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A novel chemiluminescence immunoassay for highly sensitive and specific detection of protein using rolling circle amplification and the multiplex binding system

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ABSTRACT

A simple, sensitive and specific chemiluminescence immunoassay (CLIA) strategy has been developed for protein detection by integrating rolling circle amplification (RCA), multiplex binding of the biotin-streptavidin (B-SA) system and enzymatic amplification. The cascade signal amplification methodology is initiated by specifically recognition of target protein based on sandwich immunoassay, which can combine with circular DNA for triggering RCA via biotin-streptavidin. Upon RCA, thousands of repeated sequences are generated for hybridizing with biotinylated detection probes. Then the streptavidin-horseradish peroxidases (ST-HRPs) are bound to biotinylated detection probes, which subsequently catalyze the oxidation of luminol by H_2O_2 and yield an enhanced chemiluminescence (CL) signal. Taking human prolactin (PRL) as a model, under optimal conditions, the CL intensity was proportional to the logarithm value over six orders from 10 fg mL^{-1} to 10 ng mL^{-1} with a detection limit of 0.16 fg mL^{-1}. The established approach was successfully applied for the detection of human PRL in serum samples. Thus, it might be a potential tool for protein detection in clinic biomedical application.

1. Introduction

The ultrasensitive techniques for protein detection are critically important in basic discovery research and clinical practice, because a few molecules of protein are sufficient to affect the biological functions of cells and trigger pathophysiological processes [1,2]. So far, various immunoassays, especially based on the antigen-antibody reaction [3], have been described for the determination of protein targets, including fluorescence immunoassay [4], enzyme-linked immunosorbent assay (ELISA) [5], electrochemical immunoassay [6], radioimmunoassay [7], (electro)chemiluminescence immunoassay [8], and mass spectrometric immunoassay [9]. Among them, CLIA detection combining with signal amplification is one of the most popular technique [10,11], which can be used for high throughput detection because it does not need external light source. However, the CL signal produced by the typical horseradish peroxidase-H_2O_2-luminol system for single bio-recognition event is relatively low, and difficult to be collected by a general photomultiplier tube, limiting its application in the detection of low-abundance protein in disease screening. In order to enhance the sensitivity of CL analysis, different signal amplification techniques, such as polymerase chain reaction (PCR) [12], RCA, nanomaterials [13], and biotin-streptavidin system, have been introduced into CL sensor array. Those amplification techniques can carry more enzyme molecules to catalyze the CL reaction of H_2O_2 and luminol for single bio-recognition event, thus greatly improve the practicality of CL assay in detection of protein.

RCA, an isothermal DNA amplification technique, is widely used as an effective amplification technique in the field of bioanalysis. RCA can generate a linear concatenated DNA molecule containing up to 1000 complementary copies of the circular DNA [14,15]. Compared with PCR, RCA does not need thermal cycling and strict laboratory conditions, and can also possess a great deal of merits,

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including in situ capturing target molecules, the isothermal amplification procedure, and the linear kinetic mode [16]. Therefore, RCA has attracted considerable attention as a novel tool to amplify the recognition events for ultrasensitive detections of DNA [17], RNA [18], ATP [19], and proteins [20–22].

The biotin-streptavidin system is one of the strongest non-covalent bonds known in nature [23]. The strength and specificity of the interaction has led it to be the most widely used affinity pairs in molecular, immunological, and cellular assays. Biotin is a vitamin that is present in small amounts in all living cells. Because biotin is relatively small (244.3 Da), it can be conjugated to many proteins and other molecules without significantly altering their biological activity. Streptavidin, isolated from streptomyces avidinii, has four biotin-binding sites, which can form multivalent bindings with biotinylated macromolecule derivatives and markers [24]. The conjugates of biotin with streptavidin can acquire enhanced response signal intensity. This project has following advantages, Firstly, as one streptavidin can combine with four biotins, using biotin-streptavidin-biotin (B-SA-B) "bridge" can increase the immobilization of bio-circular DNA. Secondly, the DNA detection probes modified with biotin link with the enzymes through biotin-streptavidin reaction.

Human prolactin, a 23 kDa peptide hormone, is principally released by the pituitary lactotrophs of the anterior pituitary gland [25]. Its post-translational modified variants, such as cleaved, phosphorylated and glycosylated, are present in the hypophysis [26]. With more than 100 different effects documented, prolactin is one of the most versatile hormones in terms of biological actions [27]. Research shows that human prolactin is elevated in prolactinoma, hyperprolactinaemia and hypothyroidism [28] and prolactin is an important angiogenic factor that plays a crucial role in distinguishing epileptic seizures from psychogenic non-epileptic seizures and rising following an epileptic seizure [29]. The importance of human prolactin in disease genesis and progression makes the hormone an attractive biomarker for disease diagnostics.

Here, for the first time, we fabricate a CLIA method for human prolactin by taking advantage of the high binding specificity of sandwich immunoassay and unique amplifying performance of rolling circle amplification as well as the multiplex binding system. Compared with the previous prolactin assay methods, this established approach provides a simple, cost-efficient, highly sensitive and specific platform for human prolactin analysis, which may be a potential alternative approach for protein detection in clinic biomedical application.

2. Experimental

2.1. Reagents

The chemiluminescent-immunoassay kit for PRL was purchased from Bioscience (Tianjin, China), including 96-well microplates coated with antihuman PRL monoclonal antibody, vials of human PRL solutions, and biotin labeled antihuman PRL polyclonal antibody. DNA oligonucleotides were synthesized and purified by Sangon Biotechnology Co. Ltd. (Shanghai, China), and the base sequences are listed as follow: primer, 5′-biotin-AAA AAA AAA AAA AAA CAC AGC TGA GGA TAG GAC AT-3′; circular template, 5′-p-CTC AGC TGT GTA ACA ACA TGA AGA TTG TAG GTC AGA ACT CAC CTG TTA GAA ACT GTG AAG ATC GCT TAT TAT GTC CTA TC-3′; biotinylated DNA probe, 5′-biotin-TTT TTT TCA GAA CTC ACC TGT TAG-3′. Phi29 DNA polymerase was purchased from New England Biolabs (Beijing, China). T4 DNA ligase and deoxynucleotide solution mixture (dNTPs) were obtained from Takara (Dalian, China). Streptavidin was obtained from Promega (Madison, USA). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). PBS-T buffer solution was bought from Double Helix (Shanghai, China). All other reagents (analytical grade) were purchased from standard reagent suppliers. All aqueous solutions were prepared using ultrapure water (≥18 MΩ). The physiological buffer saline (PBS) consisted of 0.15 M NaCl, 7.6 mM NaH_2PO_4, and 2.4 mM Na_2HPO_4 (pH 7.4). PBS-T buffer consisted of 0.15 M NaCl, 7.6 mM Na_2HPO_4, 2.4 mM NaH_2PO_4, and 0.05% Tween-20 (pH 7.4).

2.2. Apparatus

The chemiluminescent spectra were measured using a Synergy H1 Hybrid Reader (Gene, HK, China). Agarose gel electrophoresis (AGE) was conducted using a DYY-6C Electrophoresis Cell (LIUYI, Beijing, China) and Gel Doc-It Imaging Systems (UVP, Cambridge, UK). The images of stepwise ligation of antibody (Ab)-DNA were recorded by the atomic force microscope (AFM) IPC-208B Data System (HENGRUI, Chongqing, China).

2.3. Circularization of DNA template

One hundred nanomole of circular template oligonucleotide and 100 nM of biotinylated primer oligonucleotide were mixed in 100 μL of ligation buffer (66 mM, pH 7.6 Tris–HCl buffer, 10 mM $MgCl_2$, 10 mM dithiothreitol, and 66 μM ATP), vortexed for 1 min and incubated for 30 min. Subsequently, 6 μL T4 DNA ligase was added and incubated at room temperature for 2 h. After ligation, T4 DNA ligase was inactivated by heating the reaction mixture at 65 °C for 10 min. The resulting mixture could be used directly or stored at −20 °C.

2.4. Sandwich assay in microplate wells

PRL analytes (50 μL per well) at variable concentrations were added in microplate wells coated with antihuman PRL monoclonal antibody, and incubated for 2.0 h at 37 °C. Then, the reaction solutions were removed, and three washing steps with 300 μL of PBS-T buffer were carried out. Subsequently, 50 μL of biotin labeled anti-PRL polyclonal antibody was added in each well and incubated at 37 °C for 1 h. The resulting microplate wells were washed three times with 300 μL of washing buffer and used for the following operation.

2.5. Rolling circle amplification for chemiluminescence immunoassay

After the sandwich immunoreaction, the microplate wells were incubated with 50 μL of 15 nM streptavidin solution containing 0.2% BSA and 100 μg mL^{-1} salmon sperm DNA per well at 37 °C for 30 min. Then the reaction solutions were discarded, and three washing steps with 300 μL of PBS-T buffer of pH 7.4 were performed. Thereafter, 50 μL of circularization mixture containing 20 nM circular template DNA and 20 nM biotinylated primer DNA was added to each well and incubated at 37 °C for 1 h. Then the reaction solutions were removed, and the microplate wells were washed with 300 μL of PBS-T buffer of pH 7.4 three times. Then, the RCA reaction was carried out by addition of 1.875 units of phi29 DNA polymerase in 50 μL of reaction buffer (33 mM, pH 7.9 Tris–acetate buffer, 10 mM Mg-acetate, 66 mM K-acetate, 1 mM dithiothreitol, 0.5 mM dNTP, and 0.1% Tween 20) and continued for 1 h at 37 °C. After the reaction solutions were abandoned and the microplate wells were carefully washed, 50 μL of 500 nM complementary biotinylated DNA probes were followed to add to each well and hybridized at 37 °C for 1 h. Subsquently, the reaction solutions were discarded and the microplate wells were washed for three times again, then the system reacted with 50 μL of 5 μg mL^{-1} ST-HRP at 37 °C for 30 min and thoroughly washed with washing