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Ultrasensitive Profiling of Metabolites Using Tyramine-Functionalized Graphene Quantum Dots

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ABSTRACT: Graphene quantum dots (GQDs) are emerging fluorescence reporters attractive for optical sensing, owing to their high photostability, highly tunable photoluminescence, molecular size, atomically-thin structure, biocompatibility, and ease to be functionalized. Herein, we present a fluorometric sensing platform based on tyramine-functionalized GQDs, which is able to detect a spectrum of metabolites with high sensitivity and specificity. Furthermore, multiparametric blood analysis (glucose, cholesterol, L-lactate, and xanthine) is demonstrated. Such convenient metabolite profiling technique could be instrumental for diagnosis, study, and management of metabolic disorders and associated diseases, such as, diabetes, obesity, lactic acidosis, gout, hypertension, *etc.*

KEYWORDS: *graphene quantum dots, fluorometric biosensing, photoluminescence quenching, metabolite detection*

Graphene quantum dots (GQDs) are atomically-thin and nanometer-wide planar structures of pristine or modified graphitic carbon. Compared with the conventional fluorescent probes (organic fluorophores and semiconductor quantum dots), GQDs simultaneously possess several key merits desirable for bio-imaging and optical biosensing, including tunable photoluminescence (PL), molecular size, excellent photostability, chemical inertness, good solubility, high biocompatibility, and ease to be functionalized.¹⁻¹⁰

Quantitative measurement of metabolites in blood or other biological samples is important to diagnosis and healthcare.^{11, 12} Current methods for metabolite analysis, however, often suffer from mediocre sensitivity, low specificity, tedious or lengthy procedure, large sample consumption, or necessity for bulky equipment and high skills.¹³⁻¹⁵ To accurately assess metabolism state, profiling of multiple metabolites is necessary. But this is not feasible in typical clinic and laboratory settings because of a large amount of blood sample, different assays / instruments, a long waiting time, and high cost needed. In this work, we for the first time demonstrate the fluorometric measurement of multiple metabolites in blood using graphene quantum dots (GQDs) which is sensitive, convenient, of low-cost, and requires only a tiny amount of sample.

In addition to using GQD as the fluorescence reporter, we take advantages of its two unique properties. Firstly, due to their nanoscale lateral size and atomically-thin structure, the PL of GQDs will be largely quenched upon GQD aggregation or interaction with electron-deficient molecules. Based on such PL turn-off mechanism

of GQDs, a number of sensitive fluorescence sensors have been devised.¹⁶⁻²¹ Secondly, GQDs have been found to possess high peroxidase-mimicking catalytic activity.²²⁻²⁵ In comparison with the natural peroxidases, GQDs are stable and of low-cost. Serving as a signal amplification strategy, significant PL quenching is resulted from crosslinking of tyramine-conjugated GQDs upon triggering by their own peroxidase activities.

RESULTS AND DISCUSSION

GQDs were chemically exfoliated from carbon black as previously reported,¹⁰ followed by centrifugal ultrafiltration using a filter with a molecular-weight-cut-off at 3 kDa. The quantum yield of the purified GQDs is measured to be ~12.3% (at pH 7.0) using Rhodamine 6G as the reference, which is much higher than that of the as-synthesized GQDs (~7.0%). The average diameter and thickness of the GQDs is ~2.27 nm (± 0.25 nm, 247 samples) and ~1.2 nm (± 0.26 nm, 134 samples), indicating that the produced GQDs are uniform in size and mostly single- or double- layered (Figure 1A and B). The GQD aqueous dispersion is very stable due to the abundant oxygen-containing functional groups.¹⁰ No obvious aggregation is observed after months. As show in Figure 1C, the maximum emission from GQDs is achieved at ~524 nm while being excited at 480 nm. Therefore, this pair of emission and excitation wavelengths is used for the following experiments. Consistent with the previous observations, the excitation spectrum of our GQDs exhibits two peaks corresponding to the σ - π and π - π^* transitions originated from the carbene-like triplet state of the zig-zag edges of GQDs (Figure S1 in SI).^{18, 26, 27}

The peroxidase activity of GQDs is confirmed by chromogenic 3,3',5,5'-tetramethylbenzidine (TMB) in the presence of H_2O_2 as well as the strong absorption arising at 654 nm (Figure S2 in SI). Mimicking hydrogen peroxidase, GQD decomposes H_2O_2 into hydroxyl radical ($\bullet\text{OH}$), which is accompanied by oxidation of TMB and consequently its color change. The reaction reaches the steady state after ~20 min (Figure S3 in SI). It has been suggested that the $\text{C}=\text{O}$ groups and $\text{O}=\text{C}-\text{O}$ groups on a GQD serving as the catalytically active sites and H_2O_2 binding sites, respectively; and electron transfer occurring between the different oxidation states of GQD drives its catalytic activity.²⁸⁻³⁰

H_2O_2 is an important signaling molecule and oxidative stress indicator in biological systems.³¹ Here, a sensitive fluorometric sensor for H_2O_2 is uniquely devised based on PL quenching of tyramine-functionalized GQDs due to peroxidase activity triggered GQD crosslinking (illustrated in Figure 2). Specifically, tyramine (TYR) is covalently conjugated on GQD *via* the reaction between the carboxyl group on GQD and the amino group on TYR. And the reduction of H_2O_2 catalyzed by GQD produces hydroxyl radical ($\bullet\text{OH}$), which subsequently triggers the crosslinking between phenolic-hydroxyl (Ph) moieties on TYR through C-C and C-O coupling between aromatic rings.³²⁻³⁴

By comparing the Fourier transform infrared spectroscopy (FTIR) spectra of bare GQDs and TYR-functionalized GQDs (TYR-GQDs), the success of TYR conjugation on carboxyl-bearing GQD is evidenced by the decrease of COOH peak and appearance of CO-NH peak arisen from the amide bond between TYR and GQD

(Figure 3A). Furthermore, the zeta potential of GQDs is highly negative (-29.21 ± 1.88 mV, $n = 10$) due to the abundant negatively-charged oxygenated functional groups. After the linkage of neutral TYR molecules, the zeta potential of TYR-GQDs increases to -7.17 ± 1.3 mV ($n = 10$), indicating that the carboxyl groups on GQDs are largely consumed to form amide bonds with TYP molecules. Both bare GQD and TYR-GQD suspensions appear light yellow under daylight and emit green fluorescence under 365 nm UV-illumination (inset of Figure 3B). The conjugation of TYR causes negligible change in both emission and excitation spectra of GQDs (Figure 3B and Figure S1 in SI), suggesting that the PL properties of GQD are well-preserved after conjugation.

As demonstrated in Figure 4A, addition of H_2O_2 (150 nM) causes PL quenching over time because of induced crosslinking reaction and quenching reaches steady state after ~ 20 min (similar to the kinetics of GQD's peroxidase activity shown in Figure S3). Therefore, for all the following experiments, 20-min reaction time is used. H_2O_2 causes significant decrease of TYR-GQD PL in a dose dependent manner (Figure 4B). A trace amount of H_2O_2 (1 nM) can be detected with a signal-to-noise ratio (S/N) of 4.6, and the PL quenching linearly scales with the H_2O_2 concentration up to ~ 150 nM ($\sim 65\%$ quenching at this concentration) (Figure 4C). The theoretical limit of detection (LOD) is calculated to be 0.32 nM based on $\text{LOD} = 3 \times \sigma / m$, where σ is the standard deviation of the response at the lowest tested concentration (1 nM here) and m is the slope of the concentration dependent response (fitted line in Figure 4C). This enzyme-free fluorometric sensor is much more sensitive than the conventional

hydrogen peroxidase and TMB based colorimetric sensors and outperforms other GQD-based colorimetric, fluorometric, and electrochemical sensors (Table S1 in SI).³⁵⁻³⁷

PL quenching is resulted from the crosslinking of GQDs. This is confirmed by the dynamic light scattering (DLS) measurements. Specifically, the hydrodynamic diameter of TYR-GQD aggregates increases with more H₂O₂ being added (Figure S4A in SI). H₂O₂-induced aggregation of TRY-GQDs is further verified by atomic force microscopy (AFM) and transmission electron microscopy (TEM) (Figure S4B and S4C in SI). As a control experiment, para-aminobenzoic acid (PABA), instead of TYR, is conjugated with GQD. The only difference between TYR and PABA is that the former is terminated with phenolic-hydroxyl moiety while the latter is terminated with benzoic acid moiety. As expected, H₂O₂ (150 nM) is not able to cause appreciable PL quenching of PABA-GQDs because the oxidation product of benzoic acid (hydroxybenzoic acid) by hydroxyl radical cannot crosslink with each other (Figure S5 in SI).³⁸ This experiment further supports that phenol polymerization between TYR-GQDs induced by locally generated hydroxyl radicals underlies the detection mechanism. Such detection is specific because reactive oxygen species (ROS) generated from cell metabolism or pathological conditions are highly localized and short-lived (nanoseconds to seconds) and the previous study has shown that other ROS species (e.g., tert-butoxy radical, hypochlorite, and tert-butyl hydroperoxide) cannot trigger crosslinking of phenolic-hydroxyl moieties.³⁹

Oxidants (*e.g.*, H₂O₂) can quench GQD PL by creating surface traps.⁴⁰ However, H₂O₂ only causes weak PL quenching of bare GQDs (*e.g.*, 10 mM H₂O₂ leads to ~38% PL reduction) (Figure 4D). In comparison, 50 nM H₂O₂ can lead to ~34% PL quenching of TYR-GQDs while 1 mM H₂O₂ is needed to cause the same quenching level to bare GQDs. Therefore, TYR-GQDs are 4 orders more sensitive than bare GQDs due to the self-triggered crosslinking and resulting significant quenching effect.

Such an ultra-sensitive response of TYR-GQDs towards H₂O₂ provides a universal platform to detect any substrates of oxidoreductases as long as the enzymatic reaction produces H₂O₂, including a large variety of metabolites in human body (*e.g.* glucose, cholesterol, lactate, choline, L-lysine, pyruvate, glutamate, alcohol, xanthine, D-galactose, amino acids, sn-glycerol-3-phosphate) (Figure 2). The specificity of the detection is ensured by the necessary addition of the corresponding enzyme molecules in the TYR-GQD solution. Here as the proof-of-concept demonstration, we use TYR-GQDs to measure four metabolites in blood in parallel (namely, glucose, cholesterol, L-lactate and xanthine). Convenient metabolite profiling enabled by this technique is important for diagnosis, study, and management of metabolic disorders and associated diseases, such as, diabetes, obesity, lactic acidosis, gout, hyperuricemia, hypertension, *etc.*

To specifically detect these analytes (glucose, cholesterol, L-lactate or xanthine), their respective oxidases (glucose oxidase, cholesterol oxidase, lactate oxidase, or xanthine oxidase) are added into PBS solution (10 mM, pH 7.0) containing

TYR-GQDs (0.1 mg/mL). As shown in Figure 5A, adding glucose of various concentrations together with glucose oxidase (2.5 μ M) into TYR-GQD solution causes PL quenching to different extents. Sensitive detection at a concentration as low as 50 nM (S/N=4.5) and maximum linear response up to 2 μ M are achieved (Figure 5B). The theoretical LOD is calculated to be 7.2 nM. Glucose oxidase alone, glucose alone, or other sugar molecules (fructose, maltose and lactose) together with glucose oxidase is not able to cause appreciable PL quenching of TYR-GQDs (Figure S6 in SI), indicating the high specificity of glucose detection. This is among the most sensitive glucose sensors and superior to other GQD, graphene oxide sheet, or carbon dot based fluorometric, colorimetric, or electrochemical sensors (Table S2 in SI).^{41, 42}

Similarly, in the presence of cholesterol oxidase (10 μ M), our TYR-GQD sensor can detect cholesterol at a concentration as low as 80 nM (S/N = 3.4) with the linear response up to 10 μ M (Figure 6A and B); in the presence of lactate oxidase (5 μ M), our sensor can detect L-lactate at 200 nM (S/N = 3.8) with the linear response range up to 25 μ M (Figure 7A and B); in the presence of xanthine oxidase (5 μ M), our sensor is able to detect xanthine at 100 nM (S/N = 4.6) with the linear response range up to 18 μ M (Figure 8A and B). The theoretical LODs for the detection of cholesterol, L-lactate, and xanthine are 1.2 nM, 47 nM, 32 nM, respectively. Our sensor is among the most sensitive ones targeting on these analytes (Table S3, S4 and S5 in SI).⁴³⁻⁴⁶

Based on the determined response curves (Figure 5B, 6B, 7B and 8B), we further demonstrate the parallel measurement of glucose, cholesterol, L-lactate and xanthine in the serum samples from mice (Figure 5C, 6C, 7C and 8C). Owing to the ultra-high

sensitivity of our method, only a tiny amount of serum sample is required for each assay (0.5 μ L for glucose, 5 μ L for L-lactate, 20 μ L for cholesterol, 10 μ L for xanthine; final solution volume for measurement is 500 μ L). As shown in Table 1, our measured values are similar to that from the commercial assays. But these commercial products can only provide assay for single analyte. In addition, the UV-vis absorption measurement for cholesterol, L-lactate and xanthine based on the commercial kits take a longer reaction time (1h). And these kits are expensive.

The excellent performance of our sensor can be attributed to the intrinsic peroxidase activity of GQDs and the prominent aggregation-induced PL quenching (AIQ) effect of GQDs. Aggregation-induced or concentration-dependent quenching is common to almost all fluorophores, owing to nonradiative Förster energy transfer *via* dipole-dipole interaction significant only within a few nm distance.^{47, 48} The length of TYR-TYR pair is <2 nm. Comparing to other fluorophores, GQDs are particularly prone to AIQ because of their atomically thin-structure and also the large overlap between their emission and adsorption spectra.⁴⁹ In near field (<1 nm), AIQ can also be attributed to Dexter electron transfer.⁵⁰ Because of their atomic thickness and strong *pi-pi* interaction between each other, such nonradiative charge transfer is particularly prominent for GQDs.⁵⁰⁻⁵² AIQ is evidenced by the shortened fluorescence lifetime (from 2.62 to 1.76 ns) before and after the addition of H₂O₂ (150 nM) in TYR-GQDs suspension (0.1 mg/mL) (Figure S7 in SI). Since fluorescence lifetimes is inversely proportional to the sum of the radiative and nonradiative decay rates, its decrease is caused by nonradiative Förster energy transfer and Dexter electron

transfer arisen upon TYR-GQD aggregation.⁵³

It is arguable that quinone-like structures may be formed on TYR-GQDs which, in turn, could quench PL by acting as electron acceptors.⁵⁴ But it has demonstrated that such reaction can only be catalyzed by enzyme tyrosinase.⁵⁵ In an interesting work, a PL “turn-on” sensor based on aggregation-induced emission (AIE) of tyrosine-functionalized tetraphenylethene has been devised to detect H₂O₂ and glucose. Such AIE-based fluorometric sensor has unique advantages including low background interference and fast response time. On the other hand, TYR-GQD, which can be easily synthesized at low cost, shows several orders higher sensitivity and doesn’t require the use of horseradish peroxidase.

CONCLUSIONS

In summary, a facile fluorometric platform has been developed based on functionalized graphene quantum dots (GQDs). It allows ultrasensitive and fast profiling of a large variety of metabolites in a small amount of blood sample or other biofluids. This technique could be instrumental for diagnosis, study, and management of metabolic disorders and associated diseases. Combining with the smart-phone based or other portable fluorometers,⁵⁶⁻⁵⁸ the method developed here is also amenable for home-based healthcare.

METHODS

Synthesis and functionalization of GQDs. As previously reported, GQDs were prepared by refluxing carbon black powders (0.2 g) with nitric acid (50 mL, 6M) for

24 h. After centrifugation (5000 rpm for 10 min) to remove large particles, the solution was heat-dried to yield reddish-brown powders, followed by re-suspension in DI water. The obtained suspension was further ultra-filtered using a centrifugal filter (with a cut-off molecular weight of 3 kDa; Amicon Ultra-4, Millipore).

To preparation of TYR-GQDs, the GQD solution (0.5 mg/mL) was first mixed with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (10 mM; Sigma-Aldrich) and N-hydroxysuccinimide (10 mM; Sigma-Aldrich) for 20 min in borate buffer solution (10 mM, pH 5). Tyramine (0.5 mM; Sigma-Aldrich) was subsequently added into the above mixture for conjugation. After shaking for 4 h, the mixture was then dialyzed with cellulose ester dialysis membrane (MWCO 500-1000 Da) for 48h to remove unbounded TYR molecules, other reactants, and ions. The same protocol was also used to conjugate para-aminobenzoic acid (0.5 mM, Sigma-Aldrich) with GQDs.

Characterizations. The morphology of synthesized GQDs was examined by transmission electron microscopy (TEM; JEM 2010, JEOL) and atomic force microscopy (AFM; MFP-3D, Asylum Research). Fourier transform infrared spectroscopy (FTIR), photoluminescence (PL) spectroscopy, UV-vis absorption of bare GQDs and TYR-GQDs were characterized by Bruker spectrometer (Vertex 70), and PerkinElmer fluorescence spectrometer (LS-55), Shimadzu UV-vis spectrometer (UV-245), respectively. Their zeta potentials were measured using a Malvern Zetasizer (Nano-ZS 90).

Fluorometric detection. Phosphate buffer solution (PBS, 10 mM, pH 7.0, 500 μ l)

with 0.1 mg/mL TYR-GQDs (or bare GQDs of same concentration in control experiments) was added with the different concentrations of H₂O₂, or 2.5 μM glucose oxidase together with various concentrations of glucose, or 10 μM cholesterol oxidase together with various concentration of cholesterol, or 5 μM L-lactate oxidase together with various concentrations of L-lactate, or 5 μM xanthine oxidase together with various concentrations of xanthine, or 2.5 μM glucose oxidase together with 1 μL mouse serum, or 10 μM cholesterol oxidase together with 5 μL mouse serum, or 5 μM L-lactate oxidase together with 20 μL mouse serum, or 5 μM xanthine oxidase together with 10 μL mouse serum. The mouse serum samples were obtained by centrifuging (10,000 rpm for 5min) the blood samples taken from 8-10 week old male mice. All chemicals were purchased from Sigma-Aldrich.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Seven supplementary figures and five tables. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

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FIGURES AND TABLES

Figure 1. (A) TEM and (B) AFM images of synthesized GQDs. Inset of (B) shows the height profile along the indicated line. (C) Photoluminescence (PL) spectra of GQD suspension (in PBS buffer solution) under different excitation wavelengths (as indicated by the figure legends).

Figure 2. Illustration of fluorometric detection scheme based on tyramine-functionalized GQD (TYR-GQD).

Figure 3. (A) FTIR spectra of TYR, GQD and TYR-GQD. (B) PL spectra of GQD and TYR-GQD at the excitation wavelength of 480 nm. Inset shows photographs of TYR-GQD suspension under daylight (left) and 365 nm UV (right).

Figure 4. (A) Time-dependent (0 – 25 min) PL quenching of TYR-GQDs (1 mg/mL) in PBS (pH 7.0) containing 150 nM H_2O_2 . (B) PL spectra of TYR-GQD in PBS (pH 7.0) containing different concentrations of H_2O_2 . (C) The PL quenching efficiency vs. H_2O_2 concentration. F_0 and F_Q are the fluorescence intensity measured in the absence and presence of quencher (H_2O_2 here), respectively. The error bars indicate the standard deviation from 3 measurements. (D) PL spectra of bare GQD suspension containing different concentrations of H_2O_2 .

Figure 5. (A) PL spectra of TYR-GQD (0.1 mg/mL) in PBS (pH 7.0) containing glucose oxidase (2.5 μM) and different concentrations of glucose. (B) Quenching efficiency vs. glucose concentration. The error bars indicate the standard deviation from 3 measurements. (C) PL spectra of the test solution (TYR-GQD and glucose oxidase in PBS; 495.5 μL) before (solid line) and after (dashed line) addition of 0.5 μL serum sample.

Figure 6. (A) PL spectra of TYR-GQD (0.1 mg/mL) in PBS (pH 7.0) containing cholesterol oxidase (10 μM) and different concentrations of cholesterol. (B) Quenching efficiency vs. cholesterol concentration. The error bars indicate the standard deviation from 3 measurements. (C) PL spectra of the test solution (TYR-GQD and cholesterol oxidase in PBS; 480 μL) before (solid line) and after (dashed line) addition of 20 μL serum sample.

Figure 7. (A) PL spectra of TYR-GQD (0.1 mg/mL) in PBS (pH 7.0) containing lactate oxidase (5 μ M) and different concentrations of L-lactate. (B) Quenching efficiency vs. lactate concentration. The error bars indicate the standard deviation from 3 measurements. (C) PL spectra of the test solution (TYR-GQD and lactate oxidase in PBS; 495 μ L) before (solid line) and after (dashed line) addition of 5 μ L serum sample.

Figure 8. (A) PL spectra of TYR-GQD (0.1 mg/mL) in PBS (pH 7.0) containing xanthine oxidase (5 μM) and different concentrations of xanthine. (B) Quenching efficiency vs. xanthine concentration. The error bars indicate the standard deviation from 3 measurements. (C) PL spectra of the test solution (TYR-GQD and xanthine oxidase in PBS; 490 μL) before (solid line) and after (dashed line) addition of 10 μL serum sample.

Table 1 Multiparametric blood analysis (glucose, cholesterol, L-lactate, and xanthine): our method vs. commercial assays

	Glucose		Cholesterol		L-lactate		Xanthine	
	our method	glucose meter [†]	our method	assay kit [‡]	our method	assay kit [‡]	our method	assay kit [‡]
sample 1	6.94 ± 0.23* [#]	7.50 ± 0.12	63.90 ± 1.19	59.02 ± 0.96	281.3 ± 2.48	276.9 ± 1.33	342.7 ± 2.83	346.3 ± 2.38
sample 2	10.28 ± 0.21	11.53 ± 0.30	81.09 ± 1.32	84.26 ± 0.68	334.5 ± 1.95	339.2 ± 1.62	304.8 ± 2.31	299.0 ± 3.0

[†] The glucose meter was made by Johson & Johson Medical.

[‡] The commercial assay kits for cholesterol, L-lactate or xanthine were purchased from Sigma-Aldrich.

* All values are in units of mM

[#] The average value and standard deviation derived from 4-time measurements (n=4).

TOC graphic