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Cite this: DOI: 10.1039/c0xx00000x

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Communication

Microbial Oxidation of Graphite by *Acidithiobacillus ferrooxidans* CFMI-1

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Graphite oxide was prepared by a simple and environment-friendly bio-oxidation strategy using *Acidithiobacillus ferrooxidans* CFMI-1 as bacteria. The obtained graphite oxide nanosheets have few layers with 1.5-1.7 nm (about 3-4 layers sheets) height and 150-900 nm size.

Since its discovery in 2004, graphene has attracted a great deal of attention for fundamental studies as well as for potential applications[1,2]. As an important precursor of chemically converted graphene, graphene oxide has recently been widely studied for the applications in polymer composites, catalysts, sensors, actuators, energy conversion and storage [3].

The synthesis of graphite oxide was firstly explored by Brodie in 1859 by adding "potash of chlorate'' (potassium chlorate) to a slurry of graphite in fuming nitric acid[4]. In 1898, Staudenmaier improved Brodie's method by adding the chlorate in multiple aliquots during the course of the reaction[5]. This slight change in the procedure led to the formation of highly oxidized graphite oxide in a single reaction vessel. In 1958, Hummers developed another oxidation approach by reacting graphite with a mixture of potassium permanganate and sodium nitrate in concentrated sulfuric acid[6]. However, all three procedures inevitably generate toxic and dangerous gases, such as nitric oxides, and chlorine dioxide[7].

Recently, Tour's group at Rice University reported a new process using flakes of graphite, which is treated with potassium permanganate, sulfuric acid, and phosphoric acid[8]. This method is suited for mass production of graphene oxide without generating any gas as by-products. In 2010, Tour's group also reported that the bacteria from the common bacteria genus *Shewanella* could easily reduce graphite oxide into graphene, which then arranges itself into graphite[9]. This discovery inspires further investigation in the area of green nanochemistries. In 2013, our group firstly reported graphite bio-oxidation by nitrifying bacterial 2011.2 under aerobic conditions. We have demonstrated that the cells possess the ability to exfoliate and bio-oxidize a fraction of graphite.[10]

Here, a straight-forward and environment-friendly bio-oxidation strategy was developed to fabricate graphite oxide using *Acidithiobacillus ferrooxidans* CFMI-1 as bacteria (Supplementary Information, Figure S-1 and S-2). *Acidithiobacillus ferrooxidans* is the most widely used microorganism in the bioleaching of several sulfide minerals because of its ability to oxidize Fe^{2+} ions, elemental sulfur, hydrogen[11] and hydrogen sulfide[12-14] in acidic solution. The results show that the graphite oxide from our approach is low degree of oxidation. However, the bio-oxidation of graphite is a weak oxidation process compared to chemistry oxidation method. Thus, the great challenge is that we need bacteria of very strong oxidation ability, so that even chemically inert graphite can be oxidized.

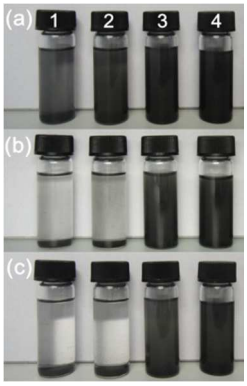

Figure 1. Digital pictures of as-prepared graphite oxide dispersed in ethanol. ((a)=1 hour after sonic dispersion; (b)=1 day; (c)=3 days. Bottles 1=blank control; 2=oxidation once; 3=oxidation of 3 times; 4=oxidation of 4 times.)

As shown in Figure 1, the purified biologically converted graphite oxide (BCGO) and blank control samples were collected and dispersed homogeneously in ethanol (Figure 1). The parts (b) and (c) of Figure 1 show the long term-stability of BCGO in ethanol solvents. After 3 days, two samples which were oxidized 3 and 4 times still showed a very good dispersion state, whereas the blank control and the oxidation once samples had low dispersibility. Good dispersion states of oxidation 3 and 4 times

samples are probably due to the microbial oxidation of graphite by *Acidithiobacillus ferrooxidans* CFMI-1.

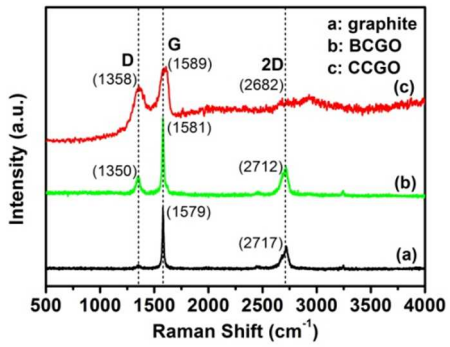

Figure 2. Raman spectra of the graphite, BCGO and CCGO.

Raman spectroscopy is one of the most sensitive and informative techniques for the characterization of carbon materials. Figure 2 shows the Raman spectra of the pure graphite, chemical converted graphene oxide (CCGO) and biologically converted graphite oxide (BCGO). As displayed in Figure 2(b) and (c), the G and D bands are assigned to the graphitized structure and local defects/disorders, respectively.[15] The peaks centered at ca. 1579 and 2717 cm^{-1} are attributed to the G and 2D bands, respectively, of pure graphite (Figure 2(a)). In comparison, the spectrum of the CCGO or BCGO shows a defect-induced mode D peak at 1350 cm^{-1}, indicating that the bacterial *Acidithiobacillus ferrooxidans* CFMI-1 has the ability to oxidize the graphite. The full width at half maximum of G band of BCGO (18.3 cm^{-1}) is narrower than that of CCGO (about 80 cm^{-1}), suggesting a lower level of disorder of the graphite oxide and a longer in-plane correlation length of graphite during the bio-oxidation process.[15, 16] Comparing with the pure graphite (I(D)/I(G) ratio is 0.05, Table S-1), the I(D)/I(G) intensity ratio (Supplementary Information, Table S-1) of BCGO is increased to 0.23, which suggests a possible decrease in the average size of the in-plane sp^2 domains.[17, 18] In addition, we see that the intensity of I(D)/I(G) ratio support our idea that the extent of oxidation of BCGO is much less than that of CCGO, reflecting that BCGO has less disordered carbon atoms and the bio-oxidation is a weaker oxidation process compared to chemistry oxidation method. This also can be attributed to the formation of further sp^2 bonds in the sheets and appearance of vicious structure such as bond-angle and edge defects.[18] Moreover, the spectrum of BCGO exhibits a 2D-band at 2712 cm^{-1}(5 cm^{-1} shift down to graphite), indicating that its number of layers of BCGO is much less than that of graphite[19].

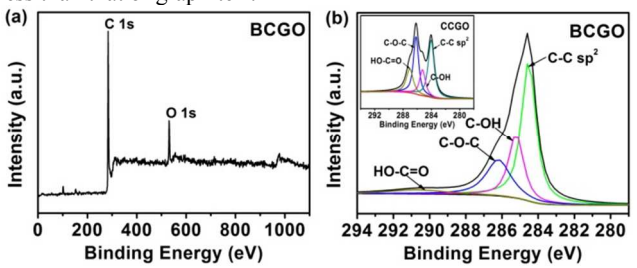

Figure 3. Wide (A) and deconvoluted (B) XPS spectra of the as-prepared BCGO. The inset in (B) is C 1s XPS spectra of CCGO.

X-ray photoelectron spectra (XPS) is also a useful technique for the elemental surface detection of variations in chemical composition and oxidation state. The XPS spectra of BCGO are shown in Figure 3. The XPS wide-scan spectrum shows the compositional elements of the sample (Fig. 3a). It can be seen that the O1s peak is apparent after incubation with bacterial. Fig. 3b shows the high-resolution XPS spectra for C 1s of BCGO. The binding energies at 290.60, 286.19, 285.22, and 284.52 eV were assigned to the C=O, C-O-C, C-OH, and C=C bonds, respectively. The XPS peak area ratios of the C=O, C-O-C and C-OH bonds to the C=C bond were calculated and listed in Table S-2. Comparing with the C 1s XPS spectra of CCGO (inset), the intensity of oxygen-containing groups (HO-C=O, C-O-C and C-OH) in the spectra of BCGO are very low. With the increase of repeated microbial oxidation times, the oxygen atom content of BCGO (bio-oxidation of 3 times) increased to 7.2% (atom %, Table S-3). These results further confirmed that the graphite has been oxidized by bacteria and the BCGO is mildly oxidized.

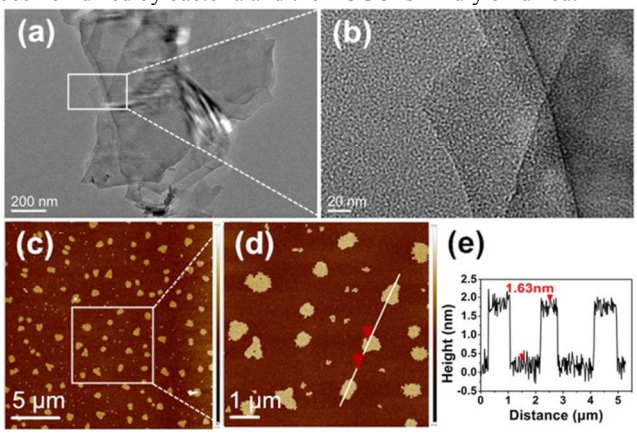

Figure 4. Typical HR-TEM (a, b) and AFM (c, d, e) images of BCGO.

The morphology and microstructure of the BCGO were investigated by FE-TEM, AFM, FE-SEM and microscope. As shown in Fig. 4(a-b) and Fig. S-3 (In the Supplementary Information), it can be clearly seen that the BCGO nanosheets are exfoliated. These pictures indicate that the BCGO samples had few-layers thickness. In Fig. 4(b), one graphene sheet was clearly displayed with several layers. A dilute suspension of BCGO was deposited onto a mica substrate and analyzed by atomic force microscopy (AFM). As shown in Fig. 4(d) and (e), the cross section analysis shows a background height of 1.63 nm which corresponds to the thickness of the 3-4 layer sheets. These nanosheets possess the average size of around 800 nm. Also, many smaller nanosheets are found in Figure 4(c) and (d), which possess the size of 150-300 nm. We have proved that the chemical composition of BCGO contains only carbon and oxygen elements in Fig. 3(a) and (b). These nanosheets probably results from the microbial oxidation or bio-eroding of pure graphite sheets by *Acidithiobacillus ferrooxidans* CFMI-1. Therefore, the BCGO samples contain graphene nanosheets with the size of 150-900 nm. These small size of BCGO consistent with those of FESEM images (Figure 5) may be caused by bio-oxidization to a certain extent. As shown in Figure 5, the surface morphology of pure graphite shows nubbly and heterogeneous distribution (Fig. 5(a)) but fragmentized and ruleless for that of BCGO (Fig. 5(b)), suggesting that the size of BCGO oxidized by *Acidithiobacillus ferrooxidans* CFMI-1 is minor-sized to that of pure graphite. Moreover, the wrinkled (Figure 5 (c)) and semitransparent thin sheets (Figure 5(d)) were observed in the direction of the arrows,

indicating that the stacking graphite powder was significantly oxidized and exfoliated which was confirmed in Figure 3 and Figure 4.

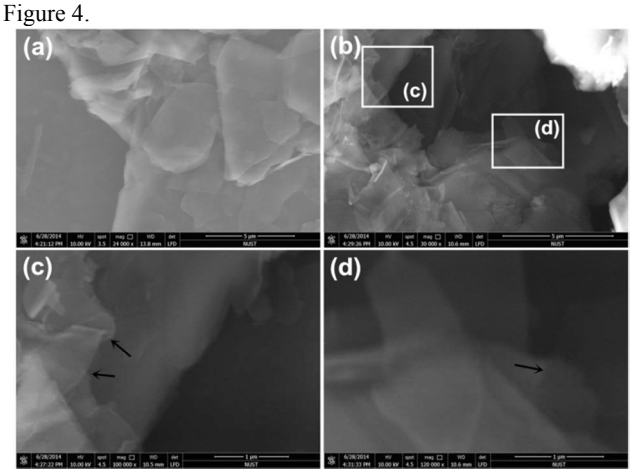

Figure 5. FE-SEM images of pure graphite powder(a) and BCGO (b, c, d).

In order to further exhibit sheet structure and observe the content and distribution of C and O, FE-TEM image and EDX analysis (Figure 6.) were accomplished. The images of Figure 6(b) and (c) indicate that the whole basal plane of BCGO sheets contain a large amount of C and O element with a uniform distribution density, evident of a facile oxidation reaction during the bio-oxidation of Fe^{2+} process by *Acidithiobacillus ferrooxidans* CFMI-1. The oxygen content of BCGO is distinctly lower than that of C, suggesting that the graphite oxidation extent is weak. The EDX spectrum (Figure S-4) is also consistent with XPS (Figure 3.), which confirmed that the graphite has been oxidized mildly by *Acidithiobacillus ferrooxidans* CFMI-1.

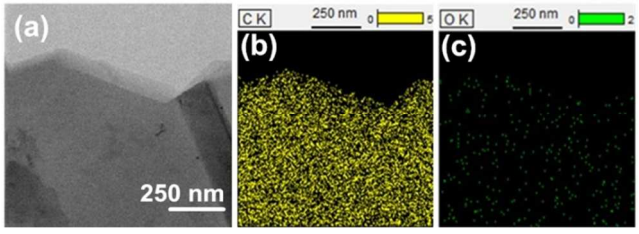

Figure 6. EDX analysis of BCGO, FE-TEM image (a) and mapping of C (b) and mapping of O (c).

To further investigate the effect of graphite on Fe^{2+} oxidation by *Acidithiobacillus ferrooxidans* CFMI-1, the concentrations of various valence of Fe in the 9K medium with and without graphite were measured at different oxidation time points. As shown in Figure S-5, the concentration of Fe^{2+} in the sample 9K medium declined faster than that of blank control without graphite. It means that the bacterial growth is not affected by the presence of pure graphite under the natural culture conditions. There may be two possible reasons in the microbial oxidation process. Firstly, the bacterial *Acidithiobacillus ferrooxidans* CFMI-1 got used to the 9K medium in the presence of graphite after two months of acclimation. Secondly, we are not excluding the possibility that the bacterial may utilize or react with the graphite in some unknown way. Therefore, further work is needed to understand the bio-oxidation of graphite by *Acidithiobacillus ferrooxidans*.

In this work, it is shown that the bacterial *Acidithiobacillus ferrooxidans* CFMI-1 not only can mildly oxidize the natural pure graphite, but also it can bio-erode the graphite and produced many few layer nanosheets during the process of bio-oxidation. In the early studies,[20, 21] *Acidithiobacillus ferrooxidans* was also proved to be the most widely used microorganism in the bioleaching of several sulfide minerals. Importantly, relative roles of direct and indirect bioleaching mechanisms in the presence of *A. ferrooxidans* have been reported.[20] Recently, several studies reported structural degradation of carbon nanomaterials using the peroxidase family of enzymes, such as lignin peroxidase (LiP), horseradish peroxidases (HRP) and myeloperoxidases (MPO).[22-26] These studies may provide a perspective on the process of oxidation of microbes to pure graphite powder. Herein, we propose a possible mechanism of microbial oxidation of graphite by *Acidithiobacillus ferrooxidans* CFMI-1 (Figure S-6) according to the literature.[27-29] The oxidation of graphite and Fe^{2+} as well as the reduction of oxygen occurred in periplasm space, rusticyanin as the first electronic receptor maybe afford another passage for electrons and very two electrons transferred could obtain a molecular ATP. In order to reveal the oxidation mechanisms, further efforts should be concentrated on the exploration of extracellular electron transfer pathways and bio-eroding mechanism for graphite oxidation.

Conclusions

In summary, we developed a straight-forward and environment-friendly bio-oxidation strategy to fabricate graphite oxide in large scale for the first time. Using *Acidithiobacillus ferrooxidans* CMFI-1 as oxidizing bacteria, graphite has been successfully bio-oxidized to the graphite oxide. Compared to chemistry oxidation method, the biological oxidation is milder and less destruction to the original graphite. The bacterial converted BCGO contains the few-layer nanosheets with the size of 150-900 nm.

Acknowledgments

We thank Gaoquan Shi and Baojin Zhou for critical reading of the manuscript. This work was supported by "National Natural Science Foundation of China (No. 51272106) ", "Research Fund for the Doctoral Program of Higher Education of China (RFDP) (No.20123219110015)", "The Fundamental Research Funds for the Central Universities (No. 30920130121001)" and "A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD, China)".

Notes and references

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† Electronic Supplementary Information (ESI) available: [Experimental section and characterization details. AFM images of *Acidithiobacillus ferrooxidans* CFMI-1, microscope photographs of graphite during the microbial oxidation, variation of concentration of Fe during the oxdation process, the proposed mechanism of microbial oxidation of graphite and the XPS data of C1s of the samples]. See DOI: 10.1039/b000000x/

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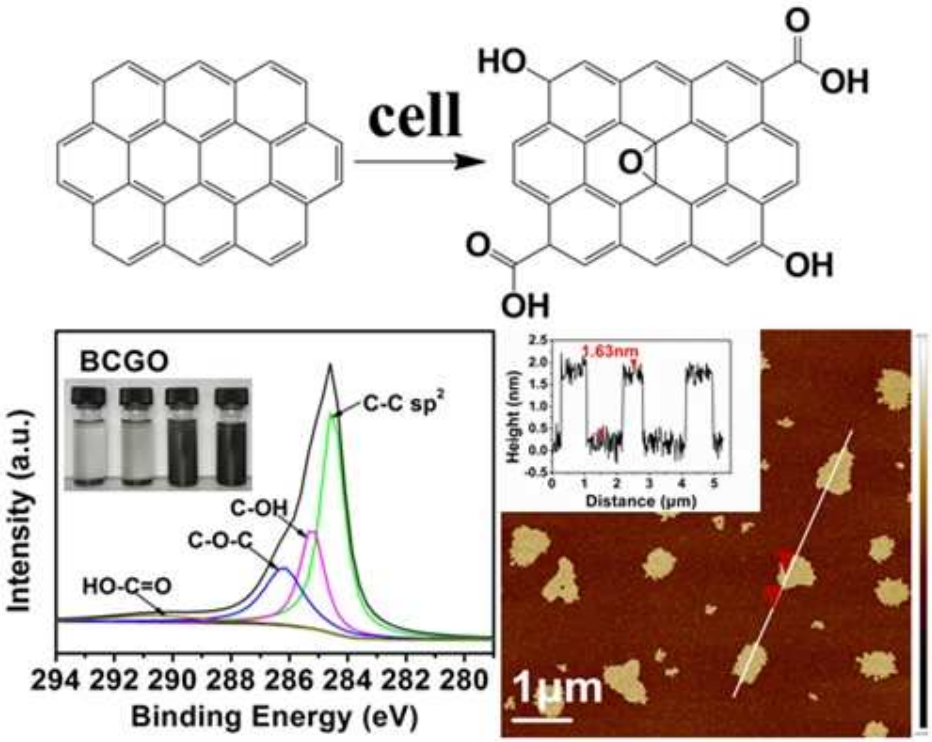
Graphical Abstract



A simple and environment-friendly bio-oxidation approach to produce graphite oxide nanosheets is described.