Plasmid HIV *p24* Gene Detection on Mercury Film Electrode using Osmium Labelling

Jiri Kudr[1], Lukas Nejdl[1,2], Sylvie Skalickova[1,2], Branislav Ruttkay-Nedecky[1,2], Miguel Angel Merlos Rodrigo[1,2], Simona Dostalova[1,2], Ana Maria Jimenez Jinemez[1,2], Dagmar Chudobova[1,2], Kristyna Cihalova[1,2], Marie Konecna[1,2], Pavel Kope1[1,2], Jindrich Kynicky[3], Vojtech Adam[1,2], Rene Kizek[1,2]*

[1] Department of Chemistry and Biochemistry, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic, European Union
[2] Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic, European Union
[3] Karel Englis College, Sujanovo nam. 356/1, CZ-602 00, Brno, Czech Republic, European Union
*E-mail: ilabo@seznam.cz

The early detection of HIV-positive individuals before infecting healthy individuals is the basic principle to fight against AIDS. Although a lot of methods has been developed to detect HIV, electrochemistry is a promising tool thanks to its high sensitivity, low cost, ease of use and mostly no antibody-based detection. The aim of our study was to perform the electrochemical detection of HIV *p24* gene for capsid protein. We utilized the ability of osmium tetroxide bipyridyl ($OsO_4(bpy)$) to create the complex with thymine and used it to label the oligonucleotide complementary to the *p24*. We also used the thiolated oligonucleotide probe to anchor the labelled *p24* gene to the magnetic particle, which served to isolate the construct from the solution of non-anchored molecules. The detection of *p24* gene was performed on the mercury film glassy-carbon electrode by the adsorptive transfer method using difference pulse voltammetry. The best mercury film on the surface of glassy carbon working electrode was received using KNO_3 as supporting electrolyte and by the set of potential -0.9 V for 300 s. We showed that also the femtomole amount of *p24* gene within linear plasmide can be absorbed to the electrode surface and detected.

1. INTRODUCTION

According to the Joint United Nations Programme on HIV and AIDS, more than 35 million persons live with the human immunodeficiency virus (HIV), which causes immune deficiency disease

(AIDS). It was estimated that since the start of the epidemics about 36 million people have died of the AIDS-related illnesses. Although the number of newly infected people is decreasing and was about 2.3 million in 2012, it still remains serious issue. A lot of methods has been developed for detection of HIV including enzyme immunoassay [1], enzyme-linked immunosorbent assay (ELISA) [2] and western blot test [3]. These antibody-based methods applied on blood samples are time-consuming, have long detection "window phase" (3 weeks to 6 months) and requires highly-trained workers, however, these are still gold standard for HIV detection. Polymerase chain reaction (PCR) based techniques have been also utilised for HIV detection and exhibited high sensitivity, but these are limited in distinguishing of single base mutations [4].

Electrochemical sensing is able to resolve these limitations and offers simple, portable, sensitive, inexpensive detection and it is useful in the detection of early HIV infection because of direct molecular recognition of HIV and its components [5]. Electrochemical detection of HIV protease, an enzyme essential to the assembly and maturation of HIV, based on the enzymatic cleavage of protease substrate from the gold electrode was suggested by Esseghaier et al. [6]. HIV reverse transcriptase and HIV p24, which appears at the earlier stage of HIV infection than antibodies, became another target molecule used for HIV detection [5,7,8]. There is also interest in the detection of short DNA sequences related to the HIV [9-11].

In this study, we designed a simple method for electrochemical detection of *p24* gene. Osmium tetroxide complex with 2,2′-bipyridine (OsO$_4$(bpy)) has been often used as a versatile chemical DNA probe [12-14]. We utilized the ability of OsO$_4$(bpy) to react with pyrimidine moieties (mostly thymine) in single stranded DNA and created the electroactive oligonucleotide probe complementary to the *p24* sequence [15]. The DNA-OsO$_4$(bpy) adduct produces a catalytic signal (about -1.2 V) at mercury electrode and other smaller signals at less negative potentials [12]. Moreover, we used the oligonucleotide probe complementary to the 5' end of *p24* sequence bounded to a gold modified magnetic particle (AuMPs) via thiol group, which enables us to separate the anchored single stranded gene from the solution of non-targeted DNA and other molecules. The created construct was adsorbed at mercury surface of the mercury film (MFE) on glassy carbon electrode (GCE) [16]. The signal of OsO$_4$(bpy) reporter molecule was analysed by differential pulse voltammetry (DPV).

2. EXPERIMENTAL

2.1. Reagents and chemicals

OsO$_4$, 2,2′-bipyridyl, Fe(NO$_3$)$_3$·9H$_2$O, NaBH$_4$, polyvinylpyrrolidone, HAuCl$_4$, trisodium citrate, Trizma base, HCl, Tris base, acetic acid, EDTA (Ethylenediaminetetraacetic acid), sodium acetate trihydrate, acetic acid, Hg(NO$_3$)$_2$, HNO$_3$, KNO$_3$, water and other chemicals were purchased from Sigma-Aldrich (USA) in ACS purity. To pipette volumes down to microlitres, pipettes used were purchased from Eppendorf Research (Eppendorf, Hamburg, Germany). Deionised water underwent demineralization by reverse osmosis using the instruments Aqua Osmotic 02 (Aqua Osmotic, Tisnov,

Czech Republic) and then it was subsequently purified using Millipore RG (Millipore Corp., USA, 18 MΩ) – MiliQ water.

2.2. Modification of oligonucleotide with $OsO_4(bpy)$ (ODN-$OsO_4(bpy)$)

The oligonucleotide complementary to the 3´ end of *p24* sequence (5´-TTTTTTTTATTGCTAAGCCAAAACCC-3´) was modified by $OsO_4(bpy)$ as described in [17]. Briefly, OsO_4 (0.25 g) in water (25 ml) and 2,2´-bipyridyl (0.156 g) were shaken until both compounds were dissolved and volume was adjusted to 50 ml. Yellow solution of $OsO_4(bpy)$ (20 mM, 8.2 mg/ml) was used for ODN labelling.

The oligonucleotide (470 µl, 100 µg/ml) was diluted with Tris-HCl buffer (200 µl, 0.1 M, pH = 7.4) and $OsO_4(bpy)$ (4.3 µl, 20 mM) was added. The mixture was shaken and heated for 3 h at 37 °C on Thermomixer comfort (Eppendorf, Hamburg, Germany). After cooling, the sample was reduced in volume and washed five times with 200 µl portions of Tris-HCl on Amicon 3k filters (Millipore, Billerica, USA) using centrifuge (6000 rpm, 20 °C, 15 min). Final volume of sample was 470 µl.

2.3. Preparation of nanomaghemit modified with gold nanoparticles

80 ml of water was poured in a 250 ml beaker and 1.5 g of $Fe(NO_3)_3·9H_2O$ was mixed on a magnetic rotor. 1.4 ml of 25% NH_3 (*v/v*) solution was mixed with 8.6 ml of water in a screw capped tube and poured in a separate beaker. 0.2 g of $NaBH_4$ was mixed with NH_3 solution. After 10 min of mixing, the solution was added to the beaker containing $Fe(NO_3)_3$. The colour of the solution became black with an initial frothing. Then it was heated at 100 °C for 2 h. The mixture was stirred overnight. The magnetic particles were separated from the solution by external magnet and washed with deionised water several times. Then, 20 ml of water was poured in the beaker and mixed with a solution of 1.5 g of polyvinylpyrrolidone (Mw 40000) dissolved in 20 ml of water. After 3 h of mixing, 25 ml of chloroauric acid ($HAuCl_4$, 1 mM) was added and mixed for 1 h. 0.75 ml of trisodium citrate (0.265 g/10 ml) was added to it and stirred overnight. Next day, the magnetic particles were separated from the solution by external magnet and washed three times with deionised water. It was collected and dried at 40 ºC. The weight of the dry sample was 0.26 g.

2.4. Cloning, sub-cloning and amplification of HIV gene p24 using polymerase chain reaction

The gene for the capsid protein p24 of human immunodeficiency virus (GenBank accession number: AJ630556.1) was synthesized and cloned into the plasmid pUC57 -Amp (GENEWIZ Gene Synthesis, Sigma-Aldrich) resulting pUC57 vector containing the HIV-cap. The chemical transformation protocol was performed following the instructions of Invitrogen using as host TOP10 chemically competent *E. coli* strain. Bacteria transformed with pUC57-HIV-cap plasmid were selected by ampicillin resistance. The positive transformants were confirmed by PCR screening. The plasmid were purified by using the Qiagen Miniprep Kit (Qiagen, Maryland, USA) and the amplification of

gene by PCR was done using a set of primers flanking the complete open reading frame (5´-ATGGTCCACCAAGCCATATC-3´ (HIV-capfw) and 5´-TTATTGCTAAGCCAAAACCC-3´ (HIV-capre)). Amplified product was analysed via agarose gel electrophoresis and the conditions were as follows: 2% agarose gel (High melt/Medium fragment, Mercury, San Diego, USA) with 1× TAE buffer, 60 V and 160 min (Bio-Rad, Hercules, USA). The 100 bp DNA ladder (New England Biolabs, Ispwich, USA) was used as a molecule size marker. Bands were visualized via UV transilluminator at 312 nm (Vilber-Lourmat, Marne-la-Vallée Cedex, France). The MiniElute PCR Purification Kit (Qiagen, Maryland, USA) was used for cleaning the PCR product.

To obtain pGEX-4T1-HIV-cap, the full-length HIV-cap was isolated from the pUC57 vector by digestion with NotI and ligated into the NotI-digested vector pGEX-4T1. Bacteria transformed with pGEX-4T1-HIV-cap were selected by ampicillin resistance. The positive transformants were confirmed by PCR screening and purified by using the Qiagen Miniprep Kit (Qiagen, Maryland, USA). Linear vector pGEX-4T1 with HIV-cap gene was obtained by digestion EcoRI following the instructions of New England Biolabs (Ispwich, USA). Linear pGEX-4T1-HIV-cap was analysed via agarose gel electrophoresis. The band corresponding to the linear vector was extracted from the agarose gel using the QIAEX II Gel Extraction kit (Qiagen, Maryland, USA).

2.5. Preparation of the construct

1 mg of magnetic particles modified by gold nanoparticles (AuMPs) was sonicated in 1 ml of deionised water for 20 min. Subsequently, it was taken 100 µl from the stock solution of the AuMPs and 2-times rinsed with ACS water. 1, 5, 10 µl of 100 µM thiolated oligonucleotide (ODN) was added to the AuMPs and shaken for 60 min. After that, the ODN-AuMPs complex was 3 times rinsed by hybridization buffer (0.1 M Na_2HPO_4; 0.1 M NaH_2PO_4; 0.6 M guanidinium thiocyanate (Amresco, USA); 0.15 M Tris-HCl (pH 7.5); NaCl (0.5 M)) to remove impurities and diluted in 100 µl of hybridization buffer. Simultaneously, 15 µl (50 µg.ml^{-1}) of the PCR product was denatured in a Thermomixer comfort (Eppendorf, Hamburg, Germany) (99 °C, 5 min) and immediately cooled. 5 µl of denatured PCR product was mixed with AuMPs in hybridization buffer and after 5 min the OsO_4(bpy)-labelled oligonucleotide (ODN-OsO_4(bpy)) was added into the mixture. After 30 min of incubation (25 °C), the complex, which consists of the ODN-OsO_4(bpy), HIV *p24* and ODN-AuMPs, was 3-times rinsed in ACS water and electrochemically analysed.

2.6. The preparation of mercury film electrode (MFE)

The glassy carbon electrode (GCE) was mechanically polished by the 0.3 µm alumina suspension (ESA Inc., Chelmford, USA) on polishing cloth to produce mirror-like surface. Then, the electrode was sonicated for 5 minutes in deionised water (25 °C) in the Sonorex digital 10 P ultrasonic bath (Bandelin, Berlin, Germany). Thin mercury film (MFE) was formed according Farias et al. with a slight modification [18]. Briefly, MFE was formed in 10 mM $Hg(NO_3)_2$ solution prepared by the dissolution of 0.4 g of mercury(II) nitrate in 100 ml of an acidified water (5 % of HNO_3, *v/v*). The cell

was provided with an Ag/AgCl/3M KCl reference electrode and platinum auxiliary electrode, and contained the plating solution, which composition was optimized. The plating conditions of GCE by mercury (deposition time, deposition potential and the composition of plating solution) were also optimized and are shown in results. After plating the MFE was washed and dried under nitrogen.

2.7. Voltammetric measurements (differential pulse voltammetry)

5 µl of sample was pipetted on the film and was absorbed to the surface of MFE for 3 minutes. Then, the surface was washed and transferred to the clean cell with 0.2 M acetate buffer. The differential pulse voltammetry was used to determine the OsO_4(bpy) redox signals. The signals were recorded by Autolab Pgstat101 (Metrohm, Herisau, Switzerland) and analysed using software Nova 1.8 (Metrohm, Herisau, Switzerland). The potentiostat was set to conditioning time 5 s, deposition potential -1.3 V, deposition time 120 s, interval time 0.1 s, equilibration time 5 s, modulation time 0.004 V, modulation time 0.1 s, initial potential -1.3 V, end potential -0.1 and scan rate 25 mV.s^{-1}. All data was obtained at ambient temperature (25 °C).
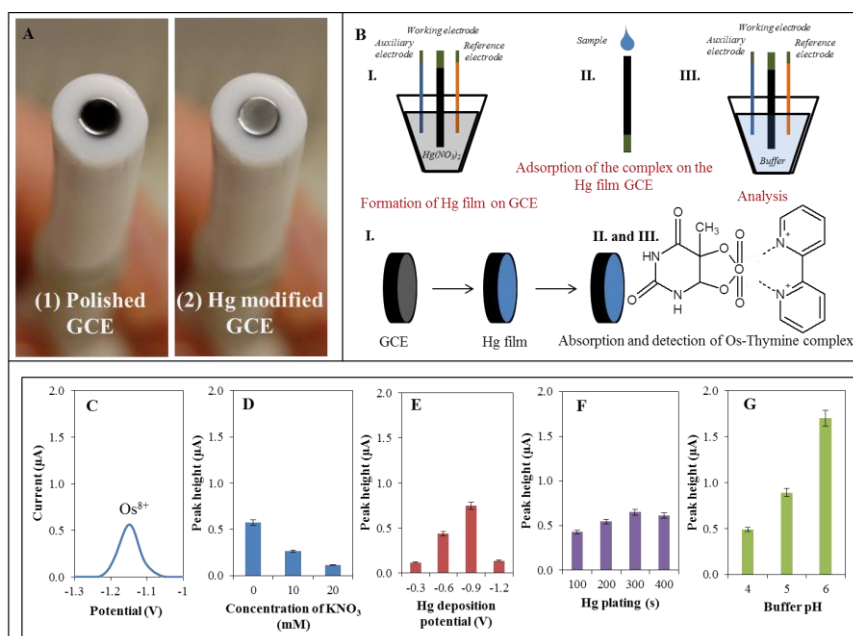
3. RESULTS AND DISCUSSION

Figure 1. (**A**) The comparison of polished glassy carbon electrode (1) and mercury film modified GCE (2). (**B**) The scheme of preparing the mercury modified glassy carbon electrode and subsequent analysis of the complex. (**C**) The electrochemical signal of OsO_4(bpy) labelled ODN (100 µg.ml^{-1}). (**D**) The influence of KNO_3 on the electrochemical signal of OsO_4(bpy) labelled ODN (100 µg.ml^{-1}). (**E**) The dependence of Hg deposition potential (-0.3 – 1.2 V) on the peak height of the OsO_4(bpy) labelled ODN (100 µg.ml^{-1}). (**F**) The time dependence of Hg plating (100 – 400 s) on the peak height of the OsO_4(bpy) labelled ODN (100 µg.ml^{-1}). (**G**) The influence of acetate buffer pH (4 – 6) on the peak height of the OsO_4(bpy) labelled ODN (100 µg.ml^{-1}).

The aim of our study was to perform the electrochemical detection of HIV gene for p24 capside protein. At the first part of experiment, the molecularly-biological methods were used to obtain the *p24* gene and the linear plasmid (pGEX-4T1) with the inserted *p24*. The creation of linear plasmid with *p24* gene and *p24* gene itself was proved by gel electrophoresis and theirs concentrations were determined by UV-vis spectrophotometry. The construct, which was analysed using mercury film electrode and adsorptive transfer method with differential pulse voltammetry detection, consisted of *p24* gene, the oligonucleotide probe to anchor the construct to magnetic particles and the second probe modified by OsO_4(bpy) to enable electrochemical detection.

3.1. Optimization of voltammetric measurements

The detection optimization of OsO_4 and 2,2′-bipyridine complex bounded to oligonucleotide (ODN-OsO_4(bpy)) was performed using DPV and three-electrode cell. The working glassy carbon electrode was modified by mercury film [19,20], which is responsible for adsorption of ODN-OsO_4(bpy) on the surface of electrode [21], like in the case of hanging mercury drop electrode [22] and it is shown in Figs. 1A and B. The Os^{+8} detection included three steps (Fig. 1A). At first, GCE was immersed to the solution of $Hg(NO_3)_2$ and by the application of negative potential the thin mercury film on the surface of electrode was created (Fig. 1A I). The rate of mercury deposition is a function of the pH of the electrolyte, deposition potential and mercury(II) ion concentration [23]. To the mercury film the sample of ODN-OsO_4(bpy) (5 μl of 50 μg.ml^{-1}) was pipetted and adsorbed to the surface of electrode for 3 minutes (Fig. 1A II). The un-adsorbed sample was washed and the electrode was transferred to the three electrode cell filled with 0.2 M acetate buffer, where the measurement was performed (Fig. 1A III). The catalytic signal of ODN-OsO_4(bpy) was analysed [24] and DP voltammogram is shown in Fig. 1D. The individual (I, II and III) steps were optimized. The effect of 0, 10 and 20 mM KNO_3 dissolved in the $Hg(NO_3)_2$ plating solution on the catalytic signal of ODN-OsO_4(bpy) was observed. The increasing concentration of KNO_3 caused the decrease of analysed signal for more than 45 % (Fig. E). The effects of deposition potentials (-0.3, -0.6, -0.9 and -1.2 V) and deposition times (100, 200, 300 and 400 s) on catalytic signal were also analysed. We found out that the best properties of mercury film were obtained by the application of deposition potential -0.9 V (Fig. 1F) for 300 s (Fig. 1G). These results are in good agreement with those published by Armalis et al. [25]. The pH 4, 5 and 6 of 0.2 M acetate buffer was also optimized. The best catalytic signal of ODN-OsO_4(bpy) was obtained by performing the measurement in buffer of pH 6 (Fig. 1H).

3.2 The voltammetric detection of the p24 gene alone

The HIV capsid protein *p24* gene was amplified by polymerase chain reaction (Fig. 2A 2) from pGEX-4T1 plasmid (Fig. 2A 1). The PCR product was analysed by gel electrophoresis, which proved the presence of target gene in the PCR solution. The 677 bp long band was observed (Fig. 2A). The unwanted compounds (salts, Taq DNA polymerase) were removed from the solution by purification (Fig. 2A 3), by which we obtained the solution of target double-stranded DNA (dsDNA). The dsDNA

was subsequently denatured (95 °C, 5 minutes) and rapidly cooled to obtain the single-stranded target DNA (Fig. 2A 4).

The gold modified magnetic particles (1 mg) were treated with 1 ml of water and sonicated for 20 minutes to divide clusters. We used the rapid creation of covalent bond between thiols and gold to capture the oligonucleotide probe to the AuMPs [26]. The 100 µl of created AuMPs mixture was after rinsing treated with 1, 5 and 10 µl of thiolated 100 µM oligonucleotide, which corresponded to 0.1, 0,5 and 1.0 nM of oligonucleotide. After removing the uncaptured probes by rinsing, to 0.1 mg of AuMPs 100 µl of hybridization buffer, 10 µl of OsO_4(bpy) modified 100 µM oligonucleotide probe (1.0 nM) and 0.6 pM of target gene sequence was added (Fig. 2A 5). The complex of OsO_4(bpy) and oligonuceotide exhibits electrocatalytic activity at the surface of mercury electrode and enables highly sensitive voltammetric detection (Fig. 2A 6) [27]. The 5 µl of obtained sample was pipetted to the surface of MFE and adsorbed to the surface for 3 minutes.
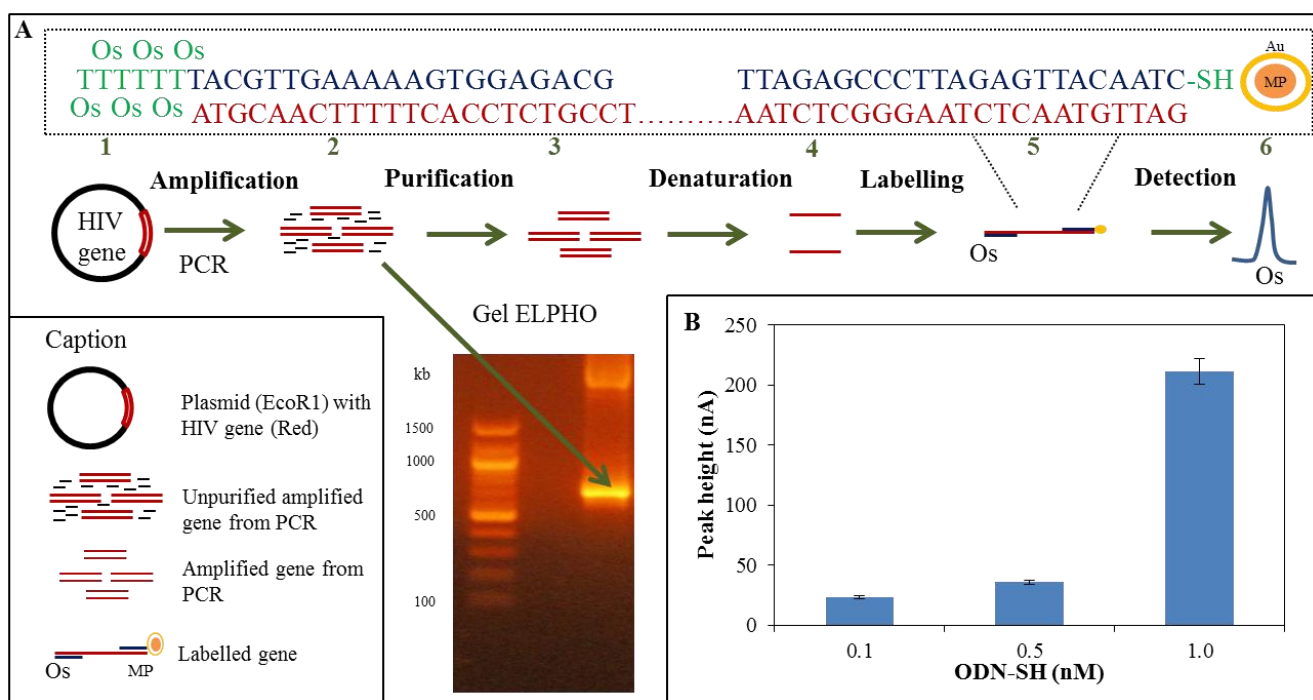

Figure 2. (**A**) Scheme of the complex preparation. Plasmid carrying the HIV gene (1) was amplified using the PCR method and analysed using the gel electrophoresis (2). Subsequently, 700 bp long amplicon was purified (3). The amplicon was denatured, afterwards labelled by thiolated oligonucleotides with bonded Au paramagnetic particle and OsO_4(bpy) modified oligonucleotide probe (5). In this way prepared complex was electrochemically analysed on MFE (6). (**B**) The dependence of the complex (I.) electrochemical signal (nA) on the concentration of thiolated ODN probe (0.1 – 1 nM).

Then, the un-adsorbed sample was washed and DPV was performed in 0.2 M acetate buffer (pH 6). We assumed that the thiolated oligonucleotide and target gene ratio (200:1, 1000:1 and 2000:1) affected the target gene detection signal (or more precisely OsO_4(bpy) oligonucleotide complex),

which was subsequently confirmed (Fig. 2B). The addition of 10-times higher amount of thiolated ODN led to the more than 9-times higher electrocatalytic signal of captured ODN-OsO$_4$(bpy). Based on these results we concluded 9-times higher capturing of target gene. The detection sensitivity of DNA (HIV) is comparable with the signal amplification of carbon nanotubes loaded with silver nanoparticles and placed on a gold microelectrode [28]. The alternative detection methods do not have comparable sensitivity [9,29].

3.3. The voltammetric detection of the linear plasmid with p24 gene

The detection of *p24* gene was also performed, when the gene was presented inside the linear plasmid. The pGEX-4T1 (4969 bp) plasmid with inserted target gene (Fig. 3A 1) was digested by the restriction endonuclease EcoRI (Fig. 3A 2). The presence of the linear plasmid in the sample was confirmed by the gel electrophoresis (Fig. 3A). The band was cut from the gel (Fig. 3A 3), purified and we obtained the solution of 10 μg.ml^{-1} of dsDNA, which was subsequently denatured.
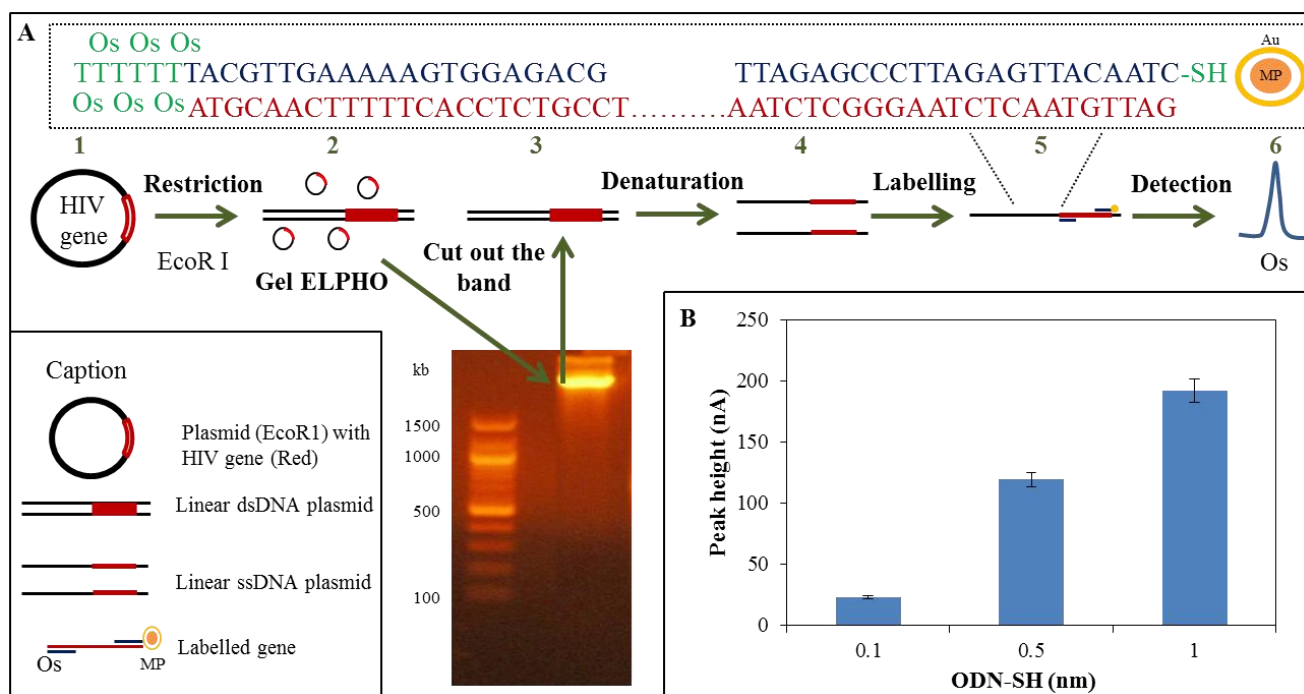

Figure 3. (**A**) Scheme of the complex II preparation. Plasmid carrying the HIV gene (1) was cleaved by restriction endonucleases (EcoRI) to linear dsDNA and analysed using gel electrophoresis (2). The band corresponding to molecular weight of linear ds DNA was cut out (3). Subsequently the sample was denatured and (4) after that the ss DNA was labelled by thiolated oligonucleotide with bounded Au paramagnetic particle and Os modified oligonucleotide probe (5). This way prepared complex was electrochemically analysed on the glassy carbon mercury film modified electrode (6). The frame shows the prepared complex. (**B**) The dependence of the complex (II.) electrochemical signal (nA) of the on the concentration of thiolated ODN probe (0.1 – 1 nM).

The samples were treated in the same manner like the sample of target gene itself and the conditions of measurement were preserved. The same volumes of samples were pipetted (5 µl) to the surface of MFE. The molar ratios of ODN-SH and target gene were 325-times higher, because no PCR was used to amplify the target gene (6500:1, 32500:1 and 65000:1). However, the detection method was able to detect the target gene in the 8 fM concentration. The increase in the amount of ODN-SH added to the MPs solution resulted in the increasing trend of detected ODN-OsO$_4$(bpy) (Fig. 3B).

Although the procedure of sample treating and detection were the same in the case of target gene itself and also gene within plasmid, and the gene concentrations were completely different. It must be pointed out that the analysed peak heights of detected ODN-OsO$_4$(bpy) are comparable (from tens to hundreds of nA). It is well known that the conformation of DNA and hybridization is influenced by temperature [30,31]. We suggest that the hybridization temperature (25 °C) did not prevent ODN-OsO$_4$(bpy) from nonspecific binding to the 677 bp long sequence of *p24* gene within plasmid. It is also possible that under particular conditions, the created construct is able to bind only limited amount of target gene.

4. CONCLUSIONS

In this study we succeeded in the preparation of nanoconstruct, which consisted of OsO$_4$(bpy) modified oligonucleotide probe hybridized to the HIV capside protein gene and its capturing to the gold modified magnetic particle. The mercury film electrode was prepared, the whole construct was adsorbed to its surface and the electrochemical signals were analysed.

CONFLICT OF INTEREST:
The authors have declared no conflict of interest.

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