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ORIGINAL PAPER

Sensitive electrogravimetric immunoassay of hepatitis B surface antigen through hyperbranched polymer bridge linked to multiple secondary antibodies

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A method for using a hyperbranched polymer (HBP) as a bridge to link multiple secondary antibodies at HBP branches to amplify the detection response signal on a quartz crystal microbalance (QCM)-based sandwich-type immunosensor is reported. Carboxyl groups were prepared at multiple branches of HBP to make possible chemical binding between HBP and secondary antibodies via the carboxyl–amine reaction. The total mass of HBP and its linked multiple secondary antibodies were used to enhance the signal on a QCM chip in comparison with a simple sandwich-type immune reaction. By contrast, the proposed method could cause one antigen to analogously react with multiple secondary antibodies as a result of the branch structure of HBP. The strategy of using HBP as a bridge to link multiple secondary antibodies succeeded in quantitatively detecting the hepatitis B surface antigen (HBsAg). By employing demonstrated HBP bridge-linking, the frequency shift on a QCM chip was approximately 5 times greater than conventional methods without modification at secondary antibodies. The limit of detection of HBsAg was achieved as 2.0 ng mL^{-1}, lower than most of the values recorded in the literature measured by the QCM technique. Taking into account the general chemical interaction of immunoreaction, this method has the potential to amplify the signal in sensing many other analytes of interest.
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Keywords: hyperbranched polymer, quartz crystal microbalance, immunosensor, antibody, antigen, hepatitis B surface antigen

Introduction

The quartz crystal microbalance (QCM) is one of the most sensitive mass-responsive and label-free analytical devices, relying on measuring the change in frequency on a piezoelectric resonator when external molecules or others are linked to its surface. This technique is especially critical in the fields of biosensing, gas sensing, drug detection and enzymatic activity characterisation, which usually involve mass change by physisorption or chemisorption of chemicals from the surrounding media onto the solid surface (Buttry & Ward, 1992; Yang & Ngo, 2000; Bayramoglu et al., 2015; Raj et al., 2015; O'Sullivan & Guilbault, 1999). Due to the high sensitivity of the QCM technique, it is a useful tool for quantitatively detecting an immune reaction event due to its inherent mass change prior to and after a reaction. In a typical immune reaction, mass increases at the QCM chip after the reaction between the in-situ immobilised antigens and the corresponding antibodies from aqueous solution (Qian et al., 2010). This change is usually linear in a certain concentration range of antigen and antibody pair, which makes possible the quantitative detection of antigens in a biological sample being tested for clinical diagnosis (Jaruwongrungsee et al., 2015). In particular, the sandwich-type immunoreaction is suitably adopted to act as a QCM-based immunosensor because the involvement of a secondary antibody provides a flexible platform for introducing the required

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modifications (Pei et al., 2013). Many research methods have demonstrated the usefulness and effectiveness of antigens quantification by combining the immune reaction and QCM technique, in which a secondary antibody has often been used to amplify a detection signal (Pei et al., 2013; Shen et al., 2011a; Cheng et al., 2012; Dultsev & Tronin, 2015; Tang et al., 2006; Luppa et al., 2001). Meanwhile, a sandwich-type immunoassay is widely adopted as a way of sensitively and selectively measuring the concentration of antigens (Pei et al., 2013). In order to improve the sensitivity of the sandwich-type immunoassay on a QCM platform, two general strategies are commonly applied. The first is to increase the number of binding sites at the primary antibody (or substrate) to enhance mass response by increasing the antigen amount and the second is to modify the secondary antibody with weighted nanoparticles or macromolecules (Pei et al., 2013; Chen et al., 2010; Mao et al., 2006; Ji et al., 2015; Wu et al., 2015; Jeong et al., 2013; Yang et al., 2015). In the present work, HBP was used for the first time as a bridge to bind multiple secondary antibodies to improve detection sensitivity. HBP is a class of branched polymers (macromolecules) with tree-like topology and high molecular mass (Gao & Yan, 2004). HBP molecules with hydrophilic carboxyl groups are very useful in biological field because they are readily soluble in water and can be covalently bound with many biological molecules containing amine groups, such as antigens and antibodies. HBP has been considered for use in immunosensors to improve detection sensitivity by anchoring HBP on the support substrate to directly increase the number of primary antibodies and further indirectly increase the amount of antigen analyte (Shen et al., 2011b, 2008). Such a strategy involved only the multiple branch property of HBP. The mass of HBP is also useful in enhancing the detection signal. In addition, direct detection of the antigen requires a relatively higher concentration of antigens, which does not contribute to achieving a lower limit of detection. In the present work, a new strategy was designed to improve detection sensitivity by using HBP as a bridge to link multiple secondary antibodies on a QCM platform. The proposed method is preferable to the reported studies (Shen et al., 2011b, 2008), and also preferable to a sole antigen-antibody covalent interaction on a QCM platform. It is apparent that heavy HBP molecules and extra antibodies on the carboxyl branches of HBP could play a central role in mass enhancement compared with the sole antigen-antibody covalent interaction in a sandwich-type immune binding as shown in Fig. 1. Accordingly, the oscillating frequency shift on the QCM chip after the HBP-involved sandwich-type immune reaction should be greater than the immune reaction with naked secondary antibodies. The proposed method has been successfully demonstrated by using a hyperbranched aromatic polyamide polymer and a hepatitis B surface antigen (HBsAg) and antibody (HBsAb) pair as a sandwich-type immune model. The limit of detection using the proposed strategy was as low as 2.0 ng mL^{-1}, much lower than the results previously reported using HBP as the substrate and the sole antigen-antibody reaction.

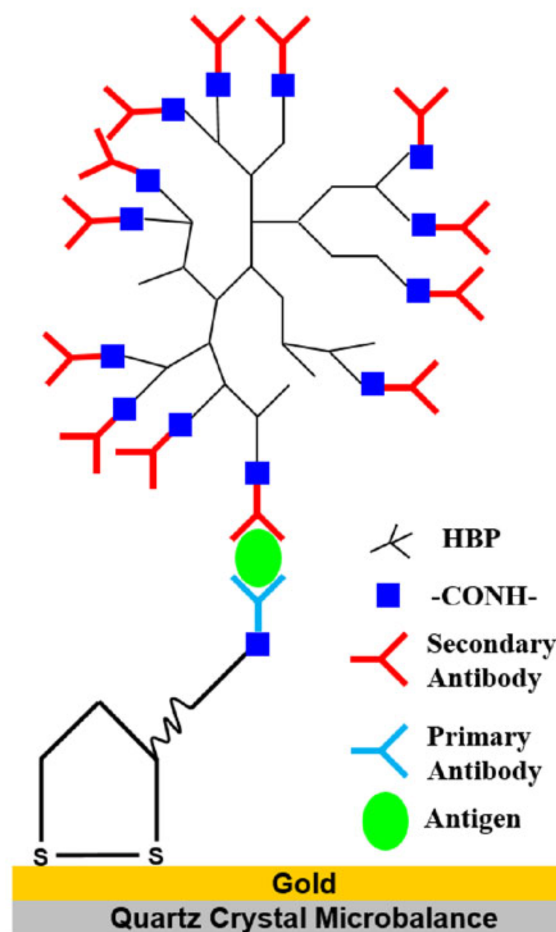

Fig. 1. Schematic illustration of mass enhancement at secondary antibody by using hyperbranched polymer (HBP) as a bridge linking multiple secondary antibodies in sandwich-type quartz crystal microbalance (QCM) immunosensor.

Experimental

General

The quartz crystal microbalance (QCM, AT-cut, 7.995 MHz, gold electrode) was obtained from Chenhua (China) equipped with a batch cell. To stabilise the frequency in the solution, one side of the QCM was sealed with an O-ring of silicone rubber covered by a plastic plate forming an air compartment isolated from the aqueous solution. The immunoassay was performed by mounting the prepared QCM in a laboratory-made reaction cell containing 3 mL of 0.1

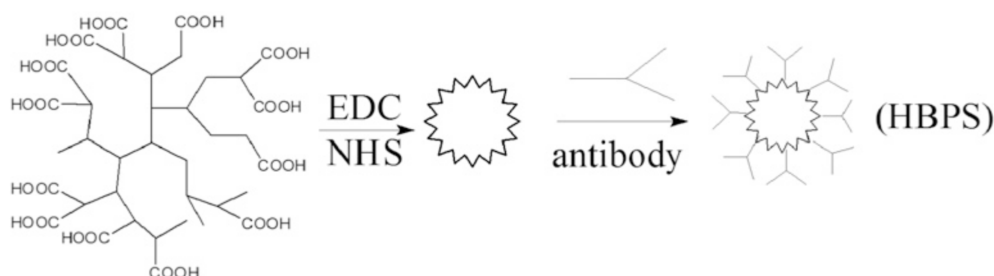

Fig. 2. Synthetic procedure of antibodies immobilisation onto HBP.

mol L^{-1} of phosphate buffer solution (PBS, pH 7.4).

^{1}H NMR spectra were recorded on an INOVA-450 NMR (Varian, USA) spectrometer. Infrared spectra were recorded using a Nicolet 470-IR Fourier transform infrared spectrometer (Nicolet, USA). An MS-3 vortex mixer and VS-15000CFN high-speed freeing centrifuge were used.

N-methylpyrrolidinone (NMP), pyridine (Py), *p*-phenylenediamine (PD), trimesic acid (TMA), and triphenyl phosphate (TPP) were obtained from Changsha Chemical Reagent Company (China). 1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and α-lipoic acid were purchased from Sigma. The hepatitis B surface antibody (HBsAb), hepatitis B surface antigen (HBsAg) and the bovine serum albumin (BSA) were obtained from Hangzhou Everlong Biotechnology Company (Hangzhou, China). All other solvents and chemicals were at least of analytical grade. Double-distilled water was used throughout the experiments. The solutions of EDC and NHS were prepared by dissolving EDC and NHS in pure water. The concentrations of the EDC and NHS solutions were 0.2 mol L^{-1} and 0.05 mol L^{-1}, respectively. Phosphate-buffered saline (PBS: 0.1 mol L^{-1} Na_2HPO_4, 0.1 mol L^{-1} NaH_2PO_4, pH 7.4) was used to maintain pH.

Synthesis of hyperbranched polymer (HBP)

In a typical experiment, 0.25 g (2.3 mmol) of PD, 0.52 g (2.5 mmol) of TMA, 2 mL of Py and 20 mL of NMP were added to a flask with a magnetic stirrer, a nitrogen inlet and a reflux condenser. The mixture containing 4 mL of TPP and 5 mL of NMP was added to the solution drop-wise. After stirring for 3 h at 80 ℃ under a N_2 atmosphere, the solution was poured into 100 mL of methanol to precipitate the product. The crude product in the NMP solution was purified by repeated precipitation from methanol three times. The product was washed with methanol three times and dried in a vacuum at 100 ℃.

Antibodies labelling to hyperbranched polymer (HBPS)

The carboxyl groups of the hyperbranched polyamides were first activated with EDC/NHS for 2 h at ambient temperature by mixing 0.2 mol L^{-1} EDC solution, 0.1 mol L^{-1} NHS solution and 1 mg mL^{-1} hyperbranched polyamides solution (EDC/NHS/HBP, $\varphi_r = 1 : 1 : 2$). Since HBP is insoluble in water (but soluble in a PBS buffer solution), the activated HBP was readily separated from the solution by centrifugation. After centrifugation, 900 µL of the phosphate buffer solution and 100 µL of 47 µg mL^{-1} of a new solution with the hepatitis B antibody were added to the cell and allowed to react under shaking for 1 h. Then the resulting solution was followed by centrifugation three times with a double-distilled water rinse. Lastly, a 1 vol. % bovine serum albumin (BSA) solution was added and shaken for 2 h to prevent the free antigen and antibody non-specific adsorption. This preparation process is shown in Fig. 2.

Gold surface treatment and disulphide self-assembled monolayer formation

The QCM crystal was first cleaned with a piranha solution (H_2SO_4/30 % H_2O_2; $\varphi_r = 7 : 3$) for 10 s. (Note: the piranha solution has strong corrosive properties, so it should be used with care). Thereafter, the Au chip was rinsed with ultrapure water and ethanol, followed by drying with N_2. The recycled Au chip should be treated with 1.2 mol L^{-1} NaCl and 1.2 mol L^{-1} HCl, and then reused following the above procedure.

2 mol $L^{-1}$$\alpha$-lipoic acid in ethanol was dropped and spread on the gold surface of the QCM for 2 h. Next, the gold surface was washed with ethanol to remove the non-adsorbed lipoic acid molecules and with distilled water to remove the ethanol residues.

Primary antibody immobilisation onto self-assembled monolayer

The mixed solution containing EDC (0.2 mol L^{-1}) and the NHS (0.1 mol L^{-1}) as coupling agent were dropped on the SAM-loaded gold surface of the QCM chip. The QCM chip was washed several times with distilled water after 2 h of incubation at ambient temperature. Next, 5 µL of 5.2 µg mL^{-1} hepatitis B antibody solution was dropped on the surface of the QCM chip and incubated at 37 ℃ for 1 h. PBS was then

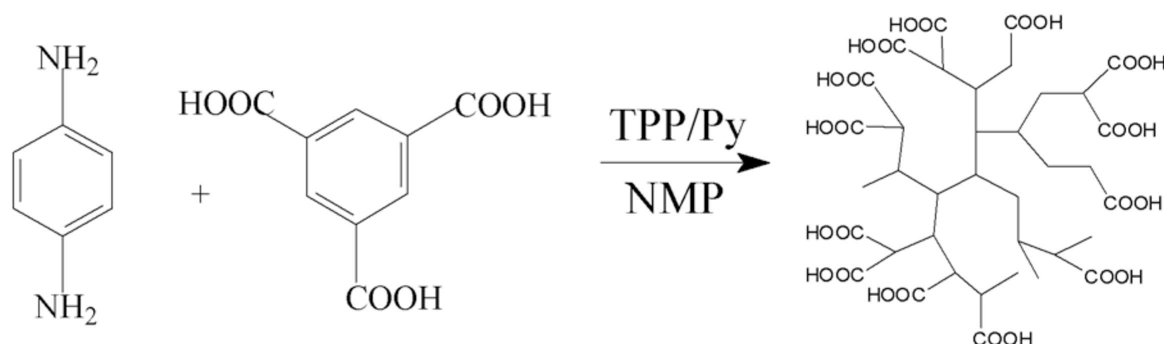

Fig. 3. Synthetic route of hyperbranched aromatic polyimide polymer (HBP).

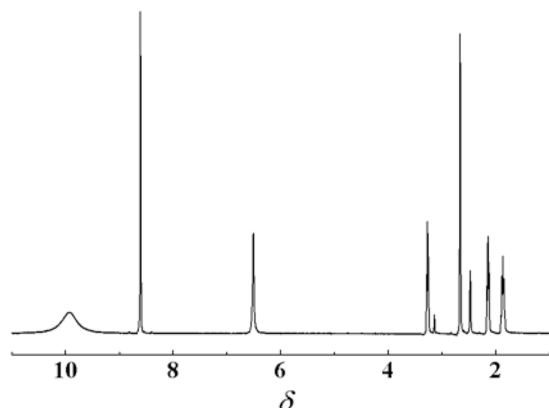

Fig. 4. ^{1}H NMR spectrum of HBP in DMSO-d_6.

used to remove the unreacted antibodies. The non-specific binding sites were blocked by adding 5 μL of 10 mg mL^{-1} BSA solution on the gold surface of the quartz crystal and incubated at 37 ℃ for 1 h. After PBS rinsing, the QCM immunosensor was dried in a refrigerator for later use.

Frequency measurements

The QCM immunosensor so prepared was inserted into a homemade Teflon cell containing 2.0 mL of buffer solution (PBS, pH 7.4). After the resonance frequency stabilisation with the first antibody and the hepatitis B antigen, the value of the frequency was recorded as f_1. Next, 0.5 mL of the HBP-linked secondary hepatitis B antibody was dropped on the surface of the quartz crystal. The frequency changes of the crystal were recorded (f_2) after approximately 60 min of the immune reaction. The frequency shift ($\Delta f = f_2 - f_1$) relates to the difference in frequencies prior to and after immunoreaction and is correlated with the antigen concentration.

Results and discussion

Characterisation of hyperbranched polymer (HBP)

The hyperbranched polymer was synthesised following the method previously reported (Ge et al., 2007). The synthetic procedure is shown in Fig. 3. The chemical reaction was related to the common condensation reaction between carboxyl and amino groups. Fig. 4 shows the ^{1}H-NMR spectrum of HBP. The resonances coupling in the region of δ 6.40–6.60 correspond to the protons of the *p*-phenylenediamine. The resonances for the –COOH functional group are located in the region of δ 9.8–10.3. The multiple peaks from δ 8.8 to δ 8.4 are assigned to the protons of all 1,3,5-trisubstituted benzene moieties.

The FTIR results in Fig. 5 show that the products bear the –OH group (3400 cm^{-1}), the presence of carboxylic groups in the HBP is supported by the strong absorption peak at 1710 cm^{-1} and the absorption at 1640 cm^{-1}, 1548 cm^{-1} suggests formation of the amide.

Electrochemical monitoring of immunosensor assembly

The proposed signal amplification for the sandwich-type QCM immunosensor using HBP as a bridge is illustrated in Fig. 1. A self-assembled monolayer (SAM) was first formed on the gold chip surface on the QCM, then the primary antibodies were bound to the SAM, followed by the target antigens conjugating with the primary antibodies. The massive secondary antibodies carried by the HBP macromolecules finally completed the sandwich-type immune system construction via specific chemical conjunction of the antigen-secondary antibody. In this strategy, the SAM is necessary to function as a mediator to link the weak-affinity organic molecules to the metallic Au surface (Tang et al., 2006). Molecules with sulphur heteroatom and carboxyl group terminated at their ends are commonly employed to act in mediating the biomolecules linkage to the metal surfaces by using their excellent affinitive characteristic brought by the free electrons in sulphur atoms and the specific conjugation capability between the carboxyl and amino groups. In the current work, disulphide molecules with carboxyl groups (e.g. α-lipoic acid) were used to form a SAM on the Au surface using sulphur moieties and linked to primary antibodies using carboxyl moi-

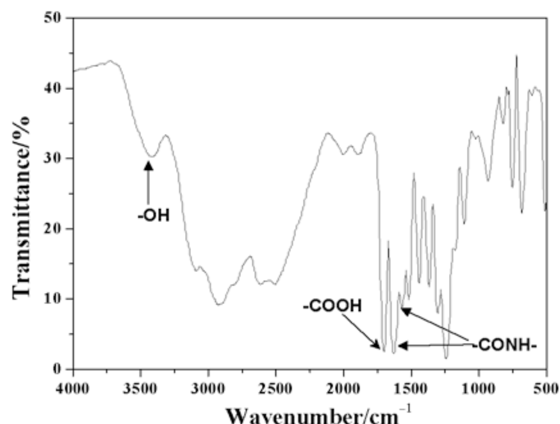

Fig. 5. IR spectrum of HBP.

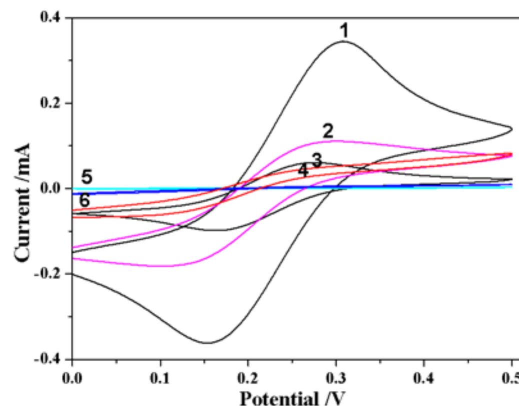

Fig. 6. Redox current levels recorded in the step-by-step immunosensor assembly: (1) bare Au electrode; (2) alpha lipoic acid self-assembled monolayer on gold electrode; (3) carboxyl activation with EDC and NHS; (4) primary antibodies immobilisation; (5) HBsAg antigens capture; (6) after secondary antibodies (on hyperbranched polymer) interacting with loaded antigens; cyclic voltammograms were recorded using electrolyte of 10 mmol L^{-1} $K_3[Fe(CN)_6]$ and 0.1 mol L^{-1} KCl. The potential was swept from 0 V to 0.5 V (vs. Ag/AgCl) with a sweep-rate of 100 mV s^{-1}.

eties. To affirm the successful step-by-step assembly of the QCM immunosensor, a cyclic voltammetric (CV) study was performed using $Fe(CN)_6^{3-/4-}$ as a redox pair to monitor the current level change (Fig. 5). With a redox system (10 mM $K_3[Fe(CN)_6]$ in 0.1 mol L^{-1} KCl), the insulating properties of the transducer were determined through the electron transfer kinetics of the $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ redox reactions. The scanning range varied from 0 V to 0.5 V with a scan-rate of 0.1 V s^{-1}. It is recognised that most biological molecules are poor electrical conductors. Thus, the electrical conductivity of the Au chip should diminish when loaded with biological materials. This is clearly indicated in Fig. 6. The current level decreased at the completion of each step. After forming the full sensor structure, the surface of the QCM chip is almost insulating, judged from the ground level of the current in the CV profile. The variation of the current levels in cyclic voltammograms affirmed the successful step-by-step formation of the proposed immunosensor system.

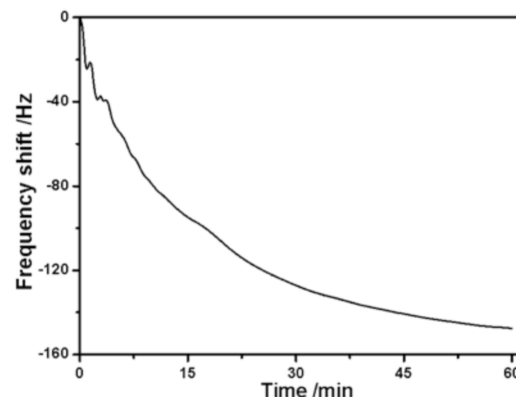

Fig. 7. Frequency response to α-lipoic acid self-assembly onto QCM gold electrode (concentration of α-lipoic acid was 0.3 mol L^{-1} in ethanol).

α-Lipoic acid self-assembly

Sulphur-containing compounds such as sulphide, disulphide and sulphydryl compounds can form a dense and ordered self-assembled monolayer on the Au substrate due to the strong interaction between the sulphur atom in an α-lipoic acid molecule and Au atoms. This kind of monolayer exhibits excellent chemical and mechanical stabilities. α-Lipoic acid can be adsorbed on an Au electrode through interaction between sulphur and Au, which was monitored by the QCM shown in Fig. 7. It was found that the frequency quickly declined at the beginning and became relatively steady after 1.5 h, suggesting that the α-lipoic acid self-assembly was almost complete.

In order to investigate the stability of the self-assembled monolayer, the electrode was immersed in a PBS buffer (pH 7.0) and the frequency change was measured. The frequency became stable with a shift of less than 1 Hz min^{-1} after 5 min. This showed the α-lipoic acid self-assembled monolayer to be stable in the PBS buffer. In the experiment, it was found that frequency increased by about 3 Hz with an error of less than 2.0 % over 2 h of self-assembly monitoring.

Antibody immobilisation time

The immobilisation time of the primary antibodies (HBsAb) influences the frequency change of the QCM immunosensor. The frequency change of the immunosensor as a function of the immobilisation time of the primary antibody is plotted in Fig. 8. The max-

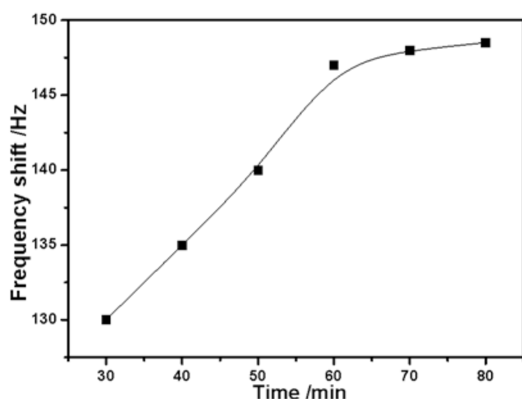

Fig. 8. Effect of immobilisation time of primary antibodies on frequency shift of immunosensor (concentration of HBsAg is 20 ng mL^{-1}).

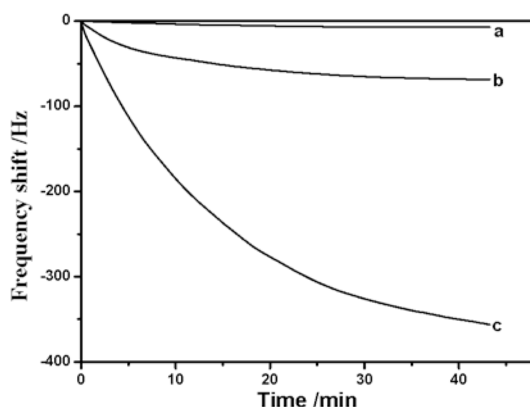

Fig. 9. Signal amplification effect using hyperbranched polymer as bridge to link multiple secondary antibodies: (a) corresponds to baseline of immunosensor; (b) compared with direct immune reaction without any modification; (c) on quartz crystal microbalance immunosensor with hyperbranched polymer bridge (concentration of HBsAg was 50 ng mL^{-1}; concentration of secondary antibody in each sample was 1 µg mL^{-1}).

imum frequency change appeared at around 60 min of the antibody-antigen binding. From 0.5 h to 1.5 h, the concentration of HBsAg was 20 ng mL^{-1} and the frequency change ranged from 131 Hz to 149 Hz. The equilibrium time was prolonged as the concentration of the antigen increased. A reduction in the frequency change was observed when the reaction time was more than 1 h. This was possibly due to steric hindrance from large numbers of primary antibodies immobilised on the SAM and from degraded activity of the primary antibody, caused by interaction over a long time.

Detection of HBsAg antigens

The proposed strategy has been successfully applied to detecting HBsAg antigens which correlate directly with liver disease (Wu et al., 2015; Xin et al., 2010). After the primary antibodies' (HBsAb) immobilisation on the Au chip, a sandwich-type immunosensor was constructed to detect HBsAg. It should be noted that the secondary antibodies were decorated with HBP molecules to increase the mass for the sensitive QCM measurements. This strategy is more applicable than those previously reported because the sensing materials containing HBP and analytes are heavier than analytes alone (Shen et al., 2011b, 2008). To emphasise the significance of the proposed sensor platform, a comparison of sensor performance is essential. Fig. 9 shows the distinctive QCM response signal between sensors without (curve b) and with (curve c) HBP molecules on secondary antibodies. It was clearly observed that the frequency shift for the QCM biosensor increased when the HBP molecules were used in the composite secondary antibodies. The enhancement factor of the proposed sensor was evaluated as approximately 5.

A series of concentrations of the HBsAg antigen analyte was tested for reproducibility. The Fig. 10 inset indicates the frequency change as a function of the HBsAg concentrations ranging from 5 ng mL^{-1} to 60 ng mL^{-1}. The calibration curve equation for HBsAg detection in the tested concentration range of the analyte was $Y = 7.163X + 0.6237$ with a good linearity with R of 0.9994, as shown in Fig. 10. The limit of detection was determined as 2.0 ng mL^{-1}. Such a performance of the QCM-based immunosensor can comply with the practical requirements in clinical diagnosis. Most importantly, this limit of detection is much lower than the concentration of HBP used at substrate (50 µg mL^{-1}) (Shen et al., 2011b, 2008).

Reproducibility and regeneration of immunosensor

Regeneration and repeatability are important parameters in evaluating the performance of an immunosensor. The reproducibility of the proposed immunosensor was examined repeatedly under the optimal experimental conditions with HBsAg concentrations of 20 ng mL^{-1} and 10 ng mL^{-1}, respectively. The corresponding results showed an acceptable repeatability with relative standard deviations (RSD) of 3.3 % and 4.2 % for five successive assays and that the immunosensor could be constructed and used for analysis with excellent reproducibility. For regeneration, the antigen could be released from the sensor by 1.2 mol L^{-1} HCl and 1.2 mol L^{-1} NaCl over the surface of the QCM sensor. Treated with the piranha solution, the chip can return to the fundamental frequency and be reused until the connection part is eroded. Under normal circumstances, the chip can be reused 20–30 times, while its fundamental frequency changed by only 6–8 Hz. After considerable use, the baseband and stability of the sensor will no longer meet the test requirements. In addition, sensors based

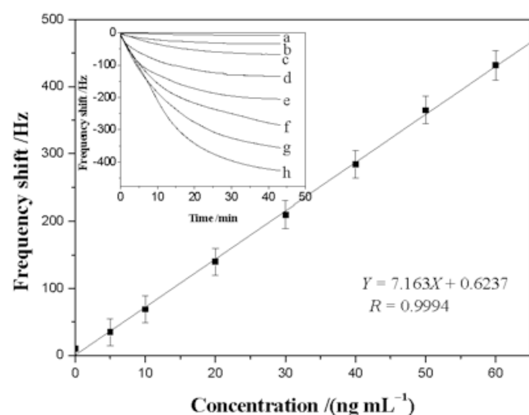

Fig. 10. Calibration curve for HBsAg detection using proposed signal amplification strategy on QCM immunosensor; inset shows corresponding frequency changes for different concentrations of HBsAg (from a to h, concentrations of HBsAg in ng mL^{-1} are 0, 5, 10, 20, 30, 40, 50, 60 respectively).

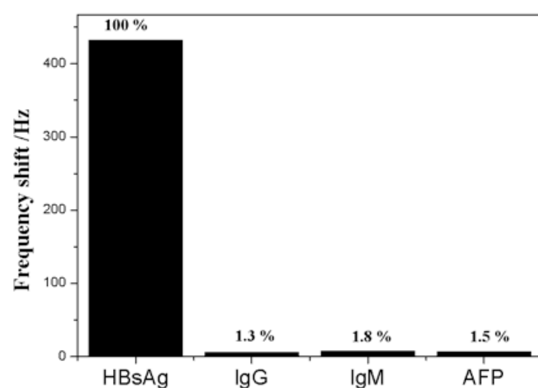

Fig. 11. Specificity of proposed QCM immunosensor (concentrations of HBsAg, IgG, IgM, and AFP in the solution were 20, 200, 200 and 200 (all in ng mL^{-1}), respectively.

on the immune reaction usually exhibit good selectivity in the detection of the analyte of interest due to the intrinsic property of immune reactions. The selectivity of the proposed sensor meets the expectations (Fig. 11). The variation in frequency signal is less than 2 % under the influence of other coexistent molecules such as IgG (1.3 %), IgM (1.8 %), and AFP (1.5 %).

Test of real samples

Five real blood serum samples were obtained from the First Hospital of Jiaxing and the concentration of HBsAg was measured using the method presented above and the ELISA (enzyme-linked immunosorbent assay) method. The HBsAg concentration in sample no. 5 was outside the linear range of this detection method, hence the no. 5 sample was diluted 10 times prior to measurement. After dilution, the result for the no. 5 sample was very similar to that obtained by the ELISA method. For the other samples, the results were consistent and had an RSD of less than 10 %.

Table 1. Results for five real samples tested by this method and ELISA method

No. of sample	1	2	3	4	5
Δf	0	130	0	0	298
This method/(mg mL^{-1})	N	18.9	N	N	420.1
ELISA/(mg mL^{-1})	0.7	20.2	N	N	416.7

Data were provided by the First Hospital of Jiaxing (China); N – denotes 'negative' for HBsAg concentration in sample of less than 0.5 ng mL^{-1}. The use of human serum samples for our purposes in this study was approved by the local ethics committee. Currently valid international guidelines and requirements have been followed for validation of the method.

Conclusions

The present study used a hyperbranched polymer (HBP) as a bridge to link multiple secondary antibodies to improve the sensitivity of a sandwich-type immune reaction on a quartz crystal microbalance (QCM) platform. A model was successfully presented on a QCM chip by using a self-assembled monolayer (SAM) film of alpha lipoic acid, and an immune system comprised of a primary HBsAb antibody, HBsAg antigen and secondary HBsAb antibody. The enhanced signal on the QCM chip was attributed to the higher total mass of HBP and the many secondary antibodies linked with it. By this strategy, a low concentration of antigen of HBsAg could yield a greater frequency shift than that achieved without modification at the secondary antibody. The proposed method exhibits high sensitivity and may be applicable to more types of immune reactions.

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