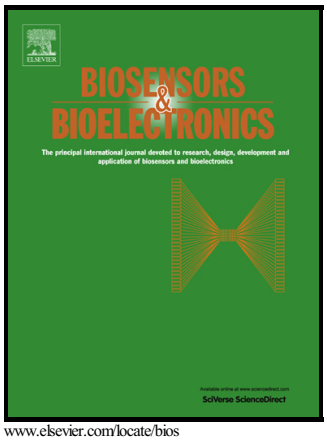

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PII: S0956-5663(14)00868-9
DOI: http://dx.doi.org/10.1016/j.bios.2014.10.069
Reference: BIOS7253

To appear in: *Biosensors and Bioelectronic*

Received date: 4 August 2014
Revised date: 14 October 2014
Accepted date: 29 October 2014

Cite this article as: Kamila Malecka, Anna Stachyra, Anna Góra-Sochacka, Agnieszka Sirko, Włodzimierz Zagórski-Ostoja, Wim Dehaen, Hanna Radecka and Jerzy Radecki, New redox-active layer create via epoxy-amine reaction-the base of genosensor for the detection of specific DNA and RNA sequences of avian influenza virus H5N1, *Biosensors and Bioelectronic,* http://dx.doi.org/10.1016/j.bios.2014.10.069

New redox-active layer create via epoxy-amine reaction - the base of genosensor for the detection of specific DNA and RNA sequences of avian influenza virus H5N1

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Abstract

This paper concerns the development of a redox-active monolayer and its application for the construction of an electrochemical genosensor designed for the detection of specific DNA and RNA oligonucleotide sequences related to the avian influenza virus (AIV) type H5N1. This new redox layer was created on a gold electrode surface step by step. Cyclic Voltammetry, Osteryoung Square-Wave Voltammetry and Differential Pulse Voltammetry were used for its characterization. This new redox-active layer was applied for the construction of the DNA biosensor. The NH_2-NC3 probe (20-mer) was covalently attached to the gold electrode surface via a „click" reaction between the amine and an epoxide group. The hybridization process was monitored using the Osteryoung Square-Wave Voltammetry. The 20-mer DNA and ca. 280-mer RNA oligonucleotides were used as the targets. The constructed genosensor was capable to determine complementary oligonucleotide sequences with a detection limit in the pM range. It is

able to distinguish the different position of the part RNA complementary to the DNA probe. The genosensor was very selective. The 20-mer DNA as well as the 280-mer RNA oligonucleotides without a complementary sequence generated a weak signal.

Keywords: Epoxide–amine „click", Phenanthroline – Epoxy - Fe(III) – (Phenanthroline Epoxy-NH_2-ssDNA)$_2$ redox active layer, Electrochemical genosensor, RNA transcripts, Avian Influenza Virus

1. Introduction

In the past two decades, much progress has been made in the development of methods of preparing electrodes with controllable surface properties. The research on self-assembled monolayers (SAMs) of organic molecules created via chemisorption of "head groups" on the surfaces of solid electrodes has become a booming field (Ulman, 1996; Smith et al., 2004; Vericat et al., 2010; Stobiecka and Hepel, 2011; Hepel and Zhong, 2012; Iost and Crespilho, 2012). Redox-active layers are an excellent platform for research including for example the kinetics of electron transfer (Albrecht et al., 2005; Zhang et al., 2008; Pobelov et al., 2008; Eckermann et al., 2010; Li et al., 2010). This very promising direction of current research is associated with electrochemical biosensors based on a redox active layer immobilized on the electrode surface. Electrochemical sensors based on redox active layers represent a relatively new trend in sensing tools development. The main advantage of this type of layers is that there is no need of using external redox active markers. The application of redox active centres for the construction of sensors provides many interesting possibilities. The redox centres located inside the active layers are responsible for converting signals from intermolecular recognition processes to an analytically useful one (Jargiło et al., 2013; Mielecki et al., 2013; Mikuła et al., 2013; Wojtasik et al., 2014; Zborowska et al., 2014).

Avian influenza (AI) is an infectious disease spreading among wild and domestic birds. It is caused by type A influenza viruses of the family *Orthomyxoviridae*. Highly pathogenic avian influenza virus (HPAIV) H5N1 is not only lethal to birds but can also pose a risk to mammals, including humans (Neumann et al., 2010; Kiilerich-Pedersen et al., 2013). Therefore, there is an urgent need for the development of rapid detection methods of the H5N1. Biosensors are cost-effective analytical devices, which do not demand sophisticated procedures and offer fast and simple analysis.

The clue of the work presented is the development of a new redox-active layer and its application for the construction of an electrochemical genosensor for the detection of specific DNA and RNA sequences of AIV H5N1. The literature describes several types of genosensors for detecting H5N1 (Table S1 - Supplementary data). The first example was based on oxidation/reduction of the peak current of electroactive oligonucleotides bases treated as an analytical signal. This concept has been originally developed by Paleček and Bartošik (2012). Electroactivity of nucleotides, such as cytosine, adenine and guanine (Zhu et al., 2009) changes upon hybridization. Any external indicator was not necessary. However, in case of ion-channel mimetic sensors, an external redox active marker is required. This type of biosensors was originally developed by Umezawa and Aoki (2004). The binding of analytes (target ssDNA) to receptors (ssDNA probe) immobilized on the electrode surfaces facilitates or suppresses the access of anionic (cationic) marker ions, present in the sample solution, to the modified surface due to electrostatic attraction or repulsion of the marker and/or distortion of the modification layer organization. This leads to changes of the electron transfer rate between the marker and electrode surface through the sensing layer (Aoki and Umezawa, 2002; Aoki et al., 2003; Umezawa and Aoki, 2004). This type of sensor based on a self-assembled layer with $[Fe(CN)_6]^{3-/4-}$ as the redox-active marker was successfully used for the detection of specific DNA sequences characteristic for the H5N1 (Kukol et al., 2008; Chung et al., 2011; Malecka et al., 2012; 2013). Intercalations of redox active compounds into DNA helix have been used for another type of genosensors. The most frequently used intercalators for detection of specific oligonucleotides sequences of the AI viruses are methylene blue (Fan et al., 2010), doxorubicin (Ting et al., 2009) or tris(1,10-phenanthroline cobalt (III) perchlorate (Liu et al., 2011). H5N1 specific sequences were also detected using genosensors attached to the electrode surface DNA probes decorated with redox-active labels (Grabowska et al., 2013; 2014a; 2014b). Electroactive molecules such as 3-iron bis(dicarbollide), ferrocene, methylene blue or Co-porphyrin were covalently attached to the DNA probes.

Joining to this intense research area, in this work we present a novel method of forming redox active layer on the gold electrode surface using the spontaneous reaction between the amine and epoxy groups. This type of reaction belongs to the larger family of "click" chemistry and it is called the nucleophilic opening of the oxirane ring (Kolb et al., 2001). The main advantages of click chemistry are good yield, the availability of a wide range of starting materials and that the process is carried out under mild reaction conditions, insensitive to oxygen and water. Therefore, these type of reactions have been recently applied

for coupling of molecules or macromolecules to surfaces (Aiello et al., 2013; Nguyen et al., 2014). The way of attaching substrates to the electrode surface to form the complex via epoxy-amine reaction is novelty. In the literature, activation of EDC/NHS of a carboxyl group for attachment to the amino group are mainly used (Malecka et al., 2013, 2014; Jarocka et al. 2014; Im et al. 2010).

We have designed the aminoethanethiol, 5,6–epoxy–5,6–dihydro–[1,10]–phenanthroline and iron (III) (AET–Phen-Epoxy–Fe(III)–$(Phen\text{-}Epoxy)_2$) redox active sensing layer and characterized it by electrochemical methods - Cyclic Voltammetry (CV), Oster Young Square Wave Voltammetry (OSWV), Differential Pulse Voltammetry (DPV). This layer was applied to the genosensor construction for the detection of the specific DNA and RNA sequences of H5N1. The NH_2-NC3 probe was attached to Phen-Epoxy–Fe(III)–$(Phen\text{-}Epoxy)_2$ redox active layer by epoxy–amine „click" reaction. The signals generated as a result of hybridization processes have been registered by OSWV. The genosensor sensitivity and selectivity were tested with two types of targets, a short (20-mer) DNA and long (ca. 280-mer) RNA sequences of oligonucleotides.

2. Materials and methods

2.1. Reagents and materials

Iron chloride (III), 2-aminoethanethiol hydrochloride (AET), potassium chloride, ethanolamine (EA), acetonitrile (AN) and phosphate buffer saline (PBS) components (137 mM NaCl, 2.7 mM KCl, 10 mM KH_2PO_4, 1.8 mM Na_2HPO_4) were obtained from Sigma–Aldrich (Poznań, Poland). Alumina slurry 0.3 and 0.05 μm was purchased from Buehler (USA). Sulphuric acid, potassium hydroxide, hydrogen peroxide, methanol (MeOH) and ethanol were supplied by POCh (Poland). 5,6–epoxy–5,6–dihydro–[1,10]–phenanthroline (Phen-Epoxy) was obtained from the University of Leuven (Belgium).

The modified oligonucleotide NH_2-ssDNA (5'-NH_2–$(CH_2)_6$-CCT CAA GGA GAG AGA AGA AG-3') was used as a probe (named NH_2-NC3) for immobilization on a gold electrode surface, while two unmodified oligonucleotides, c-NC3 (5'-CTT CTT CTC TCT CCT TGA GG-3') and nc-NC3 (5'-GGA GTT CCT CTC TCA TCA TC-3') served as complementary and non-complementary hybridization targets, respectively. The

oligonucleotides were supplied by Biomers (Germany). The region complementary to the probe is located in the region of 83-102 nt and 160-170 nt from the 5'-terminus of the RNA1 and RNA2, respectively. The RNA3 has no complementary sequence to the probe at all and was used for demonstration of the genosensor selectivity. DNA and RNA oligonucleotides were derived from the HA gene of Polish isolate of the HPAIV H5N1 (A/swan/Poland/305-135V08/2006) - Supplementary data.

The immobilization of the NH_2-NC3 probe was performed in AN. The hybridization processes were carried out in the presence of 0.1 M PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM KH_2PO_4, 1.8 mM Na_2HPO_4), pH 7.4. RNA hybridization buffer was the same (0.1 M PBS) and prepared with sterile, nuclease free water from Sigma–Aldrich (Poznań, Poland). All aqueous solutions were prepared using autoclaved Milli-Q water, with resistivity 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.

2.2. Successive steps of genosensor fabrication

The gold electrodes with a diameter of 2 mm (Bioanalytical Systems (BAS), West Lafayette, IN) were applied to genosensor preparation. They were initially cleaned mechanically by polishing with 0.3 and 0.05 μm alumina slurries (Alpha and Gamma Micropolish; Buehler, Lake Bluff, IL) on a flat pad (BAS) for 5 min each. Afterwards, they were carefully rinsed with Milli-Q water. The polished electrodes were further cleaned electrochemically by CV. At the first they were dipped in 0.5 M potassium hydroxide solution and swept with the potential between –0.4 V and –1.2 V (versus the Ag/AgCl reference electrode and the platinum wire counter electrode) with scan rate of 0.1 Vs^{-1}, number of cycles: 3, 50 and 10. Subsequently, the electrodes were cleaned in 0.5 M sulphuric acid in the potential window between –0.3 V and +1.5 V, number of cycles: 3, 10 and 3. Before modification, the surfaces of the electrodes were refreshed in 0.5 M potassium hydroxide solution for 10 cycles. After finishing of the electrochemical cleaning, each electrode was washed, and next stored in with Milli-Q water, until the next step of modification, to avoid contaminations from the air. All solutions were deoxygenated by purging with nitrogen (ultra pure 6.0, Air Products, Poland) for 15 min.

Directly after cleaning, the electrodes were rinsed repeatedly with water, MeOH and mixture of MeOH and AN (1:1) and dipped in 150 μL of modification solutions. Preparation of the genosensor has been illustrated in Scheme 1:

I. the spontaneous self-assembly to 1 mM AET layer formation – 3h, room temperature (RT)

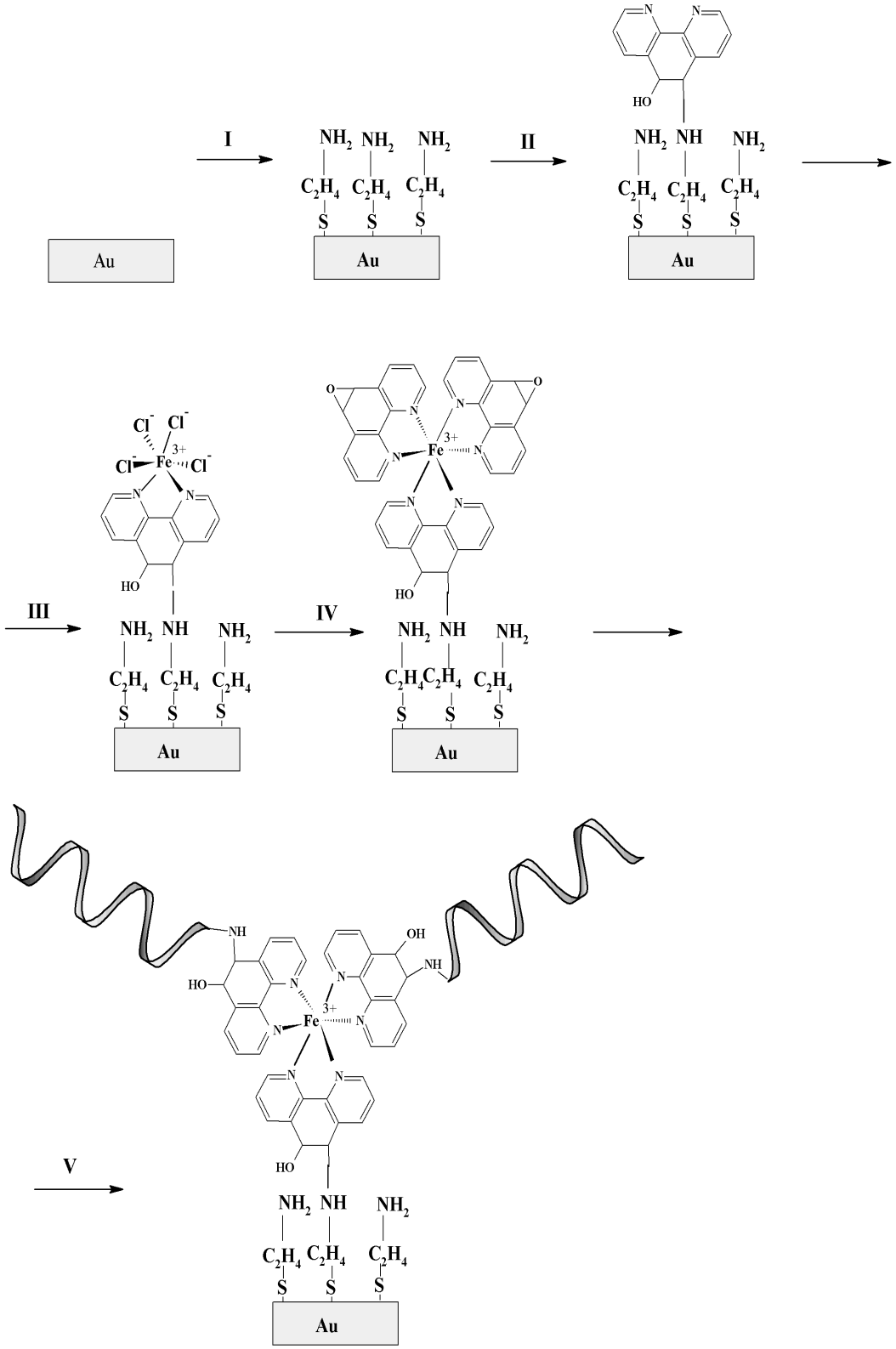
II. „click" reaction between amine groups of AET and epoxy groups of 1 mM Phen-Epoxy - 18h, RT

III. complexation of 1 mM Fe(III) metal ions by Phen-Epoxy - 6h, RT

IV. the closure of Fe(III) metal ions by 1 mM Phen-Epoxy - 18h, RT

V. „click" reaction between amine groups of 1 μM NH_2-NC3 probe (or EA) and epoxy groups of Phen-Epoxy - 6h, RT

As a solvent for I -IV steps of modification a mixture of MeOH and AN in a 1:1 ratio was used, and in case of V step AN. Then the electrodes were rinsed with 5 mL of AN and next with 0.1 M PBS. After modification the electrodes were conditioned in 0.1 M PBS overnight.



Scheme 1. Successive steps of the genosensor preparation: I - AET layer formation; II - „click" reaction between amine groups from AET SAM and epoxy groups from Phen-Epoxy layer; III - Fe(III) complexation; IV - the complexation with Phen-Epoxy in order to get Phen-Epoxy–Fe(III)–(Phen-Epoxy)$_2$ layer; V- „click" reaction between amine groups of NH_2-NC3 (or EA) and epoxy groups of Phen-Epoxy–Fe(III)–(Phen-Epoxy)$_2$ layer.

2.3. Electrochemical measurements

All electrochemical measurements were performed with a potentiostat–galvanostat AutoLab (Eco Chemie, Utrecht, Netherlands) with a three electrode configuration. Potentials were measured versus the Ag/AgCl electrode, and a Pt wire was used as the auxiliary electrode. The voltammetric experiments were carried out in an electrochemical cell of 5 mL volume.

The AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy)$_2$ redox active monolayer was characterized by electrochemical methods – CV, OSWV and DPV. In CV the potential was cycled from +0.6 V to –0.2 V with a scan rate of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 Vs^{-1}. In OSWV, a potential window from +0.4 V to −0.4 V, a step potential of 0.001 V, square-wave frequency of 25 Hz and amplitude of 0.05 V were applied. DPV measurements were performed in two cycles: with the potential from +0.5 V to –0.4 V for reduction of Fe(III) ions and from –0.4 V to +0.5 V for oxidation of Fe(II) ions. The values of the modulation amplitude and step potential were 0.05 V and 0.001 V, respectively. All measurements were carried out in the presence of 0.1 M KCl purged with nitrogen for 15 min. A gentle nitrogen flow was applied over the sample solution during all measurements.

For characterization of the AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy)$_2$ electroactive monolayer, at the last step of the modification the epoxy groups were deactivated with 1 mM solution of EA for 3h. Finally, electrodes were rinsed with AN, MeOH and 0.1 M KCl and then conditioned in 0.1 M KCl overnight.

2.4. Hybridization processes

The gold electrodes surfaces modified with a AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy–NH_2–NC3)$_2$ redox active layer were covered with 10 μL of a solution of variable concentration of the target oligonucleotides (c-NC3, nc-NC3, RNA1, RNA2 or RNA3) in 0.1

M PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM KH_2PO_4, 1.8 mM Na_2HPO_4) pH 7.4, for 30 min at room temperature. Then the electrodes were rinsed with 5 mL of PBS, pH 7.4 in order to remove the unbound targets.

The single strand nucleic acid targets, either DNA (c-NC3, nc-NC3) or RNA (RNA1, RNA2, RNA3), were diluted with the 0.1 M PBS hybridization buffer (pH 7.4) to the concentration of 100, 200, 400, 600, 800 and 1000 pM for DNA target and 10, 100 and 1000 pM for RNA target.

The hybridization processes were monitored using OSWV with a potential from +0.4 V to −0.2 V, a step potential of 0.001 V, a square-wave frequency of 25 Hz and amplitude of 0.05 V in 0.1 M PBS. The electrode responses were expressed as: $(I_n - I_0) / I_0$, where I_n is the peak current measured in the presence of the analyte and I_0 the peak current before applying the analyte (in pure buffer).

3. Results and discussion

3.1. Electrochemical characterization of Phen-Epoxy–Fe(III)–(Phen-Epoxy/EA)$_2$ and Phen-Epoxy–Fe(III)–(Phen-Epoxy/NH_2-NC3)$_2$ redox active layers

One of the goals of the presented study is the electrochemical characterization of the AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy–EA)$_2$ redox active layer prepared by means of a „click” reaction. The epoxy groups were neutralized with EA.

The presence of Fe(III) ions at the gold electrode surface was verified using CV performed in 0.1 M KCl. Representative cyclovoltammograms are presented in Fig. 1. The reduction of Fe(III) ions and oxidation of Fe(II) ions were observed at $E_{pc} = 0.248 \pm 0.009$ V and $E_{pa} = 0.149 \pm 0.007$ V, respectively, at the scan rate of 0.1 Vs^{-1}. The peaks separation $\Delta E_p = 0.099 \pm 0.009$ V indicates that the reversibility of the process is quite good. The linear relationship of the anodic and cathodic peak currents versus the scan rate from 0.05 up to 1

Vs^{-1} points out that the redox processes are not diffusion dependent and confirms the presence of the Phen-Epoxy/Fe(III)/(Phen-Epoxy–EA)$_2$ complex on the gold electrode surface.

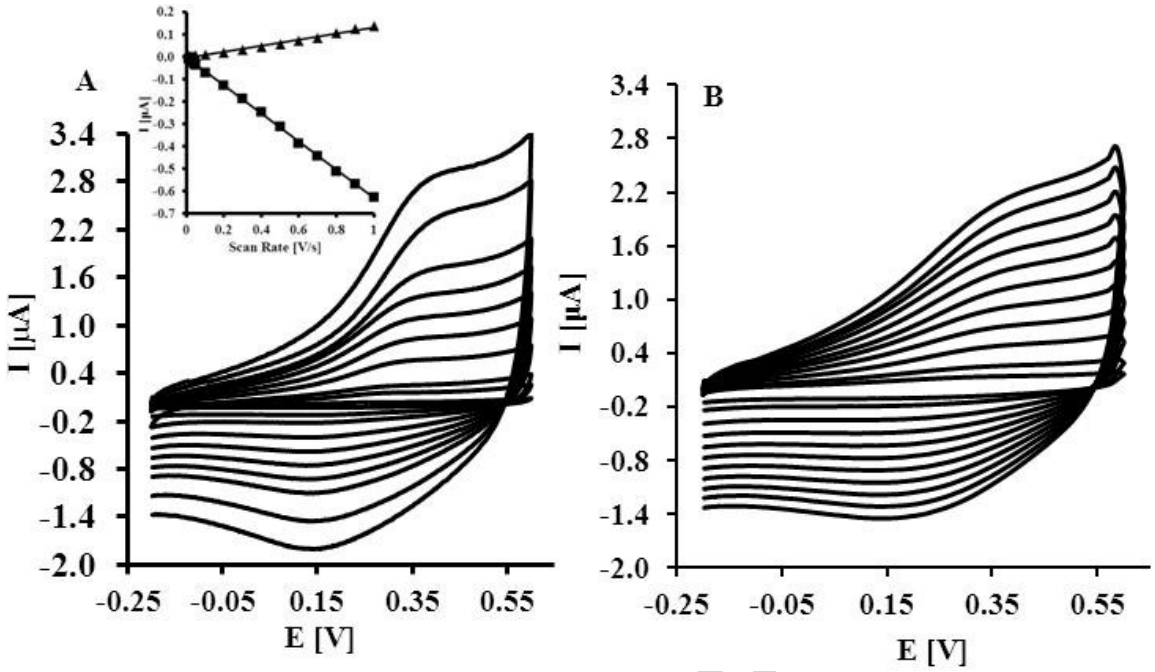


Fig. 1. An example of the CV curves obtained for the gold electrode modified with: (A) Au/AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy/EA)$_2$, Inset: plot of (▲, I_{pa}) anodic and (■, I_{pc}) cathodic peak current against potential scan rate; (B) Au/AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy/NH_2-NC3)$_2$ measured in 0.1M KCl. Scan rates: 0.050-1.0 Vs^{-1}.

The kinetic data of the electron transfer between the Fe(III) centres and the modified electrode surface were obtained from the relationship between the log scan rate versus the anodic and cathodic peak potential. The values of the electron transfer coefficient $\alpha = 0.46 \pm 0.05$ and the electron transfer rate constant $k = 0.84 \pm 0.13$ [1/s] were estimated based on the generally applicable Laviron procedure (Laviron, 1979). The results of the yield coverage determination is $\Gamma = 2.2 \pm 0.39 \times 10^{-10}$ [mol/cm^2]. The cyclic voltammograms of the gold electrode modified with AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy–EA)$_2$ (Fig. 1A) performed in 0.1 M KCl were stable during scanning, implying that the species formed by its electrochemical reduction/oxidation did not undergo ligand loss.

The OSWV, in comparison to the CV, eliminates the capacitive current and therefore is a more suitable technique for study the quasi-reversible systems. A representative Osteryoung square-wave voltammograms are presented in Fig. 2. The backward (oxidation) and forward (reduction) scans of OSWV obtained for a gold electrode modified with Au/AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy/EA)$_2$ and Au/AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy/NH$_2$-NC3)$_2$ were recorded in order to better evaluation of the total OSWV signal (Fig. 2A and B). For both modifications reduction current is higher in the comparison to oxidation current. The decrease of current value recorded after coupling of NH$_2$-NC3 probe was higher for oxidation (ca. 76 %) than for reduction (42 %) (Table S2). This indicated that in the total OSWV signal oxidation process dominated. In all cases, the current decrease was assisted with peak current potential shift into the positive value. The same tendency was observed using DPV. The representative DPV recorded for electrode modified with Au/AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy/EA)$_2$ (Fig. S1) and after coupling of ssDNA - Au/AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy/NH$_2$-NC3)$_2$ (Fig. S2) are shown in the Supplementary data. The intensity of reduction as well as oxidation current recorded with DPV were ca. ten times lower in the comparison to the values recorded with OSVW. Therefore, OSVW was selected for detection of hybridization processes.

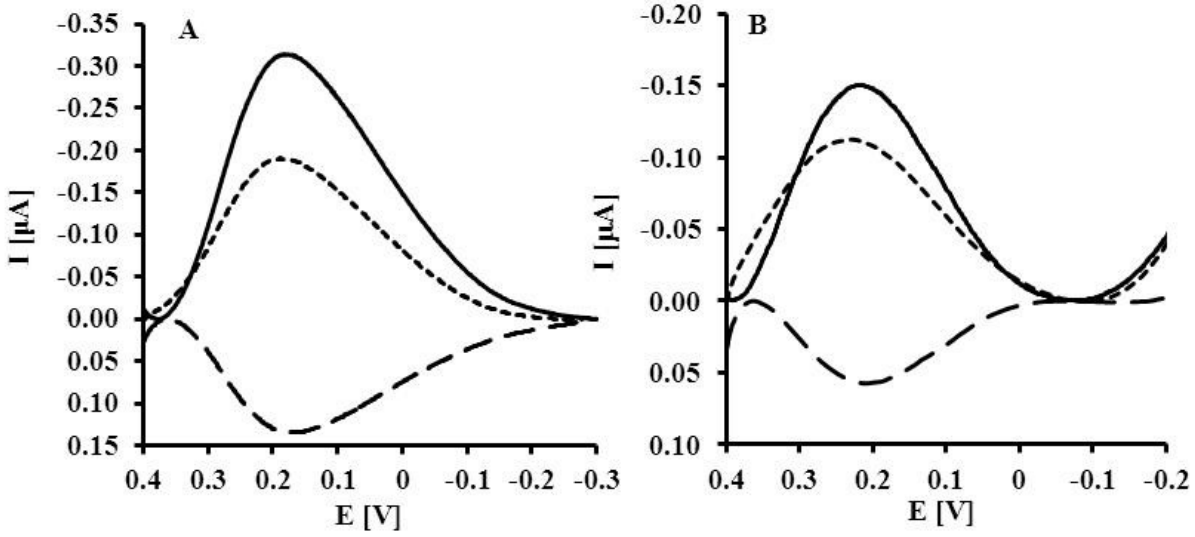


Fig. 2. An example of Osteryoung square-wave voltammograms obtained for a gold electrode modified with: (A) Au/AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy/EA)$_2$ and (B) Au/AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy/NH$_2$-NC3)$_2$.

The signals: total (—), backward (– – –) and forward (---). Measuring conditions: a step potential of 0.001 V, frequency 25 Hz, amplitude 0.05 V, solution composition: 0.1 M KCl.

Phenanthroline and its derivatives can be widely used for the complexation of numerous metals, not only Fe(III), for example: Co(II), Ni(II), Cu(I and II), Fe(II), Mn(II), Ru(II), Rh(III) (Arounaguiri et al., 2000; Mudasir et al., 2003; Wei et al., 2008; Bencini and Lippolis, 2010; Shee et al., 2011). Therefore the developed phenanthroline monolayer offers the possibility for the complexation of these metals.

3.2. Application of a genosensor based on the AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy–NH_2-NC3)$_2$ redox active layer for monitoring hybridization processes with DNA targets

The new AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy)$_2$ redox-active monolayer, displaying good electrochemical properties, was successfully applied for stable immobilization of NH_2-NC3 probe via the „click" reaction with epoxy group. The applicability of such a genosensor was tested by exploring the hybridization processes with specific 20-mer sequences of DNA related to the H5N1 using OSWV.

The representative Osteryoung square wave voltammograms are presented in Fig. 3A,B. The increase of the concentration of the complementary c-NC3 target caused a proportional decrease of Fe(III) redox current values (Fig. 3A). On the other hand, in the presence of non-complementary nc-NC3 sequence a negligible genosensor response was observed (Fig. 3B). The decrease of Fe(III) redox peak current in the presence of the highest concentration (1000 pM) of 20-mer complementary c-NC3 target was in the range 72.2 ± 4.3 % (n=6). The same concentration of nc-NC3 caused a negligible response of 0.92 ± 2.1% (n=6) (Fig. 3C). The proposed genosensor was sensitive with a detection limit of 73 pM for c-NC3.

The limit of detection was calculated based on the standard deviation of the response and the slope of a calibration curve:

$$DL = 3.3 \cdot \sigma / S$$

where σ is the standard deviation of the response and S is the slope of the calibration curve (Swartz and Krull, 2012).

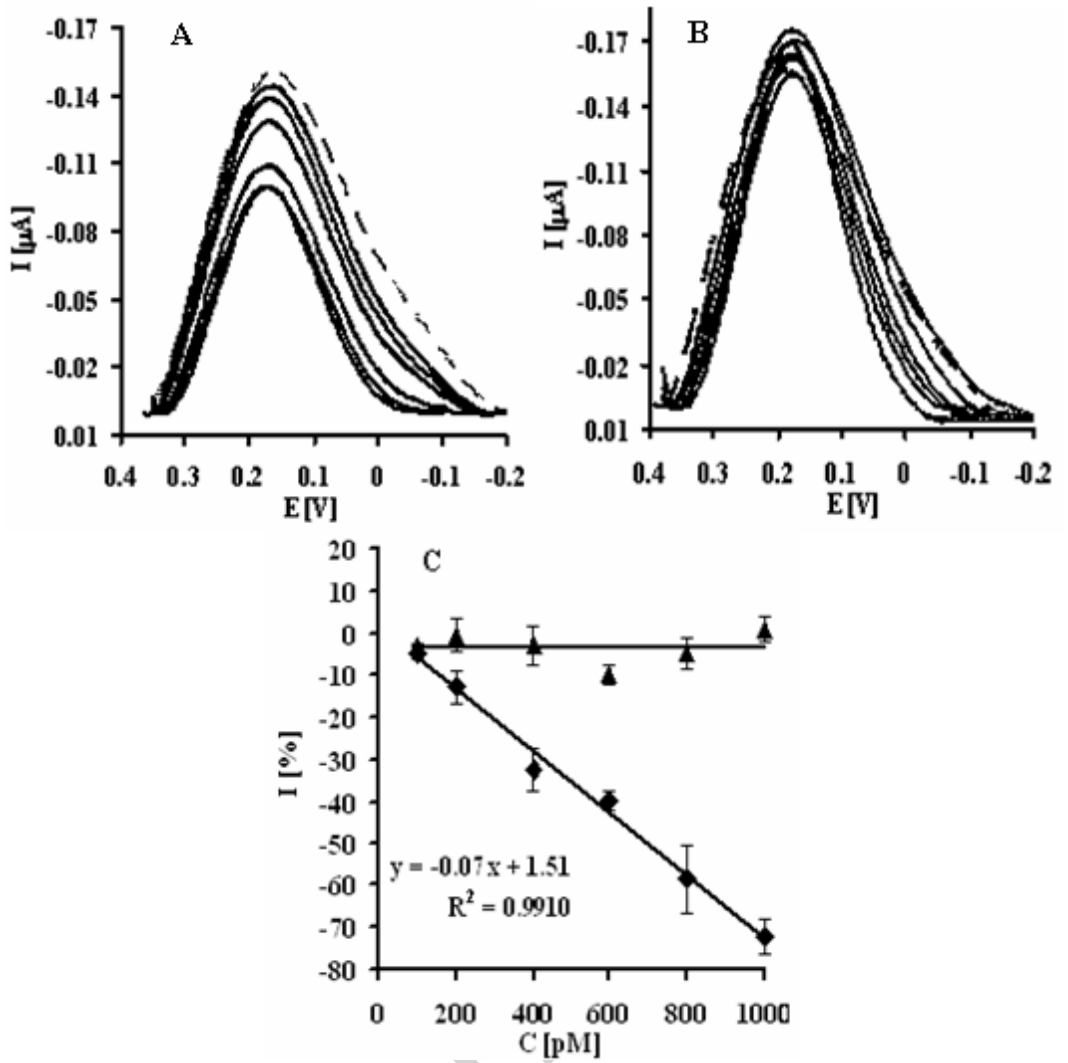


Fig. 3. Typical Osteryoung square wave voltammograms obtained for electrodes modified with Au/AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy/NH_2-NC3)$_2$ upon hybridization with 20-mer target ssDNA. Dashed curve – before hybridization and next curves upon hybridization with (**A**) c-NC3 and (**B**) nc-NC3 at concentrations: 100, 200, 400, 600, 800 and 1000 pM. Measuring conditions: see 2.3. Electrochemical measurements.

(**C**) The relationship of $I= (I_n–I_0)/I_0$ (%) vs. concentration C [pM] of (♦) c-NC3 and (▲) nc-NC3. I_n is the value of the peak current after detection of given concentration of analytes, and I_0 is the value of the peak current without presence of analyte in pure 0.1 M PBS pH 7.4 (n=6). Solution composition: 0.1 M PBS (pH = 7.4).

The literature describes genosensors for detection of the specific DNA sequences of H5N1, in which DNA probes are labeled with redox active compounds, for example cobalt porphyrins or 3-iron bis(dicarbollide) (Grabowska et al., 2014a, 2014b). In the first case, oligonucleotide

probes were bearing a metallacarborane [3-iron bis(dicarbollide)] redox label. The applied redox label was attached at the "foot" of the oligonucleotide probe, very close to the electrode surface. The second genosensor was based on the use of cobalt porphyrins as redox labels. The porphyrin was also attached to the DNA close to the electrode surface, thus the distance did not vary greatly upon hybridisation. This genosensors displayed a sensitivity in the fM range, better than the proposed in this paper (73 pM). The mechanism of the response of the AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy–NH_2-NC3)$_2$ genosensor towards a complementary target is similar to this already reported by Grabowska et al. (2014b). The formation on the electrode surface of the double helix structure as a result of the hybridization process caused changes in the thickness of the double layer at the interface surface of electrode/analysed solution. The mechanism of current decreasing when the target DNA is hybridized with DNA probe on the electrode surface is still under study in our laboratory. Upon hybridization, the minus charge of the electrode surface increase. This decrease supporting ions accessibility towards redox centres. We assume that this might be responsible for decrease of reduction/oxidation Fe(III)/Fe(II) current. In order to confirm these hypothesis, the electrochemical study on redox active layer deposited on solid support are currently performing in different supporting electrolyte.

Moreover, in contrast to genosensors incorporating labeled DNA oligonucleotides, the versatile system proposed here does not require sophisticated modifications of the DNA probe. It can be used to attach various DNA probes possessing only an amino group at 5' terminus.

3.3. Application of a genosensor based on the AET–Phen-Epoxy–Fe(III)–(Phen-Epoxy–NH_2-NC3)$_2$ redox active layer for monitoring hybridization processes with 280-mer RNA sequences

The genosensor sensitivity and selectivity were tested using three ~280-mer RNA transcripts, with (RNA1, RNA2) or without (RNA3) the 20 nucleotide (nt) region complementary to the NH_2-NC3 probe. The representative Osteryoung square-wave voltammograms recorded upon successive increasing of RNA1, RNA2 and RNA3 concentration were illustrated in Fig. 4A, 4B and 4C, respectively. A linear current decrease

was observed in the presence of complementary RNA1 and RNA2 in the concentration range from 10 pM to 1000 pM, while in the presence of the non-complementary RNA3, the opposite signal was observed (Fig. 4A-D). The strongest signal was generated by RNA1 (Fig.4A, D). It is important that genosensor recognizes complementary RNA sequence dependently on the target fragment contextual position in polynucleotide. The distance of the target sequence from the 3'-terminus is different in RNA1 and RNA2 (181 nt and 103 nt, respectively). However, the strength of the signal is affected by different target accessibility in secondary RNA structure rather than by the distance from the 5' end. The hybridization of RNAs with the probe might compete with RNA refolding. The target RNA sequence of the RNA2 molecule seems to be exposed in such a way that the rest of the molecule forms a steric hindrance during hybridization with the probe immobilized on a surface of the electrode (Fig. S3 - Supplementary Data). The same oligonucleotide sequence when presents in RNA1 seems to be better accessible to the immobilized probe. This observation opens a novel field of applications for genosensors - namely- analysis of RNA secondary structure.

The proposed genosensor was sensitive, with detection limits 0.87 pM and 0.94 pM for RNA1 and RNA2, respectively. It showed also good selectivity. The signals generated by 1000 pM RNA1 and RNA2 caused 29.5 ± 3.1% and 11.2 ± 3 % decrease of peaks current. On the other hand, in the case of RNA3 with sequence non-complementary to the probe 19.6 ± 5.0 % increase of peaks current was recorded.

In addition, the genosensor presented was able to distinguish RNA fragments having the complementary sequences in different positions The articles concerning the sensors suitable for detection of microRNA (miRNA), which is a kind of endogenous, noncoding RNAs (19-24 nt) have been already reported (Kilic et al., 2012; Yin et al., 2012; Cai et al., 2013; Liu et al., 2014). To our knowledge, there are no reports about determination of long RNA sequences. The major advantage of using the RNA sequences is the possibility for direct target detection in biological samples, without the need of transcription of viral RNA to DNA sequences. RNA, due to the presence of hard-to-inactivate enzymes – RNases requires more stringent analytical practices than DNA.

In this study the detection limit of 280-mer RNA1 transcript (0.87 pM) is much better than that of 20-mer DNA target (73 pM). The increase in target length generates larger sensor responses, because of the extra negative charges of longer target strands (Booth et al., 2011, Malecka et al., 2012 and 2013)

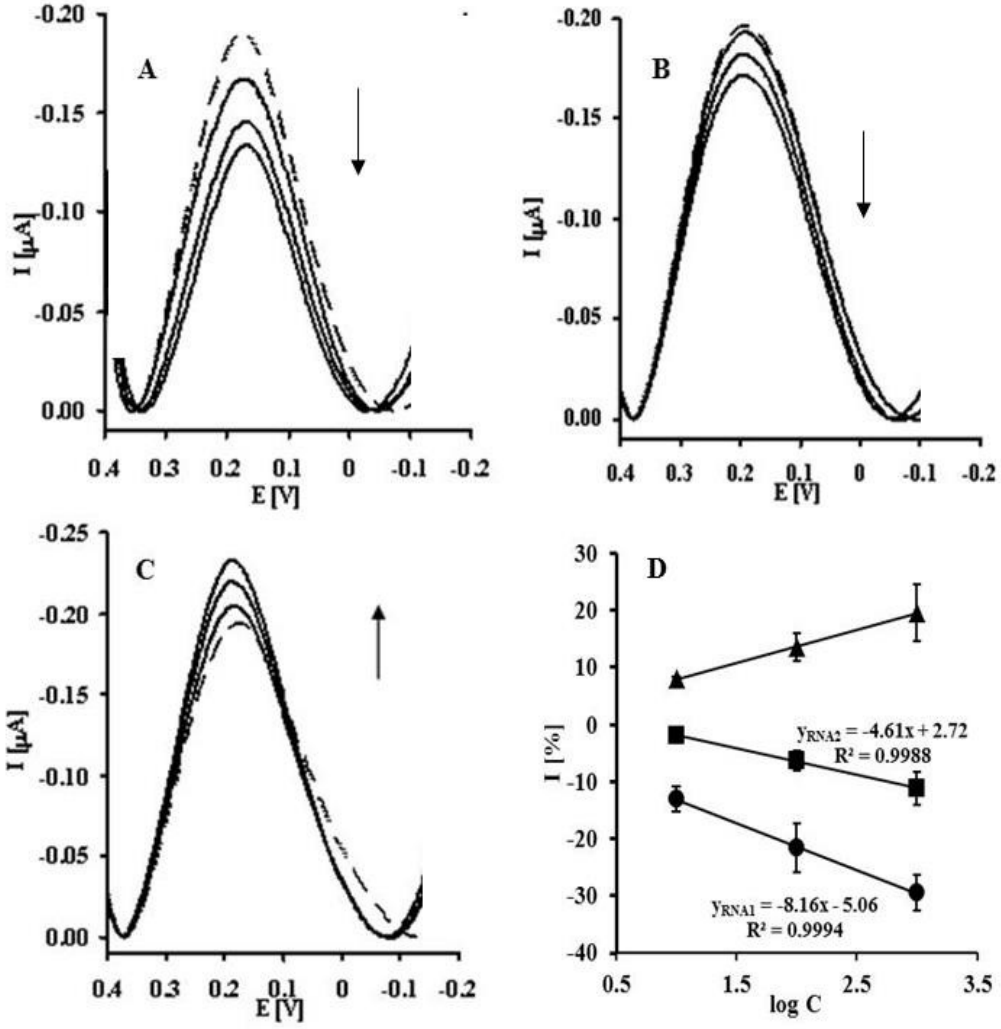


Fig. 4. Typical Osteryoung square wave voltammograms obtained for electrodes modified with Au/AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy/NH_2-NC3)$_2$ upon hybridization with RNA transcripts. Dashed curve – before hybridization and the following curves upon hybridization with target (**A**) RNA1, (**B**) RNA2 and (**C**) RNA3 at concentration: 10, 100 and 1000 pM. Solution composition: 0.1 M PBS (pH = 7.4). The measuring conditions: see 2.3. Electrochemical measurements.

(**D**) The relationship of $I=(I_n–I_0)/I_0$ (%) vs. log C [pM] of (●) RNA1, (■) RNA2 and (▲) RNA3. I_n is the value of peak current after detection of given concentration of analytes, and I_0 is the value of peak current without presence of analyte in pure 0.1 M PBS pH 7.4 (n=5÷6)

4. Conclusions

The proposed electroactive layer AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy)$_2$ is the base of the construction of DNA and RNA sensor. An efficient "click" reaction, carried out under mild conditions, between the NH_2-NC3 probe and the epoxy groups from Phen-Epoxy/Fe(III)/(Phen-Epoxy)$_2$ complex deposited on the gold electrode surface, has been successfully applied for the construction of the genosensor. This genosensor displays good sensitivity, with detection limits of 73 pM and 0.87 pM for the 20-mer c-NC3 and the 283-mer RNA1, respectively. The proposed genosensor detected RNA sequences with about one hundred times better sensitivity than the DNA sequences detection. The genosensor demonstrates also good selectivity. The 20-mer nc-NC3 without complementary sequence generated a weak response, and in the case of the 277-mer RNA3 fragment without complementary sequence the opposite signal, an increase of the Fe(III)/Fe(II) peak currents was observed. It is worth to emphasize that one of the main advantages of the presented genosensor is the suitability for determination of RNA and distinguishing of the different positions of the complementary parts. Considering the above analytically important parameters, the presented genosensor could be applied for detection of the H5N1 genetic material.

Acknowledgements

This work was supported by Innovative Economy Program, No. WND-POIG.01.01.02-00-007/08, 679/N-BELGIA/2010/0, COST Action CM10005 "Supramolecular Chemistry in Water" and Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Olsztyn, Poland.

Appendix A. Supplementary data

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HIGHLIGHTS:,

- ✓ ssDNA probe was covalently attached to the redox-active layer via click reaction
- ✓ The genosensor detects 20-mer DNA and 280-mer RNA sequences of avian influenza H5N1 at pM level
- ✓ This device distinguishes different positions of complementary to the probe RNA parts

Graphical abstract