Bare magnetic nanoparticles as fluorescence quenchers for detection of thrombin

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Rapid and sensitive detection of thrombin has very important significance in clinical diagnosis. In this work, bare magnetic ironoxide nanoparticles (magnetic nanoparticles) without any modification were used as fluorescence quenchers. In the absence of thrombin, a fluorescent dye (CY3) labeled thrombin aptamer (named CY3-aptamer) was adsorbed on the surface of magnetic nanoparticles through interaction between a phosphate backbone of the CY3-aptamer and hydroxyl groups on the bare magnetic nanoparticles in binding solution, leading to fluorescence quenching. Once thrombin was introduced, the CY3-aptamer formed a G-quartet structure and combined with thrombin, which resulted in the CY3-aptamer being separated from the magnetic nanoparticles and restoration of fluorescence. This proposed assay took advantage of binding affinity between the CY3-aptamer and thrombin for specificity, and bare magnetic nanoparticles for fluorescence quenching. The fluorescence signal had a good linear relationship with thrombin concentration in the range of 1–60 nM, and the limit of detection for thrombin was estimated as low as 0.5 nM. Furthermore, this method could be applied for other target detection using the corresponding fluorescence labeled aptamer.

Introduction

Aptamers are artificial single stranded DNA or RNA that possess high affinity and special recognition towards molecular targets ranging from small molecules and bio-macromolecules to even cells and tissues, which are selected in vitro from large combinatorial pools.1,2 Compared to the traditional recognition element antibody, aptamers have a range of unique features, such as minimal immunogenicity, synthesis convenience, ease of isolation and modification, and structural stability and flexibility, which make them an ideal alternative candidate for protein detection.3–6 Aptamer-based sensors have attracted more and more attention due to their high selectivity and affinity. In various aptamer-based analytical methods, fluorescence assays, in particular, are very attractive due to their high sensitivity, relatively short detection time and low-cost instrumentation.

Recently, nanomaterials have shown great promise in fluorescence assays because they can quench various fluorophores with high quenching efficiency.7–10 For example, graphene oxide-based multicolor fluorescent DNA as a nanoprobe was used to detect DNA targets in homogeneous solutions.11 AuNPs (gold nanoparticles) were reported as effective quenchers to detect metallic ions.12 In various nanomaterials, magnetic iron oxide nanoparticles (magnetic nanoparticles), especially, are attractive because of their superparamagnetic effect, nontoxic nature, and ease of synthesis. So far, several fluorescence analytical methods based on magnetic nanoparticles and aptamers have been developed.13–15 For example, Wu et al. invented a novel aptamer-based fluorescent biosensor that used aptamer-conjugated magnetic nanoparticles as recognition and concentration elements and employed upconversion nanoparticles as highly sensitive signal labels.16 Zhao et al. developed an aptamer-based sandwich assay for thrombin using a pair of thrombin-binding aptamers. One of them as a capture aptamer was modified on the magnetic nanoparticles and the other as a reporter aptamer on the quantum dots. Detection of thrombin was achieved by the fluorescence measurement of quantum dots in the sandwich complex. A limit of detection of 0.05 nM was accomplished.17 However, most of the above mentioned magnetic nanoparticle-based methods, in particular the methods based on fluorescence assays, required a complicated modification process of magnetic nanoparticles. The α-Fe₂O₃ nanoparticles have been used as fluorescence quenchers to detect nucleic acids, which is based on the fact that nanoscale α-Fe₂O₃ particles can strongly...
adsorb and quench the fluorescence of fluorescence-labeled single-stranded DNA (ssDNA) probes. However, the sensor platform using the bare magnetic nanoparticles as fluorescence quenchers has not been reported for detection of protein. Hence, we expect to develop a simple, rapid and sensitive fluorescent sensor for protein detection with unmodified bare magnetic nanoparticles.

In this work, we reported a rapid, simple and sensitive aptamer-based fluorescent sensor for protein detection using bare magnetic nanoparticles as fluorescence quenchers. Thrombin was chosen as the model protein because it plays many roles in the coagulation cascade-converting soluble fibrinogen into insoluble strands of fibrin as well as catalyzing many other coagulation-related reactions. The conventional methods for detecting thrombin included colorimetry, electrochemical methods, Raman spectroscopy, mass spectrometry etc. Among these methods, colorimetry is a simple method that can be observed by the naked eye, but it cannot provide high sensitivity; other methods provide high sensitivity, but they require a complicated preparation process or expensive instruments. In this design, the sensing platform based on the fact that the fluorescent dye modified thrombin aptamer (CY3-aptamer) is a polyphosphate and it can be adsorbed by bare magnetic nanoparticles in binding buffer, thus leading to fluorescence quenching.

Once thrombin was introduced, the CY3-aptamer transformed into a G-quartet structure and combined with thrombin, leading to desorption of the CY3-aptamer from the bare magnetic nanoparticles. The fluorescence signal was restored (Scheme 1). The results demonstrated that our proposal can be used to detect thrombin with high sensitivity and specificity.

### Experimental section

#### Materials

FeCl$_3$·4H$_2$O and FeCl$_3$·6H$_2$O were from Sinopharm Chemical Reagent Co., Ltd (SCRC, China). Fluorescent dye labeled aptamers were from Sagon Biotech (Shanghai, China). Fluorescent dye labeled aptamers were from Sagon Biotech (Shanghai, China). The sequence of the thrombin aptamer was 5’-CY3-ATCTAGAATTCTACGGGCTAAAGAGTGCAGAGTTACTTAG-3’. Bovine thrombin was bought from Sigma (USA). Other control proteins, including Bovine Serum Albumin (BSA), lysozyme, myoglobin, papain and hepatitis B surface antigen (HBsAg) were from Dingguo Biotech (Beijing, China). All other chemicals were of analytical grade and obtained from standard reagent suppliers. Deionized water was used to prepare all aqueous solutions.

### Preparation and characterization of bare magnetic iron oxide nanoparticles

Magnetic nanoparticles were prepared by a chemical co-precipitation method under a nitrogen atmosphere to avoid possible oxidation during the reaction. This method was based on the following chemical reaction: $\text{FeCl}_2\cdot 4\text{H}_2\text{O} + 2\text{FeCl}_3\cdot 6\text{H}_2\text{O} + 8\text{NH}_4\text{OH} = \text{Fe}_3\text{O}_4(s) + 8\text{NH}_4\text{Cl} + 20\text{H}_2\text{O}$. In a typical synthesis, 4.3 g of FeCl$_2$·4H$_2$O and 11.8 g of FeCl$_3$·6H$_2$O were dissolved in 200 ml of deionized water with vigorous stirring at a speed of 1000 rpm, then 25 ml of 25% NH$_4$H$_2$O was added after the solution was heated to 80 °C. The reaction was continued for 30 min at 80 °C under constant stirring to ensure the complete growth of the nanoparticle crystals. The resulting particles were collected by using a permanent magnet and washed several times with deionized water to remove unreacted chemicals and dried by using a freeze-dryer for 24 h. The transmission emission microscopy (TEM) images of bare magnetic nanoparticles were measured by H-7650 (Hitachi Japan) with the acceleration voltage of 80 kV.

### Determination of the optimal concentration of magnetic nanoparticles and adsorption time

To study the concentration of magnetic nanoparticles, a final concentration of the 100 nM CY3-labeled thrombin aptamer (CY3-aptamer) was dissolved in binding buffer (34 mM Tris-HCl, 233 mM NaCl, 8.5 mM KCl, 1.7 mM CaCl$_2$, 1.7 mM MgCl$_2$) and different concentrations of magnetic nanoparticles were added. After 30 minutes of incubation and magnetic separation, the fluorescence signal of the supernatant was recorded by using a fluorescence detector (Hitachi F-4600). To test the adsorption time, a final concentration of 100 nM CY3-aptamer and 0.05 mg ml$^{-1}$ magnetic nanoparticles was dissolved in binding buffer, then the fluorescence intensity of the supernatant was recorded after incubation at different incubation times and magnetic separation.

### Fluorescence recovery of aptamer-CY3 induced by thrombin

To determine the optimal time of thrombin combination with the aptamer, the kinetics was studied. The bare magnetic nanoparticles (final concentration of 0.05 mg ml$^{-1}$) and CY3-aptamer (final concentration of 100 nM) were mixed and incubated for 20 minutes, then a final concentration of 100 nM thrombin was added. After incubation of 10, 20, 30, 40, 50, 60 and 70 minutes respectively at room temperature and magnetic separation, the fluorescence intensity of each supernatant was measured.
Detection of thrombin

The bare magnetic nanoparticles (final concentration of 0.05 mg ml\(^{-1}\)) and CY3-aptamer (final concentration of 100 nM) were mixed and incubated for 20 minutes, then different concentrations of thrombin were added. After incubation of 20 minutes at room temperature and magnetic separation, the fluorescence intensity of the supernatant was measured. To further confirm that the fluorescence recovery was induced by thrombin, the control experiment was carried out to prove this process. CY3-labeled random oligonucleotides were used to replace the aptamer-CY3, and other experimental conditions were the same as mentioned above. For the specificity test, a final concentration of 10 µM control proteins (BSA, HBsAg, papain, myoglobin, lysozyme) and 100 nM thrombin was added into the bare magnetic nanoparticles (0.05 mg ml\(^{-1}\)) and CY3-aptamer (100 nM) solution, and fluorescence was recorded after 20 minutes, respectively.

Detection of thrombin in real serum sample

The serum sample was diluted 100 fold with binding buffer, then a final concentration of 1 µM thrombin was spiked in the diluted serum sample as the prepared serum. The bare magnetic nanoparticles (0.05 mg ml\(^{-1}\)) and CY3-aptamer (100 nM) were mixed and incubated for 20 minutes, and then the prepared serum sample was added into the above mixed solution and incubated for another 20 minutes. The final concentration of thrombin in the mixed solution was 10, 20, 40 and 60 nM respectively. Finally, the fluorescence intensity was recorded after magnetic separation.

Results and discussion

Principle

The synthesized bare magnetic nanoparticles were characterized by TEM, as shown in Fig. 1. The diameters of bare magnetic nanoparticles without any modification were about 10 nm and they were uniform in shape. Fig. 2 showed the nanoparticles magnetization curve and picture of magnetic separation, which suggested a good superparamagnetic effect and rapid separation process. According to the reaction formula: \( \text{Fe}^{2+} + 2\text{Fe}^{3+} + 8\text{OH}^- = \text{Fe}_3\text{O}_4(\text{s}) + 4\text{H}_2\text{O} \), the surface of bare magnetic nanoparticles held a lot of \( \text{OH}^- \) and carried a negative charge in neutral pH solution. The binding affinity of typical functional groups to a magnetite surface follows the order: phosphonate > carboxylate > hydroxy > sulfonate.\(^{38}\) An aptamer is a single stranded DNA or RNA, which consists of a phosphate backbone and different bases, leading to negative charges in neutral pH solution. In the absence of salt, the aptamer cannot be adsorbed on the surface of bare magnetic nanoparticles due to electrostatic repulsion. However, as shown in Fig. 3, in the presence of salt, the aptamer was adsorbed by bare magnetic nanoparticles through the phosphate groups and \( \text{OH}^- \) on the surface of nanoparticles and the fluorescence signal was quenched, which was different from binding to gold nanoparticles or carbon.\(^{37,40}\) There are several nanoparticles that can also adsorb oligonucleotides via phosphate groups. For example, titanium dioxide (\( \text{TiO}_2 \)) can adsorb fluorescently labeled oligonucleotides through phosphate groups and quench fluorescence signal, and the adsorption

Fig. 1 TEM image of bare magnetic nanoparticles.

Fig. 2 Room-temperature magnetization curves of magnetic nanoparticles and picture of magnetic separation.

Fig. 3 Schematic diagram of CY3-aptamer adsorption on the surface of bare magnetic nanoparticles.
can be strongly inhibited by phosphate and citrate. Cerium oxide nanoparticles (nanoceria) also can adsorb oligonucleotides via a phosphate backbone and quench fluorescence, but phosphate does not have the inhibition effect for the oligonucleotide adsorption. It has been proved that indium tin oxide (ITO) has relatively weak adsorption ability for DNA, because the DNA adsorption properties are an averaging result from its two components In$_2$O$_3$ and SnO$_2$. In$_2$O$_3$ can adsorb DNA strongly, but SnO$_2$ repels DNA at neutral pH. Compared with nanoparticles mentioned above, bare magnetic nanoparticles not only can adsorb oligonucleotides tightly and quench fluorescence but also possess magnetism, which is convenient for separation from mixed solutions. Hence, bare magnetic nanoparticles were used as adsorbents and quenchers for the CY3-aptamer. Once the thrombin was introduced, the aptamer was induced to fold into a G-quartet structure that was a stable three dimensional structure formed by an intramolecular hydrogen bond, which caused the aptamer to combine with thrombin. The thrombin aptamer contained 15 bases, which meant that the G-quartet structure resulted in the reduction of affinity between the aptamer and the surface of bare magnetic nanoparticles, furthermore, desorption from the surface of bare magnetic nanoparticles and the restoration of fluorescence. This result indicated that the affinity between the thrombin and aptamer was stronger than the aptamer and bare magnetic nanoparticles.

**Optimization of detection conditions**

To improve the sensing performance, the concentrations of bare magnetic nanoparticles and the CY3-aptamer were optimized. With a fixed concentration of the CY3-aptamer (100 nM), the concentration of bare magnetic nanoparticles was increased. As shown in Fig. 4, the fluorescence intensity was drastically decreased by increasing the concentration of bare magnetic nanoparticles from 0 to 0.5 mg ml$^{-1}$. It was found that when the concentration of bare magnetic nanoparticles was 0.05 mg ml$^{-1}$, the fluorescence signal was quenched almost completely. Thus 0.05 mg ml$^{-1}$ was chosen as the optimal bare magnetic nanoparticle concentration among the tested concentrations to quench the fluorescence of the CY3-aptamer.

Next, the optimal adsorption time was tested. As depicted in Fig. 5, the fluorescence intensity drastically decreased with time extension. The fluorescence intensity was quenched almost completely after incubation of 20 minutes. Due to the time of aptamer binding with thrombin being almost 20 minutes, 20 minutes was chosen as the optimal detection time.

**Fluorescence recovery of aptamer-CY3 induced by thrombin**

To confirm the optimal reaction time between the thrombin and aptamer, the fluorescence intensity was recorded every ten minutes after the addition of thrombin, as shown in Fig. 6.
The fluorescence intensity increased against time after the addition of thrombin, and the fluorescence intensity gradually reached the maximum after 20 minutes. This result was consistent with the reaction time between the thrombin and aptamer in homogeneous solutions.

To further understand that the fluorescence recovery was induced through interaction between the thrombin and aptamer, a control experiment was performed. A CY3-labeled random sequence was used to replace the thrombin aptamer, and the fluorescence signal was also quenched after interaction with bare magnetic nanoparticles. However, from Fig. 7, the fluorescence signal was not recovered after the addition of thrombin. This result confirmed that the random sequence cannot combine with thrombin, which led to the random sequence cannot be released from the surface of bare magnetic nanoparticles and the fluorescence was still quenched. In contrast, when thrombin was added into the solution of the CY3-aptamer and bare magnetic nanoparticles, the fluorescence signal was recovered in 20 minutes, which suggested that thrombin induced CY3-aptamer transformation to form a G-quartet structure and to move away from the surface of the nanoparticles, leading to the restoration of fluorescence.

Detection of thrombin

To quantitatively detect thrombin using this sensor, different concentrations of thrombin were mixed with bare magnetic nanoparticles and CY3-aptamer solution. From Fig. 8(A) and (B), it is shown that the fluorescence intensity gradually increased with the increasing concentration of thrombin. Thrombin can combine with the CY3-aptamer, which caused the CY3-aptamer to form a G-quartet structure and to move away from each other, leading to the restoration of fluorescence. As shown in Fig. 8 (C), the fluorescence intensity increased linearly over the thrombin concentration range from 1 nM to 60 nM, with the linear correlation coefficient of 0.997. The limit of detection was estimated to be 0.5 nM based on S/N of 3, the sensitivity was comparable or better than that in the reported thrombin detection.14-47

To test the specificity of this assay toward thrombin, Bovine Serum Albumin (BSA), lysozyme, myoglobin, papain and HBsAg were analyzed in the same binding buffer. Fig. 9 shows that none of the control proteins caused obvious fluorescence intensity restoration even with a concentration as high as 10 μM, while only 100 nM thrombin resulted in significant
In summary, we have successfully developed a simple and rapid fluorescent sensor for detection of thrombin with bare magnetic nanoparticles and a fluorescent dye labeled aptamer. Under the optimal conditions, the limit of detection of the assay was 0.5 nM, and had a good linear relationship with thrombin concentration in the range of 1–60 nM, which was comparable to or better than other fluorescence assays. Moreover, bare magnetic nanoparticles were employed as fluorescence quenchers for thrombin detection for the first time, which eliminated the complicated modification process. In addition, by substituting the thrombin aptamer with other aptamers or single stranded DNA, this strategy could be applied for detection of other proteins or DNA.

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**Notes and references**