Electrochemical biosensors based on advanced bioimmobilization matrices

Zhiai Xu, Xu Chen, Shaojun Dong

Biosensors have experienced rapid, extensive development. To maintain the bioactivity of biomolecules and to give the electrochemical output signal required, appropriate bioimmobilization matrices for biomolecules are critical.

In this review, we describe some advanced membrane materials (including hydrogels, sol-gel-derived organic-inorganic composites and lipid membranes), introduce electrochemical biosensors based on bioimmobilization materials and describe their performance.

Biosensors operating in extreme conditions and displaying direct electron transfer with electrodes based on these advanced membrane materials are attractive.

Recent developments in nanomaterials include biosensors, so we emphasize the intersection of nanomaterials with advanced membrane materials in biosensors.

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Keywords: Biosensor; Hydrogel; Lipid; Nanomaterial; Sol gel

1. Introduction

The unprecedented demand in the research and development of analytical devices for detecting, quantifying and monitoring of biologically-related species has led to the emergence of and extensive progress in biosensors.

IUPAC recently proposed a very stringent definition of a biosensor [1]: “A biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor), which is retained in direct spatial contact with a transducer element.” Biosensors based on electrochemical transducers, particularly amperometric enzyme electrodes, combine advantages offered by the selectivity of the biological recognition elements and the sensitivity of electrochemical transduction process. They have been studied widely and some have been commercialized.

One of the most important steps of building a biosensor is to immobilize the biomolecules. A successful matrix should immobilize or integrate biomolecules stably at a transducer surface and efficiently maintain the functionality of the biomolecules, while providing accessibility towards the target analyte and an intimate contact with the transducer surface. The development of a good biocompatible matrix for immobilization of biomolecules is very crucial to improving the analytical performance of a biosensor. Some favorable matrices for the immobilization of biomolecules have resulted from advanced materials [2]. The development of material chemistry provides an extensive opportunity for the design of biosensors.

In this review, we describe some biosensors based on advanced membrane materials, including hydrogels (which contain cryohydrogel, organohydrogel and grafting copolymer), sol-gel-derived organic-inorganic composites, and lipid membranes. We especially describe the recent development in biosensors for organic-phase application and protein direct electrochemistry based on lipid membranes and including nanomaterials. Excellent performances of these biosensors promise to meet the challenges raised by complex environmental and clinical samples.

2. Hydrogel

To get high stability and efficiency from a biosensor, the host matrix preferred is one that isolates the biomolecules, preventing
they from self-aggregation and microbial attack, while providing essentially the same local aqueous micro-environment as in biological media. Hence, hydrogel, which has a cross-linked network of polymers, swollen with water, is one of the ideal enzyme-immobilization materials. A large number of hydroxyl groups in hydrogel provides a biocompatible microenvironment for the enzyme to maintain its natural configuration, so biosensors based on hydrogel have high sensitivities.

Poly(vinyl alcohol) (PVA) has long been used as a matrix for immobilized enzymes because of its good biocompatibility, chemical stability and inertness to microbial degradation. However, PVA is always photopolymerized by UV radiation, and this process denatures enzymes.

A kind of polyhydroxy cellulose (PHC), a mixture of PVA and carboxymethyl hydroxyethyl cellulose, was prepared and a novel immobilization method for enzymes, cryoimmobilization, was proposed. Cryoimmobilization, represents a way of fixing biomolecules through repeated freeze-thaw treatment after the electrode has been modified with the biomolecule-containing polymer solution. This treatment results in the formation of a porous network in which polymer crystallites act as junction points, which subsequently improve the mechanical strength of the hydrogel [3]. The cross-linked, porous polymer gel material is called cryo-hydrogel. The existence of plenty of hydroxyls in PHC provides the material with good hydrophilicity and biocompatibility due to preferential hydration of enzymes.

There are three types of water in the cryo-hydrogel: “unfreezing water”; “bond water”; and, “free water”. “Unfreezing water”, which has strong interaction with the polymer, will not freeze until −40°C. “Bond water” exists near the hydrophobic group of the polymer (the amount of this kind of water is related to the component of the polymer). “Free water” has the same properties as natural water. The amount of “free water” changes with humidity, but the other types of water are quite stable.

If the cryo-hydrogel is put in a dry atmosphere, it shrinks ca. 40%, but, when in contact with a small amount of water, the material recovers to its original state. When the gel is freeze-dried with enzyme, it tends to stabilize the activity of the enzyme. This is thought to be due to the hydroxyl group in the polymer holding or substituting for the “bond water” that is necessary for retaining the tertiary structure of enzymes and the subsequent activity.

During immobilization, the enzyme is locked into a more active conformation. As an example, when a cryo-hydrogel-based tyrosinase sensor was fabricated [4a], the cryo-hydrogel immobilization distinctly improved the stability of the enzyme electrode. The specific apparent activity of immobilized enzyme was 22% higher than that of the soluble enzyme, according to the spectrophotometric measurements. The biosensor maintained 98% of the original activity after storage in a dry state at 4°C for 3 months.

More importantly, this kind of cryo-hydrogel is found to have good water-retaining ability even in the organic phase. It is a good candidate for fabricating organic phase enzyme electrodes (OPEEs) that can be applied to many water-insoluble analytes and new, challenging environments.

The maintenance of enzymatic biocatalysis in an organic solvent requires the “essential water” layer. As long as this “essential water” is localized adjacent to the enzyme molecules, replacement of the rest of the water with an organic solvent without adversely affecting the enzyme activity should be possible. However, in organic solvents, especially water-miscible solvents, this “essential” layer is easily disturbed or even lost, and the enzymes are deactivated. This can be explained by organic phase enzymology.

The type of organic solvent plays an important role in the activity of the enzyme. Based on the log P value (where P is the partition coefficient of an organic solvent in the octanol/water two-liquid phase system), organic solvents have been classified as hydrophobic (log P > 4), hydrophilic (log P < 2) and moderately hydrophobic (4 < log P < 2). Strongly hydrophobic solvents (log P > 4) will not distort the essential hydration layer around the enzyme, so the enzymes retain their catalytic activity. However, polar organic solvents (log P < 2) can strip the enzyme of this hydration layer and the active site water, which can lead to distortion and inactivation of the enzyme molecule. Organic solvent with intermediate hydrophobicity can remove some of hydration-layer water but not deactivate the enzyme due to the presence of small quantities of water in the bulk solution [5]. The more hydrophobic the solvent, the less water is lost from the cryo-hydrogel. This implies that the enzyme electrode can be used successively for a longer period of time and that the enzyme layer is easier to rehydrate.

Unfortunately, the enzyme layer becomes stiff within 30 min in 1-butanol, ethanol, and 2-propanol due to the loss of water to the hydrophilic solvent. In these solvents, the enzyme electrode can only be used three times before rehydration.

The recovery of enzyme activity varies with solvent. After the enzyme electrode is used in chlorobenzene, chloroform, octanol, 1-butanol, and ethanol, the activity can be recovered fully when the electrode is dipped in water. However, when the enzyme electrode is operated in the presence of acetonitrile, 2-propanol, benzene, and toluene, the recovered enzyme activity was only 30%, 50%, 50%, and 85%, respectively. Once the electrode is used in dimethylformamide, the activity can never be recovered again [4b].

At present, the only way of providing the essential hydration layer for enzymes immobilized on OPEEs is to add water deliberately to the organic solvent before use.
so none of the OPEEs reported so far have operated strictly in purely non-aqueous media [6]. However, the cryohydrogel can retain its water in organic solvent to some extent and that allows enzymes to function in purely organic solvents. Consequently, a new kind of OPEE – a pure organic-phase amperometric enzyme electrode – has been developed.

A reagentless H$_2$O$_2$ biosensor was fabricated by coimmobilizing a soluble mediator (potassium hexacyanoferrate (II)) and horseradish peroxidase (HRP) on a graphite electrode using a cryohydrogel [7]. The performance of the enzyme-mediator electrode was tested in different organic solvents. A rapid, sensitive response of the enzyme-modified electrode to H$_2$O$_2$ was obtained in pure chloroform at +0.03 V (see Fig. 1). A stable base current was obtained after an equilibration time of 5–15 min, which is much more rapid than that previously described (1–1.5 h) with a shorter response time of 0.5–2 min. The useful measuring range is up to 2.5 mM with a limit of detection (LOD) of $5 \times 10^{-7}$ M. Moreover, the unmediated H$_2$O$_2$ biosensor was constructed on a cryohydrogel-immobilized HRP graphite electrode [8]. The apparent direct electron transfer between a spectrograde graphite electrode and immobilized HRP was obtained in pure chloroform and pure chlorobenzene.

A cryohydrogel-immobilized tyrosinase electrode was also constructed and different phenolic substrates were determined in various pure organic solvents. Based on the enzyme-inhibition effect, thiourea, 2-mercaptoethanol and benzoic acid were determined by exploiting their noxious effect on tyrosinase immobilized in cryohydrogel in pure water-immiscible solvents [9]. Fast, sensitive responses were observed for different inhibitors. This enlarges the practical applications of biosensors, and enhances the understanding of organic-phase enzymology.

HRP and tyrosinase OPEEs prepared by cryohydrogel have been applied in various water-free organic solvents. However, due to the high hydrophilicity of the cryohydrogel, the method of cryoimmobilization is suited mainly to water-soluble analytes, such as H$_2$O$_2$ and phenols, but unworkable for hydrophobic substances, such as organic peroxides, bilirubin and cholesterol.

In order to enlarge the analytical range of biosensors for practical application, we further prepared an organohydrogel with mixture of PHC and a universal organic solvent, DMF. Apart from possessing advantages of a cryohydrogel, the organohydrogel has a higher partition coefficient for both hydrophilic and hydrophobic analytes so as to obtain high detection sensitivity, and it is also stable in a variety of solvents. Three enzymes (HRP, tyrosinase and bilirubin oxidase) were immobilized in organohydrogel to demonstrate the feasibility of these OPEEs [10].

Table 1 gives response parameters of substrates at the tyrosinase organohydrogel electrode (TOE) and the tyrosinase cryohydrogel electrode (TCE) in pure chloroform. For phenolic compounds, the TOEs have higher sensitivity than TCEs, and that is attributed to the high partition coefficient of phenolic compounds in the DMF organohydrogel and these compounds can be extracted and preconcentrated in the enzyme membrane. As shown in Table 1, different substrates have different sensitivities and $K_{app}$ values in the same solvent. In chloroform, catechol had the biggest sensitivity (55 nA/mM) and smallest $K_{app}$ (0.15 mM) value. Analytes of different solubility (e.g., organic peroxides, phenolic compounds and bilirubin) were determined using the corresponding enzyme-containing organohydrogel-modified electrode.

Recently, an organohydrogel-based biosensor prepared from agarose and DMF operated at room temperature in an ionic liquid, 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]-[PF6]) [11]. Ionic liquids are novel non-aqueous media, whose properties can be controlled by choosing appropriate cations and anions. Compared with organic solvents, they have advantages, such as negligible vapor pressure and good conductivity. Heme proteins, including hemoglobin, myoglobin, HRP, cytochrome c and catalase in the agarose, and DMF organohydrogel showed direct electron transfer with the
underlaid glassy carbon electrode. The electrocatalytic activity to trichloroacetic acid and tert-butyl hydroperoxide remained.

To further improve the activity of immobilized enzymes and to reduce the loss of enzyme activity during repeated freeze-thaw cycles using cryohydrogel immobilization, we developed a more temperate, simple method in our laboratory. It is based on a hybrid hydrogel of self-gelatinizable graft copolymer of PVA with 4-vinyl pyridine (PVA-g-PVP). Previous reports showed that poly(4-vinyl pyridine) (PVP) could adhere firmly to the electrode surface and that an additional hydrogen bond between PVA and PVP existed in a type of blended membrane. Our group synthesized the copolymer of PVA-g-PVP, which integrates the advantages of each separate component. Strong hydrogen bonds formed in the copolymer help to entrap enzymes while maintaining their activity effectively. The biosensors are constructed by simply syringing the copolymer-enzyme aqueous solution to the electrode surface and allowing water to evaporate at 4°C [12].

Tyrosinase was immobilized as described above and the amperometric biosensors obtained could successfully determine phenols in both aqueous solution and organic solvents [13]. Inspiringly, we demonstrated that the amperometric tyrosinase biosensor based on PVA-g-PVP could be exploited to monitor organic solvents, which greatly expands the range of applications of enzyme-based biosensors [14].

The factors influencing biosensor response to organic solvents were studied with in situ steady-state amperometry and quartz crystal microbalance (QCM) experiments; the mechanism is shown in Fig. 2 [14]. Experimental results ruled out the possibility of the influence of organic solvents on the essential hydration layer, and that may decrease enzyme activity or swelling of the immobilization matrix.

We demonstrated that detection was based on substrate partition equilibrium between the enzyme membrane and the water-organic solvent media. The solubility of phenols, which are the substrates of tyrosinase, is much greater in organic solvents than in water, so, when the organic solvent was added, phenols quickly concentrated in the organic solvent, which caused the decrease of the substrate concentration in the solution. Because of the dynamic balance of the substrate between water-organic solvent media and the enzyme membrane, the phenols in the enzyme membrane diffused toward the solution. This brought about the decrease of the substrate concentration in the enzyme membrane, and, accordingly, the response of the sensor decreased in proportion to the concentration of the added solvent. This kind of biosensor is especially useful for determining some non-electroactive organic solvents (e.g., acetone, acetonitrile, and tetrahydrofuran), for which no specific enzyme has been found to construct a specific biosensor.

The LODs were 0.12%, 0.040%, 0.042% and 0.075% (v/v) for methanol, acetone, acetonitrile and tetrahydrofuran, respectively [14].

The use of these hydrogels, including cryohydrogel, organohydrogel and PVA-g-PVP, for enzyme immobilization not only improves the performance of biosensors, but also provides the capabilities of electrochemical biosensing in hostile conditions [15]. However, swelling of hydrogels is often a problem. To overcome the disadvantages, inorganic sol-gel materials can be hybridized with hydrogels and a series of robust composite films of biosensors constructed.

3. Sol-gel-derived organic-inorganic composites

During the past decade, the renaissance of sol-gel chemistry has enabled sol-gel materials to play a prominent role in materials science by providing a versatile method for preparing various solids with well-controlled composition and a wide range of attractive properties. The sol-gel process involves hydrolysis and co-condensation of sol-gel precursors, such as alkoxysilanes, with acid or base catalysis. The material formed is a network of Si–O bonds. As it is inherently a low-temperature process, it provides a promising means for immobilizing bioactive molecules [16]. Sol-gel chemistry yields a matrix that retains the natural conformation and the reactivity of the protein. Besides, it possesses physical rigidity, chemical inertness, high photochemical and thermal stability, and excellent optical transparency, and it experiences negligible swelling in aqueous and organic solvents. Biosensors based on sol-gel-derived materials have been reported extensively in the past few years, especially by the group of J. Wang and O. Lev [16,18]. There have been several well-documented reviews on sol-gel materials for sensors [16–18].

Despite the many advantages of a silica sol-gel immobilization matrix, the brittleness of these matrices is a major obstacle to their wide application with biomolecules. Recently, the use of organic-inorganic composite materials has overcome this problem. Some polymers, such as poly(ethylene oxide), chitosan, polyhydroxy polymers and surfactants are introduced in the starting sol [17b,19–21]. These additives limit the shrinkage effect generally observed during the gel-to-xerogel transition. In addition, by incorporating functional organic components, special biosensors can be obtained. PVA-g-PVP hydrogel, a self-gelatinizable grafting copolymer which can firmly adhere to the electrode surface, has been hybridized into silica sol, and an organic-inorganic hybrid thin film successfully developed [22]. The sol-gel/hydrogel hybrid material combined sol-gel rigidity with the biocompatibility of a hydrogel and overcame both shortcomings (cracking of silica glass and swelling of hydrogel in solution). FTIR, SEM and QCM were
employed to characterize the hybrid thin film. The results demonstrated that the hybrid film contained a large number of hydroxyl groups and hydrogen bonds, which formed an interpenetrating network. In addition, leaching of enzymes was not observed when enzyme molecules were encapsulated in the composite film.

As a model enzyme, glucose oxidase (GOD) was first immobilized in the sol-gel copolymer hybrid matrix to develop an amperometric glucose biosensor [22]. The sensor exhibited rapid response (11 s) due to fast diffusion of the substrate molecule in the thin hybrid film. However, the enzyme electrode containing 1% (w/w) GOD also had a high sensitivity (600 nA/mM). Moreover, the biosensor had good operational and storage stability. The enzyme electrode was measured intermittently (every 2–3 days), and no apparent change of the response to 0.4 mM glucose after 150 days was found. The glucose biosensor was used practically on human plasma samples.

Based on this hybrid material, particularly, we developed an acid-stable H$_2$O$_2$ biosensor based on soybean peroxidase (SBP) purified from Chinese soybean [23a]. SBP could hold its prosthetic group tightly at pH 3, while ordinary HRP lost the heme group after being placed in a pH 3 solution for 30 min. The corresponding biosensor exhibited high sensitivity in a broad pH range (3–10). In addition, flow injection analysis (FIA) results (see Fig. 3) illustrated that the biosensor had good stability under continuous operation and could be used in on-line determination of H$_2$O$_2$ in an acidic medium. The long-term storage stability was tested over a 90-day period with no apparent decrease in the response.

A novel type of biochemical oxygen demand (BOD) biosensor was also developed for water-quality monitoring in environmental analysis by co-immobilizing microorganisms in the sol-gel-derived composite material [24].

To obtain the ion-exchange ability, ionomers (e.g., Eastman AQ and Nafion) were hybridized with sol-gel to immobilize charged mediators and improve the stability of the film at the same time [25]. Similarly, functionalized composite film was also obtained by organic modification of sol-gel matrix [26]. Sulfonated silicate sol was prepared from 3-mercaptopropyltrimethoxysilane (MPS) and methyltrimethoxysilane (MTMOS) precursors. The H$_2$O$_2$ biosensor was fabricated by simply dipping the HRP-containing functionalized membrane modified electrode into Meldola's Blue (MDB) solution. MDB was adsorbed and firmly immobilized within the membrane.

<table>
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<tr>
<th>Substrate</th>
<th>TCE</th>
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<tr>
<td></td>
<td>$K_M^{app}$ (mM)</td>
<td>Sensitivity (nA/mM)</td>
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<td>Catechol</td>
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<td>23.7</td>
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<tr>
<td>$\rho$-Cresol</td>
<td>3.5</td>
<td>3.9</td>
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<td>Phenol</td>
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Table 1. Comparison of response parameters of substrates of tyrosinase organohydrogel electrode (TOE) and tyrosinase cryohydrogel electrode (TCE) in chloroform [10].

![Figure 2. Schematic description of the mechanism of the sol-gel composite tyrosinase biosensor for organic solvent [14].](image)

![Figure 3. FIA peaks for $1 \times 10^{-5}$ M (1), $2 \times 10^{-5}$ M (2), $4 \times 10^{-5}$ M (3), and $8 \times 10^{-5}$ M (4) H$_2$O$_2$ at the sol-gel biosensor (a), and FIA peaks for $8 \times 10^{-3}$ M H$_2$O$_2$ for 9 successive injections (b). Experimental conditions: Carrier solution, 0.02 M biphthalate buffer (pH 5.0) containing 0.2 x $10^{-3}$ M methylene blue; Operating potential, -0.20 V; Flow rate, 1.05 ml/min [23].](image)
as a mediator for HRP. The biosensor had an LOD of $9 \times 10^{-7}$ M to H$_2$O$_2$.

Although the above-mentioned advantages are attractive, the sol-gel materials still have disadvantages, such as weak resistance to interference and poor conductivity. One solution to interference is to select proper, effective mediators that could mediate the detection at a potential where interfering species could not cause interference. Another way is to coat permselective films (e.g., Nafion, cellulose acetate and non-conducting polymers) [27]. Furthermore, poor conductivity could be alleviated by doping the sol-gel film with conductive constituents.

Nanomaterials constitute an emerging subdiscipline in chemistry and materials science and have numerous commercial and technological applications [28]. Due to their large surface area, high catalytic activity and good biocompatibility, metal nanoparticles have been employed in immobilizing biomolecules in constructing biosensors. Moreover, the excellent conductivity of gold nanoparticles provides the potential to construct amperometric biosensors based on redox enzymes (as sensing elements) and nanoparticle arrays (as conductive matrix onto which the enzyme molecules are implanted) [29]. Various novel gold-nanoparticle assemblies reported recently promise to realize nanoscale electronics and facilitate the development of nanosensors [30]. Combining sol-gel materials with nanomaterials could improve the conductivity of the sol-gel film and promote the electron transfer of redox enzyme with the electrode. Following this line, gold and silver nanoparticles were embedded in a sol-gel network to build sensitive biosensors [31]. For example, a nanocrystalline gold–silicate sol prepared from N-[3-(Trimethoxysilyl)propyl]ethylene diamine (EDAS) and hydrogen tetrachloroaurate was co-polymerized with MTMOS [31a]. GOD, immobilized by premixing with gold sol, retained its biocatalytic properties and the conductivity and electrocatalytic properties of the interconnected gold dispersion were maintained while the composite bioceramic material remained transparent.

Recently, our group developed a new method for fabricating a self-assembled third-generation HRP biosensor by using a hydrolyzed three-dimensional sol-gel network [30b. Fig. 4 shows the biosensor fabrication process step by step. The three-dimensional network formed by silicate monomeric precursor MPS hydrolyzation provided stereo attaching sites for gold nanoparticles. The direct electrochemistry of HRP adsorbed on the gold nanoparticles was realized because self-assembly of gold nanoparticles to silica gel provided the necessary electron transfer pathways and allowed efficient electron tunneling. Moreover, gold nanoparticles could be assembled into multilayers, so as to increase the enzyme loading. The resulting biosensor therefore possessed high sensitivity and good stability. The factors influencing the performance of the resulting biosensor were studied in detail. The resulting biosensor exhibited fast amperometric response (2.5 s) to H$_2$O$_2$. The LOD of the biosensor was 2.0 $\mu$M, and the linear range was 0.005–10.0 mM.

Carbon nanotubes (CNTs) have also recently attracted much attention in biosensor applications, due to their high conductance, tensile strength, and chemical
stability. CNTs could promote the electron-transfer reaction because of their unique structure. A sol-gel membrane doped with CNTs has improved conductivity and electrocatalysis behavior [32].

A new type of amperometric cholesterol biosensor based on sol-gel chitosan/silica and CNT organic–inorganic hybrid composite material was developed. The analytical characteristics and dynamic parameters of the biosensors with and without CNTs in the hybrid film were compared, and the results showed that the analytical performance of the biosensor could be improved greatly by introducing CNTs [33].

When it comes to good conductivity and transport properties of the immobilization matrix, conductive components, such as metal nanoparticles and nanotubes doped in the sol-gel membranes, could improve these properties, as shown above. In addition, room-temperature ionic liquids (ILs), as environmentally benign solvents, have been used as templates for the sol-gel process. Due to their negligible vapor pressure, wide potential window, good solubility and conductivity, when 1-butyl-3-methylimidazolium tetrafluoroborate (BMIM+BF4−) was doped into the silica sol-gel and HRP composite, almost no loss of the HRP activity in the hybrid film was observed at temperatures below 40°C and HRP retained about 68% of the apparent activity after thermal treatment at 60°C for 30 min. This was significantly higher than for free HRP. The specific activity of HRP was about 30-fold greater than that in silica gel without IL prepared by conventional sol-gel methods. This showed that dramatically enhanced activity and excellent thermal stability of HRP are obtained with IL, when compared with that in the silica matrix without IL [34]. This provides an alternative way of utilizing ILs in biocatalysis and biosensors.

4. Lipid membranes

Lipid membranes, imitating the basic structure of nature’s biomembrane and molecular devices, represent a relatively biocompatible structure for the development of new types of electrochemical sensors [35]. Compared with all other immobilization matrices, lipid membranes provide a natural environment for embedding biomolecules, and the biosensors display fast response times (few seconds) and high sensitivities (nanomolar LODs). Furthermore, the presence of the lipid bilayer greatly reduces background noise (interferences) and effectively excludes macromolecules that could block the electron transfer of proteins and hydrophilic electroactive compounds from reaching the detection surface. Studies on protein redox chemistry are therefore possible with cast lipid films [36]. Also, lipid-membrane systems offer more opportunity for biosensor development [35b].

Lipid membranes can be formed by casting solutions of lipids or surfactants in organic solvents or of aqueous vesicle dispersions onto solid surfaces [36]. Rusling’s group did interesting work in enzyme bioelectrochemistry in cast biomembrane-like films [37]. Myoglobin and cytochrome P450cam were entrapped in cast liquid-crystal surfactant films. Their electrochemical and catalytic behaviors were studied. The results showed that cast liquid-crystal surfactant films could facilitate electron exchange between redox enzymes or proteins and electrodes.

In our laboratory, we obtained a highly bioelectrocatalytic activity for H2O2 reduction by entrapping microperoxidase-11 into the didodecyldimethylammonium bromide (DDAB) lipid film [38]. Furthermore, we immobilized hemoproteins, HRP and hemoglobin into the supported bilayer lipid membranes (sBLMs) of dimyristoylphosphatidylcholine (DMPC) and cast lipid membranes (cLM) of DDAB and egg-phosphatidylcholine (egg-PC) on a GC electrode [39]. Because of the favorable orientation of the heme group of HRP and a biological environment provided by sBLM and cLM on the surface of GC electrode, HRP exhibited direct electrochemical behavior and retained high bioactivity in lipid membranes (see Fig. 5). The linear range of H2O2 concentration was in the range 0.5–18 mM with an LOD of 1.7 × 10−4 M. Reproducibility and storage stability of the sensor were good [39a]. The stable sBLM-HRP and cLM-HRP films on GC electrodes gave excellent models for constructing the third-generation biosensor.

The above-mentioned sBLMs can be easily formed on the surfaces of nascent metals, agar, freshly cleaved mica, polymer cushions and semiconductors. The promising support for BLM with potentially extended

![Figure 5. Cyclic voltammograms of HRP-DDAB-modified GC electrode in 5 × 10−3 M phosphate buffer (pH 5.5), (a) 0 M H2O2 (b) 5 × 10−4 M H2O2. Scan rate: 100 mV/s [39c].](http://www.elsevier.com/locate/trac)
applications needs to possess properties such as tight linkage to substrates and easily controlled thickness, while retaining hydrophilicity and electronic conductivity. Ideally, electronically conducting polymers could easily meet the requirements. Previous studies have proved that polymeric polypyrrole (PPy) shows some interesting properties for biomimetic applications. In addition, Tien and his colleagues have achieved significant results using conventional sBLM with polymeric modifiers as a tool within BLM [40a]. Based on the above result, we studied the deposition of BLM onto PPy film surface via vesicle fusion [40b]. Experimental results suggested that dimyristoyl-L-alpha-phosphatidylcholine (DMPC) and dimyristoyl-L-alpha-phosphatidyl-L-serine (DMPS) could form BLMs on PPy film surface but DMPC and DDAB cannot do so, indicating that formation of PPy-supported BLM depended on the chemical structure of the lipids used. PPy-supported BLM is to some extent comparable to conventional BLM with aqueous medium retained on two sides. As an example and preliminary application, HRP reconstituted into the sBLM showed the expected protein activity and could transfer electrons from or to the underlying PPy support for its response to electrocatalyzed reduction of \( \text{H}_2\text{O}_2 \) in solution. The system may therefore have applications in biomimetic membrane studies.

Another kind of sBLM is the hybrid bilayer membrane (HBM), which comprises both natural and synthetic components, is easily formed by self-assembly on a conductive metal surface and is stable for very long periods of time. As a result, many techniques that have not been generally applied to biological membranes are now accessible. For example, the use of a gold support means that electrochemical techniques can be used to examine the insulating characteristics of lipid layers and to assess the activity of membrane-protein pores, redox enzymes, proton translocators and ionophores. A monolayer of 2-mercapto-3-n-octylthiophene (MOT) was self-assembled on gold [41a]. A monolayer of didecanoyl-L-R-phosphatidylcholine (DDPC) from dispersions of small unilamellar vesicles was then adsorbed onto hydrophobic surfaces. HRP reconstituted into the bilayer demonstrated the expected protein activity, showing practical uses in research and biosensor applications.

Carbon materials are relatively inert but have a wide potential window in electrochemistry, so their modification is of interest to materials science and electrochemistry. We used an amino-cation-radical method to construct a hydrophobic surface of alkane amine on carbon electrodes and then self-assembled a phospholipid monolayer onto the hydrophobic surface [41b], so constructing a new kind of HBM system. By immobilizing valinomycin, a natural carrier for alkali ions, in the lipid membrane, we made a biosensor for detecting \( \text{K}^+ \).

The functions of biomembranes can be mediated by specific modifiers, which can improve the specificity and the selectivity of the biosensors. Pt nanoparticles were electrochemically deposited through lipid films [42]. The incorporation of Pt particles through the sBLMs resulted in a unique nanoelectrode array that not only enhanced the capacitance and decreased the resistance of sBLMs but also increased the sensitivity for sBLMs in electrocatalyzing the reduction of \( \text{O}_2 \) as well as rendering sBLMs as a glucose sensor when GOD was incorporated into them.

Studies of the function of biomolecules immobilized in lipid membranes could accelerate our understanding of diverse biochemical redox processes in active organisms.

5. Conclusions and prospects

Electrochemical biosensors hold great potential for determining various analytes in environmental analysis, industrial monitoring and medical diagnoses, and play an increasingly important role among the sensors currently available. Biocompatible film materials of hydrogels, sol-gel-derived organic-inorganic composites and lipid membranes provide excellent immobilization environments for proteins. We have demonstrated that the use of these advanced materials for enzyme immobilization has greatly improved the performance of electrochemical biosensors (e.g., good stability, high activity, rapid response and flexibility of use). More importantly, advanced immobilization matrices enable biosensors to operate in extreme conditions (e.g., organic phase and ionic liquids). In addition, favorable protein redox electrochemistry has been achieved, especially with lipid films and nanomaterials.

In future, protein redox chemistry and biosensor application studies will require more advanced immobilization matrices [43]. The combination of nanomaterials with enzymes has produced a large number of robust biosensors [44] and can be used to develop micro-enzyme or nano-enzyme biochips. We expect progress to be rapid, and, because electrochemical transducers are amenable to miniaturization, there is a trend towards miniature, portable biosensors [45]. Biomimetic materials that can behave as artificial receptors for molecular recognition (e.g., molecularly imprinted polymers, or MIPs) will attract more attention. The application of such materials will accelerate the commercialization of biosensors.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (Nos. 29835120, 29875028, 20275036 and 20575064).
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Shaojun Dong is currently Professor and Advisor at the State Key Laboratory of Electroanalytical Chemistry. She is the Member of the Third World Academy of Sciences. She is currently serving as a member of the editorial board of five international journals. Her research interests include biosensors, electrochemistry, chemically-modified electrodes, spectroelectrochemistry, ultramicroelectrodes and nanoelectrochemistry. She has published over 600 papers and 30 domestic patents. She received the 16th International Kharitami Award.

Zhia Xu is studying for her Ph.D. under the supervision of Prof. Shaojun Dong at Changchun Institute of Applied Chemistry, Chinese Academy of Sciences. Her research interests are biosensors and nano-electrochemistry.

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Xu Chen studied for her B.S. at Jilin University and completed her Ph.D. under the supervision of Prof. Shaojun Dong at Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, at the end of 2002. Her research interests include biosensors, electroanalytical chemistry and sol-gel materials.