Functional Protein Coatings

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Interfacial Engineering by Proteins: Exfoliation and Functionalization of Graphene by Hydrophobins**

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Graphene has attracted vast interest as a new material with many uses.[1,2] Two-dimensional, crystalline graphene has many advantageous properties, such as extremely high electric[3] and thermal[4] conductivity, high strength,[5] and a large surface area.[6] Many more useful properties can result from graphene assemblies and modification by different functionalities or additional molecules. One of the usual ways to functionalize graphene is chemical modification;[7] however, attempts to modify the surface of graphene in a noncovalent, nondestructive way have also been successful. These methods typically involve the buildup of charge on the graphene surface to enable the stabilization and assembly of the graphene sheets on the basis of electrostatic interactions.[8] In a further step towards more complex functionalities, we have now modified graphene with more specifically interacting coatings consisting of biomolecules.

One of the main challenges in the production of graphene is the scalable, controllable, and safe processing and handling of individual graphene sheets. Methods for the fabrication of graphene in a dry environment include the micromechanical cleavage of graphene sheets from graphite[9] and the epitaxial growth of graphene on certain substrates.[10,11] By these methods, very large entities of single-layer graphene can be produced, but the scalability and handling problems remain. High-yielding solution-based chemical methods[12] that enable the handling of graphene in dispersed form have been proposed; however, they involve the direct oxidation[13] of graphene, which may lower the conductivity of graphene dramatically. Recent reports on the exfoliation of graphene either in pure solvents[14] or in the presence of surfactants[15] offer promise for the production of graphene. The main benefits of solution methods are the better processability and increased safety of graphene when it is dispersed in a liquid instead of being used as a dry powder. The dispersion of graphene into aqueous solutions is especially attractive because of their nonvolatile nature.

Herein, we present a method for the exfoliation and functionalization of graphene sheets by an amphiphilic protein. It is known that a microbial adhesion protein, HFBI (Figure 1a),[16] which belongs to a class of proteins called hydrophobins,[17] interacts strongly with hydrophobic surfaces, such as graphite and silicon.[18] The protein has a strongly cross-linked fold containing four disulfide bridges. Its most striking feature is a patch of hydrophobic residues on one face of its structure. Thus, the protein resembles a typical surfactant with a hydrophilic and a hydrophobic part. In solution, hydrophobic interactions between individual proteins lead to the formation of dimers or tetramers.[20] In the vicinity of the interface between water and air, however, assembly of the protein at the interface is strongly preferred, and the protein crystallizes as a 2D lattice.[21] Lateral interactions between surface proteins at interfaces may lead

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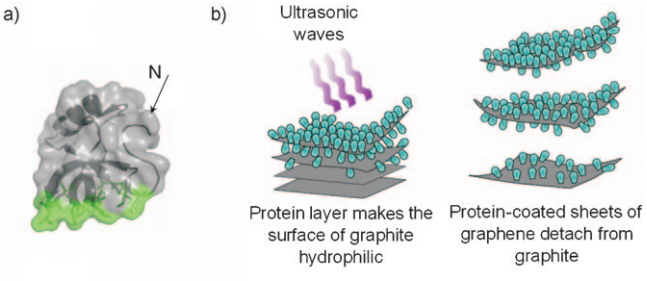

Figure 1. a) Structure of the HFBI protein.[19] The molecule has a well-defined structure with aliphatic hydrophobic side chains exposed on one face of the surface (green patch), over an area that accounts for 19% of the total surface area. The diameter of the molecule is about 2 nm, and the molecular weight is 7.3 kDa. The N terminus, to which sequences were added in the engineered variants, is indicated by an arrow. b) HFBI-facilitated exfoliation of graphene. In water, a monolayer of amphiphilic HFBI is spontaneously adsorbed on the hydrophobic surface of graphite. This process leads to a lowering of the surface energy, and improved contact between water and graphite. The detachment of graphene and ultrathin graphite sheets coated with HFBI occurs when the HFBI-modified graphite stack is disturbed with ultrasonic waves.

to strongly coherent adsorbed surface films. This behavior can be used to modify the wetting properties of hydrophobic materials, such as carbon nanotubes.[22] Lowering of the high surface tension between water and the surface of graphite has been shown to facilitate the exfoliation of graphene.[14,15] Since amphiphilic hydrophobin forms a monolayer whose sides have significantly different surface energies, the mixing enthalpy of graphene flakes coated with hydrophobin can be expressed in terms of the surface-energy differences between graphene and the hydrophobic side of the protein, and the solvent and hydrophilic side of the protein (see the Supporting Information). As these energy differences are smaller than those of a noncoated system, the adsorption of hydrophobin on the surface of graphene is expected to lead to a stable graphene dispersion.

The sonication of mixtures of HFBI and graphite produces thin graphene-like hybrid structures. The use of genetically engineered HFBI should enable specific modification of the graphene surface with both biological and nonbiological functionalities, including molecules capable of specific recognition or actuated responses. The possibility of using a layer of adsorbed molecules to affect the electrical properties of graphene makes this hybrid material especially attractive for electronic and sensor applications.[8]

Graphene exfoliation was carried out by exposing mixtures of HFBI protein and graphite to ultrasonic waves. Through opening of the basal planes and stabilization of the individual sheets (Figure 1b), this process resulted in mixtures of graphite and graphene flakes with a variety of thicknesses. Some examples of bright-field TEM images of exfoliated graphite/graphene sheets are shown in Figure 2a,b,d. Pillars of highly oriented pyrolytic graphite (HOPG) with a round shape were also used in the ultrasonic-bath exfoliation to examine the effect of the ultrasonic waves on the shape of the resulting flakes. The HOPG pillars before treatment, and an example of a resulting thin, perfectly round flake, are shown in Figure 2c,d, respectively.

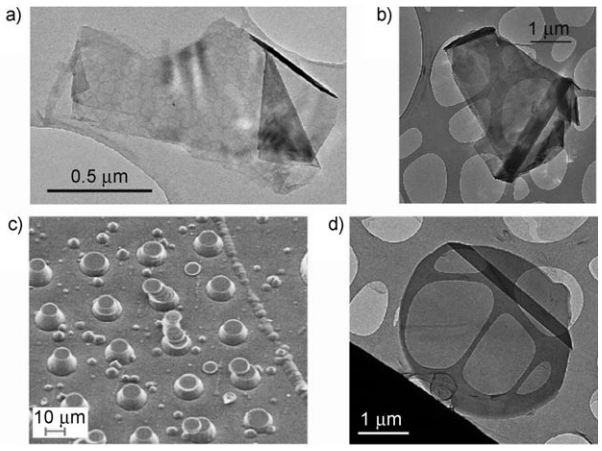

Figure 2. TEM micrographs of exfoliated thin graphene/graphite flakes. a) TEM image of a flake exfoliated from Kish graphite with HFBI by sonication for 2 min with an ultrasonic probe. The holey, networklike protein film on the graphene flake is barely visible. b) TEM image of a flake exfoliated from Kish graphite with HFBI by sonication for 40 min in an ultrasonic bath. c) SEM image of lithographically formed HOPG micropillars. d) TEM image of a thin sheet exfoliated from the HOPG micropillars. The thin membranes in the holes of the grid are formed from excess protein.

Graphene sheets of different thicknesses have special fingerprints in Raman spectra and electron diffraction that reveal the number of layers in a particular flake.[23] These characteristics were used to identify graphene/protein hybrid flakes with different thicknesses. Selected-area electron diffraction was measured for individual flakes. An example of a folded piece of single-layer graphene is shown in Figure S5 in the Supporting Information. Electron-diffraction patterns and their intensities measured at different locations on the graphene flake indicated that the flake indeed consisted of a single layer of graphene.[24]

Raman spectra of several flakes were collected by Raman mapping. An example of the D′ signal measured for a graphene flake on an SiO_2 substrate is presented in Figure 3a.

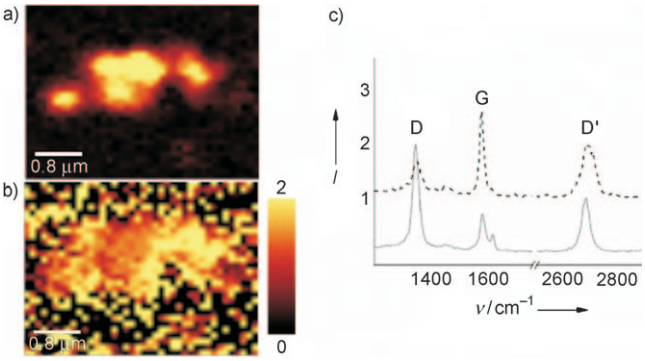

Figure 3. Confocal Raman microscopy on graphene flakes. a) Integrated intensity of the D′ peak in the scanned Raman spectra of a graphene flake. b) Ratio between the intensities of the D′ and G peaks of the same flake. The ratio 2:1 indicates less than four layers of graphene. The scale bar is 0.8 μm. c) Examples of Raman spectra of monolayer graphene (gray line) and bilayer graphene (black dashed line) from different samples.

Since the intensity ratio of D′ and G peaks is a measure of how many layers of graphene the sheet consists of,[25] we determined this intensity ratio from the data and used it to estimate the thickness of the sheets (Figure 3b). When the data was visualized as intensity ratio, the visibility of the parts of the graphene sheets that gave a signal with low absolute intensity was enhanced, especially near the edges, and at the same time, thickness variation in the flake became visible. Figure 3c shows example spectra measured for monolayer graphene and bilayer graphene from different graphene samples. The appearance of a strong D peak, indicative of a defect in the graphene structure, was associated with the edges of the flake; it is strong because of the relatively large spot size of the laser.

The functionality of the exfoliated graphene sheets was studied by using two electrostatically and chemically different variants of HFBI. The first variant was $(NCysHFBI)_2$ dimer, which has a reactive S–S group linking the two HFBI domains on its surface. To gain information on how the proteins maintain their functionality when adsorbed on the graphite/

graphene surface, we labeled flakes exfoliated by $(NCysHFBI)_2$ with gold nanoparticles coated with mercaptosuccinic acid (MSA; 3 nm). These nanoparticles were expected to bind to $(NCysHFBI)_2$ through the disulphide group and, to some extent, electrostatic interactions. Figure 4a shows part of a graphene flake exfoliated with $(NCysHFBI)_2$ and labeled with MSA-functionalized nanoparticles. The nanoparticles bind very selectively to the graphene flakes to form a monolayer; however, surface coverage by the nanoparticles was observed to vary from flake to flake. The appearance of the protein monolayer on the graphite surface was also studied by AFM. A phase-contrast image showing a protein monolayer on a 30 nm thick piece of graphite (see the Supporting Information for height data) is presented in Figure 4b.

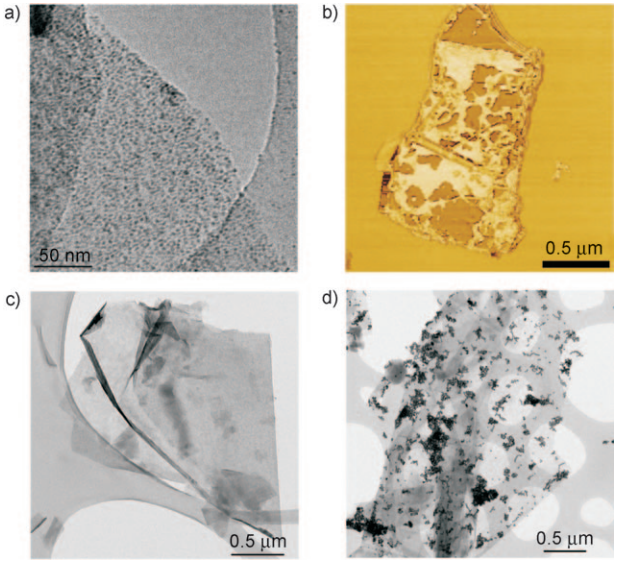

Figure 4. a) TEM image of a graphene flake exfoliated by $(NCysHFBI)_2$ and labeled with MSA-functionalized Au nanoparticles. The edge of the holey carbon film supporting the flake is visible on the far right of the image. b) AFM phase-contrast image of a thin graphite flake on an SiO_2 surface. The protein monolayer is clearly visible as light patterns against the smooth graphite surface. The color scale is 120°. c,d) Graphene flakes exfoliated with HFBI-ZE and labeled with ZR-functionalized Au nanoparticles at pH 3 (c) and pH 5 (d), under which conditions the peptides experienced electrostatic repulsion and attraction, respectively.

Another HFBI variant, HFBI–ZE, was also used in exfoliation experiments. HFBI–ZE is a hydrophobin fusion protein that includes a peptide segment that recognizes and binds a complementary peptide, ZR[26] (see the Supporting Information for details). Flakes exfoliated by HFBI–ZE were labeled with ZR-functionalized gold nanoparticles (15 nm). The interaction between ZR and ZE is very specific, but it is also very sensitive to the pH value. Thus by adjusting the pH value of the environment of graphene flakes functionalized with the HFBI–ZE protein, the attachment of ZR-functionalized gold nanoparticles could be either blocked (Figure 4c) or facilitated (Figure 4d).

We have demonstrated a one-step method for the exfoliation and functionalization of graphene by a surface-active protein. The process is unique and provides a safe and scalable way to produce graphene. The method results in a water-dispersible suspension of protein-coated flakes of graphene and ultrathin graphite. Exfoliation can also be carried out by using variants of these proteins that contain not only the surface-active hydrophobin part, which attaches to the graphite surface, but also a second part with a specified functionality. This fused portion provides the surface with new properties, as demonstrated in this study by the surface attachment of gold nanoparticles. The available functionalities are numerous, and the strategy of attaching functionalized hydrophobins can be extended, for example, to the development of biosensors. This study shows the usefulness of biomolecules for the production and functionalization of new nanomaterials, whereby the original functions of the molecules are applied in contemporary material development. The protein used is a structurally well-defined molecule, and modifications to it can be made with atomic precision by genetic engineering. This approach opens new routes towards the manufacture of atomically precise structures, which can find application in high-performance materials.

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[1] A. K. Geim, K. S. Novoselov, *Nat. Mater.* **2007**, *6*, 183–191.
[2] C. N. R. Rao, A. K. Sood, K. S. Subrahmanyam, A. Govindaraj, *Angew. Chem.* **2009**, *121*, 7890–7916; *Angew. Chem. Int. Ed.* **2009**, *48*, 7752–7777.
[3] K. S. Novoselov, A. K. Geim, S. V. Morozov, D. Jiang, Y. Zhang, S. V. Dubonos, I. V. Grigorieva, A. A. Firsov, *Science* **2004**, *306*, 666–669.
[4] A. A. Balandin, S. Ghosh, W. Bao, I. Calizo, D. Teweldebrhan, F. Miao, C. N. Lau, *Nano Lett.* **2008**, *8*, 902–907.
[5] C. Lee, X. Wei, J. W. Kysar, J. Hone, *Science* **2008**, *321*, 385–388.
[6] M. D. Stoller, S. Park, Y. Zhu, J. An, R. S. Ruoff, *Nano Lett.* **2008**, *8*, 3498–3502.
[7] S. Park, R. S. Ruoff, *Nat. Nanotechnol.* **2009**, *4*, 217–224.
[8] H. Wang, X. Wang, X. Li, H. Dai, *Nano Res.* **2009**, *2*, 336–342.
[9] K. S. Novoselov, D. Jiang, F. Schedin, T. J. Booth, V. V. Khotkevich, S. V. Morozov, A. K. Geim, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 10451–10453.
[10] T. Ohta, F. El Gabaly, A. Bostwick, J. L. McChesney, K. V. Emtsev, A. K. Schmid, T. Seyller, K. Horn, E. Rotenberg, *New J. Phys.* **2008**, *10*, 023034.
[11] M. P. Levendorf, C. S. Ruiz-Vargas, S. Garg, J. Park, *Nano Lett.* **2009**, *9*, 4479–4483.
[12] X. Li, X. Wang, L. Zhang, S. Lee, H. Dai, *Science* **2008**, *319*, 1229–1232.
[13] S. Stankovich, D. A. Dikin, R. D. Piner, K. A. Kohlhaas, A. Kleinhammes, Y. Jia, Y. Wu, S. B. T. Nguyen, R. S. Ruoff, *Carbon* **2007**, *45*, 1558–1565.
[14] Y. Hernandez, V. Nicolosi, M. Lotya, F. M. Blighe, Z. Sun, S. De, I. T. McGovern, B. Holland, M. Byrne, Y. K. Gun'Ko, J. J. Boland, P. Niraj, G. Duesberg, S. Krishnamurthy, R. Goodhue, J. Hutchison, V. Scardaci, A. C. Ferrari, J. N. Coleman, *Nat. Nanotechnol.* **2008**, *3*, 563–568.

[15] M. Lotya, Y. Hernandez, P. J. King, R. J. Smith, V. Nicolosi, L. S. Karlsson, F. M. Blighe, S. De, Z. Wang, I. T. McGovern, G. S. Duesberg, J. N. Coleman, *J. Am. Chem. Soc.* **2009**, *131*, 3611 – 3620.
[16] M. B. Linder, G. R. Szilvay, T. Nakari-Setälä, M. E. Penttilä, *FEMS Microbiol. Rev.* **2005**, *29*, 877 – 896.
[17] M. B. Linder, *Curr. Opin. Colloid Interface Sci.* **2009**, *14*, 356 – 363.
[18] P. Laaksonen, J. Kivioja, A. Paananen, M. Kainlauri, K. Kontturi, J. Ahopelto, M. B. Linder, *Langmuir* **2009**, *25*, 5185 – 5192.
[19] J. Hakanpää, G. R. Szilvay, H. Kaljunen, M. Maksimainen, M. Linder, J. Rouvinen, *Protein Sci.* **2006**, *15*, 2129 – 2140.
[20] G. R. Szilvay, T. Nakari-Setälä, M. B. Linder, *Biochemistry* **2006**, *45*, 8590 – 8598.
[21] G. R. Szilvay, A. Paananen, K. Laurikainen, E. Vuorimaa, H. Lemmetyinen, J. Peltonen, M. B. Linder, *Biochemistry* **2007**, *46*, 2345 – 2354.
[22] K. Kurppa, H. Jiang, G. R. Szilvay, A. G. Nasibulin, E. I. Kauppinen, M. B. Linder, *Angew. Chem.* **2007**, *119*, 6566 – 6569; *Angew. Chem. Int. Ed.* **2007**, *46*, 6446 – 6449.
[23] A. C. Ferrari, J. C. Meyer, V. Scardaci, C. Casiraghi, M. Lazzeri, F. Mauri, S. Piscanec, D. Jiang, K. S. Novoselov, S. Roth, A. K. Geim, *Phys. Rev. Lett.* **2006**, *97*, 187401.
[24] S. D. Bergin, V. Nicolosi, P. V. Streich, S. Giordani, Z. Sun, A. H. Windle, P. Ryan, N. P. P. Niraj, Z. T. Wang, L. Carpenter, W. J. Blau, J. J. Boland, J. P. Hamilton, J. N. Coleman, *Adv. Mater.* **2008**, *20*, 1876 – 1881.
[25] D. Graf, F. Molitor, K. Ensslin, C. Stampfer, A. Jungen, C. Hierold, L. Wirtz, *Nano Lett.* **2007**, *7*, 238 – 242.
[26] K. Zhang, M. R. Diehl, D. A. Tirrell, *J. Am. Chem. Soc.* **2005**, *127*, 10136 – 10137.

