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Self-assembled graphene quantum dots induced by cytochrome c: a novel biosensor for trypsin with remarkable fluorescence enhancement†

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On the basis of cytochrome c-induced self-assembled graphene quantum dots, we demonstrate a novel fluorescent biosensor for trypsin with remarkable fluorescence enhancement, as well as high selectivity and sensitivity.

It is critically important that assay of biomolecules is sensitive, selective, rapid, and cost-effective in clinical practice. Quantum dots (QDs), which have unique photophysical properties, such as high luminescence intensity and efficiency, stability against photobleaching, and size-controlled luminescence properties, can offer significant advantages for biosensing.[1–5] However, the biosensing applicability of QDs has been greatly limited as they need treatment or modification before they are used for assays.[4,5] Besides, QDs are composed of heavy metals (Cd, Pb, Hg, Te, As, *etc.*) which release toxic compounds leading to environmental pollution.[6] Graphene quantum dots (GQDs), a new type of carbon dots (C-dots), are single or few-layer graphenes with a tiny size of only a few nanometers.[7–11] There are some merits of GQDs, such as stable photoluminescence, high fluorescent activity, low toxicity, excellent solubility and biocompatibility, thus they can be used in the field of bioimaging and electrochemical biosensing.[12–15] However, to the best of our knowledge, as optical probes, GQDs are rarely used in the biosensor field.[16,17]

Trypsin is the most important digestive enzyme produced by the pancreatic acinar cells and it cleaves peptide bonds on the C-terminal side of lysine and arginine amino acid residues.[18] Traditional methods for the detection of trypsin or monitoring of trypsin activity mainly include radioimmunoassay, gelatin-based film techniques and a number of colorimetric, electrochemical and fluorometric methods,[19–30] among which, fluorometric methods have received great attention because of their high sensitivity, easy operation and low background noise. Fluorometric assays of trypsin based on gold nanoparticles, QDs, conjugated polyelectrolytes, fluorescence resonance energy transfer (FRET), and aggregation-induced-emission (AIE) have been reported in recent years.[25–29,31] However, these methods still have respective disadvantages, such as the complexity of conjugated polyelectrolyte synthesis, fluorophore labelling and photobleaching, insensitivity based on "turn-off" detection, the requirement for many types of reagents and the toxicity of QDs.

Herein, we demonstrate a label-free fluorescence (FL) turn-on assay of trypsin using cytochrome c (Cyt c)-induced self-assembled GQDs probes.

The green luminescent GQDs are obtained by a two-step solvothermal and separation method described in our previous reports.[32,33] The quantum yield is about 10% using quinine sulfate as a reference. The design rationale for the label-free FL turn-on sensing of trypsin through GQDs-based optical probes is schematically illustrated in Fig. 1, where Cyt c is a well-known electron transfer (ET) protein, which is able to be efficiently coupled to oppositely charged QDs by electrostatic interactions and acts as an efficient FL quencher of QDs.[34–36] Furthermore, Cyt c rich in Fe^{3+} can possibly quench FL of GQDs selectively because of the special coordination interaction between Fe^{3+} and the phenolic hydroxyl group of GQDs.[37–39] It is anticipated that Cyt c can bind to GQDs and completely quench the FL of GQDs. When trypsin is present, Cyt c can be cleaved into smaller fragments on the C-terminal side of arginine and lysine residues, and Fe^{3+} in Cyt c can possibly be reduced to Fe^{2+} with the aid of the digestive enzyme. Thus the cleaved Cyt c is unable to quench the FL of

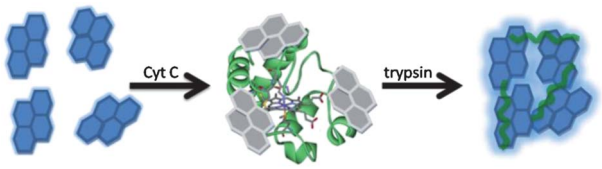

Fig. 1 Schematic illustration of the fluorescent biosensor for trypsin based-on self-assembled GQDs.

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GQDs, which results in the restoration of FL.[26,40] Moreover, trypsin cleaves the peptide bonds of Cyt c to give lysine and arginine residues, both of which chemically reduce GQDs into reducing-GQDs (r-GQDs) and induce a subsequent increase in FL intensity.[41,42] This design can lead to a label-free and FL-enhanced detection method, which is sensitive and selective to trypsin.

As the FL intensity of GQDs is tuned by its binding to and releasing from Cyt c, the structural changes of the GQDs–Cyt c complex can be directly reflected by the decrease or increase in FL signal. Fig. 2 shows the FL emission spectra of GQDs (60 μg ml^{-1}) under different conditions, indicating that GQDs exhibit FL emission owing to quantum confinement and edge effects without Cyt c. However, in the presence of Cyt c, up to 90% quenching of the FL emission at 428 nm is observed (curve 2, Fig. 2a), the result of which indicates strong adsorption of the GQDs strand on Cyt c and high FL quenching efficiency of Cyt c. This conclusion can further be confirmed by the absorption spectrum of Cyt C, which exhibited a high absorption value at 428 nm and proved that the FL emission of GQDs is absorbed by Cyt c (Fig. S1†). Besides, with the increase of Cyt c, a red-shift of the FL maximum from 428 to 456 nm can also be clearly observed (curve 2, Fig. 2b), which demonstrates the self-assembled aggregation of GQDs induced by Cyt c. Subsequently, the GQDs have a significant FL enhancement upon addition of trypsin (curve 3, Fig. 2a), showing the hydrolysis of Cyt c and the recovery of the FL. Trypsin catalyzes the hydrolysis of peptide bonds at the C side of lysine and arginine in Cyt c to give more than 15 fragments and cleaves solely at the C-terminal to arginine and lysine residues,[42] both of which chemically reduce GQDs to r-GQDs[41] and induce a subsequent increase in FL intensity (Fig. S2†). And Fe^{3+} in Cyt c can possibly be reduced to Fe^{2+} with the aid of the digestive enzyme. Therefore, the cleaved Cyt c is unable to quench the FL of GQDs.[37,43] Furthermore, the FL maximum of the GQDs–Cyt c complex is scarcely influenced by the addition of trypsin (curve 3, Fig. 2b), demonstrating the existence of GQDs aggregation states induced by Cyt c fragments (Fig. S3†). As a consequence, through cleavage of Cyt c by trypsin, the FL of GQDs can be regained and enhanced.

To examine the feasible quenching effects of Cyt c, we investigate the FL emission spectra of GQDs at different concentrations of Cyt c (0–6.0 mg ml^{-1}). In the absence of Cyt c, the FL spectrum of GQDs (60 μg ml^{-1}) in PBS buffer (pH = 8.86) shows a strong FL emission peak at 428 nm (Fig. S4†). However, with the increase in concentration of Cyt c over several minutes, the FL intensity of GQDs gradually decreases. The inset of Fig. S4† shows a 93% quenching of FL when the concentration of Cyt c reaches 6.0 mg ml^{-1}, which indicates strong adsorption of GQDs on Cyt c, thus, being a well-known ET protein, Cyt c acts as an efficient FL quencher of GQDs. Besides, a red-shift of the FL maximum from 428 to 484 nm can be clearly observed with the increase of Cyt c (6.0 mg ml^{-1}). The red-shift of the FL indicates that self-assembled aggregation of GQDs is induced by Cyt c, and the aggregation of GQDs is driven by electrostatic attraction. Cyt c is a cationic polyelectrolyte with a neutral pH[34,44–48] and thus readily forms complexes with anionic GQDs. The large amount of carboxyl groups at the edge of GQDs are considered as restraining the π–π intermolecular interactions, which eventually leads to the self-assembled packing with a specific J-type aggregation induced by Cyt c.[49]

The self-assembled aggregation of GQDs is further proven by transmission electron microscopy (TEM). The TEM analysis of Cyt c induced self-assembly of GQDs and their size distributions are shown in Fig. 3. The average diameter of the monodispersed particles is ~5 nm (Fig. 3a), which demonstrates that most of the GQDs are single layered or bi-layered.[50–52] However, after adding Cyt c, the presence of islands of small aggregates is clearly observed (Fig. 3b). Consequently, it can be concluded that Cyt c has induced self-assembly of GQDs, which is consistent with atomic force microscopy (AFM) images (Fig. S5†).

Next, we want to demonstrate that the GQDs–Cyt c complex can be employed to establish a label-free and turn-on FL sensing of trypsin. As discussed above, the initial PBS buffer solution of GQDs (60 μg ml^{-1}) and Cyt c (1 mg ml^{-1}) shows weak FL. However, the FL of the solution starts to increase with the introduction of trypsin. Fig. 4a shows the change in FL intensity of the GQDs probes with the amount of added trypsin after 24 h incubation in the GQDs–Cyt c complex solution at 37 °C. As a consequence, the recovery and sharp increase in the FL is observed after introducing trypsin into the GQDs–Cyt c complex, which suggests that Cyt c has been cleaved into small fragments.

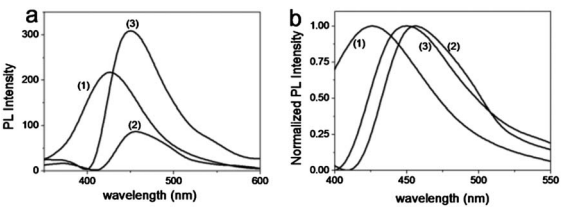

Fig. 2 FL emission spectra (a) and normalized emission spectra (b) of GQDs (60 μg ml^{-1}) under different conditions: (1) GQDs in PBS buffer; (2) GQDs + Cyt c (1000 μg ml^{-1}); (3) GQDs + Cyt c + trypsin (300 μg ml^{-1}). λ_{ex} = 320 nm.

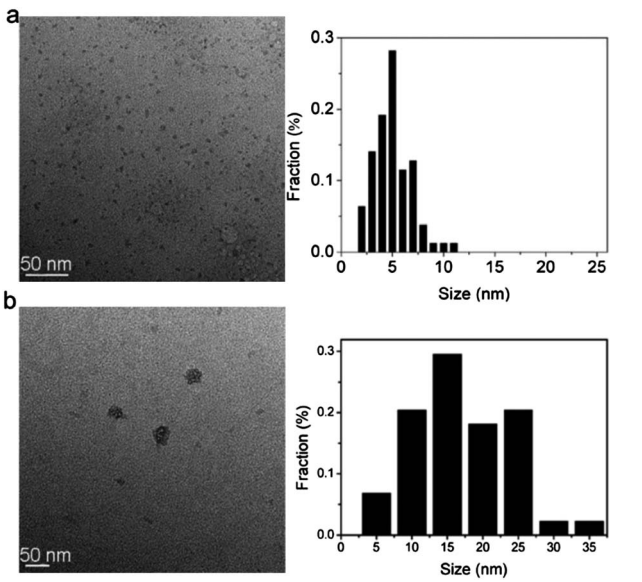

Fig. 3 TEM images of the GQDs and their self-assembled aggregates by Cyt c.

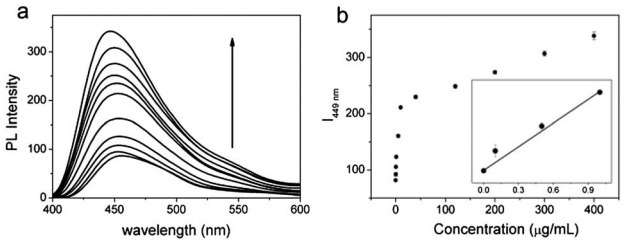

Fig. 4 Fluorescent emission spectra of the GQDs–Cyt c complex (Cyt c 1000 μg ml^{-1}) in the presence of different concentrations of trypsin, $\lambda_{ex} = 320$ nm. (a) FL spectra for analyzing different concentrations of trypsin (from bottom to top): 0, 0.1, 0.5, 1, 5, 10, 40, 120, 200, 300, 400 μg ml^{-1}. (b) Plot of $I_{449\ nm}$ against the concentration of trypsin. The error bars represent the standard deviations of three repeated measurements. Inset: expanded low-trypsin concentration region of the calibration curve.

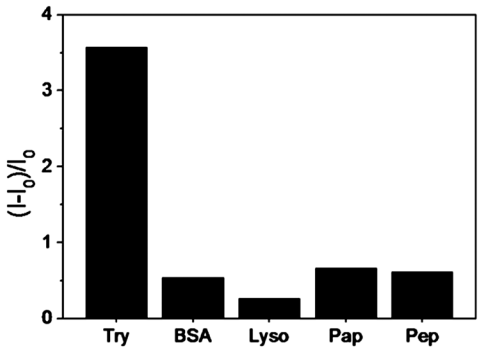

Fig. 5 Selectivity of trypsin analysis using the GQDs–Cyt c complex, all proteins are used at 300 μg ml^{-1}. $\lambda_{ex} = 320$ nm. I_0 and I are the FL intensities of the solutions at 449 nm in the absence and presence of the additional proteins.

Fig. 4b outlines the relationship between the FL intensity at 449 nm ($I_{449\ nm}$) and the concentration of trypsin. A linear relationship is observed from 0 to 1 μM ($R = 0.997$) and from 10 μM to 400 μM ($R = 0.996$). The detection limit for this biosensor at a signal-to-noise ratio (S/N) of 3 is estimated to be about 33 ng ml^{-1}, which is better than, or comparable to, that of the reported approaches.[24,25,28,53–58] Although this sensor for trypsin needs a long incubation time, the turn-on sensing mode, label-free property, low toxicity and easy preparation of GQDs can offer additional advantages of this analytical approach.

In addition, the repeatability and reproducibility of the biosensor are measured according to the same procedure. The relative standard deviation (R.S.D.) of the sensor response to 300 μg ml^{-1} trypsin is 1.2% for six successive measurements, demonstrating that the GQDs–Cyt c complex can be used repeatedly for the sensing of trypsin. The fabrication reproducibility of five sensors, made independently, shows reproducibility with the R.S.D. of 2.9% for 300 μg ml^{-1} trypsin, confirming that the fabrication method is reproducible.

To test the selectivity of the biosensor for trypsin, several control experiments were performed under the same conditions using the same concentration of control proteins such as bovine serum albumin (BSA), lysozyme (Lyso), papain (Pap) and pepsin (Pep). As shown in Fig. 5, the FL intensity changes of the GQDs–Cyt c complex at 449 nm in the absence and presence of the additional proteins were measured. None of the control proteins can significantly recover the quenched FL of GQDs. The results confirm that the FL recovery of GQDs is generated from trypsin-catalyzed enzymolysis of Cyt c, and show the high specificity of the turn-on assay for trypsin with the GQDs–Cyt c complex as the substrate.

In summary, we demonstrate that GQDs can be used as bioprobes for simple, label-free and turn-on biosensing. The protocol relies on the fact that the formation of Cyt c on GQDs is able to quench the FL of GQDs due to the ET effect of Cyt c toward GQDs as well as the possible special coordination interaction of Fe^{3+} in Cyt c, and the addition of trypsin can cause a remarkable fluorescent enhancement phenomenon due to the following reasons. Firstly, the hydrolysis of trypsin can break Cyt c into fragments so as to destroy the FL quenching effect of Cyt c. Besides, Fe^{3+} in Cyt c can also be reduced to Fe^{2+} and lead to the FL recovery with the aid of the digestive enzyme. Moreover, trypsin cleaves peptide bonds of Cyt c into lysine and arginine residues, both of which cause the chemical reduction of GQDs to r-GQDs and induce a subsequent increase in FL intensity. Such a GQDs–Cyt c complex can serve as a novel biosensor for trypsin with remarkable fluorescent enhancement, as well as high selectivity and sensitivity. Additionally, compared with other probes, this easily prepared, inexpensive, harmless, highly soluble, biocompatible GQDs are expected to be generalized for the detection of other biological analytes of interest.

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