Article

Highly Sensitive Amperometric Sensor Based on Laccase-Mimicking Metal-Based Hybrid Nanozymes for Adrenaline Analysis in Pharmaceuticals

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Abstract: Nanozymes are nanomaterials which exhibit artificial enzymatic activities and are considered as alternatives to natural enzymes. They are characterized by good catalytic activity and high stability, as well as ease and low cost of preparation. In this study, the mimetics of laccase or “nanolaccases” (NLacs) were synthesized by a simple method of chemical reduction of transition metal salts. The NLacs were tested for their catalytic activity in solution and on the electrode surface. The most effective NLacs, namely nAuCePt and nPtFe, were found to possess excellent laccase-like activities capable of oxidizing the endocrine hormone adrenaline (AD). These NLacs were characterized in detail and used for the development of amperometric sensors for AD determination. The amperometric sensors containing the best NLacs, as well as a natural fungal laccase, were constructed. The most effective nAuCePt-containing sensor had good specificity in relation to AD and improved analytical characteristics. It possessed a 384-fold higher sensitivity than adrenaline (230,137 A·M⁻¹·m⁻²), a 64-fold lower limit of detection (0.025 μM), and a broader linear range (0.085–45 μM) in comparison with the sensor based on natural laccase. The constructed nAuCePt-containing sensor was successfully used for AD analysis in pharmaceutical formulation.

Keywords: laccase-like nanozyme; adrenaline assay; laccase; amperometric sensor; pharmaceuticals

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1. Introduction

The detection of biologically active toxic pharmaceutical compounds and their metabolites in surface waters cause great concern for humanity [1,2]. Several chemicals have already been found in raw urban wastewaters at concentrations up to μg·L⁻¹, which are dangerous for health [3].

Pharmaceutical manufacturing has become a significant source of harmful chemicals as pollutants of the environment. Even after treatment facilities, the concentrations of toxic compounds in the wastewaters of these factories may be 10–1000 times higher than safe values. In addition, households and hospitals make a significant contribution to the excretion of pharmaceuticals into urban wastewaters [4].

Among the chemicals detected in terrestrial and aquatic environments, some anti-inflammatories and analgesics, catecholamines and psychiatric drugs are the most dangerous for humans and wildlife [5]. As the influence of these contaminants in the environment is clearly recognized, early detection and quantification of the mentioned pharmaceuticals is necessary, not only for controlling the quality of water, especially drinking water, but also for the diagnosis and monitoring of various health conditions [4,6].

The catecholamines—noradrenaline, adrenaline (also called, epinephrine) and dopamine—are key neurotransmitters in the sympathetic nervous system, as they stimulate adrenergic receptors in a wide variety of cells [7]. High concentrations of adrenaline (AD) correlate well with glycogenolysis in the liver, hypoglycemia, myocardial infarction, lipolysis in the adipose tissue, and the heart contraction rate [8]. AD is an important biomarker for Parkinson's disease [9] and other malignancies [10]. Thus, AD and other catecholamines are secreted in excessive amounts by pheochromocytomas (tumors of the adrenal glands).

A variety of analytical techniques for the determination of AD has been reported—among them, liquid chromatography [11], spectrophotometry [12], capillary electrophoresis [13], fluorometry [14], high-pressure liquid chromatography, and circular dichroism [15]—which are characterized by low selectivity, high costs, and time consumption. Within the last few years, electrochemical methods for the analysis of pharmaceuticals, especially catecholamines, have become very popular. However, only a limited number of papers have reported the electrochemical detection of AD. Electrochemical approaches for AD analysis are preferable to laborious instrumental methods due to the simplicity of procedures and instrumentation, fast response, sensitivity, and low cost [16].

To construct amperometric biosensors, laccase preparations and different nanomaterials were used [17–28]. The proposed laccase-based sensors have been utilized to monitor the level of AD in pharmaceuticals, even though the practical application of such biosensors is often limited due to the impact of environmental compartments on the enzyme in the recognition layer [17,18].

Artificial enzymes with pseudo-laccase activity, especially nano-size laccase-like nanozymes (NZs) or “nanolaccases (NLacs)”, have preferential properties compared to natural enzymes. Thus, the search for new effective NLacs, which are promising for construction of electrochemical sensors, appears to be an urgent task [19–28].

In the current work, we report the synthesis of NLacs, their characterization, and their application in the development of amperometric sensors for the direct measurement of AD. The most effective sensors contain metal-hybrid NZs on the surfaces of graphite electrodes. The proposed sensor was successfully tested for AD determination in a pharmaceutical product.

2. Results and Discussion

2.1. Synthesis and Characterization of the NLacs

A number of metal-based composite materials were synthesized using the method of chemical reduction. The synthesized NZs were screened for their ability to oxidize ABTS in the solution (Table 1). A natural laccase preparation from *Trametes gibbosa* 1525 was used for comparison. It was shown that several tested NZs, especially nAuCePt and nFePt, possessed significant laccase-like activities. The most catalytically active NZs in the solution were characterized by scanning electron microscopy (SEM).

Table 1. Laccase-like activities of the synthesized NZs in solution.

No	NZ	Specific Activity, U·mg ^{−1}
1	nAuCePt	2.90 ± 0.25
2	nAuCe	0.85 ± 0.15
3	nPtFe	1.40 ± 0.10
4	nFePtPd	0.70 ± 0.05
5	nAuPtPd	0.30 ± 0.02
6	nCoPtPd	0.40 ± 0.02
7	Laccase	12.0 ± 0.11

Figure 1 presents the overall morphology of the formed particles. According to SEM images, nAuCePt have the shape of prisms, with sizes varying from 20 to 100 µm (Figure 1a–c). The obtained nPtFe have the shape of cubes, with sizes varying from 4 to

10 μm (Figure 1d–f). It was shown that the characteristic peaks on the XRM images corresponded to Ce^0 , Pt^0 , Au^0 , and Fe^0 (Figure 1c,f). It was demonstrated (the data are not shown) that the sizes of all the studied compounds do not satisfy the nanoscale criterion of being less than 100 nm in all three dimensions. In some cases, they are nanoscale only in one dimension (plate), while in the others, they exceed the criterion. This is most likely due to the aggregation of the initially formed NZs. To take this factor into account, we classify NZs as materials whose nanoscale is confirmed by physical methods for at least one dimension.

Figure 1. Characteristics of the nAuCePt (a–c) and nPtFe (d–f) NZs: SEM images (a,b,d,e); X-ray spectral microanalysis (c,f).

2.2. The Development and Characterization of the NZ-Modified Electrodes

Here, we proposed a new sensor for AD determination based on metal-hybrid NLacs immobilized on a graphite surface of the electrode (further marked as NZ/GE). Figure 2 shows a scheme of the chemical processes between AD (also called epinephrine) and NLacs. The synthesized NLacs (for example, nAuCePt) catalyze the oxidation of AD to adrenochrome. The last compound is reduced on the surface of GE under the potential of 50 mV. The resulting cathodic currents correlate directly with the concentration of AD in the sample solution.

Figure 2. The principal scheme of the AD determination.

The nanozymes nAuCePt and nPtFe were deposited on the top of a GE rod electrode (marked as nAuCePt/GE and nPtFe/GE, respectively), as described in Section 3.6, and their electro-catalytic activities toward AD oxidation were compared with laccase/GE. Figures 3–5 demonstrate the electrochemical characteristics of the developed sensors as current responses upon AD addition, namely cyclic voltamperograms (CV) with a scan rate of $50 \text{ mV} \cdot \text{s}^{-1}$, chronoamperograms, and calibration curves under optimal working potentials, which were determined from the CV.

Following the chronoamperograms for the constructed amperometric sensors (Figures 3b, 4b and 5b), we plotted the calibration curves for AD determination as illustrated in Figures 3c, 4c and 5c. The linear ranges for AD determination and sensitivities were calculated for all obtained modified electrodes (Figures 3d, 4d and 5d).

The CV for the nAuCePt/GE (Figure 3a) demonstrated that the cathodic reduction peak, contributed by AD decomposition, arose at potentials of approximately -50 mV (vs. Ag/AgCl). The CV for the nPtFe/GE (Figure 4a) demonstrated that the anodic oxidation peak arose at the potential of approximately $+250 \text{ mV}$. The chronamperometric dependences and calibration graphs for AD determination are shown in Figures 3 and 4b–d respectively for both types of NZs. As a control measure, the unmodified GE was tested, and no signal was detected upon AD addition (the data not shown).

Figures 3c, 4c and 5c present the maximal current responses on AD at substrate saturation (I_{max}) and the apparent Michaelis–Menten constants (K_M^{app}) to AD. nAuCePt/GE was shown to have the highest I_{max} (27-fold increase compared to that of laccase/GE) and the lowest K_M^{app} among the sensors reported here.

The analytical characteristics of the developed electrodes on AD in comparison with the known sensors are summarized in Table 2. As demonstrated in Table 2, for AD detection, not only laccase preparations but also various nanomaterials may be used [17–28]. Some of the reported sensors exhibit excellent analytical properties, having low limits of detection (2 nM [27] or 9 nM [26]) and wide linear ranges (for example, from $0.7 \text{ } \mu\text{M}$ to $1200 \text{ } \mu\text{M}$ [22]). However, the main disadvantages of these sensors are insufficient sensitivity [20] and high working potentials ($+400 \text{ mV}$) [27]. The nAuCePt/GE proposed by us was shown to possess the highest sensitivity to AD in comparison with the reported (bio)sensors, this value showing a 384-fold increase compared to that of laccase/GE (Table 2).

As a result of our study, we can conclude that nAuCePt and nAuPtPd possess excellent sensitivities and wide linear-ranges for AD detection and may, thus, be considered as promising mimetics of laccase in amperometric sensors.

Figure 3. Characteristics of nAuCePt/GE: CV as current responses upon AD addition up to 0 mM (1, black line), 4 μ M (2, red line), 8 μ M (3, blue line), and 16 μ M (4, green line) concentrations (a); chronoamperometric response (b), dependence of amperometric signal on concentration of AD (c) and calibration graph (d) under working potential -50 mV.

Table 2. Analytical characteristics of the developed laccase-like NZ/GE as chemosensors for adrenaline in comparison with the known sensors for AD assay.

Sensing Element	Potential, mV	Sensitivity, $A \cdot M^{-1} \cdot m^{-2}$	K_M^{app} , mM	Linear Range, μ M	LOD, μ M	Reference
Laccase/PtNPs/PtE ¹	+50	3570	–	up to 55	0.4	[17]
Laccase/PPy-PVS ² /PtE	–220	8000 ³	27×10^{-6}	0.1–1.0 1–10	0.01	[18]
AuNPs/PANI/GCE ⁴	–	–	–	0.4–40	0.08	[19]
IrOx/SPE ⁵	–	30	–	0.1–15	0.03	[20]
Graphite powder: laccase:Nujol: Pt-BMI.PF6 ⁶ /CPE ⁷	–	–	–	0.1–213	0.029	[21]
Graphite powder–carbon nanotube–EBNBH ⁸ complex–paraffin/GCE ⁹	+265	–	–	0.7–1200	0.216	[22]
MWCNT/CFE ¹⁰	–100	420	–	up to 100	2	[23]
Os-(PVP) ₁₀ /Nafion/GCE	–	316	0.51	2–113	–	[24]
MWCNT-CoTSPc/GCE ⁶	–	1860	–	3.0–15	0.450	[25]

Table 2. Cont.

Sensing Element	Potential, mV	Sensitivity, $A \cdot M^{-1} \cdot m^{-2}$	K_M^{app} , mM	Linear Range, μM	LOD, μM	Reference
MXene/GPE	+100	—	—	0.02–10 10–100	0.009	[26]
Aminated graphene/AgNPs/GCE	+400	—	—	0.916–184	0.002	[27]
Na[RuL ₂]/GCE			—	27–136	35	
SP/Na[RuL ₂]/cellulose acetate/CE	+100	—	—	27–272	1.3	[28]
SP/Na[RuL ₂]/MWCNTs/CE			—	54–272	0.35	
nAuCePt/GE	−50	230,137	0.13	0.085–45	0.025	
nAuPtPd/GE	+250	10,137	0.11	0.530–32	0.5	
nPtFe/GE	+250	4900	0.73	0.530–120	0.9	Current work
nFePtPd/GE	+250	3000	0.88	1.65–53	0.9	
nCoPtPd/GE	+250	2950	0.66	1.65–61	0.9	
Laccase/GE	+100	600	1.72	5–600	1.6	

¹ PtE—platinum-rod electrode; ² PPy-PVS—polypyrrole-polyvinylsulphonate; ³ calculated from the calibration graphs; ⁴ AuNPs/PANi/GCE—Gold nanoparticles/polyaniline Langmuir-modified glassy-carbon; ⁵ IrOx/SPE—iridium-oxide modified screen-printed electrode; ⁶ Pt-BMI.PF₆—PtNPs in 1-butyl-3-methylimidazolium hexafluorophosphate; ⁷ CPE—carbon-paste electrode; ⁸ EBNBH—2,2-[1,2-ethanediybis(nitriloethylidene)]-bis-hydroquinone double-wall carbon-nanotube; ⁹ GCE—glassy-carbon electrode; ¹⁰ MWCNT/CFE—carbon-film electrode (CFE) modified with multiwalled carbon-nanotubes (MWCNTs).

Figure 4. Characteristics of nPtFe/GE: CV as current responses upon AD addition up to 0 mM (1, black line), 4 μM (2, red line) and 8 μM (3, blue line), concentrations (a); chronoamperometric response (b), dependence of amperometric signal on concentration of AD (c) and calibration graph (d) under working potential +250 mV.

Figure 5. Characteristics of laccase/GE: CV as current responses upon AD addition up to 0 mM (1, black line), 4 μM (2, red line) and 8 μM (3, blue line) concentrations (a); chronoamperometric response (b), dependence of amperometric signal on concentration of AD (c) and calibration graph (d) under working potential +100 mV.

2.3. Characterization of the Most Effective nAuCePt-Based Electrode and Its Application

In general, the specificity of a recognition element in relation to the target analyte is a valuable characteristic for its use in sensors. The results of the selectivity test for the constructed nAuCePt/GE are presented in Figure 6. The NZ-based electrode was shown to be highly specific to AD. In the case of adding structurally similar compounds as well as solutions of glucose and sodium chloride, the signals of the sensor are insignificant (less than 5%). The latter compounds are often used as solubilizers in pharmaceuticals.

Figure 6. Selectivity for nAuCePt/GE. The test was performed with 2 mM (1) and 4 mM (2) solutions of different analytes under the working potential of −50 mV. The amperometric signal on AD being the highest was chosen as 100%.

To experience nAuCePt/GE as an AD-selective sensor for determination of AD concentration, a commercial pharmaceutical product was analyzed under optimal condi-

tions. The tested sample in the ampoule was the solution for the injection of “Adrenaline (Epinephrine)”. For the analysis of AD in the sample of the pharmaceutical, the method called the “Standard Addition Test” (SAT) was used for two dilutions (Figure 7). The principles of the graphical analytical method SAT, as well as the algorithm for the calculation of an analyte’s concentration from the parameters of linear regression, were described earlier [29].

Figure 7. Determination of AD concentration in the sample of the pharmaceutical “Adrenaline (Epinephrine)” solution (for injection in the ampoule) using the nAuCePt-based chemosensor with the SAT method.

The values of AD concentration in the commercial sample, which were estimated using the developed sensor and declared by the manufacturer, are presented in Table 3. Both concentrations of AD correlate well (Table 3), with an error of less than 5%.

Table 3. Results of AD estimation in pharmaceutical “Adrenaline”.

Commercial Sample	Concentration of AD		CV, %	Producer	
	Estimated	Declared, %			
	mM				%
Adrenaline (Epinephrine) solution for injection, ampoule	5.57 ± 0.25	0.185	0.180	2.7	Pharmaceutical factory “Darnytisia”, Kyiv, Ukraine

3. Materials and Methods

3.1. Reagents

Gold(III) chloride solution (HAuCl_4), Cerium(III) hydrocarbonate ($\text{Ce}(\text{HCO}_3)_4$), chloroplatinic acid (H_2PtCl_6), palladium chloride (PdCl_3), copper(II) sulfate (CuSO_4), iron(III) chloride ($\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$), Cobalt(II) chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), adrenaline, o-dianisidine, ascorbic acid, sodium borohydride (NaBH_4), catechol, glucose, D,L-tyrosine, L-Lysine, L-glutamic acid, D,L-phenylalanine, phenol, bisphenol, NaCl, Nafion (5% solution in 90% ethanol), 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and all other reagents and solvents used in this work were purchased from Sigma-Aldrich (Steinheim, Germany). All solutions were prepared using ultrapure water obtained with the Milli-Q® IQ 7000 Water (Merck KGaA, Darmstadt, Germany).

3.2. Isolation and Purification of Laccase

Laccase was isolated from the fungus *Trametes gibbosa* 1525. The cells were cultivated at 28 °C in YPD medium supplemented with 1% sucrose and 0.05 mM Cu^{2+} for 5 days. To remove the mycelia, the fungal culture was passed through filter paper, and a cultural

liquid was used for enzyme isolation. Laccase with activity of $\geq 10 \text{ U} \cdot \text{mg}^{-1}$ was purified according to the described method [30].

The laccase activity was determined in kinetic mode spectrophotometrically at 420 nm (Shimadzu, Kyoto, Japan) as described earlier [30]. As a substrate, 0.5 mM ABTS in 50 mM sodium acetate buffer solution, with a pH of 4.5, was used.

3.3. Synthesis and Characterization of Laccase-like NZs

The tri-metallic composites of nCoPtPd, nFePtPd, and nAuPtPd were obtained according to the protocol, which was proposed for nNiPtPd synthesis [31], using NaBH_4 as a reducing agent.

To obtain nPtFe, solutions of 50 mM H_2PtCl_6 and 50 mM FeCl_3 were mixed (1:1 ratio by volume), then 100 mM NaBH_4 was added up to 5 mM while stirring for 20 min. nPtFe were shown to be formed in the reaction mixture after a 24 h incubation without stirring.

nAuCePt were obtained by the reduction of metal ions from the appropriate salts with ascorbic acid. The following scheme was used for this NZ synthesis: 5 mL 10 mM HAuCl_4 , 5 mL 10 mM $\text{Ce}(\text{HCO}_3)_4$ and 1 mL 100 mM ascorbic acid were mixed. After vigorous stirring for 5 min, 5 mL 10 mM H_2PtCl_6 and 0.1 mL 100 mM ascorbic acid were added and the reaction mixture was stirred for 20 min.

All formed NZs were collected by centrifugation, washed with water, and stored as a suspension in water until use at $+4^\circ\text{C}$, as described earlier [32,33].

The pseudo-laccase activity was determined in the same way as for natural laccase [30]. Morphological analyses of the samples were performed using scanning electron microscopy (SEM) as described in [32].

3.4. Apparatus, Measurements, and Statistical Analysis

The amperometric sensors were evaluated using constant-potential amperometry. The amperometric experiments were carried out in triplicates, using a three-electrode configuration with a graphite rod as a working electrode, a Pt-wire as a counter electrode and an Ag/AgCl/KCl (3 M) as a reference electrode. The analytical characteristics of the sensors were estimated as described earlier [29,31–33].

3.5. Functionalization and Characterization of the Electrodes

To construct the NZ-based amperometric sensor, the surface of the graphite electrode (GE) was modified with 5 μL of NLac solution (1 mg/mL) or 5 μL of enzyme solution (12 U/mL). The dried film was covered with 5 μL of 0.5% Nafion solution. After drying at room temperature, the modified GE was washed with 50 mM of acetic buffer, with a pH of 4.5. The electrochemical properties of the functionalized GEs were studied by cyclic voltammetry (CV) and chronoamperometry, and the graphs of the dependences of the amperometric signals in increasing concentrations of AD were compared. The most electroactive NLac/GE was chosen and tested as an amperometric sensor for AD determination.

3.6. Determination of AD in Pharmaceutical Formulation

nAuCePt/GE, as the most effective amperometric sensor on AD, was tested on the real sample of the pharmaceutical formulation, “Adrenaline (Epinephrine) solution for injection, ampoule” (“Darnytsia”, Kyiv, Ukraine). Each assay, being performed for two dilutions of the sample, was repeated 3 times. The analytical results were statistically processed using the OriginPro 2021 software (OriginLab, One Roundhouse Plaza, Suite 303, Northampton, MA 01060, USA). The manufacturer has declared the following composition of this pharmaceutical product: 1.8 mg/mL of adrenaline tartrate in water for injections. The tested solution additionally contained sodium metabisulfite (E223) and sodium chloride.

4. Conclusions

In the current research, a number of hybrid metal-based nanoparticles were synthesized by chemical methods and screened for their ability to oxidize AD in solution. The best catalytically active nanoparticles were chosen as artificial laccases. Their structure and electrochemical behavior on electrodes were characterized. The nAuCePt, as the most effective laccase-like nanozyme, was studied in more detail. It was successfully used as a mimetic of laccase in an amperometric sensor for AD analysis in pharmaceuticals. Due to high sensitivity and specificity, the developed nAuCePt-based sensor may be promising in the diagnosis of some diseases, including tumors of the adrenal glands or mental disorders.

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Abbreviations

ABTS	2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate)
AD	Adrenaline
CV	Cyclic voltammetry
GCE	Glassy-carbon electrode
GE	Graphite electrode
I_{max}	Maximal current response on tested analyte at substrate saturation
K_M^{app}	Apparent Michaelis–Menten constant
LOD	Limit of detection
LR	Linear range
NPs	Nanoparticles
NZ	Nanozyme
Me ¹ /Me ²	Core/shell Nanozyme; where Me ¹ —core and Me ² —shell
Laccase	Natural laccase from <i>Trametes gibbosa</i> 1525
SAT	Standard addition test
SEM-XRM	Scanning electron microscopy coupled with X-ray microanalysis
PtE	Platinum-rod electrode
PPy-PVS	Polypyrrole–polyvinylsulphonate;
GCE	Glassy-carbon electrode;
IrOx/SPE	Iridium-oxide modified screen-printed electrode;
Pt-BMI.PF6	PtNPs in 1-butyl-3-methylimidazolium hexafluorophosphate;
CPE	Carbon-paste electrode
EBNBH	2,2-[1,2-ethanediylbis(nitriloethyldiyne)]-bis-hydroquinone double-wall carbon-nanotube;
GCE	Glassy-carbon electrode
MWCNT/CFE	Carbon film electrode (CFE) modified with multiwalled carbon-nanotubes (MWCNTs)
AuNPs/PANi/GCE	Gold nanoparticles/polyaniline Langmuir-modified glassy-carbon electrode.

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