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Regular paper

Ultrasensitive electrochemical genosensor for direct detection of specific RNA sequences derived from avian influenza viruses present in biological samples*

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An electrochemical genosensor based on an epoxy-phenanthroline–Fe(III)–NH_2-ssDNA layer for the detection of RNA derived from Avian Influenza is presented. The biosensor preparation consists of: (I) modification of gold electrodes with aminoethanethiol, (II) modification of the self-assembled monolayer of aminoethanethiol with 5,6-epoxy-5,6-dihydro-[1,10]-phenanthroline using "click" chemistry, (III) a first step of complexation of Fe(III) by 5,6-epoxy-5,6-dihydro-[1,10]-phenanthroline, (IV) a second step of complexation of Fe(III) by 5,6-epoxy-5,6-dihydro-[1,10]-phenanthroline, (V) immobilization of the single stranded amino-DNA probe via "click" chemistry between epoxy and amino groups. The interactions between the ssDNA probe and RNA targets were explored with Osteryoung Square Wave Voltammetry. The genosensor showed a remarkable detection limit of 3 copies/μL (5 aM) for RNA extracted from A/swan/Poland/305/06 (H5N1) containing a fully complementary sequence. A linear dynamic range for this sequence was observed from 3.0×10³ to 3.0×10⁵ [copies/μl]. RNA extracted from A/mallard/Poland/446/09 (H7N7), containing a non-complementary sequence, generated a much weaker response. Moreover, the developed genosensor allows to distinguish RNA present in biological samples having 2, 3 and 4 mismatches. This biosensing approach can become a potential alternative tool for detecting RNA samples in biomedical research and early clinical diagnosis of avian influenza viruses.

Key words: RNA biological samples, redox-active layer, electrochemical genosensor, square wave voltammetry, mismatched base pairs

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Abbreviations: AuE, gold electrode; AET, 2-aminoethanethiol hydrochloride; AN, acetonitrile; PBS, phosphate buffer saline; Epoxy-Phen, 5,6-epoxy-5,6-dihydro-[1,10]-phenanthroline; NH_2-ssDNA, amino-terminated single stranded DNA probe; OSWV, Osteryoung Square Wave Voltammetry

INTRODUCTION

Avian influenza (AI) is a contagious disease caused by highly pathogenic (HP) viruses, especially type A viruses H5 and H7. Globally, HP-AIVs cause huge economic losses and serious risks to human health. Hundreds of millions of domestic fowl died as a result of infection and slaughter to control the escalation of the epidemic (Śmietanka *et al.*, 2017). In addition, the virus poses a risk of transferring to humans. It is extremely important to provide a device capable of detecting potential outbreaks of avian influenza viruses in an easy, fast and specific way (Fisher *et al.*, 2007; Śmietanka & Minta, 2014; Li *et al.*, 2017).

The development of DNA biosensors has become a field of great interest and is used in various areas such as medical diagnostics, environmental control, food industry, forensics and pharmacy (Bahadır & Sezgintürk, 2015; Mukama *et al.*, 2017). Genosensors are based on a highly biospecific interaction between single-stranded DNA (ssDNA, DNA probe) immobilized on a solid substrate surface and complementary DNA or RNA sequence present in the sample solution. Especially electrochemical genosensors are very promising as devices suitable for point-of-care diagnostics or multiplexed platforms for rapid, simple and inexpensive research of nucleic acids (NA) (Shojaei *et al.*, 2014; Radecka & Radecki 2015, 2016; Mehrotra, 2016; Abi *et al.*, 2018). Although significant progress has been made in the past few years, the performance of genosensors in biological samples has been appraised in only a small part of the published research papers (Paniel *et al.*, 2013; Manzanares-Palenzuela *et al.*, 2017; Ozkan-Ariksoysal *et al.*, 2017).

Various types of DNA biosensors for detecting avian influenza viruses are widely discussed in the scientific literature. Recent review papers show a wide variety in the development of genosensors, from the simplest to the most complex multi-step procedures (Li *et al.*, 2017; Tosar *et al.*, 2017; Abi *et al.*, 2018).

In recent years, our laboratory has developed several DNA biosensors for AIV detection, which are very promising for diagnostic purposes. These tools were capable of detecting not only short sequences of ssDNA, but also double-stranded PCR products (ca. 180 bp) and RNA transcripts (ca. 280 nt) containing regions complementary to the probe (Malecka *et al.,* 2012, Malecka *et al.,* 2013, Malecka *et al.,* 2015, Malecka *et al.,* 2016; Grabowska *et al.,* 2013, Grabowska *et al.,* 2014a, Grabowska *et al.,* 2014b; Kurzątkowska *et al.,* 2015; Kaur *et al.*, 2018).

This work demonstrates the electrochemical detection of RNA extracted from influenza viruses and the verification of the genosensor selectivity using viral RNAs with a different number of mismatched base pairs in the

Table 1. Strains of avian influenza viruses, which were used for hybridization processes.
Total RNA was extracted from the indicated virus strains. The probe was 100% complementary to the RNA segment encoding the C-terminal part of HA1 hemagglutinin (HA) domain of the RNA1 sample, which was isolated from the A/swan/Poland/305/06 (H5N1). The mismatches of the probe to the other RNA samples are bolded and underlined. The accession numbers of the nucleotide sequences of the respective HA regions are provided.

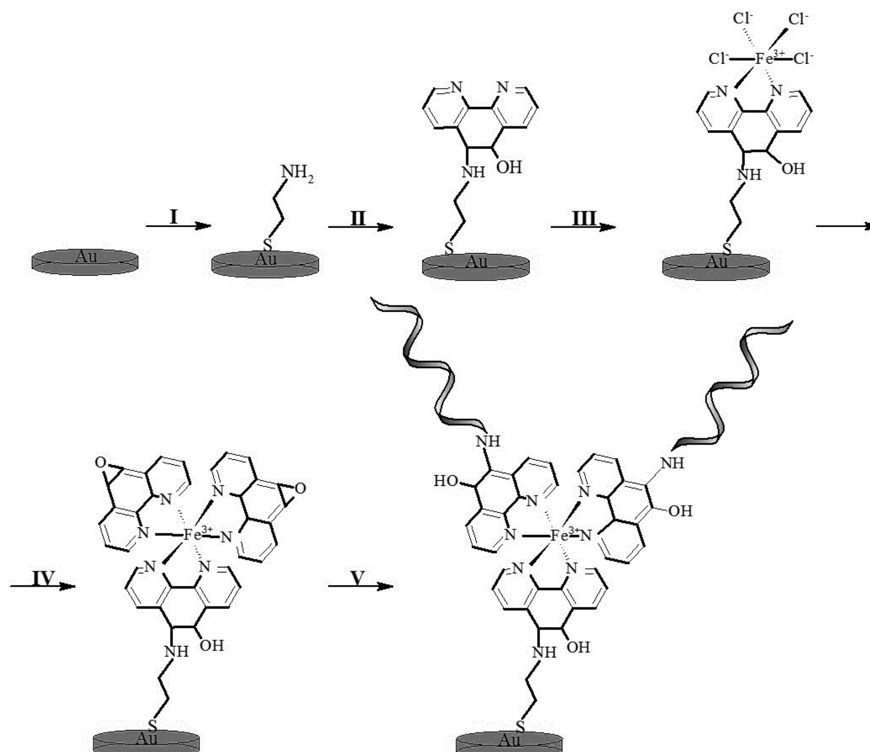
Sample	Name of the virus Sequence complementary to the probe (3'-5')	Accession number of the HA nucleotide sequence (GISAID Database)
RNA1	A/swan/Poland/305/06 (H5N1) GGAGUUCCUCUCUCUUCUUC	EPI156789
RNA2	A/turkey/Poland/R3249/07 (H5N1) GGAGUCC**C**CU**U**UCUUCUUC	EPI171604
RNA3	A/graylag goose/Poland/74/10 (H5N2) GGAGUU**U**CUCUCU**G**UUCU**C**C	EPI837534
RNA4	A/mallard/Poland/175/11 (H5N3) GG**G**GUU**U**CUCUCU**G**UUCU**C**C	EPI837712
RNA5	A/mallard/Poland/446/09 (H7N7) negative reference	EPI254381

region complementary to the probe. The electrochemical genosensor was developed based on the epoxy-phenanthroline–Fe(III) electroactive layer (Malecka *et al.*, 2015).

MATERIALS AND METHODS

Reagents and biomaterials. 2-aminoethanethiol hydrochloride (AET), acetonitrile (AN), iron chloride (III), and phosphate buffer saline (PBS) components (10 mM KH_2PO_4, 1.8 mM Na_2HPO_4, 137 mM NaCl and 2.7 mM KCl) were supplied by Sigma-Aldrich. Alumina slurries (0.3 and 0.05 μm) were obtained from Buehler (USA). Ethanol (EtOH), hydrogen peroxide (HP), methanol (MeOH), potassium hydroxide and sulfuric acid were purchased from POCh (Poland). 5,6-Epoxy-5,6-dihydro-[1,10]-phenanthroline (Epoxy-Phen) was synthesized by the group of Prof. Wim Dehaen from University of Leuven (Belgium). The modified oligonucleotide NH_2-ssDNA (5'-NH_2–$(CH_2)_6$–CCT CAA GGA GAG AGA AGA AG-3'), which was used as a probe to be attached to a surface of a gold electrode, was synthesized by Biomers (Germany).

The RNA samples were obtained from the Department of Poultry Diseases of National Veterinary Research Institute in Puławy (Poland). The influenza viruses were grown in the infected chicken embryos. RNA was extracted from viral particles present in allantoic fluid using the commercial Syngen Viral Mini Kit.



Scheme 1. Schematic representation of the genosensor preparation.

Table 2. Values of the $\Delta I=I_n-I_0$ [μA] and standard deviations (S.D.) for a given concentration of tested RNA samples (I_n is the value of the peak current measured in the presence of the RNA target and I_0 is the value of the peak current in pure PBS (before applying the analyte)).

Sample	C [copies/μL]	$\Delta I=I_n-I_0$ [μA]	S.D.
RNA1	3000	–0.054	0.006
	30000	–0.096	0.005
	300000	–0.137	0.006
RNA2	100	–0.032	0.007
	1000	–0.071	0.003
	10000	–0.100	0.004
RNA3	1000	–0.025	0.004
	10000	–0.052	0.006
	100000	–0.089	0.003
RNA4	400	–0.019	0.002
	4000	–0.047	0.001
	40000	–0.080	0.002
RNA5	500	-–0.012	0.002
	5000	–0.034	0.005
	50000	–0.061	0.004

The following RNA samples were analyzed: RNA1 – containing a region with full complementarity to the NH_2-NC3 DNA probe; RNA2, RNA3 and RNA4 – containing two, three and four mismatches in that region, respectively, RNA5 – the control RNA from different virus subtype used as a negative reference (Table 1).

The total RNA concentration in the sample was determined spectrophotometrically by measuring the absorbance at a wavelength of 260 nm on a Nanodrop spectrophotometer (Thermoscientific, USA).

The attachment of the NH_2-ssDNA probe was carried out in acetonitrile solution (AN). The hybridization processes were carried out in PBS pH 7.4 containing 10 mM KH_2PO_4, 1.8 mM Na_2HPO_4, 137 mM NaCl and 2.7 mM KCl, prepared with sterile, nuclease free water supplied by Sigma-Aldrich. All aqueous solutions used for the pretreatment of the gold electrodes were prepared using autoclaved Milli-Q water, with a resistivity of 18.2 $M\Omega \cdot cm^{-1}$ (Millipore Corporation, USA). Reagents and solvents were of analytical grade and used without further purification. All experiments were carried out at room temperature (rt).

Preparation of the genosensor. The detailed description of the genosensor preparation is included in our previous article (Malecka *et al.*, 2015). Briefly, it consists of the following steps: (I) modification of gold electrode surface with AET; (II) attachment of Epoxy-Phen to self-assembled AET monolayer using "click" chemistry; (III) the first step of complexation of Fe(III) by Epoxy-Phen; (IV) the second step of complexation of Fe(III) by Epoxy-Phen; (V) immobilization of single stranded NH_2-DNA probe (5'-NH_2–$(CH_2)_6$ – CCTCAAGGAGAGAGAAGAAG-3') *via* "click" chemistry reaction (Scheme 1).

Hybridization processes. The RNA targets were diluted in the PBS hybridization buffer (pH 7.4) according to Table 2.

10 μL of variable concentrations of RNA target solutions in PBS pH 7.4 were dropped on the surface of gold electrode modified with AuE/AET/Fe(III)(Epoxy-Phen)$_3$/NH_2-ssDNA redox-active layer for 30 minutes at room temperature. Then, the electrodes were flushed with 5 mL of PBS, pH 7.4 in order to remove the unbound targets.

The hybridization processes were controlled using Osteryoung Square Wave Voltammetry (OSWV).

Electrochemical measurements. All electrochemical measurements were carried out with the AutoLab potentiostat–galvanostat (Eco Chemie, Utrecht, Netherlands) with a three-electrode configuration. Potentials were measured versus the Ag/AgCl (saturated 3M KCl) electrode, and a platinum wire was used as the auxiliary electrode. Voltammetric measurements were performed in an electrochemical cell of 5 mL volume. In the OSWV technique, a potential window from +0.4 V to –0.15 V, a step potential of 0.001 V, square-wave frequency of 25 Hz and amplitude of 0.05 V were applied.

All measurements were carried out in PBS buffer deaerated with nitrogen for 15 min. During all measurements, a gentle nitrogen flow over the sample solution was also used.

Electrode responses are expressed as: $\Delta I=(I_n-I_0)$ [μA], where I_n is the peak current value measured in the presence of the RNA target and I_0 the peak current value in pure PBS (before applying the analyte).

RESULTS AND DISCUSSION

Preparation of the genosensor – successive steps of electrode modification

The successive steps of the genosensor preparation are shown in Scheme 1. A detailed description of the preparation of this biosensor based on the AuE/AET/Fe(III)(Epoxy-Phen)$_3$/NH_2-ssDNA redox-active layer is presented in our previous paper (Malecka *et al.*, 2015). However, in contrast to the previous work, here the genosensor was used to analyze RNA extracted directly from the biological material, which were influenza viruses amplified in chicken embryos. RNA was extracted from four avian influenza viruses (Table 1). Not only complementary (RNA1) and non-complementary (RNA5) sequences were tested, but also RNA with 2, 3 or 4 mismatches within the target region.

Detection of complementary RNA in biological samples

The genosensor selectivity and sensitivity were determined using five RNA samples, with full (RNA1), partial (RNA2, RNA3, RNA4) or without (RNA5) complementarity to the 20-mer NH_2-ssDNA probe. Representative OSW voltammograms recorded in the presence of RNA1 and RNA5 are shown in Fig. 1A and 1B, respectively. Upon increasing the concentration of RNA, a decrease of the peak current was observed. The strongest signal was generated by RNA1 (without mismatches), while the weakest signal was observed in case of RNA5 used as a negative reference.

A linear dynamic range for RNA1 was established from 3.0×10^3 to 3.0×10^5 RNA copies/μL. Limits of detection (LOD) were calculated according to the equation:

$$LOD = \frac{3.3\sigma}{S}$$

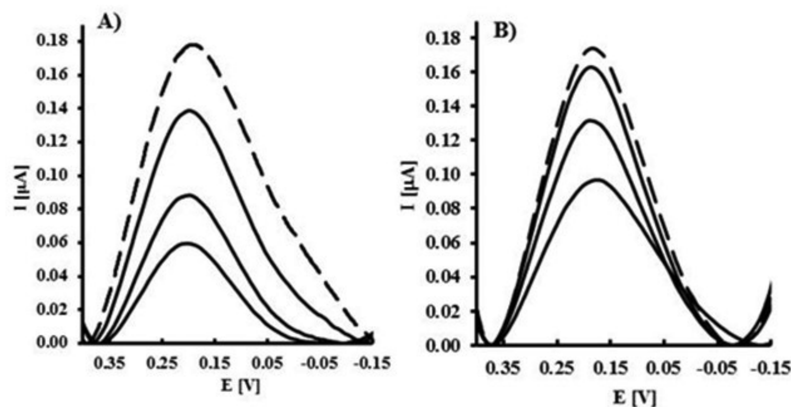


Figure 1. Typical OSW voltammograms obtained for electrodes modified with AuE/AET/ Fe(III)(Epoxy-Phen)$_3$/NH_2-ssDNA upon hybridization processes with RNA targets.
The dashed curve was registered before hybridization (pure buffer) and the next curves upon hybridization with (**A**) RNA1 and (**B**) RNA5. Measuring conditions: PBS pH 7.4 (n=5/6), RNA concentrations [number of copies/µL] – see Table 2.

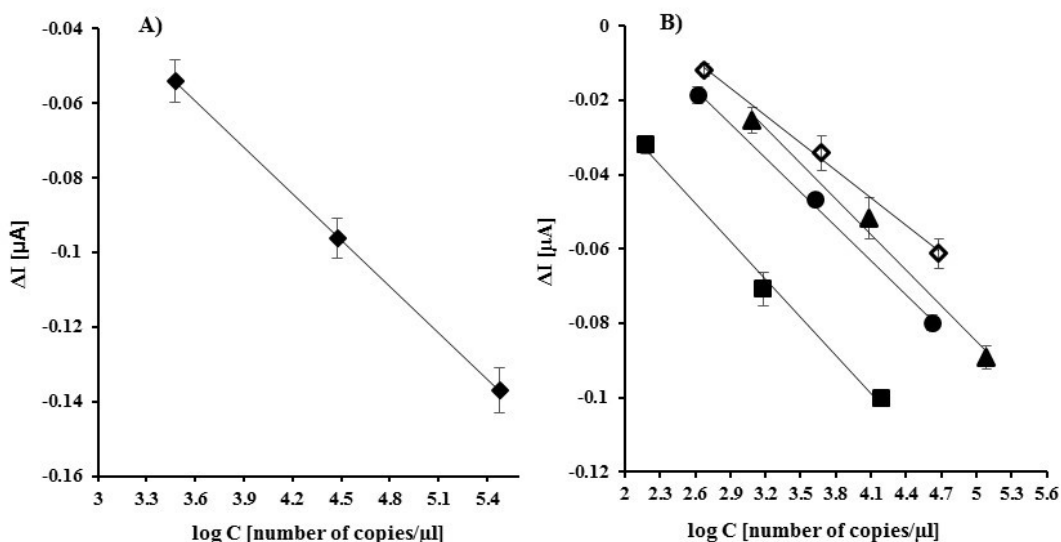


Figure 2. The relationship of $\Delta I = I_n - I_0$ [µA] *versus* log C of:
(**A**) (◆) RNA1 (y = –0.0414x + 0.0898; R^2 = 0.9999) and (**B**) (■) RNA2 (y = –0.0334x + 0.0377; R^2 = 0.9964) (▲) RNA3 (y = –0.0319x + 0.0749; R^2 = 0.9898) (●) RNA4 (y = –0.0306x + 0.0623; R^2 = 0.998) (◇) RNA5 (y = –0.0247x + 0.055; R^2 = 0.9966). Measuring conditions: PBS pH 7.4; RNA concentrations [number of copies/µL] – see Table 2; (n=5/6), I_n is the value of the peak current measured in the presence of the RNA target and I_0 is the value of the peak current in pure PBS (before applying the analyte).

where σ is the standard deviation of the response and S is the slope of the calibration curve (Swartz & Krull, 2012). A remarkable detection limit of 3 copies/µL (5 aM) was obtained for RNA1.

The genosensor also showed good selectivity (Fig. 2). The signals generated by the highest concentration of RNA1 sample caused a –0.137±0.006 [µA] decrease in the peak current. With the increase in number of mismatches in the RNA sequence, the signals became weaker. In case of RNA2, containing 2 mismatches, a decrease of –0.100±0.004 [µA] was observed. RNA3 and RNA4 (sequences with 3 or 4 mismatches, respectively) generated a decrease in the peak current of about –0.089±0.003 [µA] and –0.080±0.002 [µA], respectively. RNA5, a non-complementary sequence, generated the weakest response with a decrease in the peak current of –0.061±0.004 [µA].

The presented genosensor is able to distinguish in viral samples the complementary RNA fragments having 2, 3 or 4 mismatches within the 20-mer region hybridizing with the NH_2-ssDNA probe.

The presented genosensor is superior to the other ones already published (Table 3).

To our knowledge, there are yet no reports on detecting and distinguishing mismatches present in complementary RNA sequences extracted from the influenza viruses using electrochemical genosensors.

The majority of reported electrochemical genosensors refer to the determination of DNA point mutations in buffer solutions or real samples (Joda *et al.*, 2015; Sun *et al.,* 2015; Yang *et al.,* 2016; Rasheed *et al.,* 2017).

Publications addressing RNA sensing in biological samples are limited to miRNA (containing about 22 nucleotides) and we found no reports on longer RNA sequences sensing (Ren *et al.,* 2013; Bartosik *et al.,* 2014; Zhang *et al.,* 2015; Azimzadeh *et al.*, 2016). The main advantage of the proposed sensor is its suitability for direct determination of particular RNA sequences presence in biological material without any reverse transcription and amplification, which are a necessity when DNA is analyzed.

The designed biosensor exhibited good analytical performance towards RNA samples analysis and could be

Table 3. Comparison of the presented and already published genosensors.

Electrode modification	Measuring technique	Target	Detection limit [M]	Medium	References
AuE/AET/ Epoxy-Phen/ $FeCl_3$/ Epoxy-Phen/ NH_2-ssDNA	OSWV	ca. 280 mer RNA	8.7×10^{-13}	buffer	Malecka *et al.*, 2015
		20-mer ssDNA	7.3×10^{-11}		
AuChip/SH-ssDNA/HRP	FPA	112-mer ssDNA	–	clinical sample	Joda *et al.*, 2015
CILE/GR/NiO/ssDNA	DPV (MB)	24-mer ssDNA	3.12×10^{-14}	buffer	Sun *et al.*, 2015
		PCR products from salmonella enteritidis gene	–	biological sample	
AuE/SH-ssDNA/MCH/T/I/H1/H2	DPV (RuHex)	46-mer ssDNA BRCA1 gene	1×10^{-18}	buffer	Yang *et al.*, 2016
			–	10 pM of DNA target in a buffer:human serum (1:1)	
GCE/g-CN/ssDNA	ChA (AuNPC)	19-mer ssDNA BRCA1 gene	10^{-17}	buffer	Rasheed *et al.*, 2017
GCE/GO/AuN/SH-ssDNA/MCH	DPV (OB)	miRNA	0.6×10^{-15}	buffer	Azimzadeh *et al.*, 2016
			–	diluted in plasma	
AuE/SH-ssDNA+TGA/DSN	EIS (Ferri/ferro)	miRNA	1×10^{-15}	buffer	Ren *et al.*, 2013
			–	2.2 ± 0.53 copies/μg in serum	
HMDE/MBs/BCP	DPV	miRNA- Os(VI)bipy	–	buffer/diluted in total RNA sample	Bartosik *et al.*, 2014
AuE/C-ssDNA/MCH/H1+H2/ ST-AP/	DPV (α-NP)	miRNA	0.6×10^{-12}	buffer	Zhang *et al.*, 2015
			–	diluted in total RNA sample	
AuE/AET/ Epoxy-Phen/ $FeCl_3$/Epoxy-Phen/ NH_2-ssDNA	OSWV	RNA	5×10^{-18} (3 copies/μL)	biological sample	Present study

Abbreviations: AuE, gold electrode; ssDNA, single stranded DNA; dsDNA, double stranded DNA; MCH, 6-mercaptohexan-1-ol; DPV, Differential Pulse Voltammetry; GCE, glassy carbon electrode; EIS, Electrochemical Impedance Spectroscopy; MB, Methylene Blue; Epoxy-Phen, 5,6-epoxy-5,6-dihydro-[1.10]-phenanthroline; HRP, horseradish peroxidase; FPA, Fast Pulse Amperometry; CILE, carbon ionic liquid electrode; GR, graphene; NiO, nickel oxide; H1 and H2, hairpin probes; I, Initiator is a 48-base sequence that contains 24 bases at its 5'-end complementary to the unhybridized target sequence; TGA, thioglycolic acid; DSN, duplex-specific nuclease; g-CN, graphitic carbon nitride g-C_3N_4; ChA, Chronoamperometry; AuNPC, gold nanoparticles cluster; HMDE, hanging mercury drop electrode; MBs, magnetic beads; BCP, biotinylated capture probe; C-ssDNA, capture DNA probe; ST-AP, streptavidin-alkaline phosphatase; α-NP, α-naphthyl phosphate; GO, graphene oxide; AuN, gold nanorod; OB, oracet blue.

an alternative tool for detection of RNA in early diagnosis and biomedical research.

CONCLUSIONS

We applied an electrochemical genosensor based on AuE/AET/ Fe(III)(Epoxy-Phen)$_3$/NH_2-ssDNA redox-active layer for the direct detection of complementary RNA sequences in biological samples derived from influenza viruses. The DNA biosensor proved to be successful in the distinction of sequences containing the mismatches (2, 3 and 4) in the 20-nt target region of the NH_2-ssDNA probe. This particular trait of our genosensor may be useful in distinguishing between different, but closely related virus strains. The presented device is extremely sensitive, with the detection limit of 3 copies/μL (5 aM). The major advantage of the presented sensor is its suitability for the direct determination of the target RNA present in biological samples, without the need of its amplification. Taken together, the presented sensor has a high potential to be added to the toolkit currently used for the virus detection in various samples, or even to replace some of the methods used. The most reliable results in identification of the virus would be achieved with the use of the combination of two or three probes complementary to different viral regions, which would also enable independent monitoring of the signal

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Conflict of Interest

The authors declare no conflict of interest.

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