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Highly sensitive detection of protein and small molecules based on aptamer-modified electrochemiluminescence nanoprobe

Xiaoming Zhou, Ruixue Duan and Da Xing*

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Amplified optical detection of biomolecules using nanoparticle as the carrier has attracted considerable interest in the scientific community. In this study, a promising aptasensor was developed for highly sensitive detection of protein and small molecules based on the construction of aptamer-modified electrochemiluminescence (ECL) nanoprobe. Specifically, thrombin and ATP serve as the examples for detection. By taking advantage of sandwich binding of two affinity aptamers for high specificity, tris-(2,2′-bipyridyl)ruthenium (TBR)-cysteamine loaded in gold nanoparticle (GNP) as barcodes for signal amplification, and micromagnetic particles (MMPs) based ECL technology for rapid detection, a novel assay for biomolecules quantification was developed. The sandwich complex containing targets could be selectively captured by MMPs and then quantified by ECL intensity. We have demonstrated that the detection limits of human thrombin and ATP are 1 pM and 10 pM, respectively, with high specificity. The proposed technology is expected to become a powerful tool for biomolecule analysis.

Introduction

Low-abundance biomolecules detection plays pivotal roles in forensic science, basic discovery research and early clinical diagnosis. Substantial efforts have been directed to amplified detection of biomolecules. Focusing on the improvement of sensitivity and specificity, immuno-polymerase chain reaction (immuno-PCR),[1] immuno-rolling circle amplification (immuno-RCA),[2,3] and bead based amplification assay[3–5] have been developed. However, while each amplification strategy has distinct advantages, each also exhibits its own unique set of limitations. For example, in comparison with conventional enzyme amplified immunoassays, the immuno-PCR has offered 100–10 000 fold increase in sensitivity with the robust nucleic acid amplification.[6] Nevertheless, the requirements for thermostable enzyme and temperature cycling instrumentation have ultimately restricted the widespread adoption of immuno-PCR as an alternative to ELISA. Another representative nucleic acid amplification, immuno-RCA, has the possibility of direct quantification *via* isotherm amplification without reference to multiple standard samples. Whereas, both immuno-PCR and immuno-RCA require the procedure of preparation of antibody–DNA conjugates, which is frequently time-consuming and labor-intensive. Moreover, it is worth noting that both approaches depend on complex enzymatic reactions, which makes them more costly and potentially more difficult or less reproducible. To tackle these issues, Mirkin and coworkers have recently developed a nanoparticle-based biobarcode technique that provides PCR-like sensitivity for protein detection without a need for enzymatic amplification.[7] However, the major challenge is that it demands labor-intensive procedures, such as salt-aging step in preparation of gold nanoparticle (GNPs) probes, immobilization and repeated hybridization of barcode DNA. Another restriction associated with Mirkin's procedure may come from the utilization of polyclonal antibodies. This type of antibody can also bind to other proteins, which would result in reduction of the signal-to-noise ratio and would compromise the parallel detection of analytes. In addition, the utilization of antibodies or enzymes may encounter other draw backs caused by the difficulties in their production, storage, and modification, and therefore searching for other alternative candidates is necessary.

In this context, aptamers isolated from the systematic evolution of ligands by exponential enrichment (SELEX) are emerging.[8,9] Compared with enzymes or antibodies, aptamers have recently attracted a lot of interest due to their capability to bind not only nucleic acid targets but also proteins, metal ions and other molecules with high affinity and sensitivity.[10] Moreover, since aptamers are oligonucleotides, they can be easily modified with signal moieties and can meet the stringent requirements in bioassay. Further, aptamers are also resistant against denaturizing and are of a relatively lower cost. Up to now, a variety of assays have successfully coupled to aptamer-based analysis for biomolecule detection because they are extremely specific and suitable for use directly in complex materials.[11–14] However, such an aptasensor can only be used to detect the target that is capable of simultaneously binding two

MOE key Laboratory of Laser Life Science & Institute of Laser Life Science, College of Biophotonics, South China Normal University, Guangzhou 510631, China. E-mail: xingda@scnu.edu.cn; Fax: (+86-20) 8521-6052; Tel: (+86-20) 8521-0089

different aptamers. Efforts have been made to develop aptamer-based biosensors for sandwich assays by splitting one aptamer into two parts in recent years.[15,16] Although these biosensors could detect the target that only has one aptamer, the detection limits still need to be improved.

In addition, our group has recently demonstrated that the ECL method that introduces cysteamine as ECL barcodes has its intrinsic merits.[17–19] It is characterized not only by a low cost and a convenient procedure but also by a high sensitivity (femtomolar levels for DNA detection) without any enzyme amplification. On the basis of above researches, we here loaded the cysteamine and thrombin aptamer/split ATP aptamer on a GNP and developed a novel ECL biobarcode assay for the determination of trace amounts of thrombin and ATP. We have demonstrated that this method allows one to identify thrombin down to 1 pM concentration limit on a timescale of 1 h, and to detect ATP at concentrations as low as 10 pM in less than half an hour. Hence the technique may be generalized and applied to analysis of any other molecules that can bind to aptamer, expanding the scope of application of biobarcode ECL assay.

Experimental

Materials

Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), TPA and the chemicals to synthesize the Ru $(bpy)_3^{2+}$ *N*-hydroxysuccinimide ester (TBR-NHS ester) were purchased from Sigma (Louis, MO, USA). Streptavidin microbeads (2.8 μm diameter) were products of Dynal Biotech (Lake Success, NY, USA). Pure human α-thrombin was supplied by Haematologic Technologies Inc. BSA, trypsin, ATP, uridine triphosphate (UTP), guanosine triphosphate (GTP) and cytidine triphosphate (CTP) were obtained from Sigma-Aldrich.

The Apt29 (5′-biotin-TTT TTT TTT T AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′), Apt15 (5′-HS-$(CH_2)_6$-TTT TTT TTT T GGT TGG TGT GGT TGG-3′), Apa 13 (5′-HS-$(CH_2)_6$-TTT TTT TTT T ACC TGG GGG AGT AT-3′), and Apa 14 (5′-(biotin)-TTT TTT TTT T T GCG GAG GAA GGT-3′) were synthesized and HPLC purified by SSBE.

Preparation of 13 nm GNPs

Citrate-capped GNPs with diameter of about 13 nm were prepared by the reduction of $HAuCl_4$ according to the reported procedure.[20] Briefly, 10 mL of 38.8 mM sodium citrate was added quickly to a boiling solution containing 1.0 mM $HAuCl_4$ with vigorous stirring. The color of the solution changed from purple to blue and to red in the end, indicating the formulation of the GNPs. Boiling was continued for an additional 10 min. Then the nanoparticle solution was cooled down to room temperature with continuous stirring. The particles were examined by UV-vis absorption spectra and characterized by absorption maximum at 519 nm, which corresponds to the particle size of *ca.*13 nm.

Synthesis of TBR-NHS ester

The procedures to prepare Ruthenium bis(2,2′-bipyridine) (2,2′-bipyridine-4,4′-dicarboxylic acid) *N*-hydroxysuccinimide ester (TBR-NHS ester), according to previous published paper,[21] were divided into two parts. The first part was to synthesize Ru(bpy)$_2$(dcbpy)$(PF_6)_2$. 0.2 g Ru(bpy)$_2$C1$_2$, 0.2 g $NaHCO_3$, and 0.15 g 2, 2′-bipyridine-4, 4′-dicarboxylic were mixed in 50 mL two neck round-bottom flask, then 30 mL 80% MeOH was added into the mixture. The solution was heated at 80 °C for 10 h. Note that the water was flowing through the reflux condenser during this time. The resulting solution was cooled in an ice bath for 2 h, and the pH was adjusted with 1 M H_2SO_4 to 4.4. The formed precipitate was filtered, washed three times with 6 mL MeOH. Then the filtrate was treated with 2.5 g $NaPF_6$ in 12.5 mL H_2O, and the resulting mixtures were cooled in an ice bath and the precipitate was collected by filtration. The other part was to synthesize TBR-NHS ester. 0.23 g of dicyclohexylcarbodiimide (DCC) and 0.119 g of *N*-hydroxysuccinimide (NHS) were dissolved in 1.5 mL DMF with stirring and cooled in ice bath. 0.19 g Ru(bpy)$_2$(dcbpy)$(PF_6)_2$ was added, and the mixture was stirred for some hours, the formed precipitate was removed by filtration. The formed TBR-NHS ester in the filtrate was kept in a freezer (4 °C) for further use.

Synthesis of ECL nanoprobe

The ECL nanoprobe was made up of GNP that modified with TBR-cysteamine and aptamer. Procedures of synthesizing ECL nanoprobe were as follows. 1.4 μL of 500 mM Tris-acetate buffer (pH 8.2), 6 μL of 10 mM TCEP, and 7.2 μL of 100 μM aptamer, 7.2 μL of 100 μM cysteamine were mixed in a tube and incubated for 30 min at room temperature to activate the thiolated DNA. Then 1 mL GNP were added to the mixture and allowed to be incubated for 6 h at room temperature. Subsequently, 120 μL 0.1 M sodium boracic acid buffer (pH 9.0) and 8 μL of 11.2 mM TBR were brought to the solution. The tube was wrapped in foil and placed on an orbital shaker for 10 h. Then the solution was centrifuged at 4 °C for 30 min at 12 000 rpm and resuspended in 700 μL of GNP washing buffer (100 mM NaCl, 25 mM Tris-acetate, pH 8.2), repeated 4 times. Note that a well-synthesized ECL nanoprobe should be the same color as the unmodified GNPs with no visible aggregates.

ECL detection procedure

For thrombin detection, 5 μL of as-prepared ECL nanoprobe, 5 μL of 1 μM Apt29, and 10 μL of target thrombin at certain concentrations were mixed in a total reaction volume of 100 μL of 10 mM phosphate buffer saline buffer (pH 7.4), containing 1 mM Mg^{2+}. The mixture was placed on an orbital shaker and incubated at 28 °C for 30 min. The products of hybridization were incubated with 14 μL (10 μg μL^{-1}) streptavidin coated magnetic beads with gentle stirring for 20 min.

For ATP detection, 14 μL (10 μg μL^{-1}) streptavidin coated magnetic beads and 5 μL of 1 μM Apa 14 were incubated at 30 °C for 20 min in a total reaction volume of 100 μL of 10 mM phosphate buffer saline buffer (pH 7.4), containing 1 mM Mg^{2+}. Then 5 μL of as-prepared ECL nanoprobe for ATP and 10 μL of target ATP at certain concentrations were added into the above mixture and incubated for another 10 min.

A custom-built ECL detection system was described in detail in our previous research.[22] The complexes were isolated by washing twice with hybridizing buffer in a magnetic field, and

then mixed with 100 μL TPA. The solution was added into electrochemical reaction cell, where the magnetic beads were captured again and temporarily immobilized on the working electrode by a magnet. The photon signal was then measured, when a voltage of 1.25 V was applied.

Results and discussion

Design strategy of the aptasensor

To demonstrate the feasibility of this principle, human thrombin and ATP were our initial target. The presented ECL biobarcode assay typically involves two types of particles (Fig. 1). One is streptavidin-coated micromagnetic particle (MMP) that could function with biotin labeled aptamer. The other, ECL nanoprobe, is comprised of GNP that are modified with thiol labeled aptamer and TBR-cysteamine (barcodes) that act as reporter groups for the targets. When target (ATP or thrombin) presents, the two aptamers will bind to it, resulting in the formation of a sandwich complex (ECL nanoprobe-thrombin/ATP-MMP). A magnetic field is used to separate such complexes from the test solution. Washing the sandwiches with binding buffer twice followed by ECL detection allows one to identify the barcodes and quantify the amount of targets.

Effect of the cysteamine to aptamer ratio on ECL detection

In this system, the ECL biobarcode nanoprobe is constructed by GNP which is modified with one aptamer to recognize the targets and TBR-cysteamine to boost ECL signals. Fig. 2 represents the effect of cysteamine to Apt 15 ratio on ECL detection of thrombin. In theory, the number of TBR-cysteamine bound to a GNP should reach saturation to provide significant ECL signals, whereas the number of Apt15 on a nanoparticle should be kept minimal. Therefore, the ratio of cysteamine to Apt15 should be optimized in order to provide maximum signal amplification, thus enhancing sensitivity. However, as we can see from Fig. 2, when the ratio of cysteamine to Apt15 is 100 : 1, the absorption of ECL nanoprobes undergoes a red shift, implying an increase of self-assembly of GNPs due to the interaction between amino groups and GNP surface[23] (Fig. 2B), which is further confirmed with photos (Fig. 2A). It also shows that only weak ECL intensity is observed for the resulting ECL nanoprobe (Fig. 2C). When the ratio decreases to 1 : 1 (namely 0.72 μM cysteamine and 0.72 μM Apt15, respectively), the maximum ECL intensity signal is achieved, and the labeled ECL nanoprobe is uniform and stable (Fig. 2A and 2B). Further decrease of the ratio to 0.1 : 1 resulted in a slight ECL signal decline (Fig. 2C). This phenomenon is in good agreement with our previous study.[17] The reason for this may be that self-assembly of GNP affected the binding of cysteamine and TBR. According to Fig. 2C, we set the optimal ratio of cysteamine to Apt15 is 1 : 1. For ATP detection, we have obtained similar results and also set the optimal ratio of cysteamine to Apa13 as 1 : 1.

Optimization of detection conditions

It has been reported that suitable incubation time can further improve the efficiency of aptamer binding to thrombin because the incubation process occurs in a certain period of time.[24] The dependence of the ECL intensities on the incubation time is investigated in a range of 20–60 min, and the results are shown in Fig. 3A. The experimental results show that the formation of the sandwich format reached equilibrium in about 30 min. With the time extended, one aptamer is inclined to deplete thrombin's affinity to the other, thus dissociating the sandwich structure and decreasing ECL signals.[24,25]

Another factor taken into account for the assay optimization is the use of some common incubation solutions. During the fabrication of the aptasensor, three kinds of buffers were tested: (1) 20 mM Tris-HCl (containing 140 mM NaCl and 5 mM KCl, pH 7.4), (2) 10 mM PBS buffer (pH, 7.4), (3) PBS buffer containing 1 mM Mg^{2+} (pH, 7.4). The results show that the best performance was obtained with 10 mM PBS buffer containing 1 mM Mg^{2+}, which was likely due to the divalent ions like Mg^{2+} having a stronger impact on the binding strength of aptamers to proteins, than did monovalent ions like Na^{+} (Fig. 3B).[26] Thereby,

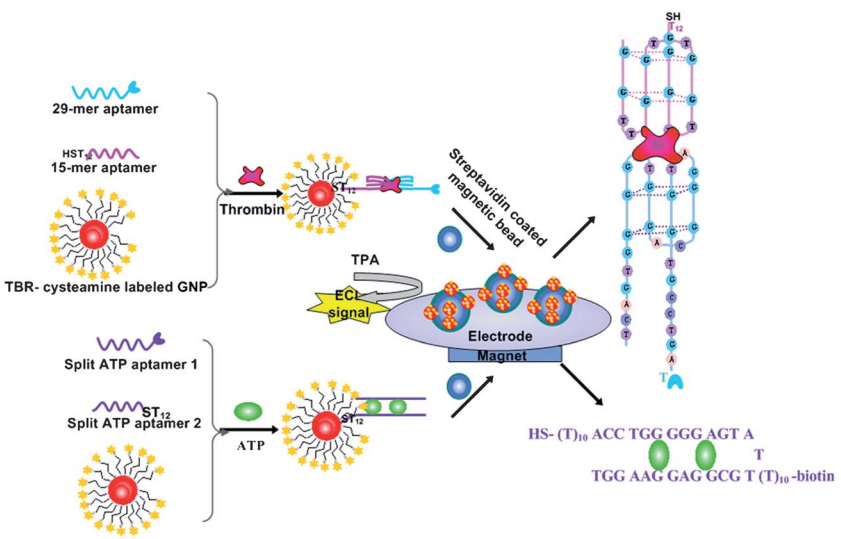

Fig. 1 Schematic description of the ECL aptasensor for human thrombin and ATP based upon the amplification of biobarcode method.

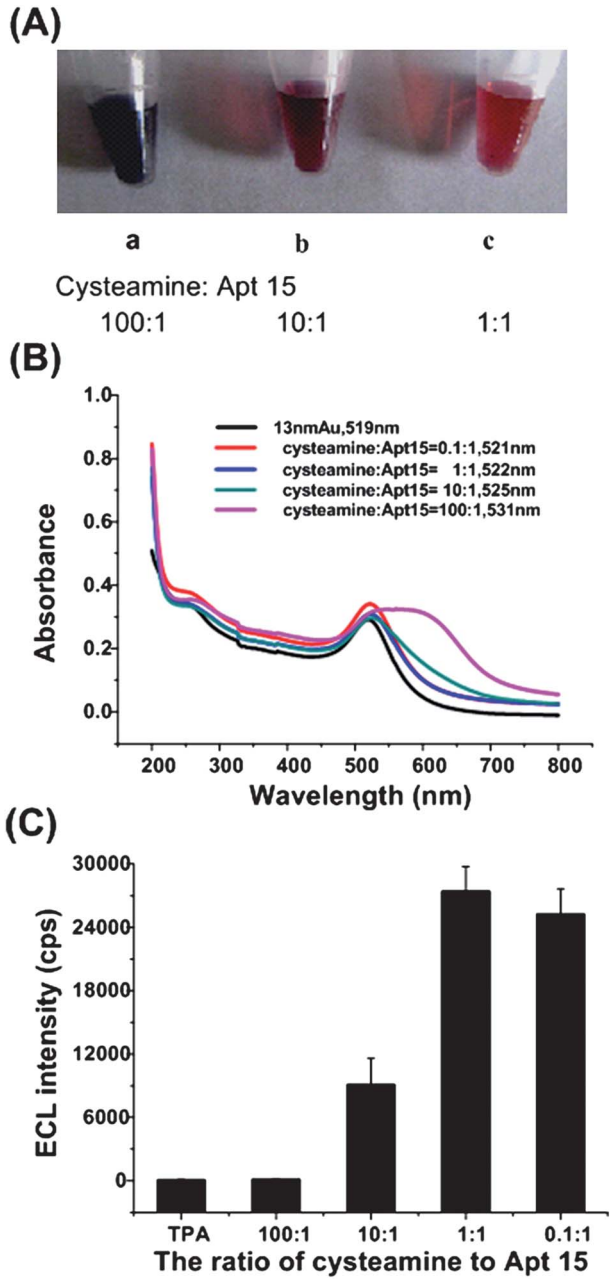

Fig. 2 The photographs (A), the absorbance spectrums (B) and ECL intensities (C) of the ECL nanoprobes from different ratios of cysteamine to Apt15. The final concentration of cysteamine is about 0.072 μM, 0.72 μM, 7.2 μM, and 72 μM, respectively, and the Apt15 is about 0.72 μM.

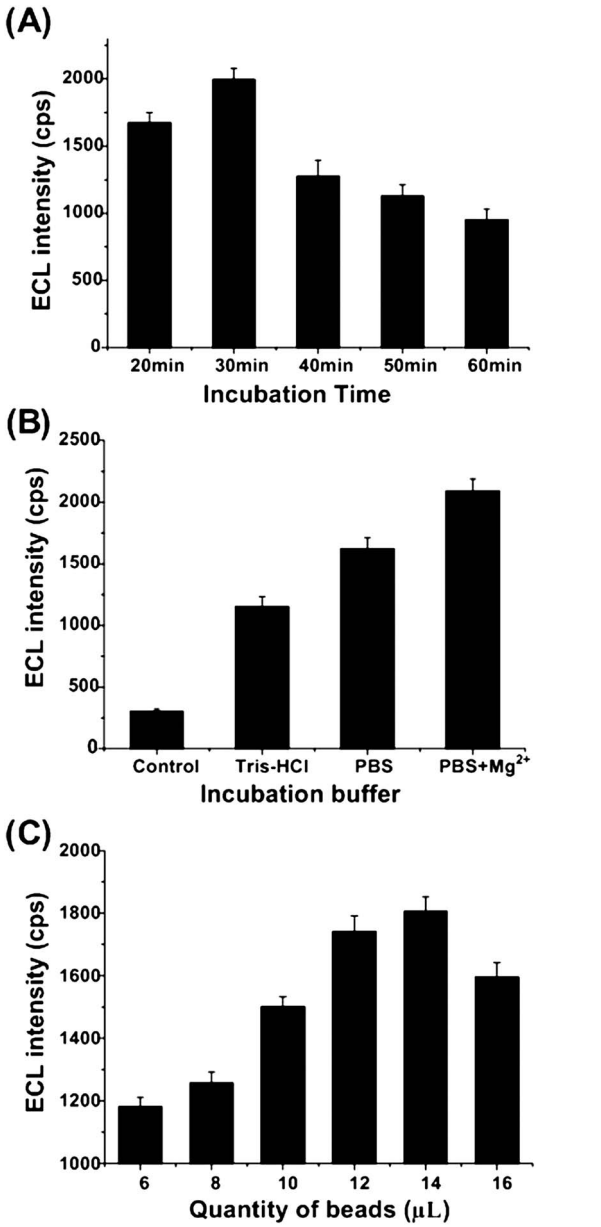

Fig. 3 Effects of incubation time, incubation buffer and the quantity of streptavidin-coated MMPs on ECL intensity. Concentrations of Apt29 and thrombin are both 10 nM.

10 mM PBS buffer containing 1 mM Mg^{2+} was chosen as incubation buffer in the experiment.

During the experimental process, we found that the quantity of MMPs played an important role to obtain ECL signals. The Apt29-thrombin-ECL nanoprobe complexes are captured by MMPs through biotin-streptavidin linkage. The appropriate MMPs can capture entire sandwich complexes, reflecting the real quantity of protein target. However, excessive MMPs would overpopulate the electrode, hence reducing the reaction of TPA and TBR on the surface of the electrode, preventing the photomultiplier tube receiving photoelectrons, and resulting in the decrease of sensitivity. In order to investigate the appropriate amount of MMPs, we conduct a series of experiments, as shown in Fig. 3C. It is observed that the optimal ECL signals are obtained when the quantity of MMPs is about 14 μL (10 μg μL^{-1}).

Based on the above optimizations, it is concluded that the optimal ECL signals for thrombin detection are obtained in 10 mM PBS buffer containing 1 mM Mg^{2+} when the quantity of MMPs is about 14 μL (10 μg μL^{-1}), and the appropriate incubation time is 30 min. in order to simplify the procedure, we applied the thrombin system to ATP detection. However, we found a background signal was produced, which may be caused by the non-specific hybridization occurring between two split parts in the absence of ATP. We then adjusted the experimental proposal, namely, the MMPs and Apa14 were incubated first,

and then the ATP and ECL nanoprobe were added to the above system to react, reducing the non-specific hybridization in the absence of target ATP.

Sensitivity study

To investigate the ability of the described strategy to sensitively quantify biomolecules, a series of different concentrations of thrombin/ATP were measured under the above optimized conditions. Fig. 4A and 4B show that while the concentration of thrombin increases gradually, the intensity of the ECL signals rises accordingly. Under the conditions studied, when further increasing the concentration of thrombin or ATP, ECL signals start to saturate. The plateau phenomenon may be due to the exhaust of ECL nanoprobes. For thrombin detection, another reason may be that the relative excess thrombin could impair the aptamer-thrombin binding,[27] leading to the slower increase of ECL signals. The threshold line was calculated by the evaluation of the average response of the control plus three times the standard deviation. According to this principle, we accessed a detection limit of 1 pM thrombin concentration and 10 pM ATP concentration, which can be clearly identified from the threshold line. Fig. 4C shows that the ECL intensity exhibits an excellent linear relationship to ATP concentration from 10 pM to 100 nM with a correlation coefficient 0.995. Meanwhile, it can be seen from Fig. 4D that, for the thrombin detection, there is a linear relationship between ECL intensities and the concentration of thrombin from 1 pM to 10 nM and the correlation coefficient is 0.996.

The detection limit of the proposed aptasensor for ATP is more sensitive than the ECL aptasensor reported by Yao *et al.*[28] It is also 1000 times lower than that of some CL approaches,[29,30] and much lower than that of colorimetric[16] or fluorescent assay.[31] It is clear that the detection limit of the ECL biobarcode technology is about 3 orders of magnitude lower than those of Dry-Reagent strip assay,[32] CE,[33] and the ECL assay based on quantum dots.[34] Moreover, the proposed approach offers more than 100-fold improvement of the detection limit as compared to electrochemistry,[12] fluorescence assay,[35] and SERS.[36] Although the presented method has a detection limit not as good as immuno-PCR,[1] immuno-RCA,[2] and the mentioned biobarcode assays,[4,37] it possesses its own superiorities as follows. Table 1 summarizes the characteristics of respective methods for protein detection. The introduction of cysteamine and aptamer, instead of costly dual labeling oligonucleotides, enzymes, and antibodies, makes procedures simpler and more efficient, and advances the precision. Furthermore, employing MMPs is another advantage of this assay. On the one hand, it facilitates the capture of targets in homogeneous solution and provides fast and efficient magnetic separation of the affinity complexes. On the other hand, using magnetic beads as solid support makes the electrode reusable, thus further reducing assay cost and detection time. More importantly, the split strategy makes the proposed approach generalizable since any aptamer-target binding events can in principle be translated to a sandwich process.

Specificity, applicability and reproducibility

In a further set of experiments, the binding specificity of the assay was evaluated. For the thrombin detection, we performed a series of contrast experiments using 1 nM BSA, 1 nM Trypsin and human serum (diluted ten times) as negative controls and a pure buffer sample without thrombin as a blank control experiment. As shown in Fig. 5A, 1 nM α-thrombin either in buffer or in

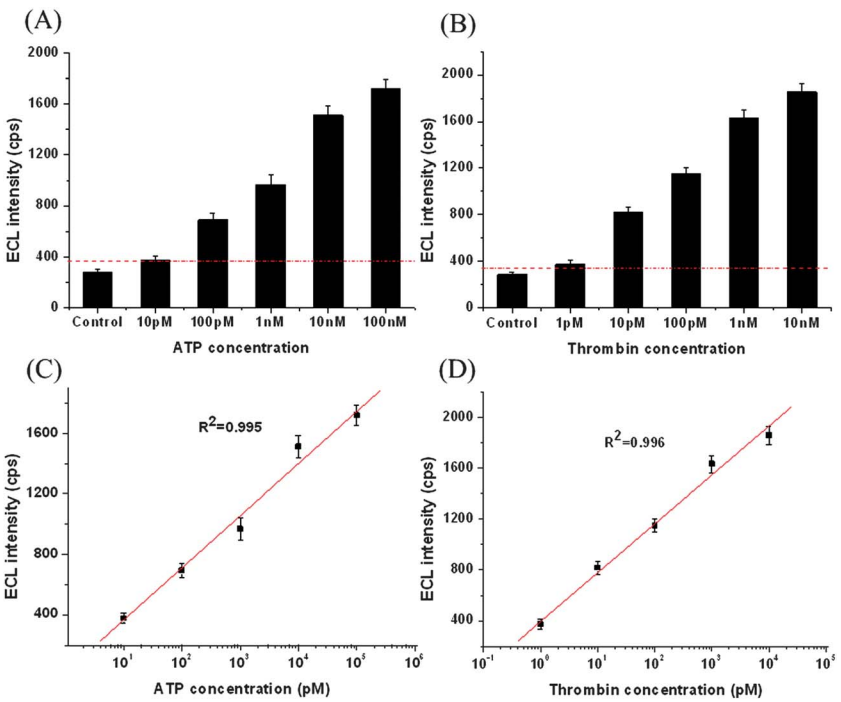

Fig. 4 ECL intensities obtained from different concentrations of ATP (A) and thrombin (B) in standard solutions based on the amplification of biobarcode. (C) and (D) represent the linear regression analysis of a plot of the ECL intensity *versus* the log of the target concentrations.

Table 1 Comparison of current method and other reported techniques

Methods	Reader	Target	Sensitivity	Electrode (resuable)
Immuo-PCR[1]	Electrophoresis	BSA	9.6×10^{-22} moles	—
Immuo-RCA[2]	Fluorescence	PSA	3×10^{-19} moles	—
Biobarcode[4]	Scanometry	PSA	30×10^{-18} moles	—
MMP based sandwich[12]	Electrochemistry	Thrombin	0.45 nM	YES
QDs biosensor[27]	Electrochemistry	Thrombin	1 pM	NO
Dry-reagent strip[32]	Strip reader	Thrombin	2.5 nM	—
Capillary Electrophoresis[33]	Fluorescence	Thrombin	9.8 nM	—
QDs ECL biosensor[34]	ECL	Thrombin	2.72 nM	No
Molecular beacon[35]	Fluorescence	Thrombin	50 pM	—
SERS[36]	Raman scattering	Thrombin	100 pM	—
Biobarcode[37]	Electrochemistry	Thrombin	6.2 nM	No
Colorimetry[38]	Absorption	Thrombin	0.83 nM	—
Pt-NP catalysis[39]	Electrochemistry	Thrombin	1 nM	No
Curren ECL assay	ECL	Thrombin/ATP	1 pM/10 pM	Yes

human serum reveals much stronger response than the blank control, while the other three samples only show similar signals to the blank control. Furthermore, the ECL intensity of thrombin in human serum is comparable to the one in buffer. In addition, the specificity of ATP biosensor was also investigated. 1 nM GTP, 1 nM CTP, and 1 nM UTP were chosen to testify the selectivity of this approach under the same experimental conditions. It can be seen from Fig. 5B that the ECL intensity in the presence of ATP is much larger than that in the presence of GTP, CTP, and UTP, respectively. It further validates that this protocol exhibits a little nonspecific adsorption and is suitable for the detection of thrombin and ATP in a real sample.

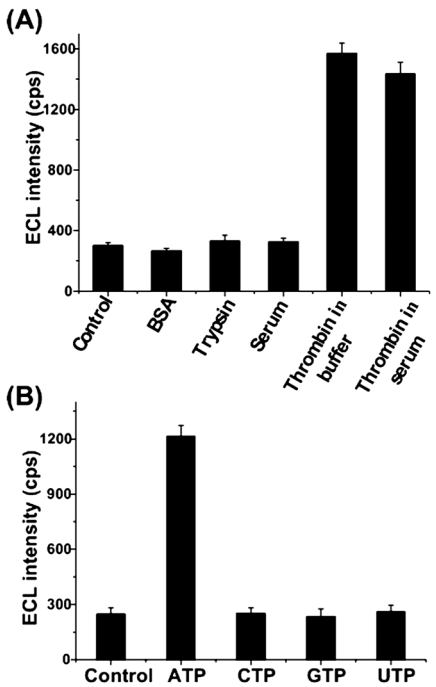

Fig. 5 Investigation of the specificity and applicability of the ECL aptasensor to human α-thrombin and ATP. (A) ECL intensity histograms from left to right stand for: a pure buffer sample without any protein, 1 nM BSA in pure buffer, 1 nM Trypsin in pure buffer, a human serum sample (diluted 10 times), 1 nM α-thrombin in the pure buffer and 1 nM α-thrombin in the human serum. The concentration of Apt29 is 10 nM. (B) ECL intensity histograms from left to right stand for: a pure buffer sample without any ATP, 1 nM ATP, 1 nM CTP, 1 nM GTP and 1 nM UTP. The concentration of Apa14 is 10 nM.

The reproducibility of the aptasensor was assessed by analyzing 4 independent 1 nM thrombin samples and 10 nM ATP samples, respectively, on the same electrode, and the corresponding R.S.D. were 3.2% and 3.8%, respectively. It is indicated that the DNA biosensor had good reproducibility. Such results obtained should be firstly attributed to the well-defined and highly reproducible magnetic collection of the sandwich complexes onto the electrode surface. Next, the proposed approach is independent of enzyme and antibody which are active biomolecules and susceptible to experimental conditions. Further, the direct detection of ECL signals without the need of any immobilization and repeated hybridization of barcodes may also improve the reproducibility as well as the sensitivity of the method.

Conclusions

In summary, we have presented a high-performance ECL biosensor conducted with the aptamer specific for biomolecules coupled to the biobarcode amplification method. The utilization of cysteamine and elimination of enzymes or antibodies for the amplified detection of biomolecule targets are certainly advantages of the proposed ECL biobarcode approach, which make the assay more efficient, cost-effective, and stable. Moreover, the detection procedure is time-saving and the electrode is reusable, which is a benefit of employing MMPs. Perhaps equally important, our approach could adapt to detection for the targets that only have single aptamers by creating a sandwich complex from two halves of a single aptamer. The demonstrated aptasensor with generalizable and extreme specificity would open the possibility of a real application to diagnostics or medical investigations.

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