JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

COMMUNICATION

pubs.acs.org/JACS

# Graphene Oxide as an Enzyme Inhibitor: Modulation of Activity of α-Chymotrypsin

Mrinmoy De,*,† Stanley S. Chou,† and Vinayak P. Dravid*

Department of Materials Science and Engineering, International Institute for Nanotechnology, Northwestern University, Evanston, Illinois 60208, United States

Supporting Information

**ABSTRACT:** We have investigated the efficacy of graphene oxide (GO) in modulating enzymatic activity. Specifically, we have shown that GO can act as an artificial receptor and inhibit the activity of α-chymotrypsin (ChT), a serine protease. Most significantly, our data demonstrate that GO exhibits the highest inhibition dose response (by weight) for ChT inhibition compared with all other reported artificial inhibitors. Through fluorescence spectroscopy and circular dichroism studies, we have shown that this protein−receptor interaction is highly biocompatible and conserves the protein's secondary structure over extended periods (>24 h). We have also explored GO−enzyme interactions by controlling the ionic strength of the medium, which attenuates the host−guest electrostatic interactions. These findings suggest a new generation of enzymatic inhibitors that can be applied to other complex proteins by systematic modification of the GO functionality.

Studies of synthetic receptor−protein interactions are of critical relevance to many broad fields, crossing biology, materials science, and pharmacology.[1] These studies have engendered successful applications ranging from enzymatic activity modulation[2] and biosensing[3] to separation[4] and production of hybrid materials.[5] The appeal of using a synthetic receptor lies largely in its tailorable features, ranging from small organic molecules to inorganic clusters that can be designed, synthesized, and functionalized according to the target molecules. In this regard, several kinds of materials have been used, ranging from metallic to oxide nanomaterials,[6] organic macromolecules,[7] macroassemblies,[8] nanotube, rods,[9] etc. Despite these broad material usages, however, biomolecular interactions with layered structures have remained underexplored. Specifically, graphene oxide (GO), despite being one of the most studied sheet-based materials,[10] has been the subject of surprisingly few reports relating to protein interactions.[11] We chose to explore the role of layered (charged) structures because GO has several advantages as a potential synthetic receptor. These include ease of synthesis, large surface area to mass ratio, surface functionalities, and a fluctuant surface that can enable induced-fit interactions for protein binding. In this communication, we report the binding and inhibition of α-chymotrypsin (ChT) activity by GO and its resultant effects on ChT secondary structure (Figure 1). The potential of GO as a protein receptor can be assessed following such investigations.

Herein, we used ChT as a model protein to study the efficacy of GO as an artificial protein receptor because ChT has a well-characterized structure and associated enzymatic activity. Deficiencies of proteolytic inhibitors have been implicated in a range of diseases including emphysema,[12] thromembolism,[12c] hereditary angiodema,[12c] diabetes, and Alheimer's disease.[13] Structurally, ChT has a ring of positively charged residues around its active pocket and patched hydrophobic "hot spots" on the surface (Figure 1a). It has been demonstrated that through its cationic residues, it can associate with anionic synthetic receptors such as polymeric micelles,[14] gold nanoparticles,[2,15] dendrimers,[16] peptides,[17] porphyrin,[18] etc., resulting in inhibition of activity toward anionic substrates. To investigate the possible binding and simultaneous inhibition of ChT using GO-based layered receptors, we synthesized a GO solution from bulk graphite using a modified Hummers method.[19] The GO layers were ∼1.1 nm thick (as measured by atomic force microscopy) and had alcohol, epoxide, carbonyl, and carboxylate functionalities on the surface and edge (Figure 1a). GO is highly hygroscopic in nature and stable in any buffer system.[20] Because of the presence of active functionalities (carboxylate, epoxide), GO can be further functionalized with diverse organic ligands and biomolecules, making it more suitable for biological applications.[21] In this report, we used native GO that was functionalized with carboxylate groups to target the cationic surface residues of ChT.

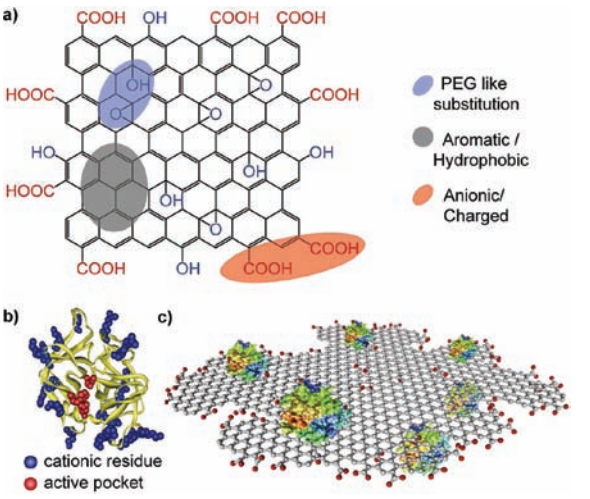


**Figure 1.** Structures of (a) graphene oxide (GO) and (b) α-chymotrypsin. (c) Schematic of GO and protein complexation.

**Received:** May 9, 2011
**Published:** September 28, 2011

ACS Publications © 2011 American Chemical Society

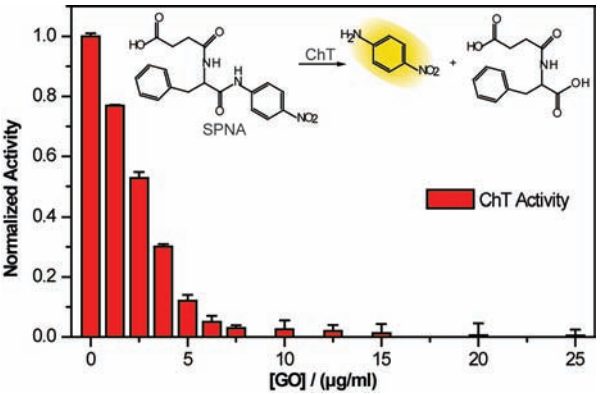


**Figure 2.** Activity of ChT plotted as a function of GO concentration in 5 mM sodium phosphate buffer (pH 7.4) using SPNA as a substrate. The activities were normalized to that of ChT.

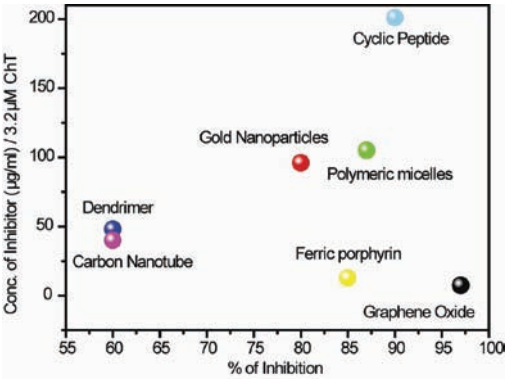


**Figure 3.** Degrees of inhibition and relative concentrations of various inhibitors used for altering the ChT activity. The right-bottom corner represents the most efficient inhibitor.

Considering the anionic functionality of GO, we expected that it would electrostatically bind with ChT at the positively charged patches around its active site, thereby inhibiting its enzymatic activity. To verify this hypothesis, activity assays were conducted to assess the inhibitory potency of the GO using *N*-succinyl-L-phenylalanine-*p*-nitroanilide (SPNA) as a substrate.

SPNA is a chromogenic substrate, and the enzyme activity was determined from its rate of hydrolysis. The studies were carried out by preincubating ChT (3.2 $\mu$M) and GO at various concentrations ranging from 0 to 25 $\mu$g/mL. Samples without ChT were considered as controls, and the activities were normalized accordingly. Here, ChT activity was suppressed to 3% with GO at a concentration of 7.5 $\mu$g/mL. Complete inhibition was observed at 20 $\mu$g/mL GO (Figure 2). A gel electrophoresis assay was performed to confirm the specific full inhibition point [see the Supporting Information (SI)]. The purpose was to establish that at 20 $\mu$g/mL GO, all ChTs in solution were bound. Here, gel electrophoresis demonstrated synergistic results, as free ChT band was not observed at 1:10 (w/w) GO/ChT (see the SI). A relatively high degree of selectivity could be achieved using this complementary electrostatic interaction. For example, when we incubated $\beta$-galactosidase, a hydrolase enzyme, with GO, no loss of enzymatic activity was observed (see the SI). Several other electrostatic selectivities have also been reported previously.[15a] Most interestingly, in comparison with other reported artificial ChT

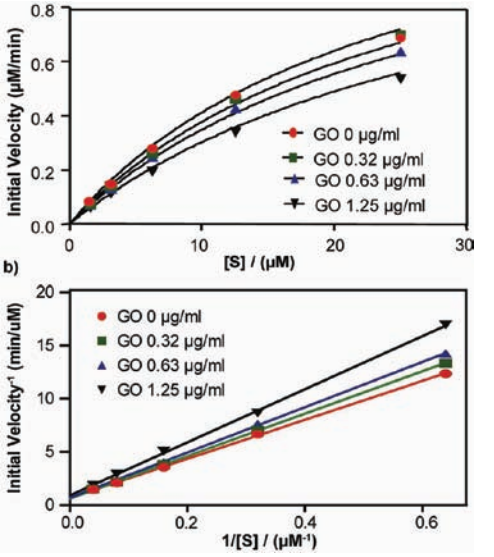


**Figure 4.** (a) Enzyme velocity as a function of substrate concentration at various fixed inhibitor (GO) concentrations. A ChT concentration of 3.2 $\mu$M was used throughout. Fitting of the data indicated competitive inhibition with $K_i$ = 2.71 $\mu$g/mL. (b) Lineweaver–Burk plots of the same data showing a common *y*-intercept, indicating competitive inhibition.

inhibitors, GO exhibited the highest inhibitory response by weight (Figure 3). After GO, there appears to be a significant dropoff in performance, with the nearest-performing materials being metal porphyrins.[16] This high degree of efficiency is presumably achieved from the flexible properties of the GO nanosheets and the presence of both hydrophobic aromatic groups and hydrophilic carboxyl groups on their surfaces. It is known that ChT has several hydrophobic patches around the active site that are attracted by hydrophobic residues; hence, the presence of the aromatic groups can enhance the affinity of the interacting substrate. Furthermore, the aromatic nature of the ChT active pocket adds the possibility of $\pi-\pi$ stacking and CH$-\pi$ interactions.[22] In view of the above criteria, GO is a good inhibitor for ChT because it contains an ideal combination of properties, namely, the coexistence of anionic, hydrophobic, and aromatic residues and a large surface area to mass ratio.

To establish the mode of inhibition and calculate the inhibition constant ($K_i$), we collected enzyme velocity data as a function of substrate (S) concentration at different fixed GO concentrations (Figure 4a). The data were fitted by nonlinear regression using GraphPad Prism 5 on the basis of the most general equation for the velocity ($V$) of an enzymatic reaction in the presence of an inhibitor (I):[23]

$$V = \frac{V_{max}[S]}{[S](1 + [I]/\alpha K_i) + K_m(1 + [I]/K_i)}$$

It can be seen that this general equation collapses into specific forms describing competitive, noncompetitive, and uncompetitive inhibitors depending on the value of $\alpha$. Specifically, $\alpha = 1$ implies that the inhibitor does not affect the binding of the substrate to the enzyme, which is noncompetitive inhibition. When $\alpha \ll 1$, inhibitor binding increases enzymatic substrate binding, and the equation describes mostly uncompetitive inhibition. When $\alpha \gg 1$, binding of the inhibitor prevents binding of the substrate, which is competitive inhibition.[24] For fitting of the data in Figure 4, we considered GO concentrations [I] as static values and obtained the


dx.doi.org/10.1021/ja208427j |*J. Am. Chem. Soc.* 2011, 133, 17524–17527

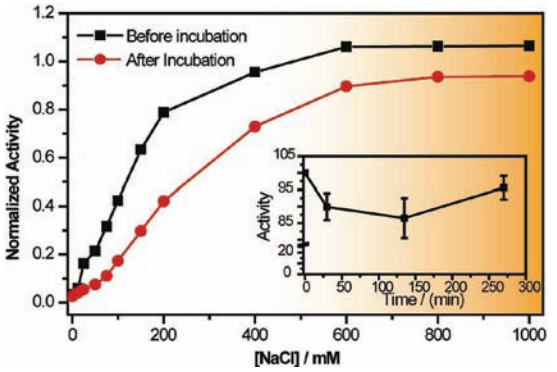


**Figure 5.** Activities of 3.2 $\mu$M ChT preincubated (red ●) and post-incubated (black ■) with 7.5 $\mu$g/mL GO in the presence of NaCl at various concentrations.

values of $V_{max}$, $K_m$, and $K_i$ as best-fit values for the data set. Here we obtained $\alpha$ = 3.94, indicating that GO inhibits ChT through competitive inhibition. The mode of inhibition can also be illustrated using a Lineweaver−Burk plot (Figure 4b). Here it can be seen that different GO inhibitor concentrations produce lines with the same *y*-intercept, indicating competitive inhibition. The mode of inhibition is therefore similar to those obtained previously using nondenaturing electrostatic-based inhibition of ChT.[17] From this data set, a $K_i$ value of 2.71 $\mu$g/mL was observed.

In addition to the inhibitory dose response, another important evaluation criterion for a potential enzyme inhibitor is the reversibility of complex formation. It was reported in earlier studies that ChT and its inhibitor interaction can be reversed by increasing the ionic strength of the medium, since the electrostatic forces can be attenuated by the presence of competitive ions.[14,25] Controlling enzymatic activity by altering the ionic strength of the medium is biologically relevant because the salt concentrations in biological systems can vary from 5 mM (bile)[26] to 250 mM (red blood cells).[27] To estimate the degree of reversibility, we ran two parallel experiments. In the first experiment, we incubated 80 $\mu$g/mL ChT with 7.5 $\mu$g/mL GO for 30 min and then added various amounts of NaCl to final concentrations of 0−1000 mM. In the second experiment, we mixed the ChT and GO in the presence of NaCl at the same concentrations as before but without the 30 min preincubation. The activity of ChT was monitored with respect to the hydrolysis of SPNA substrate. Control experiments were carried out under identical conditions without the addition of ChT. It was observed in both cases that the ChT activity could be restored with increasing salt concentrations, suggesting that the binding between GO and ChT is reversible. The maximum recovery was observed at 600 mM NaCl, but the maximum recovery differed for ChT samples preincubated with GO (after incubation) and ChT samples that were not (before incubation) (Figure 5). In the preincubated case, only 80% of the activity was recovered at 400 mM salt concentration and ∼90% at higher salt concentrations (compared with 100% recovery without preincubation). There are two possible explanations for this behavior. First, it is possible that a small amount of enzyme is bound rapidly and irreversibly to GO. Second, it is also possible that there is a two-step process, with the first step being fast and reversible and the second step slower and irreversible. To differentiate between these two possibilities, we prolonged the preincubation period for ChT and GO (up to 5 h) before addition of NaCl and observed the percentage of recovery. The fact that the results

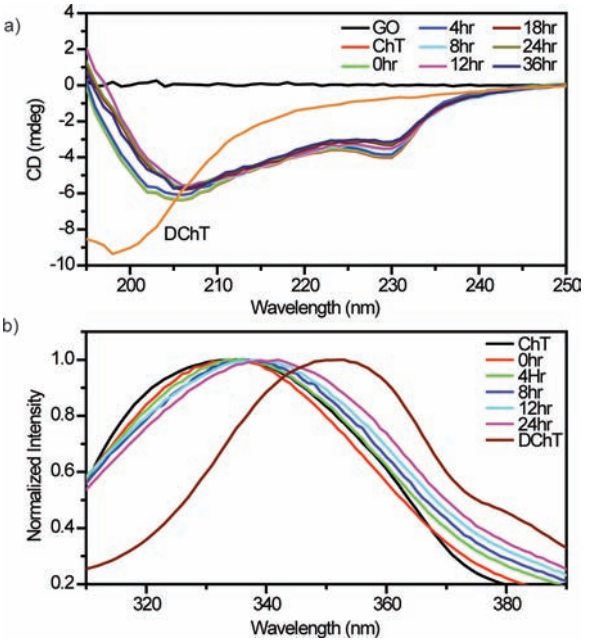


**Figure 6.** (a) Tryptophan fluorescence and (b) CD spectra of ChT (3.2 $\mu$M) with GO (7.5 $\mu$g/mL) at different times.

showed a constant ∼90% enzymatic recovery (Figure 5 inset) suggests that there are fast and high-affinity binding sites on the GO. To investigate this residual binding further, we had to discriminate between potential ChT denaturation and residual GO-ChT binding due to hydrophobic−aromatic interactions (which would not be affected by NaCl). To discriminate between these potential causes, we examined the ChT conformation (change in secondary structure) before and after prolonged GO binding. Specifically, if we were to observe that the secondary structure
of ChT remained conserved after extended interaction with GO, then the attenuated activity recovery would likely be attributable to residual binding by GO.

To investigate the effect of GO on the ChT secondary structure, we used circular dichroism (CD) and fluorescence spectroscopy. CD was used first because it can provide detailed information related to the secondary structure of a protein (i.e., combination of helix, sheet, and random coil). Natural ChT shows two characteristic minima at 232 and 204 nm,[28] while ChT with an altered conformation has its minimum at 232 nm diminished and blue-shifted to 204 nm (Figure 6a).[29] To investigate the degree of denaturation upon binding with GO, we monitored the CD spectrum of ChT bound with GO over a 36 h period and compared it with those of ChT and thermally denatured ChT (DChT). Here we observed only minimal changes in the spectrum of ChT incubated with GO. It can be seen that even after 36 h, ChT bound with GO showed a CD spectrum largely resembling that fresh ChT and nothing at all like that of DChT. To corroborate the CD data, we also investigated ChT denaturation through fluorescence spectroscopy. Here, natural ChT has a characteristic fluorescence emission peak at 334 nm.[30] After structural denaturation, the peak is red-shifted to 352 nm because of exposure of Trp residues to the aqueous environment (Figure 6b). In our fluorescence spectroscopy study over a 24 h time period, we observed a partial red shift in the spectra of both the control and the sample incubated with GO. The rates of denaturation for these two


dx.doi.org/10.1021/ja208427j |*J. Am. Chem. Soc.* 2011, 133, 17524–17527

samples were sufficiently identical that the red shift can be attributed to protein aging in solution rather than to complexation with GO (see the SI). In previous reports, it was suggested that ChT denaturation can be caused by a hydrophobic environment or strong electrostatic interactions, and this denaturation process can be prevented with either oligoethylene glycol or a suitable combination of hydrophilic and hydrophobic moieties.[15b,2a] Most materials, such as gold nanoparticles, require extensive surface modifications to meet these criteria. In contrast, GO has these properties built-in, without modification, as it is fluctuant in nature and contains surfaces with various hydrophobic and hydrophilic patches (Figure 1b). As a consequence, GO appears to prevent denaturation of the native protein structure over extended time periods. On the basis of the above results, we conclude that GO can inhibit the enzymatic activity with the highest reported efficiency without protein denaturation. We attribute this to two features. First, the flexibility of the single layer allows it to adapt to the surface curvature of the protein, in contrast to other nanomaterials. Second, the presence of ether and hydroxyl moieties mimics the oligoethylene glycol environment to minimize any nonspecific interactions.

In summary, we have demonstrated GO to be a new synthetic protein inhibitor. The prerequisites for an ideal artificial protein receptors are (i) strong and compatible binding with the target protein, (ii) reversible protein−receptor interactions, and finally (iii) no alteration of the proteins' native conformation by the receptor/inhibitor material. In this study, we have demonstrated that GO fulfills all of these requirements and thus can be utilized as a high-efficiency synthetic receptor. Further functionalization of GO can strengthen its applicability to wider target systems with more specificity and selectivity.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Preparation of GO and experimental procedures for activity assays, gel electrophoresis, CD and fluorescence spectroscopy, and denaturation kinetics studies. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Corresponding Author

m-de@northwestern.edu; v-dravid@northwestern.edu

### Author Contributions

†These authors contributed equally.

## ■ ACKNOWLEDGMENT

Absorbance measurements were performed at the Northwestern University High Throughput Analysis Facility. Fluorescence and CD measurements were performed at the Northwestern University Keck Biophysics Facility. S.C. is thankful for a DHA Graduate Fellowship. This work was funded by the National Cancer Institute Center for Cancer Nanotechnology Excellence (CCNE) Initiative at Northwestern University (Award U54CA119341).

## ■ REFERENCES

(1) (a) Katz, E.; Willner, I. *Angew. Chem., Int. Ed.* **2004**, *43*, 6042. (b) Wang, X.; Liu, L. H.; Ramstrom, O.; Yan, M. D. *Exp. Biol. Med.* **2009**, *234*, 1128. (c) Margulies, D.; Hamilton, A. D. *Curr. Opin. Chem. Biol.* **2010**, *14*, 705.

(2) (a) You, C. C.; De, M.; Han, G.; Rotello, V. M. *J. Am. Chem. Soc.* **2005**, *127*, 12873. (b) You, C. C.; De, M.; Rotello, V. M. *Org. Lett.* **2005**, *7*, 5685.

(3) De, M.; Rana, S.; Akpinar, H.; Miranda, O. R.; Arvizo, R. R.; Bunz, U. H. F.; Rotello, V. M. *Nat. Chem.* **2009**, *1*, 461.

(4) Franzreb, M.; Siemann-Herzberg, M.; Hobley, T. J.; Thomas, O. R. T. *Appl. Microbiol. Biotechnol.* **2006**, *70*, 505.

(5) Apostolovic, B.; Danial, M.; Klok, H. A. *Chem. Soc. Rev.* **2010**, *39*, 3541.

(6) De, M.; Ghosh, P. S.; Rotello, V. M. *Adv. Mater.* **2008**, *20*, 4225.

(7) Wilms, D.; Stiriba, S. E.; Frey, H. *Acc. Chem. Res.* **2010**, *43*, 129.

(8) Bally, M.; Bailey, K.; Sugihara, K.; Grieshaber, D.; Voros, J.; Stadler, B. *Small* **2010**, *6*, 2481.

(9) Liu, Z.; Tabakman, S.; Welsher, K.; Dai, H. J. *Nano Res.* **2009**, *2*, 85.

(10) Dreyer, D. R.; Park, S.; Bielawski, C. W.; Ruoff, R. S. *Chem. Soc. Rev.* **2010**, *39*, 228.

(11) (a) Yang, F.; Liu, Y. Q.; Gao, L. A.; Sun, J. *J. Phys. Chem. C* **2010**, *114*, 22085. (b) Liu, Z. F.; Jiang, L. H.; Galli, F.; Nederlof, I.; Olsthoorn, R. C. L.; Lamers, G. E. M.; Oosterkamp, T. H.; Abrahams, J. P. *Adv. Funct. Mater.* **2010**, *20*, 2857. (c) Zhang, F.; Zheng, B.; Zhang, J. L.; Huang, X. L.; Liu, H.; Guo, S. W.; Zhang, J. Y. *J. Phys. Chem. C* **2010**, *114*, 8469. (d) Zhang, M.; Yin, B. C.; Wang, X. F.; Ye, B. C. *Chem. Commun.* **2011**, *47*, 2399–2401.

(12) (a) Lomas, D. A.; Ekeowa, U. I.; Gooptu, B.; Belorgey, D.; Hagglof, P.; Karlsson-Li, S.; Miranda, E.; Perez, J.; MacLeod, I.; Kroger, H.; Marciniak, S. J.; Crowther, D. C. *Clin. Sci.* **2009**, *116*, 837. (b) Carrell, R. W.; Lomas, D. A. *Lancet* **1997**, *350*, 134. (c) Stein, P. E.; Carrell, R. W. *Nat. Struct. Biol.* **1995**, *2*, 96.

(13) Clauberg, M.; Joshi, J. G. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1009.

(14) Sandanaraj, B. S.; Vutukuri, D. R.; Simard, J. M.; Klaikherd, A.; Hong, R.; Rotello, V. M.; Thayumanavan, S. *J. Am. Chem. Soc.* **2005**, *127*, 10693.

(15) (a) Fischer, N. O.; McIntosh, C. M.; Simard, J. M.; Rotello, V. M. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5018. (b) Hong, R.; Fischer, N. O.; Verma, A.; Goodman, C. M.; Emrick, T.; Rotello, V. M. *J. Am. Chem. Soc.* **2004**, *126*, 739.

(16) Chiba, F.; Hu, T. C.; Twyman, L. J.; Wagstaff, M. *Chem. Commun.* **2008**, 4351.

(17) Park, H. S.; Lin, Q.; Hamilton, A. D. *J. Am. Chem. Soc.* **1999**, *121*, 8.

(18) Kano, K.; Ishida, Y. *Chem.—Asian J.* **2008**, *3*, 678.

(19) Hummers, W. S.; Offeman, R. E. *J. Am. Chem. Soc.* **1958**, *80*, 1339.

(20) Paredes, J. I.; Villar-Rodil, S.; Martinez-Alonso, A.; Tascon, J. M. D. *Langmuir* **2008**, *24*, 10560.

(21) Liu, Z.; Robinson, J. T.; Sun, X. M.; Dai, H. J. *J. Am. Chem. Soc.* **2008**, *130*, 10876.

(22) Shimohigashi, Y.; Nose, T.; Yamauchi, Y.; Maeda, I. *Biopolymers* **1999**, *51*, 9.

(23) Copeland, R. A. *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*; Wiley-VCH: New York, 2000; Chapter 8, p 278.

(24) Copeland, R. A. *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*; Wiley-VCH: New York, 2000; Chapter 8, pp 273−282.

(25) Verma, A.; Simard, J. M.; Rotello, V. M. *Langmuir* **2004**, *20*, 4178.

(26) Mithani, S. D.; Bakatselou, V.; TenHoor, C. N.; Dressman, J. B. *Pharm. Res.* **1996**, *13*, 163.

(27) Mouat, M. F.; Manchester, K. L. *Comp. Haematol. Int.* **1998**, *8*, 58.

(28) Cantor, C. R.; Timasheff, S. N. In *The Proteins*; Neurath, H., Hill, R. L., Eds.; Academic Press: New York, 1982; Vol. V, pp 145−305.

(29) McConn, J.; Fasman, G. D.; Hess, G. P. *J. Mol. Biol.* **1969**, *39*, 551.

(30) Lakokhin, A. S. Fluorescence Spectroscopy in Peptide and Protein Analysis. In *Encyclopedia of Analytical Chemistry*; Meyers, R. A., Ed.; Wiley: Chichester, U.K., 2000; pp 5762−5779.