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Electrochemical Aptasensor Based on Klenow Fragment Polymerase Reaction for Ultrasensitive Detection of PDGF-BB

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A novel electrochemical aptasensor based on Klenow Fragment induced extension reaction has been developed successfully for the ultrasensitive detection of platelet-derived growth factor (PDGF) protein. In the presence of target protein, the recognition probe changed its hairpin conformation and gave a 3-single-stranded sequence complementary to the biotinylated primer. In Klenow Fragment polymerase induced reaction, the recognition probe served as a template for the extension of a capture probe, then the target protein was released from replicated DNA duplex and subsequently could bind with another hairpin recognition probe to induce a new primer extension, which could produce a lot of biotin-tagged DNA duplex. Following the electrochemical signal was amplified by highly catalytic activity of streptavidin-alkaline phosphatase (ST-AP) toward enzyme substrate α-naphthyl phosphate (α-NP), producing an ultrasensitive enzyme-catalyzed electrochemical aptasensor. Under optimal conditions, the proposed aptasensor could detect PDGF protein down to 0.046 ng/mL (1.8 pM) with a linear calibration range from 0.1 ng/mL to 120 ng/mL, and was amenable to the quantification of target protein in complex serum matrixes. The simple strategy does not require special laboratory conditions, which makes it adaptable to a low-cost and robust biosensing platform for clinical diagnostic application.

Keywords: Aptasensor, Electrochemistry, Klenow Fragment, PDGF-BB

1. INTRODUCTION

Platelet-derived growth factor (PDGF), a growth factor protein found in human platelets, has growth-promoting activity toward fibroblasts, smooth muscle cells, and glial cells [1]. As a widely

used biomarker for hepatic fibrosis [2], liver cancer [3], and gastrointestinal stromal tumors [4], PDGF has been implicated in the pathogenesis of angiogenesis in these tumor types. Up to now various commonly available methods were developed for PDGF detection, such as enzyme-linked immunosorbent assay (ELISA) [5], fluorescence immunoassay [6] and chemoluminescence immunoassay [7].However, these methods are expensive, time consuming and labor-intensive [8]. Therefore, it remains a challenge to develop a new simple method with high sensitivity and specificity for the direct detection of PDGF in clinical serum specimens.

Aptamers are single-stranded DNA or RNA oligonucleotides selected from random sequence nucleic acid libraries by an in vitro evolution process of SELEX (systemic evolution of ligands by exponential enrichment) [9]. Due to their specific binding abilities and many advantages over antibodies such as small sizes, chemical simplicities, simpler synthesis, easier storage and simple modification for further immobilization procedure, aptamers are becoming ideal recognition elements for biosensor applications [10].

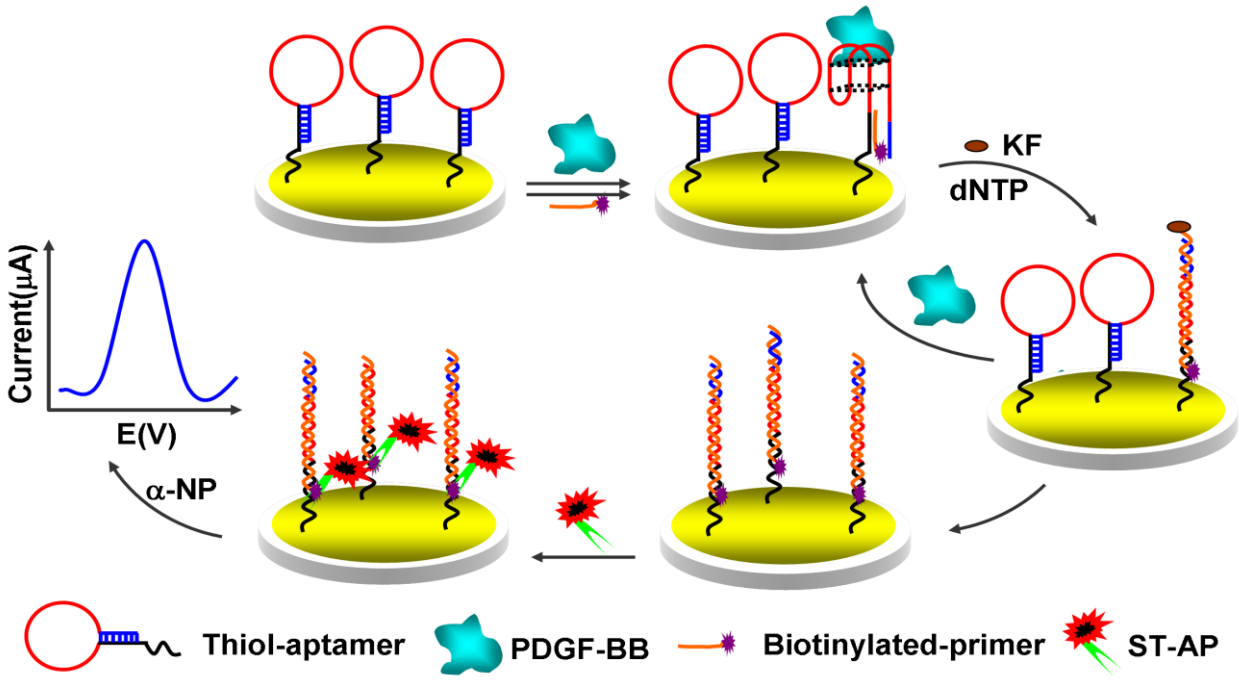

Figure 1. Schematic illustration of the electrochemical aptasensor for PDGF-BB detection based on Klenow Fragment induced extension reaction

Recently, electrochemical aptasensors based on the specificity of aptamer-target recognition have received particular attention due to their high sensitivity and selectivity, simplicity and economy [11]. In order to achieve highly sensitive electrochemical aptasensors, various nanomaterials have been applied for signal amplification, including gold nanoparticles [12], carbon nanostructures [13, 14] and quantum dots [15]. However, the application of the nanomaterials has many disadvantages, such as complicated procedures, high expenses, poor reproducibility and quantification, especially for the

complex samples [16, 17]. Thus, simple aptasensors for ultrasensitive and convenient detection of proteins are still an urgent demand.

Herein, we developed a new electrochemical aptasensor based on the Klenow Fragment polymerase induced extension reaction for PDGF-BB determination. The new biosensing process was illustrated in Figure.1. This aptasensor used a hairpin-aptamer as the capturing probe. In the presence of PDGF-BB, the hairpin-aptamer changed its hairpin conformation and gave a 3-single-stranded sequence complementary to the biotinylated primer. When the KF polymerase and dNTPs were added, the capturing probe served as a template for the primer induced extension. Subsequently, the target protein was released from replicated DNA duplex and could bind with another hairpin-aptamer probe to induce a new primer extension, which could produce a lot of biotin-tagged DNA duplex. Following the electrochemical signal was amplified by highly catalytic activity of streptavidin-alkaline phosphatase (ST-AP) toward enzyme substrate α-naphthyl phosphate (α-NP), producing an ultrasensitive enzyme-catalyzed electrochemical aptasensor. The fabricated platform could provide a pragmatic tool for convenient detection of biomarkers for the early diagnosis in the clinical examination.

2. EXPERIMENTAL

2.1. Reagents

PDGF-BB was purchased from Santa Cruz Biotechnology (USA). 6-mercapto-1-hexanol (MCH), bovine serum albumin (BSA), streptavidin-alkaline phosphatase (ST-AP) and α-naphthyl phosphate (α-NP) were obtained from Sigma-Aldrich (USA). dNTPs and Klenow Fragment polymerase were purchased from Takara (Dalian, China). All other reagents were of analytical grade. All solutions were prepared using Millipore-Q water (≥18 MΩ).

2.2. Oligonucleotides

Table 1. Oligonucleotides used in the present work.

Oligonucleotide	Sequence (5'-3')
hairpin-aptamer capturing probe:	5’-CAGGCTACGGCACGTAGAGCATCACCATGATCC TGCTCCTTACATGGTGATTT -thiol-$(CH_2)_6$-3’
biotinylated primer 1:	5’-biotin-CACCAT-3’
biotinylated primer 2:	5’-biotin-CACCATGT -3’
biotinylated primer 3:	5’-biotin-CACCATGTAA-3’

Oligonucleotides with the following sequences shown in Table 1 were synthesized by Sangon (Shanghai, China). The boldfaced regions indicate the aptamer sequences for PDGF-BB [18], while the underlined regions indicate the complementary sequences for hairpin formation and primer hybridization.

2.3. Apparatus

The electrochemical measurements were performed on a CHI 660D electrochemical analyzer (Shanghai Chenhua Instruments Co. Ltd., China) with a three-electrode system where a 3-mm-diameter gold electrode (GE) was used as working electrode, platinum wire as auxiliary electrode, Ag/AgCl electrode as reference electrode.

2.4. Preparation of electrochemical aptasensor

The bare GE was polished to a mirror using 0.3 and 0.05 μm alumina slurry respectively and ultrasonically treated in deionized water. Then, the electrode was soaked in piranha solution (H_2SO_4 : H_2O_2 = 3 : 1) for 10 min to eliminate other substances, following rinsed thoroughly with deionized water. 10 μL of 1.0 μM thiolated hairpin-aptamer capture probe was dropped onto the pretreated electrode surface and incubated at 4 °C overnight. After washed with 0.01 M pH 7.4 PBS buffer, the electrode was immersed into 1 mM MCH solution for 1 h to obtain well-aligned DNA monolayer. Finally the electrode was further treated with 1% BSA in 0.01 M pH 7.4 PBS buffer 30 min to avoid nonspecific adsorption of DNA and enzyme on the electrode surface and then thoroughly washed with 0.01 M pH 7.4 PBS buffer containing 0.05% Tween-20.

2.5. Electrochemical measurements

10 μL of PDGF-BB at variable concentrations was then dropped on the aptasensor surface and incubated at 37 °C for 60min to form the aptamer-PDGF-BB complex, then rinsed thoroughly with pH 7.4 PBS buffer contained 0.05 % Tween 20. A mixture of 5 μL biotinylated primer (2.0 μM), 2 μL 10mM dNTPs and 3U Klenow Fragment polymerase was dropped onto the modified gold electrode and incubated for 60min at room temperture. After polymerase extension, the electrode was washed with pH 7.4 Tris–HCl buffer contained 0.05 % Tween 20, then the aptasensor reacted with 10 μL 1.25 mg/mL ST-AP in pH 9.6 diethanolamine buffer (DEA, 0.1 M diethanolamine, 1 mM $MgCl_2$, 100 mM KCl, pH 9.6) for 30 min. After incubation, the sensor was washed with DEA buffer containing 0.05% Tween-20. Electrochemical measurement was performed in pH 9.6 DEA buffer containing 1 mg/mL of α-naphthyl phosphate. The differential pulse voltammetry (DPV) measurements were performed from -0.1 to 0.6 V (modulation time = 0.05 s; interval time = 0.017 s; step potential = 5 mV; modulation amplitude = 70 mV).

3. RESULTS AND DISCUSSION

3.1. Characterization of DNA sensor

Electrochemical impedance spectroscopy (EIS) was employed to monitor the interface properties of the modified gold electrode surface during stepwise modifications [19, 20]. Figure 2A

showed the Nyquist plots of $Fe(CN)_6^{3-/4-}$ containing 0.4 M KCl at different modified electrodes. The bare gold electrode exhibited an almost straight line (curve a), which was typical characteristic of a mass diffusion limiting electron-transfer process. The immobilization of the thiol-modified capture probe on the bare gold electrode surface resulted in an increase (curve b), demonstrating that the probe was successful modified on electrode surface. When the MCH was self-assembled onto the gold electrode, the Green increased remarkably (curve c). Subsequently, the Blue further enhanced to a larger value after capture aptamer hybridized with the added PDGF-BB (curve d) due to the increasing negative charge, indicating successful combination of aptamer-PDGF-BB. After the biosensor was incubated with biotinylated primer, dNTPs and Klenow Fragment polymerase, the value of Cyan increased dramaticly (curve e), demonstrating a successful extension. This can be attributed to physical coverage by the oligonucleotides and electrostatic interaction between negatively charged phosphate backbone of the single strand nucleic acid and ferricyanide anion [21]. The above results were in a good agreement with those obtained from SWV (Figure 2B), in which the peak current decreased upon the assembly and hybridization processes. Both experiment results proved the successful modifications and reactions as described in the principle scheme.

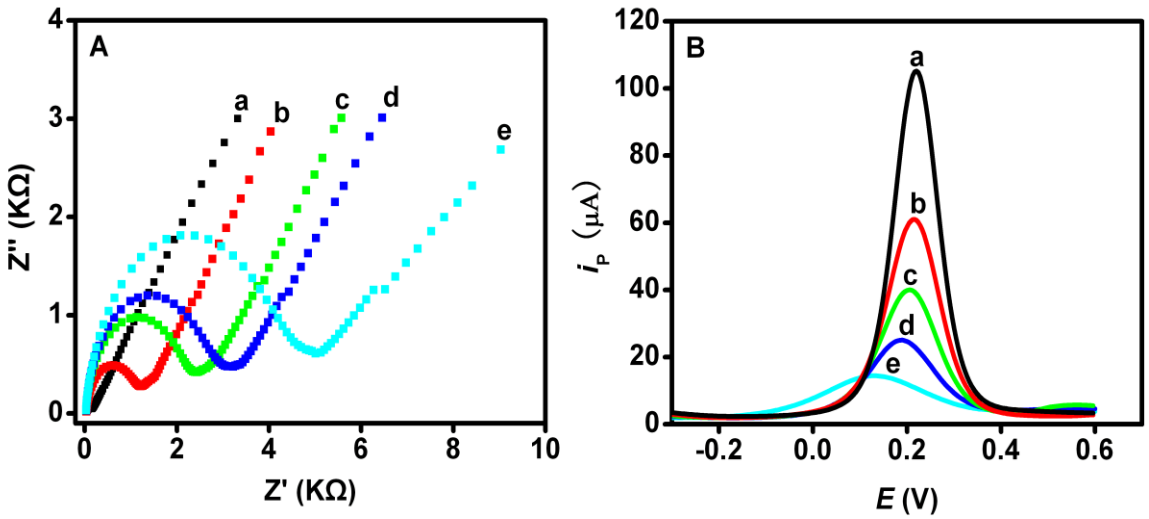

Figure 2. EIS (A) and SWVs (B) at different electrodes in 0.5 mM $Fe(CN)_6^{3-/4-}$ containing 0.4 M KCl. (a) Bare electrode, (b) electrode modified by thiolated hairpin-aptamer capture probe, (c) electrode exposure to MCH solution, (d) after reaction with PDGF-BB and (e) further incubated with biotinylated primer, dNTPs and Klenow Fragment polymerase.

3.2. Optimization of experimental conditions

The incubation time for hairpin-aptamer and PDGF-BB target protein binding was optimized. With the increase of incubation time, the DPV peak current increased sharply and tended to a steady value after 60 min and longer incubation time did not improve the response obviously (Figure 3A), so 60 min was chosen as the optimal incubation time.

To investigate the effect of primer strand length on the performance, the hairpin-probe was mixed with a specific primer with a 6-, 8-, or 10-mersegment. In Figure 3B, the DPV peak current change of the three primers with 10 ng/mL PDGF-BB target protein was monitored at room temperature. As shown in Figure 3B, primer 1 and primer 2 with only 6 or 8 nucleotides seemed to have the lower ability to hybridize with the hairpin-probes. Primer 3 provided the highest DPV peak current enhancement in the presence of target protein, which indicated successfully hybridized with the hairpin-probes. As reported by He et al, Longer nucleotides may lead to nonspecific hybridization [22], hence primer 3 was used as the optimum primer for the polymerase-induced replication.

The effect of biotinylated primer concentration on the DPV response was shown in Figure 3C. The aptasensor was incubated with primer solution for 1.0, 1.5, 2.0, 2.5 μM, and then was tested in pH 9.6 DEA buffer containing 1 mg/mL of α-naphthyl phosphate. It was found that the response current rose rapidly with increasing concentrations and then tended to level off after more than 2.0 μM. Therefore, the concentration of 2.0 μM was adopted in the subsequent work.

The performance of the electrochemical analysis was related to the concentration of α-NP in the measuring system. The DPV peak current of the aptasensor in DEA buffer increased with the concentration of α-NP from 0.6 to 1.0 mg/mL, and then maintained the maximum value at higher concentrations. Afterward, the enzymatic reaction rate depended on the amount of the ST-AP binding on the aptasensor. Therefore, the optimal α-NP concentration for DPV detection was 1.0 mg/mL (Figure 3 D).

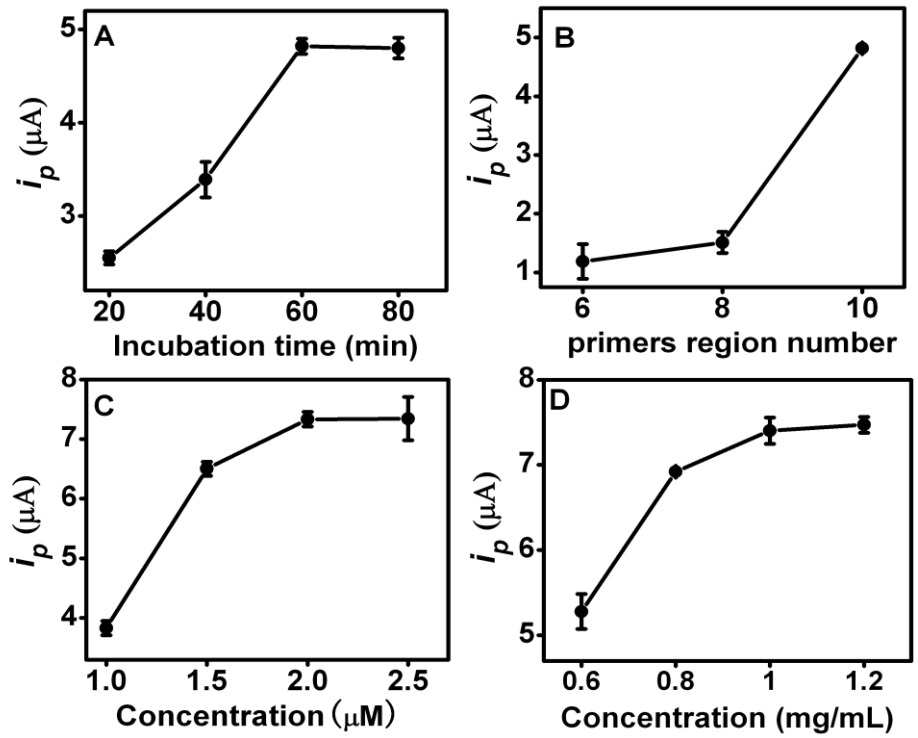

Figure 3. Dependences of DPV peak current on aptamer-protein incubation time (A) and primers varying in region number (B) at 10 ng/mL PDGF-BB, concentration of biotinylated primer (C) and α-NP concentration (D) at 120 ng/mL PDGF-BB, when one parameter changed while the others were under their optimal conditions.

3.3. Specificity of the strategy

To evaluate the selectivity of the aptasensor, 300ng/mL BSA、NSE、and IgG in human serum, which may interfere with the detection of target protein, and 100ng/mL PDGF-BB were examined respectively. The results were shown in Figure 4. Even if the concentration of interferential proteins were three orders of magnitude higher than that of PDGF-BB, there was negligible DPV peak current, only PDGF-BB produced remarkable signal amplification. The error bars represented average standard errors for three measurements. The experimental results demonstrated that the established assay method had the specific response to target protein, as expected.

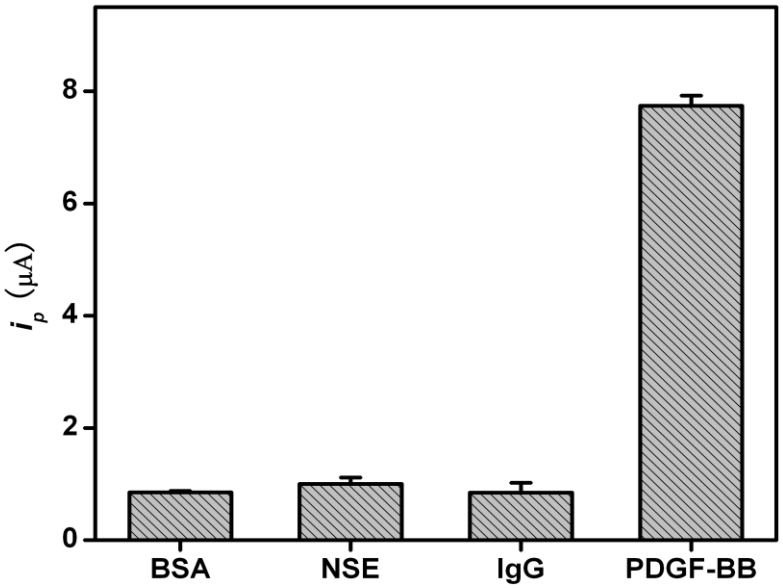

Figure 4. Comparison of the increase of the DPV peaks currents by employing different proteins (PDGF-BB, 100 ng/mL; other proteins, 300 ng/mL). The error bars represent average standard errors for three measurements.

3.4. Analytical performance of aptasensor

To elucidate the analytical performance of the designed aptasensor, experiments were carried out by adding different concentrations of PDGF-BB to the sensor to examine whether the DPV peak currents could be used for quantification (Figure 5A).Under the optimal experimental conditions, the DPV peak currents rose significantly with the increasing concentration of target PDGF-BB protein. The PDGF-BB target protein could be quantitatively detected as low as 0.1ng/mL. In Figure 5B, the graph showed a good linear relationship between the the DPV currents and the logarithm of PDGF-BB concentration from 0.1 ng/mL to 120 ng/mL with a correlation coefficient of 0.9971. The limit of detection (LOD) was calculated to be 0.046ng/mL at a signal-to-noise ratio of 3, which was much lower than clinical decision level of 17.5ng/mL [23]. This highly sensitive detection of PDGF-BB is due to the large signal amplification upon primer-induced polymerization. Compared with the reported

electrochemical methods listed in Table 2 for PDGF-BB detection, it was clear that the proposed aptasensor showed great improvement in the detection limit.

The reproducibility of the proposed sensor was investigated by detecting target protein at 10 ng/mL and 100ng/mL with five replicates, respectively. Relative standard deviations (RSD) for both concentrations were less than 5%, which indicated a satisfactory reproducibility of the designed sensor.

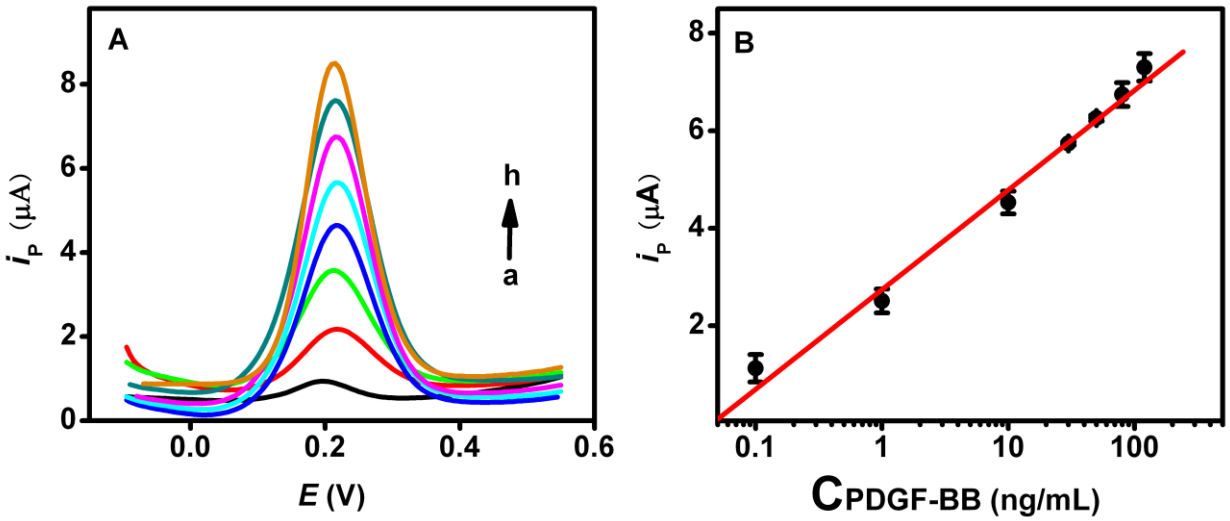

Figure 5. (A) Typical DPV curves of designed aptasensor with target protein concentrations of 0, 0.1, 1, 10, 30, 50, 80, 120 ng/mL (from a to h). (B) Plot of DPV peak current vs target protein concentration.

Table 2. Comparison between the proposed method and other reported electrochemical aptasensor for the detection of PDGF-BB

Biosensor Platform	Bio-receptor of immobilisation	Detection limits	Ref.
Electrochemical	Aptamer	40 nM	[24]
Electrochemical	Aptamer	1 nM	[25]
Fuorescent	Aptamer	0.8 nM	[26]
Electrochemical	Aptamer	50 pM	[27]
Electrochemical	Aptamer	1.8 pM	This work

3.5. Detection of human PDGF-BB in serum

To evaluate the feasibility of fabricated aptasensor for clinical diagnosis, we conducted another experiment to prove whether the aptasensor could work directly with human blood serum. Ten standards with various PDGF-BB concentrations were spiked into the blank serum samples, and the PDGF-BB contents were assayed using the electrochemical aptasensor. The results are listed in Table 3. The recoveries were among 81.4–105.1%, indicating that the electrochemical aptasensor presented

good recovery. The aptasensor, which is distinguished by its convenient miniaturization for low assay cost, high sensitivity, absence of sophisticated and expensive array detectors, showed more suitable for point-of-care testing. Therefore, the developed aptasensor could be potential tool for convenient detection of PDGF-BB in serum specimens in clinical laboratory diagnosis.

Table 3. The recoveries determined using the aptasensor via spiking PDGF-BB standards into blank cattle serum samples.

Sample No.	Spiking value (ng/mL)	Assayed value (ng/mL)	Recovery (%)
1	5	4.20	84.0
2	9	7.95	88.3
3	14	12.30	87.9
4	21	20.41	97.2
5	30	25.12	83.7
6	37	38.90	105.1
7	46	44.67	97.1
8	60	57.54	95.9
9	83	75.18	90.6
10	107	87.15	81.4

4. CONCLUSIONS

The work presented here describes a new electrochemical aptasensor that permits sensitive and convenient detection of PDGF-BB based on signal amplification under the Klenow Fragment induced extension reaction. This ultrasensitive detection of PDGF-BB is due to low background signal and large signal enhancement. In comparison to the established aptamer based PDGF-BB detection system, the amplification of DPV signals from the primer-induced replication improves the detection sensitivity greatly. Moreover, the aptasensor enables accurate target protein quantification over nearly a four log range of initial target levels. This strategy holds the advantages of excellent sensitivity, convenient detection and low cost, which would become a pragmatic tool for the detection of PDGF-BB in clinical laboratory diagnosis and could be easily extended to detect other proteins for clinical screening of cancers.

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