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Fullerene-PAMAM(G5) composite modified impedimetric biosensor to detect Fetuin-A in real blood samples

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ABSTRACT

The aim of this study is to develop a nanomaterial-dendrimer composite modified biosensor to detect Fetuin-A (HFA) in real blood samples. For this purpose, we designed an Electrochemical Impedance Spectroscopy (EIS) based anti-Fetuin-A (Anti-HFA) modified biosensor system and tested in real blood samples. Chronoimpedance was also employed. The same samples were analyzed with ELISA and the results were compared for validation of the new system. Gold screen printed electrodes (AuSPE) were used as transducer. Firstly, a self-assembly monolayer (SAM) was formed on gold surface by 4-aminothiophenol (4-ATP), Fullerene and PAMAM-NH_2 (G5), layers were formed, consecutively. Then Anti-HFA was immobilized on Au/4-ATP/Fullerene/PAMAM electrode via glutaraldehyde. The chronoimpedance test was employed to investigate the optimum analysis duration. According to the data of chronoimpedance, the total analysis time for EIS was chosen as 3 min. The new biosensor was compared with the ELISA method which required 150 min. The calibration curve was prepared electron transfer resistance of the electrode (ΔRet) per minute as ohm and 1.66–134 ng/mL.min with a R^2 = 0.9912. The LOD and LOQ of the biosensor was calculated as 0.48 ng/mL.min, 1.46 ng/mL.min, respectively. Linear regression analysis indicated that the novel developed biosensor results agreed well with that of the conventional ELISA assay.

Introduction

Biosensors are widely used to detect molecules in samples by using a proper biorecognition receptor such as enzymes, antibodies, ligand proteins or DNA and this eliminates the need for previous preparation [1–4]. The interaction between the biorecognition receptor and the analyte molecule represents the selectivity of the biosensor and forms a detectable signal. This interaction can be monitored electrochemically, optically or by piezoelectric based monitoring systems [5–13] These systems can be developed in different aspects; however, the electrochemical techniques are used more than the other methods, because of the easy applicability, low cost and less noise effects. The electrochemical biosensors which are based on the ohm law can be designed on amperometric, voltammetric or impedimetric determination fundamentals. Electrochemical impedance spectroscopy (EIS) has been used to develop different types of affinity based biosensors or to investigate transducer surface characteristics with a good sensitivity [14]. The impedimetric detection is very suitable for the affinity based biosensor systems, because the system only needs to detect the binding kinetics of the analyte molecule to the surface without a need for a label, electroactive secondary molecule or biochemical transformation. Affinity based biosensors are mostly designed by using antibodies of the analyte molecule. The immunosensor type biosensors, especially monoclonal antibody modified biosensors, have proved their specificity and sensitivity for the immunosensor development by monitoring the target molecule, usually without the need for an extra preparation phase [15–18].

In this study, we developed an affinity based impedimetric biosensor for the detection of Fetuin-A (HFA) in real blood samples. The literature attributes a great importance to this biomarker in investigation of different diseases. The new methods for the analysis of the biomolecule should work within the expect limits, must be feasible for the recognition of the HFA in blood to reduce health expenses, save time, be selective and sensitive as well as easy applicable. HFA, in other words alpha2-Heremans-Schmid glycoprotein, is a 59kDA glycoprotein released by hepatocytes. This glycoprotein is a cysteine protease and

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contains rich residues of sialic acid. Clinical studies have shown that HFA is related to hepatocellular carcinoma, atherosclerosis and other diseases and disorders about calcium metabolism. The biomarker is also a negative acute-phase reactant, of which levels decrease in case of inflammation. The normal levels of HFA are in microgram ranges in healthy humans for per liters of blood. In clinical perspective, HFA is relevant in diagnosis of diabetes, cardiovascular diseases, metabolic syndrome, obesity, multiple sclerosis, cancers, kidney diseases and neuroinflammation [19–26]. The levels of the biomarker is also important in physiological gestation [27], therefore we chose blood samples from both ectopic pregnant and healthy pregnant volunteers. This variety of the volunteers was chosen to detect different concentrations of the HFA in blood. The performance characteristics of the biosensor we designed was tested by comparing the results of the biosensor system with an ELISA test. The ELISA test is considered as a gold standard for comparison in our study. The novel biosensor provided good correlation with the ELISA results and thus can be used to detect HFA in blood samples. In this study, polyhydroxylated fullerene nanoparticles and PAMAM(G5) were used for the first time in literature to form a layer-by-layer covalent modification layers for the biosensor technology.

Materials

All chemical materials were obtained from Sigma-Aldrich (USA), electrochemical measurements were carried out by using PalmSens3 potentiostate (Palmsens BV., Netherlands). The screen-printed gold electrodes (250AT) were obtained from Dropsens (Spain). The blood samples were collected from ectopic pregnant women and women with physiological pregnancy to serve as control group. The serum samples were stored in -20^{O}C until use. All chemicals were prepared by using triple deionized water. The study was approved by Ege University, School of Medical Faculty Ethical Research Committee (17–7.2/14).

Methods

All electrode modification steps were investigated by scanning electron microscopy (SEM) and EIS. EIS was carried out in a solution of redox probe, which includes 5 mM $Fe(CN)_6^{4-/3-}$ redox couple and 100 mM KCl in 50 mM phosphate buffer (pH = 7). The EIS parameters were set as follows; frequency is chosen between 10000 and 0.05 Hz by applying 180 mV potential. Chronoimpedance detection was carried out 2000 Hz for 250 s 100 mM KCl in pH = 7.4 phosphate buffer saline. All modifications and measurements were carried out at room temperature, around 25 Celsius degrees. EIS signals showed that the electrical circuit for EIS calculation was constructed as shown in Fig. 1.

Prior to use, gold electrodes (AuE) were washed by pure ethanol and pure water, consecutively, to remove any adsorbents. EIS was performed to investigate bare gold electrodes as base line. Then the electrodes were washed with pure water and dried under nitrogen stream. AuE was soaked in 50 mM 4-aminothiophenol (4-ATP) solution in ethanol for overnight to form Self-assembly Monolayer (SAM). Then electrodes were washed by ethanol and water to remove unbonded 4-ATP from AuE surface and EIS was performed to investigate AuE/4-ATP modification. The polyhydroxy small gap fullerenes ($C_{120}O_{30}(OH)_{30}$)

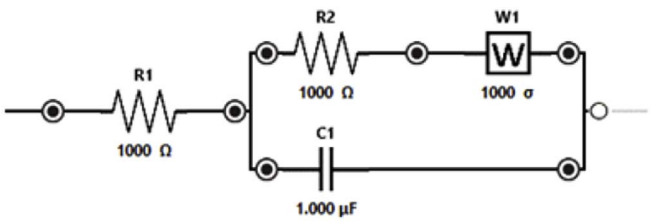

Fig. 1. Impedance spectrums fitting circuit model.

were prepared at room temperature, 4 mg/mL in HEPES buffer and sonicated until the fullerenes dispersed homogenously. 20 µL of fullerene solution which includes 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC:40 mM) and N-hydroxysuccinimide (NHS:10 mM) was incubated to activate OH groups of the fullerenes in a shaker to prevent fullerene precipitation [6]. Activated fullerene solution was dropped on the gold electrode and incubated for 2 h in an isolated moisturized environment. The AuE/4-ATP/Fullerene electrode was washed, dried and 20 µL solution of PAMAM(G5) in pH = 6.8 phosphate buffer [28] was dropped on the electrode and incubated for 1 h. The AuE/4-ATP/Fullerene/PAMAM modified electrode was washed and EIS was performed. The AuE/4-ATP/Fullerene/PAMAM electrode was soaked in 5% glutaraldehyde solution to activate amino groups of the PAMAM for 30 min and after wash process, 2 mg/mL Anti-Fetuin-A antibody solution of 20 µL was dropped on the electrode and incubated for 2 h. The electrode was washed and EIS performed to show the AuE/4-ATP/Fullerene/PAMAM/AntiFetA modified electrode modification. The unbounded glutaraldehyde groups were blocked by glycine amino acid.

The calibration curve was prepared by the standard HFA solutions, which were obtained from the ELISA kit, between the range of 5–400 ng/mL. Since the optimum analysis duration of HFA was determined as 3 min by chronoimpedimetric detection, the calibration curve results are given ng/mL per minute. Standards with different concentrations were dropped on the AuE/4-ATP/Fullerene/PAMAM/AntiHFA modified electrode for 30 min of incubation and EIS results were obtained. As base line, AuE/4-ATP/Fullerene/PAMAM/AntiHFA modified electrode EIS data was chosen and the HFA calibration curve signal axis was prepared by using HFA binded biosensors' electron transfer resistance increase (ΔRet_{HFA}). ΔRet_{HFA} was calculated as AuE/4-ATP/Fullerene/PAMAM/AntiHFA EIS signal removed from AuE/4-ATP/Fullerene/PAMAM/AntiFetA/HFA signal ($\Delta Ret_{Au/4\text{-}ATP/Fullerene/PAMAM/AntiHFA/HFA}$-$\Delta Ret_{Au/4\text{-}ATP/Fullerene/PAMAM/AntiHFA}$).

Optimization studies were carried out by differentiating the incubation times of the 4-ATP, Fullerene, PAMAM, AntiFetA. The LOQ was calculated by using $10.S_{(1.67ng/mL.min)}/m$ as 1.46 ng/mL.min and LOD result was calculated by using $3.3\ (S_{(1.67ng/mL.min)})/m$ as 0.48 ng/mL.min. Sb is the EIS data of the 5 ng/mL (1.67 ng/mL.min), the lowest concentration of the calibration curve of seven AuE/4-ATP/Fullerene/PAMAM/AntiFetA standard deviation and m is the slope of the calibration curve. The selectivity studies were directly carried out by dropping 10 µL blood samples on AuE/4-ATP/Fullerene/PAMAM/AntiFetA electrode and compared by ELISA test, which was carried out with exactly the same steps as the document provided [29]. The real blood samples were studied by ELISA test based on the manuel of the ELISA kit [29].

Results

All EIS curves of the electrode modifications are shown in Fig. 2. The AuE/4-ATP modified electrode shows less resistance than AuE EIS curve. This results from the positively charged amino groups of 4-ATP which attract the negatively charged redox probe and thus Warburg impedance becomes more dominant against resistance [30]. The fullerene incubation decreases the electron transfer resistance through the electrode by the conductivity superiority of the nanomaterial. The PAMAM layer also shows same characteristic as 4-ATP but PAMAM has more amino residues than 4-ATP for the electrode surface. The fullerene and PAMAM are used to increase electrode surface area [28] to immobilize more Anti-HFA antibodies to increase sensitivity. The Anti-HFA immobilization increases the electron transfer resistance of the surface by forming an insulating layer (Fig. 2).

As can be seen in Fig. 3, we successfully formed polyhydroxylated fullerene layer as concentrated on the electrode surface. Fullerene modification step was also a challenging step because the fullerenes are adsorbed by the cap they were activated, therefore a spectrophotometry quartz cuvette was used to incubate fullerenes to hydroxy activation.

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