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Review

Recent progress in bio-sensing techniques with encapsulated enzymes

Byung-Wook Park[a], Do-Young Yoon[b], Dong-Shik Kim[a,*]

[a] Department of Chemical & Environmental Engineering, University of Toledo, Toledo, OH 43606, United States
[b] Department of Chemical Engineering, Kwangwoon University, Seoul 139-701, Republic of Korea

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ABSTRACT

Biosensors with encapsulated enzymes have advantages of high substrate selectivity and sustained enzyme activity. Enzymes are encapsulated in various materials, such as liposome, polymer, and sol–gel or hydro-gel, depending on sensing conditions. By stabilizing the enzymes via encapsulation with new methods and materials the enzyme activity may be maintained for a longer time, and even the selectivity can possibly be enhanced. In general increased mass transfer limitation seems to be the major challenge to investigate more. Novel materials, encapsulation techniques, and sensing mechanisms have been studied intensively for encapsulated enzyme biosensors. In this review, we focus on the recent progress in encapsulated enzyme biosensors published in peer-reviewed journals over the last 10 years. The articles are categorized and reviewed in four groups based on encapsulation techniques incorporating with liposome, polymer, sol–gel or hydro-gel, and peptide. Research articles that are considered critical and noticeable are selected and compared according to these categorizations. Based on these article analyses, we suggested future direction in the encapsulated enzyme biosensor research.

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1. Introduction

Recent progress in enzyme encapsulation techniques for biosensors is reviewed based on the peer-reviewed articles published within last 10 years. The need for more accurate and stable biosensors has increased and accordingly better encapsulation techniques have been studied and made significant progress recently. Those noticeable advances will be addressed in this review to gain a better understanding on the current state of encapsulation techniques and to suggest a direction for future research.

Biosensors offer great advantages over conventional analytical techniques. Biosensors also promise sensitive, rapid, reproducible analytical tools. Although there is optimism for the potential of biosensors, the successful development of biosensors has been hampered considerably by crucial requirements, such as immobilization and stability of biological molecules. These requirements could be satisfied by making a three dimensional (3D) space for biological molecules. That is where encapsulation technique for enzymes has been emerging as a new strategy for offering a comfortable cage in biosensors that can preserve the enzyme activity in 3D matrix.

Biosensors with immobilized enzymes have been studied and developed since the first biosensor with glucose oxidase (GOx) was developed by Updike and Hicks in 1967 (Updike and Hicks, 1967a,b). Enzymes have been preferably used as sensing agents over living cells due to the problems of cell's low selectivity and

* Corresponding author at: Department of Chemical & Environmental Engineering, Mail Stop # 305, University of Toledo, Toledo, OH 43606, United States.
Tel.: +1 419 530 8084; fax: +1 419 530 8086.
E-mail address: dong.kim@utoledo.edu (D.-S. Kim).

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Table 1
Immobilization methods for the biosensor using enzyme.

Methods	Advantages	Disadvantages
Encapsulation	Activity of enzyme and specificity retained	Leakage of enzyme; high diffusion barrier
Adsorption	No modification of enzyme	Weak binding; susceptible to changes in pH temperature, ionic strength
Covalent bonding	Strong binding force; low diffusion barrier	Denaturation of enzymes obtained through covalent bonding
Cross-linking	Conjunction with entrapment to reduce loss of enzyme	Harsh chemicals in the matrix with covalent links

slow response time (Davis et al., 1995). A biological element using catalytic enzyme is very attractive for biosensor applications measuring the change in the catalytic process (Chambers et al., 2008).

Recent advances of nano- and biotechnology in chemistry, biochemistry, physics, and biology fields have made great contribution to the progress of biosensors. In medical applications, biosensors are used to recognize biological species or substrates such as glucose, virus, bacteria, neurotoxin, and cholesterol (Arya et al., 2008; Mueller, 2005; Smith, 2005). Moreover, biosensors with microelectrodes are used to examine nerve signal in brain (Dale et al., 2005). In environmental applications, biosensors are used to detect environmental hormones, pesticides such as phenol, acetylcholine, organophosphorus, carbamates, mycotoxins (Badea et al., 2008). Biosensors are widely used in food industry to examine the meat quality, fish freshness, fermentation process, and food components (Andreescu and Marty, 2006; Nakamura and Karube, 2003).

One of the greatest interests in biosensor technology development is immobilization and stabilization of enzymes for consistent and effective biosensor performance. The immobilization of enzymes on the surface of electrode, matrix, and micro/nanocapsules can be achieved through chemical and physical methods such as covalent binding, cross-linking, entrapment, encapsulation, and absorption. Thus, selection of the immobilization materials and methods should be carefully considered to maintain the enzyme activity for a long time under various conditions. Table 1 summarizes the advantages and disadvantages of enzyme immobilization methods.

Due to the rapid deactivation of biomolecules in many cases, the protection of biomolecules with various protection materials against the environmental changes becomes an important factor. Many different protection methods and materials have been investigated to improve the stability of enzyme. For example, some polymeric vesicles are known to prevent the denaturation of enzyme through encapsulation (Sukhorukov et al., 2005). More recently, an enzyme is surrounded with a porous composite organic/inorganic network, which is called single-enzyme nanoparticles (SENs) (Fig. 1) (Kim et al., 2006, 2008). Biomolecule-nanoparticles hybrid systems were also reported to have a potential to construct stable biosensors and bioelectronic systems. Metallic nanoparticles were reported to provide efficient electrical communication between a redox enzyme and electrodes with high sensitivity (Willner et al., 2007a,b).

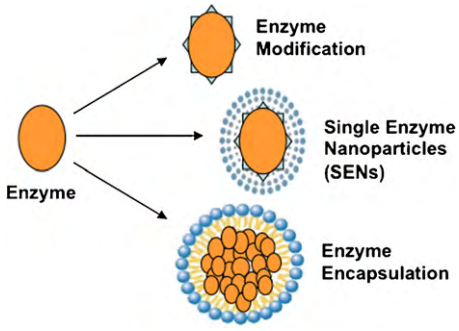

Fig. 1. Schematic representation of enzyme modification, single-enzyme nanoparticle (SEN) and enzyme encapsulation approaches.

There are four advantages why encapsulated enzyme is favorably considered to be the immobilization method for biosensor application. For the liposome encapsulation, as an example, first enzymes are immobilized with no covalent bonding and retained in a free state in a micro-space so that the chance of enzyme deformation reduces during immobilization and encapsulation. Second, with the liposome membrane that is similar to the biological cell membrane, a biocompatible environment is applied to the entrapped enzyme. This can help stabilize the conformation of enzyme entrapped in liposome due to a hydrophobic interaction between the enzyme and liposome membranes. Third, above the phase transition temperature of liposome membrane, the permeation of substrate through it can increase greatly, which results in a sharp increase in the biocatalyst activity toward the externally added substrates. Finally, the liposome membrane can protect the entrapped enzyme from outside environmental stresses such as the denaturant or the forces at the gas–liquid interface in the bubbling reaction solutions. Various encapsulation methods have demonstrated successful biomaterials stabilization under chemical and environmental perturbations of extreme pH, temperature, and ionic strength changes. List of various encapsulation methods for biosensing applications is given in Table 2. In this review, we focused on recent advances in the development of encapsulated enzyme biosensors and their specific application for sensing. The review is intended to update the current state of various enzyme encapsulation techniques including uniqueness in characterization and pros and cons in applications. We hope that the review helps readers better understand the advances in encapsulation techniques and directions for future research.

2. Current and potential sensing applications with encapsulation methods

2.1. Enzyme encapsulation with liposomal vesicles

Liposome is a model bilayer membrane system, which has the lipid fraction of cell membranes (Basu, 2002). The ability to replicate the behavior of natural cell membranes has been useful in various applications such as drug delivery, cosmetic, food science, and agriculture (Gerelli et al., 2008; Huysmans et al., 2005; Taylor et al., 2005; Thongborisute et al., 2006). A liposome vesicle is an aggregate formed from aqueous dispersion of amphiphilic molecules, such as polar lipids, which tends to construct a bilayer-structure. There are four classes of liposome vesicle, namely, the multilamellar vesicles (MLV), small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), and multivesicular vesicles (MVV) (Fig. 2) (Hermanson, 2008). The most commonly applied technique would be the participation of mechanical energy, for example, high-intensity ultrasonication, high-pressure homogenization, extrusion, and membrane homogenization. Non-mechanical methods include reverse-phase evaporation, removal of detergents from mixed detergent/lipid micelles and freeze-drying (thawing) followed by rehydration. Each method has strengths and weaknesses for the formation of liposome (Bakas, 2000; Chaize et al., 2003; Torchilin, 2003). Recently the free-thawing method has been used to treat preformed liposomes for encapsulation of the enzymes. This method may be good at improving the capsule properties

Table 2
Characteristics of various encapsulation methods for biosensing applications.

Materials	Sensing method	Enzyme	Analyte	Characteristics	Reference
Liposome	Optical (Ellman's method)	AChE	Acetylthiocholine	Free-thaw method, use of porin as a channel, pH effect, ionic strength effect studied	Chaize et al. (2003, 2004)
Liposome	Optical (Ellman's method)	AChE	Acetylthiocholine	Number of freeze–thaw cycles, lipid concentration, and stabilizers studied	Colletier et al. (2002)
Liposome (eggPC or POSP/POPE lipids)	Optical (Ellman's method)	AChE	Insecticides (paraoxon, carbaryl, methamidophos)	OmpF (porin)	Chaize and Fournier (2004)
Liposome (POPC)	Iodometry	AChE	Acethylcholine	Kinetic study in presence of porin	Winterhalter et al. (2001)
Liposome	Optical (Ellman's method	AChE	Acethylcholine	Film hydration method and BSA used to enhance stabilization	Nasseau et al. (2001)
Liposome (eggPC)	Optical (Ellman's method)	AChE	Acethylcholine Pesticides (dichlorvos)	Film hydration method and combining with sol–gel method	Vamvakaki et al. (2005, 2007)
Liposome (POPC)	Optical	YADH	Ethanol	Free-thaw method and use of NAD^+ as a cofactor	Yoshimoto et al. (2008)
Liposome (PC and cholesterol)	Optical	GOx	Glucose	Dehydration–rehydration method	
Liposome (PC)	Optical	Laccase	ABTS	Film hydration method	
Liposome (DPPC and PI)	Cyclic voltammetry	GOx	Glucose	Vesicle extrusion technique (VET)	Taylor et al. (1995, 1997)
Liposome (PC and POPC)	Electrochemical (SPE)	GOx	Glucose	Dehydration–rehydration method	Memoli et al. (2002)
Polyelectrolyte	Potentiometric and amperometric	Catalase	H_2O_2	Use QCM to confirm the formation of PE-encapsulation of catalase	Yu and Caruso (2003)
Polyelectrolyte	Optical (Fluorescence)	Urease	Ammonium	Control of the open and closed state of the PE by adding ethanol	Lvov et al. (2001)
Polyelectrolyte	Optical (fluorescence)	HRP	H_2O_2		Poulsen et al. (2007)
Polymer (hollow nanoshells)	Optical (fluorescence)	HRP	H_2O_2		Kumar et al. (2005)
Polymer (latex film)	Amperometric	GOx	Glucose		Cosnier et al. (2000)
Polymer (biomimetic silica)	Optical	Butyrylcholinesterase	Indophenyl acetate		Luckarift et al. (2004)
Polymer	Chronocoulometry	GOx	Glucose	Combine polymer with liposome vesicles	Olea et al. (2007)
Hydro-gel	Amperometric	GOx	Glucose (human serum)	Nafion film used	Hervas Perez et al. (2008)
Hydro-gel	Amperometric	GOx	Glucose	Human blood and serum	Rubio-Retama et al. (2005)
Hydro-gel	Amperometric	Cholesterol oxidase	Cholesterol (human serum)		Zhao et al. (2008)
Hydro-gel	Electrochemical	Lactate oxidase	Lactate	Dairy products (milk, yoghurt) and serum	Choi (2005)
Hydro-gel	Optical	Glutamate dehydrogenase	Ammonium		Azmi et al. (2009)
Hydro-gel	Amperometric	Glutamate oxidase and glutamate dehydrogenase	Ammonium	Wastewater samples, Clark-type oxygen electrode	Kwan et al. (2005)
Organic/inorganic hybrid gel					
Hybrid gel	Optical and electrochemical	HRP	H_2O_2	Disinfector sample, chitosan/silica gel	Li et al. (2008)
Hybrid gel	Electrochemical	HRP	H_2O_2	Direct electrochemistry of HRP performed without mediator	Feng et al. (2009)
Sol–gel	Cyclic voltammetric and amperometric	HRP	H_2O_2		Lei et al. (2004)
Sol–gel	Cyclic voltammetric and amperometric	HRP	H_2O_2		Jia et al. (2002)
Sol–gel	Optical (fluorescence)	HRP	H_2O_2		Smith et al. (2002)
Sol–gel	Optical	AChE	Dichlorvos	Detection of pesticides	Sotiropoulou and Chaniotakis (2005)
Sol–gel	Electrochemical	AChE	Acethylthiocholine chloride and thiocholine		Anitha et al. (2004)
Sol–gel	Cyclic voltammetric and amperometric	HRP	H_2O_2		Wang et al. (2003)
Sol–gel	Cyclic voltammetric and amperometric	HRP	H_2O_2		Jia et al. (2005)
Sol–gel	Optical	Urease and AChE	Heavy metals	Tap-river water and serum samples, measurements of pH, urea, acetylcholine	Tsai and Doong (2005)
Sol–gel	Optical (fluorescence)	Uricase-HRP	Uric acid	Human urine, blood, and serum	Martinez-Perez et al. (2003)
Sol–gel	Electrochemical	GOx	Glucose	Grape monitoring	Barsan et al. (2007)
Sol–gel	Amperometric	GOx	Glucose	Human plasma, Nafion and titania used	Choi et al. (2005)
Peptide nanotube	Optical (fluorescence)	Lipase	*p*-Nitrophenyl butyrate		Yu et al. (2005a,b)

rather than producing them (Taylor et al., 2005). However, the freeze–thaw could cause the denaturation of enzyme (Colletier et al., 2002).

For the liposomal encapsulation of enzymes, there are several methods used for the formation of liposomal vesicles. Among those methods, detergent dialysis and reverse-phase evaporation techniques rely on removal of the detergent by dialysis and the organic solvent by evaporation. But, denaturation of 50–70% fragile enzymes would be caused by detergents, solubilized lipids, and organic solvents during the encapsulation process (Chaize et al., 2003). Moreover, lipid hydration and sonication techniques were observed to denature the enzyme during sonication. So those drawbacks should be overcome for the retention of the enzyme activity. Hydration of a dry lipid film using freeze–thaw to convert multilamellar liposomes to unilamellar liposomes may be the most suitable for the encapsulation of enzyme. However, only 1–2% of enzymes in the solution were encapsulated although there were no harsh conditions during the procedure (Walde and Ichikawa, 2001). Fifty freeze–thaw cycles were reported as the optimum point, although the deactivation of enzyme still occurred as a number of the freeze–thaw cycles increased. The efficiency of enzyme encapsulation increased with an increase in lipid concentration (Chaize et al., 2004).

Encapsulation can be a suitable means to increase the selectivity of enzyme and to remove the matrix effect, which causes low reliability of biosensors. Encapsulation using liposomes can possibly provide an efficient barrier to hydrophilic molecules and ions reacting with active sites of enzymes. For example, acethylcholinesterase (AChE) has been selected for testing a biosensor using liposomes. The biosensors with AChE are used to measure the quantity of pesticide in environmental and food systems (Sandra et al., 2003). The sensing mechanism used here was to measure the inhibition rate of the AChE because insecticides are irreversible inhibitors of the active site of AChE. Therefore, the passing of the insecticides through the lipid bilayer was verified by investigating the irreversible inhibition of AChE. To test whether the encapsulated AChE has an improved selectivity to its hydrophobic inhibitors, hydrophilic ligands were used. The liposomes turned out to protect the active sites of enzyme from the hydrophilic molecules (Chaize and Fournier, 2004).

Consequently the enzyme encapsulation into liposomes is a technique to stabilize and prevent them from denaturation. However, the low permeability of lipid bilayer limits the use to widespread applications. In order to overcome this limitation, one of the suitable methods would be to control the permeability of liposome, thus resulting in generation of membrane channels. The AChE encapsulated in liposome and functionalized by the OmpF

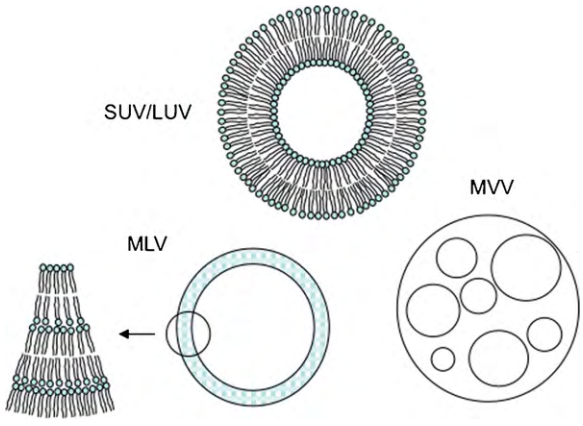

Fig. 2. Schematic representation of the structure of MLV, SUV, LUV, and MVV (Taylor et al., 2005).

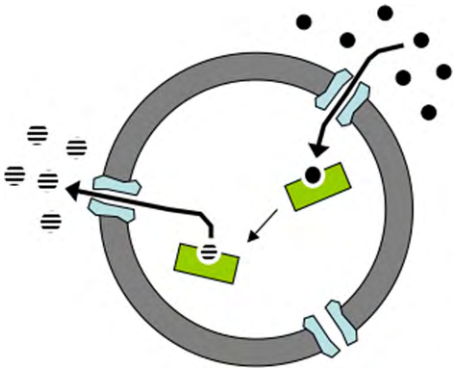

Fig. 3. Schematic representation of nano-vesicles with encapsulated enzymes. Substrates diffuse into the wall through porin channels, react with the enzymes, and then the products are released back into the solution through diffusion.

was used as a model enzyme to measure the enzyme activity in liposome. OmpF is a membrane protein, the channel from the outer cell wall of *Escherichia coli* (Fig. 3) (Sugawara and Nikaido, 1992; Winterhalter et al., 2001). The activity of encapsulated AChE in liposome with reconstituted porin OmpF was reported almost the same as that of free enzymes. However, the positively charged substrates were facilitated because OmpF is a slightly cation-selective channel. In addition to prevention of denaturation of enzymes, the use of bovine serum albumin (BSA) for the stability of enzyme has also been studied. Because the denaturation of enzyme occurred upon a dilution of the enzyme, it can be prevented by addition of BSA into the solution or by increasing the concentration of enzyme up to 100 nM (Nasseau et al., 2001). It is likely that the addition of BSA can be used for stabilizing the enzyme for other biosensor applications.

For the potential applications of liposomal vesicles with encapsulated enzymes, the liposome biosensor based on the AChE was used to examine drinking water samples, which might have the higher toxicity of pesticides. But, it is to be noted that in their work, addition of dichlorvos to the real water samples was conducted due to the fact that some heavy metal and different pesticides can inhibit the enzymatic reaction of AChE (Vamvakaki and Chaniotakis, 2007).

Regarding the storage and thermal stabilities of the encapsulated enzymes, yeast alcohol dehydrogenase (YADH) encapsulated in liposome consisted of POPC (1-palmitoyl-2-oleylsn-glycero-3-phosphocholine) were studied (Yoshimoto et al., 2008). Nicotinamide adenine dinucleotide (NAD^+) was used as a cofactor to improve the stability of YADH. The YADH/liposome, YADH/NAD^+/liposome as well as the free YADH with and without NAD^+ were compared to determine the thermal stability at 45 and 50 °C and the storage stability for 7 days. The encapsulation of enzyme was performed by the frozen/thaw method as a usual way to enhance the formation of large multilamellar vesicle in the mean diameter of 100 nm. Here it was found that YADH with NAD^+ had a higher stability than YADH with and without the liposomal system. This result indicates that NAD^+, a cofactor molecule, was bound to the active sites of YADH, thus resulting in an increase in the stabilization of enzyme against denaturation. The YADH/NAD^+ encapsulated in liposome was found to be stabilized compared to the free YADH with NAD^+. It was also found that the encapsulation in liposome would affect the interaction between enzymes and cofactors. In the case of using YADH, all the results using YADH were the same as those with HRP (horseradish peroxidase), showing that the encapsulation with liposome could possibly increase the stabilization of enzyme.

The deactivation energy values of GOx in liposome decreased with increasing temperature. At 50 °C, the enzyme in liposome

retained 100% of its activity, while only 76% of the activity of free enzymes was maintained. This result shows that enzyme deactivation may be caused by contamination with proteolytic enzymes and that the encapsulation in liposome can protect the enzyme against the proteolytic deactivation. In addition, the screen-printed electrodes (SPE) can be used in applications of liposomal biosensors to make disposable biosensors for glucose with high degree of electrochemical activity and sensitivity (Memoli et al., 2002). The biosensors using SPE offer several advantages, including cheap price, small size, and excellent electrochemical properties.

In order to improve the reproducibility of the electrochemical biosensors, various approaches of a liposomal enzyme electrode containing glucose oxidase in liposome have been studied. The effects of variations in the liposomal size and the encapsulation efficiency were characterized by electrochemical means. The biofouling problem of the lipid constituents of ruptured liposome on the response of the electrode to glucose was demonstrated. Liposome containing the enzymes was immobilized on polycarbonate membranes using poly-L-lysine as an immobilizer to set up the electrode with improving the repeatability. However, there was a loss of liposomal electrode response, which was thought to be possibly due to the aggregation in the liposome multilayer and the rupture of a very small number of liposome (Taylor and Jones, 1995).

The performance of liposomal electrode can be characterized by three factors: the amount of encapsulated enzymes, the permeability of the liposome for analytes, and the level of immobilization between the liposome containing enzymes and the electrode (Taylor et al., 1997). There are many factors to control the number of enzymes within a liposome capsule. For example, it was shown that the initial concentration of enzyme in the solution and the level of phosphatidylinositol (PI) within liposome affect the level of GOx encapsulation. However, higher concentrations of enzyme turned out to cause an unstable structure of liposome. It was also shown that the signal response time depended on the permeability of the liposome. The response time was 5 min, which is considered relatively slow. It was shown that an increase in the bilayer permeability enhanced the response of liposomal electrodes, but there was a side-effect of reducing the linear range in a working range of biosensors.

2.2. Enzyme encapsulation with polymers and polyelectrolytes

Some problems such as deactivation of enzyme, leaching, material degradation, phagocytosis, and toxicity could be overcome through the encapsulation methods with polyelectrolytes or polyions (Sukhorukov et al., 2000). A novel fluorescence biosensor with real time measurement based on a nano-assembled fluorescent microshell and capsule consisting of polymers or silica matrices has been developed. The hollow microshells have been manufactured using the layer-by-layer (LBL) assembly of polyions, which are the charged polymers (Fig. 4) (McShane, 2002; Sukhorukov et al., 2000). The LBL method is based on the alternating adsorption of cationic and anionic substances onto a charged substrate via electrostatic interaction (Levy et al., 2008; Jin et al., 2001; Caruso et al., 2000). A sodium-sensitive dye and a reference fluorophore were encapsulated in a fluorescent assay. The experiments for the stability, response, and robustness tests of the capsules were conducted to show their potential for biosensors. It was shown that the biosensor system using GOx had a broad range of glucose detection (0–100 mM) with a good linear response and that the dye molecules in the capsules retained up to 99% of fluorescence over a month (Duchesne et al., 2002a,b). Moreover, the catalase was also demonstrated to retain its electrochemical activity without the mediator through the LBL encapsulation (Yu and Caruso, 2003).

There are some challenges with the LBL method; Biofouling may take place in the graft copolymer structure. In addition, denaturation of enzymes and slow response time cannot be easily improved simultaneously because they are affected opposite by the thickness of polyelectrolytes. In order to overcome these obstacles in the system, there seems to be a possible solution that the micro channels are formed in the capsule wall.

Another solution may be found with the layer-by-layer assembly of linear polycations and polyanions. When urease is encapsulated into microshells assembled through the LBL of linear polycations and polyanions on a 5-μm diameter capsules, it preserved 13% of its activity as compared with free enzymes. It was shown that the polyion shell protected encapsulated enzymes from proteases and microbes (Lvov et al., 2001). These capsules are nonpermeable for urease in water and become permeable in a water/ethanol mixture. Thus, the transition between the open and closed states of the polyelectrolyte multilayer capsule introduced by adding ethanol in an aqueous solution is reversible. However, there is a possible problem of the denaturation of enzyme caused by media containing alcoholic solution (Olea and Faure, 2003).

In a different approach, a radical polymerization was introduced in order to encapsulate enzymes instead of the LBL technique. HRP and fluorescent dye were encapsulated into a nano-scale polyacrylamide particle by a radical polymerization in a reverse microemulsion. It turned out that the experimental data of enzyme activity in the polymer capsule showed that there were pores in the capsule wall, which were large enough for analytes and products to diffuse through the walls. Moreover, it was shown that 75% of the enzyme activity was maintained and that the enzyme was protected from proteolytic enzymes such as trypin and proteinase K. A linear dynamic range for the sensor was from 0.5 to 10 μM H_2O_2 (Poulsen et al., 2007). However, a reduced rate of enzyme kinetics was observed. It can be thought that it was due to the diffusion limitation by the walls and the leakage of enzymes from capsules through the pores.

A biosensor using hollow gold nanoparticles of the size about 100 nm diameter encapsulating HRP has been developed using reverse micelles, which demonstrated that the direct encapsulation is possible without denaturizing the enzymes (Kumar et al., 2005). From the experimental results comparing the both cases of free and encapsulated enzyme, it appears that the reaction kinetics depends on the diffusion rate. Hollow gold nanoparticle encapsulated enzyme has a potential for biosensors without bringing about any undesirable immunological reaction in the living system because the gold is biocompatible material.

In other studies, the GOx was encapsulated into hydrophilic latex matrices by the simple adsorption of enzyme-latex suspensions on the platinum electrode. The latex film functionalized by a hydroxy or a glucosamide group has been used as matrices (Cosnier, 2000; Cosnier et al., 2000). The effectiveness of thickness and temperature was monitored to estimate the performance as the biosensor with the amperometric measurement. The Michaelis constant was measured 1.25 and 1.00 mM for two films. They are relatively higher than that reported by Kumar et al. (2005). The maximum temperature for reserving 100% of enzyme activity within the latex matrices was 65 °C, which is higher than the other maximum temperatures obtained with other matrices. The response signal increased from one to two layers because the amount of enzyme immobilized in the film increased. This approach is slightly different from other encapsulation methods, which would be categorized as entrapment (Cosnier, 2000). Enzyme entrapment using biosilica is reported in the literature (Luckarift et al., 2004; Oh et al., 2006). This system was continually reusable for 2 days with gradual deactivation of enzyme. Two days are considered relatively a short time in terms of long-term sta-

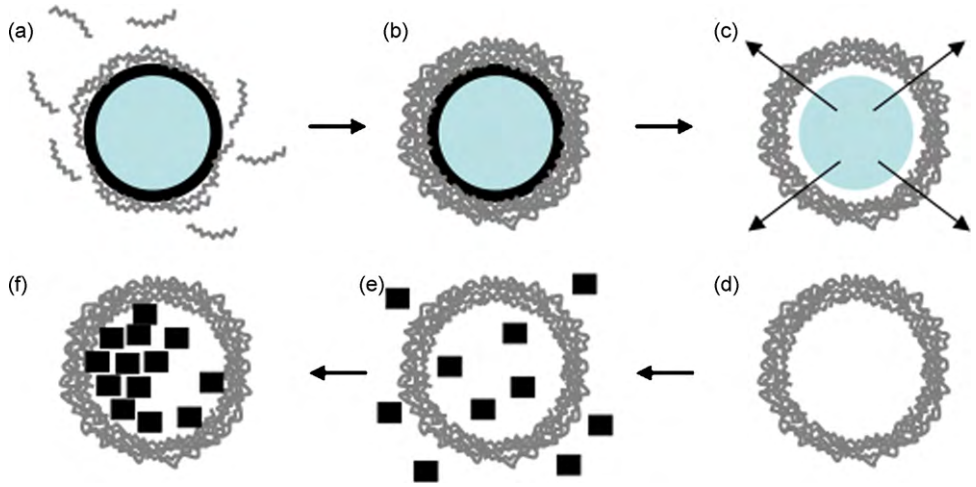

Fig. 4. Schematic representation of the polyelectrolyte layer structure and mechanism of polyelectrolyte encapsulation. The consecutive adsorption of (a) positively and (b) negatively charged polyelectrolytes onto a negatively charged colloidal particle. (c) After decomposition of the colloidal core, (d) suspension of a hollow polyelectrolyte capsule, (e) dyes and enzymes are added to the suspension of the capsule, and (f) on charging the ionic or solvent composition precipitation occurs inside the capsule (Sukhorukov et al., 2000).

bility. Moreover, the catalase can be encapsulated by LBL to retain its electrochemical activity without the mediator (Yu and Caruso, 2003).

Enzyme encapsulation in a polymer matrix has been preferred due to the low cost production of polymer. Polypyrrole (PPy) is one of the conductive polymers, which can be easily prepared in non-organic solutions by electrochemical polymerization in the ambient condition. There is a new approach, which combines conductive polymers with onion-type multilamellar vesicles produced by liposome (Olea et al., 2007). It was also observed that these liposomes did not lead to any notable denaturation because they are composed of their natural cellular medium (Olea and Faure, 2003). Here, the encapsulated enzyme (GOx-MLV-PPy) and free enzyme (GOx-PPy) were compared using the Michaelis–Menten behavior. Kinetic parameters increased with the GOx-MLV-PPy. The higher values of Michaelis constant is thought to come from higher thickness of PPy and diffusion barriers (Olea et al., 2008).

2.3. Enzyme encapsulation with sol–gel and hydro-gel

Sol–gel chemistry has offered remarkable versatility for fabricating metal-oxide, silica, and organosiloxane network for sensors, coatings, and catalysts (Gill and Ballesterous, 2000). The sol–gel processing is to form solid metal or semi-metal oxides through the aqueous processing of hydrolytically altered precursors in low reaction temperature. The sol–gel is generally formed from the oxysilane in a two-step reaction, hydrolysis and condensation, which is responsible for building the SiO_2 where enzyme can be encapsulated (Stefan et al., 2001). There are main advantages of sol–gel for immobilization of enzymes. First, the sol–gel matrix can have the characteristic of optical transparency, which is used for optical biosensors by changing in an absorbance. Second, the sol–gel matrix with mixing various mediators, polymer additives, and modified silanes could be optimum conditions for electrochemical biosensors with an electrical conductivity (Jin and Brennan, 2002). Also, one of the major advantages of sol–gel methodology for the biosensor application is that it allows the movement and stabilization of the tertiary structures of enzyme, preventing enzyme denaturation (Lei et al., 2004). However, the production of methanol or ethanol would be detrimental to enzymes and lead to significant changes in the characteristics of enzymes. The sol–gel matrices can also be used as a supporting film deposited onto an electrode to fabricate a H_2O_2 sensor (Jia et al., 2002). The gold nanoparticles combined with a sol–gel network would play a role in direct electron transfer between enzymes and electrodes. Also, this system acts as a third generation biosensor, which allows efficient electron transfer without mediators.

Organic/inorganic hybrid gel was prepared with various molar ratios of dimethyldimethoxysilane (DMDMOS)/tetramethoxysilane (TMOS) to hold an enzyme such as horseradish peroxidase (HRP). The content of a hybrid gel for the encapsulation efficiency and thermal stability of HRP were studied using spectroscopy. It was found that the proper organic/inorganic silane ratio of hybrid gel should be considered because most enzymes have different hydrophobicity and hydrophobic regions, and moreover hydrophobicity of a gel may depend upon the number of organic silane of chains of alkyl groups in the organic silane. When a specific ratio of DMDMOS and TMOS was chosen, the higher encapsulation efficiency and the best stability of HRP were demonstrated. This was likely attributed to the cooperation between a silane and mesoporous materials that forms a stable hybrid gel in the space between HRP and the pore walls (Li and Takahashi, 2000). In addition, a biohybrid hydro-gel was developed by mixing of carboxymethyl hydroxyethyl cellulose (CMHEC) and poly(vinyl alcohol) (PVA). This hybrid hydro-gel has shown that the enzyme activity of HRP was retained longer compared to free HRP, possibly because it has a large number of hydroxyl groups and can make the enzyme maintain its native secondary structure (Feng et al., 2009).

To date, an amperometric biosensor with acetylcholinesterase encapsulated in sol–gel matrices has gained attention, in particular with respect to pesticide detection such as a organophosphate. The detection is based on the inhibition of the enzyme activity, measured through amperometric detection of thiocholine. A TEOS-derived material, containing a little amount of carbon powder as a filler, was prepared without using the ethanol solvent to prevent the denaturation of enzyme (Anitha et al., 2004). First of all, the maximum sensitivity in pH 7.0 was demonstrated. Second, the increased amount of enzyme improved the sensitivity and the response time. Finally, the electrode sensitivity increased as a function of temperature at 40 °C, indicating that the sol–gel matrices did not affect the catalytic properties of enzymes. It was found that a little amount of carbon powder used for filling helped enhance the conductivity and decrease the shrinkage of the sol–gel matrix.

For optical sensor development, sol–gel matrix is also used to develop optical biosensors. A comparison between the case of AChE

incorporated with liposomes and that of AChE incorporated with the liposome-sol–gel matrix indicated that the both had similar analytical characteristics. Moreover, it turned out that the sol–gel matrix did not affect the kinetics of enzyme reaction. In other words, a sol–gel matrix did not seem to have any diffusion barriers. The enhanced mass transfer of analytes seems to be one of the advantages of sol–gel capsules over the liposome membranes. Another comparison of sol–gel biosensors with free AChE and with the liposome-encapsulated AChE was conducted because during the sol–gel process significantly detrimental problems such as deactivation of the enzyme might possibly happen. However, when incorporated with a sol–gel capsule, the activity of enzyme in the liposomal biosensor turned out stable against denaturation caused by the methanol produced by the hydrolysis process of the silicate solution (Vamvakaki et al., 2005). It is thought that the incorporation of sol–gel with liposome encapsulation has an enhanced protection from denaturizing solvents.

In addition, AChE encapsulated in a sol–gel matrix was compared with other methods that use photopolymer, photocrosslinkable poly(vinyl alcohol) bearing styrylpyridinium groups SPP-S-13(bio) (PVA-SbQ) and metal-chelate affinity. The apparent Michaelis–Menten constant of the case with the sol–gel matrix was slightly lower than other encapsulation methods (Andreescu et al., 2002). It is likely that the rate of substrate transport to the enzyme through the sol–gel matrix is relatively faster. Note that there is an exception that the apparent Michaelis–Menten constant of the enzyme encapsulated with cross-linked glutaraldehyde was lower than that with the sol–gel capsule (Bachmann and Schmid, 1999). It may possibly be proven that there is a trade off between the benefits of the simple construction procedure of a sol–gel matrix and the more selective and well-oriented enzymes with the lower detection limit in the cross-link polymers. The protection efficiency of the sol–gel matrix could provide a longer storage lifetime of the biosensors since organically modified silicates can be used to provide hydrophilic, hydrophobic, ionic, H-bonding to the material, which can enhance the stability of certain enzymes (Jin and Brennan, 2002). Also, the sol–gel possesses a wider concentration range, likely that a considerable enzyme amount was loaded on the electrode (Andreescu et al., 2002). However, the catalytic efficiency of the enzyme encapsulated in sol–gel matrices may possibly decline by two possible mechanisms; a barrier of mass transport of the sol–gel matrix causes attenuation of substrate binding and release rate, and the binding interaction such as hydrogen bonding between the enzymes and sol–gel host cause more catalyzed reaction than between the enzyme and substrates. It should also be considered that enzyme leakage could occur, but it would be minimized by the control of the matrix pore size (Smith et al., 2002).

The material derived from diglyceryl silane (DGS) was developed to increase the activity of enzymes compared with TEOS-derived sol–gel matrices, which produce methanol or ethanol, causing significant defects in the properties of enzyme. Interestingly, the enzymes used in the studies were encapsulated with a liposome prepared by 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). Then the liposomal enzymes were again encapsulated into DGS-derived materials. The reason for using the double encapsulation method seems to be the increase in the stability of enzymes. It was found that the catalytic efficiencies in both the solution and DGS-derived materials were similar, indicating that DGS-derived materials retained the activity of enzyme. The encapsulation of enzymes within the DGS-derived materials can be a useful tool, even though there is a drawback that residual glycerol in the DGS-derived material reduces the rate of substrate transport to the enzyme based on the kinetic study (Besanger et al., 2003).

Another new organic/inorganic hybrid sol–gel matrix consists of (3-aoryloxypropyl) dimethoxymethylsilane (APDMOS) cross-linked with chitosan. It was first developed to use as an amperometric hydrogen peroxide biosensor based on using a gold electrode (Wang et al., 2003). Advantages of this material are that the widely present amino groups provide a biocompatible environment for enzymes and the crucial problem of the brittleness of the silica-based sol–gel could be resolved.

In another approach, a SnO_2/gelatin composite prepared by the sol–gel method has been used to obtain a biosensor, which would be an attractive material for electrochemical studies and the development of a third generation biosensor without using a mediator just like the gold nanoparticles combined with the sol–gel network. It was found that the hybrid sol–gel material prevented the cracking problem so that it would retain the high enzyme activity and that the SnO_2 enabled the direct electron transfer between HRP and the electrode (Jia et al., 2005). It has been reported that SnO_2 has various applications in microelectronics, photoelectronics, solar cells, and gas-sensing. It has high sensitivity and fast response time as well as relatively higher conductivity than other oxide materials.

More recently various silica sol–gel films such as methyltrimethoxysilane (MTMOS), tetraethoxysilane (TEOS), 3-aminopropyltriethoxysilane (APTOS), and 3-glycidoxypropyltrimethoxysilane (GOPMOS) with different mixture ratios were compared and used in the development of glucose biosensors. The construction of a glucose biosensor was performed with a poly (neutral red) (PNR) redox mediator by electropolymerization followed by coating with sol–gel encapsulated glucose oxidase (GOx) (Pauliukaite et al., 2007). To avoid denaturation of the enzyme, no alcohol was added and vortex mixing was used instead of sonication. It was shown that GOPMOS had the most complex structure of oxysilanes with the smoother and more homogeneous characteristics of sol–gel, resulting in high sensitivity and low detection limit. Relatively homogeneous matrices indicate possibly the optimum condition for encapsulating enzymes for biosensors (Chiorcea-Paquim et al., 2008). However, this argument seems to be conflicted with the fact that the slow gelation rate with the base catalyst leads to relatively excellent matrices (Sotiropoulou and Chaniotakis, 2005). It is likely that here the acidic-catalyst was only used to compare the effects of the precursors instead of comparing the both acidic and based catalysts.

In another noticeable approach, the GOx with PNR in a sol–gel matrix made from the above precursors was applied to a flow system for fermentation monitoring of glucose in grape. GOx in the biosensor was immobilized by cross-linked glutaraldehyde (GA) or by encapsulation in a sol–gel matrix. The assays for both biosensors were conducted in the flow cell, resulting in that the biosensor with the immobilized GOx by cross-linking with GA showed better results with respect to detection limit, sensitivity, and the Michaelis–Menten constant than that with the sol–gel matrix. However, the sol–gel matrix allowed longer stability for the biosensor measurement. A higher flow rate increases the mass transfer of analytes to the electrode surface, making it possible to obtain an increase in current response (Barsan et al., 2007). In general, most nanostructures encapsulating enzymes were functionalized to be attached on the substrates through cross-linkers such as GA, which may require complex processing steps. So, a simple method for the fabrication of a biofuctionalized silicate nanoparticle containing thiol groups was introduced (Neville et al., 2009). It was shown that these nanoparticles were successfully immobilized on a gold surface. This method has a potential to use for biosensors and biocatalytic reactors. Even, the nanoparticles can be fabricated under neutral pH and at room temperature without alcohol or ammonia, which may cause denaturation of enzymes.

In particular, many engineers and scientists have been making an effort to develop glucose biosensors due to the fact that

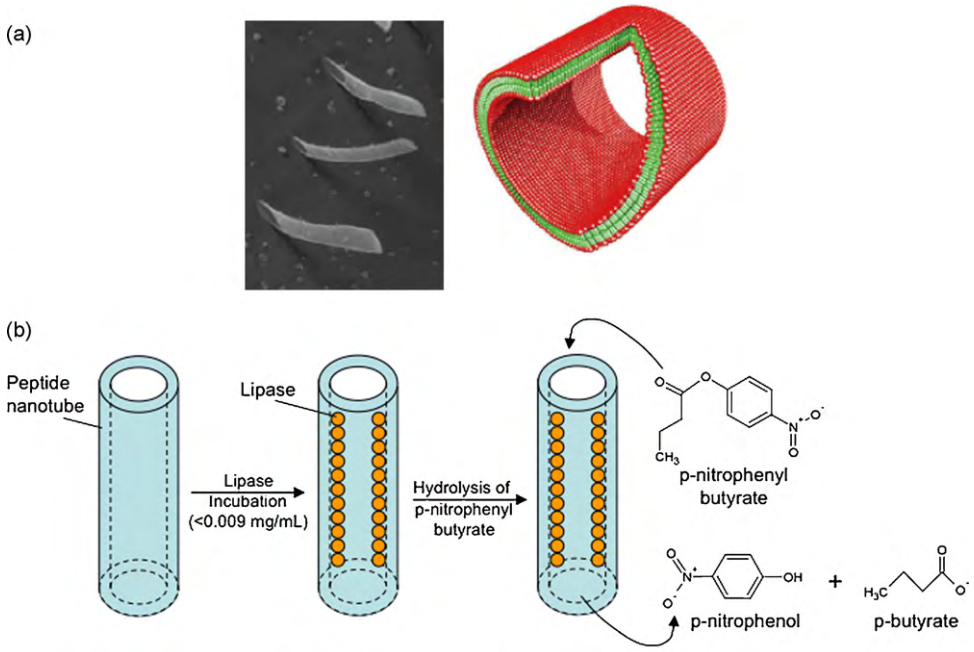

Fig. 5. (a) TEM micrograph and a molecular model of nanostructures formed by surfactant-like peptides (Zhao and Zhang, 2007). (b) Schematic illustration of the PNT-encapsulated lipase and its application. Reproduction from Yu et al. (2005b).

glucose biosensors account for around 85% of the current world market for biosensors and that there are over 170 million diabetes worldwide (Newman and Turner, 2005). For glucose detection in real samples such as human blood, plasma, and serum, the analysis with various sol–gel or hydro-gel encapsulation matrices was performed. An amperometric glucose biosensor was developed with the use of sol–gel-derived silica/Nafion and titania/Nafion (Choi et al., 2005). They have used titania (TiO_2) and Nafion since it was known that these materials are biocompatible with enzymes. Also, a deposition of Nafion film was applied onto the electrode modified with poly(ethylene glycol) methyl ether methacrylate (PEGMEM) microparticles containing GOx to improve the selectivity of the biosensor developed with human serum samples (Hervas Perez et al., 2008). The experimental results demonstrated that Nafion could prevent the negatively charged interferents such as ascorbic acid and uric acid from partitioning onto the electrode since the Nafion layer contains negatively charged sulfonate groups. They also developed a microencapsulation biosensor with hydro-gel polyacrylamide (PAA) (Rubio-Retama et al., 2005). When the negative charge of acrylic acid was applied to this sensor system, negatively charged interferents were prevented from partitioning onto the electrode.

There are some reports based on the use of sol–gel and hydro-gel matrix with encapsulated enzymes that allows the measurement of low amount of target molecules in real samples such as ammonium in a wastewater (Kwan et al., 2005; Azmi et al., 2009), lactate in dairy products and serum samples (Choi, 2005), cholesterol in human serum (Zhao et al., 2008), uric acid in biological fluids (Martinez-Perez et al., 2003), heavy metals in a tap water (Tsai and Doong, 2005), and hydrogen peroxide in disinfector samples (Li et al., 2008). In most cases, samples were directly diluted by a buffer solution and filtered by a syringe filter for the sample pre-treatment. It was an important consideration for an optimum condition in electrochemical biosensors that several possible interfering species such as ascorbic acid, uric acid, dopamine, and epinephrine in human samples should be removed to improve the selectivity of the biosensors. Although most of these sensor systems have not been marketed commercially, they have been successfully tested in the laboratory with electrochemical and optical methods.

It should be noted that there are reports that the biosensor based on a sol–gel matrix has a detrimental problem of enzyme leaching. In order to overcome this problem, polymer membrane made of carboxylated poly(vinyl chloride) (CPVC) and polyurethane (PU) have been considered to be a promising approach. The best fit concentration of a polymer solution for making a membranes should be carefully determined because the membranes made by high concentration polymers may not allow analytes to penetrate through the sol–gel matrix sufficiently, causing the diffusion barrier. It was also found that application of the polymer membrane reduced the current response to glucose. The lifetime of the biosensor was extended to more than 2.5 months. The biosensor was applied to measurement of glucose in the natural orange juice (Pauliukaite et al., 2008).

2.4. Enzyme encapsulation with nanotubes of peptides and lipid

Recently, peptide and lipid nanotubes (PNTs and LNTs) have become new powerful tools for the design synthesis and fabrication of nanodevices at the molecular scale from bottom up through molecular self-assembly (Fig. 5a) (Gazit, 2007; Gao and Matsui, 2005; Scanlon and Aggeli, 2008; Zhang, 2003). The organization of the building blocks is based on the weak and non-covalent bonds such as electrostatic interaction, hydrogen bonding, ionic bonding, hydrophobic interaction, aromatic stacking interaction, and van der Waals interaction. These overall coordinated combinations of the various molecular interactions result in the process of self-organization and building ordered structures, even thought these bonds are relatively weak in isolation (Zhao and Zhang, 2007; Zhou, 2007). There are many different types of nanotubes made of organic compounds such as Lego peptides (Zhao and Zhang, 2004), surfactant-like peptides (Vauthey et al., 2002), bola-amphiphilic peptides (Matsui et al., 2000), cyclic peptides (Hartgerink et al., 1996), dipeptides (Reches and Gazit, 2006), and lipid (Masuda and Shimizu, 2004). The diversity of compounds to construct the PNTs and LNTs through self-assembling is listed in Table 3.

Table 3
The various compounds for formation of PNTs and LNTs.

Peptides	Form	Properties	Applications	References
Lego peptides (Ionic peptides)	Hydrophobic and hydrophilic surfaces	Structural plasticity, multifaceted behavior, and spontaneously assemble to form well-ordered nanofibers self-assemble to form scaffolds	Scaffolds for tissue engineering and medicine applications	Zhao and Zhang (2004)
Surfactant-like	Hydrophilic head group and hydrophobic tail	Similar properties to other biological surfactants, good solubility and stability of peptides in the solution	Scaffolds to organize conducting and semiconducting nanocrystals and encapsulating molecules for deliveries	Vauthey et al. (2002)
Bola-amphiphiles	Two hydrophilic peptides conjugated through hydrophobic peptide linker	Amphiphile and self-assemble into well-ordered nanofibers	Scaffolds as the self-assembled nanofiber forms, fibrillar work, nano- bio- electronics, and biosensor applications	Matsui et al. (2000)
Cyclic peptides	Long cylindrical structures with an anti-parallel β-sheet structure	Stack through extensive intermolecular hydrogen bonding and adjustable internal diameter nanotubes	Antibiotic agents, nanocontainers, composite materials, and nanotubes	Hartgerink et al. (1996)
Dipeptides	Dipeptides	Combination of hydrogen bonding and π-stacking, proteolytic liability, good thermal resistivity, and electrical conductivity	Conductive nanowires and nanotubes, and sensing devices	Reches and Gazit (2006)
Lipid	A hydrophilic head group and hydrophobic tail	Amphiphilic compounds, self-assembled in water, and hydrophilic groups exposed to aqueous environment	Drug release, gene delivery, cell adhesion, antimicrobial activity, and biomolecule sensing	Masuda and Shimizu (2004)

For biosensor applications, *Candida rugosa* lipase was encapsulated in PNTs and examined its catalytic activity (Yu et al., 2005a). It was shown that the activity of enzyme inside PNTs was 33% higher as compared to that of the free enzyme in the solution at room temperature. At a higher temperature, 65 °C, the activity of enzyme inside PNTs was 70% higher compared to the free enzyme in the solution. There are two possible mechanisms to encapsulate enzymes in PNTs including the capillary effect and the hydrophobic interaction. In order to confirm whether the enzymes inside PNTs activate well, the activity of the enzyme encapsulated inside the PNTs was determined by measuring the change in the hydrolysis rate of *p*-nitrophenyl butyrate and the concentration of a product, *p*-nitrophenol (Fig. 5b). It turned out that the lipases in PNTs affected the hydrolysis rate. However, the leakage of enzyme through the ends of PNT can possibly occur due to the weak interaction between enzymes and the surface of inside wall of PNT.

3. Conclusions and future outlook of sensing technologies with encapsulated enzyme

Recent advances of encapsulated enzyme technology for biosensors are reviewed regarding the encapsulating materials and sensing methods. Encapsulation materials, liposome, polymers, and hydro-gel, have been much studied and used for biosensors to overcome the problems of enzyme deactivation and deformation. By stabilizing the enzymes via encapsulation with new methods and materials the enzyme activity may be maintained for a longer time, and even the selectivity can possibly be enhanced. Enzyme immobilization of a screen-printed electrode using metal-chelate affinity showed a promising result. It is noticeable that not only the activity and selectivity ameliorations, but also other advantages such as mediatorless sensing may be gained in liposome- or polyelectrolyte-encapsulated enzymes. Combination of different encapsulation materials is also a noticeable approach to obtain synergism of increased activity and stability, while avoiding the byproduct damage. A new material such as peptide nanotube showed great potential for stable and biocompatible encapsulation. In general increased mass transfer limitation seems to be the major challenge to investigate more.

In the future development of biosensors with encapsulation techniques, it is necessary to develop various biocompatible materials with advances in better usages of them. A better understanding of the characteristics of immobilized enzymes and improved immobilization techniques have pointed to a promising direction to the development of biosensors.

In conclusion, we believe that the encapsulation techniques will provide new opportunities to develop biosensors of better retained activity and long-term stability. PNTs and LNTs have already proved themselves to be promising candidates for diverse applications in biology, medicine, nanobiotechnology, and bionanotechnology. They will make a great asset to development of advanced biosensors in the future.

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