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Chemiluminescence enzyme immunoassay using magnetic nanoparticles for detection of neuron specific enolase in human serum

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ABSTRACT

To detect a biomarker for small cell lung carcinoma, neuron specific enolase (NSE), a sensitive and specific chemiluminescence enzyme immunoassay was developed. Fluorescein isothiocyanate (FITC) labeled NSE capture antibody connected with NSE and alkaline phosphatase (ALP) labeled NSE detection antibody in a sandwich-type detection manner. This immune complex was further reacted with anti-FITC coated magnetic beads. In a magnetic field, the complex was enriched, and the sensitivity was thus enhanced. The limit of detection (LOD) of this method was <0.2 ng mL^{-1}. The proposed immunoassay was highly selective, and not interfered by hook effect. The recovery was >83.0% and the coefficient of variation was <10.0%. Human sera from 120 patients were tested with the presented and traditional chemiluminescence enzyme immunoassay. An excellent linear relationship was obtained between two techniques. Overall, this immunoassay offers a promising alternative for NSE detection than traditional clinical examinations.

1. Introduction

In recent years, protein biomarkers have shown useful information for early diagnosis and effective treatment of cancer [1,2]. Neuron specific enolase (NSE) is a glycolytic neurospecific isoenzyme of enolase. It consists of two polypeptide chains, each with molecular weight of 39 kDa. NSE could provide information to cancer course and treatment outcome of small cell lung carcinoma (SCLC) [3] and neuroblastoma [4]. Clinically, NSE level in healthy person is below 15 ng mL^{-1}. High levels (>100 ng mL^{-1}) of NSE indicate the presence of SCLC with high probability [5]. NSE levels in patients with these two diseases could give information about the extent of the disease much earlier than chest X-ray inspection (4–12 weeks earlier). Furthermore, NSE detection is much easier and cost-effective than the current clinical examinations. So, the measurement of NSE in serum has been applied in SCLC and neuroblastoma diagnosis and the examination of the patient's recovery to treatments [6,7].

Immunoassays hold enormous potentials in diagnostic applications since they are more convenient and cost-effective than traditional analytical techniques [8]. The common immunochemical methods for NSE were radioimmunoassay (RIA) [9] and enzyme-linked immunosorbent assay (ELISA) [10]. The RIA method is sensitive (sensitivity = 3.0 ng mL^{-1}) and reliable, but it requires specialized equipments, sophisticated separation steps and long performance period. The operators may suffer from the harmful effect of radioactive labels. ELISA method is less sensitive (sensitivity = 12.5 ng mL^{-1}) and probably interfered by false negative results, especially for patients with light infection. Therefore, a more sensitive and convenient screening method is required.

The aim of this study was to develop a magnetic bead-based chemiluminescence immunoassay (MPs-CLEIA). This method is advantageous in the increased surface area for connecting antigen or antibody. It possesses the high sensitivity and specificity without using radioactive reagents [11]. MPs-CLEIA technique has been used to rapidly screen several tumor biomarkers before routine clinical examination because of its higher sensitivity and wider dynamic range [12,13]. Recently, Zhang et al. performed a magnetic beads-based chemiluminescence immunoassay for sequential dual determination of NSE and S100β for diagnosis of ischemic stroke [14]. Capture antibodies of S100β and NSE were coupled to magnetic beads. After connecting with NSE or S100β, the magnetic beads were divided into two parts. The concentration of NSE and S100β was separately determined by further reacting with detection antibodies labeled by ALP and HRP, respectively. In the "sandwich-type" detection strategy, magnetic beads (mean diameter of 1.5 μm) were used, which means the immunoreaction

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happened in an aqueous suspension. When the magnetic beads were divided for CL signal measurement, the beads in two different parts might be not same, especially in automatic analysis. In addition, the method was not actually tested in the biological samples of patients. In our research, NSE was designed connect with capture antibody and detection antibody in a liquid solution before coupling with magnetic beads, so the stability and reproducibility could be satisfactory for automated separation. The proposed MPs-CLEIA method was further applied to detect NSE in patient serum and the results obtained are in excellent linear relationship with those from traditional CLEIA method.

2. Experimental

2.1. Materials

Fluorescein isothiocyanate (FITC), luminal, cysteine, bovine serum albumin (BSA), N-Hydroxysuccinimide (NHS), Sephadex G-25 and 2-(N-morpholino)ethanesulfonic acid (MES) were from Sigma–Aldrich (St. Louis, MO, USA). The carboxylated immunomagnetic particles (diameter of 1.0 μm) were purchased from Merck (Beijing, China). Monoclonal capture and detection antibodies for NSE were obtained from HyTest Ltd. (Turku, Finland). Alkaline phosphatase (ALP) was from BBI Enzymes (Wisconsin, USA). Lumigen APS-5 was provided by Lumigen, Inc. Succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC), 1-ethyl-3-(3-dimethylaminopropyl)-carbodimide hydrochloride (EDC) and Traut's Reagent (2-iminothiolane) were from Pierce Biotechnology, Inc. (Rockford, USA).

Double distilled water was used throughout this study. Phosphate buffered solution (PBS) contained 0.01 M KH_2PO_4 and 0.01 M Na_2HPO_4. Washing buffer was phosphate buffered saline with 0.5% (w/v) Tween 20 (PBST). NSE standard solution was prepared in the 0.1 M Tris–HCl (pH 7.4) with 5% BSA.

2.2. Instrumentation

Magnetic separator was provided by Tianjin Baseline Chromtech Research Centre (Tianjin, China). ALP labeled NSE detection antibody was purified by Protein Purification System (AKTA Explorer 100, Pharmacia). Chemiluminescence (CL) signal was measured by BHP9507 Chemiluminescence Analyzer (Beijing Hamamatsu Photon Techniques Inc.).

2.3. Procedures

2.3.1. FITC labeling of NSE capture antibody

1 mg NSE capture antibody was dissolved in 0.2 mL of 1 mg mL^{-1} FITC carbonate buffer (0.2 M, pH 9.0). The reaction was allowed to proceed overnight at room temperature and the product was purified by a Sephadex G-25 column eluted with 0.1 M Tris–HCl (pH 7.4).

2.3.2. ALP labeling of NSE detection antibody

Before coupling with ALP, the primary amino groups in NSE detection antibody were activated to be sulfhydryl groups. Briefly, 1 mg detection antibody in 0.05 M PBS (pH 8.0, 5 mg mL^{-1}) was incubated with 5 μL of Traut's Reagent (12 mg mL^{-1}) at room temperature for 20 min and purified by a Sephadex G-25 column primed by 0.05 M PBS (pH 7.3).

1 mg ALP was activated by 10 μL of 7 mg mL^{-1} SMCC for 30 min at room temperature. The activated detection antibody was mixed with the pretreated ALP. Excess functional groups in SMCC were blocked by cysteine (50 μL, 10 mg mL^{-1}). The mixture was incubated at 4 °C overnight and purified by Protein Purification System (AKTA Explorer 100, Pharmacia).

2.3.3. Coating magnetic particles with FITC antibody

To conjugate with FITC antibody, 10 mg magnetic beads were mixed with 20 mg EDC and 30 mg NHS at room temperature for 30 min. Then, the beads were washed twice with 0.1 M MES buffer (pH 6.0, 2 mL) and reacted with 3 mg FITC antibody at room temperature for 2 h. After washed twice, the residual binding sites on the beads were blocked with 10 mg BSA at room temperature for 2 h. Finally, the product was washed three times with 0.1 M Tris–HCl and stored at 4 °C.

2.3.4. MPs-CLEIA analysis protocol

The schematic diagram of the MPs-CLEIA analysis is shown in Fig. 1. First of all, 50 μL of FITC labeled NSE capture antibody (0.5 μg mL^{-1}) was mixed with 50 μL of ALP conjugated NSE detection antibody (0.4 μg mL^{-1}) and 10 μL of NSE sample. The reaction was allowed to proceed at 37 °C for 15 min. Then, 60 μL of FITC antibody coated magnetic particles (MPs) was added. The mixture was incubated with shaking at 37 °C for 5 min. The FITC antibody on the surface of MPs would specifically connect with FITC labeled NSE capture antibody. The resultant precipitation was collected in a magnetic field, and the supernatant was discarded. The unbound substances were removed with 0.3 mL of washing buffer. Finally, 200 μL of Lumigen APS-5 solution was added, and the chemiluminescence signals were measured immediately.

2.3.5. CLEIA method

For comparison study, a traditional CLEIA method was built. The procedure for CLIEA analysis was similar as that for MPs-CLEIA method. After the complex was formed in a sandwich way, the microplate well was washed three times by washing buffer. 200 μL of luminol solution was added, and the chemiluminescence signal was immediately measured.

2.3.6. Interference studies

The influence of other tumor markers was evaluated in the interference studies. Five commonly tested tumor markers, alpha fetal protein (AFP), carcinoembryonic antigen (CEA), prostate specific antigen (PSA), carbohydrate antigen 15-3 (CA 15-3) and carbohydrate antigen 125 (CA 125) were analyzed by the MPs-CLEIA method. The tested concentration of these tumor markers was at least 1000 times higher than their normal clinical reference level.

3. Results and discussion

3.1. Sensitivity, accuracy and repeatability

A sandwich immunoassay was designed. Before binding with FITC antibody, the carboxylated magnetic beads were treated with EDC. As a result, FITC tagged capture antibody bound with the MPs surface after reaction with NSE and detection antibodies. The method was optimized for the concentration of magnetic beads, capture antibody and detection antibody. To optimize amount of magnetic beads, the CL response ratio was calculated according to the CL signal of positive group (300 ng mL^{-1} NSE) to that of blank group. As was shown in Fig. 2, the CL ratio reached a maximum peak when 6 mg mL^{-1} magnetic beads were used. Therefore, 6 mg mL^{-1} magnetic beads were utilized in the following experiments. The concentration of capture antibody and detection antibody was optimized to obtain higher CL response for positive group (5 and 300 ng mL^{-1} NSE) and lower CL signal for blank group. For each NSE level, as the amount of capture and detection antibody increased, the CL signal for positive groups and blank group also increased