Talanta 83 (2010) 185–189

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Time-resolved fluorescence aptamer-based sandwich assay for thrombin detection

Da-Wei Huang[a], Cheng-Gang Niu[a,b,*], Pin-Zhu Qin[a], Min Ruan[a], Guang-Min Zeng[a]

[a] College of Environmental Science and Engineering, Key Laboratory of Environmental Biology and Pollution Control, Ministry of Education, Hunan University, Changsha 410082, PR China
[b] State Key Laboratory of Powder Metallurgy, Central South University, Changsha 410083, PR China

ARTICLE INFO

Article history:
Received 14 July 2010
Received in revised form 2 September 2010
Accepted 7 September 2010
Available online 16 September 2010

Keywords:
Thrombin detection
Aptamer
Time-resolved fluorescence
Eu(TTA)3(5-NH_2-phen)

ABSTRACT

In the present study, the authors report a novel sensitive method for the detection of thrombin using time-resolved fluorescence sensing platform based on two different thrombin aptamers. The thrombin 15-mer aptamer as a capture probe was covalently attached to the surface of glass slide, and the thrombin 29-mer aptamer was fluorescently labeled as a detection probe. A bifunctional europium complex was used as the fluorescent label. The introduction of thrombin triggers the two different thrombin aptamers and thrombin to form a sandwich structure. The fluorescence intensity is proportional to the thrombin concentration. The present sensing system could provide both a wide linear dynamic range and a low detection limit. The proposed sensing system also presented satisfactory specificity and selectivity. Results showed that thrombin was retained at the aptamer-modified glass surface while nonspecific proteins were removed by rinsing with buffer solution. This approach successfully showed the suitability of aptamers as low molecular weight receptors on glass slides for sensitive and specific protein detection.

1. Introduction

In recent years, aptamer-based biochemical detection system has been widely investigated and made great success [1–3]. Aptamers are nucleic acid ligands that have been designed through an in vitro selection process called SELEX (Systematic Evolution of Ligands by Exponential Enrichment) [4,5]. Human thrombin is used to help control bleeding during surgery [6]. It is necessary to detect thrombin in blood not only for patients suffering from diseases associated with coagulation abnormalities, but also for determining the effectiveness of the therapeutic drugs after surgery or in thromboembolic disease treatments.

Thrombin has two different binding aptamers 15-mer, 5′-GGTTGGTGTGGTTGG-3′ and 29-mer, 5′-AGTCCGT GGTAGGGCAGGTTGGGGTGACT-3′. The 15-mer aptamer was the first one selected in vitro, specific for a protein without nucleic acid-binding properties [7], it can form a G-quartet structure to interact with thrombin [8,9]. The 29-mer was selected later with a K_d of 0.5 nM for thrombin [10]. The 29-mer aptamer binds to the heparin binding sites of thrombin while the previously selected 15-mer was known to bind the fibrinogen recognition sites [11]. Since two aptamers were selected for thrombin at separate binding sites, sandwich assays (two selected aptamers binding thrombin in two different, not overlapping, sites are used) became feasible.

There is a need for fast, convenient, easy-to-use analytical methods for protein detection in complex matrixes. The applicability of such methods is wide from diagnostics to basic research. Compared to the well developed electrochemical systems for aptamer-based proteins detection [3,12–16], there is increasing interest in the investigation of fluorescence sensing systems. Fluorescence-based detection, such as fluorescence anisotropy [17,18], fluorescence polarization [19–21], molecular light switch [2,22–24], and quantum dots [25–27] have become a significant area of biochemistry. A fluorescent dye is usually used as the fluorescent label, such as fluorescein isothiocyanate [28], rhodamine [29], fluorescamine [30], texas red [21], etc. However, there is a problem with background noises caused by autofluorescence from biological samples and with light scattering from solid substrates. For this purpose, one approach is to use time-resolved fluorescence with lanthanide complexes as the labels to decrease the background noises [31,32]. Fluorescent europium chelates, exhibit large stokes shifts (~290 nm) with no overlap between the excitation and emission spectra and very narrow (10 nm bandwidth) emission spectra at ~615 nm. In addition, their long fluorescence lifetimes (600–1000 μs for Eu^{3+} compared with 5–100 ns for conventional fluorophores) allow use of microsecond time-resolved fluorescence measurements, which further reduce the observed background signals [33]. To the best of the authors' knowledge, time-resolved fluorescence-based assay for thrombin detection has not been reported.

* Corresponding author at: College of Environmental Science and Engineering, Hunan University, No. 8 Lushan Nanlu, Yuelu District, Changsha, Hunan Province 410082, PR China. Tel.: +86 731 88823820; fax: +86 731 88822829.
E-mail address: cgniu@hotmail.com (C.-G. Niu).

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.09.004

Herein, the authors introduced a novel aptamer-based time-resolved fluorescence sandwich assay for thrombin detection. A europium complex, Eu(TTA)$_3$(5-NH$_2$-phen) (ETN) with long fluorescence lifetime synthesized by our group, was used as the fluorescent label [34,35]. A glass slide is first modified with aldehyde-group. Then, amino modified 15-mer aptamer probe, designed to specifically bind to the heparin binding sites of thrombin, is combined to the modified glass slide by covalent bond. The 29-mer aptamer probe, which bound to the heparin binding sites of thrombin, was fluorescently labeled. In the presence of thrombin, the two different thrombin aptamers and thrombin are more inclined to form a sandwich structure. The detection of thrombin was done by monitoring the fluorescence signals of europium complex-labeled 29-mer aptamer probe left on the glass slides surface. The detection method has advantages of easy operation and separation, short assay time, lower background signal. It also showed satisfactory sensitivity and is suitable for the detection of thrombin in complex samples. It is believed that this sensing system could be further applied to other targets of detection.

2. Experimental

2.1. Materials and reagents

The sequences of thrombin aptamers were: 5′amino modified 15-mer aptamer with polyT(20) tail, 5′-NH$_2$-(T)$_{20}$ GGTTG GTGTG GTTGG-3′ (aptamer$_1$) and 5′amino modified 29-mer aptamer with polyT(10)tail, 5′-NH$_2$-(T)$_{10}$ AGTCC GTGGT AGGGC AGGGC AGGTT GGGGT GACT-3′ (aptamer$_2$) [14]. The aptamers were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) and stored at −20 °C in 20 mM Tris buffer containing 140 mM NaCl and 1 mM $MgCl_2$, pH = 7.4.

Human α-Thrombin (T6884) was purchased from Sigma–Aldrich (Shanghai) Trading Co., Ltd. (Shanghai, China), and stored at −20 °C in PBS buffer containing 50% glycerol. Bovine serum albumin (BSA) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Human serum was purchased from Lanzhou National HyClone Bio-Engineering Co., Ltd. (Lanzhou, China). Goat anti-human immunoglobulin G (IgG) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). 3-Aminopropyltrimethoxysilane (APTES) was purchased from Acros organics (Geel, Belgium). All the materials and reagents were used as supplied without further purification and doubly distilled water was used throughout the experiments.

2.2. Preparation of buffer solutions

Tris–HCl buffer solutions (20 mM) of different pH were prepared by mixing an appropriate 0.10 M Tris base solution with 0.10 M hydrochloric acid. PBS buffer (100 mM) was prepared by mixing an appropriate content of 200 mM Na_2HPO_4 and 200 mM NaH_2PO_4. The rinse buffer was made up of certain amounts of 20 mM Tris–HCl (pH = 7.5), 100 mM NaCl, and 1 mM $MgCl_2$. The binding buffer was included appropriate of 20 mM Tris–HCl (pH = 7.4), 140 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM KCl. The composition of immobilization buffer was 100 mM PBS buffer (pH = 7.3) containing 0.9% NaCl.

2.3. Instrumentation

All fluorescence measurements were conducted on a Perkin-Elmer LS-55 spectrofluorimeter (United Kingdom) and controlled by a personal computer data processing unit. A modified home-made poly(tetrafluoroethylene) detection-cell and two arms of the bifurcated optical fiber were fixed in the detecting chamber of the spectrofluorimeter to direct the excitation and emission light. The excitation light was directed outside the spectrofluorimeter to the sensing system fixed in the detection-cell through one arm of the fiber and the emission light was collected inside the spectrofluorimeter through the other. A glass plate (side length 12.5 mm, thickness 1.5 mm) covered with thrombin–aptamer complex were fixed on the top of the flow chamber by the mounting screw nut. The time-resolved fluorescence signals of every glass slides were detected with both excitation and emission slits set at 8.0 nm. The delay time, gate time, and excitation/emission wavelength were set at 0.1 ms, 1.0 ms and 377/609 nm, respectively. All assays were performed in triplicates and all measurements were performed at room temperature (25 °C) and pressures (101 kPa).

2.4. Preparation of activated glass slides

Glass slides were activated according to the literature [36] with slight modifications. Glass slides were cleaned in chromic acid solution for 12 h then rinsed in double distilled water, followed by an immersion in ammonia water (25%) for another 12 h. The glass slides were then washed with double distilled water and dried at room temperature (25 °C). Precleaned glass slides were immersed in a 95% alcohol/water solution containing 2% APTES, and adjusted to the desired pH of 4.5 with glacial acetic acid for 30 min. Slides were ultrasonically cleaned with absolute ethanol and double distilled water. Silanized slides were incubated in PBS buffer containing 2.5% glutaraldehyde for 3 h. Afterward, the slides were rinsed with rinse buffer and double distilled water and dried at 37 °C.

2.5. Aptamer$_1$ coupling on activated glass slides

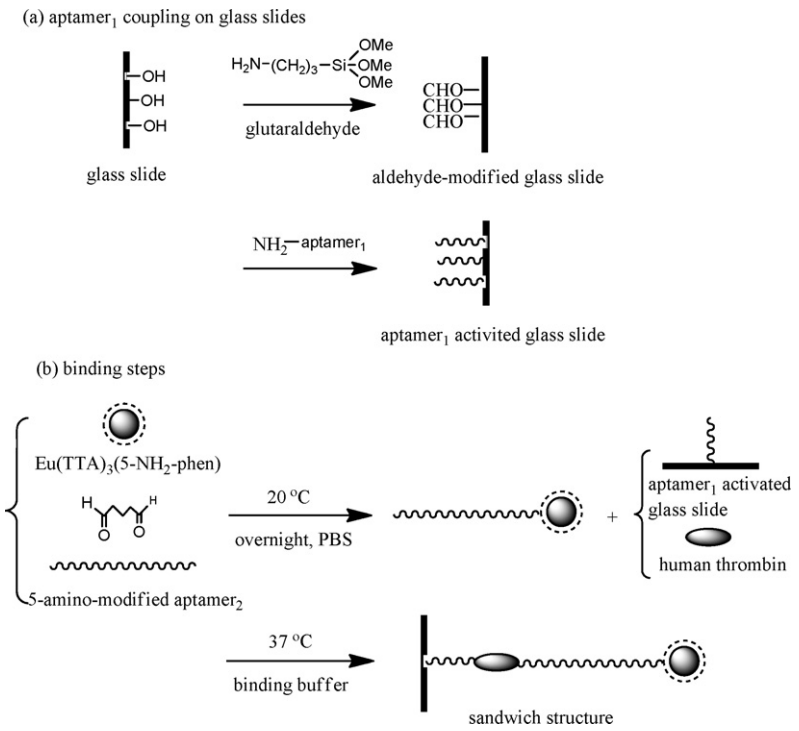
The aptamer was heated at 95 °C for 1 min and then cooled in ice for 10 min prior to coupling steps. The thermal treatment made the aptamer unfold, and the polyT(20) tail maintained the aptamer far from the surface of the sensing system, allowing the proper conformation for molecular recognition. 5′-Amino-modified aptamer$_1$ (30 μl, 1.0 μM in PBS) was dropped onto the activated glass surface and incubated overnight at 20 °C. The droplets were allowed to dry, and any non-covalently bond aptamer$_1$ was removed by washing in rinse buffer. After rinsing the glass samples three times with rinse buffer, remaining non-reacted aldehyde groups were blocked with 2% BSA at 37 °C (1 h) and then washed three times with rinse buffer. Finally, the slides were dried at 37 °C. The capture probe aptamer$_1$ was then covalently immobilized on the glass surface.

2.6. Eu(TTA)$_3$(5-NH$_2$-phen) (ETN) coupling with aptamer$_2$

ETN was suspended in PBS buffer containing 2.5% glutaraldehyde by ultrasonication and thermal agitation for 3 h, then centrifuged and washed with PBS buffer. The glutaraldehyde modified ETN was resuspended in PBS buffer, and 20 μl of 1×10^{-5} M aptamer$_2$ was added, the mixture was stirred at 20 °C overnight. After centrifuging and washing with rinse buffer to remove the unbound oligonucleotides, the ETN was coupled with aptamer$_2$, and then resuspended in 180 μl binding buffer, stored at 4 °C.

2.7. Thrombin–aptamer sandwich analysis

Once the aptamer$_1$ was covalently immobilized on the glass surface and the ETN was coupled with aptamer$_2$, the binding step with thrombin in binding buffer was realized by adding 10 μl of the thrombin solution at different concentrations. The reaction was monitored for 2 h at 37 °C, the solution was then removed, and the glass slides surface was washed with PBS buffer to eliminate the unbounded substances. Finally, all glass slides were dried at room



Scheme 1. The schematic diagram of this detection method.

temperature (25 °C). The detection was accomplished by monitoring fluorescence signals of the ETN-labeled aptamer$_2$ left on the glass slides surface. The main steps of the assay using two aptamers were shown in Scheme 1.

3. Results and discussion

3.1. Thrombin detection

Time-resolved fluorescence intensity of different concentrations of thrombin detected from glass surfaces was recorded at λ_{ex} = 377 nm, λ_{em} = 609 nm. As shown in Fig. 1, the time-resolved fluorescence intensity was increased with the increasing concentration of thrombin. Fluorescence intensity was proportional to thrombin concentration. In speculating on the results, the proposed assay can be used to detect trace thrombin with lower background signal.

The reproducibility and stability of time-resolved fluorescence signal of this assay were tested by measuring three thrombin concentrations in three independent measurements. The results were given in Table 1.

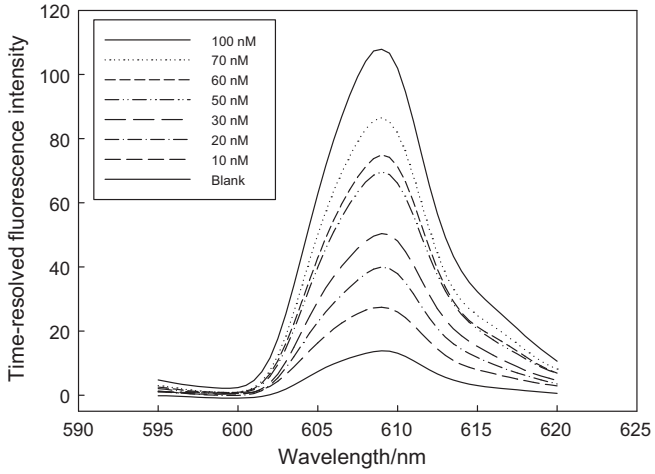


Fig. 1. Time-resolved fluorescence emission spectra for different concentrations of human α-thrombin, for time-resolved spectra: λ_{ex} = 377 nm, λ_{em} = 609 nm, delay time, 0.1 ms, gate time, 1.0 ms.

3.2. Optimization of the concentration of aptamer$_1$

The optimal concentration of aptamer$_1$ coupling onto the activated glass slides was investigated. Four different concentrations of aptamer$_1$, 5.0 μM, 1.0 μM, 0.1 μM and 0.01 μM, were employed to coupling onto the activated glass slides. The fluorescence intensity detection was then achieved using a 50 nM thrombin solution. The results were recorded in Fig. 2, which showed the different time-resolved fluorescence intensities from the different concentrations of aptamer$_1$. The concentration of the aptamer$_1$ at 1.0 μM presents a lower noise and a stable signal than others. It may be the appropriate concentration for coupling with the aldehyde group on the glass slides. Thus, the concentration of aptamer$_1$ used for all experiments was 1.0 μM.

Table 1
The reproducibility of thrombin.

$C_{thrombin}$	Intensity (609 nm)	Average	SD
0 nM	15.4, 13.8, 10.7	13.3	2.4
10 nM	27.3, 35.6, 20.6	27.8	7.5
20 nM	34.8, 40.1, 30.6	35.1	4.8
30 nM	50.4, 45.5, 53.9	49.9	4.2
50 nM	71.6, 63.9, 74.5	70.0	5.5
60 nM	74.9, 79.0, 73.3	75.7	2.9
70 nM	86.4, 93.6, 80.3	86.7	6.6
100 nM	107.8, 103.0, 112.6	107.8	4.8

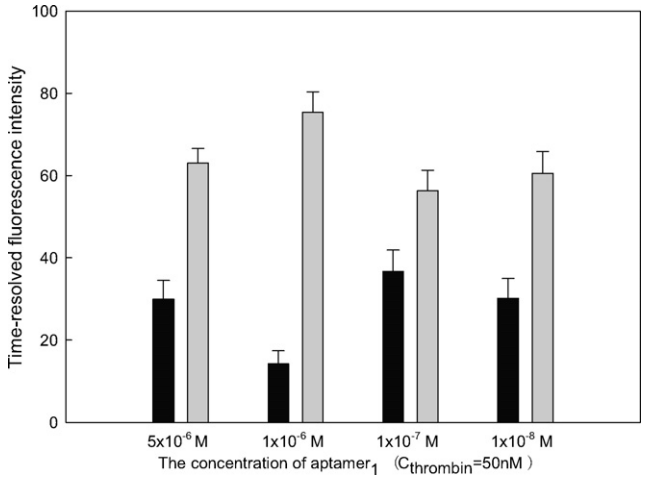


Fig. 2. Optimization of the concentration of aptamer$_1$ coupling on glass slides. The black bars represent the intensity of blanks as well as gray bars represent the samples which added in 50 nM thrombin. Every data point was the mean of three measurements. The error bars are the standard deviation.

3.3. Optimal binding time

The optimal binding time for this assay was also investigated. In the experiments, the authors discussed six different binding time, 0.5 h, 1.0 h, 1.5 h, 2.0 h, 2.5 h, and 3.0 h. As shown in Fig. 3, in the initial stage of binding time less than 2 h, the fluorescence signal increased, and then reached a steady state at a binding time longer than 2 h. On the basis of this, all the experiments were carried out for the binding time of 2 h.

3.4. Selectivity of the prepared detection system

The selectivity of the prepared detection system was studied after establishing the optimum conditions. The experiments were carried out by recording the time-resolved fluorescence intensity of different proteins employed in the detection assay. BSA, IgG at a same concentration of 100 μM, in a 2000-fold excess with respect to thrombin, were used as the control proteins. As shown in Fig. 4, a high response was observed when 50 nM thrombin and the mixture of thrombin and other proteins were tested, whereas low signals were obtained in the absence of thrombin and presence of 100 μM of BSA and IgG. The presence of a high concentration of BSA and IgG

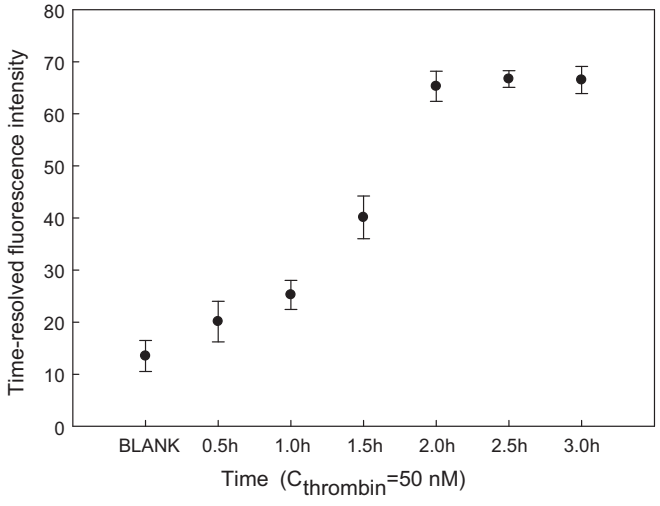


Fig. 3. Optimization of the binding time. Every data point was the mean of three measurements. The error bars are the standard deviation.

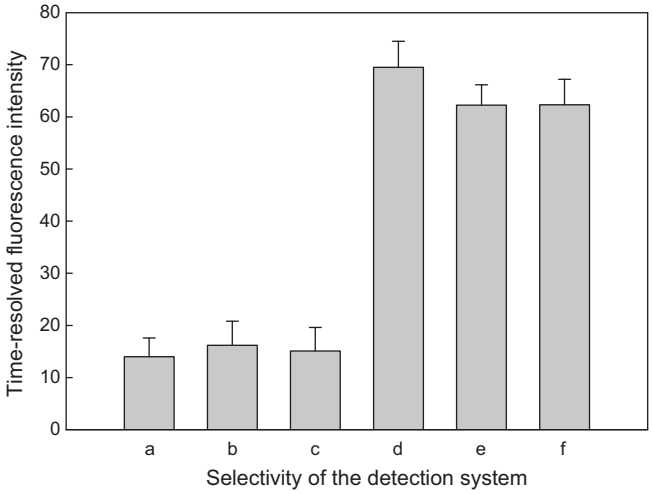


Fig. 4. Investigation of the selectivity in this method. (a) blank, (b) 100 μM BSA, (c) 100 μM IgG, (d) 50 nM thrombin, (e) 100 μM BSA + 50 nM thrombin and (f) 100 μM IgG + 50 nM thrombin). Every data point was the mean of three measurements. The error bars are the standard deviation.

did not affect the sensing system performance even the concentration of BSA and IgG was nearly in a 2000-fold excess with respect to 50 nM thrombin. The results demonstrated the high selectivity of the sensing system.

3.5. Linear response and detection limit

The linear response of the sensing system was investigated. The excitation and emission wavelengths were 377 nm and 609 nm, respectively. As given in Fig. 5, the linear response of the sensing system covered the concentrations of thrombin from 10 nM to 70 nM. The following equation was obtained by fitting the experimental data obtained:

$$I = 17.7667 + 0.9952C \, (R = 0.9856)$$

where I was the time-resolved fluorescence intensity and C was the thrombin concentration. The calibration equation can serve as the quantitative basis for the determination of trace thrombin content in buffer or complex matrixes (such as serum). The detection

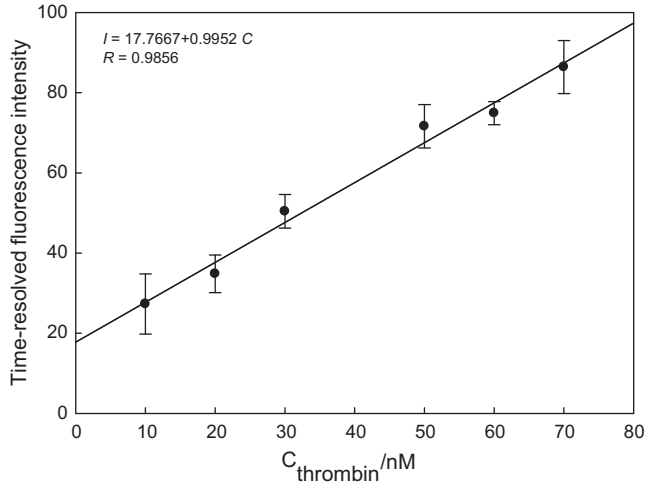


Fig. 5. The linear relationship between the time-resolved fluorescence intensity and thrombin concentration. The concentrations of thrombin were 10 nM, 20 nM, 30 nM, 50 nM, 60 nM, 70 nM. Every data point was the mean of three measurements. The error bars are the standard deviation.

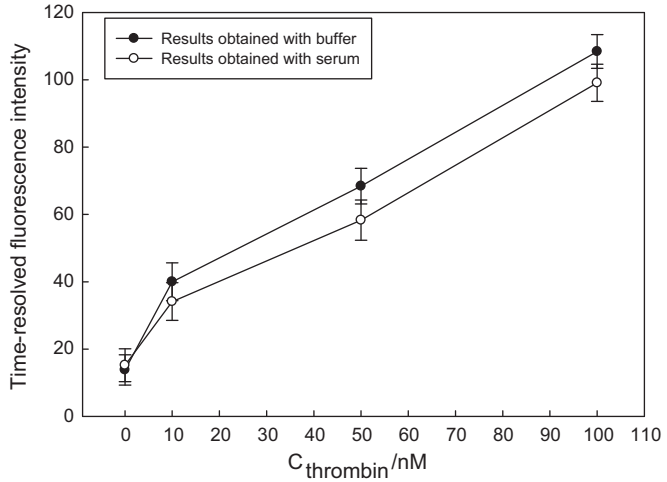


Fig. 6. Results obtained with serum samples spiked with different concentrations of thrombin and comparison with the same concentrations tested in buffer. Every data point was the mean of three measurements. The error bars are the standard deviation.

limit of 7.7 nM could be estimated by using $3\sigma + y_0$, where y_0 is the blank fluorescence intensity and σ was the standard deviation of the blank solution with 12 parallel measurements.

However, it has to be noticed that this sensing system is just a proof of principle for our proposal. Compared to the electrochemistry systems and other fluorescent chemosensor using thrombin and thrombin aptamers assays (without fluorescence labeling and/or detection in homogeneous solutions, etc), the background noises caused by autofluorescence from biological samples, the scattering light from solid substrates were avoided. In this paper, it is necessary to make the fluorophore covalently bound to the original aptamer, but it is a pity that even slight modifications on aptamers may lead to significant loss of their affinity and specificity [37,38]. The proposed assay may be a more sensitive and rapid method if it is applied to some proteins or small molecules which have high specificity and selectivity aptamers.

3.6. Determination of thrombin in human serum

The following experiments were focused on evaluating the ability of the sensing system to detect thrombin in complex matrixes. Serum is what remains from whole blood after coagulation. Its chemical composition is similar to plasma, but it does not contain coagulation proteins such as thrombin or other factors. Standard solutions of thrombin were added to human serum to test the performance of the sensing system for complex matrixes. Serum, diluted five times, was tested alone or spiked with thrombin. Comparison between the results obtained with serum and buffer was shown in Fig. 6, and demonstrated that the sensing system was also workable in complex matrixes.

4. Conclusions

The present study reported a time-resolved fluorescence detection methodology of protein using an aptamer-based sandwich assay. The ETN modified aptamer exhibited extremely long lifetime and excellent signaling ability of a trace amount of target protein. Time-resolved fluorescence signals of different concentrations of thrombin were detected and a linear response for thrombin was observed in the range from 10 nM to 70 nM with a detection limit of 7.7 nM. The optimum binding time and the concentration of aptamer$_1$ used in the experiments were 2.0 h and 1.0 μM, respectively. The selectivity of the sensing system was also illustrated. The authors believe that the proposed method can provide an alternative tool for aptamer-based protein detection due to its simplicity, rapidity, low cost, and good response characteristics.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (20977026), the National 863 High Technology Research Foundation of China (2006AA06Z407), the Research Fund for the Doctoral Program of Higher Education of China (20090161110009), the Equipment Foundation of Hunan University, and the Changjiang Scholars and Innovative Research Team in University (PCSIRT).

References

[1] S.P. Song, L.H. Wang, J. Li, J.L. Zhao, C.H. Fan, TrAC Trend Anal. Chem. 27 (2008) 108–117.
[2] Y.X. Jiang, X.H. Fang, C.L. Bai, Anal. Chem. 76 (2004) 5230–5235.
[3] J. Zheng, G.F. Cheng, P.G. He, Y.Z. Fang, Talanta 80 (2010) 1868–1872.
[4] A.D. Ellington, J.W. Szostak, Nature 346 (1990) 818–822.
[5] C. Tuerk, L. Gold, Science 249 (1990) 505–510.
[6] D.J. Cada, T. Levien, D.E. Baker, Hospital. Pharm. 43 (2008) 577.
[7] L.C. Bock, L.C. Griffin, J.A. Latham, E.H. Vermaas, J.J. Toole, Nature 355 (1992) 564–566.
[8] R.F. Macaya, P. Schultze, F.W. Smith, J.A. Roe, J. Feigon, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 3745–3749.
[9] I. Smirnov, R.H. Shafer, Biochemistry 39 (2000) 1462–1468.
[10] D.M. Tasset, M.F. Kubik, W. Steiner, J. Mol. Biol. 272 (1997) 688–698.
[11] L.R. Paborsky, S.N. McCurdy, L.C. Griffin, J.J. Toole, L.L. Leung, J. Biol. Chem. 268 (1993) 20808–20811.
[12] S. Centi, S. Tombelli, M. Minunni, M. Mascini, Anal. Chem. 79 (2007) 1466–1473.
[13] E. Suprun, V. Shumyantseva, T. Bulko, S. Rachmetova, S. Rad'ko, N. Bodoev, A. Archakov, Biosens. Bioelectron. 24 (2008) 825–830.
[14] S. Centi, G. Messina, S. Tombelli, I. Palchetti, M. Mascini, Biosens. Bioelectron. 23 (2008) 1602–1609.
[15] Y. Kang, K.J. Feng, J.W. Chen, J.H. Jiang, G.L. Shen, R.Q. Yu, Bioelectrochemistry 73 (2008) 76–81.
[16] K. Kerman, H.F. Song, J.S. Duncan, D.W. Litchfield, H.B. Kraatz, Anal. Chem. 80 (2008) 9395–9401.
[17] X.H. Fang, Z.H. Cao, T. Beck, W.H. Tan, Anal. Chem. 73 (2001) 5752–5757.
[18] G. Gokulrangan, J.R. Unruh, D.F. Holub, B. Ingram, C.K. Johnson, G.S. Wilson, Anal. Chem. 77 (2005) 1963–1970.
[19] M.Y. Song, Y.X. Zhang, T. Li, Z.X. Wang, J.F. Yin, H.L. Wang, J. Chromatogr. A 1216 (2009) 873–878.
[20] J.A. Cruz-Aguado, G. Penner, Anal. Chem. 80 (2008) 8853–8855.
[21] J. Ruta, S. Perrier, C. Ravelet, J. Fize, E. Peyrin, Anal. Chem. 81 (2009) 7468–7473.
[22] B.L. Li, C.J. Qin, T. Li, L.X. Wang, S.J. Dong, Anal. Chem. 81 (2009) 3544–3550.
[23] R. Nutiu, Y.F. Li, J. Am. Chem. Soc. 125 (2003) 4771–4778.
[24] S.J. Xiao, P.P. Hu, Y.F. Li, C.Z. Huang, T. Huang, G.F. Xiao, Talanta 79 (2009) 1283–1286.
[25] J. Zhang, X. Jia, X.J. Lv, Y.L. Deng, H.Y. Xie, Talanta 81 (2010) 505–509.
[26] V. Bagalkot, L.F. Zhang, E. Levy-Nissenbaum, S.Y. Jon, P.W. Kantoff, R. Langer, O.C. Farokhzad, Nano Lett. 7 (10) (2007) 3065–3070.
[27] J.H. Choi, K.H. Chen, M.S. Strano, J. Am. Chem. Soc. 128 (2006) 15584–15585.
[28] R.A. Potyrailo, R.C. Conrad, A.D. Ellington, G.M. Hieftje, Anal. Chem. 70 (1998) 3419–3425.
[29] Y.H. Tou, G.F. Cheng, L. Lin, J. Zheng, Z.R. Wu, P.G. He, Y.Z. Fang, Chem. J. Chin. Univ. 27 (2006) 2266–2270.
[30] E.J. Merino, K.M. Weeks, J. Am. Chem. Soc. 125 (2003) 12370–12371.
[31] A. Egorova, S. Beltyukova, O. Teslyuk, J. Pharmaceut. Biomed. 21 (1999) 585–590.
[32] P. Lode, J. Rosenberg, K. Pettersson, H. Takalo, Anal. Chem. 75 (2003) 3193–3201.
[33] E.P. Diamandii, T.K. Christopoubs, Anal. Chem. 62 (1990) 1149A–1157A.
[34] P.Z. Qin, C.G. Niu, G.M. Zeng, M. Ruan, L. Tang, J.L. Gong, Talanta (2009) 10752–10756.
[35] P.Z. Qin, C.G. Niu, M. Ruan, G.M. Zeng, X.Y. Wang, Analyst 135 (2010) 2144–2149.
[36] K. Stadtherr, H. Wolf, P. Lindner, Anal. Chem. 77 (2005) 3437–3443.
[37] S. Jhaveri, M. Rajendran, A.D. Ellington, Nat. Biotechnol. 18 (2000) 1293–1297.
[38] R. Nutiu, Y.F. Li, Angew Chem. Int. Ed. 44 (2005) 1061–1065.