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# A sensitive label-free immunosensor for detection α-Fetoprotein in whole blood based on anticoagulating magnetic nanoparticles

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ABSTRACT

Accurate values of tumor markers in blood play an especially important role in the diagnosis of illness. Here, based on the combination of three techniques include anticoagulant technology, nanotechnology and biosensing technology, a sensitive label-free immunosensor with anti-biofouling electrode for detection α-Fetoprotein (AFP) in whole blood was developed by anticoagulating magnetic nanoparticles. The obtained products of $Fe_3O_4$-ε-PL-Hep nanoparticles were characterized by fourier transform infrared (FT-IR) spectra, transmission electron microscopy (TEM), ζ-potential and vibrating sample magnetometry (VSM). Moreover, the blood compatibility of anticoagulating magnetic nanoparticles was characterized by *in vitro* coagulation tests, hemolysis assay and whole blood adhesion tests. Combining the anticoagulant property of heparin (Hep) and the good magnetism of $Fe_3O_4$, the $Fe_3O_4$-ε-PL-Hep nanoparticles could improve not only the anti-biofouling property of the electrode surface when they contact with whole blood, but also the stability and reproducibility of the proposed immunosensor. Thus, the prepared anticoagulating magnetic nanoparticles modified immunosensor for the detection of AFP showed excellent electrochemical properties with a wide concentration range from 0.1 to 100 ng/mL and a low detection limit of 0.072 ng/mL. Furthermore, five blood samples were assayed using the developed immunosensor. The results showed satisfactory accuracy with low relative errors. It indicated that our developed immunoassay was competitive and could be potentially used for the detection of whole blood samples directly.

## 1. Introduction

The keys to successful cancer therapies with better prognoses are early detection and accurate diagnostics. Biomarkers, which are defined as “molecules that can be objectively measured and evaluated as indicators of normal or disease processes and pharmacologic responses to therapeutics” by the U.S. National Institutes of Health (NIH), have great potential to play as a general tool for the effective cancer diagnostics. α-Fetoprotein (AFP), an oncofetal glycoprotein, is an important indication of liver cancer for the early diagnosis of the patients (Murray et al., 2016; Shimakata et al., 2016). For the healthy human, the level of AFP concentration is less than 25 ng/mL (Bader et al., 2004; Everman et al., 2000). But the value increases rapidly in case of pathological changes of liver. Therefore, the development of simple, rapid, sensitive and selective methods for the measurements of biomarkers has received more and more interest.

Until now, numerous techniques such as enzyme-linked immune assay (ELISA) (Gong et al., 2016; Wang et al., 2008), chemi-luminescence (Qian et al., 2010; Wenisch et al., 2014) and mass spectrometry (Crutchfield et al., 2016; Schwamborn, 2012) have been widely used for determination biomarkers in biological fluids because of good sensitivity. However, it suffers the drawbacks of labor-intensive and time-consuming. So it is necessary to develop a novel detection method of biomarkers.

Considering of benefits such as low-cost, high sensitivity, low detection limit and fast response, electrochemical immunosensors have received a lot of attention in the past few years (Chikkaveeraiah et al., 2012; Salimi et al., 2013; Stofik et al., 2009). It is based on specific interaction between antigen and antibody, and possesses the properties such as convenience and high-throughput. To avoid nonspecific adsorption of cells, proteins or other biofouling on the electrode surfaces, traditional methods always need to centrifuge the whole

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blood in advance to obtain the serum samples (Chen et al., 2013; Rivera et al., 2012; Zhang et al., 2015). This approach not only needs complex centrifugal equipment, but also wastes lots of time to prepare samples. Even more, the detection results in serum cannot express the real situation in whole blood. Hence, it is of great significance to detect the blood analyte in the whole blood.

However, it is not easy to detect the analyte in the whole blood *via* an electrochemical biosensor. When the biosensor is directly employed in whole blood, the formed biofouling on the electrode surface will act as insulators blocking the electron transfer between the probe and the electrode (Sun et al., 2013a, 2013b). Therefore, it is important to form an anti-biofouling electrode surface, which can be obtained by the anti-biofouling technique. In this paper, a novel kind of anticoagulating magnetic nanoparticles (NPs) that composed of heparin (Hep) and $Fe_3O_4$ were designed and fabricated, and used to decorate the electrode surface for detecting the AFP in the whole blood. Hep is one of the most well-known anticoagulants (Lin et al., 2004). And lots of researches based on $Fe_3O_4$ have been carried out benefited from its magnetic responsivity (Lou et al., 2012; Rostamnia et al., 2015). Whether the combination of the biocompatibility of Hep and the good magnetism of $Fe_3O_4$ could improve not only the anti-biofouling then avoid the electrode pollution, but also improve the stability and reproducibility? That is the subject of this research. At the same time, the electrochemical performance of the novel electrode we proposed for detect of AFP in the whole blood was also investigated.

## 2. Materials and methods

### 2.1. Materials

α-Fetoprotein antibody (anti-AFP) and α-Fetoprotein (AFP) were obtained from Beijing Xinkezhongjing Biological Technology Co. Ltd. (China). Porcine heparin (Hep) (Mw 8000–12000), cysteine (Cys), uric acid (UA), carcino embryonie antigen (CEA), bovine serum albumin (BSA) and β-D-(+)-Glucose (Glu) were purchased from Sigma-Aldrich Co. Ltd. (USA). ε-Polylysine (ε-PL) (MW 3500–5000) was purchased from GL Biochem Ltd. (China). Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), sodium hydroxide (NaOH), ethylene glycol (EG) and polyethylene glycol (PEG) were purchased from Sinopharm Chemical Reagent Co. Ltd (China) and used as received. All solutions were prepared by twice-distilled water. All other chemicals and reagents were of analytical grade.

### 2.2. Synthesis of the anticoagulating magnetic nanoparticles

The magnetic nanoparticles were synthesized directly through a traditional hydrothermal method with some modification. Briefly, $FeCl_3 \cdot 6H_2O$ (1.35 g, 5 mmol), NaOH (0.6 g, 15 mmol), and $PEG_{10000}$ (1 g) were dissolved in EG (40 mL) and quickly stirred for 30 min until the solution become clear. After that, the mixture was transferred into a Teflon-lined stainless-steel autoclave. Next, the oven was heated to 200 °C. After reaction for 8 h, the oven was cooled to room temperature. The black mixture was washed several times with water and ethanol, followed by being collected with a magnet. Finally, the magnetic nanoparticles were dried under vacuum (Cao et al., 2008).

Hep surface-imprinted magnetic nanoparticles were prepared as follows: ε-PL (40 mg) was dissolved in 40 mL of phosphate buffer saline (PBS, pH=7.4), and combined with $Fe_3O_4$ (400 mg). After the mixture being stirred for 2 h at 25 °C, Hep (200 mg) was added into it under stirring and reaction for 8 h continuously. Similarity, the products were washed with water for three times followed by being collected and dried.

### 2.3. Characterization of the anticoagulating magnetic nanoparticles

The structures of the anticoagulating magnetic nanoparticles we prepared were determined by Fourier transform infrared (FT-IR) spectra using a Cary 5000 Fourier transform infrared spectrophotometer (VARIAN, USA). Transmission electron microscopy (TEM) was carried out to study the morphologies of the anticoagulating magnetic nanoparticles with an H-7650 interface high-resolution transmission electron micro (HITACHI, Japan). The particle size and ζ-potential of the samples were also measured by dynamic light scattering (DLS). The hysteresis loop of the samples was obtained on Vibrating Sample Magnetometer (VSM, Lakeshore 7404, USA) at room temperature.

### 2.4. In vitro Hep release studies from the anticoagulating magnetic nanoparticles

To carry out the Hep release studies from the anticoagulating magnetic nanoparticles, the anticoagulating magnetic nanoparticles (2 mg) were dispersed in 20 mL of PBS and dispersed by ultrasonic for 1 min. Then the dispersed anticoagulating magnetic nanoparticles were moved into the dialysis membrane (Spectra/Por CE, MWCO=3000) which was immersed in 50 mL of PBS and dialysis under 37 °C. At set intervals (1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 36 h, 48 h, and 60 h), 30 mL dialysate was moved out and the same volume of PBS was added. Finally, the concentration of Hep was detected by toluidine blue O (TBO) (Li et al., 2011).

### 2.5. Blood compatibility of the anticoagulating magnetic nanoparticles

Blood compatibility of these anticoagulating magnetic nanoparticles was identified by carrying out *in vitro* coagulation tests, hemolysis assay and whole blood adhesion tests.

*In vitro* coagulation tests include three tests: activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) (Eerenberg et al., 2011). Different concentrations of magnetic nanoparticles were incubated in 1.5 mL platelet-poor plasma at 37 °C for 1 h , followed by investigating with a Semi automated Coagulometer (RT-2204C, Rayto, USA).

Hemolysis assay was worked with a microplate reader (Chen et al., 2004). Briefly, 2% red blood cells (RBCs) suspension was prepared with the PBS. Then RBCs suspension (1 mL) was added to the anticoagulating magnetic nanoparticles solutions (1 mL) at the certain concentration in tubes. All the tubes were incubated in static condition at 37 °C for 1 h. Next, the mixtures were centrifuged at 1500 r/min for 10 min, and supernatants (200 μL) were transferred into a 96-well plate. The absorbance values at 570 nm were chosen to expression the results. The calculation formula was as follow.

$$\text{Percent hemolysis}(\%) = \left(\frac{\text{sample absorbance} - \text{negative control absorbance}}{\text{positive control absorbance} - \text{negtive control absorbance}}\right) \times 100$$

Whole blood adhesion tests were carried out according to the literature (Mao et al., 2009). The surface sections of blank substrate and the anticoagulating magnetic nanoparticles modified substrate were placed into the 24-well microplates after equilibration for 24 h in PBS. Then 1 mL whole blood was added on the substrates, and the substrates were incubated at 37 °C for 30 min. After being rinsed with PBS and fixed with 2.5% glutaraldehyde, the samples were dehydrated with ethanol/water solutions (50%, 60%, 70%, 80%, 90%, 95% and 100% of ethanol) for 30 min each and air dried. The samples were coated with gold and observed under scanning electron microscope (SEM, JEOL JSM-6300, Japan) in the end.

### 2.6. Preparation of the anticoagulating lable-free immunosensor

The schematic diagram of the electrochemical immunosensor which