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Fabrication of a highly sensitive disposable immunosensor based on indium tin oxide substrates for cancer biomarker detection

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ABSTRACT

Anti-HER-3 antibody was used for the first time in a disposable immunosensor based on indium tin oxide (ITO) substrate for HER-3 quantification. Anti-HER-3 was immobilized onto ITO substrate by 3-aminopropyl)triethoxysilane (APTES) and glutaraldehyde. This highly sensitive immunosensor was capable of detecting concentrations of HER-3 down to the femtogram/ml level by investigating changes in the charge transfer resistance (R_{ct}) using electrochemical impedance spectroscopy (EIS). Construction of ITO layers was carefully investigated using a broad range of techniques such as voltammetry, EIS, atomic force microscopy (AFM), and scanning electron microscopy (SEM). Meanwhile, in an immunosensor system, the "single frequency impedance" technique was first used for characterization of interaction between HER-3 and anti-HER-3. Eventually, the proposed ITO-based immunosensor was applied to artificial serum samples spiked with HER-3.

HER-3 is a type of transmembrane growth factor receptor of the human epidermal growth factor receptor. It can activate intracellular signaling pathways in response to extracellular signals [1,2]. HER-3 itself is an incomplete receptor functionally, and consequently it is a dependent protein [3]. Many studies have reported that tumor progression and reduced survival of patients with breast [4], ovarian [5], and pancreatic [6] cancers, gastric carcinoma [7], malignant melanoma and metastases [8], and head and neck squamous cell carcinoma were considerably associated with overexpression of HER-3. Moreover, HER-3 overexpression is importantly correlated with poor prognosis [9] and worse metastasis-free survival [10] in colorectal carcinomas. For instance, it has been revealed that both HER-3 messenger RNA and protein were upregulated in human breast cancers, such that in human breast cancers HER-3 overexpression has been reported in the ratio of 50–70% [11,4,12]. In addition, it seems to be associated with metastasis and local recurrence [13,14].

In this study, because of the vital importance of HER-3, an immunosensor based on indium tin oxide (ITO)[1] substrate as a working electrode was developed. Because of its good electrical conductivity, ITO is one of the most widely used transparent conducting oxides. Transparent ITO thin films on flexible substrates such as polyethylene terephthalate (PET) have many applications. It has been reported that they can be used in plastic liquid crystal display devices, transparent electromagnetic shielding materials, flexible electro-optical devices, heat reacting mirrors, and the like [15]. Various ITO-based immobilization techniques have led to new applications in the construction of biosensors. For example, gold nanoparticles were self-assembled onto an ITO electrode to prepare a modified sandwich-type electrochemical immunoassay platform for determination of vascular endothelial growth factor [16]. In another study, ITO was covered by a poly(dopamine) layer for biomolecule immobilization [17]. However, for more reliable and stable biosensor systems based on ITO substrates, more developments are needed to create useful immobilization strategies.

Electrochemical impedance spectroscopy (EIS) has become an efficient method that is used for many chemical and physical processes. Hence, it is quite often applied in fabrication of immunosensors. In a typical EIS-based immunosensor, after any biorecognition event, charge transfer resistance (R_{ct}) usually increases, and this change in R_{ct} can be used for detection of any substances. However, in this study, a novel impedance method was applied to the immunosensor for the first time. "Single frequency impedance" was performed to reveal binding characteristics between HER-3 and anti-HER-3.

The focus of this study was on a simple immobilization procedure for anti-HER-3, development of a highly sensitive and disposable HER-3 biosensor, and its impedimetric characterization. The current study is the first to use anti-HER-3 as a bioreceptor in an immunosensor system for HER-3 analysis. We also identified

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[1] *Abbreviations used:* ITO, indium tin oxide; PET, polyethylene terephthalate; EIS, electrochemical impedance spectroscopy; SEM, scanning electron microscopy; BSA, bovine serum albumin; APTES, 3-aminopropyltriethoxysilane; CV, cyclic voltammetry; AFM, atomic force microscopy; SAM, self-assembled monolayer.

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scanning electron microscopy (SEM) features of the surfaces of each layer based on a combination of EIS in the presence of $[Fe(CN)_6]^{3-/4-}$ as a redox couple. Certain working parameters such as anti-HER-3 concentration and anti-HER-3 binding period were optimized and characterized. Finally, the immunosensor was applied to artificial serum samples for the determination of HER-3.

Materials and methods

Reagents and material

All reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). ITO-coated substrates (ITO-coated PET film) were obtained from Sigma–Aldrich. The surface resistivity and transmittance were 60 ohm/square and 550 nm (>79%), respectively. HER-3 and anti-HER-3 were also purchased from Sigma–Aldrich. HER-3, anti-HER-3, and 0.1% bovine serum albumin (BSA) were prepared in a phosphate buffer (50 mM, pH 7.0). Solutions of HER-3, anti-HER-3 and 0.1% BSA were stored at 20 °C until use. Synthetic serum solution was prepared by using 4.5 mM KCl, 5 mM $CaCl_2$, 4.7 mM D(+)-glucose, 2.5 mM urea, 0.1% human serum albumin, and 145 mM NaCl. A redox probe solution was prepared in a phosphate buffer (50 mM, pH 7.0) that contained 0.1 M KCl, 5 mM $Fe(CN)_6^{4-}$, and 5 mM $Fe(CN)_6^{3-}$.

Apparatus

Electrochemical experiments were carried out by using a Gamry potentiostat/galvanostat (Reference 600, Gamry Instruments, Warminster, PA, USA) interfaced with a PC via an EChem Analyst containing physical electrochemistry, pulse voltammetry, and EIS software (Gamry Instruments). A sheet of ITO substrate (2×15 mm) was used as a working electrode, and a silver/silver chloride reference electrode and platinum wire counter electrode were obtained from BASi (West Lafayette, IN, USA).

Fabrication of ITO-based impedimetric immunosensors

The first step was cleaning of the ITO substrates. The ITO substrates were cleaned using the following procedure. After sonication in acetone, soap solution, and ultra-pure water for 10 min each, they were dried under a stream of argon. After that, the clean ITO substrates were immersed in ultra-pure water containing hydrogen peroxide (1:7, v/v) and ammonium hydroxide (1:7, v/v) for 1 h to form hydroxyl groups on the surface of the ITO substrates in a dark and cool atmosphere. Then, the substrates were washed with ultra-pure water and dried with argon gas gently. During the next step, the ITO surface was modified with 3-aminopropyltriethoxysilane (APTES) to introduce amino terminals for covalent attachment of anti-HER-3 antibody. For this purpose, the ITO substrate was dipped into the APTES solution (1%) overnight. For covalent interaction between anti-HER-3 and ITO modified with APTES, a crosslinking agent, glutaraldehyde (0.1%), was used next. Afterward, an ITO sheet modified with anti-HER-3 was immersed in ultra-pure water to remove physically adsorbed anti-HER-3 molecules. Finally, the ITO substrate was immersed in the solution of BSA (0.1%) to block active ends. The bare (cleaned) and modified ITO substrates were denoted as ITO, ITO/APTES, ITO/APTES/anti-HER-3, ITO/APTES/anti-HER-3/BSA, and ITO/APTES/anti-HER-3/BSA/HER-3.

Experimental measurements

Cyclic voltammetry (CV) was used to characterize the steps of electrode modification and immobilization. The applied potential was varied between −500 and 500 mV (step size: 20 mV; scan rate: 50 mV/s) in the presence of a 5-M $[Fe(CN)_6^{4-}]/[Fe(CN)_6^{3-}]$ (1:1) solution that served as a redox probe containing 0.1 M KCl. For electrochemical impedance studies, an alternating wave of 10-mV amplitude was applied to the electrode over the formal potential of the redox couple (0 V). The redox couple used for the impedance studies was the same as that used for CV. Impedance spectra were collected in the frequency range between 10,000 and 0.05 Hz.

SEM and AFM studies

Structural observations of a surface modified by immobilization and HER-3 binding were performed by a field emission scanning electron microscope (FEI-Quanta FEG 250 model at 10,000-fold magnification) at the Scientific and Technological Research Center of Namık Kemal University (NABİLTEM). An acceleration voltage of 5 kV was used to acquire SEM images.

Atomic force microscopy (AFM) studies were performed by using an AFM Plus model microscope (NanoMagnetics Instruments, Turkey). Measurements were carried out by using silicon cantilevers (PPPNCLR-50 [PointProbe Plus non-contact], Nanosensors, Switzerland). Topographic images were taken in tapping mode in air and collected at a scan rate of 20 nm/s with a scan size of 40×40 and 2×2 μm.

Results and discussion

Immobilization of anti-HER-3 onto ITO sheets

The first and perhaps most important step was cleaning of the ITO surface because the further modification of ITO would definitely not have been realized without this cleaning procedure. The cleaning step mentioned above removed organic contaminants from the ITO surface, and removing these contaminants also increased the conductivity. Nevertheless, it was possible to continue immobilization after the cleaning step. All of these steps were characterized by the help of EIS and CV. A well-defined characteristic voltammogram of the redox couple, $Fe(CN)_6^{3-/4-}$, was observed on the ITO modified with hydroxyl groups. Fig. 1A shows the cyclic voltammograms obtained for the immobilization steps of anti-HER-3.

As is shown in Fig. 1A, the characteristic cyclic voltammograms of the redox couple $Fe(CN)_6^{3-/4-}$, which exhibits a nearly reversible electrode reaction without any complications of proceeding or post-chemical reactions, were not observed for the unmodified ITO surface because of its high electrical resistivity. After cleaning of the surface by the procedure mentioned above, the peak currents of the redox probe did not change considerably. However, the formation of hydroxyl groups on the ITO surface resulted in an obvious increase in anodic and cathodic currents compared with the previous ITO surfaces. This effect was probably caused by increasing the electron transfer rate of the redox probe. The ITO surface modified with hydroxyl groups was then activated with APTES, leaving a primary amine group on the surface. Most likely, these amino ends electrostatically actuated the redox probe toward the working electrode, and consequently the peak currents increased considerably. After covalent attachment of anti-HER-3, both the cathodic and anodic peak currents were decreased dramatically due to the hindering effect of anti-HER-3 on the electron transfer rate. During this process, glutaraldehyde was subsequently used to react with the amine group, yielding an aldehyde that could form an imine linkage with the primary amine group on anti-HER-3. During the last step, the addition of BSA that blocked free active ends also caused a further decrease in peak