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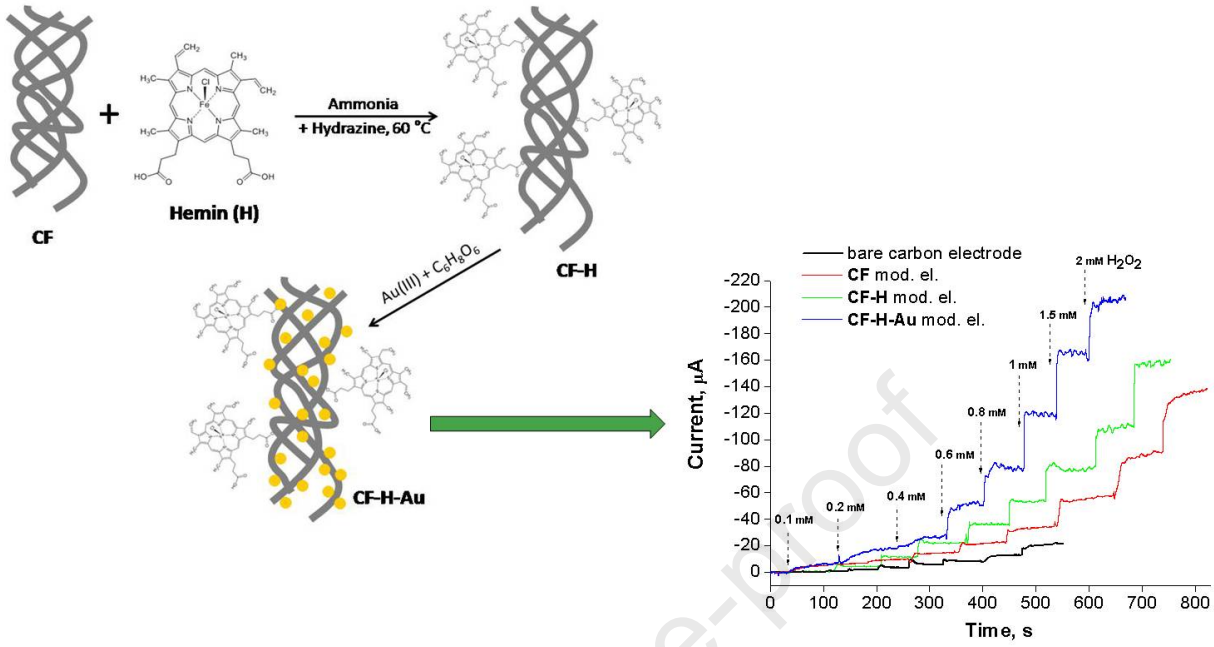

New micro/nanocomposite with peroxidase-like activity in construction of oxidases-based amperometric biosensors for ethanol and glucose analysis

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Abstract

Development of artificial enzymes, including nanozymes as an alternative for non-stable and expensive natural enzymes, is a booming field of modern Biosensorics and Biofuel Technology.

In this study, we describe fabrication and characterization of sensitive biosensors for the detection of ethanol and glucose based on new micro/nanocomposite electrodes with peroxidase-like activity (nanozyme) coupled with microbial oxidases: alcohol oxidase (AOX) and glucose oxidase (GOX). The nanozyme was synthesized by modification of carbon microfibers (CF) by hemin (H) and gold (Au) nanoparticles. The formation of gold nanoparticles on the surface of hemin-modified carbon microfibers has been confirmed by the UV-Vis and X-ray spectroscopy as well by the SEM analysis. Compared to hemin-only modified electrodes, the resulting micro/nanocomposite CF-H-Au electrodes exhibit a higher specific catalytic activity and a better affinity for H_2O_2 in solution. The H_2O_2-sensitive CF-H-Au-modified electrodes showed a higher sensitivity (1.3 – 2.6-fold) compared with the nearest carbon-derived analogs and were used for

the construction of highly sensitive ethanol and glucose biosensors. To eliminate diffusion limitation for substrates, AOX or GOX were fixed on the CF-H-Au-modified electrodes using a highly porous Nafion membrane. The main biosensors' characteristics have been investigated.

The developed biosensors were tested for ethanol and glucose analysis in the real samples of both grape must and wine. The results are in good agreement with the results obtained using enzymatic kits as reference approaches.

Keywords: Micro/nanocomposite; Peroxidase-like nanozyme; Amperometric biosensor; Ethanol; Glucose; Analysis.

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1. Introduction

Enzymes are biological catalysts that play a key role in all biological processes. They exhibit both high catalytic activity and selectivity against substrates. However, natural enzymes tend to have limited chemical and biological stability and often render high cost, due to sophisticated procedures needed for their isolation, purification, and stabilization from biological sources. This is why the investigation of artificial enzymes, mostly in the form of nanoparticles, "*nanozymes*" is a new trend of nanobiotechnology.

Artificial enzymes were invented on the landmark of the last century [1,2]. They are highly stable and inexpensive alternative catalysts for many reactions [3-5]. Novel artificial enzymes include various natural and synthetic materials: cyclodextrins, metal complexes, porphyrins, polymers, dendrimers, which mimic the structure and function of natural enzymes. More recently, it has been discovered that some nanomaterials also exhibit the catalytic properties valuable for their practical use in biosensing [5]. Among the described nanoscale materials are catalysts with different reaction specificity; first of all, these are oxidoreductases: peroxidases

[6], catalase, glucose oxidase, sulfite oxidase, superoxide dismutase, as well as proteases, endonucleases, NO-synthases, and others [4,7]. The nanomaterials with peroxidase-like activity have significant potential for a wide variety of applications, especially in biosensing and fuel cells technology.

Among the nanomaterials with peroxidase-like activity, the ferromagnetic nanoparticles were described [8]. The number of such materials is constantly growing. It includes Nafion-cytochrome *c* [9], Co_3O_4/CeO_2 nanosheets [10], Au/Ag nanowires coupled with hamin-melamine [11], Au nanoparticles [12], Fe/Au nanoparticles [13], Au/Pt nanoparticles [14], Pt nanoparticles [15], Ru and Pt/Ru nanoparticles [16,17], and Pd and Pt/Pd nanoparticles [18,19]. In the last decade, a special interest in bionanotechnology is focused on the usage of nanozymes based on carbon materials such as carbon nanotubes modified by Prussian blue [20], TiO_2 multi-walled carbon nanotubes [21], hemin-graphene hybrid nanosheets [22], hemin-graphene-Au hybrid [23], Fe_2O_3 graphene oxide [24], Cu-Ag reduced graphene oxide nanosheets [25], carbon nanofibers [26], and MoO_3 nanorods supported on gree-synthesized carbon [27]. On the other hand, direct enzyme immobilization on the surface of graphene is complicated due to its strong hydrophobicity [28]. Recently, we described the fabrication of a sensitive amperometric biosensor for wastewater phenols analysis based on microporous carbon fibers [29]. In contrast to graphene, carbon microfibers are more simple in preparation and functionalization. Moreover, they exhibit lower hydrophobicity and higher electroconductivity. All these properties of carbon microfibers make them a very promising platform for immobilization of enzymes, as well as a suitable matrix for the construction of peroxidase-like nanozyme.

This study is focused on the synthesis, characterization, and analytical application of a new micro/nanocomposite with peroxidase-like activity, which is based on carbon microfibers modified by hemin and gold nanoparticles. The application is reported of the composite as an artificial peroxidase in construction of ethanol and glucose-sensitive amperometric biosensors including their evaluation in the analysis of the corresponding analytes in beverages.

2. Experimental

2.1. Materials

Delignified cellulose Greencel purchased from Bukoza, Hencovce, Slovakia was employed without additional treatment. Alcohol oxidase (EC1.1.3.13, AOX) was purified from the mutant strain *Ogataea polymorpha* C-105 (*gcr1 catX*) [30] according to Smutok *et al.* [31]. Glucose oxidase (EC1.1.3.4, GOX) was purified from *Penicillium vitale* according to Nikolska *et al.* [32]. Nafion®, hemin, guaiacol, hydrazine sulfate, and $HAuCl_4$ were obtained from Sigma-Aldrich (Buchs, Switzerland). *D*(+)-glucose monohydrate was purchased from J.T. Baker (Deventer, The Netherlands). Na_2HPO_4, KH_2PO_4, $CaCl_2$, 28% ammonia solution, sodium citrate, and nitric acid were obtained from Merck (Darmstadt, Germany). 30% H_2O_2 was purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). The triple distilled water was applied in all procedures.

2.2. Synthesis and functionalization of CF

2.2.1. Synthesis of CF

CF were prepared from delignified cellulose by carbonization without air contact at 700 °C using the oven Elektro (Bad Frankenhausen, Germany) and boiled for 1 hour in concentrated (65%) nitric acid, washed out from acid and dried.

2.2.2. Raman spectroscopy

Raman spectroscopy of CF sample was performed using inVia Micro Raman spectrometer (Renishaw, Wotton-under-Edge, UK) using 633 nm for excitation. The laser power was set as 1.5 mW, exposure time as 10 s and 10 scans accumulations. The data were collected in the spectral range of 800–2000 cm^{-1}, with 2 cm^{-1} of spectral resolution. The spectrum was processed in the form of smoothing, baseline correction and normalization.

2.2.3. Functionalization of CF by hemin

Functionalization of CF by hemin was performed according to [22]. 5.0 mL CF suspension (0.5 mg·mL^{-1}) was ultrasonicated for 3 min to obtaining a homogenous suspension. Then, the CF suspension was mixed with 5.0 mL aqueous solution of hemin (0.5 mg·mL^{-1}) and 50 μL ammonia solution (14.76 M), followed by the addition of 10 μL hydrazine solution (15.6 M). After vigorous shaking for a few minutes, the sample was put in a water bath (60 °C) for 4 h. The obtained stable black dispersion (CF-H) was washed twice with water and centrifugated for 15 min at 9700 g; Hettich Micro-22R centrifuge.

2.2.4. Modification of CF-H by gold nanoparticles

Modification of CF-H by gold (Au^0) nanoparticles was performed by reduction of Au(III) from $HAuCl_4$ using the wet-chemical method according to [33]. 1 mL CF-H resuspended in water (0.5 mg·mL^{-1}) was premixed with 1 mL solution of $HAuCl_4$ (0.3 mM), followed by the addition of 1 mL sodium citrate (1.5 mM) at room temperature. To remove the excess of the reagents, the CF-H-Au preparation was washed twice with water followed by centrifugation for 30 min at 9700 g.

2.2.5. Analysis of peroxidase activity of the synthesized micro/nanocomposite in solution

Peroxidase activity of unmodified CF and synthesized micro/nanocomposites (CF-H and CF-H-Au), as well as pure hemin in concentration 10 μg·mL^{-1} were investigated by the spectrophotometric method (Shimadzu UV-1650 PC) using 25 mM guaiacol as a chromogen and 8.8 mM H_2O_2 in pH 5.5 at 50 °C [34].

2.3. Scanning Electron Microscopy and X-ray Microanalysis

A Scanning Electron Microscope (SEM-microanalyser REMMA-102-02, Sumy, Ukraine) was used for morphological analysis of the CF and CF-H-Au. The special cover film on the

samples with a Butvar solution B-98 (Sigma, St. Louis, MO, USA) in 1.5% chloroform was formed using an ultrasound method. The distance from the last lens of the microscope to the sample (WD) ranged from 24.7 to 24.8 mm. The accelerator voltage was 20 kV with magnification ranges from 80× to 4,000×.

2.4. Evaluation and characterization of amperometric biosensors

2.4.1. Apparatus and techniques for biosensor construction

The amperometric biosensors were constructed in a three-electrode configuration using constant-potential amperometry. The biosensor configuration included a Pt counter electrode, Ag/AgCl/KCl reference electrode, and a graphite-rod working electrode. The graphite rods (type RW001, 3.05 mm) from Ringsdorff Werke (Bonn, Germany) were used as working electrodes. Before usage, the electrodes were polished with P2000 emery paper Smirdex® (Zaporizhzhia, Ukraine). The amperometric analysis was carried out using a potentiostat CHI 1200A (IJ Cambria Scientific, Burry Port, UK) in an electrochemical cell at 25 °C under continuous stirring.

2.4.2. Modification of the working electrode by nanozyme layer

3 µL CF, CF-H, or CF-H-Au aqueous suspension (0.5 $mg \cdot mL^{-1}$) was dropped on a surface of 3.05 mm graphite-rod electrodes, followed by drying for 15 min at room temperature.

2.4.3. Formation of biorecognition layer on the top of the nanozyme layer

On the top of the working electrode modified by nanozyme layer, 5 µL enzyme solution (alcohol oxidase form *Ogataea polymorpha,* AOX or glucose oxidase from *Penicillium vitale,* GOX with the activity of 200 $U \cdot mL^{-1}$) was dropped and the formed enzyme-nanozyme layer was covered with 5 µL 1 % neutralized aqueous solution of Nafion and dried for 15 min at room

temperature. The built-up bio-functionalized electrodes were rinsed with 50 mM phosphate buffer (PB), pH 6.8 and kept at 4 °C till usage.

2.5. Real sample analysis

For evaluation of the constructed bioelectrodes, a grape must and wine *Isabella* were used. Any additional sample treatment except dilution was omitted. The samples were kept at -20 °C till analysis.

Analysis of the beverage samples by the developed bioelectrodes was performed using "*standard addition mode*", a proven measurement method for a complex sample matrix [35].

3. Results and discussion

3.1. Preparation, functionalization and characterization of peroxidase-like nanozyme bound with carbon fibers

Carbon microfibers (CF) were prepared according to our previous publications [29,36,37]. The resulting material exhibits a soft and wooly-like structure of dark black color. Microscopically the CF exhibit a twisted structure with the width about 10-15 µm and the length from several dozen to about 200 µm. The Raman spectroscopy analysis of the CF showed two intensive peaks, characteristic for carbon structures (*see supplementary material Fig. S1*). D-band at 1339 cm^{-1} corresponds to the defect-induced mode and G-band at 1595 cm^{-1} is associated with the vibration of sp^2-bonded carbon atoms in a 2D hexagonal lattice. The presence of these bands reveals the occurrence of sp^2C atoms in benzene or condensed benzene rings of amorphous (partially hydrogenated) carbon [38]. The CF crystalline structure provides an orderly charge transfer path, resulting in a good electroconductivity [39]. The surface area of CF as determined by the BET method was 450 $m^2 \cdot g^{-1}$ and their effective pore size was only 0.5-0.6 nm [37]. The pores are too small for the intrusion of hemin or gold nanoparticles, this means that there are situated exclusively on the outer surface of CF.

To study the feasibility of using the CF as a matrix platform for binding nanocomposite with peroxidase-like activity, the fibers were modified by hemin (component with a low inherent peroxidase activity) in combination with gold nanoparticles (Au) (Fig. 1).

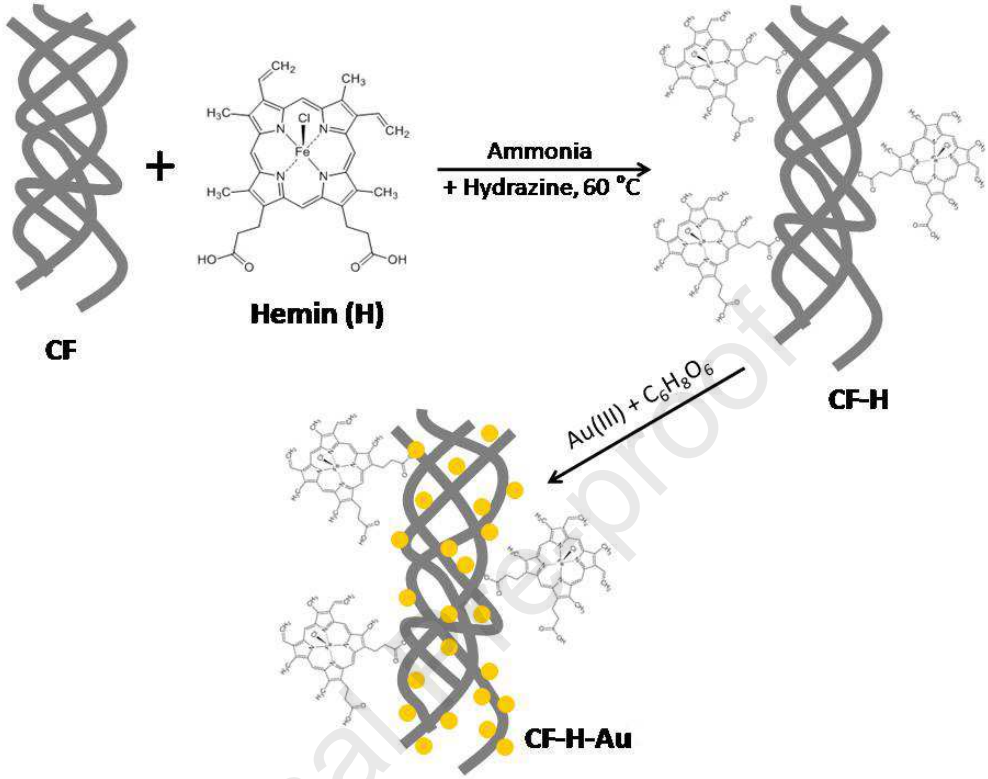

Fig. 1. The principal scheme of synthesis of micro/nanocomposites with peroxidase-like activity based on CF modified by hemin (H) in combination with gold nanoparticles (Au).

In the proposed scheme, the hemin is bound with the CF through chemical binding (Fig. 2, Fig. S3, B), while the gold nanoparticles – due to physical adsorption. The additional modification of the hemin-functionalized surface by nanostructured gold particles should, hypothetically, facilitate the electron transfer from hydrogen peroxide to hemin moiety. The electroactive CF plays a dual role: as a matrix, chemically binding hemin as a key catalytic unit, and as a transducer, providing the effective electron transfer from an electrode surface to H_2O_2 through hemin and Au.

Prior to the modification, CF was partially disintegrated using an ultrasonic bath. As a result of CF modification by hemin, in combination with Au, two different composite materials

were obtained: CF-H – a sample of CF modified by hemin and CF-H-Au – CF modified by hemin and gold nanoparticles. The obtained micro/nanocomposite samples, unmodified CF and hemin were characterized by UV-visible spectroscopy (200-700 nm) using Shimadzu UV-1650 PC spectrophotometer (Fig. 2).

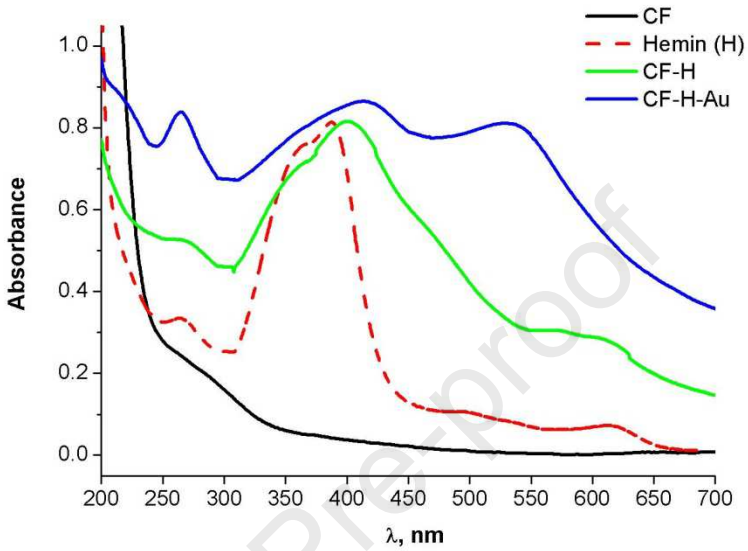

Fig. 2. UV-Vis spectra of the micro/nanocomposite materials: CF – unmodified carbon microfibers (black); H – hemin aqueous solution (red dash); CF-H – CF modified by hemin (green); CF-H-Au – CF modified by hemin and gold nanoparticles (blue).

The modification of the samples was proved by their spectral characteristics (Fig. 2). For non-modified CF, no absorption peaks in the visible spectrum were observed. The aqueous solution of hemin showed an absorption peak typical for heme (Sore peak) at 390 nm and a smaller one at 265 nm. After the modification of CF by hemin (CF-H), the spectrum of the sample exhibits typical features for hemin absorption peaks: a little shifted heme-specific peak at 400 nm and a peak at 265 nm. For the CF-H-Au sample, two absorption maxima were detected in the visible region – at 530 nm (corresponding to the surface plasmon resonance of Au) and 412 nm (heme peak). Moreover, the UV peak at 265 nm typical for the nanostructures is clearly distinguished (Fig. 2).

The detailed structural characterization of the micro/nanocomposites was performed using SEM analysis (Fig. 3).

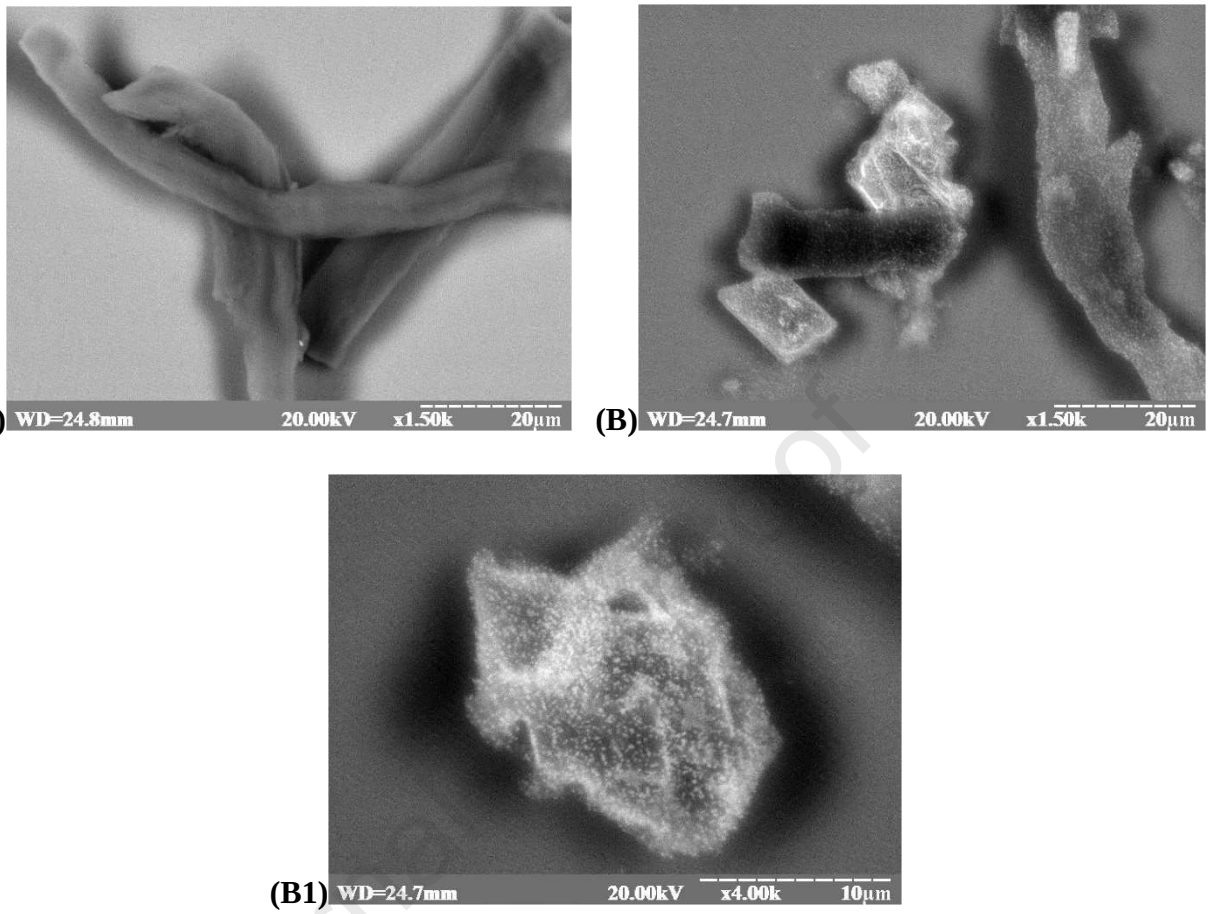

Fig. 3. SEM pictures of unmodified CF fragments (**A**) and CF-H-Au (**B-B1**) at different resolutions. *Note:* WD – the distance from the last lens of the microscope to the samples (mm); kV – accelerating voltage; x – fold magnification; μm – scale unit.

The SEM analysis confirmed the successful modification of the CF surface by Au (Fig. 3, B-B1). Moreover, it is shown that the efficiency of CF modifications by gold nanoparticles highly depends on the size of the initial fragments of the CF fibers. The formation of Au on the surface of CF fragments with a size up to 200 μm (obtained by pre-ultrasonic treatment) is much more efficient (light areas) compared to larger CF fragments (dark areas) (*see supplementary material Fig. S2*).

To confirm the formation of gold nanoparticles on the surface of hemin-modified CF, their X-ray spectral analysis was performed (*see supplementary material Fig. S3*). The X-ray spectral analysis confirmed the formation of Au of different structural types with typical peaks Kα at 2.15; 9.75 and 11.5 keV for hemin-modified CF. Moreover, the Fe peak at 6.4 keV also proves the presence of hemin (Fig. S3).

3.2. Investigation of catalytic and electrochemical properties of the synthesized carbon-based micro/nanocomposites

For a detailed study of the catalytic characteristics of micro/nanocomposites based on CF, hemin, and Au (CF-H-Au), the determination of the specific peroxidase activity ($V_{max(specific)}$) and the apparent Michaelis-Menten constant (K_M^{app}) to H_2O_2 was performed. The specific peroxidase activity of the micro/nanocomposite CF-H-Au is 1.6 fold higher than the specific peroxidase activity compared with the hemin in solution as a control (2.07 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ *vs* 1.32 $\mu mol \cdot mg^{-1} \cdot min^{-1}$). For the CF-H sample, specific peroxidase activity increased 1.3 fold (1.66 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ *vs* 1.32 $\mu mol \cdot mg^{-1} \cdot min^{-1}$) (*see supplementary material Fig. S4*). The calculated values of K_M^{app} constant toward H_2O_2 for micro/nanocomposites based on CF, hemin, and Au indicate their better affinity for H_2O_2 compared to hemin in solution. Thus, the K_M^{app} value for the CF-H-Au decreased 1.86 fold (2.87 mM *vs* 5.34 mM) and for CF-H sample – 1.14 fold (4.7 mM *vs* 5.34 mM) (*see supplementary material Fig. S4*). The comparison of the main catalytic characteristics of the micro/nanocomposites with the nearest analogs toward H_2O_2 is represented in Table S1 (*see supplementary material Table S1*). The specific peroxidase activity of CF-H-Au, as well as the affinity toward hydrogen peroxide, is higher compared with the most described analogs (*Table S1*). That opens the possibility of using the composite as a nanozyme for the construction of amperometric chemosensors (for H_2O_2) and biosensors based on oxidases, where the H_2O_2 is one of the products of the enzymatic reaction.

To study the electrochemical characteristics of the synthesized nanozymes, a voltammetric analysis of the rod graphite electrodes modified with CF, CF-H, and CF-H-Au additionally covered by 1% Nafion was performed (Fig. 4).

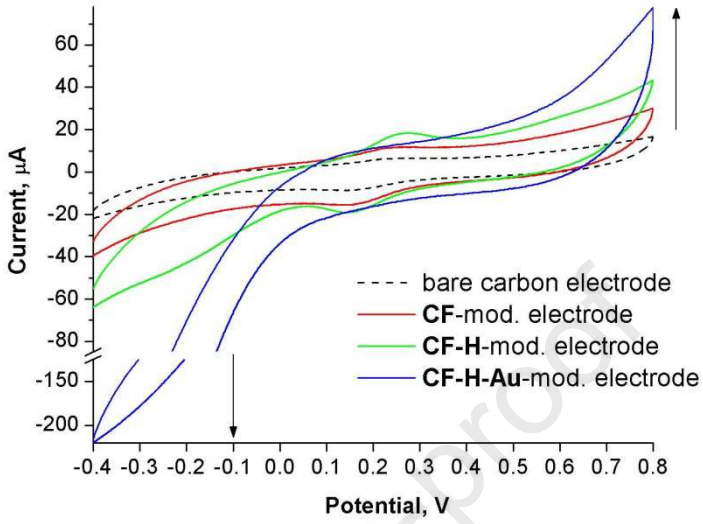

Fig. 4. Cyclic voltamperogramm of bare (dash line) and CF-modified (red line), CF-H-modified (green line), CF-H-Au-modified (blue line) 3.05 mm carbon rod electrode at addition of 5 mM H_2O_2. Conditions: scan rate 50 $mV \cdot s^{-1}$ *vs* Ag/AgCl in 50 mM PB, pH 6.8.

The results of voltamperometric analysis show that modification of rod graphite electrodes by CF, CF-H, and CF-H-Au, covered by 1% Nafion, significantly improves their electrochemical activity toward H_2O_2. This is evidenced by a significant increase in the height of the redox peaks of the electrodes modified by the nanozymes upon the addition of 5 mM H_2O_2 when compared with unmodified carbon rod electrode (Fig. 4). Additionally, the results of the voltamperometric analysis indicate that the effective electrochemical H_2O_2 reduction occurs at a potential of -0.1 V *vs* Ag/AgCl, which was selected as a working potential for performing the chronoamperometric studies of the nanozyme-modified electrodes (Fig. 4).

Chronoamperometric analysis of unmodified and nanozyme-modified 3.05 mm carbon rod electrodes upon the addition of increasing concentrations of H_2O_2 showed that all types of micro/nanocomposites are characterized by peroxidase activity (Fig. 5).

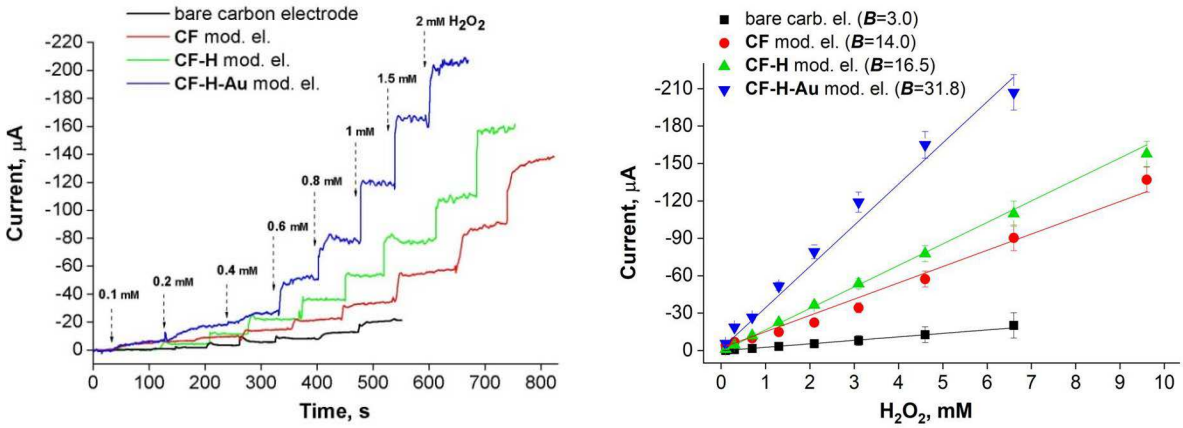

Fig. 5. Typical chronoamperometric response' currents (left) and calibration curves (right) upon subsequent additions of H_2O_2 for non-modified (black points), CF-modified (red points), CF-H-modified (green points), and CF-H-Au-modified (blue line) 3.05 mm carbon rod electrode. Abbreviation: ***B*** – graph slope. Conditions: working potential –100 mV *vs* Ag/AgCl in 50 mM PB, pH 6.8 at room temperature and continuous stirring.

The sensitivity of the constructed chemoelectrodes to H_2O_2 was calculated from the slope of the calibration curves (coefficient ***B*** in Fig. 5), considering the working area of the electrode used (7.3 mm^2). The sensitivity of CF-modified electrodes for H_2O_2 is increased 4.7-fold compared with the control unmodified (bare) electrode (1918 $A \cdot M^{-1} \cdot m^{-2}$ *vs* 411 $A \cdot M^{-1} \cdot m^{-2}$); for CF-H-modified electrodes – for 5.5 fold (2260 $A \cdot M^{-1} \cdot m^{-2}$); for CF-H-Au-modified – about 10.6 fold (4356 $A \cdot M^{-1} \cdot m^{-2}$) (Fig. 5). Consequently, the chemoelectrodes based on CF-H-Au were chosen as the most promising nanozyme for the next stage of biosensor construction. Moreover, the CF-H-Au-modified electrodes are characterized by significantly improved sensitivity compared with the latest reported analogs based on nanozymes and carbon electrodes. The sensitivity of the CF-H-Au-modified electrode toward H_2O_2 is 2.6-fold higher compared to gold nanobipyramids supported by multi-walled carbon nanotubes (AuNBP/MWCNTs) modified carbon electrodes (4356 $A \cdot M^{-1} \cdot m^{-2}$ *vs* 1706 $A \cdot M^{-1} \cdot m^{-2}$) [40]; 1.3-fold compared with ferumoxytol, Pt and reduced graphene oxide (Fer/rGO-Pt) modified carbon electrode (4356

$A \cdot M^{-1} \cdot m^{-2}$ *vs* 3400 $A \cdot M^{-1} \cdot m^{-2}$) [41]; 1.6-fold *vs* Fe_3O_4/3D graphene-supported quantum dots (Fe_3O_4/3D GNCs) modified carbon electrode (4356 $A \cdot M^{-1} \cdot m^{-2}$ *vs* 2742 $A \cdot M^{-1} \cdot m^{-2}$) [42].

3.3. Characterization of the ethanol and glucose-selective bioelectrodes based on the peroxidase-like nanozyme

The ethanol and glucose-sensitive bioelectrodes were constructed as described in section 2.4.3 of the Experimental part. The results of the chronoamperometric biosensor analysis for both substrates are represented in Figure 6.

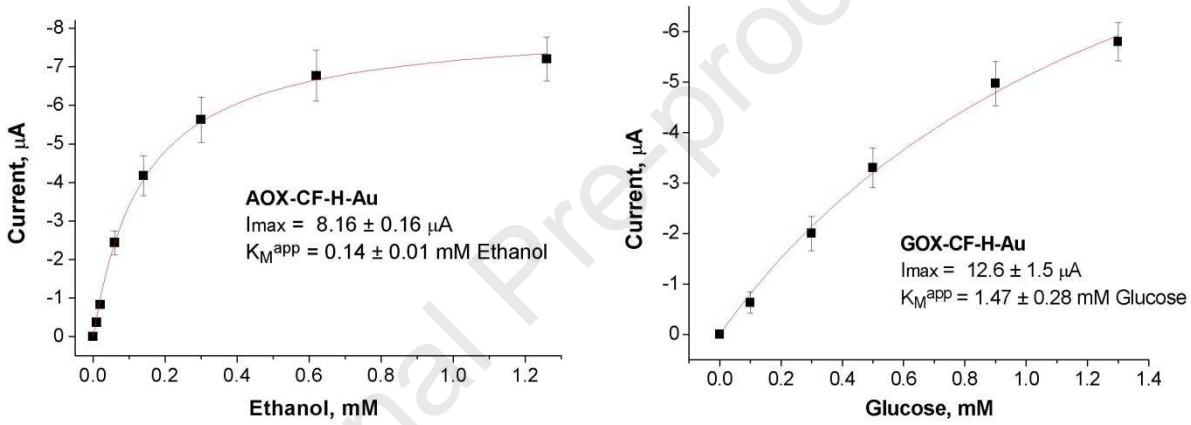

Fig. 6. Calibration curves upon subsequent additions of ethanol for AOX-CF-H-Au bioelectrode (left) and glucose for GOX-CF-H-Au bioelectrode (right).

Fig. 6 shows the typical calibration curves for ethanol and glucose derived from the corresponding chronoamperogramms of the constructed bioelectrodes. As seen from the calibration curve, the ethanol-selective (AOX-CF-H-Au) bioelectrode response (maximal current at substrate saturation I_{max}) is estimated to be 8.16 ± 0.16 μA, and the apparent Michaelis-Menten constant (K_M^{app}) to ethanol is 0.14 ± 0.01 mM (Fig 6, left). The I_{max} value for glucose-sensitive (GOX-CF-H-Au) bioelectrodes is 12.6 ± 1.5 μA and the value of K_M^{app} is estimated as 1.47 ± 0.28 mM glucose (Fig 6, right). The limit of detection (LOD) to ethanol for AOX-CF-H-Au is calculated as 0.005 mM, linear frames – 0.01-0.15 mM, and sensitivity – 4089.0 $A \cdot M^{-1} \cdot m^{-}$

^{2}. The GOX-CF-H-Au is characterized by LOD to glucose about 0.05 mM, the linearity of 0.1-0.9 mM, and sensitivity – 909.5 $A \cdot M^{-1}m^{-2}$.

The main operational characteristics of the AOX-CF-H-Au and GOX-CF-H-Au-based bioelectrodes were compared with the nearest newest analogues (*see supplementary material Table S2*). It should be noted that both of them are greatly surpassed of the analogues with sensitivity, which is higher from 2-3 fold [51-53] up to one-order [17, 45-50] value. Moreover, an additional weak point of the most of the recently described electrochemical sensors based on H_2O_2-sensitive nanozymes/electrocatalysts combined with GOX or AOX is usage of rather high or low working potentials, resulting decreasing of their selectivity due to non-specific auto-oxidation or auto-reduction of electroactive compounds of the real samples (*Table S2*). For the developed CF-H-Au-based biosensor applied potential equals -0.1 V *vs* Ag/AgCl reference electrode that eliminates possible nonspecific impact on the sensor' output at the real samples' analysis that makes it more accurate.

The constructed ethanol and glucose-sensitive bioelectrodes were tested for analysis of the corresponding analytes using the grape must and wine *Isabella* as the real samples. The biosensor analysis was performed using "*standard addition mode*" (*see supplementary material Fig. S5*) and the obtained results were compared with results acquired with help of spectrophotometric enzymatic kits "Alcotest" [43] and "Diagluc" [44] (Table 1).

Table 1. Comparison of the results of ethanol and glucose analysis in the samples of the grape must and wine *Isabella* by the bioelectrodes and by enzymatic kits "Alcotest" [43] and "Diagluc" [44].

Analyte	**Sample**	**Methods**	
		Constructed biosensors	***Enzymatic kits***
Ethanol, mM ($g \cdot L^{-1}$)	Must	0.13 ± 0.05 $(6.0 \cdot 10^{-3})$	0.14 ± 0.04 $(6.4 \cdot 10^{-3})$
	Wine	2280.0 ± 35 (104.9)	2250.0 ± 12 (103.5)
Glucose,	Must	467.5 ± 12 (84.1)	482 ± 25 (86.8)

mM ($g \cdot L^{-1}$)	Wine	99.8 ± 5.3 (18.0)	91.9 ± 2.2 (16.5)

The biosensor analysis demonstrated the following results for ethanol content in the tested samples: 0.13 ± 0.05 mM for grape must and 2280 ± 35 mM for wine, as well enzymatic kit "Alcotest" showed 0.14 ± 0.04 mM for grape must and 2250 ± 12 mM for wine, respectively (Table 1). The correlation between the results for both methods is very good (*see supplementary material Fig. S6*). Moreover, the biosensor glucose analysis of the same samples shown 467.5 ± 12 mM for grape must and 99.8 ± 5.3 mM for wine that is also well correlated (R = 0.999) with results obtained using the enzymatic kit as the reference approach – 482 ± 25 mM for must and 91.9 ± 2.2 mM for wine. This demonstrates the high accuracy of biosensor analysis of real beverage samples by the developed biosensors. It can be concluded that the developed biosensors may also be well applicable in food technologies, in medicine, as well as in other areas, where a precision analysis of ethanol and glucose is important.

4. Conclusions

The sensitive biosensors for the detection of ethanol and glucose using alcohol oxidase (AOX) or glucose oxidase (GOX) immobilized on the micro/nanocomposite with peroxidase-like activity (nanozyme) have been developed. The nanozyme was synthesized on the base of carbon microfibers modified by hemin and gold nanoparticles (CF-H-Au). The formation of gold nanoparticles on the surface of hemin-modified carbon microfibers was confirmed by the UV-Vis and X-ray spectroscopy, as well as with the SEM analysis. The resulting micro/nanocomposite exhibits 2.0 fold higher specific peroxidase activity and 2.6 fold better affinity for H_2O_2 compared to hemin in solution, as a control. The electrochemical property of the nanozyme was also significantly improved compared to the controls. For example, the sensitivity of CF-H-Au-modified electrodes for H_2O_2 is increased 10.6 fold compared with the control unmodified (bare) carbon electrode (4356 $A \cdot M^{-1}m^{-2}$ *vs* 411 $A \cdot M^{-1}m^{-2}$). The H_2O_2-

sensitive CF-H-Au-modified chemoelectrodes were used for the construction of sensitive ethanol and glucose biosensors. To eliminate diffusion limitation for substrates, AOX or GOX were immobilized on the nanozyme layer using a highly porous Nafion membrane. The apparent Michaelis-Menten constant (K_M^{app}) of constructed ethanol-selective bioelectrodes (AOX-CF-H-Au) was estimated to be 0.14 ± 0.01 mM ethanol and the K_M^{app} for glucose-sensitive bioelectrodes (GOX-CF-H-Au) was estimated as 1.47 ± 0.28 mM for glucose. The limit of detection (LOD) to ethanol for AOX-CF-H-Au was calculated as 0.005 mM, linear frames – 0.01-0.15 mM, and sensitivity – 4089.0 $A \cdot M^{-1} \cdot m^{-2}$. The GOX-CF-H-Au was characterized by LOD for glucose about 0.05 mM, the linearity of 0.1-0.9 mM, and sensitivity – 909.5 $A \cdot M^{-1} \cdot m^{-2}$.

The developed biosensors were tested for ethanol and glucose analysis in the real samples of grape must and the wine. The results exhibit a high correlation with the results obtained using enzymatic kits as reference approaches.

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►A new micro/nanocomposite with peroxidase-like activity was synthesized. ►The nanozyme is based on carbon microfibers modified by hemin and gold nanoparticles. ►It was used for construction of highly sensitive ethanol and glucose biosensors. ►The biosensors were tested for ethanol and glucose analysis in the real beverages. ►The obtained results have a high correlation with the reference approaches.

Declaration of interests

√ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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