Talanta 116 (2013) 816–821

Contents lists available at ScienceDirect

Talanta

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

An ultrasensitive chemiluminescent immunosensor for the detection of human leptin using hemin/G-quadruplex DNAzymes-assembled signal amplifier

Yuezhen He, Xiaoxun Wang, Yuzhong Zhang, Feng Gao, Yongxin Li, Hongqi Chen, Lun Wang *

Anhui Key Laboratory of Chemo-Biosensing, Key Laboratory of Functional Molecular Solids, Ministry of Education, College of Chemistry and Materials Science, Anhui Normal University, Wuhu 241000, People's Republic of China

ARTICLE INFO

Article history:
Received 10 May 2013
Received in revised form
25 July 2013
Accepted 27 July 2013
Available online 7 August 2013

Keywords:
Chemiluminescent immunoassay
Human leptin
Hemin
G-quadruplex
DNAzymes
Horseradish peroxidase

ABSTRACT

In this work, we reported a sensitive chemiluminescent immunosensor for the detection of human leptin by using hemin/G-quadruplex DNAzymes to amplify detection signal. In this sensing system, the primary antibody (anti-human leptin) was firstly bound to the 96-well plates, and human leptin and biotinylated secondary antibody were successively combined to form sandwich-type immune complex through specific interactions. Then streptavidin labeled with hemin/G-quadruplex DNAzymes was assembled to the sandwich-type immunocomplex by streptavidin–biotin interaction. The DNAzymes exhibited an excellent catalytic activity to the chemiluminescent reaction of luminol with hydrogen peroxide in strong alkaline solution, leading to significant enhancement in response signal. Under the optimum conditions, the proposed immunosensor showed high sensitivity and selectivity with a low detection limit of 1.9 pg mL^{-1} and a wide linear response range of human leptin from 10 to 1000 pg mL^{-1}. The immunosensor was used to detect human leptin in serum, and the results were in good agreement with the data obtained by conventional ELISA method.

1. Introduction

As is well known, leptin is a protein product of the obesity gene and it plays a key role in regulating energy intake and energy expenditure. Research previously shows that the level of leptin influences basal metabolism, hematopoiesis, thermogenesis, reproduction, and angiogenesis [1–3]. The circulating levels of leptin in the body are affected by many acute and chronic physiological parameters. To date, various methods for the detection of leptin have been reported including capillary electrophoresis, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) [4–6]. But these methods have several disadvantages including expensive instruments, complex sample pretreatment and requirement for professional testing staff. Hence, searching for a sensitive, rapid, simple method for the leptin detection would be of important in clinical analysis.

Chemiluminescent (CL) immunoassay, which combines the advantages of high sensitivity of chemiluminescence with the specificity of immunoreaction, is a very effective and well-accepted detection technology [7,8]. Nowadays, many enzyme-linked CL immunosensors have been reported in life science, clinical diagnosis, and environment monitoring [9–14]. Horseradish peroxidase (HRP)-catalyzed luminol–hydrogen peroxide (H_2O_2) CL system is most popular in these reported CL biosensor [15]. It is generally known that the optimum pH for HRP-related catalytic reaction is in the range of 6.0–6.5, but the optimum pH for luminol-related CL reaction is around 11. The incompatibility between the CL reaction condition and the enzyme-catalyzed reaction condition would greatly reduce the luminous efficiency of HRP-catalyzed luminol–H_2O_2 reaction, and also decrease the sensitivity of the CL assay. This problem has hampered the widespread application of CL immunoassay. In many luminol-based system, CL substrates must be added to enhancers, such as *p*-iodophenol [16]. Therefore, the development of enzymes or substrates with higher activity and better stability is the hotspot in CL immunoassay research field [17].

Hemin/G-quadruplex DNAzymes, a kind of nucleic acid artificial enzymes with peroxidase-mimicking activity, have been used as catalytic amplifiers in many biosensing events [18,19]. Compared with natural enzymes, the DNAzymes are smaller in size, easier to functionalize, higher stability against hydrolysis, and they can undergo many cycles of denaturation/renaturation [19,20]. Travascio et al. [21] investigated carefully the effect of the pH on the activity of DNAzymes to catalyze the oxidation of ABTS

* Corresponding author. Tel./fax: +86 553 5910008.
E-mail address: wanglun@mail.ahnu.edu.cn (L. Wang).

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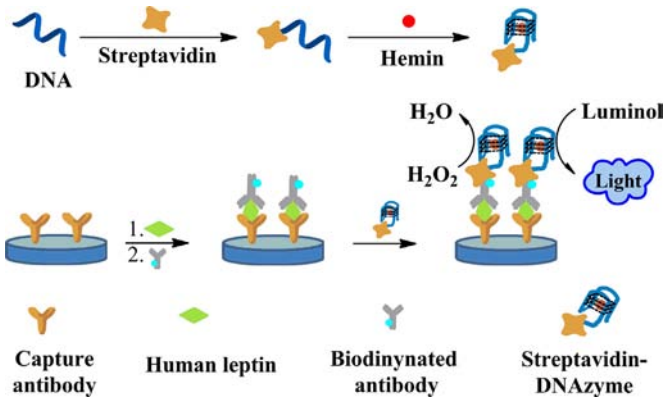
http://dx.doi.org/10.1016/j.talanta.2013.07.074



Scheme 1. Analytical procedure of DNAzymes-catalyzed chemiluminescent immunoassay.

(2, 2'-azinobis (3- ethylbenzthiazoline-6-sulfonic acid)) by H_2O_2, and they found that the activity of the DNAzymes was high at pH 9. Chen et al. [22] immobilized DNAzymes on egg shell membrane and found high catalytic activity of DNAzymes to luminol–H_2O_2 reaction in strong alkaline medium (pH 11.5).

Inspired by the above reports, we designed an ultrasensitive CL immunoassay for the detection of human leptin in serum by using hemin/G-quadruplex DNAzymes as catalytic amplifiers (Scheme 1). The fabrication process of the proposed immunosensor is schematically illustrated in Scheme 1. The pH dependence of DNAzymes catalyzing luminol–H_2O_2 CL reaction was investigated, and the experimental result showed that DNAzymes exhibited higher catalytic activity than natural enzyme HRP, which encouraged us to develop a novel DNAzymes-based CL immunoassay to sensing human leptin. In the sandwich immunoassay, human leptin and hemin/G-quadruplex DNAzymes were captured on the immunosensor, and the strong catalytic activity of DNAzymes led to a high sensitivity with a detection limit of 1.9 pg mL^{-1}. Due to unique properties of DNAzymes, the proposed method provided a promising platform for practical clinical detection of various proteins.

2. Material and methods

2.1. Reagents and apparatus

Luminol and hemin were purchased from Aladdin chemistry Co. Ltd. (Shanghai, China), and used without further purification. Stock solutions of 2.0×10^{-2} M luminol and 5 mM hemin were prepared in dimethylsulfoxide (DMSO) and stored in the dark at −20 °C. High performance liquid chromatograph (HPLC)-purified oligonucleotides and DNA precipitation solution were synthesized by Tiandz, Inc. (Beijin, China). The DNA oligomers were dissolved in 10 mM Tris–HCl buffer solution (pH 8.0) and stored at 4 °C. Dithiothreitol (DTT), streptavidin (SA), HRP (330 U mg^{-1}), sulfo-succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Human leptin, primary antibody, and biotinylated secondary antibody, human leptin ELISA kit, streptavidin-conjugated HRP were purchased from Bomei Co. Ltd. (Hefei, China). Other reagents were also obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Doubly distilled water was used throughout the experiments.

20 mM phosphate buffer saline (PBS, pH 7.4) was prepared by mixing 3.8 mM Na_2HPO_4, 16.2 mM NaH_2PO_4, 20 mM KCl, 200 mM NaCl, 0.1% Triton X-100, and 2% DMSO.

Britton–Robinson buffer solution containing 0.04 M H_3BO_3, 0.04 M H_3PO_4, 0.04 M CH_3COOH, 20 mM KCl, 200 mM NaCl, 0.1% Triton X-100, and 2% DMSO, was adjusted to the desired pH with 0.2 M NaOH.

CL measurements were performed with a BPCL-2-KIC ultra-weak Chemiluminescence Analyzer (Beijing, China) and F-4500 fluorescence spectrophotometer (Hitachi, Japan) with the xenon lamp turned off. UV–visible spectra were recorded on a U-3010 spectrophotometer (Hitachi, Japan).

2.2. Synthesis of the DNAzymes-labeled streptavidin

Under softly agitation, 60 μL of 1 M DTT were reacted with 100 μL of 30 μM of thiol-modified oligomers (1 mM EDTA, pH 7.4) at 37 °C for 2 h in the dark. The activated oligomers were separated from the excess DTT using Sephadex G15 gel filtration column. During the DNA activation, 200 μL of 100 μM streptavidin (PBS, 20 mM, pH 7.4) was activated by 60 μL of 20 mM sulfo-SMCC. Following incubation for 1 h at 37 °C in the dark, the activated streptavidin was purified by Sephadex G15 gel filtration column. The activated oligonucleotides were mixed with the activated streptavidin at the same amount of substance, followed by incubation for 1.5 h in the dark at room temperature. The products were purified by DNA precipitation solution.

2.3. Preparation of the hemin/G-quadruplex DNAzymes

G-rich DNA oligomers or DNAzymes-labeled streptavidin were diluted with 20 mM PBS (20 mM KCl, 200 mM NaCl, 0.1% Triton X-100, 2% DMSO, pH 7.4) to yield a concentration of 5 μM, and the DNA solution was incubated for 1 h at room temperature to allow appropriate folding. Hemin solution (2.5 μM) was added to the prepared DNA solution (5 μM) of an equal volume, and the mixture was allowed to incubate for 1 h in the dark at room temperature. Under these conditions, the oligomers assembled hemin to form the DNAzymes with G-quadruplex structure [23].

2.4. Preparation of sandwich-type CL immunosensor of human leptin using the hemin/G-quadruplex DNAzymes as signal amplifiers

First, 50 μL human leptin standard solution or human serum sample were pipetted into the 96-well plates onto which anti-human leptin monoclonal antibody had been bound. After incubation for 2 h at 25 °C, human leptin in sample would be captured by the coated antibody and the free others were removed by washing 5 times. Second, 50 μL human leptin biotinylated antibodies were added and incubation for 1 h at 25 °C to form the sandwich complexes, then these plates were washed 5 times with PBS buffer. Third, streptavidin-DNAzymes or streptavidin-HRP would be added to bind to the above-formed sandwich complexes for 0.5 h at 25 °C, and free streptavidin-DNAzymes or free streptavidin-HRP would be removed during the washing step. Finally 100 μL CL reagent including 5 mM luminol and 7.5×10^{-2} M H_2O_2 were added and then the CL signal was measured in the luminescence analyzer.

3. Results and discussion

3.1. Catalysis of DNAzymes and HRP to luminol–H_2O_2 system

G-quadruplex DNA binds to hemin to form hemin/G-quadruplex DNAzymes that greatly enhance peroxidase activity compared with hemin alone [24]. The interactions of hemin with the G-quadruplex DNA can be studied using UV–visible absorption spectroscopy. As shown in Fig. 1A, the Soret absorption band of hemin was around 397 nm. The absorbance of hemin/G-quadruplex composites at about

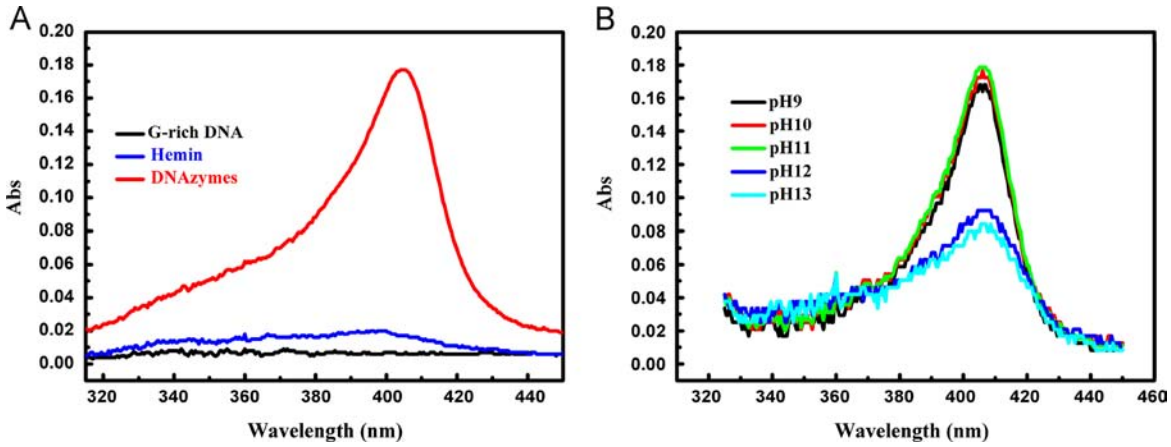
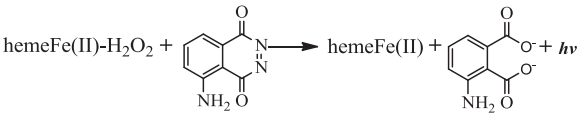


Fig. 1. (A) UV–visible absorption spectra of hemin, DNA and DNAzymes (B) UV–visible absorption spectra of DNAzymes at different pH. DNA, 5 μM; Hemin, 2.5 μM; DNAzymes, 5 μM.

400 nm was significantly higher that of free hemin. The results were presumed that hemin bound to G-quadruplex DNA [21]. The research of Travascio et al. [25] revealed that the hemin had an axial high-spin ferric heme when hemin bound to G-quadruplex DNA. The coordination of guanine to heme iron could contribute to its superior peroxidase activity. According to previous works [21,25], the CL processes that DNAzymes catalyze the oxidation of luminol by H_2O_2 could be expressed as follows:

$$\text{hemin} + \text{G-quadruplex DNA} \rightarrow \text{hemeFe(II)} \quad (1)$$

$$\text{hemeFe(II)} + H_2O_2 \rightarrow \text{hemeFe(II)}{-}H_2O_2 \quad (2)$$



(3)

The CL intensity of luminol-based reaction was strongly dependent on pH of CL reaction media, so we examined the effect of pH on the DNAzymes-catalyzed CL intensity from pH 6.0 to 12.0 in Britton–Robinson buffer solution (Fig. 2A). The maximum CL intensity of DNAzymes-catalyzed luminol–H_2O_2 CL reaction was obtained at pH 11.0, so the working buffer of pH 11.0 was used for the subsequent work. Further, we investigated the effect of pH on the structure of DNAzymes using UV–visible absorption spectroscopy. As shown in Fig. 1B, the absorption peak of the DNAzymes slowly increased with increasing pH from 7 to 11, which indicated that DNAzymes remained the G-quadruplex structure. But if the pH was over 11, the absorption peak of the DNA at 400 nm fell sharply, which indicated the destruction of G-quadruplex structure. The result indicated that the DNAzymes still retained favorable catalytic activity at pH 11 which was the optimal pH for the CL reaction of luminol. The research of Chen et al. [22] showed that the maximal response of the DNAzyme-based CL biosensor was obtained at pH 11.5, which agreed with our experiment well.

Previous studies [26] had indicated that the CL intensity of luminol increases with pH and the optimum pH was around 11.0, but the pH optimum of HRP was in the range of 6.0–6.5. The incompatibility of the CL reaction condition with the enzyme reaction condition would decrease the luminous efficiency of luminol-media CL reaction. When the pH was changed from 7.0 to 9.0, the rate constant of the reaction catalyzed by HRP decreased 100-fold [27]. Our experiment result indicated that the maximum CL intensity of HRP-catalyzed reaction was obtained at pH 8.0. This condition was not conducive to HRP-catalyzed reaction catalysis and to luminol-related CL reaction, so the luminous

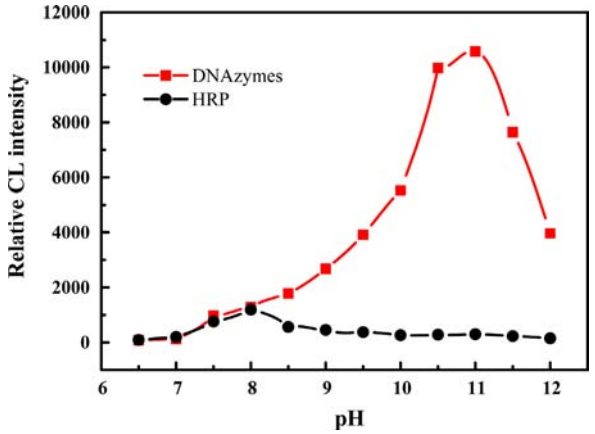


Fig. 2. The relationship between relative CL intensity and the pH of buffer the solution using DNAzymes (square) and HRP (circle) as the catalysts. Luminol, 0.5 mM; H_2O_2, 75 mM; DNAzymes, 0.25 μM; HRP, 0.25 μM; Hemin, 0.125 μM.

efficiency of HRP was not enough for CL immunoassay and some enhancers should be added to this CL system.

The above discussion demonstrated that the activity of DNAzymes was far higher than that of HRP at strong alkaline media. In view of the nature of luminol CL reaction, an alkaline medium would improve the sensitivity of the luminol-based CL immunoassay. Therefore, as an enzymic label to amplify signal in luminol-media CL biosensors, DNAzymes could be better than HRP.

3.2. Biosensor variables optimization

The concentration of luminol had a very important effect on the biosensor response. The effect of luminol concentration in the range of 5.0×10^{-6}–1.0×10^{-3} M was investigated. As shown in Fig. 3A, the optimal concentration of luminol was 5.0×10^{-4} M. The effect of H_2O_2 concentration was also investigated over the range 5.0×10^{-4}–5.0×10^{-1} M, and it was found that the optimum H_2O_2 concentration was 7.5×10^{-2} M (Fig. 3B).

The effect of the incubation time and temperature on the biosensor response was investigated. As shown in Fig. 3C, the CL intensity increased with the increase of incubation time, and then tended to level off after 2 h. So the incubation time of 2 h was chosen for subsequent experiments. The effect of the incubation temperature from 10 to 35 °C was subsequently examined. The CL signal intensity increased at first, and then decreased after 25 °C

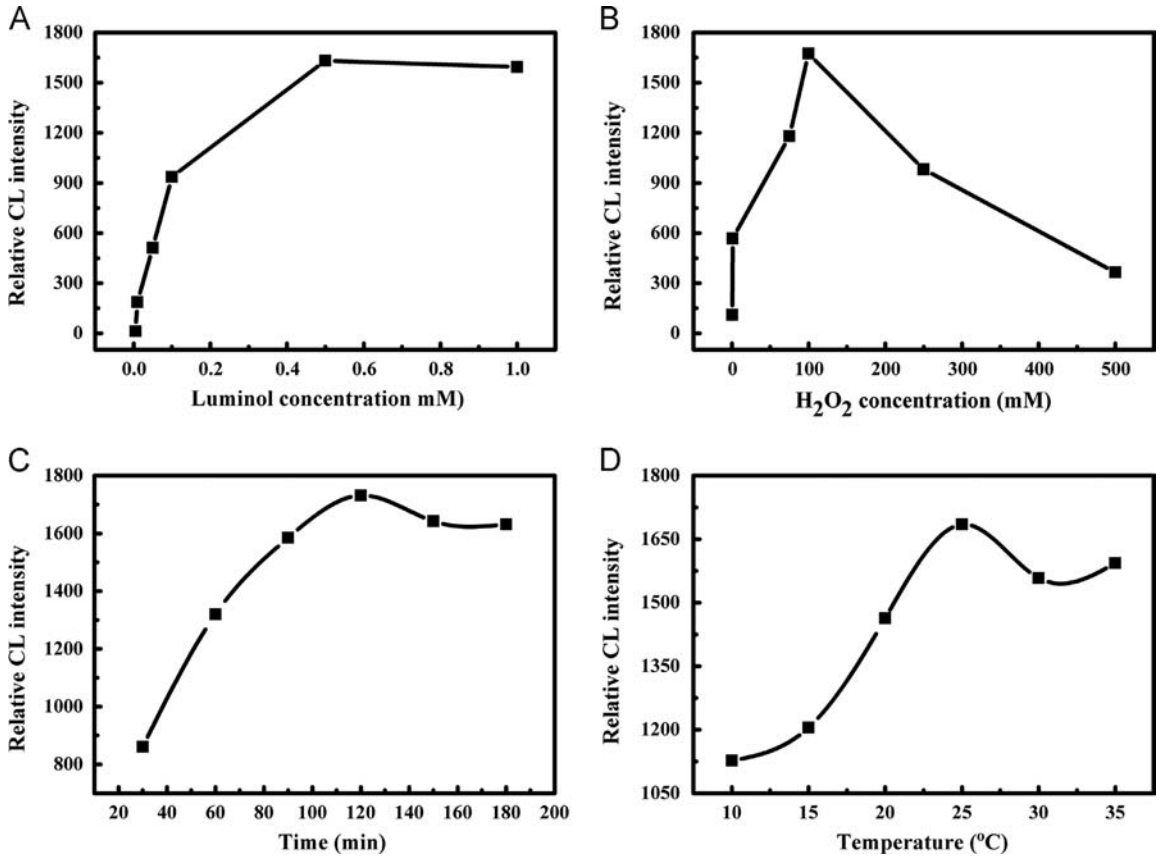


Fig. 3. Effects of (A) luminol concentration, (B) H_2O_2 concentration, (C) incubation time, and (D) incubation temperature at the proposed biosensor.

(Fig. 3D). Therefore, 25 °C was chosen as the optimum temperature for the biosensor.

3.3. Performance of the biosensor for human leptin measurements

The quantitative behavior of the proposed biosensor under the optimized conditions was assessed by monitoring the dependence of the CL intensity on the human leptin concentration. As shown in Fig. 4, CL intensities were proportional to the amount of the corresponding human leptin. A calibration curve to target concentration was linear in the range of 100 to 1000 pg mL^{-1}, and the linear regression equation was $I=4.63C-169.9$ (where I was the CL intensity and C was the concentration of human leptin, pg mL^{-1}) with a correlation coefficient of 0.999. When the concentration of human leptin was from 10 to 100 pg mL^{-1}, the linear regression equation was $I=2.64C+20.2$ (where I was the CL intensity and C was the concentration of human leptin, pg mL^{-1}) with a correlation coefficient of 0.994. The limit of detection was 1.9 pg mL^{-1} by using 3σ ($\sigma=S_b/k$, S_b, standard deviation of blank sample, $S_b=5.09$ ($n=11$) in this experiment; k, the slope of the calibration curve at low concentration, 10–100 pg mL^{-1} in this experiment.)

Compared with HRP-based CL immunoassay, the linear range of DNAzymes-based CL immunoassay was wider and its sensitivity was lower, which showed that DNAzymes-based CL immunoassay was more precise and more sensitive than HRP-based CL immunoassay for human leptin detection. Moreover, a comparison between the proposed method and other reported methods for leptin determination in limit of detection (LOD) and linear range was summed up in Table 1. It was evident that the sensitivity of this proposed method was better than most of the reported methods.

The effects of interfering substances, such as glucose (Glu), L-cysteine (L-cys), Immunoglobulin G (IgG), glutathione S-transferase (GST) and bovine serum albumin (BSA), were used to verify the selectivity of the proposed biosensor. As shown in Fig. 5, it is observed that the immunosensor showed an almost neglectable response to the interfering substances (0.6 ng mL^{-1}) compared with that of the human leptin (0.6 ng mL^{-1}). When 0.6 ng mL^{-1} of leptin and 0.6 ng mL^{-1} of another interfering substance were both added to the biosensor solution, the CL signal changed slightly in contrast to leptin alone, which indicated that the proposed immunosensor had an excellent selectivity for leptin. Furthermore, the stability of the immunoassay system was also examined. The immunosensor was stored at 4 °C for 3 weeks without obvious signal change, and it retained 90% of its initial response after a storage period of 30 days. The slow decrease in response seemed to be related to the gradual deactivation of the DNAzymes.

The reproducibility of the proposed immunosensor system was evaluated by the intra- and inter-assay coefficients of variation (CV) with four standard samples containing different levels of human leptin for five time measurements. The obtained intra-assay CVs with this method were 6.5%, 2.5%, 3.4% and 8.9% at 10, 50, 100, and 500 pg mL^{-1} of human leptin, respectively. Similarly, the inter-assay CVs was 7.7%, 3.1%, 4.8% and 9.8% at 10, 50, 100, and 500 pg mL^{-1} of human leptin. These results indicated the good precision and the acceptable reproducibility of the immunosensor.

3.4. Detection of human leptin in serum samples

The proposed CL immunoassay was applied to determine human leptin in serum samples. Five clinical human serum samples from patients were supplied by the clinical laboratory of the Yijishan Hospital (Wuhu, China). The assayed results were compared with the

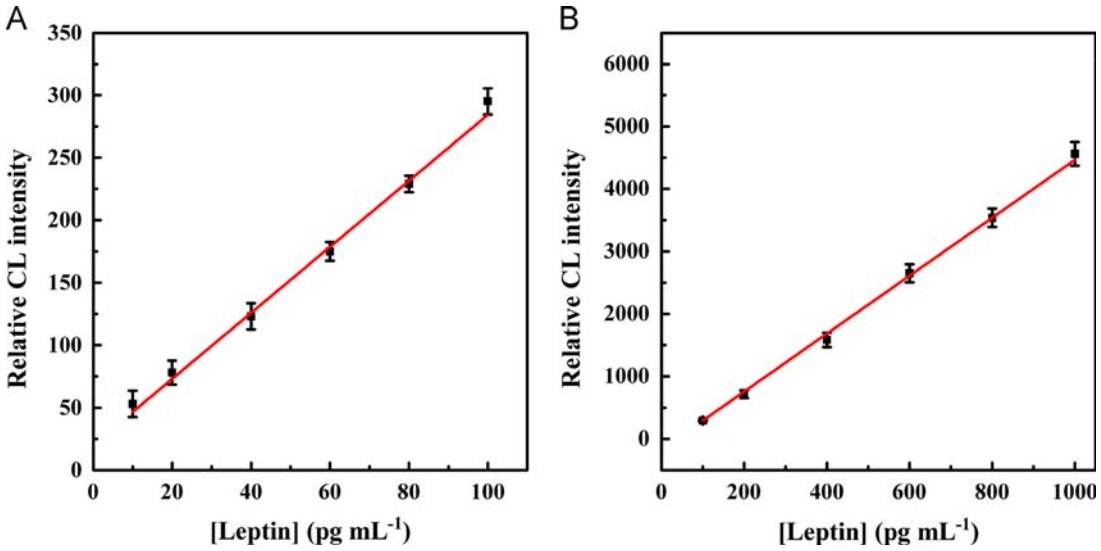


Fig. 4. The calibration curve of the proposed method for human leptin detection. (A) The concentrations of human leptin: 10, 20, 40, 80 and 100 pg mL^{-1}, (B) The concentrations of human leptin: 100, 200, 400, 600, 800 and 1000 pg mL^{-1}.

Table 1
Comparison between different methods for the detection of leptin.

No.	Detection methods	Linear range	LOD	Reference
1	Immunocapture/mass spectrometry	15.63–1000 ng mL^{-1}	15.63 ng mL^{-1}	[28]
2	Electrochemical immunosensor	10–100,000 ng mL^{-1}	10 ng mL^{-1}	[29]
3	CLEIA	0.1–1.0 pg mL^{-1}	0.1 pg mL^{-1}	[30]
4	RIA	0.5–50 ng mL^{-1}	0.5 ng mL^{-1}	[4]
5	ELISA	–	100 pg mL^{-1}	[6]
6	HRP-based CLIA	85–1000 pg mL^{-1}	20 pg mL^{-1}	
7	The proposed method	10–1000 pg mL^{-1}	1.9 pg mL^{-1}	This sensor

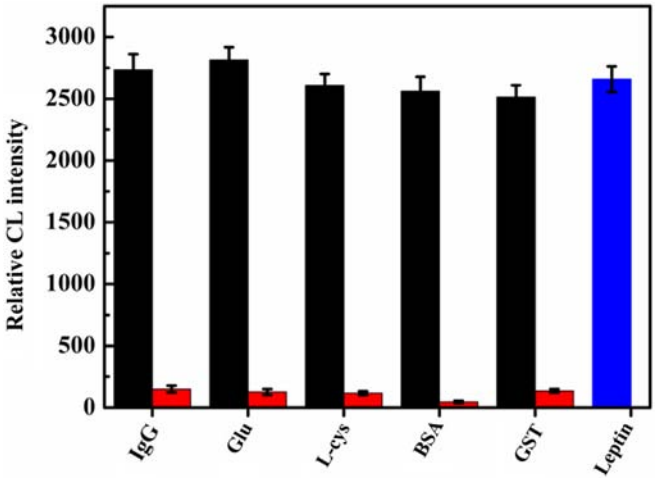


Fig. 5. The CL signals of the immunosensor in the presence of 0.6 ng mL^{-1} of leptin (*blue bar*), 0.6 ng mL^{-1} of other interfere substances (*red bars*), and mixtures of 0.6 ng mL^{-1} of leptin and 0.6 ng mL^{-1} of interfere substances (*black bars*). All experiments were performed three times.

reference values obtained by the commercialized available ELISA method. As shown in Table 2, the results were in good agreement with the date obtained by ELISA method. As confirmed by the Student t test ($P > 0.05$), the results indeed shown no significant difference between this assay and the conventional ELISA assay,

Table 2
Analysis of human leptin in serum samples (in ng mL^{-1}).

Sample	The proposed method[a,b]	ELISA
Serum1	7.77 ± 0.53	7.32
Serum2	1.23 ± 0.15	1.27
Serum 3	0.08 ± 0.005	0.075
Serum 4	0.10 ± 0.014	0.10
Serum 5	0.37 ± 0.06	0.35

[a] Serum samples were diluted 10-folds.
[b] Mean from three measurements ± S.D.

which illustrated that the DNAzymes-based CL immunosensor was sensitive and reliable for the detection of human leptin in serum samples.

4. Conclusions

In this paper, we developed a convenient signal amplification strategy for sensitive and selective CL detection of human leptin in serum samples. This method employed hemin/G-quadruplex DNAzymes as signal amplifier. Remarkably, our results indicated that DNAzymes had far higher catalytic activity to luminol–H_2O_2 CL system than natural enzyme HRP in strong alkaline medium. And, as a mimicking-peroxidase, DNAzymes have other advantages over HRP, such as smaller in size, higher in stability and easier to functionalize. Therefore, these unique properties of

DNAzymes would allow them to become a promising alternative to HRP for the detection of various proteins in clinical diagnosis and medicine.

Acknowledgments

This work was supported by the Natural Science Foundation of China (21075002, 21275008, and 21245008).

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