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Highly Sensitive Amperometric Biosensors Based on Oxidases and CeCu Nanoparticles Coupled with Porous Gold †

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Abstract: Metallic nanoparticles are usually applied in biosensors as catalysts and/or mediators of electron transfer. We describe the development of amperometric biosensors (ABSs) based on oxidases and nanoparticles of CuCe (nCuCe). nCuCe being an electro-active mediator and active peroxidase (PO) mimetic was used as a H_2O_2-sensing platform in oxidase-based ABSs. ABSs on glucose, primary alcohols, methyl amine, catechol and L-arginine, which are based on corresponding oxidases and nCuCe, were developed. These ABSs exhibit improved analytical characteristics in comparison with the appropriate bi-enzyme ABSs, containing natural PO. Including electrodeposited porous gold in chemo-sensing layer was shown to increase significantly the sensitivities of all constructed ABSs.

Keywords: electroactive nanoparticles; peroxidase-like nanozyme; oxidases; porous gold; amperometric biosensors

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1. Introduction

Metallic nanoparticles potentially have wide practical applications in various fields of science and industry. In biosensorics they usually act as mediators in electron transfer and/or catalysts (nanozymes, NZ) [1–5].

NZs are the newest class of functional nanomaterials [3–7] that have enzyme-like activity with different reaction specificities. NZs possess increased stability and greater availability due to their simpler preparation technologies. Most of reported NZs is mainly mimetics of oxidoreductases, including peroxidase (PO) [7–9].

PO catalyzes the oxidation of diverse organic compounds using H_2O_2 as the electron acceptor [8]. Many natural enzymes (oxidases) produce H_2O_2 as a byproduct of their enzymatic reaction, so the detection of the target substrate can be performed by measuring of H_2O_2 generation. Last years, a lot of reportes described the application of various PO-like NZs for H_2O_2 detection using different sensors [10–14]. The main peculiarities of PO-like NZs as catalysts are high stability, sensitivity and selectivity to H_2O_2 in extra-wide linear ranges. PO-like NZs coupled with natural oxidases are widely used in amperometric oxidase-based biosensors (ABSs) [1–3,7–10].

In our earlier works, different types of chemically and "green" synthesized PO-like NZs were described [9,10]. Nanoparticles of CuCe (nCuCe) were chosen as the effective

electroactive PO-mimetics and were characterized by Scanning electron microscopy (SEM) coupled with X-ray microanalysis (SEM-XRM) [9,15]. Our results demonstrated that the synthesized nCuCe, having an excellent sensitivity and a wide linear range for H_2O_2 detection, may be promising artificial POs for the development of oxidase-based ABSs. nCuCe were successfully used for construction of Arg-sensitive ArgO-based ABSs [15].

A lot of approaches were proposed for improving analytical characteristics of ABSs. One of them is increasing effective working surface of the electrode in order to obtain the maximal electroactive sites for immobilization of biocatalysts, including enzymes and NZs [7,8].

Micro/nanoporous gold (npAu), because of its high surface area-to-volume ratio, excellent conductivity, chemical stability, high area, electrochemical activity, easily tunable pores, and plasmonic properties, may be promising in medicine for diagnostics and drug delivery, in energy storage, in sensing and biosensing. A lot of synthetic methods for npAu obtaining was reported, including deallying, templating, sputtering, self-assembling and electrodeposition [16,17]. The last method is the most popular.
chemical inertness, physical stability, biocompatibility has attracted much interested in

The aim of the current study was to fabricate and to characterize highly sensitive ABSs using various oxidases as biorecognition elements, the nCuCe as an electroactive mimetic of PO and the electrodeposited npAu as an effective carrier of enzymes/NZs with a highly advanced surface area [16,17].

2. Materials and Methods

2.1. Reagents

Cerium(IV) bicarbonate($Ce(HCO_3)_4$), copper(II) sulfate ($CuSO_4$), L-arginine (Arg), methylamine (MA), ethanol, methanol, *o*-dianisidine, hydrogen peroxide (H_2O_2, 30%), hydrogen tetrachloroaurumate(III) $H[AuCl_4]$, D-glucose, sodium sulfide (Na_2S), ammonia chloride (NH_4Cl), Nafion (5% solution in 90% low-chain aliphatic alcohols), Horse radish peroxidase (PO, EC 1.11.1.7) from *Armoracia rusticana* (500 $U \cdot g^{-1}$), glucose oxidase (GO, EC 1.1.3.4) from *Aspergillus niger* (168 $U \cdot mg^{-1}$), and all other reagents and solvents used in this work were purchased from Sigma-Aldrich (Steinheim, Germany).

All reagents were of analytical grade and were used without further purification. All solutions were prepared using ultra-pure water obtained with the Milli-Q® IQ 7000 Water Purification system (Merck KGaA, Darmstadt, Germany).

2.2. Enzymes Isolation and Purification

Electrophoretically homogeneous yeast enzymes—alcohol oxidase (AO, EC1.1.3.13), L-arginine oxidase (ArgO, EC 1.4.3.25), methylamine oxidase (AMO, EC 1.4.3.21), laccase (EC 1.10.3.2) were used for amperometric biosensors fabrication.

Yeast AO was isolated from cell-free extract of the selected over-producing strain *Ogataea polymorpha C-105 (gcr1 catX)* by a two-step ammonium sulfate fractionation (at 30 and 70% of saturation) followed by ion exchange chromatography on DEAE-Toyopearl 650 M [18]. Purified AO with specific activity ~20 $U \cdot mg^{-1}$ of protein was kept as suspension in 70% sulfate ammonium, 50 mM phosphate buffer (PB) pH 7.5 at 4 °C.

Fungal ArgO was isolated from an extract of the fruiting body of the wild forest mushroom *Amanita phalloides* and partially purified up to ~7.9 $U \cdot g^{-1}$ of protein by a two-step ammonium sulfate fractionation (at double 70% of saturation), followed by ion exchange chromatography on Toyopearl DEAE-650M resin [15].

Activities of AO, ArgO or GO were determined by the rate of hydrogen peroxide formation in reaction with substrate (methanol, Arg or glucose) as monitored by the peroxidative oxidation of *o*-dianisidine in the presence of PO and correspondent substrates methanol [18], Arg [15] or glucose [10].

Yeast AMO was isolated from the recombinant yeast strain *Saccharomyces cerevisiae* C13ABYS86 [19]. The $(His)_6$-tagged AMO was purified from the cell-free extract by metal-affinity chromatography on Ni-NTA-agarose. Activity of AMO was determined by the rate of hydrogen peroxide formation in reaction with MA as monitored by the peroxidative oxidation of 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) in the presence of PO.

Fungal laccase was isolated from a cultural liquid of the fungus *Trametes zonata* by a two-step ammonium sulfate fractionation (up to 70% of saturation), followed by ion exchange chromatography on Toyopearl DEAE-650M [20]. Fractions with the laccase activity were pooled, concentrated by Millipore filter (10 kDa) up to specific activity of enzyme ≥10 $U \cdot mg^{-1}$ followed by precipitation with 80% sulfate ammonium.

The activity of laccase was determined by the rate of the increase in absorbance monitored spectrophotometrically at 420 nm (Shimadzu, Japan). As a substrate, 0.5 mM ABTS in 50 mM sodium acetate (NaOAc) buffer solution, pH 4.5 was used. One unit of laccase activity was defined as the amount of the enzyme required to oxidize 1 μmole of a substrate (ε_{420} = 36 $mM^{-1} \cdot cm^{-1}$) per minute at 24 °C.

2.3. Synthesis of Nanoparticles and Estimation of Their Pseudo-Peroxidase Activity

Nanoparticles of CuCe (further—nCuCe) were synthesized as described earlier [9]**.** The synthesized nCuCe were collected with centrifugation. The precipitates were rinsed twice with water and were stored as a water suspension at +4 °C until used.

Pseudo-peroxidase (PO-like) activity of the nCuCe was measured by the colorimetric method, with *o*-dianisidine as a chromogenic substrate in the presence of H_2O_2 [9]. One unit (U) of PO-like activity was defined as the amount of nCuCe releasing 1 μmol H_2O_2 per 1 min at 30°C under standard assay conditions.

2.4. Apparatus

A piece of Pt wire and an Ag/AgCI/3M KCI electrode were used as the counter and reference electrodes. 3.05 mm graphite rods (type RW001, Ringsdorff Werke, Bonn, Germany) were used as working electrodes. They were sealed in glass tubes with epoxy forming disk electrodes. Before sensor preparation the graphite electrode (GE) was polished with emery paper. Amperometric measurements were carried out with a potentiostat CHI 1200A (IJ Cambria Scientific, Burry Port, UK) in batch mode under continuous stirring in a standard 40 mL cell at a room temperature.

A SEM-microanalyser REMMA-102-02 (Lviv, Ukraine) was used for morphological analyses of the synthesized nAu-film (nAu).

2.5. Electrodeposition of Nanoporous Gold onto Graphite Electrode

A micro/nanoporous gold (npAu) was synthesized on the surface of GE in two stages. At the first stage, nAu was electrodeposited from the solution containing of 10 mM of $HAuCl_4$ in 2.5 M ammonia chloride using cyclic voltammetry in the range from 0 to +800 mV with scan rate 50 $mV \cdot min^{-1}$ during 25 cycles. On the second stages, the obtained modified electrode (npAu/GE) was re-immersed in the solution of 10 mM of $HAuCl_4$ in 2.5 M ammonia chloride using potentiostatic mode at −1000 mV at 120 s. The obtained npAu/GE was rinsed with water and was equilibrated before usage in the appropriate buffer.

2.6. Immobilization of Natural and Artificial Peroxidases onto Electrode

Natural PO and the synthesized nCuCe as artificial PO were immobilized on the surfaces of GE or npAu/GE using the physical adsorption method. For development of the nCuCe/npAu/GE, an aliquot of nCuCe solution (5–10 μL) with PO-like activity of 1 U/mL was dropped onto the surface of npAu/GE. For development of the PO/GE, an aliquot of PO solution (5–10 μL) with activity of 1 U/mL was dropped onto the surface of

GE. After drying sensing film during 10 min at room temperature, the modified GE was covered with 10 μL of 1% Nafion solution in 50 mM PB, pH7.5. The modified electrodes were rinsed with 50 mM PB, pH 7.5, and kept in this buffer with 0.1 mM EDTA at 4°C until used.

2.7. Immobilization of Oxidases onto the Modified Electrodes

To fabricate the oxidase-based amperometric biosensors (ABS), GO, AMO, AO, ArgO or laccase were immobilized onto the modified GE.

5–10 μL of enzyme solution were dropped onto the dried surfaces of the PO/GE, nCuCe/GE or nCuCe/npAu/GE. To develope ABSs on the base of ArgO, GO, MAO or laccase, the dried composites were covered by Nafion membrane as described in 2.6. To prepare 1% Nafion solution from the the stock 5% solution, the last one was diluted with appropriate buffer: with 50 mM NaOAc, pH4.5 for construction of laccase-based ABS and with 50 mM PB, pH 7.5 in other cases.

It is worth mentioning, that in the AO-based ABS, the biosensing film on electrode was fixed not with Nafion, but with dialysis membrane.

The coated bioelectrodes were rinsed with water and stored in the correspondent buffers until used.

2.8. Measurements and Calculations

Amperometric measurements were carried out using a potentiostat CHI 1200A potentiostat (IJ Cambria Scientific, Burry Port, UK) connected to a personal computer, performed in a batch mode under continuous stirring in an electrochemical cell with a 20 mL volume at 25 °C.

All experiments were carried out in triplicate trials. Analytical characteristics of the proposed electrodes were statistically processed using the OriginPro 8.5 software. Error bars represent the standard error derived from three independent measurements. Calculation of the apparent Michaelis–Menten constants (K_M^{app}) was performed automatically by this program according to the Lineweaver–Burk equation.

3. Results and Discussion

3.1. Development of Oxidase-Based Biosensors Using nCuCe and Porous Gold

We describe here the development of amperometric biosensors (ABSs) based on oxidases and nCuCe. nCuCe, being an active PO mimetic, was used here as a hydrogen peroxide sensing platform for oxidase-based ABSs.

To improve analytical characteristics of ABSs, namely, sensitivity, we have modified the surface of graphite electrode (GE) with micro/nanoporous gold (npAu).

npAu due has a high surface area-to-volume ratio, excellent conductivity, chemical stability, high area, electrochemical activity, easily tunable pores, and plasmonic properties, thus, it may be promising in sensing and biosensing.

npAu has unique properties of chemical stability, high area and electrochemical activity, thus it may be promising in biosensing. The principal scheme of bioelectrode construction is presented in Figure 1.

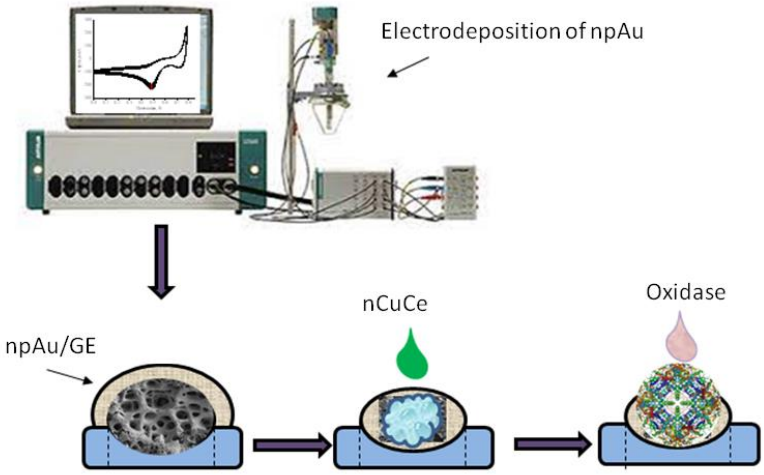

Figure 1. Scheme of electrode's modification.

Figure 2 demonstrates the results of morphological characterization of npAu using SEM technique, which provides information on the size, distribution, and shape of the tested npAu, the XRM images of showed the characteristic peaks for goal metal.

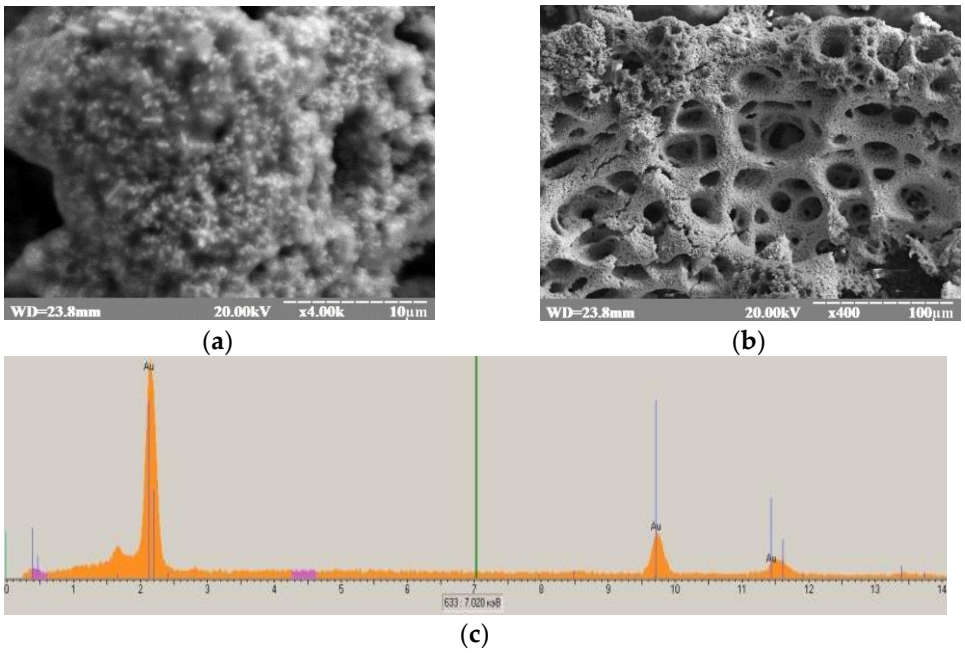

Figure 2. Characteristics of the npAu (**a**–**e**): SEM images (**a**,**b**); X-ray spectral microanalysis.

3.2. Analytical Characteristics of the Constructed Biosensors

Using GO, AO, AMO, ArgO as biorecognition elements, nCuCe as PO-like NZ or as electro-active mediator and npAu as a carrier of enzymes/NZs, the ABSs on glucose, primary alcohols, methyl amine, L-arginine and catechol, respectively, were constructed and characterized.

Figure 3 demonstrates amperometric characteristics of the developed GO-based ABS for glucose determination. Using the chronoamperograms at optimal working potentials for the modified and control electorodes, calibration curves were plotted for analytes determination by the developed ABSs. The same experiments were carried out with other oxidase-based ABSs (data not shown). It is worth mentioning that the npAu/GE as

a control electrode was also tested, and no amperometric signals were detected with any analyte addition under the chosen conditions (data not shown).

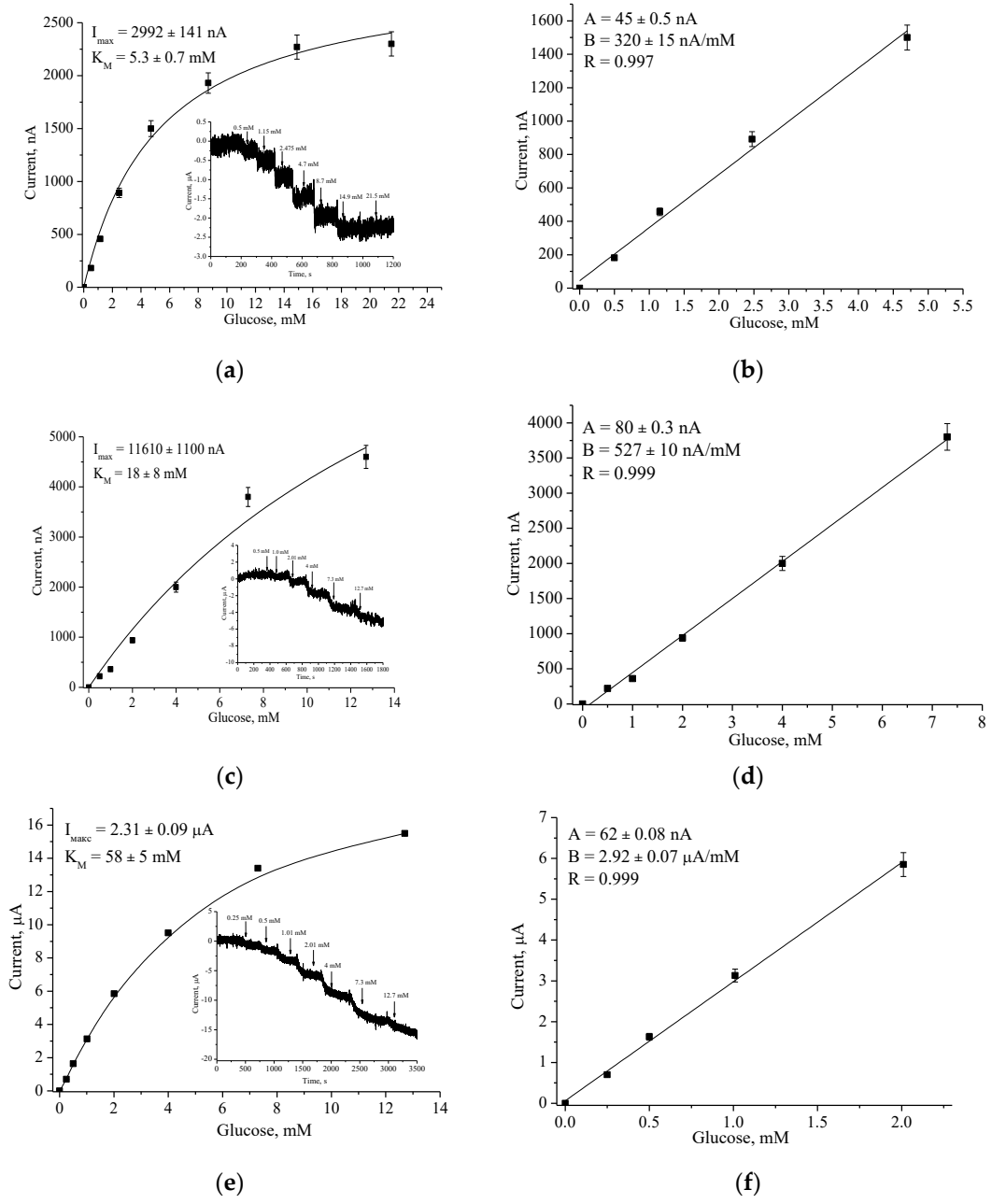

Figure 2. Amperometric characteristics of the GO/PO/GE (**a**,**b**), the GO/nCuCe/GE (**c**,**d**) and the GO/nCuCe/npAu/GE (**e**,**f**): (**a**,**c**,**e**)—chronoamperogramms (inserted) and dependences of amperometric signal on concentration of glucose; (**b**,**d**,**f**)—calibration graphs for glucose determination. Conditions: working potential –50 mV vs Ag/AgCl/3 M KCl in 50 mM PB, pH 6.0. The sensing layers contain 0.01 U of PO/PO-like activity and 0.01 U of GO.

Table 1 summarizes the main bioanalytical characteristics for the developed ABSs, which are based on the usage of various oxidases and nanomaterials. It is worth mentioning, that nCuCe plays a dual role in the developed ABSs: for laccase it is a mediator of electron transfer, for other oxidases it is an artificial PO.

Table 1. Analytical characteristics of the constructed bioelectrodes based on different oxidases, natural or artificial peroxidases and npAu.

Bioelectrode	Potential, mV	Sensitivity, $A \cdot M^{-1} \cdot m^{-2}$	Linear range, μM	LOD, μM
GO/PO/GE	–50	44	50–5000	150
GO/nCuCe/GE	–50	73	500–7300	150
GO/nCuCe /npAu/GE	–50	400	25–2000	75.7
AMO/PO/GE	–250	7	200–1700	130
AMO/nCuCe/GE	–250	35	60–1700	18
AMO/nCuCe/npAu/GE	–250	125	60–500	18
AO/PO/GE	–50	22	130–900	39
AO/nCuCe/GE	–50	32	50–2100	15
AO/nCuCe /npAu/GE	–50	102	33–500	10
ArgO/PO/GE	–150	24	75–1150	35
ArgO/nCuCe/GE	–150	113	50–2250	15
ArgO /nCeCu/npAu/GE	–150	200	100–500	33
Laccase/GE	+200	2300	8–160	2
Laccase/nCuCe/GE	+200	5055	3–40	1.5
Laccase/nCuCe/npAu/GE	+200	9280	2–40	1

As it can be seen from the Table 1, nCuCe has a significant positive effect on sensor sensitivity in comparison with electrodes, which are not modified with nanomaterials. For example, for the AMO/nCuCe/GE and for the ArgO/nCuCe/GE, sensitivities were 5-fold higher, than for corresponding GEs without nCuCe.

The presence of npAu was shown to bring additional contribution in improving analytical parameters of the ABS, especially, in their sensitivities. For example, the sensitivity of the GO/nCuCe/npAu/GE is 9.1-fold higher, than of the GO/PO/GE and 5.5-fold higher in comparison with the GO/nCuCe/GE. The same tendency, but of various levels, was demonstrated for all investigated enzymes. This fact has simple explanation: a highly advanced surface of the npAu having hierarchical pores of nano- and micro-sizes with different diameters, has the enhanced working 3D surface area of electrode. The increased surface of the modified GE leads to the enhanced adsorbtion of nanomaterials/enzymes and, so, to improved efficiency of electron transfer in ABS, in comparison with unmodified GEs.

4. Conclusions

In the present work the development of ABSs based on different oxidases and nCuCe was described. nCuCe has a dual role being an active mimetic of PO and a mediator of electron transfer. It was used as an electro-active mediator for laccase-based ABS and as a PO-like NZ in ABSs, based on other oxidases, namely, GO, AO, AMO and ArgO. The ABSs on catechol, glucose, primary alcohols, methyl amine and L-arginine respectively, were constructed and characterized. The developed mono-enzyme ABSs exhibited improved analytical characteristics in comparison with the correspondent bi-enzyme ABSs, which contained natural PO. It was demonstrated, that including electrodeposited nanoporous gold in chemosensing layer on graphite electrode allows to a significant additional increase in ABSs sensitivity. This fact may be explained by a highly advanced surface area of npAu due to pores of nano- and micro-sizes. Such hierarchical porous 3D surface leads to the enhanced adsorbtion of nanomaterials/enzymes and, so, to improved efficiency of electron transfer in ABS, in comparison with unmodified GEs.

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