Bienzyme functionalized three-layer composite magnetic nanoparticles for electrochemical immunosensors

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Abstract

The preparation, characterization and application of a three-layer magnetic nanoparticle composed of an Fe3O4 magnetic core, a Prussian Blue (PB) interlayer and a gold shell (it can be abbreviated as Au–PB–Fe3O4) for an ultrasensitive and reproducible electrochemical immunosensing fabrication were described for the first time in this work. With the employment of the Au–PB–Fe3O4 nanoparticle, a new signal amplification strategy was developed based on bienzyme (horseradish peroxidase and glucose oxidase) functionalized Au–PB–Fe3O4 nanoparticles for an electrochemical immunosensing fabrication by using Carcinoembryonic antigen (CEA) and α-fetoprotein (AFP) as model systems, respectively. The experiment results show that the multilabeled Au–PB–Fe3O4 nanoparticles exhibit satisfying redox electrochemical activity and high enzyme catalysis activity, which predetermine their utility in high sensitivity antibody detection schemes. Furthermore, this immunosensor could be regenerated by simply using an external magnetic field which ensured a reproducible immunosensor with high sensitivity.

1. Introduction

Developing the methodology of immunomolecules recognition units that reveal improved reproducibility and enhanced sensitivity is crucial for early diagnosis such as tumour and infectious diseases and also a great challenge for researchers [1,2]. As the interaction between the antigen and antibody is often inadequate to generate a highly sensitive signal for the direct electrochemical measurement, great progress is being made by using enzymes, DNA fragment, metal or semiconductor nanoparticles or inorganic metal catalysts conjugated to biorecognition events as amplifying labels [3,4]. Wang et al. [5] demonstrated a powerful bioelectronic protocol for the amplified electrical detection of proteins based on the use of DNA-functionalized polystyrene (PS) spheres as an amplified signal reporter. Haesik Yang’s group [6,7] reported a DNA-free ultrasensitive electrochemical immunosensing for signal amplification which was achieved by catalytic reduction of p-nitrophenol (NP) to p-aminophenol (AP) using gold nanocatalyst labels in the presence of NaBH4. Rushing and co-workers [8] developed a carbon nanotube amplification strategy using single-wall carbon nanotube (SWNT) forest platforms with multi-label secondary antibody–nanotube bioconjugates for highly sensitive detection of a cancer biomarker. In this perspective, a challenging topic of sensitive electrochemical immunoassay fabrication is aiming at the development of low-cost and uniform labeling and simple, nontoxic and reproducible assay system.

With their unique property—superparamagnetism, magnetic nanoparticles have become attractive for exploitation mainly in biology and medicine because they can simplify the process of proteins immobilization and separation [9]. However, due to the magnetic dipolar attraction, unmodified magnetite nanoparticles incline to aggregate into clusters and inhibit the advantage of the specific properties when they are directly exposed to the biological system [10], as a result a number of biomaterial-functionalized magnetic particles (e.g., magnetic-core/porous-shell CoFe2O4/SiO2 composite nanoparticles, which have been reported in our previous work [11,12]) have been extensively applied in a broad variety of bioelectronic applications [13]. Among them, the Prussian blue (PB) modified Fe3O4 might be greatly widened use as it combined the advantages: (1) as “artificial peroxidase” because of PB analogy with the biological family of peroxidase it can effectively catalyse the reduction of H2O2 [14]; (2) for PB’s good electrochemical behavior, which could accelerate the electron transfer between the electrode and the enzymes, it attracts the attention of the biosensor.
community [15]; (3) due to the good hydrophilic and biocompatible properties for the magnebite core of Fe3O4, it might be considered as an ideal candidate for biological applications such as a tag for sensing and imaging [16], a drug-delivery carrier or an activity agent for medical diagnostics [17]. On the other hand, gold nanoparticle is a kind of well known bio-nanomaterials because of their large specific surface area, excellent biocompatibility, strong adsorption ability, well suitability and good conductivity [18,19]; thus, multilayered nanoparticle with the structure of magnetic property core and biocompatible gold shell is an even more attractive composite system which can be stabilized more efficiently in corrosive biological conditions and readily functionalized through the well developed Au–S chemistry [20,21]. In view of the advantageous features of PB modifying and gold coating nanoparticles, herein, we present, for the first time, a new three-layer composite magnetic nanoparticle which was composed of an Fe3O4 magnetic core, a PB interlayer and a gold shell (it can be abbreviated as Au–PB–Fe3O4). Then we demonstrate an amplification strategy based on the feasibility of bienzyme, horseradish peroxidase (HRP) and glucose oxidase (GOD), functionalized three-layer Au–PB–Fe3O4 nanoparticles for an ultrasensitive and reproducible electrochemical immunosensing fabrication by using Carcinomembrionic antigen (CEA) and α-fetoprotein (AFP) as model systems, respectively (The cancer markers CEA and AFP are both the most important tumour markers from patients with gastrointestinal malignancies and hepatocellular carcinoma). The immunoassay was conducted by following the typical procedure for sandwich-type immunoreactions. Significantly, greatly amplified sensitivity was achieved by using bioconjugates featuring GOD and HRP labels and secondary antibodies linked to three-layer Au–PB–Fe3O4 nanoparticles, which exhibit attractive redox electrochemical activity and high enzyme catalysis activity when the modified electrode was detected in the working buffer containing proper amounts of glucose. Besides, comparing with the employment of hydrogen peroxide as enzyme catalysis substance, the presence of glucose, because of its inert electrochemical property, in the electrolytic cell might improve the stability and prolong the lifetime of the immunosensor.

2. Materials and methods

2.1. Reagents and materials

AFP and CEA antibody and antigen were purchased from Biocell Company (Zhengzhou China). Gold chloride ([AuCl4]−), bovine serum albumin (BSA, 96–99%), sodium citrate, chitosan, HRP and GOD were obtained from Sigma Chemical Co. (St. Louis, MO, USA); N-hydroxy succinimide (NHS) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimidehydrochloride (EDC) were purchased from Shanghai Medpep Co. Ltd (Shanghai, China). Other chemicals and solvents used were of analytical grade and were used as received. Double distilled water was used throughout this study. Phosphate buffered saline (PBS) (pH = 6.86) were prepared using 0.01 M Na2HPO4 and 0.01 M KH2PO4. The prepared solutions were kept at 4 °C for half an hour. The mean size of the prepared Au colloids was about 1–3 nm, which was estimated from transmission electron microscopy (the graph not shown).

2.2. Apparatus

Cyclic voltammetric (CV) measurements were carried out with a CH 660C electrochemistry workstation (Shanghai CH Instruments, China). A three-compartment electrochemical cell contained a platinum wire auxiliary electrode, a saturated calomel reference electrode (SCE) and the modified gold electrode (Φ = 4 mm) as working electrode. X-ray photoelectron spectroscopy (XPS) measurements were carried out using a VG Scientific ESCALAB 250 spectrometer, using Al Kx X-ray (1486.6 eV) as the light source. The sizes of nanoparticles were estimated from transmission electron microscopy (TEM) (J6000, Hitachi Instrument, Japan). The pH measurements were made with a pH meter (MP 230, Mettler-Toledo Switzerland) and a digital ion analyzer (Model PHS-3C, Dazhong Instruments, Shanghai, China). The AC impedance of the immunoelectrode membrane was measured with a Model IM6e (ZAHNER Elektrick, Germany).

2.3. Preparation of three-layer composite magnetic nanoparticle Au–PB–Fe3O4

The Fe3O4 nanoparticles were first prepared by the co-precipitation of Fe(II) and Fe(III) under a base condition according to the literature [22] with slight modification, briefly, 5.0 mL of iron ion solution containing Fe2+ and Fe3+ with the mole proportion of 1:2 was added dropwise into 50 mL 2 mol/L NaOH under vigorous mechanical stirring for 30 min at 80 °C. The precipitate was washed and collected by centrifugation and drying, then a further 10 mL of 0.10 mol/L NaOH under vigorous mechanical stirring for 30 min at 80 °C. The precipitate was washed and collected by centrifugation and drying. Next 0.10 mol/L FeCl3 solution was added drop by drop with the help of a slight excessive H2O2. The resultant mixture was continuously stirred for 30 min at 80 °C. The precipitate was washed and collected by centrifugation and drying. Then the PB–Fe3O4 nanoparticles were prepared using the Fe3O4 nanoparticles suspended solution (100 mg/mL) as the seeds under stirring. Next 0.10 mol/L K3[Fe(CN)6] containing 10 mmol/L HCl was added and stirred to form a mixed solution, then a further 10 mL of 0.10 mol/L FeCl3 solution was added drop by drop with the help of a slight excessive H2O2. The resultant mixture was continuously stirred for 30 min at 80 °C. Then the precipitate was collected by centrifugation and dried. Then the Au nanoparticles seeds coating Au–PB–Fe3O4 nanoparticles were prepared using the Fe3O4 nanoparticles suspended solution (100 mg/mL) as the seeds under stirring. Next 0.10 mol/L K3[Fe(CN)6] containing 10 mmol/L HCl was added and stirred to form a mixed solution, then a further 10 mL of 0.10 mol/L FeCl3 solution was added drop by drop with the help of a slight excessive H2O2. The final mixture was continuously stirred for 30 min at 80 °C. The precipitate was collected by centrifugation and dried.

Fig. 1. Schematic illustration of the functionalized magnetic particles fabrication process. (a) Fe3O4 nanoparticles, (b) PB–Fe3O4 nanoparticles, (c) the Au nanoparticle seeds coating PB–Fe3O4 nanoparticles and (d) Au–PB–Fe3O4 nanoparticles.
stirred for about 30 min and then collected and removed from the solution with an external magnetic field. Subsequently, the PB–Fe₃O₄ nanoparticles was surface-chemically modified by bovine serum albumin (BSA) to obtain the amido and disulfide groups modified PB–Fe₃O₄ nanoparticle (The BSA protein contains 60 amino moieties in lysine residues, 26 arginine moieties in guanidino side chains and 17 disulfide bonds with one free thiol in cysteine residues, which may be attracted to the nanoparticles for surface binding via thiolate linkages [23]). Finally the PB–Fe₃O₄ particles covalently attract 1–3 nm Au nanoparticle seeds which act in a next step as

![Diagram of immunosensor fabrication process](image)

**Fig. 2.** Schematic drawing of the immunosensor fabrication process. (a) Dropping of chitosan–nanoAu hydrogel membrane; (b) immobilizing of anti-CEA; (c) the immunoreaction of CEA and anti-CEA; (d) incubation of the solution containing multilabeled three-layer composite magnetic conjugate resulting in an amplified signal-generating detection; (e) separation of the immunocomplex using an external magnetic field permits the regeneration of the immunoosensor.

![TEM images of nanoparticles](image)

**Fig. 3.** TEMs of the different nanoparticles. (a) Fe₃O₄ nanoparticles, (b) PB–Fe₃O₄ nanoparticles, (c) the Au nanoparticle seeds coating PB–Fe₃O₄ nanoparticles and (d) Au–PB–Fe₃O₄ nanoparticles.
nucleation sites for the formation of a continuous gold outer layer by citrate reduction of Au\(^{3+}\) to Au\(^0\). Fig. 1 shows the schematic illustration of the functionalized magnetic particles fabrication process.

2.4. Preparation of HRP, GOD and antibody multilabeled Au–PB–Fe\(_3\)O\(_4\)

Immobilization of the HRP, GOD and antibody biomolecules onto the Au–PB–Fe\(_3\)O\(_4\) nanoparticle is completed in a single-step process according to the procedure in the literature [24] with modification: 100 \(\mu\)L antibody was added to 5 mL of pH-adjusted colloidal Au–PB–Fe\(_3\)O\(_4\) followed by incubation at 4 °C for 8 h. The conjugate was collected and removed from the solution by applying an external magnetic field and then dispersed in 5 mL pH 6.86 PBS. Subsequently, 1.0 mg HRP and 1.0 mg GOD were dissolved in the Au–PB–Fe\(_3\)O\(_4\) labeled antibody solution and incubated at 4 °C for 4 h. After collecting, removing and dispersing the conjugate, 1 mL 0.25% BSA solution was employed to block the unspecified sites and prevent non-specific adsorption. At last the multilabeled Au–PB–Fe\(_3\)O\(_4\) was collected and stored at 4 °C for further use.

2.5. Fabrication of the immunosensor

Gold electrode (\(\Phi = 4\) mm) was polished with 1.0, 0.3 and 0.05 \(\mu\)m alumina to obtain mirror-like surface firstly. Then it was rinsed with distilled water and put in ultrasonic bath to remove the physically adsorbed substance. Next the electrode was replaced into an electrochemical cell with 0.1M H\(_2\)SO\(_4\) until background signal stabilization for electrochemical cleaning and then allowed to dry at room temperature.

HAuCl\(_4\) solution is mixed with chitosan and chemically reduced to gold nanoparticles by adding the reducing agent citrate, which can be stabilized by chitosan and coated onto gold electrode surfaces to obtain a nature hydrogel chitosan and nanoAu composite monolayer with good biocompatibility and abundant –NH\(_2\) groups. EDC and NHS were used as coupling agents to immobilize the primary antibody on the chitosan/nano-Au modified electrode surface with the formation of amide link between the carboxyl of the antibody molecules and the amino of chitosan. Then the immobilized antibody binds corresponding antigen, which in turn, binds the secondary antibody labeled with benzyme functionalized Au–PB–Fe\(_3\)O\(_4\) nanoparticles. Significantly, the three-layer Au–PB–Fe\(_3\)O\(_4\) nanoparticles introduced

Fig. 4. XPS analysis for different nanoparticles. (a) and (b) were the Fe2p and O1s core level spectrum of Fe\(_3\)O\(_4\) nanoparticles; (c), (d) and (e) were the Fe2p, N1s and C1s core level spectrum of PB–Fe\(_3\)O\(_4\) nanoparticles; (f) and (g) were the Au4f core level spectrum and coverage of Au–PB–Fe\(_3\)O\(_4\) nanoparticles.
Fig. 5. CVs obtained for different modified gold electrodes in 0.1 M PBS (pH 6.86). (a) BSA/antibody anti-CEA/chitosan–nanoAu hydrogel film modified gold electrode, (b) the immunosensor incubated with 60.0 ng/mL CEA. The CV response after the sandwich format immunoreaction of the resulted immunosensor in the absence (c) and in the presence of 0.12 mM (d) and 1.2 mM glucose in the electrolytic cell. (Scan rate 50 mV/s).

Fig. 6. EIS obtained for different modified electrodes in PBS containing 5 mM K3Fe(CN)6·3H2O and K4Fe(CN)6 (pH 6.86). (a) BSA blocked immunosensor; the (b)–(d) show the resulted immunosensor incubated with 10.0, 20.0, 40.0 ng/mL CEA, respectively; the (b')–(d') show the EIS after the sandwich format immunoreaction of the corresponding immunosensors.

Fig. 7. The changes of the current response of the immunosensor with the different secondary labeled anti-CEAs towards different CEA concentrations: (a) Au–PB–Fe3O4 labeled anti-CEA, (b) HRP functionalized Au–PB–Fe3O4 labeled anti-CEA, (c) GOD and HRP functionalized Au–PB–Fe3O4 labeled anti-CEA in 0.1 M PBS containing 125 mM glucose under optimal conditions (pH 6.86).

A satisfying interface for enzyme and immunomolecules immobilizing through the well-developed Au–S chemistry, and the bound Au–PB–Fe3O4 nanoparticle could catalyze the reduction of the corresponding substrate of the immobilized enzyme in the presence of the PB interlayer, as a mediator, with amplified signal output. Furthermore, this immunosensor could be regenerated by simply using an external magnetic field which ensured a reproducible immunosensor with high sensitivity. Fig. 2 shows the schematic illustration of the stepwise procedure of the immunosensor fabrication and the corresponding catalysis amplifying principle of the bienzyme labeled Au–PB–Fe3O4 nanoparticles based on the sandwich immunoassay.

2.6. Experimental measurements

A standard three-electrode system used comprises a working electrode (the modified electrode), a platinum wire auxiliary electrode and a saturated calomel reference electrode (SCE). The CV scan was taken from –0.2 to 0.6 V (vs. SCE) at 50 mV/s in 0.1 M PBS (pH – 6.86).

The detection is based on the typical procedure for sandwich-type immunoreactions. When the background of the immunosensor current was stabilized, the peak of the current response was recorded as I0. Then, the sandwich immunoreaction was carried out, and after that the peak current increased to a maximum and recorded as I. The current response (ΔI) was given by

\[ \Delta I = I - I_0 \]

A series of standards of CEA and AFP were used to construct the calibration curve, which could be used for the determination of CEA and AFP in human serum samples.

3. Results and discussion

3.1. Characterization of the different nanoparticles

Transmission electron micrographs (TEMs) give the size and morphology of the as synthesized samples. The TEM image of the Fe3O4 is found in Fig. 3(a) and the average diameter of the observed Fe3O4 was less than 10 nm. Besides, the pristine Fe3O4 nanoparticles tend to aggregate as the special magnetic properties. After modification with PB, the PB–Fe3O4 became larger and the size is about 20 nm. Then the PB–Fe3O4 was surface-chemically modified by BSA to covalently attract 1–3 nm Au nanoparticle seed. Fig. 3(c) shows the Au nanoparticle seeds coating PB–Fe3O4, its morphology is somewhat irregularly shaped from oval to sphere. The Au–PB–Fe3O4 nanoparticles are presented in Fig. 3(d), it can be seen that the Au–PB–Fe3O4 nanoparticles were well formed and highly dispersed with the size about 80 nm.

To further analyze the surface composition of different nanoparticles, XPS characterization was employed. Fig. 4(a) and (b) shows the Fe2p and O1s core level spectrum of Fe3O4 nanoparticles and (c)–(e) shows the Fe2p, N1s and C1s core level spectrum of PB–Fe3O4 nanoparticles. The new peaks at 395.6 eV and around 285 eV are observed, which are the positions of N1s and C1s, indicating the presence of –CN [25]. Fig. 4(f) and (g) is the Au 4f core level spectrum and coverage of Au–PB–Fe3O4 nanoparticles. Fig. 4(f) represents the XPS signature of the Au 4f doublet (84.7 eV and 88.4 eV for the 4f7/2 and 4f5/2) for the resulting metallic gold Au0, which suggesting the form of the gold shell of Au–PB–Fe3O4.

3.2. Electrochemical characterization of the immunosensor

The electrochemical characteristics and amplification performances of the CEA immunosensor were investigated by cyclic
Thus, this result suggests that the Au–PB–Fe₃O₄ nanoparticle acts as multilabeled Au–PB–Fe₃O₄ magnetic nanoparticles have high decreased comparing with b–d. The reason might be the fact that the high frequency region of the impedance plot shows a semicircle amplify the response signals. GOD may retain high enzymatic catalytic activity and effectively bienzyme and the electrode surface, and the immobilized HRP and a good electron mediator for the charge transport between the overall process. As shown in Fig. 6, the Nyquist diagrams waves of BSA/anti-CEA/chitosan–nanoAu hydrogel film modified gold electrode and the resulted biosensor after the incubation with 60 ng/mL CEA, respectively. There are not any CV redox waves can be observed because of the lack of substance with electrochemical activity in the working potential range in working solution, which provides a low and reproducible background current. After the sandwich format immunoreaction of the resulted biosensor, a stable and well-defined redox peak at 0.18 and 0.24 V vs. SCE (curve c in Fig. 5) can be found, suggesting the efficient redox-activity of the bienzyme functionalized Au–PB–Fe₃O₄ three-layer magnetic nanoparticle immuno-probes. In order to evaluate whether or not the immobilized bienzyme can catalyze glucose, various concentrations of glucose were added into the solution. It can be readily seen that the cyclic voltammetry curve took on the appearance expected for a catalytic process mediated by the Au–PB–Fe₃O₄ magnetic nanoparticle, and the current density increased with increasing the concentration of glucose, indicating a typical electrocatalytic reduction process of H₂O₂ (Fig. 5, curves d and e). Thus, this result suggests that the Au–PB–Fe₃O₄ nanoparticle acts as a good electron mediator for the charge transport between the bienzyme and the electrode surface, and the immobilized HRP and GOD may retain high enzymatic catalytic activity and effectively amplify the response signals.

EIS has also been employed to characterize the interface properties of surface-modified immunosensors. It is well known that the high frequency region of the impedance plot shows a semicircle related to the redox probe Fe(CN)₆³⁻/⁴⁻, followed by a Warburg line in the low frequency region which corresponds to the diffusion step of the overall process. As shown in Fig. 6, the Nyquist diagrams a indicated a prepared immunosensor blocked with BSA, b–d showed the resulted immunosensors which incubated with 10, 20, 40 ng/mL CEA. The proportionate increase of the semicircle diameter with the concentration of CEA suggested that the electrode interface has captured the CEA as the immunocomplex hydrophobic protein layer would obstruct the electron transfer of the electrochemical probe. When the electrodes b–d had incubated with the bienzyme functionalized Au–PB–Fe₃O₄ labeled secondary anti-CEA via sandwich format immunoreaction, the curves b′–d′ demonstrated the EIS of the corresponding immunosensors, respectively. As shown in Fig. 5, the responses for b′–d′ were decreased comparing with b–d. The reason might be the fact that multilabeled Au–PB–Fe₃O₄ magnetic nanoparticles have high charge transfer efficiency and facilitate the electrolyte to penetrate the immunocomplex layer.

3.3. Performance of the immunosensor

In order to check up the electrocatalytic effect of the different multilabeled magnetic nanoparticles, a comparative study of the amperometric responses of immunoreaction was carried out by using three kinds of multilabeled secondary anti-CEA which were prepared as: (1) Au–PB–Fe₃O₄ labeled secondary anti-CEA, (2) HRP functionalized Au–PB–Fe₃O₄ labeled secondary anti-CEA, and (3) GOD and HRP functionalized Au–PB–Fe₃O₄ labeled secondary anti-CEA. Then the immunosensors which have been immunoreacted with various concentrations of CEA solution were transferred to incubate with the above three kinds of multilabeled secondary anti-CEA and then detected in an electrolytic cell containing 1.2 mm glucose. As shown in Fig. 7, one can find that use of GOD and HRP functionalized Au–PB–Fe₃O₄ labeled anti-CEA shows much greater amperometric changes than those obtained at the other label methods. These results indicate that the extreme enhancement of the sensitivity in Fig. 5c is due to the cooperating electrocatalytic effect of the GOD, HRP and Au–PB–Fe₃O₄ nanoparticles.

The performance of the present immunosensor was evaluated by detecting CEA and AFP standard solutions with the CV technique with the addition of 1.25 mm glucose in the electrolytic cell under the optimization conditions according to the typical procedure for sandwich-type immunoreactions, respectively. As expected for a sandwich mechanism, the catalytic CV cathodal peak current density of the immunosensor increased with the increasing antigen concentrations. The calibration plots of the changes of the cathodal peak current responses via concentration of CEA and AFP standard solution are illustrated in Fig. 8. With the amplification effect of the bienzyme catalysis towards the presence of glucose, the linear ranges span the concentration of CEA from 0.01 to 80.0 ng/mL with detection limit of 4 pg/mL (Fig. 6A, curve a) and AFP from 0.014 to 142.0 ng/mL with detection limit of 7 pg/mL (Fig. 8B, curve a), respectively. For comparison, the current responses of the proposed immunosensor were recorded without the addition of glucose. The immunosensors show linear ranges of 0.04–80.0 ng/mL for CEA (Fig. 8A, curve b) and 0.074–142.0 ng/mL for AFP (Fig. 8B, curve b) in the absence of glucose. The higher sensitivity and wider linear range of the proposed immunoassay should be taken into account as the exhibition of bienzymatic activity of GOD and HRP which performed an effective amplification property as expected.

The regeneration stability was investigated to demonstrate the further application of the immunosensor. The conventional regeneration of immunosensor usually utilizes different regeneration solutions, such as acid, alkali or electrolyte solution with high concentration to remove the target molecules (generally antigen).
from the sensing layers. In the present work the prepared immunosensor can be regenerated by simply using an external magnetic field to remove the immunocomplex of bienzyme functionalized Au–PB–Fe3O4 labeled secondary antibody bonded with the antigen. It was found that this non-chemical damaged method could regenerate the immunosensors for 18 times with the most acceptable variation coefficient of 0.37% using a constant concentration solution of CEA and for 20 times with the most acceptable variation coefficient of 0.41% for AFP immunosensor. This result confirmed that the proposed immunosensor could provide a satisfying reproducible ability with a high sensitivity.

4. Conclusions

An electrochemical immunosensor of high selectivity and good reproducibility with the employment of a bienzyme functionalized Au–PB–Fe3O4 three-layer composite magnetic particles has been successfully demonstrated in this work. The linear range and reproducible times dramatically improved. We observed a detection limit of pg/mL using the proposed immunoassay, which was about almost 50-fold more sensitive than the analogous reported works. Although this work only reported the detection of CEA and AFP in vitro, it is likely that the method based on the multilabeled functionalized three-layer Au–PB–Fe3O4 composite magnetic particles will ultimately apply in vivo and on-line detection. Therefore, the results described herein are a promising approach towards highly sensitive and reproducible assays for diseases with tumour markers detection.

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