Supplementary Material (ESI) for Chemical Communications

Supporting Information

Interaction of Peptides with Graphene Oxide and Its Application for Real-Time Monitoring of Protease Activity

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1. Materials

Graphene oxide (GO) was synthesized from natural graphite powder by modified Hummers method. Bovine serum albumin (BSA), thrombin (bovine plasma, Factor II, lyophilized powder, activity of 50-150 NIH units/mg protein), lysozyme, and hydrogen tetrachloroaurate (Ⅲ) trihydrate ($HAuCl_4 \cdot 3H_2O$) were purchased from Sigma-Aldrich (St. Louis, MO). Amino acids mixture standard solution (Type H) was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). The nucleic acids (DNA **1**, DNA **2**) and the peptides (peptide **1**, peptide **2**, peptide **3**, peptide **4**, peptide **5**, peptide **6** and peptide **7**, purity >98%) were synthesized and purchased from Songon Inc. (Shanghai, China). Sequences for nucleic acids and peptides used in this work were listed in Table S1. Other chemicals were reagent-grade and purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Unless otherwise noted, all solutions were prepared in Milli-Q water (18.2 MΩ·cm) purified by a Milli-Q water purification system (Millipore Corp., Bedford, MA). The peptides were prepared as 1 mM stock solutions in Milli-Q water. The nucleic acids were prepared as 100 mM stock solutions in Milli-Q water. The lyophilized powder of thrombin was dissolved in the phosphate buffered saline buffer (PBS buffer, 137 mM NaCl, 2.5 mM $MgCl_2$, 10 mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH 7.4).

Table S1. The information of oligonucleotides and peptides used in this work.

Name	Sequence
DNA **1**	5'-CCC TGC ACT CTT GTC TGG AAG ACG G-3'
DNA **2**	5'-AGT AGA TTG GCC AAC-3'
peptide **1**	Lys-Cys-Ala-Leu-Asn-Asn-Gly-Ser-Gly-dPhe-Pro-Arg-Gly-Arg-Ala-Lys(FITC)-OH (pI=11.01)
peptide **2**	Ala-Lys-Arg-Arg-Leu-Ser-Ser-Leu-Arg-Ala (pI=12.48)
peptide **3**	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Asn (pI=7.02)
peptide **4**	Glu-Leu-Ala-Gly-Ala-Pro-Pro-Glu-Pro-Ala (pI=3.8)
peptide **5**	Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu (pI=4.62)
peptide **6**	Tyr-Met-Glu-His-Phe-Arg-Trp-Gly (pI=6.75)
peptide **7**	Cys-Ala-Leu-Asn-Asn-Asp-His-His-His-His-His-His (pI=6.62)

2. Instrumentation

Fluorescence intensity was measured in a fluorescence microplate reader (BioTek Instruments, Winooski, VT, USA) with an excitation wavelength at 485 nm and an emission wavelength at 528 nm using a black 384-well microplate (Fluotrac 200, Greiner, Germany). The high pressure liquid chromatography (HPLC) analysis was performed by an ultra fast liquid chromatography (UFLC) system (Shimadzu, Kyoto, Japan). The mobile phases were (A) water and (B)

acetonitrile, with a gradient of % B (initial, 10%; 20 min, 50%; 25 min, 50%; 25.01 min, 10%) and with a flow rate of 1.0 mL/min. The injection volume was 20 μL, and all chromatography was carried out at an ambient temperature of 25°C with detection wavelength of 220 nm. Amino acid analysis was performed using Hitachi L-8900 high speed amino acid analyzer (Tokyo, Japan) and detected with an aplananr concave diffraction grating spectrometer at the wavelengths of 570 nm and 440 nm.

3. The procedures for preparation of peptide1-GO bioconjugate and monitoring of thrombin activity

An aliquot of peptide (10 μM) was mixed with GO solution (0.2 mg/mL) at 1:1 ratio in volume (v/v) to form peptide-GO bioconjugate solution in PBS buffer. Then the peptide-GO solution was incubated for 5 min at 25°C and diluted to corresponding concentration using PBS buffer in the following experiments. An aliquot of 98 μL substrate solutions (peptide **1** or peptide-GO bioconjugate) in PBS buffer were added into a 384-well microplate. Then, 1 μL α-iodoacetamide or sodium fluoride or H_2O was added to the corresponding wells. Subsequently, 1.0 μL thrombin was added to each wells, and the fluorescence reading was begun immediately (excitation 485 nm, emission 528 nm), following a 100 min period and a 4 min interval at 25°C. The experiments of optimization of sensing conditions were carried out under identical conditions. All experiments were repeated two times.

4. Data analysis

Data analysis was performed with GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Each sample was repeated in duplicate and data were averaged. To determine the initial velocities (v_0) at different concentrations of peptide-GO bioconjugate, the raw fluorescence intensity data were normalized and multiplied by the concentration of peptide-GO bioconjugate. The curves were fitted to a first order exponential, and the initial velocities were obtained from the slope at $t = 0$. Using the standard Michaelis-Menten equation by non-linear least squares regression, the parameters, including maximum proteolytic velocity (V_{max}), Michaelis constant (K_M), and turnover number (k_{cat}), were determined. The inhibition constants (K_i) of anti-thrombin aptamers were estimated using the Lineweaver-Burk (L-B) analysis (a plot of the inverse velocity, $1/v$, against inverse peptide-GO bioconjugate concentration, $1/c_{pep\text{-}GO}$) to identify the mechanism of enzymatic inhibition from both the changes in axis intercept values and the relative positions of the inhibited plots, which transformed the velocity data into this format to yield a value for $1/K_M$ from $y = 0$ intercept.

5. Investigation of the interaction of amino acids and GO

The binding of biomolecules onto the GO is a complicated process with various driving forces possibly involved such as hydrophobic interaction, electrostatic interaction, and hydrogen bonding via direct ionic, Van der Waals, and hydrophobic forces. We investigated the interaction of different amino acids with GO. A mixture solution of amino acids normally found in proteins was incubated with 1 mg/mL GO in PBS buffer for 10 min at 25°C with gentle shaking. Following that, the mixture was centrifuged at 13,000 rpm for 20 min to collect the supernatant and analyzed using Hitachi L-8900 high speed amino acid analyzer with a standard method. The concentrations of the tested amino acids before and after incubation with GO were calculated by compared with the standard samples and listed in Table S2.

Table S2. The concentrations of the tested amino acids (nmol/mL) before and after incubation of GO and the corresponding concentration ratios.

Amino acids	**Before incubation** (nmol/mL)	**After incubation** (nmol/mL)	**Ratio**
Asp	100.47	97.89	0.9743
Thr	44.17	43.10	0.9758
Ser	87.33	84.95	0.9728
Glu	95.67	93.36	0.9758
Gly	95.26	92.50	0.9710
Ala	97.21	94.57	0.9728
Cys	89.72	92.08	1.0262
Val	119.01	116.05	0.9751
Met	96.52	93.84	0.9722
Ile	124.70	121.40	0.9735
Gln	92.65	90.55	0.9773
Asn	102.61	100.33	0.9778
Leu	87.40	85.06	0.9732
Tyr	92.81	81.05	0.8733
Phe	94.89	89.34	0.9415
Lys	93.89	69.91	0.7446
His	77.30	55.32	0.7157
Arg	87.80	55.52	0.6323
Pro	44.22	44.37	1.0034
Trp	125.02	95.17	0.7612

6. Investigation of the interaction between histidine and GO

A series of literatures reported that peptides with higher His content bind to CNT strongly.[1] We further investigated the binding force between the His and GO. A His-rich peptide (peptide **7**: CALNNDHHHHHH) was designed as a model to test the interaction of His and GO using gold nanoparticles (AuNPs) as a marker in the agarose gel electrophoresis assay. A thiol-containing domain (CALNN), composed of electroneutrol amino acids in the experiment condition, was designed to stabilize the water-soluble AuNPs according to the reported literature.[2] In the experimental condition of pH 8.5, the peptide 7 with predicted isoelectric point (pI) of 6.62 was negatively charged and couldn't be absorbed onto the GO via electrostatic interaction, theoretically. The peptide 7-AuNPs (lane 2) had a similar electrophoretic mobility with 700 bp DNA marker, but upon incubated with GO (lane 1), the characteristic electrophoretic band as in the land 2 disappear, which meant that the absorption of peptide7-AuNPs onto GO. As shown in Fig. S1, the absorption of peptide**7**-AuNPs onto GO was mainly dependent on the His residues via π-π interactions. Taken together, a conclusion can be drawn that His interacts with GO by not only electrostatic but also π-π interaction, which was consistent with the above amino acids analysis.

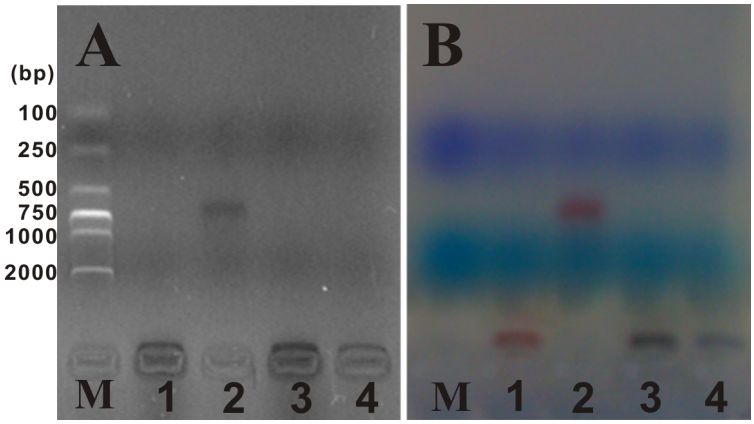

Figure. S1 Agarose gel electrophoresis for DNA ladder, AuNPs, peptide 7-AuNps and peptide 7-AuNPs-GO bioconjugate. A) Digital photo of the gel under the UV light illumination. B) Digital photo of the same gel under the visible light. Running buffer was 1×TBE buffer (89 mM tris base, 89 mM borate and 2 mM EDTA, pH 8.2 at 25°C). The GO used is 0.2 mg/mL. M: DL2000, lane 1: peptide 7-AuNPs + GO, lane 2: peptide 7-AuNPs, lane 3: AuNPs + GO, lane 4: AuNPs.

7. Investigation the effect of pH on the reaction of peptide and GO

The response of peptide-GO interaction at different pH values was studied. The peptide **3** and peptide **5** were employed to test absorption behavior upon incubated with GO in the buffer of 137 mM NaCl, 2.5 mM $MgCl_2$, 10 mM Na_2HPO_4, 2.0 mM KH_2PO_4. The pH values of the reaction buffer were adjusted by 6 mol/L HCl and 6 mol/L NaOH solutions. A series of peptide solutions with the same concentration were incubated with 1mg/mL GO in the buffer with different pH value for 10 min at 25°C with gentle shaking. After that, the mixture was centrifuged to collect the supernatant and analyzed by HPLC.

The spectrums for the original peptide solutions and supernatants were shown in Fig. S2. For peptide **3**, a completely absorption was observed in the pH values from 3 to 10. The predicted isoelectric point (pI) of peptide3 was 7.02, which means it was positively charged and was absorbed onto GO when the pH of the reaction buffer below 7.02. Whereas, the peptide **3** was negatively charged in the pH value from 7 to 10 theoretically, it was also absorbed onto GO due to His, Phe and Tyr residues, which can interact with GO via various driving forces such as π-π interaction, not electrostatic interaction in this case. The absorption of peptide **3** onto GO was dramatically decreased above pH of 10, we thought the reason was that the charge repulsion between peptide3 and GO became stronger than the π-π interaction provided by the residues in the peptide **3** when the pH value > 10. As to peptide **5** (pI 4.62), it was easy to understand that it was absorbed onto GO via electrostatic interaction as a driving force predominantly provided Arg residue with positively charged side chain when the pH value < 5. With the increasing pH values > 5, the peptide **5** was almost no absorption onto GO due to the electrostatic repulsion. In this section, we found that the absorption amount of peptide onto GO can be controlled by adjusting the pH value and changing the type of amino acids.

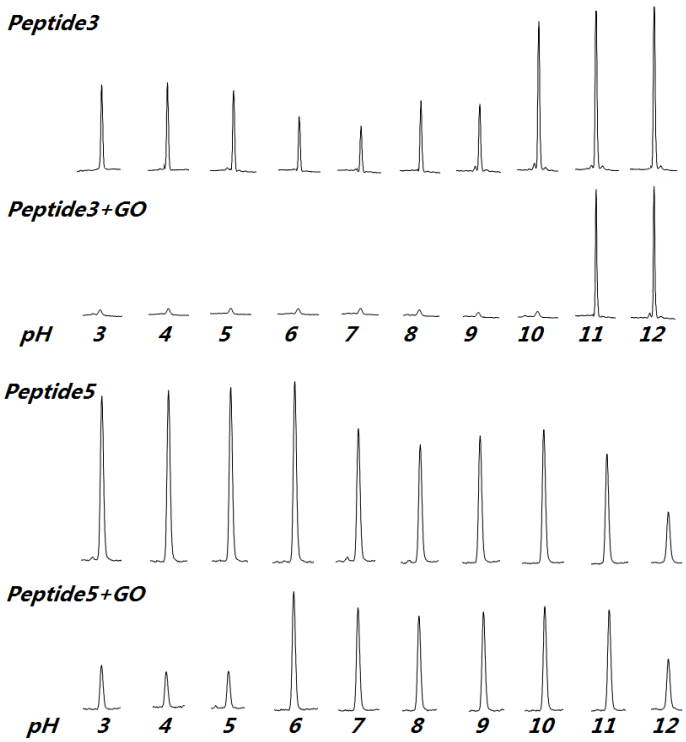

Figure S2. Spectrums of the peptide **3**-GO (peptide **5**-GO) absorption assay with different pH values using HPLC assay.

8. Investigation of kinetic behaviors of dye fluorescence quenching and absorbance stoichiometric ratio of peptide and GO

We studied the kinetic behaviors of peptide **1** and GO by monitoring the fluorescence intensity as a function of time. Figure S3A showed reaction of the fluorescence quenching of peptide **1** in the presence of GO can be completed within 1 min. Prior to the experiments of detection of protease activity, the peptide **1** adsorption onto GO was studied by evaluating the fluorescence intensity responses to the different concentrations of peptide **1** at the fixed amount of GO. An aliquot of 0.04 mg/mL GO solution were added to the increasing concentrations of peptide **1** solution from 0.2 to 2.4 μM and then incubated for 5 min at 25°C. We observed an obvious decrease of quenching efficiency at the point of 2.0 μM (Fig. S3B inset). The data clearly demonstrated that the adsorption saturation of 0.04 mg/mL GO was achieved at the 2.0 μM peptide **1**, this ratio was used in the following experiment.

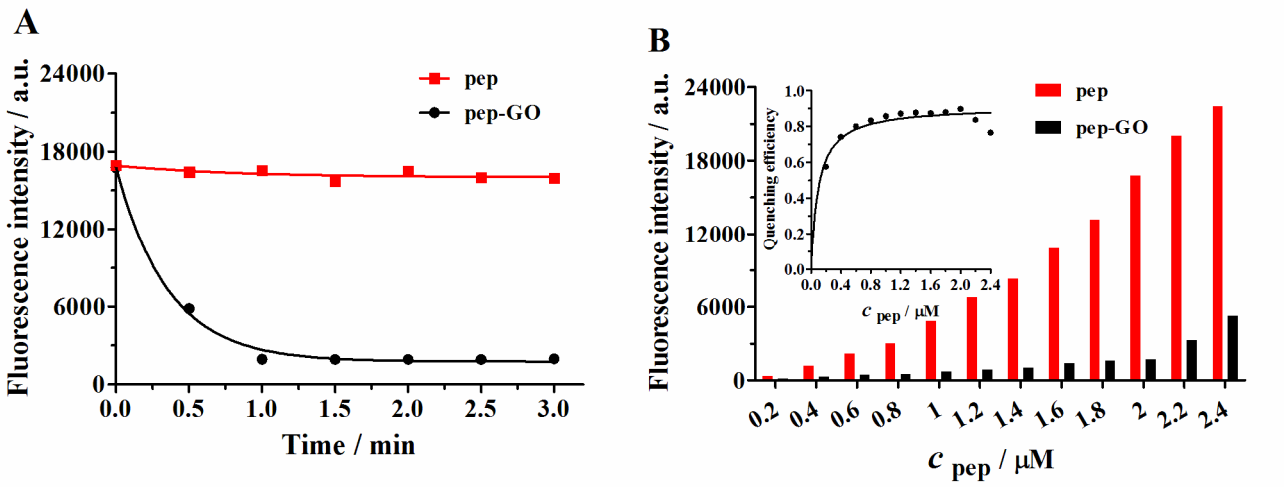

Figure S3. (A) Fluorescence intensity of peptide **1** (2 μM) in the PBS buffer as a function of incubation time (■) and fluorescence quenching of peptide **1** (2 μM) in the PBS buffer by GO (0.04 mg/mL) as a function of incubation time (●). (B) Investigation of absorbance stoichiometric ratio of peptide **1** and GO in the PBS buffer. Bars represent the fluorescence intensity of peptide **1** and peptide **1**-GO bioconjugate at increasing concentrations of peptide **1** (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4 μM). Inset: Plot of the quenching efficiency of peptide **1** with increasing concentrations (in the range of 0.2-2.4 μM) in the presence of GO (0.04 mg/mL). Define quenching efficiency (QE) as $(F_0\text{-}F)/F_0$, where F_0 and F represent the fluorescence intensity of peptide **1** in the absence and the presence of GO, respectively.

Reference

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