Anal Bioanal Chem (2016) 408:1015–1022
DOI 10.1007/s00216-015-9219-8

PAPER IN FOREFRONT

Naked-eye sensitive ELISA-like assay based on gold-enhanced peroxidase-like immunogold activity

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Received: 18 October 2015 / Revised: 22 November 2015 / Accepted: 24 November 2015 / Published online: 16 December 2015

Abstract A naked-eye sensitive ELISA-like assay was developed based on gold-enhanced peroxidase-like activity of gold nanoparticles (AuNPs). Using human IgG (H-IgG) as an analytical model, goat anti-human IgG antibody (anti-IgG) adsorbed on microtiter plate and AuNPs-labeled anti-IgG acted as capture antibody and detection antibody, respectively. Because the surfaces of AuNPs were blocked by protein molecules, the peroxidase-like activity of AuNPs was almost inhibited, evaluated by the catalytic oxidation of peroxidase enzyme substrate 3,3′,5,5′-tetramethylbenzidine (TMB), which could produce a bright blue color in the presence of H_2O_2. Fortunately, the catalytic ability of AuNPs was dramatically increased by the deposition of gold due to the formation of a new gold shell on immunogold. Under optimal reaction conditions, the colorimetric immunoassay presented a good linear relationship in the range of 0.7–100 ng/mL and the limit of detection (LOD) of 0.3 ng/mL calculated by 3σ/S for UV-vis detection, and obtained LOD of 5 ng/mL for naked-eye detection. The obtained results were competitive with conventional sandwich ELISA with the LOD of 1.6 ng/mL. Furthermore, this developed colorimetric immunoassay was successfully applied to diluted human serum and fetal bovine serum samples, and predicted a broad prospect for the use of peroxidase-like activity involving nanomaterials in bioassay and diagnostics.

Keywords Gold nanoparticles · Peroxidase-like activity · Colorimetric immunoassay · Human IgG · Gold enhancement

Introduction

Human IgG (H-IgG) plays vital roles in the human body, and its contents in serum, urine, and cerebrospinal fluid act as a significant indicator for the diagnosis of diseases, e.g., renal and nervous system dysfunction [1, 2]. Thus, the recognition and quantification of H-IgG level is worth particular concerns. As a result, it is of great importance to develop novel procedures for ultrasensitive detection of H-IgG in clinical diagnosis and pathologic analysis by utilizing new materials and advanced technologies. Owing to natural enzyme characteristics of strong substrate specificity, high catalytic activity, and mild reaction conditions, enzyme-linked immunosorbent assay (ELISA) has become the most commonly employed analytical approach for measuring trace target molecules since its introduction in 1971 [3]. For example, horseradish peroxidase (HRP) is one of most common enzymes used in the ELISA since it serves as a catalyst to facilitate a series of biological processes [4, 5]. However, natural enzyme-labeled systems have some inevitable shortcomings, such as easy denaturation and digestion by proteases, and complicated preparation, purification, and storage processes, which severely limit their routine use in diagnosis [6, 7]. Hence, it is necessary to synthesize new non-natural enzymes with high catalytic activity to overcome the restrictions of natural enzymes.

Electronic supplementary material The online version of this article (doi:10.1007/s00216-015-9219-8) contains supplementary material, which is available to authorized users.

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Meanwhile, the emergence and development of nanoscience and nanotechnology provide an alternative for the design and construction of immunoassay. Because of their simple synthetic steps, distinctive optical properties, and excellent biocompatibility [8–11], gold nanoparticles (AuNPs), especially, have been mostly chosen as signal-producing tags to develop sensitive molecule recognition-based immunoassays during the past several decades. The frequently used detection technologies for AuNPs-based immunoassays mainly include colorimetry [12], fluorometry [13], surface-enhanced Raman spectroscopy (SERS) [14], chemiluminescence [15], and electrochemistry [16]. Among them, colorimetric detection is particularly popular because of its attractive advantages, e.g., they can be easily read out with naked-eyes or low-cost instruments. For example, Zhou et al. developed an ultrasensitive naked-eye-counting strategy for quantifying target miRNAs by employing identification-miRNA-capturing AuNPs [17]. Valentini and co-workers operated an AuNPs-based colorimetric sensor for the naked-eye detection of cancer-related point mutations with picomolar sensitivity [18]. Moreover, the batch quantitative detection of analytes can be achieved by determining the absorbance of color substances on microplate reader, which is a common instrument in hospitals. Therefore, it is meaningful to research and develop immunoassay based on the combination of AuNPs and colorimetric detection.

Recently, researches in AuNPs as artificial enzyme mimics have sprung up on account of their merits, including low cost, high stability, and facile modification with biomolecules compared with natural enzymes [19–22]. Their peroxidase-like activity has been successfully applied in ELISA-like assay. For instance, Que and co-workers reported a novel competitive-type immunoassay protocol for the detection of antibiotic residue by using AuNPs tags as nanocatalysts for signal amplification [23]. The assay was implemented by monitoring the increase of absorption of 4-aminophenol, obtained by the reaction of the common enzyme substrate 4-nitrofenol with $NaBH_4$, to quantitate the increment of the molecular target. Unfortunately, the relatively low peroxidase-like activity of AuNPs could be nearly inhibited when modified with proteins, which decreases the sensitivity of this proposed method. In order to solve this problem, Zhan et al. discovered a highly sensitive colorimetric immunoassay based on Hg^{2+}-stimulated peroxidase-like activity of AuNPs-graphene oxide (AuNPs-GO) hybrids [24]. However, the unreasonable recycling of Hg^{2+} could cause environmental pollution, and long-term exposure to Hg^{2+} would harm the health of the operator. Therefore, to develop AuNPs based colorimetric immunoassays with high peroxidase-like activity and ecological friendliness is greatly desirable.

Inspired by these studies, in this work we developed a novel, simple, and environmentally friendly colorimetric immunoassay for the detection of H-IgG based on the enhanced catalytic activity of peroxidase-like AuNPs labeled on goat anti-human IgG antibody following deposition of gold. The immunoassay conditions were optimized and excellent analytical performances were presented. Furthermore, the method applicability was also investigated in real serum samples.

Materials and methods

Materials

Human IgG (H-IgG), goat anti-human IgG antibody (anti-IgG), HRP labeled goat anti-human IgG antibody (HRP-anti-IgG), and bovine serum albumin (BSA) were supplied by Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). 3,3′,5,5′-Tetramethylbenzidine (TMB) and sodium citrate dihydrate ($C_6H_5Na_3O_7{\cdot}2H_2O$) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Hydrogen tetrachloroaurate (III) hydrate ($HAuCl_4{\cdot}4H_2O$), hydrogen peroxide (H_2O_2), hydroxylammonium chloride ($NH_2OH{\cdot}HCl$), sodium carbonate anhydrous (Na_2CO_3), sodium hydrogen carbonate ($NaHCO_3$), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$), acetic acid (HAc), and sodium acetate (NaAc) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All reagents were of analytical grade and used without any futher purification.

The buffers used in the experiment are as follows: (1) coating buffer, 50 mM carbonate/bicarbonate buffer solution, pH 9.6; (2) phosphate buffered saline (PBS) buffer, containing 8 mM Na_2HPO_4, 2 mM NaH_2PO_4 and 0.9 % NaCl, pH 7.4; (3) blocking buffer (PBSB), 1 % (w/v) BSA in PBS buffer; (4) washing buffer (PBST), PBS buffer containing 0.05 % Tween-20 (v/v); (5) acetate buffer, 0.2 M, pH 4.0.

Apparatus

Solutions were prepared by double-deionized water (18.2 MΩ cm specific resistance) obtained by a Cascada LS Ultrapure Water System (Pall Corp., Port Washington, New York, USA). The morphologic evaluation was captured by scanning electron microscope (SEM, Hitachi Ltd., Tokyo, Japan). Absorption spectra were conducted on Infinite M200 PRO NanoQuant multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland). The pH of buffer solutions were adjusted on a PHS-3C meter. The solution was blended by a XW-80A vortex mixer

(Shanghai, China), and the reaction temperature was controlled by a DKB-501A constant-temperature water bath (Shanghai, China).

Preparation of AuNPs and AuNPs-anti-IgG conjugate

AuNPs were synthesized according to the reported method with slight modifications [7]. All glassware used in the preparation procedure were bathed in freshly prepared aqua regia (1:3 (v/v) HNO_3-HCl), thoroughly rinsed with double-deionized water, and dried in an oven prior to use. Typically, 10 mL of trisodium citrate (38.8 mM) was rapidly introduced into a boiling solution of $HAuCl_4$ (1 mM, 100 mL) while stirring, then the solution was refluxed for another 30 min to form a wine-red colloidal solution. The obtained solution was cooled to room temperature gradually with continuous stirring.

The following preparation of AuNPs-anti-IgG conjugate was referred to literature with some alterations [25]. One mL of anti-IgG (1 mg/mL) was added into 10 mL AuNPs suspension with pH at 9.0 (0.1 M Na_2CO_3 and 0.1 M HCl were used to adjust pH), and the mixture was incubated at room temperature for 1 h. Then 1 mL 10 % BSA solution was injected under stirring to block the unspecific binding of proteins to antibodies on AuNPs, followed by incubation at room temperature for another 0.5 h. The conjugate was centrifuged (16, 000 rpm) at 4 °C for 15 min, and the soft sediment was washed and resuspended in PBSB solution. Afterwards, the AuNPs-anti-IgG conjugate was stored at −20 °C for several months by addition of NaN_3 to a final concentration of 0.05 %.

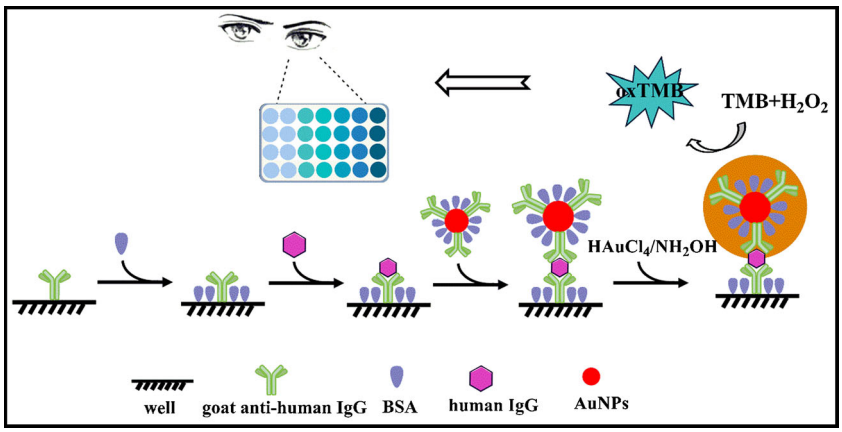
Immunoassay protocol

A typical colorimetric sandwich immunoassay was conducted as shown in Scheme 1. Initially, 100 μL of 0.1 mg/mL anti-IgG solution diluted by 50 mM Na_2CO_3-$NaHCO_3$ buffer (pH 9.6) were added to each microtiter well, followed by incubation at 4 °C overnight. The unbound antibody was washed away with PBST buffer. After washing, the wells were incubated with 150 μL blocking buffer at 37 °C for 1 h to block the unspecific binding of H-IgG. Then, the plate was washed with PBST three times and dilutions of H-IgG were pipetted into the wells and further incubated at 37 °C; 1 h later, the plate was washed with PBST followed by addition of 100 μL AuNPs-anti-IgG conjugate (1:1 dilution with PBSB). Next, six times washing with PBST for the plate was carried out after incubation at 37 °C for another 1 h. For the deposition of gold on the labeled AuNPs, 100 μL of gold enhancement solution (1:1 (v:v) mixture of 5 mM $HAuCl_4$ $4H_2O$ and 10 mM NH_2OH·HCl) was added and then incubated at 28 °C for 20 min [26]. After that, the well was washed with double-deionized water, and color development ensued in 100 μL of 0.45 mM TMB and 0.7 M H_2O_2 for 15 min incubation at 28 °C. The absorbance at 652 nm of each microtiter well was collected by using the Infinite M200 PRO NanoQuant multimode microplate reader. For comparison, HRP-anti-IgG was used as detection antibody to detect H-IgG in the substrate solution containing 0.45 mM TMB and 10 mM H_2O_2 with the same assay format after incubation at 28 °C for 15 min.

Results and discussion

Sensing mechanism of the colorimetric immunoassay

Thirteen nm AuNPs was synthesized by the citrate-mediated reduction of hydrogen tetrachloroaurate (III). The UV-vis spectra of the obtained AuNPs exhibited an intense characteristic localized surface plasmon resonance (LSPR) peak at 520 nm (see Electronic Supplementary Material (ESM) Fig. S1, red curve). Then the prepared AuNPs were labeled onto the anti-IgG (AuNPs-anti-IgG), and the UV-vis spectra of AuNPs-anti-IgG conjugates were similar to that of AuNPs (ESM Fig. S1, black curve). As shown in Scheme 1, anti-IgG fabricated on microtiter well served as capture antibody



Scheme 1 Schematic representation of the gold-enhanced peroxidase-like activity of AuNPs for colorimetric immunoassay of H-IgG

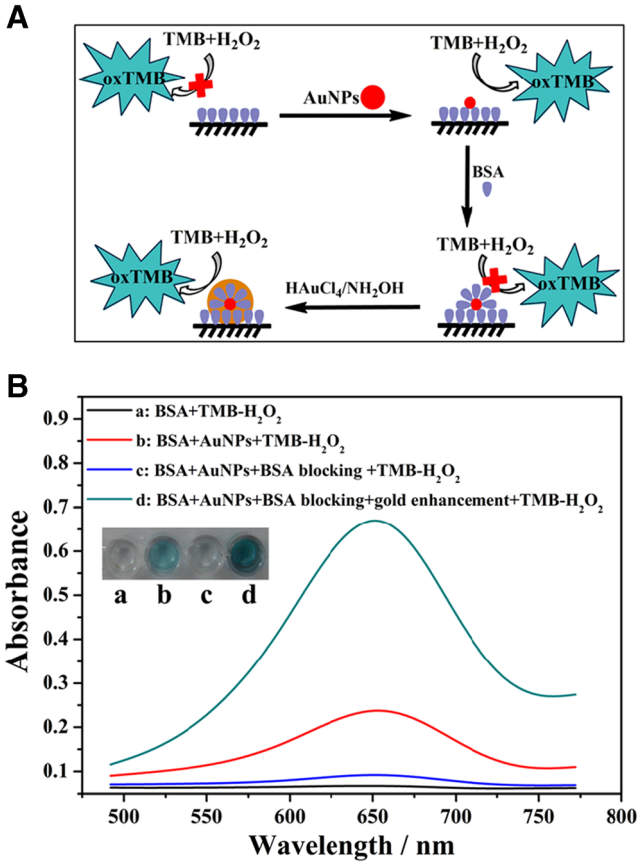


Fig. 1 **(A)** Scheme mechanism of gold-enhanced peroxidase-like activity of AuNPs and **(B)** UV-vis spectra and photo images of solutions containing (a) BSA+TMB-H_2O_2, (b) BSA+AuNPs+TMB-H_2O_2, (c) BSA+AuNPs+BSA blocking+TMB-H_2O_2, and (d) BSA+AuNPs+BSA blocking+gold enhancement+TMB-H_2O_2

to identify H-IgG upon specific antigen–antibody interactions; AuNPs-anti-IgG conjugate was added as detection antibody to bind captured H-IgG, forming a sandwich structure. Before the colorimetric measurement, $HAuCl_4{\cdot}4H_2O$ and $NH_2OH{\cdot}HCl$ were introduced. Similar to the seed-mediated synthesis of AuNPs [26–28], the addition of gold growth solution caused the continuous enlargement of the immobilized AuNPs (ESM Fig. S2), which in turn recovered their peroxidase-like activity. Because TMB could be oxidized by H_2O_2 in the presence of peroxidases or catalysts, it was adopted to visually evaluate the activity of the AuNPs-based peroxidase mimics. Therefore, the concentration of H-IgG could be detected by estimating the catalytic ability of gold-enhanced AuNPs to catalyze TMB oxidation using H_2O_2 as an electron acceptor.

To demonstrate the universality of the proposed sensing system, BSA, the most common protein, was adopted to explore the sensing mechanism instead of H-IgG. As illustrated in Fig. 1A, BSA was first incubated in a 96-well microtiter plate at 4 °C overnight, and AuNPs could bind BSA by electrostatic interaction and hydrophobic effect. Meanwhile, the peroxidase-like activity of AuNPs was evaluated by the oxidation of peroxidase enzyme substrate TMB. As shown in Fig. 1B (curve b), the bare AuNPs could catalyze the oxidation of TMB by H_2O_2 to develop a bright blue color, simultaneously showing a maximum absorbance at 652 nm. The possible principle of the catalytic reaction was that H_2O_2 could be adsorbed on the surfaces of AuNPs, making the breakup of the O–O bond into •HO radicals [19, 29]. By means of the exchange interaction between the unpaired electrons of radicals and conduction band electrons of AuNPs, the generated •HO radicals could be stabilized and become concentrated by AuNPs. Owing to the stabilization and concentration, •HO radicals triggered the •HO involved reaction, e.g., the oxidation of TMB, which further contributed to the catalytic ability of AuNPs. However, when the surface of AuNPs was blocked, the catalytic reaction was nearly inhibited (Fig. 1B, curve c). It is well known that the catalytic reactions usually take place at the surface of nanomaterials; hence, the blocking protein molecules may replace the enzyme reaction substrate, resulting in reduced catalytic activity. Interestingly, the catalytic ability of AuNPs was dramatically increased by depositing the gold shell (Fig. 1B, curve d), which was due to the occurrence of new shell with the increase of AuNPs' diameter. The photo images in Fig. 1B also confirmed the sensing mechanism.

Optimization of immunoassay conditions

The sensitivity of this proposed method was closely related to experimental parameters, including gold enhancement time, concentration of TMB and H_2O_2, and catalytic time. The value of ΔA was explored to evaluate experiments, where ΔA indicated the difference of absorbance at 652 nm of reaction solution in the presence and absence of H-IgG.

Since the gold enhancement time played a crucial role in this colorimetric strategy, it was of great necessity to study its effect. As shown in ESM Fig. S3A, the value of ΔA increased almost linearly with the increase of enhancement time in the range from 5 to 20 min; when the time was longer than 20 min, the value varied slightly. As the results indicated, 20 min of gold enhancement time was chosen to carry out the experiments.

Figure S3B in the ESM shows the effect of concentration of TMB on the catalytic reaction. The absorbance at 652 nm of reaction solution with or without H-IgG was gradually increased, respectively. On one hand, the value of ΔA reached a maximum at the concentration of 0.45 mM. On the other hand, low background color was of benefit to the conduct of colorimetric detection. In consideration of the above two factors, an optimum TMB concentration of 0.45 mM was chosen for H-IgG determination in subsequent experiments. We further investigated the effect of concentration of H_2O_2 on the value of ΔA, and the experiment was performed in the range from 0.1 to 1.1 M. As shown in ESM Fig. S3C, the value of

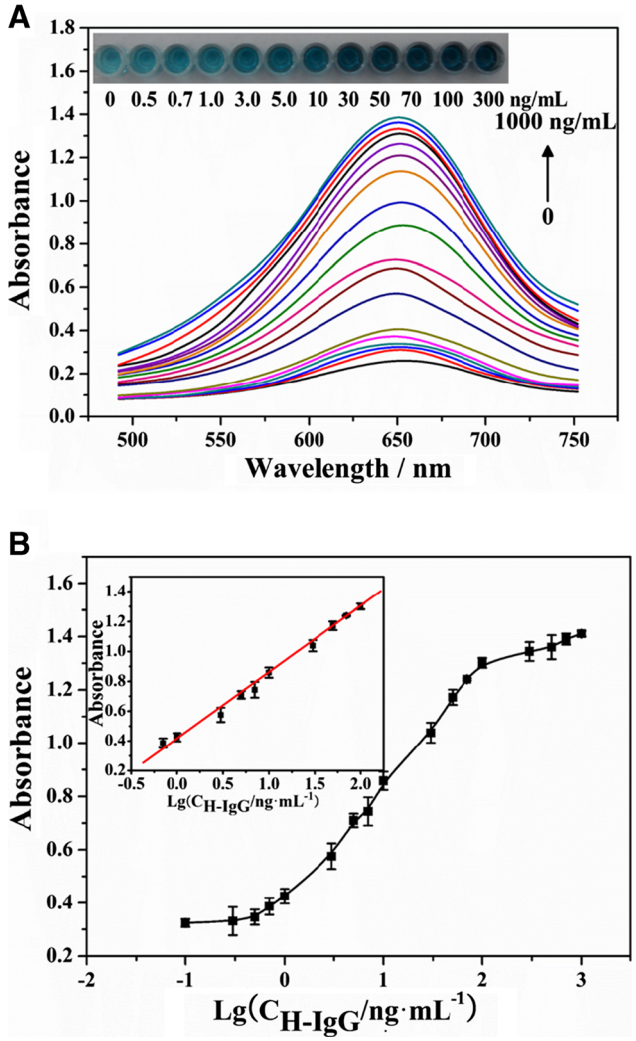


Fig. 2 **(A)** UV-vis spectra and the corresponding color change, and **(B)** a plot of UV-vis absorbance at 652 nm versus different concentrations of H-IgG. The error bars represent standard deviations obtained from three parallel experiments

ΔA became constant when the concentration of H_2O_2 reached 0.7 M. Thus, 0.7 M of H_2O_2 was used to perform the experiment.

The effect of the catalytic reaction time was also considered for improving the sensitivity toward H-IgG. We noted that the value of ΔA progressively increased upon increasing the catalytic reaction time from 5 to 15 min (ESM Fig. S3D), which revealed that the optimum time required for catalytic reaction of TMB was 15 min. Hence, all subsequent tests were carried out with a catalytic reaction time of 15 min.

Analytical performances of the colorimetric immunoassay

To investigate the analytical performances of this proposed colorimetric immunoassay, the UV-vis absorbance spectra of reaction solution with different concentrations of H-IgG were collected under optimized detection conditions. As shown in Fig. 2A, the absorbance spectra increased gradually upon addition of increasing concentrations of H-IgG with visually deeper blue color change because of the higher catalytic ability for the oxidation of TMB by H_2O_2. The absorbance at 652 nm (Fig. 2B) was employed to analyse H-IgG quantitatively. A good linear relationship was obtained in the range from 0.7 to 100 ng/mL, and the limit of detection (LOD) was 0.3 ng/mL calculated by $3\sigma/S$. In order to demonstrate the significance of gold enhancement, we compared the performance of AuNPs-anti-IgG in the absence and presence of gold deposition. As illustrated clearly in ESM Fig. S4, the detection of H-IgG in lower concentration (10 ng/mL) without gold enhancement was infeasible and no appreciable color change was observed. On the contrary, the presence of gold deposition could obtain remarkable absorbance in the detection performance, and the LOD for naked-eye detection was 5 ng/mL. The reproducibility of naked-eye detections for the proposed colorimetric immunoassay was evaluated by batch-to-batch detecting various levels of H-IgG in different polystyrene 96-well microplates six times, as shown in ESM Fig. S5. The obtained intra-batch coefficients of variation (CVs, $n=6$) were in the range from 2.2 %–9.3 % (ESM Table S1). So, the gold-enhanced AuNPs-based colorimetric immunoassay attained satisfactory reproducibility, and further verified the feasibility of batch preparation.

Table 1 Spiked recoveries for the determination of the H-IgG in real serum samples using the colorimetric immunoassay

Sample	Added (ng/mL)	Detected (ng/mL)	Recovery (%)
Fetal bovine serum	3.0	3.2±0.2	106.7±0.9
	20.0	18.6±0.4	92.9±2.0
	50.0	46.9±1.5	94.0±3.1
Human serum (10^6 folds dilution)	0	1.1±0.03	—
	10.0	12.2±0.18	111.0±1.6
	20.0	20.7±0.4	98.0±2.0

In addition, the conventional ELISA protocol with HRP-labeled antibodies was also used for comparison. As shown in ESM Fig. S6, the linear relationship was obtained in the range of 5.0–200 ng/mL, and the LOD was 1.6 ng/mL. The results revealed that our proposed method was competitive with the well-established ELISA. It has been reported that a series of peroxidase-like nanoparticles have been used in colorimetric immunoassay, such as Fe_3O_4 nanoparticles [30], Au@Pt nanostructures [31], Fe-aminoclay [32], Pt nanoparticles [33], and Pt@mSiO_2 nanoparticles [34]. By the comparison shown in ESM Table S2, our developed colorimetric immunoassay provides a potentially novel and sensitive strategy for H-IgG detection.

Practical application of the colorimetric immunoassay for detection of H-IgG

The practical feasibility of the developed colorimetric immunoassay was further explored for detection of H-IgG in real serum samples. Considering that healthy human serum generally contains H-IgG higher than 1 mg/mL [35], a high dilution strategy [33, 36] was adopted. As shown in Table 1, the human serum samples with 1,000,000-fold dilution were spiked with H-IgG, and satisfactory recoveries and accuracies were obtained in a range of 98.0−111.0 % with relative standard deviations (RSDs) of 1.6−2.0 %. It was found that the endogenous H-IgG was detected at 1.1 mg/mL in the human serum (Table 1), which was consistent with that reported [35]. As well, the recoveries for the spiked fetal bovine serum samples were 92.9−106.7 % with RSD of 0.9−3.1 %, as shown in Table 1. Therefore, these results confirmed the developed gold-enhanced AuNPs-based colorimetric immunoassay was essentially feasible for accurate determination of trace H-IgG in complex biological samples.

Conclusions

A novel colorimetric immunoassay system was established by utilizing gold-enhanced AuNPs as the enzyme mimic and TMB as the peroxidase substrate. This approach could avoid complicated instruments and allowed detecting H-IgG only by naked eyes as well as microplate reader. In addition, it also offers the outstanding features of simplicity, high sensitivity and low cost. What's more, the experimental results further provided a great possibility to the analysis of H-IgG in real samples. Thus, our developed colorimetric immunoassay paves the way for the peroxidase-like activity of nanomaterials in biochemistry and biomedical sciences.

Acknowledgments This work was financially supported by the National Natural Science Foundation of China (grant no. 21275158, 21575159), the 100 Talents Program of the Chinese Academy of Sciences, and the National Research Foundation of Korea (grant no. 2008-0061891 and 2009-00426).

Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

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