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PVP-coated graphene oxide for selective determination of ochratoxin A via quenching fluorescence of free aptamer

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ABSTRACT

In this paper, we developed a simple method to detect fungi toxin (ochratoxin A) produced by *Aspergillus Ochraceus* and *Penicillium verrucosum*m, utilizing graphene oxide as quencher which can quench the fluorescence of FAM (carboxyfluorescein) attached to toxin-specific aptamer. By optimizing the experimental conditions, we obtained the detection limit of our sensing platform based on bare graphene oxide to be 1.9 μM with a linear detection range from 2 μM to 35 μM. Selectivity of this sensing platform has been carefully investigated; the results showed that this sensor specifically responded to ochratoxin A without interference from other structure analogues (N-acetyl-L-phenylalanine and warfarin) and with only limited interference from ochratoxin B. Experimental data showed that ochratoxin A as well as other structure analogues could adsorb onto the graphene oxide. As compared to the non-protected graphene oxide based biosensor, PVP-protected graphene oxide reveals much lower detection limit (21.8 nM) by two orders of magnitude under the optimized ratio of graphene oxide to PVP concentration. This sensor has also been challenged by testing 1% red wine containing buffer solution spiked with a series of concentration of ochratoxin A.

1. Introduction

Toxins produced by pathogenic microbes as virulent factors can cause highly life-threatening diseases deleterious to the public health and life systems, by affecting eukaryotic cells by a variety of means (Schmitt et al., 1999): damaging cell membranes, inhibiting protein synthesis, activating second messenger pathways, inhibiting the release of neurotransmitters and activating the host immune response. Ochratoxin A as one of the most abundant food-contaminating mycotoxins in the world was chosen as the prototypic analyte in this paper(Petzinger and Ziegler, 2000; Pfohl-Leszkowicz and Mandervill, 2007). Human exposure to ochratoxin A occurs mainly through consumption of improperly stored food products, particularly contaminated grain and pork products, as well as coffee and wine grapes (Ahmed et al., 2007). This toxin has also been found in the tissues and organs of animals, including human blood and breast milk (Monaci and Palmisano, 2004). Ochratoxin A is potentially carcinogenic to human beings. It has been shown to be weakly mutagenic and can cause immunosuppression as well as immunotoxicity (Pfohl-Leszkowicz and Mandervill, 2007; O'Brien and Dietrich, 2005; Assaf et al., 2004). With the recognition of severe toxic effect of this fungi toxin, significant efforts (Ngundi et al., 2006) have been invested to develop simple and flexible sensing platforms to detect this fungi toxin in food and water.

Among a variety of detection methods (Clarke et al., 1993; Prieto-Simon et al., 2008; Van Der Gaag et al., 2003; Tsai and Hsieh, 2007), toxin specific antibody is always being utilized to be the molecular recognition agent. Instead of using antibody in this study, aptamer for ochratoxin A was utilized as molecular recognition agent to build a new sensing platform for detection of ochratoxin A (Cruz-Aguado and Penner, 2008a,b). Aptamers are single strand DNA that can bind target molecule with high specificity and strong binding affinity (Ellington and Szostak, 1990; Cho et al., 2009). As compared to antibody, aptamer possesses several advantages which attract extensive research in recent years (Hermann and Patel, 2000; Famulok et al., 2007; Willner and Zayats, 2007). Aptamers are easily synthesized and commercially available. It can be easily modified with a variety of chemical groups, which provides an extraordinary flexibility in different assays. Moreover, it has been recognized for many years that stability of antibody is not good as aptamer (Schmidt et al., 2004), which has prevented application of antibody in a broader range of experimental conditions. Aptamers as newly emerging molecular recognition agents have been extensively studied in recent years (Sefah et al., 2010). However, the number of biosensors based on aptamers selective for fungi toxins is still limited (Tombelli et al., 2007). Recently, an aptamer specific for ochratoxin A has

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been reported, which can fold to form antiparallel G-quadruplex structure upon exposure to ochratoxin A (Cruz-Aguado and Penner, 2008a,b; Yang et al., 2010). Inspired by the knowledge that folded DNA structure and hybrid duplex are resistant to the adsorption onto basal plane of graphene oxide which has attracted great attention in the past several years (Lu et al., 2009; Mohanty and Berry, 2008), we postulated that folded aptamer induced by binding to ochratoxin A was also resistant to adsorption onto the surface of graphene oxide. Therefore we designed a sensing strategy for detection of ochratoxin A: FAM (carboxyfluorescein) modified aptamer after binding to ochratoxin A cannot be quenched by graphene oxide; however, FAM modified aptamer without binding to ochratoxin A could be significantly quenched. Indeed, the results in our experiment also confirmed that our design strategy was very successful.

Since the discovery of graphene by professor Andre Geim in 2004 (Nobel prize winner for 2010) and the first application of graphene based advanced materials (Novoselov et al., 2004; Novoselov et al., 2005), this material has been utilized in various fields and the related references have expanded dramatically. Right now, graphene oxide can be easily synthesized by using Hummer's method (Hummers and Offeman, 1958), which has opened new avenue for biosensor (Lu et al., 2009; Balapanuru et al., 2010; Liu et al., 2010). By combining graphene oxide with aptamer modified in single end, the major advantage is that it eliminates expensive dual labeling of aptamer in comparison to conventional molecular beacon.

It is very important to note that graphene oxide based sensors have to meet one prerequisite that the analyzed target does not nonspecifically adsorb onto the surface under the experimental conditions, otherwise the sensing performance is dramatically degraded or the sensor fails to function. Very recently, Lu and coworkers (Lu et al., 2009) developed a versatile method based on unprotected graphene oxide for DNA detection. Other groups have also tested the graphene oxide (or graphene) platform to detect other analytes (Wang et al., 2010; He et al., 2010; Wen et al., 2010), such as, Ag^+, ATP, etc. In order to extend the capability of biosensor based on graphene oxide, we need to investigate the possibility of this sensor for detection of molecules which adsorb on the basal plane of graphene oxide. In this study, we choose ochratoxin A (OTA) as the object of analysis. Experimental results showed that OTA indeed adsorbed onto the surface of one-atom thick graphene oxide. In order to eliminate the adsorption of target molecules, we seek to use polymer materials (poly(vinyl pyrrolidone)) as coating materials to prevent the unspecific adsorption of target.

2. Experimental

2.1. Chemicals and materials

The sequences of OTA's aptamer (5-FAM-GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-3) were synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). DNA stock solution was obtained by dissolving oligonucleotides in 10 mM Tris buffer(pH 8.5) containing 120 mM NaCl and 5 mM KCl and 20 mM $CaCl_2$ and was stored at 4 °C before use. The concentration of oligonucleotide was determined using the absorbance at 260 nm. Tris($C_4H_{11}NO_3$)was purchased from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). Graphite was purchased from Alfa Aesar. Ochratoxin A (OTA), N-acetyl-L-phenylalanine (NAP) and warfarin was obtained from Sigma–Aldrich (St. Louis, MO, USA). Ochratoxin B (OTB) was obtained from BioAustralis Pty Ltd. (NSW, Australia). The stock solution of ochratoxin A (1 mM) was prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C. Sodium chloride (NaCl), potassium chloride (KCl), poly(vinyl pyrrolidone) (PVP) and anhydrous calcium chloride ($CaCl_2$) were purchased from Beijing Chemical Reagent Company (Beijing, China). Changyu Rose Red Wine was produced by YanTai ChangYu Pioneer wine Company Limited. All of the chemicals were of at least analytical grade. The water used throughout all experiments was purified by a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

Atomic force microscopy (AFM) was conducted with a SPI3800N microscope (Seiko Instruments, Inc.). Cary 500 Scan UV/Vis Spectrophotometer (Varian, USA) was used to quantify the oligonucleotides. Fluorescence intensities were recorded on a LS-55 luminescence spectrophotometer (Perkin-Elmer, USA). The emission spectra were recorded in the wavelength of 500–630 nm upon excitation at 492 nm with slit widths for the excitation and emission set at 2.5 and 15 nm, respectively.

2.3. Synthesis of graphene oxide

The graphite oxide (GO) was synthesized from natural graphite powder based on modified Hummers method. Then, the as-prepared graphite oxide was subjected to ultrasonication for 40 min (1000 W, 20% amplitude). Finally, a homogeneous GO aqueous dispersion (1 mg/mL) was obtained and used for further use. The graphene oxide was then characterized with tapping-mode AFM.

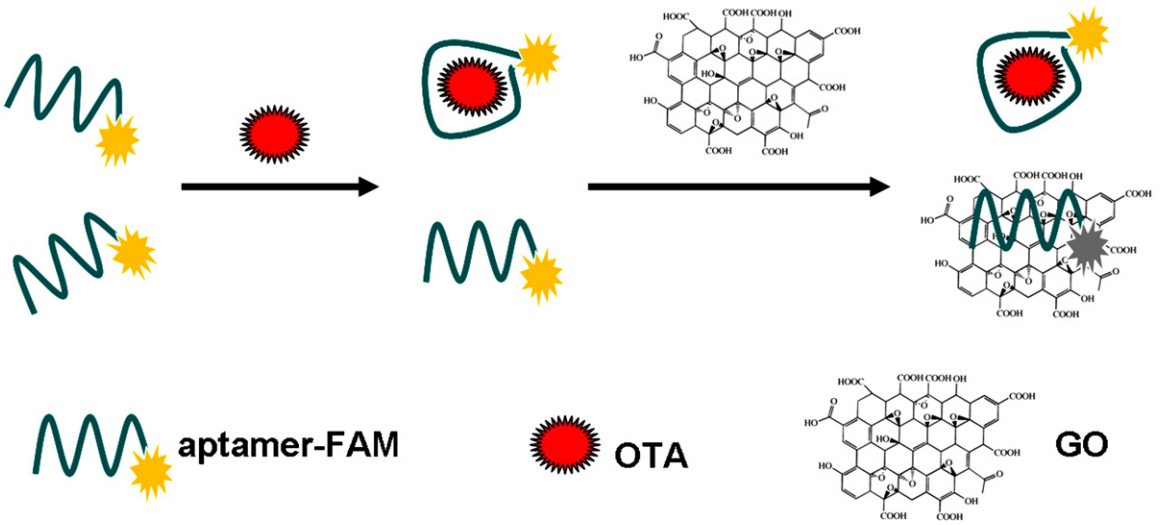
2.4. Fluorescent detection of ochratoxin A

For quantitative measurement of ochratoxin A, 400 μL solution containing 200 nM concentration of FAM-modified aptamer was mixed with different concentrations of OTA, and allowed to settle for 15 min. Then, 50 μL of solution with 96 μg/mL GO (or PVP/GO) was added. Subsequently, 150 μL of 10 mM Tris buffer was mixed with the resulting solution to give a final volume of 800 μL. After the solution was equilibrated for 30 min, fluorescence measurement was carried out.

3. Results and discussion

3.1. Design strategy for ochratoxin A detection based on graphene oxide sensing platform

Scheme 1 illustrates the sensing strategy for detection of ochratoxin A. In the absence of target molecule (OTA), FAM-modified aptamer adsorbs onto the basal plane of graphene oxide via the π–π stacking force, which is similar to the interaction between single strand DNA and carbon nanotubes (Zheng et al., 2003a,b). In addition, the electrostatic driving force is also contributable to the adsorption of DNA on the surface of graphene oxide (Lu et al., 2009; Liu et al., 2010; He et al., 2010). Consequently, the fluorescence of FAM is quenched readily via energy transfer from dye to graphene oxide. In the presence of target molecules (OTA), ochratoxin A induces conformational change of ochratoxin A specific aptamer, leading to the formation of antiparallel G-quadruplex (Cruz-Aguado and Penner, 2008a,b; Yang et al., 2010) which is resistant to adsorption onto the larger planar surface of graphene oxide. Therefore, the fluorescent intensity as a function of ochratoxin A concentration was measured correspondingly.



Scheme 1. Schematic illustration of graphene oxide sensing platform for detection of ochratoxin A.

3.2. Optimization of the concentration ratio of graphene oxide to FAM-modified aptamer

The correct concentration ratio of graphene oxide to FAM-modified aptamer is highly important. As shown in AppendixBFig. 3S, the maximum peak intensity of fluorescence spectra decreased along with increasing the concentration of graphene oxide at the fixed concentration of 100 nM FAM-modified aptamer. When the concentration of graphene oxide reached 6 μg/mL, 90% of the fluorescence intensity of FAM-modified aptamer was quenched. According to this result, we utilized the graphene oxide with concentration of 6 μg/mL in the throughout experiment. Higher concentration of graphene oxide than 6 μg/mL was not necessary, which will provide more free space on the planar surface for the adsorption of ochratoxin A, leading to worse sensing performance.

3.3. Investigation of the effect of calcium concentration on the quenching efficiency

Metal ions, especially related divalent ions, have been reported to play very important roles in the binding affinity between aptamer and target molecules. In addition, divalent metal acting as ionic bridge also enhances the electrostatic interaction force which contributes to the interaction between graphene and DNA. Since the original paper has already proved that higher calcium ion was relevant to the binding affinity of aptamer for ochratoxin A, and removal of monovalent ion (sodium and potassium) did not significantly affect the affinity between aptamer and ochratoxin A, therefore, optimal concentration of divalent metal ion is very critical in this experiment. As shown in the Fig. 1, when the calcium concentration increased from 6 mM to 20 mM, the quenching efficiency (black columns) dramatically increased. It tells us that divalent ions such as calcium ion act as bridge between the DNA and gaphene oxide whose surface is occupied by hydroxyl groups (Lu et al., 2009). Higher concentration than 20 mM has not been tested since this value is optimal for the binding interaction between aptamer and ochratoxin A as reported previously (Cruz-Aguado and Penner, 2008b). Upon addition of ochratoxin A, less amount of aptamer adsorbed onto the planar surface of graphene oxide due to the compact conformation of aptamer induced by ochratoxin A. As observed in Fig. 1 (red column), the fluorescence intensity increased in the presence of ochratoxin A, The reason is because higher amount of calcium facilitates the compact G-quadruplex formation of aptamer induced by ochratoxin A. Unless otherwise specifically stated, calcium concentration of 20 mM was used for this experiment.

3.4. Quantitative measurement of ochratoxin A based on bare graphene oxide

The premixing of aptamer/ochratoxin A is extremely important in this study (Wang et al., 2010), allowing sufficient freedom of the interaction between aptamer and ochratoxin A. As illustrated in Fig. 2A, fluorescence measurement showed that the fluorescence intensity in the solution varied with the increasing of concentration of ochratoxin A. Calibration curve of fluorescence intensity as a function of concentration down to 500 nM was plotted (Fig. 2B). The limit of detection (LOD) was defined as the concentration corresponding to the fluorescence signal at 3 times standard deviation of blank without OTA. The limit of quantification (LOQ) was defined as the concentration corresponding to the fluorescence signal at 10 times standard deviation of blank without OTA. Then the limit of detection (LOD) was calculated to be 1.9 μM and the quantifi-

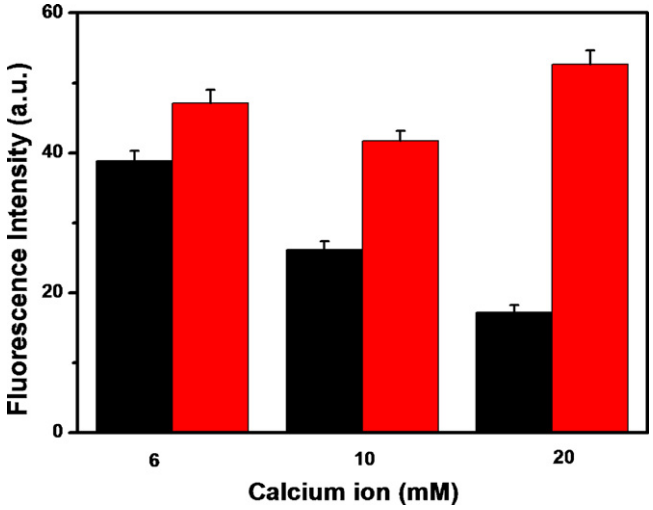


Fig. 1. Effect of calcium concentration on the quenching efficiency of graphene oxide toward FAM-modified aptamer. Black bar represents solution without ochratoxin A and red bar represents solution with ochratoxin A. Error bars were obtained from three experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

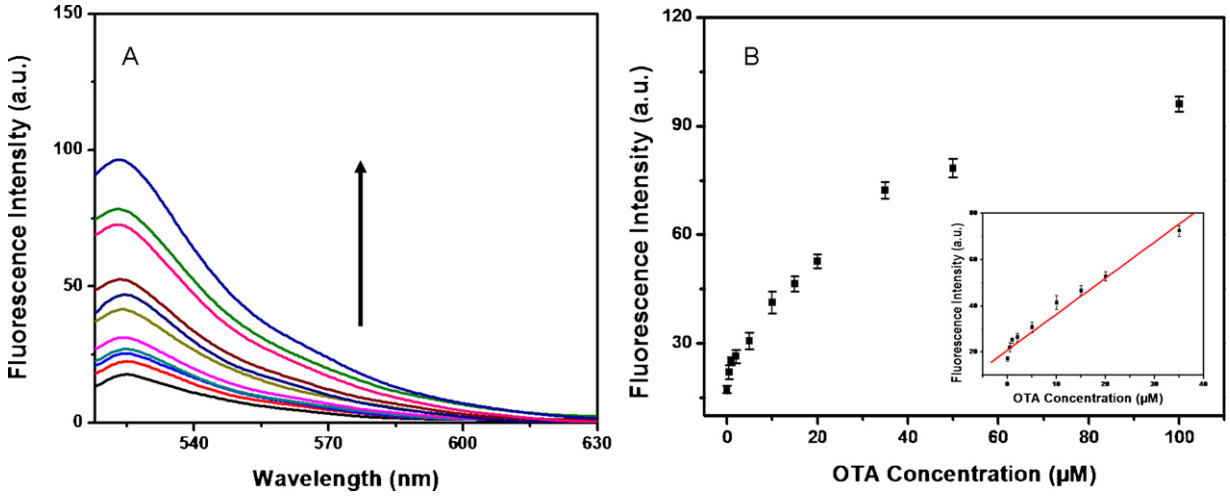


Fig. 2. (A) Fluorescence spectra of solutions corresponding to various concentrations of ochratoxin A under the condition of premixing of aptamer/ochratoxin before addition of graphene oxide. (B) The maximum peak of fluorescence spectra in (A) were plotted as a function of concentration of ochratoxin A. Inset in B: linear part of the plot in B. Error bars were obtained from three experiments.

cation (LOQ) was calculated to be 6.6 μM. With the existence of 100 nM aptamer and 6 μg/mL graphene oxide in the binding buffer solution, the linearity of the fluorescence response to concentration of ochratoxin A was located between 2 μM and 35 μM (inset in Fig. 2B). The detection limit is much higher than that obtained from fluorescence polarization displacement assay (Cruz-Aguado and Penner, 2008b) and adsorption of ochratoxin A onto the planar surface of graphene oxide may be main reason responsible for the much higher detection limit. Therefore, further optimization steps will be shown in Section 3.6.

3.5. Selectivity of graphene oxide based sensing platform against various structure analogues

In order to determine the specificity of this method, we tested the sensing platform against various structure analogues which have been characterized by UV–Vis spectrometry in the previous section. N-acetyl-L-phenylalanine whose molecular structure represents one part of ochratoxin A was used. Warfarin and ochratoxin A share the same similarity in the fact that they occupy the same binding site in human serum albumin (Il'ichev et al., 2002). Ochratoxin B that lacks the chlorine atom in the isocoumarin ring is a structural analogue of ochratoxin A. As shown in Fig. 3 (red column 2), addition of target molecule (OTA) with concentration of 20 μM induced dramatic fluorescence increase; however, other analogues with concentration of 100 μM (black columns 3, 5 and 7) did not induce apparent fluorescence increase. Therefore, this aptamer was highly specific to OTA, which was further confirmed by the slight fluorescence response to the coexistence (black column 9) of N-acetyl-L-phenylalanine (100 μM), warfarin (100 μM) and ochratoxin B (100 μM). We have also used another DNA strand which is different from this aptamer (AppendixBFig. S4) by several nucleotides, the column data in AppendixBFig. S4 shows that OTA and other structure analogues induced the same fluorescence intensity, indicating that the mutated aptamer did not bind these molecules.

At the same time, we have tested the interference effect of other structure analogues on the detection of ochratoxin A. In this experiment, we set the concentration of ochratoxin A at 20 μM, while the concentrations of other OTA analogues were set at 100 μM. As shown in the Fig. 3 (red column, 4 and 6), the presence of N-acetyl-L-phenylalanine (or warfarin) only exhibited negligible effect on the detection of ochratoxin A; addition of ochratoxin B whose molecular structure has the biggest similarity with OTA just resulted in slight increase of fluorescence intensity (red column, 8). All the data (black columns) reveal that no specific interaction existed between OTA specific aptamer and other three analogues. However, the coexistence of ochratoxin A (OTA) and ochratoxin B (OTB) cooperatively increased the fluorescence intensity (red column, 8) in the buffer solution. To take this experiment one step further, four components (warfarin, N-acetyl-L-phenylalanine, OTA and OTB) were mixed together (red column, 10), which induced much higher fluorescence in the solution as compared to that containing three components (warfarin, N-acetyl-L-phenylalanine and OTB) (black column, 9). To understand the reason behind this phenomenon, we have to understand how three elements (graphene oxide, aptamer and structure analogues) in the binding buffer solution interact with each other. Since we have precluded the possibility of interaction between structure analogues and aptamer, the interaction between graphene oxide and structure analogues had to be taken

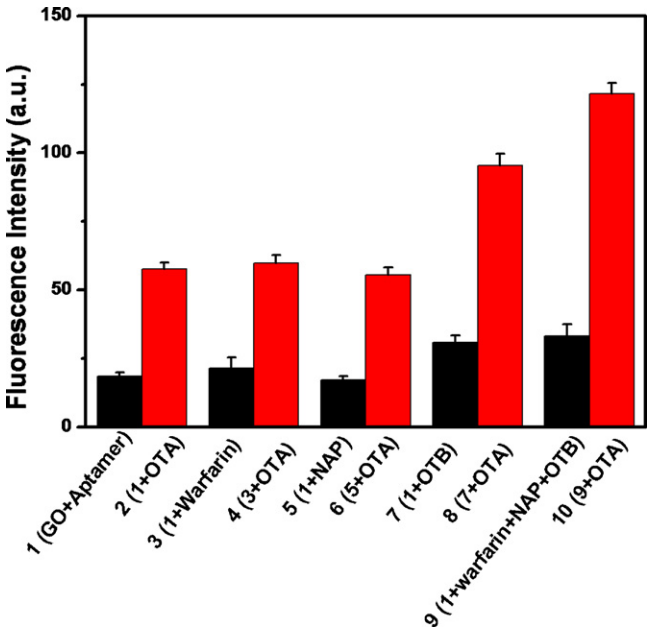


Fig. 3. Selectivity of the sensor toward ochratoxin A (20 μM) and against other structure analogues (100 μM). GO (graphene oxide), OTA (ochratoxin A), OTB (ochratoxin B), NAP (N-acetyl-L-phenylalanine) and warfarin. Error bars were obtained from three experiments.

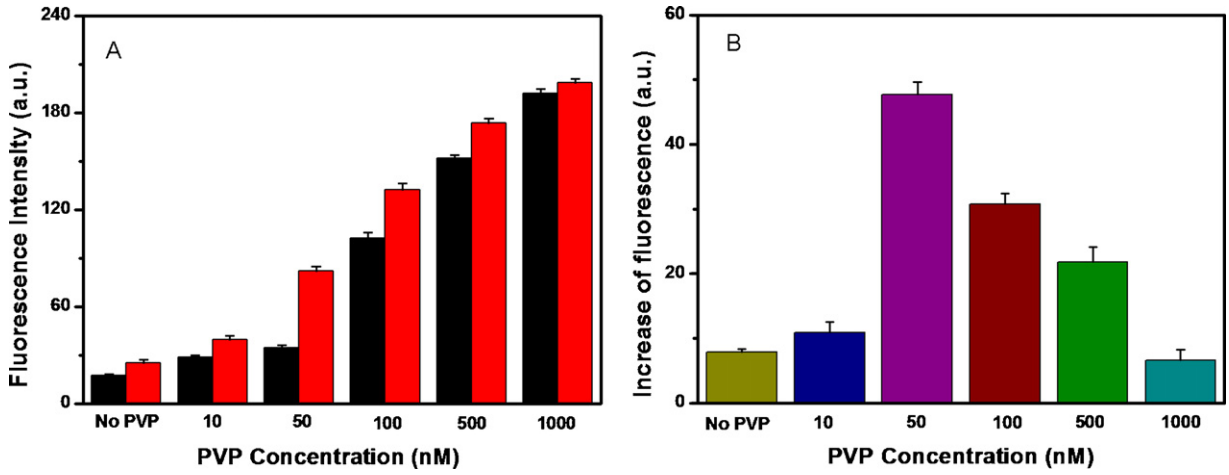


Fig. 4. (A) Effect of PVP concentration on the fluorescence intensity of graphene oxide/FAM-modified aptamer without (black bar) and with (red bar) existence of ochratoxin A. (B) column bars were obtained by subtracting the value of black column bars from the value of corresponding red column bars. Error bars were obtained from three experiments.

into account. We reasoned that the interaction between graphene oxide and OTB (or N-acetyl-L-phenylalanine, warfarin) elicit the adsorption of OTB onto the planar surface of graphene oxide in the presence of excess of mixtures of OTB plus OTA. Therefore, more amount of OTA was retained in the solution, which was confirmed by the increase of fluorescence indicated by red columns.

Motivated by this interesting discovery that small molecules coated graphene oxide has better sensitivity toward target molecules, we made efforts to find whether a commercially available polymer (PVP) can be used to coat graphene oxide, which has also been reported in previous studies (Bourlinos et al., 2009; Fang et al., 2010). Previous studies have not specifically dealt with the question if coating can improve the sensing platform based on graphene oxide.

3.6. Quantitative measurement of ochratoxin A based on PVP-coated graphene oxide

For the purpose of understanding how much PVP is needed for prevention of unspecific adsorption of ochraotixn A on the graphene oxide, Optimization steps were conducted. As shown in Fig. 4A, the fluorescence intensity of solution increased along with the addition of PVP up to 1 μM into the solution containing 6 μg/mL of graphene oxide and 100 nM FAM-modified aptamer. It disclosed that adsorption of PVP onto the graphene oxide reduced the quenching efficiency by preventing the interaction between graphene oxide and aptamer. In the meanwhile, Fig. 4B tells us that PVP coating can also efficiently block the adsorption of target molecules. Considering that best sensitivity was obtained by using PVP with concentration of 50 nM via analyzing the column bar in Fig. 4B, the following fluorescence measurements were carried out in the presence of this optimal concentration of PVP.

Fig. 5A illustrates that the fluorescence intensity in the solution varied with the increase of concentration of ochratoxin A. Calibration curve of fluorescence intensity as a function of concentration was plotted (Fig. 5B). Concentration of ochratoxin A down to 50 nM was tested. The limit of detection (LOD) was calculated to be 21.8 nM, and the limit of quantification (LOQ) was calculated to be 206.8 nM. With the existence of 100 nM aptamer, 6 μg/mL graphene oxide and 50 nM PVP in the binding buffer solution, the linearity of the fluorescence response to concentration of ochratoxin A was from 50 nM to 500 nM. Although calibration curve has a pretty narrow linear detection range (inset in Fig. 5B), the detection limit is much lower than that obtained from bare graphene oxide based assay.

We have challenged our system with red wine; the results show that our design strategy is suitable for measuring OTA in real food samples, buffer solutions containing 1% red wine were spiked

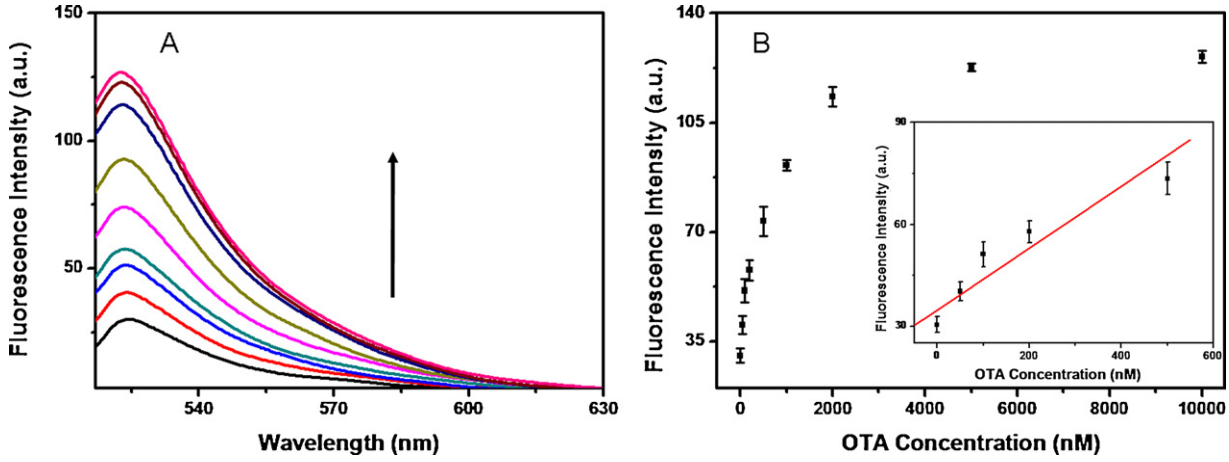


Fig. 5. (A) Fluorescence spectra of solutions corresponding to various concentrations of ochratoxin A under the condition of premixing of aptamer/ochratoxin before addition of PVP-coated graphene oxide. (B) Plot of the maximum peak of fluorescence spectra in (A) as a function of concentration of ochratoxin A. Inset: the linear part of plot in (B). Error bars were obtained from three experiments.

with various concentration of OTA (AppendixBFig. S5). The LOD is 18.7 nM; the LOQ is 88.4 nM.

4. Conclusions

In summary, this study has demonstrated that sensor assay based on bare graphene oxide, although exhibited excellent selectivity for fungi toxin OTA against other structure analogues, nevertheless showed poor sensitivity. PVP-coated graphene oxide can improve the sensing performance via quenching free unbound FAM-modified aptamer as compared to bare graphene oxide. The reason is because the adsorption of ochratoxin A onto the planar surface of graphene oxide can reduce the concentration of ochratoxin A in the solution, preventing the adsorption of ochratoxin A by PVP can result in enhancement of the concentration of the small molecules in the solution. The ratio of PVP concentration to graphene oxide is very important, lower amount of PVP is not enough to prevent the adsorption of target molecules and much higher amount of PVP will totally eliminate the DNA absorption. By means of this method, the detection limit of graphene oxide based sensing platform can be decreased. The limit of detection for ochratoxin A with concentration of 18.7 nM can be detected by using coated graphene oxide.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.01.032.

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