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

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Abstract

We have investigated the efficacy of graphene oxide (GO) in modulating enzymatic activity. Specifically, we show that graphene oxide can mimic 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 α -chymotrypsin inhibition compared to all other reported artificial inhibitors. Through fluorescence spectrometry and circular dichroism (CD) studies, we show 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 interaction by controlling the ionic strength of media, which attenuates the host-guest electrostatic interaction. These findings suggest a new generation of enzymatic inhibitors that can be applied to other complex proteins by systematic modification of GO functionality.

Study of synthetic receptor-protein interactions is of critical relevance to many broad fields, crossing biology, material science and pharmacology.¹ These studies have engendered successful applications ranging from enzymatic activity modulation,² biosensing,³ 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 which 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 macro-assemblies,⁸ nano-tubes and rods⁹ etc. Despite these broad material usages however, the biomolecular interactions with layered structures have remained underexplored. Specifically, graphene oxide (GO), despite being one of the most studied sheet based material¹⁰ has 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 induce-fit interaction for proteins bindings. 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 use ChT as a model protein for studying the efficacy of GO as an artificial protein receptor because ChT has well characterized structure and associated enzymatic activity. Deficiencies of proteolytic inhibitors have been implicated in a range of diseases including emphysema,¹² thrombembolism,^{12c} hereditary angiodema,^{12c} diabetes and

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Supporting Information Available: Preparation of GO and experimental procedure for activity assay, gel electrophoresis, CD, fluorescence and denaturation kinetics study. This material is available free of charge via the Internet at <http://pubs.acs.org>

Alzheimer'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). Through its cationic residues, it has been demonstrated that it can associate with anionic synthetic receptors such as polymeric micelles,¹⁴ gold nanoparticles,^{2,15} dendrimer,¹⁶ peptides,¹⁷ porphyrin¹⁸ etc, resulting in activity inhibition toward anionic substrates. To investigate the possible binding and simultaneous inhibition of ChT using GO-based layered receptors, we synthesized GO solution from bulk graphite using a modified Hummers method.¹⁹ GO layers are about 1.1 nm thick (as measured by AFM) with alcohol, epoxide, carbonyl and carboxylate functionalities on the surface and edges. GO is highly hygroscopic in nature and stable in any buffer system.²⁰ Due to the presence of active functionality (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 have used native GO, which is functionalized with carboxylate groups, to target the cationic surface residues of ChT.

Considering the anionic functionality of GO, we expected that it will 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 substrate.

SPNA is a chromogenic substrate and the enzyme activity was determined from its rate of hydrolysis. The studies were carried out by pre-incubating ChT (3.2 μ M) and GO with different concentration ranging from 0 to 25 μ g/ml. Samples without ChT were considered as controls, and the activity were normalized accordingly. Here, ChT activity was suppressed to 3% with GO at a concentration of 7.5 μ g/ml. Complete inhibition was observed at 20 μ g/ml GO (Figure 2). Gel electrophoresis assay was performed to confirm the specific full inhibition point (Supporting information). The purpose was to establish that at 20 μ g/ml GO, all ChT in solution will be bound. Here, gel electrophoresis demonstrated synergistic results, as free ChT band was not observed at 1:10 GO:ChT by weight (Supporting information). A relatively high degree of selectivity can be achieved using this complementary electrostatic interaction. For example, when we incubated β -galactosidase, a hydrolase enzyme, with GO, no loss of enzymatic activity was observed (supporting information). Several other electrostatic selectivity are also reported earlier.^{15a} Most interestingly, in comparison with other reported ChT inhibitors, GO exhibited the highest inhibitory response by weight (Figure 3). After GO, there appears to be a significant drop-off in performance, with the nearest performing materials being metal porphyrin.¹⁸ This high degree of efficiency is presumably achieved from the flexible properties of GOs nano-sheets and the presence of both hydrophobic aromatic groups and hydrophilic carboxyl groups on its 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 interacting substrate. Furthermore the, aromatic nature of the ChT active pocket adds the possibility of π - π stacking and CH- π interaction.²² Considering the above criteria, GO is a good inhibitor for ChT because it contains an ideal combination of properties, namely co-existence of anionic, hydrophobic and aromatic residues with large surface area to mass ratio.

To establish the mode of inhibition and calculate the inhibitor constant (K_i), we collected enzyme velocity data at different fixed GO concentrations as a function of substrate concentrations (figure 4a). The data was fitted by non-linear regression using GraphPad Prism 5 considering the most general velocity equation for an enzymatic reaction considering the presence of an inhibitor.²³

$$v = \frac{V_{\max}[S]}{[S] \left(1 + \frac{[I]}{\alpha K_i}\right) + K_m \left(1 + \frac{[I]}{K_i}\right)}$$

It can be seen that this general equation collapse in to specific forms describing competitive, noncompetitive and uncompetitive inhibitors depending on the value of α . Specifically, when $\alpha=1$, it is implied that the inhibitor does not effect binding of substrate to enzyme, and is noncompetitive inhibition. When $\alpha \ll 1$, inhibitor binding increases enzymatic substrate binding, and the equation describes mostly uncompetitive inhibition. When $\alpha \gg 1$, it is implied that binding of the inhibitor prevents binding of substrate and therefore describes competitive inhibition.²⁴ For fitting of data in figure 4, we considered GO concentrations $[I]$ as a static values and $V_{\max}$, K_m , K_i were obtained as best fit values for the data set. Here we obtained $\alpha = 3.94$, therefore it was deduced that GO inhibits ChT through competitive inhibition. The mode of inhibition can also be illustrated using a Lineweaver-Burke 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 non-denaturing electrostatic based inhibition of ChT.¹⁷ From this dataset, a K_i is 2.71 Pg/ml was observed.

In addition to inhibitory dose response, another important evaluation criterion for potential enzyme inhibitor is the reversibility in complex formation. It was reported in earlier studies that ChT and its inhibitor interaction can be reversed by increasing ionic strength of the media, since the electrostatic forces can be attenuated by the presence of competitive ions.^{14,25} Controlling the enzymatic activity by altering the ionic strength of media is biologically relevant as the salt concentrations in biological systems can vary from 5 mM (bile)²⁶ to 250 mM (RBC).²⁷ To estimate the degree of reversibility, we ran two parallel experiments. In the first experiment, we incubated 80 mg/ml ChT with 7.5 mg/ml GO for 30 min, and then added various amounts of NaCl to final concentrations of 0 mM to 1000 mM. In the second experiment we mixed the ChT and GO in presence of NaCl at the same concentrations as before, but without the 30 min pre-incubation. 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 ChT activity can 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 between ChT samples pre-incubated with GO (after incubation) and ChT samples that were not (before incubation) (Figure 5). In the pre-incubated case, only 80% of activity was recovered at 400 mM salt concentration and ~90% at higher salt concentration (compared to 100% recovery without preincubation). There are two possible explanations for this behavior. First, it is possible that a small amount of enzymes are 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 hrs) before addition of NaCl and observed the percentage of recovery. Because the results showed a constant ~90% enzymatic recovery (figure 5 inset), it suggests that there are fast and high affinity binding site on the GO. To further investigate this residual binding, we would like to discriminate between potential ChT denaturation and residual GO binding due to hydrophobic/aromatic interaction (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 observe that ChT's secondary structure remained conserved

after extended interaction with GO, then, then attenuated activity recovery can likely be attributed to residual binding by GO.

To investigate GO's effect on 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). Specifically, natural ChT shows two characteristic minima at 232 and 204 nm²⁸, while one with altered conformation has its minima at 232 nm diminished and blue shifted to 204 nm (Figure 6b).²⁹ To investigate the degree of denaturation upon binding with GO, we monitored the CD spectra of ChT bound with GO over a 36 hr period and compared it to those of ChT and thermally denatured ChT (DChT). Here, we observed only minimal change in spectrum pattern in ChT incubated with GO. It can be seen that even after 36 hr, ChT bound with GO showed CD spectra largely resembling fresh ChT, and nothing at all like DChT. To corroborate the CD data, we also investigated ChT denaturation through fluorescence spectroscopy. Here, natural ChT has characteristic fluorescence emission peak at 334 nm.³⁰ After structural denaturation the peak is red shifted to 352 nm due to exposure of Trp residues to aqueous environment (figure 6a). In our fluorescence spectroscopy study over a 24 h time period, we observed partial red shift in both the control and the sample incubated with GO. The rates of denaturation between these two samples were sufficiently identical thus that it was attributed to protein aging in solution, rather than as a consequence of complexation with GO (supporting information). In previous reports, it was suggested that ChT denaturation can be caused by a hydrophobic environment or strong electrostatic interaction, and this denaturation process can be prevented with either oligoethylene glycol or suitable combination of hydrophilic and hydrophobic moieties.^{15b, 2a} Most materials, such as gold nanoparticles, require extensive surface modifications to meet these criteria. GO, in contrast, 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 an extended time periods. Based on 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 the protein surface curvature, in contrast to other nanomaterials. Second, the presence of ether and hydroxyl moieties mimics the oligoethylene glycol environment to minimize any nonspecific interaction.

In summary we have demonstrated GO as a new synthetic protein inhibitor. The prerequisite for an ideal artificial protein receptors are, (i) strong and compatible binding with target protein, (ii) reversible protein-receptor, and finally (iii) the receptor/inhibitor material should not alter the proteins' native conformation. In this study we have demonstrated that GO fulfills all of these requirements and 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Structure of graphene oxide (GO) (a), α -chymotrypsin (b) and schematic of graphene oxide and protein complexation (c).

Figure 2. Activity of ChT plotted as a function of GO concentration in 5 mM sodium phosphate buffer, pH 7.4 using SPNA as substrate. The activity is normalized to that of ChT.

Figure 3.

Degree of inhibition and the relative concentration of various inhibitor used for altering the ChT activity. The right bottom corner represents the most efficient inhibitor.

Figure 4.

(a) Enzyme velocity as a function of substrate concentration at fixed inhibitor (GO) concentration. ChT of 3.2 μM was used through out. Fitting of data indicated competitive inhibition with $K_i = 2.71 \mu\text{g}/\text{ml}$ (b) Lineweaver-Burke plot of the same data shows common y-intercept, indicating competitive inhibition.

Figure 5. Activities of 3.2 μ M ChT pre-incubated and post-incubated with 7.5 μ g/ml GO in presence of NaCl at varying concentration.

Figure 6.

a) Circular dichroism (CD) and b) Tryptophan fluorescence of ChT (3.2 PM) with GO (7.5 Pg/ml) at different time.