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An electrochemical amplification immunoassay using biocatalytic metal deposition coupled with anodic stripping voltammetric detection

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Abstract

An electrochemical amplification immunoassay is reported using biocatalytic metal deposition coupled with anodic stripping voltammetric detection. In this method, the captured antibody was first immobilized onto a gold electrode *via* a self-assembled layer. After a sandwich immunoreaction, alkaline phosphatase-labeled antibody was bound to the gold electrode. The alkaline phosphatase on the electrode catalyzes the hydrolysis of ascorbic acid 2-phosphate to produce ascorbic acid. The latter, in turn, reduced silver ions on the electrode surface, leading to the deposition of silver onto the protein-modified electrode surface. The deposited metal was electrochemically stripped into solution and then measured by anodic stripping voltammmetry. Compared with the direct voltammetric detection of ascorbic acid, anodic stripping voltammetric detection of metal ions is more sensitive. For the amount of deposited silver relates to the amount of enzyme-generated ascorbic acid, which was controlled by the amount of enzyme bound on the electrode surface, the stripping current signal reflects the amount of target protein, achieving a linearly relationship in the range from 5 to 1000 ng mL^{-1} in a logarithmic plot with a detection limit of 2.2 ng mL^{-1}. The utilization of the high biocatalytic activity of enzyme and the sensitive anodic stripping voltammetry to detect metal ions dramatically enhanced the sensitivity in immunoassay.

Keywords: Electrochemical immunoassay; Anodic stripping voltammmetry; Biocatalytic metal deposition

1. Introduction

Immunoassays are based on the use of an antibody that reacts specifically with the substance (antigen) to be tested, and quantification is generally achieved by measuring the specific activity of a label. A challenging topic in the development of sensitive electrochemical immunoassay or DNA assay is associated with the amplification procedure and the detection methods. Many strategies, such as the using of nanoparticles [1–9], in particular ferrocence-capped gold nanoparticles [9] and liposomes encapsulated with potassium ferrocyanide [10] as electrochemical labels have been proposed to enhance the sensitivity because every label contains hundreds to millions of electroactive molecules. The utilization of the intrinsic property of gold nanoparticles to catalyze reduction of metal ions on the nanoparticles and thereby to enlarge the metallic nanoparticles is also employed to further enhance the sensitivity [11–14]. Enzyme is routinely used as the amplifier in immunoassay. The application of carbon nanotubes as label to load more enzymes has dramatically amplified enzyme-based bioaffinity electrical sensing of proteins and DNA [15].

Measuring products originated by the biocatalytic activity of enzyme-labeled immunoreagents is one of the most popular methods in immunoassay. The development of sensitive methods to detect the enzyme-generated product is, of course, very important to enhance the sensitivity. Thus, the flow system arrangement coupled with ac adsorptive stripping voltammetry was used to demonstrate detection of pneumolysin and interkeulin at picogram per milliliter levels by employing a conventional ELISA approach with alkaline phosphatase label [16]. Willner's group reported on the use of chronopotentiometry and Faradic impedance spectroscopy for the transduction

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of an amplified immunosensing scheme that consisted of the biocatalytic precipitation of an insoluble product onto a gold electrode surface [17–19]. Hwang et al. developed a DNA sensor that used stripping voltammetry to detect the accumulated silver metal reduced by enzyme-generated product [20]. The coupling of enzyme catalysis and metal deposition seems to be a promising strategy for sensitive immunoassay.

In this work, we attempted to expand the method proposed by Hwang to immunoassay. The voltammetric experiments revealed that the stripping current peak of metal deposited on protein-modified gold electrode surface was split. This makes the measurement of the current signal rather difficult. To overcome this drawback, herein we adopted two procedures to detect the metal deposited on electrode surface, i.e., the deposited metal was first stripped from electrode with a potential of 0.7 V for 1 min, and then was accumulated electrochemically on a glassy carbon electrode for anodic stripping detection. The results show that the coupling of biocatalytic metal deposition and anodic stripping voltammetric detection dramatically enhanced the sensitivity.

2. Experimental

2.1. Materials and reagents

Goat anti-human IgG antibody, human IgG, and bovine serum albumin (BSA) were purchased from Beijing Dingguo Biotechnology Development Center (Beijing, China). Cystamine dihydrochloride and alkaline phosphatase (ALP) conjugated anti-human IgG antibody was provided by Sigma–Aldrich. Different concentrations of human IgG were prepared by diluting the purchased human IgG (10 mg/ml) solution with Tris–HCl buffer containing 1% BSA. Ascorbic acid 2-phosphate (AA-p) was obtained from Express Technology Co. Ltd. (Japan). The enzyme reaction buffer (EB) was prepared containing 80 mM glycine and 1 mM $MgSO_4$ (pH 9.0). Other reagents were of analytical purity, and doubly distilled water was used throughout all experiments.

2.2. Instruments

Electrochemical measurements were performed with a three-electrode system comprising a platinum foil as auxiliary electrode, a saturated calomel electrode (SCE) as reference electrode, and the modified gold electrode or glassy carbon electrode as working electrode. All electrochemical experiments were performed on CHI660 electrochemical workstation (Shanghai Chenhua Instruments, Shanghai, China).

2.3. Au electrode modification

The gold electrode (3.0 mm in diameter) was carefully polished with alumina slurries (0.3, 0.05 μm) on microcloth pads, cleaned in distilled water, piranha solution and absolute ethanol successively, and then dried at room temperature. The cleaned gold electrode was immediately immersed in 0.01 M cystamine solution overnight at room temperature. After reaction, the modified substrates were removed from the solution and the electrode was rinsed several times with ethanol to remove any physically absorbed cystamine. An exposed active amino group was formed on the electrode surface. The electrode was subsequently immerged in 2.5% glutaraldehyde solution for 1 h at room temperature and rinsed with water. Goat anti-human IgG antibody was introduced onto the electrode surface by dispersing 20 μL of 0.25 mg mL^{-1} antibody on the gold electrode surface and incubating for 1 h. Then 20 μL of 10 mg mL^{-1} BSA was added on its surface and incubated at room temperature for 30 min to block the active binding sites for protein. Finally, the electrode was washed with PBS of pH 7.0 and water. When not in use, the modified electrode was kept at 4 °C.

2.4. Analytical procedure

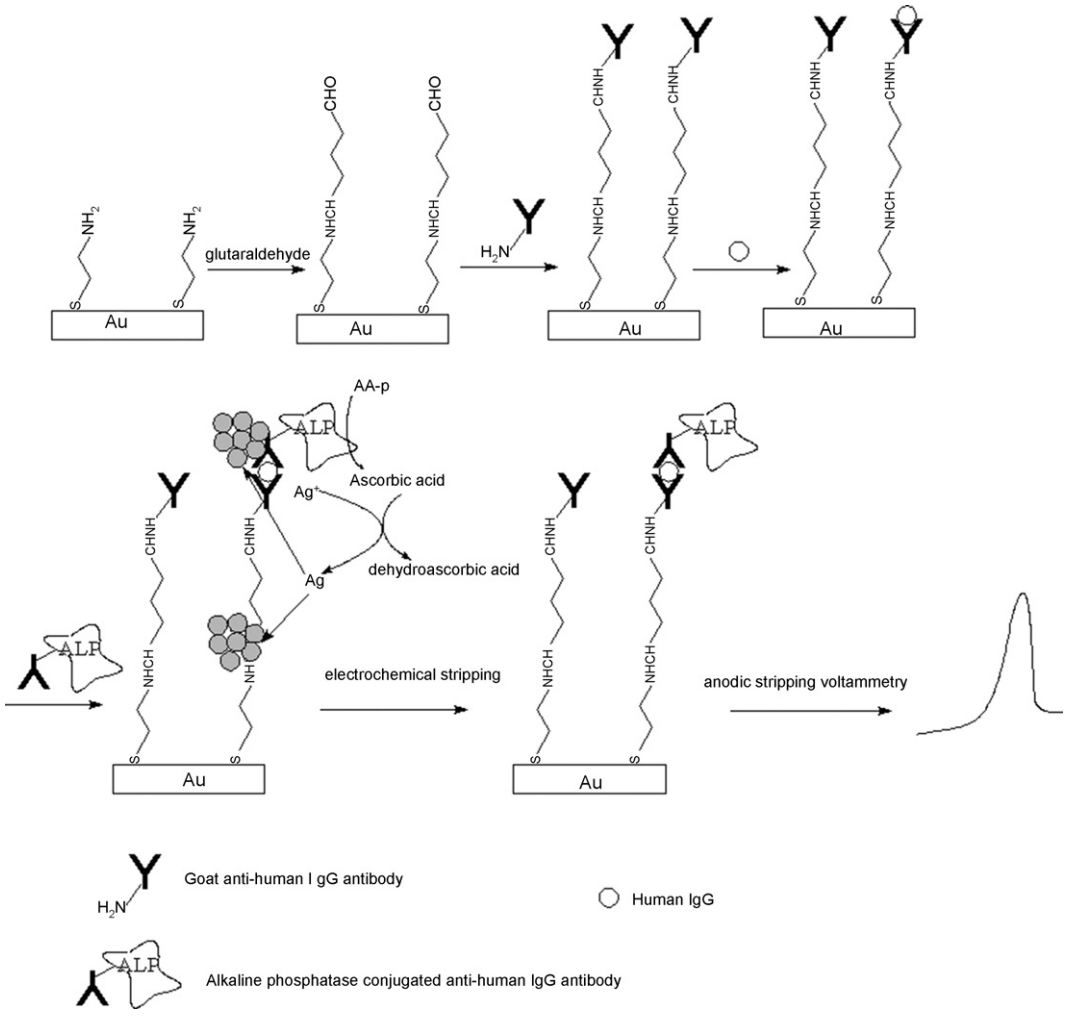
As shown in Scheme 1, the antibody-functionalized electrode was interacted with varying concentrations of human IgG for 40 min at 37 °C in Tris–HCl buffer (pH 7.4). After the attachment of the human IgG to the sensing interface, the electrode was rinsed with Tris–HCl buffer and incubated in the solution of alkaline phosphatase conjugated anti-human IgG antibody for another 40 min at 37 °C and then rinsed yielding a layer of immunocomplex on the electrode surface. The modified electrode was immersed in 0.5 mL EB solution containing 1.5 mM AA-p and 2.0 mM $AgNO_3$. Silver was deposited on the protein-modified electrode surface in a dark chamber for 20 min. After rinsing with water, the silver deposited on the electrode was stripped into 5 mL of 0.05 M H_2SO_4 solution with a potential of 0.7 V for 1 min. The released silver ions were then quantified by anodic stripping voltammetry using a glassy carbon electrode as working electrode under the following instrumental conditions: 10 min deposition at −0.5 V versus SCE and the potential scan at 100 mV s^{-1}. After every measurement, the electrode was pretreated again and the protein was immobilized as described in Section 2.3.

3. Results and discussion

3.1. Anodic stripping voltammetry experiment

We attempted to adopt stripping voltammetry to detect the silver deposited on the gold electrode directly. After the layer of immunocomplex (human IgG 1 μg mL^{-1}) was formed on the electrode surface, the gold electrode was immersed into EB solution containing AA-p and $AgNO_3$ for silver deposition. The alkaline phosphatase converts AA-p to ascorbic acid, a reducing reagent that reduces silver ions, forming a metallic silver layer on the electrode under the optimum pH value 9.0 [21]. Since the half-wave potential of ascorbic acid is 0.39 V versus NHE, and that of Ag^+/Ag is 0.7995 V, so the ascorbic acid in alkaline solution spontaneously reduces silver ions in solution. However, the higher pH value (>10.0) would cause the hydrolysis of Ag^+ ions to Ag_2O. The reduction can be explained by the following reaction:

$$\text{Ascorbic acid} + 2Ag^+ \rightarrow \text{Dehydroascorbic acid} + 2H^+ + 2Ag$$



Scheme 1. The fabrication of immunosensor and amplification immunoassay procedure.

After that, the gold electrode was taken out and the deposited silver was quantified by two methods, the direct stripping voltammetry and indirect anodic stripping voltammetry. One can see from Fig. 1 that the direct anodic stripping voltammogram of silver on protein-modified gold electrode was split (curve a). There were two peaks in the voltammogram located at 0.427 and 0.536 V, respectively. The integration of the stripping peak current (Q_p) gave an estimate of 91 μC. The appearance of two peaks in the voltammogram indicated that the protein molecules on the gold electrode would block the electron transfer and the deposited silver mainly distributed in two regions, nude gold electrode surface and the protein surface. The electrochemical oxidization of silver near to the gold surface was relatively easy, producing an oxidation peak at relative negative potential. The oxidization of silver far from the gold surface was more difficult, producing a peak at relative positive potential. Furthermore, the voltammograms for direct measurement of silver deposited on the gold electrode are not very repeatable, which would seriously influence the silver measurement. Curve b in Fig. 1 represents the anodic stripping voltammogram of silver first stripped from the gold electrode with a potential of 0.7 V for 1 min and then accumulated electrochemically on a glassy carbon electrode. It is well known that the Ox/red potential of Ag^+/Ag is 0.7995 versus NHE. Here a potential of 0.7 V versus SCE is applied to electrochemically oxidize silver deposited on protein-modified gold electrode, which is positive enough to

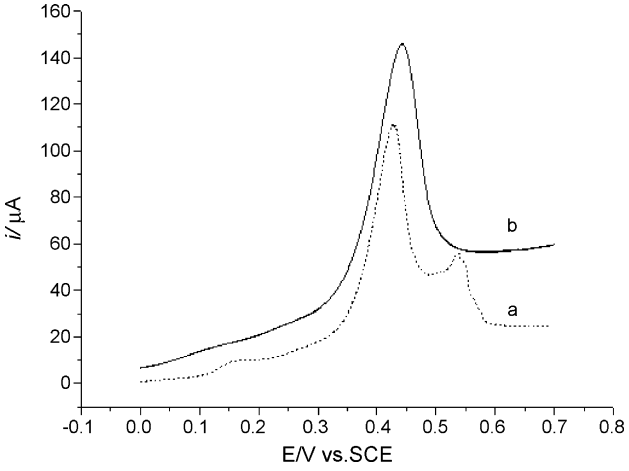


Fig. 1. Anodic stripping voltammograms: (a) direct stripped on gold electrode; (b) stripped on glassy carbon electrode after preconcentration.

electrochemically oxidize metal silver to Ag^+ ions. Obviously, the stripping current is easy to be measured and the signal can be used to quantify the biocatalytically deposited silver on the electrode. The integration of the stripping peak current (Q_p) gave an estimate of 100 μC, which is larger than the Q_p on gold electrode with direct anodic stripping voltammetry, indicating that the silver deposited on protein-modified electrode could not be stripped completely with only one scan. This phenomenon also explained why it was not appropriate to use the direct stripping voltammetry to quantify the silver metal deposited on the gold electrode using Q_p as an analytical response. In the subsequent work, all the analytical responses were recorded on a glassy carbon electrode after the biocatalytically deposited silver was stripped in the solution electrochemically. The use of glassy carbon electrode for the measurement of silver not only increased the repeatability but also improved the sensitivity of the immunoassay.

3.2. Optimization of the experimental conditions

3.2.1. Effect of the AA-p concentration

Since the amount of biocatalytically deposited silver metal is depended on the amount of enzyme-generated ascorbic acid, which is controlled by the concentration of AA-p, the dependence of the amount of deposited silver metal on the concentration of AA-p should be investigated. As shown in Fig. 2, with a fixed biocatalytic deposition time of 20 min in EB solution containing 1 mM Ag^+ ions and varying concentration of AA-p, the silver anodic stripping current for the detection of 100 ng mL^{-1} human IgG increases sharply with the increase of the concentration of AA-p in EB solution up to 1.5 mM, and then tends to level off. As a result, an AA-p concentration of 1.5 mM was selected in the subsequent work.

3.2.2. Effect of Ag^+ ions concentration

In this work, the enzyme-generated ascorbic acid should be oxidized as soon as it was produced on the electrode surface. So sufficient silver ions (I) should be added to the EB solution. The effect of the Ag^+ ions concentration on the stripping current was also investigated. As shown in Fig. 3, with a biocatalytic deposition time of 20 min in EB solution containing 1.5 mM AA-p and varying concentration of Ag^+ ions, the stripping currents for the detection of 100 ng mL^{-1} human IgG increases with the increase of Ag^+ ions concentration from 0.5 to 1.5 mM, and then tends to be stable. As a result, an Ag^+ ions concentration of 2.0 mM was selected in the subsequent work.

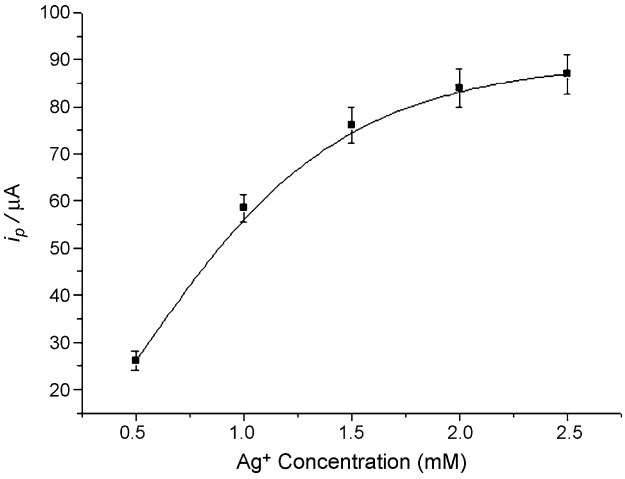


Fig. 3. The effect of the concentration of Ag^+ ions on the stripping peak current. Error bars represent S.D., $n = 4$.

3.2.3. Effect of the biocatalytic deposition time

Obviously, the amount of silver metal deposited on the detection electrode is related to the deposition time. One can predict that a longer deposition time should result in more silver deposition, but too long deposition time would lead to the enhancement of the background signal. So the deposition time that would affect the sensitivity of the immunoassay should be optimized. As shown in Fig. 4, the stripping currents for the determination of 100 ng mL^{-1} human IgG increases as the deposition time increases up to 20 min. With a deposition time more than 20 min, the stripping current tends to level off, indicating that the enzyme

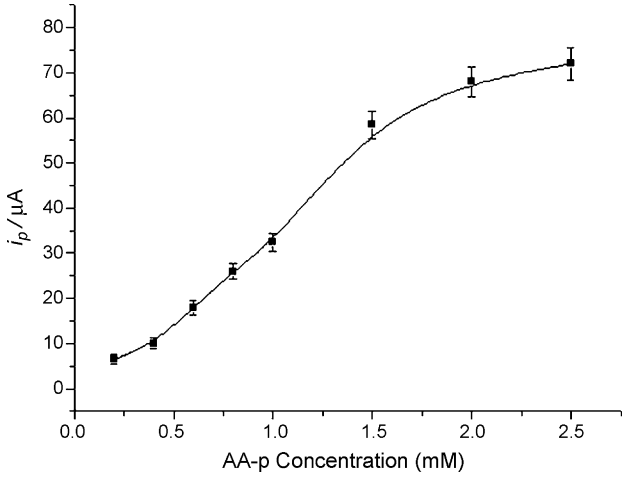


Fig. 2. The effect of the concentration of AA-p on the stripping peak current. Error bars represents S.D., $n = 4$.

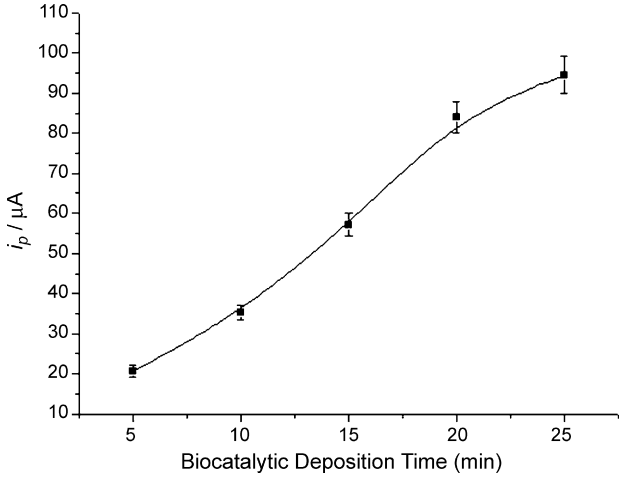


Fig. 4. The effect of biocatalytic deposition time on the stripping peak current. Error bars represent S.D., $n = 4$.

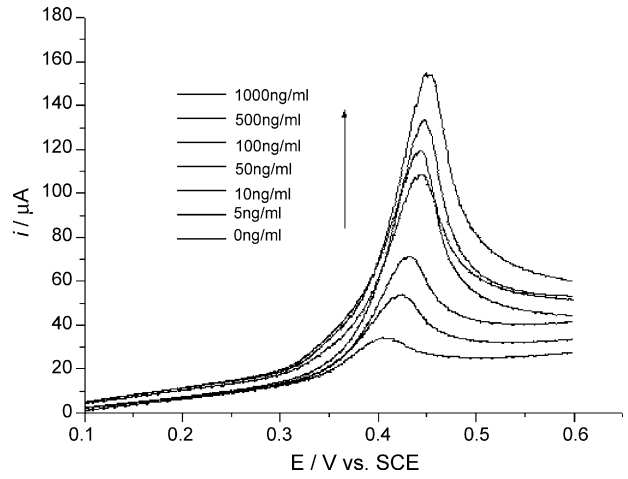


Fig. 5. Anodic stripping voltammograms for the determination of human IgG (concentrations of 0 and 5–1000 ng mL^{-1}).

activity decreases and enzyme catalytic reaction becomes very slow over 20 min. A deposition period of 20 min was adopted in this work to achieve high sensitivity.

3.3. Investigation of the nonspecific adsorption

The effect of nonspecific adsorption was investigated by incubating the antibody-modified electrode in 1% BSA solution, followed by incubating in alkaline phosphatase conjugated anti-human IgG antibody and by performing the biocatalytic metal deposition procedure. The deposited silver was stripped into the solution and quantified by anodic stripping voltammetry. It could be obtained from Fig. 5 that the peak current i_p is about 20 μA. Compared with the signal corresponding to 1 μg mL^{-1} human IgG that is approximately 120 μA, the signal arisen from the nonspecific adsorption is very low. Without much interference from nonspecific absorption, the method seems to have good selectivity.

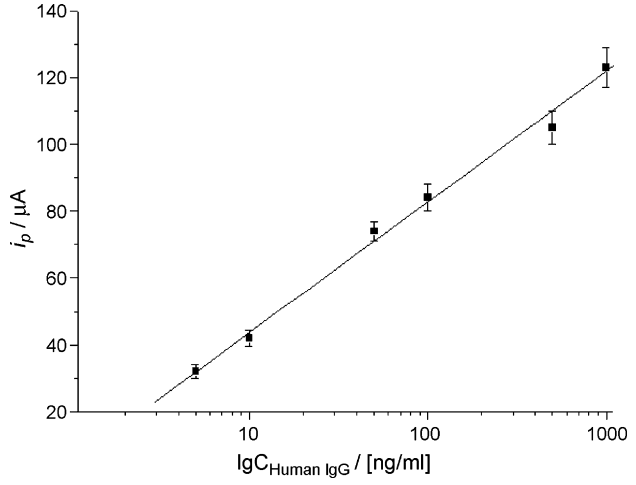


Fig. 6. The calibration curve for the determination of human IgG as the peak current plotted vs. the logarithm of the concentration of human IgG. Error bars represent S.D., $n = 4$.

3.4. Analytical performance of human IgG

The principle of immunoassay was described above. After the formation of sandwich immunocomplex on the gold electrode surface, the gold electrode was immersed in the EB solution containing 1.5 mM AA-p and 2.0 mM Ag^+ ions for silver deposition. The deposited silver was stripped into the solution and quantified by anodic stripping voltammetry. The amount of alkaline phosphatase increased with the raising concentration of human IgG, resulting in more deposition of silver. Fig. 5 illustrates this phenomenon. The peak currents were proportional to the logarithm of the concentration of human IgG in the range from 5 to 1000 ng mL^{-1} as shown in Fig. 6. The linear regression equation was i_p (peak current) = 6.31 + 37.59 log C (Human IgG concentration), with a correlation coefficient of 0.9915. The detection limit is 2.2 ng mL^{-1} calculated by the 3σ-rule.

4. Conclusion

An electrochemical amplification immunoassay was developed based on the biocatalytic metal deposition and a sensitive detection method, anodic stripping voltammetry, for the quantification of metal ions. The conversion of enzyme-generated product to metal, which can be sensitively detected by anodic stripping voltammetry, offers a substantial signal amplification of the immunorecognition events, preferable to the use of glucose oxidase as label for enzymatic electro-analysis [22] and gold nanoparticles for SERS [23] and adsorptive voltammetry [24]. This immunoassay was even comparable to those strategies using alkaline phosphatase (ALP) as labels based on iridium oxide matrices [25], alternating current voltammetry [26], amperometry at a sol–gel SPE [27] and interdigitated array electrodes voltammetry [28]. It can be expected that the proposed approach holds promise for the extended application in the field of bioaffinity assays, pharmaceutical analysis, environmental and clinical diagnosis.

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