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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.