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Quantitation of influenza A virus in the presence of extraneous protein using electrochemical impedance spectroscopy

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ABSTRACT

In an attempt to provide an insight into detection of specific viruses in biological samples, we report on quantitation of influenza A virus (IAV) in samples containing large amounts of extraneous bovine serum albumin (BSA), foetal bovine serum (FBS) and hepatitis B virus (HBV) vaccine. Detection was carried out using electrochemical impedance spectroscopy (EIS) with an antibody-neutravidin-thiol architecture immobilized on the surface of an Au electrode. A linear response of the EIS signal was observed for IAV concentrations ranging from 0 to almost 64 ng/mL. However, saturation of the EIS response was recorded for greater concentrations and only a 6% increase in signal occurred when the IAV concentration increased to 128 ng/mL. The limit of detection, determined at 8 ng/mL, remained relatively unaffected by the environment composed of 50 ng/mL of HBV and 12.5% of FBS.

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1. Introduction

Conventional methods of virus detection are based on isolation in culture and subtype identification by immunoassays. An enzyme-linked immunosorbent assay (ELISA) technique allows detection of about 1 ng of the H5 subtype of the virus [1] and 7 ng/mL of the vaccine H3N2 virus [2]. The detection of influenza A virus (IAV) at 10 and 50 copies has been reported using, respectively, individual and multiplex polymerase chain reaction (PCR) assays [3]. Although PCR methods allow for a relatively rapid detection of the virus in comparison to culture, they require the presence of specialized personnel, and are thus limited to a hospital, public health or research laboratory. A significant effort has been directed worldwide to develop alternative techniques of biosensing that could be used at point-of-care, medical practitioners' offices and in remote locations to help detect rapidly the presence of influenza virus. Examples of devices investigated for rapid identification of IAV include a microgravimetric and a quantum dot photonic immunosensors that allow detection of IAV particles at 30 ng/mL from aerosolized [4] and liquid samples [5,6]. A label-free detection of 0.5 nM of IAV DNA probes has also been reported with a conductometric multi-wall carbon nanotube sensor [7]. Using (1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide (EDC) and N-hydroxy succinimide (NHS) to fix the COOH groups of the thiol that

facilitates the attachment of the polyclonal antibody, the detection sensitivity of IAV has been reported at 5 µg/mL level using electrochemical impedance spectroscopy (EIS) measurements [8].

Most of the devices reported in the literature for biosensing applications have not been tested with real biological samples, and thus their practical value remains difficult to ascertain. In that context, it is important to carry out detection of the virus in biological fluids or those mimicking real samples. This would allow establishment of benchmarks useful in the evaluation of different biosensing devices before their introduction to the clinical environment [9–11]. We address this important problem using an EIS technique that is considered a matured technology suitable for medical, industrial and environmental analysis [12,13], even though it has not been implemented in commercial devices primarily due to its relatively slow response.

In this paper we discuss the application of the EIS technique for the detection of IAV (H3N2) mixed with different concentrations of bovine serum albumin (BSA) and foetal bovine serum (FBS) diluted in phosphate buffered saline (PBS) solutions. In addition, Hepatitis B virus (HBV) vaccine has been used for negative and selectivity tests.

2. Experimental details

2.1. Biological and chemical materials

Polyclonal antibodies against IAV coupled with biotin were obtained from ViroStat, Inc. (Portland, Maine). IAV inactivated by

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γ -radiation, with a storage concentration of 4 mg/mL, was purchased from Microbix Inc. (Toronto, Canada). Biotinylated thiol HS-(CH₂)₁₁-NH-C(O)-biotin was obtained from Prochimia Surfaces (Gdansk, Poland) and the 1-Octanethiol HS-(CH₂)₇-CH₃ was bought at Sigma-Aldrich (Oakville, Canada). Neutravidin was obtained from Molecular Probes Invitrogen (Burlington, Canada). Hepatitis B vaccine (Merck FROSST, Kirland, QC, Canada), with a storage concentration of 10 μ g/mL, was obtained from the Agence de sant e et de services sociaux de l'Estrie (Sherbrooke, Quebec). The BSA powder and liquid FBS were obtained from Sigma-Aldrich (Oakville, Canada) and Wisent (St Bruno, Canada), respectively. Disodium hydrogen phosphate and sodium chloride were obtained, respectively, from EM Chemical and EM Science (Gibbstown, USA). Potassium chloride was bought from Anachemica (Montreal, Canada), potassium dihydrogen phosphate and sodium hydroxide, both were bought from Sigma-Aldrich (Oakville, Canada) and used to prepare PBS, consisting of 1.8 mM KH₂PO₄, 0.1 mM Na₂HPO₄, 140 mM NaCl and 2.7 mM KCl, pH 7.0. All were analytical grade reagents (>99%).

2.2. Electrode and bio-sensing architecture

Gold disk electrodes with a radius of 3 mm obtained from CH Instruments (Austin, TX, USA) were polished with aluminum oxide particles of 5 μ m diameter followed by sonication in water, then polished with 0.25 μ m aluminum oxide particles followed by sonication in water, and finally polished with a soft pad followed by sonication in water. Cleaning efficiency was controlled by cyclic voltammetry.

The cleaned Au electrodes were immersed for 16 h in a mixture of 0.5 mM octanethiol and biotinylated 0.1 mM dodecanethiol in ethanol. The non-biotinylated thiol was applied as a spacer allowing to increase the efficiency of the neutravidin-biotin interaction. The thiol-modified electrode was incubated for 2 h in 200 μ g/mL solution of neutravidin to fix the neutravidin to the thiol layer via the strong biotin-neutravidin affinity [14,15]. The neutravidin layer allowed us to immobilize the biotinylated IAV antibody after incubation of the modified electrode in a 100 μ g/mL solution of antibody. This step was carried out at 4 $^{\circ}$ C, typically for 12–16 h. Different concentrations of the inactivated IAV were analysed by incubation of the functionalized Au electrode in the corresponding viral solutions. The same IAV antibody architecture was also

used to test HBV solutions with concentrations, N_{HBV} , ranging from 10 to 100 ng/mL. Three solutions were obtained by adding HBV at 50 ng/mL to 16, 64 and 128 ng/mL IAV solutions. In addition, we prepared IAV solutions ($0 \leq N_{\text{IAV}} \leq 32$ ng/mL) with 50 ng/mL of HBV containing either 2 or 4 mg/mL of BSA, or 12.5% FBS (containing 8 mg/mL of BSA, as well as some other proteins) in PBS in order to investigate samples mimicking biological environments. The biofunctionalization of Au electrodes and exposure to IAV samples were carried out in 1.5 mL volume eppendorf tubes. To determine standard deviation of Z_{Real} measured by the EIS technique for each tested sample, a series of at least 3 measurements were completed for separately biofunctionalized Au electrodes.

2.3. Electrochemical setup

Cyclic voltammetry measurements were performed in a 5 mM solution of redox couple [Fe(CN)₆]^{4-/3-} prepared in PBS solution. The scanning potential was changed in the range of ± 700 mV. Cyclic voltammetry measurements were carried out to verify the thiolation efficiency of the electrode gold surface.

We used a classical three-electrode electrochemical impedance spectroscopy (EIS) system comprising an Au working electrode, a platinum counter electrode, and a saturated calomel reference electrode. The impedance analysis was performed with a CH Instrument impedance analyser in the frequency range of 100 mHz to 100 kHz under a potential value of -200 mV. Measurements were performed in PBS buffer, pH 7. The measurements of the Z_{Real} value corresponding to the antibody level (reference) were repeated 3 times. They indicated reproducibility with a standard deviation not exceeding $0.008 \times 10^5 \Omega$ (Table 1). The antibody functionalized Au electrode was exposed to viral test solutions for 1 h at room temperature. This was followed immediately by the EIS measurements. All electrochemical measurements were carried out at room temperature and in a Faraday cage. The measurements carried out for each sample were repeated 3 times using separately prepared (functionalized) Au electrodes.

The error in determining the IAV concentration in samples mimicking a biological environment, δN (ng/mL), is defined as a difference between the concentration corresponding to the measured Z_{Real} value and the concentration that corresponds to the same Z_{Real} on the linearly fitted curve for the homogeneous fluid.

Table 1

Z_{Real} variation corresponding to different N_{IAV} and concentration errors (δN) determined in the presence of extraneous protein.

Virus concentrations (ng/mL)	0	4	8	16	24	32	64	128
Z_{Real} variation for IAV (10^2 k Ω)	0 ± 0.008	–	0.23 ± 0.01	0.35 ± 0.05	0.51 ± 0.008	0.65 ± 0.016	1 ± 0.1	1.06 ± 0.11
Z_{Real} variation (10^2 k Ω) IAV + 50 ng/mL HBV	–	–	–	0.32	–	–	1.1	1.1
δN (ng/mL)	–	–	–	0	–	–	0	0
Z_{Real} variation (10^2 k Ω) IAV + 50 ng/mL HBV + 2 mg/mL of BSA	0.06 ± 0.03	–	–	0.19	0.5	0.76	0.88	0.90
δN (ng/mL)	–	–	–	–7	–0.15	4.5	–	–
Z_{Real} variation (10^2 k Ω) IAV + 50 ng/mL HBV + 4 mg/mL of BSA	0.082 ± 0.024	–	–	0.66 ± 0.11	0.65 ± 0.05	0.71 ± 0.04	1.15	1.05
δN (ng/mL)	–	–	–	15.5	6.4	2	–	–
Z_{Real} variation (10^2 k Ω) IAV + 50 ng/mL HBV + 12.5% FBS	0.0045 ± 0.0007	0.014 ± 0.001	0.358 ± 0.052	0.402 ± 0.017	0.582 ± 0.005	0.73 ± 0.04	–	–
δN (ng/mL)	–	–	9	3	3.5	2.5	–	–

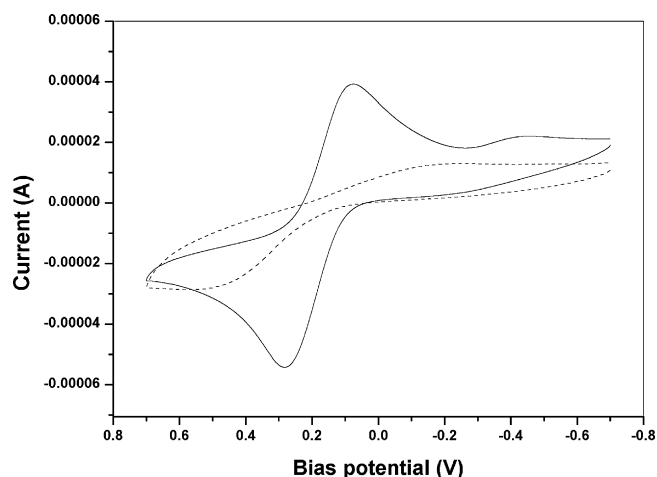


Fig. 1. Cyclic voltammogram of a bare Au electrode (continuous line) and a thiol-modified Au electrode (broken line).

3. Results and discussion

3.1. Cyclic voltammetry

Fig. 1 shows the cyclic voltammograms of the Au electrode before and after thiolation. The pre-treated Au electrodes had a symmetrically reversible voltammograms indicating a clean Au surface, with two peaks representing the oxidation–reduction potential of the Au electrode. In contrast, the electrode modified by thiol showed a quasi-flat voltammogram in which oxidation–reduction peaks were suppressed almost entirely. This indicates that the thiol monolayer acts as a barrier to electric charge transfer, confirming also the high surface coverage of the electrode. The thiol coverage of Au electrodes, θ , was investigated using the following equation [16]:

$$\theta = 1 - \frac{R_1}{R_1^*}$$

where R_1 and R_1^* is the resistance of the bare and thiol functionalized Au electrode, respectively. This allowed us to estimate that the fractional thiol coverage of Au electrodes investigated in this study was always greater than 85%.

3.2. EIS measurement in homogeneous fluids

Fig. 2 presents experimental data (lines plotted to guide the eye) of the Nyquist plots measured for the Au electrode exposed to the IAV antibody (curve 'a') and the antibody functionalized electrode exposed to solutions containing different IAV concentrations (curves 'b' to 'g'). As expected, in all cases the EIS spectra are represented by semi-circle curves. The attachment of viral particles to the functionalized Au surface leads to increased electrical impedance of the investigated electrochemical circuit, which is reflected by the increased diameter of the collected plots. The values of the real part of an impedance (Z_{Real}) measured at 100 mHz are shown by full triangles in Fig. 3 for each viral concentration. It can be seen that the method allows detection of 8 ng/mL of IAV ($Z_{\text{Real}} = 0.23 \times 10^5 \Omega$). A linear response characterizes the signal measured for viral concentrations between $0 \leq N_{\text{IAV}} < 64$ ng/mL, as indicated by the solid line obtained by the least-squares fitting procedure. The results also demonstrate that the signal at 64 ng/mL is near its saturation as there is only a 6% increase in the Z_{Real} value observed for the concentration increasing from 64 to 128 ng/mL.

The results of a negative detection test involving the exposure of the IAV antibody functionalized Au electrode to HBV diluted in

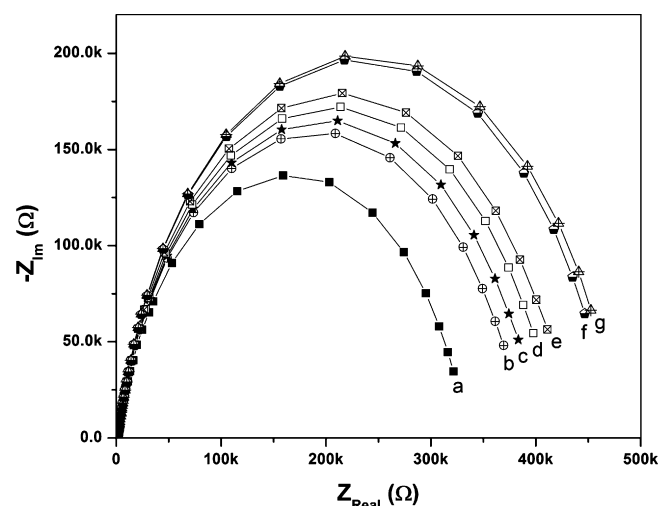


Fig. 2. Impedance spectra (Nyquist plots) measured for Au electrode with thiol/neutravidin/antibody (a), and antibody functionalized Au electrode exposed to IAV at 8 ng/mL (b), 16 ng/mL (c), 24 ng/mL (d), 32 ng/mL (e), 64 ng/mL (f) and 128 ng/mL (g). Solid lines are plotted to guide the eye.

PBS of up to 100 ng/mL are presented in Fig. 3 by full squares. This test revealed negligible EIS signal for all studied HBV concentrations. Thus, it is consistent with the expected specificity of the IAV antibody.

3.3. EIS measurement in samples mimicking a biological environment

The response of the sensor to solutions comprising 50 ng/mL of HBV and different concentrations of IAV is described in Fig. 3 by the open triangle-down symbols. For the nominal concentration of IAV at 16 ng/mL, the Z_{Real} value in this case is $0.32 \times 10^5 \Omega$, which compares to $Z_{\text{Real}} = (0.35 \pm 0.05) \times 10^5 \Omega$ measured for the homogeneous fluid. It appears that the addition of 50 ng/mL of HBV to the IAV solution does not influence the Z_{Real} value obtained for the pure IAV solution. Thus, the error in the estimation of the real concentration of IAV in this case, $\delta N = 0$. The results for solutions of IAV and 50 ng/mL of HBV mixed with 2 and 4 mg/mL of BSA and with 12.5% of FBS are shown in Fig. 3 with open circles, open triangles and open square symbols, respectively. Based on the Z_{Real} vs N_{IAV} linear fit obtained for the homogeneous fluid at $0 \leq N_{\text{IAV}} < 64$ ng/mL, we have estimated the error δN in determining the concentration of IAV in heterogeneous (simulating biological samples) solutions. This error is indicated in Fig. 3 with horizontal thin-line bars. For the clarity of this figure, no statistical errors of the measured Z_{Real} values have been indicated for most of the cases. Those errors and numeric details of the analysis are summarized in Table 1. For instance, measurements carried out for the IAV solutions comprising 16 ng/mL of IAV mixed with 50 ng/mL of HBV in 2 mg/mL of BSA yielded concentrations of IAV underestimated by 7 ng/mL, and those for the IAV solutions comprising 16 ng/mL of IAV mixed with 50 ng/mL of HBV in 4 mg/mL overestimated by 15.5 ng/mL. For greater IAV concentrations, the error δN resulting from carrying out quantitation in solutions comprising same protein concentrations did not exceed 6.4 ng/mL. This compares with an error reduced to 2.5–3.5 ng/mL measured for samples with nominal IAV concentrations of 16, 24 and 32 ng/mL mixed with 50 ng/mL of HBV and 12.5% of FBS. We also recorded the successful detection 8 ng/mL of IAV in this protein-rich environment. However, the result in that case was overestimated by 9 ng/mL compared to the detection of IAV in a homogeneous solution. This represents a three order of magnitude improvement in detection sensitivity of influenza virus in

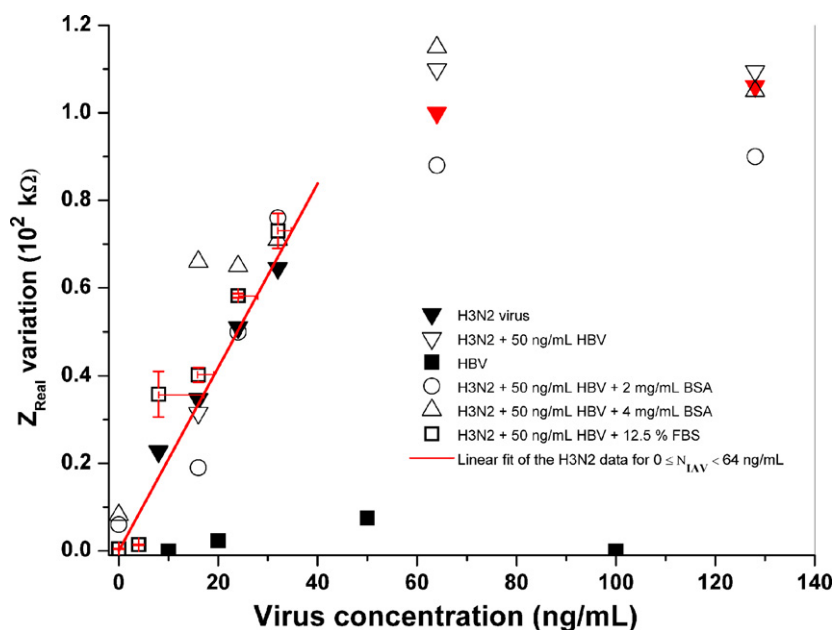


Fig. 3. Dependence of the Z_{Real} impedance variation on the concentration of IAV in pure IAV–PBS solutions (\blacktriangledown), mixtures of IAV with 50 ng/mL of HBV (∇), mixtures of IAV with 50 ng/mL of HBV containing 2 mg/mL of BSA (\circ), mixtures of IAV with 50 ng/mL of HBV containing 4 mg/mL of BSA (\triangle), mixtures of IAV with 50 ng/mL of HBV containing 12.5% of FBS (\square) and on the concentration of the HBV (negative test) in pure HBV–PBS solutions (\blacksquare). The solid line is a result of the least-squares fitting procedure applied to $0 \leq N_{\text{IAV}} \leq 32$ ng/mL in PBS solutions.

comparison to that reported with other EIS experiments [8]. The exact reason for this difference is not clear. It seems possible that the EDC-NHS procedure applied in [8] to activate thiols for the antibody attachment is less efficient for the virus immobilization than the neutravidin-biotinylated antibody architecture applied in this work. Our results suggest that the applied biosensing architecture and the EIS technique provide a satisfactory means for quantitation of IAV in the presence of extraneous protein. Nonetheless, it is feasible to expect that further improvement in both sensitivity and selectivity could be obtained using biosensing architectures comprising monoclonal IAV antibody.

4. Conclusion

To address the functioning of antibody-based biosensing architecture in the presence of large amounts of non-target protein, we investigated selectivity and sensitivity of the detection of IAV in solutions of PBS with HBV, BSA and FBS. Our results provide evidence that the electrochemical impedance spectroscopy technique is an efficient approach for detection of IAV virus in samples mimicking biological environments, as defined in this experiment, with a detection limit of 8 ng/mL. Further improvement in both sensitivity and specificity of this approach is expected for bio-architectures comprising monoclonal IAV antibodies.

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