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Electrochemical Sensors for Detections of Influenza Viruses: Fundamentals and Applications

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Abstract

Avian influenza viruses (AIVs) could cause severe diseases and, as the consequence, serious economic losses as well as a risk for potential transmission to humans. Therefore, the detection of viruses and their fragments or specific DNA sequences becomes an important approach in molecular diagnosis. Here, we present electrochemical genosensors devoted for detection of influenza virus H5N1 gene sequence. We focus our attention on ion-channel mechanism, E-DNA sensors, and genosensors based on redox-active layer. The novel dual DNA electrochemical sensor with “signal-off” and “signal-on” architecture for simultaneous detection of two different sequences of DNA derived from avian influenza virus type H5N1 by means of one electrode is presented. Immunosensors are also adequate analytical devices for detection of pathogens since antibodies are natural receptors responsible for binding of antigens. Thus, the binding selectivity and efficiency are naturally high. The immunosensors presented could be divided into two main groups: ion-channel mimetic and based on redox-active monolayer.

Keywords: Gold electrodes, Carbon electrodes, Electrochemical immunosensors, Genosensors, Influenza virus detection

1. Introduction

Infectious viral disease, which is spread among birds, in particular avian influenza (AI), could affect other animals, as well as humans [1].

The contact with infected live or dead poultry is the main source of risk of people’s AI infection [2].

Controlling the AI in animals is the first step in decreasing risks to humans. Therefore, there is a high need for the development of the analytical methods allowing the fast and reliable AI virus detection.

The real-time polymerase chain reaction (PCR) [3], enzyme-linked immunoassay (ELISA) [4], and reverse transcription-polymerase chain reaction (RT-PCR) [5] are the most frequently used methods. Their main drawbacks are being time-consuming and demanding high-quality laboratories.

The biosensors are very good alternative. They are self-contained integrated analytical instruments which are capable of providing specific quantitative or semiquantitative analytical information applying a biological recognition element, which is indirect spatial contact with a transducer element. Main parameters describing the quality of biosensors are selectivity, sensitivity, reproducibility, and time of response.

Electrochemical biosensors belong to a subclass of biosensors, which contain an electrochemical transducer responsible for converting of energetic signal coming from an intermolecular recognition process into electrical on. Their main advantages are as follows: (1) the direct conversion of a biological event to an electronic signal, (2) excellent detection limits, (3) small analyte volumes in μl range, (4) ability to be used in turbid biofluids, (5) suitability for rapid measurements of analytes from human and animal samples, and (6) easy miniaturization.

The complete electrochemical biosensor should be cheap, small, portable, and capable of being used by semiskilled operators. In order to achieve this final goal, we have been working on several types of geno- and immunosensors.

In the ion-channel mimetic immunosensors and genosensors, the presence of redox marker in the sample solution is necessary. The antigen-antibody complex formation as well as hybridization process suppresses the accessibility of redox marker toward the electrode surface. This phenomenon, which is the base of analytical signal generation by ion-channel mimetic mode, was observed using the Osteryoung square-wave voltammetry (OSWV) or electrochemical impedance spectroscopy (EIS) in the presence of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as an electroactive marker [6–14].

In the genosensors and immunosensors based on redox-active monolayer, the strategy for immobilization of the specific recognition elements involved their interactions with transition metal centers complexed on the electrode surface [15–19]. So, the presence of electroactive markers in the sample solution is not necessary. This is very important for analytical procedure involving naturally occurring molecules in which properties might be influenced by redox markers.

2. Electrochemical genosensors

In general, electrochemical genosensors monitor the DNA duplex formation at the surface of electrode through changes of current or potential values either using electrochemical labels or label-free system [20–25].

2.1. Methods for immobilization of recognition of ssDNA probe

The immobilization of single-stranded DNA (ssDNA) probe at the surface of electrode plays a crucial role for future genosensor analytical parameters. Various electrode materials such as gold, glassy carbon, carbon nanotubes, and graphene-modified electrodes have been applied for ssDNA immobilization. The physical adsorption, the simplest immobilization method, relies on the electrostatic interactions between ssDNA and surface of electrode. But such sensing layers are not stable. In addition, ssDNA strands are not well ordered, and because of this, they are not sufficiently accessible for target molecules.

The alternative method for immobilization of oligonucleotides on the electrode surface is their entrapping into polymer film deposited on the surface. The layer prepared according to this procedure is much more stable in comparison to the previous one. The weak point of this approach is the difficulty to control the flexibility of ssDNA probes and, as a consequence, their availability for target DNA.

The next method of DNA immobilization exploits the natural affinity of avidin to biotin. This method allows to create stable sensing layers with controlled density of ssDNA probes.

The most popular protocols of electrode modification are based on the formation of covalent bonds between the functional group introduced into ssDNA strand and functional group located at the surface of electrode. Therefore, the regulation of ssDNA probe density, as well as their stable (covalent) immobilization and proper orientation, is relatively easy to achieve.

2.2. Methods of hybridization process detection: selected examples of different types of genosensors

Different approaches for detection of the probe-analyte hybridization processes have been applied in various genosensors. One of them is based on changes of electrochemical activity of nucleobases upon the hybridization events. This concept has been proposed by Palecek and coworkers [26]. Oxidation of adenine (A) and guanine (G) can be readily observed using carbon electrodes or hanging mercury drop electrodes (HMDEs), which are suitable for investigation of reduction of nucleic acids. Their main drawback is background current at the relatively high potentials required for direct oxidation of DNA. In the case of reduction, the serious limitation is the necessity to use mercury electrode.

Another approach for voltammetric signal generation was presented by Umezawa and coworkers [6]. In their approach, the mechanism for generation of an analytical signal was connected with the binding event between target compound and recognition element immobilized at the electrode surface. Because of the creation of steric hindrance, as well as changing the surface charge, the accessibility of redox marker present in the sample solution toward the electrode is changed. Thus, creation of analyte-receptor supramolecular complex affected the electron transfer from marker to surface of electrode. The heterogeneous rate constant of electron transfer from marker to electrode surface became large or smaller; therefore, the redox current increased or decreased. The electrochemical sensors based on this mechanism are called ion-channel mimetic sensors.

Recently, in our laboratory, we have developed two genosensors working according to this mechanism based on the modified gold electrode intended for the detection of specific DNA sequence of avian influenza virus (AIV) H5N1 using NH_2 -ssDNA or HS-ssDNA probes for the modification of gold electrode [11, 12].

NH_2 -ssDNA probes were immobilized on the surface of mixed thioacid-thioalcohol monolayer via EDC/NHS coupling, whereas HS-ssDNA probes were immobilized directly to the gold surface via S-Au bonds. As marker ions in both cases, $[\text{Fe}(\text{CN})_6]^{3-/4-}$ was employed. When SH-NC3 probe was used, detection limit in the 10 pM range was achieved. The much lower detection limit in fM range was recorded when NH_2 -NC3 probe was applied. These data confirmed that the application of DNA probe with longer spacer part increased the hybridization signal. The better accessibility of target ssDNA toward more flexible DNA probe is the main reason of this phenomenon. But at the same time, the lower genosensor selectivity was observed. When SH-NC3 DNA probe with shorter spacer was applied, the sensor was able to distinguish between the PCR products with different positions of complementary parts, whereas the electrode modified with longer spacer molecules was not able to do this [11, 12].

Plaxo and coworkers [25] introduced another type of hybridization detection technique that exploits the difference in physical flexibility between single-stranded oligonucleotides and double-stranded ones. Upon hybridization, the physical changes in the probe structures, including the change in distances of the labeled electroactive moieties to the electrode surface, result in the switching "on/off" of the electrochemical signal.

The main advantages of E-DNA sensors are the conformational changes caused by hybridization, potential activation of redox label in the "safe" range separated from potential activation of majority of electroactive biomolecules present in the clinical and environmental samples, and detection limits in the picomoles of target DNA range.

The genosensors working on described mechanism belong to very wide "signal-off" mode family. It is worth to note that detection limit of E-DNA working according to "signal-off" mechanism is in the range of 10 pM. In case of genosensors that generated analytical signal according to "signal-on" mechanism, detection limit is around 200 pM [25].

The "signal-off" sensors have numerous advantages such as sequence specificity, reusability, and suitability for direct measurement in serum. But the suppression of signal generated after target binding is their main disadvantage, because only 100 % suppression of the original current could be detected. On the other hand, the "signal-on" sensors have a potential for great improvement of sensitivity, because the stimulation of these types of sensors with the target does not cause a limited increase of signal. A weak point of these types of E-DNA sensors is the use of rather complicated, but not very stable architectures. This approach does not work properly in the complex samples.

Today, more and more often, biosensor is required to be not only miniaturized and cost-effective but also capable of simultaneous detection of multiple analytes.

Recently, a novel dual E-DNA sensor working on "signal-off" and "signal-on" mode has been developed [20]. This sensor was suitable for simultaneous detection of two different oligonu-

cleotide sequences present in avian influenza virus (AIV) type H5N1 with using one electrode. The ssDNA probe represented by hemagglutinin was functionalized with ferrocene (ssDNA-Fc). The functionalization with methylene blue (ssDNA-MB) was applied for sequence derived from neuraminidase. Both of them were covalently immobilized on the gold electrode surface. Hybridization process going at the electrode surface was controlled by the Osteryoung square-wave voltammetry. Detection limits determined by graphic method were 4.0×10^{-8} and 2.0×10^{-8} M, for simultaneous analysis of both sequences and for single one, respectively. These values, in particular the detection limits for parallel determinations of two sequences, are very promising from diagnosing point of view. The selectivity of duo-genosensor was similar for both targets. The limits of detection were in the range of 18–21 nM. The duo-genosensor was free from interferences. The presence of oligonucleotide sequences complementary to SH-ssDNA-Fc probe does not influence the function of the probe decorated with methylene blue and vice versa. The main advantage of duo-sensor is diminishing false-positive determinations which may appear in the case of non-perfect hybridization with component(s) present in non-infected host samples. The probability for existence in native samples of components efficiently interacting with two independent DNA probes is limited.

Recently, in our laboratory, we have developed the new group of electrochemical DNA sensors in which the analytical signal generation is based on the changes of accessibility of redox center attached directly to the analytical active layer or at the “foot” of the oligonucleotide probe, very close to the electrode surface [16, 17, 20–22]. Such localization exclude the changes of distance of redox marker from the electrode surface after hybridization. As a redox center, we have used the complexes of transition metals with dipyrromethene, porphyrin, and phenanthroline.

A novel mechanism of electrochemical signal generation based on changes of the ion-barrier energy “switch-off” system has been proposed. According to this mechanism, the proposed sensors generate an analytical signal because of changes in the environment surrounding the redox center occurring as a result of hybridization processes.

For the sensor based on the Cu(II) complex, during the redox cycle, Cu(II) is reduced to Cu(I). As a consequence, a single negative charge of the reduced form appears at the surface of the electrode. The precondition of redox reaction run is the compensation of this charge by ions from the supporting electrolyte, involving transport of cations. For the electrode modified with the Co(II) complex, an oxidation process is possible, which generates an extra positive charge of the oxidized form. For its neutralization, anions from the supporting electrolyte will be involved. The mechanism of analytical signal generation by this type of sensors relies on changes in accessibility of ions present in the supporting solution to the redox centers in order to neutralize the charge occurring as a result of oxidation/reduction processes. The good illustration of such sensors is genosensor intended for detection of the sequence specific of avian influenza virus type H5N1. A 20 mer probe (NH₂-NC3) was covalently attached to the gold electrode surface.

The detection limit of 1.39 pM for fully complementary single-stranded DNA target was achieved with genosensor based on (dipyrromethene)₂Cu(II) complex. A linear dynamic range was observed from 1 to 10 pM. The good discrimination between fully complementary,

partially complementary (with only six complementary bases), and totally non-complementary to the probe was also recorded [16].

The Fe(III)-phenanthroline complex is the base of genosensor suitable for detection of target DNA as well as RNA. An efficient click reaction, carried out under mild conditions, between the $\text{NH}_2\text{-NC}_3$ probe and the epoxy groups from Fe(III)-phenanthroline complex deposited on the gold electrode surface, has been successfully applied for the genosensor construction. The good detection limits of 73 and 0.87 pM for the 20 mer c-NC3 and the 283 mer RNA1, respectively, were achieved. The sensitivity of RNA sequence detection was about one hundred times better than the DNA sequence detection. The 20 mer nc-NC3 non-complementary to the probe generated a weak response. In the case of non-complementary to the probe 277 mer RNA3 fragment, even the opposite signal, an increase of the Fe(III)/Fe(II) peak currents was observed. These data confirmed the genosensor selectivity. The main advantage of genosensor based on Fe(III)-phenanthroline complex is its suitability for determination of RNA and distinguishing of the different positions of the complementary parts. Thus, it could be applied for the detection of the H5N1 genetic material [17].

3. Electrochemical immunosensors

3.1. Methods for immobilization of recognition element

The right immobilization of the proteins as the recognition elements on the transducer surface (e.g., gold, platinum, indium tin oxide, carbon materials) is a crucial factor to fully maintain their right conformation and activity.

Physical protein immobilization is based mainly on electrostatic forces and hydrophobic interactions. The entrapping of proteins into the polymer matrix, iridium oxide films [27], and nanotextured zinc oxide [28] belongs to this strategy.

The colloidal gold layers create friendly environment for proteins. Therefore, they are frequently used in immunosensor creation [7–10, 29–31].

In this approach, proteins are mainly immobilized on colloidal gold layers based on the electrostatic interactions, which could be enhanced by selecting the proper pH conditions. The drawbacks of this method are random orientation and rather weak attachment. In order to overcome these weak points, covalent antibody immobilization has been applied. Two polypeptide chains F(ab')_2 present in immunoglobulin molecule are responsible for antigen binding. Fc domain, which is not involved in this interaction, could be removed by enzyme digestions [32]. The presence of disulfide or thiol group allow the F(ab')_2 or F(ab') fragments to covalent self-assembling on the gold nanoparticle surface [33, 34]. This approach has been successfully applied for development of immunosensor destined for selective binding of antigen rSPI2-His₆ present in the sample solution by F(ab') fragment of antibody immobilized on a surface of the electrode using electrochemical impedance spectroscopy (EIS) and surface plasmon resonance (SPR) [30] as well as for selective detection of hemagglutinin from avian influenza virus H5N1 [9, 10]. The proteins A and G possessing high affinity toward the Fc part

of immunoglobulins are frequently used for their covalent and oriented immobilization on the electrode surface [35]. The immunosensor based on glassy carbon electrode incorporating the protein A has been successfully applied to distinguish between sera of unvaccinated and vaccinated chickens against the avian influenza virus. Sensitivity of EIS immunosensor was almost 10^4 times much better than ELISA [8].

3.2. Methods of detection of immunoreaction: selected examples of different types of immunosensors

The majority of electrochemical immunosensors incorporate not only recognition element (antibody or antigen) but also secondary enzyme-labeled antibody, which follows the addition of proper enzymatic substrate. The antibody-antigen reaction is detected in no-direct manner but by electroactive molecules produced by enzymatic reaction [36, 37]. Such approach demands very precious labeled biological materials, which increase the cost of analysis. Also, it involved numerous modification steps. The no-direct detection of immunoreaction is the main drawback of sandwich type of immunoassays.

In order to overcome the above weak points, the immunosensors allowing the direct electrochemical detection of immunoreactions have been developed in our laboratories.

The whole antibodies or antibody-binding fragments (Fab') have been immobilized on the surface of gold nanoparticle layers, which create friendly environment for proteins keeping their physiological activity. The formation of immunoreactions has been detected by the changes of accessibility of redox marker present in the sample solution to the electrode surface. The deposition of antibody-antigen complex on the electrode surface increases substantially the resistance of the analytical system, and as a consequence, faradaic current is very difficult to measure by voltammetric techniques. Therefore, electrochemical impedance spectroscopy (EIS) has been widely applied. EIS is an ac method that describes the response of an electrochemical cell to a small amplitude, sinusoidal voltage signal, and a function of frequency. The most popular mode for analyzing electrochemical impedance data is the plot of the imaginary impedance component versus real impedance component at each excitation frequency (the Nyquist plot). Numerous impedimetric immunosensors have been already reported [7, 30, 31, 38]. This approach has been successfully applied for detection of fragments of H5 hemagglutinin from avian influenza viruses [9, 10]. The antibody-binding fragments have been covalently attached to the gold nanoparticle layers. Application of 4,4'-thiobisbenzenethiol self-assembled monolayer [10] improved substantially immunosensor performance in the comparison to one incorporated 1,6-hexanedithiol [9]. Taking into account the immunosensor sensitivity with detection limit of 0.6 pg/mL and specificity (negative control H7 hemagglutinin generate negligible response), they could be recommended for direct electrochemical detection of H5 hemagglutinin from influenza virus in the field conditions.

Direct impedimetric immunosensor has been successfully applied for detection of antibodies generated against H5N1 virus [8]. The base of this immunosensor was glassy carbon electrode incorporated fragment of H5 hemagglutinin. The interaction with specific antibodies was detected electrochemically by changes of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ accessibility toward electrode surface.

The immunosensor was able to distinguish the sera from vaccinated and unvaccinated hen with sensitivity 10^4 better than ELISA.

In order to avoid the necessity of the redox marker present in the sample solution, the immunosensor-incorporated redox-active layer has been developed [39].

The Cu(II) complex with dipyrromethene deposited onto the gold electrode surface plays double roles. It is a site for covalent immobilization of the His-tagged fragment of H5 hemagglutinin, as well as redox centers for sensing the antigen-antibody interaction.

This type of immunosensor was also suitable for direct antibody detection in hen sera with 200 better sensitivity than ELISA.

4. Future perspective

The analytical chemists mainly search for improving sensitivity and selectivity of sensing devices. The intensive development of nanotechnology gives wide possibility of using nanomaterial labels for signal amplification generated upon immunoreaction [18, 19, 40–43]. Nanomaterials could be used for modification of electrochemical transducers in order to improve their electrochemical properties by lowering background current and signal to noise ratio, as well as increasing electron transfer rate.

The most frequently used nanomaterials are colloidal gold and silver, semiconductor quantum dots, carbon nanotubes, and graphene.

They are very promising in the development of ultrasensitive immunosensors. Could they be applied in point-of-care and clinical diagnoses? Taking into account so intensive effort done in this research area, the answer for this question is “yes.”

5. Conclusions

The main advantage of ion-channel mimetic sensors is the possibility of their application for exploring the recognition processes occurring at the water/solid interface. It is very important from biological as well as medical point of view. The electrochemical sensors based on redox-active layer are new direction in sensing device development. Their main advantage is the lack of the necessity of using the external redox marker. The redox centers can simultaneously act as sites for host molecule immobilization and transducers.

Taking into account the following parameters of electrochemical biosensors presented such as very good sensitivity, very low sample consumption (in μl level), lack of matrix influence, simple operation, and reasonable cost, it might be concluded that they are analytical tools suitable for detection of viruses in the environmental sample.

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