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Gold nanoparticle as an electrochemical label for inherently crosstalk-free multiplexed immunoassay on a disposable chip

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ABSTRACT

This work proposed a simple, sensitive and low-cost multiplexed immunoassay by combining a disposable chip with gold nanoparticle (AuNP) as an electrochemical label. The immunosensors array as the disposable chip was firstly prepared by immobilizing capture antibodies on different screen printed carbon working electrodes by passive adsorption. With a sandwich mode, the analytes were then bound to the corresponding capture antibodies for further capture of the gold nanoparticle labeled antibodies. Gold nanoparticles were finally electrooxidized in 0.1 M HCl to produce $AuCl_4^-$ for differential pulse voltammetric detection. Using human IgG and goat IgG as model targets, under optimal conditions this method achieved linear ranges from 5.0 to 500 and 5.0 to 400 ng mL^{-1} with limits of detection of 1.1 and 1.6 ng mL^{-1}, respectively. This method eliminated completely electrochemical crosstalk between adjacent immunosensors due to the strong adsorption of the $AuCl_4^-$ on the printed carbon surfaces. The immunosensors array showed acceptable precision, reproducibility and stability, and could be readily applied to multianalyte determination for clinical diagnostics.

1. Introduction

Gold nanoparticles (AuNPs) have been attracted considerable interest in analytical and biomedical fields. The rapid and simple chemical synthesis, narrow size distribution, and convenient labeling to biomolecules have enabled AuNPs to be extensively used for the preparation of biosensors and signal amplification [1]. Primary antibody can easily be immobilized on AuNP modified electrode through electrostatic or hydrophobic interaction to construct immunosensor platforms [2–6]. The doping of AuNPs in sol–gel matrix can accelerate the electron transfer between electroactive center and electrode surface [7–9]. A great number of nanocomposites of AuNPs, including enzyme-contained nanocomposites, have been designed and synthesized as bioprobes for high sensitive detection [1,10–15] and biocatalysis [16–18]. Furthermore, AuNPs can also be used as an electrochemical label by the chemical oxidation of AuNPs in HBr/Br_2 to produce electrochemically detectable species [19,20]. This method requires a preoxidization process with highly toxic Br_2 for a relatively long time. As the improvement, an electrooxidization method of AuNPs in HCl to produce electroactive $AuCl_4^-$ has been demonstrated, which has been applied to monitor protein interactions [21–23] and DNA hybridization [24]. This work made use of this property and the strong absorption of the electrooxidation product $AuCl_4^-$ on rough carbon surface to propose a novel method for crosstalk-free multiplexed immunoassay.

Simultaneous multianalyte immunoassay that can quantitatively measure the concentrations of several analytes in a single assay is very important in clinical and environmental fields [25]. Electrochemical immunosensors arrays have gained considerable interest due to their low cost for mass produce, small sample volume, and acceptable sensitivity [26]. However, crosstalk can potentially occur due to the diffusion of electroactive product generated at one electrode to a neighboring electrode to produce an interfering amperometric response. Its elimination mainly focuses on making the distance between adjacent electrodes larger than the diffusion distance of electroactive substances [25–28] or immobilizing electron transfer mediators or electroactive enzyme on sensor surfaces to perform reagentless detection [8,9,29,30].

In this work, the advantages of AuNPs as an electrochemical label were combined with screen printed carbon electrodes to construct a disposable immunosensors array (Fig. 1). With a sandwich format for immunoassay and capture of AuNP labeled antibodies on the disposable chip, the developed multiplexed immunoassay method avoided the crosstalk and a deoxygenation process that is generally essential in amperometric immunoassays [8–11,29,30], thus simplifying greatly the detection process. This method could immediately be extended to multianalyte determination for the need of clinical diagnostics.

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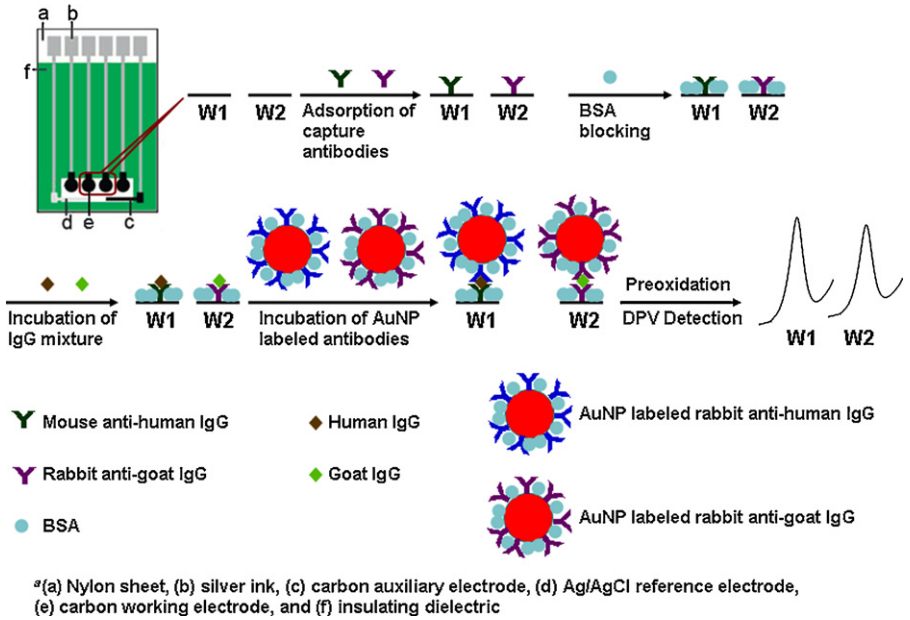

Fig. 1. Schematic representation of the preparation of immunosensors array and analytical procedure for the simultaneous detection of HIgG and GIgG.

2. Materials and methods

2.1. Materials and reagents

Human IgG (HIgG), goat IgG (GIgG) and polyclone rabbit anti-GIgG (RaG) were purchased from Beijing Solarbio Science & Technology Co., Ltd. Polyclone rabbit anti-HIgG (RaH) was purchased from Wuhan Boster Biological Technology Ltd. Monoclone mouse anti-HIgG (MaH) was purchased from Xiamen Boson Biotechnology Ltd. Bovine serum albumin (BSA) was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). All other reagents were of analytical reagent grade and used without further purification. 0.1 M Tris–HCl (pH 7.2) containing 1% BSA was used as blocking buffer. 50 mM phosphate-buffered saline (PBS) (pH 7.4) was prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4. Doubly distilled water was used throughout the experiments.

2.2. Apparatus

Preoxidation of AuNPs bound on the immunocomplexes followed by differential pulse voltammetric (DPV) measurement was automatically performed on a BAS Epsilon-EC electrochemical workstation (Bioanalytical Systems, Inc., USA). UV–vis measurements were carried out on a UV-3600 UV-vis spectrophotometer (Shimadzu, Japan).

2.3. Preparation of immunosensors array

Screen printed carbon electrodes were fabricated according to the previous literature [9]. All working electrodes with 0.5 mm edge-to-edge separation shared the same Ag/AgCl reference and graphite auxiliary electrodes, which were printed on the same chip. The insulating layer printed around the working area constituted an electrochemical microcell. As shown in Fig. 1, two neighboring working electrodes (W1, W2) were used in this work for the simultaneous detection of HIgG and GIgG.

Capture antibodies could be immobilized onto rough carbon electrode surfaces by passive adsorption [21,23,31]. 0.5 μL of MaH and RaG at 0.2 mg mL^{-1} in PBS were dropped on two working electrodes respectively. The chip was incubated in water vapor saturated environment at 4 °C overnight to allow the passive adsorption of antibodies onto the carbon electrode surfaces. After incubation, excess antibodies were rinsed with doubly distilled water. For suppression of unspecific adsorption, the electrochemical microcell was incubated with 50 μL blocking buffer for 20 min at room temperature. After rinsed thoroughly with doubly distilled water, the resulting immunosensors array was stored at 4 °C in dry air at dark prior to use.

2.4. Preparation of AuNP labeled antibodies

Gold colloids were prepared according to the previous literature with slight modification [32]. A 34-mg portion of $HAuCl_4$ was dissolved in 100 mL of Millipore purified water (1 mM) and brought to vigorous boiling with stirring. A 10-mL portion of 38.8 mM sodium citrate was added, and the stirring was continued for another 10 min. The solution changed from colorless to deep red. After 10 min, the solution was rapidly cooled in an ice water bath with stirring. This procedure produced a stable gold nanoparticle colloid solution with an average diameter of 15 nm.

Two kinds of AuNP labeled antibodies were prepared according to the previous literature [33] with slight modification. 10 μL RaH or RaG at 1.0 mg mL^{-1} was added to 200 μL colloidal gold solution adjusted to pH 9.0 with 0.1 M K_2CO_3, followed by gently shaking in room temperature for 1 h. The conjugate was then centrifuged at 8000 g for 0.5 h. The supernatant was discarded and the soft sediment was rinsed with 200 μL PBS containing 0.3% BSA. After centrifugation at 8000 g for another 0.5 h, the conjugate was resuspended in 200 μL PBS containing 0.3% BSA. The resulting gold nanoparticle labeled antibodies could be stored at 4 °C for more than 2 weeks without loss of activity.

2.5. Analytical procedure

10 μL sample containing either HIgG and GIgG or mixture of the two antigens was used to incubate two neighboring sensors for 40 min at room temperature. The sensors were then washed with doubly distilled water and incubated with 10 μL 1:1 mixture of AuNP labeled RaH and AuNP labeled RaG. After the sensors were washed with doubly distilled water, 50 μL 0.1 M HCl was dropped

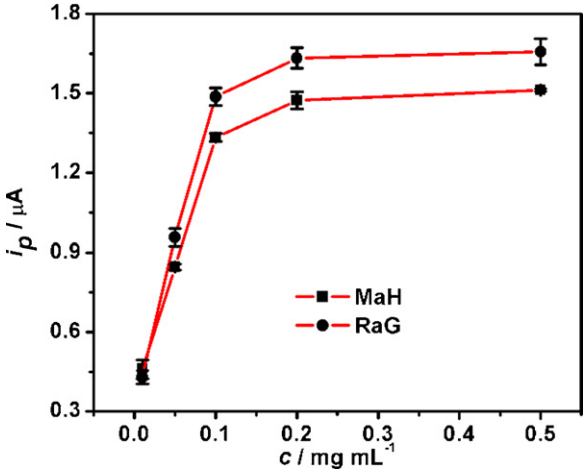

Fig. 2. Effects of MaH and RaG IgG antibody concentrations on DPV responses of HIgG and GIgG at 1 μg mL^{-1}.

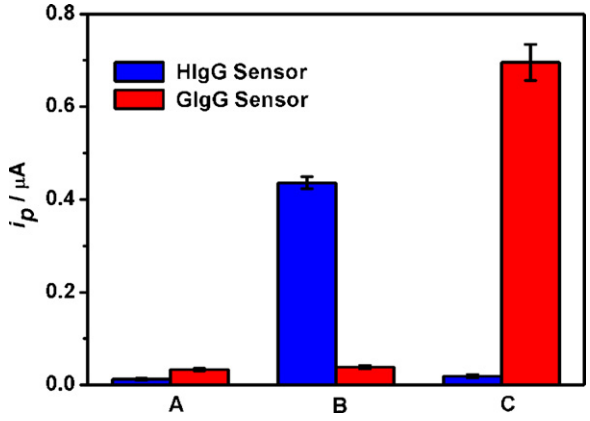

Fig. 4. DPV responses of immunosensors array to (A) blank control, (B) 100 ng mL^{-1} HIgG and (C) 125 ng mL^{-1} GIgG.

on the electrochemical microcell to perform the electrooxidation of AuNPs at a constant potential of +1.3 V for 40 s, and immediately the DPV detection from +0.6 to 0 V, with a step potential of 4 mV, a pulse amplitude of 50 mV, and a pulse period of 0.2 s.

3. Results and discussion

3.1. Preparation of gold–antibody conjugate

The surface of AuNPs should be protected by proteins in order to minimize unspecific adsorption of gold–antibody conjugate on the immunosensor surface. It was found that 10 μL 1.0 mg mL^{-1} antibody was sufficient to conjugate 200 μL as-prepared gold colloid through gold flocculation test [1,34]. After antibody solution was added to the pH-adjusted colloidal gold solution and incubated for 10 min, upon the addition of 25 μL 10% NaCl, the mixture did not show any flocculation, while pure gold colloid solution flocculated immediately after treating with NaCl. UV–vis also showed that the absorption peak of gold–antibody conjugate at 520 nm did not change after adding NaCl, indicating that the AuNPs were stabilized and protected by the antibody.

3.2. Optimization of capture antibody concentration for immobilization

The concentrations of capture antibody solutions were optimized in order that a maximum number of antibodies were immobilized on the carbon electrodes to capture the corresponding antigens. The working electrodes were coated with different concentrations of antibody solutions, followed by blocking with BSA, binding with 1 μg mL^{-1} antigens and AuNP labeled antibodies. The peak currents increased with the increasing capture antibody concentrations until the concentrations reached 0.2 mg mL^{-1} (Fig. 2). Therefore, 0.2 mg mL^{-1} of both capture antibody solutions was chosen for coating the carbon electrodes.

3.3. Selection of oxidation potential and time of AuNPs

The experiments were performed on the HIgG immunosensors and the concentration of HIgG solution was 200 ng mL^{-1}. Oxidation potential was crucial because too low potential would not be able to oxidize gold in HCl to $AuCl_4^-$, while too high potential would cause damage of the screen printed carbon electrode surfaces and further affect electrochemical signals. The oxidation potential was investigated with DPV detection after the preoxidation of the captured AuNPs in 0.1 M HCl at a constant potential for 40 s. As shown in Fig. 3a, the peak current reached the maximum value when the preoxidation potential was +1.30 V. When the potentials were at +1.35 and +1.40 V, the peak current decreased greatly and showed poor repeatability. Thus, +1.30 V was selected as the preoxidation potential.

At the preoxidation potential of +1.30 V, the oxidation time was also important. An insufficient time would lead to the incomplete oxidation of gold and a redundant time would not increase the electrochemical signal, while $AuCl_4^-$ might diffuse away slowly. As shown in Fig. 3b, the DPV response tended to a stable value at the time of 40 s. Therefore, 40 s was chosen as the preoxidation time.

3.4. Evaluation of crossreactivity and crosstalk

Multiplexed immunoassay must exclude the crossreactivity among unrelated antibodies and antigens and crosstalk between

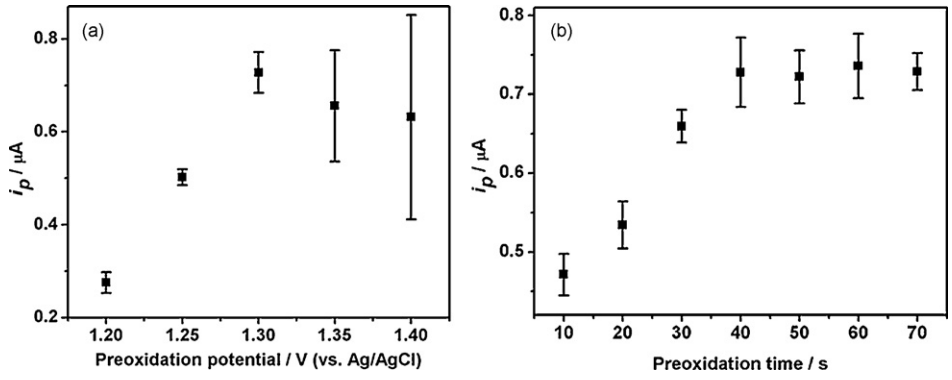

Fig. 3. Effects of preoxidation potential (a) and time (b) on DPV response to 200 ng mL^{-1} HIgG.

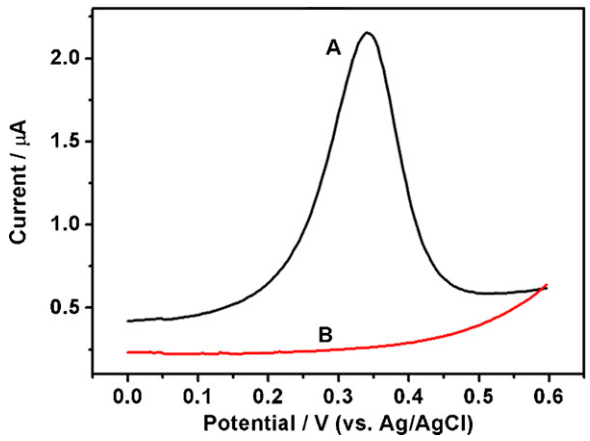

Fig. 5. DPV responses of (A) immunosensors to corresponding IgG and (B) neighboring bare electrode.

adjacent electrodes. For the evaluation of crossreactivity, 10 μL of blank solution, 100 ng mL^{-1} HIgG or 125 ng mL^{-1} GIgG was dropped on two immunosensors to perform the incubation step. Only the immunosensors prepared with corresponding capture antibodies showed reduction peak currents (Fig. 4). Increased concentration of interference antigens to some extent did not increase the currents significantly.

For the evaluation of crosstalk, 5 μL HIgG at 1 μg mL^{-1} was incubated on the chip containing a bare carbon electrode and one immunosensor prepared with MaH, and followed by incubation with 5 μL AuNP labeled RaH. Only the immunosensor showed a reduction peak (Fig. 5), indicating that potential crosstalk caused by diffusion of $AuCl_4^-$ between electrodes was completely avoided. Additionally, the possibility of the oxidation of the coexisted substances or electrode material, which might also be reduced during the DPV measurement was excluded, as no interfering signal was observed (Fig. 5, curve B).

3.5. Simultaneous detection of HIgG and GIgG

Under optimal conditions, with the increasing concentrations of HIgG and GIgG, the peak current increased, which resulted from the increasing amount of AuNPs captured on the immunosensors. As expected from a sandwich mechanism, both reduction peak currents were proportional to HIgG and GIgG concentrations. The dose–response curves obtained from HIgG and GIgG immunosensors increased linearly in the ranges from 5.0 to 500 ng mL^{-1} with a correlation coefficient of 0.995 and 5.0–400 ng mL^{-1} with a correlation coefficient of 0.993, respectively (Fig. 6). The limits of detection corresponding to the signals of 3 SD above the mean for a zero standard were 1.1 and 1.6 ng mL^{-1} for HIgG and GIgG, respectively.

3.6. Precision, reproducibility and stability of immunosensors

The inter-assay precision of the immunosensors array was evaluated using five chips. The coefficients of variation for inter-assay were 7.1 and 6.0% for 50 and 200 ng mL^{-1} HIgG, and 6.7 and 5.3% for 50 and 250 ng mL^{-1} GIgG, indicating acceptable precision and fabrication reproducibility. The immunosensors were stored in dry air at dark at 4 °C. The DPV responses were 90.1 and 92.5% of initial responses for HIgG and GIgG after a storage period of 2 weeks. Thus the storage stability of the immunosensors was acceptable.

3.7. Application of immunosensors for real samples

After human serum samples were 4000-time diluted with 50 mM pH 7.4 PBS for multiplexed immunoassay of HIgG and GIgG, different amounts of HIgG and GIgG were added into the samples for recovery tests. As human serum samples did not contain GIgG,

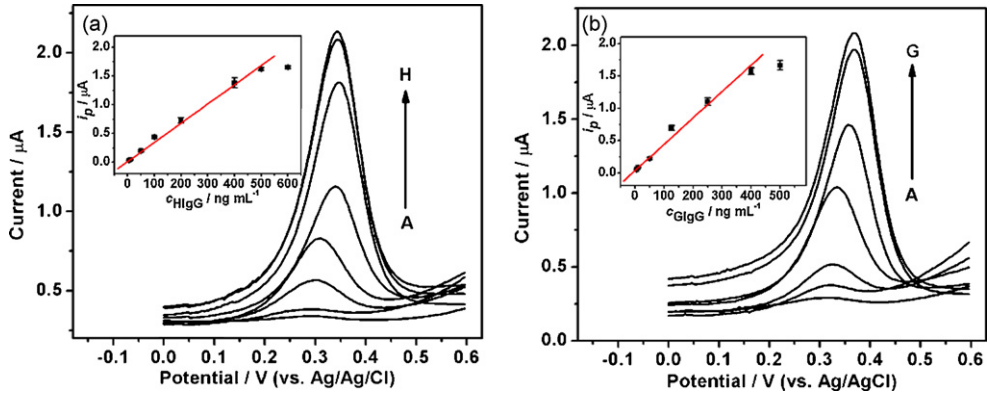

Fig. 6. DPV responses for the simultaneous multiplexed detection of (a) HIgG and (b) GIgG using AuNP as a label at HIgG concentrations of 5, 10, 50, 100, 200, 400, 500 and 600 ng mL^{-1} from (A) to (H) and GIgG concentrations of 5, 10, 50, 125, 250, 400 and 500 ng mL^{-1} from (A) to (G). Insets: corresponding calibration curves.

Table 1
Recovery tests of HIgG and GIgG in human serum samples.

	Samples	1	2	3	4	5
Detection of HIgG (ng mL^{-1})	Initial concentration	19.0	26.9	82.1	92.2	137.6
	Added	10.0	25.0	50.0	100.0	200.0
	Concentrations after addition	29.9	46.4	129.6	197.6	330.3
	Relative standard deviation (%)	9.4	3.1	8.2	6.0	4.4
	Recovery (%)	109.4	97.6	94.9	105.4	96.3
Detection of GIgG (ng mL^{-1})	Initial concentration	0	0	0	0	0
	Added	10.0	25.0	50.0	100.0	200.0
	Concentrations after addition	10.7	25.8	54.2	96.3	207.0
	Relative standard deviation (%)	6.5	4.5	3.6	7.1	5.9
	Recovery (%)	107.0	103.3	108.5	96.3	103.5

the initial concentration of GIgG was zero. The test results were listed in Table 1. The recoveries were between 94.9 and 109.4%, and the relative standard deviations were less than 9.4 for HigG and 7.1% for GIgG, indicating good accuracy of the proposed method for human serum samples.

4. Conclusions

AuNP has been used as an electrochemical label for simple, sensitive and low-cost multiplexed immunoassay on a disposable chip. The immunosensors array is prepared by simply adsorption of capture antibodies on bare screen printed carbon electrodes, and the immunoassay is performed in a sandwich format. The electrooxidation of AuNPs in HCl and the strong adsorption of the produced $AuCl_4^-$ on the rough carbon surface enable direct electrochemical detection of the amount of captured gold, and meanwhile avoid crosstalk between adjacent electrodes. For each gold nanoparticle contains thousands of atoms, the immunoassay possesses relatively high sensitivity. The proposed method shows satisfied feasibility, precision, reproducibility, demonstrating the potential extension for simultaneous detection of several biomarkers for clinical diagnostics.

Acknowledgments

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