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 3.0×10$^2$ to 3.0×10$^5$ [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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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 (Smietanka 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; Smietanka & 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 (Bahadir & 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; Mehratra, 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; Kurzatkowska 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
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$_2$PO$_4$, 1.8 mM Na$_2$HPO$_4$, 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.

Table 1. Strains of avian influenza viruses, which were used for hybridization processes.

| 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)            | GGAGUUCUCUCUCUCUCUC                      | EPI1156789                                                       |
| RNA2   | A/turkey/Poland/R3249/07 (H5N1)        | GGAGUCCUCUCUCUCUCUC                      | EPI1171604                                                       |
| RNA3   | A/graylag goose/Poland/74/10 (H5N2)    | GGAGUUCUCUCUCUCUC                         | EPI187534                                                        |
| RNA4   | A/mallard/Poland/175/11 (H5N3)         | GGAGUUCUCUCUCUCUC                       | EPI1837712                                                       |
| RNA5   | A/mallard/Poland/446/09 (H7N7)         | negative reference                       | EPI254381                                                        |

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.
The following RNA samples were analyzed: RNA1 – containing a region with full complementarity to the \( \text{NH}_2\text{NC3} \) 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 \( \text{NH}_2\text{ssDNA} \) probe was carried out in acetonitrile solution (AN). The hybridization processes were carried out in PBS pH 7.4 containing 10 mM \( \text{KH}_2\text{PO}_4 \), 1.8 mM \( \text{NaH}_2\text{PO}_4 \), 137 mM \( \text{NaCl} \) and 2.7 mM \( \text{KCl} \), 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 MΩ cm (Millipore Corporation, USA). Reagents and solvents were of analytical grade and used without further purification. All experiments were carried out at room temperature (r.t.).

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 \( \text{NH}_2\text{ssDNA} \) probe (5’-\( \text{NH}_2\text{ssDNA} \) – CCTCAA-GGAGAGAAGAAG-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.

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   | 10000         | -0.052          | 0.006|
|        | 100000        | -0.089          | 0.003|
| RNA4   | 400           | -0.019          | 0.002|
|        | 4000          | -0.047          | 0.007|
|        | 40000         | -0.080          | 0.002|
| RNA5   | 5000          | -0.034          | 0.005|
|        | 50000         | -0.061          | 0.004|

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)/\( \text{NH}_2\text{ssDNA} \) 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 Os-teryoung 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 aerated with nitrogen for 15 min. During all measurements, a gentle nitrogen flow over the sample surface was also used.

Electrode responses are expressed as: \( \Delta I=|I_n-I_0| \) [µA], where \( I_0 \) is the peak current value measured in the presence of the RNA target and \( I_n \) 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)/\( \text{NH}_2\text{ssDNA} \) 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 \( \text{NH}_2\text{ssDNA} \) 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 \( 3.0\times10^3 \) to \( 3.0\times10^6 \) RNA copies/µL. Limits of detection (LOD) were calculated according to the equation:

\[
\text{LOD} = \frac{3\times\sigma}{S}
\]
where $\sigma$ 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 $-0.137 \pm 0.006$ [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 $-0.100 \pm 0.004$ [µA] was observed. RNA3 and RNA4 (sequences with 3 or 4 mismatches, respectively) generated a decrease in the peak current of about $-0.089 \pm 0.003$ [µA] and $-0.080 \pm 0.002$ [µA], respectively. RNA5, a non-complementary sequence, generated the weakest response with a decrease in the peak current of $-0.061 \pm 0.004$ [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.

Figure 1. Typical OSW voltammograms obtained for electrodes modified with AuE/AET/Fe(III)(Epoxy-Phen)/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 ($y = -0.0319x + 0.0749; R^2 = 0.9989$) (●) RNA4 ($y = -0.0360x + 0.0623; R^2 = 0.9981$) (△) 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).
Ultrasensitive electrochemical genosensor for direct detection of specific RNA sequences

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/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.
