Biosensors and Bioelectronics 72 (2015) 237–246

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A simultaneous electrochemical multianalyte immunoassay of high sensitivity C-reactive protein and soluble CD40 ligand based on reduced graphene oxide-tetraethylene pentamine that directly adsorb metal ions as labels

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ARTICLE INFO

Article history:
Received 28 February 2015
Received in revised form
9 April 2015
Accepted 27 April 2015
Available online 30 April 2015

Keywords:
Simultaneous detection
Reduced graphene oxide-tetraethylene pentamine
Metal ion labels
High sensitive C-reactive protein
Soluble CD40 ligand

ABSTRACT

A simplified electrochemical multianalyte immunosensor for the simultaneous detection of high sensitivity C-reactive protein (hsCRP) and soluble CD40 ligand (sCD40L) that uses reduced graphene oxide-tetraethylene pentamine (rGO-TEPA) that directly adsorbs metal ions as labels is reported. rGO-TEPA contains a large number of amino groups and has excellent conductivity, making it an ideal template for the loading of Pb^{2+} and Cu^{2+}, which greatly amplifies the detection signals. The signals could be directly detected in a single run through differential pulse voltammetry (DPV), and each biorecognition event produces a distinct voltammetric peak. The position and size of each peak reflects the identity and the level of the corresponding antigen. Primarily designed for an application in a sandwich-type immunoassay based on Pb^{2+} and Cu^{2+} labels, two main challenges are accomplished with the herein presented nanosheets: fabrication of the template and the amination process for Pb^{2+} and Cu^{2+} adsorption. To further improve the analytical performance of the immunosensor, Au@bovine serum albumin (BSA) nanospheres synthesized through a "green" synthesis route were used as a sensor platform, which not only provides a biocompatible microenvironment for the immobilization of antibodies but also amplifies the electrochemical signals. Under optimal conditions, hsCRP and sCD40L could be assayed in the range of 0.05 to 100 ng mL^{-1} with detection limits of 16.7 and 13.1 pg mL^{-1} ($S/N=3$), respectively. The assay results on clinical serum samples with the proposed immunosensor were in acceptable agreement with those using the standard single-analyte test of the enzyme-linked immunosorbent assay (ELISA). This novel immunosensing system provides a simple, sensitive and low-cost approach for a multianalyte immunoassay.

1. Introduction

Cardiovascular disease (CVD) was listed as the primary cause of death globally by the World Health Organization (WHO), accounting for approximately 18 million deaths annually (Fakanya and Tothill, 2014). Currently, the standard 12-lead electrocardiogram (ECG) is the single best test to identify patients with acute CVD (Gibler et al., 2005). However, it is necessary to develop complementary chemical assay methods for the detection of acute myocardial infarction (AMI). Biomarker detection is the best method to identify patients following the ECG (Willcox et al., 2004). Several biomarkers are associated with an increased risk of cardiovascular disease, including high sensitivity C-reactive protein (hsCRP) and Soluble CD40 ligand (sCD40L) (Guldiken et al., 2011). hsCRP, a precursor to cardiovascular disease, is very sensitive to AMI (Babakhanian et al., 2015; Dieset et al., 2012; Vashist et al., 2015). sCD40L is a ligand of the glycoprotein IIb-IIIa receptor and is involved in thrombus stabilization and platelet activation. (Unek et al., 2010). There is an evidence that the sCD40L level is a strong predictor of cardiovascular risk (Erturan et al., 2014; Unek et al., 2010; Zhang et al., 2014a). Biomarkers have been proven to work better as a panel because no single biomarker is specific and sensitive enough to meet the strict diagnostic criteria of clinical diagnosis (Wu et al., 2014). In addition, different biomarkers are released into the blood stream at different pathophysiological stages of CVD and return back to their baseline levels after some time (Apple, 2007; Willcox et al., 2004). Therefore, simultaneous detection of two compounds is particularly important. Recently, the development of an immunoassay that enables the simultaneous determination of biomarkers has attracted extensive

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http://dx.doi.org/10.1016/j.bios.2015.04.088
0956-5663/

interest (Bai et al., 2012; Jia et al., 2014; Li et al., 2014, 2013; Liu et al., 2012). Among various measurement techniques, electrochemical immunoassays are an ideal strategy due to their advantages of portability, high sensitivity, rapid analysis, and low cost (Xu et al., 2014). To the best of our knowledge, there is no report on the simultaneous electrochemical detection of hsCRP and sCD40L yet. It is worth mentioning that their combined measurement may not only increase the sensitivity of the risk prediction but also provide new cardiac markers to replace cardiac enzymes for the clinical diagnosis and risk evaluation of cardiovascular events. Thus, we intend to fabricate an electrochemical immunosensor to simultaneously detect hsCRP and sCD40L in human serum.

For the development of a multiplex electrochemical immunoassay, the fabrication of label-anchored secondary antibodies for generating signals is of great importance in improving the sensitivity of the immunosensor. Numerous signal tags have been used for signal amplification such as enzymes (Zhao et al., 2014), metal nanoparticles and quantum dots (QDs) (Feng et al., 2012b). The easy inactivation and costly preparation and purification processes of enzymes restrict their applications (Zhao et al., 2014). Metal nanoparticles and QDs require a complicated and tedious preparation process (Feng et al., 2012b), as well as a time-consuming acid dissolution step and metal preconcentration before the electrochemical detection. As a result, great efforts have been made to fabricate novel labels for metallo-immuoassays to simplify the detection steps. In previous studies, Cd^{2+}-functionalised titanium phosphate nanoparticles (Feng et al., 2012b) and metal ion (Cd^{2+}, Zn^{2+})-functionalised titanium phosphate nanospheres (Feng et al., 2012a) were used as labels for electrochemical immunoassays based on the ion-exchange capability of titanium phosphate nanoparticles. However, the fabrication of the template for metal ion immobilization is a time-consuming job. Amino-capped nanomaterials showed good adsorption properties for Pd^{2+}, Ni^{2+}, Cd^{2+}, $Ag+$ and Cu^{2+} (Xu et al., 2014), and therefore, they provided another strategy for the immobilization of metal ions on immunosensor probes (Xu et al., 2014). Notably, to realize the adsorption of metal ions for amplification, the template should qualify both amino-groups and conductivity. Amino-group functionalised mesoporous Fe_3O_4 loaded with Pb^{2+} and Cd^{2+} (Zhang et al., 2014b), Cd^{2+}-functionalized nanoporous TiO_2 (Zhao et al., 2014), and amino-capped PtPNPs complexed with Cd^{2+} and Cu^{2+} (Wang et al., 2014) have been reported as labels for the detection of multiplex biomarkers. However, these modification steps are all complicated, owing to the amination process of the template nanomaterials. Ma et al. (2015) utilized metal ions as labels for the assay of proteins, but the modification steps introduced amino-group qualified materials that were nonconductive, which also made the operation procedure time-consuming. Thus, if metal ions (such as Cd^{2+}, Pb^{2+} and Cu^{2+}) could directly conjugate with an amino-group qualified, conductive nanomaterial, the amination process could be eliminated, avoiding the above-mentioned problems.

A novel material, rGO-TEPA (reduced graphene oxide-tetraethylene), which combines reductive graphene oxide (rGO) with tetraethylene pentamine through covalent bonding, has been developed (Wu et al., 2014; Zhang et al., 2014c). This combination possesses the bulk properties of rGO but with improved stability (Guo et al., 2015; Wu et al., 2014, 2015). Most importantly, rGO-TEPA contains a large number of amino groups and has excellent conductivity, which makes it an ideal template for the loading of metal ions. To the best of our knowledge, rGO-TEPA adsorbed metal ions as probes for electrochemical immunoassays have not been reported in the literature. Usage comparison of rGO-TEPA in the electrochemical immunoassay is shown in Table S6. Therefore, to further simplify the routine modification steps, a functionalised rGO nanomaterial, rGO-TEPA, was introduced in the fabrication of a multiplex electrochemical immunosensor.

To further increase the sensitivity for biomarker detection, great efforts have been made toward bio-component immobilization and signal amplification for sandwich-type immunoassays. Therefore, a nanomaterial-based sensor platform with a large surface area and superior conductivity can be used. Au@BSA nanospheres are an ideal nanomaterial owing to their good biocompatibility and excellent conductivity (Cao et al., 2015; Ma et al., 2015). In addition, they can be synthesized through a “green” synthesis route by using the benign reductant ascorbic acid in place of hydrazine monohydrate for the preparation of core–shell nanomaterials (Qu et al., 2013). The resulting unique three-dimensional architecture not only provides a biocompatible microenvironment for the immobilization of antibodies but also amplifies the electrochemical signals.

Herein, we designed a novel and facial sandwich-type immunoassay for the simultaneous detection of hsCRP and sCD40L by employing Au@BSA and rGO-TEPA for dual amplification. The application of Au@BSA not only provided a biocompatible microenvironment for the immobilization of antibody but also accelerated the electron transfer rate to amplify the electrochemical signals. Meanwhile, rGO-TEPA contains a large number of amino groups and has excellent conductivity, which makes it an ideal template for loading numerous metal ions such as Pb^{2+} and Cu^{2+} to form rGO-TEPA-metal ion labels. hsCRP and sCD40L antibodies were conjugated with rGO-TEPA-Pb^{2+} and rGO-TEPA-Cu^{2+} to fabricate anti-hsCRP- rGO-TEPA-Pb^{2+} and anti-sCD40$_L$-rGO-TEPA-Cu^{2+} probes, respectively. The metal ions in the bioconjugates can be detected by differential pulse voltammetry (DPV) without the acid dissolution and preconcentration required by stripping voltammetry, which greatly simplifies the detection steps and reduces the detection time. This proposed strategy exhibited good stability, precision, and reproducibility, suggesting a wide range of potential diagnostic applications.

2. Materials and methods

2.1. Materials and reagents

hsCRP, mouse monoclonal capture anti-bodies (Ab_1) and signal antibodies (Ab_2) of antigen were purchased from Linc-Bio Science Co., Ltd. (Shanghai, China). sCD40L, mouse monoclonal capture anti-bodies (Ab_1) and signal antibodies (Ab_2) of antigen were purchased from Abcam (USA). hsCRP and sCD40L ELISA kits were obtained from Linc-Bio Science Co., Ltd. (Shanghai, China). $HAuCl_4 \cdot 3H_2O$ was obtained from Sigma-Aldrich (St. Louis, USA, www.sigmaaldrich.com). L-ascorbic acid (AA) was bought from Aladdin Ltd. (Shanghai, China). Reduced graphene oxide–tetraethylene pentamin (rGO-TEPA) were purchased from Nanjing XFNANO Materials TECH Co., Ltd. (China). $Pb(NO_3)_2$, $Cu(NO_3)_2$ and Bovine Serum Albumin (BSA), potassium ferricyanide ($K_3Fe(CN)_6$) and potassium ferrocyanide ($K_4Fe(CN)_6$) were purchased from Beijing Chemical Reagents Company (Beijing, China). Glutaraldehyde (GA) was ordered from Sinopharm Chemical Reagent Company Limited (China). Clinical serum samples were obtained from a local hospital and stored at 4 °C. Phosphate buffered solution (PBS) (pH 7.4, 0.1 M) was prepared with NaH_2PO_4 and Na_2HPO_4. HAc/NaAc solutions with different pH values were prepared by mixing the stock solutions of HAc and NaAc. All other reagents were of analytical reagent grade and used without further purification. Ultrapure water ($>$ 18.2 MΩ) obtained from a Millipore Mill-Q purification system was used throughout the experiment.

2.2. Apparatus

All electrochemical experiments were carried out on a CHI660D electrochemical workstation (Chenhua Instruments Co., Shanghai, China). Transmission electron microscopy (TEM) investigations