Enzyme-free gold-silver core-shell nanozyme immunosensor for the
detection of haptoglobin

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Abstract

Nanoparticles have been widely developed and shown to have intrinsic enzymatic ability and used in biosensors. Compared to biological enzymes used in biosensor, which are expensive and tedious to harvest, enzyme-mimic nanoparticles or nanozymes are both more stable and sensitive. An important area in this work is the development of a simple detection principle of immunosensor based on one-step synthesis of silver nanoparticle seeded onto gold core. The gold-silver core-shell nanoparticle act as a peroxidase mimic, which enable them to oxidise 3, 3’, 5, 5’ – tetramethylbenzidine (TMB) with H$_2$O$_2$ giving a colourimetric response. Herein, the analytical performance of the nanozyme is exploited to detect haptoglobin as a model analyte in a 96-well plate and measured the colourimetric product using spectrophotometer. The sensitivity of the immunosensor was as low as 100 pg/mL. The viability of our immunosensor was shown with good selectivity and satisfactory recovery in real serum sample.

**Keywords:** Enzyme-free, peroxidase, gold nanoparticles, silver, TMB

Graphical Abstract
Introduction

Biological enzymes have been widely integrated in immunosensors for reaction cascade and amplification. Additionally, the biological enzymes can further contribute to the selectivity of enzyme substrates in immunosensors. However, its utilisation in immunosensors may be limited because of environmental instability such as denaturation at extreme temperatures and pH, requires tedious immobilisation methods, and can be expensive to purify. These greatly limit the development and application of biological enzymes in immunosensors. There has been an advancement in the utilisation of nanoparticles in immunosensor as an alternative to biological enzymes where the employment of nanoparticles has been shown to increase the sensitivity and amplify the detection signal of the immunosensor.

Nanoparticle have been developed and utilised in drug delivery systems\(^1\), medical therapy\(^2\), catalysis\(^3,4\), and electroanalysis\(^5-8\) to mention a few. Its utilisation in biosensors is advantageous as nanoparticles have a high surface-to-volume ratio for bioconjugation of multiple moieties. This property also allows for easy surface modification for further surface functionalisation, and increased biosensor sensitivity and biorecognition\(^9-11\). Additionally, nanoparticles are diversely used in modification of electrochemical electrodes as nanoparticles have shown excellent tendency to enhance electron transfer rate and widely used in nanoscale catalysis\(^12,13\).

Gao et al. (2007) discovered that the nanoparticle iron oxide (Fe\(_3\)O\(_4\)) possesses intrinsic enzyme mimetic activity and was able to oxidise hydrogen peroxide\(^14\). The Fe\(^{2+}/\)Fe\(^{3+}\) ions played a part in breaking down the hydrogen peroxide and thus, it has peroxidase ability. The Fe\(_3\)O\(_4\) nanoparticles not only possessed intrinsic enzyme catalytic activity, their large surface area to volume ratio offers a better catalytic efficiency than natural enzymes. Furthermore, enzyme-mimic nanoparticles or nanozyme offer more sensitivity compared to the biological enzyme used in immunosensor, with more durability in harsh conditions such as high temperatures and extreme pH. Metallic nanozyme have attracted particular attention because of their defined mechanism, profound synthesis methods, easy surface modification, and good bio-compatibility (As reviewed in\(^15\)). These novel properties of nanozymes
make them perfectly suited for biosensor applications with increased efficiency and improved limit of detections.

Gold nanoparticles (AuNPs) are one of the most commonly used nanoparticles in the application of biosensors. AuNPs have been widely explored, with well-known properties and established principles, hence its wide application in biosensors. Gold nanoparticles are very stable in character and non-toxic in nature, and are easily conjugated to biorecognition molecules such as antibodies, DNA, and aptamers. Furthermore, the ability of gold nanoparticles to provide stable immobilization of biomolecules whilst retaining their bioactivity is highly advantageous in the preparation of biosensors. These aforementioned properties of gold nanoparticles have seen them being used as a seed or core in the synthesis of core-shell nanoparticle structure and as a template for nanoparticle growth.

Gold seeding enables the growth of the nanoparticle shell to be tuned to the shape of the gold seed itself, thus allowing the shape, size and thickness of the nanoparticle to be controlled during the synthesis. During the reaction, heterogenous nucleation on the gold seed surface takes place whereby metal ion precursors are reduced to its metallic state and subsequently, more metal is deposited onto the seed.

Both gold and silver nanoparticles have been widely demonstrated to have catalytic abilities and they have both been extensively utilised in detection biosensors. Chen et al. (2015) used silver nanoclusters in electrochemical biosensing and was able to detect picomolar levels of protein. Additionally, studies have shown that silver nanocomposite possess intrinsic enzymatic activities. Furthermore, Jiang et al. (2012) showed that silver-chitosan nanocomposites possessed intrinsic peroxidase-like mechanism towards peroxidase substrates, 3, 3’, 5, 5’ – tetramethylbenzidine (TMB) and o-phenylenediamine dihydrochloride (OPD), in the presence of H$_2$O$_2$, and was able to detect nanomolar range of glucose in a complex sample. Li et al. (2015) also demonstrated that silver nanoparticles exhibit both peroxidase- and catalase-like activity at different pH. This demonstrated that nanomaterials as an enzyme-mimic can be used at a wide range of pH without the risk of denaturation or deactivating the enzymatic activity.

Haptoglobin (Hp) is a 100 kDa positive acute phase protein, which will increase several-fold following local or systemic inflammation reaction. The primary role of Hp is to scavenge and form a complex
with free haemoglobin (Hb) to neutralise the damaging radicals from free Hb. Haptoglobin is usually measured as a clinical biomarker when a patient is suspected to have anaemia in correlation with a decreased red cell count and haemoglobin, and increase in immature red blood cells or liver malfunction. There are three common methods for measuring haptoglobin in the clinical setting: Spectrophotometry, immunocomplex method, and utilising gel electrophoresis. Nevertheless, these methods include limitations such as interference from components in serum during spectrophotometry measurement, the use of hazardous substances such as cyanide, or requiring manual execution which can be tedious.

Recent research has looked into the development of novel detection methods coupled with nanozymes. In this study, an immunosensor was developed using silver nanoparticles as the nanozyme instead of the biological enzyme using a simple one-step synthesis of silver nanoparticles deposition. Gold nanoparticles were used as the seed platform and to enhance the sensitivity of the sensor by increasing the surface area for seeding of silver nanoparticles, as well as for the conjugation of detecting antibodies which will result in the higher likelihood of detecting the target protein. Herein, we have designed a quantitative immunosensor where the growth of silver nanoparticles onto gold nanoparticle cores can be correlated to the availability of gold cores conjugated with detection antibodies. Furthermore, with the use of nanozymes, it is postulated that the sensitivity of the assay will be amplified and improved. Moreover, silver was used here because of its cost-effectiveness as compared to other metallic nanozyme such as gold, palladium, or platinum. Additionally, the immunosensor performed on the spectrophotometric 96-well plate advantageously exhibit simple instrumentation and high throughput testing. The peroxidase-mimic silver nanoparticles can exhibit colourimetric product from TMB-H$_2$O$_2$ system. The experimental conditions of the immunoassay were optimised and colourimetric determination of a range of concentrations of haptoglobin in the serum sample were presented, which holds a great potential in clinical settings.
Experimental section

Chemicals

Polyclonal mouse anti-Hp, haptoglobin (Hp) from human plasma, bovine serum albumin (BSA), tween-20, sodium chloride (NaCl), and hydrogen peroxide (H₂O₂) solution were obtained from Sigma. Sodium carbonate (Na₂CO₃), L-ascorbic acid (C₆H₈O₆), and glacial acetic acid (C₂H₄O₂) were all obtained from BDH chemicals. Sodium acetate (NaAc), and silver nitrate (AgNO₃) were obtained from VWR chemicals. Tris-HCl was obtained from 1st base. Polyethylene glycol (PEG)-20,000 was obtained from Wako Chemicals. K-Blue® Aqueous 3, 3’, 5, 5’ – tetramethylbenzidine (TMB) substrate was obtained from Neogen and was used as is. Gold nanoparticle (AuNP) stabilised in citrate was obtained from BBI solution.

The buffers used in the experiment are as follows: (1) TBS buffer, (2) conjugating buffer, containing 0.2M Na₂CO₃, pH 9.8, (3) washing buffer TBST, (4) blocking buffer for conjugation, PBS buffer containing 10% BSA (w/v), (5) blocking buffer for immunoassay, TBS buffer containing 1% PEG (w/v), (6) Acetate buffer, 0.2M, pH 3 adjusted using acetic acid.

Instrumentation

Absorbance was measured using spectrophotometry by employing the Thermo-Labsystem Multiskan™ FC absorbance plate reader using the filter wavelength of 650 nm. The experiments were run in a high-binding polystyrene 96-well microtiter plate. The pH of buffers was adjusted using the Accume™ AB15 pH meter, with 1M HCl and 1M NaOH used to maintain either pH, unless otherwise stated. GLS Aqua 12 plus water bath was used to maintain temperatures. Ultrapure water was used and obtained from a Milipore water purification system (18MΩ, Milli-Q, Millipore). The Implen nanophotometer P360 was used to obtain UV-visible spectrophotometry wavescan to observe the localised surface plasmon resonance.
Preparation of silver nanoparticles seeded onto gold seed nanoparticles

Silver nanoparticles with gold seed were synthesised according to literature \(^\text{24}\) with little modification. In a 50-mL flat-bottomed flask, 10 mL distilled water and 50 µL 10mM ascorbic acid were mixed using the magnetic stirrer. After that, 150 µL of AuNPs were added and stirred for 2 minutes. Finally, with stirring, 15 mL of 0.01M AgNO\(_3\) was added dropwise and stirring continued for 30 minutes at room temperature. The colour change was observed from a colourless to yellow solution.

Preparation of AuNPs-Anti-HP antibody conjugate

The preparation of AuNPs-Anti-Hp antibody conjugate was followed according to literature with slight modifications \(^\text{36}\). In brief, 1mL of polyclonal anti-Hp (1µg/mL) was added into 10mL of 1µM 15nm citrate-stabilised gold nanoparticles in 0.1M carbonate buffer (pH9.8). The mixture was then incubated for 1 hour at room temperature with slight stirring. Following that, 1mL of 10% BSA-PBS was added into the mixture and incubated for 30 minutes at room temperature. After that, the mixture was centrifuged for 20 minutes at 14,000 rpm. The soft sediment was resuspended in 1.2 mL 1% BSA-PBS and afterwards, stored at 4ºC. The BSA acts as a stabilising agent for the AuNPs-Anti Hp conjugate \(^\text{54}\).

Silver nanoparticle immunoassay protocol

A direct immunoassay was carried out. The illustration is shown in Figure 1. Briefly, 100 µL of Hp antigen was coated onto 96-well plate and incubated at 4ºC overnight. After that, the wells were washed with 200 µL TBST three times, followed by 300 µL 1% PEG-TBST for blocking non-specific binding sites on the wells. The plate was then incubated for 1 hour at 37ºC. Following that, the wells were washed three times with TBST and 100 µL of the AuNPs-anti Hp conjugate were added into the coated wells, followed by incubation at 37ºC for 1 hour. The plate was washed with TBST three times and once with distilled water to remove the bubbles. Subsequently, 50 µL of 0.1M silver nitrate and 50 µL
of 10mM ascorbic acid were introduced onto the wells and were incubated for 5 minutes. Following that, the wells were washed thoroughly with distilled water two times. After that, 100 µL of TMB-H₂O₂ in 0.2M sodium acetate buffer (pH 3) were added into the wells. The reaction was incubated for 6 minutes in the dark at room temperature. Finally, the absorbance was read using a plate reader at 650 nm. The reaction was not stopped during observation of the colour development.

[Place Figure 1 here]

Results and Discussion

Sensing mechanism of the colourimetric immunosensor

From Figure 2A(a), a characteristic peak of gold nanoparticle was observed. The conjugation was proved to be successful from the UV-visible spectrum, in which, the characteristic gold nanoparticle localised surface plasmon resonance peak at 520 nm was red-shifted with the peak broadening. This is due to the conjugation of antibody on its surface and increase in diameter of the gold nanoparticles (curve (c) in Figure 2A). A red-shift of the spectrum peak is an effect from the increasing particle size of the binding of the antibody to the gold nanoparticle surface. From this indication, the conjugation was considered successful with a low polydispersity index (PDI = 0.355). Furthermore, the characteristic UV-vis absorption peak of gold nanoparticles at 520 nm has disappeared showing that gold core seed was completely coated by silver nanoparticles with characteristic peak at 430 nm (curve (b) in Figure 2A). Additionally, the silver nanoparticle shell was shown to also be successfully seeded onto the antibody-conjugated gold nanoparticle (curve (d), Figure 2A) with the peak shifted to a longer wavelength compared to curve (b). The shift implied an increase in diameter compared from the successful conjugation of antibody on the gold seed nanoparticle. From the SEM images (Figure S1 (Supporting Information)), the gold nanoparticles can be observed to have an increase in diameter suggesting successful coating of silver nanoparticles onto its surface. Furthermore, EDX analysis showed that the peaks of Au disappeared after the seeding of silver onto gold nanoparticles (Figure S2 (Supporting Information)).
A direct immunoassay was carried out by directly incubating haptoglobin antigen onto the well. The antigen binds onto the surface of the well through electrostatic interactions. The AuNPs-Anti-Hp antibody conjugate was then introduced into the well to detect the haptoglobin and form an immunocomplex. Silver nitrate and ascorbic acid were introduced and the silver nanoparticles synthesised in situ were seeded onto the gold conjugate and eventually grow, forming a shell as schematically illustrated in Figure 1. This mechanism is widely known as the gold-seed meditated growth of nanoparticles \(^{57}\). After incubation, the wells were washed thoroughly to remove any excess silver nitrate as to avoid any false positives with TMB \(^{58,59}\).

To demonstrate the ability of silver nanoparticles to act as a peroxidase mimic in this mechanism, gold seeds without in situ growth of silver nanoparticles were tested to compare the absorbance of TMB between the presence and absence of silver nanoparticle in the system. A range of haptoglobin concentrations were incubated and detected using the immunosensor, with and without the synthesis of silver nanoparticles. As shown in Figure 2B, the gold seeds without the presence of seeded silver nanoparticles did not yield any colour compared to when in the presence of silver nanoparticles. Although gold nanoparticle is also a well-known nanozyme, the gold core nanoparticle was observed to not have any peroxidase activity because the surface of the gold nanoparticle may be inhibited when coated with proteins (Graph (b) in Figure 2B). It was established that the catalytic reaction occurs on the surface of the nanoparticles \(^{28,60}\), hence blocking the surface impede the reaction. When silver nanoparticles were seeded onto the gold nanoparticle, the catalytic ability of the nanoparticle can be observed. Hence, the peroxidase-like activity to oxidise TMB to blue was as a result of the seeded silver nanoparticles.

Such activity is well-observed in horseradish peroxidase (HRP), whereby hydrogen peroxide is broken down by peroxidase and the highly oxidising hydroxyl radical is presented to TMB. In metallic silver nanoparticles, it was postulated that hydrogen peroxide is adsorbed onto the surface of silver nanoparticles and eventually, the products of hydrogen peroxide decomposition i.e. the highly oxidising hydroxyl radical, is stabilised onto its surface. The mechanism of the enzyme mimetics on the surface
of silver nanoparticle is illustrated in Figure S3 (Supporting Information). A typical Michaelis-Menten curve was observed for H$_2$O$_2$ (Figure S2 (Supporting Information)) with the nanoparticles. The $K_m$ and $V_{max}$ values ($K_m^{H_2O_2} = 20.595$ mM, $V_{max}^{H_2O_2} = 1.71 \times 10^{-5}$ Ms$^{-1}$) were obtained from the Lineweaver-Burk plot and compared to HRP (Table S1 (Supporting information)).

Optimisation of experimental parameters of immunosensor

Several parameters of the reaction conditions were optimised. These include blocking agent of the 96-well microtiter plate, incubation time of $in situ$ synthesis of silver nanoparticles, catalytic reaction time, H$_2$O$_2$ concentrations, and optimal pH. Blocking a surface reduces non-specific adsorption between the antibodies and surface of the platform, and acts as a stabiliser by reducing the effect of denaturation on solid-phase assays. Blocking is significant to ensure low background signal while enhancing the specificity and sensitivity of the designed immunosensor$^{61}$. Two types of blocking agents, bovine serum albumin (BSA) and polyethylene glycol (PEG) were tested. BSA is one of the most common blocking agents used in biosensing, but it may cause non-specific signals$^{62,63}$. PEG is a non-ionic polymer and reported to effectively block plastic surfaces and biosensor platforms$^{64-66}$. When BSA was used, a blue colour was observed. This phenomenon has been reported by Dasgupta et al. (2016), where silver nanoparticles are able to be adsorbed onto BSA$^{67}$. When PEG was employed, it was observed that non-specific seeding of silver nanoparticles and non-specific binding of antibodies on the surface of the well were successfully blocked (Figure 3A).

Furthermore, the incubation time for the synthesis of silver nanoparticles was optimised in 1% PEG blocking buffer. After blocking, silver nitrate and ascorbic acid was incubated at 5, 10, 15 and 20 minutes. It was found that incubation longer than 5 minutes may give rise to non-specific settling of the silver nanoparticles onto the surface of the well (Figure 3B). Thus, to reduce such non-specific adsorption on the surface of the plate from longer incubation time$^{68}$, 5 minutes incubation time was chosen as a colourless reaction was maintained and therefore will reduce any false positive results.
during analysis. Additionally, optimising the catalytic activity of the silver nanoparticles is crucial in augmenting the sensitivity of the immunosensor. Thus, effects of pH, concentration of H$_2$O$_2$, temperature, and catalytic incubation time were investigated. Different H$_2$O$_2$ concentrations were tested to investigate the optimal H$_2$O$_2$ concentration for the immunosensor, and as shown in Figure 4A, 1M H$_2$O$_2$ was chosen as the optimal condition. The optimal pH of sodium acetate buffer for maximum catalytic activity was found to be pH 3 (Figure 4B). It is also noted that the silver nanoparticle showed relative activity in acidic conditions (pH 1 – 4). This supports the study by Li and colleagues (2015) that silver nanoparticles exhibit peroxidase-like activity in acidic conditions, and catalase-like catalytic mechanism in alkaline conditions$^{41}$. They postulated that in acidic conditions, H$_2$O$_2$ preferentially decomposes into H$_2$O$^•$ and O$^•$, and these highly oxidising radicals readily abstract hydrogen from the organic TMB, resulting in a colour change of the TMB from colourless to blue. The optimal temperature for the assay was at 22.5ºC (room temperature) with the nanozyme still having activity at relatively higher temperatures (Figure 4C). The optimal time for catalytic incubation was tested with 1M H$_2$O$_2$ and as noted in Figure 4D, the absorbance progressively increased with time. The optimal time was chosen at 360 seconds (6 minutes).

Calibration curve and analytical performance

To investigate the analytical performance of the gold-silver core-shell nanoparticle immunosensor, Hp at different concentrations (0 to 5000 pg/mL) was tested for detection under the optimised parameters. As shown in Figure 5, it can be observed that the measured absorbance was directly proportional to the increasing concentration of Hp (with increasing contrast of blue coloured TMB visually observed). As more antigen is detected, more gold seeds were available for silver nanoparticles to be adsorbed onto, and eventually more peroxidase-mimic was able to react with TMB-H$_2$O$_2$. This phenomenon is in accordance with the Beer-Lambert law. This immunosensor can detect Hp concentrations as low as 100 pg/mL, which is more sensitive than other types of immunosensor for similar detection (Table S2 (Supporting Information)). The equation of the calibration curve was $Y = 2.7033 \times 10^{-4} X + 0.2614$ with a correlation coefficient of 0.9748. The increase in sensitivity may be attributed to the increase in active
sites for H$_2$O$_2$ decomposition on the surface of the silver nanoparticle, and eventually increased oxidation of TMB. This is in contrast to biological enzymes, where the active sites may undergo conformational changes, limiting its ability for further reactions \(^{69}\). Furthermore, despite high catalytic ability of biological enzymes, they exhibit drawbacks such as denaturation in extreme pH and temperatures, expensive purification, and tedious conjugation method \(^{70}\). In contrast, nanoparticles exhibit and expose more active site for enzymatic reaction because of the large surface area. Additionally, bimetallic nanoparticles have been demonstrated to have improved catalytic activity compared to single metal nanoparticles \(^{71,72}\). The bimetallic nanoparticle carries discrete domains of each nanomaterial hence, it exhibits enhanced electronic properties through the synergistic effect of both nanomaterials in the same structure \(^{73}\). This was demonstrated in a study by Liu et al. (2016) where silver was alloyed with a palladium lattice, and used to boost the electrocatalytic activity of oxidation of formic acid thus improving the electro-performance of the assay \(^{34}\). They concluded that with bimetallic nanomaterials, the formic acid intermediate adsorption and the electrostatic attraction to polar molecules were enhanced thus increasing the catalytic efficiency of the reaction. Furthermore, nanoparticles possess easy conjugation reaction with biological molecules and are more stable in harsh conditions \(^{74,75}\).

In this work, it should also be noted that a low concentration of anti-Hp (1 µg/mL) was conjugated onto the gold nanoparticle. Thus, it is speculated that an increase in antibody concentration conjugated onto the gold nanoparticle may further enhance the sensitivity of the immunosensor by increasing the binding domains between antibody and antigen \(^{76}\), and thus enable the detection of a lower concentration of Hp whilst simultaneously increasing the dynamic range. Furthermore, the immunosensor employed a direct immunoassay as compared to the sandwich immunoassay, which is more sensitive.

[Place figure 5 here]

**Selectivity assay**

In order to analyse the specificity and selectivity of the developed immunosensor, the gold-silver core-shell immunosensor was tested against different serum proteins. The assay was carried out with the
optimised parameters and as in Figure 6, it can be observed that the absorbance for 1000 pg/mL of Hp was higher compared to the other serum proteins. These results indicate that the developed immunosensor was show to be sufficiently selective and specific to Hp.

[Place figure 6 here]

**Practical application of the colourimetric immunosensor for the detection of haptoglobin**

To investigate the analytical performance of the immunosensor in a complex matrix, a spike-and-recovery assessment was carried out to test its performance in real serum sample. Considering that a healthy human serum sample already contains Hp level between the range of 0.3 - 3 mg/mL [77], a high dilution strategy was carried out [36,78]. Thus, haptoglobin was still expected to be detected without spiking. Moreover, for the designed immunosensor to detect the healthy Hp serum range, the serum was diluted to the dynamic range of the immunosensor. As shown in Table 1, serum samples were diluted by 3,000,000-fold and spiked with 200 pg/mL and 500 pg/mL Hp. The immunosensor was able to obtain satisfactory recovery of the spiked Hp with accuracies between 75 – 100 %. Furthermore, the level of normal Hp in serum was able to be detected within the general range of 0.3 – 3 mg/mL. Therefore, the results concluded that the designed immunosensor was possible to accurately detect haptoglobin in a complex serum matrix.

[Place Table 1 here]

**Conclusion**

In summary, we demonstrated a simple enzyme-free immunosensor with silver nanoparticles as peroxidase-mimic utilising the seeding method onto gold nanoparticle core with TMB as the colourimetric substrate. The immunosensor can successfully catalyse TMB-H$_2$O$_2$ reaction without the utilisation of biological peroxidase with high sensitivity. The immunosensor is economical and avoids the use of sophisticated instruments. In addition, the immunosensor was able to detect pico-concentrations of haptoglobin at 100 pg/mL with very good selectivity. Furthermore, clinical application is possible as it provided satisfactory recovery in real serum samples.
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Supporting Information

Supporting information available.

References


Figures and Captions

Figure 1 Schematic representation of the immunosensor. (a) Haptoglobin incubation onto the 96-well plate, (b) Blocking of the wells with 1% PEG-TBST, (c) AuNPs-Anti Hp conjugate was introduced to detect the haptoglobin, (d) Silver nitrate and ascorbic acid were introduced into the immunosensor and TMB-H$_2$O$_2$ was introduced to colourimetrically detect haptoglobin at 650 nm.
Figure 2 (A) UV-vis absorbance spectra of (a) 15nm gold nanoparticles, (b) gold core seed nanoparticles with silver nanoparticles shell, (c) gold conjugated antibody, and (d) antibody conjugated to gold nanoparticle with silver shell. Peaks maximum are (a) 520 nm, (b) 425 nm, (c) 525 nm, and (d) 430 nm; (B) The analysis of absorbance of TMB activity of gold seed (a) with addition and (b) without addition of in situ silver nanoparticles. Error bars indicate standard deviation (n = 3)
Figure 3 Optimisation of parameters of the immunosensor. The effect on the absorbance of TMB at 650 nm with (A) different blocking buffer used to block the surface of plate; and (B) different incubation time of silver nitrate and ascorbic acid. Experiments A and B were carried out by blocking the plate with 1% BSA or 1% PEG. Then, 50 µL of silver nitrate and 50 µL ascorbic acid were incubated. Finally, TMB and H\textsubscript{2}O\textsubscript{2} were introduced and absorbance was measured. Error bars indicate standard deviation (n = 3).
Figure 4 Optimisation of parameters of the immunosensor. The percentage relative activity with (A) different H₂O₂ concentrations; (B) different pH; (C) different temperature; and (D) catalytic incubation time. Experiments were carried out by blocking the plate with 1% PEG. Then, 50 µL of silver nitrate and 50 µL ascorbic acid were incubated. Finally, TMB and H₂O₂ were introduced and absorbance was measured. Experiments (A – D) were carried out by incubating the plate with 10 000 pg/mL of haptoglobin. Then, immunosensor protocol was carried out with (A) different concentrations of H₂O₂ (0 – 1.2M); (B) different pH level of sodium acetate buffer (pH 1 – 8); (C) different TMB incubation temperature; and (D) 1M H₂O₂ in pH 3 sodium acetate buffer with time monitoring for 12 minutes. Error bars indicate standard deviation from three experiments.
Figure 5 Standard calibration plot of absorbance at 650 nm against different concentrations of haptoglobin. Absorbance below baseline was colourless. The error bars represent standard deviations from three experiments.
Figure 6 The specificity of anti-Hp gold-silver core-shell immunosensor towards serum proteins, Hp (1000 pg/mL), AFP (1000 pg/mL), CEA (1000 pg/mL), B2M (1000 pg/mL), HCG (1000 pg/mL), cortisol (1000 pg/mL), DHEA (1000 pg/mL), leptin (1000 pg/mL), IgA (1000 pg/mL), and CRP (1000 pg/mL). Error bars indicate standard deviations (n = 3).

Table and Caption

Table 1 Spike-and-recovery assay using the immunosensor

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