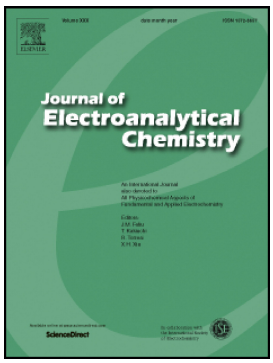

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Anti-human albumin monoclonal antibody immobilized on EDC-NHS functionalized carboxylic graphene/AuNPs composite as promising electrochemical HSA immunosensor

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Abstract

Diagnosis and treatment of some important diseased and metabolic disorders is based on successful detection of albumin. In this work, we aim to develop a simple immunosensor for the

detection of human serum albumin in biological fluids. Anti-human albumin antibody was covalently attached to the activated surface of screen-printed carbon electrodes enriched with carboxyl graphene/gold nanoparticles composite. Microstructure (TEM, FE-SEM, XRD) and electrochemical (CV, EIS) characterization methods were used to investigate composite properties and to confirm the successful modification of the electrodes. Under the optimal conditions, linear working range and limit of detection were 2.5-500 μg/ml and 1.55 μg/ml, respectively. Additionally, the effect of some possibly interfering compounds was investigated and the immunosensor was used for real sample analysis. The results showed that the sensor exhibited accurate, precise and sensitive characteristics and can be promising replacement to the convention methods for albumin detection in clinical practice.

Keywords: human serum albumin; electrochemical biosensor; immunosensor; screen-printed carbon electrode

1. Introduction

Human serum albumin (HSA) is the liver secreted protein, most abundant in the circulatory system. The main functions of albumin include maintaining the osmotic pressure in the plasma, the transfer of fatty acids, steroids, hormones and certain drugs [1]. Normally, the concentration of HSA in serum is 35–50 g/L and less than 20 mg/L in urine of healthy persons [2, 3]. However, these values can significantly deviate in the case of some diseases or metabolic disorders [4]. Chronic hepatitis, cirrhosis and liver failure can be indicated with the low presence of albumine [5, 6] and, in another case, high concentration of albumin can be pointer for kidney failure [7]. Therefore, urinary albumin, alone or accompanied with other urinary compounds, can be used as an important clinical biomarker for various renal and cardiovascular diseases [8].

Detection of proteins and hormones in clinical analysis requires the development of rapid and reliable methods for detecting very low concentrations of these analytes with great precision. Electrochemical sensors and biosensors are powerful analytical tools thanks to portability, self-control and low cost [9]. Among these sensors, immunosensor have received major attention, because they combine the specificity of an immunoreaction with the very high sensitivity of different electrochemical detectors [10, 11]. Immunosensors are analytical devices used to detect the binding between antibody (Ab) and antigen (Ag) with formation of a stable complex [12, 13]. This event is dominantly followed with changes in potential, current, ion concentration, conductivity, capacitance or impedance in the electrochemical converters to which either the antibody or antigen is immobilized.

For the biosensors development and construction several important criteria must be followed: sensitivity, selectivity, user friendliness, accessibility, rapidity, equipment simplicity and deliverability to the end-user. Screen printed electrodes (SPEs) are especially attractive for this

purpose because they exhibit disposability, robustness, stability and low cost for mass production. These electrodes are usually prepared from conductive inks based on platinum, silver, copper or carbon [14]. With the wide expansion of material science and production of nanomaterials and composites for modification of SPEs and improvement of their characteristic these electrodes are dominantly used in the field of biosensor sciences.

High surface area and excellent electrical and mechanical properties are important parameters that set up graphene and similar carbonaceous materials frequently used in electroanalytical chemistry [15–17]. Possibility of functionalization of graphene using different functional groups covalently or non-covalently bonded offers increment of the material characteristics [18]. One of these modified forms of graphene is carboxylated graphene which can form well-dispersed aqueous colloids [19, 20]. Also, the carboxylic acid groups can react with proteins or carbohydrates via amide or ester linkages [19, 20] which results in increased attractiveness of this material in the development of new biosensors. Furthermore, the properties of this material often can be additionally improved by its decoration with metal or metal-oxide nanoparticles.

Precious metal nanoparticles, such as gold (Au), silver (Ag), platinum (Pt), palladium (Pd) and their corresponding alloys or cores, are primarily used to develop electrochemical sensors for in vivo and in vitro biomedical analysis due to their physical, chemical and electrochemical properties, as well as good biocompatibility [21]. Gold nanoparticles satisfy some important criteria such as chemical stability, working potential range, surface-to-volume ratio, catalytic activity and good biological compatibility and based on this they become very attractive for construction of biosensors [22–26].

Among various types of combination for the construction of immunosensors functionalization of the electrode active surface is still the key issue toward the development of robust and stable

immunosensors. This problem has been addressed by different working groups by using standard chemistry and several functionalization steps including a high number of chemical reagents. Recently, our research group showed that successful combination of novel materials significantly improves the characteristics of NADH sensing [27]. In this study, our goal was to develop a disposable electrochemical immunosensor for urinary albumin detection. For this purpose, we used disposable SPEs enriched with composite made of functionalized carboxylic graphene and gold nanoparticles. The main idea was introducing a novel material, carboxylic graphene, in order to reduce the functionalization time and the number of chemical reagents, and gold nanoparticles for enhanced conductivity in combination with multi-step amperometry in order to enable real time monitoring of antibody–antigen interactions.

2. Materials and methods

2.1. Instrumentation and reagents

Electrochemical experiments involving electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV) and multi-step amperometry (MA) were performed using a potentiostat/galvanostat PalmSense3 (PalmSens BV, Houten, The Netherlands). Ag/AgCl electrode (3M KCl) as reference electrode and a Pt plate as counter electrode were used in standard 25 ml working cell. For pH measurements, a pH meter, model Orion 1230 equipped with a combined glass electrode model Orion 9165BNWP (USA), was used.

The morphology of materials and nanocomposites was examined using a field emission-scanning electron microscope FE-SEM MIRA3 (Tescan, Czech Republic) coupled with an EDS analyser (Oxford, UK) operated at 30 kV and a scanning transmission electron microscope STEM (JEOL-TEM 2100F) operated at 200 kV. A drop of an aqueous suspension of the particles was placed

onto a carbon-coated copper grid and left to dry at room temperature for FE-SEM observations. The size of Au nanoparticles was determined by manual measurement of particles using the public domain software Image J.

Microstructural properties of the materials were examined by X-ray powder-diffraction (XRPD) performed on a high-resolution SmartLab® X-ray diffractometer (Rigaku, Japan) with a CuKα radiation source, an accelerating voltage of 40 kV and a current 30 mA. Samples were prepared by flattening dried powders with a zero-background silicon wafer and diffraction patterns were collected within 5-70° 2θ range.

All chemicals were of analytical grade and were used as supplied without any further purification. N-Hydroxysulfosuccinimide sodium salt (sulfo-NHS), 1-ethyl-3-(3-di-methylamino-propyl) carbodiimide hydrochloride (EDC), bovine serum albumin (BSA), human serum albumin (HSA; lyophilized powder, ≥97%) and mouse anti-human albumin monoclonal antibodies (anti-HSA) were purchased from Sigma-Aldrich Corp (St. Louis, MO, USA). Gold nanoparticles (5 nm diameter, stabilized suspension in 0.1 mM phosphate buffer solution (PBS), reactant free) were obtained from Merck, Germany, while carboxylated graphene (CGR, water dispersion, 5 mg/ml) was obtained from ACS Material (Pasadena, CA, USA). Uric acid, ascorbic acid, glucose, dopamine and paracetamol were obtained from Sigma Aldrich (St. Louis, MO, USA). Potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium ferrocyanide ($K_4[Fe(CN)_6]$) and potassium chloride were all supplied by Merck (Germany). All measurements were performed in phosphate buffer solution (PBS; 0.1 M) which was obtained by dissolving corresponding amounts of phosphate salts (potassium di- and hydrogen phosphate, KH_2PO_4 and K_2HPO_4, Merck, Germany) in ultra-pure water.

Carbon ink (No. C2030519P4, Gwent, Pontypool, UK) was used for the preparation of screen-printed carbon electrodes. Electrodes were prepared using printing procedure developed in our previous studies [27, 28].

2.2. Preparation of working electrodes

2.2.1. Preparation of Au@CGR-SPCE electrodes

For the preparation of the Au@CGR-SPCE modified electrode, firstly we prepared the composite made of gold nanoparticles (Au, nanoparticles 5 nm diameter, stabilized suspension in 0.1 mM phosphate buffer solution) and carboxylated graphene (CGR, water dispersion, 5 mg/ml). These two components were mixed in volume ratio 1:3 (Au:CGR) and sonicated for three hours [29]. After this period, the composite (10 μL) was applied on the surface of activated SPCE and allowed to dry at room temperature. Activation of SPCEs was achieved by electrochemical pre-treatment. Several potential cycles between 1 and -1.5 V (scan rate 100 mVs^{-1}) were applied using 0.5 M H_2SO_4 solution until the characteristic cyclic voltammogram for a clean SPCE surface was obtained.

2.2.2. Preparation of the immunosensor

Anti-human albumin antibodies (anti-HSA) were covalently attached to the carboxyl groups at the electrode surface through EDC–NHS chemistry. The Au@CGR-SPCEs were thoroughly rinsed with large amounts of ultra-pure water. Then, the electrodes were immersed in 10 mM PBS solution (pH 6) containing 2 mM of EDC and 5 mM of sulfo-NHS for 30 minutes at room temperature. After rinsing, on every electrode 10 μL of the anti-HSA solution was applied [13]. The anti-HSA solution was obtained by diluting (1:1000) monoclonal anti-human serum albumin antibodies in 10 mM PBS solution (pH 7.2). The electrodes were left at 4°C for 45 minutes and then rinsed with water and immersed in 10 mM PBS solution (pH 7.2) containing 1 % of BSA

for 1 hour, in order to block non-specific, remained binding sites. Then, the electrodes were rinsed with PBS solution and ultra-pure water to remove unbound BSA from the electrode surface and left at 4°C until use.

3. Results and discussion

3.1. Characterization of working electrodes

The FE-SEM micrographs of carboxyl graphene and gold nanoparticles composites with a magnification of 50.000 times are shown in Fig. 1A and B. Carboxyl graphene (Fig. 1A) is an almost transparent thin film with large amount of folds and wrinkles which possess desirable properties for anchoring small Au nanoparticles. Aggregation of gold nanoparticles on top of the carboxyl graphene layers enable a larger surface area and subsequently better conductivity in comparison with CGR (Fig. 1B). In Fig. 1C a high magnification image of a single gold nanoparticle is depicted. It shows that the Au nanoparticles are spherical in shape with an average diameter of 17±2 nm in a well-arranged crystal structure. Small Au nanoparticles were rather homogeneously dispersed over the thin film of carboxyl graphene layer (Fig. 1D).

>>>>> ***Preferred position for Fig. 1*** <<<<<<<

The XRD pattern of the obtained composite is shown in Fig. 2. A diffraction of 11.5° of 2θ is characteristic for the (001) layer of carboxyl graphene [30]. Due to small the concentration of the composite the diffraction pattern possesses high background. The most intensive diffraction peak at 38.5° can be indexed to the (111) layer of Au nanoparticles, confirming the formation of the composite [31].

>>>>> ***Preferred position for Fig. 2*** <<<<<<<

3.2. Electrochemical characterization of the working electrodes

Fig. 3. shows the characterization of the electrode surfaces using electrochemical methods. Cyclic voltammetry was selected for the electrochemical characterization of electrodes surfaces after each assembly step. All measurements were performed in 250 mM KCl solution containing 5 mM of the redox probe $[Fe(CN)_6]^{3-/4-}$ with a scan rate of 50 mV/s in the potential range from -0.5 V to +1.0 V. As can be seen from the voltammograms (Fig. 3A), characteristic anodic and cathodic peaks were observed with all electrodes. In case of bare SPCEs $[Fe(CN)_6]^{3-/4}$ exhibited a peak separation of ~0.7 V. The electrode modified with the composite made of carboxyl graphene and gold nanoparticles presents higher faradaic current and lower peak separation (ΔEp ~ 0.5V) (curve b) in comparison with a bare SPCE (curve a), which confirms that the composite material improves the electrochemical characteristics of a bare SPCE. After activation with EDC/NHS the faradic currents in the cyclic voltammogram increase and the peak separation (ΔEp) decreases (curve c). This may be due to a decrease in the number of free carboxyl groups on the electrode surface because of their EDC-NHS coupling. Because of this depletion the repulsive force between the free carboxyl groups and the $[Fe(CN)_6]^{3-/4-}$ anions is decreased. Covalent immobilization of anti-HSA onto the activated surface of an Au@CGR-SPCE results in a decrease of the peak current (curve d). Concomitantly the anodic and cathodic peaks become wider. The reason for this is that the immobilized anti-bodies represent an electron transfer blocking layer (as non-conductive organic layer) and interferes with the diffusion of the redox probe $[Fe(CN)_6]^{3-/4-}$ to the surface of the electrode. These results demonstrate successful immobilization of antibodies at the electrode surface.

>>>>> ***Preferred position for Fig. 3*** <<<<<<<

Electrochemical impedance spectroscopy (EIS) was used for further investigation of the electrochemical performance of the proposed immunosensor. Each step of the surface modification of the electrode (as described above) was characterized by EIS. Fig. 3B shows the Nyquist plots of impedance spectra of different electrodes in 250 mM KCl solution containing 5 mM of the redox probe $[Fe(CN)_6]^{3-/4-}$ at a potential of 0 V. The frequency range varied from 0.01 to 100000 Hz and the amplitude was 5 mV. The semicircle diameter in the Nyquist plot at higher frequencies corresponds to the electron-transfer resistance (Rct), and the linear part at lower frequencies corresponds to the diffusion process [32]. A similar trend in CV was observed. The Nyquist plot shows a semicircle with a charge transfer resistance (Rct) of 5858 Ω for the bare SPCE. After modification with the composite of AuNp and CG, Rct was slightly lowered to 5604 Ω. Similar to results obtained from CV these data confirm an improvement of the electrochemical properties of the modified electrode. Activation of terminal carboxylic groups, by EDC/NHS, results in a decrease of Rct (5420 Ω). Higher Rct (6202 Ω) was observed after immobilization of anti-HSA on the electrode surface (curve d) by the generation of a protein layer at the electrode surface, which results in a higher electron transfer resistance observable as enlarged diameter of the semicircle in the corresponding Nyquist plot. These results are coherent with the results obtained from CV measurements and confirm successful functionalization of the modifying composite and immobilization of the anti-HSA on the electrode surface.

3.3. Optimization of experimental variables for HSA detection

3.3.1. Optimization of antibody immobilization time

Some important parameters that can affect the performance of the immunosensor were optimized. Firstly, the time needed for linking anti-HSA with amino-groups at the activated Au@CGR-SPCE surface using cyclic voltammetry was studied. All measurements were done in

250 mM KCl solution containing 5 mM of the redox probe $[Fe(CN)_6]^{3-/4-}$. The potential ranged from -0.5 to 1.0 V with a scan rate of 50 mV/s. The reproducibility of different electrodes was determined by cyclic voltammetry of each electrode before and after immobilization of antibodies comparing the resulting differences in oxidation current peaks. The results are summarized in Fig. 4. as mean value from three independent tests. An optimal incubation period of 60 minutes was chosen. After this period there were no significant changes in the peak current (Fig. 4A) which indicates that during this time the majority of available amino-groups for anti-HSA binding on the electrode surface has been utilized.

>>>>> ***Preferred position for Fig. 4*** <<<<<<<

3.3.2. Optimization of antigen binding time

The incubation time for the immunoreaction was investigated in the range from 15 to 120 min with four different concentrations of antigen HSA (10, 50, 100 and 250 μg/ml). For every examined concentration, cyclic voltammograms were recorded for each electrode before and after corresponding antigen binding time. Cyclic voltammograms were recorded in 250 mM KCl solution containing 5 mM of redox probe $[Fe(CN)_6]^{3-/4-}$. The results (Fig. 4B) uncover a stable current with negligible changes after 45 minutes of immunoreaction which was selected as optimum for the HSA immunosensor.

3.4. Analytical performance of the immunosensor

Multistep amperometry (MA) was used for the detection of albumin utilizing the immunosensor under optimal experimental conditions in order to improve its selectivity. First, possible interfering substances are oxidized at a lower potential; then the analyte of interest is detected at higher potential [13, 33]. Standard HSA solutions were prepared by mixing a 10 mM phosphate

buffer solution (pH 7.2) containing 5 mM of redox probe $[Fe(CN)_6]^{3-/4}$ with a proper amount of HSA. For every standard HSA solution, amperograms were recorded using three different immunosensors. The initial potential in multistep amperometry was 0.2 V while the detection potential was 0.3 V, each with a duration time of 20 s. As expected, the response of the anti-HSA/EDC+sulfo NHS/Au@CGR-SPCE immunosensor decreased with increasing the HSA concentration in solution due to the enhanced immunocomplex formation at the electrode surface. Some typical recorded amperograms are presented in Fig. 5.

>>>>> ***Preferred position for Fig. 5*** <<<<<<<

There was a linear relationship between the immunosensor response (steady current response at 40 s) and the logarithm of HSA concentration in the range of 2.5 to 500 µg/ml with a linear regression equation of I (µA) = 4.48-0.70 log C_{HSA} (µg/ml) and a correlation coefficient of 0.9945 (Fig. 6). The limit of detection was calculated according to the 3 s_a/b criterion [34] as 1.55 µg/l. Comparing these results (Table S1, Supplementary material) with literature data [3, 13, 35–38], it can be noticed that the approach described here offers comparable or better characteristics. The immunosensor presented in this work possesses a wider linear range and a very good limit of detection. Although there are sensors described with lower LODs in the literature the analytical performance of the new device is perfectly sufficient and satisfactory for clinical analysis.

>>>>> ***Preferred position for Fig. 6*** <<<<<<<

3.5. Selectivity and reproducibility

To investigate the selectivity of the immunosensor, its response to the possible interferents expected in the tested sample (uric acid, UA; glucose, Glu; bovine serum albumin, BSA; and

paracetamol, Para) was examined. All selected compounds were added to a solution of PBS containing 50 µg / ml HSA, while the concentration of each test compound was 1 mg / ml. Based on the results (Fig. S1, Supplementary Material), it can be concluded that the presence of all test compounds had negligible effects (signal changes below 10%) on the immunosensory response to HSA, although their concentration was 20 times higher than the concentration of HSA in model solutions.

The immunosensor reproducibility was assessed by measuring the amperometric response of 50 µg / ml HSA with five electrodes produced under the same conditions (Figure S2, supplementary material). The relative standard deviation (RSD) was below 7%, confirming that the production of the immunosensor shows acceptable reproducibility. The sensor retained about 70% of its original response after storage at 4 ° C for 1 week, demonstrating acceptable long-term stability of functionalized carboxyl-graphene / Au nanoparticles on the surface of the screen-printed carbon electrode. In conclusion, the above results confirm the fact that the immunosensor can be considered as a progressive electrochemical device for the sensitive and accurate quantification of HSA in biological samples under selected experimental conditions.

3.6. Real sample analysis

The urine of three healthy volunteers was used as the real sample in this study. Samples were centrifuged for 5 minutes at 5000 rpm and then diluted 2 times with 10 mM phosphate buffer solution (pH 7.2) containing 5 mM $[Fe(CN)_6]^{3-/4}$ probe. All measurements were performed in triplicate. The results are summarized in Table S2 (Supplementary Material). Data and recovery experiments show that the developed approach including the manufacturing process and the detection procedure can be applied to the clinical determination of HSA in biological fluids with satisfactory recovery and accuracy.

4. Conclusion

A new immunosensor has been proposed for the rapid and accurate detection of HSA. The biosensor is effectively transduced by a combination of carboxyl-graphene that allows covalent binding of the antibody to the electrode surface with gold nanoparticles that increase conductivity. The electrochemical immunosensor had very good HSA detection performance with a wide linear operating range (2.5 to 500 µg / ml) and a detection limit of 1.55 µg / mL. The accuracy and precision of the proposed approach were confirmed by experiments with urine samples. These results and developed immunosensor indicates the great potential of synthesized materials for the production of reliable electrochemical immunosensors as a promising platform for the construction of medical home care products.

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Figure captions:

Fig. 1. FE-SEM micrographs of (A) carboxylated graphene (CGR), (B) Au nanoparticle-modified carboxylated graphene (Au@CGR), (C) HR-TEM micrograph of a Au nanoparticle, and (D) TEM micrograph of Au@CGR.

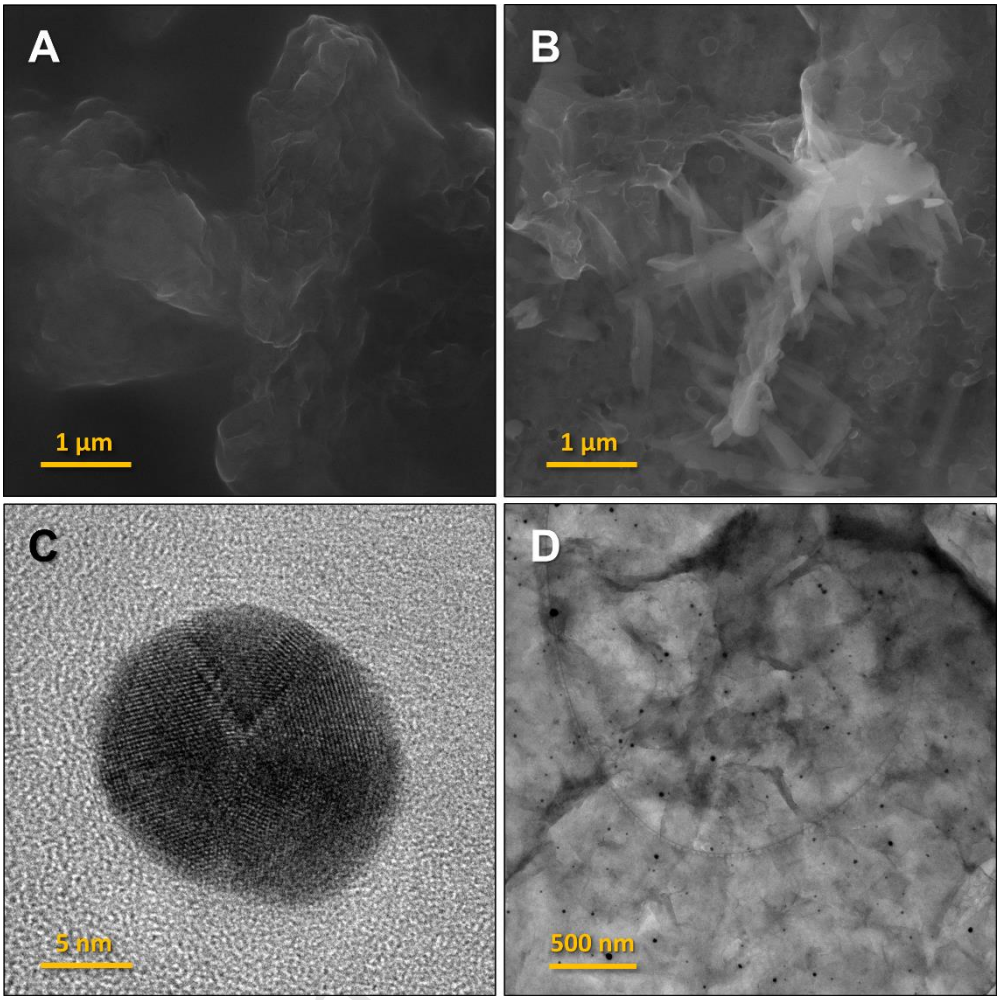
Fig. 2. XRD pattern of the Au@CGR composite

Fig. 3. (A) Cyclic voltammograms and (B) EIS measurements of a 5 mM $[Fe(CN)_6]^{3-/4-}$ probe in 250 mM KCl with a) bare SPCE b) Au@CGR-SPCE, c) EDC + sulfo NHS/Au@CGR-SPCE and d) anti-HSA/EDC+sulfo NHS/Au@CGR-SPCE

Fig. 4. Dependence of (A) antibody immobilization time and (B) antigen binding time monitored by cyclic voltammetry in 250 mM KCl solution containing 5 mM of $[Fe(CN)_6]^{3-/4-}$; scan rate 50 mV/s.

Fig. 5. Typical amperograms for various concentrations of HSA. Multistep amperometry, initial potential 0.2 V, detection potential 0.3 V

Fig. 6. Dependance of the immunosensor response on the concentration of HSA. The inset shows the relationship between the current and the logarithm of the HSA concentration.



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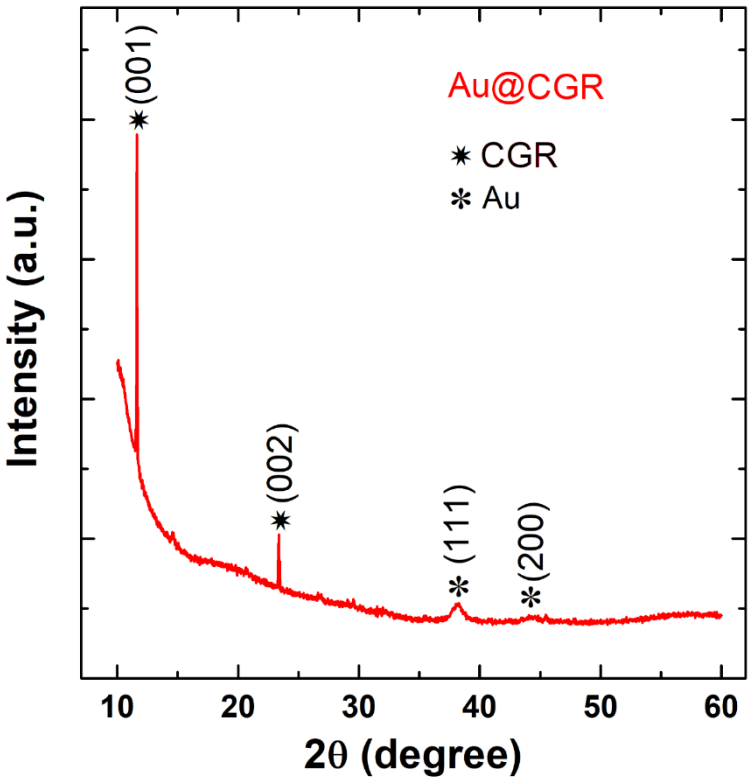

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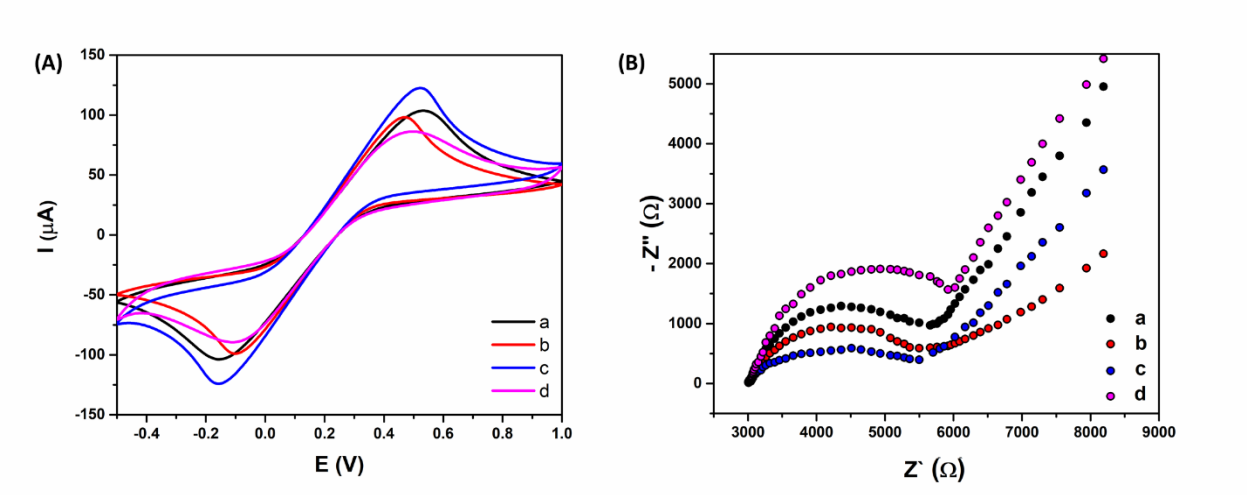

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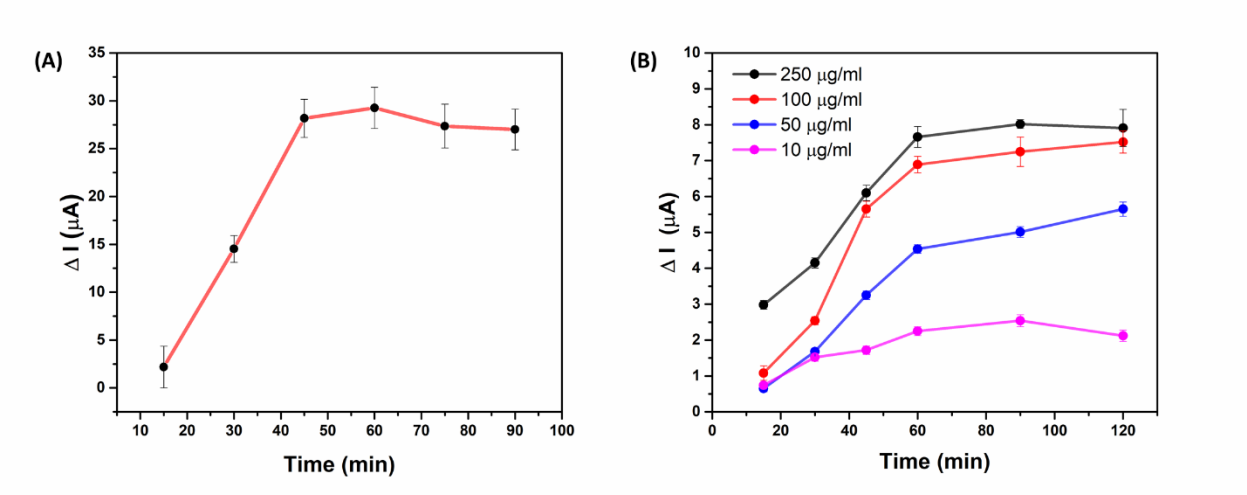

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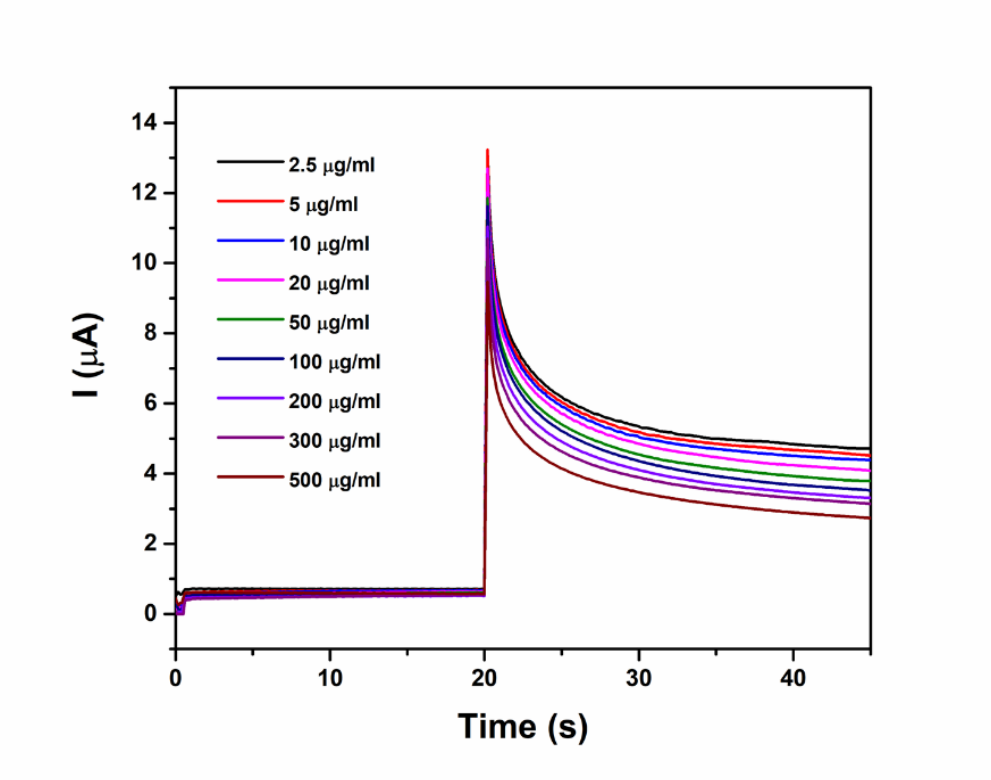

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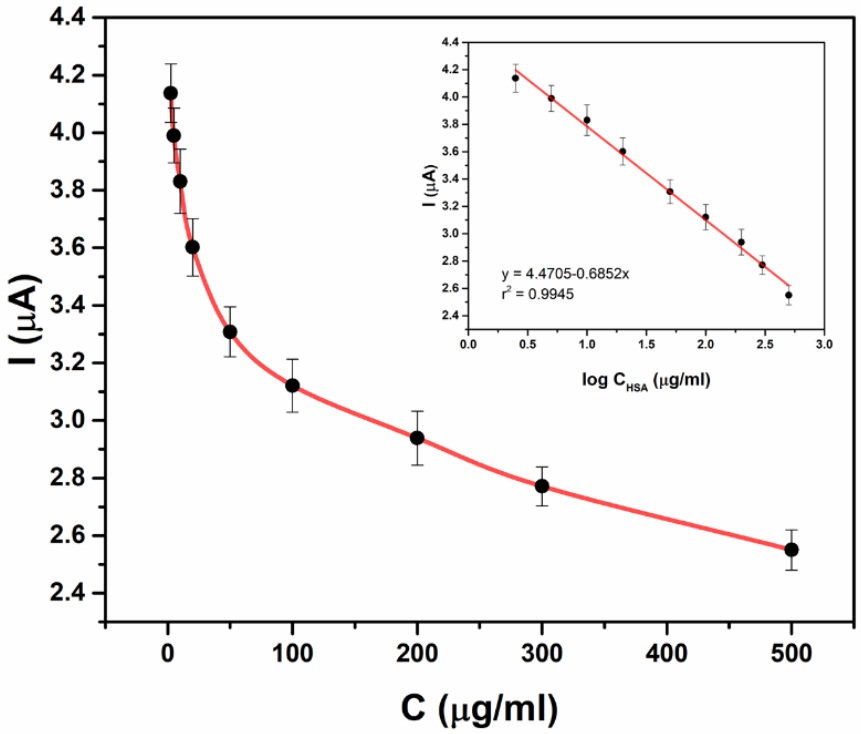

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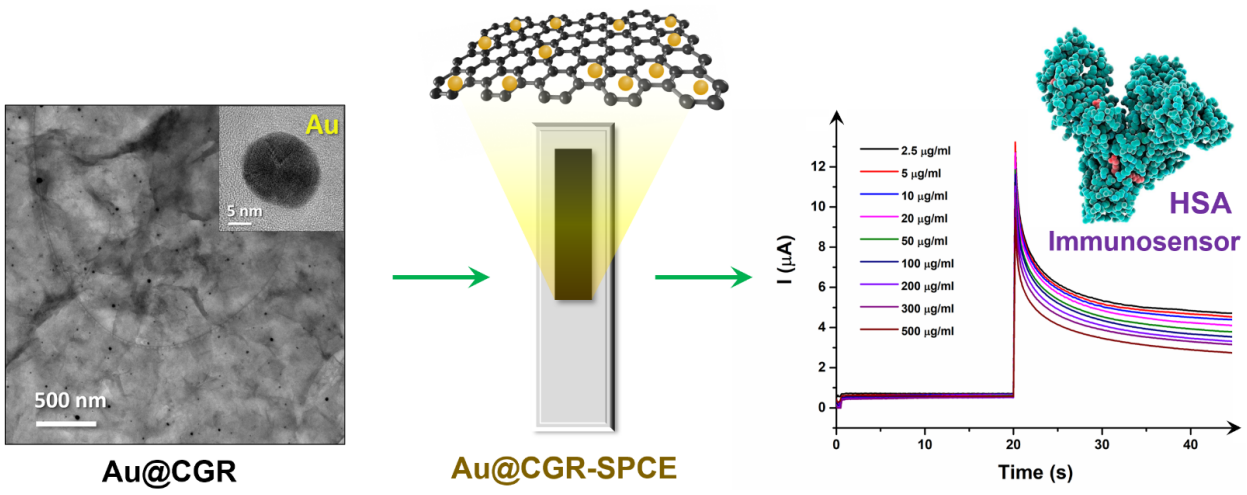
Vesna Stanković and Slađana Đurđić did electrochemical measurements. Miloš Ognjanović and Bratislav Antić did synthesis of materials and characterization. Eda Mehmeti did laboratory work. Jelena Mutić did writing - review & editing. Kurt Kalcher and Dalibor Stanković supervised work and did writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



Au – GOLD NANOPARTICLES CGR – CARBOXYLATED GRAPHENE
SPCE – SCREEN PRINTED CARBON ELECTRODE HSA – HUMAN SERUM ALBUMIN

Graphical abstract

Highlights

- New immunosensor for the determination of human serum albumin is proposed.
- Disposable immunosensor based on carboxylic graphene enriched with gold nanoparticles supported on screen printed electrode
- Satisfactory selectivity, sensitivity and precision of proposed method are obtained.