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Amperometric biosensors based on oxidases and PtRu nanoparticles as artificial peroxidase

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ABSTRACT

Catalytically active nanomaterials have several advantages over their natural analogues when used as artificial enzymes (nanozymes), namely, higher stability and lower cost. Nanozymes with metallic nanocomposites are promising catalysts for biosensing applications. The aim of the current research is to construct oxidase-based bioelectrodes for food analysis using nanozymes as peroxidase mimetics. Bimetallic PtRu nanoparticles (nPtRu) coupled with alcohol oxidase (AO) and methylamine oxidase (AMO) were chosen to construct amperometric biosensors (ABSs) for primary alcohols and methylamine (MA). Both ABSs show high sensitivities (336 $A{\cdot}M^{-1}{\cdot}m^{-2}$ for the AO-ABS and 284 $A{\cdot}M^{-1}{\cdot}m^{-2}$ for the AMO-ABS), broad linear ranges (25–200 μM ethanol and 20–600 μM MA) and satisfactory storage stabilities. Practical feasibility of the constructed ABSs was demonstrated on food samples. High correlation between contents of MA and ethanol in foods determined by the ABSs and reference methods was observed.

1. Introduction

The first report on electrochemical enzyme biosensor was published 55 years ago. More than 100,000 articles described this problem during these years, but very few biosensors have reached practical application and commercialization (Reyes-De-Corcuera, Olstad, & García-Torres, 2018). Combining nanobiotechnology with enzyme or cell-based electrochemical biosensors has become a crucial strategy for the fabrication of simple and reliable analytical systems. Unique optical, electrical, and electrochemical properties of nanomaterials provide promising alternatives to conventional biosensor development with improved stability, sensitivity and selectivity.

Artificial enzymes as highly stable and low-cost alternatives to natural enzymes have been discovered over the past decade (Karyakin, Karyakina, & Gorton, 2000). Nanomaterials with enzyme-like characteristics (nanozymes) are the next-generation artificial enzymes. Nanozymes, consisting of bi- and trimetallic nanoparticles (NPs) of noble and transition metals are particularly promising due to large surface area and high catalytic activity. The majority of traditionally constructed amperometric biosensors with oxidases are based on detection of hydrogen peroxide (H_2O_2), generated as a result of oxidase reaction with its own substrate. To achieve the detection of H_2O_2 under low reducing potential, peroxidase (PO) is usually considered when designing the biosensor. Although PO has high specificity and sensitivity, the application of natural enzyme is limited by its high cost. Novel artificial PO-mimetics hold great promise for successful assay of various real samples including food and biological liquids. PO-like nanozymes of different nature (organic, inorganic or combined) and composition (mono-, bi- or trimetallic NPs stabilized by block copolymers, organic ligands, surfactants and dendrimers) were shown to decompose effectively H_2O_2. PO-like metallic NPs demonstrate an outstanding electrochemical catalytic activities and usually are coupled

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with carbon, graphene, natural polysaccharides or synthetic polymers (Zou, Xiang, Sun, & Xu, 2008; Zhai et al., 2013; Rothwell, Killoran, & O'Neill, 2010; Kung et al., 2014; Reyes-De-Corcuera et al., 2018; Dong et al., 2018).

AuPt nanostructures, AuNPs, Pt-based nanomaterials, cobalt(II)-selenide NPs, PtRuNPs (Kung et al., 2014), nanoclusters, that contain Cu or SnO_2, nickel zinc ferrite were shown to possess intrinsic PO-like activities. Combination of metallic NPs with different forms of carbon and graphene improves catalytic properties of the artificial PO (Kung et al., 2014). Some of the listed PO-mimetics have also the properties of oxidases and catalases (Liu et al., 2015), but dual specificity of such NPs makes it impossible to use them for real biological samples analysis. It is also worth mentioning that the optimal pH for the most of these NPs is 3.0–4.0, thus under physiological pH 7.0–7.4 these NPs are catalytically inactive.

Various nanomaterials were shown to be effective "artificial POs" as transducers for H_2O_2 detection in oxidase-based biosensors: AuNPs, AuAg nanorods (Han, Li, Zhang, Lang, & Liu, 2015), Fe_3O_4NPs, CeO_2NPs, Co_3O_4NPs (Xie et al., 2013), V_2O_5 nanowires, TiO_2 nanotubes, V_2O_3 -ordered mesoporous carbon composite (Han, Zeng, Wei, Li, & Liu, 2015), nanocomposites of NiFex (x = 0, 1, 2), embedded in ordered mesoporous carbon (Xiang et al., 2015) and others. Analytical characteristics of some oxidase-based methods with PO-like nanozymes are compared in Table 1. One of the most effective inorganic artificial PO was shown to be Prussian blue-based NPs. This catalyst has high stability, sensitivity and selectivity to H_2O_2 in extra-wide linear range $1{\cdot}10^{-9}$–$1{\cdot}10^{-2}$ M (Karyakin et al., 2000).

The aim of current research is to design oxidase-based amperometric biosensors (ABSs) showing potential for food analysis by using nanozymes as PO mimetics. In this paper we describe ABSs based on yeast alcohol oxidase (AO) and methylamine oxidase (AMO), as well as on NPs of PtRu (further – nPtRu) as artificial PO, for the detection of ethanol and methylamine (MA), respectively. AO and MAO were selected due to using them in our previous works related to construction of yeast over-producing strains (Gonchar, Ksheminska, Hladarevska, & Sibirny, 1990; Sigawi et al., 2014) and development of biosensors and enzymatic kits for assay of ethanol/methanol and MA (Gonchar, Maidan, Pavlishko, & Sibirny, 2001; Smutok et al., 2006, Stasyuk, Smutok, Zakalskiy, Zakalska, & Gonchar, 2014).

2. Materials and methods

2.1. Reagents

Sodium ethylenediaminetetraacetate (EDTA), $CaCl_2$, NaCl, KH_2PO_4, Na_2HPO_4, NH_4Cl, ruthenium(III) chloride hydrate, chloroplatinic acid, MA, dimethylamine (DMA), trimethylamine (TMA), methanol, ethanol, propanol, glycerol, glucose, formaldehyde, ammonium chloride and hydrogen peroxide were purchased from Sigma-Aldrich.

The cathodic polymer GY 83–0270 0005 was from BASF Farben und Lacke (Munster, Germany). All buffers and standard solutions were prepared with Milli-Q (Millipore) water.

2.2. Enzymes isolation and purification

Electrophoretically homogeneous yeast enzymes – alcohol oxidase (AO, EC 1.1.3.13) and methylamine oxidase (AMO, EC 1.4.3.21; primary amine:oxygen oxidoreductase (deaminating) or amine oxidase) were used for ABSs fabrication.

AO was isolated from cell-free extract of the selected over-producing strain *Hansenula 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 (Gonchar et al., 1990; Shleev et al., 2006). Activity of AO was determined by the rate of hydrogen peroxide formation in reaction with methanol as monitored by the peroxidative oxidation of *o*-dianisidine in the presence of PO. Purified AO with specific activity 20 $U{\cdot}mg^{-1}$ of protein was kept as suspension in 70% ammonium sulfate, 50 mM phosphate buffer (PB) pH 7.5 at 4 °C.

As a source of AMO, the recombinant yeast strain *Saccharomyces cerevisiae* C13ABYS86 constructed by us earlier was used (Sigawi et al., 2014). The $(His)_6$-tagged enzyme was purified from the cell-free extract

Table 1
PO-like nanozymes as the effective platforms in oxidase-based analytical approaches.

Analyte/Enzyme	Composition of nanozyme	Characteristics of analytical method			References
		Mode of signal detection	Linear range, mM	LOD, μM	
Glucose/Glucose oxidase	Pt	Amperometry	0.01–8	0.7	Zhai et al. (2013)
			0.001–23	1	Zou et al. (2008)
			up to 10		Rothwell et al. (2010)
	Pd		up to 12		Lim, Wei, Lin, Li, and Kuayou (2005)
	Co		0.0025–0.030	0.156	Dong et al. (2018)
	mixed-valence manganese oxides		up to 3.5	100	Cui, Liu, and Lin (2005)
	ZnO		0.01–6.5	4.5	Hwa and Subramani (2014)
	$NiFe_2$-ordered mesoporous carbon		0.049–12.5	2.7	Xiang et al. (2015)
	Co_3O_4	Spectrophotometry	0.001–0.1	1	Xie et al. (2013)
	NiO		0.05–0.50	20	Liu et al. (2015)
	Au@Ag nanorods		0.05–20		Han, Li et al. (2015)
	V_2O_3-ordered mesoporous carbon		0.01–4		Han, Zeng et al. (2015)
Cholesterol/Cholesterol oxidase	Cu	Chemiluminescence	0.05–10	1.5	Xu et al. (2016)
	Cu_2O		0.0006–0.013	0.17	Hong, Liu, Li, Chen, and Lin (2013)
	AuPt	Amperometry	0.05–11.2	90.7	Safavi and Farjami (2011)
Ethanol/Alcohol oxidase	Au		0.01–4.7	7	Chinnadayyala, Santhosh, Singh, and Goswami (2015)
	PO/Au		0.005–3	2.3	Chinnadayyala, Kakoti, Santhosh, and Goswami (2014)
	Prussian Blue	Amperometry Paper-based screen-printed biosensor	up to 10	520	Cinti, Basso, Moscone, and Arduini (2017)
Glutamate/Glutamate oxidase		Amperometry	0.1–100		Karyakin et al. (2000)

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 ABTS in the presence of PO. Purified AMO with specific activity 16 U·mg^{-1} of protein was stored in 20% glycerol, 30 mM PB pH 7.5 at −20 °C.

2.3. Apparatus

A piece of Pt wire and an Ag/AgCI/3M KCl 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) and an AFM-microanalyser Solver P47-PRO (NT-MDT, Netherlands) were used for morphological analyses of the PtRu-film (nPtRu).

2.4. Synthesis of metallic NPs and testing their ability to react with hydrogen peroxide

Mono- and bimetallic NPs were synthesized on the surface of a GE by electrodeposition of 1 mM solutions of Pt^{4+}, Pd^{2+}, Ru^{3+} or Cu^{2+} salts in 0.1 M PB, pH 7.0 using cyclic voltammetry in the range from −1000 to +1000 mV with scan rate 50 mV·min^{-1} during 10 cycles. The obtained modified electrodes were rinsed with water and equilibrated before use in 30 mM PB pH 7.5.

The electro-activities of the synthesized NPs were studied by cyclic voltammetry under the same conditions and the profiles of amperometric signals at increasing concentrations of H_2O_2 were compared. The most electroactive NPs, that have the highest PO-like properties, were chosen for further investigation.

2.5. Preparation and morphological analysis of the nPtRu/GE

The most effective nPtRu was synthesized from aqueous solution containing of 25 mM ruthenium(III) chloride hydrate and 0.5 mM chloroplatinic acid by electrodeposition during 24 cycles under described conditions (see subdivision 2.4).

Both SEM and AFM were used to characterize a thickness and morphology of the nPtRu film on a GE. For AFM the tapping mode with a resonance frequency of 160 kHz, scan rate of 1 Hz/s and resolution of 256 × 256 pixels was used.

2.6. Immobilization of enzymes on the surface of nPtRu/GE

AMO or AO solutions were dropped on nPtRu/GE and covered by commercial polymer GY 83-0270 0005 (CP). The immobilization procedure was as follows: 3 μL of enzyme solution with activity 15–35 U·mL^{-1} in 30 mM PB, pH 7.5 was dropped on the top of the nPtRu/GE. After drying for 2 min at a room temperature the layer of enzyme was covered with 4 μL CP for polymer layer formation. The prepared bio-functionalized electrode was rinsed with 30 mM PB pH 7.5 and kept before use in this buffer with 0.1 mM EDTA at +4 °C.

2.7. Preparation of food samples for biosensor analysis

The samples of wine “Chianti” dry red (Corte Alle Mura, Italia) and Kefir “Halychyna” 2.5% of fat (Halychyna, Ukraine) were used for ethanol analysis. All samples were diluted stepwise by 50 mM PB pH7.5 (the dilution factors were 6020 and 12020, 76 and 151, respectively). The analytical results were statistically processed using the OriginPro 8.5 software.

For MA assay, the frozen sea fish samples of tilapia and pigfish were used. Protein-free extract was prepared as described earlier (Stasyuk et al., 2014).

2.8. Reference methods for MA and ethanol determination

Analysis of MA was performed by a colorimetric method in the multiple standard addition variant on a Shimadzu UV-1650 PC spectrophotometer (Shimadzu, Kyoto, Japan) at 545 nm relative to MA-free control sample. The concentration of MA in the tested samples was determined from a calibration curve (Stasyuk et al., 2014).

Ethanol assay was carried out using analytical kit “Alcotest” by the measurement of the dye-product accumulation in peroxidative oxidation of chromogen by H_2O_2 generated from ethanol in alcohol oxidase reaction (Gonchar et al., 2001). Because of high sensitivity of the method, the “fixed reaction time” was used at the incomplete conversion of analyte.

2.9. Statistic analysis

All the measurement’s results and the level of correlation between results obtained by the different analytical methods were calculated by computer program Origin 8.0 and Microsoft Excel.

3. Results and discussion

3.1. Selection and characterization of the best nanocomposite as peroxidase-like nanozyme

The electroactivities of the synthesized NPs, electrodeposited on the surface of GE, were tested by cyclic voltammetry as described in 2.4. The amperometric responses of different NPs/GEs to the added H_2O_2 (up to final concentration 1 mM) were compared (See Supplementary information, Fig. SI.1). Fig. SI.1 demonstrates that nPtRu/GE was the most effective artificial PO, thus it was studied in more detail as chemosensor for H_2O_2 detection.

To improve the electrodeposition of nPtRu on graphite electrode, 24 cycles were experimentally chosen. The resulted nPtRu/GE turned dark brown-golden due to formation of the nPtRu-film. The oxidation (at −0.05 V) and reduction (at −0.25 V) peaks were observed and the current was increased (Supplementary Information, Fig. SI.2).

Morphological properties of the PtRu-film were studied (Fig. 1). The SEM micrographs of the film formed by a potentiodynamic mode during 10 min in the $RuCl_3$ and H_2PtCl_6-containing solution are presented on Fig. 1. *a, b*. The Gaussian distribution by size (Fig. 1.*d*) obtained from the AFM data (Fig. 1.*c*) proved that an average thickness of the layer is 60 nm, thus the PtRu-film is really nanosized.

The XRM images of the PtRu-film show the characteristic peaks for Pt^0 and Ru^0 indicating the formation of nPtRu (Fig. 1.*e*).

The electrochemical characteristics of nPtRu/GE as effective chemosensor for H_2O_2 were studied (Fig. 2). The cyclic voltammogram (Fig. 2.*a*) demonstrates that a cathodic reduction peak contributed from H_2O_2 arises at a potential of approximately −100 mV (vs Ag/AgCl) for 0.15 and 0.6 mM H_2O_2 solutions. The chronoamperometric dependence and calibration graph for H_2O_2 determination at −100 mV are shown on Fig. 2(*b* and *c*, respectively). As a control, unmodified GE was tested and no signal was detected upon H_2O_2 addition. The maximum current response of the nPtRu/GE to H_2O_2 was 2020 ± 90 nA with a K_M^{app} = 0.77 ± 0.08 mM ($I_{50\%}$ at 0.77 ± 0.08 mM). Other analytical characteristics of H_2O_2-sensitive layer based on nPtRu are compared with results of other scientists using nPtRu as PO-like nanozyme in Table SI.1 (Supplementary information).

The aim of our work was to use nPtRu/GE as PO-mimetic for ABSs based on AO and AMO. We studied the ability of nPtRu/GE to react with the substrates of AO (methanol, ethanol, formaldehyde) and AMO (MA). Fig. 2.*d* demonstrates the absence of any signals on the tested

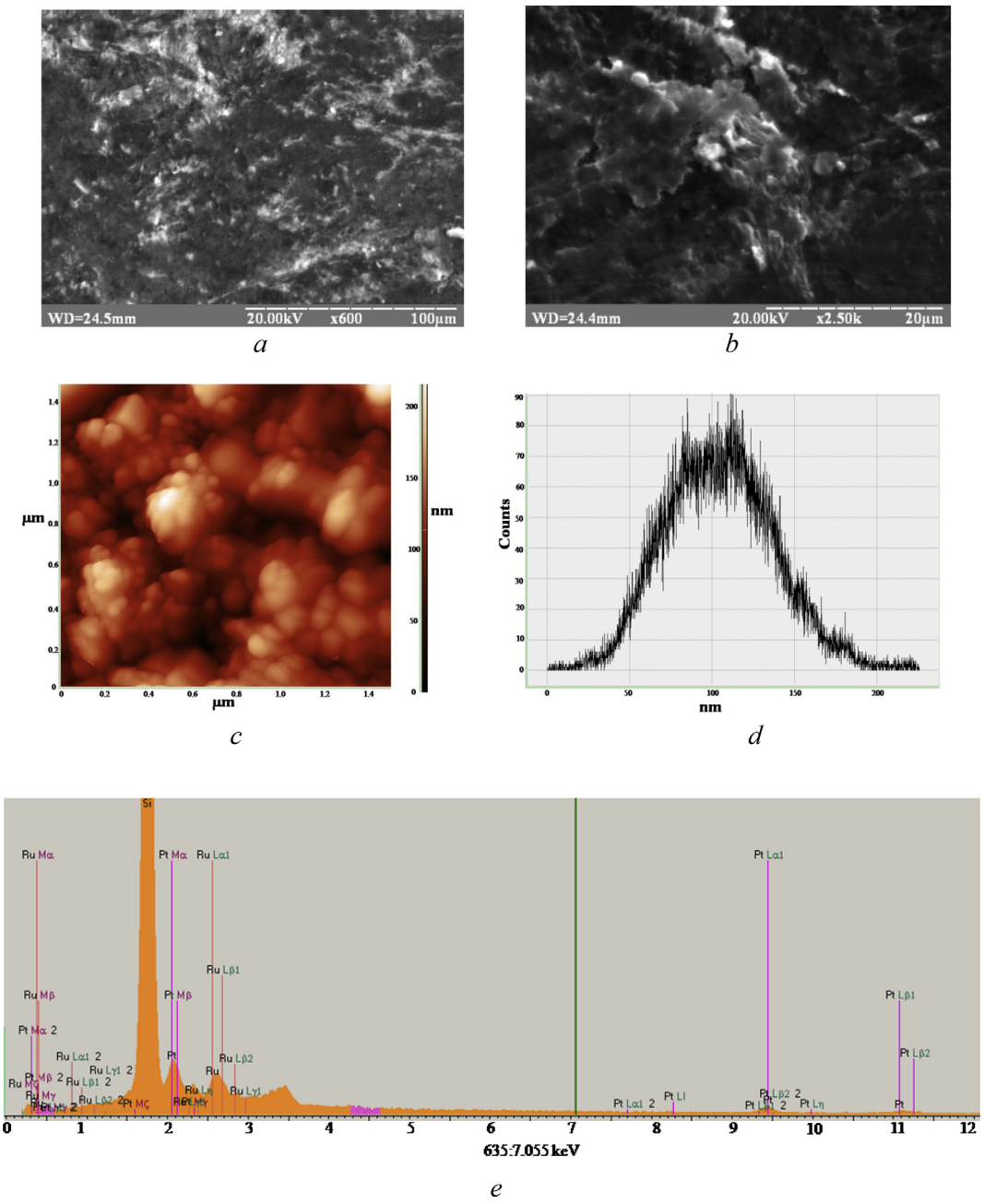

Fig 1. Characteristics of PtRu-film formed on the top of a GE after 24 cycles of electrodeposition. *a*, *b* – Scanning Electron Microscopy images; *c* – AFM micrograph; *d* – The Gaussian distribution curve of the film's thickness; *e* – X-ray spectral microanalysis of nPtRu.

analytes in the range from 0.05 to 5 mM with the step of 0.1 mM. Under the chosen conditions nPtRu/GE was a highly selective to H_2O_2.

The first bimetallic NPs catalyst comprising a Ru core covered with a shell of Pt atoms (a Ru@Pt core–shell NPs) was proposed by Alayoglu, Nilekar, Mavrikakis, and Eichhorn (2008). nPtRu had predictable catalytic properties that are markedly different from NPs of 'bulk' PtRu alloys or monometallic Pt and Ru mixtures of identical loadings and compositions (Kua & Goddard, 1999).

Among platinum based bimetallic nanocatalysts, nPtRu was shown to be an effective PO-like nanozyme (Kung et al., 2014). It is worth mentioning that the nature of supporting materials co-immobilized with nPtRu is crucial for the electrochemical oxidation of H_2O_2, namely for sensitivity of the electrode and limit of detection (LOD).

Different carbon nanomaterials (carbon powder, graphene, and graphene foam or porous grapheme) positively affect the electroactivity of nPtRu due to enhanced availability of electrochemical active surface resulting in higher mass transport of reactants. The characteristics of nPtRu bimetallic nanocatalysts on various forms of carbon toward the H_2O_2 detection are presented in Table SI.1 (Supplementary Information). The most effective was the porous graphene.

The nPtRu-based electrodes proposed by Kung (Table SI.1) having a superior conductivity, a low LOD and a high sensitivity to H_2O_2 provide a new opportunity for electrode materials with enhanced performance in biosensing. But these electrodes have a narrow linear range and a rather high (+0.32 V) applied potential. To avoid interfering effects during analysis of real samples using enzymatic ABSs, the lowest applied potential is desirable.

Thus, a number of mono- and bimetallic NPs were synthesized on the surface of GE by electrochemical deposition and their ability to react with H_2O_2 was tested. The most effective nPtRu/GE chemosensor

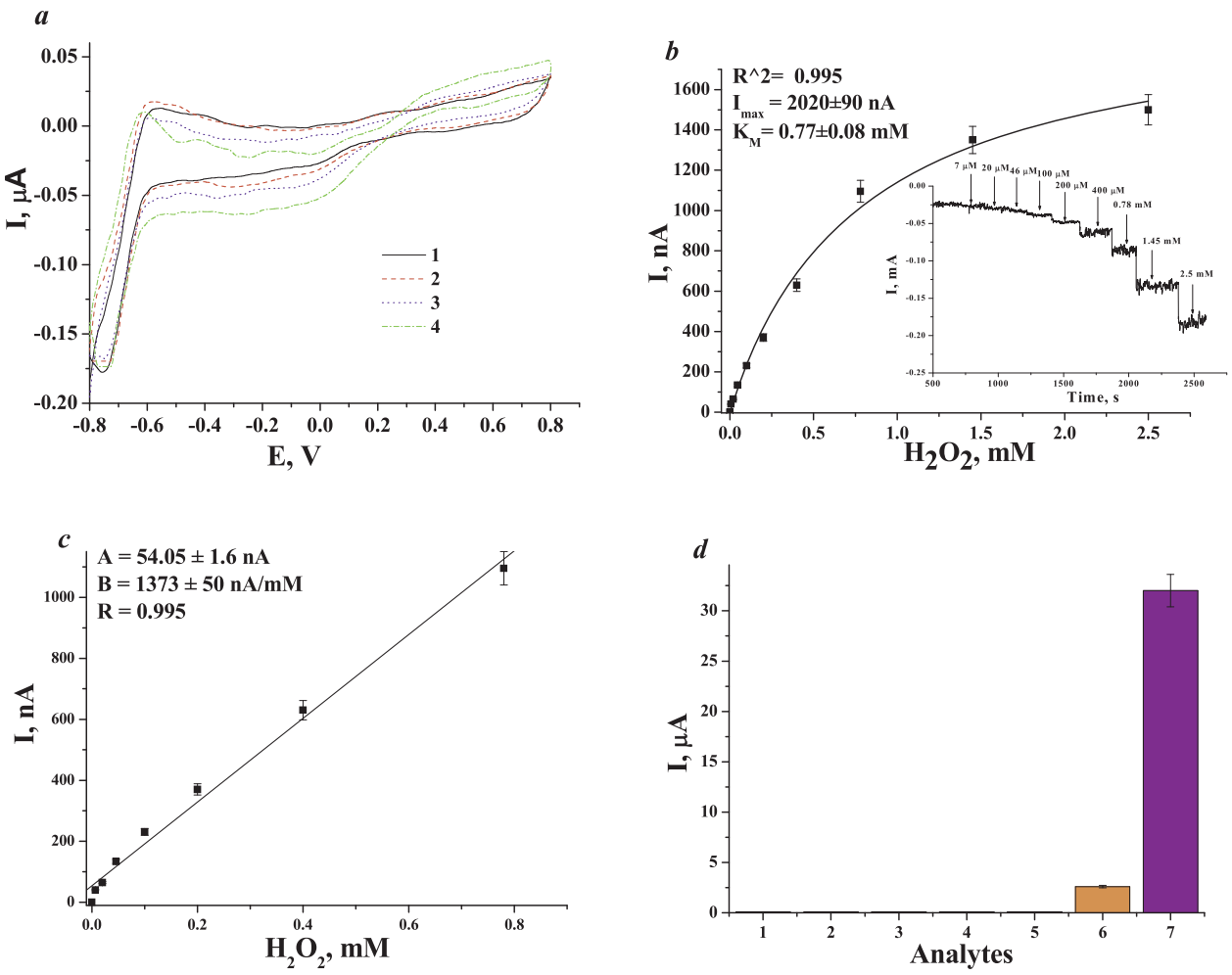

Fig. 2. Amperometric characteristics of nPtRu/GE: *a* – the cyclic voltammograms of H_2O_2. The final concentrations of analyte (mM) in 30 mM PB, pH 7.5 were 0 (**1**); 0.15 (**2**); 0.3 (**3**); 0.6 (**4**); *b* – dependence of amperometric signal and chronoamperometric response (the insert) on concentration of H_2O_2 upon the subsequent additions of analyte; *c* – calibration graph for H_2O_2 determination; *d* – selectivity to different 1 mM chemicals: **1** – MA; **2** – methanol; **3** – ethanol; **4** – formaldehyde; **5** – glucose; **6** – ammonium chloride and **7** – H_2O_2. Remark to (d): GE with the diameter 9 mm was used.

for H_2O_2 was selected and characterized.

3.2. Fabrication and characterization of bioelectrodes

Selective to hydrogen peroxide nPtRu/GE coupled with AO or recombinant AMO appear to be a good candidate for ABSs on alcohols and MA (Supplementary Information, Fig. SI.3).

To form an enzymatic layer, an aliquot of enzyme solution was dropped on the surface of the nPtRu/GE and covered with a cathodic polymer (CP) according to the experimental part (2.6). The calibration curves for the developed ABSs (see subdivision 2.6) and the corresponding chronamperograms (insert) are shown in Fig. 3*a–d*. The calibration was performed by the stepwise addition of a standard analyte solution. The bioanalytical characteristics of both ABSs are presented in Table 2.

The ABSs were tested in the range of concentrations up to 1.8 mM for both substrates (Fig. 3*a*, *c*).

Stability and selectivity of the developed ABSs are presented in Fig. SI.4 (Supplementary Information). To test a stability of the AO-ABS, the current responses to 0.2 mM ethanol in 50 mM PB, pH 7.5 for a period of 16 days were detected. The current response of the AO-ABS was shown to decrease by 80% for a period of 14 days and the half-life of this ABS was about 10 days. Storage stability of the AMO-ABS was measured by addition of 0.5 mM MA. A half-life of this ABS is 14 days (Fig. SI.4. a). It is worth mentioningthat the keeping free enzymes in 50 mM PB, pH 7.5 leads to a quick loss of their enzymatic activities. These conditions were shown to be non-optimal for AO and AMO storage, see subdivision 2.2. Besides, the inactivation rates of free enzymes in buffer solution are 2-fold higher, than in the case of immobilized enzymes in ABS.

A selectivity of the ABS to the target analyte is of a great importance, especially for analysis of real samples. In this paper, a selectivity of the constructed ABS was estimated in relative units (%) as a ratio of the detected signal to the value of the highest current response for the natural substrate (Fig. SI.4.*b, c*).

In case of the AO-ABS, neither glycerol nor glucose elicited any signals while some primary alcohols and formaldehyde induced essential analytical signals (Fig. SI.4.*b*). It should be noted that possible impact of methanol, propanol and formaldehyde on ethanol assay is not important due to a very low content of the listed compounds in natural foods, including wine, yogurts, *etc*. (Rehm et al., 2014; Tulashie, Appiah, Torku, Darko, & Wiredu, 2017). DMA and TMA, which are structurally similar to MA, did not produce positive signals with AMO-ABS (Fig. SI.4.*c*).

The novel mono-enzyme AO and AMO-based ABSs for ethanol and MA determination have some advantages over the conventional bienzyme- and cell-based ABSs constructed by us earlier with the same enzymes (Table 2). The proposed ABSs are highly sensitive, stable, easy-to-use and cost-effective, in addition, the AMO-ABS is very selective to its natural substrate MA.

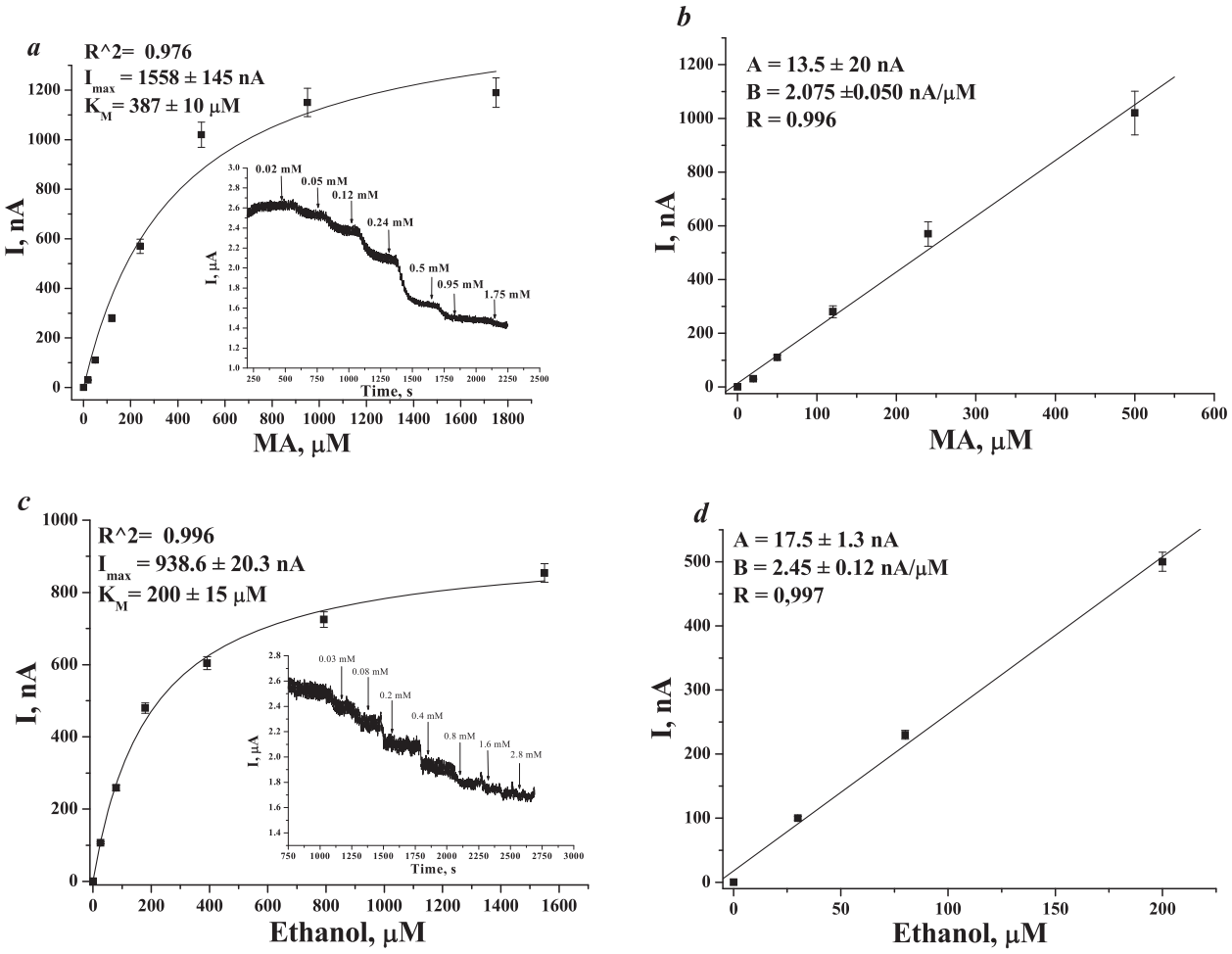

Fig. 3. Bioanalytical characteristics of AMO/nPtRu/GE (*a*, *b*) and AO/nPtRu/GE (*c*, *d*) under applied potential of −100 mV at 22 °C. Chronoamperometric current responses (the insert) and calibration curves upon subsequent additions of MA (*a*, *b*) and ethanol (*c*, *d*).

It is worth mentioning that nPtRu-containing electrodes were fabricated earlier as chemosensors for methionine (Tavakkoli, Soltani, & Khorshidi, 2017) and for ethanol (Xiao, Zhao, Zeng, & Zeng, 2009; Hajian et al., 2015). Both sensors for ethanol have long-term stabilities and large linear ranges for ethanol determination in model solutions but there are no data about the possibility of their application for real samples analysis. Besides, these electrodes have similar sensitivities to ethanol ($A{\cdot}M^{-1}{\cdot}m^{-2}$): 566 (Hajian et al., 2015) and 210 (Xiao et al., 2009) in comparison with 336 (our AO/nPtRu/GE).

3.3. Assay of MA and ethanol in food samples

The assay of aliphatic amines and ethanol in environmental, food and biological samples is crucial due to their use in industry and their presence in living organisms. MA is a result of natural degradation of proteins, amino acids, and other nitrogen-containing compounds (Tseng & Graves, 1998). MA usually accumulates in high amounts in fish, especially of *Gadoid* species. If such food is consumed in combination with nitrate-containing products, dangerous carcinogens N-nitroso derivatives are formed (Rehbein et al., 1995). The standard approaches for analysis of amines are HPLC, colorimetry, laser spectroscopy, fluorometry, gas chromatography coupled with mass spectrometry, chromatography as well as enzymatic methods (Ordóñez, Troncoso, Carmen, Raquel, & Callejón, 2016; Biji, Ravishankar, Venkateswarlu, Mohan, & Srinivasa Gopal, 2016). The usage of enzymatic methods is limited because of the absence of commercial AMO

Table 2
Comparison of the developed amperometric biosensors on alcohols and methylamine.

Biocomponent	Analyte	Potential, mV	Linear range, mM	LOD, μM	K_M, mM	Sensitivity, $A{\cdot}M^{-1}{\cdot}m^{-2}$	Response time, s	Stability (50%), days	Reference
AO-PO/Os polymer	Ethanol	−50	0.5–2.0	15	1.94	29	60	16	Smutok et al. (2006)
AO/nAu/PO- permeabilized yeast cells	Methanol	−50	Up to 2.0	N.d.	1.93	N.d.	80	30	Karkovska et al. (2017)
AO/nPtRu	Ethanol	−100	0.025–0.20	3	2.0	336	70	10	Current paper
AMO-PO	MA	−50	0.015–0.06	2.8	0.039	1450	10	15	Stasyuk et al. (2014)
AMO-PO nAu		−50	0.015–0.15	6	0.15	700	10	30	
AMO/nPtRu		−100	0.02–0. 60	2.5	0.387	284	60	14	Current paper

N.d. – Not detected.

Table 3
Determination of ethanol and MA in the samples of food products using bioesensor and reference methods.

Samples	Analyte	Difference, %	Biosensor, mM	Reference, mM
Pigfish	MA	0.582 ± 0.014	0.550 ± 0.008	+5.8
Fish "Tilapia"		0.276 ± 0.002	0.290 ± 0.014	−4.8
Wine "Chianti"	Ethanol	2620 ± 110	2710 ± 90	−3.3
Kefir "Halychyna"		23.4 ± 1.10	22.6 ± 1.31	+3.5

preparations. In previous paper, we have described for the first time the MA-selective bi-enzyme AMO/PO-ABS (Stasyuk et al., 2014).

Ethanol is a component of many commercial goods (spirit drinks, biochemicals, bioplastics, pharmaceuticals, cosmetics, solvents, paints) and a biofuel (Rehm et al., 2014). We have reported earlier about the development of AO-based methods for ethanol determination (Gonchar et al., 2001; Smutok et al., 2006; Karkovska, Stasyuk, Gayda, Smutok, & Gonchar, 2017).

In order to demonstrate the practical feasibility of the constructed ABSs, contents of MA and ethanol were determined in food samples using standard addition method (SAT). SAT is a type of quantitative analysis approach often used in analytical chemistry where a standard is added directly to the aliquots of the analyzed sample. Graphical SAT method is used in situations where sample components also contribute to the analytical signal. Estimation of MA or ethanol in the initial sample from the parameters of linear regression of the correspondent bioselective electrode was done, taking into account factors of dilution (Eq. (1)); calculation of the average concentration from two values:

$$C = \frac{A}{B}N, \quad (1)$$

where C – concentration of MA or ethanol, A and B – parameters of linear regression; N – dilution factor of the tested sample, shown in graph.

The results of MA assay in protein-free extracts of the frozen samples of fishes and ethanol assay in beverages are shown in detail in Fig. SI.5 (Supplementary Information).

The average contents of the analytes determined by the developed ABSs and the reference methods are presented in Table 3. The differences less than 5.8% between the results of both methods were observed. The contents of MA, determined in the protein-free extracts of fish samples (Table 3) and recalculated on the fish muscles tissues, were shown to be 5.0 and 3.8 mg/kg for pigfish and tilapia, respectively. These values are correlated with published data (Neurath, Dünger, Pein, Ambrosius, & Schreiber, 1977).

The concentrations of the tested analytes that were estimated using two methods have strong (R = 1) correlations (Fig. SI.6. *a, b*).

The developed ABSs were evaluated using statistical analysis. The Student's *t*-test did not reveal significant difference between the experimental values obtained in the sample analysis by the two methods. The calculated t-values for MA assay in pigfish (t_{calc} = 3.5) and in tilapia (t_{calc} = 1.75) as well as for ethanol assay in kefir (t_{calc} = 0.56) and in wine (t_{calc} = 2.12) were found to be less than the critical t-value (t_{crit} = 4.3) at 5% significance level. The assessment of the validation parameters of the method is given in Table SI.2 (See Supplementary Information).

Thus, the results of the accuracy research of the biosensors approach for MA and ethanol determination was shown to meet requirements for the permissible limits of ± 10.0%. These results demonstrate the possibility of application of the developed ABSs – AMO/nPtRu/GE and AO/nPtRu/GE for the analysis of MA and ethanol in food.

4. Conclusions

In summary, mono- and bimetallic NPs were synthesized on the surface of GE by electrochemical deposition and characterized as electroactive chemosensors for H_2O_2. The most effective PO-mimetic (nPtRu) was chosen for construction of two ABSs coupled with yeast oxidases: the AO and the recombinant AMO.

The novel oxidase/nPtRu-based ABSs have been developed for alcohols and MA detection. ABSs exhibit high sensitivities (336 $A \cdot M^{-1} \cdot m^{-2}$ to ethanol for AO-ABS; 284 $A \cdot M^{-1} \cdot m^{-2}$ to MA for AMO-ABS), broad linear ranges (25–200 μM ethanol and 20–600 μM MA), satisfactory storage stabilities and good selectivities toward natural substrates. We have demonstrated that the substitution of PO by nPtRu as a artificial PO in biosensing layer provides an improvement in the electrochemical properties of the electrodes and detection with the wider linear ranges.

The constructed ABSs were tested on the real samples of food: AO-ABS – for ethanol assay in wine and kefir, AMO-ABS – for MA assay in fish tissue extracts. High correlations of the obtained results and of the reference methods were demonstrated for both target analytes.

Declaration of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.01.117.

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