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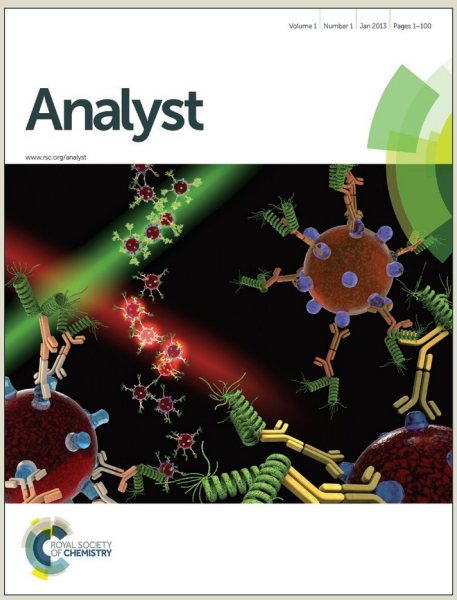

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A novel fluorescent assay for inorganic pyrophosphatase based on modulated aggregation of graphene quantum dots

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A simple and highly sensitive fluorometric method has been developed for inorganic pyrophosphatase (PPase) activity detection based on the disaggregation and aggregation of graphene quantum dots (GQDs). Copper ions can trigger the sever aggregation of GQDs with rich carboxyl groups, which results in effective fluorescence quenching. While, with the addition of pyrophosphate (PPi), the quenched fluorescence is effectively recovered owing to the strong interaction between PPi and Cu^{2+}. Furthermore, under the catalytic hydrolysis of PPase, the complex of PPi-Cu^{2+}-PPi is rapidly disassembled, the fluorescence re-quenched. This method is highly sensitive and selective for PPase detection, with a linear correlation between the fluorescence intensity and the PPase concentration in the range from 1 to 200 mU/mL with a detection limit down to 1mU/mL (S/N=3). Additionally, the inhibition effect of NaF on the PPase activity is also studied. Thus, the proposed mothed may hold a potential application in diagnosis of PPase-related diseases and screening of PPase inhibitors, to evaluate the function and inhibition of PPase in biological systems.

Introduction

Pyrophosphate (PPi) is one kind of the most important biological anions, which is the product of adenosine triphosphate (ATP) hydrolysis under cellular conditions,[1] playing an important role in pathological, energetic and metabolic progresses.[2] Soluble inorganic pyrophosphatase (EC 3.6.1.1; PPase) is a ubiquitous enzyme that can provide phosphate substrates and energy for biosynthesis reactions such as RNA, DNA and protein syntheses by hydrolyzing PPi,[3] which also plays an vital role in controlling the level of PPi in cells.[4] PPase has been proved to be relevant to evolutionary events, phosphorus metabolism and carbohydrate metabolism, which is of vital importance for cell growth and differentiation.[5] Furthermore, the level of PPase is connected to several diseases, such as hyperthyroidism, lung adeno-carcinomas and colorectal cancer.[6] Thus, the analysis of PPase activity is of fundamental importance and significance. Additionally, PPases are strong metal-dependent enzymes,[7] they need divalent ions such as Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+} and Cd^{2+} for catalytic reactions, with Mg^{2+} conferring the highest activity.[4a,c,5c,8] Up to now, several assays such as colorimetric,[9] enzymatic[10] and fluorometric[11] have been developed for the detection of PPase activity. However, it has shown that these methods suffer from their own drawbacks such as time-consuming, high cost, sophisticated instrumentation, demand of specialized skills, high detection limit and poor stability in aqueous solution for noble metal nanoclusters etc. Thus, a convenient, simple yet effective method for monitoring the PPase activity is still urgently needed.

In theoretical and experimental studies, graphene quantum dots (GQDs) whose diameters are less than 10 nm can exhibit quantum-confinement effects, and thus, wavelength-dependent and photobleaching-resistant photo-luminescence can be observed.[12] GQDs, as a class of zero-dimensional graphitic nanomaterials, display special optical and electrical phenomena comparing with other kinds of quantum dots.[13] With regard to conventional dye molecules and semiconduction quantum dots, they have many advantages such as good photostability, low toxicity, excellent water solubility, easy modulation, excellent biocompatibility, high electrical and thermal conductivity, which make them promising candidates for future applications in such fields like fluorescent probe, optoelectronic devices, bioimaging and photocatalysts.[13a-c,14] Based on these superiorities, a multi-function fluorescence sensing platform was developed to achieve selective detection of DNA targets[14g], as well as H_2O_2, glucose,[14i,14k] metal ions,[14a,14e] and proteases[14f].

Herein, we report the development of a novel fluorometric method for highly sensitive detection of PPase using the GOD nanoprobe. This method relies on our new finding that carboxyl-modified GQDs can be assembled by coordination with Cu^{2+} into large aggregates, which can be reversely

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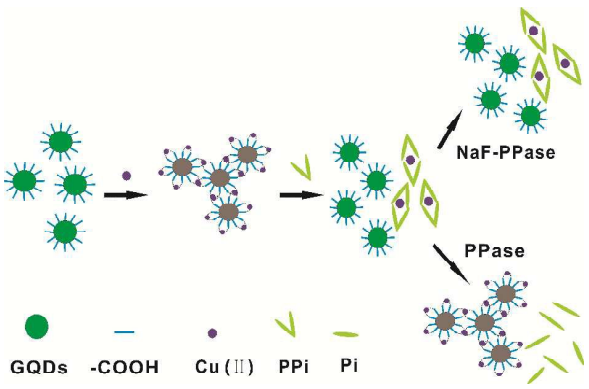
disaggregated into GQDs by the addition of PPi. The reversible modulation of fluorescence signal by Cu^{2+} and PPi in the aggregation and disaggregation enables sensitive detection of PPase, as illustrated in Scheme 1. In the presence of Cu^{2+}, GQDs are assembled into aggregates due to the crosslinked coordination between Cu^{2+} and carboxyl group, which results in substantial fluorescence quenching because of self-quenching between GQDs. After the addition of PPi, the competition of PPi with GODs for Cu^{2+} due to strong interaction between PPi and Cu^{2+} induces disaggregation of GODs, recovering the fluorescence of GODs. The presence of active PPase catalyzes the hydrolysis of PPi into phosphate, which releases Cu^{2+} and, in turn, mediates re-aggregation of GQDs with a quenched fluorescence signal. Therefore, the modulation of fluorescence in response to PPase gives a quantitative measure for the assay of activity or inhibition of PPase.



Scheme 1. Illustration of biosensing strategy for the detection of PPase activity based on aggregation and disaggregation of graphene quantum dots.

Experimental

Chemicals and Materials

Baker's yeast inorganic PPase (EC 3.6.1.1, 100 U) was purchased from Sigma-Aldrich (shanghai). One unit of Baker's yeast inorganic PPase can liberate 1.0 μmol of inorganic orthophosphate per minute at pH=7.2 at 25 ℃. Sodium pyrophosphate (PPi) was acquired from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai). HEPES was obtained from HeFei BoMei Biotechnology Co. Ltd. (Hefei). $MgCl_2{\cdot}6H_2O$, $Cu_2SO_4{\cdot}5H_2O$ and NaF were all bought from Sinopharm Chemical Reagent Co. Ltd. (Shanghai). All other regents used in this work were at least analytical grade and directly used without any further purification. The solutions were prepared with ultrapure water (resistance>18.2MΩ) that was obtained from a milli-Q water purification system (Billerica, MA).

The fluorescence emission spectra of GQDs were obtained from F-7000 and Fluoromax-4 spectrofluorometers. The UV-vis absorption was recorded on a UV-2450 spectrometer. Transmission electron microscopic (TEM) images were purchased from JEM-2100F with an accelerating voltage of 200kV. Zeta potential and dynamic light scattering (DLS) analysis were performed by Malvern Zetasizer Nano ZS90.

Fluorescent PPase Activity Assay

Different activity units of PPase, ranging from 0 to 600 mU/mL, were separately added to the mixed solution that contains GQDs, Cu^{2+} (100 μM), Mg^{2+} (0.1 μM), PPi (160 μM) and HEPES buffer (10 mM), the final volum was 100 μL. After an incubation time of 60 min in a water bath at 37 ℃, the fluorescence spectra of the mixture were recorded respectively.

Inhibition Efficiency of F^- on PPase Activity

For evaluating the inhibition efficiency of F^- on PPase activity, we first separately added PPase (200 mU/mL) to the aqueous solution of NaF with different concentrations. After 15 min at room temperature, the NaF-treated PPase was added to the mixed solution containing GODs, Cu^{2+} (100 μM), Mg^{2+} (0.1 μM), PPi (160 μM) and HEPES buffer (10 mM), the final volum was 100 μL. After another 60 min in a water bath at 37 ℃, the fluorescence spectra of the mixture were obtained respectively.

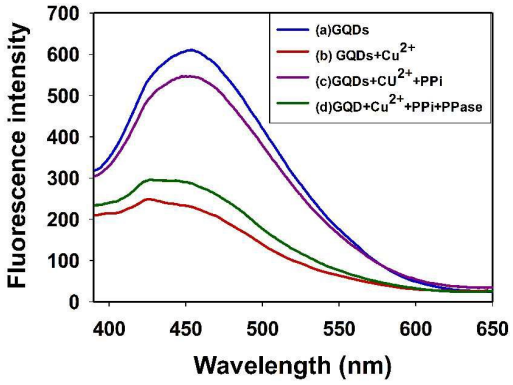


Fig. 1. Fluorescence emission spectra of (a) single GQDs (blue curve); (b) GQDs + Cu^{2+} (100 μM) (red curve); (c) GQDs + Cu^{2+} (100 μM) + PPi (160μM) (purple curve); (d) GQDs + Cu^{2+} (100 μM) + PPi (160μM) + PPase (600 mU/mL) (green curve) in 10 mM HEPES buffer at pH 7.2 (E_{ex}= 368nm).

Results and Discussion

Mechanism of PPase Activity Detection

Fluorescent carboxyl-modified GQDs can be facilely synthesized and purified based on the previous procedure.[13e] The fluorescence emission spectrum of the GQDs showed a peak at 460 nm, while the excitation spectrum exhibited a peak at 368 nm (Figure 1, blue curve, and Figure S1 in Supporting information). The fluorescence quantum yield of GQDs was determined to be 5.58% by comparing the wavelength integrated intensity of the samples to that of standard quinine sulfate (Φ_{ST} = 0.577) (Figure S2 in Supporting

information). After the addition of Cu^{2+}, appreciable quenching of the fluorescence was observed (Figure 1, red curve). It is reasonable to assume that the quenching mechanism should be the coordination of Cu^{2+} with carboxyl group on the surface of GQDs, which eventually leads to the aggregation of GQDs. We can also prove the proposed mechanism of aggregation by comparing the TEM image of Cu^{2+}-GQDs complex with that of GQDs. We observed that the GQDs were well-dispersed and gave a diameter of ~3 nm. In contrast, the Cu^{2+}-GQDs complex was found to form clear aggregates with a diameter of ~20nm (Figure 2A,B). This observation gave immediate evidence for the induced assembly of GQDs into large aggregates triggered by Cu^{2+}. Further evidences from zeta potential analysis revealed that the zeta potential of the Cu^{2+}-GQDs complex showed positive shift with comparison to that of GQDs, indicating the formation of Cu^{2+}-GQDs complex (Figure S3 in Supporting information).

On adding PPi into the solution of Cu^{2+}-GQDs complex, the fluorescence spectrum was found to be recovered up to 90% (figure 1, purple curve). This was ascribed to the disaggregation of the Cu^{2+}-GQDs complex into to free GQDs, because the binding affinity between Cu^{2+} and PPi was reported to be higher than that between Cu^{2+} and carboxyl group,11b,15 The TEM image in figure 2C manifested that most of GODs were restored to monodispersity in this case, confirming that PPi mediated disaggregation of Cu^{2+}-GQDs complex into GQDs. After PPase incubated for 30 minute with the solution containing Cu^{2+}, PPi and GQDs, the fluorescence was quenched again (figure 1, green curve). This result was attributed to the hydrolysis of PPi into Pi catalyzed by PPase. Because Pi had much weaker interaction with Cu^{2+}, the coordination between Cu^{2+} and GQDs became dominant, which caused re-aggregation of GQDs with quenched fluorescence. The TEM image in figure 2D also manifested that most of GODs were restored to monodispersity in this case, testifying the presumed mechanism for our assay.

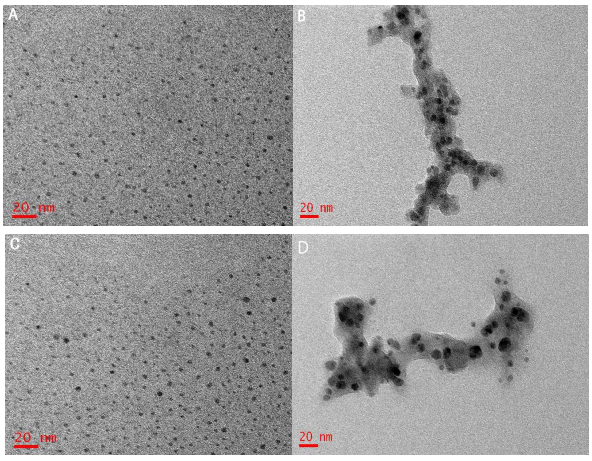


Fig. 2. (A)TEM images of single GQDs, (B) Cu^{2+}-GQDs complex, (C) re-disaggregated GQDs and PPi-Cu^{2+} complexes, and (D) re-aggregated GQDs with copper ions complexes in the presence of PPi and PPase.

Optimization of the Experimental Conditions

We first studied the effect of Cu^{2+} concentration on the sensing system. As shown in figure 3A and 3B, different concentrations of Cu^{2+} were mixed into the GQDs aqueous solution with the incubation time of 30 min, leading to gradual quenching of the GQDs fluorescence. When it reached 100 μM, the fluorescence of the mixture solution changed little with the further increase of Cu^{2+} concentration, thus, the Cu^{2+} concentration of 100 μM was adopted in the following experiments.

Figure 3C exhibited the fluorescence spectra of the GQDs solution containing 100 μM Cu^{2+} and different concentration of PPi from 0 to 180 μM, as we can see from figure S4 (Supporting information), 30 min later, the addition of 160 μM PPi made the fluorescence intensity close to that initial intensity, and further addition caused little change in it. Moreover, the fitted linear date between fluorescence intensity and concentrations of PPi in the range of 2 - 100 μM (Figure 3D), and the equation can be expressed as $y = 1.1356x + 256.58$, $R^2 = 0.9913$. This strategy can also be used to detect PPi.

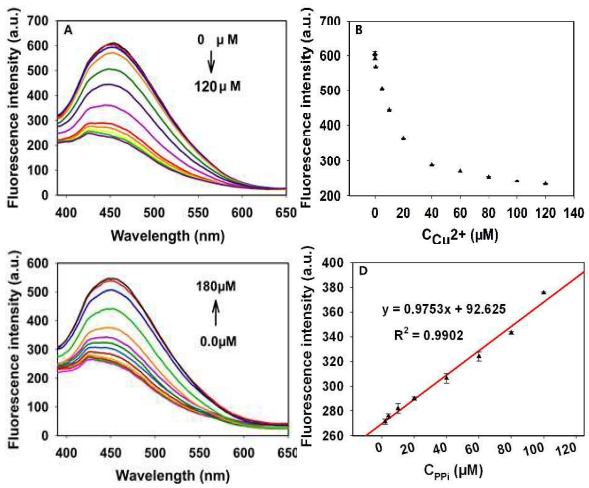


Fig. 3. (A) Fluorescence emission spectra of the GQDs with addition of different Cu^{2+} concentrations ranging from 0 to 120 μM (up to down). (B) Fluorescence intensity (I at 449nm) for GQDs with respect to the Cu^{2+} concentrations. (C) Fluorescence emission spectra of the Cu^{2+}-GQDs complexes with addition of different PPi concentrations ranging from 0 to 180 μM. (down to up). (D) The linearity of peak intensity with respect to PPi concentrations.

Sensitivity and Selectivity

Under the optimized experimental conditions, the activity of PPase was tested in the GQDs aqueous solution containing Cu^{2+} (100 μM), Mg^{2+} (0.1 μM), PPi (160 μM) and HEPES buffer (10 mM). As displayed in the figure 4A and figure S5 (Supporting information), the mixed solution was respectively added to different activity units of PPase in the range of 0 - 600 mU/mL, 60 min later in a water bath at 37 ℃, the decrease of fluorescence intensity was recorded, and there is a linear correlation between the fluorescence intensity and the

concentration of PPase in the range of 1 - 200 mU/mL. The linear equation can be expressed as $y = -0.9569x + 569.44$, $R^2 = 0.9961$ (figure 5B), and the limit of detection (LOD) based on $3\sigma/s$ was calculated to be about 1 mU/mL.

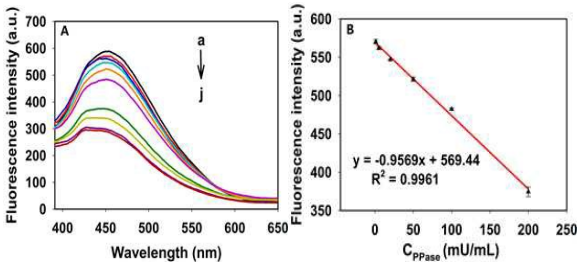


Fig. 4. (A) Fluorescence emission spectra of the system at different PPase concentrations, ranging from a to j: 0, 1, 5, 20, 50, 100, 200, 300, 400, and 600 mU/mL (B) The linearity of peak intensity with respect to PPase concentrations.

The developed assay was compared with literature reports. From Table S1 (Supporting information), it can be seen that our method exhibits comparable or even better performances than the published methods.

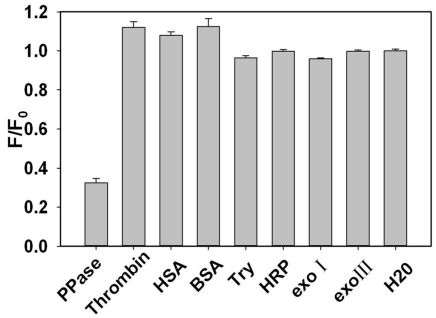


Fig. 5. Response of the method to different of proteins in 10 mM HEPES buffer at pH 7.2, 37 ℃. The concentration of PPase is 0.4 U/mL, [thrombin] 2×10^{-6} M, [HAS] 2.680×10^{-5} M, [BSA] 3.303×10^{-5} M, [Try] 1.91 U/mL, [HRP] 1.028 U/mL, [exo□] 5 U/mL, [exo□] 10 U/mL.

Before applying the proposed biosensor for real sample analysis, we investigated its selectivity comparing with several representative interfering proteins such as thrombin, human serum albumin (HAS), bovine serum albumin (BSA), trypsin (Try), horseradish peroxidase (HRP), exonuclease Ⅰ and exonuclease Ⅱ. As displayed in figure 5, the relative fluorescence intensities of these proteins did not decrease evidently in the absence of PPase, although their concentrations is much higher than PPase. The result indicated that these representative interfering proteins could not influence the quenching effect of PPase assay, that is to say, the proposed assay shows superior selectivity for PPase activity detection, which is promising to be applied in real biological samples.

Inhibition study

According to recent crystallographic results, the fluoride ion is a forceful and specific inhibitor of cytoplasmic pyrophosphatase due to the formation of the F^--PPase intermediate which results in an instant decrease in enzyme activity.[7a,10b,11a,16] To attest this result further, PPase with final concentration of 200 mU/mL was separately added into the aqueous solution of NaF with different concentrations (i.e., 0, 0.1, 0.5, 1, 5, 50, 200, 400, 500 μM), incubated for 15 min at room temperature. Then different concentrations of NaF-treated PPase were respectively mixed into the solution containing diluted GQDs, Cu^{2+} (100 μM), Mg^{2+} (0.1 μM), PPi (160 μM) and HEPES buffer (10 mM), incubated for another 60 min at 37 ℃. As shown in figure 6, the fluorescence intensity of the system increased gradually with the increase of NaF concentrations. Furthermore, the IC_{50} (the half maximal inhibitory concentration) values of 200 mU/mL PPase was calculated to be about 7.730 μM, which is in accord with the previous report.[9a] These results suggest that the proposed method can also be applied to evaluate the inhibition efficiency of PPase.

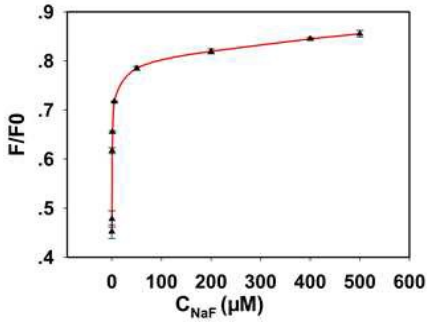


Fig. 6. Effect of NaF inhibition of PPase activity by measuring the fluorescence intensity of the GQDs in 10 mM HEPES buffer at pH 7.2.

Conclusions

In summary, we have reported a new finding on the aggregation and disaggregation of GQDs mediated by Cu^{2+} and its interacting anion PPi, which enables the development of a simple and highly sensitive fluorometric method for PPase activity and inhibition assay. To our knowledge, the proposed method is the first analytical system based on a fluorescence GQDs sensor that can quantitatively, selectively and sensitively detect PPase activity with a detection limit of 1mU/mL. This method can also be applied to screening inhibitors of PPase. In virtue of these advantages, the developed method may have great potential for the ascertainment of PPase activity and its potential inhibitors for basic biology and clinical diagnostics for PPase-related diseases and PPase-targeted drug discovery.

Acknowledgements

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