Recent advances in electrochemiluminescent enzyme biosensors

Xiao-mei Chen, Bing-yuan Su, Xin-hong Song, Qing-ai Chen, Xi Chen, Xiao-ru Wang

Electrochemiluminescence (ECL) has received considerable attention due to its versatility, simplified optical set-up, and good temporal and spatial control. ECL enzyme biosensors (also called enzyme-based ECL biosensors) offer selective, sensitive detection of analytes (e.g., glucose, alcohol, hypoxanthine and choline).

This review gives an overview on the recent developments of ECL enzyme biosensors. It briefly covers:

- **the three main enzymatic ECL reactions (luminol, $Ru(bpy)_3^{2+}$ and quantum dot);**
- **important factors in fabrication of the containing materials, methods and electrodes; and,**
- **analytical applications in electro-optical enzyme arrays, enzyme biochips, and enzyme immunosorbent assay.**

In addition, we discuss a non-enzymatic ECL biosensor, which we compare with enzyme biosensors.

Keywords: Biosensor; Electrochemiluminescence; Electro-optical enzyme array; Enzyme; Enzyme biochip; Enzyme immunosorbent assay; Luminol; Quantum dot; $Ru(bpy)_3^{2+}$; Tris(2,2′-bipyridyl)ruthenium(II)

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1. Introduction

The first enzyme-based biosensor was reported in 1962 [1]. Since then, efforts have been focused on the development of enzyme-based biosensors. In the recent past, publications related to enzyme-based biosensors became plentiful. From 1990 to 1999, there were about 760 such scientific publications, and, in the past 10 years, the number increased almost six-fold to 4030. Such an impressive number of publications undoubtedly suggests a continuing bright future for research and development activities in enzyme-based biosensors.

The use of enzymes as the biological recognition element was very popular in the development of biosensors due to their commercial availability or ease of isolation and purification from different sources. Among various enzymes, glucose oxidase (GOD), horseradish peroxidase (HRP), choline oxidase (ChOD) and acetylcholinesterase (AChE) have been employed in most biosensor studies [2–4]. For a practical enzyme-based biosensor, the most important issues were significant improvements in terms of selectivity and detection sensitivity, at least under well-controlled environments.

Electrochemiluminescence (electrogenerated chemiluminescence, ECL) is a means of converting electrochemical energy into radiative energy at the surface of an electrode through an applied potential. Luminescence signals can be obtained from the excited states of an ECL luminophore generated at the electrode surfaces during the electrochemical reaction. Because of its simplified set-up, low background signal, and high sensitivity, ECL has received considerable attention from many researchers in recent decades [5,6].

In the development of enzyme-based biosensors, ECL has some unique advantages. The electrochemical reaction in ECL allows the time and the position of the light-emitting reaction to be controlled. By controlling the time, light emission can be delayed until enzyme-catalyzed reactions have taken place. The control over position can be used to confine light emission to a region that is precisely located with respect to the detector, improving sensitivity by

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Table 1. Typical enzyme catalytic reactions

Analytes	Enzyme reaction process	Ref.
Ethanol	$\text{Alcohol} + \text{NAD}^+ \xrightarrow{\text{alcohol dehydrogenase}} \text{acetaldehyde} + \text{NADH} + \text{H}^+$ $\text{acetaldehyde} + \text{NAD}^+ \xrightarrow{\text{alcohol dehydrogenase}} \text{acetlc acid} + \text{NADH} + \text{H}^+$	[22,33,35–37,39,40]
Choline	$\text{Choline} + \text{O}_2 \xrightarrow{\text{Choline oxidase}} \text{betaine aldehyde} + \text{H}_2\text{O}_2$	[4,8,11,18,22,27–29]
Acetylcholine	$\text{Acetylcholine} \xrightarrow{\text{acetylcholine esterase}} \text{choline} + \text{acetate}$ $\text{Choline} + \text{O}_2 \xrightarrow{\text{choline oxidase}} \text{betaine aldehyde} + \text{H}_2\text{O}_2$	[4,19]
Glucose-6-phosphate	$\text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP}$ $\text{Glucose-6-phosphate} \xrightarrow{\text{glucose-6-phosphate dehydrogenase}} \text{gluconolactone-6-phosphate} + \text{NADH} + \text{H}^+$ $\text{Glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{glucose-6-phosphate dehydrogenase}} \text{gluconolactone-6-phosphate} + \text{NADPH} + \text{H}^+$	[22]
Glucose	$\text{Glucose} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + \text{H}_2\text{O}_2$	[12–15,26,36,58]
Proline	$\text{Glycylproline} + \text{H}_2\text{O} + \text{O} \xrightarrow{\text{prolidase}} \text{glycine} + \text{proline}$	[47,51]
Hypoxanthine	$\text{Hypoxanthine} + \text{O}_2 \xrightarrow{\text{xanthine oxidase}} \text{xanthine} + \text{H}_2\text{O}_2$ $\text{xanthine} + \text{O}_2 \xrightarrow{\text{xanthine oxidase}} \text{xanthine} + \text{H}_2\text{O}_2$	[21,67]
Pyruvate	$\text{Phosphoenolpyruvate} + \text{HCO}_3^- \xrightarrow{\text{phosphoenolpyruvate carboxylate}} \textit{oxalacetate} + \text{HPO}_4^-$ $\text{oxalacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{malate dehydrogenase}} \text{malate} + \text{NAD}^+$ $\text{Acetate} + \text{ATP} \xrightarrow{\text{acetate kinase}} \text{acetatylphosphate} + \text{ADP}$ $\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} \text{pyruvate} + \text{ATP}$ $\text{Pyruvate} + \text{HPO}_4^{2-} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{pyruvate oxidase}} \text{acetatylphophate} + \text{HCO}_3^- + \text{H}_2\text{O}_2$	[7,22]
Cholesterol	$\text{Cholesterol} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest-4-en-3-one} + \text{H}_2\text{O}_2$	[7]

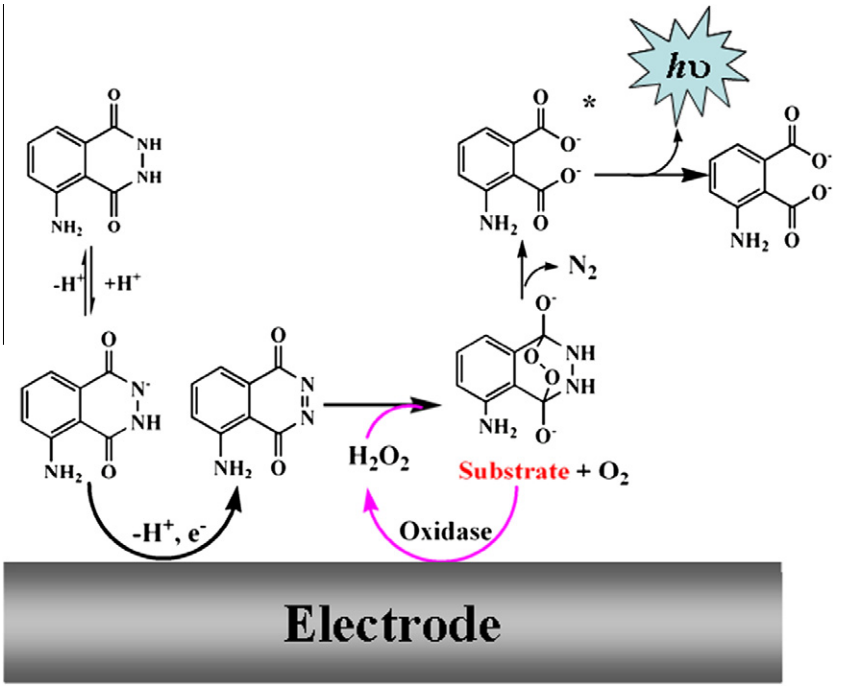

Figure 1. Luminol-based electrochemiluminescence (ECL) reaction mechanism for detection of hydrogen peroxide (H_2O_2)-generating oxidase substrates.

increasing the signal-to-noise ratio. However, control over position can also be used to determine the results of more than one analytical reaction in the same sample by interrogating each electrode in an array, either in sequence or simultaneously using a position-sensitive detector.

Jameison et al. reported significant research on ECL enzyme biosensors in 1996 [7]. In their research, many classical chemistry analytes were commonly quantified by coupling them to enzyme systems that either utilized β-nicotinamide adenine co-factors or produced H_2O_2. Their ECL biosensor systems mainly included ECL detection of NADH using dehydrogenases and H_2O_2 using oxidases. Although various ECL enzyme biosensors have been developed recently, most are still based on these two major types.

This review focuses on the development of ECL enzyme biosensors. We summarize three typical systems based on various enzyme catalytic reactions of these biosensors. We discuss the important fabrication factors and some novel applications. In addition, we also discuss a non-enzymatic ECL biosensor, which we compare with enzyme biosensors. Finally, we briefly address the future trend of ECL enzyme biosensors.

2. ECL enzyme biosensors

2.1. Typical enzyme-based ECL systems and their mechanisms

A considerable number of ECL enzyme biosensors have been discovered in the past 20 years. These biosensors can be summarized as three main types – luminol; $Ru(bpy)_3^{2+}$; and, quantum dot (QD) – as set out in Table 1.

2.1.1. Luminol-based enzyme catalytic ECL systems [7–30]. As a classic organic luminophore, luminol (2,3-aminophthalhydrazide) received great interest after its ECL was first reported in 1928 [31]. In an alkaline or neutral medium, luminol is electrochemically oxidized to form an anion. In the presence of hydrogen peroxide (H_2O_2), the diazo compound undergoes further oxidization to produce the excited state of 3-aminophthalate. The excited state then goes back to its ground state and gives the characteristic ''luminol'' emission at 425 nm. H_2O_2 participates in this ECL reaction in the form of the peroxide anion HOO^- or an electrochemically formed superoxide radical $O_2^{\cdot-}$. Because many enzymes can produce H_2O_2 during their substrate-specific enzymatic reaction, ECL enzyme biosensors are made possible by coupling the luminol light-emitting reaction with enzyme-catalyzed reactions generating H_2O_2 (Fig. 1).

Sakura proposed that the theoretical ratio of photons produced to H_2O_2 consumed is 1 for the luminol/H_2O_2 system, higher than the 0.5 for the peroxidase-catalyzed reaction [32], indicating that H_2O_2 is more efficient in luminol ECL than using the peroxidase-catalyzed reaction. As mentioned above, simple auxiliary H_2O_2-generating reactions and multi-enzymatic systems leading to the production of H_2O_2 can be used for sensitive, selective detection of H_2O_2-generating enzymes and their precursors.

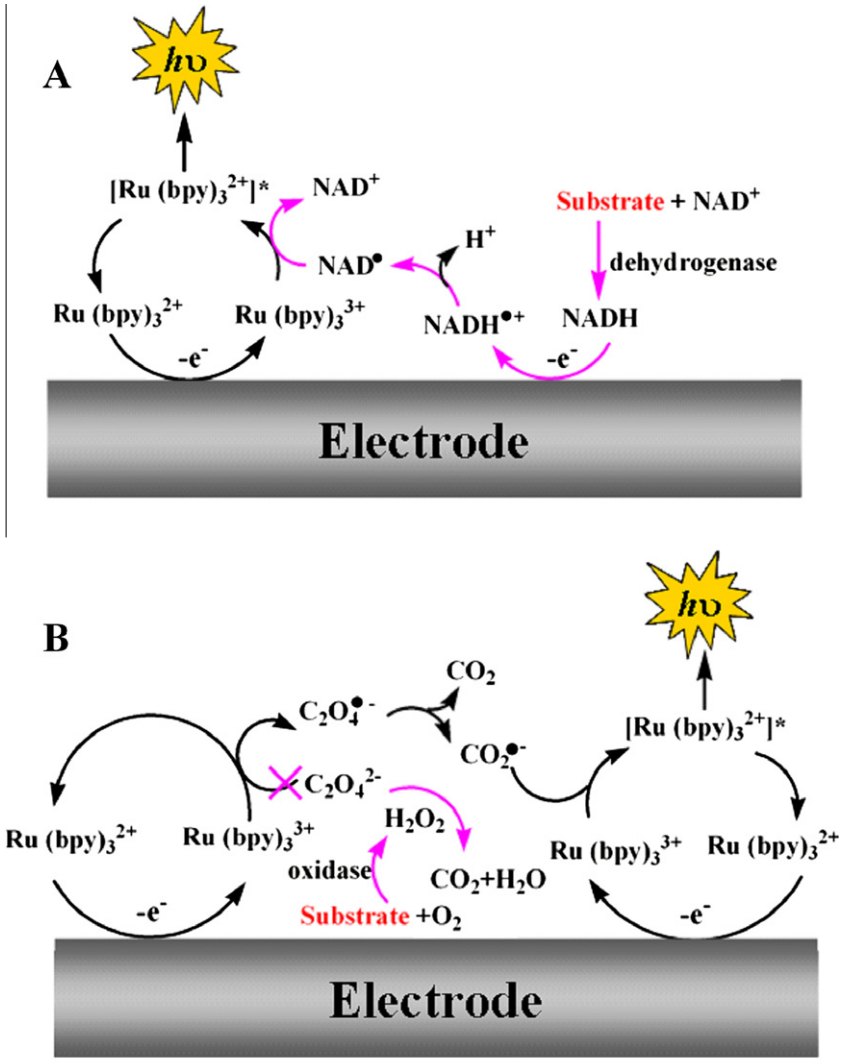

Figure 2. (A) $Ru(bpy)_3^{2+}$-based electrochemiluminescence (ECL) reaction mechanism for detection NAD^+-dehydrogenase substrates; and (B) $Ru(bpy)_3^{2+}$-based ECL reaction mechanism for detection of oxidase substrates through ECL Inhibition.

In the development of choline biosensors, ChOD and biocompatible titanate nanotubes (TNTs) are immobilized on a chitosan (CHIT)-modified glassy carbon electrode (GCE) via electrostatic adsorption and covalent interaction [8]. In this work, ChOD catalyses H_2O_2 production in the presence of oxygen and choline. H_2O_2 is involved in the ECL reaction of luminol and gives rise to a light signal proportional to the choline concentration over a definite range. TNTs in this biosensor not only provide a biocompatible microenvironment for the immobilized enzyme, which results in excellent stability and long lifetime of the ECL biosensor, but also greatly enhance luminol ECL, and, thus, lead to a significant improvement in the sensitivity of the ECL biosensor. The proposed biosensor is able to detect choline with a limit of 1×10^{-8} M and the reproducibility of ECL intensity obtained at the enzyme/TNT/CHIT-modified electrode for 1×10^{-4} M choline is 1.33%, which is better than most previous choline biosensors [9–11].

Generally, the GOD-based biocatalyzed luminol ECL system is applied in the detection of glucose [12–15]. Further research results indicate that a DNA sensor can also be constructed using this system. Recently, a DNA sensor for sequence-specific DNA detection was designed [16] with a GOD-labeled sandwich-type DNA sensor built on the non-fouling surfaces of mixed self-assembled monolayers incorporating thiolated oligonucleotides and oligo(ethylene glycol) (OEG) thiols. The sequence-specific DNA sensing was accomplished by the ECL signal of luminol with the in-situ generated H_2O_2. The protein-resistant non-fouling surfaces significantly suppressed the non-specific adsorption of the enzyme label on the electrode and reduced the background noise of this sensor. As a result, this sensor was able to detect target

DNA as low as 1 pM. Satisfactory results were also obtained when employing this biosensor in complicated biological fluids (e.g., human serum). The performance was superior to the conventional sandwich-type DNA sensors with mercaptohexanol-coated surfaces.

2.1.2. $Ru(bpy)_3^{2+}$-based enzyme catalytic ECL reactions [7,33–54]. Tris(2,2′-bipyridyl)ruthenium (II) ($Ru(bpy)_3^{2+}$) has proved to be the most valuable inorganic luminophore in ECL since its discovery in 1972 [55]. The most attractive characteristics of $Ru(bpy)_3^{2+}$ are its strong luminescence, good solubility in a variety of aqueous and non-aqueous solvents, and its ability to be regenerated in its native form after having completed the light-emission-reaction sequence.

The photochemical properties of $Ru(bpy)_3^{2+}$ can be affected obviously by co-reactants, so the overwhelming majority of $Ru(bpy)_3^{2+}$-based enzyme ECL biosensors are based on $Ru(bpy)_3^{2+}$/co-reactant systems. Because the reduced form of nicotinamide adenine dinucleotide (NADH) contains a tertiary amine, NADH has been selected as a co-reactant for $Ru(bpy)_3^{2+}$ ECL. In a typical $Ru(bpy)_3^{2+}$/NADH system, NADH and $Ru(bpy)_3^{2+}$ are both oxidized at the surface of an electrode. The one-electron-oxidized cation radical $NADH^{\cdot+}$ loses a proton to become a strongly reducing radical, $NAD^{\cdot}$, which subsequently reacts with $Ru(bpy)_3^{2+}$ to generate excited state $Ru(bpy)_3^{2+*}$ in situ on the working electrode. $Ru(bpy)_3^{2+*}$ emits a photon when it decays to the ground state, $Ru(bpy)_3^{2+}$ (Fig. 2A).

In dehydrogenase-type enzyme-catalyzed reactions, the addition of NAD^+ is required as a cofactor for the enzymatic reaction. With substrate oxidation, NAD^+ is simultaneously reduced to NADH. The ECL response is increased in proportion to the concentration of the substrates. By contrast, an oxidase-type enzyme consumes NADH during the enzymatic reaction, resulting in a decreased ECL response in the presence of substrates. In the annihilation model of ECL, signals might suffer from false positives when directly deploying complex sample matrices, and, moreover, their signal gain is limited since the analytes can suppress 100% of the original ECL intensity. There is no doubt that the sensitivity and the selectivity of the $Ru(bpy)_3^{2+}$/NADH ECL system based on oxidase-type enzymes is seriously affected. Most of the $Ru(bpy)_3^{2+}$/NADH systems in enzyme-based biosensors involve dehydrogenase-type enzymes.

In the work from Dong's group, an alcohol ECL enzyme biosensor was developed by self-assembling alcohol dehydrogenase (ADH) to $Ru(bpy)_3^{2+}$-AuNP aggregates (Ru-AuNPs) on an indium-tin oxide (ITO) electrode surface [33]. In this design, positively-charged $Ru(bpy)_3^{2+}$ was stably immobilized on the electrode surface with negatively-charged AuNPs in the form of aggregate via electrostatic interaction. ADH was immobilized by covalent attachment of its cysteine residues and amine groups with AuNPs. In the ECL sensing of ethanol, NAD^+ was reduced to NADH simultaneously with ethanol oxidation in the presence of ADH. Then, NADH could react with $Ru(bpy)_3^{2+}$ to produce excited state $Ru(bpy)_3^{2+*}$. The enhancement of the ECL response was in proportion to the concentration of the ethanol substrate. It is worth mentioning that AuNPs evidently improved the sensitivity of this biosensor because they could act as tiny conduction centers to facilitate the transfer of electrons. The biosensor displayed a wide linear range (10^{-5}–10^{-2} M), high sensitivity (3.33×10^{-6} M) and good stability in the determination of alcohol.

However, further research indicated that this method has some disadvantages. Generally, when ADH and $Ru(bpy)_3^{2+}$ are immobilized in the same layer, ADH activity obviously decreases because the high concentration of $Ru(bpy)_3^{2+}$ increases the hydrophobicity of the layer microenvironment. In addition, the leakage of $Ru(bpy)_3^{2+}$ during continuous determination causes the modified electrode to be unstable.

Wang's group has proposed a new method to immobilize $Ru(bpy)_3^{2+}$ on the electrode surface [34]. In their study, $Ru(bpy)_3^{2+}$-doped silica nanoparticles (RuSiNPs) were synthesized and applied in ECL. Since the modification of the RuSiNPs could improve their biocompatibility and prevent leakage of $Ru(bpy)_3^{2+}$, RuSiNPs could

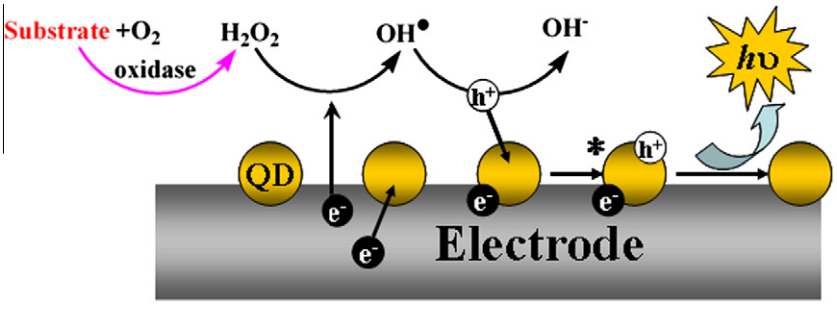

Figure 3. Quantum-dot-based electrochemiluminescence (ECL) reaction mechanism for detection of hydrogen peroxide (H_2O_2)-generating oxidase substrates.

readily be used as efficient, stable ECL-tag materials in bioanalyte sensing.

Our group further investigated an alcohol ECL enzyme biosensor based on RuSiNPs cross-linked to ADH [35]. The cross-linking immobilization method for enzymes maintained both activity and stability of the ADH–RuSiNP composite. The biosensor exhibited excellent performance during ethanol determination with a wide linear range (10^{-7}–10^{-2} M), low limit of detection (LOD) (5.0×10^{-8} M) and good stability.

Another $Ru(bpy)_3^{2+}$-based enzyme ECL biosensor used the $Ru(bpy)_3^{2+}$/oxalate ($C_2O_4^{2-}$) system (Fig. 2B). In this case, the reaction occurring between oxalate and H_2O_2, leading to the production of H_2O and CO_2 could be used to detect H_2O_2 [56]. The ability of the H_2O_2 to diminish the $Ru(bpy)_3^{2+}/C_2O_4^{2-}$ ECL was shown to be a possible route for H_2O_2-generating enzymes.

Only a few biosensors have been built based on the $Ru(bpy)_3^{2+}/C_2O_4^{2-}$ system [7]. In glucose detection, GOD was applied to produce H_2O_2. An inverse relationship was observed between glucose concentration and ECL intensity, but on a very narrow range (e.g., 1–12 mM). Moreover, this system was not set up as a real biosensor (i.e. including immobilized enzyme), and, even then, it consumed a great deal of reagent.

Similarly to the work presented above for the detection of glucose through the $Ru(bpy)_3^{2+}/C_2O_4^{2-}$ system, cholesterol has been detected using cholesterol oxidase. In addition, a correlation between the ECL intensity decrease and the cholesterol concentration was found over the limited range 2–10 mM.

2.1.3. QD-based enzyme catalytic ECL reactions [4,57–60]. QDs have attracted considerable attention due to their unique electrical, magnetic and optical properties. The ECL study of QDs was first reported in 2002 for SiNPs, where ECL was generated from both annihilation and co-reactant oxalate and persulfate systems in acetonitrile (MeCN) [61]. In enzyme-based ECL bioanalysis, QDs have been extensively studied in aqueous medium containing co-reactants (e.g., H_2O_2, $S_2O_8^{2-}$ and O_2). Both cathodic and anodic ECL processes are important to QD-based enzyme catalytic ECL reactions. In the cathodic ECL process, taking H_2O_2 as a representative co-reactant, both QDs and H_2O_2 are reduced at the working electrode. H_2O_2 is reduced to be a strong oxidizing agent ($OH^{\cdot}$), which can inject a hole into QD species (QD^{+}). Excited state QD^{*} can be produced by oxidized and reduced QD species, and also by direct injection of a hole into a reduced QD by $OH^{\cdot}$ (Fig. 3). Most enzymes producing H_2O_2 during their substrate-specific enzymatic reaction can enhance QD ECL.

Several research groups have made contributions to the development of ECL biosensors based on QDs. Ju's group is one of the earliest groups investigating this field [57–59]. They cast a solution of CdSe QDs onto the surface of a paraffin impregnated graphite electrode (PIGE). This system was used to detect H_2O_2 down to a level as low as 0.1 μM [57]. The ability of H_2O_2 to act as a cathodic ECL co-reactant was the basis of transduction. It followed that the ability to detect H_2O_2 enabled the development of a glucose biosensor [58]. A mixture of GOD and mercaptoacetic acid-coated QDs was successively cast onto a PIGE. During the ECL process, the activity of GOD in the presence of glucose converted dissolved oxygen into H_2O_2. Although both oxygen and H_2O_2 were able to act as ECL co-reactants, the electron-transfer kinetics of oxygen were much faster than those of H_2O_2, which made oxygen a more efficient co-reactant. In the presence of glucose, the conversion of dissolved oxygen to H_2O_2 caused a decrease in the ECL intensity of the QDs. The response was linear with the increase of glucose concentration over the range 25–3000 μM, and the LOD is 4 μM.

Ju et al. have also developed QD-based enzyme ECL biosensors utilizing anodic QD ECL [59,60]. They first achieve anodic ECL with CdTe QDs on an ITO working electrode in air-saturated solution [59], but the relatively high anodic potential (+1.17 V *versus* Ag/AgCl) and alkaline condition (pH 9.3) limited the application of this approach in biosystems, since ECLs are generally weak in a neutral medium and that makes the quenching effect undetectable for analytical performances. They further studied the effect of co-reactants on the anodic ECL emission of CdTe QDs in aqueous solution. In the potential sweep range 0 to +1.10 V, QDs and sulfite were oxidized to hole-injected QDs (QD^{+}) and sulfite radical anions ($SO_3^{\cdot-}$). Then, $SO_3^{\cdot-}$ further reacted with dissolved oxygen to form the superoxide anion ($O_2^{\cdot-}$). The superoxide anion injected an electron into the QDs, so excited-state QDs were produced.

As described previously, dissolved oxygen plays an important role during the ECL-emission procedure [60]. Using tyrosine as a model compound, the oxidized o-quinone product of tyrosine efficiently quenched ECL (at +0.90 V *versus* Ag/AgCl). Although anodic oxidation of the tyrosine was possible, the addition of tyrosinase increased the rate of oxidation, and consumed dissolved oxygen during the enzymatic catalyze reaction, resulting in stronger quenching. Without tyrosinase, the LOD was 46 nM; with tyrosinase, the LOD decreased to 0.1 pM.

Most QD-based enzyme ECL biosensors depend on annihilation of ECL, involving interaction between analytes and the species in the ECL process. These signal-off ECL sensors might suffer from some problems (e.g., false positives in complex sample matrices and limited signal gain from the original ECL intensity).

Recently, Xu et al. discovered a signal-on ECL enzyme biosensor based on CdS nanocrystals (CdS NCs) formed in situ on the surface of multi-walled carbon nanotubes (MWCNTs) [4]. The MWCNT–CdS reacted with H_2O_2 to generate strong, stable ECL emission in neutral solution. Compared with pure CdS NCs, the MWCNT–CdS en-

Table 2. Selected papers on electrochemiluminescence (ECL) enzyme biosensors

Analytes	Matrix	Interaction	Electrodes	Applications	Comments		Ref.
					LR[a]	LOD[b]	
Acetylcholine	MWCNT-CdS/AChE-CHO	Cross-linking	PIGE[c]	ECL	3.3×10^{-6}–2.16×10^{-4} M	1.7×10^{-6} M	[4]
	LB film/AChE	Entrapment	Graphite	ECL	4.0×10^{-7}–7×10^{-5} M	4×10^{-7} M	[19]
Bicarbonate	–	–	–	ECL	1.9×10^{-2}–2.9×10^{-2} M	–	[7]
Cholesterol	–	–	–	ECL	0–1.0×10^{-2} M	–	[7]
Choline	MWCNT–CdS/ChO	Cross-linking	PIGE	ECL	1.7×10^{-6}–3.32×10^{-4} M	0.8×10^{-6} M	[4]
	PVA-SbQ/oxidases	Electrostatic	GC	Biochip-ECL	2×10^{-6}–2×10^{-4} M	2×10^{-6} M	[22]
	ChO/TNTs/CHIT	Electrostatic, Covalent	GC	ECL	1.0×10^{-7}–5×10^{-4} M	1×10^{-8} M	[8]
	PVA-SbQ/ChO	Electrostatic	SPE[d]	FIA-ECL	2.0×10^{-8}–1×10^{-4} M	–	[11]
	ChO/TNTs/Nafion	Electrostatic, Covalent	GC	ECL	1.0×10^{-7}–5×10^{-4} M	7×10^{-8} M	[18]
	CHO/PVA-SbQ/ Polyluminol	Entrapment	SPE	FIA-ECL	8×10^{-8}–1.3×10^{-4} M	2×10^{-7} M	[27]
	ACPG/ChO	Covalent	GC	FIA-ECL	0–2×10^{-6} M	5×10^{-8} M	[28]
	sol-gel ChO polyluminol	Entrapment	SPE	FIA-ECL	4×10^{-2}–1.3×10^{-4} M	–	[29]
DNA	SH-DNA/OEG/GOD	Covalent	Au	ECL	No reported	1×10^{-12} M	[16]
Ethanol	Ru-AuNPs ADH	Electrostatic	ITO	ECL	10^{-5}–10^{-2} M	3.33×10^{-6} M	[33]
	RuSiNPs/ ADH	Cross-linking	GC	ECL	10^{-7}–10^{-2} M	5×10^{-8} M	[35]
	AQ/ADH/$Ru(bpy)_3^{2+}$	Ion-exchange	Pt	FIA-ECL	2.5×10^{-5}–1.5×10^{-3} M	–	[36]
	Sol–gel/chitosan PSS	Entrapment	GC	ECL	2.79×10^{-5}–5.78×10^{-2} M	9.35×10^{-6} M	[37]
	AuNPs/PSSG/ $Ru(bpy)_3^{2+}$	Entrapment	Au	ECL	5×10^{-6}–5.2×10^{-3} M	1.2×10^{-11} M	[39]
	Sol–gel/Nanfion/ $Ru(bpy)_3^{2+}$/ADH	Entrapment	GC	ECL	2.5×10^{-5}–5.2×10^{-2} M	1×10^{-5} M	[40]
	PVA-SbQ/oxidases	Non-covalent	GC	Biochip-ECL	2×10^{-5}–2×10^{-3} M	2×10^{-5} M	[22]
Glucose	–	–	ITO	FIA-ECL	0–1×10^{-2} M	4.195×10^{-4} M	[13]
	–	Incorporate	CNTP[e]	ECL	1.0×10^{-6}–2.0×10^{-3} M	5.0×10^{-7} M	[12]
	PDDA-CH1T/GOD	Cross-linking	GC	ECL	5×10^{-10}–4.0×10^{-5} M	1×10^{-10} M	[14]
	Sol–gel film/GOD	Entrapment	GC	FIA-ECL	5×10^{-5}–1×10^{-2} M	2.6×10^{-5} M	[15]
	Electrodeposition paint/GOx	Entrapment	Glass slide	SECM/SECL	–	–	[26]
	AQ/GDII	Ion-exchange	Pt	FIA-ECL	0.2–5×10^{-3} M	–	[36]
	TGA-CdSe QDs/GOD	Covalent	PIGE	ECL	2.5×10^{-5}–3×10^{-3} M	4×10^{-6} M	[58]
Glutamate	PVA-SbQ oxidases	Electrostatic	GC	Biochip-ECL	1×10^{-6}–5×10^{-4} M	1×10^{-6} M	[22]
Hypoxanthine	BSA/XOD	Cross-linking	HCPE	ECL	6×10^{-7}–2×10^{-4} M	1×10^{-7} M	[21]
	BSA/XOD/Nation	Cross-linking	HITO[f]	ECL	3×10^{-7}–2×10^{-4} M	1.5×10^{-7} M	[67]
Lactate	PVA-SbQ Lox	Electrostatic	GC	Biochip-ECL	2×10^{-6}–2×10^{-4} M	2×10^{-6} M	[22]
	Methocel/luminal/Lox/ BSA	Entrapment	Graphite	ECL	1×10^{-4}–5×10^{-4} M	5×10^{-6} M	[20]
	AQ/LDH/$Ru(bpy)_3^{2+}$	Ion-exchange	Pt	FIA-ECL	5×10^{-5}–2.5×10^{-3} M	–	[36]
Lysine	PVA-SbQ/oxidases	Electrostatic	GC	Biochip-ECL	1×10^{-6}–5×10^{-4} M	1×10^{-6} M	[22]
Pentaerythritol	–	Antibody–antigen	Au	FIA-EIA-ECL	–	19.8 ppb	[24]
Proline	–	–	Pt	CE-ECL	1×10^{-5}–3.75×10^{-3} M	5×10^{-6} M	[47]
	–	–	Pt	CE-ECL	5×10^{-6}–1×10^{-3} M	4×10^{-6} M	[51]
Tyrosine	MPA-CdTe QDs/ tyrosinase	Covalent	PIGE	ECL	1×10^{-13}–1×10^{-10} M	5×10^{-6} M	[60]
Urate	PVA-SbQ/oxidases	Electrostatic	GC	Biochip-ECL	1×10^{-6}–2.5×10^{-5} M	1×10^{-6} M	[22]
2,4,6-Trinitroto	–	Antibody–antigen	Au	FIA-EIA-ECL	No reported	0.11 ppb	
2,4,6-Trinitroto		Antibody–antigen	Au	FIA-EIA-ECL	0–100 ppb	12 ppb	[25]

[a]LR: linear range.
[b]LOD: limit of detection.
[c]PIGE: paraffin impregnated graphite electrode.
[d]SPE: screen-primed electrodes.
[e]CNTP: carbon nanotube paste.
[f]HITO: heated indium-tin-oxide.

hanced ECL intensity by 5.3-fold and moved the onset ECL potential more positively by about 400 mV, which reduced H_2O_2 decomposition at the electrode surface and increased the detection sensitivity of H_2O_2. Furthermore,

the ECL intensity was less influenced by the presence of oxygen in solution. On the basis of these properties, they fabricated a series of signal-on, enzyme-based biosensors by cross-linking ChOD and/or acetylcholine esterase with glutaraldehyde on MWCNT–CdS modified electrodes for the detection of choline and acetylcholine. The resulting ECL biosensors showed wide linear ranges of 1.7–332 mM and 3.3–216 mM with lower LODs of 0.8 mM and 1.7 mM for choline and acetylcholine, respectively. Furthermore, both ECL biosensors possessed satisfactory reproducibility and acceptable stability.

2.2. Fabrication of ECL enzyme biosensors

The immobilization of enzyme and/or luminophore for interfacial assays has several advantages over homogeneous solution-phase assays:

(1) it can save on expensive reagents (e.g., enzymes and $Ru(bpy)_3^{2+}$);
(2) the enzymes immobilized at an interface need less strict requirements for their solubility, so there is potentially greater versatility in the selection of coatings, coupling chemistries, and solvent systems that can be used for the assembly of ECL enzyme biosensors; and,
(3) the immobilization can simplify the experimental design.

Many different methods and materials have been developed to fabricate ECL enzyme biosensors (Table 2).

2.2.1. Materials. In recent years, there has been extensive interest in polymer-modified electrodes, which possess many advantages. One of the most significant advantages is that the multilayer-polymer coating yields a three-dimensional reaction zone at the electrode surface, which results in an increase in the rates of reaction occurring at the surface of the electrode. The response sensitivity of the electrodes can therefore be greatly improved.

The solid-state ECL using cation-exchange polymer Nafion reported by Rubinstein and Bard [62,63] has been extended by many researchers [64–66]. Although Nafion has served as a perm-selective membrane to exclude electroactive anionic species and deter fouling, common with electrochemical measurement in physiological fluids, it is not well suited for mixing with enzymes. Commercially-available Nafion (in solution) is in the acid form and its alcoholic solvent denatures enzymes upon casting.

Martin and Nieman [36] developed an ECL sensor by the fixation of $Ru(bpy)_3^{2+}$ and Eastman AQ polymers, which are relatively high molecular-weight, amorphous polyesters with sulfonic groups on aromatic dicarboxylic acid units. The polyester backbone of these polymers results in a greater hydrophilic characteristic than that of Nafion (with a fluorocarbon backbone) and consequently AQ is easily dispersed in aqueous solutions. Moreover, these polymer solutions are generally at pH 5–6, which is more compatible with enzymes.

In the above work, the AQ polymer-based biosensors was applied successfully in the determination of glucose, L-lactate and ethanol. Further studies found that another polymer-chitosan (CHIT) was superior to most other polymers for the design of biosensors [8,35,37]. CHIT is a naturally-occurring biopolymer with unusual combination properties (e.g., excellent membrane-forming ability, high permeability towards water, and good adhesion). Dai et al. prepared a glucose biosensor by immobilizing GOD to a CHIT matrix [14]. Since the CHIT matrix provided good biocompatibility and a stable microenvironment around the enzyme, this glucose biosensor showed excellent sensitivity, good reproducibility and stability for glucose detection.

As mentioned above, polymers have been widely used in the construction of ECL enzyme biosensors, but, in pure polymer film, the rate of charge transfer is quite low, and that seriously influences the sensitivity and the stability of the biosensors. Many novel electron-conductive materials have therefore been introduced into polymer films to improve the performance of the biosensors. For example, Lin et al. reported production of an ECL biosensor through entrapping GOD into the MWNTs/Nafion mixture [17]; and, Dai et al. developed an ECL biosensor by immobilizing biocompatible titanate nanotubes (TNTs) and ChOD on a CHIT-modified GCE [8]. All these sensors revealed better properties than those of the pure polymer film-modified electrodes.

2.2.2. Methods. Methods for immobilizing enzymes and/or luminophores have been developed in many studies. Physical entrapment, covalent attachment, electrostatic attraction, cross-linkage, and other affinity interactions are generally applied for enzyme immobilization. Selected examples from the literature include:

(1) encapsulating ChOD into TNTs and Nafion composite film for choline sensing [18];
(2) cross-linking ChOD and/or AChE with glutaraldehyde on MWCNT–CdS modified electrodes for the fabrication of signal-on enzyme-based ECL biosensors [4];
(3) electrostatic attraction and covalent attachment between positively-charged $Ru(bpy)_3^{2+}$, negatively-charged citrate-capped AuNPs and NADH on an ITO electrode for the detection of alcohol [33]; and,
(4) the biotin–avidin interaction between biotinylated antibodies and avidin-AChE to assemble an ECL-ELISA biosensor [38].

Other technologies have also been introduced to fabricate ECL enzyme biosensors. There are many reports concerning enzyme immobilization. In general, the selected enzyme-immobilization method must address particular criteria (e.g., the degree of enzyme and/or luminophore loading at the interface, the maintenance

of enzyme activity and the stability of the immobilization). Godoy et al. designed a highly organized proteolipidic nanostructure using interfacial liposome fusion and Langmuir-Blodgett techniques [19]. This Langmuir-Blodgett film could sequester AChE in a suitable orientation and maintain enzyme activity for several months. Wei et al. presented a layer-by-layer assembly method to fabricate RuSiNPs with lysozyme [34]. Their results indicated that this effective, versatile approach improved the particle biocompatibility and inhibited leakage of $Ru(bpy)_3^{2+}$.

Dong's group [37,39,40] improved upon incorporation of enzymes into sol-gels. They developed a stable, sensitive, alcohol ECL biosensor based on the synthesis of a new sol-gel material to co-immobilize $Ru(bpy)_3^{2+}$ and ADH [39]. In that work, a partial sulfonated (3-mercaptopropyl)-trimethoxysilane sol-gel (PSSG) film was chosen and acted as both ion exchanger to immobilize $Ru(bpy)_3^{2+}$ and matrix to immobilize AuNPs and ADH. The novel film promoted electron transfer between $Ru(bpy)_3^{2+}$ and the electrode, resulting in higher sensitivity than those using sol-gel-based ECL enzyme biosensors.

2.2.3. Electrodes. Besides the various types of immobilization materials and methods, many kinds of electrode have been introduced in fabricating ECL enzyme biosensors. Different from common electrodes, screen-printed (SP) electrodes appear attractive, small tools that are mass-produced at low cost with good reproducibility. Blum and co-workers were the first to describe an SP multi-parametric ECL biosensor [11]. Based on ECL detection principles of enzymatically produced H_2O_2, the SP electrode-based sensor was close to being a disposable device. Claver et al. further developed a disposable SP electrochemical cell to detect lactate [20], in which the reagents (e.g., luminol, lactate oxidase and bovine serum albumin), the electrolyte and the buffer were immobilized by a Methocel membrane placed on the working electrode assembled in the SP electrochemical cell. The ECL measurement was made possible via a photocounting head when 50 μL of sample was placed into the SP cell with a circular container containing the disposable sensing membrane. Moreover, by comparing the results against an enzymatic reference procedure, this disposable ECL biosensor was validated for lactate analysis in human saliva.

The activity of most enzymes greatly depends on temperature, so a well-controlled temperature for the sensors is helpful in increasing their sensitivity and reproducibility. The application of heated electrodes is an effective approach, which has attracted great interest from theoretical and practical points of view in recent years.

Lin's group was the first to report the application of a heated electrode in ECL enzyme biosensors [21]. In this work, xanthine oxidase (XOD) was modified on the surface of a heated carbon-paste electrode (HCPE) to detect hypoxanthine (HX). Because the XOD activity greatly depended on temperature, the biosensor was very sensitive to the temperature change of the electrode. In addition, since the temperature of the electrode might also affect diffusion and convection of the luminescent compounds near the electrode surface, a suitable temperature for XOD/HCPE had to be controlled to achieve the best ECL signal. The key feature of the biosensor that they designed was that the temperature of the electrode was controllable, so the most suitable temperature for the enzyme reaction could be obtained. The results obtained showed that the ECL enzyme biosensor exhibited its best sensitivity at an electrode temperature of 35°C for the detection of HX. Analogue research was developed in another of their works, based on a heated ITO electrode [67].

2.3. Analytical applications

ECL enzyme biosensors can not only be used in stationary solution, but also be coupled to flow systems (e.g., for flow injection analysis, high-performance liquid chromatography, and capillary electrophoresis). More than this, their analytical applications can extend to new techniques [e.g., electro-optical enzyme arrays, biochips, and enzyme immunoassay (EIA)].

2.3.1. Electro-optical enzyme arrays. Traditionally, ECL analysis was based on detection of an electrotriggered, weak light, invisible to the eye, using a photomultiplier tube (PMT) to achieve highly-sensitive detection. However, if not coupled to other separation equipments, this analytical system is suited for the determination of a certain analyte only, and application of individually addressable array electronics to achieve high-throughput analysis is impossible. Recently, a charge-coupled device (CCD) was used instead of a PMT to image the weak ECL phenomenon and to improve its analytical capacity.

Rusling's group first demonstrated the application of ECL arrays for high-throughput in vitro genotoxicity screening [41]. They designed a prototype ECL array with a single 2.5-cm^2 pyrolytic graphite-block electrode in a cell that was placed in a dark box with a CCD camera for detection. The array with 50 small individual spots containing DNA, various human cytochrome P450 (cyt P450) enzymes, and $[Ru(bpy)_2(PVP)_{10}]^{2+}$ (RuPVP) was exposed to H_2O_2 to activate the enzymes. Upon application of 1.25 V (*versus* SCE), the electrochemically-oxidized RuPVP generated light from each spot by oxidizing guanines in the DNA. As in all catalytic DNA-detection schemes, larger signals were obtained from damaged DNA because of better accessibility of the guanines to the catalyst as the ds-DNA unraveled. Rates of DNA damage from metabolites produced by individual

cyt P450s were estimated simultaneously from the increase of ECL intensity with reaction time.

ECL arrays can be configured to measure the time course of reactions catalyzed by a single enzyme, or multiple enzyme reactions for a single substrate can be followed simultaneously. Data obtained with the array can be re-organized and presented in any way desired by computer software. Based on this work, Rusling's group further proposed a platform, based on thin enzyme/DNA films, which could be used in two-tier screening of chemicals for reactive metabolites capable of producing toxicity [42]. Microsomes, which are vesicles of fragmented endoplasmic reticulum containing lipids, collections of cyt P450 enzymes, and their natural reduction partner (cyt P450 reductase) were used as sources of cyt P450 enzymes in these devices. Initial rapid screening involved ECL arrays featuring spots containing RuPVP, DNA, and rat-liver microsomes, or bicistronically-expressed human cyt P450 2E1 (h2E1). As the presence of the reductase in microsomes allowed for cyt P450 activation by protocols featuring electron donation from NADPH via the reductase, cyt P450 enzymes were activated via the NADPH/reductase cycle. When bioactivation of substrates in the films gave reactive metabolites, they were trapped by covalent attachment to DNA bases. The rate of increase in ECL with enzyme reaction time reflected relative DNA-damage rates.

In addition, our group has reported a colorimetric approach to fabricate an ECL sensor, based on homogeneous, strong ECL emission from RuSiNPs, which was then associated with a green reference light to transform the ECL into a distinct variety of colors [68]. On the basis of this design, the change in concentration of analytes could easily be monitored directly using the naked eye or a commercial CCD camera. Compared with the necessary of a cooling system for the highly sensitive CCD detector, this greatly simplified technique holds promise for the application of ECL enzyme arrays.

2.3.2. ECL enzyme biochips. Designing a multiple-analyte biosensor generally requires a suitable immobilization method adaptable to different enzymes, as well as performing the detection with a transducer enabling easy concomitant measurement of the different spots. As a ''lab-on-a-chip'', a biochip allows researchers to analyze DNA, RNA and protein samples on a microchip, providing a good technique for the design of such biosensors.

Many applications of ECL enzyme biochips were reported by Blum and co-workers [9,22,23]. They developed a multi-parametric biochip for choline, glucose and lactate based on a luminol/H_2O_2 ECL system [22]. The sensing layers comprised enzyme-bound beads co-entrapped in a photopolymer with luminol-charged beads and spotted at the surface of a GCE. Three different oxidases were non-covalently immobilized on imidodiacetic acid chelating beads (GOD only) or on diethylaminoethyl anion-exchanger beads, and spotted on the surface of glassy-carbon foil (25-mm^2 square), entrapped in a PVA-SbQ photopolymer. Luminol immobilization enabled achievement of micro-biosensors at the surface of the same GCE and free of lateral contamination between each spot by the other reaction products. Biosensing chip measurement was performed by capturing a numeric image with a CCD camera under selected experimental conditions: the integration time of 3 min, and applied potential at +850 mV. After optimization, the multi-biosensor enabled detection of glucose, lactate and choline in ranges 20 μM–2 mM, 2 μM–0.2 mM and 2 μM–0.2 mM, respectively.

This ECL biochip was extended to detect acetate using a tri-enzymatic sensing layer based on kinase-oxidase activity. A reaction sequence using acetate kinase, pyruvate kinase, and pyruvate oxidase was shown to enable the production of H_2O_2 in response to acetate injection in the range 10 μM–100 mM. Based on a similar entrapment concept of enzyme and luminol in PVA–SbQ photopolymer, a microarray of nine SP graphite electrodes was used to develop multi-parametric ECL biochips [23].

2.3.3. Enzyme immunosorbent assay. The development of a highly sensitive detection system for trace levels of disease markers is important for the early diagnosis of fatal disorders. EIA is a trace-analysis technology that combines the amplification of enzyme and the specificity of the immunologic reaction. Because a relatively large EIA signal can be easily obtained by optimizing the incubation time of the labeled enzyme, due to the catalytic effect of the labeled enzyme, which produces a large number of optically-measurable molecules. EIA, especially enzyme-linked immunosorbent assay (ELISA), is currently one of the most frequently used techniques for determining various disease markers.

Wilson et al. first reported ECL EIAs for the detection of 2,4,6-trinitrotoluene (TNT) and pentaerythritol tetranitrate (PETN) [24]. Haptens corresponding to these explosives were covalently attached to high-affinity dextran-coated paramagnetic beads. The beads were then mixed with the corresponding Fab fragments and samples. After adding a second HRP-labeled antispecies-specific antibody, the mixture was pumped into an electrochemiluminometer where beads were concentrated on the working electrode magnetically. The concentration of analyte in the sample was determined according to the light-emission intensity when H_2O_2 was electrochemically generated in the presence of luminol and an enhancer. It should be mentioned that the sensitivity in this assay could be greatly enhanced using Fab fragments rather than whole antibodies. The LODs for TNT and PETN were down to 0.11 ppb and 19.8 ppb, respectively.

Analogous work was designed using a computer-controlled flow-injection electrochemiluminometer [25]. In this assay, a re-usable immunosorbent dextran surface was anchored to a gold surface in the flow cell by chemiadsorbed thiol groups. Antibodies were labeled with enzyme GOD and used in competitive immunoassays, in which the separation step was carried out by concentrating unbound antibodies on the immunosorbent surface. H_2O_2 generated by the enzyme label when glucose was pumped through the flow cell was detected using luminol ECL. Based on this method, the LOD for TNT was low to 2.3 ppb.

Recently, Kurita et al. reported that, as an enzymatic reaction product between acetylcholinesterase and acetylthiocholine, thiocholine is a very useful co-reactant with $Ru(bpy)_3^{2+}$ for bright ECL emission [38]. Based on this result, they achieved a highly-sensitive ECL ELISA. Using the enzymatic reaction between an acetylcholinesterase-labeled antibody and a thiocholine monolayer modified on the gold electrode, the emission of $Ru(bpy)_3^{2+}$ was obviously enhanced. Two monoclonal anti-tumor necrosis factor-α (anti-TNF-α) antibodies were used for a sandwich immunoassay. One was a capture antibody, and the other was a detection antibody labeled with an acetylcholinesterase via an avidin–biotin interaction. Compared with the directly labeled detection antibody with a co-reactant or luminophore of previous reports, this method greatly enhanced the immunoassay signal, since a large number of co-reactant molecules could be generated by the enzymatic reaction. As a result, high sensitivity and a low LOD of 0.2 pM were successfully achieved for TNF-α analysis.

3. Non-enzyme based ECL sensors

In ECL enzyme biosensors, enzymes are necessary and generally immobilized in a suitable matrix. Although these enzyme-based sensors show good selectivity and high sensitivity, originating from the enzyme characteristics, the most common, serious problem with enzymatic sensors is insufficient long-term stability. In addition, since the sensor sensitivity essentially depends on enzyme activity, reproducibility is still a critical issue in quality control.

Inspired by the non-enzymatic amperometric glucose sensor, our group designed a novel non-enzymatic ECL sensor [69]. In this work, we prepared PdNPs and attached them onto FCNTs using spontaneous redox reaction between the metal ion and the functional defects of the FCNTs [70]. The PdNP-FCNT-Nafion film-modified electrode displayed high electrocatalytic activity towards the oxidation of glucose. The free radicals generated from the glucose oxidation reacted with the luminol anion, and enhanced the ECL signal. As PdNPs are highly resistant towards the poisoning of chloride ion [71], the novel method exhibited very high, sensitive ECL responses to glucose, even in the presence of a high concentration of Cl^-. Under optimized conditions, the linear response of ECL intensity to glucose concentration was valid in the range 0.5–40 μM with an LOD (S/N = 3) of 0.09 μM.

4. Conclusions

This review presents recent advances in ECL enzyme biosensors. Based on the attractive properties of ECL and enzyme, selective, sensitive detection can be successfully achieved using ECL enzyme biosensors. In contrast to the numerous ECL enzyme biosensors produced, only a few are commercially available.

The usefulness of ECL enzyme biosensors in a real detection system depends on particular factors [e.g., the method of immobilization], the chemical and physical conditions of use (e.g., pH, temperature, ionic strength of the sample, long-term stability of the biocomponent, and interfering species), the activity and the stability of the enzyme once immobilized, the stability of the electrochemical sensor, the response time, and the storage conditions.

Practical application of ECL enzyme biosensors is a most important field for future research.

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