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Universal biosensor for detection of influenza virus

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A B S T R A C T

Influenza is a contagious disease caught by humans caused by viruses belonging to the family *Orthomyxoviridae*. Each year, the influenza virus infects millions of people and kills hundreds of thousands of them. Traditional diagnostic methods, such as virus propagation and isolation, antigen capture immunoassays and molecular methods are not sufficient for the detection of the influenza virus. Development of a valid diagnostic assay for quick detection (in less than an hour) of the virus, with high sensitivity, is a challenge for researchers all over the world.

Here we present a new, universal immunosensor for detection of the influenza A virus. By using electrochemical impedance spectroscopy (EIS) and direct attachment of antibodies to the gold electrode the assay allows detection of the pathogen with sensitivity similar to molecular methods in relatively short time. Application of universal anti-M1 antibodies allows detection of all serotypes of influenza A virus.

The simple design of the sensor facilitates miniaturization of the device and its implementation for routine diagnostics during first contact with the patient, before applying a proper treatment.

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1. Introduction

Fast, efficient, effective, and low-cost detection of pathogens is a challenge for scientists all over the world. Considering the spreading (infection) rate of the influenza virus during the pandemic of 2009 (WHO Website, 2013), it is clear that we need to develop an effective system for diagnosing the influenza virus at the early stages of infection, when the titer of virus in the throat is low.

During three pandemics in the 20th century, influenza virus (family *Orthomyxoviridae*) killed an estimated 100 million people. Each year the virus infects around 1 billion people and causes 250,000 deaths. High variability of the virus and its easy spread make it one of the world's most dangerous pathogens (de Jong and Hien, 2006; WHO Website, 2013).

Standard diagnostic methods for detection of the influenza virus seem to be insufficient. The traditional methods are based on virus isolation from tissue cultures (MDCK, Vero) or embryonating chicken eggs. This effective and sensitive technique requires several days of analysis involving labor intensive and time-consuming procedures (Storch, 2000). There are ELISA tests for detection of virus antigens in the infected organism (He et al., 2007). However, this method is relatively time-consuming and multistage which

makes it significantly more difficult to perform. Moreover, ELISA provides relatively low sensitivity and may produce false negative results.

In order to provide maximum sensitivity and specificity of the method while maintaining a short time of analysis (a few hours), currently, the most frequently used methods are based on detection of the virus genetic material. Molecular methods based on PCR, RT-PCR, and Real-time PCR are more specific, more sensitive, and consume less time, compared with traditional methods. However, a big disadvantage of these methods is the necessity to isolate the genetic material and to take special care not to contaminate the sample. Moreover, these methods require the use of equipment which is not available in outpatient clinics, but only in diagnostic or scientific laboratories.

There are several bedside tests which allow a relatively quick (up to 30 min) detection of viral antigens. Unfortunately, these tests provide low sensitivity and often produce false negative results, especially during later stages of the disease development. Low sensitivity is the main reason why these tests are seldom used in routine diagnostics of influenza virus (Storch, 2003; Woolcock and Cardona, 2005).

State of the art diagnostic methods combine elements of biology, chemistry, engineering, and electrochemistry. Methods based on the most modern technology allow significant reduction of analysis time, while maintaining high sensitivity and specificity.

A few modern biosensors are known, whose operation is based on quartz crystal oscillation (Hewa et al., 2009; Li et al., 2011), refraction changes (Xu et al., 2007), interferometry (Farris et al.,

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2010) carbon nanotubes (Lee et al., 2011), imaging ellipsometry (Qi et al., 2010) or surface plasmon resonance (Park et al., 2012; Su et al., 2012). However, these methods require application of highly sophisticated equipment, and are often difficult to perform, analyze, and interpret. Moreover, to date, no simple and universal sensor was developed that would be able to detect all types of the influenza A virus.

In this paper we present a new, universal immunosensor for detection of influenza virus in swabs from the human throat. The biosensor is based on impedance electrochemical spectroscopy and its design involves direct attachment of antibodies to the electrode.

The antigen used for detection by the developed sensor is the most conserved and popular structural protein in the virion (M1). This approach allows quick detection of all types of the influenza A virus with a sensitivity comparable to molecular methods.

2. Materials and methods

2.1. Viruses propagation

Avian influenza A/ostrich/Denmark/96-72420/96 (H5N2), A/GraylagGoose/Poland/MW74/10 (H5N2) and A/Afri.Star./Eng-Q/938/79 (H7N1) (kindly provided by Z. Minta and K. Smietanka, Department of Poultry Diseases, National Veterinary Research Institute, Pulawy, Poland) and human influenza A viruses (pandemic H1N1 strains) from the collection of the Department of Recombinant Vaccines, University of Gdansk, Poland were propagated in Madin-Darby canine kidney cells (MDCK) cultured at 37 °C under 5% CO_2 in Dulbecco's Modified Eagle's Medium (D-MEM) (Sigma-Aldrich), supplemented with 2 mM L-glutamine, 0.2% bovine serum albumin, 25 mM HEPES buffer, 100 U/ml of penicillin, 100 μg/ml of streptomycin in the presence of 2 μg/ml TPCK (L-1-Tosylamide-2-phenylethyl chloromethyl ketone) – trypsin (Sigma-Aldrich). Viral stocks were stored at −70 °C and titrated by plaque assay before use.

2.2. Production of recombinant M1 in the bacterial expression system

The M1 gene of influenza A virus A/England/195/2009 (H1N1) was PCR amplified using plasmid DNA (pPol-M1) as a template and oligonucleotides (For-M1-BamHI 5′ TT*GGATCC* AGTCTTC-TAACCGAGGTCGAA 3′ and Rev-M1-EcoRI 5′ TTT*GAATTC* CTTGAATCGCTGCATCTGC 3′) as primers. The obtained PCR product of 770 bp was digested with BamHI and EcoRI enzymes and cloned into a commercial vector, pGEX 2TK (GE Healthcare). The resulting plasmid, pM1-GST was verified by restriction analysis and nucleotide sequencing. pM1-GST was used to transform BL21 *Escherichia coli* strain, and the recombinant strain was used to overproduce M1-GST after addition of IPTG (final concentration 1 mM). The 54 kDa protein was visualized in a blue-coomassie-stained gel and purified by affinity chromatography on glutathione resin (GE Helathcare). 0.2 mg of pure M1-GST protein was obtained from 0.25 l of culture.

2.3. Production of recombinant M1 in a baculovirus expression system

We generated a recombinant strain of *Autographa californica* nuclear polyhedrosis virus (AcNPV) expressing M1 protein of influenza virus A/England/195/2009 (H1N1), using the Bac-to-Bac baculovirus expression system by Invitrogen (Luckow et al., 1993). M1 sequences of influenza virus were amplified by PCR using the pPol-M1 plasmid as the template and the following

primers: For-FB-M1-BamHI 5′ TT*GGATCC*ATGAGTCTTCTAACCGAG-GTCGAA 3′ and Rev-FB-M1-EcoRI-6xHis 5′ TT*GAATTC***TCA***GTG-GTGGTGGTGGTGG*CTTGAATCGCTGCATCTGC 3′. The resulting 790 bp-long product was double-digested and cloned into BamHI and EcoRI sites of the baculovirus transfer vector pFastBac1. The recombinant baculovirus, Bac-M1, expressing M1 protein was generated by transposition-mediated recombination. To obtain overexpression of M1-His protein, Sf9 (*Spodoptera frugiperda*) insect cells were transfected with Bac-M1 baculovirus and then cultured for 96 h. The resulting 28 kDa M1-His protein was visualized in a blue-coomassie stained gel and purified by affinity chromatography on Ni-NTA resin (Life Technologies).

2.4. Production of polyclonal anti-M1 antibodies

For antibody production, six C57BL/6J mice were immunized intraperitoneally with 30 μg of purified M1-GST protein mixed with incomplete Freund's adjuvant in a total volume of 100 μl per mouse. The injections took place on day 0, 14, 35, and 56. On days 24 and 45, sera samples were taken by tail bleeding. On day 66, total blood was collected. The obtained sera was tested for purified protein and optimal dilution of anti-M1 sera for western blot analysis was established as 1:100,000. Universality and selectivity of the antibodies were confirmed using western blot and slot blot methods. Anti-M1 antibodies were purified by affinity chromatography on CNBr-activated Sepharose 4B coupled with M1-His protein.

2.5. Fabrication of the immunosensor

2.5.1. Preparation of electrodes

Gold electrodes were cleaned mechanically by polishing in a water suspension of aluminum oxide (alumina). The electrodes were then cleaned electrochemically in a 0.5 M solution of potassium hydroxide by using potential sweep in the range of −1200 mV to −400 mV (in relation to Ag/AgCl as the reference electrode) at the rate of 100 mV/s, number of cycles: 3, 50, and 5. The electrodes were then put one by one in a 0.5 M solution of sulfuric acid and potential sweep was applied in the range of −300 mV to 1500 mV, at the rate of 100 mV/s, number of cycles: 3, 10, and 5. Finally, the surface of the electrodes was reconditioned in a KOH solution by using 10 cycles.

2.5.2. Modification of electrodes.

Clean gold electrodes were rinsed with ethanol and put in a 10 mM solution of 1,6-hexanedithiol (HDT) in ethanol for 20 h at room temperature (Sigma-Aldrich). The test tubes containing the electrodes in HDT solution were sealed with a teflon tape and parafilm in order to avoid solvent evaporation. Next, the electrodes were rinsed, first with ethanol, and then with deionized water, and turned upside down. Then, 10 μl of colloidal gold particles (GCP, 5 nm) was applied on the electrodes for 18 h at +4 °C temperature (Sigma-Aldrich). During the next step, the electrodes were rinsed with 0.1 M PBS buffer pH 5.5 (145 mM NaCl, 2.7 mM KCl, 8.4 mM NaH_2PO_4, and 1.6 mM Na_2HPO_4) and 10 μl of anti-M antibodies solution of 28 μg/ml in 0.1 M PBS buffer was applied on the electrodes for 2 h at +4 °C. During the final step, 10 μl of 0.5% (w/v) BSA solution in 0.1 M PBS, pH 5.5, were applied on the electrodes surface for 2 h at +4 °C. Finally, the electrodes were rinsed with PBS buffer and stored at +4 °C until use.

Individual steps of the modification were characterized using cyclic voltammetry and impedance electrochemical spectroscopy using ferrocyanides ($Fe(CN)_6^{3-/4-}$) as redox-active markers. In Figs. 1 and 2 we present cyclic voltammograms and impedance spectra obtained after consecutive modification steps.
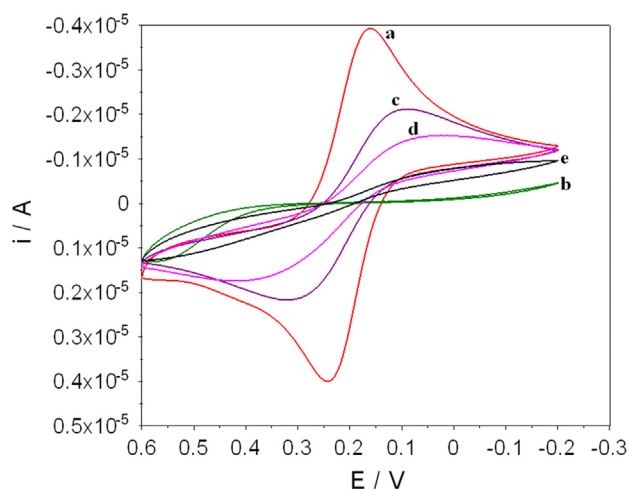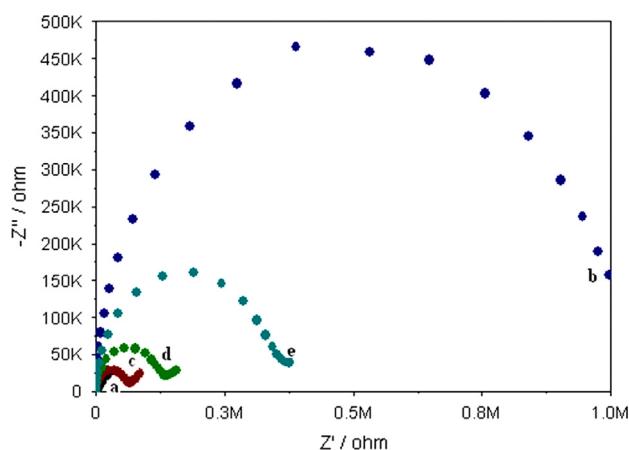
Fig. 1. Cyclic voltammograms recorder for consecutive steps of the immunosensor preparation: (a) clean gold electrode; (b) 1,6-hexanedithiol; (c) GCP; (d) anti-M antibodies; (e) BSA. Measurement conditions: 1 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ in 0.1 M solution of PBS pH 5.5; scan rate 100 mV/s. GCP – gold colloidal particles.

Fig. 2. Impedance spectra recorded for consecutive steps of the immunosensor preparation: (a) clean gold electrode; (b) 1,6-hexanedithiol; (c) GCP; (d) anti-M antibodies; (e) BSA. Measurement conditions: 1 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ in 0.1 M solution of PBS pH 5.5; scan rate 100 mV/s.; potential 0.17 V. GCP – gold colloidal particles.

2.6. Detection of M1 protein

All electrochemical measurements were performed with a potentiostat–galvanostat AutoLab (Eco Chemie, Utrecht, Netherlands) with a conventional three-electrode configuration with gold electrodes unmodified or covered by different layers as working electrodes. The prepared immunosensor was used to detect influenza virus M protein. The virus/protein was suspended in 0.1 M PBS buffer pH 5.5 and shaken vigorously. On the surface of the electrodes, 10 μl of solution was applied for 30 min. Next, the electrodes were rinsed with PBS buffer and put into an electrochemical cell. Measurements of impedance electrochemical spectroscopy were performed in the presence of ferrocyanides ($Fe(CN)_6^{3-/4-}$) as redox-active markers. We observed changes in electron transfer resistance.

2.7. Universality of the antibodies, sensitivity and selectivity of the assay

To confirm the universality of the antibodies obtained, we performed a slot blot analysis using different influenza virus

strains (A/ostrich/Denmark/96-72420/96(H5N2), A/GraylagGoose/Poland/MW74/10 (H5N2), A/Afri.Star./Eng-Q/938/79 (H7N1), A/H1N1v/32u/10, A/H1N1v/47u/10). In order to make sure that the test does not show any unspecific detection of antigens present in the oral cavity, we tested a throat swab from a healthy patient.

To analyze the sensitivity of the test, serial dilution of recombinant M1 protein in the range of 10–100 pg/ml was prepared and measured.

3. Results

3.1. Detection of M1 protein

After preparation, the sensor was put into a buffer containing virus particles and impedance electrochemical spectroscopy measurements were performed in the presence of ferrocyanides ($Fe(CN)_6^{3-/4-}$) as redox-active markers. With increasing concentration of M protein (amount of the virus) we observed an increase in electron transfer resistance (($R_i - R_0$)/R_0) (Fig. 3). Values higher than 5% were considered as valid results. Values lower than 5% were rejected due to a possible measuring error.

3.2. Universality of the antibodies, sensitivity, selectivity and reproducibility of the assay

The universality of the method was confirmed through a slot blot analysis of several human and avian viruses. Specificity of the test was assessed through analysis of a throat swab from a healthy patient (Fig. 4).

We observed a positive reaction during analysis of all viruses, whereas no reaction was observed during analysis of the throat swab from a healthy patient. We confirmed the specificity and universality of the used antibodies and thus, the method itself.

Sensitivity of the method was assessed through analysis of serial dilutions of M protein. Differences in resistance lower than 5% were rejected. This value was calculated as standard deviation of the blank control multiplied by 5. The detection limit corresponding to a difference in electron transfer resistance greater than 5% was obtained for the concentration of 20 pg/ml (Fig. 3), which corresponds to around 80–100 virus particles/μl. This result is comparable to the sensitivity of molecular methods (e.g. Real-time PCR).

The reproducibility was determined by testing a known concentration of M1 protein (60, 80 and 100 pg/ml) using 5 different
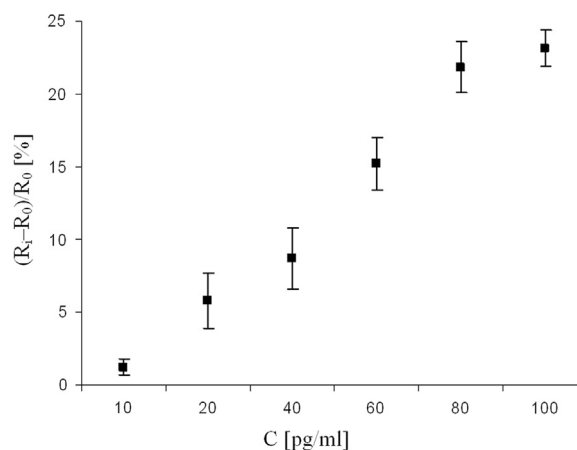

Fig. 3. Plots of the relationship between ($R_i - R_0$)/R_0 and C [pg/ml] – concentration of standard M protein solutions in 0.1 M PBS pH 5.5. R_i is the value of resistance of electron transfer through a double layer before immobilization of protein particles.
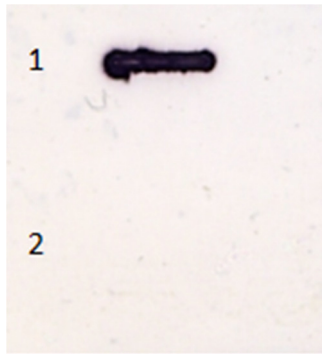
Fig. 4. Slot blot analysis of influenza virus (1 – A/H1N1v/32u/10) and a throat swab from a healthful patient (2).

electrodes during five days. The results show good reproducibility because the coefficient of variation was lower than 9% for each concentration (8.9%, 7.2% and 5.2%).

4. Discussion

Development of a universal and sensitive test for quick detection of influenza virus is an important element of international efforts to fight this pathogen. Traditional diagnostic methods, like virus propagation and isolation, antigen capture immunoassays, and molecular methods do not meet the requirements dictated by current diagnostic needs. The biosensors developed so far allowed detection of viruses of selected serotypes (He et al., 2007; Li et al., 2011; Xu et al., 2007), which restricted their applicability to selective analyses. Other methods require sophisticated research equipment and are often restricted to laboratory use (Hewa et al., 2009; Li et al., 2011; Qi et al., 2010). The presented immunosensor was developed with the intention to be used (after miniaturization) in practice, in routine diagnostics during visits to the general practitioner.

Due to the application of antibodies against conserved virus antigens – anti-M1, the presented test allows detection of all serotypes of influenza A virus. Moreover, application of polyclonal antibodies allows a much broader spectrum of antibodies, thus reducing the risk of false negative results (which could occur if monoclonal antibodies were used).

Electrochemical impedance spectroscopy (EIS) is a very sensitive and, at the same time, very well understood method. It is an effective way to detect the formation of antigen–antibody on electrode surfaces and formation of biosensors (Chang and Park, 2010; Lisdat and Schäfer, 2008). Its use for development of biosensors has already been shown (Yang et al., 2004; Kim et al., 2012). It guarantees simplicity of analysis. Direct attachment of antibodies to the gold electrode is the simplest possible solution, which reduces the need to transfer signals through proteins (e.g. protein A) or other intermediate substances. Thus, occurrence of noise and distortions, which can affect sensitivity and specificity of the method, is reduced. Such solution greatly facilitates the design process of the biosensor and also its future miniaturization.

Detection of virus antigens lasts only 30 min and does not need isolation of the virus, genetic material, or any other complicated procedures associated with sample preparation. A throat swab or a saliva sample is enough to perform the analysis. The sample is suspended in PBS buffer and shaken. The complete sample preparation procedure lasts less than 1 min. The developed sensor is characterized by high sensitivity (80–100 virions/μl), comparable to the sensitivity of molecular methods, very high specificity, and very short analysis time (around 30 min.).

5. Conclusions

In this study we present the universal immonosensor for the detection of all serotypes of the influenza A virus. Using ESI and direct immobilization it is possible to miniaturize the device down to the size of a pen or a glucose meter strip. Application of a reader (central unit) and exchangeable elements (for each patient) will allow significant reduction of the production costs of the device and will increase its mobility, thus enabling its application in practice.

Universality and simplicity of performing the test will not only allow its application for diagnostics in humans, but also in animals. The high sensitivity of the method allows for quick detection of the virus at its early stages of infection in birds, which can greatly contribute to taking suitable sanitary measures at early stages of an epidemic.

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