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Biomolecular detection of influenza virus based on the electrochemical impedance spectroscopy using the nanocrystalline boron-doped diamond electrodes with covalently bound antibodies

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Abstract

New rapid pathogen detection methods with improved cost-effectiveness and efficiency are currently in the focus of the scientists from all over the world. Based on the experiences from the rapid spread of the influenza virus pandemic in 2009 it is clear that the development of the system for early diagnosis of this infection is essential. The crucial stage of the treatment is the detection of the viral infection during its initial development phase when approximately only several dozens to hundreds of virus particles are present in the pharynx of the infected individual.

The present study describes the new universal antibody-modified nanocrystalline boron-doped diamond biosensor for the direct detection of the viral particles at ultralow concentrations. Polyclonal anti-M1 antibodies against the M1 protein, the universal biomarker of influenza virus, are attached to the surface of the diamond electrode. Capturing the M1 protein results in electrochemical impedance spectra changes.

Achieved limit of detection for the approach using M1 biomarker in saliva buffer is 5×10^{-14} g/mL, i.e., several virus particles per sample. Additionally, this assay may be soon developed into the first commercial test utilizing diamond electrodes for the detection of the influenza infection.

Keywords: impedance spectroscopy; influenza virus; antibodies; M1 protein; nanocrystalline boron-doped diamond electrodes; diamond electrode modification

1. Introduction

The influenza virus (*Orthomyxoviridae family*) is responsible for three pandemics in 20th century, which altogether led to approximately 100 million deaths worldwide. Additionally, every year brings about

1 billion new infections and 250,000 deaths all over the world. Influenza virus is extremely dangerous because of its quick accumulation of the mutations and its easy spread {Citation}. Inoculation of the influenza virus in embryonated chicken eggs or in the tissue cultures (MDCK Vero) are one of the traditional methods for its detection. However, these methods, despite high sensitivity and effectiveness, are not sufficient in the modern diagnostic laboratories because of the high workload and time requirements {Citation}. Molecular techniques such as PCR, real-time PCR, and reverse transcriptase PCR exhibit highest sensitivity and specificity. In addition, the duration of this assay does not exceed few hours, what in combination with higher specificity and sensitivity gives a significant advantage over the traditional methods. The main drawback of these techniques is the isolation of the genetic material, which is a laborious procedure requiring special measures to keep samples uncontaminated.

Currently available point-of-care tests for virus infection can also be used with the results in less than 30 minutes, however, these kind of tests are not reliable due to often false negative results and low sensitivity, especially during last stages of the disease. These tests are rarely used in the routine influenza virus diagnostics mostly due to lack of sensitivity {Citation}.

Combination of the highest possible level of specificity and sensitivity with low time-consumption of analyses are the characteristics of available new technologies. Based on new technologies it is

possible to develop new tools that combine complementary elements of chemical engineering, electrochemistry, and biology.

The biosensors are the example of these novel methods, however, only a few of them have been developed so far. They are based on the refraction changes {Citation}, surface plasmon resonance {Citation}, electric properties of carbon nanotubes {Citation}, interferometry {Citation}, imaging ellipsometry {Citation}, UV-Vis {Citation}, or quartz crystal oscillation {Citation}. Biosensors offer many benefits in diagnostics, however data interpretation, analysis, and conduction of the tests are still highly problematic. These tests require extremely sophisticated and expensive equipment and additionally up to now, there is no universal biosensor available for the detection of all types of influenza A virus.

Impedance biosensors are the simplest and most sensitive comparing with other types of sensing approaches due to the lack of acoustic or optical components what offers crucial advantages in case of the portable devices. {Citation}. Thanks to this feature impedance biosensors are ideal for the environmental monitoring of disrupting chemicals, drugs, specific proteins or DNA strains {Citation}. Electrochemical impedance spectroscopy (EIS) in comparison with other electrochemical methods often used for the molecular recognition, e.g. differential pulse voltammetry or cyclic voltammetry, is less destructive for the measured interactions between biological moieties. This is the result of EIS spectra registration in very narrow range of small potentials. Thanks to the high sensitivity of the method it is possible to monitor the changes on the surface of the electrode at each modification step and during the final detection {Citation}.

The boron-doped diamond (BDD) is a highly promising material for the third generation biosensor due to its wide potential window, high stability, low background current, biocompatibility, and chemical inertness {Citation}. The result of this combination is high sensitivity and specificity with additional advantages of modern microelectronics. The sensors equipped with modified BDD electrodes exhibit fast response what rapidly caught the attention of scientist {Citation}. Härtl *et al.* in the leading publication in the field {Citation} presented enzyme-based amperometric biosensor with bovine liver catalase enzyme attached to the BDD film surface via photochemically introduced TFA-amine. Microwave plasma enhanced chemical vapor deposition (MW PE CVD) is the method selected for the preparation of B:NCD electrodes which are dominantly hydrogen-terminated (H-B:NCD) {Citation}. As the result of this method, the diamond surfaces obtain the hydrophobic characteristic what helps to avoid the non-specific adsorption of biomolecules and the protein denaturation difficulties.

The standard boron-doped diamond utilized as biosensing electrodes are thick microcrystalline grown at 700-900°C consuming lots of time and energy. In this report, we are utilizing ultra-thin, low

temperature nanocrystalline boron-doped diamond (B:NCD) films exhibiting the very low electrical noise and background level {Citation}. The nanocrystalline structure of electrode surface reveals highly efficient modification by polyclonal anti-M1 antibodies towards detection of the viral particles at ultralow concentrations.

The recognition of the biomolecules was based on the interactions between antibody and antigen after conjugation of the non-specific bovine serum albumin to the surface of the electrode by means of impedance spectra measurements. Taking into account the repeatability of the obtained results and multiplicity of the performed tests, it was possible to develop a rapid diagnostic method for the detection of M1 proteins from samples containing real viruses.

2. Experimental section

2.1. Infection of the host cells using influenza virus

Canine kidney cells Madin-Darby (MDCK; ATCC CCL-34) were cultured using Dulbecco's Modified Eagle's Medium (D-MEM) (Sigma–Aldrich, St. Louis, MI, USA) with 0.2% bovine serum albumin, 2 mM L-glutamine, 100 µg/mL of streptomycin, 100 U/mL of penicillin, 25 mM HEPES buffer at 37°C under 5% CO_2.

Following viruses, Human influenza A/Virginia/ATCC1/2009 (H1N1) and A/Aichi/2/68 (H3N2), were grown in cultures of MDCK cells with 2 µg/mL TPCK (l-1-Tosylamide-2-phenylethyl chloromethyl ketone)-trypsin (Sigma–Aldrich) in the culture medium. Virus plaque assay was used to obtain the viral titers.

2.2. M1 protein – cloning and expression

The amplification of the gene encoding M1 protein was conducted using cDNA of Influenza A virus structural gene M1 based on the H1N1 strain (A/England/195/2009/H1N1) using PCR with the forward primer (5'- TTGGATCCAGTCTTCTAACCGAGGTCGAA-3') and the reverse primer (5'-TTTGAATTCCTTGAATCGCTGCATCTGC -3') containing BamHI and EcoRI restriction endonuclease sites, respectively. The PCR product was ligated into the BamHI/EcoRI sites of the commercial vector pGEX 2TK (GE Healthcare) after previous digestion of the product with given restriction enzymes. After ligation, the obtained plasmid pM1-GST was subjected to confirmation using nucleotide sequencing and restriction enzyme analysis. Subsequently, given plasmid was used for the transformation of BL21 cells. The protein expression was induced using IPTG in 1 mM final concentration. Resulting M1-GST protein was subjected to purification using affinity chromatography with glutathione resin and analyzed using electrophoresis (SDS-PAGE).

2.3. Polyclonal anti-M1 antibodies – preparation and purification

Purified M1-GST (300 µg) was added to the incomplete Freund's adjuvant obtaining total volume of 1 mL. Subsequently, the mixture was injected intramuscularly (IM) into the New Zealand white rabbit. The further portions, second and third, were injected on days 21 and 42. Blood samples were taken from rabbits after the last immunization and the titer of antiserum was calculated based on the Western Blot analysis. Subsequently, the antisera were purified based on the affinity chromatography using CNBr-activated Sepharose 4B beads conjugated with M1-His protein when the titer reached the ratio of 1:10,000. These purified anti-M1 antibodies were later utilized for the production of the biosensor.

2.4. Manufacturing of the immunosensor

2.4.1. Manufacturing of nanocrystalline electrodes

The deposition of B:NCD electrodes was conducted on a mirror polished p-type Si(100) substrates (10 mm x 10 mm) using MWPACVD system (SEKI Technotron AX5400S, Japan). Truncated cone-shaped substrate holder prepared manually was utilized to improve the formation of nanocrystalline diamond films {Citation}. For this purpose, the mixture of gases, H_2, CH_4, and B_2H_6 (diborane) as the source of boron, was used. More details concerning growth parameters were described elsewhere {Citation}. The seeding of the substrates was conducted based on the spin-coating method in DMSO-PVA-DND slurry according to {Citation}. The obtained boron content of the diamond film was of the order of 1.37×10^{20} cm^{-3}. After 3 h of the film growth using this method nanocrystalline diamond films of semimetal characteristics with thickness of approx. 250 nm and 0.3 Ω cm resistivity were obtained.

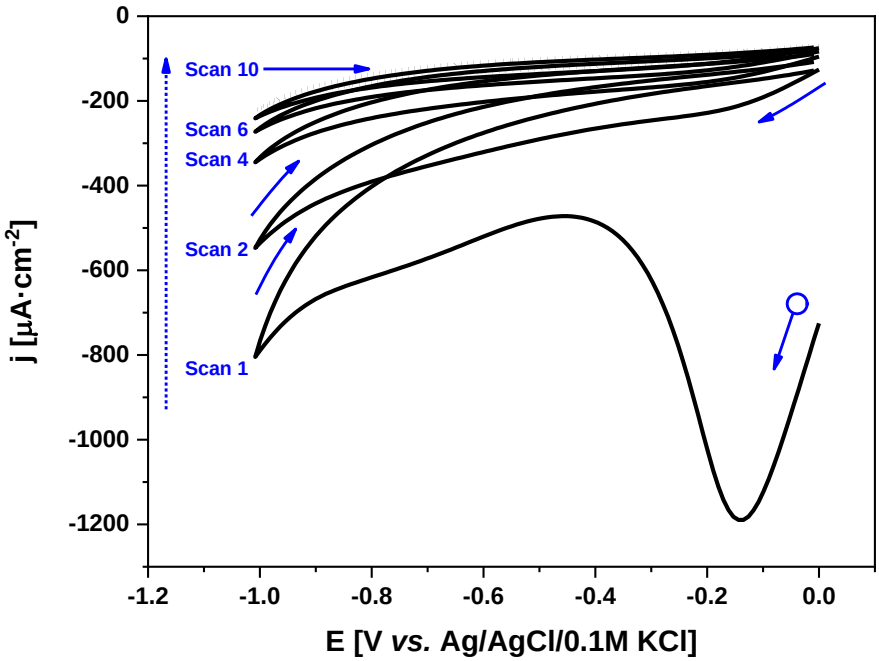


Figure 1. B:NCD electrode cyclic voltammograms (10 scans, scan rate – 100 mV/s) conducted in the 4-aminobenzoic acid diazonium salt solution in diluted HCl.

2.4.2. Modification of electrodes

Subsequently, to obtain the influenza sensor the modification of the surface in multiple stages was performed. In the first step, the 4-aminobenzoic acid diazonium salt was reduced electrochemically and covalent bond was formed between modifier and B:NCD surface electrode. This approach prepared the foundation for further utilization of the chemical reactions for the surface modification. The immobilization of the anti-M1 antibodies was achieved using two chemical agents, a coupling reagent N-hydroxysuccinimide (NHS) and a cross-linking reagent 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC).

2.4.2.1. Modification of B:NCD surface using the 4-aminobenzoic acid diazonium salt

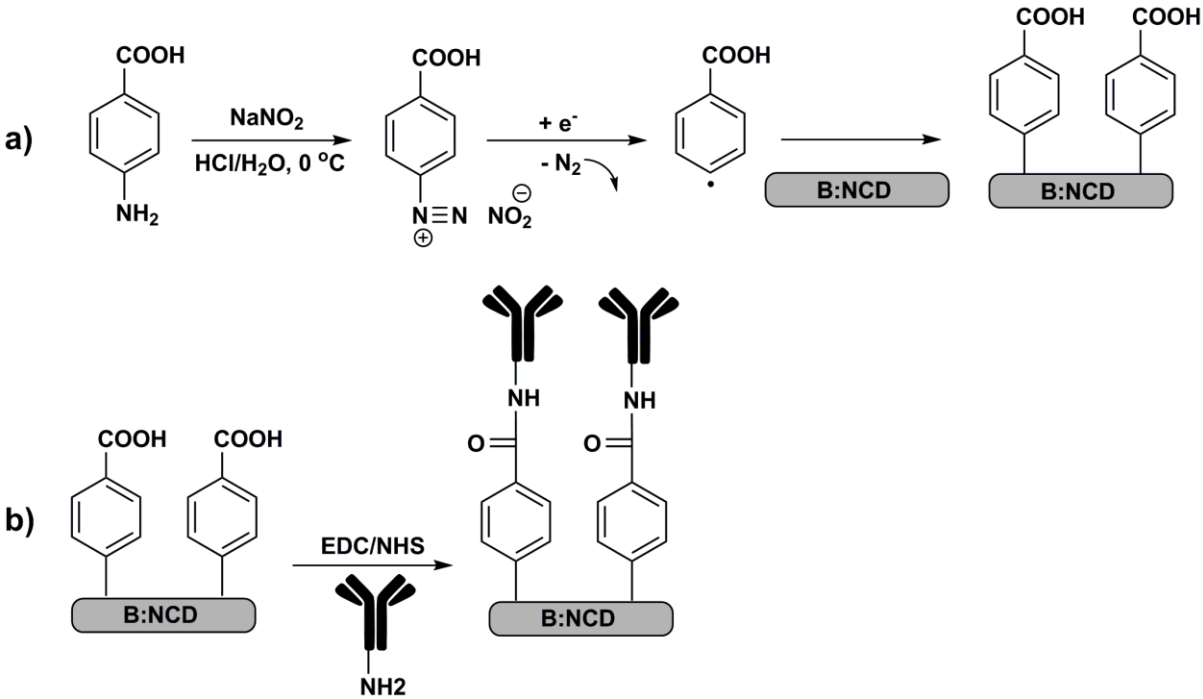
The previously described procedure was used to modify the B:NCD electrode B:NCD electrode {Citation}. 4-aminobenzoic acid (Sigma Aldrich) (10 mg, 0.073 mmol) was dissolved in 2 mL of a mixture consisting of concentrated hydrochloric acid and water (1:1). Subsequently, the obtained solution of the 4-aminobenzoic acid was cooled using an ice-water bath. After 10 minutes the further step was the dropwise addition of the 1.5 mL of the sodium nitrate aqueous solution (12.5 g,

0.1811 mmol) while stirring and subsequently exposed area of B:NCD was covered with 0.5 mL of the 4-aminobenzoic acid diazonium salt using Pt wire and Ag/AgCl electrode as the counter and reference electrode, respectively. After the deposition was complete, the cyclic voltammetry (CV) measurements in the range from 0 to -1.0 V were performed (scan rate – 100 mV/s; Figure 1). The last step was the washing of the electrodes using water and methanol and drying.

2.4.2.2. Attachment of the anti-M1 antibody to the B:NCD surface

Scheme 1 presents the method for the attachment of antibodies to the surface of B:NCD electrode. The anti-M1 antibody was covalently attached via amine group to the B:NCD electrode using the mixture of coupling agents N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Sigma Aldrich) after modification with an aminobenzoic acid diazonium salt. The mixture of EDC (0.18 mmol) and NHS (0.18 mmol) was prepared using 5 mL of DMF (dimethylformamide) with subsequent stirring for 10 min at room temperature.

The B:NCD electrode after previous modification with 4-aminobenzoic acid diazonium salt was incubated in 1 mL of coupling mixture (EDC/NHS) and subsequently in 6 µg/mL phosphate buffer solution, pH = 5.5 of the anti-M1 antibody was added to the coupling mixture containing the modified electrode. The electrodes were incubated in the mixture for 24 h at 4°C and after 4 hours were rinsed with distilled water and dried.



Scheme 1. Preparation of B:NCD electrode surface using 4-aminobenzoic diazonium salt for the attachment of antibodies (a) Electrografting of the B:NCD electrode surface – reduction of

4-aminobenzoic acid diazonium salt (b) Attachment of the antibodies to the B:NCD electrode based on the EDC/NHS chemistry.

2.4.2.3. Attachment of BSA to the B:NCD-aM1 electrode

Before utilization of the B:NCD-aM1 electrode for the biosensing purposes its free surface must be blocked with bovine serum albumin (BSA). For this purpose electrode was incubated in 40 µL of the 0.5% BSA solution in 0.1 M PBS for 1 h, at 4°C. B:NCD electrode after attachment of the antibodies on its surface and blockage with BSA becomes the sensing electrode (SE).

2.4.3. Preparation of measurements

The surface of the electrode was washed gently with 0.1 M PBS and rinsed with 100 µL of the electrolyte solution at room temperature after the incubation with various sample solutions in 4°C is finished. Thermal equilibration of the electrode with the external condition was required to initiate the electrochemical measurements. Each measurement of the sample containing electrolyte with ferricyanide redox active species was followed by washing of the electrode surface using fresh 0.1 M PBS.

2.5. Electrochemical measurements

Modified B:NCD and pristine surfaces were used as a working electrode for measurements using electrochemical impedance spectroscopy (EIS) and the cyclic voltammetry (CV). These tests were conducted using standard three-electrode assembly based on the PGSTAT302N potentiostat/galvanostat system (Methrom, Autolab, Netherlands). Sample exposed to the electrolyte had 0.1256 cm^2 in its surface geometric area and the shape was circular. In this approach Ag/AgCl/0.1M KCl was utilized as a reference electrode and Pt wire was utilized as a counter electrode.

All measurements were conducted in deaerated 1 mM $K_3Fe(CN)_6$ / 0.1 M PBS.

Optimization of the volume, incubation time, concentration of the protein in a large number of iterations was performed before utilization of the three protocols described below. The present study shows only these optimum values which exhibited fastest response time and highest sensitivity.

In case of the electrochemical impedance spectroscopy measurements (EIS) the frequency ranged from 20 kHz to 1 Hz with 50 points. The amplitude of the AC signal was 10 mV. Obtained impedance

spectra were recorded at the redox reaction formal potential (E_F). E_F value was calculated based on the redox peaks positions present on the CV voltammograms for the pristine B:NCD electrode. Each potential was held constant for 60 s before each measurement to obtain steady-state condition. Obtained data were subjected to the analysis using EIS Spectrum Analyzer according to the proposed electric equivalent circuit (EEQC). The amplitude weighting, r_a, was used for the modified Powell algorithm:

$$r_a(\omega, P_1 ... P_M) = r_c^2 / (N - M) \quad (1)$$

Where M is the number of parameters, ω is the angular frequency, $P_1 ... P_M$ are parameters, and N is the number of points.

Following formula presents the definition of the r_c:

$$r_c^2 = \sum_{i=1}^{N} \frac{\left(Z_i^{'} - Z_{i_{calc}}^{'}\right)^2 + \left(Z_i^{''} - Z_{i_{calc}}^{''}\right)^2}{Z_i^{'2} + Z_{i_{calc}}^{'\ 2}} \quad (2)$$

where N is the number of points, i_{calc} is attributed to the calculated values, and i corresponds to the impedance measured values.

Following three aspects, control measurements, M1 protein detection in very low concentrations, and measurement of the M1 protein of the viral origin (H1N1 and H3N2), they are the subjects of the experimental protocols.

2.5.1. Control measurements

B:NCD-aM1-BSA electrode was immersed in the substrate solution based on 0.1 M PBS and then subjected to tests in order to eliminate the response to unspecific interactions. The control was prepared using biological material from an oral cavity of a healthy patient.

2.5.2. Detection of the M1 protein

Incubation of the sensing electrode (SE) in the solutions with a various concentration of M1 protein was conducted to test the sensitivity of the electrode. First incubation was conducted for 30 min at 4°C using 40 µL of the solution with 50 fg/mL of the M1 protein concentration. Before next EIS measurements, the electrode was washed using 0.1 M PBS, immersed in deaerated 1 mM $K_3Fe(CN)_6$ / 0.1 M PBS, and equilibrated to the room temperature. After the measurements the electrode surface was once again washed using 0.1 M PBS and then incubated for 30 min at 4°C using 40 µL of the solution with 100 fg/mL M1 protein concentration. Subsequently, the whole experimental procedure, including washing with PBS and EIS measurements in ferricyanide solution, was performed once again after the incubation is finished. The next step were the electrochemical

measurements in order to detect the M1 protein in 40 µL of the solutions with following M1 protein concentrations: 500 fg/mL, 4 pg/mL, and 4 pg/mL. Due to this fact 650 fg/mL, 4.65 pg/mL, and 8.65 pg/mL were chosen as the total concentration of the M1 protein in the last three steps.

2.5.3. Detection of the M1 protein in viral samples

The 30 min equilibration (room temperature) of the electrode in 40 µL of 5% Triton X-100 mixture in 0.1 M PBS was performed to eliminate any response of the electrode during contact with Triton X-100. Subsequently, the wash with 0.1 M PBS was conducted. The E_F was applied to the working electrode to obtain the EIS spectra using ferricyanide solution. According to the procedure, the electrode was subsequently rinsed with 0.1 M PBS and immersed in 40 µL of H1N1 for 5 minutes. Second B:NCD-aM1-BSA electrode was utilized for the detection of the M1 protein from H3N2 virus which was suspended in the solution of 5% Triton X-100 in 0.1 M PBS. The viral solutions were vigorously shaken before incubations.

3. Results and discussion

3.1. B:NCD electrode and its characteristics

The reversible behavior of the electrode towards $[Fe(CN)_6]^{3-/4-}$ redox system is presented in Figure 2 based on the results of cyclic voltammetry studies. The pristine B:NCD electrode gave a standard reduction and oxidation current set what is related with changes in the iron oxidation state {Citation}.

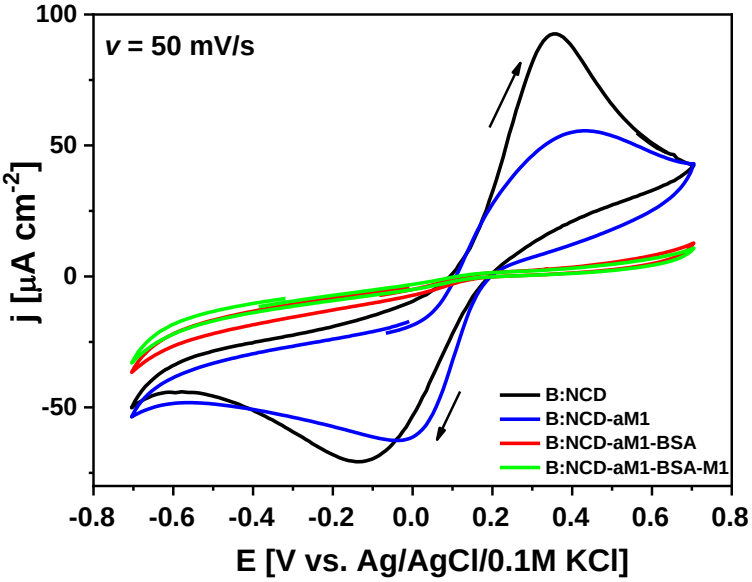


Figure 2. Curves for cyclic voltammetry obtained for modified and unmodified B:NCD electrode – 1 mM $K_3Fe(CN)_6$ / 0.1 M PBS buffer, 50 mV/s scan rate.

The obtained difference between potential peaks for oxidation and reduction is ΔE = 0.39 V. Coupling of the anti-M1 antibodies to the surface of B:NCD electrode still led to obtaining of the reversible response, however the peak current decreased while ΔE increased up to 0.57 V. Incubation of the electrode with antibodies in the BSA solution resulted in no reduction or oxidation peak – only small charging current was observed on CV curve obtained after this modification. The blockage of the electron transfer between redox active species from the solution and the electrode was the main cause of such a significant difference observed for given CV curve {Citation}. As the result, almost no significant changes were observed concerning the shape of the curve after incubation of the B:NCD-aM1-BSA electrode with M1 protein in the 50 fg/mL concentration solution. Based on these results, the cyclic voltammetry curves give the possibility to monitor the changes concerning aM1 modification and also the unspecific binding of BSA, however still these measurements cannot be accepted as the diagnostic method to confirm the protein anchoring status.

The formal potential of redox reaction for $Fe(CN)_6^{3-/4-}$, E_f = 0.13 V vs. Ag/AgCl/0.1 M KCl, was used to record the electrochemical impedance spectra for the monitoring of all electrode resistance changes occurring at each step of the electrode surface modification. Electrochemical impedance spectroscopy is accepted as the technique which is highly sensitive to any changes within the surface of the electrode. This method is commonly used to detect the interactions between antibody and antigen on the surface of the electrode, what can be utilized in the biosensor response {Citation}. Figure 3 presents the Nyquist representation of impedance spectra of unmodified B:NCD surface, and modified using anti-M1 antibodies and BSA. Additional two spectra represent the electrode after incubation in 0.1 M PBS only and the electrode after incubation with a sample subjected to dilution obtained from a swab of a healthy patient (NEG). Based on the literature reports {Citation} and shape of given spectra the analysis of the unmodified B:NCD electrode spectrum was performed using electric equivalent circuit $R_e[R_{ct}(CPE\mathrm{W})]$. This circuit contained electrolyte resistance R_e connected in series with R_{ct}, charge transfer resistance, and in parallel with *CPE*, constant phase element, and W, Warburg element (diffusional resistance). The Warburg element, however, was not utilized in the case of the modified B:NCD electrode due to the lack of the 45-degree line on the impedance spectra. Table 1 presents the values of each *EEQC* element. Normalized fitting errors were obtained on the 10^{-5} level using the fitting procedure in the optimal solution. Incorporation of the surface heterogeneity effect along and through the interface of electrode and electrolyte is achieved using constant phase element accepted as a heuristic method {Citation}. CPE impedance is characterized by $Z = Q^{-1}(i\omega)^{-n}$, where Q is *CPE* parameter, n is the exponential (c.a. 0.96 for CPE elements), and ω is the angular frequency.

Taking into account the high-frequency regime for the electrolyte resistance, the range of 390-420 Ohm was accepted as the range for the ohmic resistance element and was similar for all materials subjected to tests. The significant change in R_{ct} resistance values can be observed based on the comparison of spectra obtained for unmodified B:NCD, antibody-modified electrode, and BSA-modified electrode. This elevation of the R_{ct} can be interpreted as a successful attachment of the antibody to the surface of the pristine B:NCD electrode and subsequent attachment of BSA to the modified electrode. Coupling of the anti-M1 antibody and BSA caused the effective reduction of the charge transfer, resulting in a substantial elevation of R_{ct}, but the electrochemical response of the B:NCD-aM1-BSA electrode was not affected in a significant manner after incubation with 0.1 M PBS or biological sample. Modification of the charge transfer resistance did not exceed 8% in all five tested B:NCD-aM1-BSA electrodes comparing the R_{ct} value before and after the incubation with 0.1 M PBS or biological sample. Based on these results, such a system can be potentially utilized as the sensing electrode for the detection of given protein in the samples to be analyzed.

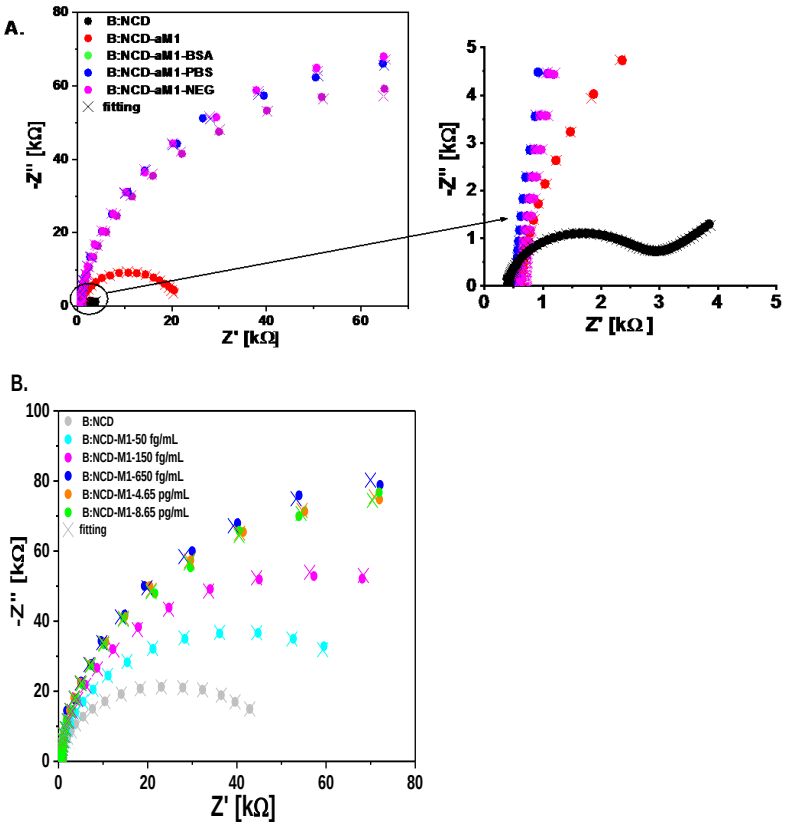


Figure 3. (A) Impedance spectra – modified and unmodified B:NCD electrodes for solutions with various concentrations. Recorded in 1 mM $K_3Fe(CN)_6$ + 0.1 M PBS at E_f = + 0.13 V *vs*. Ag/AgCl/0.1 M KCl; (B) Impedance spectra of sensing electrode (SE) in solutions with various M1 concentration. Recorded in 1 mM $K_3Fe(CN)_6$ + 0.1 M PBS at E_f = + 0.13 V *vs*. Ag/AgCl/0.1 M KCl.

Table 1. The values of the elements obtained using *EEQC* – unmodified B:NCD electrode and electrode modified with various solutions.

Sample	Re [Ω]	Q_{dl} [$\mu\Omega^{-1}s^{n}$]	n	R_{ct} [10^5 Ω]	AW [$\Omega s^{-0.5}$]
B:NCD	398.72	1.172	0.934	0.023	3147
B:NCD-aM1	405.52	1.491	0.934	0.207	0
B:NCD-aM1-BSA	399.32	1.297	0.951	1.335	0
B:NCD-aM1-BSA-PBS	418.64	1.297	0.951	1.422	6.5
B:NCD-aM1-BSA-NEGATIVE	394.05	1.290	0.951	1.437	7.5

3.2. Detection of viral particles

Two analytical procedures were proposed in the Scheme 2 based on the specific detection of the M1 protein using the sensing electrode (SE). The electrodes were incubated for 5 minutes in the samples containing viral particles. All steps of the procedures were conducted at the room temperature. Each procedure requires utilization of a new electrode. Subsequently, the particular electrodes were separately incubated with the samples containing H1N1 and H3N2 viral particles. Determination of the electrochemical characteristics was performed in 0.1 M PBS, 1 mM $K_3Fe(CN)_6$ as previously mentioned in the experimental section.

FIRST PROCEDURE (P1)

Step	Preparation of the electrode – description	Probe code	R_{ct} [MΩ]
P1.1	Blank measurement – electrode prior to incubation	1-SE	0.1762
P1.2	Incubation of the electrode in 5% Triton-X 100 in 0.1 M PBS solution – utilized for virus preparation to eliminate the possible interferences	1-SE-Triton	0.188
P1.3	Incubation of the electrode with the M1 protein obtained from the sample containing H1N1 viral particles	1-SE-Triton-H1N1	0.3911

SECOND PROCEDURE (P2)

Step	Preparation of the electrode – description	Probe code	R_{ct} [MΩ]
P2.1	Blank measurement – electrode prior to incubation	2-SE	0.16
P2.2	Incubation of the electrode with the M1 protein obtained from the sample containing H1N1 viral particles	2-SE-Triton-H3N2	0.3372

Scheme 2. Description of two analytical procedures covering the detection of the viral M1 protein with the comparison of R_{ct} (charge transfer resistance) values.

The R_{ct} obtained after control measurement (Triton X addition) was only 10% higher than the initial value obtained for SE. Additionally, one should also take into account that the shapes of spectra were unchanged. These characteristics show that solutions with given composition can be potentially used for the extraction of M1 protein from viral particles for further measurements. The enormous rise of charge transfer resistance was observed for the M1 protein present in both virus types – M1 from H1N1 sample gave 127% rise of R_{ct} comparing with the value before incubation and in case of the M1 from H3N2 sample the comparison gave 95% difference in the charge transfer resistance.

The specificity of the R_{ct} changes can be attributed to the selective protein binding on the surface of the electrode excluding any unspecific interactions, measurement errors or instabilities of the electrode. This conclusion is based on the comparison of the R_{ct} significant differences before and after incubation using solution of M1 protein and based on the minor R_{ct} value variations during control measurements.

3.3. Immunosensor sensitivity

Further verification of the modified B:NCD electrode concerning its potential use as the biosensor included incubation in the M1 protein diluted solution with measurement of the response using electrochemical impedance spectroscopy. A detailed testing protocol was presented in the experimental section. Figure 3.B shows the impedance spectra for the sensing electrode as the response for the incubation using M1 protein solutions with increasing concentrations. Figure 4 presents values of the elements utilized for the fitting procedure using *EEQC*. Only permanently attached proteins remain on the electrode surface due to the fact that after each incubation step the electrode was rinsed using fresh wash solution (0.1 M PBS). The significant disturbances in the shape of EIS spectra can be observed during incubation of the electrode with the M1 protein solutions of the following concentrations: 50 fg/mL, 150 fg/mL, 650 fg/mL. The values presented in Figure 4 show that the electrode R_{ct} value change reached 77% during contact with 50 fg/mL M1 protein solution, 145% during contact with 150 fg/mL M1 protein solution, and finally 272% during contact with 650 fg/mL M1 protein solution. All these values are compared with the electrode resistance with any M1 protein attached to the electrode surface. These changes were the results of the proceeding saturation of the active attached antibodies with M1 protein what led to the generation of the compact protein layer on the sensing electrode surface. Increased R_{ct} value was the result of the hampering of the electron transfer between electrolyte redox active species and the electrode. Saturation of the active sites by M1 proteins prevented the further changes in the resistance during incubation of the electrode in more concentrated solutions {Citation}, thus the incubation with the

M1 protein solution of 650 fg/mL concentration led to the complete blockade of all available active sites.

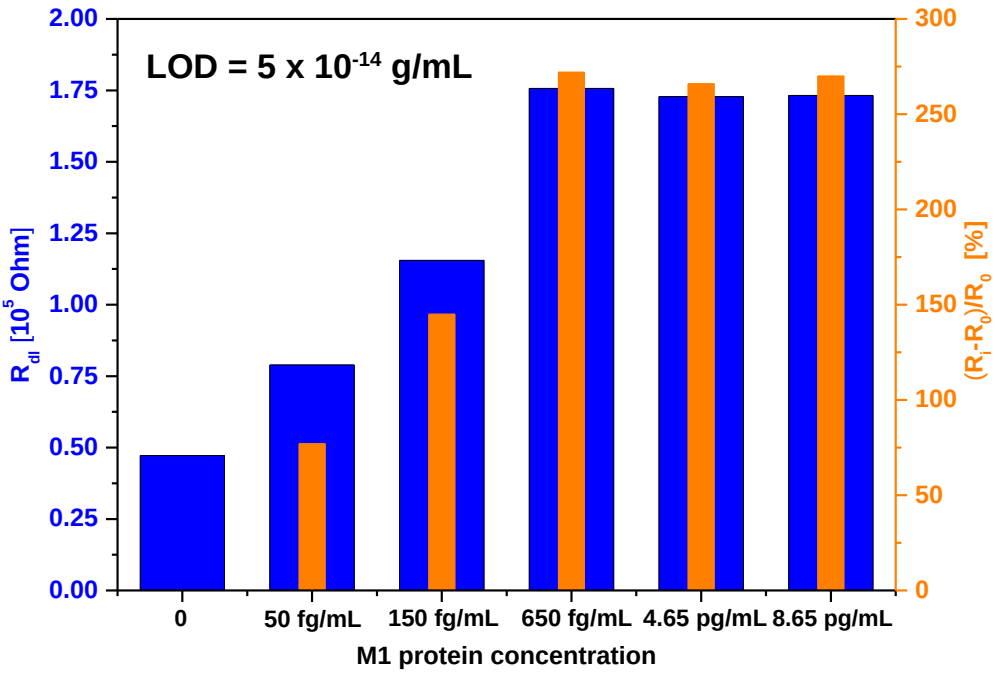


Figure 4. Values of the EEQC-calculated elements for the sensing electrode (B:NCD) subjected to incubations with M1 protein solutions of various concentrations.

4. Discussion

The techniques currently used for the detection of influenza virus, such as molecular methods, antigen capture immunoassays, virus propagation and isolation, do not offer the quality which is required for the rapid analytical detection of the influenza virus. Due to this fact, the efforts concerning control of the influenza infections focused mainly on the development of the new versatile detection platform with the ultra-high sensitivity. Currently available biosensors still experience problems concerning the limited detection range – only selected serotypes can be analyzed using these technologies {Citation}. Alternative detection methods, however, are still dependent on the sophisticated laboratory equipment which can be operated only in the specialized laboratories {Citation}. The main advantage of the immunosensor presented herein is its applicability in the non-laboratory environment, e.g. consultation room, after the development of the scale-down device.

The detection method presented in this study is suitable for the detection of all Influenza A virus serotypes due to the versatility of the detection antibodies directed against the conserved viral

antigen, M1 protein. This detection strategy is additionally based on the utilization of polyclonal antibodies which exhibit significantly higher epitope detection heterogeneity for given antigen comparing with monoclonal antibodies.

Table 2. Comparison of the influenza virus detection method used currently with the biosensing technology demonstrated in the present article.

Detection method	Limit of Detection [virions]	Time of analysis [h]	References
BDD-click chemistry	20	0.6	{Citation}
Real-time PCR	10	3	{Citation}
Virus Isolation	10	48	{Citation}
Reverse transcriptase PCR	100	4	{Citation}
ELISA test	1000	5	{Citation}
EIS at Au electrode	1000	1	{Citation}
Rapid tests	1000	1	{Citation}
B:NCD impedance	250	0.1	Present study

This results in lower risk of obtaining the false negative results. The electrochemical impedance spectroscopy (EIS) is one of the most effective methods for the detection of interactions between the antigen and the antibody. EIS is the well-understood method and offers both high sensitivity and simplicity of the analysis {Citation}. Additionally, there are few examples of biosensors developed previously which successfully utilize given technology {Citation}. Attachment of the antibodies directly to the surface of the electrode is the most appropriate and simplest method for the present application. The implementation of this detection method is also easier in case of the design process for given biosensor. Additionally, the scale-down of the device will be much easier in case of this technology. The whole analysis is planned to be finished within 5 minutes without utilization of any complicated, time-consuming, and laborious procedures such as obtaining of the genomic material from the previously isolated viral particles. This procedure also avoids cumbersome sample preparation steps – only saliva sample or throat swab is needed for the analysis and whole sample preparation procedure can be performed in less than a minute. Sample taken from the patient is just suspended in a buffer and shaken. The detection using the biosensor strategy gives the extremely high sensitivity and specificity (virions/mL order of magnitude) comparable with the sensitivity parameters of the alternative molecular detection methods.

5. Conclusions

We can hereby conclude that the new label-free method for detection of the M1 influenza virus protein offering high sensitivity and simplicity has been developed. The electrode utilized in this method is based on the B:NCD-substrate approach including the chemical modification of its surface to facilitate the anti-M1 antibody attachment via covalent bonds. The characteristics of this biosensor scaffold, such as low background current, inherent biocompatibility, large potential window, and extremely high stability, were the key factors which underlay its selection. The detection protocol was carefully prepared taking into account variables in the electrode response in the case of the various protein concentrations, incubation time, non-specific interactions, and temperature conditions.

A significant charge transfer resistance change was recorded after the electrode was incubated with a sample containing real viruses. However, no specific response was observed after incubation with PBS solution, Triton X-100/PBS solution, or a negative biological sample obtained from a healthy individual. Based on these results the modified B:NCD substrate can be accepted as a promising biosensing electrode. The tests performed herein confirmed the universality and specificity of selected antibodies for given approach. This, in turn, proves that given detection system can be used for the reliable detection of influenza virus particles on a 50 fg/mL concentration level. Having regard to the short incubation time, low detection limit, and recommended room temperature conditions, the electrode described herein can be accepted as the valuable biosensor designed for the influenza virus detection.

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References

{Citation}