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Label-free immunosensor based on graphene/polyaniline nanocomposite for neutrophil gelatinase-associated lipocalin detection

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ABSTRACT

A novel label-free electrochemical immunosensor for neutrophil gelatinase-associated lipocalin (NGAL) detection has been developed. The immunosensor has been constructed by immobilization of NGAL capture antibodies to electropolymerized aniline deposited on top of an electrosprayed graphene/polyaniline (G/PANI) modified screen printed carbon electrode. Electrospraying of G/PANI increases the electrode surface area while electropolymerization of aniline increases the number of amino groups ($-NH_2$) for antibody immobilization. The factors affecting the sensor sensitivity (i.e. aniline concentration, scan number and scan rate of electropolymerization) have been optimized. In a prior report, Kannan et al. reported a broad oxidation peak in cyclic voltammetry upon the binding between NGAL with its antibody. In this study, a dramatic increase (58-fold) in the oxidation current upon the binding between NGAL and its antibody is obtained when compared to an unmodified electrode, verifying a substantial improvement in the electrochemical sensitivity of this system. Under optimal conditions, this system exhibits high sensitivity with a limit of detection (LOD) of 21.1 ng mL^{-1}, wide linearity (50–500 ng mL^{-1}) and high specificity toward NGAL detection from small samples (10 μL). As an example application, the sensor is tested for the detection of NGAL in human urine, and the results correspond well with the values obtained from a standard ELISA. Compared to the ELISA method, our system requires less analysis time ($\lesssim$ 30 min/sample), less sample and less operating cost.

1. Introduction

Acute kidney injury (AKI) has been reported in 5 to 7% of hospitalized patients worldwide (Cruz et al., 2007; Himmelfarb and Ikizler, 2007; Mandelbaum et al., 2011). AKI results in loss of kidney function within hours, days or weeks. Importantly, AKI increases the risk of end-stage renal disease in the elderly and death after cardiac surgery (Ishani et al., 2009; Rosner, 2012). Thus, the diagnostic approach for AKI has been continually developed to improve the accuracy and sensitivity for an earlier diagnosis of patients. The standard diagnostic method for AKI is relied on the determination of serum creatinine (SCr) (Bagshaw et al., 2009; Weisbord et al., 2006; Zappitelli et al., 2009a, 2009b). Unfortunately, the use of SCr has a practical limitation i.e. the concentration of SCr will not significantly changed unless the kidney has lost at least 50% of its function (Wagener et al., 2006). Recently, several biomolecules have been used as better, alternative biomarkers for early diagnosis of AKI including urinary interleukin-18 (IL-18), kidney injury molecule-1 (KIM-1), cystatin C and neutrophil gelatinase-associated lipocalin (NGAL) (Devarajan, 2007; Hall et al., 2010; Nguyen and Devarajan 2008). Among all, NGAL is one of the most promising biomarkers for AKI diagnosis (Devarajan, 2010a, 2010b). NGAL is identified as a 25 kDa protein, found in association with gelatinase from neutrophils. This protein is expressed at very low concentrations in human tissues such as kidney, lung, stomach, and colon (Dent et al., 2007; Gabbard et al., 2010; Mishra et al., 2005). In AKI, urinary NGAL concentration is highly associated with SCr concentration (Wagener et al., 2006). After surgery, it usually takes 1–3 days before a diagnosis of AKI can be made using an SCr level, but this diagnosis can be reached within 2–6 h using an increase in urine NGAL level. Given the importance of NGAL as a biomarker, we have explored a new approach for detecting this protein as an early indicator of AKI.

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Various analytical techniques have been used for the determination of NGAL, including immunoblotting (Wagener et al., 2006) and enzyme-linked immunosorbent assays (ELISA) (Dent et al., 2007; Hirsch et al., 2007); however, these techniques require expensive laboratory equipment and are time-consuming. To solve these problems, electrochemical techniques have been considered as alternative tools for NGAL detection due to its high robustness, ease of use, low cost and rapid analysis (Kannan et al., 2012). Moreover, it can be used for both qualitative and quantitative analyses. Electrochemistry has been used for sensitive protein detection (Cai et al., 2013; Cheng et al., 2012), for biomarkers such as cytochrome C, a heme-containing protein that transfer electron in human cells (Furbee et al., 1993). It was reported that the electrochemical detection of NGAL relies on the increasing of oxidation peak current upon antibody-NGAL binding (Kannan et al., 2012); however, the mechanism was not reported (Iannetti et al., 2008; Schmidt-Ott et al., 2007). One possible reason for the enhanced signal is that NGAL binds iron and thus electron transfer could be facilitated upon antibody-NGAL binding.

In an electrochemical biosensor, a working electrode is usually miniaturized to make the biosensor portable and compatible with small biological samples (i.e. urine). Nonetheless, a small working electrode inevitably limits the surface area and therefore the sensitivity of the developed sensor. Further modification of the working electrode is required to improve the electrochemical sensitivity of a sensor. In recent years, carbon-based nanomaterials, such as carbon nanotubes (Zhang et al., 2013), carbon nanofibers (Promphet et al., 2015; Rodthongkum et al., 2013), carbon nanodots (Dai et al., 2012) and graphene have been used to modify working electrodes to improve their electrochemical performance. Graphene (G) has attracted considerable attention due to its large specific surface area, high electrochemical conductivity, high stability, and relatively low cost. G possesses a single layer of carbon atoms in a closely-packed honeycomb two-dimensional lattice (Zhang et al., 2013). To prevent the agglomeration of G, conducting polymers are used along with G for electrode surface modification. A nanocomposite between G and a conducting polymer makes this hybrid nanomaterial more suitable for electrode fabrication and biofunctionalization than a pure G. It has been reported that the use of G/conducting polymer nanocomposite-modified electrodes significantly enhance the electrochemical sensitivity of sensors (Ruecha et al., 2015; Tirawattanakoson et al., 2016). Different conducting polymers have been used for electrode surface modification, such as polyaniline (PANI) (Fan et al., 2011; Radhapyari et al., 2013), polypyrrole (PPy) (Bora and Dolui, 2012; Xing et al., 2012) and poly(3,4-ethylenedioxythiophene) (PEDOT) (Wisitsoraat et al., 2013; Zhang et al., 2012). Among these, PANI is the most appealing material due to its low cost, easy synthesis, good environmental stability, reversible redox properties and high biocompatibility (Bo et al., 2011; Fan et al., 2011).

G/PANI nanocomposite-modified electrodes are fabricated by electrospraying since this technique can create well-defined nanodroplets on the electrode surface. G/PANI nanodroplet-modified electrodes show higher specific surface area than unmodified and thin-film modified electrodes, leading to enhanced electrochemical sensitivity (Thammasoontaree et al., 2014). Unfortunately, G/PANI nanodroplets produced by electrospraying are randomly distributed on the electrode surface, resulting in suboptimal exposure of amino groups (-NH_2). Thus, developing a method that helps organize and increase the number of amino groups on the electrode surface for biomolecule immobilization is required. In this study, electropolymerization of aniline on the top of G/PANI nanodroplets is carried out to increase the number of amino groups on the electrode surface. Then, NGAL capture antibodies were conjugated to the active amino groups via peptide bonds using EDC/NHS coupling chemistry. The resulting immunosensor was tested for NGAL detection and applied for the analysis of NGAL in complex biological fluids (i.e. normal human urine VS pooled patient urine).

2. Materials and methods

2.1. Chemicals and materials

Graphene nanopowder was purchased from SkySpring Nanomaterials Inc, (Houston, TX, USA). Recombinant NGAL and NGAL-selective antibody were obtained from R&D Systems, Inc. (Minneapolis, MN, USA) and used as manufacturer instruction. Polyaniline emeraldine base (M_w=65,000), (+)-camphor-10-sulfonic acid (CSA), polystyrene (M_w=180,000), potassium ferricyanide ($K_3[Fe(CN_6)]$), potassium ferrocyanide ($K_2[Fe(CN_6)]$), 1-ethyl-3-(3-dimethyaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (St, Louis, MO, USA). Potassium dihydrogen phosphate (KH_2PO_4), chloroform, dichloromethane, *N,N*-dimethylformamide (DMF) were obtained from Carlo Erba Reagents (Milano, Italy). Disodium hydrogen phosphates (Na_2HPO_4) and potassium chloride (KCl) were purchased from Merck (Darmstadt, Germany). Carbon ink and silver/silver chloride ink were obtained from Gwent group (Torfaen, UK). Filter paper grade no.1 (size, 46×57 cm^2) was purchased from Whatman International, Ltd (Maidstone, UK). All solutions were prepared in Milli-Q water (Millipore, USA, $R \geq 18.2$ MΩ cm^{-1}). A phosphate-buffered solution was prepared by dissolving 0.144% (w/v) Na_2HPO_3, 0.024% (w/v) KH_2PO_4 in Milli-Q water.

2.2. Apparatus

All electrochemical measurements, including cyclic voltammetry and amperometry, were performed on a μAUTOLAB type III potentiostat (Metrohm Siam Company Ltd, Bangkok, Thailand) controlled by General Purpose Electrochemical System (GPES) software. A three-electrode system was used. The working electrode (WE) was fabricated by modification of a screen-printed carbon electrode surface (4 mm in diameter) with G/PANI nanodroplets via electrospraying and electropolymerized aniline via cyclic voltammetry. A JSM-6400 field emission scanning electron microscope (Japan Electron Optics Laboratory Co., Ltd, Tokyo, Japan) with an accelerating voltage of 15 kV, a JEM-2100 transmission electron microscope (Japan Electron Optics Laboratory Co., Ltd, Tokyo Japan) and atomic force microscope (Bruker, Karlsruhe, Germany) were used for electrode surface characterization.

2.3. Electrospraying of G/PANI nanocomposites on screen–printed carbon electrodes

A three-electrode system (working, counter, and reference) was fabricated on a polyvinyl chloride (PVC) substrate using a screen-printing technique. The patterned electrode was designed by Adobe Illustrator and an ink-blocking stencil was fabricated by Chaiyaboon Co. (Bangkok, Thailand). First, silver/silver chloride ink was printed on the PVC substrate for all electrodes. The reference electrode and conductive pads were used without further modification. Next, carbon ink was printed on top of one silver/silver chloride layer to generate the working and counter electrodes, respectively. Finally, the screen-printed electrode was dried at 50 °C for 1 h to remove the residual solvent.

For electrode modification, G/PANI nanodroplets were created on the working electrode using electrospraying. The composite solution of G/PANI was prepared as follows. G nanopowder and PVP (2:2 mg) were dispersed in 1 mL DMF using an ultrasonicator for 24 h at a room temperature. PANI (0.4 g) was doped with CSA