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Determination of C-reactive protein by PAMAM decorated ITO based disposable biosensing system: A new immunosensor design from an old molecule

Münteha Nur Sonuç Karaboğa[a,*], Mustafa Kemal Sezgintürk[b]

[a] Namık Kemal University, School of Health, Tekirdağ, Turkey
[b] Canakkale Onsekiz Mart University, Faculty of Engineering, Bioengineering, Canakkale, Turkey

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ABSTRACT

This paper demonstrates a new and sensitive electrochemical immunosensor for the analysis of C-reactive protein, an important marker of inflammation. Indium Tin Oxide (ITO) disposable sheets were modified by using 11-cyanoundecyltrimethoxysilane (CUTMS) and PAMAM dendrimers (G:1 amino surfaces) for the first time to immobilize the anti-CRP antibody via covalent interactions. Cyclic voltammetry (CV), and electrochemical impedance spectroscopy (EIS), as well as square wave voltammetry (SWV) methods, were applied to characterize the immobilization stages of anti-CRP and to determine the CRP concentrations. Charge transfer resistance changes were highly linear and sensitive to CRP concentration in the range 21–6148 fg mL^{-1} and were associated with a limit of detection of 0.34 fg mL^{-1}. The system had acceptable repeatability (6.45%, n = 18) and good storage stability (4.5% loss after 6 weeks). Moreover, the binding characterization of CRP to anti-CRP was monitored by a single frequency impedance technique. The amount of CRP in human serum samples was analyzed with a fabricated biosensor to determine the feasibility of the biosensing system for medical purposes. We suggest that 11-CUTMS, a new silanization agent, is ideal for biosensor applications.

1. Introduction

C-reactive protein is an effective plasma protein of hepatic origin that responds to the inflammatory process and has homologues not only in vertebrates but also in many invertebrates. Its plasma concentration increases at an obvious rate during the inflammation process, making it possible to use it for clinical purposes [1,2]. This systemic marker with high clinical susceptibility was the first acute phase protein to be identified. While the average concentration of C reactive protein in healthy individuals is 0.8 mg/L, the serum levels can increase up 10,000-fold rapidly during the development of inflammatory conditions [3]. The most important feature of this protein belonging to the pentraxins family is that it is widely used as a prognostic or diagnostic biomarker especially in cardiovascular disease [4–7], obesity [8,9], diabetes mellitus [10,11], renal diseases [12], some types of cancer [11,13–16] and neurological disorders [17–21]. Currently, CRP analysis in the clinical field is widely performed using turbidimetric, nephelometric techniques or enzyme-linked immunosorbent assay (ELISA) kits. However, these techniques have some disadvantages such as low sensitivity, amount of time involved and high cost [22]. To detect CRP, several methods such as surface plasmon resonance [23,24], chip-based systems [25], optical biosensors [26], quartz crystal microbalance sensors [27,28] and electrochemical sensors [29–31] were developed. Among these, electrochemical techniques become prominent from the point of view of sensitivity, low cost, and practicality.

Poly(amidoamine) (PAMAM) dendrimers consisting of branched monomers can be used effectively in the immobilization process of biorecognition elements thanks to their large surface areas and the high number of functional groups they contain [32]. There are several effective studies in the literature that have used a PAMAM platform for biosensor design [33–36].

ITO-PET electrodes are unique materials in terms of being disposable, cheap, allowing different chemical modifications on the surface, being flexible, and providing reproducible and highly accurate results. Many methods can be used to immobilize the biorecognition element onto the ITO surface. Among these, the most effective and most commonly used method is to use silanization agents such as 3-Glycidoxypropyltrimethoxysilane (3-GOPS) [37], 3-glycidoxypropyltriethoxysilane (3-GOPE) [38] and, 3-aminopropyltriethoxysilane (APTES) [39].

In this study, a new biosensing system was developed to detect CRP, an important biomarker of inflammation. The design of the biosensor

* Corresponding author.
E-mail address: mnsonuc@nku.edu.tr (M.N. Sonuç Karaboğa).

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was planned on the basis of a combination of 11-CUTMS and PAMAM dendrimers. 11-CUTMS is used to form the self-assembled monolayers (SAMs) on the surface, while PAMAM is intended for use as an agent to increase active amino terminals. The immobilization steps of the biosensor were evaluated by cyclic voltammetry (CV), square wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS). The developed biosensor surface morphology was visualized by scanning electron microscopy (SEM). In order to achieve a biosensor system with a high applicability, extensive optimization studies were carried out. Repeatability, reproducibility and selectivity studies were also completed. The kinetic behavior of the bond between anti-CRP and CRP was followed effectively by an electrochemical technique called single frequency impedance. Analysis of the concentration of CRP in blood serum samples with the developed biosensor and comparison of the results with reference methods were the end points of this study.

2. Materials and methods

2.1. Chemicals and apparatus

The reference electrode and counter electrode were purchased from BASi (West Lafayette, IN, USA), disposable ITO-coated PET films (The transmittance and surface resistivity are 550 nm (> 79%) and 60 Ω/square, respectively and geometry area 0.25 cm^2) Standard anti-CRP and CRP antigen were acquired from Sigma Aldrich (St. Louris, MO, USA) CRP, anti-CRP portions were prepared at certain concentrations in 50 mM pH 7.0 phosphate buffer and stored at −20 °C. until use. 11-cyanoundecyltrimethoxysilane was purchased from abcr GmbH&Co. (Karlsruhe, Germany). Amino surfaces PAMAM (generation:1), and the other reagants were supplied from Sigma Aldrich (St. Louris, MO, USA). Ultrapure water (18.2 MΩ/cm) was obtained from a Elga LC134 system and used throughout.

All electrochemical experiments were carried out in an electrochemical cell (three electrode system), consist of a sheet of ITO film as a working electrode, a platinum wire as a counter electrode and a silver/silver chloride as a reference electrode which has a volume of 10 mL 1 M KCl, 5 mM $[Fe(CN)_6]^{4-}$ and 5 mM $[Fe(CN)_6]^{3-}$ redox probe. The blood samples were obtained by research ethics committee approval with the number of 2013/86/07/05 from Namık Kemal University, Faculty of Medicine. All the electrochemical measurements, including electrochemical impedance spectroscopy, were performed by a Potentiostat/Galvanostat (Gamry Interface 1000 Gamry Instruments, Warminster, USA). The measurements were checked by a personal computer running the electrochemical software program of Gamry Instruments (Echem Analyst) for data collection, monitoring of optimization parameters, and processing.

2.2. Preparation of ITO-based electrodes

The first step in the design of the biosensor is the cleaning of the ITO electrodes. The ITO-PET electrodes were polished by sonicating with acetone, soap solution and ultrapure water respectively for 10 min. Before proceeding further, all the electrodes were gently dried under ultra-pure argon gas. In order to construct the silanization agent on the ITO electrodes, hydroxide groups must first be formed on the surface of each cleaned electrode. For this, the electrodes were incubated at room temperature for 90 min in a solution containing hydrogen peroxide, ammonium hydroxide and ultrapure water (1:1:5). The hydroxyl-group-formed electrodes were gently washed with ultrapure water and dried with ultrapure argon gas.

2.3. Fabrication of immunosensor

After cleaning procedure and hydroxyl group formation on the surface, the ITO electrodes were immersed into 0.5% 11-CUTMS solution prepared in pure toluene-ethanol mixture and allowed to incubate overnight at room temperature. Then, in order to remove the physically adsorbed 11-CUTMS molecules, the electrodes were washed with ultra pure water and gently dried with ultra pure argon gas. In the next step, the ITO-based electrodes were immersed in the 1.0% PAMAM solution prepared in methanol and allowed to incubate for 60 min at room temperature. PAMAM-modified electrodes were washed with ultrapure water and dried again with argon gas. Afterwards, PAMAM-modified electrodes were immersed in 1% pH 7.0 PBS solution of glutaraldehyde, a cross-linker commonly used in the activation of amino groups, and allowed to incubate for 15 min. ITO substrates modified with 11-CUTMS and PAMAM were dried with argon gas gently and immersed into 100 µL of 63.4 ng mL^{-1} anti-CRP solution in a dark and moist medium as quickly as possible for 60 min. After incubation with anti-CRP antibody, electrodes were washed with ultra-pure water to remove unbound antibody molecules. Finally, anti-CRP antibody-immobilized ITO electrodes were treated with BSA (0.5%) for 60 min to block the active ends. At the end of this, the electrodes were washed with ultrapure water and gently dried with ultra pure argon gas. With this last step, the now-prepared biosensor was stored at +4 °C until the CRP measurements were performed. The bare (cleaned) and modified ITO substrates are indicated as ITO, ITO-OH, ITO/CUTMS, ITO/CUTMS/antiCRP, ITO/CUTMS/anti-CRP/BSA and ITO/CUTMS/antiCRP/BSA/CRP, respectively.

2.4. Electrochemical measurements

Immobilization steps, optimization assays and analytical properties of the developed biosensor were monitored with electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV), and square wave voltammetry (SWV).

All electrochemical experiments were carried out in a solution containing 5 mM K3[Fe(CN)6]/K4[Fe(CN)6] (1:1) as a redox probe. The redox probe solution also contained 0.1 M KCl to decrease the resistance of probe solution.

In cyclic voltammetry experiments, the potential was differentiated between −500 mV and 1000 mV (step size: 10 mV, scan rate: 100 mV/s). Electrochemical impedance measurements were performed by applying an alternating potential of 5 mV to the working electrode. The formal potential applied in the impedance studies was 0 V. Another important parameter in impedance measurements was the frequency range from 50,000 to 0.05 Hz. SWV were carried out with potential scan range: 0–1.2 V, and pulse size: 25 mV.

Morphological observation of the different surfaces obtained during the fabrication process of the biosensor used a field emission scanning electron microscope (FEI-Quanta FEG 250) operated at the Scientific and Technological Research Center of Namık Kemal University (NABILTEM). 5 kV was used as an acceleration voltage to obtain SEM images.

2.5. Measurement principle of C-reactive protein

Each of the 11-CUTMS and PAMAM-modified ITO-based biosensors were incubated with standard CRP solutions prepared at different concentrations for 60 min at room temperature and in darkness. After the incubation period, the biosensor was gently immersed into ultrapure water to remove physically adsorbed CRP molecules. Electrochemical impedance spectroscopy and cyclic voltammetry measurements of each CRP concentration were performed by immersing the working electrodes into the redox probe. EIS and CV measurements were recorded via EChem Analyst software program.

K3 [Fe (CN) 6] / K4 [Fe (CN) 6] is a redox couple that acts as an electrochemical mediator between the biological element and the surface of the modified electrode.

In electrochemical impedance spectroscopy, charge transfer resistance (Rct) is a demonstration of two effects: (1) potential energy associated with the oxidation or reduction event at the electrode. (2) the formation of an electrostatic repulsion or steric barrier with the