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Cascadic Multienzyme Reaction-Based Electrochemical Biosensors

Yong Duk Han, Yo Han Jang and Hyun C. Yoon

Abstract Since the first glucose biosensor was developed by Clark and Lyons, there have been great efforts to develop effective enzyme biosensors for wide applications. Those efforts are closely related to the enhancement of biosensor performance, including sensitivity improvement, elevation of selectivity, and extension of the range of analytes that may be determined. Introduction of a cascadic multienzyme reaction to the electrochemical biosensor is one of those efforts. By employing more than two enzymes to the biosensor, its sensitivity and accuracy can be enhanced. Also, the narrow application range that is a typical limitation of single enzyme-based biosensor can be overcome. This chapter will discuss the fundamental principles for the development of cascadic multienzyme reaction-based electrochemical biosensors and their applications in clinical and environmental fields.

Keywords Biosensors · Cascadic multienzyme reaction · Coupled enzyme assay · Electrochemical analysis · Enzyme electrode

Abbreviations

ACh	Acetylcholine
AChE	Acetylcholine esterase
ALT	Alanine transaminase
ALP	Alkaline phosphatase
AST	Aspartate transaminase
AuNP	Gold nanoparticle
BS^3	Bis(sulfosuccinimidyl) suberate
CE	Cholesterol esterase
Ch	Choline
ChOx	Choline oxidase
CK	Creatine kinase

Y. D. Han · Y. H. Jang · H. C. Yoon (✉)
Department of Applied Chemistry and Biotechnology and Department of Molecular Science and Technology, Ajou University, Suwon 443-749, Republic of Korea
e-mail: hcyoon@ajou.ac.kr

CNT	Carbon nanotube
CO	Cholesterol oxidase
Cyt-c	Cytochrome complex
DAP	Diaphorase
GA	Glutaraldehyde
GalDH	Galactose dehydrogenase
GOx	Glucose oxidase
G3PO	Glycerol-3-phosphate oxidase
HRP	Horseradish peroxidase
LDH	Lactate dehydrogenase
MP	Magentic microparticle
MWCNT	Multi-walled carbon nanotube
NAD	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NHS	N-hydroxysuccinimide ester
OAC	Oxaloacetate decarboxylase
OP	Organophosphorous
PANI	Polyaniline
p-AP	p-Aminophenol
PASA	Polyaniline sulfonic acid
PDMS	Polydimethylsiloxane
PLL	Poly-L-lysine
POx	Pyruvate oxidase
PPY	Polypyrrole
p-QI	p-Quinonimine
SAM	Self-assembled monolayer
SO	Sulfite oxidase

Contents

1 Introduction

Curiosity and the desire to understand the composition of certain objects have been strong driving forces for advances in analytical science. As the scientific knowledge about physics, chemistry, and biology has grown, analytical chemists and biochemists have developed various analytical methods to detect the amounts of biochemical components in complex systems such as the human body. Those analytical methods for chemical or biochemical assays have been performed in professional laboratories and facilities by experts using additional equipment. Rationally, we have sought more convenient and efficient biochemical assay methods that require minimal professional skills and equipment. These needs triggered the development of a brand-new analytical methodology: the biosensor [1].

A biosensor can be generally defined as an analytical device that is composed of a biological recognition part (the so-called bioreceptor) and a physico-chemical transducer to convert an observed biospecific response into a measurable signal whose intensity is proportional to the concentration of a target analytes. Briefly, biosensors can be classified as affinity biosensors and catalytic biosensors. In affinity biosensors, biomolecules having target-specific binding capabilities—such as antibodies, oligonucleotides, and cellular receptors—are used as bioreceptor elements. If the target analytes are composed of protein or oligonucleotide, one can select an affinity biosensor for an assay. In catalytic biosensors, enzymes possessing a biocatalytic activity with target analytes are employed as a bioreceptor element. Generally, this catalytic biosensor is used in detection of non-protein biochemical metabolites.

According to the utilized transducer type, biosensors also can be divided into several groups such as electrochemical, optical, piezoelectric, and calorimetric biosensors [1–5]. Among these various transducing strategies, electrochemical transducers are most widely used as biosensors due to their cost efficiency, operation convenience, good portability, and simplicity of construction. Also, the electrochemical transducer (especially an amperometric transducer) can be easily combined with catalytic bioreceptors (enzymes) generating bioelectro-catalytic signals when the target analytes recognized [2–4]. Figure 1 shows a scheme of a typical amperometric enzyme biosensor principle.

Since the first glucose biosensor using a catalytic biosensing concept was introduced by Clark and Lyons in 1962 [6], many researchers have struggled to develop efficient and sensitive enzyme-based electrochemical biosensors that can be applied to various fields, such as healthcare [1–3, 7–11], environmental monitoring [12–18], food analysis [16–18], and bioreactor monitoring [19]. By these efforts, there has been an explosive growth of research activities in the biosensor development area. Although there were huge advances in the academic fields, few commercially successful biosensor products exist, except for the blood-glucose biosensor in the industrial field [8].

Commercialized glucose biosensors employ an amperometric enzyme electrode as a biosensing platform. On the amperometric enzyme electrode, the enzyme–

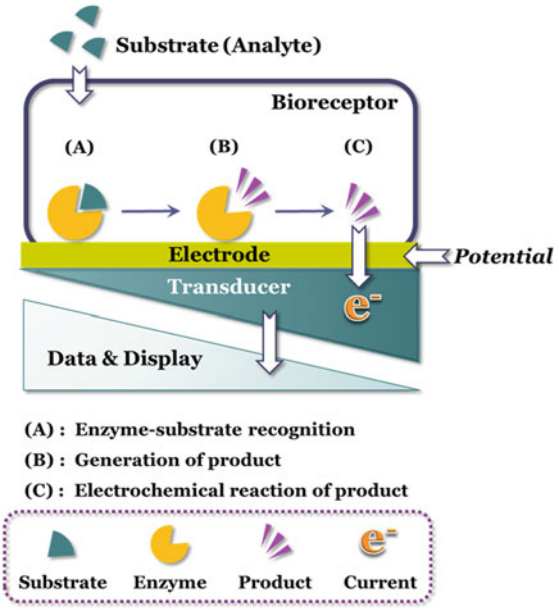


Fig. 1 Schematic illustration of a typical amperometric enzyme biosensor

substrate biocatalytic reaction results in an alteration of redox-active species concentrations. During an appropriate electric potential applied to the electrode, oxidation or reduction of these redox-active species generates measurable current whose magnitude has a stoichiometric relationship to the concentration of the analyte [7, 8]. The success of this glucose biosensor was affected by its comparably simple electrocatalytic reaction principle that requires only one redox enzyme to measure the concentration of blood glucose. Unfortunately, many analytes in nature require more complicated enzymatic assay methods involving more than two enzymes. Only a few enzymes that have the ability to directly generate or consume electroactive species. These limited numbers of analytes can be measured by a mono-enzymatic biosensor and thus hinder the expansion of biosensor applications [20].

To overcome this limitation, introduction of cascadic multienzyme-modified electrodes that couple the bioelectrocatalytic activities of different enzymes in a sequential manner should be considered [20, 21]. For instance, determination of triglycerides in serum sample cannot be achieved by using a monoenzymatic assay method because there is no enzyme that can directly oxidize the analytes and produce electroactive substances. However, triglycerides can be hydrolyzed into fatty acids and glycerol by the enzyme reaction of lipase. Sequentially, by using a glycerol kinase (GK) reaction, the generated glycerol is converted into glycerol-3-phosphate that can be oxidized by glycerol-3-phosphate oxidase (G3PO). Based on this three-enzyme involved cascadic reaction, triglycerides can be electrochemically determined [22].

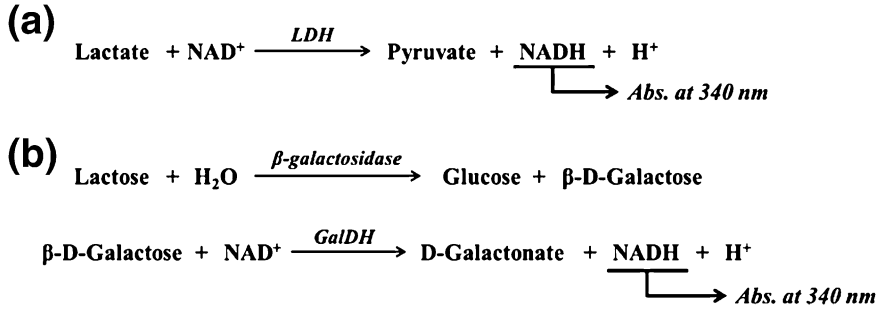
This cascadic multienzyme-involved biosensing strategy provides not only an expansion of the biosensor application range but also an enhancement of the selectivity and sensitivity of the biosensor. To analyze the very low concentrations of target analytes, enhancement of enzymatic biosensor sensitivity is required. In this regard, Wollenberger et al. suggested a multienzyme system employing the recycling of substrates and electroactive species [20]. For example, a very sensitive measurement of glutamate has to be accomplished by employing an enzyme-modified carbon electrode that is comprised of glutamate dehydrogenase and alanine aminotransferase [23]. On the bienzyme-modified electrode, a sequential enzyme reaction allows the recycling and regeneration of the glutamate, a target analyte, and it provides amplified electrochemical signals that guarantee higher sensitivity. As discussed previously, the employment of cascadic multienzyme reactions to the electrochemical biosensor offers great advances to its sensitivity and extended usage to industrial fields. This chapter reviews the principles of the development of multienzyme-based electrochemical biosensors and their practical applications in various fields.

2 Basic Principles of the Development of Cascadic Multienzyme Reaction-Based Electrochemical Biosensors

Simply, the multienzyme-modified electrochemical biosensor might be considered as extra enzyme reactions added to the mono-enzymatic electrochemical biosensor. However, the practical realization of multienzyme reaction-involved biosensor demands more complicated conditions, such as appropriate strategy selections for immobilization and arrangement of different enzymes on the electrode, diffusion controls of substrate products in multienzyme-modified layers, adjustments of different enzyme activities, and electrochemical signaling efficiencies. On the basis of these considerations, this section will consider some essential knowledge for the development of cascadic multienzyme-modified electrochemical biosensor including (1) the kinds of analytes that require a cascadic multienzyme reaction-based assay method and their multienzyme reaction mechanisms, (2) electrochemical signaling principles, and (3) multienzyme immobilization strategies.

2.1 Cascadic Multienzyme Reaction for Enzymatic Analysis

From the 1960s, a full-scale analytical use of enzymes began, especially in the medical analysis field, and their accumulated experience has influenced other industrial fields, such as food analysis and environmental analysis [24].



Scheme 1 Examples of conventional enzymatic analytical method. **a** Monoenzymatic assay for lactate determination. **b** Coupled enzymatic assay for lactose determination

In the most frequently used enzymatic analytical methods, stoichiometric measurements of targeted enzyme-substrate reactions are achieved by spectrophotometric observation of the optical density alterations of cofactors such as $NAD(P)^+$ or NAD(P)H, which are involved during enzyme-substrate reactions. For example, the determination of pyruvate involves the reaction of lactate dehydrogenase (LDH) and nicotinamide adenine dinucleotide (reduced; NADH) as shown in Scheme 1a.

By the LDH reaction, pyruvate is converted into lactate in the presence of NAD^+, and NAD^+ is reduced to NADH. By reading the appearance of NADH by an ultraviolet and visible absorption spectrophotometer, concentrations of pyruvate can be determined. When the target analytes have no adequate enzymes to directly catalyze them with cofactor-involving reactions, additional enzymes are employed to convert the target analytes into an accessible form that can be catalyzed by adequate cofactor-dependent enzymes. This enzymatic method, based on the coupling of conversion purpose-enzymes and indication-purpose enzymes, is called *coupled enzymatic assay* [24–27]. The measurement of lactose, for instance, can be accomplished by employing cascadic bienzyme reactions of *β*-galactosidase and galactose dehydrogenase (GalDH), as given in Scheme 1b. To our best knowledge, there is no redox enzyme (oxidase or dehydrogenase) that directly reacts with lactose and generates signals. However, a coupled enzyme reaction enables the lactose determination. By the reaction of *β*-galactosidase as a conversion-purposed enzyme, lactose is decomposed into glucose and galactose. In the presence of NAD^+, GalDH is employed as an indicating enzyme converts galactose into galacturonic acid. During the indication-purposed enzyme reaction, NAD^+ is reduced into NADH. Finally, the measured optical density of appeared NADH reflects the concentration of lactose in tested samples. A number of such coupled enzymatic assay methods for various analyte determinations are listed by Rudolph et al. [27] and some important analytes are listed in Table 1.

As shown in Table 1, many biochemicals require coupled enzymatic assay methods to be measured. The concept and principle of these coupling enzyme assay methods provide important clues to the development of cascadic-multienzymatic

Table 1 Examples of coupled enzyme assays

Target analyte	Coupling enzymes	Indication
Aspartate	AST, MDH	Disappearance of NADH
Alanine	ALT, LDH	Disappearance of NADH
Fructose	Hexokinase, G6PD	Formation of NADPH
Citrate	Citrate lyase, LDH or MDH	Disappearance of NADH
Creatine	CK, PK, LDH	Formation of NADH
Glycerol	GK, PK, LDH	Formation of NADH
TG	Lipase, GK, PK, LDH	Formation of NADH
ATP	Hexokinase, G6PD	Disappearance of NADPH
ADP	PK, G6PD	Disappearance of NADPH

electrochemical biosensors. First, most indicating enzymes used in coupled enzymatic assays are redox enzymes, and those are also essential components of electrochemical biosensors for bioelectrocatalytic signaling. The only difference between coupled enzymatic assays and electrochemical enzymatic biosensors is the type of redox enzymes that are frequently used in indication and signaling. In the case of traditional coupled enzymatic assays, $NAD(P)^+$ and NAD(P)H-dependent enzymes are predominantly used for optical indications. Contrary to this, enzymatic electrochemical biosensors employ the oxidase type of enzymes, using oxygen as an electron acceptor for signaling. Therefore, for the implantation of coupled enzyme reactions to an electrochemical biosensor, adjustment and modification of $NAD(P)^+$ and NAD(P)H-dependent enzyme couples into the type of enzyme couples to enable easy and efficient signaling should be considered [28]. A number of examples of enzyme couples that can be applied in a multienzymatic electrochemical biosensor are listed in Table 2.

As shown in Table 2, the cascadic enzyme reaction couples employed in a multienzymatic electrochemical biosensor can be briefly classified into two categories: non-redox enzyme/redox enzyme couple and redox enzyme/redox enzyme couple [29]. In the cascadic reaction of non-redox enzyme/redox enzyme couple, non-redox enzymes convert the analytes into a certain form that can be oxidized by the following redox enzyme reaction. As a non-redox enzyme, any possible enzymes such as kinase, transferase, invertase, and hydrolase can be employed for an analyte conversion reaction. For instance, maltose can be measured by using glucosidase-glucose oxidase (GOx) coupled reaction. Glucosidase hydrolyzes maltose into glucose that can be oxidized by GOx. Cascadic enzyme reactions of these non-redox and redox enzyme couples produce hydrogen peroxide (or reduced electron transferring mediators) as an electroactive product. By the electrochemical oxidation (or reduction) of these electroactive products, electrochemical signals can be generated on the electrode. This type of enzyme reaction is frequently used for determination of target analytes that have no directly related redox enzymes. Appropriate selection of these types of enzyme couples gives a strong chance to extend the biosensor application ranges. The schematic illustrations of non-redox enzyme and redox enzyme coupling reaction are depicted in Fig. 2a.

Table 2 Examples of enzyme couples used in multienzymatic electrochemical biosensors

Target analyte	Enzyme coupling type	Involved enzymes	Enzyme reaction mechanism
Ethanol	Redox/redux	AOx, HRP	$\text{Ethanol} + O_2 \rightarrow (\text{AOx}) \rightarrow \text{Acetaldehyde} + H_2O$ $2H_2O_2(\text{HRP}) \rightarrow H_2O$
Glucose	Redox/redox	GGx, HRP	$\text{Glucose} + O_2 \rightarrow (\text{GOx}) \rightarrow \text{Gluconic acid} + H_2O_2$ $2H_2O_2 \rightarrow (\text{HRP}) \rightarrow H_2O$
L-lactate	Redox/redox	LDH, DAP	$\text{L}-\text{Lactate} + NAD^+ \rightarrow (\text{LDH}) \rightarrow \text{Oxaloacetate} + \text{NADH} + H^+$ $\text{NADH} + H^+ \rightarrow (\text{DAP}) \rightarrow NAD^+$
L-malate	Redox/redox	MDH, DAP	$\text{L}-\text{Malate} + NAD^+ \rightarrow (\text{MDH}) \rightarrow \text{Oxaloacetate} + \text{NADH} + H^+$ $\text{NADH} + H^+(\text{DAP}) \rightarrow NAD^+$
β-D-glucan	Non-redox/redox/ redox	β-Glucanase, GGx, HRP	$\beta\text{-D-Glucan} + H_2O \rightarrow (\beta\text{-glucanase}) \rightarrow \beta\text{-D-Glucose} + H_2O$ $\beta\text{-D-Glucose} + O_2 \rightarrow (\text{GOx}) \rightarrow \text{Gluconic acid} + H_2O_2$ $2H_2O_2 \rightarrow (\text{HRP}) \rightarrow H_2O$
ACh	Non-redox/redox	AChE, ChOx	$\text{ACh} + H_2O \rightarrow (\text{AChE}) \rightarrow \text{Acetic acid} + \text{Ch}$ $\text{Ch} + O_2 \rightarrow (\text{ChOx}) \rightarrow \text{Betain} + H_2O_2$
Arginine	Non-redox/redox	Arginase 1, urease	$\text{L-Arginine} + H_2O \rightarrow (\text{Arginase I}) \rightarrow \text{L-Ornitine} + \text{Urea}$ $\text{Urea} + 2H_2O + H^+ \rightarrow (\text{Urease}) \rightarrow 2NH_4^+ + HCO_3^-$
ALT	Non-redox/redox	POx	$\text{ALA} + \alpha\text{-Ketoglutarate} \rightarrow (\text{ALT}) \rightarrow \text{Glutamate} + \text{Pyruvate}$ $\text{Pyruvate} + O_2 + H_2PO_4^- \rightarrow (\text{POx}) \rightarrow \text{Acetylphosphate} + H_2O_2$
AST	Non-redox/redox	OAC, POx	$\text{ASP} + \alpha\text{-Ketoglutarate} \rightarrow (\text{AST}) \rightarrow \text{Glutamate} + \text{Oxaloacetate}$ $\text{Oxaloacetate} \rightarrow (\text{OAC}) \rightarrow \text{Pyruvate} + CO_2$ $\text{Pyruvate} + O_2 + H_2PO_4^- \rightarrow (\text{POx}) \rightarrow \text{Acetylphosphate} + H_2O_2$
ATP	Non-redox/redox	GK, G3PO	$\text{Glycerol} + \text{ATP} \rightarrow (\text{GK}) \rightarrow \text{Glycerol-3-phosphate} + \text{ADP}$ $\text{Glycerol-3-phosphate} + O_2 \rightarrow (\text{G3PO}) \rightarrow \text{Dihydroxyacetone phosphate} + H_2O_2$

(continued)

Table 2 (continued)

Target analyte	Enzyme coupling type	Involved enzymes	Enzyme reaction mechanism
Total cholesterol	Non-redox/redox	CE, CO	Cholesterol ester → (CE) → Cholesterol + Fatty acid Cholesterol + O_2 → (CO) → Cholestenone + H_2O_2
Creatine	Non-redox/redox	CI, SOx	Creatine + H_2O → (CI) → Sarcosine + Urea Sarcosine + H_2O + O_2 → (SOx) → Formaldehyde + Glycine + H_2O_2
Creatinine	Non-redox/redox	CA, CI, SOx	Creatinine + H_2O → (CA) → Creatine Creatine + H_2O → (CI) → Sarcosine + Urea Sarcosine + H_2O + O_2 (SOx) → Formaldehyde + Glycine + H_2O_2
Glycerol	Non-redox/redox	GK, G3PO	Glycerol + AFP → (GK) → Glycerol-3-phosphate + ADP Glycerol-3-phosphate + O_2 → (G3PO) → Dihydroxyacetone phosphate + H_2O_2
D-lactosc	Non-redox/redox	β-Galactosldase, GalOx, GOx	D - Lactose + H_2O → (β-Galactosidase) → β-D-Glucose + D-Galactose β-D-Glucose + O_2 → (GOx) → Gluconic acid + H_2O_2 D-Galactose + O_2 → (GalOx) → Galactohexodialdose + H_2O_2
Sucrose	Non-redox/redox	Invertase, mutarotase, GOx	Sucrose + H_2O → (Invertase) → β-D-Glucose + Fructose α-D-Glucose → (Mutarotase) → β-D-Glucose β-D-Glucose + O_2 → (GOx) → Gluconic acid + H_2O_2
TG	Non-redox/redox	Lipase, GK, G3PO	TG + H_2O → (Lipase) → Fatty acid + Glycerol Glycerol + ATP → (GK) → Glycerol-3-phosphate + ADP Glycerol-3-phosphate + O_2 → (G3PO) → Dihydroxyacetone phosphate + H_2O_2

AChE Acetylcholine esterase; *AOx* Alcohol oxidase; *CA* Creatinine amidohydrolase; *CE* Cholesterol esterase; *ChOx* Choline oxidase; *CI* Creatine amidinohydrolase; *CO* Cholesterol oxidase; *DAP* Diaphorase; *G3PO* Glycerol-3-phosphate oxidase; *GalOx* Galactose oxidase; *GK* Glycerol kinase; *GOx* Glucose oxidase; *HRP* Horseradish peroxidase; *LDH* Lactate dehydrogenase; *MDH* Malate dehydrogenase; *OAC* Oxaloacetate decarboxylase; *POx* Pyruvate oxidase; *SOx* Sarcosine oxidase

The other cascadic enzyme reaction used in enzymatic biosensor is the redox enzyme/redox enzyme coupling reaction. In the cascadic reaction of the redox enzyme/redox enzyme couple, a primary redox enzyme reacts with a target analyte in the presence of electron acceptor (or donor) molecules, such as oxygen, and produces oxidized substrate and reduced (or oxidized) electroactive species, such as hydrogen peroxide. A secondary redox enzyme oxidizes the produced electroactive species and generates an electrochemical signal on the electrode [29]. This enzyme coupling of a redox enzyme is generally used for the enhancement of sensitivity and accuracy. For example, redox enzymes such as GOx can be combined with a peroxidase such as horseradish peroxidase (HRP). The oxidase-substrate reaction yields an oxidized substrate and hydrogen peroxide by oxygen involved in the electron transferring reaction. Sequentially, the oxidation of hydrogen peroxide by HRP occurs and it initiates generation of an electrochemical signal on the electrode. The electrochemical signaling of HRP-induced hydrogen peroxide oxidation can be accomplished under a relatively low potential range that can prevent signal interference by the overpotential of ascorbic acid, uric acid, and acetaminophen [30].

Similar to the oxidase–peroxidase couple, NAD^+-dependent dehydrogenase such as LDH can be coupled with diaphorase (DAP), which oxidizes NADH. As a result of the dehydrogenase-analytes reaction, oxidized substrates and NADH are generated. DAP re-oxidizes NADH into NAD^+ and generates electrochemical signals on the electrode. In this case, the employed DAP reaction efficiently recycles NAD^+/NADH and finally provides an amplified electrochemical signal [31]. In both cases, a secondary redox enzyme reaction can be combined with appropriate electron transferring mediators to provide an amplified signal, as will be discussed in the next section. The reaction schemes of the oxidase-HRP couple and dehydrogenase-DAP couple are illustrated in Fig. 2b, c respectively.

As a minor case, the determination of the enzyme itself in the sample can be considered. When the target analyte is a certain enzyme form, its own reaction and related substrate should be induced on the appropriate oxidase-modified electrode. In this case, careful preparations of conditions for the enzyme-substrate reaction, such as substrate solution and pre-reaction time, are required. For example, alanine transaminase (ALT) quantification can be achieved by using a pyruvate oxidase (POx)-modified electrode [32]. In the presence of α-ketoglutarate and alanine, ALT produces pyruvate as an oxidizable product in the solution phase. After the pre-reaction solution is applied to the POx-modified electrode, electrochemical signals can be registered by the POx-pyruvate reaction.

According to the target analytes, the non-redox enzyme/redox enzyme couple and redox enzyme/redox enzyme couple can be mixed and used for biosensing. For example, GK, G3PO, and HRP involving a cascadic multienzyme reaction are employed to quantify creatine kinase (CK) concentrations in serum. In this case, GK takes the conversion reaction and the G3PO-HRP couple performs the coupled redox enzyme reaction [33].

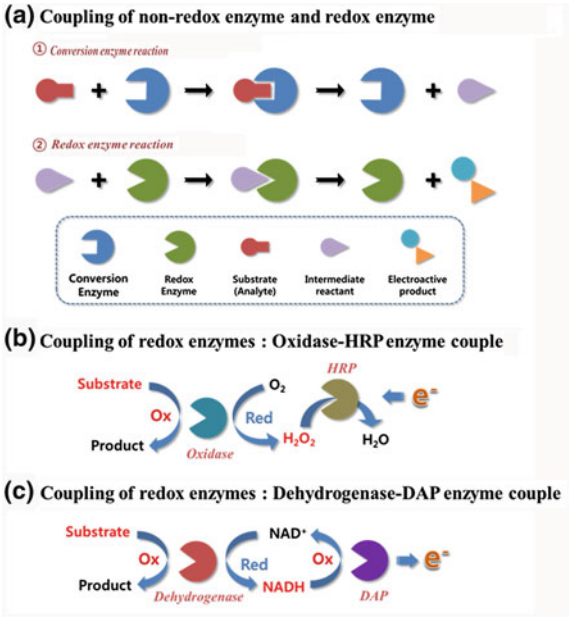


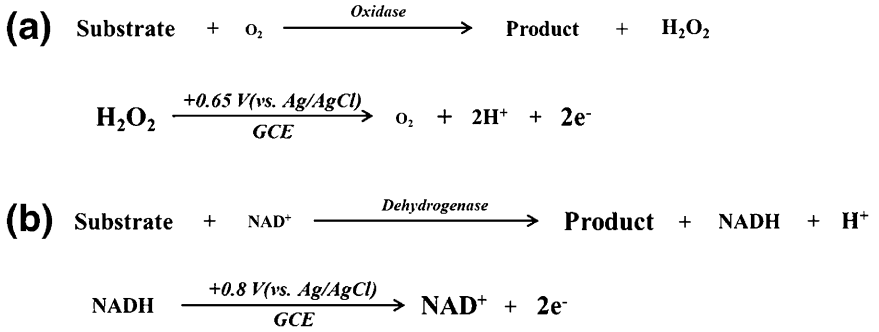
Fig. 2 Types of cascadic multienzyme reactions. **a** Cascadic reaction of non-redox enzyme and redox enzyme couple. **b** Cascadic reaction of oxidase-HRP enzyme couple. **c** Cascadic reaction of dehydrogenase-DAP enzyme couple. *DAP*, Diaphorase; *HRP*, Horseradish peroxidase

2.2 *Principles of the Electrochemical Signaling in a Cascadic Multienzymatic Electrochemical Biosensor*

The electrochemical signaling of an enzymatic electrochemical biosensor is mainly dependent on the reaction of redox enzymes with their corresponding substrate. In this section, principles of bioelectrocatalytic signaling using redox enzymes on the electrochemical biosensing surface will be discussed.

As described in the previous section, for the generation of electrochemical signals, most catalytic biosensors employ redox enzymes, such as oxidases, which produce hydrogen peroxide and dehydrogenases that produce NAD(P)H during the course of catalytic reaction with the enzyme substrate of interest. The enzymatic products from the redox enzyme reactions, such as hydrogen peroxide and NAD(P)H, can be directly oxidized on the biosensing electrode by applying a certain electrical potential, and this direct electrocatalytic oxidation provides a measurable electrical signal.

As shown in Scheme 2, products from redox enzyme reactions such as hydrogen peroxide and NADH can be measured amperometrically by electro-oxidation at the solid electrode (e.g., glassy carbon electrode), polarized at more than +0.65 and +0.8 V, respectively. This direct electrocatalytic oxidation provides a simple and convenient biosensor transducing strategy. However, in a complex sample such as human blood, at those potential levels where the direct electrocatalytic oxidation of hydrogen peroxide and NADH is accomplished, co-oxidation of various organic compounds, such as ascorbic acid and uric acid, can



Scheme 2 Scheme for redox enzyme reactions and the direct electrocatalytic oxidations of electroactive products from enzyme reactions. **a** Oxidase enzyme reaction and direct electrocatalytic oxidation of hydrogen peroxide at electrodes. **b** Dehydrogenase enzyme reaction and direct electrocatalytic oxidation of NADH at electrodes

also occur and cause poor biosensor selectivity. It is a serious problem for practical applications of biosensor in clinical fields and bioreactor monitoring. Additionally, the high oxidative potential results in the fouling of the solid electrode surfaces. Although careful selection and use of biosensing electrode materials may solve this problem, it cannot be a fundamental solution.

To overcome the overpotential problems in direct electrocatalytic oxidation of hydrogen peroxide and NADH, the use of additional redox enzymes and electron transferring mediators has been studied [12, 28–31]. Generally, these additional redox enzymes and mediators are used to replace the natural electron acceptor. Electron transferring mediators are employed to participate in the electrocatalytic reaction of redox enzyme as an artificial electron acceptor instead of a natural electron acceptor such as oxygen or NAD^+(P). Those mediators were reduced by the redox enzyme reaction and oxidized on the electrode at relatively low oxidative potentials, which could prevent the co-oxidation of non-target interfering molecules. Also, during the bioelectrocatalytic reaction of the redox enzyme on the polarized electrode, mediators are regenerated in a reduced form by a cycling reaction of repetitive reduction and oxidation. The regeneration of the mediator results in the amplification of amperometric signals and a higher sensitivity of biosensors. Moreover, especially in the oxidase-based biosensor, the mediator recycling reaction minimizes the signal hindrance that may be caused by the oxygen depletion in the sample solution.

One thing that should be noted in the use of a mediator is the possible side reaction between the mediator and non-target molecules in the sample, which causes a signal interference during the biosensor operation. A detailed review for the required conditions that should be satisfied by an electron transferring mediator was reported by Turner [34]. A lot of work has been done in mediated biosensing systems, and the advantages from the use of mediators have been reported in comprehensive reviews [35, 36]. For the electron transferring mediators of amperometric enzyme biosensors, ferrocene derivatives, ferricyanides, organic

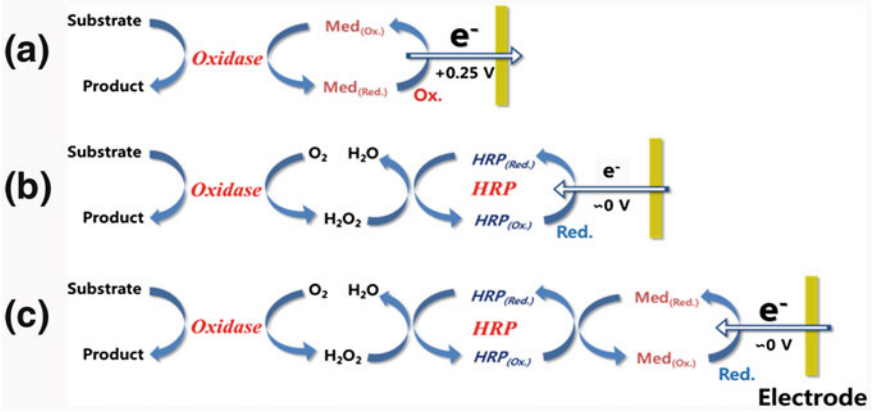


Fig. 3 Schematic illustrations for the electrocatalytic signaling of an oxidase-based biosensor. **a** Mediated mono-oxidase reaction. **b** Nonmediated oxidase-HRP cascadic enzyme reaction. **c** Mediated oxidase-HRP cascadic enzyme reaction. *HRP*, Horseradish peroxidase

dyes, quinone derivatives, and metal-conductive polymer complexes can be used. These mediators can be prepared by adsorption onto the enzyme electrode or by mixing with sample pre-treatment solution. In addition, mediators can be covalently immobilized on the electrode as mediator–polymer complex formats.

Similar to the use of electron transferring mediators, the use of additional redox enzymes lowering the electrocatalytic potential range is another promising route. This redox enzyme coupling was mentioned in the previous section. In this case, additional redox enzymes (e.g., HRP and DAP) catalyze products from the primary redox enzyme reaction (e.g., hydrogen peroxide or NADH) and accept the electrons to their own redox active site. Those activated additional redox enzymes can generate amperometric signals on the electrode by direct electrocatalytic oxidation with a certain electrocatalytic potential or again by mediator-involved electrocatalytic reaction. By using these combined redox enzyme couples, their electrocatalytic potential levels for the amperometric signal generation also can be reduced similarly to the case of a single redox enzyme-mediator system. Moreover, the high substrate specificity and fast catalytic rate of these additional redox enzymes provide enhanced signaling efficiency and accuracy.

These approaches employing mediators and extrinsic redox enzymes to minimize the side effects caused by high electrocatalytic working potential for direct oxidation of electroactive species can be classified into oxidase-based biosensing systems and dehydrogenase-based systems, as shown in Figs. 3 and 4, respectively.

In oxidase-based biosensing, the amperometric signaling methods could be divided into three cases: (1) Mediator-involved bioelectrocatalytic signaling using single oxidase-modified electrodes, (2) Direct electrocatalytic reduction of activated HRP using oxidase-HRP redox enzyme couple-modified electrodes, and (3) Mediator involved electrocatalytic signal generation using oxidase-HRP redox

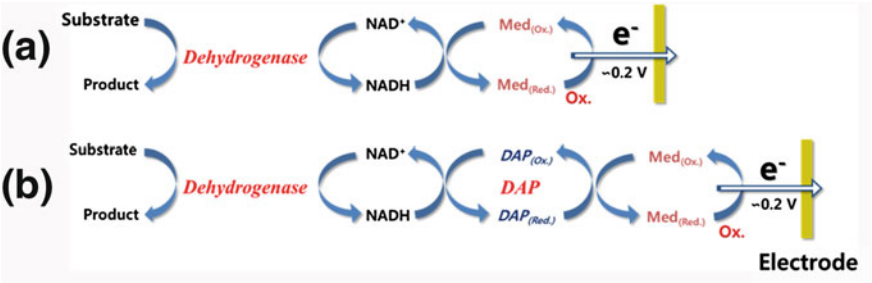


Fig. 4 Schematic illustrations of electrocatalytic signaling of a dehydrogenase-based biosensor. **a** Mediator-involved indirect measurement of NADH. **b** Mediated dehydrogenase-DAP cascadic enzyme reaction. *DAP*, Diaphorase; *NADH*, Nicotinamide adenine dinucleotide (reduced)

enzyme couple-modified electrodes. Also, distinctively in the dehydrogenase-based biosensing, the amperometric signaling methods follows in two cases: (1) Mediator-involved bioelectrocatalytic oxidation of NADH signaling using single dehydrogenase-modified electrodes and (2) Mediator-involved electrocatalytic signal generation using dehydrogenase-DAP redox enzyme couple-modified electrodes. All the cases that use mediators or extrinsic redox enzymes include regeneration and recycling of redox active species causing an amplification of signal. However, when redox enzyme couples and mediators are used together, the type of mediators should be carefully chosen to avoid possible distracted electron transfer, because some mediators participate in both coupled redox enzyme reactions and may generate false signals. For example, the most widely used mediator, ferrocenemethanol, can react with flavin adenine dinucleotide (oxidized)-dependent oxidase and HRP simultaneously. The distracted electron transfer reduces the signal accuracy and sensitivity of the biosensor. Similar problems appear in the mediated dehydrogenase-DAP system because some mediators can react with NADH and DAP simultaneously. To address these issues, rate constants of the redox enzymes and mediators should be carefully considered for the selection of mediators [28, 34].

On the basis of these bioelectrocatalytic reaction principles, recent advances in materials science and nanotechnology offer a great possibility to enhance the signaling performance of biosensors. A number of studies have reported on the application of newly developed nanomaterials having novel electrochemical properties that may improve of the performance of enzymatic biosensors [37–42]. For example, nanocarbon materials (e.g., carbon nanotubes [CNT], graphene) that show good electrical and mechanical properties—including high conductivity, stability under a high potential level, minimized surface fouling, high surface area, and biocompatibility—were used as electrode substrates or electrode additives for the fabrication of enzyme biosensors [39, 40]. Also, carbon-based nanomaterials remarkably decreased the oxidation potential level of NADH, which was ascribed to the edge-plane sites or defects present in the pyrolytic graphite and CNTs [43]. Another example of nanomaterial applications in biosensors is the use of metal

nanoparticles [37, 41, 42, 44]. Among the various metal nanoparticles, gold nanoparticles (AuNPs) are most widely used in enzymatic biosensors because of their convenient synthesis, biocompatibility, and novel electrical properties. The use of AuNPs in the enzyme electrode provides an enhanced electron transferring (hopping) between redox enzymes and electrode surfaces. These improvements in electrochemical activities are induced by AuNPs' own characteristics, such as the high surface-to-volume ratio and high surface energy. The effectively small size of AuNPs (a few nanometers) offers an opportunity to approach the active site of redox enzymes and facilitate the electron transfer. Also, AuNPs play a role as electro-conducting pathways between electroactive sites in redox enzymes and the electrode surface. Pingarron et al. reported a review on AuNP-based electrochemical biosensors [44]. Besides carbon-based nanomaterial and metal nanoparticles, nano metal-oxide materials (e.g., ZnO and TiO_2) and conducting polymers (e.g., polyaniline [PANI] and polypyrrole [PPY]) are also applied to the enzyme biosensor to improve the electrochemical signaling [45–48]. Although these nanomaterials provide an enhancement of electrochemical signaling performance, ill-prepared use of nanomaterials can cause some problems, including complicated sensor construction steps, an increase of background signals, and uneconomical fabrication costs. To develop a highly sensitive multi-enzymatic biosensor for practical use, appropriate selection and organization of enzyme couples and mediating scenarios are more important than the employment of new material.

2.3 Enzyme Immobilization Strategies for the Construction of Multienzyme-Modified Electrodes

To develop enzyme-based biosensors, enzymes have to be properly attached to the transducer surface. This process is known as immobilization. The optimal immobilization method retains the activity of biomolecules with long-term stability regarding their function [5]. Generally, there are a number of factors to be considered for the enzyme immobilization, including the characteristics of the supporting material, functional moiety on the enzyme surface, molecular weight of the enzyme, isoelectric point and polarity of enzyme, substrate diffusivity toward enzyme layer, and the transfer of electroactive product toward the electrode surface [5, 49–51]. To carry out the multienzyme immobilization, those factors for each enzyme should be individually satisfied. Certainly, if the characteristics of different enzymes to be immobilized are similar, optimization of those factors can be rather simple. Otherwise, careful selection of immobilization conditions for each enzyme, such as pH and the ionic strength of the treating solution, is required.

In addition, the efficient diffusion of an intermediate reactant that is generated from a primary enzyme reaction to the secondary enzyme location should be kept in mind for the multienzyme immobilization [5, 49–51]. Also, the amount of each

enzyme and the ratio for immobilization are critical factors for sensitive biosensor fabrication. These factors are related to the catalytic reaction of each enzyme, which is represented as the K_m value, exhibiting the lag period in the coupled reaction. Thus, it is highly necessary to evaluate the K_m values of each enzyme to fabricate the most effective multienzyme biosensor. The correlation between the K_m values of each enzyme and the total coupled enzyme assay was demonstrated by Tipton in 2002 [25]. Based on the evaluation according to the product generation, the total reaction rate of the cascadic coupled enzyme reaction is proportional to the reaction rate of the primary enzyme. In the enzyme biosensor, reaction of the final redox enzyme for signal generation is equivalently important as the primary enzyme reaction. Therefore, when the reaction rates of each enzyme are different, the ratio of immobilized enzymes would be changed to compensate for this imbalance. Thus, by understanding the enzyme reaction rate, we can calculate the optimal amount of enzymes to be immobilized, which provides an efficient multienzyme reaction and biosensing.

In addition to the ratio control, the compensation of enzyme reaction rates can be achieved by the arrangement of individual enzymes during the immobilization. When each enzyme is separately immobilized on the electrode, the delayed diffusion of the intermediate from primary enzyme location to the next enzyme layer occurs. This delayed diffusion provides time to produce a sufficient amount of intermediate that acts as a substrate for secondary enzymes. Regarding biosensor sensitivity, an intense immobilization of the redox enzyme that generates an electrochemical signal may enhance the electrochemical signal. Although this approach is not required for every multienzyme system, it is useful when the involved primary enzyme is remarkably slow in the cascadic multienzyme reaction. The mathematical modeling studies about the kinetic behavior of two enzyme reactions on the multienzyme-modified electrode were summarized by Baronas et al. [21].

The immobilization methods used for enzyme biosensors can be divided into physical and chemical approaches (Fig. 5) [5, 49–51]. Physical methods include adsorption, entrapment, and encapsulation. Chemical methods can be briefly categorized into cross-linking and covalent bonding. Adsorption is the simplest and involves minimal preparation. In this case, enzymes are randomly attached to surfaces of supporting material by low energy bonds such as electrostatic interaction, hydrogen interaction, van der Waals forces, and hydrophobic interactions [52]. However, the bonding is relatively weak. Therefore, this method is only suitable for exploratory work over a short timespan. In this method, arrangement of individual enzymes after immobilization cannot be achieved.

In entrapment and encapsulation methods, enzymes are held in three-dimensional polymeric lattices, polymeric membranes, and micellar polymers. Entrapment is normally achieved by forming a networked polymer gel, such as a starch gel, polyacrylamide gel, or PEG-based hydrogel, around the enzymes. Also, a sol–gel matrix can be applied to this method, such as tetraethyl orthosilicate [53, 54]. Conducting polymers including PPY and PANI are also useful [47, 48]. The polymeric network can be formed by traditional polymerization and electro-

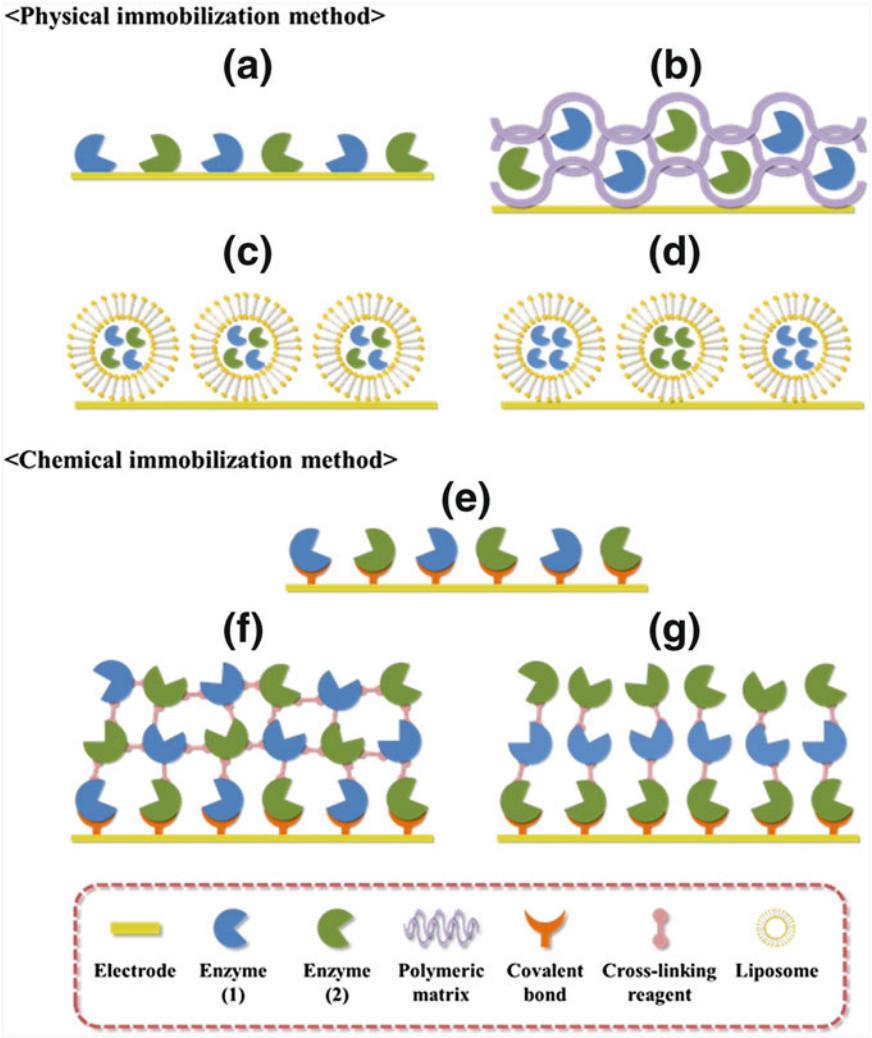


Fig. 5 Methods of enzyme immobilization. **a** Adsorption **b** Entrapment **c** Liposome-based encapsulation-random immobilization **d** Liposome-based encapsulation-isolated enzyme immobilization **e** Covalent bonding **f** Blended method using covalent bonding and cross-linking **g** Layer-by-layer (LBL) formation and LBL associated with covalent bonding

polymerization for entrapping enzymes [40]. Simple polymer membranes such as polyvinyl chloride, nylon, and Nafion also have been employed to hold enzymes [22]. In addition, electrostatic interaction-based multienzyme layer formation using linear polyelectrolytes such as polyethyleneimine, poly-L-lysine (PLL), and polyaniline sulfonic acid (PASA) are frequently used [55]. The entrapment method provides a definite enzyme immobilization with minimized leaching of enzymes as

a function of time. Moreover, by repeating the polymerization with different enzymes, separated enzyme layers can be achieved.

Encapsulation is similar to the entrapment method. In cases of encapsulation, enzymes are immobilized in a polymeric matrix having isolated pores, room, or film structure [56]. In the early type of biosensors, enzymes were held in place behind a permeable membrane such as a dialysis membrane, which allowed selective substrate and product penetration. Recently, enzyme micelle and enzyme liposome structures have been developed and used. However, during the polymerization step, enzyme activity can be reduced by uncertain denaturation. In addition, the polymeric networks such as polymeric matrix, micelle structures, and polyelectrolyte layers may act as barriers against the diffusion of substrates and intermediates; thus, reduction or loss of the cascadic multienzyme reaction rate can occur. To address this issue, polymerization degree and polymerization conditions should be carefully controlled [57].

Cross-linking and covalent bonding are the most stable immobilization methods for enzyme electrode fabrication [49–51]. In these methods, enzymes are bound to an activated electrode surface or other enzyme molecules by forming a covalent bond. Functional moieties of the enzyme surface, such as amine groups and carboxyl groups, are involved in covalent bonding. To attach the enzymes onto the electrode surface, electrode surfaces are preactivated by additional treatments such as silanisation of ITO glass, oxidation of carbon, and formation of self-assembled monolayers (SAM) on gold electrode surfaces. By this surface activation, various functional groups, such as amine groups, carboxyl groups, hydroxyl groups, thiol groups, N-hydroxysuccinimide ester (NHS) groups, and maleimide groups, can be exposed on the electrode. To these functional groups of electrode surfaces, functional moiety of the enzyme surface can be immobilized by direct covalent bonding or bifunctional cross-linking reagents (e.g., succinimidyl-4-[N-maleimidomethyl]cyclohexane-1-carboxylate, glutaraldehyde (GA), and bis[sulfosuccinimidyl] suberate [BS^3]). The reaction between electrode surface and enzyme was simply done by dipping the activated surface into an enzyme solution or applying a drop of this solution onto the surface.

To the enzyme immobilized electrode that is fabricated by covalent bonding, one can immobilize more enzymes repetitively by using a bifunctional cross-linking reagent. Using this strategy, one can construct an enzyme electrode in a layer-by-layer (LBL) format [32]. Cross-linking can be formed among enzymes in the same layer after LBL formation. This covalent bond-based enzyme immobilization necessarily causes a reduction of enzyme activities due to the covalent bonding. Therefore, the selection of relatively mild conditions of enzyme immobilization treatments such as low ionic strengths, low temperatures, near physiological pHs, and the selection of water-soluble chemical reagents are very important [57]. Recently, nanomaterials with unique properties such as high surface-to-volume ratio, mesoporous nanostructures, and good electrochemical activities (e.g., CNTs, mesoporous metal oxides, metal nanoparticles) have been applied to the enzyme immobilization for the construction of highly concentrated enzyme layers and sensitive multienzyme electrodes.

3 Practical Applications of Cascadic Multienzyme-Modified Biosensing Electrodes in Various Fields

The efforts of scientific and engineering research provide us with the knowledge to develop accurate and effective electrochemical multienzymatic biosensors. The theories and techniques include coupled enzyme reaction mechanisms, electrochemical signaling techniques, and enzyme immobilization strategies. On the basis of these findings, a number of studies about the development of biosensors employing cascadic multienzyme reactions have been reported. They are used in clinical diagnostics, food analysis, and environmental monitoring [1–3, 10–19]. This section discusses the practical applications of multienzyme-based electrochemical biosensors in various fields.

3.1 Applications in Clinical Diagnostics

Among many achievements in enzyme biosensor research and development, the most successful is the diagnostic use of blood glucose sensors. Although there exist many important target metabolites that need to be measured by biosensors, the list of commercialized enzyme biosensors is very short. Recent advances in multienzyme reactions involving biosensing techniques may offer an opportunity to overcome this situation. By using cascadic multienzyme-modified biosensing electrodes, more clinically significant analytes that require complicated analysis procedures for detection—including biochemical metabolites, cofactors, and metabolic enzymes—can be analyzed more efficiently. Moreover, a sensitive electrochemical immunoassay can be accomplished by using a multienzymatic reaction as a label of antibody molecules. D'Ozario reviewed the biosensing technologies for clinical chemistry [2].

Many researchers reported the development of multienzymatic electrochemical biosensors to quantify the concentration of biochemical metabolites such as acetylcholine [58, 59], arginine [60], cholesterol [52, 61], creatinine [62, 63], glucose [64–66], and triglycerides [22, 67]. Ahmadalinezhad et al. proposed a total cholesterol biosensor using a titanium electrode modified with three enzymes and nanoporous gold coating [61]. As enzymatic constituents, cholesterol esterase (CE), cholesterol oxidase (CO), and HRP were employed and were immobilized onto a nanoporous gold-coated Ti electrode by simple adsorption and chitosan-based entrapment. As shown in Fig. 6, the cascadic enzyme reaction for cholesterol detection is composed of non-redox enzyme/redox enzyme couple and redox/redox enzyme couple. The multienzyme-modified biosensing electrode (Ti/AuNP/CE-CO-HRP) exhibited a high sensitivity (29.33 $\mu A/mM \cdot cm^2$) and a wide linear range to 300 mg/dL. In addition, the apparent K_m constant of the proposed biosensor was very low (0.64 mM). The researchers explain that the effective enzyme immobilization strategy and the nanoporous structure of the electrode surface are the possible reasons for the observed biosensor performance.

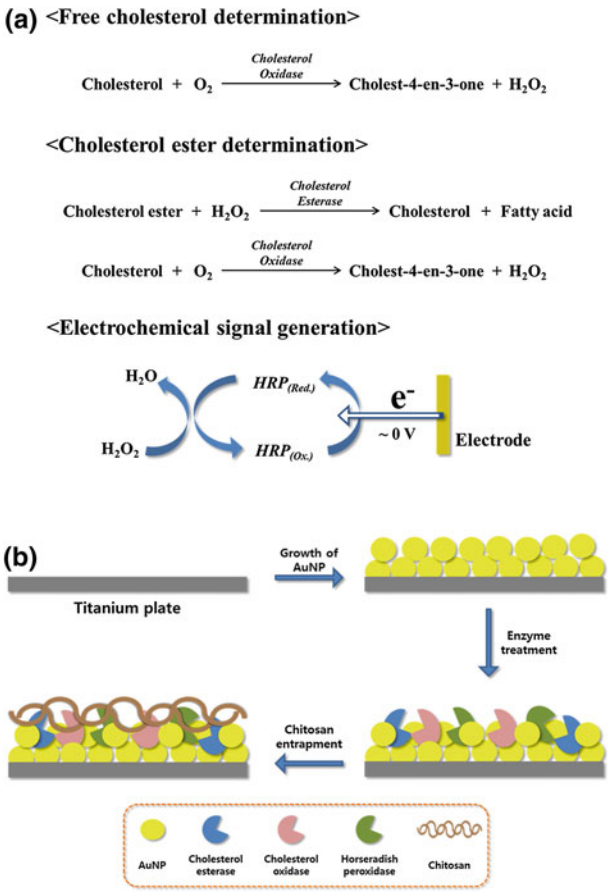
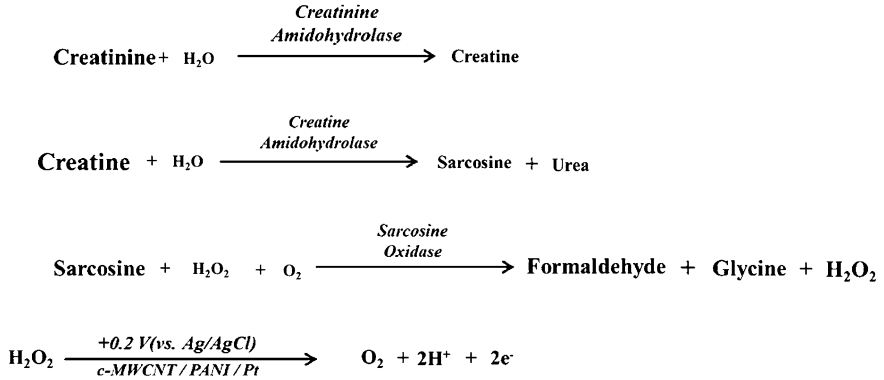


Fig. 6 Schematic illustration of cascadic tri-enzyme reaction mechanism and multienzyme electrode fabrication for the total cholesterol biosensor proposed by Ahmadalinezhad et al. [61]. **a** Cascadic multienzyme reaction mechanism and electrochemical signaling mechanism **b** Multienzyme electrode fabrication procedures. *AuNP*, Gold nanoparticle; *HRP*, Horseradish peroxidase

Yadav et al. reported a sensitive and stable serum creatinine biosensor employing carboxylated multiwalled carbon nanotube (c-MWCNT) and a PANI-modified electrode [63]. For the multienzyme-modified electrode fabrication, c-MWCNT/PANI nanocomposite film was electrodeposited over the surface of a platinum (Pt) electrode. To the carboxyl group on the activated electrode, enzymes (creatinine amidohydrolase, creatine amidinohydrolase, and sarcosine oxidase) for creatinine detection were covalently immobilized by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/NHS chemistry. The cascadic enzyme reaction and electrochemical signaling mechanism are shown in Scheme 3.



Scheme 3 Cascadic tri-enzyme reaction employed in electrochemical creatinine biosensor (Yadav et al. [63])

In this study, as an electrochemical signaling strategy, direct oxidation of hydrogen peroxide, which was generated from the cascadic enzyme reaction, was employed. The proposed biosensor exhibited a linear response range from 10 to 750 μM creatinine with a limit of detection of 0.1 μM. Also, Yadav et al. described that the proposed biosensor was successfully employed for the determination of creatinine in human serum samples. According to their report, the developed biosensor showed a 15 % loss in its initial response after 180 days storage at 4 °C.

Recently, the development of multienzymatic biosensors for the analysis of clinically meaningful metabolic enzymes including CK [33] and transaminase [32, 68] have been reported. Han et al. investigated a new method for the electrochemical detection of human serum transaminases (e.g., aspartate transaminase [AST] and ALT) by using a bienzyme-modified electrode [32]. For the electrochemical detection of AST and ALT, POx and oxaloacetate decarboxylase (OAC) were immobilized on the gold electrode surface using an amine-reactive SAM (DTSP) and a homobifunctional cross-linker (BS^3). AST and ALT were pre-reacted with a substrate such as L-aspartate, L-alanine, or α-ketoglutarate. Enzyme reactions of AST and ALT generated pyruvate as an end product. To determine the activities of AST and ALT, electroanalyses of pyruvate were conducted using POx/OAC-modified multienzyme electrode with ferrocenemethanol as an electron mediator. Generated biosensor signals from the multienzyme-mediated reaction were correlated to AST and ALT levels in human plasma. The calibration results for AST and ALT concentrations from 7.5 to 720 units/L in human plasma-based samples were found useful, covering the required clinical detection range for the liver disease diagnosis. For the enzyme immobilization, Han et al. employed an LBL technique associated with covalent bonding to enhance and control the biosensor sensitivity (Fig. 7).

Cascadic multienzyme reactions also can be applied to an affinity biosensor system such as immunoassays [69] and aptamer-based protein analyses [70, 71].

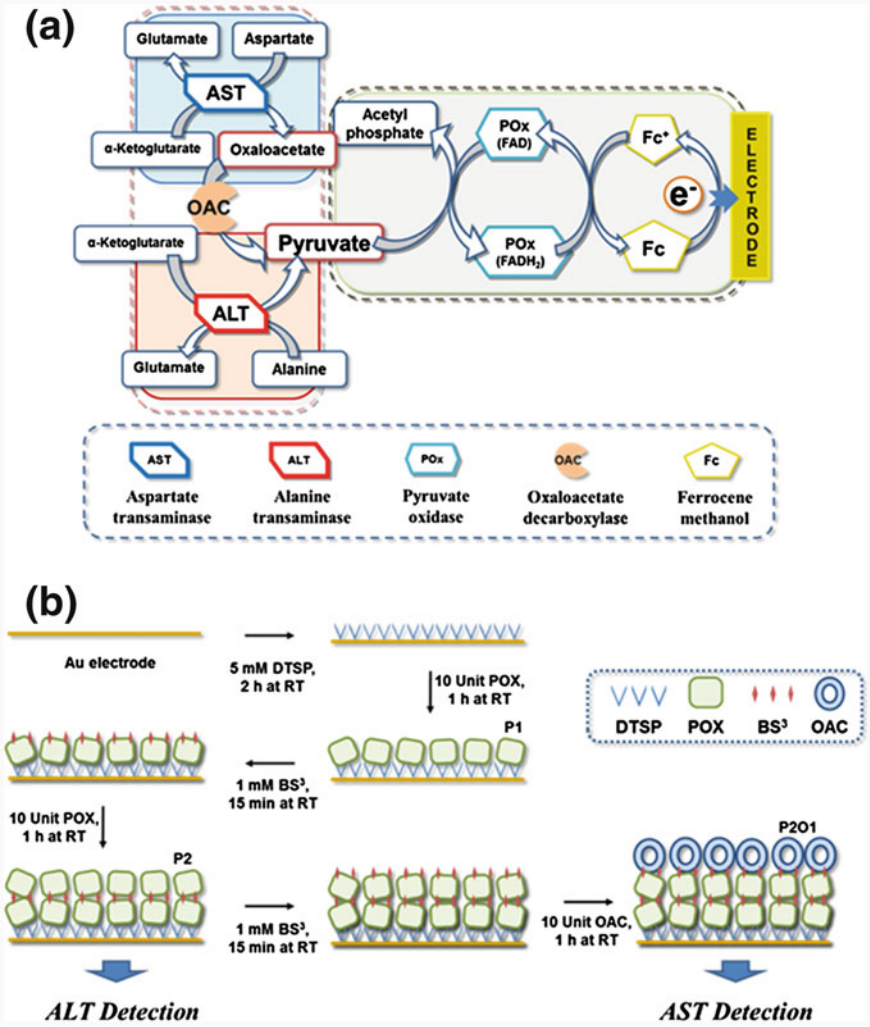


Fig. 7 Schematic illustration of a multienzyme reaction mechanism and electrode fabrication for an electrochemical transaminase biosensor proposed by Han et al. [32]. **a** Cascadic multienzyme reaction mechanism and electrochemical signaling mechanism **b** Multienzyme electrode fabrication procedures

Xiang et al. suggested an electrochemical sandwich-type aptasensor for the monitoring of proteins through a multienzyme reaction-based signal amplification strategy [70]. In this study, thrombin was employs as a model analyte, sandwiched between an electrode surface immobilized aptamer and an aptamer enzyme (alkaline phosphatase [ALP])-CNT bioconjugate. By coupling the ALP-DAP bienzymatic signal amplification with the biocatalytic signal enhancement from redox-recycling, the resulting analytical signal was significantly amplified. In the

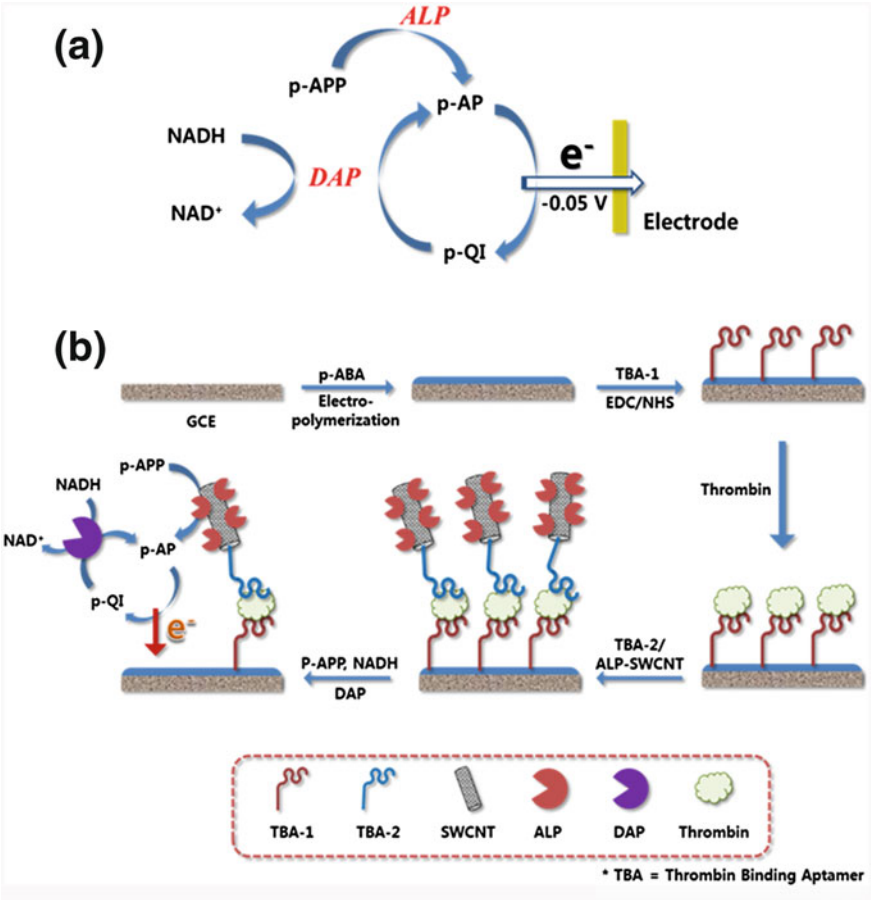


Fig. 8 Schematic illustration of an electrochemical aptasensor suggested by Xiang et al. [70]. **a** Cascadic bienzyme reaction-based electrochemical signaling **b** Fabrication and operation procedures of electrochemical aptasensors. *ALP*, Alkaline phosphatase; *DAP*, Diaphorase; *EDC*, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; *GCE*, Glassy carbon electrode; *NADH*, Nicotinamide adenine dinucleotide (reduced); *NHS*, N-hydroxysuccinimide ester; *p-AP*, p-Aminophenol; *p-QI*, p-Quinonimine; *p-APP*, p-Aminophenylphosphate; *SWCNT*, Single-walled carbon nanotube; *TBA*, Thrombin-binding aptamer

presence of DAP, p-aminophenylphosphate, and NADH, the electrochemically oxidized product p-quinonimine (p-QI) is reduced back to p-aminophenol (p-AP) by DAP, and NADH reduces the oxidized form of DAP to its native state. A redox-recycling between p-AP and p-QI is made accordingly, resulting in an increased electrocatalytic signal (Fig. 8). Xiang et al. demonstrated that the detection limit of the proposed aptasensor was as low as 8.3 fM, and it showed highly improved sensitivity for thrombin detection compared to a conventional single ALP-based aptamer assay.

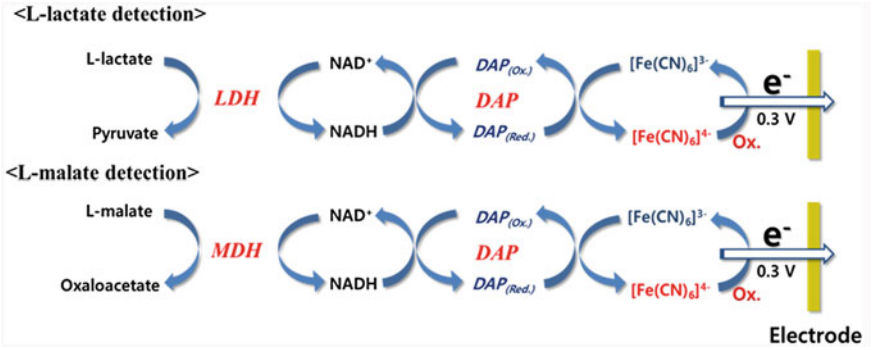


Fig. 9 Schematic illustration of a cascadic bienzyme reaction employed in an electrochemical L-lactate/L-malate biosensor suggested by Katrlík et al. [73]. *DAP*, Diaphorase; *LDH*, Lactase dehydrogenase; *MDH*, Malate dehydrogenase; *NAD*, Nicotinamide adenine dinucleotide (oxidized); *NADH*, Nicotinamide adenine dinucleotide (reduced)

3.2 Applications in Food Analysis and Environmental Monitoring

Accurate monitoring of contaminants in food and the environment, such as chemical compounds and toxins, is a significant issue to assess and prevent risks for human and environmental health. Moreover, in the food industry, the analysis of nutrients in a product is essential for quality control. On the basis of these requirements, a number of multienzyme-based biosensors for monitoring the food and environmental conditions have been developed [16–18].

Glucan [72], L-lactate [73], L-malate [73], polyphenol [74], and sulfites [55] are typical target analytes for the reported multienzyme-based biosensors for food analysis. Katrlík et al. presented bienzymatic electrochemical biosensors for the selective detection of L-lactate and L-malate in wine [73]. The developed electrode comprised a solid binding matrix (SBM) having a hydrophobic skeleton such as 2-hexadecanone, graphite, and NAD^+. As the enzymatic component, malate dehydrogenase or LDH and DAP were applied to the prepared electrode and covered by a dialysis membrane, which substantially reduced interferences from easily oxidizable compounds such as polyphenols in wine. As an electron transferring mediator, hexacyanoferrate (III) was used for DAP-involved bioelectrocatalytic signaling. During the biocatalytic reaction, the substrate (L-malate or L-lactate) is oxidized and NAD^+ is reduced to NADH. The reduced cofactor NADH is then reoxidized by DAP, which reacts with the electron mediator hexacyanoferrate (III). The reduced mediator is electrochemically oxidized on the electrode and generates a signal (Fig. 9). The response of the proposed bienzymatic biosensing electrode were found to be linear up to 1.1 mM for L-malate and 1.3 mM for L-lactate, respectively. The biosensing results obtained from wine samples were in good agreement with those obtained by liquid chromatography.

Spricigo et al. investigated an electrochemical biosensor for the detection of sulfite, which is a hazardous food additive [55]. An enzyme electrode was

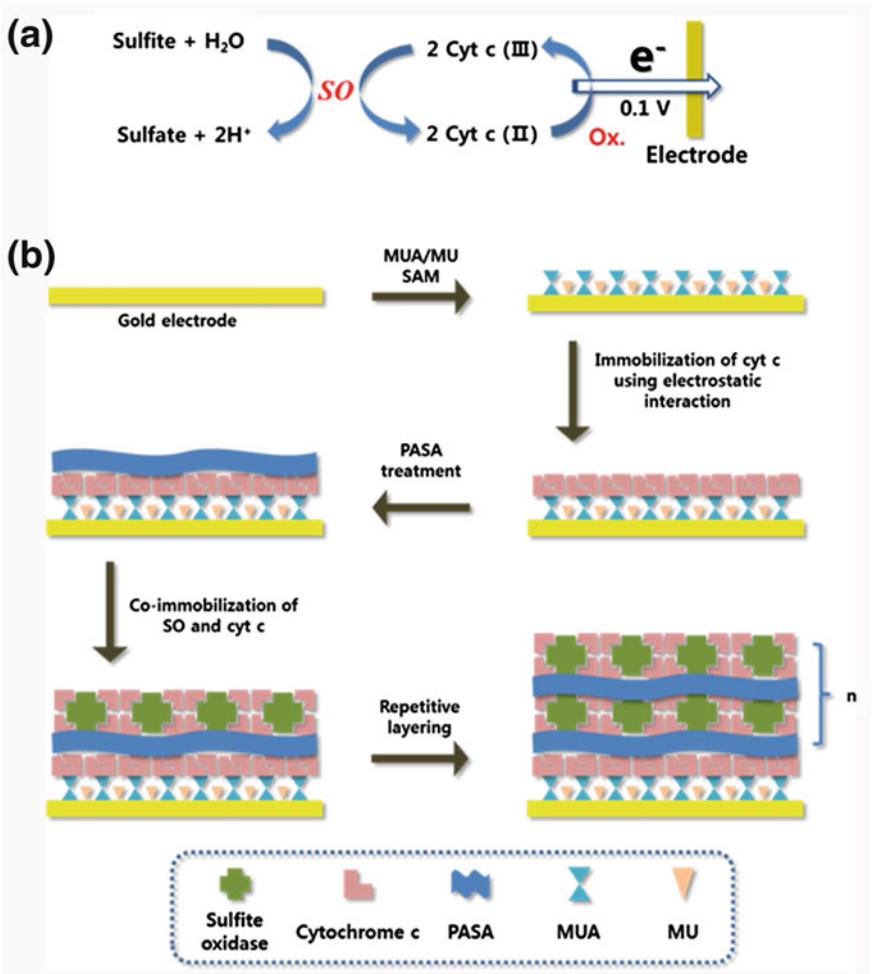


Fig. 10 Schematic illustration of a sulfite biosensor proposed by Spricigo et al. [55]. **a** Mechanism of SO/Cyt-c enzyme reaction and electrochemical signaling mechanism **b** Multienzyme electrode fabrication procedures. *Cyt-c*, Cytochrome compound; *MU*, 11-Mercapto-1-undecanol; *MUA*, 11-Mercapto-1-undecanol acid; *PASA*, Polyaniline sulfonic acid; *SAM*, Self-assembled monolayer; *SO*, Sulfite oxidase

fabricated by co-immobilizing sulfite oxidase (SO) and cytochrome complex (Cyt-c) with PASA on the gold wire electrode as an LBL assembly (Fig. 10). An electrostatic interaction between positively charged enzymes and negatively charged PASA was used as the driving force for immobilization. SO is a metalloprotein containing both the molybdenum cofactor (Moco) and a cytochrome b5-type heme. Sulfite is oxidized at the Moco cofactor of the SO enzyme. The resulting electrons from Moco are transferred to the heme b5 domain in the same SO and then transferred to the nearest Cyt-c molecule that is immobilized. Then,

the Cyt-c transfers received electrons further to the neighboring Cyt-c molecules and ultimately to the electrode, generating the sensor signal. A electro-oxidation current was measured in the presence of sulfite at the enzyme/cytochrome t-modified electrode under the working potential of +0.1 V versus Ag/AgCl, which is a relatively low potential level and advantageous in sensor operation. According to Spricigo et al., a 17-bilayer deposited electrode exhibited a linear range between 1 and 60 μM sulfite, with a sensitivity of 2.19 mA/M. The resulting enzyme electrode was found to be stable for extended uses (5 days of operational stability and 2 months of storage stability). By the sulfite testing using unspiked and spiked samples of red and white wine, Spricigo et al. verified that the suggested biosensor is applicable for real sample analysis.

In the environmental monitoring field, the development of a multienzyme reaction-based biosensor is focused on the detection of toxic chemicals such as pesticide [14, 15, 75] and formaldehyde [76]. Among them, the detection of organophosphorous (OP)-type pesticides has been one of the most popular subjects for multienzymatic biosensor development because it requires complicated enzyme inhibition assay procedures to be conducted. In a number of studies about OP compound determination, acetylcholine esterase (AChE)-related enzyme pathways have been widely employed because they are specifically inhibited by the OP compounds.

Recently, Han et al. developed an integrated microsystem for the OP detection using AChE and choline oxidase (ChOx) cascadic bienzyme bioelectrocatalysis [75]. The electrocatalytic signaling mechanism of the AChE–ChOx bienzyme reaction for OP determination is depicted in Fig. 11. Acetylcholine (ACh) is decomposed into choline (Ch) and acetic acid by the AChE reaction, and Ch is then used as a substrate for ChOx, generating an amplified electrochemical current based on its electrocatalytic reaction with mediators such as ferrocenemethanol. The changes in AChE activity by the irreversible inhibition of OP compounds can be tracked to determine the OP concentration. For field applications, a microsystem containing an enzyme biosensor and microfluidic sample treatment part was designed and fabricated. By using the microfluidic channel and chamber, all the required procedures of an OP test, including enzyme immobilization, sample injection, and washing procedures, are accomplished without any other external preparation.

In this report, enzymes were immobilized on the biosensing microchip in two different ways. As shown in Fig. 11, ChOx was immobilized on the electrode using PLL, GA, and an amine-rich interfacial surface such as cystamine SAM. In the other method, AChE was attached on the magentic microparticle (MP) surface via imine bond formation, and the enzyme-modified MPs were localized on the working electrode using a magnet installed under the microfluidic channel. By utilizing the developed OP biosensing chip, calibration results for OP from 0.05 to 0.8 ppm of diazinon were obtained. The concentrated enzyme arrangement and reduced distance between the enzymes participating in the cascade signaling reactions improved the biosensing performance. The suggested system is a good example of an integrated multienzyme biosensor for field use.

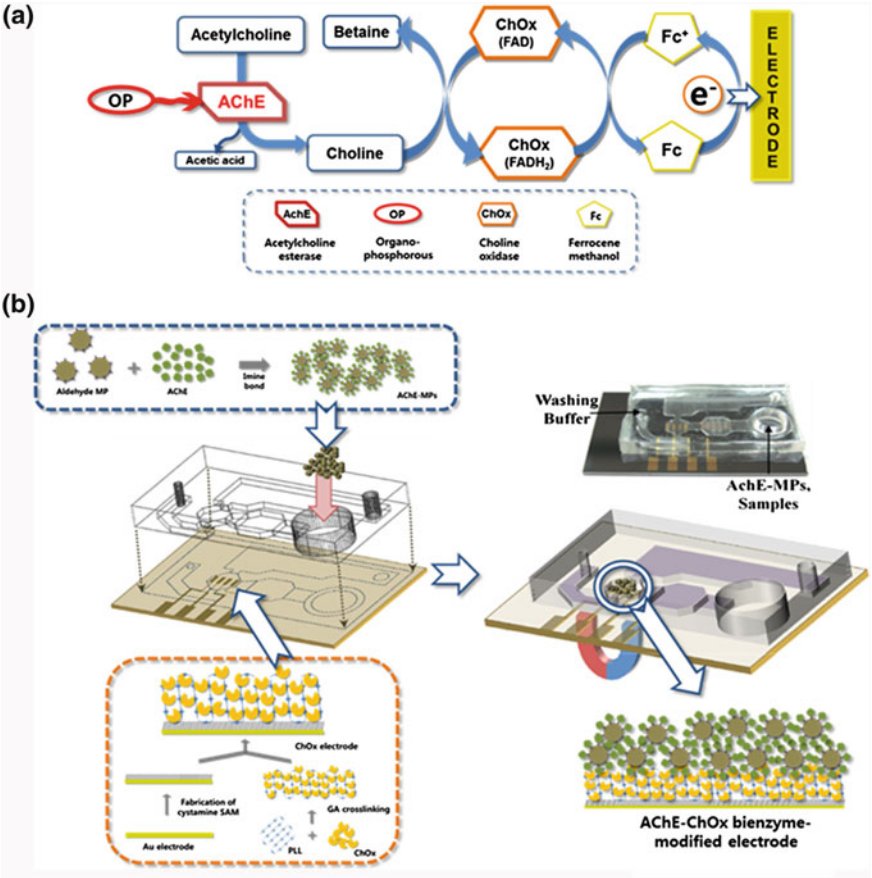


Fig. 11 Schematic illustration of a biosensor for OP-type pesticides proposed by Han et al. [75] **a** Mechanism of bienzyme reactions and electrochemical signaling for OP **b** Procedures of multienzyme electrode fabrication and integrated microchip construction. *OP*, Organophosphorous

4 Conclusion

This review described fundamental principles for multienzyme biosensor development and its applications in various fields. As mentioned in the text, an understanding of the enzyme reaction mechanisms is the most important factor in the development of effective biosensors. The selection of appropriate enzyme couples based on the mechanisms and molecular characteristics is the starting point of multienzyme reaction-based biosensor research.

The potential values and advantages of multienzyme reaction-based biosensors are clear. To expand the pipeline of enzyme biosensors, research on multienzyme reaction-based biosensors is highly important. For the commercialization of

multienzyme biosensors, a number of critical problems should be solved. First, signal interferences induced by non-target molecules should be determined. When employing more than two enzymes for the analysis, one should consider possible side reactions of the enzymes and chemical substances. Another issue is the stability of the biosensor. Because the sensor stability is directly related to the enzyme stability being used, one should find a way to maintain or enhance the enzyme stability. Also, the development of genetically engineered enzymes with improved stability can be another promising solution to the problem. A final concern is the cost of biosensor fabrication. By using new materials and microfabrication technologies, sensors containing small amounts of immobilized enzymes will be made to solve the issue. For the commercialization of biosensors, balanced approaches toward the biosensor performance and fabrication costs are required.

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