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Review

Electrochemical biosensors based on multienzyme systems: Main groups, advantages and limitations – A review

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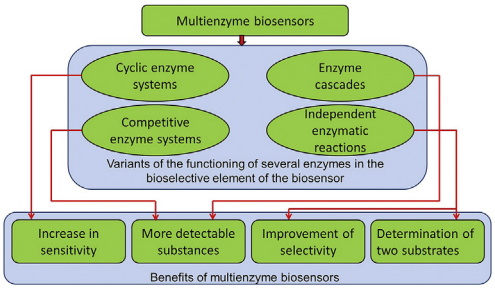
HIGHLIGHTS

- Biosensors based on cascade, cyclic and competitive enzyme systems are described.
- Advantages and limitations of multienzyme electrochemical biosensors are analyzed.
- Multienzyme systems allow an extension of the spectrum of detectable substances.
- An improvement of the biosensor sensitivity and selectivity is possible.

GRAPHICAL ABSTRACT



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ABSTRACT

In the review, the principles and main purposes of using multienzyme systems in electrochemical biosensors are analyzed. Coupling several enzymes allows an extension of the spectrum of detectable substances, an increase in the biosensor sensitivity (in some cases, by several orders of magnitude), and an improvement of the biosensor selectivity, as showed on the examples of amperometric, potentiometric, and conductometric biosensors. The biosensors based on cascade, cyclic and competitive enzyme systems are described alongside principles of function, advantages, disadvantages and practical use for real sample analyses in various application areas (food production and quality control, clinical diagnostics, environmental monitoring). The complications and restrictions regarding the development of multienzyme biosensors are evaluated. The recommendations on the reasonability of elaboration of novel multienzyme biosensors are given.

Contents

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1. Introduction

The electrochemical biosensors based on immobilized enzymes are one of the most popular and commercially successful groups of biosensors. The field of their utilization ranges widely - clinical diagnosis, agriculture, food industry, biotechnology, environmental monitoring, home appliances, etc. [1–5]. The electrochemical biosensors are popular due to their low cost, relatively quick and easy procedure of analysis, small size of the measuring unit (which is often portable). The development of screen printed planar electrodes which are cheap, sensitive and capable of miniaturization also contributes to the success of biosensors [6,7]. Depending on the type of transducer the electrochemical biosensors are divided into amperometric, potentiometric, conductometric, impedimetric, and other groups [8–12].

Commonly, enzymes serve as sensitive elements of electrochemical biosensors [13,14]. This is due to the fast catalysis of reactions, high specificity to the substrate and relatively long-term enzyme stability [15]. A large assortment of commercially available purified enzymes allows the creation of biosensors based on a wide variety of biochemical reactions. For these reasons, the enzyme biosensors are under very intensive investigation [16].

However, many enzymes do not consume or generate electrochemically active substances during catalysis and for this reason the enzyme-catalyzed reaction cannot be directly registered by the electrochemical transducer. Thus, the number of available enzymes for the biosensor development is limited as well as the range of target compounds. To solve the problem, several enzymes are often added to the biorecognition element of the biosensors, while the product of only one enzymatic reaction is being detected [14]. In traditional clinical diagnosis, cascade enzyme reactions have been used for more than 40 years in so-called coupled enzyme assays for the spectrophotometric determination of various substances, from the concentration of amino acids to the activity of biomarker enzymes [17,18]. Multienzyme systems are also utilized to create the aptamer- or antibody-based highly sensitive affine biosensors, in which the enzymatic reactions are used to detect the binding of the receptor molecule to the target substrate [19]. Besides analytical applications, the artificial multienzyme complexes (immobilized and free) are widely used in various biotechnological processes to catalyze the effective conversion of the initial substrate into the target product [20].

Application of the multienzyme systems in biosensors is aimed not only at the extension of the spectrum of target substances but also at the improvement of the biosensor performance (sensitivity in the first place). Furthermore, some enzymes can be inhibited by the products of the reaction, and multienzyme systems (i.e., enzymes cascades) can be helpful to prevent the enzyme inhibition. In enzyme cascades the product of one enzymatic reaction is immediately used by another enzyme, and thus the enzymes are not inhibited by the products of their reactions.

This paper presents an overview of existing biosensors based on immobilized multienzyme systems and analysis of their advantages and disadvantages. This work is structured according to different principles of coupling enzymatic reactions (sequential, cyclic, competitive or independent reactions). It is rather difficult to cover the whole range of commonly used multienzyme systems, therefore, only the most typical or interesting examples of multienzyme systems are under consideration in each section.

2. Biosensors based on enzyme cascades

Biosensors based on the cascade of enzymatic reactions are the most common group of multienzyme biosensors. The enzymes in this case are involved in a single sequence of reactions: the first enzyme (E_1) converts substance A into substance B, the second enzyme (E_2) converts substance B to C, and so on. The sequence ends when an electrochemically active product is formed, which can be registered by the biosensor directly or via a mediator:

$$A \xrightarrow{E_1} B \xrightarrow{E_2} C \rightarrow (\text{mediator}) \rightarrow \text{electrode}$$

Sometimes, different functions of the enzymes in cascade are highlighted. Enzymes at the beginning of cascade are called conversion or conversion-purpose, whereas the last enzyme, the reaction product of which is detected – indicator or indicator-purpose [21]. Yet, these terms are not used in this paper.

Although a multistage substances conversion involving multiple enzymes is possible, in most cases the number of enzymes is restricted to two, since usage of each extra enzyme complicates the biosensor and causes additional problems (see section 6 for details).

The examples of biosensors based on enzyme cascades are given in Table 1.

2.1. Enzyme cascades for carbohydrates detection

One of the most widespread groups of multienzyme biosensors are biosensors for the determination of carbohydrates. The reason is that redox enzymes (oxidases or dehydrogenases) mainly use monosaccharides as substrates, whereas oligo- or polysaccharides should be first split into monosaccharides. Commonly, glycoside hydrolases split the complex carbohydrate to glucose, which is oxidized by glucose oxidase (GOx) to form gluconolactone and hydrogen peroxide. The latter is detected amperometrically. Conductometric biosensors and biosensors based on pH-sensitive field-effect transistors detect the product of additional reaction:

Table 1
Examples of biosensors based on enzyme cascades.

Target substance	Type of transducer	Enzymes and catalyzed reactions	Working range	Limit of detection	Supposed application	Ref.
Proteins	Conductometric Au IDE	Proteinase K: protein → protein fragments Pronase: protein fragments → protein fragments + amino acids	TOC: 2.0 −11.0 μg L^{-1} Norg: 0.8–4.0 μg L^{-1}	TOC: 0.583 μg L^{-1} Norg: 0.218 μg L^{-1}	Biological research	[141]
Sucrose	Conductometric Au IDE	Invertase: sucrose + H_2O → β-D-fructose + α-D-glucose Mutarotase: α-D-glucose → β-D-glucose GOx: β-D-glucose + O_2 → D-gluconolactone + H_2O_2	0.002–5.0 mM	0.001 mM	Control of food quality	[142]
Lactose	Conductometric Au IDE	β-galactosidase: lactose + H_2O → galactose + α-D-glucose Mutarotase: α-D-glucose → β-D-glucose GOx: β-D-glucose + O_2 → D-gluconolactone + H_2O_2	Up to 1.0–3.0 mM	0.001 mM	Control of food quality	[37]
Maltose	Conductometric Au IDE	α-glucosidase: maltose + H_2O mutarotase: α-D-glucose → β-D-glucose GOx: β-D-glucose + O_2 → D-gluconolactone + H_2O_2	Up to 1.0–3.0 mM	0.001 mM	Control of food quality	[37]
L-malic acid	Amperometric Au disk electrode	L-malate dehydrogenase: L-malic acid + NAD^+ → oxaloacetate + NADH diaphorase: NADH + $2TFF^+$ → NAD^+ + 2TFF	5.2×10^{-7} – 2.0×10^{-5} M	5.2×10^{-7} M	Monitor malolactic fermentation for winemaking	[64]
L-lactic acid	Amperometric Au disk electrode	L-lactate oxidase: L-lactic acid + O_2 → pyruvic acid + H_2O_2 Horseradish peroxidase: H_2O_2 + 2TFF→ $2TFF^+$ + H_2O	4.2×10^{-7} – 2.0×10^{-5} M	4.2×10^{-7} M	Monitor malolactic fermentation for winemaking	[64]
Glycerol	Amperometric Au disk electrode	Glycerol dehydrogenase: glycerol + NAD^+ → dihydroxyacetone + NADH diaphorase: NADH + $2TFF^+$ → NAD^+ + 2TFF	1.0×10^{-6} – 2.0×10^{-5} M (0.092 −1.84 mg L^{-1})	4.0×10^{-7} M	Control of wine quality	[65]
Glycerol	Amperometric Au disk electrode	Glycerol kinase: glycerol + ATP → glycerol 3-phosphate + ADP glycerol-3-phosphate oxidase: glycerol 3-phosphate + O_2 → dihydroxyacetone-phosphate + H_2O_2 horseradish peroxidase: H_2O_2 + 2TFF→ $2TFF^+$ + H_2O	4.0×10^{-7} – 1.0×10^{-5} M (0.037 −0.92 mg L^{-1})	3.1×10^{-7} M	Control of wine quality	[65]
Cholesterol & its esters	Amperometric glassy carbon electrode	Cholesterol esterase: cholesterol ester + H_2O → cholesterol + fatty acid cholesterol oxidase: cholesterol + O_2 → cholestenone + H_2O_2 horseradish peroxidase: H_2O_2 + ferrocene → H_2O + ferrocenium	Up to 39 mg L^{-1}	No data	Blood analysis	[143]
Citric acid	Amperometric Pt electrode	Citrate lyase: citrate → oxaloacetate + acetate Oxaloacetate decarboxylase: oxaloacetate → pyruvate + CO_2 pyruvate oxidase: pyruvate + phosphate + O_2 → acetylphosphate + CO_2 + H_2O_2	1 pM–1 mM	0.5 pM	Foodstuffs analysis, monitoring of fermentation processes	[87]
Proteins	Amperometric Pt electrode	Trypsin: protein → L-amino acid + peptides Leucine aminopeptidase: peptides → L-amino acid + oligopeptide L-amino acid oxidase: L-amino acid + O_2 + H_2O → 2-keto acid + NH_3 + H_2O_2	0.1–4.0 g L^{-1}	0.1 g L^{-1}	Investigation of bioprocesses	[55]
Phosphate	Amperometric Pt disk electrode	Maltose phosphorylase: maltose + phosphate → α-D-glucose +β-D-glucose-1-phosphate Mutarotase: α-D-glucose → β-D-glucose GOx: β-D-glucose + O_2 → D-gluconolactone + H_2O_2	1–50 μM	1 μM	Ecological monitoring	[111]
Creatine	Potentiometric NH_4^+-selective electrode	Creatinase: creatine + H_2O → sarcosine + urea Urease: urea + H_2O → $2NH_3$ + CO_2	0.1–30 mM	$<10^{-5}$ M	Medical diagnostics	[144]
Arginine	Potentiometric NH_4^+-selective electrode	Arginase: L-arginine + H_2O → L-ornithine + urea Urease: urea + H_2O → $2NH_3$ + CO_2	0.1–30 mM	$<10^{-5}$ M	Medical diagnostics	[144]

ADP – adenosine diphosphate; ATP – adenosine triphosphate; GOx – glucose oxidase; IDE – interdigitated electrode; TTF – tetrathiafulvalene; TOC - total organic carbon; Norg - organic nitrogen.

gluconolactone spontaneously turns into gluconic acid, which dissociates into the acid residue and hydrogen ion. The latter causes a change in the pH and conductivity of the solution. Noteworthy, GOx is specific to β-D-glucose, whereas the hydrolysis of complex carbohydrates results in formation of α-D-glucose, which is converted into β-D-glucose by the intermediate enzyme mutarotase. A spontaneous α-to β-form transformation is also possible but it is relatively slow (tens of minutes). Therefore, mutarotase is required to obtain the biosensor with a reasonable response time.

One of the earliest amperometric biosensors of this type had a β-galactosidase-GOx system and was intended for the lactose determination [22]. The biosensor was characterized by a wide linear range – from 0.02 to 3.00 mM and was successfully used for lactose determination in food and urine. Other lactose-sensitive biosensors based on this enzyme system are also known [23–27]. A non-typical biosensor was based on the determination of the pressure that was created in the enclosed working cell during the hydrolysis of lactose and subsequent oxidation of glucose [28]. The operation of this biosensor was not affected by typical interferents, such as electrochemically active substances or substances with optical properties. The β-galactosidase-based biosensors were recently reviewed in detail [29]. Sensitivity to glucose is a significant drawback of the mentioned biosensors – glucose is common in most food products, which complicates detection of lactose. Galactose oxidase can be used instead of GOx as the second enzyme to eliminate the sensitivity of the biosensor to glucose and thus improve its selectivity [30].

More complex biosensors for sucrose determination contain three enzymes: invertase (splits sucrose to α-D-glucose and fructose), mutarotase, and GOx [31]. Such biosensors were also applied for the determination of mercury ions by inhibitory analysis [32]. Sucrose biosensor based on invertase/GOx cascade without mutarotase was described recently [33].

Instead of GOx, glucose dehydrogenase is sometimes used at the end of the enzymatic cascade. The dehydrogenase oxidizes β-D-glucose to D-gluconolactone and reduces $NAD(P)^+$ [34]. The

disadvantage of this reaction is that NAD^+ should be added to the working buffer. Moreover, amperometric detection of this reaction is rather difficult because NADH can be oxidized on the electrode and detected only at a high potential. Nevertheless, as a result of the gluconolactone spontaneous conversion described above, the solution conductivity and pH are being changed that allows development of potentiometric biosensors using pH-sensitive field-effect transistors [35]. Furthermore, a biosensor based on invertase and fructose dehydrogenase was proposed for the determination of sucrose [36].

Noteworthy, despite good characteristics of biosensors for carbohydrates determination (sufficient sensitivity and linear range, good selectivity and successful application for real samples), the significant disadvantage is their sensitivity not only to the target carbohydrate but also to glucose. Since GOx is the last link in the cascade of reactions, the sensitivity to glucose is higher than to the target analyte, thus, a total response to glucose and target carbohydrate is generated. Glucose is a common substance in both biological and food samples, therefore, to ensure accuracy of the biosensor operation, it is necessary either to remove glucose by the sample pretreatment, or to determine the glucose concentration separately. In the latter case, it is possible to determine the contribution of the target carbohydrate to the total biosensor response if the sensitivity of the biosensor to glucose is known. Biosensor arrays for the simultaneous determination of several carbohydrates work on this principle [37]. In this work an array of conductometric biosensors was developed for simultaneous determination of four carbohydrates (lactose, maltose, sucrose and glucose) in solution. Direct enzyme analysis carried out by the developed biosensors was highly sensitive to the corresponding substrates. The analysis lasted 2 min. The dynamic range of substrate determination extended from 0.001 mM to 1.0–3.0 mM.

2.2. Enzyme cascades for lipids determination

Multienzyme systems are used in biosensors for the detection of many groups of lipids (triglycerides, phospholipids, cholesterol esters), since there are no enzymes for the direct oxidation of these substances.

Triglycerides (fats) should be first hydrolyzed to fatty acids and glycerol. Next, glycerol is detected after several more enzymatic reactions. One of the first works in this direction described a biosensor based on a cascade of three enzymatic reactions for the determination of triglycerides in blood serum [38]. Triacylglycerol lipase split fats into glycerol and fatty acids. Glycerol dehydrogenase oxidized glycerol and reduced NAD^+, which in turn was oxidized by diaphorase with the simultaneous reduction of the mediator 2,6-dichlorophenolindophenol. The mediator was oxidized on the working electrode generating the biosensor response. In this work, only glycerol dehydrogenase and diaphorase were immobilized on the biosensor collagen membrane, whereas triacylglycerol lipase was added free to the test solution before measurements. The range of triglycerides detection was from 0.1 to 5 mg mL^{-1}, the analysis time was less than 15 min.

Subsequent biosensors included completely immobilized enzymes, e.g. lipase, glycerol kinase and glycerol-3-phosphate oxidase [39]. Lipase split fats to form glycerol, glycerol kinase phosphorylated glycerol (using ATP) to glycerol-3-phosphate, which was oxidized by glycerol-3-phosphate oxidase to form hydrogen peroxide. This biosensor had a response time of only 40 s and was successfully used to determine the content of triglycerides in blood serum of healthy and sick people. No decrease in the biosensor response was observed over 25-day multiple usage, which indicates high stability of the immobilized enzymes. In the next work, the authors improved response time of the biosensor to 2.5 s and storage stability to 240 days [40]. Recently, the biosensor based on the same three-enzyme system has been adapted to determine the fat in sebum [41]. To improve the availability of fats for enzymatic hydrolysis, they were transferred to the micellar form using a surfactant. Other works in this direction are also known [42].

Interestingly, glycerol oxidase can be used for the direct oxidation of glycerol instead of the two enzymes, but it is commercially inaccessible and it is not used in biosensors. Biosensors for the determination of triglycerides were reviewed in detail recently [43–45].

Biosensors for the cholesterol determination contain cholesterol oxidase. However, since cholesterol is often present in the form of esters, it is necessary to add the second enzyme – cholesterol esterase, which removes fatty acids from cholesterol and allows the biosensor to determine the total level of cholesterol and its esters in the blood [46–50]. Cholesterol was released from its esters by cholesterol esterase and was further oxidized by cholesterol oxidase to generate hydrogen peroxide. The latter reduces the silver ions to metallic silver, which deposited on the surface of gold nanoparticles. This reaction was detected by anodic stripping voltammetry. The biosensor had excellent analytical characteristics – linear range of the biosensor was from 0.00001 mg mL^{-1} to 5 mg mL^{-1}, LOD – 0.000001 mg mL^{-1}, and showed low interference from glucose, DL-cysteine, ascorbic acid, BSA, and uric acid. Such biosensors can be useful for medical diagnostics and food industry.

Alternatively, a biosensor based on cholesterol dehydrogenase instead of cholesterol oxidase was proposed, and the signal was detected amperometrically through a mediator [51]. The biosensor showed the linearity for 0.50–5 mg mL^{-1} cholesterol acetate. The minimum detection limit of the cholesterol was 0.50 mg mL^{-1}. While determining the cholesterol concentration in real samples the results were in good correlation with those obtained by commercial colorimetric test strip and clinical/laboratory methods.

2.3. Enzyme cascades for determination of peptides and proteins

Biosensors for the determination of peptides and proteins are based on several sequential enzymatic reactions [52]. The cascades of hydrolases and L-amino acid oxidase are most commonly used for the sequential splitting of the protein into amino acids and their following oxidation [53,54]. For example, the biosensor for the protein determination contained trypsin (hydrolyzed proteins to peptides), leucine aminopeptidase (hydrolyzed proteins to amino acids), and L-amino acid oxidase (oxidized amino acids and produced hydrogen peroxide) [55]. Detection of hydrogen peroxide was carried out using platinum electrode at a potential of +0.65 V (vs Ag/AgCl). The conditions of separate or simultaneous immobilization of leucine aminopeptidase and L-amino acid oxidase were studied in detail (trypsin was immobilized in a separate membrane). The linear range of detection of casein (used as a protein substrate) was 0.1–4 mg mL^{-1} and the response time was 4–9 min depending on the protein concentration. The biosensor response decreased by 20% relative to the initial value after one-month storage, by 75% – after 3 months. Noteworthy, due to the presence of L-amino acid oxidase, the total concentration of proteins and amino acids in the sample is actually determined.

2.4. Multienzyme biosensors for evaluation of enzyme activity

Some biosensors based on cascades of enzymatic reactions were used to evaluate the activity of free enzymes. A bienzyme (based on GOx and mutarotase) biosensor for the determination of the activity of free and immobilized invertase was proposed [56]. Free invertase split sucrose to glucose and fructose, and glucose was

converted by mutarotase and GOx to hydrogen peroxide. Actually, the biosensor was sensitive to α- and β-D-glucose, but it was applied for the evaluation of the invertase activity with the linear range from 10 to 70 U (per 50 ml). This biosensor was proposed for monitoring enzymatic processes in the food industry.

In the clinical diagnosis, the evaluation of activity of biomarker enzymes is a common assay. For example, an increased creatine kinase activity in blood serum is a marker of muscle damage, in particular myocardial infarction. Creatine kinase uses adenosine diphosphate (ADP) and creatine phosphate to form adenosine triphosphate (ATP) and creatine. To evaluate the creatine kinase activity, the biosensors based on two variants of sequential enzymatic reactions were proposed, each biosensor actually determines one of the products of creatine kinase [57]. The enzymatic scheme of biosensor is presented in Fig. 1. In one sequence, creatine was hydrolyzed with creatinase to sarcosine and urea. Sarcosine was further oxidized by sarcosine oxidase to glycine, formaldehyde and hydrogen peroxide. In another sequence, ATP was used by glycerol kinase to synthesize glycerol-3-phosphate, which in turn was oxidized with glycerol phosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. In both cases, hydrogen peroxide was detected amperometrically as a result of its decomposition on a working electrode at +0.6 V. To obtain higher sensitivity to hydrogen peroxide, the planar gold electrodes were metallized with platinum. To improve the biosensor selectivity, semipermeable poly(phenylenediamine)-based membrane was used, through which only hydrogen peroxide and similar small molecules could reach the electrode surface. The creatine kinase activity was measured using both variants of biosensor, the second variant was found to have a higher sensitivity. The enzyme activities in the range 95–2000 mU/ml were determined in 2–15 ml standard solution and artificial serum samples.

Later, the biosensor based on glycerol kinase/glycerol phosphate oxidase was described, with the addition of horseradish peroxidase (HRP) and a redox polymer containing Os(II/III), which allowed usage of low working potential (−100 mV) [58]. Low-cost disposable transducers were produced by vacuum deposition of gold electrodes on a polycarbonate substrate. One polycarbonate plate was sufficient to obtain 200 transducers, three electrodes on each. The working and auxiliary electrodes were metallized with platinum. This biosensor was disposable and demonstrated a high reproducibility of the measurement results. The authors investigated the biosensor analytical characteristics and measured the creatine kinase activity in one sample of blood serum.

Other important enzymes for clinical diagnosis are alanine aminotransferase (ALT) and aspartate aminotransferase (AST), the activities of which in the blood and their ratio indicate liver and heart diseases. To determine the activity of both enzymes, a two-biosensor system was proposed [59]. One of them contained pyruvate oxidase, which oxidized pyruvate and transmitted the resulting electrons to the electrode via a mediator. Pyruvate was formed as a result of the ALT catalyzed transamination of α-

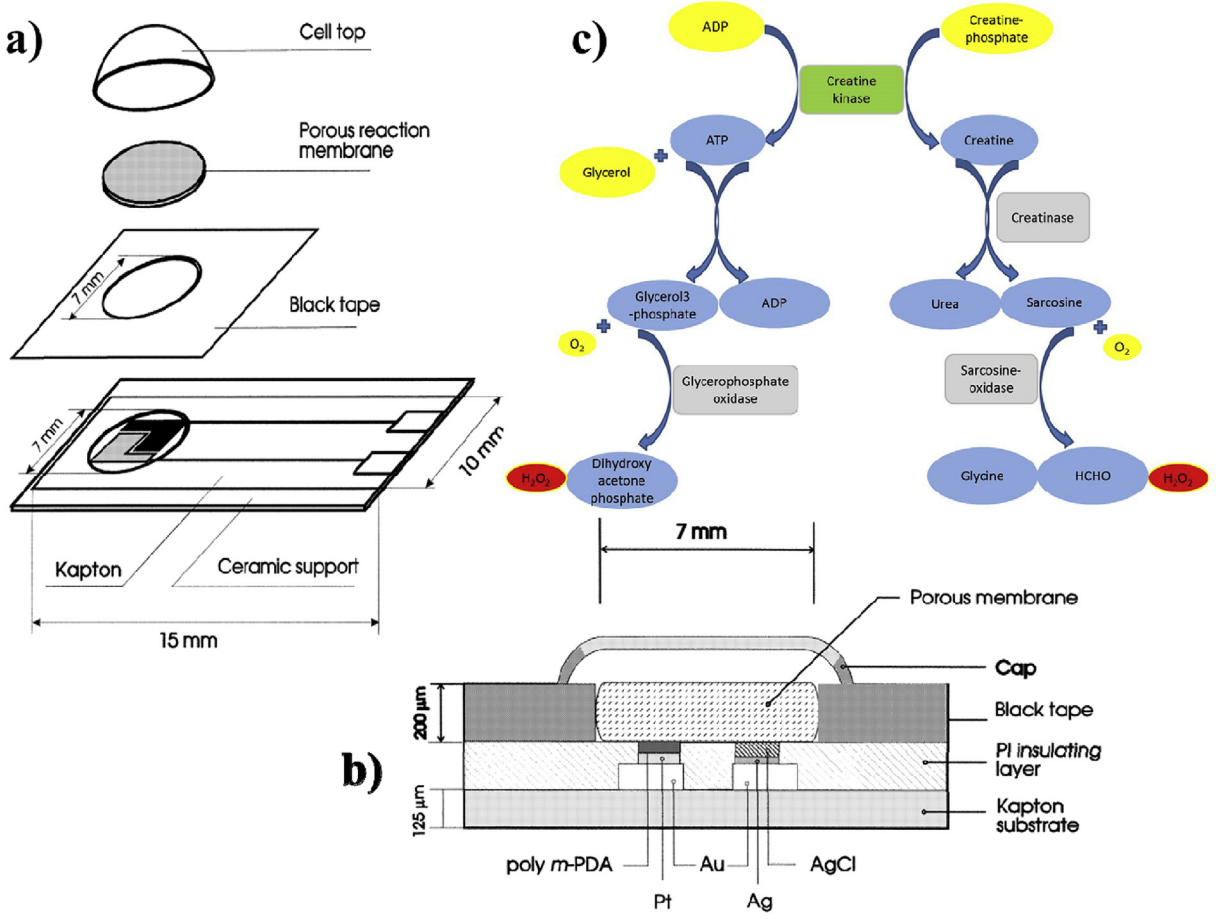

Fig. 1. Structure of an amperometric cell prepared by photolithography (a, b) and enzyme cascades (c) used in the biosensor for CK activity determination. Colors: green – target enzyme, yellow – additional substrates present in working buffer, red – reaction product directly detected by the biosensor, blue – other substances. Reprinted from Ref. [57], Copyright (1998), with permission from Elsevier. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

ketoglutarate and alanine. For the AST determination, a bienzyme system consisting of oxaloacetate decarboxylase and pyruvate oxidase was used. AST formed oxaloacetate during transamination of aspartate and α-ketoglutarate. Oxaloacetate decarboxylase split off carbon dioxide from oxaloacetate to form pyruvate, which was detected by biosensor using pyruvate oxidase. Thus, this biosensor actually determined the total activity of AST and ALT. When the ALT activity was measured by the first biosensor, it was possible to calculate the AST activity from response of the second biosensor. Multilayer immobilization of the enzymes on the electrode surface was used: two pyruvate oxidase monolayers were immobilized followed by a monolayer of the third enzyme (pyruvate oxidase for the ALT biosensor, and oxaloacetate decarboxylase for the AST biosensor). The biosensors were successfully used for the determination of the AST and ALT activities in blood serum. The linear ranges of detection were 30–240 U L^{-1}, and 43–480 U L^{-1}, respectively.

2.5. Multienzyme biosensors with horseradish peroxidase

HRP is often the last link in the cascades of reactions, in which hydrogen peroxide is produced. HRP accelerates the spontaneous reaction of the hydrogen peroxide decomposition and thus decreases the biosensor response time. Sometimes it is accompanied with an increase of the biosensor sensitivity.

A lactose biosensor with a three-enzyme system (β-galactosidase/GOx/HRP) immobilized on the surface of a glassy carbon electrode by polyethyleneimine was described [60]. The biosensor had lower limit of detection (LOD) of lactose about several micromoles per liter and fast response (2–4 s). It was successfully used to determine lactose in fresh milk.

The enzyme system consisting of cholesterol oxidase, cholesterol esterase and HRP has been used to create biosensors for the determination of cholesterol and its esters [61,62]. The biosensor based on a simpler version of this enzyme cascade without cholesterol esterase is also known, although it is not capable of detection of cholesterol esters [63].

Additionally, HRP can be an intermediate link to transfer electrons between the mediators and hydrogen peroxide, thereby decreasing the biosensor's working potential to the values of oxidation or reduction of the corresponding mediator. The potential decrease, in turn, slows down the oxidation of interfering substances on the biosensor surface and the accuracy of analysis increases. For example, using this principle, a lactate biosensor was developed, which was based on lactate oxidase, HRP and mediator tetrathiafulvalene [64]. The biosensor had a linear range in the region of low lactate concentrations – from 0.42 μM to 20 μM. The highest sensitivity to lactate was observed at a potential of −50 mV (vs Ag/AgCl). However, this mediator is also efficient in the electron transfer between an electrode and ascorbic acid, therefore this biosensor cannot be applied to samples containing ascorbic acid and lactate in comparable concentrations. However, this was not a problem when determining lactate in wines – 15 samples were analyzed, the results were confirmed with the commercial enzymatic method.

The same research group proposed a three-enzyme glycerol biosensor, which was based on glycerol kinase, glycerol phosphate oxidase and HRP, with the same mediator tetrathiafulvalene [65]. A potential of +0.0 V (vs Ag/AgCl) was used, and strong sensitivity to ascorbic acid was also observed. Nevertheless, glycerol concentration was analyzed in 12 samples of white and red wines. However, this biosensor showed rather poor characteristics – it had a narrow linear range of determination (from 1 to 10 μM) and low storage stability (after 8-day storage the response decreased twofold).

A biosensor based on invertase, GOx and HRP for the determination of sucrose was proposed, which was distinguished by multilayer immobilization of enzymes using high-affinity interactions [66]. GOx/HRP cascade was also used in other works [67–70]. Heavy metal ions can be also detected by these biosensors by evaluation of GOx inhibition [71]. Mathematical models of functioning of GOx/HRP system were described, which can be useful to find the optimal ratio of the two enzymes in the biosensor [72].

Thus, in most cases, an addition of HRP and a mediator accelerated the hydrogen peroxide decomposition and resulted in an improvement of the biosensor characteristics. However, a mediator and low applied potential do not always eliminate the impact of interfering substances. For biosensors based on platinum electrodes, the use of HRP is less relevant due to rapid oxidation of hydrogen peroxide on the platinum surface [73].

A biosensor based on D-amino acid oxidase and HRP was used for the detection of D-amino acids in bacterial samples [74]. Enzyme immobilization was improved by the modification of screen-printed electrode surface with multi-walled carbon nanotubes and gold nanoparticles with subsequent covalent immobilization of the enzymes.

Butyrylcholinesterase and HRP were used in an amperometric biosensor for the detection of malathion (pesticide) [75]. Butyrylcholinesterase catalyzed hydrolysis of butyrylthiocholine to thiocholine. The latter was oxidized by oxygen with a formation of two molecules of hydrogen peroxide. HRP reduced hydrogen peroxide and generated electrochemical signal. Malathion inhibited butyrylcholinesterase and thus biosensor response decreased proportionally to the pesticide concentration. The biosensor could reliably measure gas phase malathion concentrations between 6 and 25 ppbv and could be stored during 6 weeks without cold storage requirement. A similar approach was used for the detection of pesticide monocrotophos using acetylcholinesterase/HRP system [76].

HRP can transfer electrons to the redox hydrogels. For example, the acetylcholinesterase/choline oxidase/HRP system was used in the biosensor for acetylcholine determination [77]. In this system, acetylcholinesterase catalyzes the acetylcholine hydrolysis to choline. Choline oxidase catalyzes the oxidation of choline with formation of H_2O_2, and HRP transfers electrons from H_2O_2 to the working electrode via osmium-containing hydrogel. This enabled using a very low working potential of −50 mV (vs Ag/AgCl). However, the biosensor sensitivity to interfering substances was not evaluated. This enzyme system can be also used in biosensors to detect various toxins, which are the acetylcholinesterase inhibitors.

2.6. The use of diaphorase in the enzyme cascades with NAD(P)H formation

Diaphorase is another common terminal enzyme in biosensor enzymatic cascades. It is used together with the enzyme, which produces NADH or NADPH. Noteworthy, the name "diaphorase" is actually a designation for two separate enzymes – NADH dehydrogenase (EC 1.6.99.3) and NADPH dehydrogenase (EC 1.6.99.1). Diaphorase oxidizes NAD(P)H and simultaneously reduces the mediator, which is in turn detected by the biosensor. Therefore, diaphorases are the popular enzymes in biosensors based on NADH-producing dehydrogenases such as formaldehyde dehydrogenase [78], malate dehydrogenase [79,80], D-lactate dehydrogenase [81,82], L-lactate dehydrogenase [83], formate dehydrogenase [82], and others [84].

In particular, a malate biosensor was proposed, which is based on malate dehydrogenase, diaphorase, and mediator (tetrathiafulvalene) [64]. Malate dehydrogenase oxidized malate along with reduction of NAD^+. Diaphorase oxidized NADH and reduced

mediator, which was oxidized on the working electrode at a potential of +100 mV (vs Ag/AgCl). The linear range of the malate determination was from 0.52 μM to 20 μM. However, the disadvantage of the biosensor was effective oxidation of ascorbic acid by the mediator. Thus, the biosensor sensitivity to ascorbic acid was several times greater than that to malate. Nevertheless, the concentration of malate was determined in 9 samples of wine.

Diaphorase was used as a component of an amperometric biosensor array for control of fermentation processes [85]. The array consisted of biosensors for ethanol, formate, D- and L-lactate detection that were based on corresponding dehydrogenase and diaphorase. Practical applicability of the array was demonstrated by analysis of spiked sludge samples collected from biogas plants.

NADH oxidase is an alternative to diaphorase. This enzyme oxidizes NADH (and often NADPH) to NAD^+ with the formation of hydrogen peroxide, which is detected [86].

2.7. Other biosensors based on cascades of enzymatic reactions

To determine citrate in food samples and fermentation broth a biosensor was proposed based on citrate lyase (split citrate to oxaloacetate and acetate), oxaloacetate decarboxylase (split oxaloacetate to pyruvate and carbon dioxide) and pyruvate oxidase (oxidized pyruvate by O_2 to form hydrogen peroxide), which were co-immobilized in the gelatin membrane [87]. A platinum electrode was used, with the applied potential of +0.6 V for H_2O_2 detection or −0.6 V (vs Ag/AgCl) for O_2 detection (both detection methods showed close results). The LOD of citrate was 0.5 μM, and the linear range – from 1 μM to 1 mM. The concentration of citrate was determined in various food products (juices, puree and sauces) and fermentation broth (product of *Claviceps purpurea* fermentation) and confirmed by a photometric method of citrate detection. Since the biosensor was sensitive not only to citrate, but to oxaloacetate and pyruvate, the concentrations of the latter two substances were measured by an additional biosensor without citrate lyase, and the results were taken into account when working with the citrate biosensor. The storage stability of the biosensor was rather low: the response decreased by 75% after 18 days probably because of poor stability of pyruvate oxidase.

A number of biosensors were proposed for the determination of arginine based on arginase-urease bienzyme system [88]. Arginase splits arginine to ornithine and urea, whereas urease splits urea to two ammonium ions and a carbonate acid ion. In the urease reaction, the solution pH and conductivity change, which allows the creation of conductometric and field effect transistors-based biosensors for the arginine determination [89,90]. The enzymes were immobilized on the reduced graphene oxide via electrostatic layer-by-layer procedure. First, the electrode surface was covered with negatively charged sodium 1-pyrenesulfonate. Next, the electrode was immersed in a solution with polyethyleneimine (positively charged polymer) and urease (first cycle) or polyethyleneimine, urease and arginase (second cycle). The linear range of the biosensor was 10–1000 μM, the LOD – 10 μM. The proposed method for controllable multilayer enzyme immobilization can be used for the development of other multienzyme biosensors. Arginase/urease system was also applied for the detection of manganese (II) ions [91]. The biosensor had a linear range from 1 μM to 6.5 μM, a limit of detection of 0.15 μM and a response time – 2.5 min.

Ammonium-sensitive electrodes were also used to develop amperometric biosensors based on these enzymes [92]. Principle of work of the most recent biosensor for arginine determination is presented in Fig. 2. The biosensor was characterized by a low applied potential (−200 mV), fast response to the analyte (10 s), broad linear dynamic range (0.07–0.6 mM), high sensitivity (110 ± 1.3 nA/mM*mm^2) and a low limit of detection (0.038 mM).

A biosensor for the determination of adenosine monophosphate (AMP) and inorganic phosphate was one of the first based on four sequential enzymatic reactions [93]. In the presence of phosphates (substrate) and AMP (allosteric activator), glycogen phosphorylase split glycogen to form glucose 6-phosphate. Glucose 6-phosphate was detected by a tri-enzyme system consisting of alkaline phosphatase (split off phosphorus group from glucose 6-phosphate to produce α-D-glucose), mutarotase (converted α-D-glucose to β-D-glucose) and GOx (oxidized glucose to form hydrogen peroxide, which was detected). It was possible to determine AMP in the range from 5 to 150 μM, and phosphates – from 0.05 to 1 mM. Additionally, a possibility to determine the glycogen phosphorylase activity in the range 5–200 U L^{-1} was shown. The biosensor stability has not been investigated.

Glycerol kinase/glycerol phosphate oxidase system is used in the biosensors for ATP detection [94–96]. Glycerol kinase phosphorylates glycerol by transferring a phosphate group from ATP. Then glycerol phosphate oxidase oxidizes glycerol phosphate and generates amperometric response. The main drawback of these biosensors is the necessity in stable and known concentration of glycerol in the analyzed sample.

The longest enzyme cascades found in biosensors consisted of five enzymes. For example, a glycerol biosensor contained a system of five enzymes in the receptor element: glycerol kinase/creatine kinase/creatinase/sarcosine oxidase/peroxidase [97]. The principle of this biosensor work is schematically shown in Fig. 3. The biosensor had good analytical characteristics, in particular, the LOD was 2 μM, the linear range was 5–640 μM, and no loss in sensitivity was observed over 90 sequential measurements. The biosensor was successfully used for the glycerol analysis in various samples of white and red wines. Storage stability of the biosensor declared by the authors (10% loss of activity after 15-month storage at room temperature) was very high. In contrast, the biosensor is known also based on creatine kinase, which had a simpler design, but much worse storage stability [98].

A biosensor with a long chain of sequential enzymatic and non-enzymatic reactions was proposed for creatinine determination [99]. The target substrate (creatinine) was sequentially hydrolyzed by creatininase and creatinase to sarcosine, which in turn was oxidized by sarcosine oxidase to formaldehyde, glycine and hydrogen peroxide. The latter was reduced by HRP with the simultaneous oxidation of ferrocene to ferricinium cation, which was reduced at a potential of 0.0 V (vs Ag/AgCl) on a composite electrode consisting of a mixture of Teflon® paste, golden nanoparticles (AuNPs) and multi-walled carbon nanotubes (MWCNTs). The use of such complex reaction sequence allowed the creation of a very sensitive biosensor (the LOD was 0.1 μM), which practically did not undergo an impact of the interfering substances due to the neutral potential of the working electrode. The disadvantage of the biosensor was its sensitivity to creatine, which is also present in blood serum in a high concentration. However, the authors did not discuss this issue, and the biosensor was successfully used for the determination of creatinine in human blood serum. Storage of the biosensor over two weeks resulted in the about 50% loss of activity, but probably this can be significantly improved by changing storage conditions.

Unusual biosensor for determination of amygdalin (a compound causing the toxicity of fruit stones, in particular, almonds) was proposed in Ref. [100]. The biosensor contained three enzymes. Two of them catalyzed a sequential reaction: β-glucosidase split amygdalin to glucose and mandelonitrile, mandelonitrile lyase split mandelonitrile to benzaldehyde and hydrogen cyanide. The third enzyme was HRP, which catalyzed the oxidation of ascorbic acid and the reduction of hydrogen peroxide (both substances were

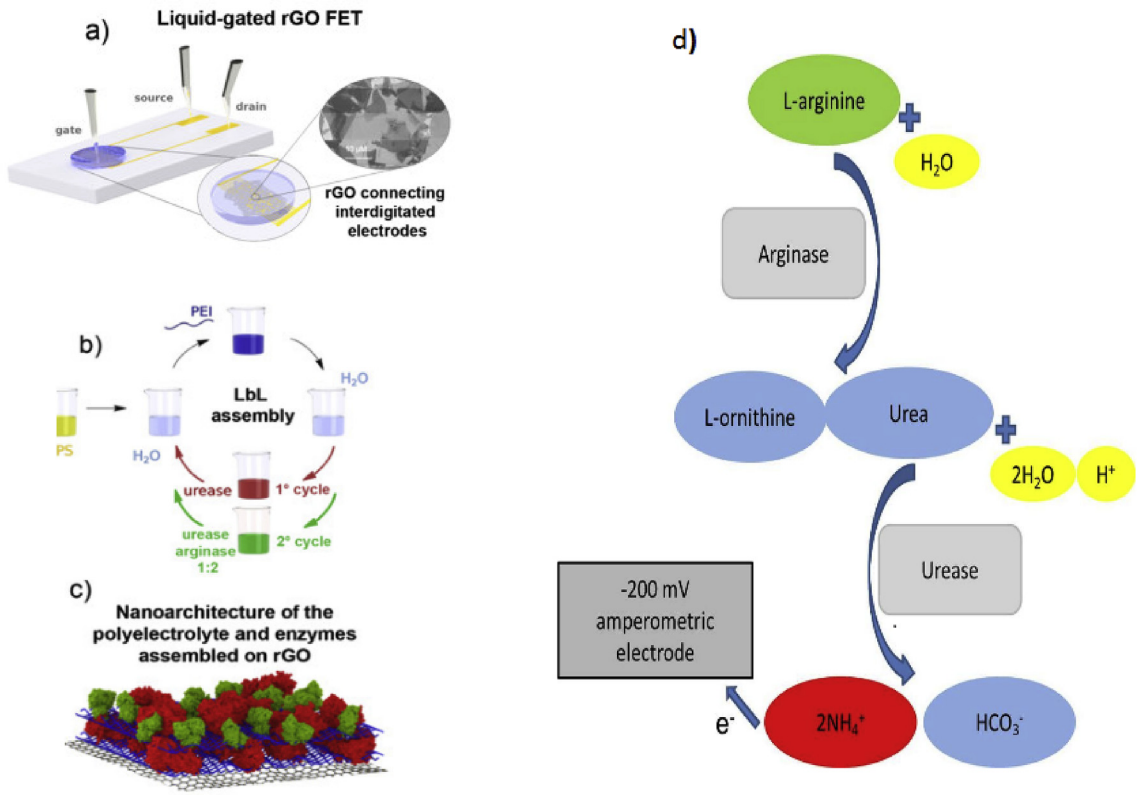

Fig. 2. Scheme of the bienzyme amperometric biosensor for the arginine determination: structure of working biosensor (a), process of layer-by-layer (LbL) enzyme immobilization (b) and resulting enzyme layer (c), and enzyme cascade (d). Colors: green – target substrate, yellow – additional substrates present in working buffer, red – reaction product directly detected by the biosensor, blue – other substances. Reproduced from Ref. [90] under Creative Commons CC-BY license. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

added to the working buffer in a sufficient concentration). This reaction resulted in a change of the solution pH, which was recorded by the ion-selective field-effect transistor (ISFET). Hydrogen cyanide formed from amygdalin inhibited HRP. Thus, the biosensor response decreased proportionally to the amygdalin concentration in the range of 10–300 μM. Noteworthy, the HRP inhibition was very fast - the maximum level was observed one minute after amygdalin was added. This biosensor was proposed for the control of amygdalin and similar components in food products.

3. Biosensors based on cyclic enzymatic reactions and recycling of substrate

An increase of sensitivity is one of the main challenges in the creation of enzyme-based biosensors. This is due to very small (sub-micromolar) concentration of many important analytes to be measured, whereas the LOD of conventional enzyme-based biosensors usually ranges from 1 to 100 μM. One of the approaches for measuring lower concentrations is the recycling of substrate via cyclic enzymatic reactions, in which the substrate is recycled and regenerated. Such biosensors were intensively studied by the Frieder Scheller group in Germany in the 1980s and 90s. In 1993, they published the first review devoted to this topic [101]. The examples of biosensors, which utilize cyclic enzymatic reactions, are given in Table 2.

Based on this approach, the first biosensor was described in 1986 [102]. It was developed for the glutamate determination and based on glutamate dehydrogenase and glutamate-pyruvate transaminase (now known as alanine transaminase). The scheme of functioning is shown in Fig. 4. Glutamate dehydrogenase oxidized and deaminated glutamate to α-ketoglutarate along with the reduction of NAD^+. In turn, alanine aminotransferase re-synthesized glutamate from α-ketoglutarate by the transferring amino group from alanine, without using NADH. Thus, in the presence of glutamate and alanine in the solution, a cyclic reaction began. Due to the glutamate recycling, glutamate dehydrogenase produced a significantly larger amount of NADH than it would be in unidirectional reaction. Two detection methods were used: (1) NADH was directly oxidized on the working electrode (LOD was 0.5 μM), and (2) NADH was oxidized using a mediator and oxygen. The drop of oxygen concentration was detected by the oxygen electrode (LOD was 0.1 μM). The biosensor had significantly higher sensitivity compared to other glutamate biosensors.

The principle of the substrate recycling was realized in the biosensor for the determination of L-phenylalanine with the purpose of diagnosis of hereditary disorder phenylketonuria [103]. The biosensor was based on a cascade of three enzymatic reactions. L-phenylalanine dehydrogenase oxidized L-phenylalanine to phenylpyruvate with the simultaneous NAD^+ reduction to NADH. The latter was used by salicylate hydroxylase for the catechol synthesis from salicylate. The third enzyme, tyrosinase, oxidized catechol to quinone using oxygen. Quinone was reduced back to catechol at the working electrode at a potential of −50 mV (vs Ag/AgCl) – this reaction generated the biosensor response. The formed catechol was again oxidized by tyrosinase. Thus, actually a multiple oxidation of the same molecule took place on the electrode surface, which led to a significant increase in the biosensor sensitivity. In comparison with the biosensor with no tyrosinase (direct catechol oxidation on the electrode), recycling of catechol resulted in 33-fold higher sensitivity of the biosensor to NADH. The LOD was 5 μM, and

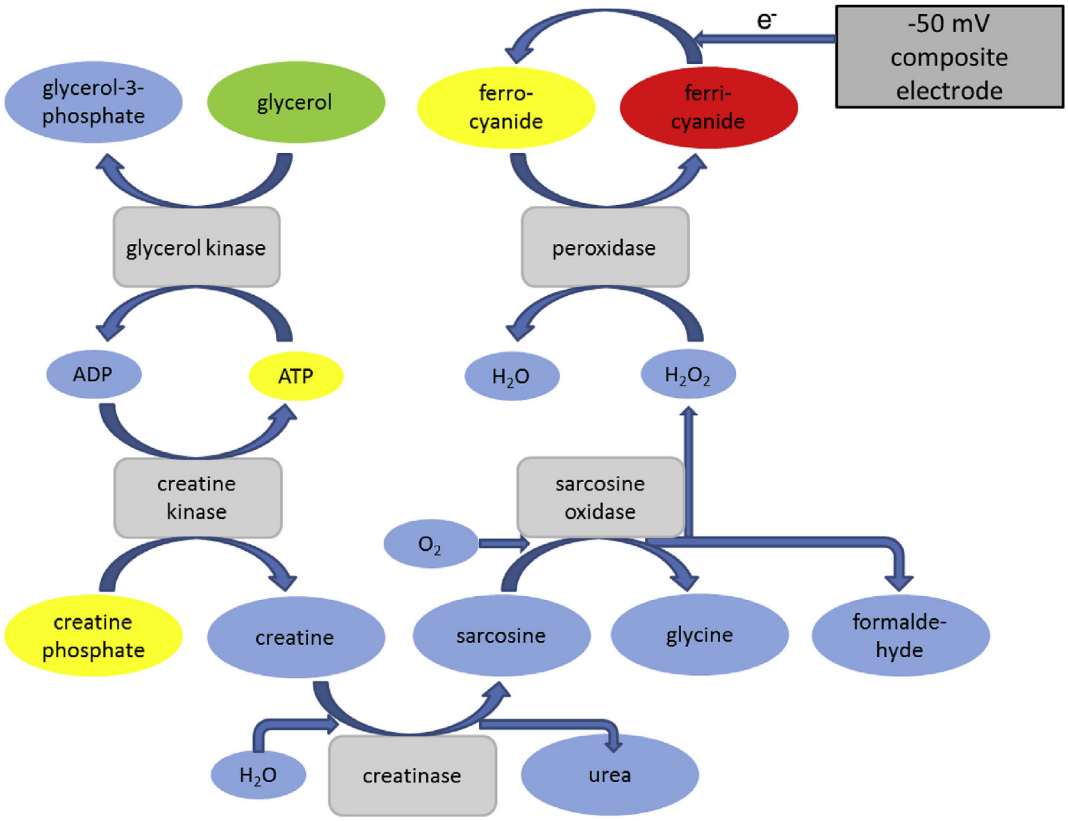

Fig. 3. Scheme of the five-enzyme cascade used in the biosensor for glycerol determination. Colors: green – target substrate, yellow – additional substrates present in working buffer, red – reaction product directly detected by the biosensor, blue – other substances. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the linear range was 20–150 μM. Due to low working potential, the biosensor was successfully tested with the samples of serum and even whole blood. However, the authors noted that the presence of ascorbic acid in physiological concentration (200 μM) is a cause of a drop in the biosensor sensitivity by 14% and interference from paracetamol is also possible. Noteworthy, this system can be used for the development of other highly sensitive biosensors based on dehydrogenases.

A biosensor based on lactate oxidase and lactate dehydrogenase for the lactate determination was described. In the presence of oxygen in sufficient concentration and NADH, a cyclic lactate-pyruvate-lactate conversion took place, with the formation of hydrogen peroxide by lactate oxidase [104]. Interestingly, the cyclic system, though increased the sensitivity, caused the biosensor reaction to the second substrate (pyruvate), often present in the samples. Actually, it was a biosensor for the simultaneous determination of lactate and pyruvate concentrations, but it was impossible to distinguish them. This restricted the biosensor utilization to the samples, in which one of the substrates is absent.

A bienzyme biosensor involving recycling of the substrate was proposed for the determination of catecholamines in very low concentrations (nano- and picomolar range). Laccase oxidized catecholamine to the corresponding quinone, in the presence of oxygen [105]. The reaction was detected by measuring the concentration of oxygen consumed by laccase. Quinone acted as an electron acceptor during the catalysis of oxidation of glucose with glucose dehydrogenase, which enabled returning the substrate to the initial state and its recycling. In the first paper, this biosensor was used for the determination of *p*-aminophenol with a LOD 100 pM [106]. Under optimum conditions (dry at + 4 °C) the biosensor was stored over one month with no loss of activity. However, laccase is a relatively nonselective enzyme and able to oxidize a wide range of compounds. In particular, the biosensor efficiently detected adrenaline, norepinephrine, L-dioxyphenylalanine (DOPA), ferroceneacetic and ferrocenecarboxylic acids, etc. In view of such non-selectivity, this enzyme system was proposed for usage as a detecting component in immunoassays. In the next work, the biosensor was adapted to determine adrenaline, norepinephrine and dopamine [107]. The LOD of adrenaline was 0.5 nM. The biosensor was sensitive in the range of physiological concentrations of ascorbic and uric acids, which complicates using the biosensor in practice for measurement in biological samples (the authors proposed to pre-eliminate these substances by adding the ascorbate and urate oxidases to the sample). In the recent work, genetically modified laccase was used, with increased stability and ability to work in a wide range of pH [108]. However, the main problem, a low selectivity of the biosensor, was not resolved.

A biosensor based on the similar system was used to determine the total concentration of phenolic compounds [109].

A combination of cyclic and cascade reactions was used to create a phosphate-sensitive biosensor based on four enzymes (Fig. 5) [110]. Maltose phosphorylase in the presence of phosphate split maltose into α-D-glucose and β-D-glucose-1-phosphate. Acid phosphatase split the formed glucose phosphate into β-D-glucose and phosphate, thus regenerating the phosphate molecule for the next cycle. Mutarotase converted α-D-glucose into β-form, and GOx oxidized two β-D-glucose molecules to form hydrogen peroxide, which then was detected by the amperometric platinum electrode. Thus, the combination of several enzymes doubled the biosensor signal due to the formation of two glucose molecules and

Table 2
Examples of biosensors based on cyclic enzymatic reactions and enzymes competition for the substrate.

Target substance	Type of transducer	Enzymes and catalyzed reactions	Working range	Limit of detection	Supposed application	Ref.
Glutamate	Graphite rod electrode	Glutamate dehydrogenase: L-glutamate + NAD^+ + H_2O → α-ketoglutarate + NH_4^+ + NADH Alanine transaminase: α-ketoglutarate + L-alanine → L-glutamate + pyruvate	5×10^{-7} – 1×10^{-4} M	5×10^{-7} M	–	[102]
L-phenylalanine	Carbon paste electrode	L-phenylalanine dehydrogenase: L-phenylalanine + H_2O + NAD^+ → phenylpyruvate + NH_3 + NADH + H^+ Salicylate hydroxylase: salicylate + NADH + $2H^+$ + O_2 → catechol + NAD^+ + H_2O + CO_2 tyrosinase: catechol + O_2 → quinone + H_2O	20–150 μM	5 μM	Diagnosis of phenylketonuria	[103]
lactate	Indium tin oxide electrode	L-lactate oxidase: lactate + O_2 → pyruvic acid + H_2O_2 Lactate dehydrogenase: pyruvate + NADH → lactate + NAD^+	0.1–1 mM	1×10^{-5} M	Medicine and biotechnology	[104]
Catecholamines	Oxygen electrode	Laccase: catecholamine + O_2 → quinone + H_2O Glucose dehydrogenase: D-glucose + quinone → D-glucono-1,5-lactone + catecholamine	0.5 –100 nM	Adrenalin: 5 nM Noradrenalin: 5 nM Dopamine: 5 nM	Enzyme immunoassays	[106]
Phosphate	Pt electrode	Maltose phosphorylase: maltose + phosphate → α-D-glucose + β-D-glucose-1-phosphate Acid phosphatase: β-D-glucose-1-phosphate + H_2O → β-D-glucose + phosphate mutarotase: α-D-glucose → β-D-glucose GOx: β-D-glucose + O_2 → D-gluconolactone + H_2O_2	0.1 – l μM	0.1 μM	Monitoring of water pollution	[110]
ATP	Oxygen electrode	Hexokinase: glucose + ATP → glucose-6-phosphate + ADP GOx: β-D-glucose + O_2 → D-gluconolactone + H_2O_2	Up to 1 mM	No data	Biological research	[145]
ATP	Graphite electrode	Cellobiose dehydrogenase: glucose → D-gluconolactone + e^- Hexokinase: glucose + ATP → glucose-6-phosphate + ADP pyruvate kinase: phosphoenolpyruvate + ADP → pyruvate + ATP	0.1–1 mM	63.3 nM	Biological research	[124]

multiplied the signal due to cyclic use of phosphate. This allowed obtaining a LOD of phosphate 10 nM, which is a very good result for enzyme biosensors. The linear range of phosphate determination was 0.1–1 μM. To reduce the cost and complexity of the biosensor, a similar biosensor was developed with no acid phosphatase and thus without the recycling of the substrate [111]. This resulted in deterioration of the LOD and the shift of the linear range towards higher phosphate concentrations (1–50 μM), indicating a significant effect of acid phosphatase on the biosensor characteristics.

The biosensors were also proposed based on other cyclic enzymatic reactions, e.g. horseradish peroxidase/glucose dehydrogenase (recycling of NAD^+), GOx/glucose dehydrogenase (recycling of glucose), cytochrome *b*2/lactate dehydrogenase (recycling of pyruvate) [112]. The fundamentals of the substrate recycling for the improvement of biosensor sensitivity were considered in detail in a three-part work devoted to the biosensor for the catechol and phenol determination [113–115]. This biosensor was based on a single enzyme and therefore it is beyond the scope of this review. Nevertheless, the biosensor employs the cyclic catechol to quinone oxidation and the reverse quinone reduction, which makes it similar to the multienzyme biosensors with substrate recycling. The results of investigation carried out for this biosensor (including the influence of mass transport, partition coefficient, enzyme and electron transfer kinetics, etc.) may be useful for the development of other biosensors based on the substrate recycling.

4. Biosensors based on enzyme competition for substrate

It can be a problem to choose several enzymes so that they sequentially catalyze the reactions to form the electrochemically active product. To overcome this limitation sometimes it is possible to select two enzymes specific to the same substrate, one of which generates an electrochemically active product whereas another does not. In this case, both enzymes will compete for the substrate. A typical example can be the biosensor for the ATP determination based on GOx/hexokinase system. Hexokinase uses ATP to phosphorylate glucose to glucose-6-phosphate, thus the glucose concentration in the near-electrode layer is being reduced (Fig. 6). Simultaneously GOx oxidizes glucose and produces hydrogen peroxide, which is detected with an electrochemical transducer. The greater the ATP concentration in the solution, the larger the amount of phosphorylated glucose will be produced by hexokinase, thus the smaller amount of hydrogen peroxide will be generated by GOx.

The transducers used in the biosensors based on this enzyme system are platinum electrodes [116–118], glassy carbon electrodes [119], platinum microelectrodes [120,121], and Pt nanoflowers decorated micro-needle tip [122]. The highest sensitivity to ATP was achieved in the case of using glassy carbon electrodes: the range of ATP detection was 0.5–20 μM. The biosensor lost 35% of the initial activity after 22 days when stored in 0.1 M phosphate buffer, pH 7.4, at 4 °C. The biosensors with platinum electrodes had a wider range of ATP determination (50–500 μM [116] and 10–200 μM [117]), but a poorer storage stability (60% loss after 2 weeks). The difference in storage stability of different biosensors can be explained by diverse types of immobilization, storage conditions, stability of the enzyme itself, etc. To prevent the oxidation of interfering substances, in most cases the electrode surface was covered with a poly(phenylenediamine) membrane, which allowed the hydrogen peroxide diffusion to the electrode and its next oxidation, but was impermeable for larger molecules. The ATP concentration was evaluated in real samples (human erythrocytes hemolysate [116], gastrointestinal tract tissues [121], and pharmaceuticals [118]), which demonstrated sufficient efficiency of the GOx/hexokinase system.

The main problem of such biosensors is the dependence of their sensitivity to ATP on the glucose concentration. Since the latter in biological samples is high (several millimoles per liter), ATP cannot be determined in the micromolar range due to insufficient biosensor response. Additionally, the glucose concentration should

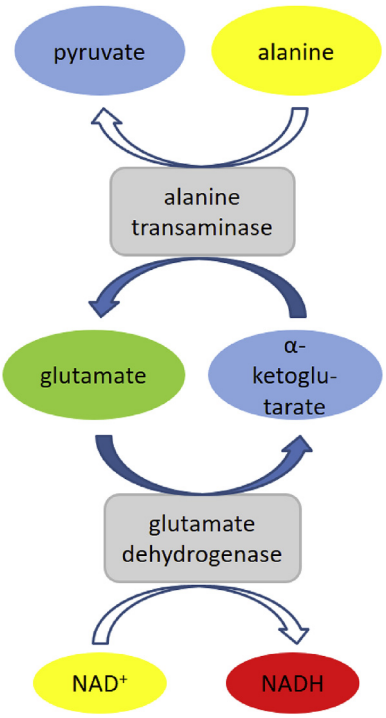

Fig. 4. Scheme of cyclic enzymatic reactions underlying the work of biosensor for glutamate determination. Colors: green – target substrate, yellow - additional substrates in the solution, red –reaction product directly detected by the biosensor, blue – other substances. The cycle of substrate recycling is highlighted with blue arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

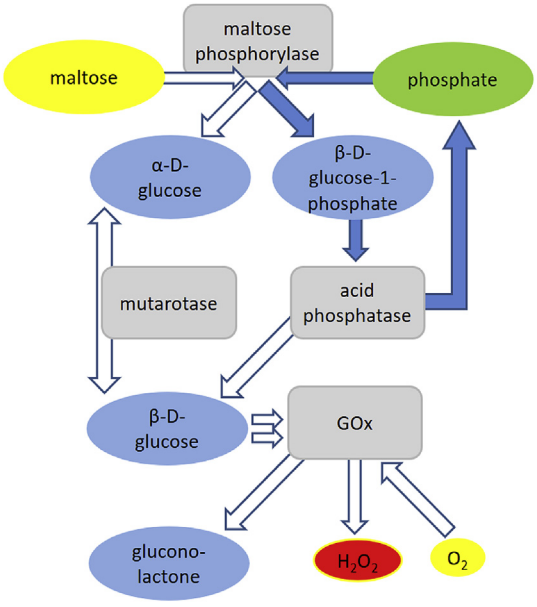

Fig. 5. Scheme of enzymatic reactions underlying the work of the biosensor for phosphate determination. Colors: green – target substrate, yellow – additional substrates in the solution, red – product of reaction directly detected by the biosensor, blue – other substances. The cycle of substrate recycling is highlighted with blue arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

be stable and known throughout the measurements. This is a significant restriction for the practical use of such biosensors.

The application of such biosensors does not restrict to the ATP determination only. For example, a biosensor for determination of the activity of the biomarker enzyme creatine kinase was described in 1986 [123]. This amperometric biosensor was based on the hexokinase/GOx bienzyme system. The biosensor response inversely depended on the creatine kinase activity: the higher activity (and thus higher ATP production by the enzyme), the lower the biosensor response. However, the purpose of this work was to show a possibility of the development of such biosensor. The authors neither evaluated the biosensor analytical characteristics nor offered the procedure of measuring the creatine kinase activity in real samples. Recently this biosensor was further elaborated and the procedure for the determination of creatine kinase activity in blood serum was proposed [98].

An alternative three-enzyme system was recently proposed for the creation of ATP-sensitive biosensor [124]. The feature of this biosensor was a combination of the enzyme competition for the substrate and the substrate recycling. Cellobiose dehydrogenase (mutated, with high glucose specificity) oxidized glucose and directly transferred electrons to the working electrode. On the other hand, hexokinase phosphorylated glucose by transferring the phosphate group from ATP. Thus, an increase in the ATP concentration resulted in a decrease in the concentration of available glucose. Pyruvate kinase provided the ATP recycling: ADP formed by hexokinase was phosphorylated back to ATP by transferring the phosphate group from phosphoenolpyruvate. Compared to other biosensors for ATP determination, this biosensor used a low working potential (−100 mV), did not need oxygen for functioning and was characterized by high sensitivity to ATP. The LOD of ATP was 63.3 nM, which is significantly better compared to the biosensors based on the hexokinase/GOx system (usually >1 μM). The biosensor disadvantage was an emergence of the sensitivity to ADP as a result of ADP participation in the hexokinase/pyruvate kinase cycle along with ATP. The sensitivity to ADP was half of that to ATP.

5. Biosensors based on independent enzymatic reactions

There are a number of biosensors based on several independently acting enzymes. In this case, one enzymatic reaction provides the detection of the target analyte whereas the others result in a decrease of the concentration of untargeted substances, the presence of which interferes with the measurement. An example of such approach is the use of ascorbate oxidase, the enzyme converting electrochemically active ascorbic acid into relatively stable dehydroascorbic acid. Thus, ascorbic acid is split by the enzyme before diffusion to the electrode, which enables the measurement in biological fluids commonly containing ascorbic acid. This approach was used in the development of amperometric microbiosensors for the determination of lactate, glutamate and glucose based on corresponding oxidases [125]. In this work, the biosensor selectivity was ensured by a poly(phenylenediamine)-based semipermeable membrane. However, low electrode sensitivity to ascorbic acid still remained and vanished completely only after the co-immobilization of ascorbate oxidase in the biosensor biorecognition element. The activity of ascorbate oxidase was probably insufficient to oxidize ascorbic acid completely. Nevertheless, the enzyme decreased the local concentration of ascorbic acid so that the phenylenediamine membrane could block its effect.

In another work, ascorbate oxidase was used to improve the selectivity of a lactate biosensor based on lactate oxidase (Fig. 7) [126]. Layer-by-layer enzyme immobilization on Pt electrode was used, and the best results were obtained with three layers of lactate oxidase/polyethylenimine and three layers of ascorbate oxidase/

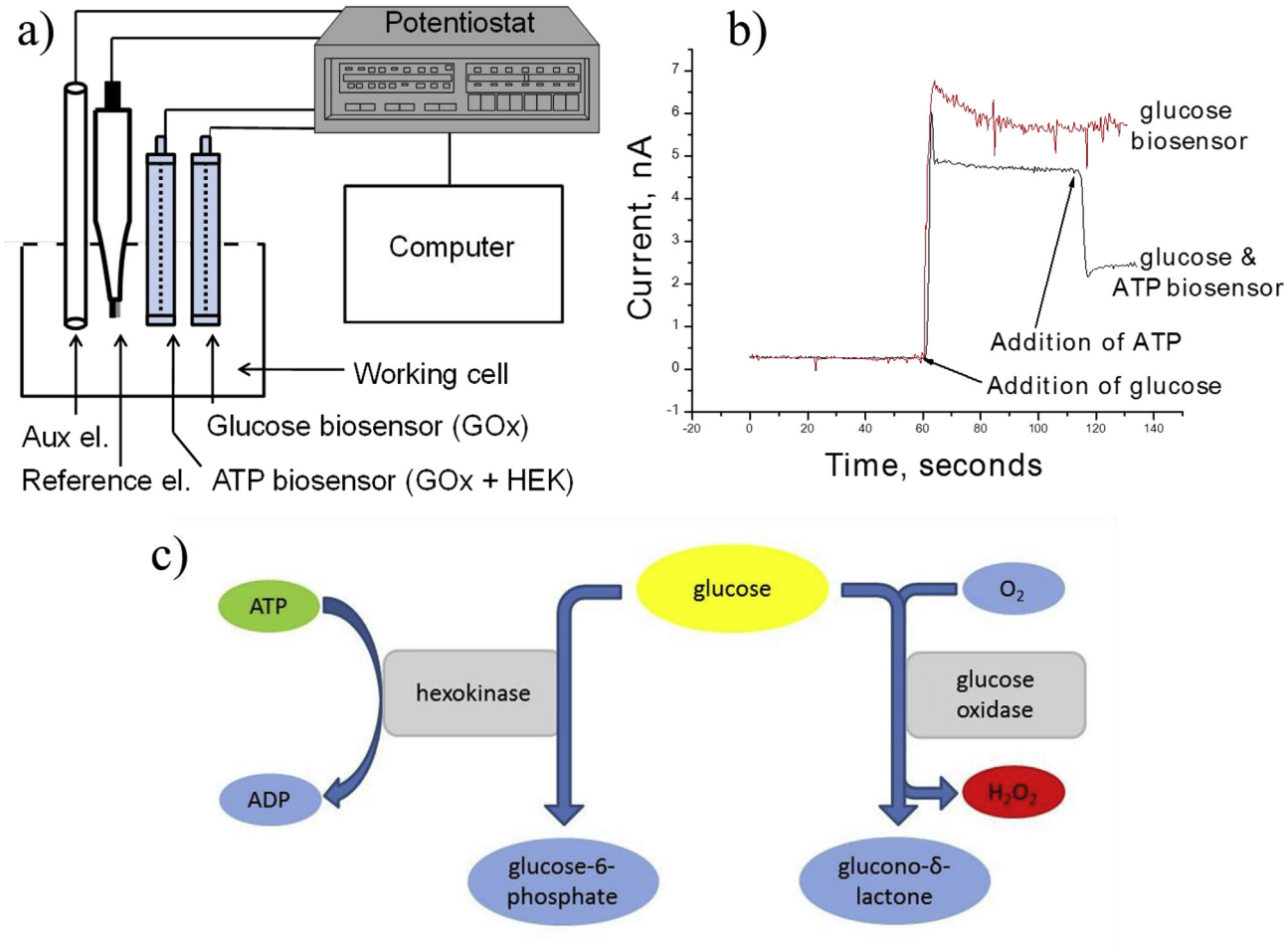

Fig. 6. Scheme of the biosensor array for glucose and ATP detection (a). The array consists of the biosensor based on GOx and the biosensor based on competition between GOx and hexokinase, and the competition leads to signal decrease in the presence of ATP (b). The enzymatic reactions that allow ATP determination are presented in (c). Colors: green – target substrate, yellow – additional substrates present in working buffer, red – reaction product directly detected by the biosensor, blue – other substances. Reprinted with permission from Ref. [118]. Copyright (2014) American Chemical Society. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

polyethyleneimine. However, a significant decrease in the dissolved oxygen concentration was observed because of ascorbate oxidase activity, which interfered with lactate oxidase activity. Therefore, cerium nanoparticles were added to the biosensor – the particles probably absorbed oxygen and served as its source during the analysis. With such biosensor structure, no interference was observed from ascorbic acid. Positively charged polyethylenimine decreased interference (not completely) from other negatively charged molecules such as uric acid. Lactate concentration in human serum samples was successfully measured. Lactate oxidase in this biosensor can be easily replaced by other enzymes, which allows construction of other interference-free biosensors.

The use of ascorbate oxidase for the same purpose is described in other works, in which positively charged polymers polyethyleneimine or Nafion® reduced the impact of positively charged interferents [127,128]. However, it is also possible to pre-treat samples using the ascorbate oxidase by adding it to the sample or passing the sample through a column with this enzyme [129].

To reduce the impact of uric acid on the biosensor work, it was proposed to immobilize urate oxidase (uricase) that cleaves this compound [130]. However, this method has not become widespread, since the enzyme forms hydrogen peroxide, which generates a signal in most electrochemical biosensors.

Another (very rare) purpose of using several independent enzymatic reactions is to expand the range of substances detectable by the biosensor. For example, a biosensor was created on the basis of co-immobilized urease, HRP and butyrylcholinesterase to determine a wide range of toxic substances [131]. Various enzymes are inhibited by different toxins, so the three-enzyme biosensor detected heavy metal ions (which inhibit urease), cyanides (inhibit peroxidase), and pesticides (inhibit butyrylcholinesterase). Separate addition of the substrates made it possible to determine the level of inhibition of each enzyme and to identify certain toxins. It would be impossible to determine such a wide range of toxic substances using a single-enzyme biosensor. However, since enzymes are inhibited by a rather wide range of toxic compounds with different sensitivity, the exact concentration of toxins can be determined only in a model solution with one known inhibitor. In mixtures and real samples this and other enzyme-based biosensors are more suitable for the estimation of overall toxicity of the solution.

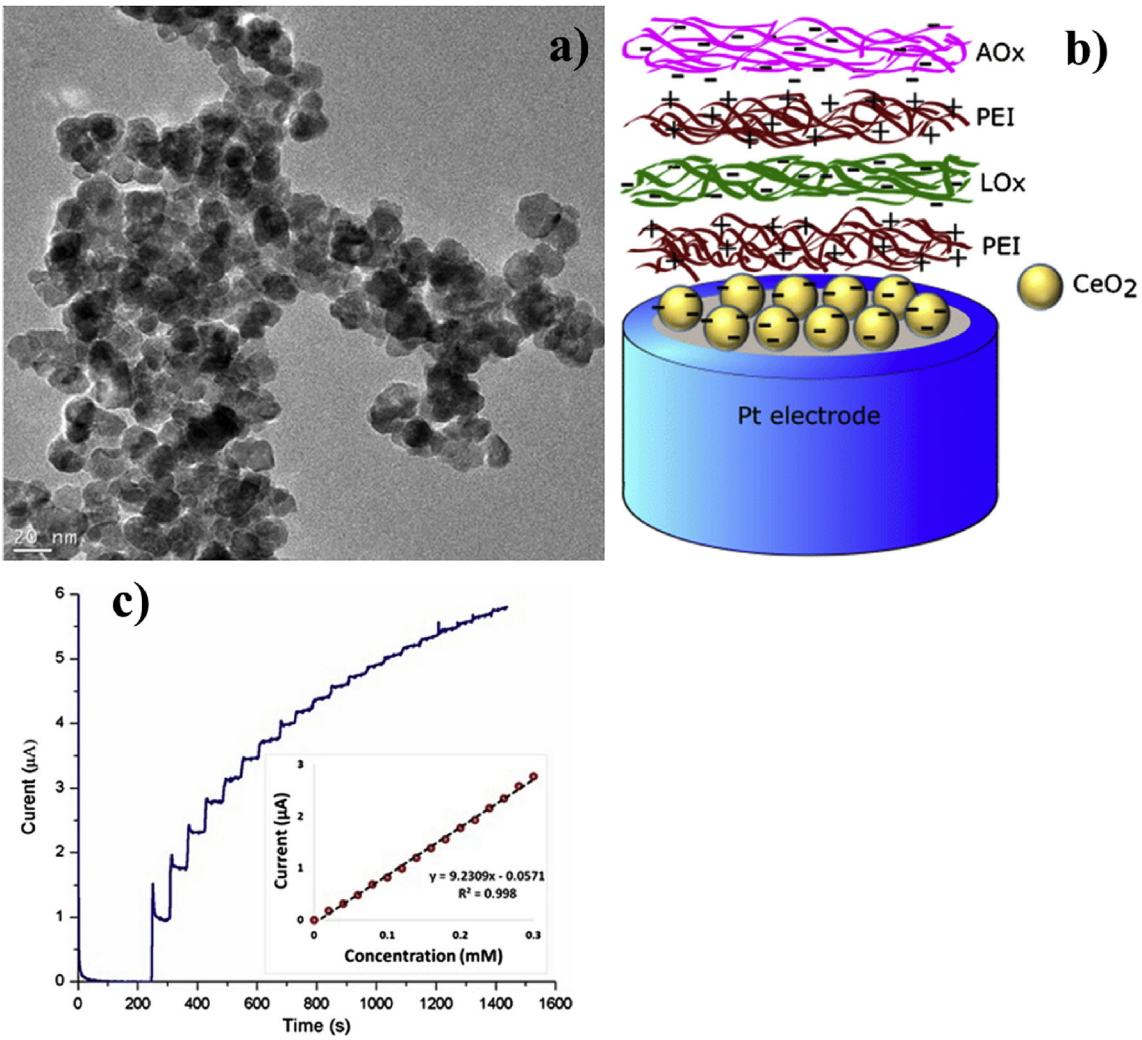

Fig. 7. Scheme of the lactate biosensor based on ceria (CeO_2) nanoparticles (a), lactate oxidase (LOx) and ascorbate oxidase (AOx) immobilized electrostatically via polyethylenimine (PEI) (b), and the calibration curve of the biosensor (c). Reprinted by permission from Springer Nature [126], Copyright 2016.

6. Limitations of multienzyme biosensors and suggestions for further development

Rapid growth of biosensor applications in lab-on-a-chip devices, wearable electronics, and point-of-care systems leads to the development of new enzyme-based biosensors [132]. Biosensors based on multienzyme systems are being actively investigated, but their development and practical applications are restricted by several issues.

1) *The biosensor sensitivity to several substances.* A biosensor based on several enzymes is sensitive simultaneously to the substrates of all enzymes. This makes impossible to determine the concentration of different substrates in their mixtures using a single biosensor. Therefore when creating the biosensor, the composition of multienzyme system should be chosen so that the corresponding substrates were absent in the analyzed samples (of course, except for the target one). Another possible salvation can be the development of multiplexed systems and arrays of a number of biosensors, one of which is sensitive to a single substance whereas others – to several. Thus it is possible to determine the concentrations of all substances in the sample by analyzing the data from several biosensors [118].
2) *The requirement of the same conditions of enzyme immobilization.* Usually enzymes have different optimal immobilization conditions. In case of multienzyme biosensors, the selected immobilization conditions should be a compromise and suitable for all the enzymes used. However, such "compromise" immobilization can lead to a worse activity of enzymes. There are some approaches of layer-by-layer immobilization of several enzymes. Nevertheless, a much simpler procedure of enzymes co-immobilization in a single matrix or on a carrier is mostly used. The simultaneous immobilization of several enzymes in biosensors was reviewed in detail in Ref. [133–135].
3) *Necessity of the functioning of different enzymes under the same conditions* (pH, type of buffer, the presence of cofactors). Alike the previous issue, the "compromise" composition of the working buffer may decrease the enzyme activity compared to that in optimal conditions.
4) *A longer-time procedure of the biosensor development.* Additional experiments are necessary to overcome the above-mentioned issues. Moreover, it is necessary to determine the enzymes ratio ensuring the most effective functioning of the biosensor.

5) *The effectiveness of biosensor storage is defined by the least stable enzyme.* Enzymes have different storage stability, but in multienzyme systems only the least stable enzyme matters. For example, GOx retains its activity for many months (sometimes up to one year) whereas hexokinase inactivates in 1–2 months. Thus, the storage time for the ATP-sensitive biosensors based on the GOx/hexokinase system is usually no more than 2 months.
6) *An increased response time of the biosensor.* In case of multiple sequential reactions, the biosensor response time often increases [136]. Similar effect is observed in coupled enzyme assays – a lag phase is present before the linear steady state phase with maximum and stable reaction rate. The duration of the initial lag phase depends on the conditions for the enzyme reactions, the better the conditions are fulfilled, the shorter the lag [21].
7) *More complex manufacture and a higher cost of biosensor.* There are a lot of possible enzyme combinations to be used when creating biosensors based on new multienzyme systems. However, multienzyme biosensors are more complex and often more expensive then the single-enzyme ones. Thus, it should be taken into account that the biosensors are devices for practical use, therefore low cost, easy-to-manufacture technology, and sufficient time of storage are actually the vital matter, and there is no need to develop a complex multienzyme biosensor if it will be novel but completely inapplicable.

In order not to waste resources for creation of would-be inefficient and inapplicable biosensors, the following questions should be analyzed before the elaboration of new biosensors based on multienzyme systems:

1) Have been the biosensors already developed to identify the same substance using one enzyme only? If so, what are the benefits of the more complex and probably more expensive multienzyme system?
2) Can all enzymes of the system function under the same conditions (pH, the presence of cofactors, etc.)? Do all enzymes have sufficiently high activity for the rapid flow of all reactions? Of course, the properties of immobilized enzymes somewhat change compared to intact enzymes, but if the enzymes in nature function under essentially different conditions, it seems unreasonable to combine them into one system.
3) What is the storage stability of each of the enzymes in the system? If at least one enzyme quickly loses its activity when stored, the biosensor as a whole will have poor storage stability.
4) Can real samples contain the substrates of the enzymes (other than the target substrate)? If so, how is it possible to determine the target substance selectively?

In some cases, other methods can be used to avoid the need for additional enzymes. For example, HRP is often utilized to transfer electrons from hydrogen peroxide to a mediator for further transfer to the working electrode at a low applied potential. Theoretically, this prevents the oxidation of interfering substances on the electrode, but often these substances (in particular, ascorbic acid) are well oxidized with the mediator, which results in a false signal of the biosensor. Therefore, it is possible to refuse from using HRP and the mediator in favor for another method to avoid nonspecific oxidation – the deposition of semipermeable membranes (in particular, phenylenediamine-based) on the electrode, which effectively prevent the electrochemically active substances from the penetration to the surface whereas make no barriers for small molecules like hydrogen peroxide [137,138]. The same membranes can be often used to avoid ascorbic and uric acids instead of ascorbate and urate oxidases.

The presented variants of multi-enzyme systems used in the described biosensors mimic the possible metabolic situations (cascades, pathways) in a living cell and thus provide better sensitivity, a wider range of concentrations of the measured substances and high selectivity of the biosensor systems developed, that was previously described [139]. Additionally, application of highly selective materials sensitive to biochemical signals in biosensorics allows the use of various enzymatic reactions to control the properties of adaptive materials and systems with built-in Boolean logic, which can be an effective way to manufacture "smart" multi-sensor systems that can operate without communication with external computers revolutionizing many biotechnological applications [140].

7. Conclusions

Different types of multienzyme electrochemical biosensors and their use in various areas were reviewed. The largest group of the biosensors is based on a cascade of enzymatic reactions. They are primarily developed to expand the range of application of electrochemical biosensors. The biosensors based on cyclic enzymatic reactions and substrate recycling are less widespread due to a few possible combinations of enzymes despite these biosensors have the best sensitivity among electrochemical enzyme biosensors.

On the other hand, it is shown that the addition of several enzymes to the biosensor complicates the biosensor and may impose significant restrictions on its characteristics and possibilities of application. A key aspect in the elaboration of a multienzyme biosensor is careful selection of the enzyme system to prevent its sensitivity to other than target substances and to ensure sufficient biosensor stability. Thus, the possible positive and negative consequences should be assessed prior to use multienzyme systems in the development of biosensors.

Declaration of competing interest

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.

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