer via electroactive metabolites (Rabaey et al., 2005). EET in a direct process is considered to be more favorable for practical applications due to its higher efficiency and suitability for continuous systems. However, it has been noticed that the EET is dominated by the cells attached directly on the electrode surface rather than planktons (Fan et al., 2008). Thus, the fabrication materials of the electrode play a vital role in affecting bioelectrochemical systems (BESs) performance by determining the actual accessible electrode surface area for exoelectrogens and influencing the interfacial EET between microbes and the electrode (Zhang et al., 2011). Recently, the major efforts for facilitating EET were the use of various materials to modify electrode surface. It has been reported that non-conductive peptide chain would wrap the active sites of out-membrane c-type cytochrome (c-cyts), which strongly hinder direct EET of exoelectrogens towards electrode (Wu et al., 2011). Thus, the purpose of modifying electrodes was to stimulate bioelectrochemical reaction by directing the active center of proteins towards the electrode surface. Nano-structured materials with unique electrical and structural properties, e.g. carbon nanotubes, carbon nanoparticles, palladium nanoparticles, and iron oxide nanoparticles, were found to be efficient modifiers for such function (Liang et al., 2011; Yuan et al., 2011a; Wu et al., 2010; Nakamura et al., 2009).

Graphene, one-atom-thick two-dimensional layer of sp2-bonded carbon, has attracted significant scientific and technological interest because of their unique mechanical, electronic, and thermal properties since their discovery in 2004 (Novoselov et al., 2004). The special properties of graphene provide excellent opportunity to improve the performance of BESs, in which the blocked heterogeneous electron transfer was stimulated or the electron transfer was greatly facilitated with the formed graphene/biomolecules biocomposites (Huang et al., 2011).
Currently, the chemical reduction of graphene oxide (GO) with hydrazine/hydrazine derivatives was widely used to produce graphene. However, great care and caution is always required due to the use of highly toxic and dangerously unstable hydrazine. Very recently, Salas et al. (2010) and Wang et al. (2011) discovered that GO could be microbially reduced to graphene by Shewanella respiration under both anaerobic and aerobic conditions. They had claimed that the direct electron transfer at exoelectrogens/GO interface involved the reduction process. However, very little is understood about the electron transfer process of the bacteria/microbially reduced graphene (MRG) networks, particularly the long-distance EET process stimulated by graphene, and the further application of the resulted material is currently remained blank.

Herein, we presented a simple method to build graphene scaffolds in the anode biofilm via in situ conversion of GO to graphene by the extracellular respiration of bacterial cells. In such a design, GO was microbially reduced to graphene by the anode biofilm, meanwhile, the resulted graphene was assembled automatically onto the biofilm to form a bacteria/MRG network. In the present study, we made a special effort to investigate the EET process of the self-constructed bacteria/MRG network with various electrochemical techniques, including cyclic voltammetry, electrochemical impedance spectroscopy, and Tafel plots. The potential application of the bacteria/MRG network in bioenergy production was investigated with MFC.

2. Methods

2.1. Graphene oxide preparation

GO was prepared from graphite powder based on a modified Hummer’s method (Hummers and Offeman, 1958). Graphite powder (1.0 g) was mixed with 10 mL HNO₃ and 40 mL H₂SO₄ in an ice bath. Then, 3.0 g of KMnO₄ was slowly added to the mixture with stirring. The solution was heated at 35 °C for 3 h, and then diluted with 80 mL of distilled water. The solution was further diluted by slow addition of 200 mL distilled water in 2 h. Thereafter, H₂O₂ (30%, 3.0 mL) was added dropwise into the mixture. The solution was isolated by centrifugation and washed with water till the supernatant became neutral, and then re-suspended in distilled water. The aqueous GO solution was then sonicated for 2 h to facilitate the exfoliation of GO to sheets. Finally, a homogeneous GO aqueous dispersion (1 mg/mL) was obtained and used for the further microbial and chemical reductions.

2.2. Microbial fuel cell construction and operation

Air-cathode single-chamber MFCs with a liquid volume of 10 mL were constructed as previously reported (Yuan et al., 2011b). The cylindrical MFC chamber was made of plexiglass with a length of 1.7 cm and a diameter of 3.0 cm in cathode side and 1.8 cm in anode side. The cathode surface area (7 cm²) was larger than that of the anode (2.5 cm²) in order to minimize the effect of cathode size on energy output. As described by Zhang et al. (2009), the cathodic Pt catalyst (20% Pt/C, E-Tek) was brush-coated onto a piece of carbon membrane (type A) at a loading of 0.5 mg/cm². The coated carbon cloth was uni-axially hot-pressed onto one side of the proton exchange membrane (CEM, Zhejiang Qianqiu Group Co., Ltd., China), forming a membrane cathode assembly (MCA). The MCA was then assembled on the MFC reactor. The side coated with catalyst was exposed to air to avoid the possible effect of graphene on the cathode.

MFC reactors were inoculated with 2.0 mL activated anaerobic sludge (Liede Sewage Treatment Plant, Guangzhou, China) and 10 mL sodium acetate (1000 mg L⁻¹) culture medium solution. The culture medium solution contained: NaH₂PO₄·2H₂O (2.77 g L⁻¹), Na₂HPO₄·12H₂O (11.40 g L⁻¹), NH₄Cl (0.31 g L⁻¹), KCl (0.13 g L⁻¹), a vitamin stock solution (12.5 mL L⁻¹) and a mineral stock solution (12.5 mL L⁻¹). Power density curves were obtained by changing the circuit resistor from 5000 Ω to 50 Ω, with a single resistor used for a full batch cycle. All tests were conducted in batch mode in a 30 °C incubator. The power was normalized by the projected surface area of the anode. All tests were conducted in duplicate, and mean values are presented.

2.3. Microbial reduction of GO and formation of the bacteria/MRG network

A one-pot method was employed to reduce GO and decorate the anode. The operation was as follows: a fresh acetate medium amended with a milliliter of GO solution was used to replace the depleted substrate after the voltage outputs from the well operated MFCs were below 50 mV at 1000 Ω. When the solution in the reactor turned black, it was again replaced by a fresh acetate medium without GO. The resulted black species were used for further analysis. For control, GO was also chemically reduced to graphene as suggested by Tang et al. (2009).

2.4. Characteristics of MRG

Scanning electron microscopy (SEM) was applied to confirm the decoration of the anode biofilm by MRG. Prior to SEM measurements, the anode samples were fixed in 2.5% glutaraldehyde solution for 1 h, followed by an ethanol dehydration series (i.e., 25%, 50%, 75%, and 100% v/v EtOH, 0.5 h each treatment), and then dried at CO₂-critical point for 3 h. The resultant specimens were coated with gold using a coating device (Emitech K550X; UK) and observed under SEM (JEOL JSM-6330F; Japan) at 20 kV. As a comparison, the SEM image of the chemically-reduced graphene was also taken by being brush-coated on the carbon cloth. For Raman spectroscopy, the MRG suspension was spread on a class substrate and dried in vacuum. Raman spectra were collected with a Niclet Almega XR dispersive Raman spectrometer (laser wavelength 488 nm).

2.5. Electrochemical measurements

Cyclic voltammograms (CVs) of the anodic biofilms were carried out with a CHI660D system (CH Instruments, Inc.) in a three-electrode conventional cell. The MFC anode was kept as the working electrode, saturated calomel electrode (SCE) as the reference electrode, and the cathode as the counter electrode, respectively. For the electrochemical measurements of the biofilms in the absence of acetate, the electrolyte was not amended with nutrition and mineral solution to avoid interference. Potentiodynamic polarization (Tafel plots) of the anode biofilms were performed from −0.8 to −0.4 V at 0.5 mV s⁻¹ (vs. SCE). All measurements were conducted at 30 °C in duplicate and the typical curves are presented. Electrochemical impedance spectroscopy (EIS) was employed to analyze anode resistance of the MFCs from 1 × 10⁵ to 0.01 Hz. Impedance measurements were performed using a potentiotstat (Autolab PGSTAT 30, ECO CHEM) at open-circuit potential with a sinusoidal perturbation of 5 mV amplitude. Data from EIS were obtained as Nyquist plots and fitted to an equivalent electrical circuit using Autolab impedance analysis software (FRA, Eco Chemie, The Netherlands). Before starting each impedance measurement, the MFC was operated with a 1000 Ω external load discharging for over 1 h and then pre-polarized at 300 mV for at least 15 min to reach the static state.

The conductivity of MRG was characterized using electrochemical methods. MRG or GO was coated on a glassy carbon electrode (GCE) with a loading of ca. 0.5 mg cm⁻². Prior to the coating, the
GCE was firstly polished to form a mirror surface with alumina slurry and then sonicated in water and ethanol each for several minutes. CV and EIS were performed to evaluate the electrical conductivity of the MRG. CV and EIS were carried out in a 0.1 M KCl solution containing 2.5 mM K$_3$[Fe(CN)$_6$]/K$_4$[Fe(CN)$_6$] (1:1) as the redox probe.

3. Results and discussion

3.1. Formation and characterization of the bacteria/MRG network

A one-pot method for GO reduction and decoration of the biofilm in the anode was employed. Fig. S1A shows a scheme of the bacteria/MRG network formation in the anode biofilm and the possible EET processes was. In such a design, GO was randomly inserted into the biofilm, meanwhile, GO was reduced to graphene mediated by microbial respiration of the biofilm. Wang et al. (2011) demonstrated that both direct electron transfer and electron mediators were involved in the GO reduction, which was similar to that in microbial iron(III) reduction. To verify the formation of the bacteria/MRG network, SEM was performed to characterize the morphology of the anode surface. Rod-type microorganisms were attached upon the surface of carbon cloth before the modification (Fig. S1B). After 48-h incubation with GO and sodium acetate solution, the biofilm closely associated with the MRG that consists of randomly aggregated, thin, crumpled sheets with a worm-like appearance (Fig. S1C). The appearance was exactly similar to the chemically-reduced graphene (CRG) as shown in Fig. S1D.

It has been reported that GO and reduced GO exhibit distinctive G bands, located at ~1593 and 1580 cm$^{-1}$, respectively, on the Raman spectroscopy (Zhu et al., 2010). Fig. S2A shows the Raman spectra collected from GO, MRG, and CRG. In the Raman spectrum of GO, two distinctive peaks at ~1351 and 1600 cm$^{-1}$ were appeared, corresponding to the D and G bands of in-phase vibration of graphite lattice. As expected, both MRG and CRG also showed the two peaks, but an apparent red shift of the G band from ~1600 cm$^{-1}$ to ~1580 cm$^{-1}$ was observed, suggesting the transition from GO to graphene as mediated by anodic bacteria or chemical reagents. Salas et al. (2010) discovered that the electrochemical activity of MRG was comparable to those forms of CRG. The electrochemical conductance of the resulted MRG in the anode was also confirmed by electrochemical measurements. Fig. S2B shows the voltammograms recorded with bare GCE, GO/GCE, and MRG/GCE in the absence and presence of 2.5 mM potassium ferricyanide/2.5 mM potassium ferrocyanide couple. In the absence of the redox couple, the CV at MRG/GCE showed the highest background current, and followed by the GCE and GO/GCE. The increased background current was likely due to the increase of the surface area after the coating of nano-structural MRG. The low background current of the GO/GCE was probably because of the poor conductivity and the negatively charged oxygen containing moieties of GO. The similar trend was discovered from the CVs in the presence of ferricyanide/ferrocyanide couple. The anodic peak current (I$_{pa}$) and the cathodic peak current (I$_{pc}$) observed on GO/GCE (I$_{pa}$ = 9.7 μA and I$_{pc}$ = 11.5 μA) are significantly lower than that with bare GCE (I$_{pa}$ = 22.9 μA and I$_{pc}$ = 24.2 μA), while the MRG/GCE (I$_{pa}$ = 34.8 μA and I$_{pc}$ = 32.5 μA) showed higher redox peak currents than that on bare GCE. The result demonstrated that MRG had the highest conductivity. EIS is another decisive and directive parameter to reflect the changes of conductive features of electrode/electrolyte interface (Guo et al., 2009). As shown in Fig. S2C, the Nyquist plot of EIS measurements shows typical semicircles in the high-frequency region. When GO is coated on the GCE surface, the semicircle was quite large compared with the MRG/GCE. This suggested that the attachment of GO upon the electrode might have a blocking effect and inhibit the charge transfer due to its low conductivity, whereas the MRG could accelerate electron transfer between the electrochemical probe and the electrode due to the increased surface area and the high conductivity.

3.2. Extracellular electron transfer of the bacteria/MRG network

To investigate the influence of MRG on the EET of the electrochemically active biofilm, we carried out electrochemical measurements as suggested by Yuan et al. (2011b) and Fricke et al. (2008). As depicted in Fig. 1, MRG shows the apparent effect on CVs of the biofilm in both turnover and non-turnover states. Two couples of redox peaks with formal potentials of ~0.30 and ~0.38 V (vs. SCE) appeared in the CVs. It was worth noting that the voltammetric behavior of the acetate-enriched biofilm was very similar to that of Geobacter sulfurreducens biofilm reported in literatures (Fricke et al., 2008). These two couples of redox peaks ascribed to OmcB (outer membrane c-type cytochrome B) and OmcZ (outer membrane c-type cytochrome Z), which mediated EET of exoelectrogen to solid electrode in BESs (Liu et al., 2008). We found that the peak currents were slightly increased after the biofilm was associated with MRG in the non-turnover state. For example, the anodic peak current at the potential of ~0.27 V (vs. SCE) increased from 0.02 to 0.04 mA cm$^{-2}$. This confirmed that the presence of MRG might stimulate the electron transfer of electrochemical active species whose electron transfer was blocked due to the long distance from the electrode surface as illuminated in Fig. S1A. As suggested by Jain et al. (2011), the anode biofilm could be subdivided into three regions, including electrochemically active inner core, electron acceptor limitation zone, and metabolically inactive zone. The electron transfer process of the biofilms was mostly contributed from the inner core and partially from the intermediate zone, whereas very little from the top layers. Fig. 1B shows
slow-scan CVs of biofilms in the presence of acetate. Sigmoidal CVs were observed, demonstrating the catalytic oxidation of acetate by these two biofilms. However, catalytic currents were varied from each other, and the catalytic current from the MRG-biofilm was significantly higher than that from the O-biofilm. OmcZ has been confirmed to play an extremely important role for mediating the electron transfer from bacterial cells to electrode, while pili networks are essential for the long-distance electron transfer of bacterial cells in biofilm (Lovley and Nevin, 2011). In our study, the conductive bacterial pili was invisible, which suggested that the short-distance electron transfer mediated by the surface accumulated OmcZ must have the most contribution for current generation. As expected, MRG was produced in the anode and randomly embedded into the biofilm to form a 3D-like network of bacteria/MRG, in which the top layers of bacterial cells were also accessible for EET from microbes to electrode through the MRG scaffolds (See Fig. S1A). As a result, the long-distance electron transfer became possible via the wiring of these graphene scaffolds, same as the network of bacterial pili. In addition, the porous 3D structure provided by the MRG-biofilm composites was favorable for substrate transport in the biofilm, resulting in an increased anolyte-biofilm-anode interfacial area (Xie et al., 2012).

3.3. Electron transfer kinetics of the bacteria/MRG network

We found that the increase of current resulted not only from the graphene mediated long-distance EET (from the intermediate or top layers of the biofilm to electrode), but also from the facilitation of the electron transfer. As shown in Fig. 1A, the oxidative/reductive peak separations of the MRG-biofilm were obviously smaller than those of the O-biofilm, suggesting that the presence of graphene scaffold in the anode biofilm could also improve the kinetics of electron transfer between cell surface proteins and electrode. To further elucidate the roles of the MRG in the EET process, the electron transfer kinetics of the bacteria/MRG network were studied in terms of electron transfer rate from bacterial cells to the electrodes, exchange current densities and electrochemical impedances. First, CVs with varied scan rates were carried out to get an insight into the dependence of the peak currents on the scan rates. Fig. S3A–C display that the peak currents of biofilms increased linearly with increasing the scan rates, indicating typical surface-controlled electrochemical processes as expected for immobilized biofilms. Then, the dependence of redox species’ peak potential on the scan rates was investigated by plotting ($E_p - E^0$) against $\ln v$ (Fig. S3D). According to Laviron theory, the apparent electron transfer rate constant ($k_{app}$) can be determined by the extrapolated intercept at $E = 0$ which is a measure of the maximum current that can be extracted at negligible polarization) and the transfer coefficient (from the slope), were obtained (Raghavulu et al., 2012). As shown in Fig 2A, the polarization behaviors of the biofilms revealed that the $i_0$ of the MRG-biofilm had a higher value of 7.6 $\mu$A m$^{-2}$, whereas the O-biofilm only exhibited a value of 3.4 $\mu$A m$^{-2}$. In terms of anodic electron transfer coefficient ($b_a$), the MRG-biofilm showed a lower value than the O-biofilm (Table 1). The electron transfer will be facilitated when $b_a$ is low and $i_0$ is high. Based on these results, the MRG-biofilm exhibited facile electron transfer kinetics compared with the O-biofilm. The similar changes in the kinetics parameters of anodic EET were previously observed with addition of graphite flakes to lake bed sediment in a sediment-type MFC (Babu and Mohan, 2012). Finally, the anode impedances were analyzed using EIS (Fig. 2B). The ohmic resistances and charge transfer resistances of the MRG-biofilm anode were lower than those of the O-biofilm (Table 1). A large potential gradient could be caused by the low biofilm conductivity, which consequently resulted in an increase in potential loss and slowed the kinetics (Torres et al., 2008). The decrease in ohmic resistance suggested that the biofilm conductivity was improved due to the presence of graphene scaffolds, same as the role of pili nanowires of exoelectrogens for enhancing the conductivity of biofilm. Furthermore, the significant difference in the charge-transfer resistance also implied the facile electron transfer pathway between exoelectrogens and the electrode in the presence graphene scaffold. The similar results

![Fig. 2.](image)
had been discovered in other carbon nanomaterials associated bacterial composites, such as carbon nanotubes and carbon nanoparticles (Liang et al., 2011; Yuan et al., 2011a).

### 3.4. Enhanced performances of MFCs with the bacteria/MRG network

As an example of application, we examined the performance of an MFC using the MRG-biofilm anode. The reduction of GO by the anode biofilm was well reflected by the voltage versus time curve. As shown in Fig. 3A, with a 1000 Ω external resistor, the voltage output of the original MFC was 420 mV, while it was decreased to 86 mV after GO was added into the anode chamber. It was the reason that electrons produced by anode biofilm was directly consumed by GO for graphene reduction. It should be noted that two electron acceptors, GO and oxygen, were coexisted in such a process. GO seemed to be the favorable electron acceptors compared with oxygen in this case, concluding from the significant voltage decay. Theoretically, oxygen is a thermodynamically more feasible electron acceptor than GO, however, the electron delivery to oxygen was limited by the external resistance (Raghavulu et al., 2011), which made the GO reduction possible in such a system. The reduction of GO to graphene was also evidenced by the color change of the solution (from brown to dark, as shown in the inset graph of the Fig. 3A). After the complete reduction of GO, the anodic solution was replaced by the fresh culture medium in the absence of GO. Surprisingly, the voltage output of the MFC started to recover. After two cycles of substrate replacement, the voltage reached 478 mV. For better evaluating the enhanced performance of MFCs after biofilm decoration with MRG, the power density versus current density curves were examined as well. As shown in Fig. 3B, the maximum power density produced from the MFC with the MRG-modified anode was 1905 ± 80 mW m⁻², which was higher than that from the original MFC (1440 ± 52 mW m⁻²). Meanwhile, the columnic efficiency (CE) increased from 30% to 54% after the modification. The results agreed with the findings of the facilitated EET process of the graphene-biofilm, and further supported the fact that the introduction of the MRG in the biofilm could enhance the performance of the BESs.

### 4. Conclusions

A graphene-bacterial network was fabricated by a simple approach by which the graphene oxide was microbially reduced to graphene via bacterial respiration and the graphene scaffolds were self-assembled in the biofilm. As a result of the increased actual accessible active exoelectrogens and accelerated EET kinetics, the MRG-biofilm showed apparent enhancement of the catalytic activity towards acetate oxidation. Applying the MRG modification in MFCs, the maximum power density and columnic efficiency was increased by 32% and 80%, respectively. The findings provided a potential alternative to increase MFC energy output.

### Acknowledgements

This study was supported by the National Nature Science Foundation of China (41101211, 41171205, and 21177030), the Nature Science Foundation of Guangdong Province, China (104510 65003005012), and The Team Project of Guangdong Natural Science Foundation (S201103002882).

### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biotech.2012.03.118.

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