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APPLIED METHODOLOGY

Development of magnetic separation and quantum dots labeled immunoassay for the detection of mercury in biological samples

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ABSTRACT

A rapid and sensitive immunoassays of mercury (Hg) in biological samples was developed using quantum dots (QDs) and magnetic beads (MBs) as fluorescent and separated probes, respectively. A monoclonal antibody (mAb) that recognizes an Hg detection antigen (BSA-DTPA-Hg) complex was produced by the injection of BALB/c mice with an Hg immunizing antigen (KLH-DTPA-Hg). Then the ascites monoclonal antibodies were purified. The Hg monoclonal antibody (Hg-mAb) is conjugated with MBs to separate Hg from biological samples, and the other antibody, which is associated with QDs, is used to detect the fluorescence. The Hg in biological samples can be quantified using the relationship between the QDs fluorescence intensity and the concentration of Hg in biological samples following magnetic separation. In this method, the detection linear range is 1–1000 ng/mL, and the minimum detection limit is 1 ng/mL. The standard addition recovery rate was 94.70–101.18%. The relative standard deviation values were 2.76–7.56%. Furthermore, the Hg concentration can be detected in less than 30 min, the significant interference of other heavy metals can be avoided, and the simultaneous testing of 96 samples can be performed. These results indicate that the method could be used for rapid monitoring Hg in the body.

Introduction

Mercury (Hg) is a lasting, ubiquitous environmental toxin that threaten the health of humans about neurological, renal, cardiovascular, reproductive and immune system [1–4]. China consumes about half of the global Hg supply and is believed to be responsible for a quarter of anthropogenic Hg emission in the world, and the incidents of human Hg poisoning have been reported from time to time [5,6]. Therefore, a sensitive, specific and rapid analytical technique to measure the concentration of Hg in the body is highly desirable. At present, analytical methods for the detection of Hg include atomic fluorescence spectrometry (AFS), cold-vapor atomic absorption spectrometry and dithizonecolorimetric analysis, which are sensitive and reliable techniques [7]. However, these methods require complicated sample preparation, which makes them unsuitable for rapid detection in the field. In 1985, Reardan et al. [8] developed antibodies against metal chelates. Afterward, a large numbers of monoclonal antibodies were identified, which are specific against heavy metal species [9]. In recent decades, immunoassays have been applied to the detection of heavy metals [10,11].

In biosensing research, magnetic beads (MBs) and quantum dots (QDs) are two important tools [12,13]. Because MBs can be used to capture analytes from a complex sample with antibodies on the surfaces and finely separate in a complicated system using a magnetic field, MBs-based methods possess higher sensitivity and analytical speed and are easier to integrate into diverse assay processes in high-performance tests [14]. QDs are semiconductor nanocrystals, made of a semiconductor core and a cap or shell, whose diameter is approximately 2–100 nm. Due to their specific structural, thermal and optical properties [15], surface-functionalized QDs are used as signal-transduction probes and applied to label captured analytes using biotin–streptavidin or antibody–antigen interactions [16] The combination of QDs and MBs for signal transduction and biorecognition is believed to be a promising approach for biosensing applications, and it has been reported for the detection of various pathogenic bacteria and disease biomarkers [17–20]. In these reported studies, MBs with

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capture-antibodies were used to capture and separate analytes from samples, and they were further incubated with detection-antibody-conjugated QDs to form sandwich immune-complexes (MBs–analyte–QDs). However, there have not been reports on the detection of metals based on labeled QDs coupled with immune-magnetic separation. This work demonstrates a high-throughput and sensitive method for detecting Hg in biological samples without extensive sample preparation. Furthermore, the specific Hg can be detected in less than 30 min, the significant interference from other heavy metals can be eliminated, and 96 samples can be tested at the same time.

Materials and methods

Materials

The chemicals and reagents used included p-SCN-Bn-CHX-A″-DTPA (Macrocyclics), PEG4000 (Merck), mercury(II) atomic absorption metal standard (1000 μg/mL) (Chinese National Center of Analysis and Testing for Nonferrous Metals and Electronic Materials), bovine serum albumin (BSA) (Sigma), keyhole limpet hemocyanin (KLH) (Sigma), Freund's complete adjuvants (FCA) (Sigma), Freund's incomplete adjuvants (FIA) (Sigma), quantum dot-585 streptavidin conjugates (QD585-streptavidin, Invitrogen), and magnetic beads (Dynabeads M-280, Invitrogen). The instruments used included an enzyme-linked immune detector (Stat Fax-2100, Awareness Inc.), fluorescence microplate reader (FLx800, Biotek), amicon centricon 30 K (Millipore), ultraviolet spectrophotometer (UV-2660, Shimadzu), atomic fluorescence spectrometer (AFS) (PF6, Purkinje General), and −80 °C refrigerator (Thermo).

Protein-chelate conjugates

Protein-chelate conjugates were prepared essentially as described in Ref. [21]. Hg(II)-DTPA-protein conjugates were prepared at a final volume of 1 mL containing 10 mg of BSA/KLH, 1.7 mmol/L p-SCN-Bn-CHX-A″-DTPA, 2.0 mmol/L $Hg(NO_3)_2$, and 47 mmol/L triethylamine in 0.1 mol/L HEPES buffer (pH 9.5). The reactions were stirred for 24 h at 25 °C. Unreacted low-molecular-weight molecules were removed by buffer exchange using amicon centricon 30 K (Millipore). The degree of lysine substitution was determined using the trinitrobenzenesulfonic acid method described [22]. The antigen protein content detection was performed using a BCA protein assay kit. The Hg concentration was determined by AFS. The detection antigen and immune antigen were scanned using a UV-2660.

Mouse immunization

Eight 6-week-old female BALB/c mice were purchased from Beijing Vital River Laboratories (scxk(Jing) 2012–0001) were used for immunization [11]. The mice were housed in a ventilated, temperature-controlled, and standardized sterile animal room with a 12 h day/night cycle at the China Capital Medical University. The mice were allowed to acclimate to the animal room for 7 days prior to experimentation. All procedures used in this study were performed in accordance with animal welfare protocols that had been approved by the Capital Medical University Animal Care and Use Committee. A 1.6-mg portion of the immunogen KLH-DTPA-Hg prepared in 1.6 mL phosphate buffered solution (PBS, 0.15 mol/L, pH 7.4) was mixed with 1.6 mL FCA by the double-push method and subsequently injected into the multipoint of the hypodermic of six BALB/c mice. Every two weeks after that for a total of four rounds, each mouse received 200 μg FIA. One week after the third immunization, tail vein blood was collected for Hg-mAb titer testing by indirect ELISA, with unimmunized mice as the control.

Hybridoma production and Hg-mAb purification

For hybridoma production the mouse that exhibited the highest serum reactivity was selected. Five days before cell fusion, the mouse was "hyperimmunized" by the intraperitoneal injection of 200 μg of KLH-DTPA-Hg diluted in 200 μL of PBS. The SP2/0 cells with high viability and rapid growth were cultured in RPMI 1640 medium supplemented with 15% fetal bovine serum (FBS), 1% nonessential amino acid and 1% penicillin–streptomycin. Then the mouse spleen cells were harvested aseptically, washed in RPMI 1640 medium, and fused with SP2/0 cells at a ratio of 9:1 (spleen/myeloma). The cell pellets were suspended in HAT selective medium (20% FBS + HAT), and then added dropwise to 96-well cell culture plates (100 μL/well) coated with mouse peritoneal cavity cells as feeder cells. The plates were incubated at 37 °C in an atmosphere with 5% CO_2 for 7 days. The HAT medium was changed every other day and switched to HT medium and the complete medium when successive fused. BALB/c mice pretreated with aseptic paraffin were injected into the peritoneal cavity with hybridoma cells. After approximately 10 days, ascites were collected, partially purified by saturated $(NH_4)_2SO_4$ solution and purified through Sephacryl S-300 before being stored at −20 °C. The protein concentration in the purified antibody preparations was determined by the BCA protein assay, and the Hg-mAb was detected by SDS-PAGE. The immunoglobulin subclasses of the antibodies were determined using a mouse monoclonal antibody isotyping kit. Furthermore, the affinity constant (Kaff) of the Hg-mAb was calculated to identify the specificity of Hg-mAb against BSA-DTPA-Hg [11].

Performance for Hg detection in urine

Before detecting Hg in the urine, the Dynabeads M-280 was labeled according to the instrument book by forming a mixture of conjugated Hg-mAb. The total analytical procedure comprised three steps. First, we reacted for 15 min at 37 °C the following: 13 μL of 10 μg/mL p-SCN-Bn-CHX-A″-DTPA, 1 μL of 2.17 mg/mL BSA and 1 μL of 4.6×10^{-4}% TEA in 0.1 mL urine. Next, we performed immune-magnetic separation: 2 μL of anti-Hg-MBs were added to the mixture and incubated for another 15 min at 37 °C. Afterward, the mixture was immune magnetically separated, and then 4 μL of biotinylated anti-BSA and 1 μL of 1 mmol/L QD585-streptavidin were added. The third step was the measurement of fluorescence. Following the final immune-magnetic separation, the Hg that contained QDs and MBs were resuspended in 200 μL of borate buffer solution for the fluorescence measurement (Ex = 405 nm, Em = 585 nm). The calibration curve for Hg was constructed using standards of 1, 10, 100, 500, and 1000 ng/mL in urine. 5, 50, 500 ng of Hg were added to 0.1 mL of normal urine from the occupational poisoning and diseases medical center of Beijing Chaoyang Hospital respectively, the concentrations of Hg in urine were detected three times in one day. The standard addition recovery rate was calculated by the ratio of the measured quantity and the addition quantity. The relative standard deviation (RSD) values were calculated. The specificity of the Hg-mAb produced was investigated by a cross-reactivity (CR) experiment. Various cations, including Pb^{2+}, Hg^{2+}, Mn^{2+}, Bi^{2+}, and Ni^{2+} were selected for CR testing. The standard solutions of the cross-reacting chemicals were prepared in the concentration range of 0.001–1000 ng/mL. The CR was expressed as the percent IC_{50} values based on 100% response of Hg, e.g., $(CR) = IC_{50}(Hg)/IC_{50}$ (for the competing metal) × 100%.