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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

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 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].

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

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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-

ated at the Scientific and Technological Research Center of Namık Kemal University (NABILTEM).

2.5. Measurement principle

After preparing the biosensor as mentioned above, EIS and CV measurements were performed and recorded via EChem Analyst software program. Afterwards, varying concentrations of a 100 μL standard solution CRP were introduced to each modified ITO electrode based biosensors for an hour in dark and moist medium. After incubation period, the biosensor was gently immersed into the ultrapure water to remove physically adsorbed CRP molecules. Finally the working electrode with ITO again put into the cell containing the $Fe(CN)_6^{4-/3-}$ redox probe solution and the electrochemical measurements of each electrode taken and recorded separately as described previously.

3. Results and discussion

3.1. Immobilization of anti-CRP on ITO surface

Self-assembling monolayers (SAM) are often used to control the physical and chemical properties of the solid surface. When we consider electrochemistry, SAM is very common in selective electrode design for electroanalytical applications. In the immobilization of biomolecules, the ITO surface is usually modified by a silane reactant. The chemically modified ITO electrode is used as a working electrode for the detection of biomolecules.

For covalent immobilization of anti-CRP antibody onto ITO surface, a silanization agent conjugated with indium tin oxide was needed at hydroxylated terminals. Organosilanes with hydrogen, oxygen and a functional group directly attached to the silicon atom are very reactive to form covalent attachment with other substrates [20]. The first reaction that happens in solution is condensation of the silane coupling agents to form a monolayer with —Si—O—Si bonds. Simultaneously, the growing silane polymers interact with the ITO substrate through the formation of a hydrogen bonding network with the —OH groups on its surface.

Cyanopropylsiloxanes are among the most useful organosilanes as they have both polar and polarizable features. The cyano group with an unshared electron pair in the nitrile nitrogen provides enhanced affinity as it may compose intermolecular hydrogen-bonds with suitable hydrogen donor sample molecules such as the biorecognition element bearing p-electrons [21].

Moreover, organo-functional silanes possess low surface tension that provides good surface wetting and ensures an increase in adhesion between dissimilar materials because of their reactivity to different surfaces. They may form interactions and an adequate transition interphase between the monolayer and the substrate to be bonded [22]. CPTMS molecules consist of three parts: the head group, the alkyl chain and the end group. The head group, trimethoxy- is responsible for the anchoring of the molecules onto the substrate. The alkyl chain provides stability of the single layer due to van der Waals interactions and has a significant effect on the regulation of SAM; the terminal group brings chemical functionality to the monolayer system and is important for the general properties of the surfaces. Terminal group (-cyano) of CPTMS having an unshared electron pair on the nitrile nitrogen, provide high functionality to the structure [23]. Consequently, in this study 3-cyanopropyltrimethoxysilane was used effectively as SAM agent due to the above mentioned features. To form a stable monolayer on the surface, incubation was performed overnight with 3-CPTMS. Afterwards, anti-CRP was covalently immobilized to SAM modified ITO surface as quickly as possible. If there were functional open ends after immobilization, BSA was used as blocking agent.

The step-by-step formation of the constructed biosensor was monitored by EIS and CV techniques. K3[Fe(CN)6]/K4[Fe(CN)6] is a redox couple including a redox reaction between the biological element and modified electrode surface as an electrochemical mediator.

Many biosensor devices are used for development of a recognition complex between the biosensing material and the analyte in a monolayer or thin film. Any change in the conductive or semiconductive surface results in alteration of the capacitance and the resistance at the surface electrolyte interface. Moreover, the growth of biosensing material on the conductive or semiconductive surface induces capacitance and resistance properties on the surface-electrolyte interface. All these changes are effectively reflected in electrochemical impedance spectroscopy results. Hence, EIS is a very powerful method to understand interfacial changes and give detailed information about biorecognition element events on the surface. The impedance spectra for each immobilization step of anti-CRP are shown in Fig. 1A. and on CV voltammograms in Fig. 1B

The semicircle diameter in an impedance spectrum indicates the electron transfer resistance; R_{ct}. Electron-transfer kinetics of the redox probe at the electrode-solution interface are controlled by electron transfer resistance. The charge transfer resistance (Rct) value of bare ITO was assessed as 43130 Ω. As seen from impedance spectra, the formation of —OH groups on the ITO surface causes more conductive behavior toward the electrode that was proven by a decrease in charge transfer resistance (695.5 ohm). After SAM of CPTMS was formed, negatively charged cyanide groups at pH 7.0 were pushed to the negative redox probe and did not allow it to easily diffuse to the surface which might be reflected in the impedance spectra results as a significant increase in the semicircle diameter (950 ohm).

In the next step, the anti-CRP is immobilized on the modified surface through chemical covalent bonding by the reaction between the cyano group and the amino group of anti-CRP, the structure of a secondary ketimine is formed (Graphical abstract) resulting in an obvious increase in charge transfer resistance (1272 ohm). This apparent increase is an indication that the layer was successfully formed by anti-CRP on the surface and partially permits diffusion of the redox probe due to the generation of an insulating protein layer on the ITO surface. The high affinity of amino groups for active cyanide groups without need any cross linker is already defined the literature [24]. Likewise, BSA used for blocking active nitrile ends also has the effect of enhancing the electron resistance on the surface (1410 ohm).

Cyclic voltammograms were also applied to investigate the electrochemical behavior of the anti-CRP based immunosensor. The impedance spectra and cyclic voltammogram results are compatible with each other. The electrostatic repulsive force between negatively charged cyanide groups of CPTMS and the negatively charged redox probe is the reason for the reduction in the signal at peak current after the formation of self-assembled mono layers of CPTMS on the ITO surface. As seen from Fig. 1B, after the covalent immobilization of anti-CRP onto the CPTMS modified ITO surface there was a decrease in both anodic and cathodic peaks due to preventing effects of the immobilized layers between the redox probe and electrode surface.

3.2. Optimization studies for the CRP biosensor

Because antigens and antibodies are mostly charged protein structures, their affinities to each other are influenced by the formation and charge on the electrode surface. Therefore, it is possible to understand the capacitance variations of bioaffinity complex formation by determining optimal conditions.

Since the concentration of the self-assembled monolayer agents on the surface can significantly affect binding of the biorecog-

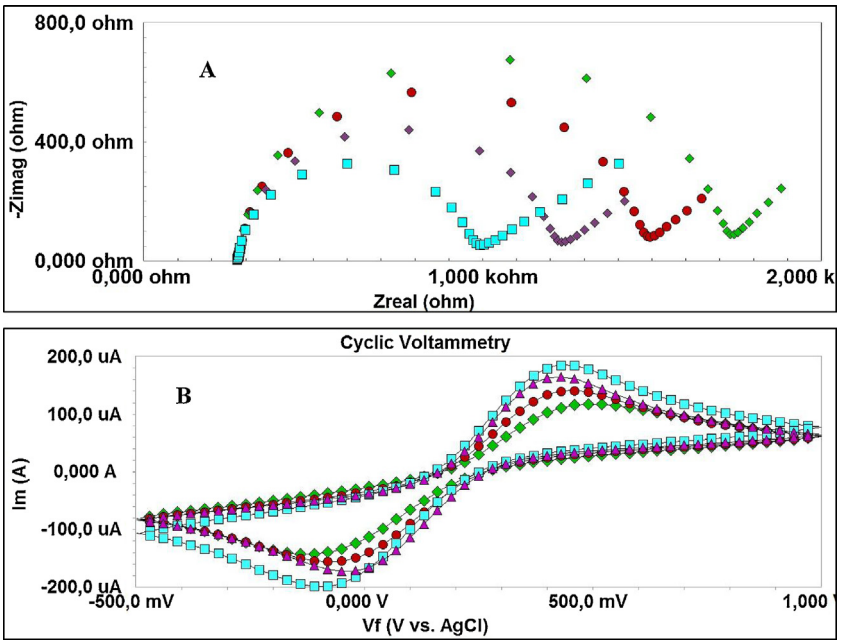

Fig. 1. EIS spectrum (**A**) and cyclic voltammogram (**B**) of immobilization steps (-■-■-): ITO/OH (-▲-▲-): ITO/OH/CPTMS (-●-●-):ITO/OH/CPTMS/antiCRP, (-♦-♦-): ITO/OH/CPTMS/antiCRP/BSA.

nition element, the first step when designing the biosensor is to elucidate the concentration of the SAM. Moreover, since 3-cyanopropyltrimethoxysilane is a very new silanization agent used for the first time, the response of the monolayer covered electrodes was carefully monitored by EIS and CV. For this purpose, different concentrations of CPTMS (0.5%, 1%, 1.5%, 2%, 2.5%) were applied on hydroxylated ITO electrodes overnight at room temperature. Since the stacking of the layer on the surface is influenced more by the concentration, different concentrations of CPTMS exhibited different impedimetric behaviours. As seen from calibration curves (Supplemental data, Fig. 1S) obtained from EIS data, while CPTMS of 1.5% has a relatively high charge transfer resistance and a low coefficient of determination (R^2*: 0.9685*), 2.5% CPTMS has low charge transfer resistance and a relatively high coefficient of determination (R^2*:0.9803)*. However, results for 1% and 0.5% CPTMS are close to each other showing high charge transfer resistance and high coefficient of determination. As the concentration of CPTMS increased, there was an irregularity in the immobilization of the anti-CRP. Thus, 0.5% CPTMS was chosen for SAM formation for the optimal immunological recognition of the designed biosensor at the selected silane concentration.

A wide range of antibody concentrations (3.17, 15.85 ng mL^{-1}, 0.317, 1.585, 3.17, 6.34, 12.56, 25.34 μg mL^{-1}) were assayed to optimize the immunosensor response (Fig. 2S).

At higher antibody concentrations (for 25.34 μg mL^{-1} of anti-CRP $y = 359.24$ $R^2 = 0.978$), a decrease in sensitivity of the immunosensor was observed, probably due to the higher steric barrier and the destructive effect of protein-protein interactions inhibiting the recognition of CRP. A decrease in sensitivity was also noticed using anti-CRP solution of 3.17 ngmL^{-1} because it is not enough for effective binding of CRP onto the anti-CRP immobilized surface. Therefore, anti-CRP solution of 15.85 ng mL^{-1} was optimized for further experiments.

Another important parameter for a sensitive immunosensor is determination of incubation time for the formation of antigen-antibody bioaffinity complexes. For this purpose, modified ITO electrodes were incubated in anti-CRP solutions for different periods of 30, 45, and 60 min. The immunosensor response increased slightly with increasing incubation time from 30 to 60 min. This result shows that 60 min of anti-CRP incubation time is not necessary for the formation of the immuno complex, while 30 min of incubation time is enough to obtain sensitive immunosensor response (Fig. 3S).

3.3. Analytical performance of designed biosensor

The analytical characteristics of the designed biosensor were specified by measuring standard CRP solution with different concentrations under optimal conditions. The relationship between antibody concentration and electron transfer resistance was determined by the equivalent circuit model inserted in Fig2A. This equivalent circuit model, which is frequently used in impedimetric immunosensor studies, is composed of resistive and capacitive elements as well as Warburg element. *Rct* is the charge transfer resistance, *Rst* indicates resistance of the working solution of system, CPE indicates the constant phase element of the bioactive layer and Z_w is the Warburg element. The most remarkable change among these elements belongs to charge transfer resistance (R_{ct}). This circuit was applied to describe the electron transfer from an electrolyte to an electrode.

Based on this information, the calibration graph of the CRP immunosensor was drawn using the following equation.

$$\Delta R_{ct} = R_{ct}(CRP) - R_{ct}(BSA)$$

where $R_{ct(CRP)}$ is the value of electron transfer resistance after antibody-antigen binding between anti-CRP and CRP. Rct(BSA) is the value of the semicircle diameter of the blocking step of the constructed biosensor with BSA. Nyquist plots and cyclic voltammograms of the fabricated biosensor incubated in increasing concentrations of the CRP are presented in Fig 2A and B. As the CRP concentration increased, a linear increase in the diameter of the semicircle on Nyquist plots was observed. The increase in R_{ct} could be due to the generation of an immunocomplex that causes distinction in the conductivity properties of the electrode surface. Likewise, Fig. 2B showed that the CV peak currents decreased with increasing concentrations of CRP. The reason for this is the growth of the immunocomplex with increasing CRP concentration on the surface enhanced the electron transfer barrier significantly. The cal-

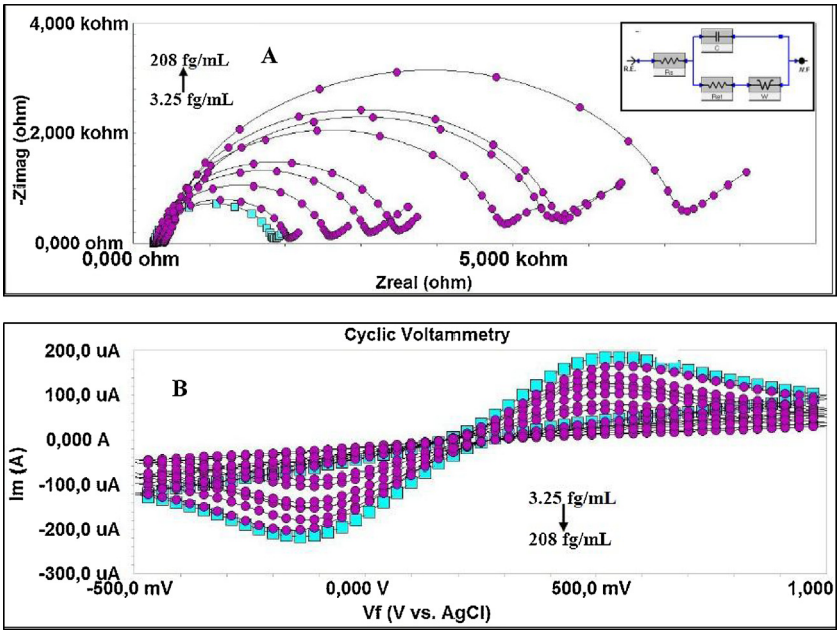

Fig. 2. Impedance spectra (A), cyclic voltammograms (B) obtained for different concentrations of CRP (-■-■-):ITO/OH/CPTMS/antiCRP/BSA(-●-●-):ITO/OH/CPTMS/antiCRP/BSA/CRP.

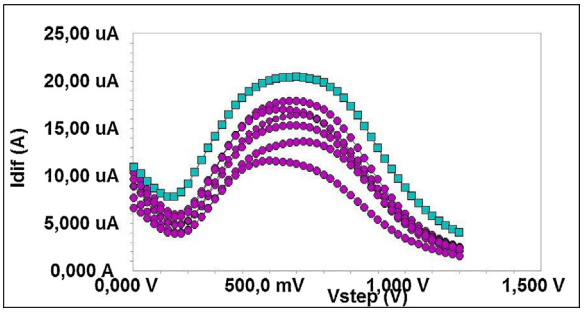

Fig. 3. Square Wave Voltammograms obtained for different concentrations of CRP, and (-■-■-):ITO/OH/CPTMS/antiCRP/BSA (-●-●-): ITO/OH/CPTMS/antiCRP/BSA/CRP.

ibration values (Table 1) showed a linear correlation in the range from 3.25–208 fg mL^{-1} with a regression of $R^2 = 0.9862$. The linear equation of this calibration graph is $y = 25.576x + 143.58$.

LOD (limit of detection) and LOQ (limit of quantification) values were determining according the IUPAC recommendations [25] as 0.455 fg mL^{-1} and 1.517 fg mL^{-1} respectively. These results emphasize that the developed biosensor shows high sensitivity in CRP assay.

There are several commercial and non-commercial biosensors developed for identification of CRP. Table 2 compares these biosensors with the constructed biosensors in terms of determination range. The developed biosensor is superior with features such as femtogram level sensitivity, single used biosensing system, wide detection range, low-cost and simple preparation method [26–29].

The characterization steps of the developed immunosensor were monitored not only by EIS and CV techniques but also by SWV, which is another electrochemical technique depending on frequency like EIS. SWV consists of the combination of a series of potential signals between the cathodic pulse and the anodic pulse at the same amplitude. While the cathodic pulse is applied, the analyte is diffused to the surface and rapidly reduced. In the course of the anodic pulse, the reduced analyte is re-oxidized immediately. Therefore, there is a linear relationship between the decrease in peak current as the CRP concentration increases (Table 3). As seen from linear equation of CRP biosensor calibration curve obtained by plotting ΔIdif versus CRP concentration is as $y = 0.0349 + 2.9061$ with regression of 0.9708 the SWV results have similar sensitivities with EIS. The advantage of SWV to EIS is that the potential scan is completed within a few seconds whereas a longer period is needed for EIS.

Repeatability studies were carried out under optimum conditions by measuring CRP with the same concentration (52 fg/mL) with 20 independently prepared ITO electrodes. For these repeatability experiments, the relative standard deviation (RSD) was calculated as 3.88%.

The reproducibility of ITO-based CRP immunosensor was evaluated by monitoring the responses of 10 biosensor systems prepared with the same procedure in the 3.25–208 fg/mL determination range. Reproducibility experiments were carried out at different

Table 1
Concentration-dependent calibration values for CRP and selectivity results for HER-3, Haptoglobin, HSP-70 obtained by the biosensor based on anti-CRP at optimal working conditions.

CRP (fg/mL)	ΔRp (ohm)	Standard Devitation (SD) n = 4	ΔRp (ohm) (HER-3)	ΔRp (ohm) (Haptoglobin)	ΔRp (ohm) (HSP-70)
3.25	137	±0.0065	22	41	48
6.5	312	±0.013	32	36	66.5
13	518	±0.032	45	45	72
26	985	±0.052	55	52.5	88
52	1585	±0.104	62	77	77
104	2631	±0.211	72	82	78
156	3714	±0.312	80	94.5	90
208	5813	±0.335	102	122	132.5

Table 2
The results for the sample analysis and comparison of methods used for CRP.

Samples	*Clinical Results from NKU hospital*[*] (mg/L)	*Found by Biosensor* (mg/L)	*Relative Difference*	*Recovery* (%)
1	4.1	3.99	2.68	97.3
2	1.1	1.42	29.27	129.27
3	2.4	2.57	7.08	107.08
4	2.1	2.41	14.57	114.57
5	4.7	5.45	15.95	115.95
6	2.3	2.12	7.83	92.17
7	1.4	1.5	7.14	107.14
8	3.3	2.8	15.15	84.85

Methods	Measurement Principle	Detection Range	LOD	References
Graphene oxide-nanoparticle	Impedimetric	$1–10^9$ fg/mL	80×10^3 fg/mL	[26]
Aptamer based sandwich assay	Electrochemical	$0–10^{12}$ fg/mL	54×10^6 fg/mL	[27]
Parlyene-n film	SPR	$10^6–10^9$ fg/mL	–	[28]
Carbon nanofiber	Electrochemical	5×10^7 fg/mL–5×10^9 fg/mL	11×10^6 fg/mL	[29]
The proposed biosensor	Electrochemical	3.25–208 fg/mL	0.455 fg mL^{-1}	

[*] Obtained from the reference methods used in the Namık Kemal University hospital.

Table 3
SWV results of different concentration of CRP at optimal working condition.

CRP(fg/mL)	ΔIdif ($\Delta Idif_{bsa}$-$\Delta Idif_{CRP}$, **A**)
3.25	1.39
6.5	2.46
13	3.35
26	3.81
52	4.72
104	6.53
156	8.35
208	10.16

times by ten different researchers working in our laboratory. The responses of these immunosensors were found to represent similar linearity in the range between 3.25–208 fg mL^{-1} for CRP. The relative standard deviations were also calculated by using the slopes and the intercepts of the linear equations obtained from these 3.39% and 2.21%. Results of reproducibility and repeatability studies emphasize that ITO electrodes modified with a new silanization agent named 3-cyanopropyltrimethoxysilane provide great working surface to immobilize anti-CRP antibody for the determination of CRP with the newly constructed immunosensor system.

The storage stability of the fabricated ITO-based disposable biosensor was specified by monitoring the impedimetric response of the immunosensor at the end of specific periods. Immunosensors prepared under optimal conditions were stored at 4 °C and CRP detection was performed in every 7 day period. For the first eight weeks immunosensors almost completely retained their initial activity (99.6%), whereas at the end of the nineth week the immunosensor response reduced significantly due to some destructive effects and non-specific bindings (Fig. 4).

Fig. 4 Regenerating biosensors is critical in order to reduce costs through reusable features and accelerate the commercialization process. The regeneration step is carried out under extreme conditions such as acidic and alkaline solution treatment as well as by high potential application. Regeneration of the constructed immunosensor was achieved by immersion of CPTMS-modified ITO electrodes in 10 mM HCl for 5 min, to dissociate the CRP antibody-antigen immunocomplex. The immunosensor retained 91.4% of the original response after 11 regeneration cycles. The reason for this minor decrease in activity may be due to antibody denaturation or destruction of CPTMS monolayer during continuous processing with the regeneration solution.

Fourier-transform infrared spectroscopy (FTIR) spectra of CPTMS modified ITO surface and immobilization of anti- CRP, are shown in Fig. 5. Fig. 5A shows CPTMS modified ITO electrode surface. The observation of medium band at ~2250 cm^{-1} clearly

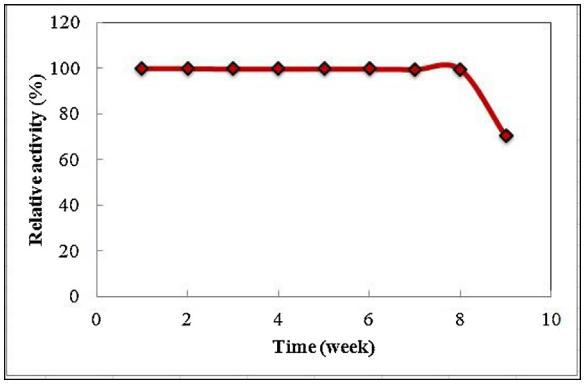

Fig. 4. The storage stability of the developed biosensing system.

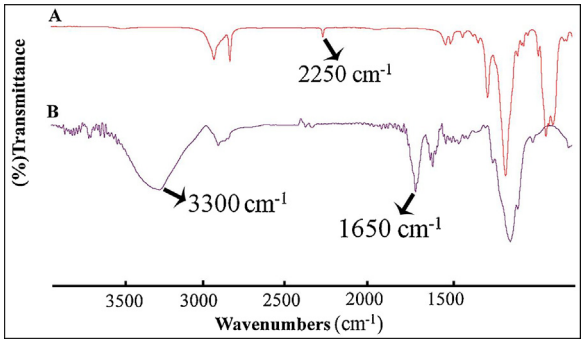

Fig. 5. FTIR spectra of CPTMS modified (A) and after anti-CRP immobilization (B) of ITO based biosensor.

indicates the presence of C≡N groups in CPTMS. Fig. 1B illustrates the FTIR spectrum of the same surface after immobilization of anti-CRP on CPTMS modified ITO surface. Around 1650 cm^{-1} intense and strong band indicate the interaction between CPTMS and anti-CRP and the resulting imine group. Also, the broad band between 3350 cm^{-1} corresponded to secondary amine groups [30].

3.4. Single frequency impedance (SFI) experiments

One of the effective electrochemical methods for monitoring the kinetic behavior of antibody/antigen binding is single frequency impedance. This method is useful to better understand alteration on the electrical surface. For this purpose, the potentiostat was set up at a fixed frequency of 45 Hz which was defined with the assistance of Bode plots. While the Bode plots provide important information

about the frequency of the system, the Nyquist plot is weak on this point. we performed single frequency impedance experiments in pH 7.0 PBS solution containing CRP, so it is non- faradaic procedure. The variation in impedance value was measured at the fixed frequency as a function of time and phase angle for 30 min. In Fig. 6, time-dependent change in impedance is presented by a blue curve while the change in the phase angle as a function of time is shown by the red curve. When the CRP interacts with its target anti-CRP, an extra hindrance effect occurs on the designed surface and this is reflected as a decrease in phase angle and an increase in impedance value of the circuit. The advantage of this method is to characterize surface events such as attachment of immunocomplex through a time-dependent system. The principle of this technique is based on surface changes and kinetic parameters during interaction of antigen-antibody with the fixed frequency. The time-dependent increase in impedance can be regarded as a sign of specific interaction. However, single frequency impedance is strongly affected by environmental factors like electromagnetic waves.

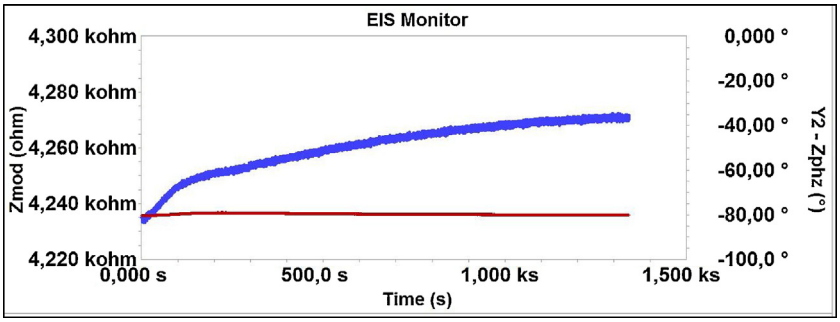

Fig. 6. Single frequency impedance measurement of biosensor based on CRP (■ variation is phase angle; red • variation is impedance; blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

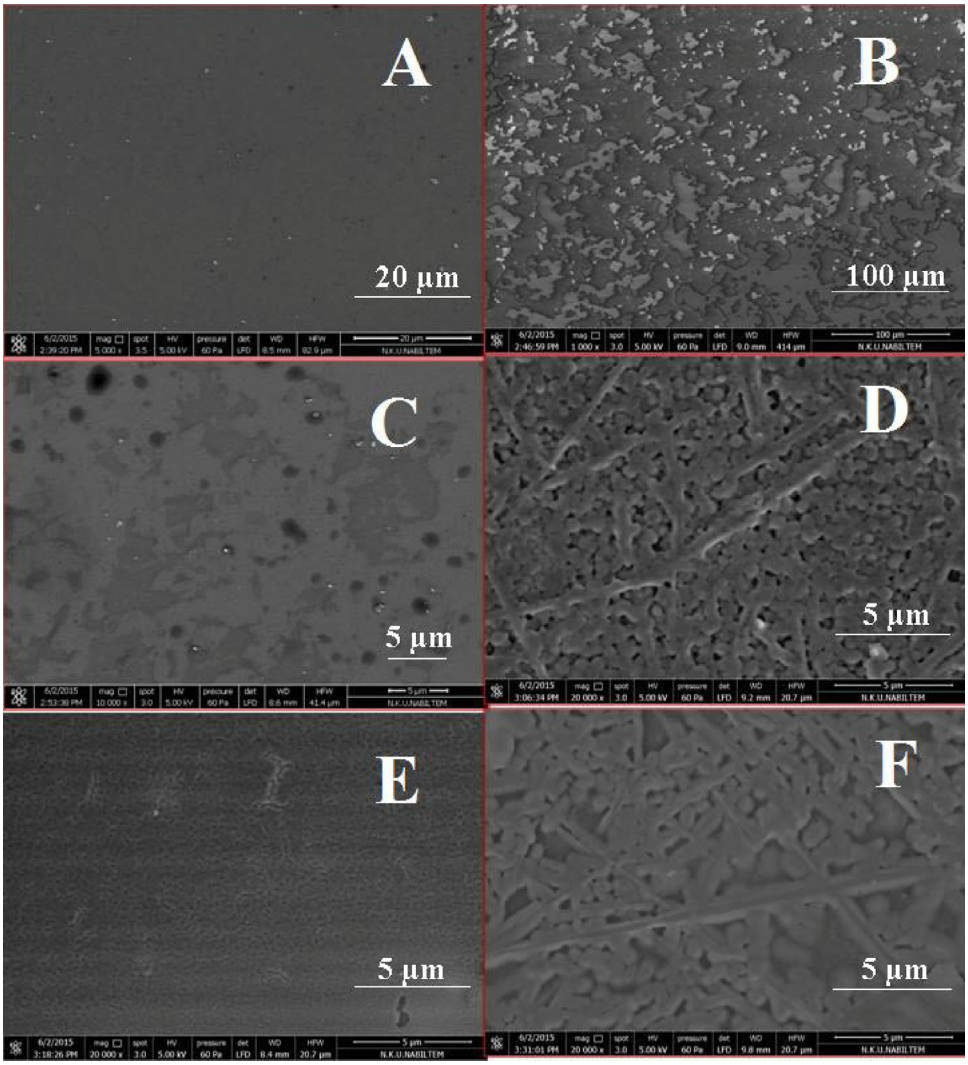

Fig. 7. SEM images related with the morphological changes of the biosensor surfaces. [(A) ITO/BARE; (B) ITO/OH/; (C) ITO/OH/CPTMS; (D) ITO/OH/CPTMS/anti-CRP; (E) ITO/OH/CPTMS/anti-CRP/BSA; (F) ITO/OH/CPTMS/anti-CRP/BSA/CRP].

3.5. Application of immunosensor for CRP detection in clinical serum samples

The fabricated immunosensor was used for detection of CRP in eight human serum samples containing different concentrations of CRP. The serum samples were obtained with research ethics committee approval number 2013/86/07/05 from Namık Kemal University, Faculty of Medicine. The results for the use of the constructed biosensor for CRP detection are compared with serum CRP levels determined at the hospital by a reference technique (Table 2). CRP measurements at the hospital were performed by Roche Diagnostics model no: cobas c 501 autoanalyzer using an immunoturbidimetric method. After the CRP measurements of the serum samples were performed at the hospital with the technique indicated, the serum were brought to the our research laboratory without loss of time. Until the determination of CRP by construction biosensing system were made, it was kept at −20C in certain portions. Human serum samples were first diluted 10^7 times because the CRP content of serum from a healthy person has higher concentration than the proposed biosensor detection range. As seen from the results (Table 2), good agreement and accuracy was observed between the reference method of the Medical Faculty Hospital and the proposed immunosensor.

3.6. Morphological analysis by SEM

The chemical and physical changes on the surface can be monitored morphologically step by step with scanning electron microscopy. Tracing these changes provides superior information about the biosensor's motion. The SEM images for the constructed biosensor are shown in Fig. Fig. 7A shows the smooth surface of an ITO electrode typically. The formed hydroxylated surface is illustrated Fig. 7B. Morphological change in the surface after modification with SAM of CPTMS is shown in Fig. 7C When anti-CRP was immobilized onto the electrode surface, a more globular form is obtained due to the pentraxin structure of protein (Fig. 7D). In the BSA blocking step, a denser surface was formed as seen from Fig. 7E. After interaction between CRP and anti-CRP, a bean-like surface was observed (Fig. 7F).

3.7. Selectivity

The selectivity of the constructed biosensor towards target CRP was investigated by monitoring ΔRct of the biosensor for a nonspecific interaction, if any, with the nonspecific protein antigens, human HER-3, haptoglobin, HSP-70 over the same concentration range of 3.25–208 fg mL^{-1}. Table 1 shows that the non spesific of HER-3, haptoglobin, and HSP-70 to the biosensor has a very small contribution. This negligible contribution of the nonspecific interactions to the total Rct response refered a good selectivity of the biosensor to the specific target CRP.

4. Conclusion

In current study, a low cost, easy prepared, disposable biosensing system for the determination of CRP has been successfully developed. It was suggested that CPTMS provided an effective new immobilization material for the sensitive and practical immobilization of biorecognition elements onto ITO substrates. Due to its chemical structure, no need for cross-linking agents during immobilization is superior of the current study. Moreover, CPTMS based biosensing system allowed reproducible and repeatable with high sensitive analysis of CRP. Impressive LOD (0.455 fg mL^{-1}) and LOQ (1.517 fg mL^{-1}) values and a wide determination range (3.25 fg mL^{-1}–208 fg mL^{-1}) were obtained by using the simple, disposable, rapid electrochemical immunosensor based on flexible ITO electrodes. CRP levels of human serum sample experiments showed good agreement with reference method through CPTMS modified disposable ITO material should prevent undesirable physical boundings to the electroactive surface and provide selective results of CRP. Moreover, the lifetime of fabricated biosensor relatively long which is a considerable parameter for future prospective studies in medical field. Besides this, constructed biosensing system was regenerated several times and it point out that analysis of CRP can be performed more than once without surface degradation. The Indium tin oxide coated PET sheets that we used sizes are 1 ft × 1 ft × 5 mil. One package include 5 sheets. The price of a package is 112 euros (Sigma-Aldrich, 639303) We use only 2 × 0.5 cm size electrode. It means that an electrode is about **0. 021** euros. We use a limited number of chemicals to develop the biosensor. This makes the cost of our work quite affordable and feasible. Finally, it is proposed that CPTMS is a very new silanization agent should be used in biosensor construction processes due to its excellent suitability with ITO substrates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jpba.2018.03.016.

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