Int. J. Electrochem. Sci., 12 **(2017)** 5233 – 5242, doi: 10.20964/2017.06.45

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

Short Communication

Development of Electrochemical Sensor for Coronary Heart Disease Biomarker MMP-9 Analysis

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Received: 28 February 2017 / *Accepted:* 2 April 2017 / *Published:* 12 May 2017

This work explored the preparation of a sensitive amperometric immunosensor for MMP-9. The in situ deposition of Au nanoparticles (Au NPs) on the polydopamine functionalized silica nanosphere and subsequent signal antibodies tagging were carried out for the preparation of Au nanoprobe. The fabrication of immunosensor was achieved while the capturing antibodies were covalently immobilized onto the glassy carbon electrode. Cyclic voltammetry (CV) was adopted for studying the modified electrode traits at diverse modification phases. Besides, particular investigation of the behaviors of immunosensor was herein carried out. Apart from the significant sensitivity in detecting MMP-9, the final immunosensor features a favorable correlation in terms of MMP-9 detection with a range (0.1-150.0 ng/mL) and a limit of detection (0.06 ng/mL) estimated at a ratio of 3 (signal-noise). The one-step immunoassay detection of MMP-9 is available with the proposed approach which is potential to be applied in diagnosing coronary artery disease at an early stage.

Keywords: Polydopamine; AuNPs; MMP-9; Silica; Electrochemical sensor; Coronary heart disease

1. INTRODUCTION

Matrix metalloproteinase-9 (MMP-9) exerts crucial influences on pathological processes like neuroinflammation in multiple sclerosis which troubles more than 2.3 million people in the world as a progressive auto-immune disease of central nervous system [1]. It is well accepted that the clinical attacks or relapses onset occurs after the inflammation in MS. The treatments concerning inflammation

inhibition are just of partial effectiveness [2]. Currently the detection of subclinical disease behavior in MS is achieved by serial magnetic resonance imaging (MRI) investigations, which is costly and has some other disadvantages like the restricted examination area of hospital for people with MS (pwMS). In addition, with the consideration of not missing early inflammation stage and reoccurring, rather constant monitoring is worthy of being conducted. In correlation with relapsing-remitting multiple sclerosis (RR MS) related MRI investigations, MMP-9, the peripheral biomarker of neuroinflammation has been researched more thoroughly than its counterparts [3]. An active form (65 kDa and 82 kDa) of MMP-9 exists in RR MS, together with its inactive form (92 kDa) [4]. High levels of MMP-9 could be detected in patients suffering RR MS, according to myriads of researches. At present, the active and/or inactive forms of MMP-9 could be detected through zymography [4], enzyme-linked immunosorbent assay (ELISA) [2, 5-8] and other biochemical assays. The detection of active MMP-9 by Avolio and co-workers was conducted at the level of 326.5±140.3 ng/ml ((mean±SD)), 529.1±370.7 ng/ml and 647.2±333.2 ng/ml respectively for control specimens, long course of disease and short course of disease. In contrast to the patients without response to the therapy (442±265.4 ng/ml), a decline in total MMP-9 level (357.7±272 ng/ml) was monitored for the MS patients with response to the treatment [2].

It is expensive and entails qualified professionals and plenty of time for the assays above to be conducted for the detection of MMP-9, in spite of their sensitivity. However, such disadvantages could be eliminated by the adoption electrochemical sensors which feature rapid real-time detection of analytes without various preparing stages needed in biochemical assays. Besides, electrochemical devices are promisingly characteristic of easy operation, cost-effectiveness and miniaturization. To detect MMP-9, scientists have explored myriads of electrochemical sensors with diverse recognition elements. The activity sensors and immunosensors constitute sensors. The former one was coated by synthetic/natural substrates degraded by MMP-9, and the latter modified by antibodies specific to MMP-9 [9-11]. An impedimetric immunosensor with a detection limit of 1.1 nM (100 ng/ml) fabricated by Ciani and co-workers [12] entailed 20 min incubation in the presence of enzyme and a subsequent washing for 5 min. Thus instead of a household device, the above method is more applicable in the construction of a point-of-care instrument. Moreover, a sensor for the detection of active MMP-9 (65 kDa) which exerts great influence on MS is necessary, since this immunosensor was employed to detect the inactive MMP-9. That MMP-9 (65 kDa) existed exclusively exists in patients suffering RR MS was discovered by Rossano and co-workers [4]. This finding indicated that the truncated form is in correlation with the process of inflammation, instead of routine physiological processes within body. A hanging mercury drop electrode provided a platform for the chronopotentiometric stripping analysis to be conducted for MMP-9 detection with or without the addition of collagen with a range of 1-10 nM and a detection limit of 100 pM. However, this system had some drawbacks. For instance, the sensor is possibly vulnerable to interference from other proteases that exist in the blood samples; meanwhile there are large numbers of cleavage sites in collagen and other natural substrates. In addition, real-time MMP-9 detection was not practical, since prior to the beginning of measurement, there were enzyme accumulation and rinsing processes.

Featuring low refractive, density & dielectric constant, desirable biocompatibility, expanded surface area and uniform size of pores, mesoporous materials have recently been appealing to many

scientists as the nanomedicine developed. Mesoporous SiO_2 (MS) is of vital importance due to its combined properties of mesoporous materials and silica [13-17]. Fluorescent molecules, noble metals and other materials are possible to be combined with silica chemistry which is versatile. Thus, the fields of targeted drug delivery and drug transport have been witnessing the wide application of functionalized MS.

Another vital factor in gaining significant immunosensor sensitivity is signal amplification. In the electrochemical immunoassays, signal transduction is normally carried out by antibodies tagged Au NPs [18-20]. Due to strengthened surface free energy, desirable biocompatibility and expanded specific area, Au NP could strongly adsorb the antibody. Taking a significant physiological part in mammalians as a chemical carrier, dopamine is viewed as a crucial catecholaminergic neurotransmitter. The self-polymerization of dopamine could be achieved under weakly alkaline circumstance, and spontaneous formation of a multifunctional biocompatible polydopamine (PDA) layer on basically any surface, as shown by recent studies. Therefore, it is likely for in situ deposition of metallic nanoparticles to be conducted and the biological molecules to be immobilized in the presence of this PDA functionalization that features superb versatility [21-24].

In this work a novel gold nanoprobe was successfully prepared based on in situ deposition of Au NPs on the PDA functionalized silica nanosphere (PDA/silica) followed by the labeling of MMP-9 antibodies. The number of receptor molecules immobilized on the surface of the sensor reveals the immunosensor sensitivity. An enlargement of the sensor area would allow for an increase of the binding capacity, hence a larger amount of immobilized receptor molecules. The proposed immunosensor was successfully used for determination of MMP-9 in the range of 0.1-150.0 ng/ml with a detection limit of 0.06 ng/ml. Moreover, the proposed immunosensor was also successfully used for MMP-9 determination in serum sample.

2. EXPERIMENTAL

2.1. Materials

Biocell Company located in Xiamen of China was the material source for pro-MMP9 and anti-MMP. Human serum albumin (HSA), dopamine, chloroauric acid ($HAuCl_4$) and bovine serum albumin (BSA, 96–99%) were commercially available from Sigma. The mixture of 50 mM NaH_2PO_4 and Na_2HPO_4 stock solutions contributed to a phosphate-buffered saline (PBS) (pH 7.4). The blocking solution and washing buffer were respectively prepared by a 50 mM pH 7.4 PBS containing 2% (w/v) BSA and a 50 mM pH 7.4 PBS containing 0.05% (w/v) Tween-20 (PBST). Besides, PDA functionalization was achieved with a 50 mM pH 8.5 Tris–HCl buffer.

The purchased ProMMP-9 took the role of inactive forms due to the obstruction of active site in these enzymes by their propeptide domains. As a famous compound, *P*-aminophenyl mercuric acetate (APMA) excels in breaking the bond between zinc and propeptide in the active domain. After the active site that relies on zinc is unblocked, myriads of autolytic cleavages would occur. The truncated form of MMP-9 (67 kDa) was reported to be formed as MMP-9 was activated with APMA.

APMA optimized MMP-9 activation, and parameters indicating the most significant MMP-9 stability & activity were selected. The MMP-9 activation was achieved as APMA was added and dissolved in NaOH (0.1 mM) to obtain a mixture with a concentration of 2 mM, with a volume ratio (APMA:proMMP-9) of 1:10. The specimens went through 37 °C incubation for 30 min, then put into 5 °C storage. 50 mM Tris buffer, pH 7.5 containing 0.05% Brij-35 (w/v), 150 mM NaCl and 10 mM $CaCl_2$ provided platform for testing MMP-9 activity. The fluorescence substrate degradation was observed at 25 °C for more than 8 min as follows: Mca–Lys–Pro–Leu–Gly–Leu–Dpa–Ala–Arg–NH_2 (Mca–KPLGL–Dpa–AR) (10 μM).

2.2. Apparatus

A CHI 660 electrochemical workstation in CH Instruments located in the USA provided a platform for the whole set of electrochemical immunoassays. The working electrode, reference electrode and auxiliary electrode were the modified electrode, a saturated calomel electrode (SCE) and a platinum wire respectively, constituting a triple-compartment electrochemical cell. UV–vis absorption spectra were measured with SM-240 CCD spectrophotometer (CVI spectral instruments, Putnam, CT, USA). The EDX spectrum was recorded using a EDX coupled scanning electron microscopy.

2.3. Preparation of gold nanoprobe

The Au nanoprobe is prepared through the following procedures. To begin with, the synthesis of monodispersed silica nanosphere was conducted on the basis of previous literatures [25]. The in situ deposition of Au NPs on the PDA/silica surface was carried out for the preparation of Au NPs functionalized PDA/silica nanocomposite. To be brief, the mixture of silica nanosphere dispersion (1.0 mL, 5.0 mg/mL) (in 50 mM pH 8.5 Tris–HCl buffer), together with dopamine (5.0 mg) went through 5h stirring & reaction at ambient temperature. The redispersion of the as-prepared PDA/silica into 2.0 mL, 0.25% (w/v) $HAuCl_4$ containing 10 mg citrate was conducted after centrifugation and washing for three times by water. Subsequently as-prepared mixture went through stirring for reaction (2 h). Then the final Au NPs functionalized PDA/silica nanocomposite was fabricated after a second round of centrifugation and washing by water for three times. Eventually the dispersion of the as-fabricated composite into 2.0 mL water was carried out for its future application.

2.4. Fabrication of the immunosensor

To begin with, the removal of adsorbed organic substance was achieved by polishing GCE with a diameter of 4 mm using 0.05, 0.3 and 1.0μm alumina slurries to a mirror-like finish on a micro cloth pad. The electrode went through ultra bath cleaning with ethanol & water after the trace alumina was removed from the surface of the electrode. Then the cleaned electrode went through drying at ambient temperature. The pipette Au NPs-silica composite solution (25 μl) onto the surface of GCE went

through drying overnight at a temperature of 4 °C. The 12 h immersion of as-prepared electrode (Au NPs-silica/GCE) after water washing was conducted at 4 °C in anti-MMP-9 solution (10 mM phosphate buffer solution (pH 7.0) containing 0.1 mM anti-MMP-9). Eventually, to prevent non-specific adsorption and obstruct potential residual active sites, the incubation of the electrode in BSA solution (w/w, 0.25%) was conducted at the temperature of 35 °C for roughly 1 h. and the resulting immunosensor was put into 4 °C storage if not in application.

2.5. Experimental measurements

An unstirred electrochemical cell provided a platform for the electrochemical measurements to be conducted with a scan rate (50 mV/s) and a potential range (− 0.2-0.6 V) (in contrast to SCE) at a temperature of 35 °C. The immunoreaction was carried out through the incubation of the immunosensor in 0.01 M PBS containing diverse concentrations of MMP-9 (pH 7.0) for 20 min at 35 °C. The MMP-9 level detection was conducted through the detection of oxidation current response (ΔI) variations preceding & succeeding the immunoreactions in 5.0 mM $[Fe(CN)_6]^{4-/3-}$ solution (pH 7.0) containing 0.1 M KCl.

3. RESULTS AND DISCUSSION

The characterization of Au NPs generation on PDA/silica surface was also achieved the UV–vis spectroscopy. There were not absorption peak detected for PDA/silica (Figure 1A), while after in situ chemical deposition reaction a significant absorption peak was observed at 504 nm. This peak corresponds to characteristic plasmon absorption peak of colloidal Au NPs formed with traditional citrate reduction approach. The average size of the formed AuNPs was around 23 nm based on the peak location [26]. The slight red-shift of the maximum absorption peak caused by the surface effect of Au NPs deposited on the core/shell nanosphere. Moreover, that Au exists after the in situ deposition of Au NPs on PDA/silica was indicated through EDX spectrum in Figure 1B. Such results provided convincing evidences that Au NPs functionalized PDA/silica nanocomposite had been successfully prepared. Based on the intensity information, the roughly percentage of AuNPs in the composite materials is 23%. The fabrication of a novel Au nanoprobe for nonenzymatic electrochemical immunoassay was hence achieved as the biomolecules of antibody interacted inherently with Au NPs. Apart from offering a convenient and eco-friendly technique for the high-content Au NPs to be loaded on the nanocarriers, this in situ deposition approach also made the Au nanoprobe fabrication more controllable and repeatable.

As revealed in Figure 2, diverse designed electrodes in a 5 mM $[Fe(CN)_6]^{4-/3-}$ solution containing 0.1 M KCl were characterized via CVs. On the original GCE, a reversible CV was shown for redox-label $Fe(CN)_6^{4-/3-}$. An obvious rise in peak current was deetcted with the modification of GCE by Au NPs functionalized PDA/silica nanocomposite. The peak current enhancement can be ascribed as the excellent electro-conductivity of the AuNPs [27]. There was a decline in current response, as the membrane performed worse in conductivity after the immobilization of anti-MMP-9

on the surface of the electrode. A sharp drop of peak current could be monitored with 20 min incubation of the immunosensor into MMP-9 solution, where anti-MMP-9/MMP-9 immunocomplex was formed. Herein, the immunocomplex impedes the diffusion of ferricyanide to the surface of the electrode with the role of inert electron and mass transfer barrier layer.

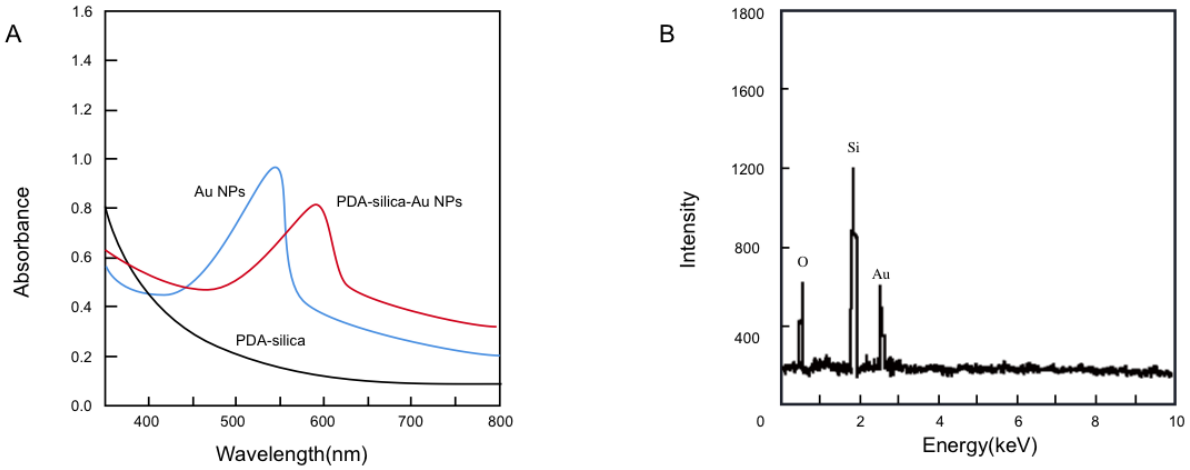

Figure 1. (A) UV–vis spectra of colloidal Au NPs, PDA/silica and the as-prepared Au NPs functionalized PDA/silica nanocomposite. (B) EDX spectrum of the as-fabricated Au NPs functionalized PDA/silica nanocomposite.

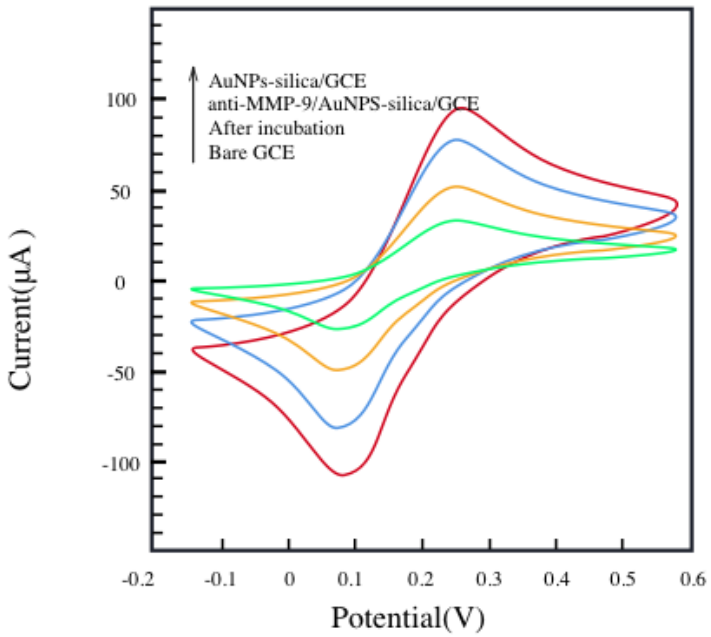

Figure 2. CVs of the electrode at diverse phases: bare GCE, Au NPs-silica/GCE, anti-MMP-9/Au NPs-silica/GCE and after incubated in the solution containing MMP-9 for 20 min. Supporting electrolyte: 5 mM $Fe(CN)_6^{4-/3-}$ solution containing 0.1 M KCl; scan rate, 50 mV/s.

The incubation time period & temperature and supporting electrolyte pH are viewed as experimental variables that influence the amperometric response of immunosensor. This work studied the impact of solution pH (6.0-8.5) on the immunosensor performance. As shown in Figure 3, it can be seen that the current response was gradually increased from pH 6 to 7.5 and reached the maximum in the pH 7.5. Further increasing of pH value results a decreasing of peak response. Thus, $Fe(CN)_6^{4-/3-}$ (pH 7.5) solution was applied to the whole set of experiments in this work.

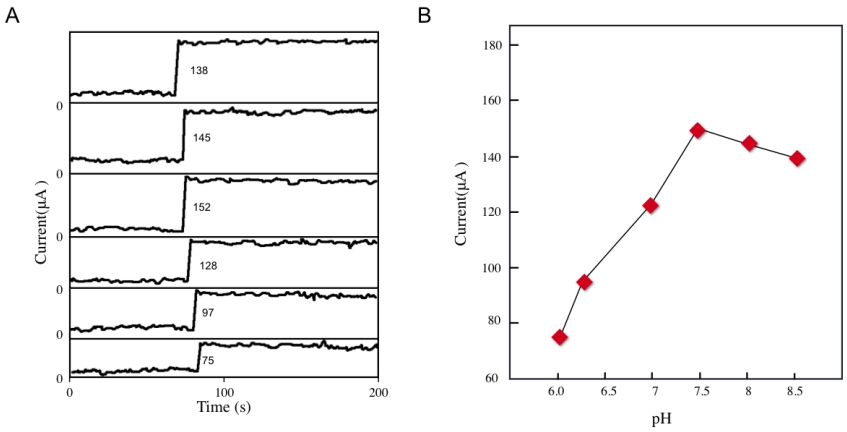

Figure 3. Influence of the pH of the solution on the response of the immunosensor incubated with 20 ng/ml MMP-9 in 5 mM $Fe(CN)_6^{4-/3-}$ solution containing 0.1 M KCl. All potentials are given vs. SCE and the scan rate was 50 mV/s. (A) Amperometric response curves. (B) Current response trend.

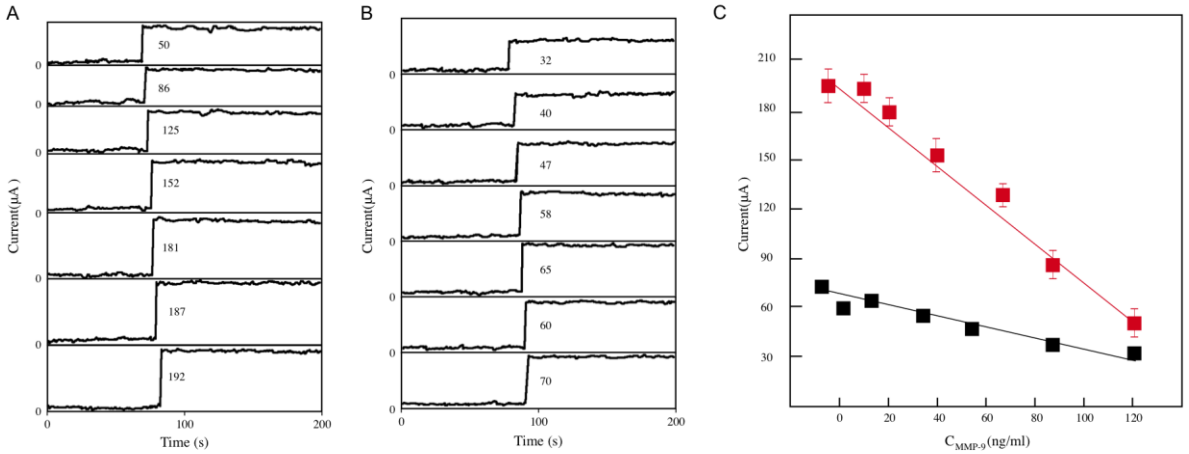

Figure 4. (A) Amperometric response curves of anti-MMP-9/GCE and (B) anti-MMP-9/Au NPs-silica/GCE. (C) Calibration plots of the anodic peak current response vs. concentration of anti-MMP-9/GCE and anti-MMP-9/Au NPs-silica/GCE with the different immunosensors under optimal conditions.

The analytical curves of immunosensors fabricated by the diverse approaches were compared in Figure 4. The calibration plots of anti-MMP-9/Au NPs-silica/GCE and anti-MMP-9/GCE were characterized through profiles a–c (Figure 4), where those of the proposed immunosensor was indicated to be more sensitive and expanded dynamic measurement range than those of anti-MMP-9/GCE. With a significant sensitivity (778 nA/ng/ml) for MMP-9 detection, the final immunosensor

correlates with MMP-9 detection with a range (0.1-150.0 ng/ml), and the limit of detection is 0.06 ng/ml. The sensitivity of the anti-MMP-9/Au NPs-silica/GCE was compared with that of other reported modified electrodes and the results were presented in Table 1.

Table 1. Comparison of the present anti-MMP-9/Au NPs-silica/GCE with other MMP-9 determination methods.

Electrode	Linear detection range	Detection limit	Reference
Triple-helical peptide optical probe	0.04 to 10 pg/ml	6 ng/ml	[28]
Enzyme immunoassay	0.5 to 7 pg/ml	0.22 pg/ml	[29]
Immunocapture assay	5 to 30 pg/ml	2.4 pg/ml	[30]
anti-MMP-9/Au NPs-silica/GCE	0.1-150.0 ng/ml	0.06 ng/ml	This work

During the consecutive human serum specimen analysis, the immunosensor selectivity is of vital significance. In order to study the immunosensor selectivity, the influence of potential interferences in human serum, hepatitis B core antigen (40 ng/ml), hepatitis B surface antigen (40 ng/ml) andα-1-fetoprotein (40 ng/ml) was put into respective investigations with MMP-9 (40 ng/ml). The variation of peak current responses is below 5.6%, as indicated via their CVs. The application of this approach in determining MMP-9 in human samples is practical due to the specificity of the system to MMP-9, as manifested through these results. The excellent selectivity of the proposed immunosensor can be also ascribed to the high specific surface area of the Au NPs-silica, which provide a platform for MMP-9 and anti-MMP-9 loading.

The immunosensors are potential to be applied and developed, mostly because they could be regenerated. The immersion of prepared immunosensor in a urea solution (5 mM) for roughly 10 min, together with its removal to be water-washed succeeding every determination could conveniently achieve the immunosensor regeneration. With 8 rounds of successive measurements on the electrode, 3.7% was obtained as the relative standard deviation (R.S.D.). This simple regeneration method could be used for lowering cost of product.

Since the $Fe(CN)_6^{4-/3-}$ mediator would be leaked onto the electrode and the protein became gradually deactivated, it is most likely for the modified electrode to turn unsteady step by step after being stored and used for a period of time. Herein the steadiness of the consecutive assays was investigated. 4.1% was obtained as the R.S.D. after 50 CV rounds of measurements in the working buffer, together with 60-day investigation of long-time stability of the immunosensor. After 30 days storage at 4 °C, the immunosensor indicated a favorable storage steadiness with 91.6% of initial remaining, and 87.8% of initial remaining after 60 days storage at 4 °C. Herein the desirable storage stability is revealed for the immunosensor through the results. In terms of routinely diagnosing coronary artery disease at an early stage, the proposed immunoassay approach is a desirable candidate for determining MMP-9 in human serum due to its favorable storage stability revealed by this result.

Table 2. Experimental results of different methods obtained in serum samples

Serum samples	1	2	3	4	5	6
Immunosensor (ng/ml)	0.47	2.27	5.96	10.36	15.91	42.09
ELISA (ng/ml)	0.48	2.21	6.03	10.21	15.69	40.50
Relative deviation (%)	4.6	5.3	3.7	5.5	6.0	6.3

The results concerning five serums were gained respectively from ELISA as an effective and significant approach to analyzing serum and this approach. And the examination of the MMP-9 determination accuracy was carried out through the comparison between the results of the two approaches. Table 2 indicates the data and relative deviations between above two approaches, with their relative error of lower than 7 %, where the favorable agreement was revealed in them. Therefore the requirement of MMP-9 immunoassays in clinically diagnosing coronary artery disease at an early stage could be met by the current approach.

4. CONCLUSIONS

A novel Au nanoprobe was herein fabricated on the basis of in situ deposition of high-content Au NPs on the PDA/silica to serve the ultrasensitive electrochemical immunoassay on an immunosensor. Herein the in situ deposition approach features its convenience and controllability for the fabrication of this novel Au nanoprobe. Meanwhile, the immunosensor is characteristic of favorable storage stability, satisfactory accuracy, low limit of detection and significant sensitivity. In conclusion, it is promising of the proposed approach to be further improved for its future engagement in clinically detecting serum MMP-9 level.

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