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Influence of a polymer membrane on the electrochemical determination of insulin in
nanomodified screen printed carbon electrodes

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Abstract

Diabetes mellitus can be considered one of the most widespread diseases globally. Hence, the diabetes research is currently focused on developing an effective, low-cost sensor having high stability and suitable analytical characteristics. Screen printed carbon electrodes (SPCEs) embody ideal candidates for insulin determination due to the small area of the working electrode eliminating the solution volume required for the given purpose. Modification of SPCEs by using nanoparticles resulted in an increase of the working electrode surface area and formation of a higher number of active species. The aim of this paper is to examine the impact of a chitosan membrane on the electrochemical determination of insulin on NiO nanoparticles (NiONPs) and multi-walled nanotube (MWCNTs) modified SPCE (NiONPs/MWCNTs/SPCE). This study is primarily conceived to compare the analytical characteristics and stability of NiONPs/chitosan-MWCNTs/SPCE and NiONPs/MWCNTs/SPCE. An electrode modified with chitosan displays a wider linear range, one of 0.25 μM - 5 μM (R^2 0.997); a lower limit of detection, 94 nM; a high sensitivity (0.021 $\mu\text{A}/\mu\text{M}$) and better stability than that of an electrode without chitosan. According to these characteristics, the polymer is considered a necessary compound of the electrochemical insulin sensor, improving the sensor's analytical characteristics.

Keywords: insulin determination, screen printed carbon electrodes, polymer membrane, nickel oxide nanoparticles, chitosan

1. Introduction

Insulin as a main anabolic hormone [1] regulating the blood glucose level in the body [2]. It is produced by small spherical structures called islets of Langerhans, located in the pancreas [3-6]. Insulin triggers the transport of glucose to cells, mainly to the liver, muscle, and fat tissues [7]. The secretion or resistance disorders lead to the widespread disease called diabetes mellitus [8, 9].

The enzymatic glucose sensors currently commercially used to diagnose diabetes [10, 11] exhibit some drawbacks arising from the dependence of the used enzyme's activity on the temperature, humidity, and pH [12]. Therefore, insulin determination is intensively studied as one of the alternative techniques for diabetes diagnostics [13-15]. In general, insulin determination methods are classified into the immune and the non-immune groups. The former set includes radioimmunoassay [16], enzyme immunoassay (EIA) [17], and luminescent immunoassay (LIA) [18,19]; the disadvantages then comprise low sensitivity, long-running analysis, and pre-treatment of the samples with fluorogenic labels [20]. Capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) are non-immune methods requiring a complete pre-treatment stage and expensive instrumentation [21]. Thus, a highly sensitive, small, rapid, selective, accurate, and cost-effective electrochemical sensor is a much desired component [22]. Using electrochemical assays for insulin determination can overcome the shortcomings of the methods mentioned above because electrochemical assays display a wide linear concentration range, a low limit of detection, high sensitivity, and inexpensive instruments [23].

Different carbon electrodes, such as glassy carbon (GCE) [24], carbon paste (CPE) [25], edge plane pyrolytic graphite (EPPGE) [26], screen printed carbon (SPCE) [27], and pencil graphite ones (PGE) [28] have been extensively used for electrochemical insulin determination. In a previous study, the electrochemical determination of insulin on a PGE modified by a combination of chitosan, MWCNTs, and Ni nanoparticles and NiONPs was investigated. The PGE modified by MWCNTs, chitosan, and NiONPs displayed a low detection limit (260 nM), a wide linear range (0.05 μM – 5 μM), and good sensitivity (0.123 $\mu\text{A}/\mu\text{M}$) [28]. The current work is centred on the electrochemical determination of insulin on a nanomodified SPCE, used instead of a PGE. SPCEs modified by chitosan, MWCNTs, and NiONPs displayed a lower detection limit (94 nm) and better stability compared to a PGE modified in the same manner. This was probably caused by additional impurities, contained in the PGE. Dropsens-made SPCEs type DS 110 embody one of the most appropriate candidates for insulin determination because of the small size of the working electrode (4 mm diameter). Miniaturization has also lead to a reduction in the insulin volume necessary for

electrochemical measurements. SPCEs have been widely employed in electrochemical assays also due to their excellent properties, such as the high chemical stability and low background current [29]. Although carbon is considered the optimal electrode material for an electrochemical sensor to determine insulin [30], the bare carbon electrode has some drawbacks, including insufficient sensitivity and slow kinetics of the electrochemical reaction [22]. In an effort to improve the electrochemical characteristics of bare carbon electrodes, dedicated researchers have modified their surfaces by using different nanoparticles, such as metal nanoparticles (Ni, Cu, Co) or metal oxide nanoparticles (NiO, RuO, CoO), including widely used MWCNTs [27, 22], which enlarge the active surface area and create more active sites [31, 32]. Moreover, the catalytic activity of different metal (Ni, Cu, Co) or metal oxide (NiO, RuO, CoO) nanoparticles towards insulin oxidation has been intensively studied [33]. Ni-based nanoparticles ensure high electrical conductivity, low cost, and excellent catalytic activity towards insulin oxidation because of the presence of NiO(OH) active species formed in an alkaline solution [27].

Also, the utilization of different polymer materials, such as Nafion, chitosan, or polyethylene glycol, which formed a polymer membrane on the electrode surface, has been discussed. An electrochemical sensor towards insulin oxidation with superior properties has been designed via a combination of the polymer membrane, MWCNTs, and metal nanoparticles [27]. Using a polymer membrane prevents the occupation of active sites with Cl⁻ ions present in the phosphate-buffered saline and fixes the nanoparticles on the electrodes' surface during the electrochemical measurements, thus improving the stability of the electrodes [34].

Herein, the impact of chitosan as a polymer material on the electrochemical determination of insulin on NiONPs and MWCNTs-modified SPCEs is analysed. The electrochemical properties, morphology, and stability of NiONPs/chitosan-MWCNTs/SPCE and NiONPs/MWCNTs/SPCE were compared to evaluate the respective impact of chitosan. The morphology of the electrodes was examined by transmission electron microscopy (TEM), scanning transmission electron microscopy, (STEM), scanning electron microscopy (SEM), and EDX analysis. Cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), and chronoamperometry were employed to study the electrochemical properties of modified SPCEs. All the measurements were carried out in a phosphate-buffered saline (PBS) solution simulating the presence of Cl⁻ ions close to their concentration in the human body. Also, the influence of interferences (ascorbic acid, glucose, sucrose, and lactic acid) on insulin oxidation was studied via the cyclic voltammetry method.

2. Experiments

2.1 Chemicals and Materials

The employed Insulin, Human Recombinant (5807.6 g/mol) was purchased from MP Biomedicals (France, Illkrich). The MWCNTs (diameter: 3 nm – 10 nm, length: 1 μ m - 10 μ m) were obtained from BOC SCIENCES Creative Dynamics Inc. (USA, New York). The phosphate-buffered saline (PBS D8662, sterile filtered), nickel nitrate hexahydrate ($\text{Ni}(\text{NO})_3 \cdot 6\text{H}_2\text{O}$, 99.9%), sodium hydroxide (NaOH, 99%), potassium ferrocyanide trihydrate ($\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$, 99.95%), potassium ferricyanide trihydrate ($\text{K}_3[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$, 99.95%), nitric acid (HNO_3 , 65%), sulfuric acid (H_2SO_4 , 96%), ethanol ($\text{CH}_3\text{CH}_2\text{OH}$, 99.5%), glucose ($\text{C}_6\text{H}_{12}\text{O}_6$, 99.5%), sucrose ($\text{C}_{12}\text{H}_{22}\text{O}_{11}$, 99.5%), ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$, 99%), lactic acid ($\text{C}_3\text{H}_6\text{O}_3$, 98%) and chitosan (from shrimp shells, 75%) were ordered from Sigma Aldrich (USA, Missouri). The insulin solutions were freshly prepared by dissolving powdered insulin in 0.1 M NaOH in PBS before each electrochemical measurement. Deionized water ($\rho = 18.2 \text{ M}\Omega \cdot \text{cm}$) was used to dissolve other chemical substances. All the measurements were performed at room temperature and atmospheric pressure.

2.2 Apparatus

All the electrochemical experiment, except for the EIS measurement, were performed by using AUTOLAB type IME663 (Metrohm, Switzerland). The EIS measurements were materialized with AUTOLAB type PGSTAT302N (Metrohm, Switzerland). In the measurements, we utilized SPCEs type DS110 (Dropsens, Spain); these consist of three electrodes (a working electrode (WE) = a carbon electrode, an auxiliary electrode (AE) = a carbon electrode, and a reference electrode (RE) = a silver electrode) printed on a ceramic substrate ($L = 33 \text{ mm} \times W = 10 \text{ mm} \times H = 0.5 \text{ mm}$). The printed reference Ag electrode represents a pseudoelectrode and it was not modified prior to the electrochemical measurements. Many articles [27, 29] have used the printed Ag electrode as a pseudoelectrode because of the simplification it allows for SPCE use without additional pre-treatment. The surface morphology of the SPCEs was characterized by a TEM with EDX analysis (FEI Tecnai F20 S, CEMAS, Ohio), a STEM (FEI Magellan 400 L, CEMAS, Ohio), and a SEM (Jeol JSM 7000 N, Japan).

2.3 Fabrication of modified SPCEs

The bare SPCEs were rinsed with ethanol to remove possible impurities before every modification cycle [27]. The MWCNTs were activated in a solution containing nitric acid and sulphuric acid at the ratio of 3:1, according to Erdelyi et al. [35]. The powdered chitosan was dissolved in a solution containing the PBS and 0.1 M acetic acid with $\text{pH} = 3$. Thereafter, 2 mg of the activated MWCNTs were dispersed in 1 ml of the PBS. In the NiONPs/chitosan-MWCNTs/SPCE modification,

1 μl of a chitosan solution was also added. Both mixtures were ultrasonicated for 1 hour to produce a homogenous suspension. Then, 10 μl of the suspension, with or without chitosan, were dropped on the SPCE surface and dried at room temperature [28].

The deposition of the NiONPs on the MWCNTs/SPCE or the chitosan-MWCNTs/SPCE was performed from a 40 mM $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ solution. The pH of the solution was adjusted to pH = 2 by using nitric acid. The pulsed electrodeposition of the NiONPs on the SPCE was carried out according to Rafiee et al. [27], using an optimized double-pulse sequence of potentials: $E_1 = 0.4 \text{ V}$ (vs. Ag) for $t_1 = 0.3 \text{ s}$ and $E_2 = 0.0 \text{ V}$ (vs. Ag) for $t_2 = 2.5 \text{ s}$. To study the mechanism of insulin oxidation on NiONPs/chitosan-MWCNTs/SPCE different deposition times of NiONPs were used. In the first case t_1 was 0.3 s and t_2 was 2.5 s, secondly t_1 was 0.6 s and t_2 was 2 s and in the third case t_1 was 0.9 s and t_2 was 7.5 s. The prepared electrodes were activated in a 0.1 M NaOH solution via the cyclic voltammetry method by potential scanning between + 0.1 V and + 0.7 V at the scan rate of 100 mV/s for 10 cycles.

2.4 Electrochemical measurements

The active surface area of the NiONPs/MWCNTs/SPCE and the NiONPs/chitosan-MWCNTs/SPCE was determined by the chronoamperometry method in 1 mM $\text{K}_4[\text{Fe}(\text{CN})_6]/\text{K}_3[\text{Fe}(\text{CN})_6]$ and 1 M KCl at the potential of 0.25 V. The stability measurements were executed in the same solution by means of cyclic voltammetry in the potential range from -0.4 V to 0.6 V during 50 cycles at the scan rate of 100 mV/s. The different insulin concentrations were determined in the 0.1 M NaOH and PBS in the potential range from 0 V to 1 V at the scan rate of 100 mV/s. The cyclic voltammetry experiments at different scan rates were performed using 2 μM insulin in the 0.1 M NaOH and PBS at the potential range from 0 V to 1 V. The EIS measurements were recorded at 0.51 V in the frequency range of 0.1 Hz to 100 kHz in the presence of 2 μM insulin and the 0.1 M NaOH in PBS. The solutions with different concentrations of insulin were prepared by dissolving the required amount of powdered insulin in a solution containing the PBS and 0.1 M NaOH. Since the printed Ag reference electrode was used for all electrochemical measurements, potential values for the oxidation and reduction processes can be shifted as compared with Ag/AgCl, but with no influence on anodic peak currents corresponding to insulin oxidation.

3. Results and discussion

3.1 Surface characterization

Figures 1 A and B show TEM micrographs of the NiONPs/MWCNTs/SPCE and NiONPs/chitosan-MWCNTs/SPCE, respectively. Both of the TEM images (Figure 1 A and B) display

MWCNTs with the length of several micrometres and diameter of about 20 nm. In the NiONPs/MWCNTs/SPCE (Figure 1A), only smooth MWCNTs lacking polymer material were observed. Compared to the NiONPs/MWCNTs/SPCE, the surface of the NiONPs/chitosan-MWCNTs/SPCE (Figure 1B) displays visible changes. The chitosan is present on the electrode surface as a polymer layer on the MWCNTs (Figure 1 B). The chitosan layer on the NiONPs and MWCNTs modified SPCE was not completely homogeneous. There are visible dark areas present on the NiONPs/chitosan-MWCNTs/SPCE surface, corresponding to a higher chitosan concentration (Figure 1 B); this effect could be attributed to insufficient chitosan dissolution in the nanotubes suspension.

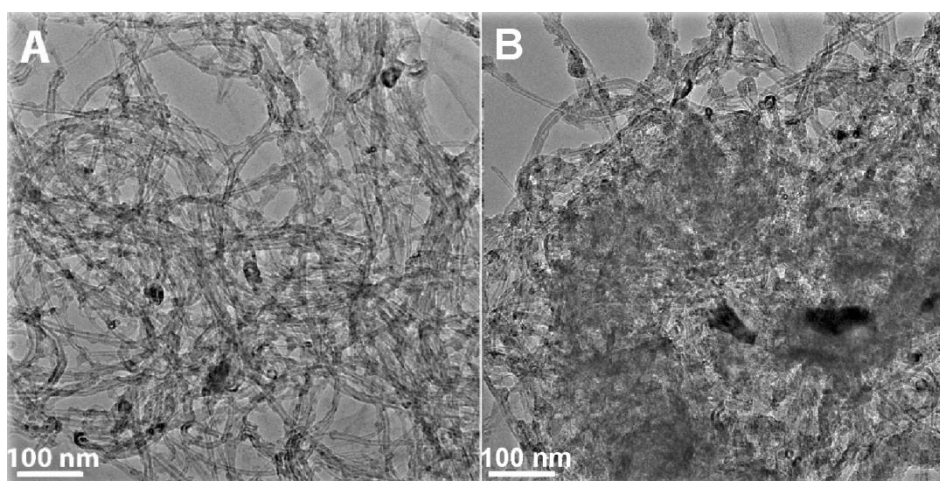


Figure 1. The TEM image of the NiONPs/MWCNTs/SPCE (A) and NiONPs/chitosan-MWCNTs/SPCE (B).

Figure 2 shows the detailed SEM image of the NiONPs/chitosan-MWCNTs/SPCE. The spherical NiONPs are located mainly on the undulations or endings of the MWCNTs with a diameter of 5 nm – 10 nm. The NiONPs form nanoparticle aggregates with a size of 50 nm – 100 nm. EDX analysis was used to determine the chemical composition of the NiONPs/chitosan-MWCNTs/SPCE, confirming the presence of 59 % of carbon (C), 18 % of nickel (Ni), and also additional elements like magnesium (Mg), calcium (Ca), silicon (Si), phosphorus (P), and chlorine (Cl). The presence of the additional compounds was caused by the use of the PBS for the MWCNTs dispergation.

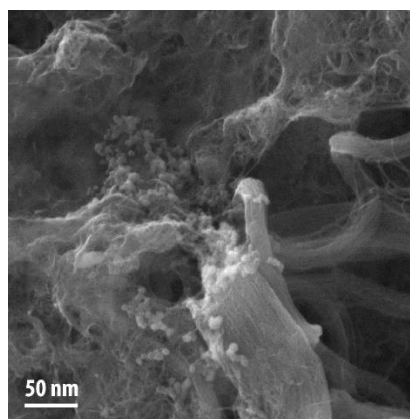


Figure 2. The detailed SEM image of the NiONPs/chitosan-MWCNTs/SPCE.

The active surface area of the NiONPs/MWCNTs/SPCE and NiONPs/chitosan-MWCNTs/SPCE was determined by using the chronoamperometry method in 1 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ and 1 M KCl. The active surface area measured via chronoamperometry was calculated using the Cottrell equation:

$$I = \frac{nFAD^{1/2}c}{\pi^{1/2}t^{1/2}} \quad (1)$$

where I is the measured current in A; F denotes the Faraday constant; n is the number of exchanged electrons; A represents the surface area of the working electrode in cm^2 ; c stands for the concentration of $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ in mM; D is the diffusion coefficient in cm^2s^{-1} ; and t denotes the time in s. The diffusion coefficient used to compute the surface area equalled $7.6 cm^2s^{-1}$ [36].

The plots of the oxidation peak current versus t for the NiONPs/chitosan-MWCNTs/SPCE (full line) and the NiONPs/MWCNTs/SPCE (dashed line) obtained in 1 mM $K_4[Fe(CN)_6]/1 mM K_3[Fe(CN)_6]$ in 1 M KCl are shown in Figure 3. The active surface area of the NiONPs/MWCNTs/SPCE without the chitosan was determined to be $0.89 cm^2$, and the active surface area of the NiONPs/chitosan-MWCNTs/SPCE corresponded to $0.77 cm^2$. The use of the polymer layer caused a reduction of the active surface area due to the low electrical conductivity of chitosan ($1.7 \times 10^{-2} S.cm^{-1}$) [37]. The experiments were performed three times, with the relative standard deviation (RSD) of 5 % for the NiONPs/MWCNTs/SPCE and 4 % for the NiONPs/chitosan-MWCNTs/SPCE.

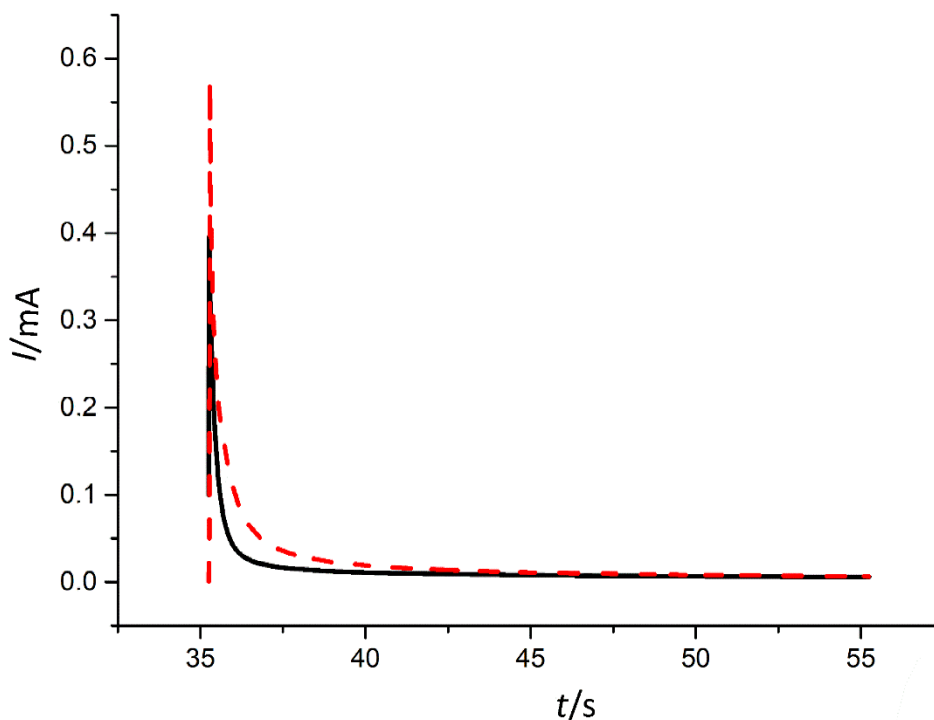


Figure 3. The plot of the oxidation peak current versus t for the NiONPs/chitosan-MWCNTs/SPCE (full line) and NiONPs/MWCNTs/SPCE (dashed line).

3.2 Stability tests

The stability of the NiONPs/MWCNTs/SPCE and NiONPs/chitosan-MWCNTs/SPCE was examined in different ways to confirm the stabilization of the NiONPs with the chitosan on the electrode surface during the electrochemical measurements.

First, we obtained the EDX spectra for the NiONPs/MWCNTs/SPCE (Figure 4 A, B) and NiONPs/chitosan-MWCNTs/SPCE (Figure 4 C, D) before and after one insulin electrochemical determination cycle performed in the 0.1 M NaOH and PBS via cyclic voltammetry in the potential range of 0 V to 1 V at the scan rate of 100 mV/s. Before the electrochemical measurement, Ni was present on both electrodes' surfaces in approximately the same amount (18 %). After the electrochemical experiments, Ni was observed only in the EDX spectrum of the NiONPs/chitosan-MWCNTs/SPCE (Figure 4 D), where 18 % of Ni was detected. The Ni volume is the same as that detected before the electrochemical measurement (Figure 4 C). The presence of Ni was not observed on the surface of the NiONPs/MWCNTs/SPCE without the chitosan after the electrochemical measurement (Figure 4 B). This effect confirms the ability of the chitosan to stabilize the NiONPs on the electrode surface during the electrochemical process.

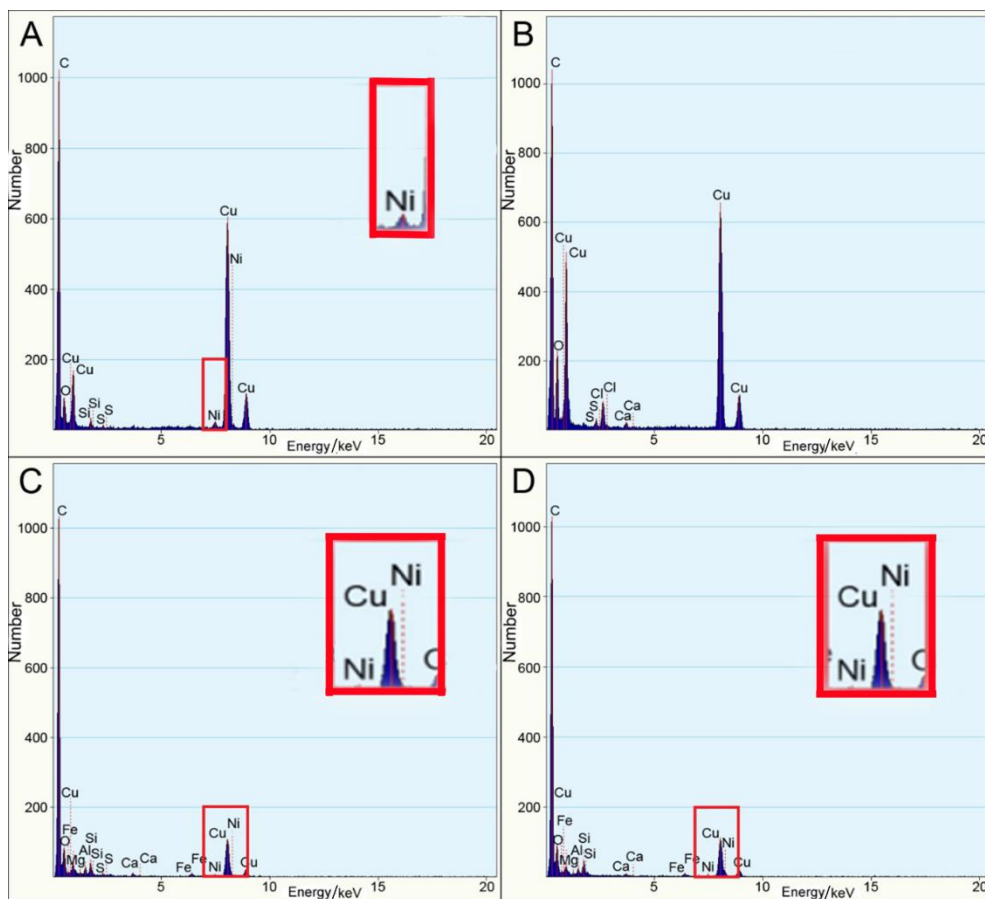


Figure 4. The EDX spectrum of the NiONPs/MWCNTs/SPCE (A, B) and NiONPs/chitosan-MWCNTs/SPCE (C, D) before (A, C) and after (B, D) one insulin electrochemical determination case using the cyclic voltammetry method. The insert comprises a detailed part of the EDX spectrum showing the presence of Ni.

The stability of the NiONPs/MWCNTs/SPCE and NiONPs/chitosan-MWCNTs/SPCE was also electrochemically determined in 1 mM $K_4[Fe(CN)_6]$ /1 mM $K_3[Fe(CN)_6]$ in 1 M KCl by using cyclic voltammetry. Since, the electrodes for stability tests were not preconditioned, oxidation potentials can be a little shifted. A decrease of the maximum current value attributed to the oxidation of $K_4[Fe(CN)_6]$ after 50 cycles was observed. Figure 5 shows the cyclic voltammograms of 1 mM $K_4[Fe(CN)_6]$ /1 mM $K_3[Fe(CN)_6]$ in 1 M KCl on the NiONPs/MWCNTs/SPCE (Figure 5 A) and NiONPs/chitosan-MWCNTs/SPCE (Figure 5 B). In the NiONPs/MWCNTs/SPCE, a 16 % decrease of the maximum current value after 50 cycles was detected (Figure 5 A); this value is higher than the maximum current value decrease after 50 cycles on the NiONPs/chitosan-MWCNTs/SPCE (3 %). Stability measurements were performed on three different electrodes, with the RSD of 3 % for the NiONPs/MWCNTs/SPCE and 2 % for the NiONPs/chitosan-MWCNTs/SPCE. These measurements demonstrated better electrochemical stability of the NiONPs/chitosan-MWCNTs/SPCE as well as improved RSD, and they confirmed the results obtained from the EDX analysis. The presence of the

chitosan on the electrode surface enhanced the electrochemical stability of the electrode and stabilized the nanoparticles on the electrode surface during the electrochemical measurement.

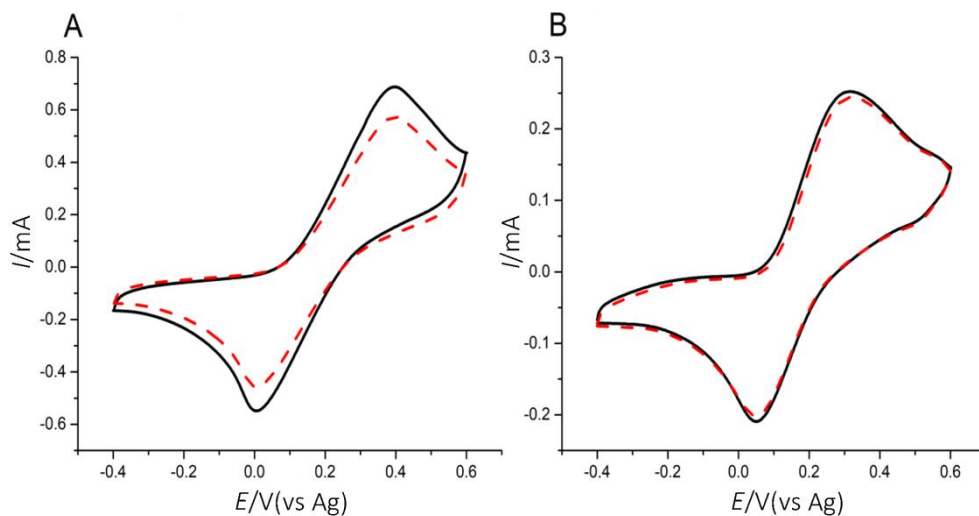


Figure 5. The cyclic voltammograms of 1 mM $K_4[Fe(CN)_6]$ /1 mM $K_3[Fe(CN)_6]$ in 1 M KCl on the NiONPs/MWCNTs/SPCE (Figure 5 A) and NiONPs/chitosan-MWCNTs/SPCE (Figure 5 B) display the 1st (full line) and 50th (dashed line) measurement cycles.

3.3 Insulin oxidation

Insulin is a polypeptide hormone whose structure comprises 51 amino acids divided into A and B chains. These chains are composed of 21 and 30 residues, respectively, linked by two disulphide bridges at positions A7 – B7 and A20 – B19 [38]. Three regions of the A chain, namely, residues 1 – 3, 12 – 17, and 19, are important for the insulin structure and its function. Similarly, in the B chain the active insulin region includes residues 8–25 [39]. These active regions of the insulin molecule are highlighted in Figure 6 (red frame). Another study proposes that tyrosine (Tyr) and tryptophan (Thr) are the only two amino acids with the possibility of oxidizing on the carbon electrode surface [40]. Considering this argument, we can exclude Thr oxidation because Thr is not located in the active part of the insulin molecule. These results then promote the theory of Tyr oxidation in the insulin molecule on a carbon surface because three Tyr molecules are located in the active regions of insulin (Figure 6, green frame).

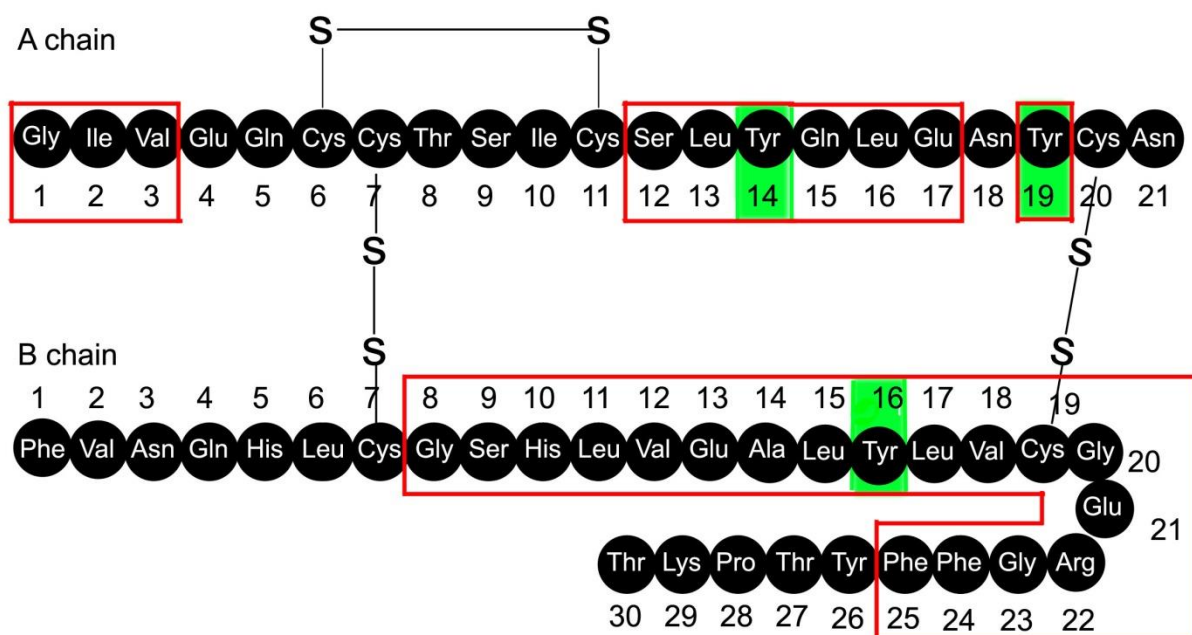


Figure 6. The structure of human insulin.

3.3.1 Mechanism of insulin oxidation on the NiONPs-modified SPCE

To examine the insulin oxidation mechanism, the electrochemical behaviour of the NiONPs/chitosan-MWCNTs/SPCE (Figure 8 A, full line) and chitosan-MWCNTs/SPCE (Figure 8, dashed line) under insulin oxidation in the 0.1 M NaOH and PBS was studied via the cycling voltammetry method (CV) in the potential window ranging from 0 V to 1 V and at the scan rate of 100 mV/s. As shown in the related voltammograms, the current response towards the oxidation on the chitosan-MWCNTs/SPCE (Figure 8 A, dashed line) is lower, and there is no apparent peak for the oxidation, confirming that the SPCE without the NiONPs has no electrocatalytic activity towards the insulin oxidation in alkaline solutions. Conversely, the insulin oxidation process on the NiONPs/chitosan-MWCNTs/SPCE occurred in two steps, and thus two oxidation peaks were observed (Figure 8 A, full line). The first anodic peak at the potential $E = 0.20$ V, detailed within the inset in Figure 8 A, is attributed to the oxidation of NiO to NiO(OH) (peak I.) according to Equation 2 [27]. This oxidation process is controlled by the adsorption of the OH⁻ ions from the alkali solution. The second oxidation peak at the potential $E = 0.51$ V is assigned to the direct oxidation of insulin (peak II.) according to Equation 3 [27]. Tyr is an insulin molecule aminoacid whose oxidation occurs at the carbon electrode, and the relevant mechanism is shown in Figure 7.



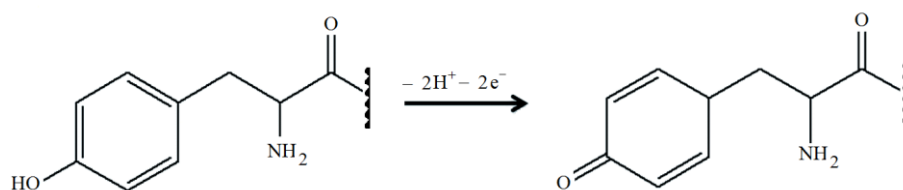


Figure 7. The oxidation of tyrosine.

The reduction peak at the potential $E = 0.24$ V corresponds to the reaction described by Equation 4 [41] due to the extremely high sensitivity of NiO(OH) to electron exposure.



The distance between the oxidation and the reduction peaks $\Delta E = 0.26$ V indicates an irreversible electrochemical process.

In an effort to study oxidation and reduction peak at potential $E = 0.20$ V and $E = 0.24$ V, respectively attributed to the oxidation of NiO to NiO(OH) and its consecutive reduction, blank experiments in alkaline solution with NiONPs/chitosan-MWCNTs/SPCE were done. Three different modified NiONPs/chitosan-MWCNTs/SPCEs were prepared using double pulsed method as was mentioned in part 2.3 but different deposition times of NiONPs were used. Figure 8 B shows cyclic voltammograms of the 0.1 M NaOH in PBS without insulin on NiONPs/chitosan-MWCNTs/SPCEs. As indicate in these voltammograms oxidation peak ($E = 0.20$ V) corresponding to Ni to NiO(OH) oxidation (Equation 2) increased with the prolonged deposition time. The same trend was observed for mentioned reduction peak which confirms that this peak is attributed to reduction of NiO(OH) in alkaline solution (Equation 4).

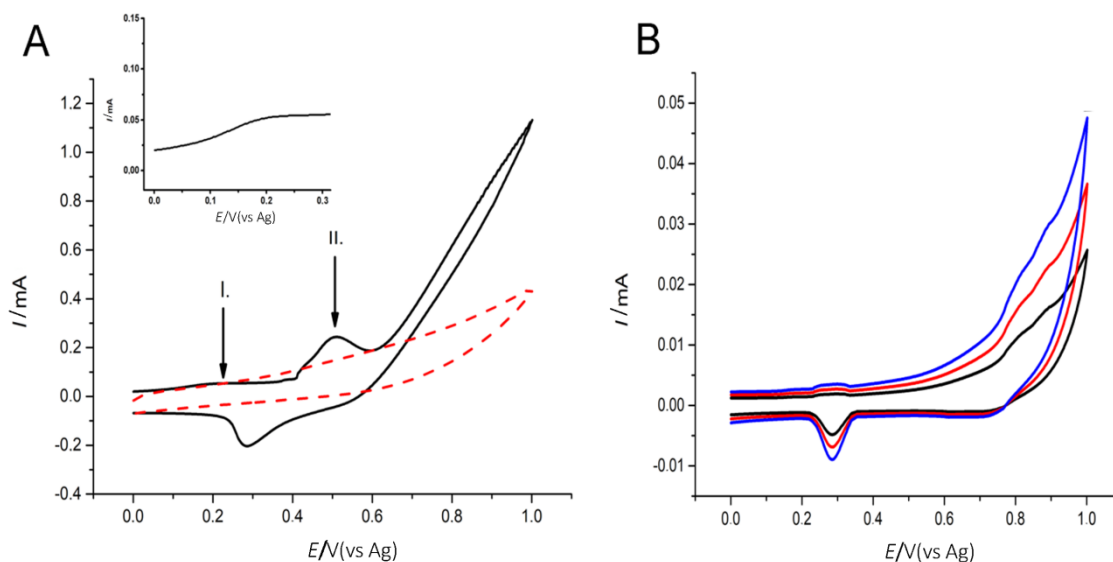


Figure 8. The cyclic voltammograms of the 2 μM insulin in the 0.1 M NaOH and PBS on the chitosan-MWCNTs/SPCE (dashed line) and NiONPs/chitosan-MWCNTs/SPCE (full line). Peak I. NiO to NiOOH oxidation and peak II. direct insulin oxidation. Inset: a detail of peak II at the potential 0.20 V (Figure 8 A). Cyclic voltammograms of 0.1 M NaOH in PBS on NiONPs/chitosan-MWCNTs/SPCE prepared by different deposition time of NiONPs (Figure 8 B).

3.3.2 Insulin oxidation on the NiONPs/MWCNTs/SPCE and NiONPs/chitosan-MWCNTs/SPCE

The electrochemical behaviour of the NiONPs/MWCNTs/SPCE and NiONPs/chitosan-MWCNTs/SPCE towards the insulin oxidation in the 0.1 M NaOH and PBS was studied by using the cycling voltammetry method in the potential window ranging from 0 V to 1 V at the scan rate of 100 mV/s. The impact of the chitosan on the electrochemical insulin determination was examined. Figure 9 A shows the cyclic voltammograms recorded in the 0.1 M NaOH and PBS for the NiONPs/chitosan-MWCNTs/SPCE (Figure 9 A, dashed line) and NiONPs/MWCNTs/SPCE (Figure 9 A, full line) in the presence of the 2 μM insulin. As indicated in these voltammograms, the current response towards the oxidation of the 2 μM insulin on the NiONPs/MWCNTs/SPCE (Figure 9 A, full line) is higher compared to the current response towards the insulin oxidation on the NiONPs/chitosan-MWCNTs/SPCE (Figure 9 A, dashed line). The lower current response towards the oxidation on the NiONPs/chitosan-MWCNTs/SPCE is due to the reduced conductivity after polymer application. This effect was also confirmed by examining the active surface area, which decreased in the NiONPs-modified SPCE after application of the chitosan.

The lower conductivity of the NiONPs/chitosan-MWCNTs/SPCE was also confirmed by electrochemical impedance spectroscopy (EIS), an important electrochemical method used to investigate the resistance of the NiONPs/MWCNTs/SPCE and NiONPs/chitosan-MWCNTs/SPCE surfaces. According to Ohm's law, resistance is indirectly dependent on conductivity. Figure 9 B

shows the Nyquist plots recorded at 0.51 V in the frequency range of 0.1 Hz to 100 kHz in the presence of the 2 μM insulin and 0.1 M NaOH in PBS and fitted with the most suitable equivalent circuit shown in Figure 9 B; in the electrochemical system presented therein, R_s is the solution resistance, R_{ct} is the charge transfer reaction resistance, and CPE denotes the constant phase element.

As shown in Figure 9 B, a higher R_{ct} value ($R_{ct} = 3.02 \times 10^{10} \Omega$) was observed for the NiONPs/chitosan-MWCNTs/SPCE (Figure 9 B, b), where the chitosan application caused an increase in the R_{ct} . The higher conductivity and lower R_{ct} ($R_{ct} = 1177 \Omega$) were achieved by modifying the SPCE via the NiONPs and MWCNTs without the chitosan (Figure 9 B, a).

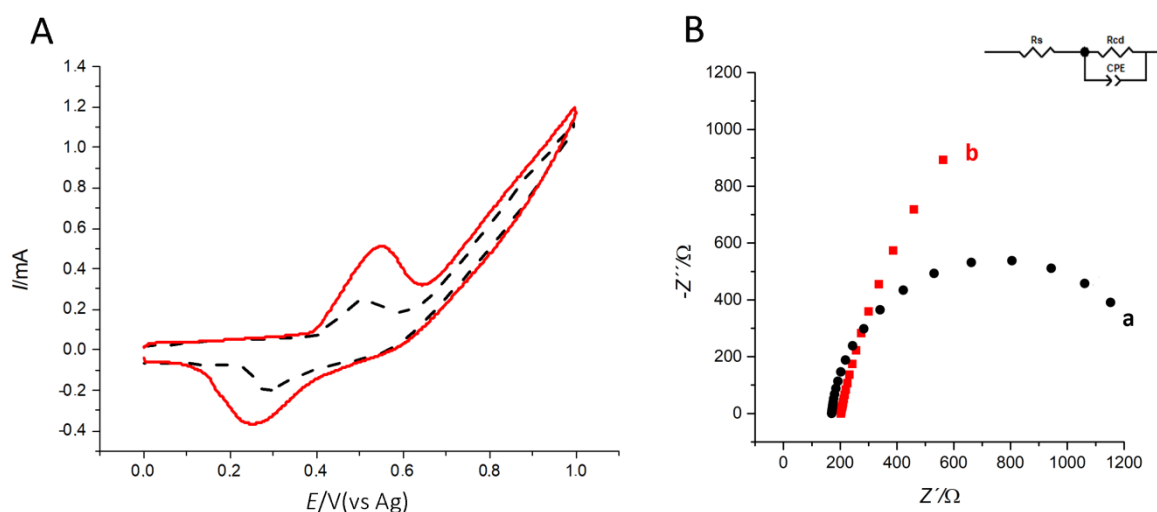


Figure 9. The cyclic voltammograms of the 2 μM insulin in the 0.1 M NaOH and PBS on the NiONPs/MWCNTs/SPCE (A, full line) and NiONPs/chitosan-MWCNTs/SPCE (A, dashed line), and the Nyquist plots of the NiONPs/MWCNTs/SPCE (B, a line) and NiONPs/chitosan-MWCNTs/SPCE (B, b line) in the presence of 2 μM insulin in the 0.1 M NaOH and PBS. The applied potential equalled 0.51 V, and the frequency range was from 0.1 Hz to 100 kHz. Inset: the equivalent circuit used for the data fitting.

3.3.3 Kinetics of insulin oxidation on the NiONPs/MWCNTs/SPCE and NiONPs/chitosan-MWCNTs/SPCE

The 2 μM insulin oxidation kinetics were studied via the cyclic voltammetry method in the 0.1 M NaOH and PBS at various scan rates from 10 mV/s to 200 mV/s. Figure 10 shows the cyclic voltammograms for the 2 μM insulin in the 0.1 M NaOH and PBS on the NiONPs/chitosan-MWCNTs/SPCE (Figure 10 A) and NiONPs/MWCNTs/SPCE (Figure 10 B) at various scan rates with different linear regression (Figure 10 C – D). Figure 10 also shows the dependence of the maximum current value corresponding to the insulin oxidation (I) on the scan rate (ν) in the NiONPs/chitosan-MWCNTs/SPCE (Figure 10 C) and NiONPs/MWCNTs/SPCE (Figure 10 D). Figures 10 E and F show the dependence of $\log I$ on $\log \nu$ in the NiONPs/chitosan-MWCNTs/SPCE (Figure 10 E) and

NiONPs/MWCNTs/SPCE (Figure 10 F), fitted with a linear function. Both of the dependences were fitted with a linear function. The peak current increased linearly with the scan rate ($R^2 = 0.999$) in the NiONPs/chitosan-MWCNTs/SPCE and ($R^2 = 0.971$) for the NiONPs/MWCNTs/SPCE; therefore, the dependence of $\log I$ on $\log \nu$ was plotted and fitted with a linear function (Figure 10 E, F). The linear dependences of $\log I$ on $\log \nu$ with linear regression equations were $I = 0.931\nu - 2.37$ and $I = 0.701\nu - 1.71$ for the NiONPs/chitosan-MWCNTs/SPCE and NiONPs/MWCNTs/SPCE, respectively. The obtained linear regression equations indicate that the processes are controlled by adsorption on the NiONPs/chitosan-MWCNTs/SPCE and diffusion on the NiONPs/MWCNTs/SPCE. All the measurements were carried out on three different electrodes, exhibiting the excellent RSD of 3 % for the NiONPs/MWCNTs/SPCE and 1 % for the NiONPs/chitosan-MWCNTs/SPCE.

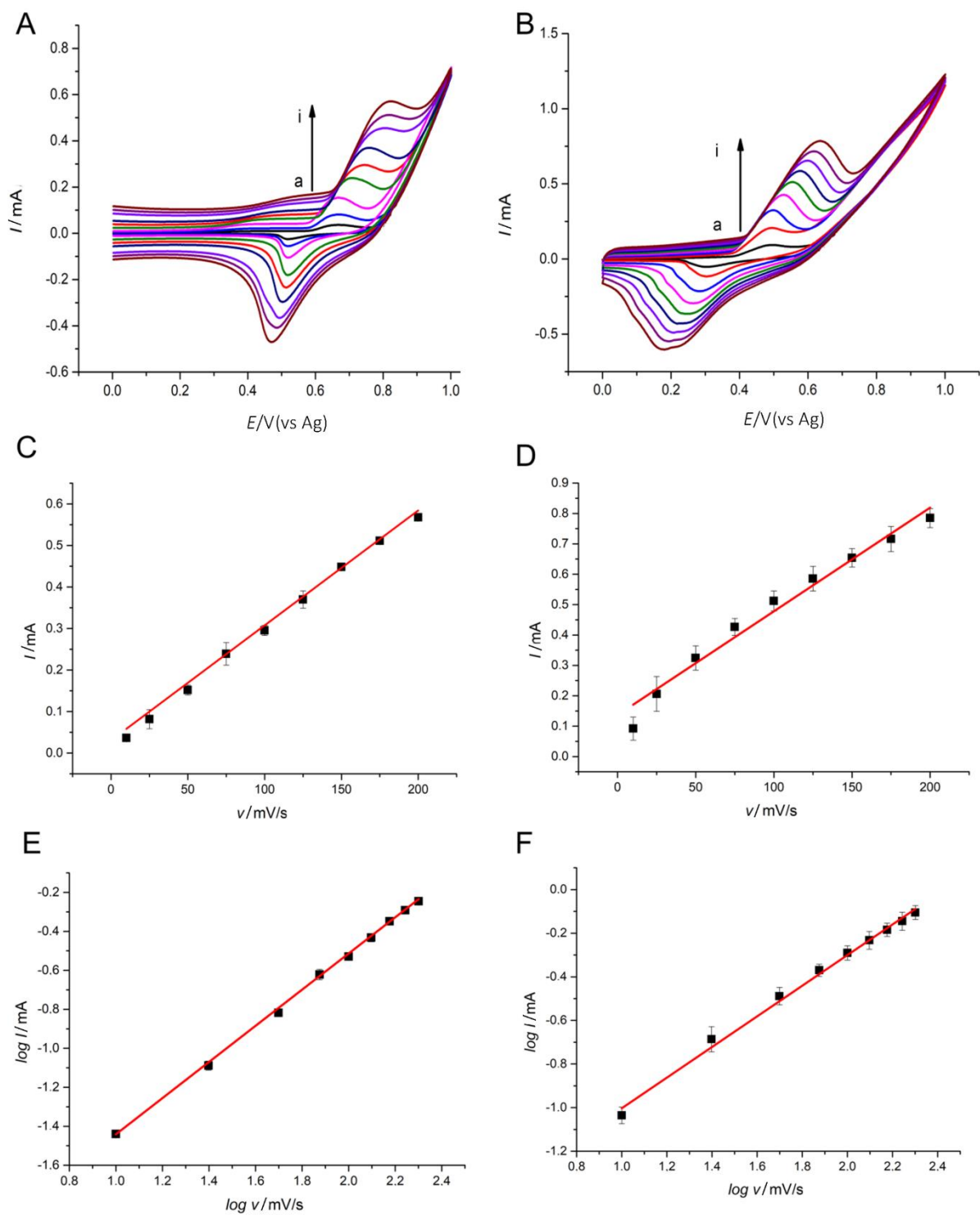


Figure 10. The cyclic voltammograms of the 2 μ M insulin in the 0.1 m NaOH and PBS at various scan rates from 10 mV/s to 200 mV/s (a-i) with the NiONPs/chitosan-MWCNTs/SPCE (A) and NiONPs/MWCNTs/SPCE (B). The dependence of the insulin oxidation-related maximum current value on the scan rate for the NiONPs/chitosan-MWCNTs/SPCE (C) and NiONPs/MWCNTs/SPCE (D). The dependence of the insulin oxidation-related maximum current value log on the log scan rate in the NiONPs/chitosan-MWCNTs/SPCE (D) and NiONPs/MWCNTs/SPCE (E).

3.4 Analytical characteristics

The analytical characteristics of the NiONPs/chitosan-MWCNTs/SPCE and NiONPs/MWCNTs/SPCE were studied via the CV method in the 0.1 M NaOH and PBS at the scan rate of 100 mV/s (Figure 11). Figure 11 shows the effect of the various insulin concentrations (0.25 μM – 5 μM) on the anodic peak current (0.51 V) for the NiONPs/chitosan-MWCNTs/SPCE (Figure 11 A) and NiONPs/MWCNTs/SPCE (Figure 11 D) in the 0.1 M NaOH and PBS. The maximum current value in the insulin oxidation on the NiONPs/chitosan-MWCNTs/SPCE increased linearly by raising the hormone's concentration (Figure 11 B); the effect was confirmed through determining the correlation coefficient ($R^2 = 0.997$) and residuals analysis, where R^2 was 0.997 (Figure 11 C). The plot of the current response versus the insulin concentration on the NiONPs/chitosan-MWCNTs/SPCE is linear over the wide concentration range of 0.25 μM – 5 μM (Figure 11 B, C). The sensitivity of the NiONPs/chitosan-MWCNTs/SPCE determined from the calibration plot with the correlation coefficient of 0.997 corresponded to 0.021 $\mu\text{A}/\mu\text{M}$. The detection limit of the NiONPs/chitosan-MWCNTs/SPCE, evaluated at the signal-to-noise ratio of 3:1 and calculated according to Equation 5 [42], was found to be 94 nM.

$$LOD = \frac{3x \text{ standard intercept error}}{\text{slope}} \quad (5)$$

Also, the insulin oxidation (0.25 μM - 5 μM) in the 0.1 M NaOH and PBS on the NiONPs/MWCNTs/SPCE (Figure 11 D) was studied. No linear dependence was observed, and the correlation coefficient of the dependence of the maximum current value according to the insulin oxidation on the NiONPs/MWCNTs/SPCE in the concentration range from 0.25 μM to 5 μM could not be established (Figure 11 E). The unlinear dependence was confirmed also with residuals analysis (Figure 11 F) with R^2 of only 0.285.

Moreover, the sensitivity and limit of the NiONPs/MWCNTs/SPCE detection could not be calculated because of the absolutely unlinear dependence of the maximum current value on the insulin concentration in the NiONPs/MWCNTs/SPCE; therefore, this electrode is not convenient for the insulin determination within the concentration range required to facilitate such a procedure in the human blood.

Hence, the application of the chitosan membrane on the NiONP/MWCNTs/SPCE improved the analytical characteristics of the discussed electrode and is a precondition for the insulin determination. The detection limit, sensitivity, and linear calibration plot for the determination in the NiONPs/chitosan-MWCNTs/SPCE are comparable with those of the other common modified electrodes [11, 30, 43] summarized in Table 1. Compared to the PGE modified with the

NiONPs/chitosan-MWCNTs and SPCE modified with MWCNTs-Nafion/NiONPs [27] a lower detection limit (only 94 nM) was achieved [28]. Also, the linear range (0.25 μM – 5 μM) in the NiONPs/chitosan-MWCNTs/SPCE proved to be wider than that of the chitosan-CNTs/GCE (0.1 μM – 3 μM) [45].

A prominent advantage of the prepared NiONPs/chitosan-MWCNTs/SPCE consists in using the SPCE for the modification because of the small area of the working electrode; this property makes the given SPCE type an ideal candidate for insulin detection in real samples. Unlike other commonly used carbon electrodes employed in the electrochemical procedure, such as the GCE [44, 45] or PGE [28], the SPCE facilitates rapid system miniaturization while the amount of the analyte required for the determination is reduced from the approximately 50 ml needed to enable the procedure in the GCE [44, 45] or PGE [28] down to only 50 μl . Further, no complex equipment has to be acquired to run the process in the NiONPs/chitosan-MWCNTs/SPCE, and the measurements do not need to be realized in a three-electrode electrochemical cell, because all the electrodes (WE, AE, RE) are pre-printed on a ceramic substrate. Therefore, no complicated pre-measurement preparation is required.

Also, compared to other methods commonly used for the determination [18, 19, 20], the electrochemical procedure on a nanomodified SPCE is considered a suitable method because of the fast running analysis that lasts only a few seconds. The electrochemical determination of insulin on the NiONPs/chitosan-MWCNTs/SPCE did not necessitate sophisticated sample preparation before the measurement either.

Table 1. The analytical insulin determination parameters in the different modified electrodes.

Electrode material	Method	Detection limit	Sensitivity	Dynamic range	References
NiONPs/chitosan-MWCNTs/SPCE	Cyclic voltammetry	94 nM	0.021 $\mu\text{A}/\mu\text{M}$	0.25 μM – 5 μM	This paper
Chitosan-CNTs/GCE	Amperometry	30 nM	135 mA/mM	0.1 μM – 3 μM	[44]
MWCNTs-Nafion/NiONPs/SPCE	Amperometry	6.1 nM	1.83 $\mu\text{A}/\mu\text{M}$	20 nM – 260 nM	[27]
CNTs/GCE	Amperometry	14 nM	48 nA/ μM	-	[30]
NiONPs/Chitosan-MWCNTs/PGE	Amperometry	260 nM	0.642 $\mu\text{A}/\mu\text{M}$	0.05 μM - 5 μM	[28]
Ru/Methalodendrimers/GCE	Amperometry	2 nM	225 nA/ μM	6 nM – 0.4 μM	[45]

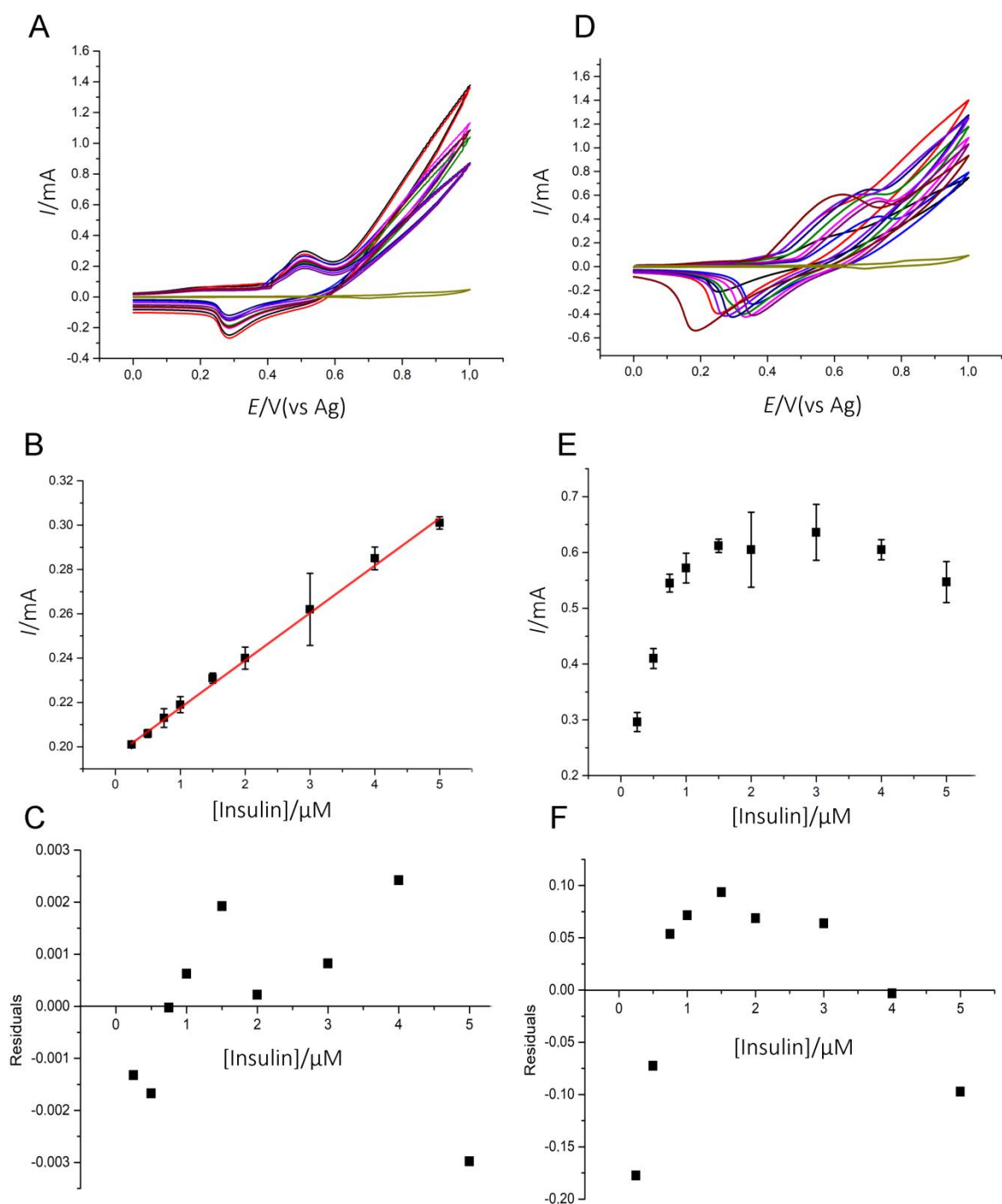


Figure 11. The cyclic voltammograms of 0.25 μM – 5 μM insulin in the 0.1 m NaOH and PBS on the NiONPs/chitosan-MWCNTs/SPCE (A) and NiONPs/MWCNTs/SPCE (D). The linear plot of the insulin oxidation peak current versus the insulin concentration on the NiONPs/chitosan-MWCNTs/SPCE (B) and NiONPs/MWCNTs/SPCE (E). The residuals analysis on NiONPs/chitosan-MWCNTs/SPCE (C) and NiONPs/MWCNTs/SPCE (F).

3.5 Interference study

Due to inappropriate analytical characteristics of the NiONPs/MWCNTs/SPCE for the insulin determination in real samples, the interference tests were performed only for the NiONPs/chitosan-MWCNTs/SPCE. In order to study the impact of the interferences present in blood on the insulin determination in the NiONPs/chitosan-MWCNTs/SPCE, 2 μM of the hormone was determined in a solution consisting of 0.1 mM ascorbic acid, 0.1 mM sucrose, 5 mM glucose, and 0.1 M lactic acid in the 0.1 M NaOH and PBS (Figure 12). Three measurements were executed, with the RSD of only 1 %. None of the compounds showed a specific current peak in the potential area related to the insulin oxidation on the NiONPs/chitosan-MWCNTs/SPCE, and the cyclic voltammogram was the same as that of the 2 μM insulin the in 0.1 M NaOH and PBS. All the electrochemical measurements were also performed in the PBS, simulating the concentration of Cl^- ions in biological samples.

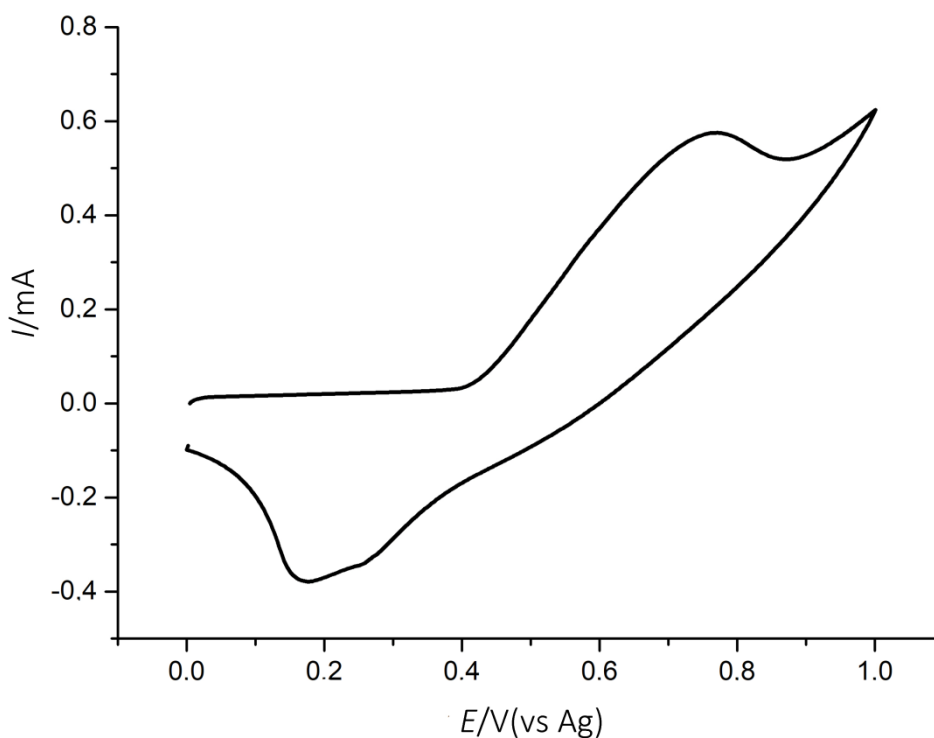


Figure 12. The cyclic voltammograms of the 2 μM insulin in the solution consisting of 5 mM glucose, 0.1 M ascorbic acid, 0.1 M lactic acid, 0.1 M sucrose, and 0.1 m NaOH and PBS on the NiONPs/chitosan-MWCNTs/SPCE at the scan rate of 100 mV/s.

4 Conclusions

The impact of chitosan as a polymer membrane on the insulin determination in NiONPs and MWCNTs-modified SPCEs was investigated. Although the chitosan layer on the NiONPs and MWCNTs-modified SPCE was not completely homogeneous, the polysaccharide stabilized the NiONPs on the MWCNTs-modified SPCE, increased the sensitivity, and fixed the NiONPs on the SPCE during the electrochemical measurements. Thus, the spin-coating method, instead of simple dropping of the MWCNTs-chitosan suspensions onto the electrodes, will be studied in the near future to eliminate formation of heterogeneous zones on the electrode. This coating procedure is capable of producing a thinner layer and accelerating the drying process, as confirmed by the EDX analysis of the NiONPs/MWCNTs/SPCE and NiONPs/chitosan-MWCNTs/SPCE before and after the electrochemical measurements and also by the electrochemical stability measurements. Even though the application of chitosan decreased the current response towards the insulin oxidation on the NiONPs and MWCNTs-modified SPCE, it substantially improved the analytical characteristics. The NiONPs/chitosan-MWCNTs/SPCE offers a lower detection limit, higher sensitivity, and sufficient linear range for the insulin determination in real samples. Moreover, the electrochemical procedure involves fast analysis lasting only a few seconds. Respecting the ion concentrations in real samples, all the measurements were performed in PBS to simulate the concentration of Cl^- ions in the human blood. Having in mind the possible interaction of the substances that can be contained in real samples, the influence of the interferences (sucrose, glucose, ascorbic acid, and lactic acid) on the insulin determination in the NiONPs/chitosan-MWCNTs/SPCE was studied. The cyclic voltammograms of the above-mentioned compounds did not show any measurable interference in the detection. Due to the small area of the working electrode in the SPCE and appropriate analytical characteristics of the NiONPs/chitosan-MWCNTs/SPCE, further research will focus on the electrochemical determination of insulin in the human blood with the NiONPs/chitosan-MWCNTs/SPCE.

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References

- [1] C. Kanaka-Gantenbein, G. Mastorakos, G.P. Chrousos, Endocrine-related causes and consequences of intrauterine growth retardation, *Ann. N. Y. Acad. Sci.* 997 (2003) 150–157. doi:10.1196/annals.1290.017.

- [2] G. Van Den Berghe, How does blood glucose control with insulin save lives in intensive care ? Greet Van den Berghe Find the latest version : Science in medicine How does blood glucose control with insulin save lives in intensive care ?, 114 (2004) 1187–1195. doi:10.1172/JCI200423506.
- [3] O. Cabrera, D.M. Berman, N.S. Kenyon, C. Ricordi, P.-O. Berggren, A. Caicedo, The unique cytoarchitecture of human pancreatic islets has implications for islet cell function, *Proc. Natl. Acad. Sci.* 103 (2006) 2334–2339. doi:10.1073/pnas.0510790103.
- [4] Y. Dor, J. Brown, O. Martinez, A. Melton, Adult pancreatic B-cells are formed by self-duplication rather than stem-cells differentiation, *Nature*. 229 (2004) 41–46. doi:10.1016/j.molliq.2016.05.027.
- [5] M. Shahidul, Islets of Langerhans: cellular structure and physiology, in: *Chronic Allograft Failure: Natural History, Pathogenesis, Diagnosis and Management*, 2008. pp. 1-4.
- [6] M.N. Poy, L. Eliasson, J. Krutzfeldt, S. Kuwajima, T. Tuschl, N. Rajewsky, P. Rorsman, M. Stoffel, A pancreatic islet-specific microRNA regulates insulin secretion, 432 (2004) 2–6. doi:10.1038/nature03076.
- [7] H.E. Lebovitz, Insulin resistance: definition and consequences, 109 (2001) 48-135. doi:10.1055/s-2001-18576
- [8] American Diabetes Association, Diagnosis and classification of diabetes mellitus., *Diabetes Care*. 29 (2006) 43. doi:10.2337/dc10-S062.
- [9] A. Bascones-Martínez, J. González-Febles, J. Sanz-Esporrín, Diabetes and periodontal disease. Review of the literature, *Am. J. Dent.* 27 (2014) 63–67. doi:10.4317/medoral.17032.
- [10] S. Park, H. Boo, T.D. Chung, Electrochemical non-enzymatic glucose sensors, *Anal. Chim. Acta*. 556 (2006) 46–57. doi:10.1016/j.aca.2005.05.080.
- [11] G.X. Zhong, W.X. Zhang, Y.M. Sun, Y.Q. Wei, Y. Lei, H.P. Peng, A.L. Liu, Y.Z. Chen, X.H. Lin, A nonenzymatic amperometric glucose sensor based on three dimensional nanostructure gold electrode, *Sensors Actuators, B Chem.* 212 (2015) 72–77. doi:10.1016/j.snb.2015.02.003.
- [12] K. Tian, M. Prestgard, A. Tiwari, A review of recent advances in nonenzymatic glucose sensors, *Mater. Sci. Eng. C*. 41 (2014) 100–118. doi:10.1016/j.msec.2014.04.013.
- [13] S.L.C. Geijselaers, S.J.S. Sep, M.T. Schram, M.P.J. van Boxtel, R.M.A. Henry, F.R.J. Verhey, A.A. Kroon, N.C. Schaper, P.C. Dagnelie, C.J.H. van der Kallen, C.D.A. Stehouwer, G.J. Biessels, Insulin resistance and cognitive performance in type 2 diabetes — The Maastricht study, *J. Diabetes Complications*. 31 (2017) 824–830. doi:10.1016/j.jdiacomp.2017.01.020.
- [14] A. Salimi, A. Noorbakhash, E. Sharifi, A. Semnani, Highly sensitive sensor for picomolar detection of insulin at physiological pH, using GC electrode modified with guanine and electrodeposited nickel oxide nanoparticles, *Biosens. Bioelectron.* 24 (2008) 792–798. doi:10.1016/j.bios.2008.06.046.
- [15] R. Soffe, V. Nock, J.G. Chase, Towards Point-of-Care Insulin Detection, 4 (2019) 3-19. doi:10.1021/acssensors.8b01253.
- [16] H. Murayama, N. Matsuura, T. Kawamura, T. Maruyama, N. Kikuchi, T. Kobayashi, F. Nishibe, A. Nagata, A sensitive radioimmunoassay of insulin autoantibody: Reduction of non-specific

- binding of [125I]insulin, *J. Autoimmun.* 26 (2006) 127–132. doi:10.1016/j.jaut.2005.11.003.
- [17] F. Mizutani, E. Ohta, Y. Mie, O. Niwa, T. Yasukawa, Enzyme immunoassay of insulin at picomolar levels based on the coulometric determination of hydrogen peroxide, *Sensors Actuators, B Chem.* 135 (2008) 304–308. doi:10.1016/j.snb.2008.08.037.
- [18] J.L. West, N.J. Halas, Engineered Nanomaterials for Biophotonics Applications: Improving Sensing, Imaging, and Therapeutics, *Annu. Rev. Biomed. Eng.* 5 (2003) 285–292. doi:10.1146/annurev.bioeng.5.011303.120723.
- [19] S.M. Taghdisi, N.M. Danesh, P. Lavaee, Aptamer Biosensor for Selective and Rapid Determination of Insulin, (2014) 37–41. doi:10.1080/00032719.2014.956216.
- [20] J. Hovancová, I. Šišoláková, R. Oriňaková, A. Oriňak, Nanomaterial-based electrochemical sensors for detection of glucose and insulin, *J. Solid State Electrochem.* 21 (2017) 2147–2166. doi:10.1007/s10008-017-3544-0.
- [21] N.F.C. Visser, M. Van Harmelen, H. Lingeman, H. Irth, On-line SPE-CE for the determination of insulin derivatives in biological fluids, *J. Pharm. Biomed. Anal.* 33 (2003) 451–462. doi:10.1016/S0731-7085(03)00292-9.
- [22] B.B. Prasad, R. Madhuri, M.P. Tiwari, P.S. Sharma, Imprinting molecular recognition sites on multiwalled carbon nanotubes surface for electrochemical detection of insulin in real samples, *Electrochim. Acta.* 55 (2010) 9146–9156. doi:10.1016/j.electacta.2010.09.008.
- [23] S.K. Arya, S.P. Singh, B.D. Malhotra, *Electrochemical Techniques in Biosensors*, 2007. doi:10.1002/9780470061565.hbb032.
- [24] V. Serafín, L. Agüí, P. Yáñez-Sedeño, J.M. Pingarrón, Electrochemical immunosensor for the determination of insulin-like growth factor-1 using electrodes modified with carbon nanotubes-poly(pyrrole propionic acid) hybrids, *Biosens. Bioelectron.* 52 (2014) 98–104. doi:10.1016/j.bios.2013.08.021.
- [25] M. Jaafariasl, E. Shams, M.K. Amini, Silica gel modified carbon paste electrode for electrochemical detection of insulin, *Electrochim. Acta.* 56 (2011) 4390–4395. doi:10.1016/j.electacta.2010.12.052.
- [26] C.E. Banks, R.G. Compton, New electrodes for old: From carbon nanotubes to edge plane pyrolytic graphite, *Analyst.* 131 (2006) 15–21. doi:10.1039/b512688f.
- [27] B. Rafiee, A.R. Fakhari, Electrocatalytic oxidation and determination of insulin at nickel oxide nanoparticles-multiwalled carbon nanotube modified screen printed electrode, *Biosens. Bioelectron.* 46 (2013) 130–135. doi:10.1016/j.bios.2013.01.037.
- [28] I. Sisolakova, J. Hovancova, R. Orinakova, A. Orinak, D.R. Garcia, O. Shylenko, J. Radonak, Comparison of Insulin Determination on NiNPs / chitosan- MWCNTs and NiONPs / chitosan-MWCNTs Modified Pencil Graphite Electrode, (2019) 103–112. doi:10.1002/elan.201800483.
- [29] J. Wang, Z. Xu, M. Zhang, J. Liu, H. Zou, L. Wang, Improvement of electrochemical performance of screen-printed carbon electrodes by UV/ozone modification, *Talanta.* 192 (2019) 40–45. doi:10.1016/j.talanta.2018.08.065.
- [30] J. Wang, M. Musameh, Electrochemical detection of trace insulin at carbon-nanotube-modified electrodes, *Anal. Chim. Acta.* 511 (2004) 33–36. doi:10.1016/j.aca.2004.01.035.

- [31] F. Qu, M. Yang, Y. Lu, G. Shen, R. Yu, Amperometric determination of bovine insulin based on synergic action of carbon nanotubes and cobalt hexacyanoferrate nanoparticles stabilized by EDTA, *Anal. Bioanal. Chem.* (2006). doi:10.1007/s00216-006-0642-8.
- [32] G.A. Rivas, M.D. Rubianes, M.C. Rodríguez, N.F. Ferreyra, G.L. Luque, M.L. Pedano, S.A. Miscoria, C. Parrado, Carbon nanotubes for electrochemical biosensing, *Talanta*. 74 (2007) 291–307. doi:10.1016/j.talanta.2007.10.013.
- [33] E. Martínez-Periñán, M. Revenga-Parra, M. Gennari, F. Pariente, R. Mas-Ballesté, F. Zamora, E. Lorenzo, Insulin sensor based on nanoparticle-decorated multiwalled carbon nanotubes modified electrodes, *Sensors Actuators, B Chem.* 222 (2016) 331–338. doi:10.1016/j.snb.2015.08.033.
- [34] A.I. Gopalan, K.P. Lee, K.M. Manesh, P. Santhosh, J.H. Kim, J.S. Kang, Electrochemical determination of dopamine and ascorbic acid at a novel gold nanoparticles distributed poly(4-aminothiophenol) modified electrode, *Talanta*. 71 (2007) 1774–1781. doi:10.1016/j.talanta.2006.08.026.
- [35] B. Erdelyi, A. Oriňak, R. Oriňaková, J. Lorinčík, M. Jerigová, D. Velič, M. Mičušík, M. Omastová, R.M. Smith, V. Girman, Catalytic activity of mono and bimetallic Zn/Cu/MWCNTs catalysts for the thermocatalyzed conversion of methane to hydrogen, *Appl. Surf. Sci.* 396 (2017) 574–581. doi:10.1016/j.apsusc.2016.10.199.
- [36] S.J. Konopka, B. Mcduffie, Diffusion Coefficients of Ferri- and Ferrocyanide Ions in Aqueous Media , Using Twin-Electrode Thin-Layer Electrochemistry IqT, 42 (1970) 1741–1746. doi:10.1021/ac50160a042.
- [37] D. Permana, L.O. Ahmad, Conductivity Improvement of Chitosan Membranes through Modification Conductivity Improvement of Chitosan Membranes through Modification with Lithium for Lithium Polymer Battery Application, (2016).
- [38] Y. Wang, J. Li, *Analytica Chimica Acta* A carbon nanotubes assisted strategy for insulin detection and insulin proteolysis assay, 650 (2009) 49–53. doi:10.1016/j.aca.2009.02.029.
- [39] D.R. Mayer John, Zhang Faming, Insulin Structure and Function, *Mem. Fukui Prefect. Dinosaur Museum*. 88 (2007) 687–713. doi:10.1002/bip.
- [40] B. Malfoy, J.A. Reynaud, Electrochemical investigations of amino acids at solid electrodes. Part II. Amino acids containing no sulfur atoms: Tryptophan, tyrosine, histidine and derivatives, *J. Electroanal. Chem.* 114 (1980) 213–223. doi:10.1016/S0022-0728(80)80448-7.
- [41] J.M. Chem, Fabrication and electrochemical performances of hierarchical porous, (2012) 11494–11502. doi:10.1039/c2jm30221g.
- [42] R. Article, Methods for the determination of limit of detection and limit of quantitation of the analytical methods, 2 (2011) 21–25. doi:10.4103/2229-5186.79345.
- [43] M. Feng, H. Han, J. Zhang, H. Tachikawa, Electrochemical sensors based on carbon nanotubes, *Electrochem. Sensors, Biosens. Their Biomed. Appl.* (2008) 459–501. doi:10.1016/B978-012373738-0.50017-9.
- [44] M. Zhang, C. Mullens, W. Gorski, Insulin oxidation and determination at carbon electrodes, *Anal. Chem.* 77 (2005) 6396–6401. doi:10.1021/ac0508752.
- [45] L. Cheng, G.E. Pacey, J.A. Cox, Carbon Electrodes Modified with Ruthenium Metallodendrimer

Multilayers for the Mediated Oxidation of Methionine and Insulin at Physiological pH, 73
(2001) 5607–5610.