Int. J. Electrochem. Sci., 7 **(2012)** 11564 - 11577

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

A Signal-Amplified Piezoelectric Sensor for the Detection of hs-CRP Using HRP Doped Magnetic Core-Shell Fe_3O_4@SiO_2@Au Nanostructures as Labels

Ning Gan[1,], Lihong Wang[2], Hankun Zhou[1], Tianhua Li[1],Weiguo Sang[3], Futao Hu[3,*], Yuting Cao[1]*

[1] The state key laboratory base of novel functional materials and preparation science, Faculty of material science and chemical engineering,Ningbo University, Ningbo 315211, China
[2] The faculty of science, Ningbo University, Ningbo 315211, PR China
[3] The faculty of marine, Ningbo University, Ningbo 315211, PR China
[*]E-mail: ganning@nbu.edu.cn; hufutao@nbu.edu.cn

Received: 4 September 2012 / *Accepted:* 6 October 2012 / *Published:* 1 November 2012

A highly sensitive and signal-amplified electrochemical immunoassay for high sensitivity C reaction protein (hs-CRP) has been developed based on an enzyme-catalyzed amplification due to the formation of an insoluble precipitate on the surface of a piezoelectric electrode. A novel horseradish peroxidase (HRP) and CRP antibody co-immobilized magnetic core-shell Fe_3O_4@SiO_2@Au nanostructures (Fe_3O_4@SiO_2 @Au-HRP-anti CRP-HRP) was synthesized and employed as labels for detection of CRP. After sandwich-type immunoreactions between CRP and labels on 96-well micro-titer plate, the immunocomplex on the bottom was subsequently exposed to 3-Amino-9-ethylcarbazole (AEC). The HRP labeled on labels can biocatalyze AEC in the presence of H_2O_2, resulting in an insoluble product. When the product solution was dropped on the piezoelectric electrode's surface, it can achieve an obviously decreased of frequency(△f). The amount of precipitation was found proportional to CRP within a certain range of concentration from 0.01 to 200 ng mL^{-1} with a low detection limit of 3 pg mL^{-1}. Comparing with the conventional ELISA, the immunoassay with the proposed amplified procedure shows improved sensitivity due to the significantly elevated amount of HRP labeled which can significantly amplify the subsequent enzyme catalysis reaction. It can be used to quantitatively determine other biomarker in the sample of human serum if changed the detection antibody.

Keywords: Magnetic core-shell Fe_3O_4@SiO_2@Au nanostructures, hs-CRP, piezoelectric sensor, electrochemical immunoassay

1. INTRODUCTION

The development of new methods for accurate and sensitive detection of disease-related proteins, such as high sensitivity C-reactiveprotein(hs-CRP) in cardiovascular disease, remains a challenge to the scientists in various fields [1,2]. In recent years, some biosensors have been reported as alternatives to the conventional methods, which are reliable but time-consuming or complicated, for rapid detection of biomarkers for cardiovascular disease[3]. Enzyme-linked immunosorbent assay (ELISA) is the most widely used in clinical laboratory. However, the concentrations of tumor related proteins are very low at early stage of cancers, which is often beyond the detection limit of ELISA[4]. Thus, new methods that can monitor tumor related proteins of low concetraion level are highly desired. Electrochemical immunoassays, based on specificity of antigen-antibody interactions with electrochemical transducers, have attracted considerable interest due to their intrinsic advantages, such as high sensitivity, simple pretreatment procedure, low cost and fast analysis [5].

The piezoelectric crystal detector can be a very powerful analytical tool because of the relationship shown for the change in frequency to the analyte concentration with high sensitivity. Piezoelectric immunosensors, which are based on a combination of highly specific immuno-recognition and ultra-sensitive QCM mass detection, also offer such opportunities for direct detection of biomarkers[6]. In 1959, Sauerbrey developed an empirical equation for AT-cut quartz crystals vibrating in the thickness shear mode that describes the relationship between the mass of thin metal films deposited on quartz crystals and the corresponding change in resonant frequency of the crystal[7]:

$$\Delta F = -2.3\times10^{6}F^{2}\frac{\Delta M}{A}$$

Where, F = frequency change in oscillating crystal in Hz, F = frequency of piezoelectric quartz crystal in MHz, □M = mass of deposited film in g, and A = area of electrode surface in cm^2. These relationships not only apply to film deposition but also to particulate deposition. The above explanation shows that the crystal detector indiscriminately changes frequency due to the deposition of mass of any material on its surface. Thus, it is the task of the researcher to choose a coating that will undergo a highly selective immunocomplex binding with the substance to be detected. Thus it can a highly selective sensor be constructed that will be sensitive to the subject to be detected.

Generally, primary or capture antibodies are immobilized onto an AT-cut quartz crystal, adsorption of bacteria onto the immobilized antibodies results in an increase in the surface mass loading of the crystal, and this will decrease the sensor's resonant frequency.According to Sauerbrey equation, the frequency decrease is proportional to the mass change,which in turn is linked to the biomarkers' concentration. A number of piezoelectric immunosensors have been developed for rapid detection of biomarkers including CEA,AFP etc [8,9]. and this has been summarized in several reviews. However, the usage of piezoelectric immunosensors for CRP are rarely reported.

Antibody immobilization is vital in successful development of a piezoelectric immunosensor, and the present immobilization methods are mainly based on silanized layer,SEM layer etc. However,

all the above methods are quite complex for the immobilization of antibody on the electrode which restrict its real application. So if the antibody modification procedure can be excluded, there will be a good prospect of application of piezoelectric method. Moreover, some of the color reaction product in sandwich ELISA was precipitation, so it can be directly measured on QCM electrode[10-13].

Itamar Willner[8have used a series of dye substrates such as o-phenylenediamine (AEC), 3,3'-diaminobenzidine(DAB), in immunocytochemistry(IHC) for immunoassay by piezoelectric method. Enzyme labels change their substrates into enzymatic reaction products that can be easily detected either by their electrochemical properties. The precipitation of an insoluble product of AEC or DAB on a tricomponent enzyme-layered electrode has been used in the development of a new electrochemical biosensor to detect acetylcholine [14]. The insoluble enzyme-catalyzed precipitates of immunoreacted complexes which accumulated on the transducers can be used to develop QCM sensors. QCM analyses were then used to probe the precipitation of the insoluble product on the transducer. AEC (3-Amino-9-EthylCarbazole) is one of these reagents, which can produce a positive immunoreaction precipitation product after exposure to HRP enzyme conjugated to an antibody reagent in IHC. Its immunocytochemical visualization of specific antigens of interest can induce a more specific stain by DAB in IHC . So, in the article, ACE was used for a diagnosis.

Nevertheless, this method does not sufficiently meet the sensitivity requirements to determine CRP for clinical analysis, particularly in the early stages of cardiovascular disease. It's because in this kind of electrochemical immunoassays, HRP labeled on a signal tags catalyzed the oxidation of dye substrates by H_2O_2, generating the precipitation product. While the labeled amount of HRP enzyme on each secondary antibody of conventional ELISA was limited(only about one HRP in one HRP linked antibody), restricting higher detection sensitivity. Therefore, in order to improve the sensitivity, signal amplification strategy for mass detection techniques by enhancing the HRP label amount in signal tags have been shown to be an effective method.

Nanotechnology-based immunoassays are rapidly emerging as convenient and high sensitive tools for a variety of immune assays [15-22]. Among them, immunomagnetic beads have been the focus of researchers because of their special magnetic properties enable the immunoconjugates to be readily separated from reaction mixtures by means of an external magnetic field [16]. Fe_3O_4 is one of the most extensively studied magnetic nanomaterials due to its good bio-compatibility, superparamagnetism, low toxicity and easy prepation. Unfortunately, iron nanostructures with a large surface area are easily oxidized and also react between themselves to form aggregates [17]. To avoid such problems, magnetic core-shell nanostructures with Fe_3O_4 as core and silicon oxide(SiO_2) as shell is proposed. The shell coating can make naked core particles stable, more easily dispersed and tune their properties [18].Gold nanostructures(Au NPs) can provide a natural environment for bimolecular immobilization and facilitate the electron-transfer of biosensor because of their high surface area, excellent electrochemical properties and good biocompatibility [19-22]. Therefore, if Au NPs can be immobilized on the magnetic core-shell nanostructures Fe_3O_4 @SiO_2 have stimulated a vast amount of research due to its fascinating properties. In the article, a new hybrid spherical nanostructure Fe_3O_4@SiO_2@Au was employed for immobiliztion of biomoleculars with a size of about 150~200 nm was designed, with a spherical Fe_3O_4 nanostuctures core surrounded by a thick silica shell and further decorated with gold nanostructures.

In this work, we report an enzyme-catalyzed signal amplification approach for the electrochemical detection of CRP in human serum using a QCM electrode. core-shell Fe_3O_4@SiO_2@Au nanocomposites were used as the immobilization substrate for the co-conjugation of HRP and CRP secondary antibody (HRP/anti-CRP). In this way, a nano-bioconjugate named Fe_3O_4@SiO_2@Au-HRP-anti-CRP containing multiple secondary antibodies and HRP molecules was successfully prepared and acted as a detection antibody. The analytical procedure consists of the immunoreaction of the antigen (CRP) with anti-CRP coated on the 96-well microtiter plates followed by binding Fe_3O_4@SiO_2@Au-HRP-anti-CRP_2. The formed HRP-labeled immunoconjugate showed good enzymatic activity for the oxidation of AEC by H_2O_2, and CRP could be quantitatively detected by the frequency changing response of the precipitation product of AEC. The usage of Fe_3O_4@SiO_2@Au-HRP-anti-CRP as a detection antibody with a high HRP label amount can greatly enhance the sensitivity. In addition, this strategy combines the merits of electrochemical method and traditional ELISA method, and smoothes away the cumbersome electrode modification process in electrochemical method and low sensitivity in ELISA.

2. EXPERIMENTS

2.1. Chemicals and Materials

HRP-labeled monoclonal mouse anti-human CRP and CRP test kit were obtained from Sigma. Santa Cruze Co.Lte (USA). 3-Mercaptopropyltriethoxysilane (3-MPTES), horseradish peroxidase (HRP EC 1.11.1.7, RZ>3.0, A>250 U/mg) were purchased from Aldrich(USA).Tetraethyl orthosilicate(TEOS) 、 Glycidoxypropyltrimethoxysilane (GPTES) 、 3-Aminopropylt riethoxysilane(APTES), Hydrogen tetrachloroaurate (III) tetrahydrate ($HAuCl_4 \cdot 4H_2O$), hydrogen peroxide (H_2O_2) and bovine serum albumin (BSA) were obtained from Sinopharm Group Chem. Re. Co, Ltd. (Shanghai, China). All reagents used were of analytical-reagent grade, and all solutions were prepared with double-deionized water. Phosphate buffer solution (PBS, 0.1M) of various pHs were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4, and then the pH was adjusted with 0.1 M NaOH and H_3PO_4.

2.2. Apparatus

Electrochemical quartz crystal microbalance (CHI-440A, Shanghai Chen hua instruments Ltd), gold-plated 8 MHz quartz oscillator (Crystal diameter of 13.6mm, gold electrodes 5.3 mm diameter, Shanghai Chen hua instruments Ltd).The morphology of the nanostructures was characterized by an H-7650 transmission electron microscope (Hitachi, Japan). UV-Vis spectra were recorded with a TU-1901 spectrophotometer (Beijing Purkinje General Instrument Co, China). Electrochemical immunoassay measurements were performed on a CHI 660 electrochemical analyzer (CHI, USA) with a conventional three-electrode system comprising a platinum wire as the auxiliary electrode, a

saturated calomel electrode (SCE) as the reference and a glass carbon electrode (GCE) as the working electrode.

2.3. Preparation of core-shell Fe_3O_4@SiO_2@Au nanostructures

To prepare the composite core-shell nanostructures Fe_3O_4@SiO_2@Au, Fe_3O_4@SiO_2 nanostructures were first prepared according to the classical Frens synthesis method [17]. The magnetic nanostructures of Fe_3O_4 (Fe_3O_4 NPs) were synthesized by co-precipitation method. Then, the particles were coated by TEOS (300 μL) and ammonium hydroxide (500 μL) under vigorous stirring in order to the following deposition of the SiO_2 shell. The reaction mixture was heated to 60℃ and kept for 6 h under N_2 atmosphere. The resulting precipitate was separated by magnetic separation and washed for three times with double-deionized water and ethanol respectively to give Fe_3O_4@SiO_2 NPs. The thickness of SiO_2 shell was increased by the hydrolysis and condensation of silica alkoxides. Au nanostructures were adsorbed on Fe_3O_4@SiO_2 NPs surface via the interaction between the negative charge on the Au nanostructures which was reductively synthesized by citrate acid and the positive charge of $-NH_3^+$ on the SiO_2 shell modified by 3-aminopropyltriethoxysilane (APTES,300 μL)[21]. After magnetic separation, the obtained Fe_3O_4@SiO_2@Au nanostructures were obtained and rinsed with double-deionized water. The procedures are shown in Figure.1

2.4. Preparation of Fe_3O_4@SiO_2@Au-HRP-anti CRP-HRP labels

The procedure to prepare Fe_3O_4@SiO_2@Au-HRP-anti CRP signal tags was shown in Figure.1. About 1.0 mL of Fe_3O_4@SiO_2@Au nanostructures suspension was initially adjusted to pH 8.2 using Na_2CO_3, and then 1.0 mL of the original HRP-anti CRP was added into the mixture and incubated for 12 h at 4 ℃ with slightly stirring. After magnetic separation, the obtained Fe_3O_4@SiO_2@Au-HRP-anti CRP labels were incubated with 1 mL of 1.0 wt% HRP for 1 h to block the nonspecific sites on the uncovered surface of Fe_3O_4@SiO_2@Au.

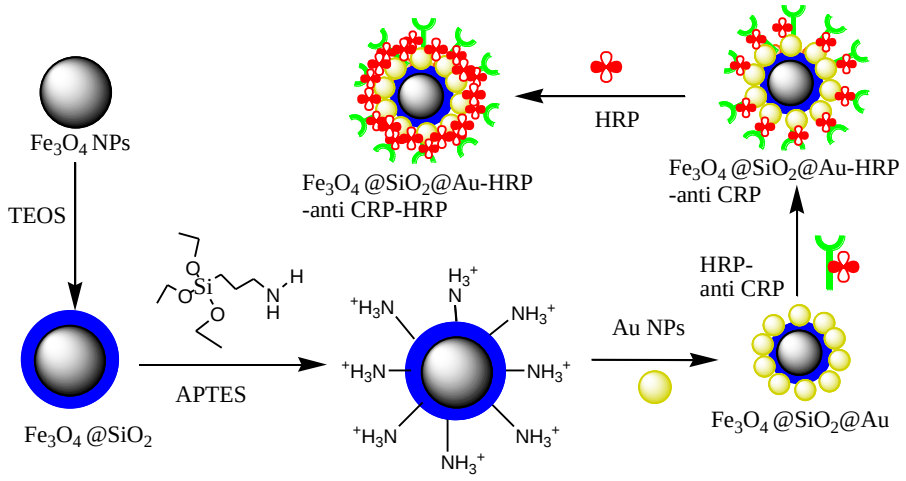

Figure 1. Schematic of the preparation of Fe_3O_4@SiO_2@Au-HRP-anti CRP conjugation.

The synthesized Fe_3O_4@SiO_2@Au- HRP-anti CRP-HRP signal tag was stored in pH 7.4 PBS at 4 ℃ when not in use.

2.5. Immunoassay procedures

A schematic representation of the steps used to perform the electrochemical ELISA was shown in Figure. 2 up. An anti CRP pre-coated polystyrene 96-well microtiter plate was incubated with 100 μL of different concentrations CRP for 30 min at 37 ℃. After incubation, the wells were washed six times with pH 7.4 PBS containing 0.05% (W/V) Tween-20. Then 100 μL of Fe_3O_4@SiO_2@Au-HRP-anti CRP conjugations (1:10 dilution with PBS containing 1 wt.% BSA) were pipetted into each well and incubated at 37 ℃ for 30 min. After the wells were rinsed, 100 μL of 1mmol/L AEC and H_2O_2 mixture solution were added to each well, and the enzymatic reaction was allowed to proceed for 10 min at 37 ℃. 20μL of the resulting reaction solution was transferred and dropped onto QCM electrode with 5 μL PBS (pH 7.0,Figure.2 down).

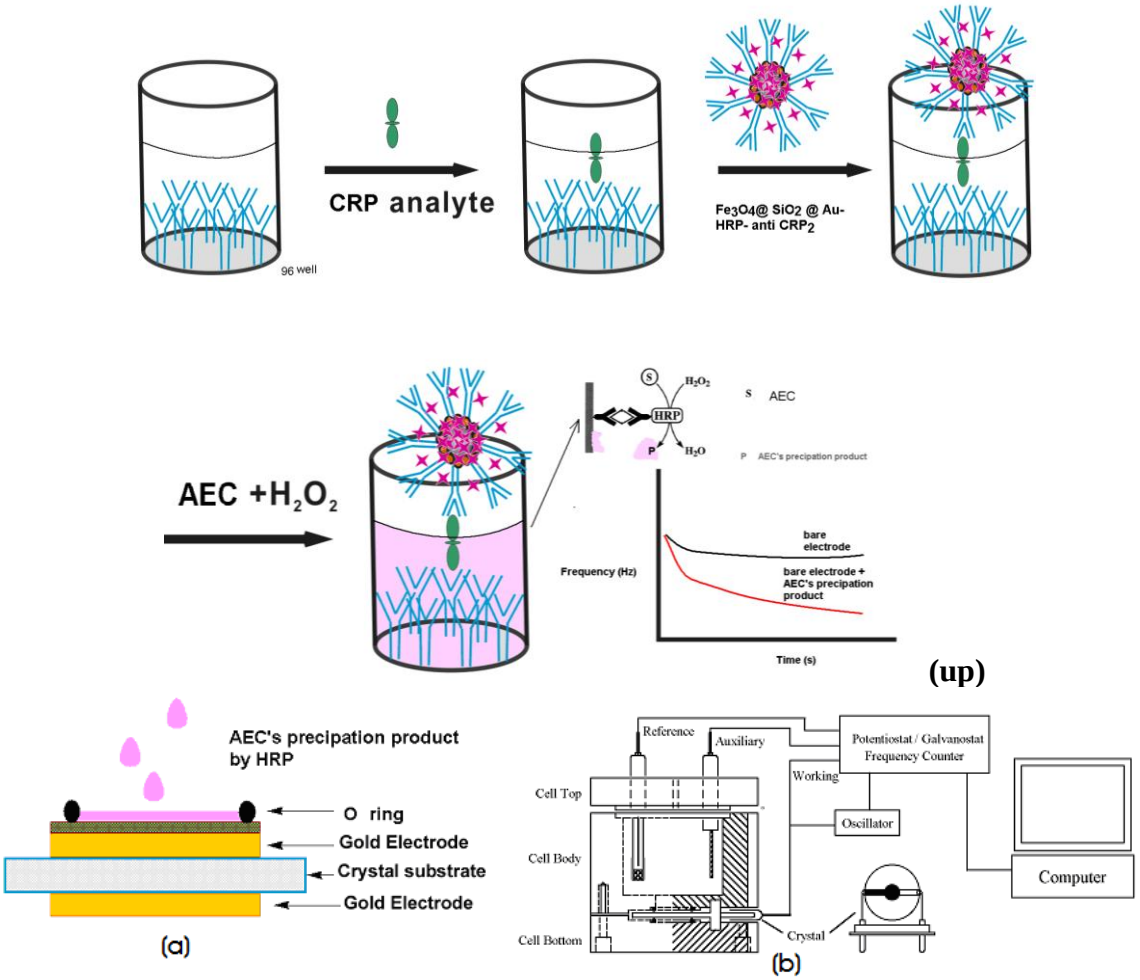

Figure 2. Schematic of the preparation of Fe_3O_4@SiO_2@Au-HRP-anti CRP-HRP conjugation and the electrochemical enzyme-linked immunoassay (ELISA) procedure with the application of Fe_3O_4@SiO_2@Au-HRP-anti CRP-HRP nano conjunctures as the signal enhancer(up) and the piezoelectric electrode(a) and detection system(b).

3. RESULTS AND DISCUSSION

3.1. Characterization of Fe_3O_4@SiO_2@Au-HRP-anti CRP nano-bioconjugation

The core-shell Fe_3O_4@SiO_2@Au nanostructures was used to label HRP-anti CRP because its high specific surface area may enhance the immobilization amount of HRP-anti CRP and HRP, and thus a nano-bioconjugation with mutiple anti CRP sites and HRP molecules can be obatined, which favors the HRP enzymatic oxidation of AEC by H_2O_2 and hence provides amplified signal in the electrochemical ELISA.

Transmission electron microscopy(TEM) images showed that both Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2@Au nanostructures were of well spherical structure and preferable mono disperisity in size. The average diameter of Fe_3O_4 nanostructures and core-shell Fe_3O_4@SiO_2@Au nanostructures were about 100 nm (Figure. 3a) and 120 nm (Figure. 3b), respectively. Upon deposition of gold shell to Fe_3O_4@SiO_2 nanostructures, the diameter of the Fe_3O_4@SiO_2 particle increased by about 20 nm, demonstrating that the Au shell was about 10 nm thick. As shown in Figure. 3c, Fe_3O_4@SiO_2@Au-HRP-anti CRP-HRP suspension was homogeneously dispersed in the solution. Once an external magnetic field was applied, the Fe_3O_4@SiO_2@Au-HRP-anti CRP-HRP was attracted quickly toward the magnet, leaving the bulk solution clear and transparent, indicating the magnetic activity of the Fe_3O_4@SiO_2@Au-HRP-anti CRP-HRP (Figure. 3d).

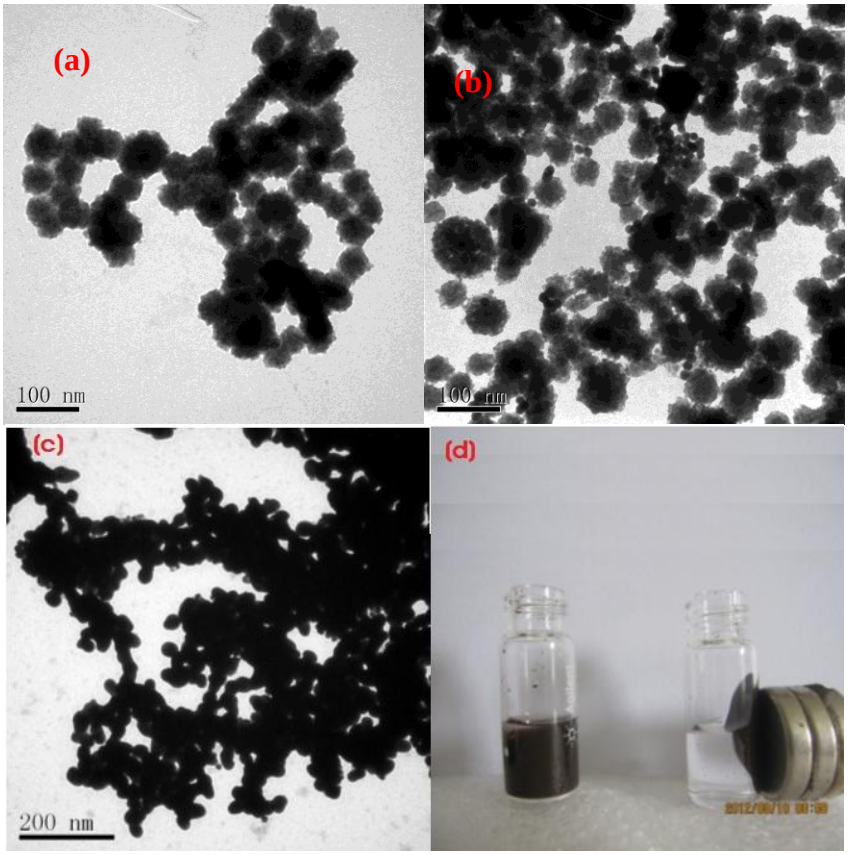

Figure 3. TEM images of (a) Fe_3O_4@SiO, (b) Fe_3O_4@SiO_2@Au (c) Fe_3O_4@SiO_2@Au -HRP-anti CRP (d) c in absence (right) and presence (left) of external magnetic field.

The XRD patterns were applied on characterization of the Fe_3O_4@SiO_2 nanostructures and Fe_3O_4@SiO_2 @Au nanostructures. The diffraction peak positions at 2θ = 30.0°, 35.3°, 42.6°, 53.4°, and 62.5° can be attributed to the (220), (311), (400), (422), (511), and (440) planes of Fe_3O_4 in a cubic phase , respectively. While in Fe_3O_4@SiO_2 , @Au, the diffraction peak positions at 2θ = 38.1°, 44.2°, 64.5°, 77.5°, and 81.6° can be attributed to the (111), (200), (220), (311), and (222) planes of Au, respectively. Other peaks can be attributed to Fe_3O_4.There is no crystal peak of SiO_2, it's because SiO_2 existed in the presence of amorphous. The simultaneous observation of both Fe_3O_4@SiO_2 and gold peaks verified successful assembly of Fe_3O_4@SiO_2 @Au nanostructures.

The magnetic properties of the nanostructures are illustrated in Figure 4. It can be seen that a small coercivity or remanence existed at room temperature, indicating the Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2 @Au have super paramagnetic properties. As a result, the Fe_3O_4@SiO_2 @Au could be isolated from solution by an adscititious magnet. It can also be seen that the saturation magnetization for Fe_3O_4@SiO_2 @Au is smaller than that for Fe_3O_4@SiO_2.The saturation magnetization values were 2.68 emu/g and 1.79 emu/g for Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2 @Au nanostructures, respectively.

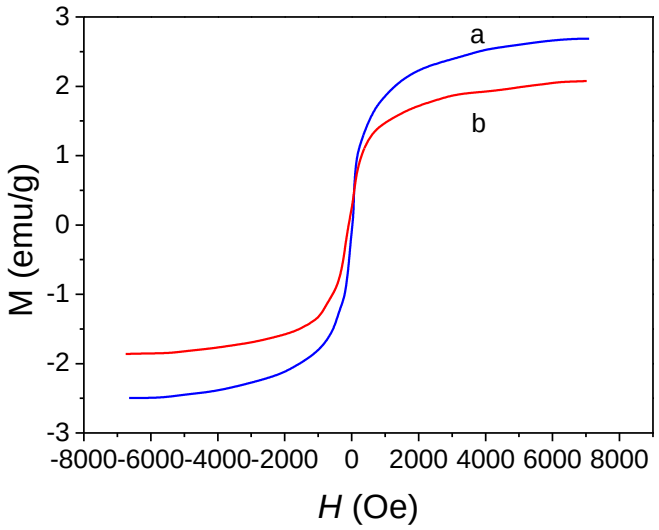

Figure 4. Hysteresis loops of magnetic silica nanoshperes with (a)Fe3O4@SiO_2 and (b) Fe_3O_4@SiO_2 @Au at 300K,respectively

UV-Vis absorption spectrometry was also employed to characterize the nano-bioconjugation of Fe_3O_4@SiO_2@Au-HRP-anti CRP. As could be seen, the Fe_3O_4 nanostructures showed no characteristic absorption in the examined range (Figure. 5a). When gold nanostructures were deposited on the surface of nano Fe_3O_4, a new absorption band centered at 520 nm resulted from gold nanostructures was observed (Figure. 5b), indicating the Fe_3O_4@SiO_2@Au nanostructures were successfully prepared, which was in good agreement with the results of Qiu et al[17]. After the HRP-anti CRP(Figure.5c) molecules being labeled onto the surface of the Fe_3O_4@SiO_2@Au nanostructures, the obtained Fe_3O_4@SiO_2@Au-HRP-anti CRP nano-bioconjugate exhabited two absorption peaks at 280 and 410 nm(Figure.5d). The peak at 520 nm originated in Au peaks on the Fe_3O_4@SiO_2@Au

nanostructures, and the peak at 280 nm and 410nm was attributed to the absorption of HRP-anti CRP proteins as judged from Figure. 4c. On the basis of the above results, it can be concluded that Fe_3O_4@SiO_2@Au-HRP-anti CRP nano-bioconjugation was successfully prepared and could be used in the electrochemical ELISA of CRP.

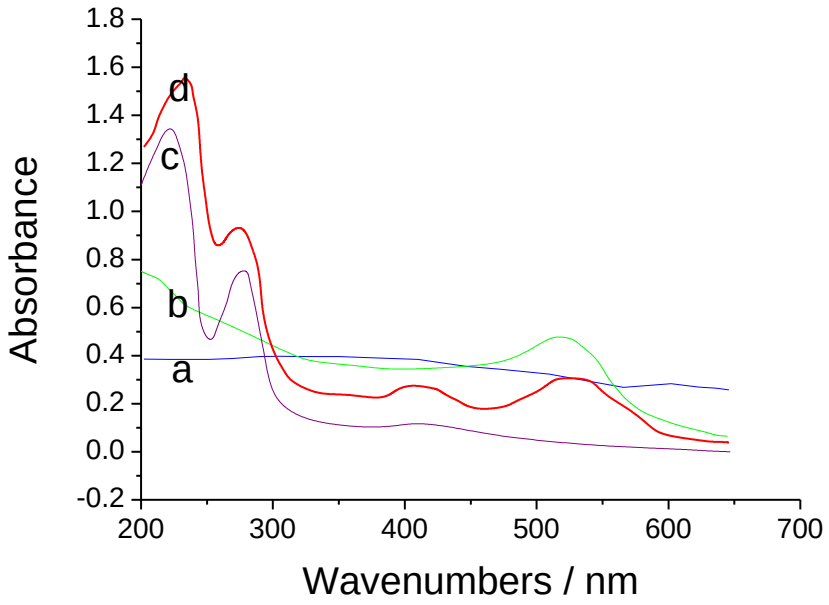

Figure 5. UV-Vis absorption spectra of (a) Fe_3O_4 nanostructures, (b) Fe_3O_4@SiO_2@Au nanostructures, (c) HRP-anti CRP, (d) Fe_3O_4@SiO_2@Au-HRP-anti CRP conjugation.

3.2.The characterization of signal amplification by different labels

To clarify the advantages of the QCM methods using Fe_3O_4@SiO_2@Au-HRP-anti CRP-HRP bioconjugation as the detection antibodies, we constructed two types of detection antibodies as labels including HRP-anti CRP, and Fe_3O_4@SiO_2@Au-HRP-anti CRP. 5 ng/mL of CRP was used as an example for the evaluation of the electrochemical signal response. The judgment was based on the current change slope (Δf) between QCM data of the oxidation product of AEC generated in the electrochemical immunosensor and the background current of QCM(Figure.5, dotted line). As shown in Figure. 5, the use of Fe_3O_4@SiO_2@Au-HRP-anti CRP as the detection antibody (Δf=22.1 Hz/logC A, Figure. 6a) exhibited much higher current response than that of applying HRP- anti CRP (Δf =6.4 Hz/ logc, Figure. 6b). The former is about 3.5 folds of the latter. This showed that the immunosensor by Fe_3O_4@SiO_2@Au-HRP-anti CRP-HRP can generate significant signal amplification for the development of ultrasensitive amperometic immunoassay method than HRP-anti CRP. The reason might be the fact that binding amount of HRP is much more in Fe_3O_4@SiO_2@Au-HRP-anti CRP-HRP than HRP- anti CRP. When one Fe_3O_4@SiO_2@Au-HRP-anti CRP-HRP detection antibody reacted with the antigen. Other HRPs can also participate the enzymatic reaction, which facilitated the

oxidation of AEC by H_2O_2 to produce more precipitation product for signal amplification and improved sensitivity.

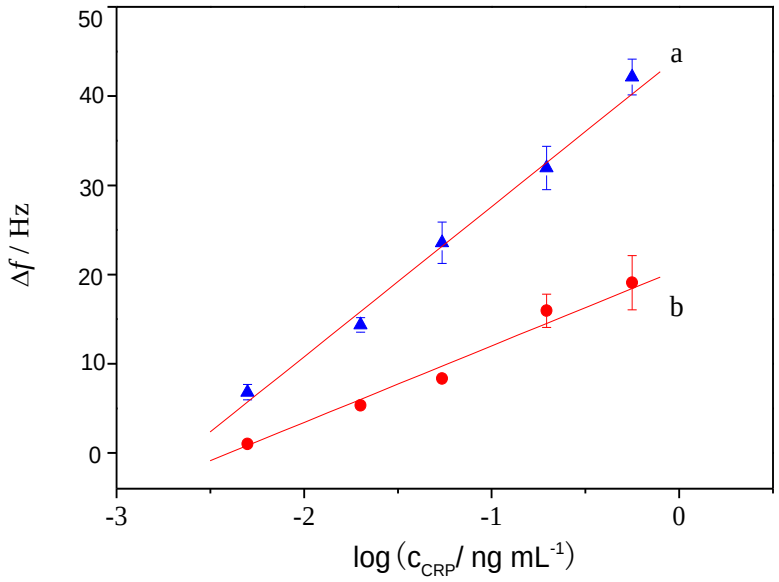

Figure 6. The calibration curve of the immunosensor modified with Fe_3O_4@SiO_2@Au-HRP-anti CRP (a),HRP-anti CRP (b) immunocomplexes.

3.3. Optimization of experimental conditions

When using the Fe_3O_4@SiO_2@Au-HRP-anti CRP-HRP nano-bioconjugate as a detection antibody, the ratio of HRP-anti CRP and HRP on the surface of Fe_3O_4@SiO_2@Au would have important effect on the amount of HRP, which determined the interaction efficiency. To determine optimal ratio of HRP and anti CRP, various volume ratios of 1 mg·mL^{-1} HRP and original anti CRP including 20:1~300:1, were used for the preparation of Fe_3O_4@SiO_2@Au-HRP-anti CRP-HRP. After incubated with 10 ng·mL^{-1} CRP, the immunoconjugates were allowed to react with HRP substrate AEC. The reaction product was measured by QCM value increases with increased to the plateau at the ratio of 1:100(Figure.7a). Thus, HRP to anti CRP 1:100 was chosen as the optimal volume ratio to prepare Fe_3O_4@SiO_2@Au-HRP-anti CRP-HRP.

The concentrations of H_2O_2 and AEC are important parameters that influence the activity of HRP. Using 10.0 ng·mL^{-1} CRP as a model, we monitored the effect of concentrations of H_2O_2 and AEC on the current response of the electrochemical ELISA. The reduction peak current of the enzymatic oxidation product increased with the increasing the concentrations of H_2O_2 (Figure. 7b) and AEC(Figure.7c) maintained the maximum value at higher concentrations. Afterward, the enzymatic reaction rate depended on the amount of the labeled HRP. Therefore, the optimal concentrations of 9.8 mmol/L for AEC and 2.33 mmol/L for H_2O_2 were used for the immunosensor.

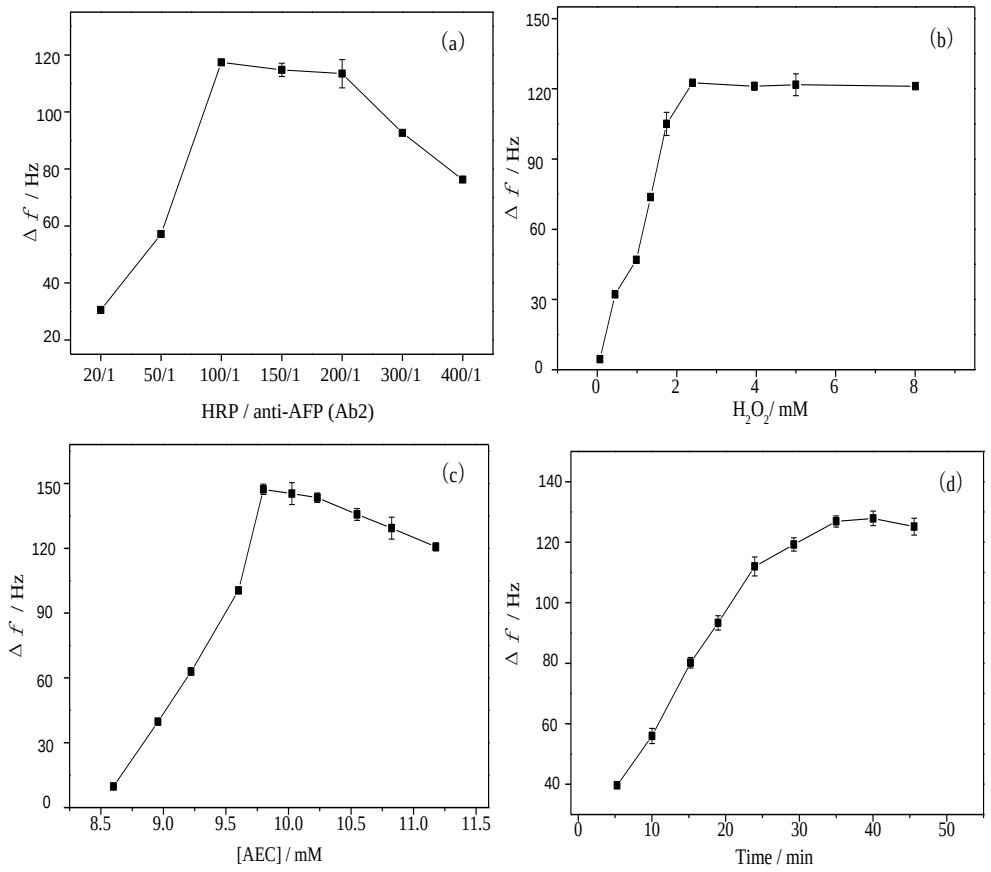

Figure 7. (a) Optimization of volume ratio of HRP to HRP-anti CRP for preparation of Fe_3O_4@SiO_2@Au-HRP-anti CRP. Effects of concentration of AEC(b), H_2O_2(c), and pH of PBS on cyclic voltammetric response at the immunosensor(d).

Incubation time and incubation temperature also affected the performance of the activity of enzyme(Fig 7d). The optimum incubation temperature of antigen-antibody reaction should be close to the normal temperature of human body (37 ℃), so all the experiments were carried out at 37 ℃. At this temperature, the enzymatic reaction equilibrium can be achieved in 30 min. So the optimal reaction time was selected as 30 min.

3.4. Analytical performance

Under the optimal conditions, the electrochemical ELISA was carried out to analysis various concentrations of CRP standard solution and the CV response was recorded. The Δf ofQCM response increased with the increasing of CRP concentration. The linear range was from 0.01~200 ng·mL^{-1}.The linear regression equation was Δf(Hz)= 22.1logC_{CRP}(ng·mL^{-1})+0.12 with a coefficient of 0.9923, it's detection range was between 0.01 to 200 ng mL^{-1} with a limit of detection(LOD) of 5 pg·mL^{-1}.

To highlight the advantages of the Fe_3O_4@SiO_2@Au nanoparticles label based electrochemical ELISA for CRP detection, the analytical properties of the developed immunoassay were compared with those of other CRP immunoassay reported previously. The results are listed in Table 2. It is

obvious that the linear range was broader and the LOD was comparably low. This result indicated that use of Fe_3O_4@SiO_2@Au nanoparticles as labels could obviously improve the sensitivity and dynamic range of the immunoassay.

Table 1. Comparison of analytical properties of various CRP immunosensors and immunoassays

Immunoassay	Linear range (ng•mL^{-1})	LOD (ng·mL^{-1})	Detection antibody	Ref.
ELISA	300–10000	100	HRP -labeled antibody	[23]
Electrochemistry	6.6-66000	2.1	silicon nanowire array-antibody	[24]
SPR-based immunosensor	2000-5000	650	CRP-antibody	[25]
Electrochemiluminescence	100 -10000	100	CRP Ab2 labeled on liposomes containing $Ru(bpy)_3^{2+}$	[26]
	10-10000	3.2	$[Ru(bpy)_3]^{2+}$ containing polystyrene microspheres	[27]
This method	0.01-200	0.003	Fe_3O_4@SiO_2@Au-HRP-antibody	This work

3.5. Specificity, reproducibility and stability

To evaluate the specificity of the proposed electrochemical ELISA for CRP detection, various biomarkers including carcinoma antigen 125 (CA 125), carcinoembryonic antigen (CEA), human IgG (HIgG), and prostate-specific antigen (PSA) were tested. The electrochemical signals were recorded in 10 ng·mL^{-1} CRP with and without the interfering agents. No significant difference of currents was observed in comparison with the result obtained in the presence of only CRP, indicating the specificity of the proposed electrochemical ELISA was acceptable.

The reproducibility of the immunoassay was evaluated by using the coefficients of variation of intra- and inter-assays. Taking 10 ng·mL^{-1} CRP for examples, the intra-assays and inter-assays of the electrochemical was evaluated using one electrochemical ELISA for five repeat assays and five copies of electrochemical ELISA for one time assay. The coefficients of variation of the intra-assay and inter-assay were 3.4% and 2.4%, respectively. Thus, the reproducibility of the electrochemical ELISA is satisfactory.

The stability of the synthesized nano-bioconjugate of Fe_3O_4@SiO_2@Au- HRP-anti CRP was also examined. When not in use, it could be stored in pH 7.0 PBS for at least 2 weeks without obvious signal change. Moreover, it retained 92.4% of its initial response after a storage period of 3 weeks. The slow decrease in the current response may be attributed to the gradual deactivation of the immobilized biomolecules on the surface of the nanostructures.

3.6. Determination of CRP in human serum samples

In order to investigate the possibility of the developed method for clinical analysis, some of patient human serum samples were examined by the developed method and the results were compared with the classical ELISA method. The concentration of hs-CRP in three samples were deterrmined to be consistent with the results obtained by the classical ELISA method(Table 2), indicating that the proposed immunoassay could be satisfactorily applied to the clinical determination of hs-CRP.

Table 2. Comparison of hs-CRP levels in patient human serum samples (n=3) determined using two methods

Serum samples	The developed method concentration($ng \cdot mL^{-1}$)	The classical ELIAS concentration($ng \cdot mL^{-1}$)
Sample 1	10.2±0.13	10
Sample 2	5.27±0.12	5.5
Sample 3	21.1±0.22	22

4. CONCLUSIONS

In this study, an ultrasensitive electrochemical ELISA method was developed for the detection of CRP by using a newly designed detection antibody. The detection antibody was prepared by loading high-content HRP and HRP-anti CRP antibody on core-shell Fe_3O_4@SiO_2@Au nanostructures. The signal was greatly amplified to detect hs-CRP comparing with other ELISA mehtod. The results described above demonstrate that amplification proceeds via Fe_3O_4@SiO_2@Au-HRP-anti CRP binding to a anti CRP/CRP complex, and that the biocatalyzed AEC to produce the precipitation of an insoluble product on the surface of the QCM electrode can significantly extend the sensitivity, leading to an unprecedented detection limit for CRP of 5pg/mL. With a sandwich-type immunoassay format, ultrahigh sensitivity was achieved by the enzymatic signal amplification using AEC as substrate. The use of core-shell Fe_3O_4@SiO_2@Au nanostructures as signal enhancers not only resulted in better performance, but also favored the rapid separation and purification of detection antibody on magnetic field. The detection limit of the proposed electrochemical ELISA was 100 times lower than that of the traditional ELISA method. The proposed method has a broader linear range, high sensitivity, convenient preparation and low detection limit, which provided a promising potential in clinical diagnosis.

ACKNOWLEDGEMENTS
This work was supported by the Natural Science & Scientific and technological research Foundation of Zhejiang province and Ningbo (LY12C20004, 2011A610018, 2012C23101, 2011C50038, Y3110479, 2011C23126, 2012A610144, 2011B82014, 2011A610006, Y3110478, 2011A610026), the K.C. Wong Magna Fund in Ningbo University.

References

1. R.P. Smith, B.J. Lipworth, A. I. Cree, E.M Spiers, J.H.Winter. *Chest*. 108 (1995)1288-1291
2. S. Black, I. Kushner, D. Samols. *J of Biological Chem*, 279(2004)48487-48490
3. V-L.Inger, M. A. Willem. *Biosensors and Bioelectronics*. 21 (2006)1141–1148
4. G.J. Wolbink, M.C. Brouwer, S. Buysmann, I.J.T. Berge, C.E.Hack. *J of Immunology*. 157(1996)1473-479
5. J.G Hou, N Gan, F.T Hu1, L.Zheng, Y.T Cao, T.H Li. *Int. J. Electrochem. Sci.,* 6 (2011) 2845 - 2858.
6. B. Attili, A. Suleiman.*Microchem. J.* 54(1996)174–179
7. G.G. Sauerbrey.*Z Phys,* 155(1959)206-212
8. A. Bardea, E. Katz, I. Willner. *Electroanalysis.* 12(2000)1097-1106
9. T.N. Ermolaeva, E.N. Kalmykova, *Russ. Chem. Rev.* 75(2006)397-412
10. M.Pohanka, O. Pavlis, P. Skladal. *Sensors.* 7(2007)341-353
11. Z.Y. Wu, C.P He, S.P. Wang, G.L. Shen, R.Q. Yu. *Chem J of Chin Univs.* 22 (2001)542–546
12. Z.Z. Chen, K.M. Wang, Y.K. Chen. *Chem J of Chin Univ*. 23(2002) 1044–1046
13. E. Briand, M.Salmain, C. Compere . *Biosensors and Bioelectronics,*22*(*2007) 2884–2890.
14. Z.Y. Wu, J. Wu, S.P. Wang, G.L. Shen, R.Q. Yu. *Biosensors and Bioelectronics,* 22(2006) 207–212.
15. N.L. Rosi, C.A. Mirkin. *Chem. Rev*, 105(2004)1547–1562
16. F.Lacharme, C.Vandevyver, M.A.M. Gijs. *Anal. Chem*, 80(2008) 2905–2910
17. D. Tang, R.Yuan, Y. Chai. *Clin. Chem,* 53(2007)1323–1329.
18. N.Gan, H.J. Jin, T.H. Li, L. Zheng. *Int J of Nanomedicine,* 6(2011) 3259-3269
19. L.P. Bicelli, B. Bozzini, C. Mele, L. D'Urzo. *Int. J. Electrochem. Sci.,* 3 (2008) 356 - 408
20. X.H. Huang, I.H. El-Sayed, W. Qian, M.A. El-Sayed. *J. Am. Chem. Soc*, 128(2006) 2115–2120
21. M.E. Khosroshahi, L. Ghazanfari. *Materials Chemistry and Physics.* 133(2012)55–62.
22. H.F. Chen, D.P. Tang, B. Zhang, B.Q. Liu, Y.L. Cui . G.N. Chen.*Talanta*, 91(2012)95-102.
23. W.L. Roberts, L. Moulton, T. C. Law, G. Farrow, M. C. Anderson, J. Savory, N. Rifai.*Clinical Chemistry.* 47(2001)418-425
24. M.H. Lee, K.N. Lee, S.W. Jung, W.H. Kim, K.S. Shin, W.K. Seong.*Int J Nanomedicine.* 3(2008)117–124.
25. H.F.M. Martin, H. Markus, K. Michael. *Biosensors and Bioelectronics*,21(2006)1987–1990.
26. W Zhan,A. J. Bard. *Anal. Chem*., 79 (2007) 459–463
27. W.J Miao,A.J. Bard. *Anal. Chem.*, 76 (2004)7109–7113