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A novel silanization agent based single used biosensing system: Detection of C-reactive protein as a potential Alzheimer's disease blood biomarker

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ABSTRACT

This paper illustrates a new and sensitive electrochemical immunosensor for the analysis of C-reactive protein. Indium Tin Oxide (ITO) disposable sheets were modified by using 3-cyanopropyltrimethoxysilane (CPTMS) self-assembled monolayers (SAMs) for the first time for immobilizing the anti-CRP antibody via covalent interactions without the need for any cross-linking agent. Cyclic voltammetry (CV), and electrochemical impedance spectroscopy (EIS), as well as square wave voltammetry (SWV) methods were applied to characterize immobilization steps of anti-CRP and to determine the CRP concentration. The optimization of the fabricated parameters and the analytical performance of the biosensor were widely evaluated. Charge transfer resistance changes were highly linear and sensitive with CRP concentration of 3.25–208 fg mL^{-1} range and associated with a limit of detection of 0.455 fg mL^{-1}. This impedimetric biosensing system have excellent repeatability, reproducibility and reusability. 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 were analyzed by fabricated biosensor to determine the feasibility of the biosensing system in medical purposes. We suggest that CPTMS, a new silanization agent, is ideal in biosensor applications.

1. Introduction

C- reactive protein (CRP) is well known immune response protein that belongs to pentraxin protein family. The C- reactive unit occurred in very early stage of infections and found high concentrations in the blood during inflammatory conditions. Different from many other acute-phase proteins, CRP level rises and falls more rapidly and more strikingly thus, it has been long used clinical purposes as diagnostic and prognostic biomarker as well as response to treatment [1,2].

As well-known neurodegenerative disorder, Alzheimer's disease (AD) is most common form of dementia and that is diagnosed after age of 65 years. Considerable studies proof that inflammation process is associated with Alzheimer's disease (AD) pathology [3,4]. CRP level is increased in the brain and serum of patients with Alzheimer's disease (AD), and has been associated with risk of progression dementia especially in oldest old people [5]. Some pentraxins include the C-reactive protein associated with AD brain lesions [6] and up regulated by pyramidal neurons in the AD brain while normally produced in liver [7]. Although CRP has regulator role in inflammation and autoimmunity, it may be cause tissue damage due to destructive role in the pathogenesis of AD.

Because of diagnostic importance of CRP as a biomarker for inflammation there are a lot of methods commonly used in literature such as well-known Enzyme-Linked Immunosorbent Assay Technique (ELISA [8,9], microchip assay system [10], quantum dot-labeled microplate immunoassay [11], and different types of biosensors. Most of these methods are expensive and applicability are difficult in medical field. ELISA test are often expensive and prolonged preparations. In addition, the sensitivity of the method may vary according to the ELISA kit used and limited to elevated concentrations of CRP. In order to minimize ELISA based CRP analysis problem such as high false-positive rate due to non-specific binding, the surface plasmon resonance (SPR) biosensor assay has been suggested for immunological assays. This technique has several advantages in detection applications but In the SPR technique, it is time-consuming to prepare the sensor surface and it is costly compared to our work because materials such as gold are used [12].

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The applications of indium tin oxide (ITO) electrodes in biosensor fields is remarkable because of their potential to different surface modification and amplification [13,14]. ITO substrates are characterized by high electrical conductivity, low capacitive current, electrochemical activity over a wide potential range, and stability exhibited by their physical and electrochemical properties [15]. Due to the high adhesiveness between ITO and PET materials, the stability and flexibility of ITO-PET materials is very high [16]. From this point, the purpose of this research is to develop a novel, easily prepared, low cost immunosensor to detect CRP as a potential Alzheimer disease blood biomarker.

Our research group developed ITO based biosensor systems for various purposes by different modification techniques previously [17].

During the past 50 years the synergism between organic and silicon chemistries has been examined to develop many organo-functional silanes that are necessity today in many applications. Organo-functional silanes are composed of two different reactive groups on their silicon atom thus, they can react and couple with very different materials. Organo-functional silanes are used for surface modification [18].

When a molecular scale device is desired to be developed, a molecular level controlled surface modification means are needed. A widely used method of achieving molecular level control of surface modification is to apply self-assembled monolayers [19]. In current study, the self-assembled monolayer (SAMs) was formed by 3-cyanopropyltrimethoxysilane (3-CPTMS) which is very new and first used organo-functional silanes agent for improving biosensor.

To monitor immobilization steps of biosensor, cyclic voltammetry (CV), square wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) techniques were applied. Surface morphology was characterized by scanning electron microscopy (SEM). In order to obtain applicable biosensor, extensive optimization studies were performed. Repeatability and reproducibility parameters were also studied. Interaction between anti-CRP and CRP were observed in real time by a valuable technique – single frequency impedance –. The behavior of the developed biosensor against regenerative solutions was also studied. At the end of study, the quantities of CRP in human serum samples were measured with the designed biosensor and the results were compared with the results obtained with a reference method.

2. Materials and methods

2.1. Materials and apparatus

ITO-coated Polyethilenteraftalat(PET) films (The transmittance and surface resistivity are 550 nm (>79%) and 60 Ω/square, respectively and geometry area 0.25 cm^2), The reference electrode and counter electrode were purchased from BASi (West Lafayette, IN, USA) and the other reagents were acquired from Sigma-Aldrich (St. Louis, MO, USA). 3-CPTMS from Flourochem Ltd (Graphite Way, Hadfield, England) CRP, anti-CRP and bovine serum albumin (BSA, 1%) were prepared in 50 mM pH 7.0 potassium phosphate buffer.

All electrochemical experiments were carried out in an electrochemical cell (three electrode system), consist of a sheet of ITO thin film (2×0.5 cm) 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 real 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 self-assembled monolayers (SAMs) of 3-cyanopropyltrimethoxysilane

The first step of biosensor construction is cleaning procedure of ITO substrates by following process, sonicated in pure acetone, soap solution and ultra-distilled water respectively for 10 min. Before using each cleaned electrodes were dried under an ultrapure stream of argon. To form conductive hydroxyl groups onto ITO surfaces, electrodes were immersed into ultrapure water containing hydrogen peroxide (1/7, v/v), and ammonium hydroxide (1/7, v/v) for 90 min at room temperature in dark ambient. After that, electrodes were washed thoroughly with ultrapure water and gently dried by using ultra-pure argon gas. Self-assembled monolayers were generated by 3-cyanopropyltrimethoxysilane which has functional – cyano groups for covalently attachment to anti-CRP antibody. ITO electrodes are incubated in 3-CPTMS (prepared in toluene/ethanol mixture) overnight. Then they were rinsed with ethanol/toluene solution mixture and ultrapure water respectively to remove physically adsorbed CPTMS molecules.

2.3. Covalent immobilization of anti-CRP onto SAM of 3-CPTMS

ITO substrates modified with 3-CPTMS were dried argon gas gently and immersed into 100 μL of anti-CRP solution in a dark and moist medium as quickly as possible. After incubation with anti-CRP antibody, electrodes were washed with ultra-pure water to remove unbounded antibody molecules. Lastly, anti-CRP immobilized ITO sheets were treatment with 0.5% BSA solution to close the active 3-CPTMS ends. The bare (cleaned) and modified ITO substrates were indicated as ITO, ITO-OH, ITO/CPTMS, ITO/CPTMS/antiCRP, ITO/CPTMS/anti-CRP/BSA and ITO/CPTMS/antiCRP/BSA/CRP.

2.4. Electrochemical measurements

Immobilization steps, optimization studies and analytical characteristics of constructed biosensor were performed following electrochemical methods, 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 KCl to decrease the resistance of probe solution.

In cyclic voltammetry experiments, the potential was differentiated between −500 mV and 1 V (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. The other important parameter in impedance experiments was the frequency range that was in the range between 50,000 and 0.05 Hz. SWV were carried out by potential scan range: 0–1.2 V, pulse size: 25 mV

Morphological observation of the different surfaces obtained when the fabrication process of the biosensor, a field emission scanning electron microscope (FEI-Quanta FEG 250) was 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.

FTIR spectra in the range 4000–400 cm^{-1} were recorded in order to investigate the nature of the chemical bonds formed and oper-