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Label-free fluorescent detection of thrombin activity based on a recombinant enhanced green fluorescence protein and nickel ions immobilized nitrilotriacetic acid-coated magnetic nanoparticles

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ABSTRACT

Herein, a novel label-free fluorescent assay has been developed to detect the activity of thrombin and its inhibitor, based on a recombinant enhanced green fluorescence protein (EGFP) and Ni^{2+} ions immobilized nitrilotriacetic acid-coated magnetic nanoparticles (Ni^{2+}–NTA MNPs). The EGFP, containing a thrombin cleavage site and a hexahistidine sequence (His-tag) at its N-terminal, was adsorbed onto Ni^{2+}-NTA MNPs through Ni^{2+}-hexahistidine interaction, and dragged out of the solution by magnetic separation. Thrombin can selectively digest EGFP accompanied by His-tag peptide sequence leaving, and the resulting EGFP cannot be captured by Ni^{2+}-NTA MNPs and kept in supernatant. Hence the fluorescence change of supernatant can clearly represent the activity of thrombin. Under optimized conditions, such assay showed a relatively low detection limit (3.0×10^{-4} U mL^{-1}), and was also used to detect the thrombin inhibitor, Hirudin, and further applied to detect thrombin activity in serum. Combined with the satisfactory reusability of Ni^{2+}-NTA MNPs, our method presents a promising candidate for simple, sensitive, and cost-saving protease activity detecting and inhibitor screening.

1. Introduction

Proteases are quite important in wide scope of physiological processes, such as simple protein catabolism and highly regulated cascades, etc. Thrombin is a kind of serine proteases, which can cleave peptide bonds containing arginine residue [1]. As the last protease involved in the coagulation cascade, thrombin converts fibrinogen to insoluble fibrin, which forms the fibrin gel [2]. Also, thrombin plays a pivotal role in the process of thrombiosis, platelet activation, and various cardiovascular diseases [3], and regulates many processes in inflammation and tissue repair at the vessel wall [4]. Recently, many thrombin detecting methods have been reported, including various aptamer-based assays. Most of these aptamer-based assays are sensitive and selective, and some fluorescence sensors have fast response [5,6]. However, the experiment process of several electrochemical and electrochemiluminescence assays based on aptamer-nanomaterials complexes is complicated, requiring electrode modification and multistep washing processes [7–12]; gold nanoparticles and aptamer-based colorimetric detecting mehtods [13–15] can rarely be applied to real samples; the aptamer modified nanosilver resonance scattering spectral probe [16] cannot be applied in high-throughput screening (HTS). Furthermore, these thrombin detection systems, which depend on the interaction between aptamer and thrombin, are only able to display the overall quantity of thrombin molecule, rather than its enzyme activity. Hence, developing an easy-conducting method to detect thrombin activity is in great need.

Fluorescence proteins (FPs) are one kind of autofluorescence proteins which can emit fluorescence without any substrate or cofactor. As coded in different vectors and expressed by prokaryotic or eukaryotic cells [17,18], FPs gene can be fused to other genes through molecular cloning technique, which is widely used in fluorescent imaging [19] and other areas, such as protein interaction detecting [20,21], DNA and RNA labeling [22,23], and biosensors for numerous biological processes in transgenic animals [24]. These applications prove that FPs are ideal fluorescent reporters in various research areas, but reports about thrombin activity detection based on FPs are scarce.

Magnetic nanoparticles (MNPs) are one kind of attractive nanoparticles, because of their physical and chemical properties, such as small size, high surface/volume ratio, good dispersion, fast binding of biomolecules, reversible and controllable flocculation, and easy separation from reaction mixtures in an external magnetic field. Accordingly, MNPs are superior to conventional micrometer-sized resins or beads in the fields of DNA hybridization detection [25,26], protein and enzyme immobilization [27,28], cell separation [29,30],

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and drug delivery [31–33]. In recent years, nickel ions immobilized nitrilotriacetic acid-coated magnetic nanoparticles (Ni^{2+}–NTA MNPs) are commercialized, and are used for His-tagged proteins collection through chelation between nickel ions and hexahistidine. Because His-tag can be fused to various peptides and proteins, Ni^{2+}–NTA MNPs have the potential to be used in protease activity measurements, which is highly ignored.

Here, in our work, we demonstrate a novel, simple and label-free method to detect the activity of thrombin based on a recombinant EGFP and Ni^{2+}-NTA MNPs. The *egfp* gene is inserted in pET-28(a+) vector, so that the expressed EGFP would directly harbor a His-tag with an interval of thrombin recognition site at the N-terminal from the vector, without additional genetic modification. Through the interaction between His-tag and Ni^{2+} ions, EGFP would couple onto Ni^{2+}-NTA MNPs and be extracted out of the solution after magnetic separation. Such interaction between EGFP and Ni^{2+}-NTA MNPs would be disturbed by thrombin digestion. Hence, the fluorescence intensity variation of the solution can represent the thrombin activity. Compared to the prior thrombin detection methods, which are mostly based on thrombin aptamers [7,8,10,12,16], this EGFP and Ni^{2+}-NTA MNPs-based method exhibits predominant merits. Firstly, as the fluorescent signal change in this method depends on the digestion of the recombinant EGFP by thrombin, it can measure the thrombin activity rather than thrombin molecules. Secondly, our method can be applied to thrombin inhibitor detection and screening, while aptamer-based assays cannot, for the aptamer is one kind of inhibitors itself. Finally, due to the effective and selective interaction between Ni^{2+}-NTA MNPs and EGFP, this method is applicable in serum for thrombin detection. These advantages show the great potentiality of our method to be used in diagnosis and anticoagulation drugs screening. Thus, a label-free, selective and widely applicable thrombin detection method is established.

2. Experimental section

2.1. Chemicals and materials

Thrombin was purchased from Sigma-Aldrich (St. Louis, MO). Ni^{2+}-NTA MNPs were obtained from the MagExtractor@-His-tag-fusion Protein purification kit purchased from Toyobo Co., Ltd. (Osaka, Japan). Thrombin inhibitor, Hirudin, was purchased from Sango Co., Ltd. (Shanghai, China). Tris, yeast extract, sodium chloride, tryptone, imidazole, ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2) and all other chemicals were of analytical grade and purchased from Sangon Co., Ltd. Serum sample was received from Blood Center of Hunan University Hospital. Thrombin was dissolved in sterile ultrapure water. All samples and buffers were prepared with ultrapure water from Milli-Q water purification system, and were stored at 4 °C.

2.2. Heterologous expression of gene egfp in E. coli and purification of the recombinant EGFP

The plasmid pET28(a)-*egfp*, donated by Dr. Lei Liu in Yale University, was electroporated into *E. coli* BL21 (DE3) cells to highly heterologous express *egfp*. Briefly, *E. coli* BL21 cells were grown overnight at 37 °C in 3 mL Luria–Bertani (LB) medium, and were transferred to 100 mL fresh LB for another 2-h cultivation till the OD_{600} reached 0.6. Then 0.4 mM Isopropyl β-D-1-Thiogalactopyranoside (IPTG) was added to induce *egfp* expression for another 6 hours at 30 °C. Cells were harvested by centrifugation at 7000 × g for 3 min, washed once with purified water and twice with ice-cold 10 mM Tris–HCl buffer (pH 7.5), and resuspended in the same buffer, disrupted by sonification in an ice-water bath. After centrifugation at 12,000 rpm for 10 min to remove cell debris, the clear green supernatant containing EGFP was obtained.

The green color supernatant was filtered through Mixed cellulose ester (MCE) syringe filter, and purified with HisTrap HP column (GE Healthcare, China), followed by being desalted with HisTrap Desalting column (GE Healthcare, China) to conserve EGFP in 10 mM Tris–HCl. The solution was then quantified by the EGFP chromophore spectral absorbance at 480 nm (extinction coefficients for EGFP at 480 nm is 55,000 M^{-1} cm^{-1}) [34] and stored at −20 °C before use.

2.3. Detection of the activity and inhibition of thrombin based on EGFP and Ni^{2+}-NTA MNPs

Before detection of the activity and inhibition of thrombin, influences of buffers and Ni^{2+}-NTA MNPs volume on the assay were assessed. The manipulation is similar to the following process. Besides, 10 mM Tris–HCl (pH 7.5), phosphate buffered saline (PBS, pH 7.4) and Binding Buffer (20 mM Na_3PO_4, 0.5 M NaCl, 20 mM imidazole, pH 7.4) were used to dilute EGFP solution, respectively, and different volumes of Ni^{2+}-NTA MNPs ranging from 0 to 10 μL were used to collect EGFP. The thrombin activity assay was conducted in 0.2 mL Polymerase Chain Reaction (PCR) tubes containing EGFP (90 nM) and thrombin at final concentration ranging from 1.0×10^{-4} U mL^{-1} to 1.0 UmL^{-1}. One sample without thrombin was used as a negative control. Then the volume of reaction solution was adjusted to 100 μL by adding the buffer. The mixture was incubated at 37 °C for 60 min [35,36] and the fluorescence intensity was measured at 508 nm, I_{F1}, with Synergy™ Mx multimode microplate reader (BioTek Instruments, Inc.) at an excitation wavelength of 480 nm. Subsequently, 3 μL Ni^{2+}-NTA MNPs were added into each reaction system and stirred for 10 min. Bead/Fluid (B/F) separation was conducted with a magnet, and 100 μL supernatant was transferred to a 96-well plate and the fluorescence intensity at 508 nm, I_{F2}, was immediately read again by Synergy™ Mx multimode microplate reader. Fluorescence intensity variation was obtained according to the equation $\Delta I_F = I_{F1} - I_{F2}$.

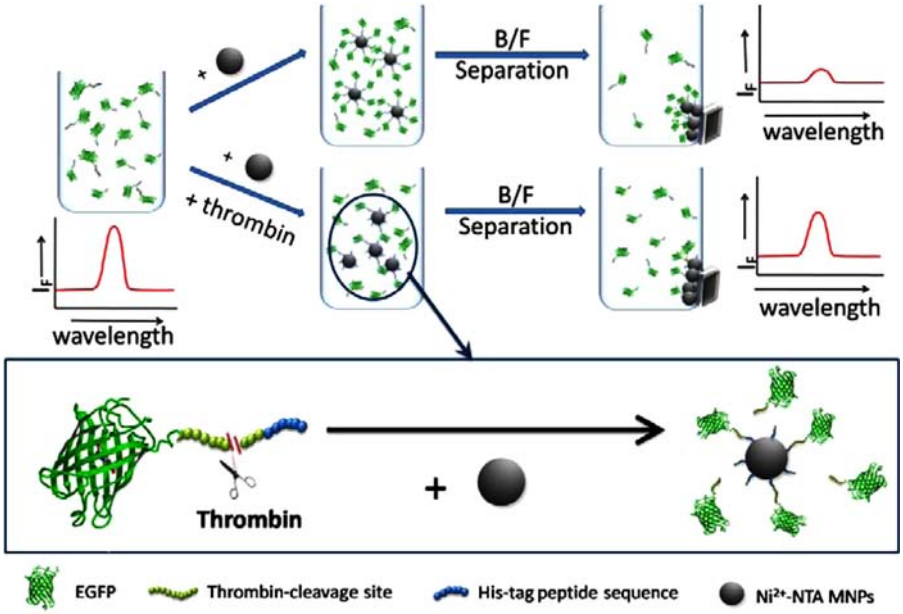
The inhibition of thrombin by Hirudin was measured in the similar way as of thrombin assay, except for the involvement of 1×10^{-2} U mL^{-1} thrombin and different concentrations of Hirudin in the reaction solutions.

2.4. Analysis of the thrombin activity in serum

The applicability of this biosensor in real sample was evaluated by the standard addition method in serum. The 100 μL digesting solution was consisted of 90 nM EGFP, 10 μL blood serum and different concentrations of thrombin. The following measurement procedures were the same as those aforementioned in thrombin assay.

2.5. Reutilization of Ni^{2+}-NTA MNPs

To test the reutilization, the used Ni^{2+}-NTA MNPs were washed and recovered as follows. The Ni^{2+}-NTA MNPs were firstly washed with stripping buffer (20 mM Na_3PO_4, 0.5 M NaCl and 50 mM EDTA, pH 7.4), and then rinsed twice with purified water. After reacting with 0.1 M $NiSO_4$ for 30 min and again washed with purified water to remove unbound nickel ions, the recovered MNPs were collected and stored in 20% ethanol at room temperature before use. The following separation ability testing procedures are the same as that of thrombin assay.



Scheme 1. Schematic illustration of the strategy using Ni^{2+}-NTA MNPs and the recombinant EGFP to detect the activity of thrombin.

3. Results and disussion

3.1. Strategy design for thrombin detection based on a recombinant EGFP and Ni^{2+}-NTA MNPs

Recombinant proteins fusing several functional peptides or proteins are very usable in biosensor design [37]. Here, the gene *egfp* in pET-28(a+) vector enables the expressed recombinant EGFP directly acquires a His-tag followed by a thrombin cleavage site at the N-terminal from the vector, without any additional genetic modification. Based on this recombinant EGFP and Ni^{2+}-NTA MNPs, a novel thrombin activity detecting method was developed.

Scheme 1 shows the mechanism of EGFP and Ni^{2+}-NTA MNPs-based method for thrombin activity detection. The N-terminal His-tag enables EGFP to be selectively trapped on the surface of Ni^{2+}-NTA MNPs through chelation between hexahistidine and Ni^{2+}. In the external magnetic field, the His-tagged EGFP can be rapidly aggregated and separated from the solution, which leads to a dramatic decrease of fluorescence intensity of the fluid. After thrombin digesting at the thrombin recognition site, the resulting EGFP, which losses the structure of His-tag peptide sequence, cannot be adsorbed by Ni^{2+}-NTA MNPs and would remain in fluid, and the fluorescence of solution would keep stable. In this way, the change of supernatant fluorescence would selectively respond to the thrombin activity. A proof-of-principle study was performed and is shown in Fig. 1. A notable reduction in fluorescence intensity of the solution, which diminished to approximately 17% of that of the beginning EGFP solution, was observed after Ni^{2+}-NTA MNPs addition and magnetic separation, indicating that the His-tagged EGFP was effectively trapped out of the reaction system. Conducting magnetic separation after thrombin digestion, however, gave much less decrease in fluorescence intensity of the solution (remains 70% of the initial datum). Excessive other proteins, e.g., lysozyme, hemoglobin, β-glucosidase, and myoglobin, respectively, were also added into the system. Comparatively, no obvious recovery of the fluorescence intensity was observed (Fig. 1), suggesting that the recombinant EGFP was not cleaved by these proteins, except for thrombin. Because imidazole can compete with His-tag to bind with Ni^{2+} ions, it is used in the purification of His-tagged proteins in immobilized metal affinity chromatography (IMAC) to elute His-tagged proteins bound. When our reaction was performed in binding buffer (binding buffer of Ni^{2+}-NTA MNPs kit, containing

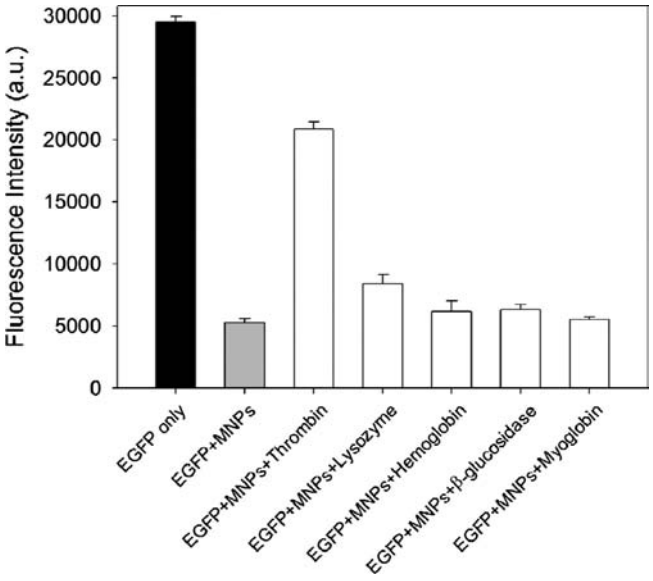


Fig. 1. Fluorescence response of EGFP solutions with different kind of proteins/enzymes. 1.0×10^{-2} U mL^{-1} thrombin, 1.0 μM Lysozyme, 1.0 μM Hemoglobin, 1.0 U mL^{-1} β-glucosidase, and 1.0 μM Myoglobin, were added respectively.

20 mM Na_3PO_4, 0.5 M NaCl, 20 mM imidazole, pH 7.4), there was no fluorescence decline of the supernatant after magnetic separation (Fig. S1), indicating that the competition of imidazole may prevent EGFP from attaching to the surface of Ni^{2+}-NTA MNPs, and proved that it was the interaction between His-tag and Ni^{2+}-NTA MNPs that caused EGFP to be trapped out of the fluid. Hence, these results indicate that EGFP and Ni^{2+}-NTA MNPs-based system is appropriate to selectively detect thrombin activity.

3.2. Thrombin detection

In order to improve the efficiency and specificity of the method, several factors that may affect the activity of thrombin, the fluorescence of EGFP, and the separation capacity of Ni^{2+}-NTA MNPs were considered, respectively. First of all, different buffers were evaluated (Fig. S1). The result implies that although Ni^{2+}-NTA MNPs have better

aggregating ability in PBS than that in Tris–HCl, considering the much higher activity of thrombin in Tris–HCl, 10 mM Tris–HCl was chosen as the assay buffer. Secondly, different volumes of Ni^{2+}-NTA MNPs was added into reaction system, from 0 to 10 μL, to find out the least volume of Ni^{2+}-NTA MNPs that can fully capture the EGFP in solution, by measuring the fluorescence of supernatant after magnetic separation. As anticipated, with the increasing amount of Ni^{2+}-NTA MNPs added into the EGFP solution, more His-tagged EGFP was trapped out of the fluid after magnet separation, and the fluorescence of supernatant decreased correspondingly (Fig. S2). When 3 μL or more Ni^{2+}–NTA MNPs was added, no more decrease of supernatant fluorescence was observed. Therefore, 3 μL of Ni^{2+}–NTA MNPs was used in the following experiments. Thirdly, in binding buffer, not only fluorescence of supernatant shows no fluorescence decline after magnetic separation (because of the competition of imidazole in the buffer), but also an abnormal high fluorescence, which is higher than that of EGFP in the buffer, was observed after thrombin digestion and MNPs separation (Fig. S1). It was further found that the fluorescence of EGFP and thrombin mixture rose slowly with the increasing concentration of thrombin in the reaction buffer (Fig. S3). Both of these two phenomena indicate that thrombin may enhance the fluorescence of the reaction system. Such phenomenon was also found when EGFP was mixed with other proteins, such as BSA (data not shown). To eliminate such interference and unspecific adsorption between protein and Ni^{2+}-NTA MNPs, a fluorescence change ΔI_F ($\Delta I_F = I_{F1} - I_{F2}$), by subtracting the fluorescence of solution after thrombin digestion and magnetic separation (I_{F2}) from that before adding Ni^{2+}–NTA MNPs (I_{F1}), is calculated, and the normalized ΔI_F ($\Delta I_F/\Delta I_{F0}$), where ΔI_{F0} ($\Delta I_{F0} = I_{F10} - I_{F20}$) is the fluorescence change of the solution before (I_{F10}) and after magnetic separation (I_{F20}) without thrombin, is used to represent the relative enzyme activity of thrombin.

After evaluating these influence factors in reaction system, detection of thrombin activity was conducted under optimized conditions. From Fig. 2A, it can be clearly seen that the fluorescence intensity of the solution increases with the rising of thrombin concentration, which indicated that the higher the concentration of thrombin, the more EGFP were cleaved and remained in the solution. Meanwhile, the normalized ΔI_F value decreased (Fig. 2B). When more than 5.0×10^{-2} U mL^{-1} thrombin was added, the fluorescence intensity of the solution did not increase any more, and the normalized ΔI_F kept stable, suggesting that almost all EGFP were cleaved by thrombin. The remained fluorescence may be derived from the unspecific chelation between Ni^{2+} ions and carboxyl group in the protein [38]. In the range from 3.0×10^{-4} U mL^{-1} to 5.0×10^{-2} U mL^{-1} of thrombin, the normalized ΔI_F is linear to the logarithmic concentration of thrombin, with the correlation coefficient of 0.9903. The lowest linear detection concentration, 3.0×10^{-4} U mL^{-1}, is much lower than some aptamer based detection methods [5,11,15]. The relative standard deviation (RSD) of 1.4%, obtained from three replicate determinations of 5.0×10^{-4} U mL^{-1} thrombin, indicates a good reproducibility of our method. Compared with the prior thrombin detection method which was based on aptamer-functionalized materials, this EGFP-MNPs detection system provides a much more precise way to detect the activity of thrombin rather than measuring the overall amount of thrombin in reaction solution [11].

3.3. Thrombin inhibitor assay

Screening protease inhibitors has significant meanings in drug development, and attracts increasing interest these years. To further demonstrate whether this method can be extended to inhibitor assay, Hirudin, one of the most powerful inhibitors of thrombin, was used to detect this potential application of our system. It was reported that Hirudin and thrombin would form a tightly binding complex, by interaction both at the active site of the enzyme and at secondary binding sites distant from the active site [39]. Hence, coaddition of Hirudin and thrombin into the reaction system would inhibit the function of thrombin, and protect the recombinant EGFP from being cleaved, which results in EGFP being trapped out of fluid by magnetic separation, and reducing the fluorescence of the solution to a considerably low level. The inhibition percent of thrombin was calculated with the following equation:

$$\text{Inhibition Percent} = (\Delta I_{Fi} - \Delta I_F)/(\Delta I_{F0} - \Delta I_F) \quad (1)$$

where ΔI_{Fi} is the fluorescence change of inhibition reaction system (EGFP, Hirudin, and 1.0×10^{-2} U mL^{-1} thrombin) before and after magnetic separation.

Hirudin can intensively inhibit the activity of thrombin (data not shown), and when the inhibition percent of thrombin versus the Hirudin concentration is plotted (Fig. 3), one clearly sees that inhibition percent rises with the increasing concentration of Hirudin, and reaches plateau over 1.4×10^{-10} M Hirudin (equal to 1.0×10^{-2} U mL^{-1}). This result is well matched with the definition of Hirudin biological activity, that 1 unit of Hirudin is defined as the amount of Hirudin neutralizing 1 unit of thrombin [39,40]. These

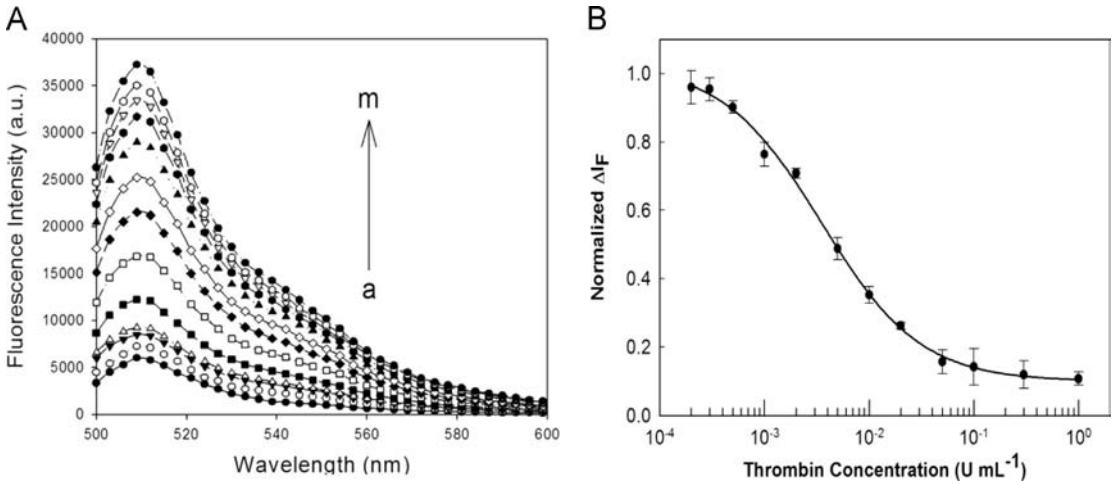


Fig. 2. (A) Fluorescence responses of supernatants with different concentrations of thrombin (U mL^{-1}): (a) 0; (b) 2.0×10^{-4}; (c) 3.0×10^{-4}; (d) 5.0×10^{-4}; (e) 1.0×10^{-3}; (f) 2.0×10^{-3}; (g) 5.0×10^{-3}; (h) 1.0×10^{-2}; (i) 2.0×10^{-2}; (j) 5.0×10^{-2}; (k) 1.0×10^{-1}; (l) 3.0×10^{-1} and (m) 1.0. (B) Plot of the normalized ΔI_F of supernatants versus the concentration of thrombin. The normalized ΔI_F is defined as $\Delta I_F/\Delta I_{F0}$, where ΔI_F is the fluorescence change of the solution with thrombin before and after magnetic separation, and ΔI_{F0} is the fluorescence change of solution without thrombin.

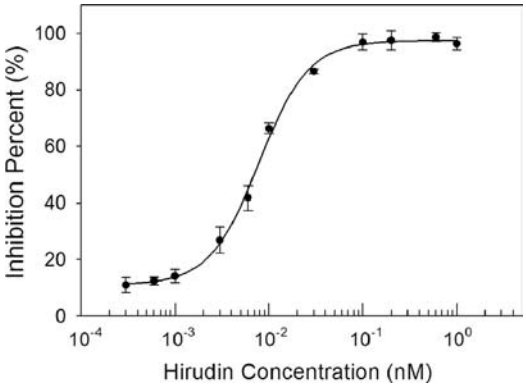


Fig. 3. Inhibition of Hirudin to thrombin (1.0×10^{-2} U mL^{-1}).

Table 1
Detection of thrombin in human blood serum with this method.

serum sample	Concentration of thrombin added (U mL^{-1})	Concentration obtained with this method (U mL^{-1})	RSD (%)	Recovery (%)
1	7.0×10^{-4}	6.5×10^{-4}	1.8	92.8
2	2.0×10^{-3}	1.8×10^{-3}	1.1	90.0
3	8.0×10^{-3}	8.6×10^{-3}	1.5	107.5
4	3.0×10^{-2}	2.6×10^{-2}	2.1	86.7

data, in return, show that the EGFP-MNPs-based method is viable to detect thrombin inhibitor and apply in inhibitor drug screening.

3.4. Detection of thrombin activity in real sample

Thrombin is an essential protein in various life procedures, so thrombin activity detection in real sample is essential for diagnosis. Herein, we used serum as a complex biological matrix to evaluate the practical application of our thrombin assay, and standard addition method was performed since there is no thrombin but prothrombin in serum. A series of samples were prepared by adding different concentrations of thrombin into human blood serum, and analyzed by our method. From the data displayed in Table 1, the recovery (86.7–107.5%) and the relative standard deviation (RSD) values (1.1–2.1%) were obtained, which indicates the acceptable accuracy and the satisfactory precision of the EGFP-MNPs-based method, respectively, and implies that this analysis system may have a promising application for detecting thrombin in complex bio-samples.

3.5. Reusability of Ni^{2+}-NTA MNPs

Since recombinant proteins are attached to Ni^{2+}-NTA MNPs through His-tag-Ni^{2+} interaction, and Ni^{2+} ions, even binding with proteins, can be chelated by EDTA, such binding proteins can be easily stripped from MNPs by EDTA chelating, and Ni^{2+}-NTA MNPs can be recovered after reacting with $NiSO_4$. Reusability of Ni^{2+}-NTA MNPs was evaluated by different concentrations of thrombin with different recycle times. As shown in Fig. 4, normalized ΔI_F decreases with the increasing concentration of thrombin in reaction system, exhibiting a similar protein-capture capacity between new Ni^{2+}-NTA MNPs and the recycled ones. This proved that Ni^{2+}-NTA MNPs is reusable, and able to construct the EGFP-MNPs complex to detect thrombin activity for several times. Compared with other methods, like QDs/AuNPs-based thrombin measurements [12–15], which cannot be recovered after use, our

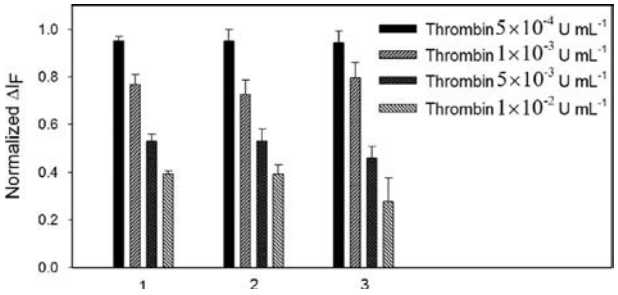


Fig. 4. The normalized ΔI_F of supernatant when EGFP and thrombin reaction systems were mixed with the new and recycled Ni^{2+}-NTA MNPs, respectively. Group 1, new Ni^{2+}-NTA MNPs; Group 2, Ni^{2+}-NTA MNPs recovered once; Group3, Ni^{2+}-NTA MNPs recovered twice.

EGFP- MNPs-based system is a cost-saving way to detect protease activity.

4. Conclusions

We have developed a label-free, simple and sensitive fluorescent detecting method of thrombin activity, using a recombinant EGFP and Ni^{2+}-NTA MNPs. Not only the thrombin activity and inhibition in buffer, but also the thrombin activity in serum, can be sensitively detected by our method. With notable advantages such as simple synthesis of the recombinant EGFP and reusability of Ni^{2+}-NTA MNPs, this method is a desirable alternative to aptamer-based means, which can only analyze thrombin amount. Moreover, the measurement in multiwell plates reflects this assay a promising technique for high-throughput manipulation in thrombin detection and anticoagulation drugs discovery.

In addition, changing protease recognition peptides between EGFP and His-tag can extend this detection system to other proteases analysis, as here reported for thrombin. Meanwhile, extensive range of EGFP variations enables a multiple targets assay with different colors of fluorescence proteins at the same time. All these outstanding properties ensure our EGFP and Ni^{2+}-NTA MNPs-based system more potential biological applications, and it is expected to be used in diagnosis of protease-associated disease in the future.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.07.017.

References

[1] R.C. Becker, F.A. Spencer, J. Thomb., Thrombolysis 5 (1998) 215–229.
[2] C.A. Holland, A.T. Henry, H.C. Whinna, F.C. Church, FEBS Lett. 484 (2000) 87–91.
[3] M.T. Stubbs, W. Bode, Trends Biochem. Sci. 20 (1995) 23–28.
[4] H. Inuyama, T. Saito, J. Takagi, Y. Saito, J. Cell Physiol. 173 (1997) 406–414.
[5] B. Li, H. Wei, S.J. Dong, Chem. Commun. 1 (2007) 73–75.
[6] H.X. Chang, L.H. Tang, Y. Wang, J.H. Jiang, J.H. Li, Anal. Chem. 82 (2010) 2341–2346.
[7] J. Zheng, G.F. Cheng, P.G. He, Y.Z. Fang, Talanta 80 (2010) 1868–1872.
[8] C.Y. Deng, J.H. Chen, Z. Nie, M.D. Wang, X.C. Chu, X.L. Chen, X.L. Xiao, C.Y. Lei, S. Z. Yao, Anal. Chem. 81 (2009) 739–745.

[9] P.L. He, L. Shen, Y.H. Cao, D.F. Li, Anal. Chem. 79 (2007) 8024–8029.
[10] X.R. Zhang, B.P. Qi, Y. Li, S.S. Zhang, Biosens. Bioelectron. 25 (2009) 259–262.
[11] H.M. So, K. Won, Y.H. Kim, B.K. Kim, B.H. Ryu, P.S. Na, H. Kim, J.O. Lee, J. Am. Chem. Soc. 127 (2005) 11906–11907.
[12] H.P. Huang, J.J. Zhu, Biosens. Bioelectron. 25 (2009) 927–930.
[13] C.K. Chen, C.C. Huang, H.T. Chang, Biosens. Bioelectron. 25 (2010) 1922–1927.
[14] T. Li, E. Wang, S. Dong, Chem. Commun. 31 (2008) 3654–3656.
[15] H. Wei, B. Li, J. Li, E. Wang, S. Dong, Chem. Commun. 36 (2007) 3735–3737.
[16] S.W. Huang, J.S. Li, A.H. Liang, Z.L. Jiang, Acta Chim. Sinica 69 (2011) 183–189.
[17] H.W. Ai, K.L. Hazelwood, M.W. Davidson, R.E. Campbell, Nat. Methods 5 (2008) 401–403.
[18] K. Boeneman, J.B. Delehanty, K. Susumu, M.H. Stewart, J.R. Deschamps, I. L. Medintz, Adv. Exp. Med. Biol. 733 (2012) 63–74.
[19] L.M. DiPilato, X. Cheng, J. Zhang, Proc. Natl. Acad. Sci. USA 101 (2004) 16513–16518.
[20] V.A. Romoser, P.M. Hinkle, A. Persechini, J. Biol. Chem. 272 (1997) 13270–13274.
[21] A. Miyawaki, J. Llopis, R. Heim, J.M. McCaffery, J.A. Adams, M. Ikura, R.Y. Tsien, Nature 388 (1997) 882–887.
[22] B.I. Lindhout, P. Fransz, F. Tessadori, T. Meckel, P.J.J. Hooykaas, B.J. Zaal, Nucleic Acids Res. 35 (2007) 107.
[23] D.L. Beach, E.D. Salmon, K. Bloom, Curr. Biol. 9 (1999) 569. (S1).
[24] O.V. Stepanenko, V.V. Verkhusha, I.M. Kuznetsova, V.N. Uversky, K.K. Turoverov, Curr. Protein Pept. Sci. 9 (2008) 1389.
[25] P. Hu, C.Z. Huang, Y.F. Li, J. Ling, Y.L. Liu, L.R. Fei, J.P. Xie, Anal. Chem. 80 (2008) 1819–1823.
[26] J. Wang, A.N. Kawde, A. Erdem, M. Salazar, Analyst 126 (2001) 2020–2024.
[27] H. Gu, K. Xu, C. Xu, B. Xu, Chem. Commun. 9 (2006) 941–949.
[28] J.W. Choi, K.W. Oh, J.H. Thomas, W.R. Heineman, H.B. Halsall, J.H. Nevin A.J. Helmicki, H.T. Henderson, C.H. Ahn, Lab Chip 2 (2002) 27–30.
[29] D. Wang, J. He, N. Rosenzweig, Z. Rosenzweig, Nano Lett. 4 (2004) 409–413.
[30] R.S. Molday, D. Mackenzie, J. Immunol. Methods 52 (1982) 353–367.
[31] J. Dobson, Drug Dev. Res. 67 (2006) 55–60.
[32] M. Arruebo, P.R. Fernández, M.R. Ibarra, J. Santamaría, Nano Today (2007) 22–32.
[33] S.J. Son, J. Reichel, B. He, M. Schuchman, S.B. Lee, J. Am. Chem. Soc. 127 (2005) 7316–7317.
[34] S.R. McRae, C.L. Brown, G.R. Bushell, Protein Express Purif. 41 (2005) 121–127.
[35] C. Guarise, L. Pasquato, V.D. Filippis, P. Scrimin, PNAS 103 (2006) 3978–3982.
[36] B.C. Yin, M. Zhang, W. Tan, B. Ye, Chem. Bio. Chem 11 (2010) 494–497.
[37] H. Mattoussi, J.M. Mauro, E.R. Goldman, G.P. Anderson, V.C. Sundar F.V. Mikulec, M.G. Bawendi, J. Am. Chem. Soc. 122 (2000) 12142–12150.
[38] G.R. Brubaker, D.H. Busch, Inorg. Chem. 5 (1966) 2110–2113.
[39] M.G. Grütter, J.P. Priestle, J. Rahuel, H. Grossenbacher, W. Bode, J. Hofsteenge, S.R. Stone, EMBO J. 9 (1990) 2361–2365.
[40] T.J. Rydel, A. Tulinsky, J. Mol. Biol. 221 (1991) 583–601.