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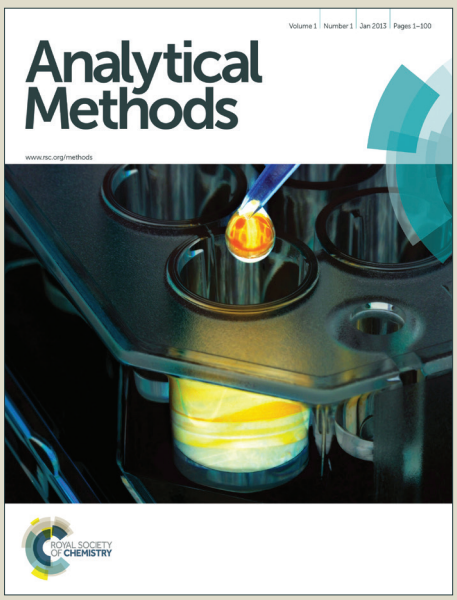

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Fluorescent detection of Mucin 1 protein based on aptamer functionalized biocompatible carbon dots and graphene oxide

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ABSTRACT: An ultrasensitive aptasensor for detection of Mucin 1 (MUC1) protein based on fluorescence resonance energy transfer (FRET) between carbon dots (CDs) and graphene oxide (GO) was reported. Taking advantage of strong fluorescence and good biocompatibility of CDs, the MUC1 aptamer was covalently conjugated to CDs (aptamer-CDs) to capture MUC1 protein through high affinity interaction between aptamer and MUC1 protein. The FRET process between aptamer-CDs and GO is easily achieved due to their efficient self-assembly through specific π–π interaction, in which the fluorescence of CDs was efficiently quenched. In the presence of target MUC1 protein, the association constant between aptamer-CDs and MUC1 is bigger than aptamer-CDs and GO, leading to the release of the aptamer-CDs from GO, and resulted in the recovery of CDs fluorescence. This was shown to detect MUC1protein specifically and sensitively in a linear range from 20.0 to 804.0 nM with a detection limit of 17.1 nM. The developed aptasensor is highly biocompatible and nontoxicity, which can be easily modified for the detection of other protein biomarker.

Keywords: aptasensor, fluorescence resonance energy transfer, carbon dots, graphene oxide, Mucin 1

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1. Introduction

Biomolecular detection has shown more and more critical application in early diagnostics and treatment of diseases, which has promoted intensive interest in the development of trustworthy, simple and low-cost biosensors for proteins, nucleic acids and other biomolecules.[1, 2] Aptamer, a kind of single-stranded oligonucleic acids or peptide molecules, shows high affinity to specific targets like proteins, peptides, organic molecules, metal ions, as well as cells, and thus is well recognized as a useful tool for binding target biomolecules.[3,4] Aptamer-functionalized nanomaterials integrated the advantages of both aptamer and nanomaterials, and displayed great potential applications in the construction of biosensors,[5] biosystem imaging[6] and targeted in vivo drug delivery.[7] Recently, novel nanomaterials have attracted great attention and have been intensively studied in biological analysis and detection. Due to their quantum effects proceeding from the large surface area-to-volume ratio, nanomaterials including Au nanoparticles, semiconductor QDs and carbon-based nanomaterials possess unique optical, electrical, catalytic and magnetic properties, and thus are widely applied to the detection of biological species.[8-10]

Fluorescence resonance energy transfer (FRET) from an excited state of a donor to a proximal ground state acceptor, is widely used in biological analysis.[11,12] Excellent donor–acceptor pairs and good biocompatibility of donor–acceptor pairs are two significant factors to improve the efficiency of FRET and the resulting analytical performance. However, current-used fluorescent probes including organic dyes and inorganic semiconductor quantum dots have their own drawbacks including poor

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photostability, easy photobleaching, small Stokes shifts, short lifetimes and significant toxicity even at relatively low concentrations,[13, 14] which may prove prohibitive to patient studies. Therefore, the search for better alternatives has continued. Carbon is hardly considered as an intrinsically toxic element. Of particular interest and significance was the recent finding that small carbon nanoparticles could be surface-passivated by organic or biomolecules to become strongly fluorescent.[15, 16] These fluorescent carbon nanoparticles named "carbon dots" were found to be physicochemically and photochemically stable and nonblinking. Carbon dots (CDs) are quasi-spherical nanoparticles with good biocompatibility and tunable surface functionalities. These superior properties afforded the use of CDs in broad fields such as bio-imaging[17] and sensing.[18, 19]

Graphene oxide (GO) possesses oxygen containing functional groups[20] and an unique microstructure composed of sp^2 carbons surrounded by sp^3 carbons, which give it an excellent aqueous solubility and hence their potential fluorescence quenching ability[21]. Recently, it has been employed as a good energy acceptor that could quench the fluorescence of semiconductor quantum dots, metal nanoclusters and organic dye and so on. Wei and co-workers[12] constructed a GO FRET aptasensor for MCF-7 breast cancer cells detection based on the dye -labeled aptamer assembled on GO.

In this paper, CDs and GO were designed respectively as the energy donor and acceptor for the FRET system. This system used Mucin 1 **(**MUC1) aptamer labeled CDs (aptamer-CDs) as a probe. MUC1 is a tumor marker encoded by the MUC1 gene

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in humans.[22] It is often overexpressed on the surface of cancer cells like breast, ovarian, lung and pancreatic cancers.[23] A 25-base oligonucleotide (aptamer) has specific binding properties for MUC1 peptide.[24] The fluorescence of the aptamer-CDs could be quenched efficiently by GO due to the FRET. As shown in Scheme 1, high quenching efficiency of fluorescence occurred when the aptamer-CDs was bound to GO, due to decrease in distance between GO and CDs. Upon recognition of the aptamer to the target MUC1 protein, the fluorescence increased significantly since the formation of the aptamer–MUC1 complex led to the release of aptamer-CDs from the surface of GO, which increased the distance between GO and CDs, hindered the energy transfer, and recovered the fluorescence of CDs. This system was demonstrated to possess high sensitivity and displayed promising applications in biomolecule detection.

2. Experimental

2.1. Materials and reagents

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), MUC1 protein and IgG were purchased from Abcam Company (Cambridge, UK). The MUC1 aptamer, complementary strand and a three-base mismatched strand DNA (mDNA) were synthesized by Sangon Biological Engineering Technology & Co. Ltd. (Shanghai, China) and purified using high-performance liquid chromatography. Their sequences were:

MUC1 aptamer: 5'-NH_2-GCAGTTGATCCTTTGGATACCCTGG-HPO_4-3'

Complementary strand (cDNA): 5'-CCAGGGTATCCAAAGGATCAACTGC-3'

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A three-base mismatched strand (mDNA):

5'-CCAGGGTATGCGACGGATCAACTGC-3'

Ethylenediamine, citric acid, nitric acid and sodium carbonate (Na_2CO_3) were obtained from Sinopharm Chemical Reagents Co., Ltd. Phosphate buffer solution (PBS) was prepared using 0.01M Na_2HPO_4 and 0.01M KH_2PO_4. All other chemicals were of analytical grade and used as received.

Fluorescence spectra were measured by an FL-4600 spectrometer (Hitachi, Japan). UV-Visible absorption spectra were collected on a Shimadzu UV-2450 spectrophotometer. Transmission electron microscopy (TEM) images were obtained from T20 FEI TECNAI G2 (America, FEI). Samples were prepared by dropping aqueous suspensions of aptamer-CDs, GO and aptamer-CDs/GO onto Cu TEM grids coated with a holey amorphous carbon film and following solvent evaporation in a dust protected atmosphere.

2.2. Synthesis of carbon dots (CDs)

CDs were synthesized according to the reference.[19] 1.05 g Citric acid and 335 μL ethylenediamine were dissolved in 10 mL deionized water. Then the solution was transferred to a Teflon-lined autoclave (30 mL) and heated at 250 °C for 5 h. Subsequently, the crude CDs suspension was mixed with concentrated nitric acid and heated at reflux for 12 h to obtain abundant carboxyl groups on the surface of CDs. After cooling to room temperature, the obtained solution was first neutralized by Na_2CO_3 solution. Then, the carbogenic nanoparticles obtained above were dialyzed against Milli-Q water for 3 days to remove all salts. The suspension appeared

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homogeneous light yellow and was finally stored at 4 °C in a refrigerator. The nanosized carbon dots were of 5 nm as estimated from TEM images.

2.3. Preparation of aptamer–CDs probe

The 3'-phosphate of the MUC1 aptamer was covalently combined to the carboxyl group of CDs as in previous reports.[25] Generally, the 3'- phosphate group of the MUC1 aptamer was first activated by adding 200 mL of a 0.10 M imidazole solution (pH 6.8) to 2 OD (optical density, 1 OD at 260 nm ≈ 4.31 nM ssDNA) of the aptamer for 30 min. Simultaneously, 1-ethyl-3-(3-dimenthylamino propyl)-carbodiimide (EDC) (120 μL, 0.4 M) and N-hydroxysulfosuccinimide sodium salt (NHS) (120 μL, 0.1 M) were added to the above CDs solution to activate the surface carboxylic group for 30 min at room temperature with continuous stirring. Subsequently, the activated CDs and aptamer (15.0 μL, 200.0 μM) were mixed together and allowed to react overnight at room temperature with continuous stirring. The aptamer–CDs bioconjugates were obtained by centrifugation and washing with PBS buffer three times to remove the free nonconjugated complex and by-products.

2.4. Synthesis of graphene oxide (GO).

GO was synthesized by natural graphite oxidation using a modified Hummer's method. Generally, 12 mL concentrated H_2SO_4 was put into a round bottom flask and cooled to 4 ^oC by using an ice bath. Then, added 0.4 g graphite powder and 0.2 g $NaNO_3$ under intense stirring for 30 minutes, followed by the addition of 1.2 g $KMnO_4$ in many times in 2 h. During these processes, the temperature of the mixture was maintained at 4 ^oC. Then it was heated to 35 ^oC by using oil bath for 4 h. After

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that, the mixture was diluted with 40 mL H_2O slowly and the temperature was increased to 90 ^oC. Then 4 mL 30 % H_2O_2 was added drop by drop after cooling the above mixture for about 0.5~ 1 h. After being cooling down to room temperature, the mixture was centrifuged and washed with 5 % HCl five times, and then with deionized water to neutral, and finally dried in air to obtain the GO.

2.5. Determination of MUC1 protein

The quenching effect of GO on fluorescence of aptamer-CDs was carried out as follows: 100 μL of aptamer-CDs (45.0 μg mL^{-1}) suspension in PBS was prepared at first, then, a series of 20 μL GO solutions with various concentrations were added and the final mixture solution was diluted to 300 μL with PBS. After 30 min of sonification and another 30 min of standing to ensure sufficient adsorption of the aptamer-CDs on GO, the fluorescence intensities of resulting mixtures were recorded using fluorescence spectrometer. For MUC1 protein detection, a series of 20 μL target MUC1 protein with different concentrations was incubated with the aptamer-CDs/GO mixture at 37 ^oC for 60 min. The resulted solutions were finally diluted to 300 μL with PBS and the fluorescence intensity was measured under the optimal excitation wavelength of 390 nm. The fluorescence dependent on time of quenching and recovery were conducted by monitoring fluorescence intensity at different incubation times.

3. Results and discussion

3.1. Conjugation of aptamer with carbon dots (CDs)

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After activation of the 3' phosphate group with imidazole, the aptamer was reacted with the carboxyl group on the surface of CDs in the presence of EDC and NHS to produce the aptamer-CDs conjugates. The conjugation of the CDs and aptamer was illustrated by UV-vis absorption spectra. Fig.1 shows the UV-vis absorption spectra of the CDs, aptamer, and aptamer - CDs in 0.01 M pH 7.4 PBS. The UV-vis absorption spectra of CDs shows a maximum adsorption peaks at ~340 nm which corresponds to π–π* transition of C=C bonds and respectively to n–π* transitions of C=O bonds (Fig. 1a). A characteristic absorption peak at ~260 nm is observed for the MUC1 aptamer (Fig. 1c). The aptamer-CDs maintained the characteristic absorption peaks of both the MUC1 aptamer and the CDs, indicating the successful labeling of the aptamer with CDs (Fig. 1b).

3.2. Characterization of CDs, aptamer-CDs, graphene oxide (GO) and aptamer-CDs/GO

The successful synthesis of GO and CDs are demonstrated by FT-IR spectra (Fig.2). The FT-IR spectrum of the as-synthesized GO display the characteristic vibrations that was reported in previous work, [26] including a broad and intense peak of an O–H group at 3443 cm^{-1}, an OH deformation peak at 1387 cm^{-1}, a C–O stretching peak at 1094 cm^{-1}, and a peak attributed to the vibrations of unoxidized graphitic skeletal domains and the adsorbed water molecules at 1631 cm^{-1} (Fig.2. curve GO). Also, as can be seen from the curve CDs in Fig.2, The peaks at about 1650 $cm^{-1}$and 1390 cm^{-1} indicate the existence of COO^{-} of the as-synthesized CDs, while the peak at 3434 cm^{-1} corresponds to the OH stretching mode.

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The representative TEM images and the size distribution plots of CDs, aptamer-CDs, GO and aptamer-CDs/GO are shown in Fig.3. As can be seen from Figure, the size of CDs and GO are quite uniform with diameters of about 5 nm (Fig. 3a, Fig. 3c), and the size of aptamer-CDs conjugates is about 30 nm (Fig. 3b). Compared with Fig.3b and Fig.3c, however, a clear evolution on the microstructure can be observed for the aptamer-CDs/GO mixture (Fig.3d), in which the diameter and morphology drastically changed and the particle size distribution become broad. It is demonstrated that aptamer-CDs conjugates successfully assembled with GO particles.

3.3. Detection of MUC1 protein with FRET

CDs possess good biocompatibility, strong fluorescence, and thus were chosen as the fluorophore in this aptasensor. GO with unique two-dimensional structure and good dispersion in water after moderate oxidation was used as electron acceptor and quenching agent in FRET. Scheme 1 displays the schematic illustration of MUC1 protein detection strategy based on FRET between MUC1 aptamer labeled CDs (aptamer-CDs) and GO. For detection of MUC1 protein, aptamer-CDs is firstly synthesized by conjugating aptamer and CDs via covalent coupling using EDC and NHS. Secondly, aptamer-CDs probe is mixed with GO so that aptamer-CDs can be adsorbed on the surface of GO through π–π stacking interaction to assemble aptamer-CDs/GO structure. Fig.4 demonstrates the strong quenching effect of GO on the fluorescence of aptamer-CDs and subsequent fluorescence recovery by MUC1 protein. As can be seen that the fluorescence aptamer–CDs still exhibits strong fluorescence (Fig.4b) although it is little smaller than that of pure CDs (Fig.4a) and it

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is scarcely influenced by the addition of specific target MUC1 protein in the absence of GO (Fig.4c), however, it can be largely quenched by addition of GO without target MUC1 protein (Fig.4d), indicating that fluorescence energy transfer between aptamer-CDs and GO in the self-assembly process. The formation of aptamer-CDs/GO results in nearly complete quenching of fluorescence of aptamer-CDs probe through FRET. The quenched fluorescence can be efficiently recovered by addition of target MUC1 protein (643 nM) (Fig.4e), which is due to the affinity interaction between the aptamer-CDs and MUC1 protein. After addition of MUC1 protein, the aptamer in aptamer-CDs conjugates should form the specific configuration and MUC1 protein is captured by aptamer-CDs to produce the MUC1/aptamer-CDs through specific base pairing. The formation of MUC1/aptamer-CDs complexes decreased the interaction of the aptamer-CDs and GO, and thus led to liberation and detachment of MUC1/aptamer-CDs from GO, resulting in fluorescence recovery of CDs. In addition, GO itself exhibits no fluorescence emission, and thus make no contribution to whole fluorescence intensity of each sample measured.

In order to develop this analytical method based on FRET between aptamer-CDs and GO, optimal conditions including concentration of added GO, incubation time of fluorescence quenching and recovery were studied. Fig.5A shows the quenching effect with various concentrations of GO. The fluorescence gradually decreases with the increasing concentration of GO, and remains stable at 120 μg mL^{-1} GO, when almost 95 % of the initial fluorescence is quenched. Further increase the GO

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concentration leads to fluorescence intensity unchanged. Thus, 120 μg mL^{-1} was chosen as the optimal concentration of GO. Additionally, the quenching effect with GO and recovery efficiency with MUC1 protein as a function of incubation time are displayed in Fig.5B. One can note that the fluorescence is gradually quenched with time and remains nearly constant after 60 minutes in the absence of MUC1 protein (Fig.5B, curve a). The curve for fluorescence recovery by MUC1 protein (643.0 nM) shows a rapid growth in fluorescence intensity in the first 30 min, and then keeps nearly unchanged in fluorescence intensity after 60 min (Fig.5B, curve c). In the presence of target MUC1 protein, the fluorescence intensity decreased in the first 30 min, however, it tended to stabilize at higher value (Fig.5B, curve b). These time dependent experiments clearly indicated that the MUC1 protein had a better binding affinity to aptamer than GO, and thus the resulting MUC1/aptamer-CDs complex prevented the fluorescence of CDs from being quenched by GO. So, 60 min were chosen for the both optimal fluorescence quenching time and recovery time in the following determination of MUC1 protein.

Under the optimal conditions, the sensing performance of this FRET system was evaluated by adding various concentrations of MUC1 protein into aptamer-CDs /GO mixture. The fluorescence comes from MUC1/aptamer-CDs formed between aptamer–CDs and MUC1 protein after addition of MUC1 protein into aptamer-CDs /GO mixture solution. As illustrated in Fig. 6A, the fluorescence intensity of MUC1/aptamer-CDs is increased gradually with increasing concentration of MUC1 protein from 20.0 to 804.0 nM because more and more amounts of

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MUC1/aptamer-CQD formed and escaped from GO surface during this process. The plot of fluorescence intensity as a function of MUC1 concentration is shown in Fig.6B. The linear equation can be expressed as y =2.20x+119.83, where R^2= 0.972. The limit of detection (LOD) was estimated about 17.1 nM according to the 3σ (standard deviation) rule, which was lower than the previously reported biosensors.[12]

The specificity of the proposed aptamer-functionalized CDs probe was further carried out by using different DNA sequences and proteins. The comparison of results between the perfectly complementary strand (cDNA) and three-base mismatched strand DNA (mDNA), MUC1 protein and IgG protein were shown in Fig.7. Upon addition of analyte, the restored fluorescence intensity for the target MUC1 protein (643 nM) showed the highest value (1500 a.u), while the fluorescence value for the same concentration of IgG protein was only150 a.u. Moreover, the response of the cDNA (643 nM) was slightly lower than that of the target MUC1 protein with a fluorescence value of 1280, while the response of the same concentration of mDNA (180 a.u.) and the blank control without target (50 a.u) were very slow. These results demonstrated that the proposed aptamer-functionalized CDs probe could easily discriminate between the perfectly complementary strand and the MUC1 protein from the randomly mismatched strand and other protein, which indicates that the proposed approach has high specificity for the target detection.

4. Conclusion

In conclusion, a novel and effective aptasensor based on FRET between CDs and

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GO for MUC1 protein detection has been established. The MUC1 aptamer-functionalized CDs were prepared and has strong fluorescence, good biocompatibility and resistance to photobleaching. FRET can be easily achieved because of efficient self-assembly between CDs and GO. This aptasensor can distinguish complementary nucleic acid sequences and MUC1 protein from other biomolecules like three-base-mismatched nucleic acid sequences and IgG with high sensitivity and good specificity. It shows a quite broad linear scope and low detection limit of 17.1 nM for MUC1 protein. Since all the materials involved in the sensing system are of excellent biocompatibility, it is expected that this proposed approach would be used in vivo and in vitro, and promotes the application of carbon-based nanomaterials in bioassays.

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Figure captions:

Scheme 1. Schematic illustration of detection of MUC1 protein based on fluorescence energy transfer (FRET) between aptamer–functionalized carbon dots (CDs) and graphene oxide.

Fig.1. UV–vis spectra of (a) CDs, (b) MUC1aptamer-CDs and (c) MUC1 aptamer in 0.01M pH 7.4 PBS.

Fig.2. FT-IR spectra of carbon dots (CDs) and graphene oxide (GO).

Fig.3. TEM images of (A) CDs, (B) aptamer–CDs, (C) graphene oxide and (D) mixture between graphene oxide and aptamer–CDs.

Fig.4. The quenching effect of GO on the fluorescence of aptamer–CDs and following fluorescence recovery MUC1 protein, (a) CDs, (b) aptamer–CDs, (c) aptamer–CDs + MUC1 (643.0 nM), (d) aptamer–CDs + GO (120 μg mL^{-1}), (e) aptamer–CDs + GO (120 μg mL^{-1}) + MUC1 (643.0 nM).

Fig.5. (A) The fluorescence quenching of aptamer-CDs with different GO concentrations of 0, 10, 30, 50, 80, 120, 150 μg mL^{-1} in 0.01M PBS (pH 7.4). Each data point represents the average of the fluorescence responses of triplicate measurements**. (B)** Time dependence of the fluorescence quenching by 120 μg mL^{-1} GO without (a) and with (b) MUC1 (643.0 nM), and (c) the fluorescence recovery with 643.0 nM MUC1 protein.

Fig.6. (A) The fluorescence recovery of aptamer–CDs/GO system after incubation with 0, 20.0, 160.0, 300.0, 352.0, 400.0, 440.0, 566.0, 643.0, 700.0, 804.0 nM MUC1. **(B)** The linear relationship between the fluorescence intensity and concentration of

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MUC1 protein. Each data point represents the average of the fluorescence responses of triplicate measurements

Fig.7. Fluorescence intensity of aptamer-CDs after incubated with 643.0 nM of (a) MUC1, (b) complementary DNA stand, (c) three-base mismatch stand (mDNA), (d) IgG protein, (e) no target in the presence of 120 μg mL^{-1} GO. Each data point represents the average of the fluorescence responses of triplicate measurements

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Scheme 1

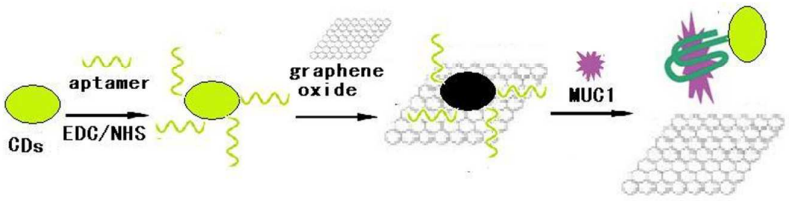

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Fig.1

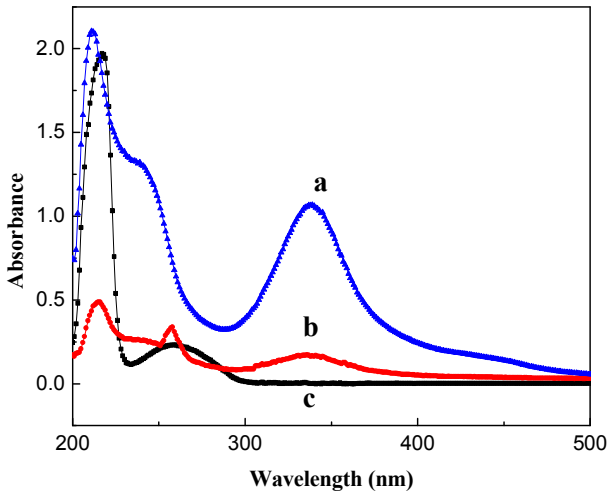

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Fig.2

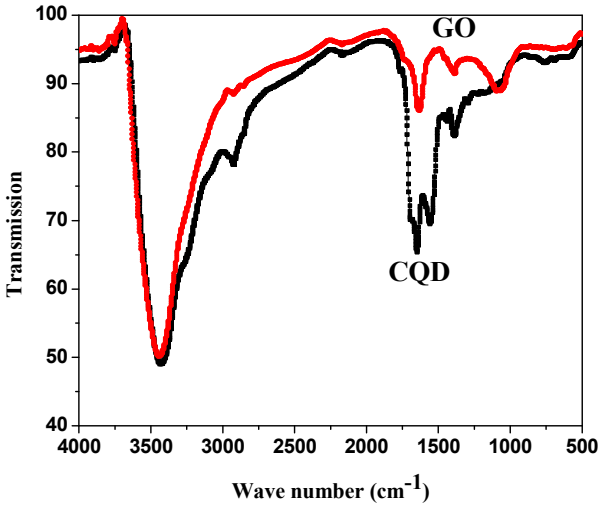

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Fig.3

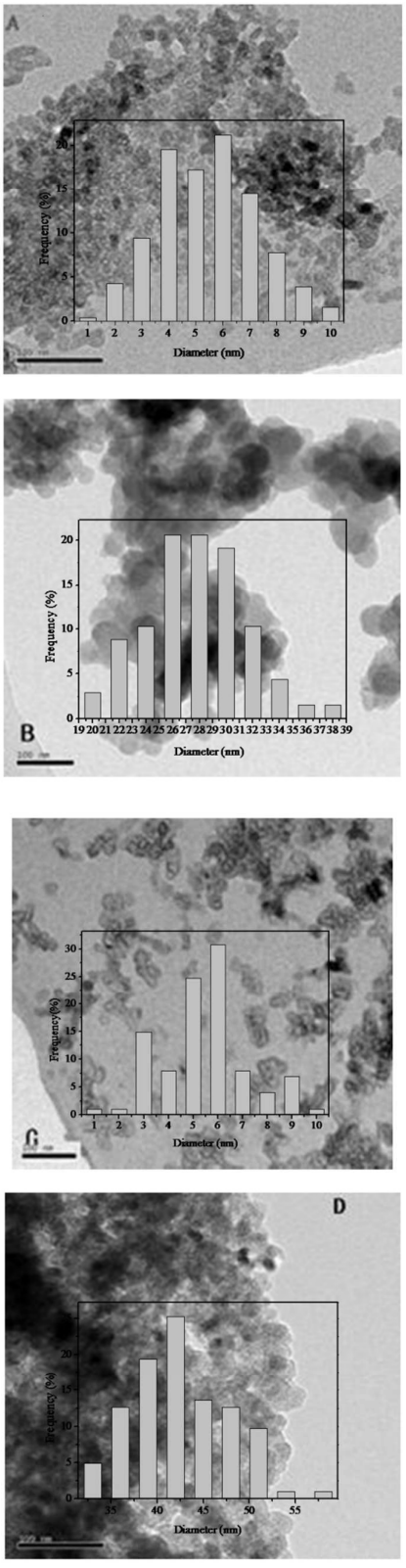

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Fig.4

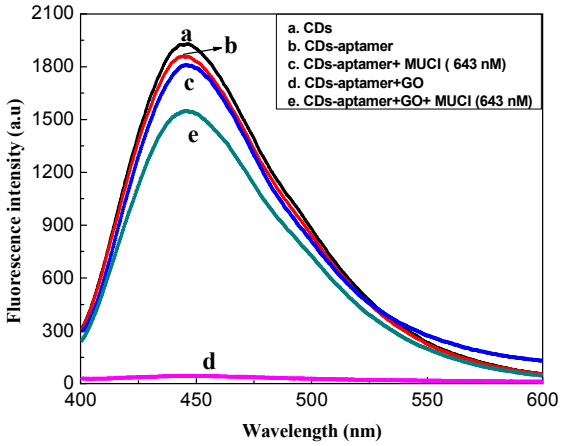

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Fig.5

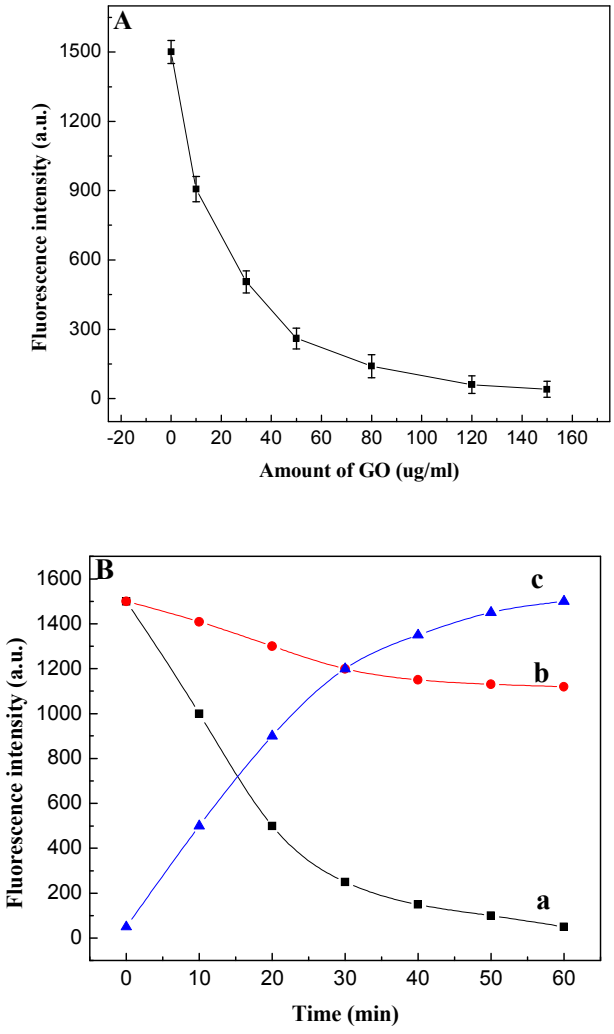

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Fig.6

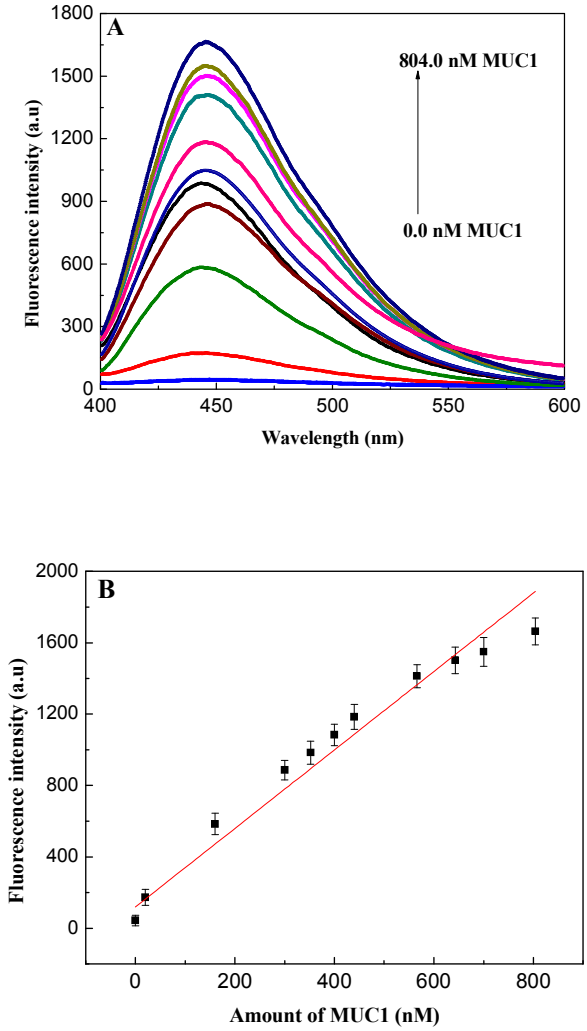

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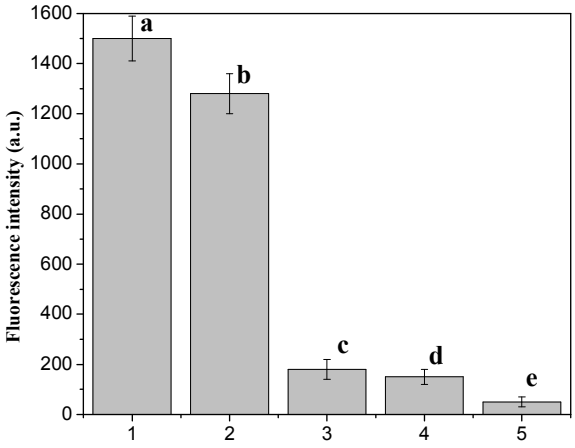
Fig.7.



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