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Review

Electrochemical biosensors for detection of avian influenza virus — current status and future trends

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Electrochemical biosensors have emerged as reliable analytical devices suitable for pathogen detection. Low cost, small sample requirement and possibility of miniaturization justifies their increasing development. Thus, we report in this review on the state of the art of avian influenza virus detection with genosensors and immunosensors working by an electrochemical mode. Their working principles focusing on the physical properties of the transducer, the immobilization chemistry, as well as new trends including incorporation of nanoparticles will be presented. Then, we critically review the detection of avian influenza virus in the complex matrices that use electrochemical biosensors and compare them with traditionally applied methods such as ELISA or Western blot.

Key words: electrochemical biosensors, genosensors, immunosensors, electrochemical detection, avian influenza virus

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INTRODUCTION

Avian influenza virus (AIV), especially type H5N1 has become nowadays a very dangerous, poultry threatening pathogen. Although, H5N1 is an AIV and usually does not spread among people, 650 human infections with highly pathogenic H5N1 virus have been confirmed in 15 countries since 2003, with about 60% of deaths (WHO, 2012). If a human who is already ill with seasonal flu becomes infected with avian influenza, the H5N1 virus may exchange genetic information with the human flu virus and acquire ability to spread from human-to-human. An easily human-transmissible AIV strain could have devastating consequences. Thus, there is a need for highly sensitive, accurate and rapid tests for diagnosis of AIV infection which would allow early antiviral therapy. Conventional tests that employ cell culture and serological testing require from two days up to two weeks and therefore are unsuitable for fast detection. On the other hand, molecular tests for influenza's diagnosis, such as reverse transcription-polymerase chain reaction (RT-PCR) (Ng *et al.,* 2005), and enzyme-linked immunosorbent assay (ELISA) (Ho *et. al*., 2009) are sensitive and rapid, but at the same time are expensive and rather complicated. Therefore, in order to minimize the social and economic costs, the development of rapid diagnostic tests is essential (Prasad, 2014). It is believed, that these tests should meet the following requirements: (1) high throughput, (2) possibility of multiple target detection, (3) accuracy: specificity and sensitivity, (4) speed, (5) ease of use, (6) suitability for on-site use in field investigation and (7) be inexpensive. In our opinion, such criteria are met by electrochemical biosensors. The development of biosensors began in the early twentieth century, when the first glass pH electrode was introduced. The first commercial biosensor, a glucose biosensor, was introduced by Yellow Springs Instruments in 1957. From then on, there has been a phenomenal growth in this field with emphasis on nanotechnology application (Turner, 2013). Generally, biosensor is defined as an analytical device that converts a biological response into an electrical signal. This device is composed of a biological recognition element (often called bioreceptor) and directly interfaced signal transducer. The selective and reversible process of interaction between the analyte and bioreceptor is transduced into a measurable signal, for example electrical signal, which is proportional to concentration or activity of analyte in any type of sample (Reyes De Corcuera *et al*., 2003). A schematic structure of a biosensor is presented in Fig. 1. Biosensor classification can be based on the transduction element or the biological element. According to the basic principles of signal transduction, biosensors can be electrochemical, optical, colorimetric and acoustic/gravimetric. Furthermore, the electrochemical transducers can be: potentiometric, amperometric, conductometric, voltammetric, polarographic, impedimetric, capacitive, piezoelectric (Spichiger-Keller, 1998). In this review, we will primarily concentrate on voltammetric or impedimetric detection of AIV. In a voltammetric transducer, controlled variation of the potential is applied between electrodes and an electrochemical current produced by the oxidation or reduction of electroactive species is measured. The change in the current is correlated with the concentration of analyte. In the case of impedimetric transducer, the response of electrochemical cell is recorded as a small amplitude sinusoidal voltage signal with a function of frequency. The choice of electrode material is a very important step in biosensor design, because it should be inert at the potential at which the electrochemical reaction takes place. In recent years, solid electrodes made of metals, such as

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Abbreviations: AIV, avian influenza virus; BSA, bovine serum albumin; CdSe, cadmium selenide; CV, cyclic voltammetry; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EIS, electrochemical impedance spectroscopy; ELISA, enzyme-linked immunosorbent assay; Fc, ferrocene; GNPs, gold nanoparticles; HA, hemagglutinin; Mab 6-9-1, anti-H5 HA monoclonal antibody; MB, methylene blue; MWNTs, multi-walled nanotubes; NHS, N-hydroxysuccinimide; OSWV, Osteryoung square wave voltammetry; PP-NWS, polypyrrole nanowires; QDs, quantum dots; RT-PCR, reverse transcription-polymerase chain reaction; SWNTs, single-walled nanotubes.

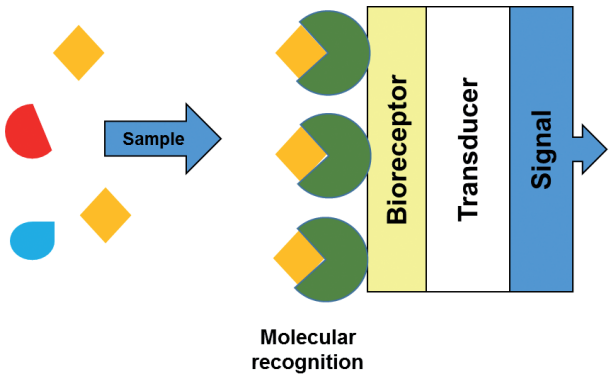

Figure 1. Schematic structure of a biosensor.

platinum, gold, silver, nickel, copper, mercury or various forms of carbon based materials have become very popular. A biosensor usually consists of three electrodes: a working electrode, a reference electrode and a counter electrode. Depending on the biological element incorporated, different types of biosensors are constructed, such as an enzymatic electrode, DNA sensors (genosensors), immunosensors or microbial sensors.

In this review, we focus on recent development of electrochemical biosensors: genosensors and immunosensors for detection of avian influenza virus type H5N1. Although avian influenza virus shows rapid evolutionary dynamics, consistent with a high background mutation rate and rapid replication (Chen & Holmes, 2006), electrochemical biosensors for its detection are highly recommended. The redox active monolayers, acting as the transducer, could serve as universal platforms suitable for attaching the selected recognition elements, specific antibody or specific oligonucleotide sequences for developing electrochemical immunosensors and genosensors. Such strategy allows for making simple and rapid changes of specific recognition elements and solves the problem associated with high mutation rate of the avian influenza virus.

ELECTROCHEMICAL GENOSENSORS FOR DETECTION OF AIV

In the case of a genosensor (DNA biosensor), the biologically active component is usually a single stranded DNA (ssDNA) that is used for hybridization of complementary ssDNA. The key step in genosensor preparation is a proper immobilization methodology applied on the surface of a transducer. Till now, various methods of ssDNA probe immobilization onto solid substrates have been reported, such as self-assembling sulfur-containing ssDNA probes onto gold electrodes (Liu *et al.*, 2005; Malecka *et al.*, 2012; Reddy *et al.*, 2012; Grabowska *et al.,* 2013; Grabowska *et al.*, 2014a), attachment of amino-terminated ssDNA probes to carboxy terminated thiols *via* EDC/NHS activation (Chen *et al.*, 2009; Loget *et al.*, 2013; Malecka *et al.*, 2013; Grabowska *et al.*, 2014b), biotinylated DNA probe linking through biotin-avidin interactions on the electrode surface (Davis *et al.*, 2003; Bonanni *et al.*, 2007; Stobiecka *et al.*, 2007; Chung *et al.*, 2011), just to name of few examples.

Generally, in the genosensors developed up to now, a few different approaches for the observation of the hybridization processes are applied (Drummond *et al.*, 2003). In the concept designed originally by Palecek, the changes in the electrochemical activity of nucleobases, such as guanine, cytosine or adenine on the surface of mercury or carbon electrodes are taken into account (Palecek *et al.,* 2012). To this group belongs an electrochemical biosensor for determination of DNA sequence related to AIV genotype reported by (Zhu *et al.,* 2009). There, a label-free determination of DNA sequence derived from AIV based on glassy carbon electrode modified with DNA probe covalently bound to poly(amidoamine) (PAMAM) dendrimer and containing multi-walled carbon nanotubes-cobalt phthalocyanine nanocomposite for signal amplification has been presented. Guanine oxidation signal of the probe before and upon hybridization has been monitored by differential pulse voltammetry (DPV), in the complementary target concentration from 0.01 to 500 ng/mL. The detection limit of 1.0 pg/mL has been estimated.

In a further approach, changes in electrochemical parameters of the interface electrode/aqueous solution caused by hybridization event have been monitored (Zhang *et al.*, 2008; Malecka *et al.*, 2012; Malecka *et al.*, 2013). In this type of biosensors, the mechanism of the signal generation, originally developed by Umezawa, is related to an ion-channel mimetic system (Umezawa & Aoki, 2004). In this system, upon the binding of complementary ssDNA to the ssDNA probe immobilized on the electrode surface, the access of anionic (or cationic) marker ions present in the sample solution to the modified surface is changed. A scheme of the working principle of the ion-channel mimetic genosensor, where anionic marker ions exist in the sample solution, is shown in Fig. 2.

Kukol and co-workers used the gold electrode surface modified with ssDNA oligonucleotide probes for detection of DNA targets derived from AIV H5N1. This device is based on impedimetric determination system in

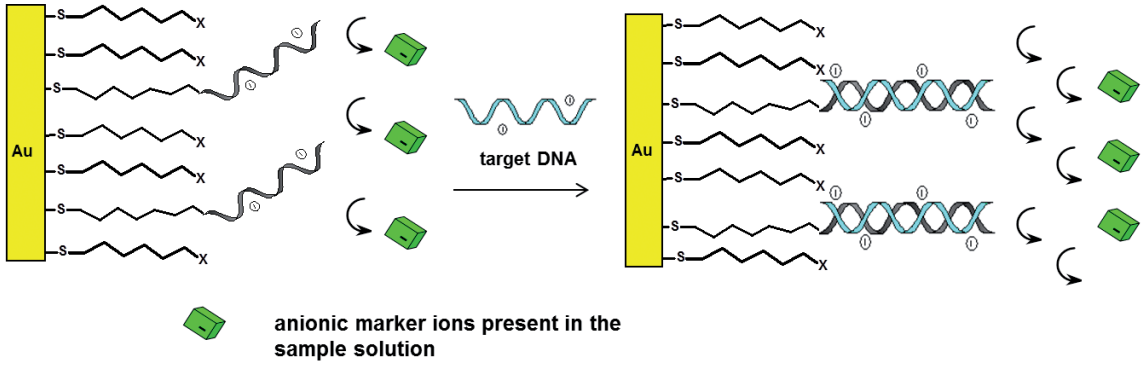

Figure 2. A scheme of the working principle of the ion-channel mimetic genosensor.

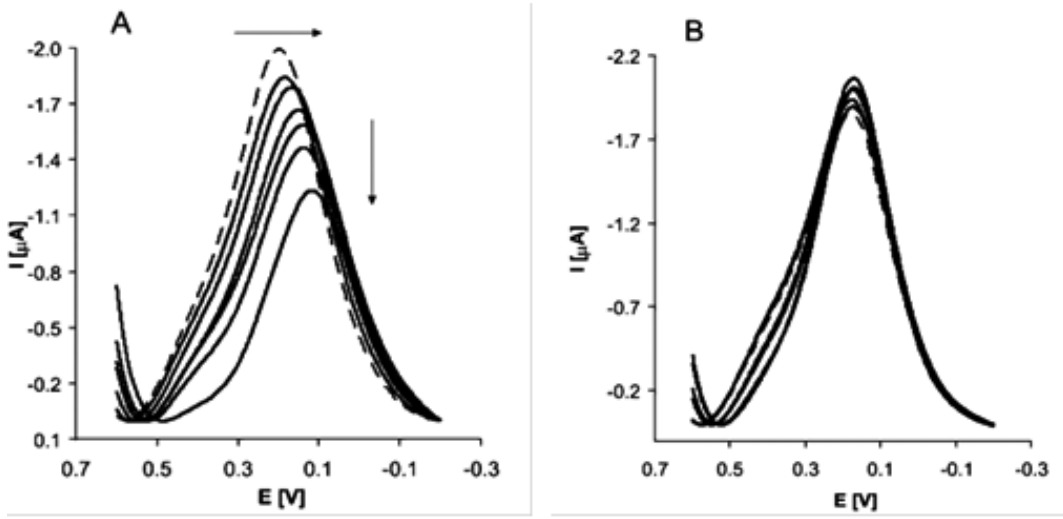

Figure 3. Typical Osteryoung square wave voltammograms obtained for electrodes modified with HS-ssDNA and 6-mercaptohexan-1-ol upon hybridization.
Dashed curve pertain to state before hybridization, the solid curves — upon hybridization with PCR1 (**A**) and PCR4 (**B**), in the concentration range of 10, 20, 40, 60, 80 and 100 pM. Solution composition: 1 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$, 0.1 M PBS (pH 7.4). Measurement conditions: three electrode configurations — Au working electrode, Ag/AgCl reference electrode and Pt counter electrode. Reprinted from Malecka *et al.*, 2012, with permission. Copyright 2012, WILEY-VCH.

the presence of a redox couple $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ with the detection limit below 200 fmol (Kukol *et al.*, 2008). The DNA biosensor, in which NH_2-ssDNA probe has been deposited on the gold electrode surface for detection of specific oligonucleotides sequence of AIV type H5N1 has been recently proposed (Malecka *et al.*, 2013). The detection techniques used Osteryoung square wave voltammetry (OSWV) and electrochemical impedance spectroscopy (EIS) in the presence of $[Fe(CN)_6]^{3-/4-}$ as a redox marker. This system displays good selectivity and sensitivity, with a major advantage being the ability to determine presence of 180-base pair (bp) PCR products with the detection limit in the fM range. Another fabricated genosensor based on self-assembling thiolated ssDNA probe on the gold electrode surface applied for detection of 20-mer oligonucleotides sequence and PCR products has been developed (Malecka *et al.*, 2012). In this case, the hybridization processes have been recorded using voltammetric techniques in the presence of a redox active marker $[Fe(CN)_6]^{3-/4-}$, with the detection limits in the 10 pM range. In comparison to other genosensors, the ability to distinguish between different positions in the complementary parts of the PCR products should be highlighted here. The 20-bp region complementary to the probe is located at the 3'-end of PCR1, at the 5'-end of PCR2, in the middle (83–103) of PCR3, and non-complementary — in PCR4. Typical Osteryoung square wave voltammograms recorded upon successive increase in concentration of PCR1 and PCR4 are presented in Fig. 3A and 3B. The linear decrease of current and potential shift observed in the presence of different complementary PCR1 products as well as non-complementary PCR4 is presented in Fig. 4A and 4B.

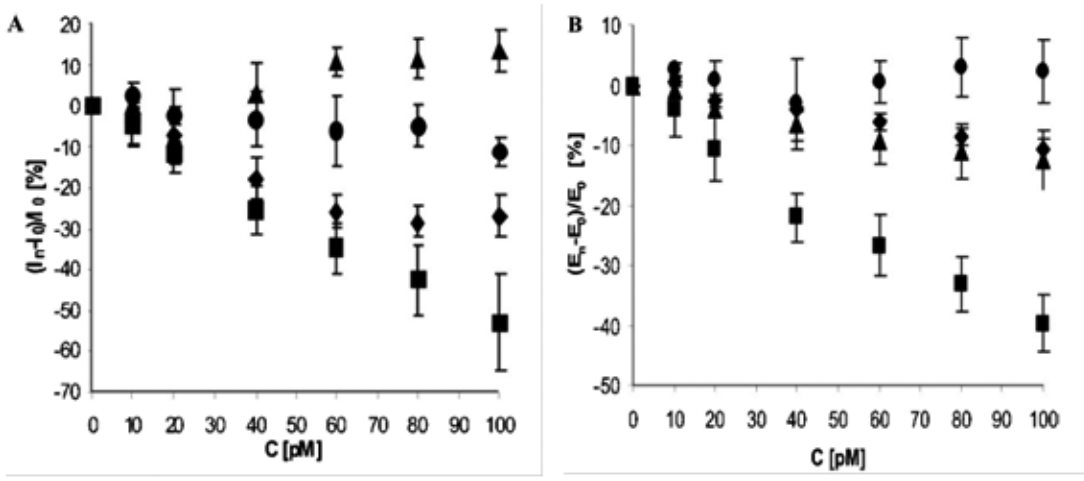

Figure 4. (A) The relationship of $(I_n–I_0/I_0)$ [%] *vs.* concentration C [pM] of (■) PCR1, (●) PCR2, (◆) PCR3 and (▲) PCR4.
I_n is the value of the peak current after detection of the given concentration of PCR product, and I_0 is the value of the peak current without analyte in pure 0.1 M PBS pH 7.4 (n=5÷6).
(B) The relationship of $(E_n–E_0)/E_0$ [%] *vs.* concentration C [pM] of (■) PCR1, (▲) PCR2, (◆) PCR3 and (●) PCR4.
E_n is the value of the peak potential after detection of the given concentration of PCR products, and E_0 is the value of the peak potential without analyte in pure 0.1 M PBS pH 7.4 (n=5÷6). Reprinted from Malecka *et al.*, 2012, with permission. Copyright 2012, WILEY-VCH.

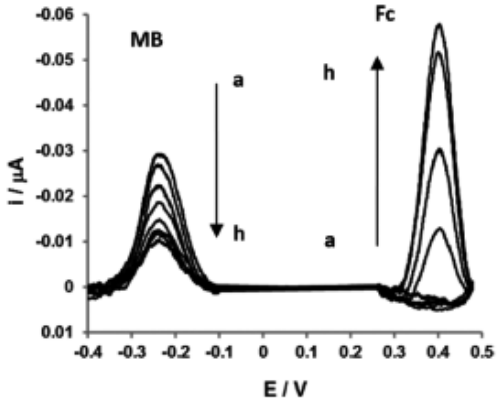

Figure 5. Representative Osteryoung square wave voltammograms obtained for electrodes modified with (a) SH-ssDNA-Fc probe and SH-ssDNA-MB probe and after hybridization with (b) 8 nM, (c) 10 nM, (d) 20, (e) 40, (f) 60, (g) 80 and (h) 100 nM oligonucleotides sequences complementary to SH-ssDNA-Fc and SH-ssDNA-MB.
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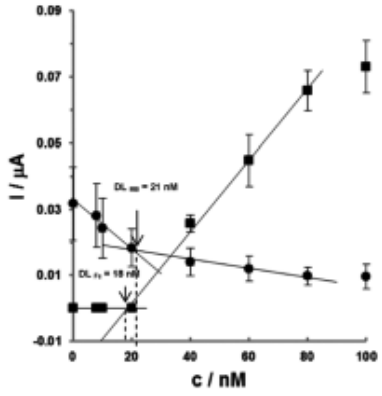

Figure 6. Change of peak current recorded for electrodes modified with (■) SH-ssDNA-Fc probe and (●) SH-ssDNA-MB probe *vs*. concentration of particular complementary sequences: from 8 to 100 nM (n=4).
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The above described DNA sensors also have some unfavorable features. Genosensors based on electroactivity of the nucleobases display rather weak analytical signals. However, ion-channel DNA biosensors require well ordered, free of pinholes monolayers attached on a gold electrode surface and the presence of a redox marker in the sample solution (Finklea, 1996). Because of that, another class of genosensors has been developed. In this type of devices the redox molecules are covalently bonded to the DNA probe. In most cases, the redox labels are attached to the oligonucleotide probes on one terminus, with the other end linked to the electrode surface (Fan *et al*., 2003). Two types of signal generation: "signal-on" or "signal-off" could be identified. Depending on the structure of the probe and biosensor design, the increasing ("signal-on") or decreasing ("signal-off") current upon hybridization could be observed. It might be due to the significant alteration in the distance between the label and the electrode surface (Immos *et al*., 2004; Farjami *et al*., 2011). Redox labels used the most frequently for this purpose are ferrocene (Aoki *et al*., 2010) as well as methylene blue (Liu *et al*., 2010). Recently, a multiplexed detection became very desirable because it allows measurement of multiple analytes (two or more) in a single run at the same time (Kang *et al*., 2012). An interesting approach worth highlighting is the single electrode genosensor for simultaneous determination of AIV type H5N1 (Grabowska *et al*., 2013). The signals generated as a result of hybridization processes are registered by the OSWV technique. In this genosensor, two different ssDNA probes decorated with ferrocene (Fc) and methylene blue (MB) are covalently immobilized on the gold electrode surface. This genosensor has been used for simultaneous detection of two different oligonucleotide targets derived from genes encoding hemagglutinin and neuraminidase of the AIV H5N1. The detection limits of both targets are in a similar range of 18-21 nM. The mechanism of the described duo-genosensor is based on a dual mode: "signal-on" and "signal-off". The presence of targets complementary to two different ssDNA probes labeled with Fc or MB causes increase of the Fc current and decrease of the MB current, respectively. The representative Osteryoung square wave voltammograms recorded for an electrode modified with a mixture of Fc and MB decorated probes with the changes of peak current *vs*. concentration of particular complementary oligonucleotides sequences are presented in Fig. 5 and Fig. 6.

The most desirable features of redox labels used for covalent attachment to the probes are reversible redox properties or applicability of these labels for measurements in appropriate potential windows. Thus, a great deal of effort is devoted to searching for alternative redox labels. The complex of 3-iron bis(dicarbollide) holds a great promise to serve as a suitable redox label, chemically and electrochemically stable with well-defined electrochemical properties. A remarkable approach is that developed for electrochemical determination of DNA sequence of AIV, type H5N1. The 5'-terminus NH_2 oligonucleotide probes bearing 3-iron bis(dicarbollide) redox label were immobilized on the gold electrode via NHS/EDC coupling to 3-mercaptopropionic acid SAM, previously deposited on the gold surface (Grabowska *et al*., 2014b). The changes in the redox activity of Fe(III) centres recorded upon hybridization by OSWV have been used as the analytical signal. The developed system is selective and very sensitive with an excellent detection limit of 0.03 fM. Moreover, the genosensor proposed is able to distinguish PCR products with different location of the complementary region. Recently, a new type of the electrochemical genosensor has been presented with cobalt-porphyrin used as a redox label (Grabowska *et al*., 2014a). This redox label is covalently attached to the DNA and located very close to the surface of the gold electrode. A novel mechanism of the analytical signal generation has been proposed, which takes into account the changes in the hydrophobic environment of the label upon hybridiza-

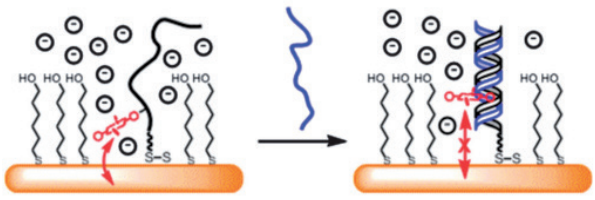

Figure 7. Schematic illustration of the signal generation mechanism of the Co-porphyrin-DNA genosensor.
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tion, without the "signal on-off" architecture. The AIV H5N1-based DNA sequences have been determined with the detection limit of 21 fM. An illustration of the signal generation mechanism of the Co-porphyrin-DNA genosensor is presented in Fig. 7.

After the hybridization process, the electrode surface environment has been changed, because the Co-porphyrin centre is more embedded in DNA duplex, more polar in comparison with ssDNA. In such a way, some hindrance occurs for the anionic counter ions transported to the redox centre, and, because of this, the decrease of Faradic current was observed.

These novel electrochemical genosensors present very recent efforts in the area of electrochemical avian influenza viruses detection.

Very promising trends appear in application of redox active monolayers in the genosensor construction. In these cases, redox centers play a double role. They are sites for covalent attachment of oligonucleotide probes. In addition, they act as a transducer of electrochemical signal generated upon hybridization. Their main advantage is the lack of the necessity of using an external redox marker, which is very important for future miniaturization and practical application.

Incorporation of novel advanced materials for improving genosensor's analytical parameters currently attracts attention of many scientists (Krejcova *et al.*, 2012, Krejcova *et al.*, 2014; Prasad 2014; Hepel & Zhong, 2012).

Novel electrochemical genosensors displaying sensitivity in the fM range could become alternative sensing techniques which might replace the PCR methods. The low sample consumption (in µl range) and easy way of miniaturization make them very attractive sensing devices. In addition, they posses the ability to differentiate between the complementary sequences position. This opens a novel field of applications for genosensors — namely-analysis of the RNA secondary structure (Malecka *et al.* 2012; Grabowska *et al.* 2014b; Patent No: P.408351).

ELECTROCHEMICAL IMMUNOSENSORS FOR DETECTION OF AIV

Immunosensors are biosensors based on an immunoreaction between antigen and antibody, where a stable complex is formed. Here, the electrochemical, optical or microgravimetric detection methods are the most frequently applied (Luppa *et al.*, 2001; Wąsowicz *et al.*, 2010; Jarocka *et al.*, 2011; Jarocka *et al.*, 2013). Selection of the suitable biorecognition element as well as its immobilization strategy is very important for the fabrication of the immunosensor with desired sensitivity, selectivity and stability (Arya *et al.*, 2013). Specific antibodies are usually used as recognition elements in the immunosensor platform (Chen *et al.*, 2013). However, because production of specific antibodies is difficult, expensive and time-consuming, as well as the fact that antibodies undergo frequent inactivation, alternatives are being pursued. One way to overcome these problems is using aptamers. Recently, Wang and co-workers have been selecting and characterizing DNA aptamers that can specifically bind to AIV H5N1 (Wang *et al.*, 2013).

Another specific receptor that has been proposed in the development of immunosensors is a two-stranded α-helical coiled-coil peptide (CCP). CCP has an advantage over antibodies because of a lower production cost and better stability. The construction of an ultrasensitive biosensor for HA-antibody detection was achieved with an electrode modified with CCP containing HA-antibody specific peptide sequence. This device is characterized by a detection limit of 1 pg/mL and selectivity against BSA and different antibodies (Arya *et al.*, 2014). Another new strategy is based on the tailoring of receptors similar to those found in target cells as selective recognition elements of biosensors. Wicklein and co-workers have recently published a protocol of novel type of impedance biosensor incorporating biomimetic sialic acid-galactose receptor that allows fast discrimination of phenotypes of influenza virus isolates. This biosensor is very selective for influenza virus phenotype because of the affinity between the specific sialic acid groups and the viral hemagglutinin. Thus, it is able to successfully detect and distinguish between human and avian (H5N1) isolates (Wicklein *et al.*, 2013).

Another possibility, which has been already successfully applied, is incorporation of a specific antigen as receptor for the detection of specific antibodies. It is very important from the application point of view, since vaccination has been employed in many countries in order to prevent and control AI. Lately, Jarocka and coworkers (Jarocka *et al.*, 2014) had presented a sensitive and selective immunosensor for the detection of antibodies against hemagglutinin (HA) from highly pathogenic avian influenza (HPAI) virus H5N1 using electrochemi-

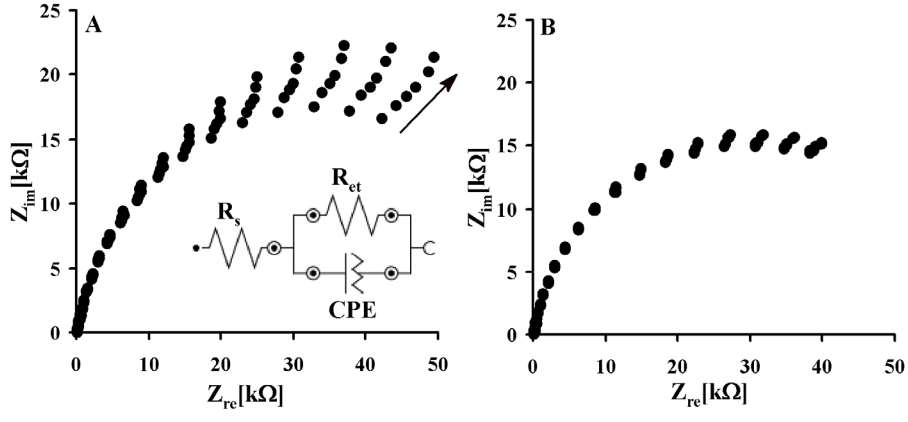

Figure 8. Electrochemical impedance spectra of BSA/long His_6-H5 HA/anti-His/protein A modified electrode (a) in buffer solution and after treatment with diluted hen serum with PBS buffer: (b) 7 x 10^7, (c) 7 x 10^6, (d) 7 x 10^5, (e) 7 x 10^4, (f) 7 x 10^3.
(**A**) vaccinated hen serum; (**B**) unvaccinated hen serum. Measurement conditions: three electrode configurations–GC working electrode, Ag/AgCl reference electrode, and Pt counter electrode; a bias potential of 0.2 V; the frequency range from 0.1 Hz to 10 kHz. Circuit model used for fitting Nyquist plots in inset: R_s — solution resistance, R_{et} — electron transfer resistance, CPE — constant phase element. Reprinted from Jarocka *et al.*, 2014, with permission from Elsevier.

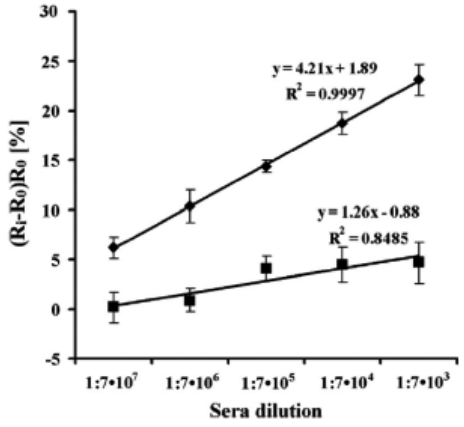

Figure 9. The relationship of relative electron transfer resistance changes $(R_i–R_0)/R_0$ *vs.* sera dilutions of (◆) vaccinated and (■) non-vaccinated hens (n=8).
R_0 — electron transfer resistance of fully modified electrode, measured in the presence of PBS before antibodies detection and R_i — electron transfer resistance of fully modified electrode measured in the presence of sera from vaccinated or non-vaccinated hen diluted with PBS. Reprinted from Jarocka *et al.,* 2014, with permission from Elsevier.

cal impedance spectroscopy (EIS) in the presence of $[Fe(CN)_6]^{3-/4-}$ as an electroactive marker with the detection limit of 2.1 pg/mL. Glassy carbon electrodes were modified in order to immobilize recombinant His-tagged hemagglutinin (His_6-H5 HA). The antibodies against His_6-H5 HA found in the serum of vaccinated chickens could be diluted up to 7×10^7-fold, which is almost 10^4 times better than for ELISA method. It is worth to emphasize that the main advantage of this immunosensor is its suitability for determination of antibody directly in the diluted hen sera. An example of a typical response of the immunosensor towards the hen sera dilution measured by EIS is shown in Fig. 8A, B. This system is able to selectively distinguish between the sera of vaccinated and non-vaccinated hens against AIV. The electrochemical impedance spectra recorded with an electrode which incorporated long version of His_6-H5 HA in the presence of the diluted vaccinated hen sera, displayed an increase of electron transfer resistance (Fig. 8A). However, the spectra recorded in the presence of unvaccinated hen sera did not change (Fig. 8B). The slope of relative electron transfer resistance $[(R_i–R_0)/R_0]$ for an unvaccinated hen serum dilution was about 3 times lower in comparison to the slope recorded for vaccinated hen serum dilution rate (Fig. 9).

A label-free immuno-biosensing concept based on the modulation of amperometric signals of surface bound redox species has been described (Liu *et al.,* 2008). In this concept, a glassy carbon electrode is modified with a derivative of ferrocene, followed by an epitope (biotin) with which an antibody is complexed. Upon this complex formation, a redox probe is immersed in a protein environment and this causes a significant attenuation of the current derived from the ferrocene, due to the restriction of counter ions access to the redox probe to balance the charge. As a result, hindering in the electron transfer was observed (Khor *et al.*, 2011; Liu *et al.*, 2013).

Electrochemical biosensors based on redox active layers have been developed in our laboratory (Jargilo *et al.*, 2013; Mikula *et al.*, 2013). This approach is based on the oriented immobilization of a his-tagged protein via covalent bonds between nitrogen atom(s) from histidine tag and the Cu(II) center present in the dipyrromethene complex deposited on an electrode surface. This strategy has been applied for the development of an immunosensor destined for detection of a specific antibody against avian influenza virus. This work is currently in progress in our laboratory.

It might be concluded that electrochemical immunosensors are very promising tools for rapid and reliable detection of influenza. They are very effective regarding parameters such as: sensitivity (several orders of magnitude better then ELISA), very good selectivity (matrix form the sera have no influence on the immunosensor's performance), very low sample volume requirement (in a μl range) and relatively easy way of miniaturization. Thus, they have a high potential to be widely applied for early detection of the avian influenza viruses.

NANOMATERIALS IN BIOSENSOR DEVELOPMENT

A new trend in the biosensor construction is the use of nanomaterials which exhibit unique and attractive chemical, physical and electronic properties (Park *et al.,* 2009; Bonanni and del Valle, 2010; Hepel and Zhong, 2012; Krejcova *et al.*, 2012; Luz *et al.*, 2013; Krejcova *et al.*, 2014). Various nanomaterials have been recently used as an electrode platform in highly sophisticated electroanalytical biosensing devices (Hepel & Zhong, 2012; Hernandez & Ozalp, 2012). The working electrodes (actual physical transducers) upon modification with these materials gain large effective surface area, high catalytic capabilities and high conductivity. Thus, these transducers could act as effective mediators and facilitate electron transfer between an active site on the receptor and the electrode surface. Nanoparticles (Peng *et al.*, 2006), carbon nanotubes (Tam *et al.,* 2009; Viswanathan *et al.* 2009), gold nanorods (Manelli and Marco, 2010; Wąsowicz *et al.* 2010) or quantum dots (Krejcova *et al.*, 2013) enhance sensitivity and selectivity of the electrochemical detection (Liu *et al.*, 2009). Among the variety of metal nanoparticles, gold nanoparticles (GNPs) have been extensively utilized in recent years, mainly because of their nanoscopic size, good conductivity and biocompatibility. One of the most commonly used are single-walled (SW) or multi-walled (MW) carbon nanotubes (CNTs) as well as graphene and graphene nanosheets. Thanks to their fast electron transfer ability, mechanical strenght, chemical stability, catalysis effect, thermal and electrical conductivity, they are attracting much interest among all application technologies.

Tam and co-workers described a DNA immobilization using multi-walled carbon nanotubes for direct and label-free detection of an influenza virus (type A). This genosensor can detect as low as 0.5 nM of target DNA based on changes in the conductance on the surface of the sensor (Tam *et al.*, 2009). Recently, Liu and co-workers (Liu *et al.*, 2011) have developed a sensitive electrochemical method for the detection of AIV H5N1 gene sequences using a DNA aptamer immobilized on a hybrid nanomaterial-modified electrode. At the same time, these authors modified electrodes with MWNTs, polypyrrole nanowires (PPNWs) and GNPs in order to obtain a porous structure with a large effective surface area combined with highly electrocatalytic activities and electronic conductivity. A novel electrochemical immunosensor that can sensitively detect AIV captured by graphene oxide-H5-polyclonal antibodies-bovine serum albumin (GO-PAb-BSA) nanocomposite has been reported recently, whereby use of graphene oxide allowed

256-fold increase in detection sensitivity compared to the immunosensor without GO-PAb-BSA (Xie *et al*., 2014).

Another type of nanomaterial which lately has attracted much attention is the semiconductor chalcogenide, with the cadmium selenide (CdSe) as the most extensively used in biosensor construction (Dyadyusha *et al*., 2005), due to their excellent electronic and optical properties. Fan *et al.* have presented a method for the controllable synthesis of different morphologies of CdSe nanostructures, and their application for the detection of AIV DNA sequences. An electrode modified with CdSe nanostructures shows a good selectivity for detecting the AIV DNA sequences (Fan *et al*., 2010).

Application of other quantum dots (QDs) such as CdS, ZnS and/or PbS for the influenza detection has been recently reported by Fialova and co-workers. These authors developed a method of low-cost isolation and detection of specific influenza protein, hemagglutinin (HA), labelled with CdS, PbS and CuS quantum dots. CdS QDs appeared to be the best label for viral protein determination via concentration of cadmium (II) ions, contained in the quantum dots, using stopped flow injection analysis with electrochemical detection (Fialova *et al*., 2013).

The use of silver nanoparticles conjugated with a well-known DNA intercalator — doxorubicin, has been reported (Ting *et al.,* 2009). This report described an electrochemical biosensor for the detection of short DNA oligonucleotides of the AIV H5N1. Cyclic voltammetry in 0.3 M KCl solution was performed and the silver nanoparticles were detected as a result of a highly characteristic solid-state Ag/AgCl redox process. A detection limit of 1 pM has been achieved.

Research on novel conducting materials and nanoparticles belongs to one of the fastest growing fields. These new advanced materials possess a very high potential for improving biosensor parameters, in particular their sensitivity.

CONCLUSIONS

Electrochemical biosensors have attracted tremendous interest in the early diagnostics of many diseases. They are characterized by good selectivity and sensitivity with a wide dynamic range from subfemtomolar to nanomolar, easy and rapid experimental protocol, reasonable cost and usage of the sample volume in a µl range. Most of the electrochemical biosensors are superior in comparison with the traditional methods, such as ELISA, PCR or Western blot. They have a comparable or better sensitivity and selectivity, with no need for usage of radioactive labels, toxic dyes or very expensive equipment. Rapid development of nanotechnology has opened a new way for construction of biosensors with even better features. A wide range of nanomaterials, such as gold and carbon nanoparticles have been successfully applied in the biosensor design in order to improve their performance. On the other hand, there is not a lot of information about biosensor validation in multicomponent biological or real samples, or long-term storage stability, which is very important in diagnostic tests. Therefore, there are still many challenges waiting for scientist working in this extensive field of research.

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