Label-free Electrochemical Immunosensors for Viruses and Antibodies Detection-Review

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Abstract. This article reviews the electrochemical immunosensors developed by the successive modification of gold as well as glassy carbon electrodes. Antibody or antigen fragments have been applied as the sensing elements. The complex between virions and specific antibody adsorbing on a surface of an electrode forms an insulating layer. This phenomenon, which is a base of ion – channel mimetic type of immunosensors, can be monitored by Osteryoung square-wave voltammetry (OSWV) and electrochemical impedance spectroscopy (EIS) in the presence of $[Fe(CN)_6]^{3-/4-}$ as a redox marker. Another type of immunosensors are based on redox active layers incorporated di-pyrromethene -Cu(II) or phenanthroline – Epoxy - Fe(III) complexes. Changes of electrochemical parameters of redox centres upon target analyte binding are the basis of analytical signal generation. Both type of immunosensors displayed better sensitivity in comparison to ELISA as well as being very selective. The matrix has no influence on the immunosensors performance. These devices could be recommended for the direct electrochemical detection of viruses as well as antibodies in physiological samples.
Key words: electrochemical immunosensors; electrode modification; redox active monolayers; viruses detection; antibodies detection; natural matrixes.

Resumen. Este artículo presenta una revisión acerca de inmunosensores electroquímicos desarrollados mediante la modificación sucesiva de oro así como de electrodos de carbón vítreo. Como elementos sensores, se incorporaron fragmentos de antígenos o anticuerpos a las superficies. El complejo formado entre los viriones y los anticuerpos específicamente adsorbidos sobre el electrodo genera una capa aislante. Este fenómeno, el cual es fundamental para la operación de inmunosensores miméticos de canales iónicos, puede ser monitoreado por voltamperometría de onda cuadrada de Osteryoung y por espectroscopía de impedancia electroquímica en presencia de $[Fe(CN)_6]^{3-/4-}$ como marcador redox. Otro tipo de inmunosensores presentados está basado en capas electroactivas incorporadas como complejos de di-pirrometheno-Cu(II) o fenantrolina-epoxi-Fe(III). Los cambios en los parámetros electroquímicos de los centros redox durante el enlace de los analitos objeto es la base de la generación de señales analíticas. Ambos tipos de inmunosensores mostraron mejor sensibilidad al compararlos con pruebas tipo ELISA así como una alta selectividad. No se observó influencia de la matriz en el desempeño de los inmunosensores. Dichos dispositivos pueden ser recomendados para la detección electroquímica de virus así como de anticuerpos en muestras fisiológicas.
Palabras clave: inmunosensores electroquímicas; modificación de electrodos; monocapas electroactivas; detección de virus; detección de anticuerpos; matrices naturales.

Introduction

In recent years, much attention in virology has been focused on developing of affordable and adequate methods for detection of whole viruses or their fragments. Immunosensors are a promising technique for detection of pathogens since antibodies are natural receptors meant for binding of antigens harmful for the organism. Thus the binding selectivity and efficiency are naturally high.

Conventional methods which are frequently used for detection of antibodies against influenza virus are enzyme-linked immunosorbent assays (ELISA), hemagglutination inhibition (HI) and Western blot assay (WB). Nevertheless, they are often laborious and time-consuming or need expensive instruments. Therefore, there is still significant need to create simple, sensitive, and low cost diagnostic methods for detection of antibodies against different type viruses. Immunosensors incorporating specific antigen are a promising alternative systems for the detection of antibodies. The low sample consumption, reasonable cost of instrumentations and good possibility for miniaturisation are the main reasons for extensive development of electrochemical immunosensors.

The most frequently used sandwich – type immunoassay involves a couple of match antibodies. Primary antibodies are usually immobilized on a solid support, and sandwiched immune-complexes are formed between the immobilized primary antibodies and labelled (signal) antibodies. The detectable signal mainly depends on labeled signal tags. Therefore, a great scientific effort is devoted for developing new nano-labels with improved signal amplification properties, such as noble nanoparticles, carbon nanomaterials, semiconductor

nanoparticles, metal oxides nanostructures, hybrid nanostructures, just to name a few examples [1-6].

Although sandwich – type immunoassays possess obvious advantages, they have also some weak points. First of all, their fabrication involves numerous steps, which make them labor and chemical demanding. In addition, the antigen concentration is not measured in a direct way, but rather based on the signal generated by either the label or the enzyme–label product. Therefore, there is a high need to develop label – free immunosensors suitable for the direct detection of antibody-antigen complex formation. We have been involved in this research direction and our recent achievements will be reviewed.

The immunosensors presented can be divided into two main groups: ion-channel mimetics and those based on redox active monolayers [2]. In the ion-channel mimetic immunosensors, the presence of redox marker in the sample solution is necessary. The antigen-antibody complex formation suppress the accessibility of redox marker towards the electrode surface. This phenomena, which is the basis of analytical signal generation by ion-channel mimetic mode was observed using Osteryoung square-wave voltammetry (OSWV) or electrochemical impedance spectroscopy in the presence of $[Fe(CN)_6]^{3-/4-}$ as an electroactive marker (Fig. 1).

In the immunosensors based on redox active monolayers, the strategy for immobilization of the specific recognition elements involved the interaction between transition metal centres forming complexes on the electrode surface and nitrogen atoms from the histidine–tag present in the protein molecule. In this type of immunosensors, the presence of electroactive markers in the sample solution is not necessary. This is very important for analytical procedures involving naturally occurring molecules, whose properties might be influenced by redox markers.

The antigen- antibody complex formation occurring at the electrode surface influence the accessibility of ions from supporting electrolyte towards the redox centres embedded into sensing layers. The consequences of this phenomenon are changes of redox centres properties, which is the basis of analytical signal generation and have been explored electrochemically (Fig. 2).

Several immunosensors destined for detection of *Plum pox virus* (PPV) [7,8], and for avian influenza virus [9-11] will be presented. The influence of transduction layers and the way of immobilization of sensing elements on the immunosensors performance will be discussed. Both type of immunosensors are suitable for detection of specific antiobodies as well as viruses or their fragments.

Immunosensors Based on ion-channel mimetic mode

It has been already reported that natural antibody activity can be retained on gold nanoparticles´ built surfaces. Deposition of antibody on the colloidal gold layer relies on ions' electrostatic attraction. Colloidal gold nanoparticles are formed in the presence of citrate salt; therefore, these nanoparticles are surrounded by citrate anions which are negatively charged. For efficient immobilization of antibodies molecules, a positive charge is necessary. This can be gained by providing a suitable pH condition. The specific pathogen-antibody reaction changes the redox marker accessibility to the electrode surface. This is the working principle of ion-channel mimetic electrochemical immunosensor (Fig. 1).
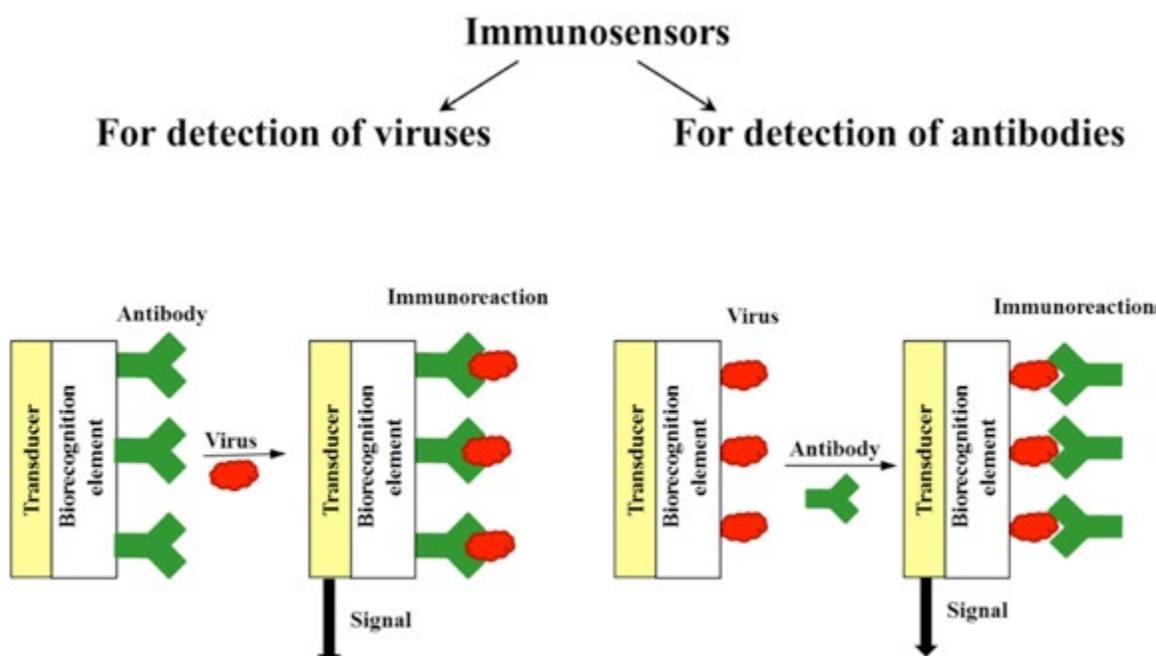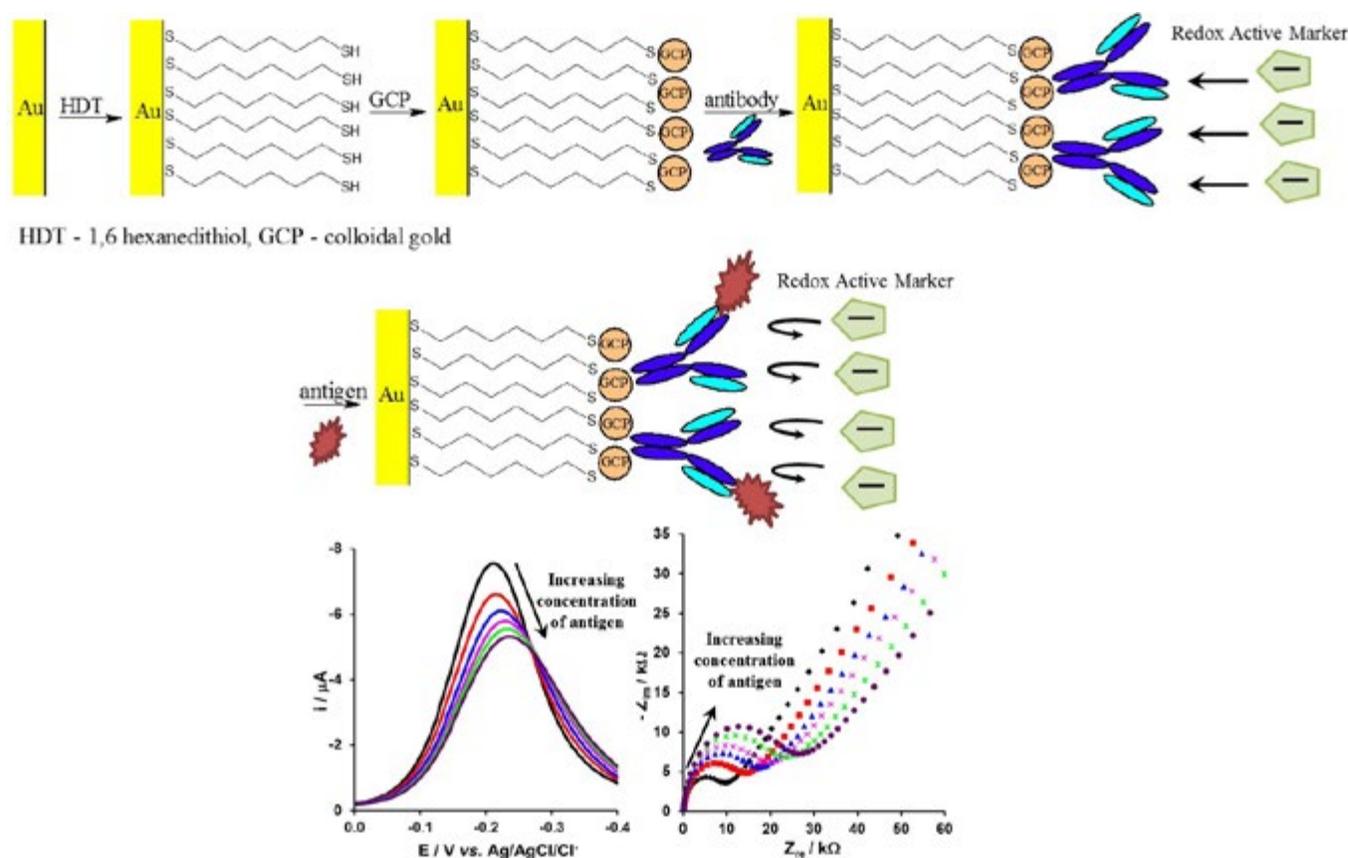

Fig. 1.

HDT - 1,6 hexanedithiol, GCP - colloidal gold

Fig. 1 (cont.). Working principle of immunosensor based on ion –channel mimetic mode and its responses towards antigen recorded with Osteryoung square-wave voltammetry (OSWV) and electrochemical impedance spectroscopy (EIS) in the presence of $[Fe(CN)_6]^{3-/4-}$ as a redox marker.

Our first successful application of gold nano-particles for antibody immobilization was done creating an immunosensor destined for detection of histidine-tagged proteins in culture medium [12]. Among two type of gold nanoparticles -colloidal and nanorods-, the second ones display better transduction properties, which generate better immunosensor parameters. The anti his-tag antibody, AH-IgG, exist as a positively charged species below its isoelectric point (pI = 8.6) in PBS buffer pH 7.4. This facilitates its immobilization via electrostatic interactions over the citrate capped gold nano-particles. It could be remarked that the performances of immunosensors were significantly better than immunoblotting technique, taking into account advantages such as simplicity, label free assay, low sample volume, a 5 pg/ml limit detection and suitability for *lab-on-chip* devices. The immunosensor was totally free form the matrix influence. Its analytical parameters recorded in buffer or in the *Pichia Pastoris* culture media were the same. Thus, it could be recommended for screening the culture media used in biotechnology.

Gold nanoparticle layers have been incorporated for the fabrication of immunosensors used for detection of *Plum pox virus* (PPV) in plant extracts [3]. This virus affected stone fruit trees, causing significant economic losses. Therefore, development of affordable methods for its quick dectection is very desirable. The immunosensor displayed very good analytical

parameters: very low detection limit (10 pg PPV/ml) and very good dynamic range from 10 pg PPV/ml to 200 pg/ml. The presence of matrix –healthy plum leaves extract– had no apparent influence on the immunosenor's response toward PPV virus. There is no difference between calibration curve for PPV obtained in the presence of PBS buffer and in the presence of healthy plum leaves extract. This makes the immunosensor proposed a promising device for fast, reliable and simple PPV detection.

Surface immobilization of antibodies is key parameter for immunosensor sensitivity. The most important aspect is to identify a surface chemistry that allows optimal orientation, but also appropriate density and accessibility of the antibody binding sites. Using direct physical adsorption via electrostatic forces of whole antibodies on the surface of gold nanoparticles as well as carbon nanotubes, which creates a suitable environment for keeping protein activity [2], does not allows a proper antibodies orientation on the electrode surfaces. To avoid this problem, covalent immobilization of the antibodies F(ab') fragments, self-assembled on the nano-gold surfaces through covalent bonds between Au and disulfide or thiol groups from the hinge region of immunoglobulin G, was applied. This strategy have been used in immunosensors destined for detection of His-tagged proteins in culture medium [13].

Immunosensor incorporating Anti-His IgG F(ab') fragment and gold nanorods for detection of his-tagged proteins is able to detect 10 pg/mL (0.13 pM) of antigen. The recognition process takes place on the surface, not in solution. Therefore, the required amount of F(ab') fragments is very small (6×10^{-11} mols of F(ab') per electrode). It also requires no labels, which is a distinctively advantage. These traits make it an easy to use and relatively cheap method of detecting antigens in complex medium. The limit of detection remains almost the same in culture medium as was observed in PBS buffer. Analytical response range for the immunosensor proposed is between 10 pg/mL (0.13 pM) and 1 ng/mL 13.1 pM) which is better in the comparison of other already reported. Immunosensor incorporating F(ab') fragments is more reusable than one incorporating whole antibody IgG.

A sensitive and selective impedimetric immunosensor for the detection of peptides derived from avian influenza hemagglutinin H5 has been developed using Fab' immobilized on a gold electrode surface via colloidal gold nanoparticles [10]. This device is able to recognize three different His-tagged fragments of HA: Qinghai, HA/Nde and Vietnam. The strongest response was observed for Qinghai, with a detection limit of 2.2 pg/mL and a dynamic range from 4.0 pg/mL to 20.0 pg/mL. This immunosensor proved that gold colloidal nanoparticles may be used for the creation of a very good underlayer of Fab' oriented immobilization. This allows fragments of antibodies to retain their activity and constitute a good electron conductive layer for electrochemical sensors. Considering its good selectivity and sensitivity in the pg/mL range, the proposed immunosensor was superior in comparison to others already reported, therefore, it could be recommended for the rapid, simple and direct electrochemical detection of avian influenza virus H5N1.

In the immunosensors described above, 1,6-hexanedithiol (HDT) Self Assembled Monolayer (SAM) has been used for covalent immobilization of gold nano-particles [3,4,6,8,9]. The main disadvantage of this approach is the very high resistance of HDT SAMs. In order to avoid this problem, 4,4'-thiobisbenzenethiol (TBBT) SAM was deposited on the gold surface [11]. This SAM possess superior parameters regarding reproducibility and stability, as well as a three times lower electron transfer resistance in the comparison to 1,6-hexanedithiol (HDT) SAM. The TBBT SAM was suitable for covalent deposition of colloidal gold nanoparticles, which function as excellent environment for scFv immobilization. Such modification was the basis for building sensitive and selective electrochemical immunosensor for the detection of peptides hemagglutinin from avian influenza viruses. This device is capable to recognize two His-tagged variants of H5. The strongest response was observed for the longer variant ("Qinghai") with a detection limit of 0.6 pg/mL and dynamic range from 4.0 pg/mL to 20.0 pg/mL. A negative control (H7 hemagglutinin), generated only a weak response. The miniaturized system was able to detect "Qinghai" with a detection limit of 0.9 pg/mL. Therefore, this could be the method of choice for the rapid, simple and direct electrochemical detection of H5 hemagglutinin from influenza virus in the field conditions [11].

Another approach suitable for oriented and stable antibody immobilization relies on protein A. This protein is a cell wall protein consisted of single polypeptide chain produced by most strains of *Staphylococcus aureus* [14] and is extremely suitable for a direct antibody immobilization because of its natural affinity towards the Fc region of immunoglobulin, such as IgG. The Fab binding sites of IgG antibody are thus oriented away from the solid phase [15]. We have fabricated two immunosensors with protein A of *Staphylococcus aureus* to modify the glassy carbon electrode surface followed by immobilization of specific antibody. One of them was destined for Prunus necrotic ringspot virus (PNRSV) in plant extracts [8]. The immunosensor was capable of discriminating between samples from healthy plants and samples containing 0.01% of extract from infected plant material. The presence of constant concentration (50 pg/ml) of PPV influences a little on PRNSV impedimetric responses. The proposed immunosensor was effective regarding the following parameters: good sensitivity towards PRNSV, good selectivity and very small volume (10 μl) of analyzed sample.

Another example of an immunosensor incorporating protein A was employed for detection of hemagglutinin antibodies present in hen sera [9]. Its preparation consists of successive modification steps of glassy carbon electrodes: (i) creation of COOH groups, (ii) covalent immobilization of protein A with EDC/NHS coupling reaction, (iii) covering with anti-His IgG monoclonal antibody, (iv) immobilization of the recombinant His-tagged hemagglutinin (His_6-H5 HA), (v) filling free space with BSA. The interactions between two variants of recombinant HA (short and long) from highly pathogenic avian influenza virus H5N1 and the anti-H5 HA monoclonal antibody (Mab 6-9-1) have been explored with electrochemical impedance spectroscopy (EIS). The impedimetric immunosensor displayed a very good detection limit (LOD) of 2.1 pg/mL, a quantification limit (LOQ) of 6.3 pg/mL and a dynamic range from 4 pg/mL to 20 pg/mL. In addition, this analytical device was applied for detection of antibodies against His_6-H5 HA in serum of vaccinated hen using serial 10-fold dilutions of serum. The immunosensor was able to detect antibody in hen serum diluted up to 7×10^7-fold. Its sensitivity was about four orders of magnitude better than ELISA. The presented system is able to safely distinguish between sera of non-vaccinated and vaccinated chickens against the avian influenza virus. Similar sensors, for example those equipped with a variant of AIV antigen absent in the applied vaccine, could be also used to differentiate vaccinated individuals from the infected ones. Therefore, it could be very effective in detection of antibodies for immune surveillance and monitoring the efficiency of poultry of vaccination programs.

As concluding remarks, it could be stated that immunosensors belong to ion-channel mimetic mode are superior in the comparison to widely used immunoassay approaches. First of all their fabrication procedure is simple, with very low chemical and analysed sample consumption. The need of application of redox active markers in the sample solution could be in some cases a disadvantage. The solution of this problem will be presented in the next chapter.

Immunosensors based on redox-active monolayers

Self-assembled monolayers (SAMs) obtained based on the co-valent interactions of thiols, disulfides, sulfides, and other related molecules with the surfaces of noble metals, particularly gold, as well as platinum and mercury [16,17] are excellent ways for creation of numerous functional surfaces, which are widely applied in biosensing approaches. Despite of the promising properties of SAMs, they also have some weak points. The most important is the presence of pinholes and defects, which affect the faradaic response of blocking monolayers. In consequence, the ion channel type sensors based on such surfaces could loss their sensitivity and selectivity. Attaching redox centers to SAMs is a possible solution of the above described problem. Such prepared surface could play a double role, both as an analytically active element of sensor as well as transducer. They display many advantages in comparison to monolayers without redox centres. The main one is the close packing prevents motion of the redox centres toward the electrode, toward a pinhole or a defect. Therefore, the faradaic current due to redox reaction at (or near) pinholes and defects becomes a negligible component of the total faradaic current. The electroactive centers are combined with the electrode surface, therefore the diffusion does not have any influence on cyclic voltammetric responses. Thus, the double-layer correction for the surface concentration versus the bulk concentration is not necessary.

On the other hand, the new double-layer effect becomes important due to the changing oxidation state of the surface confined redox centres. The voltammetric waves frequently deviate from the ideal behavior, depending on such factors as the dielectric constants of film and solution, the concentration of electroactive species, supporting electrolyte composition and the film thickness [17,18]. Distortions caused by double-layer effects are less significant when the distances between redox active units are greater and when the electrolyte concentration is high. Thus, the formation of mixed SAMs with a relatively low concentration of redox sites and working in concentrated electrolyte is highly recommended.

We have developed a method for creation of electroactive monolayers based on the two step complexation reaction of Cu(II) performing on the surface of a gold substrate, previously modified with dipyrromethene (DPM) or pentetic acid (DPTA) derivative [19-21]. Again, redox centres play a double role: they are sites for recognition elements immobilization, as well as for analytical signal transduction. These monolayers were suitable for stable immobilization of his-tagged proteins via histidine coordination with Cu(II) sites. Such approach have been applied for development of several biosensors destined for screening of interactions between kinase and potential inhibitors [21, 22], domains of Receptor for Advanced Glycation end Products (RAGE) and its ligands (Aβ peptides and protein S100B) [23-26]. The binding affinity of His-tagged protein to the dipyrromethene-Cu(II) complex has been confirmed using surface plasmon resonance (SPR) [27]. The DPM-Cu(II) layer incorporated with His_6-protein was stable during several hours. Also, we did not observe any changes of electrochemical parameters recorded in the buffer solution before starting of analyte measurements.

Very recently, DPM-Cu(II) redox active monolayer has been applied for electrochemical immunosensor destined for detection of antibodies against avian influenza virus H5N1 [28]. This immunosensor incorporated His-tagged hemagglutinin (His_6-H5 HA) allowed for precise detection of antibodies against hemagglutinin (HA) from the HPAI virus H5N1. A specific interaction between the His_6-H5 HA and the appropriate monoclonal antibody was observed using Osteryoung square-wave voltammetry (OSWV). The device proposed showed the low LOD and LOQ equal to 2.4 pg/mL and 7.2 pg/mL in buffer, respectively. The monoclonal anti-IL-2 antibodies, used as a negative control (unspecific to the His_6-H5 HA), generated weak response. This system detected specific antibodies in a selective way. In addition, this biosensor was able to detect a humoral response in the sera of hens immunized with DNA vaccine based on the sequence of HA from the H5N1. The limit of antibodies detection in serum from vaccinated hen was $1 : 7 \times 10^6$ dilution range. So, immunosensor sensitivity is about 200 times better than ELISA. The high sensitivity and
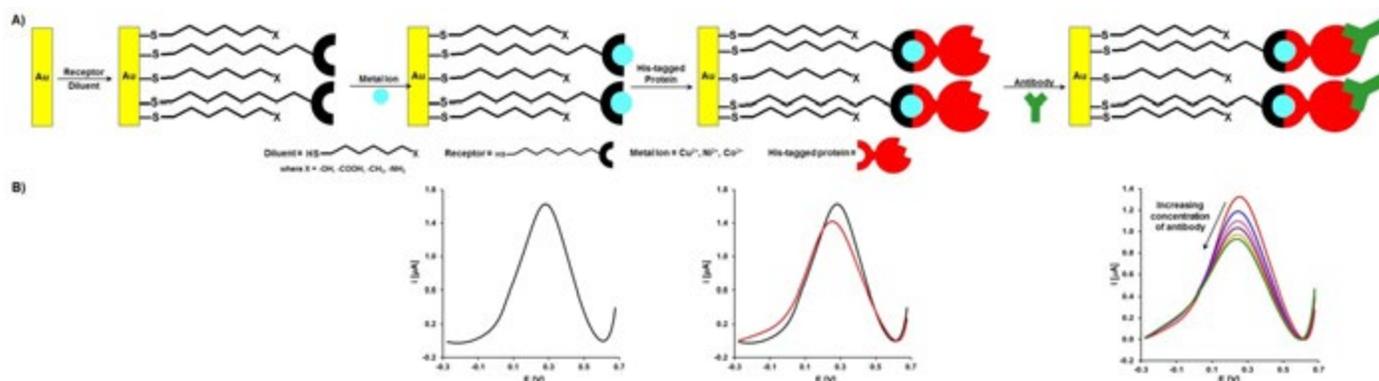

Fig. 2. (A) Scheme of fabrication of immunosensor based on redox active monolayer for antibodies detection; (B) Electrochemical responses of immunosensor for increasing concentration of analyte recorded by Osteryoung square-wave voltammetry (OSWV).

selectivity of the system presented allowed distinguishing hens vaccinated from non-vaccinated ones against AI virus. Consequently, it could be very effective in detection of antibodies for immune surveillance and monitoring of the efficiency of poultry vaccination programs.

The main advantages of electrochemical biosensors based on redox active monolayers are the following: (i) no need of using redox marker in the sample solution, (ii) lack of influence of pinholes in the monolayers carring the receptors on sensors performance, (iii) redox active centers can simultaneously act as host as well as a transducer of electrochemical signals generated upon analyte recognition. The incorporation of gold as well as carbon nanoparticles into fabrication of sensors and biosensors based on redox active monolayers might be very beneficial. Their presence facilitates the electron transfer from redox centers to the electrode surface and improves sensors performance [2]. There is still much room for scientific research and application of nanostructures. This strategy is currently developing also in our laboratories. Transition metals complexes with porphyrines, dipyrromethenes, terpyridines deposited onto gold nanoparticles as well as carbon nanostructures such as single-walled carbon nanorods and graphene, will be applied for proper receptor proteins and ssDNA probe immobilization. The biosensors prepared in such way will be tested for sensing of target analytes important for medical diagnosis as well as for environmental monitoring.

Conclusions

The two main types of electrochemical sensors: based on ion-channel mimetic mode and based on redox active layers were presented. The main advantage of ion-channel mimetic sensors, besides from their high sensitivity and selectivity, is the possibility for application in the investigations of recognition processes occurring at the water/solid interface. This is very important from the biological as well as the medical point of view. The main disadvantage of this type of sensors, particularly from an analytical point of view, is the need of using the redox markers in solution, which may have toxic effects on biomolecules tested.

The electrochemical sensors based on redox active layer are relatively a new direction in sensing devices development. Their main advantage is the lack of the necessity of using the external redox marker. The application of redox centres in such sensor allows very interesting possibilities. They can simultaneously act as host molecules and as well as transducers.

Taking into account the following parameters of immunosensors presented such as: very good sensitivity (improvement in several orders of magnitude than ELISA), very low sample consumption (in the μL Level), lack of matrix influence, simple operation and reasonable cost, it might be concluded that they are analytical tools suitable for medical diagnosis and environment and food quality control.

Acknowledgements

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