Supplementary Material (ESI) for Analyst
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Supporting Information

The potential of magnetic nanocluster and dual-functional protein-based strategy for noninvasive detection of HBV surface antibodies

Hengyao Hu[1]，Hao Yang[1], Ding Li[2], Kan Wang[1], Jing Ruan[1], Xueqing Zhang[1], Jun Chen[1], Chenchen Bao[1], Jiajia Ji[1], Donglu Shi[1,3], Daxiang Cui[1]*

Experimental

Materials

EG (Ethylene glycol), ferric chloride, citric acid, anhydrous sodium acetate, polyvinya alcohol (PVA), bovine serum albumin (BSA), Isopropyl-l-thio-β-d-galactopyranoside (IPTG), sodium borohydride and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) were obtained from Sigma. IgG Sepharose 6 Fast Flow was obtained from GE Healthcare Company. Recombinant HBsAg and anti-HBs monoclonal antibodies were provided by Center of Biological Diagnosis and Therapy of No. 261 Hospital of PLA. Oral fluid sample buffer and oral fluid sample collection swabs were the components in Aware™ HIV-1/2 OMT Test kit from Beijing Calypte Biomedical Corporation. Dual-labeled double chain nucleic acid substrates were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. The sequences were as follows: FAM-5'-AAACATGATCAAGCCC-3'-Eclipse and 5'-GGGCTTGATCATGTTT-3' as the complementary chain and the restriction

enzyme cutting site were underlined in each sequences. pET Duet-1 vector and *E coli* BL21(DE) strain were purchased from Merck KGaA. Other materials and chemicals unmentioned were purchase domestically.

Collection and simulation of OMT specimens

The OMT specimens were collected according to the instruction of Aware™ HIV-1/2 OMT Test. In our test, 20 specimens from the subjects for healthy examination whose sera were negative for infection of Hepatitis B (HBV), Hepatitis C (HCV), *Trepanema pallidum,* and Human Immunodeficiency Virus (HIV) were collected and mixed in one 50 ml tube. The mixture was used to simulate diseased specimens by adding different amount of anti-HBs monoclonal antibodies to a final concentration of 0 ng/mL, 0.1 ng/mL, 0.5 ng/mL, 2.5 ng/mL, 12.5 ng/mL, 62.5 ng/mL, and 125 ng/mL respectively. All work with human OMT was approved by Ethics Committee of both Shanghai Jiao Tong University and No. 261 Hospital of PLA.

Preparation of functionalization of magnetic nanocluster

0.1 M $FeCl_3 \bullet 6H_2O$, 0.001 M citric acid and 0.05M polyvinyl alcohol (PVA) were completely dissolved in 35 mL ethylene glycol by the aid of ultrasonication. The solution was sealed in a 50 mL Teflon lined stainless-steel autoclave and then heated at 200 °C for 10 h. After cooling down to the room temperature, the black sediment was separated magnetically and washed with ethanol and deionized water for 3 times respectively to eliminate organic and inorganic impurities, and then dried in a vacuum

at 60 °C. A JEOL2010 transmission electron microscope (TEM) and JEOL scanning electron microscope (SEM) were used for taking images of MNCs. X-Ray diffraction (XRD) was used to confirm the crystalline phase of MNCs. Fourier transform infrared (FTIR) spectra were obtained using a PerkinElmer spectrum 100 to testify the adsorption of carboxyl groups on the MNCs surface. The mass fraction of magnetite in MNCs powder was determined by a Mettler Toledo TGA/DSC 1/1600 theromgravimetric analyzer, by heating MNCs powders from 30 °C to 1000 °C at 10 °C/min under nitrogen flow. Magnetization of MNCs powders was carried out on a Lakeshore 7300 vibration sample magnetometer (VSM).

Coupling of magnetic nanocluster with recombinant HBsAg

20 mL of 1 mg/mL MNCs and 20 mL of 1 mg/mL HBsAg were mixed respectively, and then 2 mL 1 mg/mL EDC were added and blended by pipetting up and down. Meanwhile, 2 mL de-ionized water was taken as control of coupling. The resulting solution reacted at the room temperature for 3 h with continuous mixing in roller mixer and then separated magnetically. BSA was added into the solution at a concentration of 1 mg/mL and incubated at the room temperature for 3 h. The HBsAg-labelled MNCs were then separated magnetically and the supernatant was discarded. 20 mL PBS [Phosphate-Buffered Saline (PBS); 0.2 mg/mL KCl, 1.44 mg/mL Na_2HPO_4 , 0.24 mg/mL KH_2PO_4 , 8 mg/mL NaCl, pH 7.4] with 0.5 % tween-20 (v/v) and 1 % BSA was used to resuspend and wash HBsAg-labeled MNCs for 3 times. The HBsAg-labelled MNCs were finally dispersed in 20 mL PBS with 0.5 % tween-20 (v/v) and 0.5 % BSA and kept at 4□ until use. The coupling

efficiency was obtained by measuring the OD (Optical Density) at 280 nm of pre- and post-coupling HBsAg solution.

Design and construction of dual-functional protein

The design of dual-functional protein is shown in Scheme 2. The coding sequence includes 2 synthetic "ZZ" domains based on the "B" IgG binding domain of protein A, linker and Mbo I endonuclease. [1-3] The designed sequence was synthesized by Shanghai Generay Biotechnology Company and cloned into pET Duet-1 vector at Multiple Cloning Sites (MCS)-1 between Bam H I and Not I restriction enzyme cutting sites. In order to protect the nucleic acid of the expression strain, we also inserted the sequence of Mbo I methyltransferase A into the MCS-2 between Nde I and Xho I restriction enzyme cutting sites.[4] The constructed pET Duet-1 vector was characterized with corresponding restriction enzyme and named pET Duet-1 DP.

Expression and identification of dual-functional protein

pET Duet-1 DP was transformed into BL21 (DE3) expression strain and inoculated in 10 mL LB broth supplemented with 50 μg/mL ampicillin. Overnight cultures were transferred to 500 mL of the same medium and cultivated at 37°C until OD_{600} of 0.8. IPTG was added to a final concentration of 1 mM for induction. After that, cells were harvested by 3, 000×g centrifugation for 15 min at 4°C, and the pellet was washed once with 50 mM NaH_2PO_4 (pH 8.0). Cells were resuspended in 50 mM Tris (pH 7.5) and disrupted by sonication on ice. Cell debris was separated from supernatant by 10, 000×g centrifugation for 20 min at 4°C to obtain the crude extract. The crude extract containing the dual-functional protein was purified by immuno-affinity column filled

with IgG Sepharose 6 Fast Flow. Recombinant protein was eluted with 0.5 M lithium diiodosalicylate dissolved in sterile deionized water. Eluted protein was dialyzed against 10 mM Tris-HCl (pH7.5) and 1 mM DTT(DL-Dithiothreitol), Purity was determined by SDS polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out in gels containing 12% (w/v) polyacrylamide according to standard protocols using Bio-Rad Mini-PROTEAN® equipment. Gels were stained with Coomassie Brilliant Blue R-250 and destained with methanol: acetic acid: water (5:1:4 v:v:v).

Protein concentration was determined using a Beijing Tiangen® protein quantitative assay kit with BSA as standard. The endonuclease activity of purified dual-functional protein was assayed by endonuclease digestion of known 525 bp gene fragment of *Helicobacter pylori* CagA gene kept in our lab (Genbank Accession: DQ011620, from nt 844 to nt 1370), which possesses 3 Mbo I cutting sites at nt 849, nt 1175 and nt 1323 respectively. The digestion reaction system was approximately 200 ng 525 bp CagA gene fragment in 20 ul reaction buffer (10 mM Tris-HCl (pH8.5), 10mM $MgCl_2$, 100mM KCl and 0.1mg/mL BSA) with 10 U of commercial Mbo I endonuclease and 100 ug of dual-functional protein. The mixture was incubate at 37°C for 1h. 5 ul of resultant product was used for 1% agarose gel electrophoresis.

Detection of OMT specimens

In a typical assay process, 1 mL of OMT sample was mixed with 1 mg MNCs and incubated at 37 °C for 30 min. After the MNCs were washed by TS buffer [10 mM Tris, 150 mM NaCl, pH 8.0] with 0.05% Tween-20, 1 mL of 100 ug/mL

dual-functional protein was used to redisperse MNCs. The mixture was incubated at 37 °C for 30 min and washed by TS buffer with 0.05% Tween-20. 5 pmol of dual-labeled double chain nucleic acid substrates dissolved in 200 uL of reaction was used to redisperse MNCs and incubated at 37 °C for 1 h. The reaction was stopped by magnetic separation and the supernatant was used to analyze fluorescence intensity. The signal amplification of reaction was observed by measuring the fluorescence intensity of 520 nm at 0, 20, 40, 60, 80 and 100 min.

Result

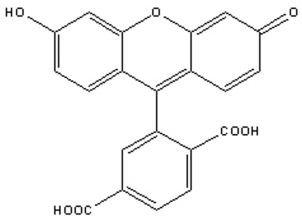
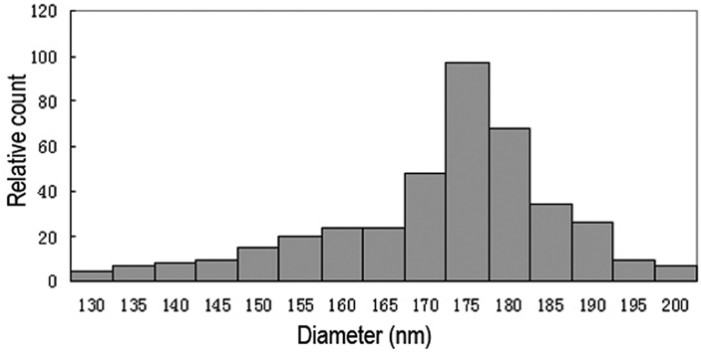


Figure S1 Structure of 6-FAM dye



Average diameter: 172nm size variation: ± 14.06 (±8.2%)

Figure S2 Size distribution of as-prepared magnetic nanoclusters

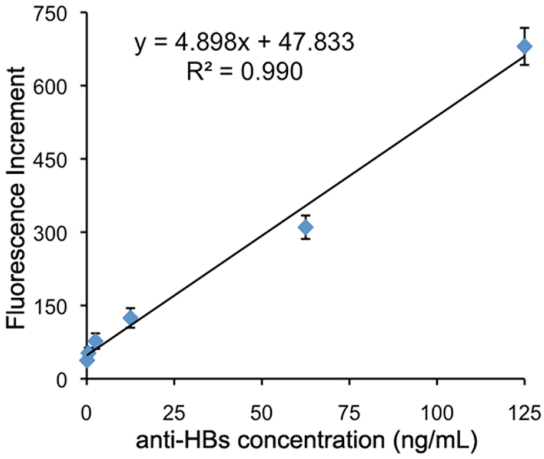
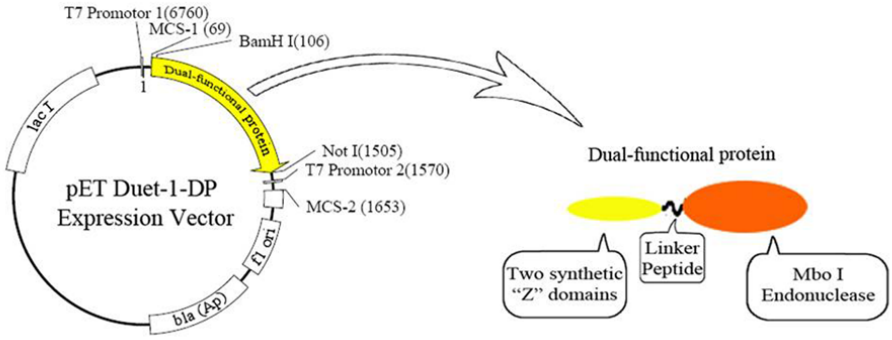


Figure S3 Linear correlation between anti-HBs concentration and fluorescent intensity. Anti-HBs concentration range from 0.1 ng/mL to 125 ng/ mL

OMT samples were mimic ones, the experiment data of digestion for 1h are added in supporting materials as follow:

Table1 Result of OMT simulated samples by using established method

Anti-HBs concentration (ng/mL)	0	0.1	0.5	2.5	12.5	62.5	125
Fluorescent Intensity at 520 nm	426±7	471±12	455±13	485±9	532±16	714±21	1065±33



Scheme 2. Schemetics of dual-functional protein.

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