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Magnetic immunoassay coupled with inductively coupled plasma mass spectrometry for simultaneous quantification of alpha-fetoprotein and carcinoembryonic antigen in human serum

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The absolute quantification of glycoproteins in complex biological samples is a challenge and of great significance. Herein, 4-mercaptophenylboronic acid functionalized magnetic beads were prepared to selectively capture glycoproteins, while antibody conjugated gold and silver nanoparticles were synthesized as element tags to label two different glycoproteins. Based on that, a new approach of magnetic immunoassay-inductively coupled plasma mass spectrometry (ICP-MS) was established for simultaneous quantitative analysis of glycoproteins. Taking biomarkers of alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA) as two model glycoproteins, experimental parameters involved in the immunoassay procedure were carefully optimized and analytical performance of the proposed method was evaluated. The limits of detection (LODs) for AFP and CEA were 0.086 μg L^{-1} and 0.054 μg L^{-1} with the relative standard deviations (RSDs, n = 7, c = 5 μg L^{-1}) of 6.5% and 6.2% for AFP and CEA, respectively. Linear range for both AFP and CEA was 0.2–50 μg L^{-1}. To validate the applicability of the proposed method, human serum samples were analyzed, and the obtained results were in good agreement with that obtained by the clinical chemiluminescence immunoassay. The developed method exhibited good selectivity and sensitivity for the simultaneous determination of AFP and CEA, and extended the applicability of metal nanoparticle tags based on ICP-MS methodology in multiple glycoprotein quantifications.

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1. Introduction

Glycosylation is one of the most ubiquitous post-translational modifications for proteins and often plays key roles in a lot of physiological and pathological processes including cell adhesion, signal transduction, tumor immunology, inflammation, and so on [1–3]. Recent researches indicated that tumor progression, cancer metastasis, and other immunodeficiency diseases are likely to be related to the changes of glycans on proteins [4]. As a result, some glycoproteins have been identified as tumor markers for biomedical researches and clinical diagnosis, such as carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), transferring (Tf), prostate specific antigen (PSA), carbohydrate antigen 19-9 (CA19-9), etc. Accurate quantification of these low abundant glycoproteins in biological samples is of great importance for the early diagnosis of cancers and the monitoring of the treatment effect. In particular, simultaneous determination of multiple glycoproteins would be more beneficial since biological samples such as human serum and biopsy tissues are usually precious and limited.

Because of the complex matrix of biological samples and low-level content of target glycoproteins, separation/preconcentration of glycoproteins prior to detection is required. In our previous work, lectin conjugated magnetic nanoparticles (NPs) were applied to capture glycoproteins [5]. Lectins can recognize specific group of carbohydrate and thus be widely used as probe to carbohydrate. Boronic acid is also well known to reversibly react with cis-diols on glycans and form stable complexes in alkaline conditions [6,7]. Unlike lectins' specific non-covalently binding to carbohydrate with certain moiety, boronic acid binds to diols on glycans in a covalent way regardless of the structure of glycans, making it more versatile with a wider range of glycan binding coverage. In addition, as small molecule, boronic acid is more stable than lectins which are proteins [8]. Boronic acid functionalized materials were widely employed for the enrichment of glycopeptides and glycoproteins in molecular mass spectrometry (MS) techniques [9–11]. Shen et al. [12] prepared core–shell boronic acid functionalized NPs SnO_2@Poly (HEMA-co-St-co-VPBA) for selectively enriching glycopeptides, followed by matrix assisted laser desorption/ionization mass spectrometry (MALDI–MS) analysis. Yeap et al. [9] modified detonation nanodiamond with aminophenylboronic acid for the selective capture of glycoproteins. The nonspecific binding of proteins was effectively eliminated by insertion of an alkyl spacer chain to form a molecular

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shell around the nanodiamond, ensuring a good selective binding affinity for glycoproteins. Zhang et al. [11] synthesized a core-satellite-structured composite material, which was composed of a silica-coated ferrite "core" and numerous "satellites" of Au NPs with lots of "anchors" 3-aminophenylboronic acid for capturing target molecules. The composite magnetic material was highly specific and sensitive for the enrichment of both glycosylated peptides and proteins with satisfactory recovery and adsorption capacity.

Molecular tandem MS techniques are powerful for the characterization of glycoprotein structure and identification of glycosylation sites. However, the quantitative determination of glycoprotein in a complex sample is still a challenge. Although a number of promising quantification strategies such as stable isotope tags in synthetic amino acids treated cell culture (SILAC), isotope-coded affinity tags (ICATs), or isobaric tags for relative and absolute quantification (iTRAQ), have been used for quantitative analysis by molecular MS technique [13]. The main limitation was that a lot of standards need to be synthesized to achieve the absolute quantification. The newly developed elemental tags based inductively coupled plasmamass spectrometry (ICP-MS) methods provide an alternative ofmolecular MSfor quantitative analysis of biomolecules, and are able to simultaneously measure multi-proteins [14–21]. ICP-MS based assays are featured with simple quantification concepts, high sensitivity, low matrix effects, large dynamic range, and outstanding ability for multiple elements analysis. With various elemental tags including endogenous heteroatom S, P, Se etc. or exogenous metal chelates, metal-containing polymers or metal NPs, ICP-MS based approach is prosperous for protein quantification in recent years with the merits of absolute quantification, high sensitivity, multi-protein measurement [22–26]. Lelpold et al. [27] labeled multiple lectins with lanthanide-chelating polymers, and developed a multiplexed lectin-based assay to investigate the glycosylation patterns on a panel of glycoproteins. It indicated that the application of ICP-MS could be extended in glycoprotein study, showing the potential to identify individual bacterial cells as well as to investigate differences in cellular glycosylation between diseased and nondiseased cells.

In this article, we reported an ICP-MS based magnetic immunoassay for the simultaneous quantification of glycoproteins in complex biological samples (using AFP and CEA as model glycoproteins), by using boronic acid functionalized magnetic for glycoproteins capture and AuNP and AgNP antibodies for labeling. The sensitivity of the method will be improved by using metal NPs as element tags which contains more atoms than metal-chelates or metal-contained polymers. Boronic acid functionalized magnetic beads can selectively capture different kinds of glycoproteins in one round and separate them from the complex matrix of biological samples, which is more convenient and lesser cost than using different kinds of antibodies conjugated magnetic beads. After the optimization of the whole magnetic immunoassays, the developed method was applied for the simultaneous quantification of AFP and CEA in human serum.

2. Experimental

2.1. Instrumentations

An X Series II quadrupole (Q) ICP-MS (Thermo Fisher Scientific, MA, USA) was used for the determination of silver (Ag^{107}) and gold (Au^{197}) with integral parameter mode. Further details of the instrumental parameters are given in Table 1. An Agilent 7500a ICP-MS (Agilent, Tokyo, Japan) with a Babington nebulizer was also applied in the optimization of experimental parameters.

The morphology of Ag NPs, Au NPs and magnetic beads was characterized by transmission electron micrograph (TEM) using an EM2010 electron microscope (JEOL, Tokyo, Japan). Fourier transform infrared (FT-IR) spectra (4000–400 cm^{-1}) in KBr were recorded on a Magna-560 spectrometer (Nicolet, WI, USA). Magnetic properties of the materials were characterized by a PPMS-9 vibrating sample magnetometer (Quantum Design Inc, CA, USA). An Nd–Fe–B magnet (15.0 cm × 6.0 cm × 1.6 cm) purchased from a local market (Wuhan, China) was used for magnetic separation. The absorption spectra were recorded on a UV-2550 UV–Vis spectrophotometer (Shimadzu, Kyoto, Japan). An XS105 Dual Range microbalance (Mettler Toledo Instruments Co. Ltd., Shanghai, China) and BS110S electronic balance (Beijing Sartorius Instrument Systems Inc., Beijing, China) were used for reagents weighing.

Table 1
Operating parameters of ICP-MS with a micro-concentric nebulizer.

ICP-MS	Parameters
Rf power	1400 W
Plasma gas flow	14 L min^{-1}
Auxiliary gas flow	0.8 L min^{-1}
Nebulizer gas flow	0.82 L min^{-1}
Sampling depth	117 steps
Sampler/skimmer diameter orifice	Nickel 1.1 mm/0.7 mm
Peak pattern	Peak-jumping
Dwell time	100 ms
Integration mode	Peak area
Monitored isotope	Au^{197} Ag^{107}

2.2. Standard solutions and reagents

Human AFP antigen, human CEA antigen, monoclonal mouse anti-human AFP antibodies and anti-human CEA were all purchased from Shanghai Linc-Bio Science Co. Ltd. (Shanghai, China). Bovine serum albumin (BSA, 97%) was purchased from Beijing Biosynthesis Biotechnology Co. Ltd. (Beijing, China) and skimmed milk was purchased from BD Biosciences (NY, USA). Horse radish peroxidase (HRP), ovalbumin (OVA, 98%) and 4-mercaptophenylboronic acid (4-MPBA, 90%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human serum samples were provided by Zhongnan Hospital of Wuhan University (Wuhan, China), they do not have any identifying information about the participants that were included in this study. The ethics committee of Zhongnan Hospital of Wuhan University reviewed and approved the informed consent forms provided by all participants according to ethics requirements.

The phosphate buffer solution (PBS, pH 7.4) consisted of 0.01 mol L^{-1} phosphate buffered saline. Blocking solution was 1% (w/v) skimmed milk in PBS (pH 7.4). Tetraethoxysilane (TEOS) and γ-mercaptopropyltrimethoxysilane (γ-MPTS) were purchased from Chemical Plant of Wuhan University (Wuhan, China). $AuCl_4 \cdot 4H_2O$ was obtained from Shanghai Chemical Reagent Co. (Shanghai, China). $FeCl_3$, $AgNO_3$, sodium acetate, poly(ethylene glycol) (PEG, MW 2000) and formic acid were of analytical grade and purchased from Sinopharm Chemistry Reagent Co. Ltd (Shanghai, China). High purity deionized water (18.25 MΩ·cm, Milli-Q Element, Millipore, Mulheim, France) was used throughout this work.

2.3. Synthesis of boronic acid functionalized magnetic beads (MBs)

The procedure for the synthesis of boronic acid functionalized MBs is illustrated in Fig. 1. The Fe_3O_4 MBs were prepared via a solvothermal method with slight modification [28]. Briefly, 13.5 g of anhydrous sodium acetate and 2 g of PEG (MW 2000) were dissolved in 120 mL glycol, and then mixed with 5.06 g of $FeCl_3 \cdot 6H_2O$ in 30 mL glycol under mechanical stirring. Then the mixture was stirred vigorously for 30 min and transferred into a Teflon-lined autoclave and reacted at 200 °C for 12 h. After cooling down to room temperature, the resulting black Fe_3O_4 MBs were recovered by a magnet, washed with ultrapure water and ethanol and stored in ethanol for further use.

The silica-coated MBs were prepared via a sol–gel method. Fe_3O_4 MBs (0.75 g) were dispersed in a mixture of ethanol (150 mL) and ultrapure water (12 mL) under ultrasonication for 5 min. Concentrated

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