Int. J. Electrochem. Sci., 8 **(2013)** 689 - 702

International Journal of ELECTROCHEMICAL SCIENCE
www.electrochemsci.org

Using of Paramagnetic Microparticles and Quantum Dots for Isolation and Electrochemical Detection of Influenza Viruses' Specific Nucleic Acids

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Received: 30 October 2012 / *Accepted:* 28 November 2012 / *Published:* 1 January 2013

The aim of this study was to suggest isolation protocol based on paramagnetic microparticles and multicolour electrochemical detection of three various influenza oligonucleotides (H1N1 influenza subtype, H3N2 influenza subtype and H5N1 influenza subtype) modified with nanoparticles (NPs) called quantum dots (QDs), which are made from zinc, cadmium or lead. Particularly, we described hybridization assay based on paramagnetic microparticles (MPs) and coupled with detection of three different influenza derived oligonucleotides (ODNs) labelled with QDs. Hybridization efficiency between NPs conjugated anti-senses and target nucleic acid hybrids was optimized and evaluated by the electrochemical analysis. The highest response of ODN-SH-NPs was determined for the hybridization temperature of 25 °C. Further, we tested detection of the complexes via peak coming from the presence of ODNs and/or from the presence of metal ions in QDs. Under the optimized conditions, LODs (3 S/N) based on the determination of metal ions in QDs label were estimated as follows: 0.1 ng/ml of ODN-SH-CdNPs, 0.5 ng/ml of ODN-SH-ZnNPs and 1 ng/ml of ODN-SH-PbNPs. Besides this we also correlated the results from ODNs and metal ions signals.

Keywords: Influenza; Nanoparticles; Quantum Dots; Multi Target Detection; Voltammetry; Magnetic Particles; Hybridization; Automated Separation; Electrochemistry

1. INTRODUCTION

Influenza is an infectious disease, which affect respiratory system and is often associated with the high morbidity in the common population and mortality in a group of high-risk patients

(immunosuppressed, geriatrics or very young etc.). Currently, vaccination is the most common way of the influenza control. Influenza vaccine exists, however, it must be prepared year after year again because of the constant mutational changes in the antigenic structure of the influenza virus (hemagglutinin and neuraminidase antigens). In collaboration with World Health Organization (WHO), two vaccines are prepared every year in a six-month interval between the Southern and Northern hemisphere due to seasonal occurrence in the winter months [1]. Except the common subtypes that are responsible for mortality only in a high-risk patients group, highly virulent subtypes, such as H5N1 (highly pathogenic avian influenza) with mortality of about 60%, occur [2,3]. Constant mutational changes of antigens represent a high risk, especially in the connection with a possibility of origination of the highly virulent subtype(s). Global travelling can successfully potentiate extension of the global pandemic. Rapid and well-timed diagnostics is one of the most effective tools in the fight against emergence and spread of the epidemic. Multi target analyses have a great potential as a tool for the diagnosis of commonly occurring and new influenza subtypes [4-6]. Obviously applied analytical methods for identification of nucleic acids (NAs) require highly sensitive analytical tools. Microarrays, as one of the tested tools, are based on the fluorescent probes; however, they are limited by their sensitivity, range of the emission spectrum and demands on data evaluation [7-11]. In the light of the above-mentioned facts, methods of nucleic acids detection and isolation have become the crucial part of nucleic acids analysis [12-19]. Presently, paramagnetic particles are widely used in the NAs isolation [20-25]. They may be applied for the isolation of RNA, DNA and proteins as well as for the isolation of specific nucleic acids or specific antigens [11,24,26-29]. Paramagnetic particles (MPs) enable automation of the whole process; in addition, paramagnetic particles' surface may be effectively modified by interactions with target biomolecules [23]. Target DNA is usually isolated and detected by probe hybridization and by using different compounds such as biotin-avidin system, substrate-enzyme systems, antigen-antibody system and fluorescent dyes or quantum dots (which has vast spectrum of application in microarrays as fluorescent probes) [30-32]. Recently, a plenty of assays uses oligo (probe) conjugated MPs for the isolation of target DNA [26,33-35].

Nanoparticles (NPs) have significantly improved the techniques based on the paramagnetic particles. Through the nano size (scale) dimensions ranging from 1 to 100 nm, NPs have a quite large active surface, high mechanical resistance, excellent electrical, catalytic and eventually magnetic properties with the possibility to interact with a wide range of biological as well as chemical compounds [36-41]. They extended possibilities in the NAs isolation and especially detection [12,14,23,24,29,42-46]. Gold, silver and iron NPs are the most used NPs for this purpose [14,47,48]. However, isolation itself is not sufficient for the diagnosis. Isolated targeting NA must be detected by the use an appropriate method. Detection of hybridised target NANAs is important for a diagnosis of viral and bacterial pathogens as well as diagnosis of genetic diseases or for forensic analyses [11,49]. Nowadays, research is directed to the finding the methods for the simultaneous detection of multiple target DNA. The research is focused on the methods using optical detection with application of fluorescently active labels [50-52]. Wang et al. describes multi-target electrochemical DNA detection based on the application of different quantum dots tags, multi-target electrochemical detection scheme incorporates the high sensitivity and selectivity advantages of the NPs-based electrochemical assays [53]. The great advantage of electrochemical methods is based on their high sensitivity, low costs and

possibility of automation and connection with other analytical methods [54-59]. In addition, they can be miniaturized into nanoscale dimensions [60-64].

The aim of this study was to suggest isolation protocol based on paramagnetic particles and multicolour electrochemical detection of three various influenza ODNs (H1N1 influenza subtype, H3N2 influenza subtype and H5N1 influenza subtype) modified with nanoparticles (NPs) called quantum dots, which are made from zinc, cadmium or lead according to scheme shown in Fig. 1/Part 1.

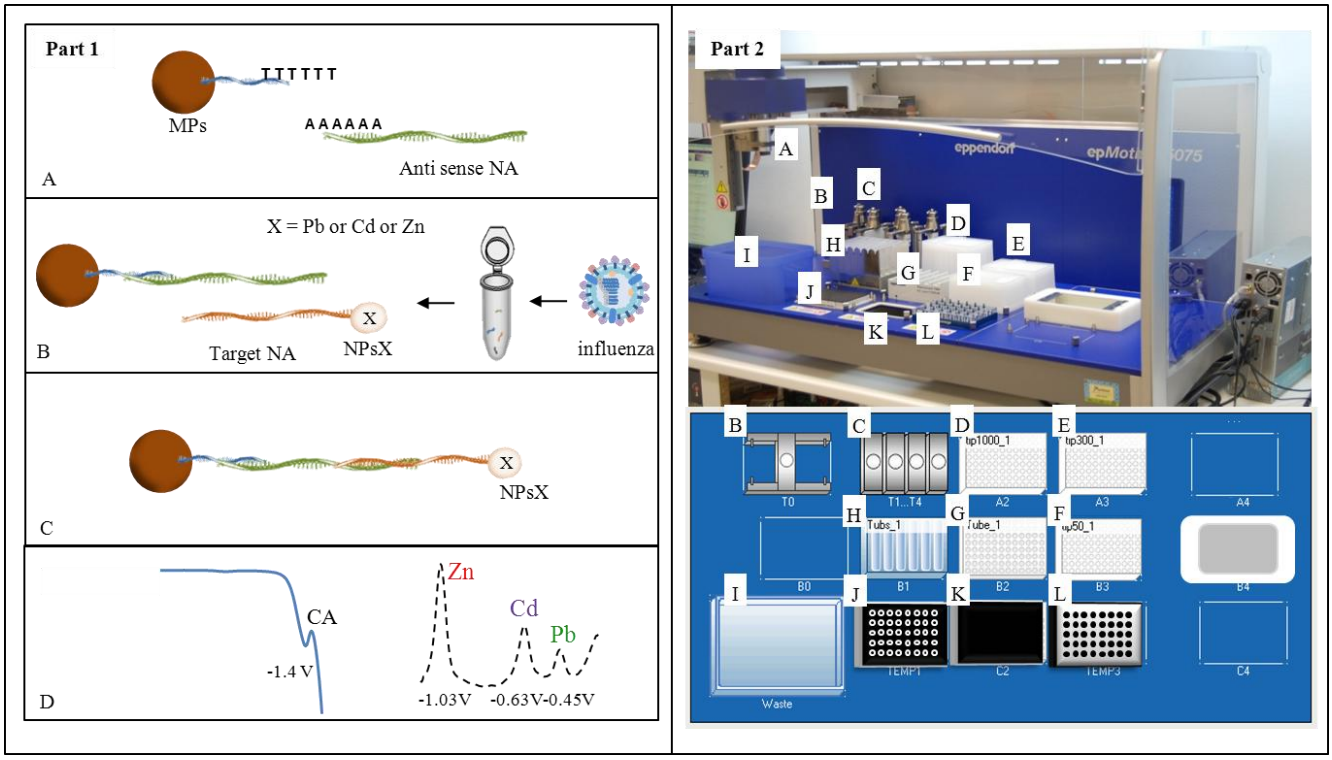

Figure 1. (**Part 1**) Scheme of isolation and detection of specific influenza sequences by using paramagnetic microparticles (MPs), nanoparticles labels as quantum dots (NPs) and electrochemistry. (**A**) Paramagnetic particle modified by poly thymine strand oligonucleotide and addition of probe modified by poly adenine strand. (**B**) Addition of target to: ODN-SH-XNPs. X= Pb or Cd or Zn. (**C**) Hybridization ODN-SH-XNPs to anti sense on MPs. (**D**) Electrochemical detection of XNPs and target influenza ODN. (**Part 2**) (**A**) arm for tips and microplate holder with optic sensor, (**B**) microplate holder, (**C**) tips holders, (**D**) tips (1000 µl), (**E**) tips (300 µl), (**F**) tips (50 µl), (**G**) rack tubes (Eppendorf 1.5 ml, MPs, antisense, specific ODN labelled by NPs, hybridization buffer, phosphate buffer II), (**H**) reservoir holder (30 ml, phosphate buffer I and waste), (**I**) container for used tips, (**J**) thermo adapter, (**K**) start position for microplate – PCR plate 96, (**L**) magnetic adapter.

2. EXPERIMENTAL PART

2.1 Preparation of nanoparticles (NPs)

All chemicals were purchased from Sigma Aldrich (USA) in ACS purity and used without further purification. CdS QDs were prepared with a slightly modified method published in [34].

Briefly, cadmium nitrate tetrahydrate $Cd(NO_3)_2 \cdot 4H_2O$ (0.03085 g, 0.1mM) was dissolved in ACS water (25 ml). 3-mercaptopropionic acid (35 µl, 0.4mM) was slowly added to stirred solution. Afterwards, pH was adjusted to 9.11 using 1M NH_4OH (1.5 ml). Sodium sulphide nonahydrate $Na_2S \cdot 9H_2O$ (0.02402 g, 0.1mM) in 23 ml of ACS water was poured into the first solution with vigorous stirring. Obtained yellow solution was stirred for 1 h. CdS QDs were stored in dark at 4ºC. ZnS QDs were prepared similarly to CdS QDs; zinc nitrate hexahydrate $Zn(NO_3)_2 \cdot 6H_2O$ (0.02975 g, 0.1 mM) was used for this preparation. Obtained colourless solution was stirred for 1 h and stored in dark at 4ºC. PbS QDs were prepared by modified method of Hennequin [65]. Lead acetate trihydrate $Pb(OAc)_2 \cdot 3H_2O$ (0.03794 g, 0.1mM) was dissolved in ACS water (25 ml). 3-mercaptopropionic acid (60 µl, 0.69mM) was slowly added to stirred solution. White precipitate was formed, which disappeared after addition of 3.8 ml of 1M NH_4OH (pH = 9.88). $Na_2S \cdot 9H_2O$ (0.01201 g, 0.05mM) in 21.2 ml of ACS water was added with vigorous stirring. The obtained brown solution was stirred for 1 h and stored in dark at 4 ºC.

2.2 Preparation of NPs-labelled oligonucleotide (ODN-SH-NPs)

ODN-SH (100 µl, 100 µg/ml, Tab. 1) was mixed with a solution of NPs (100 µl). This mixture was shaken for 24 h at room temperature (Vortex Genie2, Scientific Industries, USA). Subsequently, solution was dialysed against 2000 ml of miliQ water (24 h, 4ºC) using a Millipore membrane filter 0.025 µm VSWP. During dialysis the sample was diluted to 800 µl. Diluted sample was concentrated to the final volume of 500 µl on a centrifuge filter device Amicon Ultra 3k (Millipore, USA). Centrifuge 5417R (Eppendorf, Germany) set to the following parameters: 15 min, 4,500 rpm, 15 ºC.

Table 1. Probes and targets oligonucleotide (ODN) [66].

Probes	Target oligonucleotide
H1N1- 5′ (AAAAA) CCA TTG GTT C 3′	H1N1- 5′ (Th) GAA CCA ATG G 3′
H3N2- 5′ (AAAAA) CCC GTT ACA C 3′	H3N2- 5′ (Th) GTG TAA CGG G 3′
H5N1- 5′ (AAAAA) CCT CAA GGA G 3′	H5N1- 5′ (Th) CTC CTT GA GG 3′

2.3 Automatic isolation of ODN-SH-NPs

Automatic pipetting station epMotion 5075 (Eppendorf, Germany) with original devices (microplate holder, tips holder, tips (1000, 300, 50 µl), rack tubes, reservoir holder, container for used tips, thermo adapter, PCR plate 96, magnetic adapter) was used for the fully automated target nucleic acids isolation process (Fig. 1/Part 2). Volume of 10 µl of Dynabeads Oligo $(dT)_{25}$ (Invitrogen, Oslo) was dispensed in each well in the plate (PCR 96, Eppendorf, Germany). The plate was subsequently transferred to the magnet and NPs storing solution was aspirated to waste. Subsequently, beads were further washed three times with 20 µl of phosphate buffer I (pH = 6.5, 0.1 M NaCl + 0.05 M Na_2HPO_4 + 0.05 M NaH_2PO_4). The *first hybridization* according to the following protocol was the next step. 10 µl of polyA-modified anti sense oligonucleotide and 10 µl of hybridization buffer (0.1 M phosphate

buffer, 0.6 M guanidinium thiocyanate, 0.15 M Tris, pH = 7.5) were added in each well and then the plate was incubated (15 min, 25°C, mixing). This procedure was followed by a washing (three times) with 20 µl of phosphate buffer I. The *second hybridization* according to the following protocol was the next step. 10 µl of NPs-labelled oligonucleotide and 10 µl of hybridization buffer (0.1 M phosphate buffer, 0.6 M guanidinium thiocyanate, 0.15 M Tris, pH = 7.5) were added to each well and the plate was incubated (15 min, 25°C, mixing). Procedure was followed by a washing (three times) with 20 µl of phosphate buffer I. Then, 30 µl of elution solution (phosphate buffer II - 0.2 M NaCl + 0.1 M Na_2HPO_4 + 0.1 M NaH_2PO_4) was added into each well and plate was subsequently incubated (5 min, 85°C, mixing). After elution step, the plate was transferred to the magnet, and elution product from each well was transferred to a separate well.

2.4 Method for detection of CA and metal peak of ODN-SH-NPs

Electrochemical measurements were performed with 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder (4 °C). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and glassy carbon electrode was auxiliary electrode. GPES 4.9 supplied by EcoChemie was employed for data processing. All measurements were performed in the presence of acetate buffer 0.2M CH_3COOH + 0.2M CH_3COONa (pH 5.0) at 25°C. The analysed samples were deoxygenated prior to measurements by purging with argon (99.99%) for 120 s.

For detection of CA signal, square wave voltammetry (SWV) method was applied [23]. The parameters of the electrochemical determination were as follows: initial potential 0 V; end potential - 1.85 V; frequency 10 Hz; potential step 0.005 V; amplitude 0.025 V. Differential pulse voltammetry (DPV) was applied for electrochemical detection of metal part of the complex. In this case the parameters of electrochemical determination were as follows: Cd (initial potential -0.9 V; end potential -0.45 V); Zn (initial potential -1.2 V; end potential -0.85 V); Pb (initial potential -0.6 V; end potential -0.25 V); others parameters were the same: deposition potential -0.9 V; duration 240 s; equilibration time 5 s; modulation time 0.06 s; time interval 0.2 s; potential step 0.002 V; modulation amplitude 0.025 V.

2.5 Mathematical treatment of data and estimation of detection limits

Mathematical analysis of the data and their graphical interpretation was realized by MICROSOFT EXCEL® (USA). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise (EXCEL®). The detection limits (3 signal/noise, S/N) were calculated according to Long and Winefordner [67], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

3. RESULTS AND DISCUSSION

We suggested and developed hybridization kit for the isolation and detection of three different influenzas' nucleic acids sequence from one sample. Procedure is based on the detection of metal-based NPs (ZnNPs, PbNPs and/or CdNPs) complex with NAs. Generally, metals can be very sensitively and selectively determined using the electrochemical techniques. NPs from different metals provide signals at different potentials -1.03 ± 0.005 V (Zn), -0.63 ± 0.005 V (Cd) and -0.45 ± 0.005 V (Pb) [40,63,68,69]. The suggested procedure is shown in Fig.1/Part 1. Firstly, separation step as isolation of target sequences from sample was provided by anti-sense modified MPs (Fig. 1/Part 1A). After hybridization of target sequence to MPs, the washing step followed. After removing the interfering compounds (unbound MPs, proteins and other residues of matrix), detection of the elution of bound target influenzas' sequences could be carried out. However, we would obtain information only about the total amount of isolated NAs without details about their sequence or influenza subtypes. For the simultaneous three influenza subtypes differentiation and their quantification, target NAs (influenzas' derived oligonucleotides) were incubated with the ZnNPs, CdNPs, and PbNPs (Fig. 1/Part 1B). MPs used in isolation part were modified with three different probes (anti sense chain), which are able to bind the target sequences by hybridisation reaction between anti sense chain and target influenza ODN (Fig. 1/Part 1C). Complex NPs with target ODN was designated as ODN-SH-ZnNPs; ODN-SH-CdNPs and/or ODN-SH-PbNPs (connection due to-S-S- bond). After complex formation due to recognizing the specific target sequence, this can be determined electrochemically (Fig. 1/Part 1D).

ODN-SH-NPs enable simultaneous quantification of three different target NAs. Application of two different electrochemical methods (SWV and DPV) brought two diverse signals. The first one corresponds to ODN-SH (CA peak) measured by SWV and the second one to metal part of QDs (metal peak) measured by DPV. In the case of application of both methods, we obtain information about the presence of NPs corresponding to one of the three target molecules (influenza subtype) which coresponded to one of three specific influenzas' derived ODNs. However, in the case of the only one signal detection, we can assume that the probe has not been linked with the target sequence or the linkage has failed. Signal of metal part NPs cannot be evaluated in this case. In addition to above-mentioned facts, all steps were fully automated by the use of an automated pipetting system modified for the hybridisation assay and for manipulation with paramagnetic and nano particles (Fig.1/Part 2).

3.1 Electrochemical detection of ODN-SH-NPs – CA and metal peaks

In general, electrochemical determination of oligonucleotides is based on the measurement of electroactivity of DNA (respectively cytosine and adenine bases), or the measurement of electroactivity of labels that has been used for modifying the oligonucleotides. Measurement of direct electroactivity (label-free) of oligonucleotides is the easiest and low cost variant, which is available [58,70-73]. The second possibility of DNA detection is DNA modifying by the electroactive label (indirect detection). The widely used compounds are methylene blue [74], Meldola's blue [75],

ferrocene and organic ligand complexes with osmium, ruthenium and copper [76-78]. Recently, quantum dots were used for this purpose also [30,79,80].

Labeling of oligonucleotides with nanoparticles as quantum dots is the basic requirement for theirs detection in our suggested system. Therefore, it was necessary to optimize all steps for the correct function of hybridization kit. Isolation of target sequences using anti sense modified MPs was carried out in accordance with the following paper [23]. Due to this fact, we focused on the optimization of ODN-SH-NPs detection. Effect of ODN-SH-NPs accumulation and ODN-SH accumulation on the working electrode is shown in Fig. 2A. We detected CA signal at -1.4V. This signal corresponds to reduction of adenine and cytosine bases, why it is called CA peak. The highest signal of CA peak was obtained at detection of ODN-SH without NPs (ODN-SH signal in blue) linkage at the time of accumulation 120 – 180 s (Fig. 2A). Signals of all ODN-SH-NPs are app. 50% lower compared to ODN-SH at the same time of accumulation (Fig. 2A). However, they provide sufficient signal (signal of metal part NPs) for their determination. Therefore we detected signals of individual NPs corresponding to metals (Zn, Cd and Pb) at potentials of -1.03V (Zn), -0.63V (Cd) and -0.45V (Pb). The main advantage of the NPs detection (compared to CA signal determination) is the fact that this way of detection is more sensitive with the lower limit of detection. Evaluated signals corresponding to individual NPs metals (Zn signal in red); (Pb signal in green) and (Cd signal in violet) are demonstrated in Figs. 2B, C and D. The increasing time of accumulation lead to the increase in response. Various NPs achieved saturation of electrode surface at various times. The highest responses were detected at time of accumulation 900 s for Zn and at 1000 s for Pb (Figs. 2B and C). ODN-SH-CdNPs required the shorter time of accumulation. The highest response of Cd peak was determined at 390 s (Fig. 2D).

We were subsequently focused on the possibility to bind various amounts of ODN onto NPs and to detect these amounts. Effect of ODN concentration on the CA peak height is shown in Figs. 3A and B. We used ODNs, which was linked to NPs via -SH groups, within the concentration range from 0.002 to 2 μg/ml. The increasing concentration led to the increase of the detected signal in all targets. The dependences had polynomial character in all cases (Fig. 3A). However, in lower concentration range (0.002 μg/ml to 0.125 μg/ml) it was possible to detect linear course too (Fig. 3B). Regression equations for individual probes with the coefficient of determination R^2=0.99 are shown in Fig. 3A. If we compare ODN-SH and ODN-SH-NPs, we can determine the effect of bounded NPs on the response of CA signal. The most significant effect on the signal response was determined for PbNPs, the least for CdNPs. Due to differences in the sensitivity, LODs (3 S/N) were estimated as follows: 0.1 ng/ml of ODN-SH-CdNPs, 0.5 ng/ml of ODN-SH-ZnNPs and 1 ng/ml of ODN-SH-PbNPs.

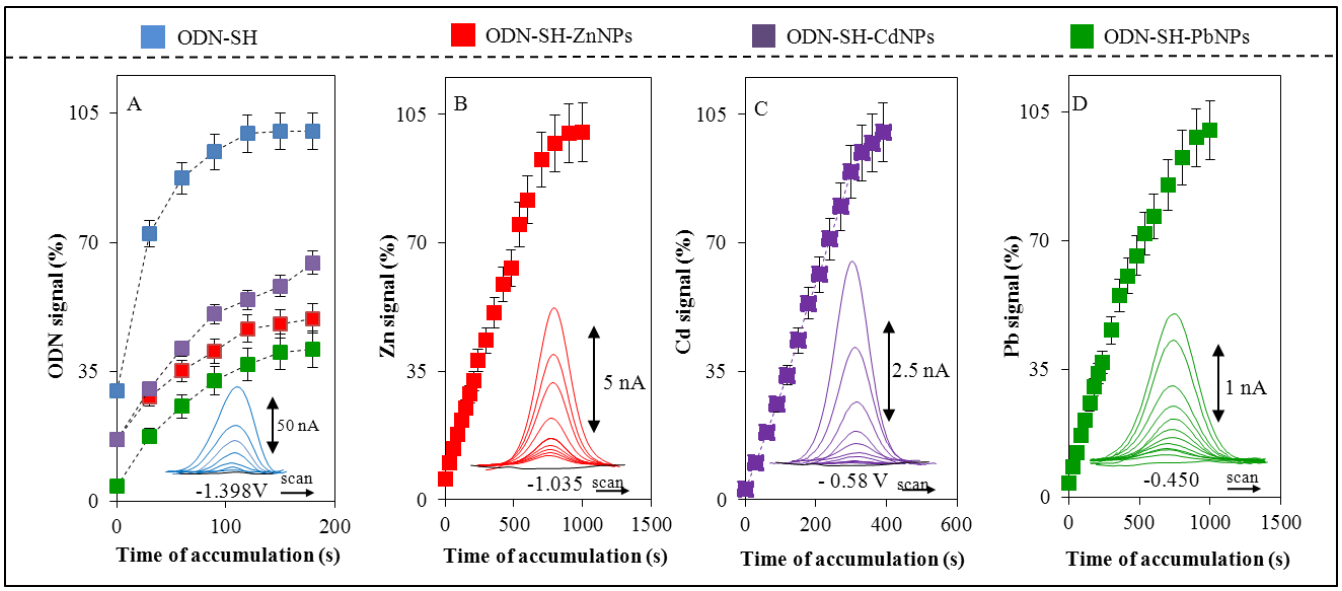

Figure 2. Optimization of accumulation time: (**A**) CA peaks of ODN-SH (♦); ODN-SH-ZnNPs (♦); ODN-SH-PbNPs (♦); and ODN-SH-CdNPs (♦). (**B**) Zn peak of ODN-SH-ZnNPs (♦). (**C**) Pb peak of ODN-SH-PbNPs (♦). (**D**) Cd peak of ODN-SH-CdNPs (♦); concentration of ODN-SH and ODN-SH-NPs was 2 µg/ml. CA peak was measured by SWV. Zn, Cd and Pb peaks were measured by DPV.

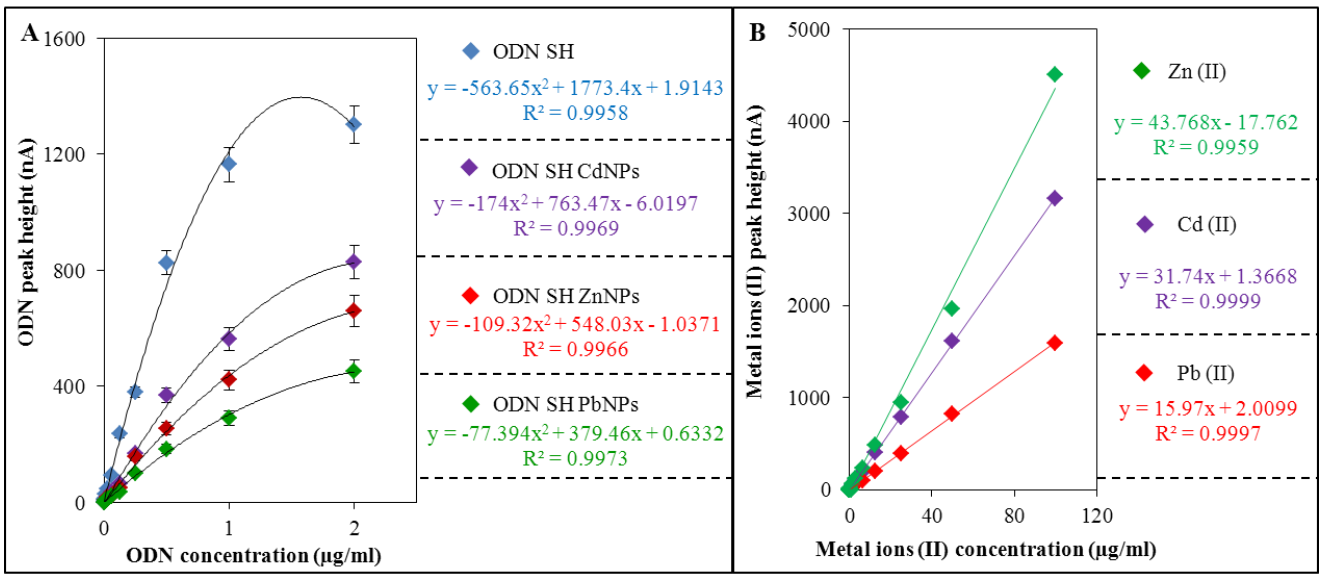

Figure 3. (**A**) Dependence of CA peak height on concentration of ODN (µg/ml) as ODN-SH (♦); ODN-SH-ZnNPs(♦); ODN-SH-PbNPs(♦) and ODN-SH-CdNPs (♦). (**B**) Dependence of metal ions peak height on concentration of metal ions (µg/ml) as Pb(II): (♦); Zn(II): (♦) and Cd(II): (♦).

3.2 Optimization of hybridization temperature

It was necessary to determine the effect of hybridization temperature on the binding of ODN-SH-NPs with the target NAs linked with magnetic NPs for the optimum hybridization (Fig. 4). Target

NAs interacted for 30 min with 2.5; 5; 10 and 20 µg/ml of ODN-SH-NPs at the temperature of 15 °C, 20 °C, 25 °C and 30 °C. Subsequently, the CA signal (Figs. 4. A, C and E) and Cd, Zn and Pb responses were determined (Figs. 4 B, D and F). It clearly follows from the results shown in Fig. 4 that the highest response of ODN-SH-NPs was determined for the hybridization temperature of 25 °C. On the other hand, the lowest ODN-SH-NPs response was detected for the hybridization temperature of 30 °C. ODN-SH-CdNPs represented exception. In this case, hybridization temperature of 30 °C led to the binding of the second higher amount of labelled oligonucleotides in the studied concentrations 10 and 20 µg/ml compared to other temperatures. We expected that the higher temperature of hybridization increased hybridization effect. This direct correlation can be applied only to the temperature of 25 °C because the oligonucleotide T_m was 28 °C.

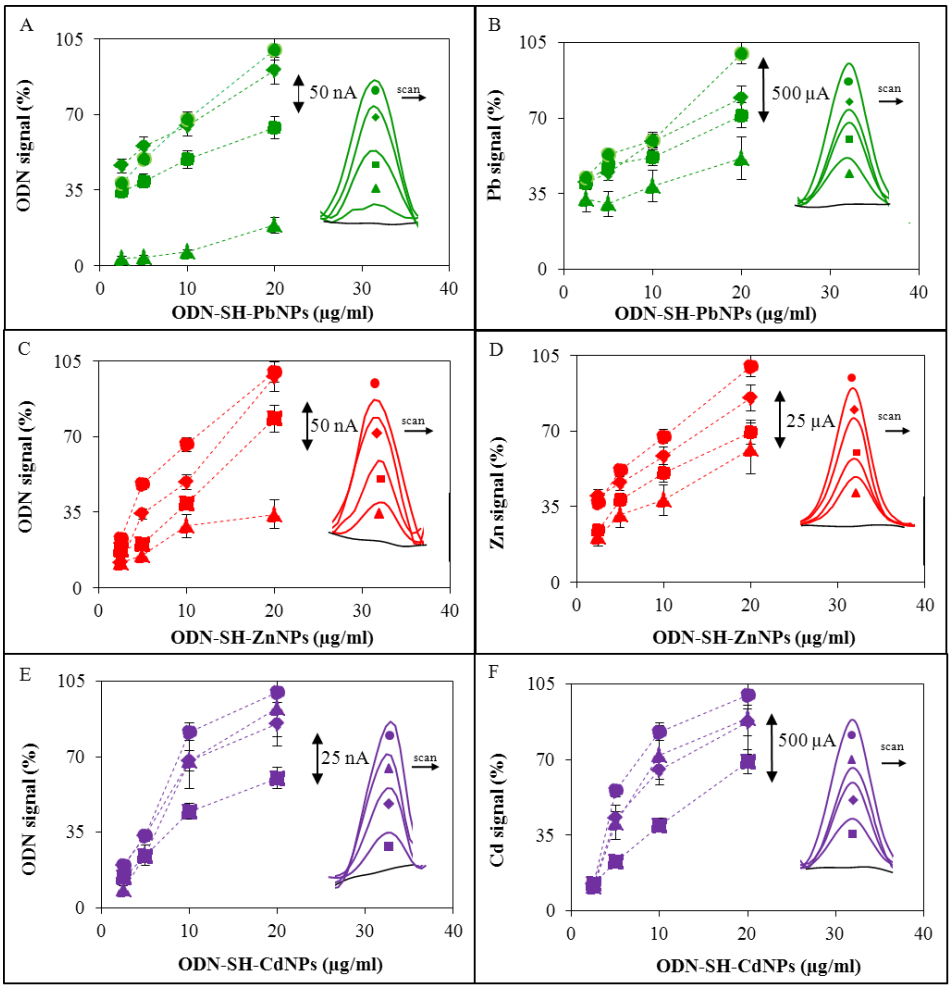

Figure 4. Dependence of relative peak heights (%) on concentration of ODN-SH-NPs (µg/ml), (left side – ODN peak height; right side – metal peak height); (**A+B**) ODN-SH-PbNPs; (**C+D**) ODN-SH-ZnNPs; (**E+F**) ODN-SH-CdNPs. Hybridisation temperatures: ■15 °C; ♦20 °C; ● 25 °C; and ▲30 °C.

3.3 Correlation Analysis

For the evaluation of dependence between CA signal and signal corresponding to NPs, correlation analysis was carried out (Fig. 5/Part 1/A, B and C). We used percentage values of signals

for individual NPs, temperatures and concentrations. The correlation was 77% in the case of PbNPs, 89% at ZnNPs and 91% at CdNPs. Further, the Spearman Rank-Order Correlation analysis was proved. In this case, the correlation coefficient for PbNPs was 0.90, for ZnNPs 0.89 and for CdNPs 0.96. Correlation analysis provided sufficient data, which showed that measuring both ODN-SH and NPs gave us similar results.

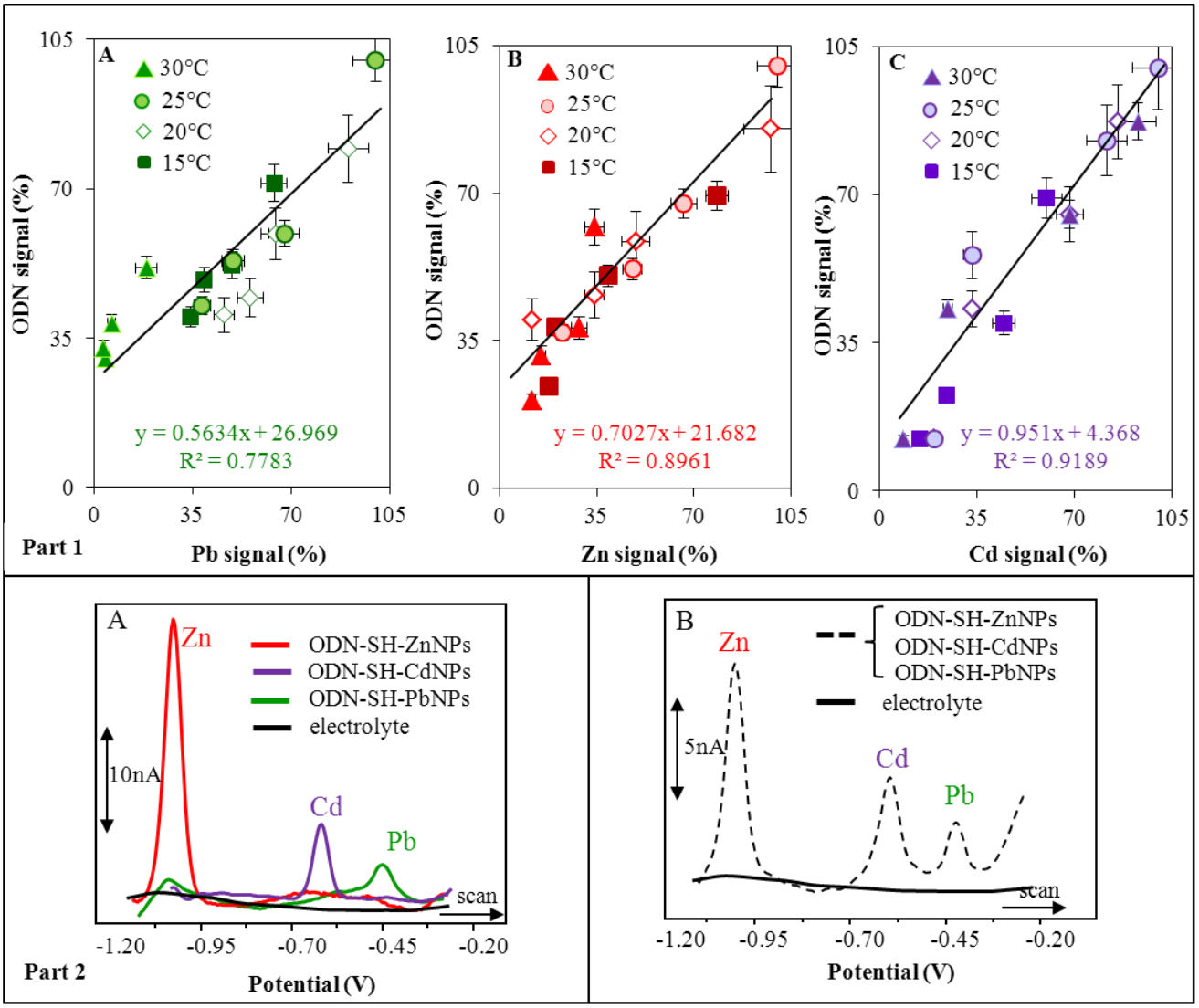

Figure 5. (**Part 1**) Correlation between the peak height of ODN and metal ions peak for hybridization temperatures: 15 °C, 20 °C, 25 °C and 30°C and concentrations of ODN-SH-NPs: 2.5; 5; 10 and 20 µg/ml for (**A**) ODN-SH-PbNPs (♦); (**B**) ODN-SH-ZnNPs (♦) and (**C**) ODN-SH-CdNPs. (**Part 2**) (**A**) voltamograms of Zn, Cd and Pb peak from complex ODN-SH-ZnNPs (-); ODN-SH-PbNPs (-) and ODN-SH-CdNPs (-), measured by DPV (concentration of ODN was 2 µg/ml). (**B**) Mixture of oligonucleotides (concentration of all ODNs was 2 µg/ml).

The results showed that both ODN-SH-ZnNPs and ODN-SH-PbNPs had optimal hybridization temperature 25 °C for 25 µg/ml. Further, ODN-SH-CdNPs provided optimal hybridization temperature as 25 °C also, but the other hybridization temperature provided almost similar data like hybridization at 25 °C. This we observed only in ODN-SH-CdNPs for 25 µg/ml concentration. We conclude from

the results that hybridization between ODN-SH and NPs within the range from 20 °C to 25 °C is suitable for the whole tested concentration interval.

3.4 Multi target determination

All above-mentioned experiments led to the optimization of the experimental conditions for individual experimental steps of the whole experiment. After it, we applied this procedure on the preparation samples containing mixture of specific probes and target sequence for the given specific sequence of ODN labelled by NPs. Test tubes with these reagents were placed in the automated pipetting system, where the isolation of target influenzas' ODN using the MPs, hybridization and washing steps take place. The resulted samples were determined using DPV. Voltammograms of individual signals for individual NPs are shown in Fig. 5/Part 2A. Red curve indicates record of ZnNPs with Zn signal in the potential area of -1.03 ± 0.005V. Violet curve indicates record of CdNPs and Cd signal at the potential of -0.63 ± 0.005V. Green curve represents PbNPs and Pb signal in the potential area of -0.45 ± 0.005V. The obtained signals are well separated and they are symmetric and well repeatable. In addition, we focused on the detection of individual ODN-SH-NPs in the mixture in test tube. Voltammogram of this measurement is shown in Fig. 5/Part 1B. In given potential areas all three signals are well evident and they correspond to ZnNPs – H1N1 influenza subtype (on the left), CdNPs – H3N2 influenza subtype (in the middle) and PbNPs H5N1 influenza subtype (on the right). This result confirmed our main goal of this study to suggest electrochemical nanobiosensor that enables identification of three specific influenza sequences without demands on special apparatus equipment.

4. CONCLUSIONS

We suggested fully functional hybridization kit for the identification of specific nucleic acids based on the hybridization and electrochemical detection. Due to the various NPs based on the different metals that provide signals at the specific potential areas, we can simultaneously determine three different influenzas subtypes in one sample. Connecting between paramagnetic particles, nanoparticles, automated pipetting system and electrochemical detection enable proposition of unique tool for the detection of specific NAs sequences. Electrochemical bionanosensors brings new possibilities into this field. High sensitivity of the suggested sensing device with the possibility of miniaturization, compatibility with modern technologies, low price and low demands on the power (possibility of *in situ* application) represent the excellent properties for detection of specific NAs in the field of pathogen and genetic disease diagnostics.

ACKNOWLEDGEMENTS
Financial support from NANIMEL GA CR 102/08/1546, NanoBioTeCell GA CR P102/11/1068 and CEITEC CZ.1.05/1.1.00/02.0068 is highly acknowledged.

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