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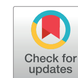
Ultrasensitive determination of receptor tyrosine kinase with a label-free electrochemical immunosensor using graphene quantum dots-modified screen-printed electrodes



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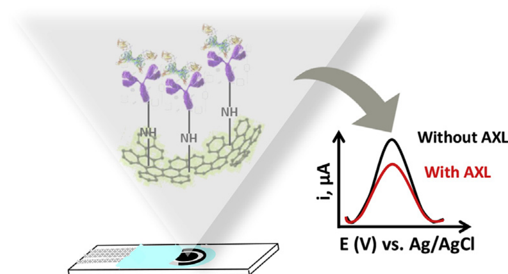
HIGHLIGHTS

- Sensitive label-free voltammetric immunosensor for receptor tyrosine kinase (AXL) determination.
- Antibody immobilization via Schiff reaction on amine functionalized graphene quantum dots.
- LOD of 0.5 pg mL^{-1} far below the cut-off value for heart failure diagnosis in serum.
- Accurate determination of AXL in heart failure patients' sera without matrix effect after just a dilution.

GRAPHICAL ABSTRACT



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ABSTRACT

A new label-free electrochemical immunosensor is constructed for the selective and sensitive determination of the clinically relevant biomarker receptor tyrosine kinase (AXL) in human serum. The disposable immunosensing platform is prepared by immobilization of the specific anti-AXL antibody onto amine functionalized graphene quantum dots (fGQDs)-modified screen-printed carbon electrodes (SPCEs). The affinity reactions were monitored by measuring the decrease in the differential pulse voltammetric (DPV) response of the redox probe $Fe(CN)_6^{3-/4-}$. All the experimental variables involved in the preparation of the modified electrodes and in the immunosensor performance were optimized. The as prepared immunosensor exhibits an improved analytical performance with respect to other electrochemical immunosensors reported so far, with a wider range of linearity and a lower detection limit, 0.5 pg mL^{-1}, which is more than one hundred thousand times lower than the established cut-off value for heart failure (HF) diagnosis in serum (71 ng mL^{-1}). The developed immunosensor was successfully applied to the determination of the endogenous content of AXL in serum of HF patients without any matrix effect observed after just a sample dilution.

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1. Introduction

Heart failure (HF) has a high impact on health systems and society as a whole because of its incidence, prevalence, and high

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mortality rate. The number of admissions due to HF has quadrupled in the last 30 years. Therefore, prognostic biomarkers able to be measured with minimally invasive methodologies are needed to improve the management of the HF epidemic. AXL is a receptor tyrosine kinase discovered by Bryan and co-workers in 1991. The proteolytically processed extracellular fraction of this protein (sAXL) is considered to be a relevant biomarker in cancer [1], inflammatory processes [2] and pathophysiology of HF [3]. The sAXL concentration in serum is elevated in HF patients compared to controls, with a cut-off value of 71 ng mL^{-1} being established [4]. Therefore, the determination of sAXL by means of simple, low cost and fast methodologies able to be easily integrated in point-of-care (POC) devices and direct applicability in body fluids is highly demanded. Several sandwich-type commercial ELISA kits using biotin-labeled secondary antibodies and streptavidin-peroxidase conjugates are available. These kits provide calibration plots for AXL in concentration ranges covering from some tens to several thousands of pg mL^{-1}. Apart from the relatively poor sensitivity, ELISAs suffer from other important drawbacks such as the relatively long assay time, which in some cases takes more than five hours, and the poor reproducibility with relative standard deviation (RSD) values around 10% or higher. It is worth to mention that the only electrochemical immunoassays for sAXL existing so far have been reported by our group. The developed methods used sandwich-type configurations involving covalent immobilization of anti-AXL antibody on polypyrrole propionic acid-modified SPCEs [5], or carboxyl-functionalized magnetic micro- or nanoparticles [6] and amperometric transduction at SPCEs using the HRP/H_2O_2/HQ system.

As it is well-known, label-free electrochemical immunosensors have some advantages compared which the sandwich configurations, mainly in terms of protocols simplification and cost per assay. Label-free strategies allow reducing the number of steps involved in the assay avoiding the need for tedious immobilization processes and the use of secondary antibodies. However, the preparation of label-free immunosensors requires careful consideration of two important aspects such as the immobilization of large immunoreagent loadings without increasing the resistance of the electrode surface and with optimal orientation and spacing for an efficient immunoreaction, and the use of a powerful amplification for the electrochemical response.

Among the quantum dot nanostructures, graphene quantum dots (GQDs) (<20 nm) have demonstrated to be attractive materials with unique features such as good water solubility and large surface-to-volume ratio [7]. Compared to semiconducting quantum dots (QDs), GQDs are superior in biocompatibility and low toxicity [8]. Moreover, unlike carbon dots (CDs), the graphene structure inside the GQDs endows them some of the excellent properties of graphene [9]. Taking advantage of their electrochemical properties, similar to graphene, GQDs have been used as modifiers of the electrode surface [10] and as carrier tags for signal amplification [11] in the development of electrochemical immunosensors. The abundance of hydrophilic edges and the presence of functional groups (-OH, -COOH) can enhance the loading of immunoreagents on the GQDs surface thus greatly improving the sensitivity.

In this work, a new label-free electrochemical immunosensor for AXL using SPCEs modified with amino-group functionalized GQDs is reported. Functionalization of GQDs/SPCE was accomplished by electropolymerization with 2-aminobenzyl amine (2-ABA). The affinity reaction was monitored by measuring the decrease in the differential pulse voltammetric (DPV) responses of $Fe(CN)_6^{3-/4-}$ redox probe recorded at the modified SPCEs upon addition of AXL. The developed immunoplatform fulfilled the requirements of sensitivity, selectivity and reproducibility needed in clinical applications and was successfully employed for the determination of AXL in human sera from follow-up patients with heart diseases.

2. Experimental

2.1. Apparatus and electrodes

Voltammetric experiments were made with a PGSTAT101 potentiostat (Methrohm Autolab) using the Nova 1.8 electrochemical software (EcoChemie B.V.). Electrochemical impedance spectroscopy measurements were carried out with a μ-Autolab type III potentiostat (Ecochemie, The Netherlands) with FRA2 software. Screen-printed carbon electrodes (SPCEs) (DRP-110, DropSens) consisting of a 4-mm diameter carbon working electrode, a carbon counter electrode and an Ag pseudo-reference electrode were used as electrochemical transducers. A specific cable connector (DRP-CAC, DropSens, S.L.) acted as an interface between the potentiostat and the SPCE. All measurements were performed at room temperature.

Field emission scanning electron microscopy (FESEM) of GQDs was carried out with a Tescan Mira3 FESEM equipped with gold coating and an EDX system. X-ray powder diffraction (XRD) was done with a Rigaku D/max-2200X-Ray Diffractometer (Bruker AXS) using a Cu Kα source ($\lambda = 0.154056$ nm). Fourier transform infrared (FTIR) analysis was carried out using a Bruker FTIR spectrometer at room temperature (25 ± 1 °C). The photoluminescence (PL) spectra were recorded with a Shimadzu RF−540S fluorescence spectrometer. UV−visible studies were made with an Avantes diode array spectrophotometer. The transmission electron microscopy (TEM) characterization was performed using a Phillips CM 12 microscope operating at an accelerating voltage of 120 kV.

2.2. Reagents and solutions

The main reagents, including citric acid, potassium ferro- and ferricyanide, 2-aminobenzyl amine (2-ABA) and sodium borohydride, were purchased from Sigma-Aldrich. Bovine serum albumin (BSA) was from Gerbu. Sodium chloride, potassium chloride, disodium hydrogen phosphate and sodium dihydrogen phosphate were from Scharlab. Anti-human AXL mouse monoclonal antibody (anti-AXL) and AXL were obtained from the Human Total AXL DuoSet®IC ELISA kit (R&D Systems, Inc. Catalog Number DYC 1643-2). Phosphate buffer saline (PBS) consisting of 0.1 M phosphate buffer and NaCl solutions with adjusted pH at 7.4 was used as a washing buffer and supporting electrolyte for preparing AXL solutions. All chemical reagents used were of analytical grade and all aqueous solutions were prepared with deionized water. Anti-AXL antibody solution was prepared in 0.1 mol L^{-1} PBS solution of pH 7.4 containing also 3 mg mL^{-1} sodium periodate (Sigma-Aldrich). Human cardiac troponin T (cTnT, Cat 8 T13, HyTest, Ltd.), human C-reactive protein (CRP, Audit Diagnostics), lipoprotein(a) (Lp(a), kindly provided from Audit Diagnostics), human interleukin 8 (IL-8, from IL-8 human Elipair Kit, Abcam, Cat. ab48483), human tumor necrosis factor alpha (TNF-α, from Duoset ELISA human TNF-α, R&D Systems, Inc. Catalog Number DY210-05) and human cardiac troponin I (cTnI, Life Diagnostics) were tested as potential interfering species to evaluate the selectivity of the developed immunosensor.

2.3. Procedures

2.3.1. Synthesis of GQDs

GQDs were synthesized by direct pyrolysis of citric acid (CA) according to the method described by Dong et al. [12] with slight modifications. Briefly, 2 g of CA were heated until the temperature

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