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Chitosan–graphene oxide based aptasensor for the impedimetric detection of lysozyme

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ABSTRACT

An impedimetric detection of lysozyme (LYS) was performed for the first time in this study at the surface of chitosan–graphene oxide (CHIT–GO) modified sensor based on the specific interaction process between DNA aptamer and its cognate protein, LYS.

The amino linked DNA aptamer (APT) was covalently immobilized without using any chemical agents onto the surface of pencil graphite electrode (PGE). These PGEs are inexpensive and simple to use, and thus, they can be furtherly developed for a single-use application in a portable protein chip device. The electrochemical impedance spectroscopy (EIS) technique was used herein to analyze (i) the surface characterization of unmodified PGE and CHIT–GO modified PGE, and also (ii) the interaction between APT and LYS. The limit of detection (DL) was found as 0.38 μg/mL (equals to 28.53 nM). This impedimetric LYS aptasensor exhibited a higher selectivity toward thrombin and bovine serum albumin, even in the mixture samples.

1. Introduction

Graphene with monolayer of sp^2 carbons has 2D nano structures, which exhibits the properties including excellent electrical conductivity, high surface area, good mechanical strength, high thermal conductivity and high mobility of charge carriers [1–3].

Graphene and graphene oxide (GO) have been used as the advanced nanomaterials for development of sensors due to their high conductivity and providing a larger surface area [4,5]. In particular, the unique surface properties of GO (oxygenated functional groups on the basal planes and edges), large surface area; layered structure, and easy exfoliation into monolayers under water mean that GO is a suitable building block for fabricating versatile functional materials via covalent, or non-covalent approaches [6,7].

Chitosan is the semi-synthetically derived amino-polysaccharides, that have unique structures, multidimensional properties, highly sophisticated functionality and a wide range of applications in biomedical and other industrial areas [8–10]. In addition, chitosan molecule is a copolymer composed of N-acetyl-D-glucosamine and D-glucosamine units available in different grades depending upon the degree of acetylated moieties [11]. It is a polycationic polymer that has one amino group and two hydroxyl groups in the repeating glucosidic residue [12].

Aptamers are artificial single-stranded DNA, or RNA oligonucleotides (typically smaller than 100 mer), which are selected from large randomized oligonucleotide libraries by SELEX (systematic evolution of ligands by exponential enrichment) [13,14]. Different types of target molecules including proteins [15,16]; cells [17,18]; viruses [19] and bacteria [20], small molecules such as organic dyes; metal ions [21] and amino acids [22,23] can specifically bind with aptamers [24]. It is in analogy to affinity between antibodies and antigens. However, aptamers have presented some superior features such as high specificity of binding affinity, better stabilization, and longer shelf life comparison to antibodies. Moreover, the aptamers can reversibly capture and release their target protein. It is facile for the aptamer to transduce the recognition events into the detectable signals.

The fabrication of novel nanostructures with improved properties is an alluring prospect of nanotechnology. Recently, the electrochemical techniques have received a considerable attention due to their advantages such as rapid and reliable response, low cost, practical operation, rapidity, high sensitivity and good selectivity. Especially, the impedimetric biosensors, which are based on the change at the electron transfer resistance in the presence of redox probe couple, $[Fe(CN)_6]^{3-/4-}$, can provide an attractive tool for the analysis of interfacial changes induced from biomolecular interactions at the electrode surfaces.

Lysozyme is an abundant protein widely distributed in nature. So far, it has been found in mammalian tissues and secretions, and in organisms as diverse as insects, bacteria, viruses and plants [25].

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In the human body, it has physiological and pharmaceutical functions, such as anti-inflammatory, anti-viral, immune modulatory, anti-histaminic and anti-tumor activities [26]. The quantification of lysozyme amount could be a marker of some health problems. Reduced lysozyme levels have been associated with bronchopulmonary dysplasia in newborns [27]. Children fed infant formula lacking lysozyme in their diet have three times the rate of diarrheal disease comparing to children fed lysozyme-rich milk in their diet [28]. Since lysozyme is a natural form of protection from gram positive pathogens like *Bacillus* and *Streptococcus*, a deficiency due to infant formula feeding can lead to increased incidence of disease. Whereas the skin is a protective barrier due to its dryness and acidity, the conjunctiva (i.e., the membrane covering the eye) is protected by secreted enzymes, mainly lysozyme and defensin. However, when these protective barriers fail, consequently the conjunctivitis occurs. Additionally, it was reported that lysozyme concentration in serum and urine increased in case of leukemia [29] and several kidney problems [30].

Many researches have been performed in the progress of aptamer-based electrochemical sensors, "aptasensor" for the determination of various proteins by using EIS technique. Rodriquez et al. [31] designed an aptasensor based on indium tin oxide as working electrode by using electrochemical impedance spectroscopy technique. Chen et al. [32] developed an impedimetric aptasensor for lysozyme detection by using gold nanoparticles deposited onto the surface of gold electrode. Another impedimetric aptasensor was developed by electrodeposition of Fe_2O_3/GO nanocomposite onto the surface of glassy carbon electrode and utilized for indicator-free detection of LYS by Du et al. [33]. One of the recent studies on impedimetric aptasensor for LYS detection was introduced by Rohrbach et al. [34] using multi walled carbon nanotube modified screen printed electrodes with the detection limit of 12.09 μg/mL.

To the best of our knowledge, there has been no reports on the development of impedimetric detection of 129 amino acids, long globular protein, lysozyme (LYS) using the DNA aptamer immobilized chitosan–graphene oxide (CHIT–GO) modified PGE. The CHIT–GO modified PGE was also applied for the first time herein for sensing of interaction between aptamer and its target protein LYS toward different proteins, thrombin (THR) and bovine serum albumin (BSA).

2. Experimental

2.1. Apparatus

Each experimental measurement was carried out by using AUTOLAB-PGSTAT 302 electrochemical analysis system supplied with a FRA 2.0 module for impedance measurements in the Faraday cage (Eco Chemie, The Netherlands). The conventional three electrode system consisted of PGE as the working electrode, an Ag/AgCl/3M KCl as the reference electrode (BAS, Model RE-5B, W. Lafayette, USA) and a platinum wire as the auxiliary electrode.

The surface characterization of the modified electrodes (bare PGE, CHIT modified PGE and CHIT–GO modified PGE) were performed by using Quanta 400 FEI, field emission scanning electron microscope (FE-SEM) (Tokyo, Japan).

2.2. Chemicals

The amino-linked single-stranded DNA (ssDNA) aptamer was purchased from Ella Biotech (Germany).

Anti-lysozyme DNA aptamer (APT):

5′-NH_2-ATC TAC GAA TTC ATC AGG GCT AAA GAG TGC AGA GTT ACT TAG-3′

The stock solution of the DNA aptamer was prepared with fresh ultrapure triple-distilled water, and stored at −20 °C. The diluted solutions of the aptamer were prepared with Tris-buffered saline (TBS: 5 mM Tris-HCl buffer supplemented with 20 mM NaCl, pH 7.0).

LYS, BSA and THR were purchased from Sigma. The stock solutions were prepared by dissolving them in the fresh ultrapure triple-distilled water, and stored at −20 °C. The diluted solutions of proteins were prepared in 50 mM phosphate buffer solution (PBS, pH 7.4) [32].

Graphene oxide was kindly donated from Nanoinnova Technologies SL (Spain). The further details of GO is available at web site as: www.nanoinnova.com.

Chitosan and other chemicals were supplied from Sigma (USA) and Merck (Germany) in analytical reagent grade.

2.3. Procedure

2.3.1. Preparation of CHIT–GO solutions

2000 μg/mL of chitosan (CHIT) was dissolved in 1% acetic acid solution by using a sonicator during 45 min. Then, a required amount of GO was added into the CHIT solution, and this mixture was once again sonicated during 15 min.

2.3.2. Preparation of CHIT–GO modified PGE

A Tombow pencil was used as a holder for each new graphite lead. Electrical contact with the lead was obtained by soldering a metallic wire to the metallic part. The pencil was held vertically with 14 mm of the lead extruded outside (10 mm of which was immersed in the solution).

The PGEs were electrochemically pretreated by applying +1.40 V for 30 s in acetate buffer solution (ABS). Each pretreated pencil lead was immersed into the Eppendorf tubes containing 110 μL of composite, including 2000 μg/mL CHIT and 2000 μg/mL GO during 15 min in order to form CHIT–GO layer onto the surfaces of PGEs. Then, CHIT–GO modified PGEs were allowed to dry for 15 min at upside down position.

2.3.3. APT immobilization onto the surface of CHIT–GO PGE

CHIT–GO modified electrodes were immersed into the vials containing 110 μL of amino linked DNA Aptamer (APT) solution in TBS during 30 min by the formation of covalent coupling between the carboxylic groups of the GO and the amino groups of APT without using any chemical agents for covalent binding such as *N*-(3-dimethylamino)-propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) similarly to our previous work [34]. Each of the electrodes was then rinsed with TBS for 10 s to remove unbound APT from electrode surface.

The modification of PGE surface with CHIT–GO, and the immobilization of APT onto CHIT–GO PGE was represented in Scheme 1.

2.3.4. Interaction with LYS

APT modified PGEs were immersed into the vials containing LYS (or THR, BSA) solution prepared in PBS during 15 min. Each electrode was then rinsed with PBS for 10 s.

2.3.5. Impedance measurements

The EIS measurements were performed in the presence of 2.5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture as redox probe prepared in 0.1 M KCl. The impedance was measured in a frequency range from 10 mHz to 1000 kHz at the open circuit potential of +0.23 V versus Ag/AgCl with a sinusoidal signal of 10 mV. The respective semicircle diameter corresponds to the charge transfer resistance (R_{ct}), the values of which are calculated using the fitting program AUTOLAB 302 (FRA version 4.9, Eco Chemie).