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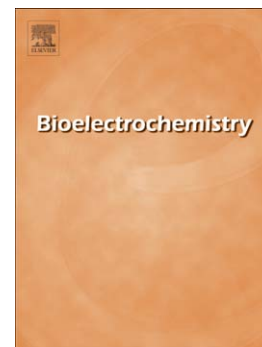
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A novel bioelectrochemical interface based on in situ synthesis of gold nanostructures on electrode surfaces and surface activation by Meerwein's salt. A bioelectrochemical sensor for glucose determination.

Konstantin Nikolaev^{&§}, Sergey Ermakov[§], Yuri Ermolenko[§], Elena Averyaskina[§],
Andreas Offenhäusser[&], Yulia Mourzina^{&*}

[&]Peter Grünberg Institute 8, Forschungszentrum Jülich GmbH and Jülich-Aachen Research Alliance - Fundamentals of Future Information Technology (JARA-FIT), 52428 Jülich, Germany

[§]Institute of Chemistry, St. Petersburg State University, Universitetskaya nab. 7/9, 199034 St. Petersburg, Russia

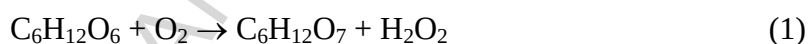
*Corresponding author: Tel.: +49(0)2461612364. E-mail address: y.mourzina@fz-juelich.de (Dr. Yu. Mourzina)

Abstract. A novel effective bioelectrochemical sensor interface for enzyme biosensors is proposed. The method is based on in situ synthesis of gold nanostructures (5-15 nm) on the thin-film electrode surface using the oleylamine (OA) method, which provides a high-density, stable, electrode interface nanoarchitecture. New method to activate the surface of the OA-stabilized nanostructured electrochemical interface for further functionalization with biomolecules (glucose oxidase enzyme) using Meerwein's salt is proposed. Using this approach a new biosensor for glucose determination with improved analytical characteristics: wide working range 0.06–18.5 mM with a sensitivity of $22.6 \pm 0.5 \mu\text{A mM}^{-1}\text{cm}^{-2}$, limit of detection 0.02 mM, high reproducibility, and long lifetime (60 days, 93 %) was developed. The surface morphology of the electrodes was characterized by scanning electron microscopy (SEM). The electrochemical properties of the interface were studied by cyclic voltammetry and electrochemical impedance spectroscopy using a Fe(II/III) redox couple. The studies revealed an increase in the electroactive surface area and a decrease in the charge transfer resistance following surface activation with Meerwein's reagent. A remarkably enhanced stability and reproducibility of the sensor was achieved using in situ synthesis of gold nanostructures on the electrode surface, while surface activation with Meerwein's salt proved indispensable in achieving an efficient bioelectrochemical interface.

Keywords: bioelectrochemical sensor, glucose oxidase, gold nanowire, gold nanoparticle, Meerwein's reagent, oleylamine

Introduction

Bioelectrochemical application of enzymes has attracted much attention for the development of practical devices such as enzyme biosensors for monitoring of biological analytes, for example, neurotransmitters and metabolites and enzymatic biofuel cells [1-7]. Enzymatic sensors for glucose determination in blood are important for clinical analysis and home measurements. The necessary blood tests must be fast and accurate. Optical and electrochemical methods are the most frequently used methods for glucose determination. Electrochemical methods have better analytical characteristics (sensitivity, selectivity, and reliability), in situ real time monitoring capacity, portability, and the required sensors can be manufactured in a simple process at low cost [8]. Since the development of the first sensor for glucose determination by Clark and Lyons in 1962 [9], which determined the amount of oxygen consumed in the glucose oxidase-catalyzed reaction of glucose oxidation (1):



three generations of electrochemical biosensors for glucose determination have been developed. The first generation of biosensors was based on the natural physiological electron acceptor oxygen co-substrate and the detection of hydrogen peroxide as a product of the glucose oxidation. The disadvantage of this method is the need to apply a relatively high anodic potential (about +0.6 V against Ag/AgCl) to detect hydrogen peroxide. Electrochemical biosensors of the second generation use nonphysiological electron acceptors (synthetic mediators, e.g., potassium hexacyanoferrate, hydroquinone, and ferrocene) for the electron transfer from the redox center of glucose oxidase (FAD/FADH₂) to the electrode surface. The third generation of biosensors will eliminate the use of mediators, e.g., by the direct electron transfer between enzyme and electrode surface [8, 10, 11] or by employing organic semiconductors [12-15]. Although a number of approaches to create third-generation sensors with direct electron transfer exist, the fabrication of these sensors requires sophisticated and expensive techniques and the results are insufficiently reproducible. Further investigations are required to enable noninvasive sensing of glucose levels at home [16, 17]. Critical aspects for electrochemical applications of enzymes are properties of the interface between biological molecules and electrical transducer and preservation of enzyme activity in a device for a reasonable length of time [18].

Over the last decade the electronic nanomaterials advanced the development of bioelectrochemical sensors and systems [10, 16, 19-25] by offering excellent prospects for interfacing biological recognition events with electronic signal transduction schemes to design new bioelectrochemical interfaces and enzyme sensors [26]. Modifying electrodes with nanostructures allows a unique range of properties to be conferred to the electrode surface from a high surface area to electrocatalytic properties in redox reactions. Specifically for biosensors,

nanostructures [27] may facilitate electron transfer between the catalytic redox center of an enzyme and an electrode surface, prevent denaturation of molecules on the electrode surface, and increase the surface-to-volume ratio, and therefore the amount of the immobilized enzyme [28-30]. Stabilization of the immobilized enzyme on nanosupports may be attributed to the “three-dimensional enzyme immobilization”, combining several stabilization factors including confinement effects and multipoint attachment [31]. Nanostructuring electrodes can be regarded as a general strategy to control the electrode architecture at the nanoscale [32]. A number of nanomaterials have been applied for the development of biosensors: noble metal nanostructures, carbon-based nanomaterials, metal oxides, and sulfides [33]. Nanostructures can be immobilized by reagents using covalent cross-linking, nanostructure adsorption on the electrode surface, and layer-by-layer methods [34].

Gold nanostructures are one of the most attractive materials for electrochemical biosensors [22, 35] due to their low electrical resistivity ($2.44 \cdot 10^{-8} \Omega \text{ m}$), relatively wide electrochemical potential window, catalysis of redox reactions of molecules such as O_2 , H_2O_2 , NO , and NADH [32, 36], and the availability of numerous techniques to prepare them. Chemical fabrication methods enable large quantities of nanostructures to be produced quickly, easily, and at low cost. Gold nanowire geometries are constrained by the conditions of the reactions, which produce them. Generally, such syntheses proceed in nonaqueous media in order to produce nanowires with small diameters of a few nm [36-43]. Most synthetic routes employ long hydrocarbon molecules containing a coordinating headgroup as a ligand, such as oleylamine, which sterically stabilize the nanostructures and play a role as both a reducing and stabilizing agent [39, 44, 45]. The oleylamine method for preparing gold nanostructures is the most economic and fastest method, producing a high yield of the final product. This method is based on the reduction of Au (III,I) to Au (0) by oleylamine, leading to the formation of gold nanoparticles and nanowires of various diameters and lengths. The oleylamine method is characterized by the possibility of obtaining gold nanostructures with a diameter of 2–15 nm and nanowires with lengths of up to several micrometers. Modifying the electrode surface with these nanostructures increases the electroactive electrode surface area and thus enhances the sensor signal. Among the disadvantages of the oleylamine method is a limited use of gold nanostructures in the electrolyte medium because the stabilizing organic molecules, such as oleylamine and its oligomers, prevent charge transfer and block and deactivate the surface of the electrode, where bare nanocrystal surfaces are desirable. Coordinating ligands, which are generally acquired by nanostructures during their chemical synthesis, have been optimized to control the structural and morphological characteristics of the nanostructures. However, they are often undesirable in the final applications of nanostructures, since they are insulating compounds

and constitute a barrier for charge transport [46]. The present paper describes the development of a new bioelectrochemical sensor based on oleylamine-stabilized gold nanostructures and Meerwein's reagent, which is used to activate the nanostructured electrode surface and introduce functional molecules to the surface of the nanostructures [47, 48]. Using this approach, an enzyme biosensor for glucose determination with improved analytical characteristics was developed.

2. Experimental part

2.1. Reagents

Glucose oxidase (from *Aspergillus niger*, type X-S, lyophilized powder, 100,000-250,000 units/g solid, EC number 1.1.3.4), oleylamine (70 %), glutaraldehyde (grade I, 25% in H₂O), disodium phosphate, sodium dihydrogen phosphate, toluol, acetone, ethanol, propanol, D(+)-glucose, L-ascorbic acid, 2-aminoethanethiol (cysteamine, CA), triethyloxonium tetrafluoroborate, acetonitrile, and other chemicals employed in the synthesis and for electrochemical measurements were purchased from Sigma-Aldrich and used as received. Solutions of glucose in phosphate buffer were allowed to mutarotate for 24 h at room temperature before being used in the experiments. All solutions were prepared using distilled or deionized water (Merck Millipore: Milli-Q Advantage A10 Ultrapure Water Purification System).

2.2. Methods

For the structural characterization of the nanostructures and electrodes, a scanning electron microscope Magellan™ XHR SEM was used. All electrochemical measurements were performed using a potentiostat-galvanostat AUTOLAB PGSTAT302. The electrochemical cell included a silver/silver chloride reference electrode (Ag/AgCl, 3 M KCl, World Precision Instruments), a coiled platinum auxiliary electrode, and a desired working electrode (sections 2.3–2.5). Electrochemical measurements were performed in 0.1 M phosphate buffer solution at pH 6.8. The oxygen was removed by purging the electrochemical cell with argon. Diameter of the working electrodes in electrochemical cells were 0.5 cm (for experiments with sulfuric acid and EIS) and 0.3 cm (for experiments with glucose oxidase sensors). The measurements were performed at room temperature 21±1 °C.

2.3. Working electrode fabrication

A silicon substrate was coated with silicon dioxide (1 µm) by thermal oxidation. An adhesion layer of titan (10 nm) and a thin film of gold (300 nm) were deposited on a Si/SiO₂ substrate by e-beam evaporation. The gold electrodes were first chemically cleaned in acetone,

2-propanol, distilled water, a mixture of sulfuric acid and hydrogen peroxide (2:1), and distilled water. The gold electrodes were further subjected to electrochemical cleaning in 0.1 M H₂SO₄ solution under potential cycling conditions from 0 V to 1.5 V with a scan rate of 0.05 V s⁻¹ with a start and end potential of 0 V (Ag/AgCl, 3 M KCl). The surfaces of these electrodes were modified with gold nanostructures.

2.4. Synthesis of gold nanostructures

Gold nanostructures were produced using two methods: oleylamine and gold(I) chloride in hexane using a previously published protocol (method I; [39]) and gold(III) chloride in toluene (method II; [45]) (Fig. 1). In method II, oleic acid was replaced by OA, according to the report on the effect of OA and oleic acid on the aspect ratio of nanostructures [40]. Moreover, in method II, the thin-film gold electrodes prepared as described in section 2.3 were immersed in the gold(III) chloride in toluene before synthesis and the nanostructures were grown in situ on the electrode surfaces. The solutions were heated at 80 °C (in hexane, method I) for 4 h or near the boiling temperature of toluene 110 °C (in toluene, method II) for 45 min, respectively. In method II, ascorbic acid was added to the solution after the solution was cooled to room temperature to facilitate the growth of the nanowires via the oriented attachment mechanism [45]. The nanostructures prepared by method I were centrifuged and washed with hexane several times to remove excess OA.

2.5. Modification of gold electrodes with gold nanostructures

The nanostructures prepared by method I were dropped onto the surface of a thin-film gold electrode (50 µL nanostructure solution in hexane) and left to dry. The procedure was repeated five times and the electrodes were left overnight to allow the nanostructures to adhere. In method II, the thin-film gold electrodes were first immersed in the toluene solution before the nanostructures were grown on the electrode surface during synthesis. Finally, the electrodes were washed with hexane (method I) or toluene (method II) and distilled water in series. Modified electrodes and solutions of nanostructures were stored in a refrigerator at 4 °C. The electrodes prepared by both methods were structurally and electrochemically characterized and method II was selected for the final fabrication and characterization of the enzymatic sensor. The results of the experiments on the structural and electrochemical characterization are presented to emphasize the advantages of the in situ growth of the nanostructures on the electrode surfaces. The characteristics of the glucose biosensors based on the nanostructured electrodes prepared by method I were not advantageous in comparison with the characteristics of the glucose biosensors prepared by method II and therefore were not extensively statistically evaluated. The nanostructured electrode interface prepared by the in situ growth of nanostructures on the electrode surface had a much higher density and stability than the electrodes with adhered

nanostructures prepared by method I (Figure 1 B, C and D). In method II, gold nanowires of 2 to 15 nm diameter and up to several micrometer length are co-produced with gold nanoparticles of about 12 nm diameter, Fig. 1D. The amount of gold nanoparticles is significantly higher than the amount of the nanowires due to the presence of a large number of gold nucleation centers on the gold thin-film substrate. Some NWs were grown on the Au NPs surfaces, however, we didn't separate the effect of NPs and NWs in this study.

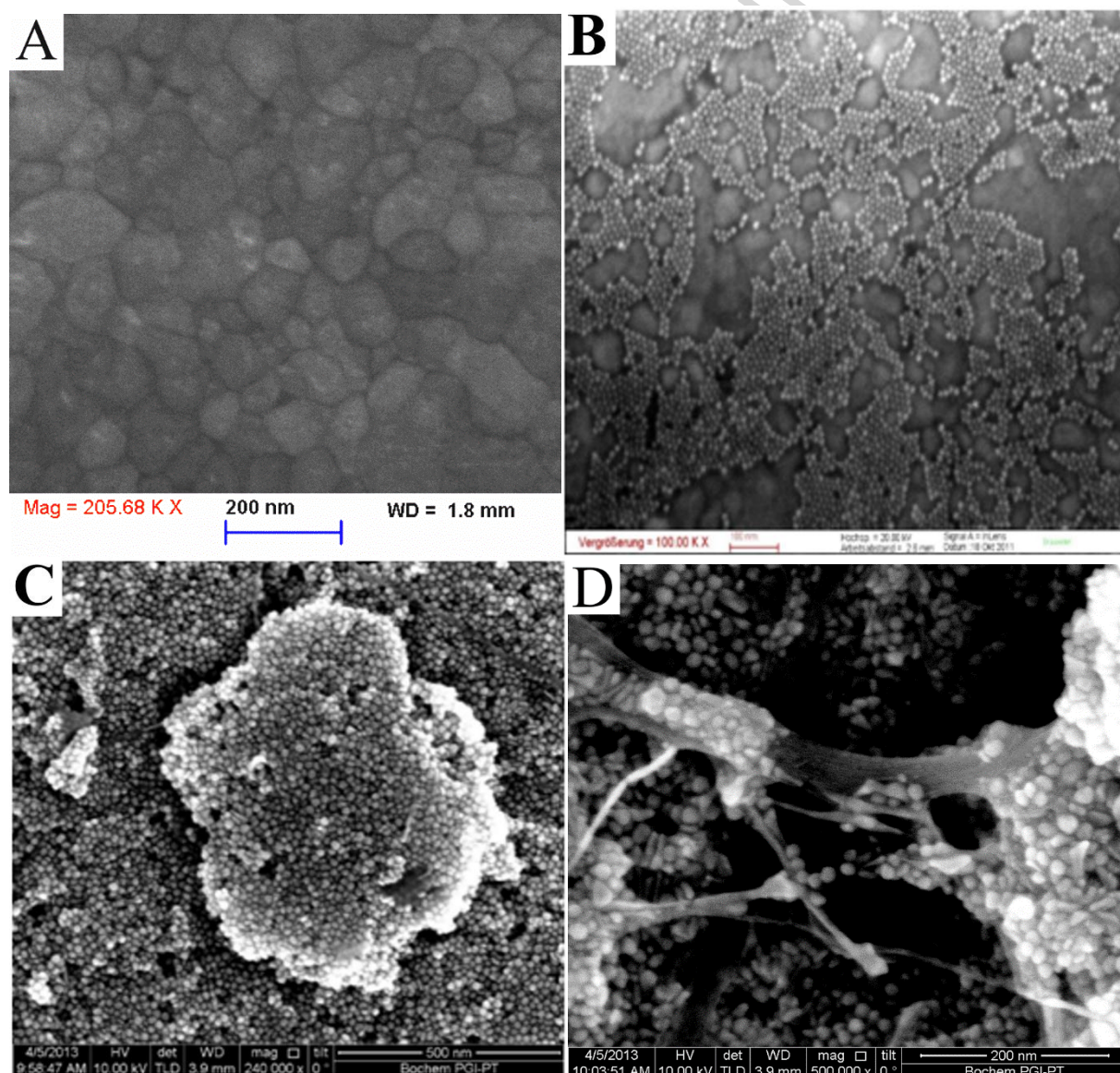


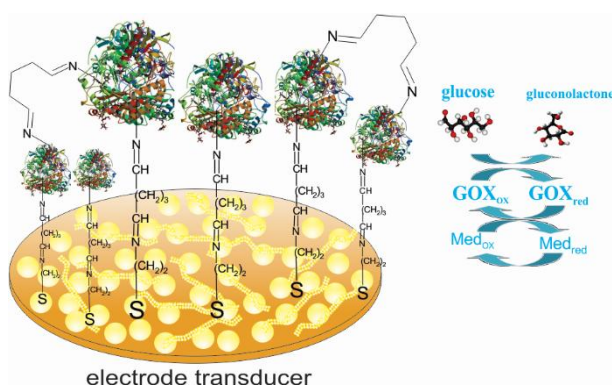
Figure 1. SEM images of a gold electrode surface: (A) planar polycrystalline thin-film gold electrode, (B) gold electrode as shown in (A) modified with gold nanostructures synthesized by method I, (C, D) gold electrode as shown in (A) with high-density gold nanostructures grown in situ using method II, some Au NWs are also co-produced on Au NPs surface.

2.6. Surface treatment with Meerwein's reagent

Meerwein's reagent, triethyloxonium tetrafluoroborate ($[(\text{CH}_3\text{CH}_2)_3\text{O}]\text{BF}_4$), was used as a soft oxidizing agent for oleylamine. 0.1 M Meerwein's salt in acetonitrile was used. Surface treatment was carried out for 10 min. The samples were washed with acetonitrile and water.

2.7. Functionalization of electrodes

Covalent bonding of the enzyme to the electrode surface consisted of three steps. Firstly, the $-\text{NH}_2$ -terminated monolayers of 2-aminoethanethiol (CA) on gold were prepared. Self-assembled monolayers (SAMs) of thiol were deposited on the gold electrodes (modified and unmodified with gold nanostructures) using chemisorption of thiols on gold from 5 mM thiol solutions in ethanol [49]. The process was carried out for 2 h. The electrodes were washed with ethanol and deionized water to remove excess thiol. In a second step, aldehyde carrier was prepared via a reaction with glutaraldehyde. The reaction was carried out in a 2.5 % v/v solution of glutaraldehyde in 0.1 M phosphate buffer at pH 7 at room temperature for about 2 h, followed by thoroughly washing out the excess glutaraldehyde with water. The resulting aldehyde carrier was used for covalent attachment of glucose oxidase. Glucose oxidase was covalently immobilized from 12 mg/ml solution of the enzyme in 0.1 M phosphate buffer at pH 7 with the addition of 0.05% v/v glutaraldehyde at room temperature for about 2 h. The aldehyde groups of glutaraldehyde bound to the amino-groups of cysteamine and glucose oxidase [50]. Some amino-groups of glucose oxidase might be additionally cross-linked with each other by glutaraldehyde during immobilization, however, we used a low concentration of glutaraldehyde in the third step to prevent an excessive cross-linking of the enzyme molecules to each other. For smaller enzymes, like horseradish peroxidase (ca. 44 kDa), we could immobilize the enzyme without adding glutaraldehyde in the second step [51]. However, addition of a low concentration of a cross-linking agent was necessary in our experiments with glucose oxidase. We assume that this can be explained by a larger size of the enzyme (ca. 160 kDa) as well as different distributions of amino-groups on the surfaces of various enzymes. After immobilization the sensors were washed with 0.1 M phosphate buffer solution, pH 7. The sensor was stored at 4 °C with a thin layer of a phosphate buffer. A scheme of the sensor is presented on Scheme 1.



Scheme 1. Assembly of oxidoreductase enzyme glucose oxidase on the nanostructured electrode interface (structures of glucose oxidase molecule was adapted from the Protein Data Bank <http://www.rcsb.org/pdb>).

3. Results and discussion

3.1. Surface modification with Meerwein's reagent

The oleylamine stabilizing shell can be removed with chemical oxidizing agents that destroy the carbon chain of oleylamine. In our previous studies, we used oxygen plasma treatment (200 W, 1–5 min) [51, 52] to remove oleylamine and improve electron transfer between redox proteins and electrode surfaces. Although significant improvement was obtained by this treatment, the electron transfer rate to nanostructured electrode surfaces was slightly lower than for thin-film flat electrodes. However, the amount of immobilized protein increased significantly by using the nanostructures.

In this work, we used an oxidizing Meerwein's reagent to counteract the insulating shell of long hydrocarbon organic molecules on the surface of gold nanostructures in electrochemical measurements and to develop (bio)electrochemical sensors and bioelectronics devices. Recently, Meerwein's reagent was used to treat semiconductor nanoparticles. It was applied as a general reagent for mild reactive stripping of oleate ligands [47, 48]. Meerwein's reagent acts as an oxidizing agent for the oleylamine stabilizing shell according to N-alkylation [53, 54], Figure 2E.

After treatment with Meerwein's reagent, the contrast in the SEM images of the electrode surfaces was improved significantly because the oleylamine layer had been removed [55]. The electrode surfaces before and after treatment with Meerwein's reagent are shown in Figures 1 and 2, respectively. After the treatment, the electrodes were used for electrochemical studies and for further surface functionalization, as discussed below.

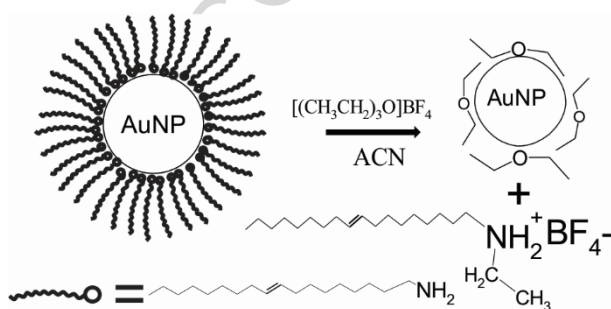
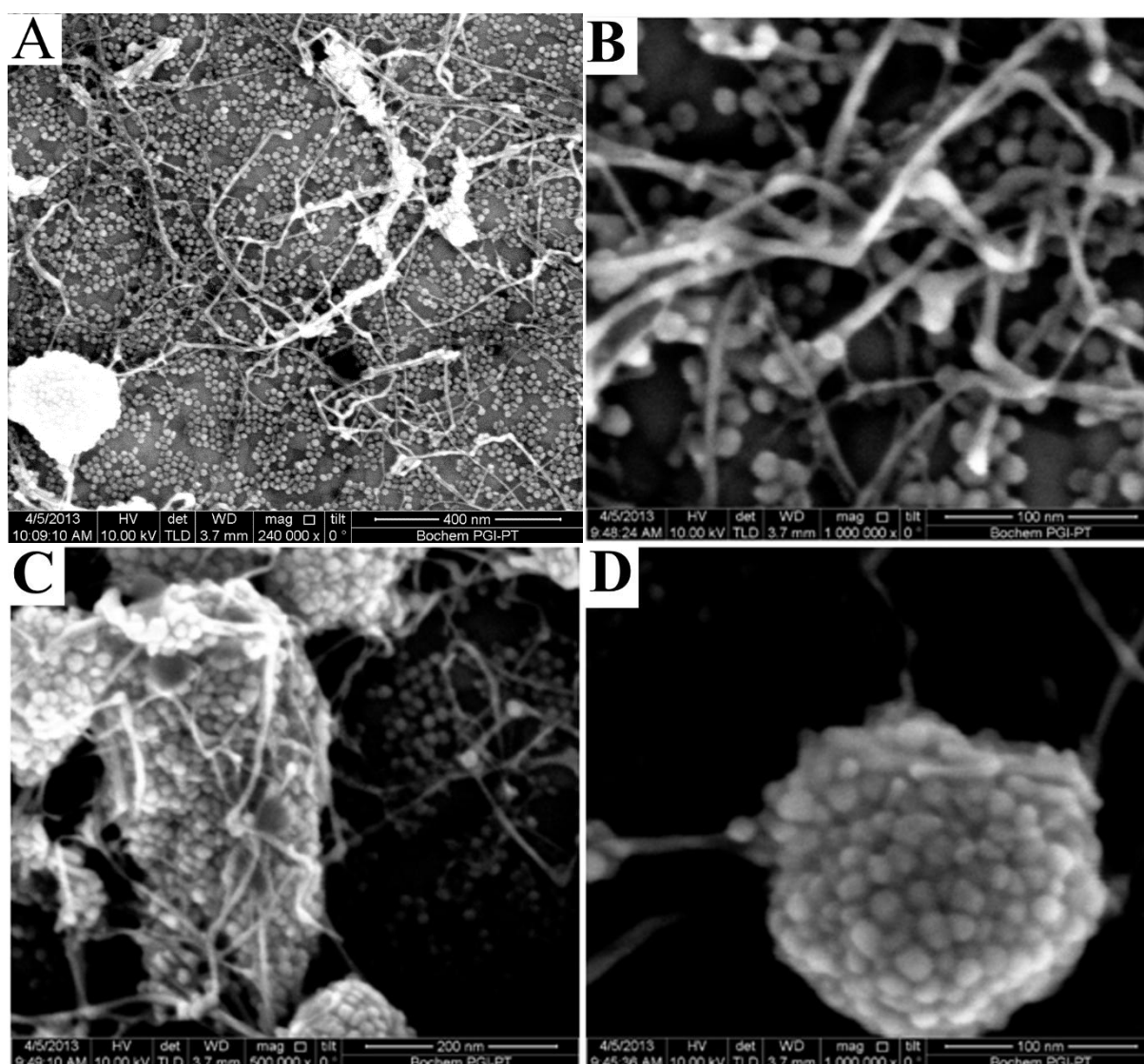


Figure 2. A-D: SEM image of a gold electrode surface with gold nanostructures grown in situ (method II) after treatment with Meerwein's reagent. E: scheme of oleylamine oxidizing reaction on the surface of gold nanostructures with Meerwein's reagent according to N-alkylation [53, 54].

3.2. Determination of the electroactive surface of the electrode

Several electrochemical methods can be used to quantify the electroactive surface of the electrode [56]. They can also be used to verify the possibility of using electrodes modified with nanostructures for the construction of electrochemical sensors. The electroactive surface of electrodes modified with nanostructures is an important parameter, which quantifies an increase in the electroactive surface area of the electrode after modification with nanostructures compared to the flat electrode surface area.

3.2.1. Determination of the electroactive surface of the electrodes using gold oxide cathodic reduction in cyclic voltammetry

To determine the electroactive electrode surface, we used the cyclic voltammograms of the gold electrodes in a potential range of 0–1.5 V (against Ag/AgCl, 3M KCl) [56]. The electroactive surface area of the electrodes was calculated by integrating the gold oxide reduction peak into the cyclic voltammogram using the formula [57]:

$$\Gamma = \frac{A}{v \cdot 482 \mu\text{C cm}^{-2}} \quad (2),$$

where Γ is the electroactive area (cm^2), A is a cathodic peak area in cyclic voltammogram (I-V), v is a scan rate (V s^{-1}), and $482 \mu\text{C cm}^{-2}$ is the charge density per unit area for the electrochemical reduction reaction of a monolayer of chemisorbed oxygen on polycrystalline gold.

The analysis of the experimental data showed that the electroactive area of the gold electrodes with gold nanostructures grown in situ using method II (0.858 cm^2) increased about three times in comparison with the electroactive surface area of the unmodified planar thin-film gold electrode (0.288 cm^2) for the same projected area (area of the electrochemical cell, where the working electrode was in contact with an electrolyte), Figure S1. The electroactive surface area increased about five times (1.423 cm^2) for the gold electrodes with gold nanostructures grown in situ (method II) after treatment with Meerwein's reagent, Figure S1.

Figure S1 shows subsequent scans of a thin-film electrode and thin film electrodes with two kinds of nanostructures (prepared by methods I and II) during electrochemical cycling in sulfuric acid. One can see that the reduction peak of gold monoxide, which is used to evaluate the electroactive surface area of the gold electrode, continuously decreases from initial to subsequent scans of the gold electrode prepared by the drop casting method (method I). This implies that the electroactive area of the electrode diminishes. In contrast, the reduction peaks of gold monoxide in the thin-film electrodes and electrodes prepared by in situ synthesis (method II) remain stable from initial to subsequent scans. The decrease in the electroactive area of the electrode prepared by method I is explained by the fact that the nanostructures prepared by the

drop casting method detach more easily from the surface than nanostructures prepared by in situ synthesis. This observation is supported by the EIS experiments (section 3.2.2).

3.2.2. Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) was applied to determine the properties of the electrode-electrolyte interface of the electrodes modified with nanostructures in comparison with the unmodified gold electrode. Figure 3 shows electrochemical impedance spectra of the unmodified gold electrode, gold electrodes modified with nanostructures prepared by methods I and II, and a gold electrode modified with the same nanostructures after treatment with Meerwein's reagent.

The impedance spectra were analyzed on the basis of the equivalent circuit model shown in Figure 3 B using a Zview 3.2© program (Scribner and Associates Inc.). For modeling interfacial electrochemical reactions in the presence of semi-infinite linear diffusion of electroactive species to the electrodes in the supporting electrolyte Randles circuit, insert in Figure 3, is an adequate model. This model is recommended and widely used to describe and analyze the EIS spectra of similar electrochemical systems [58-66]. The equivalent circuit consists of an active electrolyte resistance, R_s , in series with a parallel combination of the double-layer capacitance, C_{dl} , and the faradaic impedance, which is represented by a serial connection of the charge-transfer resistance, R_{ct} , and the mass-transfer impedance.

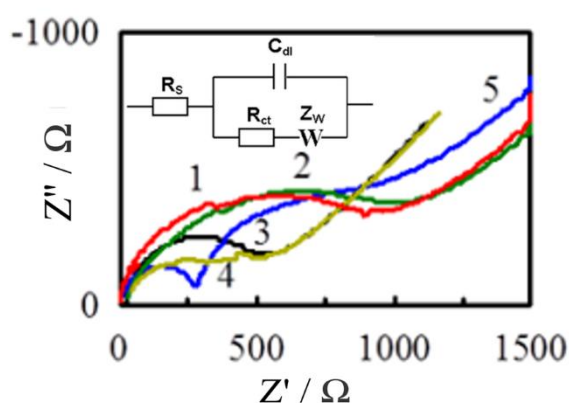


Figure 3. A: Nyquist plot showing the gold working electrode interface for: (1) the electrode prepared by in situ growth of nanostructures using method II, (2) the electrode prepared by method I, (3) the electrode prepared by method II after treatment with Meerwein's reagent, (4) the electrode prepared by method I after treatment with Meerwein's reagent, and (5) the unmodified gold electrode. The measurements were carried out in a solution of 5×10^{-4} M potassium hexacyanoferrate(III) in 0.1 M KNO_3 background electrolyte solution and a frequency

range of 1 Hz – 10^4 Hz, ac voltage of 0.01 V, dc voltage 0.225 V. Insert in Figure 3 shows the equivalent circuit for the analysis of the impedance spectra of the electrochemical cell comprising a working electrode, platinum counter electrode, and a Ag/AgCl (3 M KCl) reference electrode, 5×10^{-4} M $\text{K}_3[\text{Fe}(\text{CN})_6]$ in 0.1 M KNO_3 .

In the Randles circuit, the resistance to the ionic migration current in the aqueous bulk solution of the electrolyte within the frequency range of a typical impedance experiment in EIS is simplified by a small “solution resistance” component R_s [60]. When the electrode is immersed in the electrolyte solution, an electrochemical double layer is formed at a phase boundary between the electrolyte and surface of an electrode with a non-uniform distribution of charges and electrochemical potential gradients [60, 67]. The electrical double layer gives rise to a capacitive element with a double-layer capacitance, C_{dl} , producing a parallel “energy storage” electrical pathway. Therefore, the double-layer capacitance is placed in parallel with the faradaic impedance. A typical electrochemical (faradic) reaction is composed of both the mass-transport processes of redox species to the electrode surface and their discharge at the interface. The faradaic impedance can thus be represented by a series combination of mass transfer impedance, which is given by a specific electrochemical element of diffusion W , referred to as a Warburg element, and the charge-transfer resistance. At the electrode-electrolyte interface, the charge carriers change and there is a transition from ionic to electronic conductance. The transfer of electric charge across the electrode-electrolyte interface, where heterogeneous charge-transfer exchange between the solution ions and electrons in the electrode takes place, is accompanied by electrochemical discharge of the redox species at the interface, which is determined by the electrochemical potential of the electrode. The impedance of the system to the current generated by this discharge process occurring in a nanometer-thick “Helmholtz” layer of the solution immediately adjacent to the electrode is represented by the charge-transfer resistance [60]. The Warburg impedance manifests itself in EIS spectra as the straight line with a slope of 1 (an angle of 45°) in the low-frequency region, which is indicative for a purely diffusion-controlled reaction [64]. The Randles equivalent circuit is one of the most common cell models describing processes at the electrochemical interface [58], although, in some cases, more complicated circuits have been used to describe a multi-layer structure of the double layer at the electrode-electrolyte interface [63]. The EIS spectra shown in Figure 3 were obtained with a dc voltage corresponding to a redox potential of the $\text{Fe}^{3+}/\text{Fe}^{2+}$ couple, since the charge-transfer process should be evaluated. Application of the dc potential influences the electrode polarization and formation of ion layers on the electrode surface [62]. Therefore, the values of the double-layer capacitance

and evaluation of the surface area may deviate from the values of the double-layer capacitance obtained at 0 V dc voltage.

The values of capacitance found using EIS verified an increase of more than two fold in the electroactive surface area for the electrodes modified with gold nanostructures at $5.9 \mu\text{F cm}^{-2}$ (method I, $R_{\text{ct}}=3460 \Omega \text{ cm}^{-2}$) and $6.8 \mu\text{F cm}^{-2}$ (method II, $R_{\text{ct}}=3768 \Omega \text{ cm}^{-2}$) in comparison with the unmodified gold electrode at $2.9 \mu\text{F cm}^{-2}$ ($R_{\text{ct}}=1467 \Omega \text{ cm}^{-2}$) and for the electrodes modified with gold nanostructures prepared by method II (in situ synthesis) after treatment with Meerwein's reagent: $6.6 \mu\text{F/cm}^2$ ($R_{\text{ct}}=1808 \Omega \text{ cm}^{-2}$). Furthermore, the values of the charge-transfer resistance confirmed that the hydrophobic oleylamine shell was destroyed significantly after treatment with Meerwein's reagent. Somewhat larger values of the charge-transfer resistance for the electrodes prepared by method II after treatment with Meerwein's reagent in comparison with a thin-film electrode might be due to some remaining molecules of OA at the interfaces between nanostructures and between nanostructures and the thin-film support. This effect was not apparent, however, in the cyclic voltammogram of the mediator, as will be shown below.

In the case of the electrodes modified with nanostructures prepared by the drop casting method (method I), treating the electrodes with Meerwein's reagent resulted in a capacitance decrease down to $2.8 \mu\text{F cm}^{-2}$ ($R_{\text{ct}}=1994 \Omega \text{ cm}^{-2}$), approaching the values of the thin-film electrodes. We assume that this decrease in the capacitance value is due to the detachment of nanoparticles during Meerwein's reagent treatment. This observation agrees with the results of the electrochemical cycling experiments, where detachment of the nanoparticles was manifested in a decrease in the electroactive surface area (section 3.2.1 and Figure S1). These observations support our conclusion that in situ synthesis produces more stable nanostructured surfaces and is a way of minimizing the detachment of nanostructures from the substrate.

Thus, the electroactive surface area was found to double while the charge transfer resistance decreased at the electrode-electrolyte interface after surface activation using Meerwein's reagent (sections 3.2.1–3.2.2). This shows that Meerwein's reagent significantly improves the electrochemical properties of the nanostructured interface.

3.3. Application of the gold electrode modified with nanostructures for glucose determination

3.3.1. A mediator for glucose determination

The principal criteria for selecting the redox mediator were faster reaction kinetics and peak current value (under constant mediator concentration). Three redox mediators [68-70] were

investigated here: ferrocenemethanol, hydroquinone, and potassium hexacyanoferrate(III). Cyclic voltammograms of these mediators on an electrode prepared by method II are shown in Figure 4 for mediator solutions with a mediator concentration of $5 \cdot 10^{-4}$ M. We selected ferrocenemethanol for further investigation as a mediator because we observed better kinetics of the Fe(II/III) redox reaction (Figure 4).

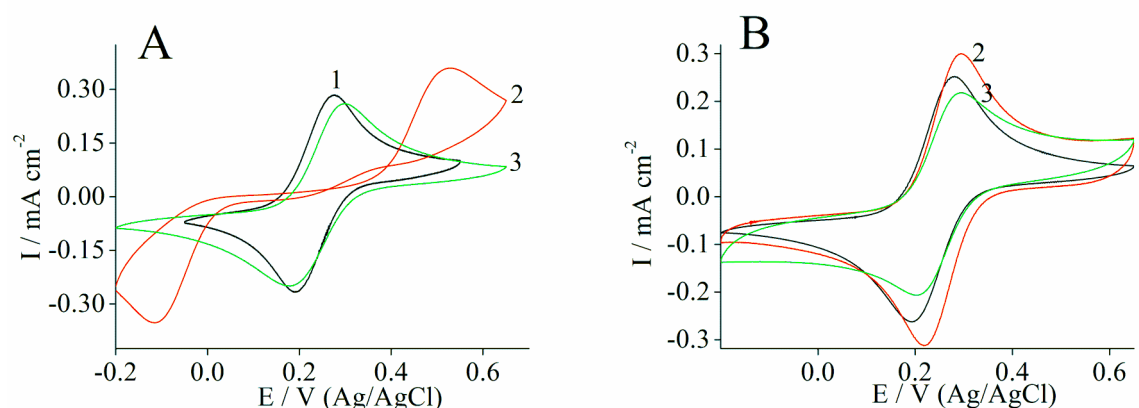


Figure 4. A: Cyclic voltammograms of the redox mediators: (1) ferrocenemethanol, (2) hydroquinone, and (3) potassium hexacyanoferrate(III) on a nanostructured electrode (fabricated by method II). B: Cyclic voltammograms of 5×10^{-4} M potassium hexacyanoferrate(III) solution on three different electrodes: (1) electrode prepared by method II, (2) electrode prepared by method II after Meerwein's reagent, and (3) planar thin-film gold electrode. Other conditions: concentrations of mediators 5×10^{-4} M, 0.1 M background electrolyte KNO_3 , scan rate 50 mV s^{-1} .

As can be seen in Figure 4, cyclic voltammograms of the diffusion-controlled redox mediators showed no significant increase in current density for the nanostructured electrodes in comparison with the planar gold electrode. This is because the distance between the nanostructures was smaller than the diffusion layer thickness (the nanostructures were closely attached to each other; see Figures 1 and 2) and semi-infinite linear diffusion limited the observed current densities, which agrees with previous observations reported [56, 71]. These observations additionally confirm that redox systems with diffusion limitation are unsuitable for evaluating the effective electroactive surface area of electrodes, where surface-confined reactions should be used.

3.3.2. Glucose determination

Covalent immobilization methods allow at least 1000 measurements to be conducted per one electrode, while physical immobilization allows fewer measurements to be made (up to 200 measurements) [72]. As such, covalently immobilized enzyme electrodes have a higher stability and longer lifetime (up to 14 months) in comparison with physically immobilized enzymes on

electrodes. Thus, covalent immobilization (section 2.7) of the enzyme was used in this study, similar to our previous studies on enzyme sensors [49, 51], Scheme 1.

The response of the sensor in glucose test solutions were measured by cyclic voltammetry and the sensor performance was statistically evaluated. Cyclic voltammograms were obtained for unmodified gold electrodes and nanostructured gold electrodes in solution with varying glucose concentrations. Figure 5 demonstrates the dependence of the oxidation currents of a mediator ferrocenemethanol on the concentration of glucose. Electrodes with nanostructures grown in situ after surface activation using Meerwein's reagent achieved the best performance. Such sensors had a detection limit of 0.020 mM and a working range of 0.06–18.5 mM with a sensitivity of $22.6 \pm 0.5 \mu\text{A mM}^{-1}\text{cm}^{-2}$ (measurements were performed at room temperature, $21 \pm 1^\circ\text{C}$), Table 1. The concentration range for commercial blood glucose monitors is 0.5–15 mM.

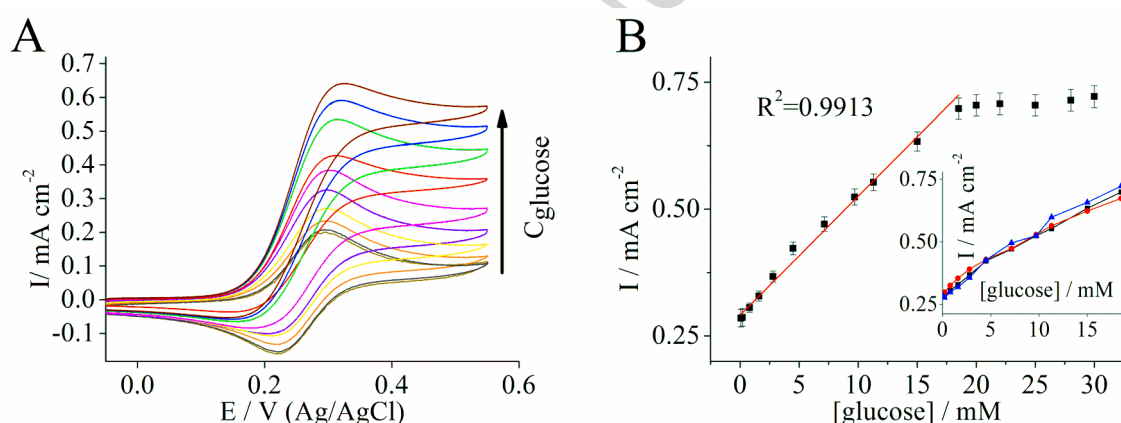


Figure 5. A: Dependence of the sensor response on the concentration of glucose in analyzed solution for the modified electrode (method II) after treatment with Meerwein's reagent (concentration range 0–20 mM), and (B): a calibration curves for the electrode modified with gold nanostructures (method II) after treatment with Meerwein's reagent; the insert in B shows calibration curves for three electrodes modified with gold nanostructures (method II) after treatment with Meerwein's reagent. Other conditions: $5 \cdot 10^{-4}$ M ferrocenemethanol in 0.1 M phosphate buffer solution with pH=7, scan rate 50 mV s^{-1} , $21 \pm 1^\circ\text{C}$.

Table 1. Analytical parameters of glucose biosensors based on enzyme glucose oxidase immobilized on nanostructured electrodes.

biosensor composition	Mediator/ Signal transduction scheme	Linear range, mM	Sensitivity, $\mu\text{A} \cdot \text{mM}^{-1} \text{cm}^{-2}$	LOD, mM	Method	Stability	Ref.
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GOx/GA/cys teamine/AuN S/Au	FcMeOH	0.06 - 18.5	22.6±0.5	0.02	CV	2 months	this work
GOx/AuNPs/ ESM	O ₂ sensor*	0.008-0.96	-	0.0035	A	1.5 months	[73]
GOx/cystea mineAuNPs/ dithiol/Au	FcMeOH	0.02-5.7	8.8±0.22	0.0082	A	1 month	[74]
GOD/chitosa n/AuNPs/PP y/RGO	O ₂ reduction at -0.67 V (SCE)	0.2-1.2	123.8	-	CV	2 weeks	[75]
GOx/AuNPs/ GR	PMS	0.1 - 10	101.02	0.083	A	1 week	[62]
GOx/hPG	H ₂ O ₂ detection at 0.52 V (SCE)	0.05-10	22.7±0.1	0.025	CV	48 h	[25]
GOx/AuNPs/ cysteamine/ Au	Tetrathiaful valene (TTF)	0.01-10	1.02 ± 0.06 **	0.0067	A	28 d	[76]
(AuNPs/GOx /cysteamine) ₆ /Au	FcMeOH	0.01-13	5.72	0.008	A	4 weeks	[66]
[Chit ⁺ (NG+ GOx)/PSS ⁻ /c hit ⁺ (NG+ GOx)] _n /AuQ C	-	0.2-1.8	10.5	0.064	A	2 weeks	[77]
PB/GOx/ MBA/Au	Prussian blue	0-25	0.11	-	A	4 d	[65]

(PEI/MP-11) ₂ /PEI/(GOx + POPG + DPPG) ₁	MP-11	0.125-0.475	0.91	0.0086	A	-	[78]
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Glucose concentration range in human blood: 0.5-15 mM

Symbols: CV, cyclic voltammetry; A, amperometry; RGO, reduced graphene oxide; GR, graphite rod electrode; hPG, highly porous gold; NS, nanostructures; ESM, eggshell membrane; NG, Nitrogen-doped graphene; AuQC, Au quartz crystal; chit, chitosan; PB, Prussian blue; MBA, 4-mercaptobenzoic acid; MP-11, microperoxidase-11; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol; DPPG, 1,2-dipalmitoyl-phosphatidylglycerol; FeMeOH, ferrocenemethanol; PMS, N-methylphenazonium methyl sulphate; MP-11, microperoxidase-11; *, monitoring O₂ consumption by the O₂ sensor (a Clark-type electrode) at a working voltage of 0.70 V; **, the data are given in A·mM⁻¹ [76].

The selectivity of these sensors is realized by the selectivity of the enzymatic reaction of glucose oxidation catalyzed by the enzyme. The effect of electroactive species such as ascorbic acid, ethanol, and uric acid on the analytical signal of the biosensor was investigated (Table 2). Increase of the sensor signal in the presence of ascorbic acid has been observed, which is probably, explained by the reduction of a mediator by this substance and due to the direct effect of this electroactive compound. The pH working range for these sensors depends on the pH activity range of glucose oxidase with a broad pH range of 4–7, pH_{optimum} 5.5 [79] and buffer capacity (change in the pH of the solution in the electrochemical cell after addition of 0.5 mL 0.1 M; solution of glucose was 0.0083 molar equivalent L⁻¹).

Table 2. The effect of electroactive species on the response of the proposed GOx-biosensor, concentration of glucose in the tested solutions was 1.5 mM.

Interferent	% of sensor signal without interfering substance (n=3)
	GOx-AuNS
Uric acid, 2.5 mM	98
Ethanol, 0.1 v/v %	101
Ascorbic acid, 2.5 mM	111

The sensors had a high reproducibility and long lifetime. The reproducibility of the sensors was evaluated by measuring the sensor response in 500 μM glucose solution. The relative standard deviation of six successive measurements was about 3%. The stability of the sensors was evaluated by measuring the sensor response to the test solutions of glucose during a period of 60 days (storage at 4 $^{\circ}\text{C}$). Figure 6 shows the sensitivity of the biosensors in a concentration range 0.06–18.5 mM. The biosensors based on in situ synthesis of gold nanostructure and Meerwein's reagent treatment retained about 93% of their initial biocatalytic response in the concentration ranges 0.06–18.5 mM, respectively, thus demonstrating a high stability. A remarkably enhanced stability and reproducibility of the sensor was achieved by a new approach of in situ synthesis of gold nanostructures directly on the electrode surface. This significantly enhanced the stability and reproducibility of the nanostructured electrode interface and minimized the detachment of nanostructures from the electrode surface, while covalent immobilization of the enzyme on the nanostructured electrode surface contributed to a higher stability of the enzyme layer. At the same time, surface activation with Meerwein's salt proved indispensable in achieving an efficient electrochemical interface.

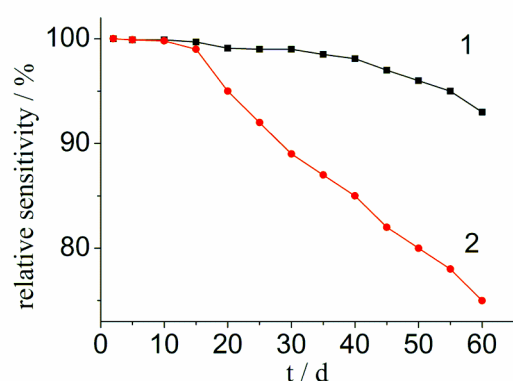


Figure 6. Sensitivity of a glucose oxidase biosensor in a concentration range 0.06–18.5 mM as a function of time: (1) - biosensor based on in situ synthesis of gold nanostructure and Meerweins reagent treatment, and (2) - biosensor based on a thin film gold electrode. Enzyme electrodes were stored at 4 $^{\circ}\text{C}$ with a thin layer of a phosphate buffer. Relative sensitivity was calculated as the ratio of sensitivity at each point to the initial sensitivity of the sensor.

3.4. The apparent Michaelis constant

We used the apparent Michaelis constant to characterize the catalytic activity of the enzyme on the nanostructured electrode surfaces. The constant was calculated from the dependence of the inverse value of the ferrocenemethanol oxidation current on the inverse value of glucose concentration in the analyzed solution [69] in the linear range using the equation (3):

$$\frac{1}{I_{ss}} = \frac{K_m^{app}}{I_{max}} \frac{1}{C} + \frac{1}{I_{max}} \quad (3),$$

where I_{ss} is the steady-state current for the corresponding glucose concentration, I_{max} is the maximum current under the saturated concentration of glucose, C is the max glucose concentration in the analyzed solution, and K_m^{app} is the apparent Michaelis constant.

Glucose oxidase is specific for β -D-glucose with a K_m of 33 – 110 mM for glucose oxidase purified from *Aspergillus niger* [80, 81]. Free enzymes generally exhibit 10 – 1000 times higher volumetric activity ($U\ g^{-1}$) than the immobilized enzyme [31]. Many experimental data have shown that the relationship between K_m^{app} and K_m^{free} varies under various conditions of immobilized enzyme reaction [82]. The apparent Michaelis constants for a number of electrodes modified with glucose oxidase are also given in the literature: 4.3 mM [74], 6.7 mM [69], 10.5 mM [83], 14.9 mM [84], 20 mM [85], and 6.3 mM [25]. The apparent Michaelis constant for the nanostructured electrodes synthesized in situ with surface activation by Meerwein's reagent was found to be 10.5 mM. This value is comparable with the values of the apparent Michaelis constant for other glucose biosensors as shown above. Thus, glucose oxidase in this case preserved its affinity and enzymatic activity for glucose after immobilization on the electrode.

3.5. Determination of glucose in a human blood serum sample.

To illustrate the feasibility of the developed electrochemical biosensor in practical analysis, it was employed to determine glucose level in human blood serum samples using a spike procedure [86]. Serum was diluted ten times with a 0.1 M phosphate buffer, pH=7, containing $5 \cdot 10^{-4}$ M ferrocenemethanol. Known amounts of glucose were added to the serum samples and the concentration of glucose in the spiked samples was determined, Table 3. The recovery results demonstrate that the developed glucose electrochemical biosensor can be used for the selective determination of glucose level in real samples.

Table 3. Recovery of glucose in a human blood serum samples using electrochemical enzymatic sensor based on in situ synthesis of gold nanostructures and Meerwein's reagent surface activation.

Glucose content in a diluted human blood serum sample, mM, *	Glucose concentration added, mM	Glucose concentration determined by the biosensor, mM	Recovery, %, **
0.64	0.5	1.13	98
0.64	3	3.52	96
0.64	6	6.58	99
0.64	12	12.45	98

* - determined by the spectrophotometric method in hospital; ** - three replicates were performed.

4. Conclusions

In situ synthesis of gold nanostructures on the electrode surface was proposed for the development of a bioelectrochemical sensor platform. A remarkably enhanced stability and reproducibility of the biosensor was achieved using this method, minimizing detachment of nanostructures from the electrode surface. Treatment with Meerwein's reagent was introduced as an effective method of activating surfaces modified with gold nanostructures prepared using the oleylamine method for electrochemical studies. These nanostructures can be used for electrochemical measurements in aqueous solutions. The oxidoreductase enzyme glucose oxidase retained a high electrocatalytic activity after immobilizing the enzyme on the proposed interface, making this approach prospective for protein immobilization and biosensor development. A new biosensor for glucose determination demonstrated the following sensor characteristics: wide working range 0.06–18.5 mM with a sensitivity of $22.6 \pm 0.5 \mu\text{A mM}^{-1}\text{cm}^{-2}$, limit of detection 0.02 mM, high reproducibility, and long lifetime. The proposed method is therefore an attractive route for designing biosensors, enzymatic bioreactors, and biofuel cells.

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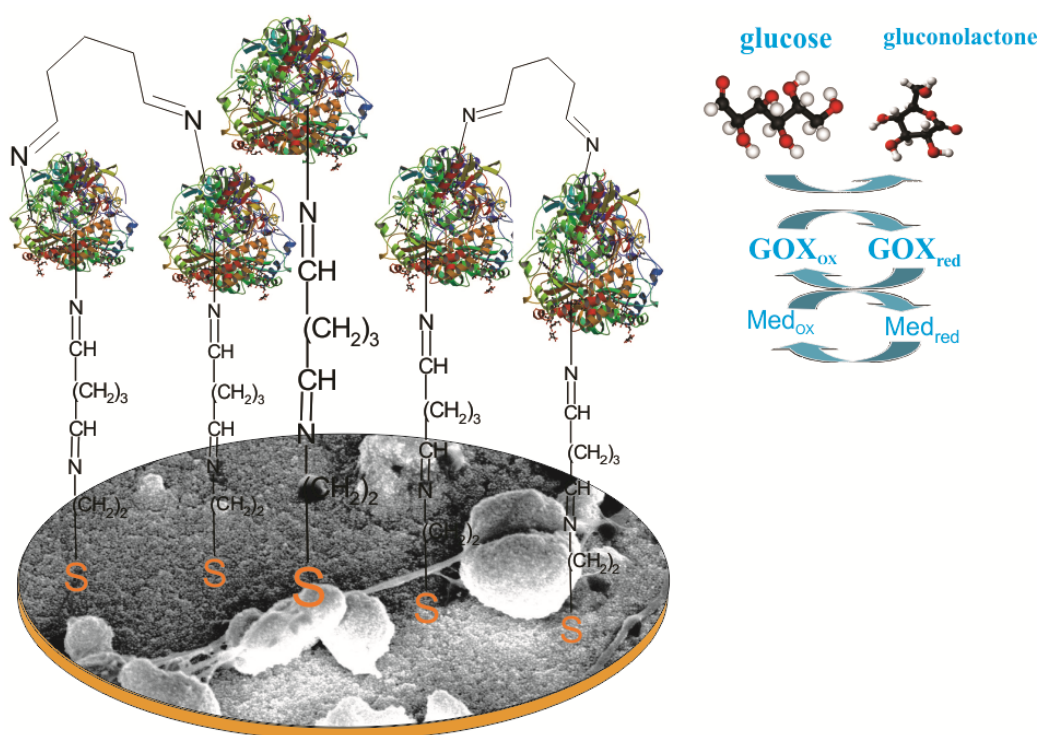
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Graphical abstract

Highlights

- In situ synthesis of gold nanostructures on electrode surfaces.
- Surface activation with Meerwein's salt for an efficient electrochemical interface.
- A new enzyme biosensor for glucose with a limit of detection of 0.02 mM.
- Working range 0.06–18.5 mM with a sensitivity of $22.6 \pm 0.5 \mu\text{A mM}^{-1}\text{cm}^{-2}$.
- Apparent Michaelis constant is 10.5 mM.