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Developing an Electrochemical Biosensor for the Detection of Hemagglutinin Protein of Influenza A virus Subtype H1N1 in Artificial Saliva †

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Abstract: Influenza A virus belongs to the Orthomyxoviridae family and to date is one of the most important pathogens causing acute respiratory infections, such as the recent pandemic of 2009. Hemagglutinin (HA) is one of the surface proteins of the virus that allow it to interact with cellular molecules. Due to the reason that is the most abundant protein in the virus capsule; make it the best target in the detection of Influenza A H1N1 virus through biosensing devices. Our aim is to develop an electrochemical biosensor to detect H1 by modifying carbon screen printed electrodes with gold nanoparticles and further functionalization with monoclonal antibodies specific to this protein. The electrodes were characterized by the means of cyclic voltammetry, differential pulse voltammetry and electrochemical impedance spectroscopy. The electrode is coupled to a 3D printed droplet guidance microfluidic system to ensure homogeneous distribution across the electrode. Our preliminary results suggest that the selected monoclonal antibodies have acceptable affinity and bind effectively to the H1 protein and that the electrodes have a wide potential window in the presence of $[Fe(CN)_6]^{3-/4-}$. In the future, we will continue to develop this biosensor in hope that it will be commercialized and be common in medical procedures during flu seasons and future influenza pandemics.

Keywords: Influenza virus; voltammetry; screen printed electrodes; hemagglutinin/HA protein; thiol chemistry

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1. Introduction

In 2009, a novel H1N1 influenza A virus caused a pandemic leading to the death of 151,700-575,400 people worldwide [1, 2]. H1N1 influenza is a subtype of influenza A virus that was previously detected in swines, which causes upper and in some cases lower respiratory tract infections in its host [1, 3, 4]. Influenza A virus causes one of the most common respiratory diseases globally, seasonal flu, and together with Influenza B,

C, and D is a part of the virus family Orthomyxoviridae [3, 4]. Moreover, influenza A virus belongs to the single-stranded RNA viruses [3, 4]. It has a segmented genome that encodes several viral proteins that are important for the pathogenesis of the virus [3, 4]. Two of these proteins are important for detecting the virus in human specimens; these are hemagglutinin (HA) and neuraminidase (NA), which are the surface proteins of the virus involved in host invasion [5]. HA is the major protein of H1N1 and it's the protein with which the virus binds to the host's cells and invades them, while NA helps in the viral spreading from cell to cell [5].

So far, most of the detection methods for influenza A virus are characterized by a long detection time, expensive instruments and reagents, and the need of trained technicians, thus creating an inconvinience for both the patients and the healthcare workers [1, 6]. The development of sensitive and rapid detection methods, such as biosensors is now the focus of many research groups and could be a great solution to the aforementioned problem. A lot of different biorecognition elements can be used for the detection of an analyte. However, antibodies seem to be the mostly used type of these elements.

Antibodies are specialized, Y-shaped proteins that identify pathogens by selectively binding to their membrane [7]. Due to their high specificity and sensitivity, antibodies are ideal biorecognition elements for biosensors [8]. The focus of this paper is the development of an electrochmical antibody-based biosensor for the detection of the influenza A surface protein H1.

2. Materials and Methods

2.1. Reagents and Materials:

HA H1N1 protein, mouse monoclonal antibodies (mAbs) and rabbit polyclonal antibodies (pAb) were purchased from Sinobiological (Germany). Secondary goat anti-Rabbit IgG antibodies Alexa Fluor 568 were purchased from Thermofisher (US), Chloroauric acid ($HAuCl_4$), Sulphuric acid (H_2SO_4), 4 aminothiophenol (4-ATP), ethanol, potassium hexacyanoferrate (II) trihydrate and Potassium hexacyanoferrate(III) were purchased from Sigma Aldrich (Germany). Carbon screen printed electrodes (CSPE) were provided by Zimmer & Peacock (Norway).

2.2. Electrochemical Measurements:

The EmStat Pico Module potentiostat controlled by computer software PSTrace 5.8 was employed for all cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), chronoamperometric electrodeposition and differential pulse voltammetry (DPV), experiments. The CSPE was used as a three-electrode cell system comprised of a carbon working electrode (WE), carbon counter electrode (CE) and Ag/AgCl reference electrode (RE). EIS measurements were made at 6 mV ac amplitude in the frequency range of 5.0 mHz to 50 kHz and the equivalent circuit models were fitted using PSTrace software.

2.3. Electrodeposition of Gold Nanoparticles on CSPE:

A modified method from the literature was employed [9], an aqueous solution containing 2 mM $HAuCl_4$ and 0.5 M H_2SO_4 was used to cover the CSPE, a chronoamperometric method using a constant potential of -0.25 V for 60 seconds was used to deposit gold nanoparticles on top of the CSPE, the electrode was washed with abundant deionized water, left to dry at room temperature and identified as AuNP-CSPE.

2.4. Modification of AuNP-CSPE Electrodes with 4-ATP:

A reported method was adapted [10], in a typical experiment, the working electrode was covered with 10 μL of 10 mM 4-ATP solution in ethanol at room temperature (22 °C) during 15 minutes. Nonspecifically adsorbed molecules were flushed off by care-

ful rinsing with ethanol and deionized water. The electrode was dried under a stream of nitrogen and identified as NH_2-AuNP-CSPE. The amine functionality in the electrode can be used later on to form an amide bond [11] and immobilize the mouse monoclonal antibodies against HA H1N1 protein.

2.5. Testing of mAb Specificity and Sensitivity:

The enzyme-linked immunosorbent assay (ELISA) was used for this purpose. The protocol used for this indirect sandwich ELISA assay is according to the mAb provider [12].

3. Results and Discussion

3.1. Electrodeposition of Gold Nanoparticles:

The CSPEs offered a reasonable potential window to study the redox reaction of the $[Fe(CN)_6]^{3-/4-}$ system and showed a symmetric shape, the distance between the oxidation and the reduction peaks was of 727 mV (Figure 1), this value is much higher than the prediction of the Nerst equation for single electron transfer reactions and it has been attributed to a potential drop due to the resistance of the carbon material [13]. When the CSPEs were modified with gold nanoparticles, the reversibility of the $[Fe(CN)_6]^{3-/4-}$ redox system increased as the distance between the oxidation and reduction peaks was 280 mV on the voltammogram, this is attributed to the increase in the surface area of the electrode and to the high conductivity of metallic gold nanoparticles.

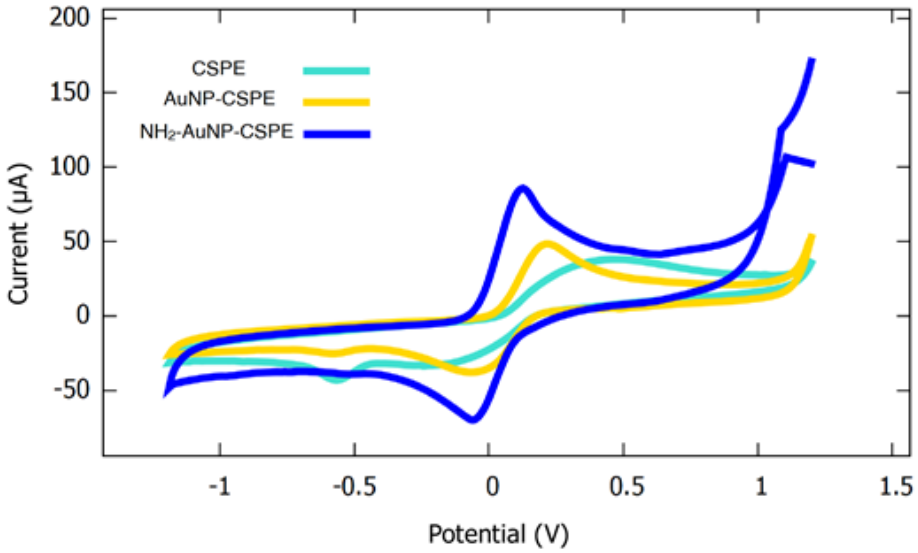

Figure 1. Cyclic voltammogram of CSPE, AuNP-CSPE and NH_2-NP-CSPE in the presence of $[Fe(CN)_6]^{3-/4-}$ obtained at a scan rate of 100 mV/s.

3.2. Electrodeposition Length:

Further study into the gold electrodeposition process as a function of time (Figure 2) showed that longer reaction times than one minute don't increase neither the current response of the electrode or the reversibility of the system.

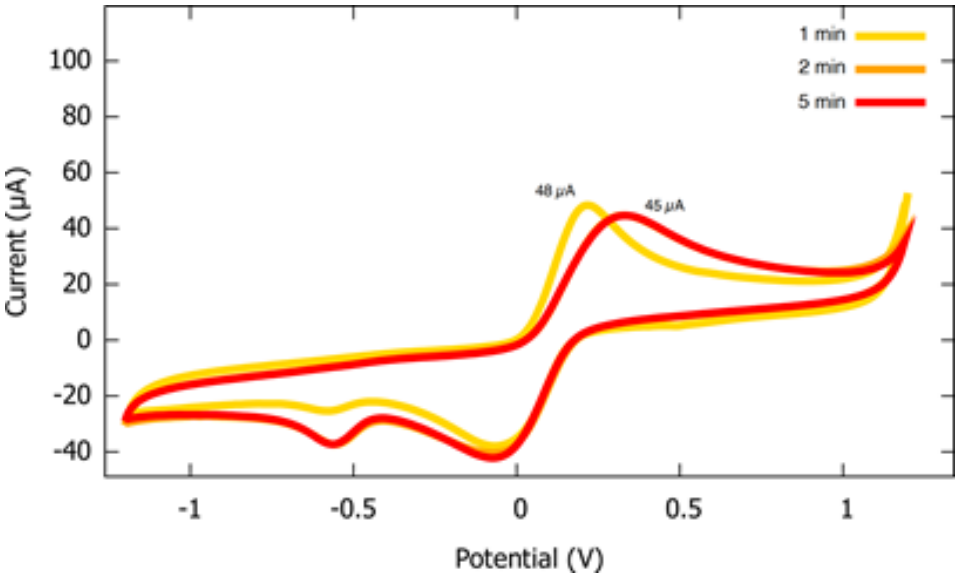

Figure 2. Cyclic voltammogram of modified AuNP-CSPEs using different times of electrodeposition, the experiment was conducted in the presence of $[Fe(CN)_6]^{3-/4-}$ at a scan rate of 100 mV/s.

3.3. Characterization of NH_2-AuNP-CSPE:

The cyclic voltammogram showed promising results after functionalizing the nanoparticles with the 4-ATP linker molecule (Figure 1), as the reversibility of the system increased and the electron transfer process for the reduction and oxidation reactions of $[Fe(CN)_6]^{3-/4-}$ was facilitated on these modified electrodes. The electrochemical impedance spectroscopy tests indicated a decrease in impedance after modification of the electrodes with gold nanoparticles and 4-ATP linker. The Nyquist plot of the bare CSPE can be fitted to a equivalent circuit for a simple electron-transfer reaction and NH_2-AuNP-CSPE can be fitted to the classical Randles equivalent circuit composed by the ohmic resistance of the electrolyte solution (R_Ω), the charge transfer resistance (R_{CT}), in series with the Warburg impedance element (diffusion controlled impedance) and in parallel to a double layer capacitance (C_{DL}) (Figure 4). The modified NH_2-AuNP-CSPE showed a significant smaller R_{CT} and is therefore highly conductive compared to the bare CSPE.

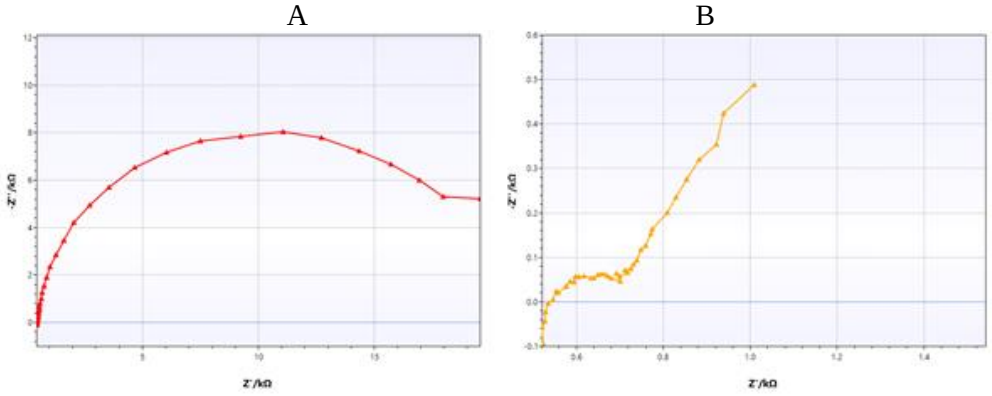

Figure 3. Nyquist plot of CSPE (**A**) and NH_2-AuNP-CSPE (**B**) using frequency range of 5.0 mHz to 50 kHz.

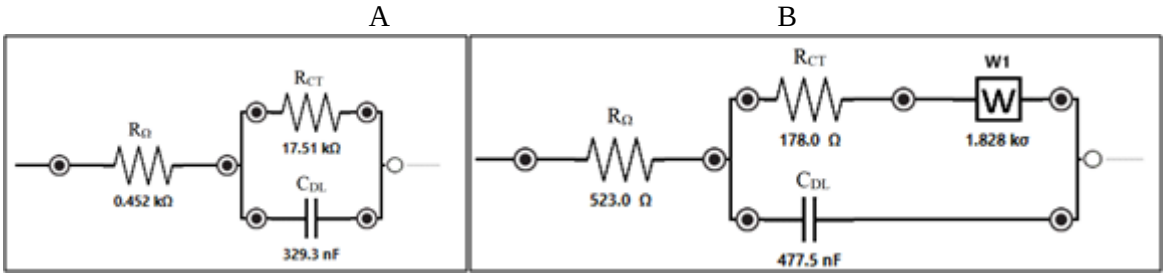

Figure 4. Fitting of CSPE to equivalent circuit for for a simple electron transfer (**A**) and fitting of NH_2-AuNP-CSPE Randles equivalent circuit (**B**).

3.4. Effect of the $[Fe(CN)_6]^{3-/4-}$ Concentration:

The effect of $[Fe(CN)_6]^{3-/4-}$ concentration over the current response was evaluated by the means of CV and DPV (Figure 5), a higher concentration of the electroactive species in solution implies that more $[Fe(CN)_6]^{3-/4}$ molecules can reach the surface of the NH_2-AuNP-CSPE to undergo oxidation and reduction respectively, experimentally higher current responses were observed at high concentration of $[Fe(CN)_6]^{3-/4}$. This experiment provided visual basis of the expected effect on the final design of the biosensor, where the NH_2-AuNP-CSPE is going to be coupled to monoclonal antibodies against H1 protein and a blocking effect over $[Fe(CN)_6]^{3-/4}$ (lowering the current response) could take place once the H1 protein is binded to the antibody modified electrode.

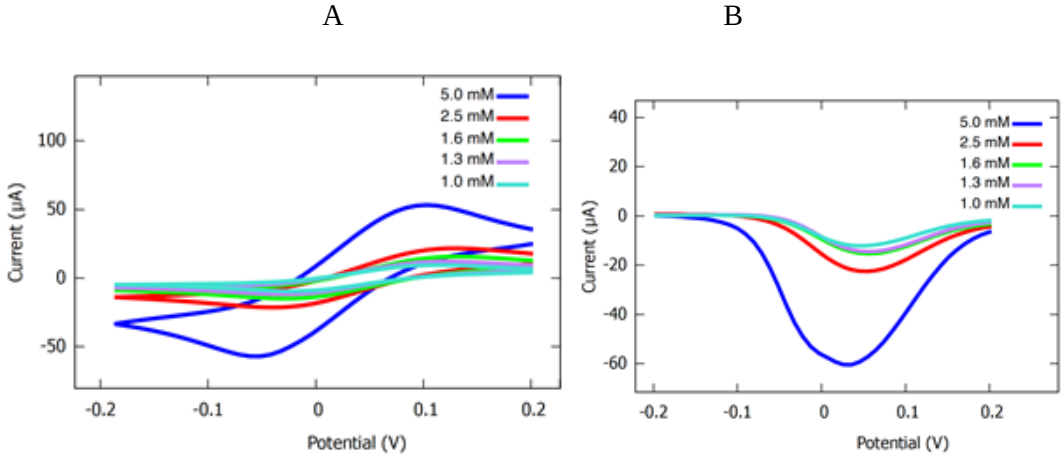

Figure 5. Cyclic voltammogram (**A**) and differential pulse voltammogram (**B**) for NH_2-AuNP-CSPE as a function of the $[Fe(CN)_6]^{3-/4-}$ concentration.

3.5. mAb Characterization:

To characterize the specificity of the monoclonal antibody targeting the H1 protein, an indirect sandwich ELISA was done. It was shown that the antibody can detect the H1 protein specifically. (Figure 6) The approach was tested for different H1 concentrations and the lowest detectable concentration was 10 ng/mL.

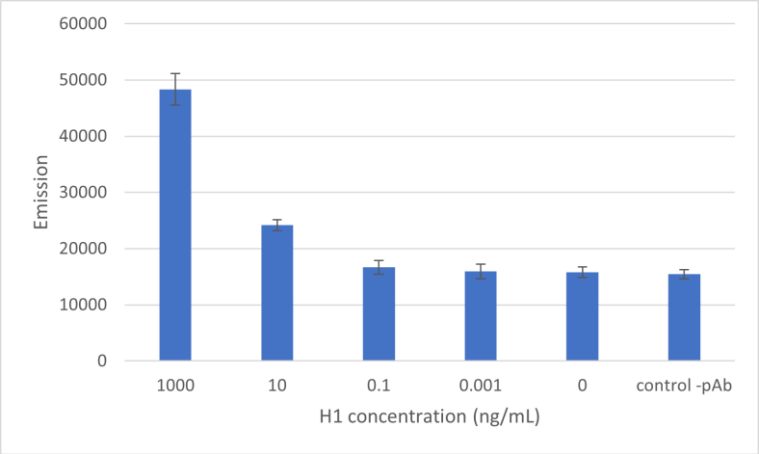

Figure 6. Indirect sandwich ELISA results using 1:500 dilution of mAb, 1:1000 dilution of pAb and different H1 concentrations as shown in the x axis; y axis shows the emission measured after the addition of the secondary antibody. Experiments were done twice with replicates of 2-6.

4. Conclusions

Cost effective CSPE have been modified with gold nanoparticles and 4-ATP, the electrodes have been characterized by the means of electrochemical methods in the presence of $[Fe(CN)_6]^{3-/4-}$ complexes. The redox system $[Fe(CN)_6]^{3-/4-}$ is more reversible in the modified electrodes AuNP-CSPE and NH_2-AuNP-CSPE than in the bare CSPE. The modified NH_2-AuNP-CSPE showed a decrease in impedance compared to CSPE indicating that the electron transfer process is more favorable in the modified electrode. With the indirect sandwich ELISA, it was shown that the monoclonal antibody targets specifically the HA H1N1 protein and can be further used in the biosensor setup. The amine functionality on the modified electrodes can be exploited to couple mouse monoclonal antibodies against HA H1N1 protein in future work of this project. In addition to this, future work aims to detect the HA H1N1 protein in artificial saliva using DPV, establish a protocol using bovine serum albumin (BSA) to avoid non-specific binding and determine the sensibility and detection limits of the biosensor.

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