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Highly sensitive amperometric biosensor based on alcohol dehydrogenase for determination of glycerol in human urine

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ABSTRACT

In this paper we report the development of a highly sensitive amperometric glycerol biosensor based on alcohol dehydrogenase from *Pseudomonas putida* immobilized on graphite electrode modified with carbon nanotubes and a redox mediator tetrathiafulvalene. The designed biosensor demonstrates very high sensitivity towards glycerol ($29.2 \pm 0.9\ \mu A\ mM^{-1}\ cm^{-2}$), low limit of detection (18 μM), linear range from 0.05 to 1.0 mM, high selectivity and satisfactory stability. Biosensor has been successfully used for the determination of glycerol concentration in buffer solutions as well as in the human urine samples. Received results shows a satisfactory agreement with the control measurements carried out using colorimetric commercially available glycerol determination assay kit, thus developed biosensor can be successfully applied for measurements of glycerol concentration in human urine and may be a fast, attractive and non-invasive tool for the determination of glycerol.

1. Introduction

Glycerol (1,2,3-propanetriol) is one of the most abundant chemical compounds in nature as well as a key compound participating in many biochemical pathways. A determination of glycerol is essential both in human blood serum as well as urine. It has been shown, that increased concentration of glycerol in human blood serum may be related to serious medical conditions, e.g., type 2 diabetes [1], could interfere with clinical assays, which may result in an inaccurate diagnosis [2] and, in addition, measuring glycerol concentration in human serum is critical for the control of the level of triacyl glycerides [3]. Measuring glycerol concentration in human urine is important for the detection and diagnosis of hyperglycerolemia [4], which in turn may cause deadly diseases such as acute pancreatitis [5]. Moreover, the accurate assay of glycerol is important for the anti-doping control tests used by World Anti-doping Agency (WADA), since glycerol expands plasma volume and masks prohibited doping usage [6]. Standard methods for glycerol determination usually include liquid/gas chromatography [7] as well as mass spectrometry, which are usually expensive as well as requires highly trained personnel. Those issues have raised the importance for the development of the fast, accurate, easy-to-use and inexpensive biosensors for the determination of glycerol.

One of the best type of sensors are amperometric biosensors, since they are rapid, precise, and can be used to monitor the concentration of the analyte in real time [8,9]. Hence, an amperometric glycerol biosensors based on glycerol dehydrogenase, NAD and a redox mediators including Meldola Blue, Nile Blue or Toluidine Blue as well as the polyaniline film in the presence of hexacyanoferrate have been designed successfully and used for the determination of glycerol concentration in buffer solutions and/or grape juice [10,11]. In addition, two consecutive enzymes (glycerokinase and glycerol-3-phosphate oxidase) have been applied for an amperometric glycerol assay [12]. Other amperometric glycerol biosensors were developed also [13–15]. However, in many cases, issues regarding the operation of glycerol biosensors still limit their performance capabilities. In some cases, detection of glycerol was carried via oxidation of produced hydrogen peroxide, which may result in interference due to high oxidation overpotential of hydrogen peroxide [11]. The usage of soluble mediators (e.g., hexacyanoferrate(II)/hexacyanoferrate(III)) may cause their dissolution from the electrode surface and thus result in complex kinetics [16], while a dual enzyme system, though usually increases the sensitivity, however, may require a complex immobilization matrix for the stabilization of both enzymes [17]. Still a major challenge is the application of developed biosensors for clinical monitoring, i.e., measurements of glycerol concentration in real-world clinical samples, especially, urine. Due to a complex composition of human urine (e.g., wide pH range, high conductivity, wide number of redox-active compounds), multiple non-specific signals

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may arise, and thus result in unreliable measurements of biosensor [18].

In this work we report an amperometric biosensor for the determination of glycerol based on the alcohol dehydrogenase from *Pseudomonas putida*, a redox mediator tetrathiafulvalene and single-walled carbon nanotubes. A single enzyme biosensor for glycerol determination involving bacterial ADH, capable of two-step glycerol oxidation to glyceric acid, co-immobilized onto CNT film modified graphite rod electrodes was addressed for the first time in this work. Moreover, our designed biosensor demonstrated very high sensitivity towards glycerol (29.2 ± 0.9 μA mM^{-1} cm^{-2}), wide linear range of 0.05–1.0 mM, low operation potential (0.05 V *vs* SCE), high selectivity and satisfactory stability. Moreover, the performance of biosensor was tested in human urine samples and received results were compared with commercially available glycerol determination assay kit demonstrating a satisfactory agreement.

2. Materials and methods

2.1. Materials

Graphite rods with diameter of 3 mm were obtained from Sigma. Biotech CE dialysis tubing (MWCO 100–500 Da) were purchased from Spectrum Laboratories. Tetrathiafulvalene (TTF), single–walled carbon nanotubes (CNT), Nafion® 117 solution, Free Glycerol Reagent, bovine serum albumin, inositol, sorbitol, mannitol, galactitol, glycerol, glucose, L-ascorbic acid, urea, acetylsalicylic acid and $K_3Fe(CN)_6$ were purchased from Sigma-Aldrich. 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS) and anhydrous $CaCl_2$ were obtained from Serva and Roth, respectively. Acetic acid, acetonitrile, NaOH and HCl were purchased from Riedel-de Haën. All chemicals were analytical grade and were used in this study without any further purification. Buffers and other solutions were prepared using deionized water (18 MΩ cm) purified with Crystal E Ultrapure system from ADRONA SIA. Suspension of CNT was prepared as followed: 3.5 mg of CNT were dispersed in 1 ml of deionized water and sonicated for 2 h using 500 W ultrasonic homogenizer from Cole-Parmer. All used solutions were diluted using deionized water unless stated otherwise.

Heme containing PQQ-dependent alcohol dehydrogenase IIG (ADH) was purified from *Pseudomonas putida* HK5 [19] cells as described [20]. The crystal data of ADH is available from the Protein Data Bank (reference No. 1YIQ) [21]. The final concentration of the enzyme in stock solution was estimated by Lowry method with bovine serum albumin as a standard and was equal to 20 mg ml^{-1} [22]. The specific activity of purified enzyme was 500 U mg^{-1} towards glycerol (K_M 2.4 mM), it is also known that ADH is capable to oxidize ethanol (K_M 33.2 mM) [19].

2.2. Measurements

All measurements were performed at 25 ± 0.1 °C maintained using thermostat from Lauda and were carried out in a buffer solution containing 50 mM acetic acid, 50 mM TRIS and 1 mM $CaCl_2$. This solution is further referred as a working buffer solution unless stated otherwise. pH value of working buffer solution was adjusted by addition of NaOH or HCl.

The electrochemical measurements were carried out in a conventional three-electrode system using saturated calomel electrode (SCE, 0.244 V *vs* NHE) as a reference electrode. All potential values in the text are presented *vs* SCE. Platinum plate was used as a counter electrode. Measurements were carried out using Gamry Reference 600™ potentiostat in the 2.4 ml or 20 ml cells under stirring, unless stated otherwise.

Current densities (further in the text referred as current) were calculated by dividing the measured current by geometrical surface area of graphite rod electrode (surface area approximately 0.071 cm^2).

Apparent Michaelis constant (K_M) and turnover number (k_{cat}) of the enzyme in a solution were calculated by observing the decrease of absorbance of $K_3Fe(CN)_6$ at 420 nm using Thermo Evolution 300 spectrophotometer with 10 mm quartz cuvette at 25 °C. Reaction mixture contained 0–25 mM of glycerol, 1 mM of $K_3Fe(CN)_6$, 192 nM ADH and buffer solution pH 7.5.

Measurements using *Free Glycerol Reagent* (Sigma-Algrich) were carried out at 30 °C according to protocol from the supplier with a slight modification. Briefly, 400 μl of *Free Glycerol Reagent* was mixed with 5 μl of standard/sample solution. The mixtures of solutions were kept for 10 min allowing reaction to occur resulting in a violet product. The calibration curve was carried by observing absorbance intensity at 540 nm after the addition of fixed concentration of glycerol. Afterwards concentration of unknown sample solutions was calculated according to the calibration curve.

2.3. Electrodes preparation procedures

For electrochemical measurements a cylindrical surface of graphite rod electrodes was isolated using heat-shrinking tube and a planar side was polished using emery paper (200 μm). Then the electrodes were thoroughly rinsed with deionized water and dried using pure argon. Four types of electrodes were prepared. The first type of electrodes (C/ADH) were prepared when 2.5 μl of ADH solution was dropped on planar side of dried rods and left to dry at room temperature. The second type of electrodes (C/TTF/ADH) were prepared by dropping 10 μl of solution containing 0.2% (*w/w*) TTF in acetonitrile and let for solvent to evaporate at room atmosphere. Later 2.5 μl of ADH solution was dropped on the electrode and left to dry at room temperature. The third type of electrodes (C/CNT/ADH) were prepared by placing 10 μl solution containing 3.5 mg ml^{-1} CNT in water and leaving to dry at room temperature. Afterwards 2.5 μl of ADH solution was placed on the electrode and left to dry at room temperature. The fourth type of electrodes (C/CNT/TTF/ADH) were prepared by dropping 10 μl solution containing 3.5 mg ml^{-1} CNT in water and leaving to dry at room temperature. Afterwards, 10 μl solution containing 0.2% (*w/w*) TTF in acetonitrile were placed on the dried rod and allowed solvent to evaporate at room atmosphere. Then 2.5 μl of ADH solution was placed on the electrode and left to dry at room temperature. Finally, all of the electrodes were modified by placing 10 μl solution containing 0.5% Nafion® on the surface and allowed to dry at room temperature. Before measurements all electrodes were thoroughly rinsed with working buffer solution and covered by 500 Da cut-off dialysis membrane. The enzyme was immobilized by a simple adsorption method due to simplicity of the method [23]. Moreover, this method did not require any additional reagents which could affect catalytical performance of the enzyme and may also increase time required for preparation of the electrodes. The dialysis membrane served as protective layer for immobilized enzyme, which prevented electrode from the leakage of the enzyme [24]. It also protected the surface of the electrode from other macromolecules which could cause electrochemical interference during operation of the electrode [24].

3. Results and discussion

3.1. Electrochemical activity of enzyme electrodes

At beginning, the electrode composed of ADH immobilized on graphite electrode (C/ADH) was created and analyzed (Fig. 1A). The CV of C/ADH electrode at pH 7.5 did not show any significant electrochemical process from –0.1 to 0.4 V. The addition of 50 mM glycerol increased anodic current at electrode potential higher than 0.08 V. The anodic current reached maximum value of 0.31 mA cm^{-2} at 0.4 V. The subtraction of the background current produced bioelectrocatalytic current of 0.044 mA cm^{-2}. This experiment demonstrated that ADH is capable of direct electron transfer (DET) with graphite electrode. However, the DET was not as effective as using modified gold electrode

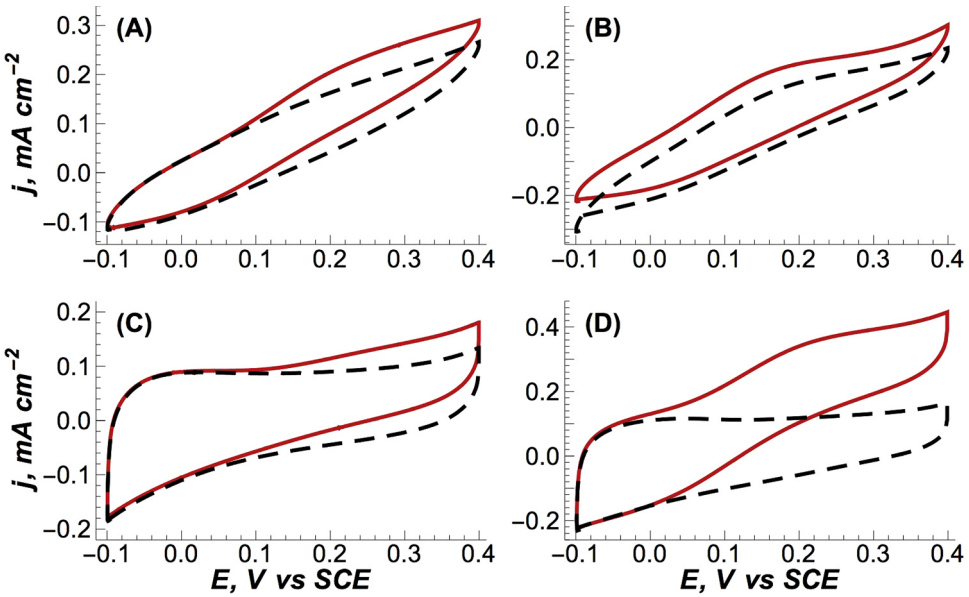

Fig. 1. Cyclic voltamperograms (CV) of C/ADH (A), C/TTF/ADH (B), C/CNT/ADH (C) and C/CNT/TTF/ADH (D) electrodes in the absence (dashed) and in the presence (solid) of 50 mM of glycerol at pH 7.5. During measurements, solutions were not stirred. Potential scan rate was 10 mV s^{-1}.

[25]. To improve the performance of the electrode a redox mediator, i.e., tetrathiafulvalene (TTF) was used. It was demonstrated previously that TTF is an effective electron transfer mediator in glucose oxidase catalysis [26]. The first oxidation step of TTF takes place from 0.0 to 0.2 V and results in formation of TTF^+. At higher than 0.2 V potential a second oxidation step starts, leading in a formation of water soluble TTF^{2+} [27]. TTF was successfully used for creation of various biosensors at operational potential up to 0.2 V [13,28].

The CV of the electrode composed of TTF and ADH (C/TTF/ADH) showed an insignificant current change neither in absence nor presence of glycerol at electrode potential from –0.1 to 0.4 V (Fig. 1B). Most likely the adsorbance of TTF limited the available surface area for enzyme and resulted in no bioelectrocatalytic process. For this reason, we introduced single-walled carbon nanotubes, which should significantly increase the effective surface area.

The adsorbtion of single-walled carbon nanotubes on a graphite electrode followed by the immobilization of ADH changed the enzyme electrode response. The CV of the electrode containing CNT and ADH (C/CNT/ADH) in the absence of glycerol demonstrated no significant electrochemical process (Fig. 1C). However, the difference in current between forward and backward scans was significantly noticeable, indicating a capacitive current which is typical after the immobilization of high surface area CNTs [29]. Hence, the C/CNT/ADH electrode demonstrated an increased anodic current at a potential greater than 0.1 V in the presence of 50 mM of glycerol. The anodic current reached a maximum value of 0.18 mA cm^{-2} at 0.4 V. The bioelectrocatalytic current calculated as a difference between the currents in the presence and in the absence of glycerol was only 1.07 times greater compared to C/ADH electrode.

Finally, the addition of TTF (C/CNT/TTF/ADH) significantly improved the response of electrode. The CV of C/CNT/TTF/ADH electrode showed a low 0.11 mA cm^{-2} average current at potential range from 0.2 to 0.4 V in the absence of glycerol (Fig. 1D). The addition of 50 mM of glycerol resulted in a significant increase of the anodic current at a potential greater than 0 V. To add more, the anodic current became almost constant at a potential higher than 0.2 V. The average value of reached anodic current was 0.39 mA cm^{-2}. The bioelectrocatalytic current was calculated to be 0.28 mA cm^{-2} after the subtraction of background current. Thus, we demonstrated that the application of CNT in combination with TTF was the most effective method for the development of high-performance electrode.

The C/CNT/TTF/ADH electrode was chosen for further chronoamperometric analysis as a possible glycerol biosensor due to the highest bioelectrocatalytic currents demonstrated as well as the lowest operational potentials.

The activity dependence of the C/CNT/TTF/ADH electrode on an applied potential was determined by measuring an electrode sensitivity towards glycerol in the range of 0–0.4 mM at different electrode potential ranging from 0 to 0.2 V (Fig. 2A). At least three independent measurements were made at fixed potential to ensure statistical significance. In a whole potential range C/CNT/TTF/ADH electrode did not demonstrate any significant response change. The response was almost constant in a potential range from 0.025 to 0.175 V, thus small alterations in a potential value should not affect the performance of the

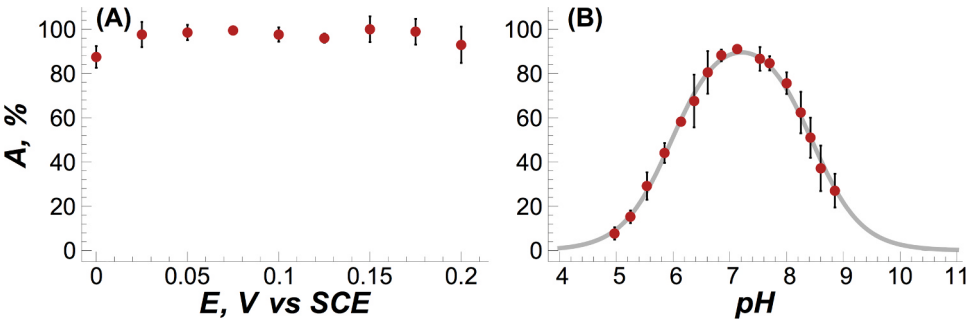

Fig. 2. Dependence of relative activity of C/CNT/TTF/ADH electrode on potential (A) and pH (B). Error bars represents standard deviation (n = 3).

electrode. Consequently, a potential of 0.05 V was chosen for the further analysis due to a high electrode activity as well as minimization of possible interference of other substances present in real samples. Since TTF has a broad redox potential (0–0.2 V) depending on electrode type as well as composition and pH of buffer solution, we showed that 0.05 V potential is sufficient for the re-oxidation of formed TTF during enzymatic reaction. We measured the open circuit potential of C/CNT/TTF/ADH electrode with 20 mM glycerol and found it to be around − 0.08 V. Thus, to ensure that TTF can be oxidized and reduced we carried chronoamperometric measurements when oxidation potential was set to 0.05 V and reduction potential to − 0.08 V (Supporting Information (SI) **Fig. S1**). It can be seen from the curve that at 0.05 V an anodic current is present indicating oxidation of TTF to TTF^+. Afterwards, the immediate change in potential to − 0.08 V induces a negative current indicating the reduction of TTF^+ back to TTF. Thus, from those measurements in can be seen that TTF can be oxidized at 0.05 V and this potential is suitable for the operation of biosensor. An electrode without TTF did not demonstrate similar redox pattern.

The dependence of C/CNT/TTF/ADH electrode activity on pH was determined by measuring electrode sensitivity towards glycerol in the range of 0–0.4 mM at 0.05 V electrode potential and pH ranging from 5.0 to 8.9 (Fig. 2B). At least three independent measurements were made at each pH to ensure statistical significance. The electrode exhibited a bell-shaped response dependence on pH. Experimental data were fitted to two proton transfer model with three protonated forms of enzyme in a whole pH range [30]. The calculated pK_a values were 5.86 ± 0.06 and 8.55 ± 0.06 demonstrating a broad operation range (at pH range from 6.6 to 7.7 the electrode exhibited higher than 80% relative activity). Consequently, pH 7.5 was chosen for further analysis due to a high electrocatalytic activity as well as similarity to pH of most physiological media.

To determine apparent parameters of the C/CNT/TTF/ADH electrode (K_M and j_{max}) the dependence of an electrode current on glycerol concentration at 0.05 V was measured (Fig. 3). Once a steady blank current was observed, the concentration of glycerol was gradually increased from 0.2 to 23.4 mM and a time-current relationship was recorded. Afterwards a curve demonstrating the dependence of current on glycerol concentration was fitted to Michaelis-Menten equation (Fig. 3 Inset) and apparent parameters of the electrode (K_M and j_{max}) were determined. The apparent K_M was 4.0 ± 0.1 mM and j_{max} was 74 ± 3 μA cm^{-2}. The calculated K_M value was higher than K_M for ADH in a solution (2.30 ± 0.05 mM). The increased K_M may be associated with the mass transfer limitations [31], which are favorable in biosensing applications.

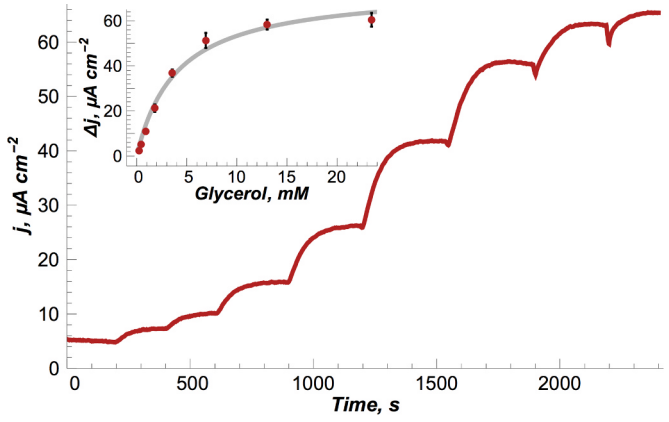

Fig. 3. The dependence of current of C/CNT/TTF/ADH electrode on glycerol concentration (0.2–23.4 mM) at 0.05 V potential and 7.5 pH. The inset shows the dependence of current change on concentration of glycerol fitted to Michaelis-Menten model. Error bars represents standard deviation (n = 3).

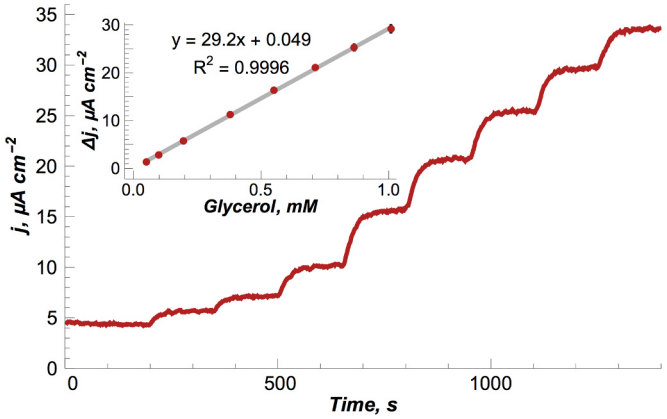

Fig. 4. Calibration curve of glycerol biosensor (C/CNT/TTF/ADH electrode) at 0.05 V potential and pH 7.5. Error bars represents standard deviation (n = 3).

3.2. Calibration curve, sensitivity and detection limit of glycerol biosensor

To determine crucial parameters (linear range, sensitivity, limit of detection) of glycerol biosensor based on C/CNT/TTF/ADH electrode the dependence of biosensor current on glycerol concentration at 0.05 V and pH 7.5 was measured (Fig. 4). Once a steady blank current was observed, the concentration of glycerol was linearly increased from 0.05 to 1 mM and a time-current curve was recorded. Afterwards a calibration curve demonstrating the dependence of current on glycerol concentration was fitted to linear model (Fig. 4 Inset) and sensitivity of the biosensor was determined to be 29.2 ± 0.9 μA mM^{-1} cm^{-2}, which was one of the highest reported in the literature for this type of biosensor.

The effect of dialysis membrane on biosensor sensitivity and response time was evaluated using C/CNT/TTF/ADH electrode covered with and without dialysis membrane. The dependence of current on glycerol concentration (0.05 V and pH 7.5) was measured (SI **Fig. S2** and **Fig. S3**). Once a steady blank current was observed, the concentration of glycerol was linearly increased from 0.05 to 0.5 mM and a time-current curve was recorded. Afterwards calibration curves demonstrating the dependence of current on glycerol concentration were fitted to linear model (SI Fig. S2 Inset and Fig. S3 Inset) and sensitivity of C/CNT/TTF/ADH electrode with and without dialysis membrane were compared. The sensitivity of C/CNT/TTF/ADH electrode with membrane was determined to be only 8% lower than the electrode without dialysis membrane. The effect of dialysis membrane on response time was calculated by measuring time which was needed to reach 95% of steady current after a change in glycerol concentration. The response time for C/CNT/TTF/ADH electrode with and without dialysis membrane were calculated to be 41 ± 4 s and 20 ± 3 s, respectively. Results indicated that dialysis membrane did not cause a significant decrease in neither sensitivity nor response time of the electrode.

The limit of detection (LOD) was calculated according to the $3 \cdot s_b / m$ criteria, where m is sensitivity and s_b is the standard deviation of the blank current before the addition of glycerol. The calculated value of LOD for created biosensor was 18 μM.

For comparison, the parameters of other amperometric biosensors for glycerol determination are represented in Table 1. Most of the compared biosensors were targeted to the determination of glycerol in wines or during fermentation processes and their operational potential varied from –0.35 to 1.3 V; while majority operated in a range from –0.05 to 0.2 V. However, to minimize the interference of other substances, while measuring real samples, operational potential is desired to be around 0 V. Gamella et al. created a glycerol biosensor by using two/three enzymes and TTF as a redox mediator to determinate glycerol concentration in wine at the potential of 0 V. Despite one of the

Table 1
Amperometric biosensors for the determination of glycerol reported in literature and their characteristics.

Electrode	Enzyme(s)	Redox mediator	Operational potential, V	Linear range, mM	Sensitivity, μA mM^{-1} cm^{-2}	Limit of detection, μM	Stability, % of remaining activity	Sample	Ref
Platinum	GK/GPOx	Oxygen	0.65 *I*	0.002–2	–	0.5	–	Alcoholic fermentation	[12]
Carbon paste	GlyDH	–	0.05 *II*	0.001–0.1	2.04	0.4	80 (after 3 days)	Plant–extract syrup	[32]
Carbon rod	PQQ-GlyDH	Phenazine methosulphate	0.3 *II*	up to 8	4.8	–	50 (after 3 days)	Wines	[14]
Carbon rod	GlyDH	Os-complex	0.2 *III*	0.001–0.2	31.69*	1	80 (after 30 days)	Wines	[33]
Carbon film	GK/GPOx	Poly-(neutral red)	–0.35 *IV*	0.01–0.14	6.37	7.12	63 (after 37 days)	Wines	[34]
Screen printed	GlyDH/ NADHOx	Prussian Blue	–0.05 *V*	0.001–0.04	9.73*	0.11	70 (after 120 injections)	Alcohol fermentation from grapes	[35]
Screen printed	GlyDH	$Zr(HPO_4)_2$, Meldola blue and Reincke salt	0.1 *V*	0.01–0.1	0.37*	2.6	34 (after 14 days)	Fermentation of grape juice	[36]
Gold disk	GlyDH/DP	TTF	0.15 *VI*	0.001–0.02	21	0.4	87 (after 51 day)	Wines	[13]
	GK/GPOx/HRP		0 *VI*	0.001–0.01	25	0.31	46 (after 8 days)		
Gold disk	GlyDH	DET via FeS	1.3 *I*	1–25	0.79	168	–	–	[15]
		DET via PQQ			0.45	336			
Carbon rod	PQQ–ADH	TTF	0.05 *IV*	0.05–1.0	29.2	18	20 (after 30 days)	Human urine	This work

GK – glycerokinase; ***GPOx*** – glycerol-3-phophate oxidase; ***PQQ-GlyDH*** – PQQ dependent glycerol dehydrogenase; ***GlyDH*** – NAD-dependent glycerol dehydrogenase; ***DP*** – diaphorase; ***HRP*** – horse radish peroxidase; ***PQQ-ADH*** – PQQ-dependent alcohol dehydrogenase.
I – *vs* Ag/AgCl; *II* – *vs* Ag/AgCl (saturated); *III* – *vs* Ag/AgCl (0.1 M KCl); *IV* – *vs* SCE; *V* – *vs* Ag/AgCl (pseudo); *VI* – *vs* Ag/AgCl (3 M KCl); * – FIA mode.

best sensitivities (25 μA mM^{-1} cm^{-2}) among all published glycerol biosensors, it had a narrow linear range which may cause extra work while preparing samples before measurement, as well as used a complex bienzyme/trienzyme systems which may increase the cost of biosensor [13]. Our created biosensor among others had a wide linear rage at low 0.05 V potential as well as exhibited second highest sensitivity. Probably the most remarkable achievement is a high sensitivity while using single enzyme – alcohol dehydrogenase, as well as utilization of the biosensor in human urine. Typically, high sensitivity biosensors use complex multiple enzyme systems or involves techniques for signal amplification. It can be seen from Table 1 that biosensors based on single dehydrogenases usually have lower sensitivities.

3.3. Scheme of biosensor action

The principal scheme of biosensor design is demonstrated in Fig. 5A and implies oxidation of glycerol to glyceric acid catalyzed by ADH [25], thus avoiding the reactive by-product glyceraldehyde. During the oxidation of glycerol, the reduction of PQQ takes place and inner electron transfer occurs. Consequently, a *c*-type heme group receives the electrons from the active site of the enzyme [37]. Finally, the heme is oxidized by TTF^+ resulting in a formation of TTF. The electrochemical re-oxidation of formed TTF is detected by electrode and depends on the concentration of glycerol (Fig. 5B).

3.4. Interference

Some compounds, which may cause interference on the biosensor response, were investigated at 0.05 V electrode potential and pH 7.5. The influence of inositol, sorbitol, galactitol, mannitol, acetylsalicylic acid (aspirin), urea, glucose, ethanol and L-ascorbic acid was tested (SI Fig. S4). None of these substances resulted in a noticeable interference with the exception of ethanol and L-ascorbic acid. The addition of 0.1 mM of ethanol caused an increase in the current by 0.49 μA cm^{-2} and the addition of 0.1 mM of L-ascorbic acid resulted in 1.43 μA cm^{-2} increase in the current. Nevertheless, biosensor was 6 and 2 times more sensitive towards glycerol than to ethanol and L-ascorbic acid, respectively. These results represent that the complex samples should be analyzed with extra care due to a couple limitations: (i) urine samples should be collected from sober patient; (ii) to ensure more accurate analysis, urine samples should be tested for L-ascorbic acid if higher concentration of L-ascorbic acid is suspected. We have also investigated the effect of mediator (TTF) on all the substances and only L-ascorbic acid had small interference (data not shown).

3.5. Glycerol concentration measurements

To confirm the reliability and practical application of biosensor for the determination of glycerol, the analysis of artificial and real samples was carried out. Artificial samples were produced by diluting stock solution of glycerol using working buffer solution to prepare solutions containing 0.25, 0.5 and 0.75 mM of glycerol. Once the steady blank current was reached, 50 μM of glycerol was added and amperometric response observed. Then the steady–state current was reached again, a sample was added and a change in current was registered.

Urine sample was taken from a male subject without any known addictions or diseases. In a healthy non-athlete human an average glycerol concentration in urine is around 17 μM [38], thus the LOD of our created glycerol biosensor should be improved to ensure the accurate analysis of glycerol in healthy subjects. However, it is known that when glycerol concentration in plasma reaches value of 0.33 mM or higher (normal values 0.016–0.145 mM) urinary glycerol concentration starts to increase and linearly corelates with glycerol concentration in serum [38,39]. In clinical cases (e. g. hyperglycerolemia) concentration of glycerol in serum ranges from 2.6 to 7.8 mM [4]. In sports glycerol is used by athletes to mask prohibited blood doping thus

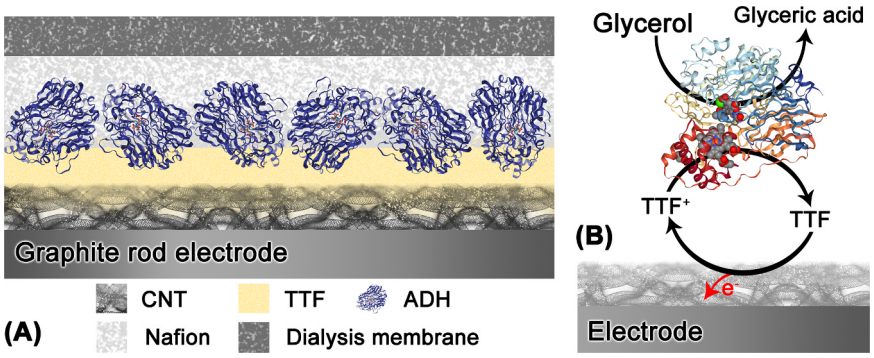

Fig. 5. A principal design scheme of biosensor (A) and a catalytical oxidation of glycerol (B).

Table 2
Determination of glycerol using glycerol biosensor and colorimetric method.

Sample	Glycerol added, mM	Glycerol found, mM		Recovery, %
		Glycerol biosensor	Commercial kit	
1☆	–	0.23 ± 0.02	0.25 ± 0.03	92.00
2☆	–	0.52 ± 0.03	0.51 ± 0.02	104.00
3☆	–	0.80 ± 0.03	0.79 ± 0.04	106.67
4★	–	Lower LOD (< 0.018 mM)	Not found	–
5★	1	1.04 ± 0.06	1.1 ± 0.1	104.03
6★	3	3.0 ± 0.2	3.1 ± 0.3	101.15
7★	5	5.3 ± 0.3	5.3 ± 0.4	105.01

☆ – Diluted solution of glycerol; ★ – Human urine.

according to WADA regulations concentration of glycerol in urine should not exceed value of 2.2 mM [40]. Therefore, our glycerol biosensor is suitable for the analysis of urine glycerol in clinical and anti-doping cases. To demonstrate reliability of biosensor we have measured concentration of glycerol in real samples using addition method (SI Fig. S5–S9). Measurements were carried out as described above. For comparison, a concentration of glycerol solutions was determined using commercially available *Free Glycerol Reagent*. Obtained results are presented in Table 2.

It is clear that the created biosensor is suitable for glycerol determination in human urine, since the measured concentrations of glycerol agree very well with the concentrations determined using the commercial kit.

3.6. Stability of glycerol biosensor

We also evaluated a storage stability of the glycerol biosensor by measuring the decrease of an electrode relative current at 0.05 V potential and pH 7.5 for 29 days (SI Fig. S10.). Between the measurements biosensor was kept in working buffer solution at 4 °C. Biosensor demonstrated a satisfactory stability. During the second day of operation electrode retained around 86% of initial activity. Apparent half-time of the biosensor response decrease was about 7 days. After nearly a month (29 days) biosensor still demonstrated more than 20% of the initial activity. Experimental data was fitted using exponential first order decay model [41] and showed a good agreement. A few methods could be used to increase stability of biosensor as a future challenge, however they have their own limitations. One of the key steps to increase the stability is stabilization of the enzyme. It could be done be multi-point covalent attachment of enzyme to the electrode using glutaraldehyde, thus helping to increase a thermal stability and stability towards exposure to organic solvents [42]. Despite pros of covalent attachment, it has negative impact on enzyme catalytical performance which could lead to higher LOD and narrowed linear range [43]. Another way to increase the stability of biosensor is to change the path of electron transfer. Partly soluble mediator (TTF) could be changed to conducting nanofibers or DET between enzyme and electrode could be achieved [25,44].

4. Conclusions

In summary, an amperometric glycerol biosensor was created based on alcohol dehydrogenase. The electrochemical analysis of enzymatic electrodes was carried out and the best design of enzymatic electrode was chosen, once ADH was immobilized on single-walled carbon nanotubes film modified by tetrathiafulvalene. A single enzyme biosensor for glycerol determination involving bacterial ADH, co-immobilized onto CNT film modified graphite rod electrodes was addressed for the first time in this work. It was demonstrated that biosensor had a wide linear range (0.05–1.0 mM), exhibited high sensitivity (29.2 ± 0.9 μA mM^{-1} cm^{-2}) with 18 μM detection limit at 0.05 V potential and 7.5 pH and was applicable for determination of glycerol in human urine.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2019.03.063.

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