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High performance system for protein assays: synergistic effect of terminal protection strategy and graphene oxide platform

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Abstract

In this work, a novel fluorescent method for protein detection has been developed based on terminal protection of small molecule-linked DNA by target protein and the difference in affinity of graphene oxide (GO) for single-stranded DNA (ssDNA) containing different numbers of bases in length. A probe ssDNA, which is labeled carboxyfluorescein (FAM) at the 5' end and a small molecule at the 3' end, is designed for the detection of target protein. In the absence of target protein, the probe ssDNA can be hydrolyzed into mononucleotides by Exo I. The introduction of GO into the sensing solution results in weak quenching of the fluorescence of FAM due to the weak affinity of the short FAM-labeled oligonuleotide fragment to GO. Conversely, and very importantly, in the presence of target protein, the specifically binding of target protein to the small molecule of probe ssDNA can protect probe ssDNA from the Exo I-catalyzed digestion. Then the adsorption of the probe ssDNA on GO makes FAM close proximity to GO surface resulting in

high efficiency quenching of fluorescence of FAM, and the fluorescence intensity gradually decreases with increasing concentration of target protein. Taking folate receptor (FR) as an example in this work, we can determine the protein in a linear range from 1 to 80 ng/mL with a detection limit of 0.81 ng/mL. Besides satisfactory sensitivity, the developed strategy also shows high selectivity, excellent reproducibility, and low cost, implying that this technique may have great potential applications in the future.

Keywords: graphene oxide; fluorescence; folate receptor; terminal protection

1. Introduction

Molecular diagnostics and therapeutics would greatly benefit from novel techniques for detecting proteins because of the tremendous importance of proteins in almost all functions of life. Monoclonal antibody-based specific immunoassays have been used during the past decades for proteins detection[1]. However, there have been recent advances in the use of aptamers[2, 3] for the detection and analysis of proteins. Compared with antibodies, aptamers possess significant advantages, such as simple synthesis, easy labeling, good stability, and design flexibility.[4-6] Nevertheless, some notable limitations still restrict the further application of aptamers in protein detections. For instance, some aptamers have low association constants to their ligands[7]; chemical modification-induces loss of binding activity[8]; some proteins only have RNA aptamers that are unstable and easily degraded by ribonucleases; aptamers of some proteins have not been screened out yet.[9-11]

Recently, Jiang's group had a great discovery that small-molecule-linked DNA can be protected from exonuclease degradation by target protein specific binding[12, 13]. This finding may represent an ideal option for the detection of proteins. The DNA part provides the chimeras with

versatile ability for sequence-dependent separation, amplification and detection, and the protein-binding small molecule moiety offers the capacity of selective capturing the target proteins.[14, 15] For example, Jiang's group firstly used this strategy developing a sensitive biosensor for electrochemical detection of folate receptor (FR) via selective carbon nanotube assembly.[12] Cao further developed an amplified electrochemical method for FR detection based on the nicking endonuclease-assisted amplification strategy with a detection limit of 0.19 ng/mL.[16] Other electrochemical methods also achieve ultra-high sensitivity for protein detection.[17-19] However, electrochemical strategies are not only time-consuming and tedious but also usually require deliberate modifications of electrode surfaces and laborious optimization procedures. Recently, several fluorescence-based approaches for specificity measuring proteins have been developed, including using nucleic acid dyes as signal probe-based label free assays[13,20], DNA-templated metal nanoparticles-based assays[21], and fluorescence resonance energy transfer (FRET) technology assay[15, 22, 23]. However, these strategies are compromised to low sensitivity or expensive double fluorophore-labeled DNA substrates. Therefore, to overcome these shortcomings, the development of a simple and sensitive method for proteins analysis should be of general interest.

As a kind of promising single-atom thick and two-dimensional nanomaterial, graphene has received much attention for its remarkable electronic, mechanical, and thermal properties.[24, 25] Graphene oxide (GO), resulting from exfoliation of graphite oxide[26], has shown great potential for biosensing applications because of its good water-solubility compared with graphene and excellent fluorescence quenching ability[27, 28]. Meanwhile, it is found that single-stranded nucleic acids adsorb strongly on GO, while duplex DNAs cannot bind to GO stably[29-31]. Combined the

above features, GO has been widely exploited[32-36], for instance the detection of nucleic acids[29, 37-41], proteins[33, 35, 36], metal ions[34, 42, 43] and enzymes activity[30, 44, 45].

In addition, there is a big difference in affinity of GO for ssDNA containing different numbers of bases in length. And short ssDNA had weaker affinity to GO than long ssDNA.[42, 46] Based on this remarkable affinity difference, the GO-DNA complex has emerged as a novel bioassay platform to kick-start ultra-high sensitive mental ions[47], antibiotics[48], and nucleases[49] detection via DNA(RNA) cleavage reaction, which greatly increasing the sensing application of GO. Inspiring by this special characteristic of GO, herein, by taking the advantage of the difference in affinity of GO for ssDNA containing different numbers of bases in length and terminal protection of small molecule-linked DNA strategy, a simple and rapid GO-based sensing platform is constructed for fluorescence detection of proteins.

2. Experimental

2.1. Chemicals and materials

Probe single-stranded DNA (ssDNA), with a sequence of 5'-FAM-TATATGGATGATGTGGTATT-folate-3', was synthesized by Takara Biotechnology Co., Ltd. (Dalian, China). Graphite oxide was purchased from Sinocarbon Materials Technology Co., Ltd. (China). Folate receptor was purchased from Sino Biological Inc. (Beijing, China). Exonuclease I (Exo I) was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The buffer solutions used in this work are as follows: Exo I buffer consisted of 67 mM Glycine-KOH (pH 9.5), 1 mM DTT, and 6.7 mM $MgCl_2$, and the Tris-HCl buffer consisted of 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 50 mM NaCl. Milli-Q purified water was used to prepare all the solutions.

2.2. Apparatus

Fluorescent emission spectra were performed on Varian cary eclipse fluorescence spectrophotometer, Varian Medical Systems, Inc. (Palo Alto, American). The sample cell is a 700-μL quartz cuvette. The luminescence intensity was monitored by exciting the sample at 480 nm and measuring the emission at 520 nm. The slits for excitation and emission were set at 5 nm, 10 nm respectively. The fitting of the experimental data was accomplished using the software Origin 8.0.

2.3. Optimization of the concentration of GO

The purchased GO[50] was sonicated in Milli-Q purified water for 5 h to give a homogeneous black solution and stored at 4 ºC for use. To optimize the concentration of GO, 2 μL probe ssDNA (10 μM), and 0, 5, 10, 15, 20, and 25 μL GO solution (250 μg/mL) as prepared were mixed. The mixed solution was diluted with Tris-HCl buffer to 500 μL. The above prepared solution was incubated for 10 min at room temperature. Finally, the fluorescence intensity of the incubated solution was measured at 520 nm with excitation at 480 nm.

2.4. Optimization of the reaction time between probe ssDNA and GO

To optimize the reaction time between probe ssDNA and GO, 2 μL probe ssDNA (10 μM), and 15 μL GO (250 μg/mL) solution were mixed. The mixed solution was diluted with Tris-HCl buffer to 500 μL and incubated for 0, 1, 2, 5, 10, 15, 20, 25 and 30 min at room temperature. Finally, the fluorescence intensity of the incubated solution was measured at 520 nm with excitation at 480 nm.

2.5. Optimization of the concentration of Exo I

To optimize the concentration of Exo I, 2 μL of probe ssDNA (10 μM), and 0, 1, 2, 3, 4, 5, and 6 U Exo I solution were mixed and incubated for 30 min at 37 ºC with gentle shaking. Then the solution was added into the GO solution and diluted with Tris-HCl buffer to 500 μL and incubated for 10 min at room temperature. Finally, the fluorescence intensity of the incubated solution was measured at 520 nm with excitation at 480 nm.

2.6. *Optimization of the reaction time between probe ssDNA and Exo I*

To optimize the reaction time between probe ssDNA and Exo I, 2 μL probe ssDNA (10 μM), and 4 U Exo I solution were mixed. The above prepared solution was incubated for 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min at 37 ºC with gentle shaking. Then the solution was added into the GO solution and diluted with Tris-HCl buffer to 500 μL and incubated for 10 min at room temperature. Finally, the fluorescence intensity of the incubated solution was measured at 520 nm with excitation at 480 nm.

2.7. Performance of FR detection:

For quantitative measurement of FR, 2 μL probe ssDNA (10 μM) was treated with different concentrations of FR (0, 1, 2, 4, 8, 10, 20, 40, 80, 160 and 320 ng/mL) and shaken gently 1 h at 37 ˚C. After that, 4 U Exo I was added to the solution, the mixed solution was incubated for 30 min at 37 ˚C. Finally, the above mixture was added to the GO solution and incubated for 10 min with gentle shaking at room temperature. Then the fluorescence intensity was measured at 520 nm with excitation at 480 nm.

3. Results and discussion

3.1. Design Strategy

A 20-mer single-stranded DNA (ssDNA), which was labeled carboxyfluorescein (FAM) at the 5' end and a small molecule at the 3' end, was used as probe DNA for the detection of target protein. Folate receptor (FR), a highly selective biomarker that is overexpressed by many primary and metastatic cancers, is used as the model target. Figure 1 illustrates the sensing strategy for the detection of FR. In the absence of target molecule (FR), probe ssDNA is hydrolyzed into mononucleotides by Exo I. The weak affinity between mononucleotides and graphene oxide (GO) making the system shows high intensity of fluorescence. In the presence of FR, the specifically binding of FR to the small molecule of probe ssDNA can protect probe ssDNA from the Exo I-catalyzed digestion. Then the probe ssDNA is adsorbed onto the GO sheet by π-π stacking, making the fluorophore close proximity to GO surface, thus GO significantly quenches the fluorescence of FAM, and the fluorescence intensity gradually decreases with increasing concentration of FR. Therefore, the fluorescence intensity of FAM as a function of FR concentration is measured correspondingly.

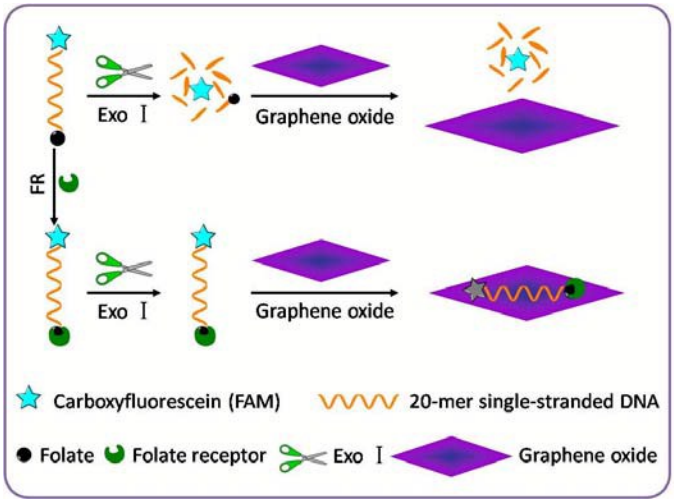

Figure 1 Scheme for the mechanism of GO-based biosensor for folate receptor (FR) detection.

The process of fluorescence changing of the GO-based biosensor for FR detection is shown by fluorescence spectra. Figure 2 shows the fluorescence emission spectra of the sensing system

under various conditions. The fluorescence spectrum of probe ssDNA in Tris-HCl buffer shows strong fluorescence intensity due to the presence of the FAM (curve a). However, in the presence of GO, up to 94% quenching of the fluorescence emission was observed (curve b), which indicates strong adsorption of probe ssDNA on GO and high fluorescence quenching efficiency of GO. Upon the reaction of probe ssDNA with 4 U Exo I, the introduction of GO into the sensing solution exhibits significant fluorescence intensity (Figure 2, curve c). However, after probe ssDNA react with the target protein FR, and then react with 4 U Exo I, the fluorescence of the probe ssDNA was quenched significantly by GO (curve d). This result implies that the specific binding of FR to the small molecule folate of probe ssDNA could protect probe ssDNA from Exo I-catalyzed digestion. Therefore, FR detection could be easily realized by monitoring the change of fluorescence signal.

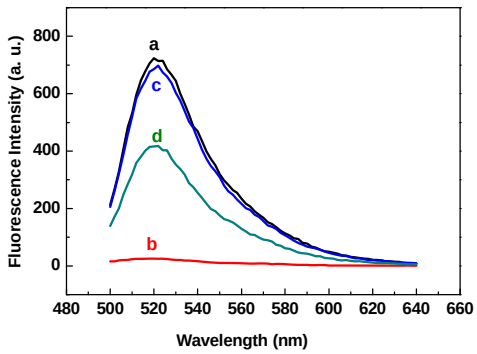

Figure 2 Fluorescence emission spectra of probe ssDNA under different conditions: (a) probe ssDNA in Tris-HCl buffer; (b) probe ssDNA + GO; (c) probe ssDNA + Exo I + GO; (d) probe ssDNA + FR + Exo I + GO. Probe ssDNA, 40 nM; GO, 7.5 μg/mL; Exo I, 4 U. Excitation: 480 nm.

3.2. Optimization of detection strategy

In order to establish optimal conditions for the fluorescence assay, relevant experimental parameters affecting the reaction were assessed and optimized. SI, Figure S1 shows the effect of GO concentration on the fluorescence intensity of probe ssDNA. From Figure S1, we find that

when GO is introduced, the fluorescence signal is gradually reduced. And when GO is up to 7.5 μg/mL (probe ssDNA, 40 nM), the fluorescence intensity of FAM is quenched down to 6% of the original fluorescence signal. As a result, 7.5 μg/mL was used as the optimized concentration for GO. SI, Figure S2 shows the fluorescence quenching of FAM-labeled probe ssDNA in the presence of GO as a function of incubation time. Probe ssDNA adsorption on the surface of GO is very fast at room temperature. The process of adsorption reaches equilibrium in 5 min. SI, Figure S3 shows the effect of Exo I concentration on the fluorescence intensity of the sensing system. From SI, Figure S3, we find that fluorescence intensity of probe ssDNA increases sharply as the concentration of Exo I increases from 0 to 6 U in the sensing solution. When Exo I concentration is up to 4 U, fluorescence intensity of FAM is nolonger increased. As a result, 4 U was taken as the optimized concentration for Exo I. The control experiments indicated that Exo I itself could not result in the fluorescence intensity change (shown in SI, Figure S4). The reaction time is a crucial parameter for the Exo I-digested ssDNA reaction process. It was optimized via measuring the fluorescence emission spectra of probe ssDNA as a function of time in the sensing solution. SI, Figure S5 displays the changes of fluorescence intensity with incubation time. It was observed that the fluorescence signal increased gradually with the increase of the reaction time and then reached equilibrium in 30 min, suggesting the complete cleavage process. Thus, the optimal reaction time of Exo I was chosen to be 30 min.

3.3. FR detection

For the sake of evaluating the activity of FR, different concentrations of FR were applied in the sensing system based on the optimal assay conditions. Figure 3 shows the fluorescence responses for different FR concentrations. It is observed that the fluorescence signals gradually

decreased as the concentrations of FR varied from 1 ng mL^{-1} to 320 ng mL^{-1}. The amount of FR was reflected by the change of fluorescence signal, which originated from the target induced terminal protection of probe ssDNA and FRET between FAM attached probe ssDNA and GO. The fluorescence signal was linearly decreased with FR concentration in the range from 1 ng mL^{-1} to 80 ng mL^{-1} with linear equation y = -5.5x + 661.5, where y is the fluorescence intensity of FAM at 520 nm and x is the concentration of FR (regression coefficient R^2=0.9894). The detection limit is estimated to be 0.81 ng mL^{-1} ($3S_0/S$, in which S_0 is the standard deviation for the blank solution, n=11, and S is the slope of the calibration curve), which is comparable to previous reported FR biosensors[13-21]. For each concentration of the protein, the measurement has been repeated for at least three times independently. The average RSD is 4.5%, indicating that the precision and reproducibility of the method are acceptable. These results demonstrates that the target protein induced terminal protection of samll-molecule-linked DNA system combing with the GO based platform can be applied to sensitive protein activity analysis in a wide concentration range and good precision and reproducibility.

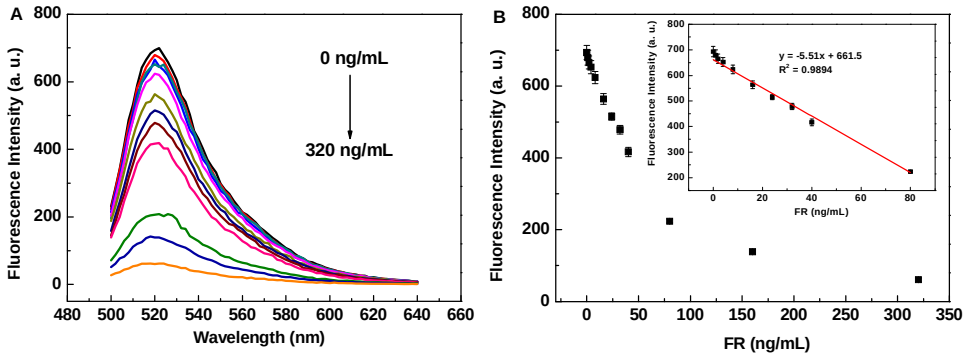

Figure 3 Fluorescence emission spectra of GO-based biosensor in the presence of increasing amount of FR and calibration curve for FR detection. (A) Fluorescence emission spectra of this GO-based biosensor upon the addition of different concentrations of FR. (B) Calibration curve for FR detection. Probe ssDNA, 40 nM; Exo I, 4 U; GO, 7.5 μg/mL. Excitation: 480 nm.

3.4. Specificity

In this study, the specificity of the GO-based biosensor was also studied by using other interfering proteins such as BSA, thrombin, IgG and hemoglobin, as shown in Figure 4. All the detections were carried out under identical conditions. It was revealed that the non-specific protein could not prevent Exo I from digesting the probe ssDNA and no significant fluorescence signal decrease could be obtained. Therefore, the protection of folate-linked ssDNA was specific to the FR binding event and these interfering proteins do not interfere with the detection of FR, implying high specificity of the GO-based terminal protection assay for FR detection.

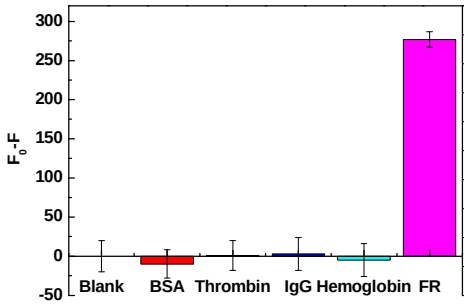

Figure 4 Fluorescence intensity of this GO-based biosensor in the presence of different proteins: blank control (without FR); BSA (40 ng mL^{-1}); thrombin (40 ng mL^{-1}); IgG (40 ng mL^{-1}), hemoglobin (40 ng mL^{-1}) and FR (40 ng mL^{-1}). Excitation: 480 nm.

3.5. FR detection in human serum

In order to evaluate the practicability of the proposed design, the detection of FR activity in serum, a kind of fluid sample with complicated biological matrix, was also performed. The interaction between GO and probe ssDNA in 2% serum was examined (SI, Figure S6). The fluorescence intensity of probe ssDNA is still efficiently quenched by GO in 2% serum, and upto 88.2% fluorescence is quenched when 50 μg/mL GO is added. The fact that more GO is needed to quench equivalent probe ssDNA in 2% serum than that in Tris-HCl buffer, may be attributed to

the interaction between GO and some biomolecules in serum, which results in the reduction of interaction area between the GO and probe ssDNA. The detection process of FR in 2% serum was the same as in the Tris-HCl buffer. As shown in SI, Figure S7, there is still a good linear relationship between the fluorescence intensity and the concentration of FR. This clearly shows this GO-based biosensor can be used to detect FR in practical samples sensitively.

4. Conclusion

In conclusion, a simple, selective and sensitive strategy for FR detection using GO-based biosensor is developed in this work. This method is simply utilizing terminal protection of small molecule-linked DNA by target proteins and the difference in affinity of GO for single-stranded DNA containing different numbers of bases in length. With such a straightforward design, quantitative determination of FR can be performed with quite good analytical performances. In addition, the new strategy is featured with high simplicity. Therefore, we can reasonably expect that this method hold considerable potential for molecular diagnostics, genomic research, and drug development.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 21405125), Natural Science Foundation Project of CQ (No. CSTC2014JCYJA80041), Fundamental Research Funds for the Central Universities (No. XDJK2015C090 and SWU113099), the National Key Technology R&D Program (No. 2009BADB7B04) and China Agriculture Research System (No. CARS-27).

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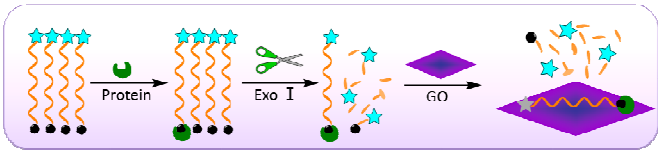
Graphical abstract

High performance system for protein assays: synergistic effect of terminal protection strategy and graphene oxide platform

Yue He* and Bining Jiao



A straightforward biosensor for protein assay has been developed based on terminal protection of small molecule-linked DNA by target protein and the difference in affinity of graphene oxide for ssDNA containing different numbers of bases in length.