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Flow Injection Electrochemical Analysis of Complexes of Influenza Proteins with CdS, PbS and CuS Quantum Dots

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Due to threat coming from viral diseases, mainly from influenza, new assays and devices are investigated. This study is aimed at developing of low-cost isolation and detection of specific influenza protein hemagglutinin (HA) labelled with CdS, PbS, and CuS quantum dots. The influenza protein was determined via concentration of cadmium(II), lead(II), and copper(II) ions contained in quantum dots using stopped flow injection analysis with electrochemical detection (SFIA-ED). The main attention was devoted to detection of quantum dots and optimization of the whole analytical system. A particular aim was focused on an application of streptavidin-coated paramagnetic particles that were modified with biotinylated glycan. In addition, their selective binding properties to the influenza virus were monitored. Based on the results it can be concluded that CdS QDs were the best label for viral protein determination.

Keywords: Nanoparticles; Quantum Dots; Differential Pulse Voltammetry; Stopped Flow Injection Analysis; Glassy Carbon; Biosensors; Electrochemical Detection; Influenza Virus H5N1; Hemagglutinin

1. INTRODUCTION

Nanotechnology is currently one of the most discussed technologies with possible application in many branches [1], which depends on the size of the nanomaterials being developed and used [2,3]. Nanotechnology is widely considered as being such a general-purpose technology and becomes a common technology for almost all sectors because of its ability to create greatly-functional properties

of materials at nano-scale level. These nanomaterials are based on the large surface of active area [4,5]. Nanomaterials also include fluorescent semiconductor nanocrystals, commonly known as quantum dots (QDs). Quantum dot is a bounded conductive region of very small dimensions with large band gap energy [6,7]. Optical properties of QDs, i.e. the ability to emit light, belong to the one of the most important features. This ability is linked to the size of quantum dots. QDs with higher diameter emit the red light while smaller QDs emit the blue light. This ability is again associated with the distribution of energetic layers in the dot and its ability to absorb not only electrons but also photons [6,8,9]. Generally, the band gap energy determines the energy and thus colour of the fluorescent light and is inversely proportional to the size of QDs.

Due to tuneable properties of QDs, it is not surprising that these nanomaterials have numerous applications in biosensors and bioassays [10-12] including those aimed at detection of viruses [13]. Influenza virus represents one of the greatest threats [14,15]. Based on this, World Health Organization (WHO) initiated the Global Influenza Program, which provides member states with technical support, strategic guidance and coordination of activities essential to better prepare healthcare systems against seasonal, zoonotic, and pandemic influenza threats to populations and individuals (http://www.who.int/influenza/en/) [13,15-22].

This study is aimed at developing of low-cost isolation and detection of specific influenza protein hemagglutinin (HA) labelled with CdS, PbS, and CuS quantum dots. Influenza virus contains two major surface proteins, hemagglutinin (HA) and neuraminidase (NA). HA, an antigenic homotrimeric integral membrane glycoprotein, mediates glycan receptor binding. Neuraminic acid (known also as sialic acid) is generally presented on the surface of target cells as a part of glycoproteins. Neuraminic acid mediates the sticking the viral particles onto the surface of the cells and membrane fusion for cell entry of influenza virus. NA functions as a receptor destroying enzyme in virus release and is necessary for influenza virus replication [23]. It cleaves both terminal neuraminic acid groups from glycoproteins on the surface of the infected cells and neuraminic acid residues from viral proteins, which prevents aggregation of viruses. Here, the influenza protein was determined via concentration of cadmium(II), lead(II), and copper(II) ions included in quantum dots employing stopped flow injection analysis with electrochemical detection (SFIA-ED). This system worked with a glassy carbon electrode as a working electrode. The sample was pumped into a measuring cell and then drained away. The great advantage of this system consists in the minimal volume of applied sample and rapidity of setting and measurement [14,24-26]. The main attention was devoted to the detection of quantum dots and optimization of the whole analytical system. A particular aim was focused on (1) an application of streptavidin-coated paramagnetic particles that were modified with biotinylated glycan and (2) monitoring their selective binding properties to the influenza virus.

2. EXPERIMENTAL PART

2.1 Chemicals and material

All chemicals with ACS purity were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise noted. Stock solutions were prepared using ACS water. pH value and conductivity

were measured using an inoLab Level 3 (Wissenschaftlich-Technische Werkstatten; Weilheim, Germany). Deionized water underwent demineralization by reverse osmosis using an Aqua Osmotic 02 (Aqua Osmotic, Tisnov, Czech Republic) and was subsequently purified using a Millipore RG (MilliQ water, 18 MΩ, Millipore, Billerica, MA, USA). Deionized water was used for rinsing, washing, and buffer preparation.

2.2 Preparation of QDs (CdS, PbS and CuS)

All chemicals were purchased from Sigma-Aldrich and used without further purification. CdS QDs were prepared using a slightly modified method published in [27]. Cadmium nitrate tetrahydrate $Cd(NO_3)_2 \cdot 4H_2O$ (0.031 g, 0.1 mM) was dissolved in ACS water (25 mL). 3-mercaptopropionic acid (35 μL, 0.4 mM) was slowly added to stirred solution. Afterwards, pH was adjusted to 9.11 with 1M NH_4OH (1.5 mL). Sodium sulphide nonahydrate $Na_2S \cdot 9H_2O$ (0.024 g, 0.1 mM) in 23 ml of ACS water was poured into solution of cadmium nitrate under vigorous stirring. CuS QDs were prepared by reaction of copper acetate monohydrate $Cu(OAc)_2 \cdot H_2O$ (0.020 g, 0.1 mM) dissolved in ACS water (25 mL) with mercaptosuccinic acid (0.080 g, 0.53 mM). Subsequently, 0.5 ml of 1M NH_4OH was added under constant stirring to yellow solution, followed by addition of sodium sulphide nonahydrate $Na_2S \cdot 9H_2O$ (0.012 g, 0.05 mM) in 24.5 ml of ACS water. Yellow colour of solution turned to light brown. Finally, colourless solution was obtained. PbS QDs were prepared by modified method of Hennequin et al. [27]. Lead acetate trihydrate $Pb(OAc)_2 \cdot 3H_2O$ (0.038 g, 0.1 mM) was dissolved in ACS water (25 mL). 3-mercaptopropionic acid (60 μL, 0.69 mM) was slowly added to stirred solution. White precipitate was formed, which disappeared after addition of 3.8 mL of 1 M NH_4OH (pH = 9.88). Sodium sulphide nonahydrate $Na_2S \cdot 9H_2O$ (0.012 g, 0.05 mM) in 21.2 mL of ACS water was added with vigorous stirring. Colour of solution was brown. All QDs solutions were stored in dark at 4 ºC.

2.3 Preparation of viral protein-QDs complexes

A/H5N1/Vietnam/1203/2004 protein (accession no. ISDN 38687, Prospec-Tany TechoGene, Tel Aviv, Israel), (200 μL, 100 μg/mL) was mixed with a solution of CdS, PbS, and CuS QDs (100 μL). This mixture was shaken for 24 h at room temperature (Vortex Genie2 [Scientific Industries, Bohemia, NY, USA]). The volume of solution was reduced to 100 μL on Amicon Ultra 3k centrifugal filter device (Millipore). Centrifugation using 5417R centrifuge (Eppendorf, Hamburg, Germany) was performed under the following parameters: 15 min, 6000 rpm, 20 °C. The obtained concentrate was diluted with 400 μL of ACS water and subsequently reduced to 100 μL on centrifuge. The process was repeated five times. The washed sample was diluted to 300 μL and used for subsequent measurements.

2.4 Separation using magnetic nanoparticles

Streptavidin Dynabeads M-270 were purchased from Life Technologies (Carlsbad, CA, USA). Biotinylated multivalent glycans (01–078 [Neu5Ac_2–3Gal_1–3GlcNAc_1-PAAbiotin]) were

obtained from GlycoTech (Gaithersburg, MD, USA). Soluble forms of HA expressed in baculovirus derived from newly emerging influenza viruses were purchased from Prospec-Tany TechnoGene. Streptavidin Dynabeads M-270 (10 μL) were pipetted to microplates (PCR 96, Eppendorf), and then transferred to a magnet plate. Stored solution was drained from the magnetic particles. Magnetic particles were washed three times with 100 μL of phosphate buffer (0.3 M, pH 7.4, made from NaH_2PO_4 and Na_2HPO_4). 20 μL of biotinylated glycan were added to each of the wells and incubated (30 min, 25 °C, 400 rpm). After the incubation, the sample was washed three times with phosphate buffer (0.3 M, pH 7.4). Subsequently 20 μL of H5N1- Cd-labelled protein were added. This solution was further incubated (30 min, 25°C, 400 rpm) and washed with 100 μL of phosphate buffer (0.3 M, pH 7.4). 35 μL of phosphate buffer (0.3 M, pH 7.4) were added and the sample was treated with an ultrasound needle (2 min). The plate was transferred to the magnet plate and the supernatant (product) was measured using the differential pulse voltammetry. The detected substance was identified as cadmium, or lead, or copper (QDs).

2.5 Stopped flow injection analysis (SFIA)

Microfluidic system for fully automated electrochemical detection (SFIA-ED) was suggested. The system consisted of a solvent delivering automated analytical syringe operating in the working volume range of 1–50 μL under variable flow rate from 1.66 to 50.00 μL/s (Model eVol, SGE Analytical Science, Australia), a three-way two-position selector valve (made from six-way valve) (Valco, Instruments, USA), and a dosing capillary that directly enters to an electrochemical flow cell (CH Instruments, USA). To prepare a fully automated system, a switching valve enabling switching between off (waste) and sample flow positions was placed into the system. The sample (10 μL) was injected by an automated syringe (SGE Analytical Science, Australia) through a flow cell under flow rate of 1.66 μL/s. The flow cell was cleaned by rinsing with 200 μL of ethanol in water (75%, *v*/*v*), then with 200 μL of 100% methanol and stabilized with 200 μL of the supporting electrolyte (0.2 M acetate buffer, pH 5). Cleaning step was applied after 50 measurements. An electrochemical flow cell includes one low-volume (1.5 μL) flow-through analytical cell (CH Instruments), which consisted of a doubled glassy carbon (GC) working electrode, an Ag/AgCl electrode as the reference electrode and output platinum tubing as an auxiliary electrode. The electrochemical flow cell was connected to a miniaturized potentiostat 910 PSTAT mini (Metrohm, Switzerland) as a control module (Fig. 1). Differential pulse voltammetry (DPV) as a measuring method was used and the parameters were as follows: initial potential -1.2 V, end potential -0.2 V, modulation amplitude 0.05 V, step potential 0.001 V. All experiments were carried out at room temperature. Acetate buffer (0.2 M, pH 5) was used as a supporting electrolyte. Each measured sample consisted of 15 μL of sample solution, which was diluted with acetate buffer. The data obtained were processed by the PSTAT software 1.0 (Metrohm).

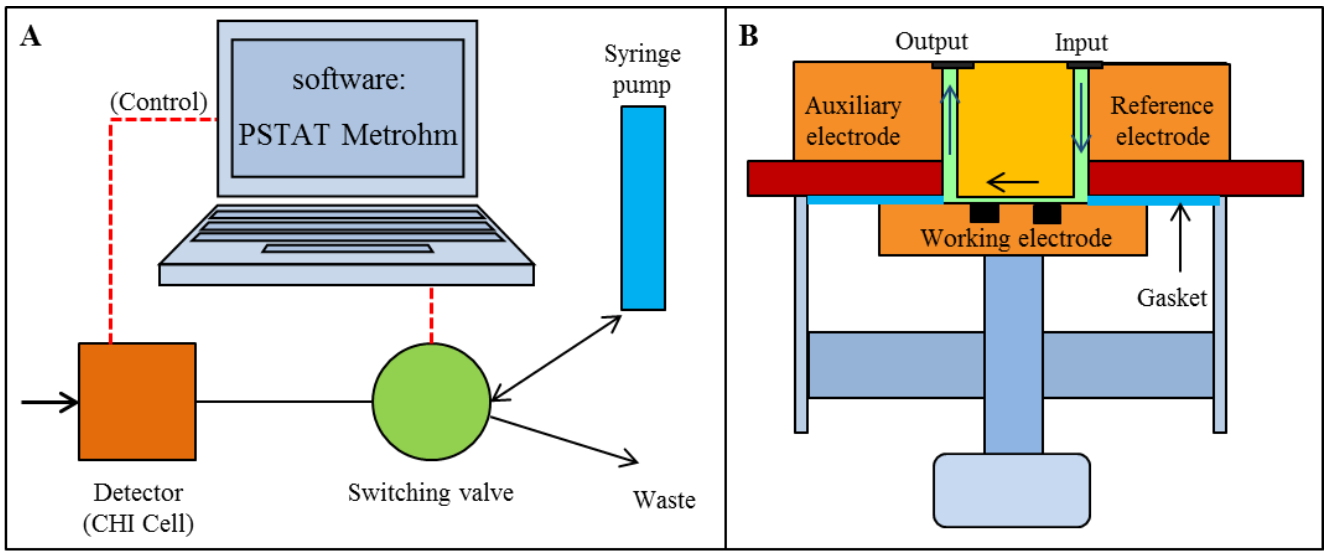

Figure 1. Scheme of stopped flow injection analysis (SFIA). (**A**) The sample was injected into the electrochemical cell by an injection pump. A valve enables switching between a waste and an electrochemical cell. Data were recorded using a potentiostat, controlled by a computer. (**B**) Scheme of the electrochemical cell. As a reference electrode an Ag/AgCl 3M KCl was used, as auxiliary electrode a platinum wire was employed and a glassy carbon served as a working electrode. The seals were placed between the electrodes with volume of 500 nL for a sample.

2.6 Characterization of fluorescence of QDs

Fluorescence monitoring was performed using an In vivo Xtreme system by Carestream (Rochester, NY, USA). This instrument was equipped with a 400 W xenon light source and 28 excitation filters (410–760 nm). Emitted light was captured by a 4MP CCD camera. The excitation wavelength was set at 410 nm and the emission was measured at 700 nm. The exposure time was 6 s, Bin 2x2, fStop 1.1, field of view 15 cm. Samples of volume of 100 µL were placed in a Nunc microplate MaxiSorp (Thermo Fisher Scientific, Roskilde Denmark). Concentration of QDs varied within the interval from 5 to 80 µM.

2.7 Descriptive statistics and estimation of detection limit

Data were processed using MICROSOFT EXCEL (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). All results were expressed as mean ± SD unless noted otherwise. Detection limits (3 signal/noise, S/N) were calculated according to Long and Winefordner [28], whereas N was expressed as a standard deviation of noise determined in the signal domain unless stated otherwise.

3. RESULTS AND DISCUSSION

Quantum dots, metal nanocrystals, are frequently used for the construction of biosensors. Their specific properties, especially optical and electrochemical activity, are very advantageous and enabled

us to apply them in optical or electrochemical detection systems. In this study we focused on the detection of QDs using differential pulse voltammetry in combination with microfluidic injection system, which is of great interest due to the creation of miniaturized sensing systems [13,24,29-31].

3.1 Determination of cadmium(II), lead(II) and copper(II) ions using the SFIA system

Electrochemical detection of Cd(II), Pb(II) and Cu(II) ions using the differential pulse voltammetry method was used to quantify QDs used in our experiments. This method was optimized for the standard solution of $Cd(NO_3)_2 \cdot 4H_2O$ and $Pb(NO_3)_2$ within the concentration range of metal ions from 0.02 to 1.56 µM and for $Cu(NO_3)_2 \cdot 3H_2O$ within the concentration range of metal ions from 0.1 to 1.56 µM. Various time of accumulation (30, 60, 120, 180, 240, 360 and 420 s) and deposition potential (-1.2, -1.1, 1.0; -0.9, -0.8, and -0.7 V) were tested. The results are shown in Fig. 2A, B and C for cadmium(II), lead(II) and copper(II) ions, respectively. Based on the results, the accumulation time of 240 s was selected as the optimal value to measure sufficient signal during reasonable time. Optimized deposition potentials increased linearly with the decreasing potential. The highest signal was achieved at -1.2 V for all metal ions.

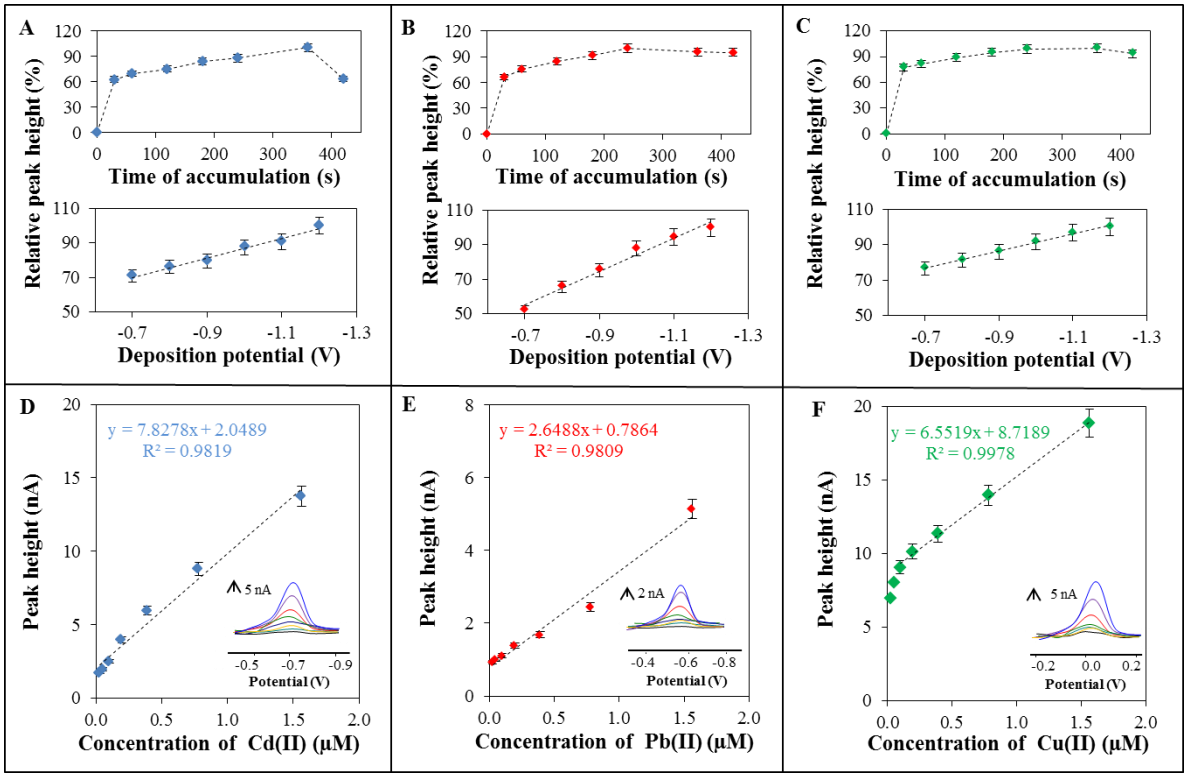

Figure 2. Optimization of differential pulse voltammetric determination of cadmium(II), lead(II) and copper(II) as electrochemical active parts of the synthesized QDs. Influence of accumulation time (0–460 s) and deposition potential (from -0.7 to -1.2 V) on microfluidic determination of (**A**) cadmium(II), (**B**) lead(II) and (**C**) copper(II). Concentration of metal ions was 12.5 µM. Calibration curves of (**D**) cadmium(II), (**E**) lead(II) and (**F**) copper(II); in insets: characteristic signals determined at -0.7 V (cadmium(II)), -0.6 V (lead(II)) and 0.03 V (copper (II))

Therefore, the accumulation time of 240 s and deposition potential of -1.2 V were selected for the following experiments. Further, the calibration curves were measured. The obtained concentration dependence for cadmium(II) ions was linear within the range from 0.02 μM to 1.56 μM and equation of dependence was y = 7.828x+2.049; R^2 = 0.982, n = 3, R.S.D = 3.6 % (Fig. 2D). The obtained concentration dependence for lead(II) ions was linear within the range from 0.02 μM to 1.56 μM and equation of dependence was y = 2.649x+0.786; R^2 = 0.981, n = 3, R.S.D = 2.2 % (Fig. 2E). The obtained concentration dependence for copper(II) ions was linear within the range from 0.1 μM to 1.56 μM with equation y = 6.5519x+8.7189; R^2 = 0.998, n = 3, R.S.D = 4.3 % (Fig. 2F). Limits of detection (3 S/N) were estimated as 0.02 μM for cadmium(II), 0.02 μM for lead(II) and 0.08 μM for copper(II) ions. The typical signals of cadmium(II), lead(II) and copper(II) are shown in insets in Figs. 2D, E and F, respectively.

3.2 Preparation of QDs and fluorescence characterization of QDs

Preparation of CdS, PbS, and CuS QDs is shown in Fig. 3A. QDs were prepared from $Cd(NO_3)_2 \cdot 4H_2O$, $Pb(OAc)_2 \cdot 3H_2O$, and $Cu(OAc)_2 \cdot H_2O$ according to the procedure presented in the Experimental part. The stability of the prepared qQDs was app. 1 month and was determined according to the fluorescence measuring using In vivo Xtreme system. This fact was confirmed by our another study [32]. Fig. 3B shows the dependence of emission intensity of prepared CdS QDs on their concentration in half part dilution monitored by fluorescence detection.

3.3 Preparation of QDs complex with protein and detection of its metal part

Miniaturization of suggested system and its application as a biosensor assume the ability to detect the complex of protein and QDs. For the verification of this assumption, the mixtures of individual types of QDs and HA influenza protein were prepared. The detection of metal parts of QDs was performed to determine the QDs components in the prepared complexes. Determined concentration of metal ions (metal part of QDs) was compared with the applied concentration of QDs, which was used for the preparation of complex (Fig. 3C). The obtained dependences had polynomial character for all QDs. As it is evident from the obtained dependences, lead based ODs were influenced by protein interaction only minimally. On the other hand, QDs containing cadmium showed the lower detected concentration of metal in the mixture, which likely means that these QDs maximally interacted with the protein. The concentration of metal in QDs based on copper stand in between of the above-mentioned QDs. Lead based QDs showed the best properties as a protein label. On the other hand the sensitivity of electrochemical detection of lead based QDs was the worst among all three measured metal ions. The other two metal ions, copper and cadmium, had the similar sensitivity of electrochemical detection. Therefore, cadmium based QDs could be considered to be the best option for the construction of biosensor, especially due to wider applicable concentration range and possibility of detection by fluorescence methods compared to other two types. In the following part of the experiment, all three types of QDs were further tested.

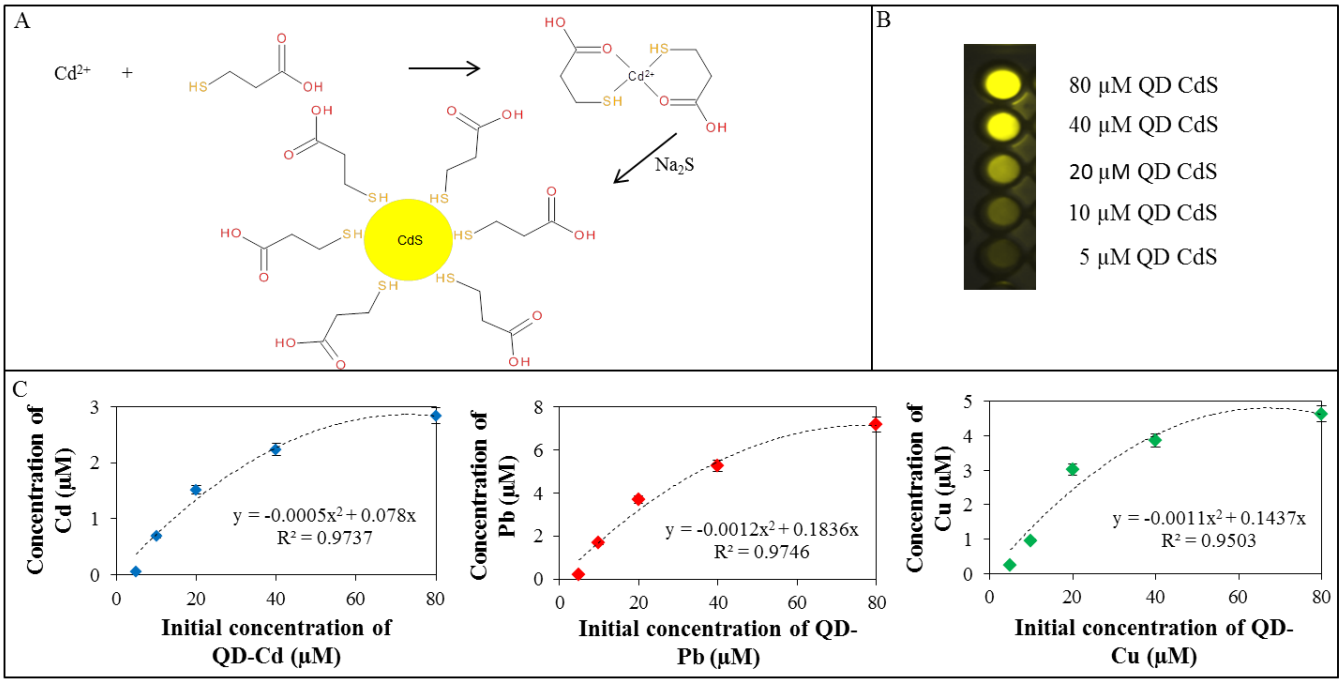

Figure 3. Scheme of QDs preparation. (**A**) Formation of CdS QDs covered by 3-mercaptopropionic acid. Possible structure of intermediate chelate formed in the reaction of Cd^{2+} and 3-mercaptopropionic acid is depicted. (**B**) Detection of fluorescence of CdS QDs. Carestream In-Vivo Xtreme Imaging System, excitation filter: 410 nm, emissions filter: 700 nm. (**C**) Dependence of Cd(II), Pb(II) and Cu(II) ions concentration on the applied concentration of QD-Cd, QD-Pb, and QD-Cu (concentration, which was used for the preparation of sample, namely complex H5N1 protein with CdS, PbS, CuS quantum dots) measured on glassy carbon electrode.

3.4 Magnetic particles for viral isolation

In the isolation procedure, a microfluidic instrument was used to perform the detection of paramagnetic particles. Streptavidin-modified paramagnetic particles were mixed with biotinylated glycan. Glycan-binding proteins are often expressed by viruses, bacteria and protozoa on their surface to facilitate the attachment to host cells, and to establish the colonization and infection. The first key step in the process of infection, transmission and virulence of influenza viruses involves an expression of HA, a trimeric glycoprotein, on the membrane of influenza virus [33]. It binds to a host cell's surface glycans via terminal sialic acid (Sia) with α(2-3) and α(2-6) linkages [34,35]. Fig. 4 schematically shows the process of viral protein isolation. This procedure is based on the linkage between biotinylated glycan and streptavidin paramagnetic particle (due to biotin-streptavidin affinity, Fig. 4A), and binding HA influenza protein (labelled with Cd QDs) with glycan (Figs. 4A and B). The created complex is broken by ultrasound and then metal part of QDs is electrochemically determined (Fig. 4C). Cadmium(II), lead(II) and copper(II) ions were detected by differential pulse voltammetry according to our previously published works [36-41].

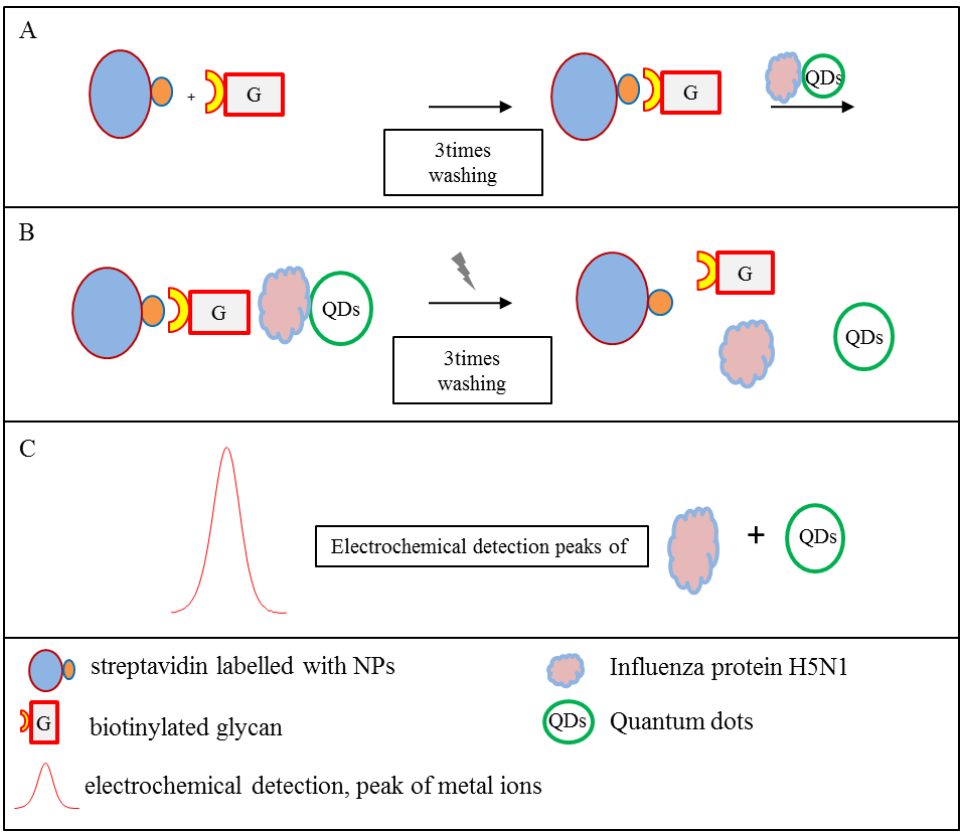

Figure 4. Scheme of electrochemical detection of protein-QDs complex. (**A**) Streptavidin labelled NPs and biotinylated glycan (G) binding to streptavidin magnetic particle (due to biotin-streptavidin affinity), (**B**) binding HA influenza protein with QDs, (**C**) electrochemical detection of HA protein and metal ions (Cd(II), Pb(II) and Cu(II)).

3.5 Electrochemical detection of metal part of quantum dots from isolated protein complexes

Isolation procedure of influenza protein using specific glycan binding had the effect on the amount of QDs bond to protein. Decrease of detected electrochemical signal was expected, especially because the individual steps through the procedure were not wholly effective. The subject of our interest was to investigate this phenomenon. Fig. 5A, B and C show the effect of applied cadmium, lead and copper based QDs concentration, namely of theirs metal parts, on the detected amount of individual metals related to the amount of protein. The blue columns represent the concentration of individual metal ions detected in the product of isolation process. These dependences had a linear character for all three types of QDs, i.e. $y = -0.0002x + 0.0009$, $R^2 = 0.998$ for cadmium; $y = -0.0008x + 0.0041$, $R^2 = 0.998$ for lead, and $y = -0.0002x + 0.0009$, $R^2 = 0.966$ for copper. It is obvious that these dependences had very similar equations (with minimum differences) because the detected metal concentration was related to the amount of protein.

The red columns represent the purified part of the mixture, concentration of metal, which was lost during the isolation process. The dependence for all QDs had a polynomial character with reliability $R^2=0.99$. Particularly, the cadmium dependence was $y = 0.0145x^2 - 0.1322x + 0.3155$, $R^2 = 0.994$; dependence for lead $y = 0.0145x^2 - 0.1315x + 0.3122$, $R^2 = 0.994$; and for copper $y = 0.0145x^2 -$

0.1322x + 0.3156, R^2 = 0.994. The dependences for cadmium and copper ions showed very similar equations. On the other hand this equation for lead ions differed from the others (mainly in the second member of the equation). This fact is in accordance with the difference in the linear equations of the detected concentrations of metals (blue columns), where the direction of lead based QDs differs from the others.

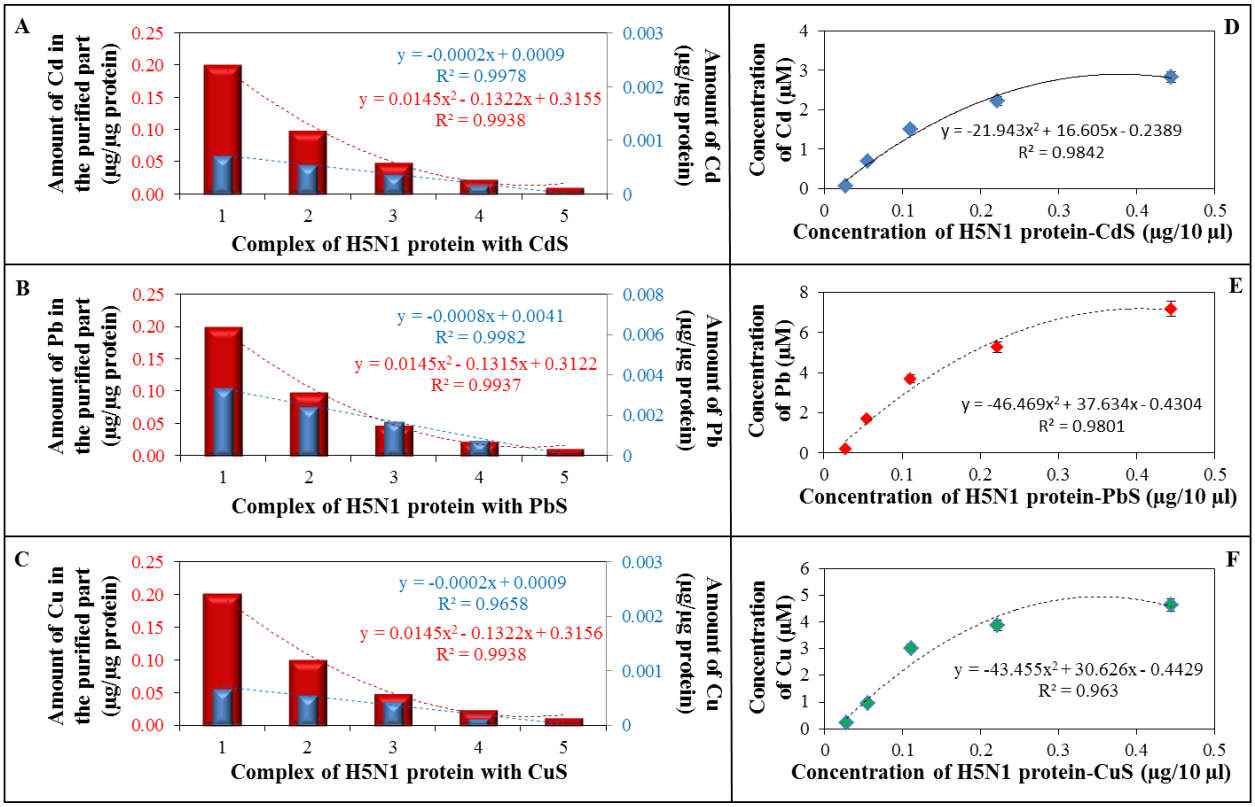

Figure 5. Dependences of (**A**) cadmium, (**B**) lead and (**C**) copper concentration (related to the amount of protein) on various concentrations of complex H5N1 protein (protein concentration is 0.0028, 0.0055, 0.0111, 0.0222 and 0.0444 µg/µL) with CdS, PbS and CuS QDs (concentrations: 0.0127, 0.0253, 0.0506, 0.1013 and 0.2025 µg/µg of protein). Blue part corresponds to measured signal of Cd(II), Pb(II) and Cu(II). Red part represents concentration of Cd(II), Pb(II) and Cu(II) in the purified part of solution. Dependences of (**D**) Cd(II), (**E**) Pb(II) and (**F**) Cu(II) detected concentration on H5N1 protein complex.

Figs. 5D, E and F show the dependences of determined concentrations of metals on the applied concentration of protein, which was used to create the complex with individual QDs for cadmium, lead and copper based QDs. The amount of protein was related to volume of 10 µL, which corresponds to one dosage of mixture in the electrochemical cell in SFIA system. The obtained dependences had a polynomial character, for cadmium y = -21.943x^2 + 16.605x - 0.2389, R^2 = 0.98; for lead y = -46.469x^2 + 37.634x - 0.4304, R^2 = 0.98, and for copper y = -43.455x^2 + 30.626x - 0.4429, R^2 = 0.96. The increasing concentration of protein caused an increase of detected metal concentration, but the protein concentration higher than 0.25 µg per 10 µL resulted only in small enhancement of detected signal of metal. The polynomial dependences had local maxima, which were as follows: 0.38

(cadmium), 0.41 (lead), and 0.35 μg per 10 μL (copper). These results indicated that these concentrations of protein represent limits to obtain the highest signals of metals after the isolation process. In this way, lead based QDs seemed to be the most advantageous because (1) they had the highest direction of concentration dependence curve, and (2) the highest concentration of detected metal. On the other hand, lead based QDs complex with protein had the highest protein concentration needed to achieve the maximum efficiency in the isolation process. Copper based QDs had the lowest optimal protein concentration, while cadmium based QDs had the concentration optimum of 8 % higher.

4. CONCLUSIONS

Electrochemical biosensors and bioassays have attracted considerable interest as a detection system due to their high performance, miniaturized construction, and low costs [2,36,39,40]. In spite of the fact that QDs are currently utilized especially for in vivo and in vitro imaging, their application in the field of biosensing devices could be also considered [42]. The application of flow injection electrochemical analysis for the determination of influenza characteristic protein, hemmaglutinin, due to various sulphide QDs detection is presented. One may suggest that QDs could find a place in a rapid detection of various viruses.

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