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Nano-biotinylated liposome-based immunoassay for the ultrasensitive detection of protein biomarker in urine

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ABSTRACT

With the development of proteomics and the continuous discovery of biomarkers of trace proteins, it is important to accurately quantify low abundance protein, especially in urine for clinical diagnostics. In this paper, we reported a novel nano-biotinylated liposome-based immuno-loop-mediated isothermal amplification (LI-LAMP) for the ultrasensitive detection of REG1A (a biomarker for pancreatic ductal adenocarcinoma (PDAC) in urine) with high specificity. The detection range was 1 μg/mL to 1 fg/mL, with a detection limit of 1 fg/mL, and no cross-reactivity was observed to occur in this assay. Compared with the amount of REG1A added, REG1A recovery using this method was 130% and 89%. Detection of REG1A concentrations using the LI-LAMP assay from real samples were in good agreement with those determined using ELISA, and relative deviations were not more than 10%. LI-LAMP shows good potential as a clinical diagnostic assay.

1. Introduction

In many clinical diagnoses, the ability to accurately quantify specific antigens within a relatively low concentration range is essential [1]. Many protein biomarkers in clinical specimens occur at levels lower than nanograms per milliliter in range, especially in urine [2]. At present, urine is the most attractive sample for clinical proteomics research because it can be obtained in a completely non-invasive way and is much more stable than other body fluids. Hence, it is great diagnostic significance to develop a kind of novel assay for the urine protein and applied in clinical practice [3].

Different protein detection platforms have been established in recent years, including the agglutination test, immunoprecipitation test, radioimmunoassay, fluoroimmunoassay, enzyme immunoassay, chemiluminescence immunoassay, solid-phase membrane-based immunoassay, immunohistochemical techniques, and flow cytometry [4]. While, most of which the sensitivity of protein detection ranges within nanogram levels; femtogram levels of protein cannot be detected using these typical protein detection methods.

Liposomes are spherical vesicles consisting of phospholipid bilayers surrounding an aqueous volume. Liposomes can wrap different markers, combining with biological recognition molecules of various types for measurement in dual-signal amplification liposome immunoassays. Liposome-encapsulated markers have been successfully demonstrated for the detection of *Escherichia coli* [5], *Botulinum* toxin [6], *Cholera* toxin [7], carcinoembryonic antigen [8], and prostate-specific antigen [9]. Compared with conventional ELISA, liposome-encapsulated signals can produce 500–1000-fold signal enhancement [10].

A liposomes immunoassay using signaling molecule-loaded liposomes whose surfaces have been tagged with the protein of interest have been developed for a variety of assays, particularly for flow-injection automated procedures [11]. Many marker systems based on chemiluminescence [12], electroactivity [13], and enzymes [14] have been developed for liposomes immunoassay. The biotinylated liposome is one such system. This system has a hollow closed-shell nanosphere comprising a phospholipid bilayer with biotin-labeled polyethylene glycol (PEG) phospholipid conjugates incorporated into the outer bilayer leaflet, and has the advantage of a long shelf-life, is easy to prepare, and shows stable performance. The signal can be further amplified by an avidin–biotin bridge. This system greatly simplifies the preparation and purification of detection liposomes.

Immuno-PCR was first described by Santo et al. in 1992 and is a process by which a specific DNA molecule is used as a marker [15]. Immuno-PCR shows an increase in detection sensitivity of approximately 10^5 compared with ELISA. Nam et al. have reported a nanoparticle-based bio-bar code approach to detect a protein target [16],

Abbreviations: LAMP, loop-mediated isothermal amplification; SUV, small unilamellar vesicles; PEG, polyethylene glycol

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with the limit of detection increasing from ng/mL to pg/mL, and low abundance proteins are able to be detected. For example, the prostate-specific antigen limit of detection is 5 pg/mL using real-time immuno-PCR and *C. botulinum* neurotoxin type A has a limit of detection of 50 fg/well [17]. Despite the success of the system, there are also some challenges, such as non-specific adsorption of nucleic acid amplification, which greatly increases the false-positive rate [15].

Loop-mediated isothermal amplification (LAMP) is an outstanding gene amplification procedure in which the reaction can be processed at a constant temperature by one type of enzyme, and its rapid and simple features define it from existing genetic tests [18]. The LAMP method uses four primers that recognize six or four regions on the target DNA, thus the specificity and sensitivity are high extremely compared with PCR [19]. Real-time LAMP shows improved quantitative accuracy compared with end-point LAMP.

In this study, we developed another type of assay, biotinylated liposome-based immuno-loop-mediated isothermal amplification (LI-LAMP), for the ultrasensitive detection of REG1A (a biomarker for pancreatic ductal adenocarcinoma in urine [20]) with high specificity. As we known, pancreatic ductal adenocarcinoma is one of the most lethal cancers, and no significant improvements have been obtained in non-invasive early diagnosis with high sensitivity until now [21]. In this process, we use a DNA reporter encapsulated in liposomes that cannot only double the signal amplification but can also reduce non-specific adsorption caused by free DNA. So, in this paper, we utilized REG1A as a model protein. This method presented can also be generalized for the testing of any other low-abundance proteins in urine, and has important application in clinical diagnostics.

2. Material and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol, bovine serum albumin (BSA), Triton-X 100, hydroxymethyl methyl aminomethane (Tris), human interleukin-6 (IL-6), and phosphotungstic acid were purchased from Sigma-Aldrich (St. Louis, MO). 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol) − 2000] (DSPE-PEG2000), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[biotinyl (polyethylene glycol) − 2000)] (DSPE-PEG2000-biotin) were obtained from Ponsure Biotechnology (Shanghai, China). Phosphate buffered saline (10 mM; pH 7.4) (PBS), dialysis bags (300 kD), and bovine pancreatic DNase I were manufactured by Solarbio Life Sciences (Shanghai, China). Chloroform, methanol, and HCl were provided by Sinopharm Chemical Reagent (Shanghai, China). Encapsulated DNA, primers, goat anti-rabbit biotin, coating buffer (50 mM bicarbonate; pH 9.6), and avidin were synthesized by Sangon Biotech (Shanghai, China) (Supporting Information, Table S1). Anti-REG1A antibodies (mouse monoclonal antibody, rabbit polyclonal antibody), recombinant human REG1 alpha protein, and recombinant human MUC1 protein were from Abcam (Cambridge, MA). High-binding microwell plates (Corning Costar, 96-wells) were from Sigma-Aldrich. The REG1A ELISA kit was from Gefan Biotechnology (Shanghai, China). LAMP was generated in-house. All reagents were analytical grade. Millipore ultrapure water (18.2 MΩ cm) was used throughout the experiment.

2.2. Clinical specimens

Experiments were carried out in accordance with approved international guidelines and ethical standards. The Ethics Committee of Changhai Hospital, Second Military Medical University approved the research and informed consent was obtained from each donor.

For the LI-LAMP assay for the recovery rate of REG1A in human urine, urine from healthy individuals was obtained from Changhai Hospital, Shanghai. All samples were REG1A-negative. The method described in this study was used to analyze REG1A in human urine samples. The assay was performed by adding the recombinant REG1A to REG1A-negative human urine.

Detection of REG1A in real samples using LI-LAMP was compared with the traditional horseradish peroxidase-ELISA. Three REG1A-positive urine samples were collected from a patient with pancreatic ductal adenocarcinoma. All urine specimens were collected using the same standard operating procedures, which included midstream urine, collected in the morning after a 12-h fast, frozen within 1 h of collection, and stored at −80 ℃ until analyzed. Samples were diluted 10-fold for LI-LAMP and 100-fold for traditional horseradish peroxidase-ELISA using 1 × tris-buffered saline (TBS; 20 mM Tris-HCL, 150 mM sodium chloride, pH 7.4) before analysis.

2.3. Preparation of DNA-encapsulating liposomes

For the REG1A assay, we chose a 286-base pair gene segment as the DNA reporter, which comprised a sequence not likely to be found in human DNA. LAMP primers were designed using LAMP primer designing software (Primer Explorer v4; http://primerexplorer.jp/e/) (Supporting Information, Table S1). DNA-encapsulating liposomes were prepared using the film hydration method [22,23]. A mixture of DPPC (47.5 mol%), cholesterol (47.5 mol%), DSPE-PEG2000 (4.5 mol%), and DSPE-PEG2000-biotin (0.5 mol%) (36.5 mg in total) was dissolved in 4 mL chloroform and methanol (6:1 vol ratio) followed by sonication (SB-80; Scientz, Ningbo, China) for 10 min. The mixture was placed in a 100-mL round-bottomed flask, then evaporated under N_2 and high vacuum for at least 4 h at 35 °C. The reporter DNA (1 μg) was diluted in 1 × TBS and used as an encapsulate at 45 °C, with vigorous rotation of the flask (HH S11-2-S; Cimo, Shanghai, China) until the lipid film peeled off the inside wall. To obtain small unilamellar vesicles (SUV), multilamellar liposomes underwent sonication with a probe-tip sonicator (10 cycles of 4-min on/1-min off) (IID; Scientz, Ningbo, China) in an ice-water bath. The resulting SUV were centrifuged at 630 × *g* for 15 min to remove undispersed lipids and titanium from the probe tip. The liquid supernatant was extruded at room temperature through 0.45-μm membranes, followed by extrusion 20 times through two 100-nm polycarbonate membranes using a LiposoFast-Basic extruder (LF-1; Avestin Inc., Ottawa, Canada). Unencapsulated DNA oligonucleotides were removed by dialysis against 2000 volumes of 1 × TBS (300 kD MWCO) for 24 h at 4 °C with three changes of buffer. The purified liposome detection reagents (~10 mg lipid/mL) were stored under N_2 in a sealed dark vial at 4 °C. Blocking SUV were used as a blocking reagent in microwell plates. These SUV were prepared and dialyzed as described above. However, the encapsulate no contained DSPE-PEG2000-biotin in liposome phospholipid bilayers and no DNA in 1 × TBS. Storage conditions and shelf-life for the blocking SUV were the same as those for DNA-encapsulating liposomes.

2.4. Liposome lipid concentration test

Liposomes were assayed for their phospholipid content by measuring phosphorus using an inductively-coupled plasma atomic emission spectrometer (ICP-AES, Optima 8000; PerkinElmer, Waltham, MA). For the assay, 50 μL liposomes were digested to inorganic phosphates with 150 μL aqua regia reagent for 24 h at 25 °C. Then the mixture was diluted to 2 mL with 1 × TBS using ICP to test for phosphorus content.

2.5. Determination of liposome size

The hydrodynamic diameter of liposomes was determined at 25 °C by dynamic scattering (Nano ZS90; Malvern Instruments, Malvern, UK) using a 1:200 dilution of the liposome detection reagent in 1 × TBS. Scattered light was measured at a 90° angle, a 6.7 μ/s channel width, and with a refractive index of 1.333.