African Journal of Biotechnology Vol. 8 (19), pp. 4724-4733, 5 October, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Review

Recent advance in the support and technology used in enzyme immobilization

Tian Xie*, Anming Wang, Lifeng Huang, Haifeng Li, Zhenming Chen, Qiuyan Wang and Xiaopu Yin

Research Center for Biomedicine and Health, Hangzhou Normal University, No. 222, Wenyi Road, Hangzhou 310012, P. R. China

Accepted 4 September, 2009

In the industrial biotechnology, the skill to prepare the immobilized enzyme with stability has proven to be one of the key steps in rendering an enzymatic process that is economically viable. Availability of the immobilized enzyme biocatalyst with improved activity and stability is also expected to reduce the expense of products. If properly designed, enzyme immobilization is also a very powerful tool to enhancing almost all enzyme properties, e.g., activity, stability, specificity and selectivity, reduction of inhibition. Due to the above advantages, enzyme immobilization has been a focus for biologists, chemists and enterprisers. This review is focused on advances over the last several years in enzyme immobilization. Several new type of carriers and technology have been implemented to improve traditional enzyme immobilization, which aimed to enhance enzyme loading, activity and stability to decrease the enzyme biocatalyst cost in industrial biotechnology. These include cross-linked enzyme aggregates (CLEAs), microwave-assistant immobilization, click chemistry technology, mesoporous support and single enzyme nanoparticle. In addition, with the growing attention paid to cascade enzymatic reaction and *in vitro* synthetic biology, it is possible that multi-enzyme co-immobilization would be one of the next goals in the future.

Key words: Enzyme immobilization, cross-linked enzyme aggregates (CLEAs), microwave irradiation, mesocellular siliceous foams (MCFs), click chemistry, single enzyme nanoparticle.

INTRODUCTION

A substantial increase in political and financial investment that aligns plant and industrial biotechnology will pay dividends for sustainable energy and materials production (Bevan and Franssen, 2006). Industrial biotechnology, or white biotechnology (sustainable chemical processes built on renewable resources and biocatalysts carried out in “bio-refineries”) builds not only on fermentation using metabolically engineered microorganisms, but as much on enzymes improved by protein engineering techniques (Soetan, 2008). However, native enzyme application is often hampered by its lack of long-term stability under process conditions and also by difficulties in its recovery and reuse. To date, biologists and chemists have been searching for the ideal and suitable biocatalyst through recombinant DNA technology, medium engineering and immobilization technology (Boller et al., 2002; Bornscheuer, 2003; Burton et al., 2002; Hegedus and Nagy, 2009; Mateo et al., 2007; Yan et al., 2006).

In the industrial biotechnology, the skill to stabilize and re-use an enzyme catalyst through immobilization has proven one to be of the key steps in rendering an enzymatic process that is economically viable (Cao and Schmid, 2005; Zhou, 2009). Availability of the immobilized enzyme catalyst with improved activity and stability is also expected to reduce the expense of products (Parmar et al., 2000). Moreover, enzyme immobilization has been revealed in the last times as a very powerful tool to enhancing almost all enzyme properties, if properly designed: e.g., stability, activity, specificity and selectivity, reduction of inhibition (Blanco et al., 2007).

To improve the enzyme immobilization, various new carriers and technologies have been used to immobilize enzymes. We will try to make a brief summary of the

*Corresponding author. E-mail: tianxiehz@hotmail.com. Tel.: +86-571-28861623. Fax: +86-571-28865630.

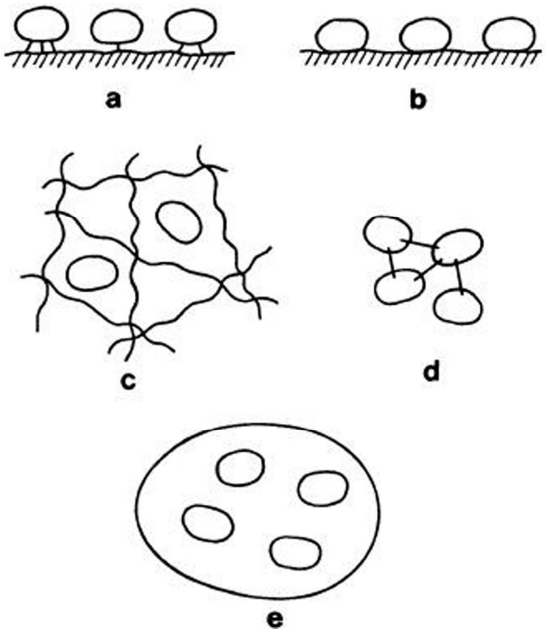

Figure 1. Methods of enzyme immobilization: (a) covalent attachment to solid supports, (b) adsorption on solid supports, (c) entrapment in polymeric gels, (d) intermolecular crosslinking and (e) encapsulation.

most interesting and recent advances in these topics such as new carriers and new protocols. The trend of the topics will also be discussed.

TYPES OF IMMOBILIZATION

The traditional and most frequently used immobilization techniques fall into 5 categories (Figure 1) (Klibanov, 1983). As for the types of enzyme immobilization, entrapment and encapsulation were often combined together into one type (Bornscheuer, 2003). In addition, with the development of chemistry and materials science and the increasing knowledge of enzyme structure, all the above approaches are a compromise between maintaining high catalytic activity while achieving the advantages of immobilization.

Recently, cross-linked enzyme crystals (CLECs) (Quiocho and Richards, 1964; Stclair and Navia, 1992) and cross-linked enzyme aggregates (CLEAs) (Cao et al., 2000) have been introduced as the new modes of enzyme preparation. They were often called carrier-free immobilization, for no support was used in the immobilization (Sheldon, 2007). In the preparation of cross-linked enzyme crystals, enzyme was first crystallized in an aqueous solution at suitable temperature, then crystals of enzyme were cross-linked with glutaraldehyde (Roy and Abraham, 2004). CLECs often present higher activity and enantioselectivity in organic solvents than they do in aqueous solution. The activity of CLECs of subtilisin in decane was 780 times greater than that in triethylamine. CLECs of subtilisin preferred L-enantiomer in the transesterification between N-acetyl-phenylalanine ethyl ester and n-propanol, and the $(k_{cat}/K_M)_L/(k_{cat}/K_M)_D$ ratio was 20000 in cyclohexane (Noritomi et al., 2007). Sometimes, CLECs also give an increased enantioselectivity relative to its native enzyme (Lalonde et al., 1995). This was mostly attributed to the removal of a less selective isoenzyme during CLECs preparation. Cross-linked enzyme crystals (CLECs) retain catalytic activity in harsh conditions, including temperature and pH extremes, exposure to organic or aqueous-organic mixtures, which may result from the stabilization of the crystalline lattice and its constituent enzyme molecules after chemical cross-linking of enzyme crystals (Stclair and Navia, 1992).

Enzyme crystallizes when preparing the CLECs, which is often a laborious procedure requiring enzyme of high purity. Additionally, protein molecules would physically aggregate and hold together by non-covalent bonding without perturbation and denaturation of their tertiary structures when salts, water-miscible organic solvents or non-ionic polymers were added into aqueous solutions of proteins. Cross-linked enzyme aggregates (CLEAs) (Figure 2) (Cao et al., 2000; Sheldon, 2007), which are obtained by precipitation of proteins followed by cross-linking with glutaraldehyde, might represent an easy alternative. The CLEAs of penicillin acylase not only had the same activity as the CLECs in the synthesis of ampicillin, but also the cross-linked aggregate catalyzed the reaction in a broad range of organic solvents. However, carrier-free immobilization may not be suitable for enzyme which catalyzes the hydrolysis or synthesis of macromolecular substrate or product, which results from low diffusion in the narrow channel in enzyme aggregations. Moreover, something lacking in perfection was that CLEAs are too soft and hence may exhibit poor stability when used in stirred tanks or in packed bed reactors (Roy and Abraham, 2004). If it were encapsulated in large porous support or a very rigid poly (vinyl alcohol) network through a suitable immobilization technique, it would be used widely as a sturdy process biocatalyst.

NEW CARRIERS USED IN THE IMMOBILIZATION

In the early enzyme immobilization, support was used to insolubilize the enzyme and thus to facilitate its separation and reuse, which provides easy control over the non-catalytical properties of the obtained immobilized enzyme. In the search for suitable support for enzyme to attach, it was found that physical and chemical properties (e.g. pore size, hydrophilic/hydrophobic balance, aquaphilicity and surface chemistry) of support could exert effect on enzyme immobilization and its catalytic properties (Boller et al., 2002; Cao et al., 2003). Thus, with the increased understanding of the correlation of enzyme property with structure and microenvironment (Wang et

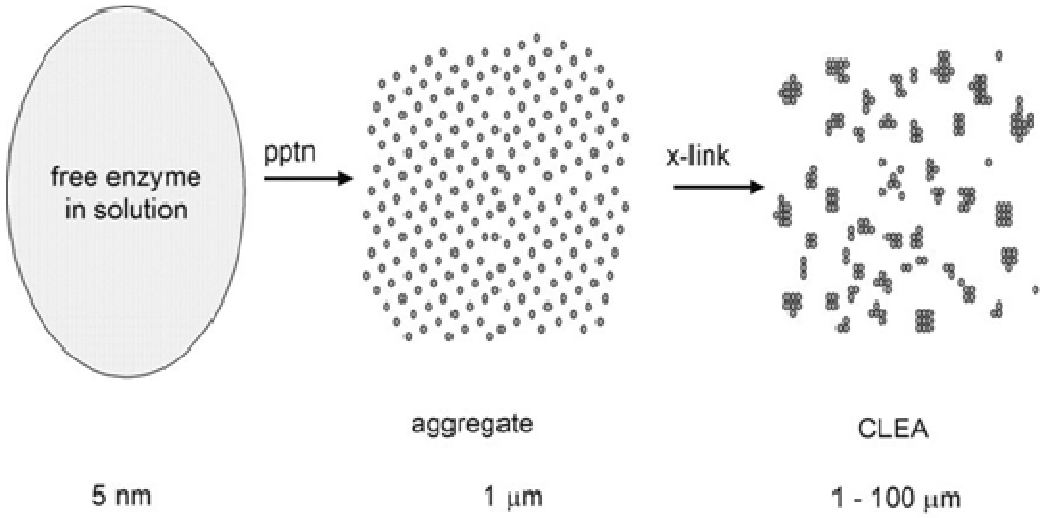

Figure 2. Preparation of a CLEA.

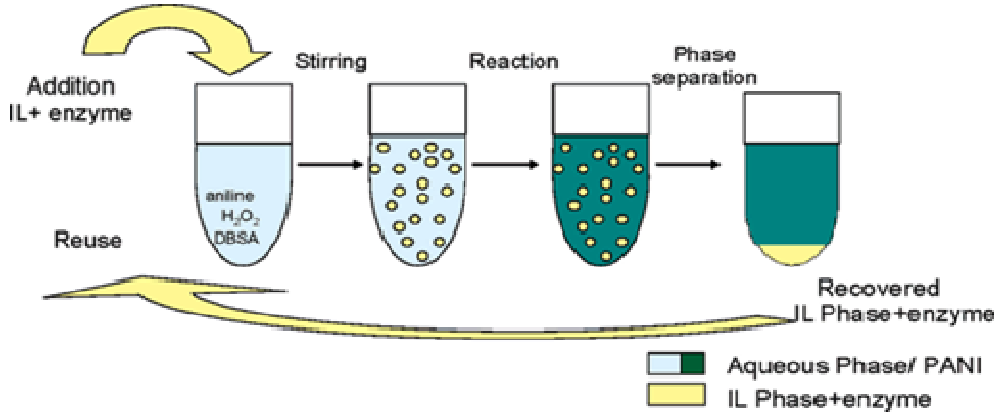

Figure 3. Attachment of enzyme to ionic liquid (IL) and catalysis in bi-phase system.

al., 2008c; Wang et al., 2009a), a great number of synthetic or natural carriers of tailor-made chemical and physical properties, with different shapes/sizes, porous/ non-porous structures, different aquaphilicities and binding functionalities, have been specifically designed for various bioimmobilization and bioseparation procedures (Chong and Zhao, 2004; Gemeiner, 1992; Luckarift et al., 2004; Sun et al., 2006; Wang, 2006; Wang et al., 2009b).

Molecular support

Ionic liquids are composed entirely of ions. In the patent and academic literature, the term “ionic liquid” now refers to liquids composed entirely of ions that is fluid around or below 100°C. Room-temperature ionic liquids are frequently colorless, fluid and easy to handle (Rogers and Seddon, 2003). Versatile biphasic systems could be formed by controlling the aqueous miscibility of ionic liquid (Gutowski et al., 2003), which was conveniently used in the enzyme biocatalysis to separate the enzyme and reaction system (Sheldon et al., 2002; van Rantwijk et al., 2003). Based on a biphasic catalytic system where the enzyme is immobilized into an ionic liquid (IL), Mecerreyes and co-workers (Rumbau et al., 2006) have reported a new method which allows recycling and re-using of the HRP enzyme in the biocatalytic synthesis of PANI. The IL 1-butyl-3-methylimidazolium hexafluorophosphate was used as immobilization media by dissolving the HRP enzyme (Figure 3). The IL/HRP phase acts as an efficient biocatalyst and can be easily recycled and reused several times. Due to the immiscibility between the IL and water, the immobilized HRP could be simply recovered by liquid/ liquid phase separation after the biocatalytic reaction. This new method is faster and easier than the classical immobilization of HRP into solid supports.

In bio-electrochemistry field, carbon ionic liquid elec-

trode (CILE) was immersed in a solution containing Hb and ionic liquid, octylpyridinium chloride ([OcPy][Cl]) to directly immobilize hemoglobin (Hb) on CILE. Cyclic voltammetry of modified electrode exhibited quasi-reversible peaks, corresponding to Hb. Hb retained its bioactivity well on modified electrode and showed excellent electrocatalytic activity towards oxygen, hydrogen peroxide and nitrite, which resulted in the determinable hydrogen peroxide content in the range of $1.0 *10^{-4}$-$5.0*10^{-3}$ M (Safavi et al., 2008). This novel method would not be widely applied to the industrial production because ionic liquid is still an expensive chemical in the coming future.

Mesoporous support

Now, one of the most promising carriers for enzyme immobilization is the mesoporous material (Chen et al., 2007b; Kim et al., 2007; Rosales-Hernandez et al., 2007; Wang et al., 2008a; Wang et al., 2007). The exploitation of novel carriers that enable high enzyme loading and activity retention has become the focus of recent attention (Boller et al., 2002; Cao et al., 2003). The large surface areas and greater pore volumes of these materials could enhance the loading capacity of an enzyme. By now, the highest loading of a-chymotrypsin (CT) in the mesoporous support reached 570 mg/g on immobilization surfaces (Kim et al., 2007). Since the pore volume of HMMS is 1.34 mL/g, the theoretical maximal CT loading value in the HMMS was approximately 904 mg/g, indicating that the enzyme loading is 63% of the theoretical maximum value. Obviously, the use of immobilized enzymes with high enzyme loading and high volumetric capacity is beneficial because high productivity and space–time yields can be obtained (Cao, 2005). When mesoporous material is used as support, the large pores in the support facilitate transport of substrate and product (Chong and Zhao, 2004).

Functional mesoporous material with the unique mesoporous structure and surface chemistry could also provide both high affinity for the charged protein molecules and a favored microenvironment. This microenvironment resulted in exceptionally high immobilization efficiency (> 200%) with enhanced stability, while conventional approaches yielded far lower immobilization efficiency (Lei et al., 2002). Additionally, the increase in the thermal stability of immobilized enzyme indicated that protein inside a confined space could be stabilized by some folding forces which did not exist in proteins in bulk solutions (Wang et al., 2008b). Confinement of the support nanopore could be similar to the macromolecular crowding (Cheung and Thirumalai, 2006), and could also stabilize the enzyme at high temperature.

In the family of mesoporous materials, nanoporous gold (Szamocki et al., 2007) and nanotube (Chen et al., 2001; Wan et al., 2008) have also been used to immobilize enzyme and most of the obtained immobilized enzymes were used in the electrode preparation and biosensor applications. Porous electrodes with increased surface area have been prepared using a template route via colloidal crystals. The modified porous gold electrode shows an overall increased signal, and therefore a better detection limit and higher sensitivity when used as sensors. It is a potentially higher power output when employed in biofuel cells (Szamocki et al., 2007). The greatly enhanced sensitivity makes this type of electrodes well suited to improve existing sensing devices and the overall higher current densities also open up promising applications as efficient electrodes in biofuel cells. In the immobilization using this support, it is very important that physical chemistry of support surface exerts great influence on properties of the immobilized enzyme (Wang et al., 2009a). Looking for a way to regulate the physical chemistry of support surface suitable for enzyme is necessary for efficient immobilization.

Cloth and sponge

A porous wool keratin sponge (Hideno et al., 2007) has been used to immobilize lysozyme and prepare a unique biomaterial. In the obtained immobilized systems, the activity of lysozyme linked to the sponge through disultide bond was gradually released, while lysozyme through thioether bond was stably maintained. Prior to keratin sponge, polyurethane foam has also been used to immobilize crude phosphotriesterase and more than 50% of the initial enzyme specific activity was retained after immobilization (LeJeune and Russell, 1996).

To reduce the cost of support, cotton fabric was used to immobilize enzyme (Albayrak and Yang, 2002; Li et al., 2007; Wang et al., 2008d). The fabric was first oxidized with sodium periodate, and then employed to immobilize catalase. The staining test and reusability showed that the catalase was fixed covalently on the oxidized cotton fabric. The oxidation condition affected the covalent immobilization, which perhaps resulted from the excessive covalent linking between the enzyme and support. A protocol of enzyme immobilization on nylon film with relatively inexpensive and non-toxic reagents, involving acid hydrolysis, glutaraldehyde, has been reported (Isgrove et al., 2001). This provided a method having potential in bioreactor applications to combine biocatalysis with bioseperation. The main advantage for this support is low cost and we need not to spend much time to produce them. In addition, separation of immobilized enzyme from reaction system is very easy.

Magnetic hybrid support

The applications of magnetic support to enzyme immobilization are mainly based on the magnetic properties of the solid-phase that enables a rapid separation in a

magnetic field. Moreover, the magnetic supports can be easily stabilized in a fluidized bed reactor for continuous operation of enzyme by applying an external magnetic field and the use of magnetic supports can also reduce the capital and operation costs (Bayramoglu et al., 2008). Due to the functionalization (Dyal et al., 2003) of enzyme and its suitable microenvironment (Bayramoglu et al., 2008), magnetic materials were often embedded in organic polymer or inorganic silica to form hybrid support (Liu et al., 2005).

Recently, because of the low enzyme loading on the conventional magnetic beads (Liu et al., 2005), more and more attention was paid to the magnetic mesoporous support (Sadasivan and Sukhorukov, 2006). Magnetite-mesoporous silica hybrid support was fabricated by depositing magnetite and MCM-41 nanoparticles onto polystyrene beads using the layer-by-layer (LBL) method. The incorporation of magnetite gives an additional magnetic property to the hollow mesoporous silica shells, which resulted in the perfect combination of mesoporous materials properties with magnetic property to improve the enzyme immobilization (Kim et al., 2005).

Microchannel in the microfluidics device

Micro-scale device fabrication, micro-fluidics and lab-on-chip technology were used firstly for chemical and biochemical analysis in the chemical and pharmaceutical industry. Integration of flow channels and reaction chambers in these microanalytical systems enable the dramatic acceleration, automation and miniaturization of traditional benchtop analytical tools with enhanced performance and dramatically reduced sample sizes. Recently, more complex microfluidic devices have been developed for bioprocess operations, enzyme immobilization and catalysis (Ku et al., 2006; Lee et al., 2003; Luckarift et al., 2007). Dordick and co-workers (???) pre-packed Ni-NTA agarose beads attached with 1,3,6,8-tetrahydroxynaphthalene synthase (THNS) into a first microfluidic channel and covalently immobilized type III polyketide synthase (PKS) on the walls of a second micro-fluidic channel pre-coated with a reactive poly (maleic anhydride) derivative (Ku et al., 2006). Using this method, they efficiently performed *in vitro* metabolic pathway reconstruction or modification to synthesize novel natural product derivatives using a simple and likely expandable micro-fluidic platform. Then, they expanded the method to the combinatorial synthesis of 2-aminophenoxazin-3-one (APO) (Luckarift et al., 2007). The microfluidic device system stabilized the enzymes in the channel and proved suitable for synthesis of a complex natural product (APO) from a simple substrate (nitrobenzene) under continuous flow conditions. The conversion of reactants in the microfluidic channel bioreactor was often found to be higher than that in the batch slurry reactor, used as a control.

NEW TECHNOLOGY FOR ENZYME IMMOBILIZATION

Microwave irradiation

In the immobilization, especially in porous support, enzyme molecules are difficult to distribute throughout the porous carriers because of diffusion limitations (Buchholz, 1979). And they often remain only on external channels (Chen et al., 2007a), using non-microwave-assisted protocols, which are currently the method of choice for immobilizing enzymes. The mass transfer is even slower for enzymes having large dimensions, such as penicillin acylase. The immobilization of such enzyme to porous materials can prove tedious using conventional techniques (van Langen et al., 2002). In our study, microwave irradiation was applied in the immobilization of enzyme in the mesocellular siliceous foams (MCFs) (Wang et al., 2008b; Wang et al., 2009a). In our work, microwave irradiation technology was used to immobilize papain and penicillin acylase (PA) into MCFs. Time for immobilization was decreased significantly and the enzyme loading was dramatically improved. 80 and 140 s were enough for papain and PA to attach on the wall of MCFs, respectively. The maximum loading of papain reached 984.1 mg/g, 1.26 times of that obtained from the conventional method which was non-microwave-assisted. The activities of papain and penicillin acylase immobilized with microwave-assisted method were 779.6 and 141.8 U/mg, respectively, 1.86 and 1.39 times of those with conventional method. The results showed that microwave irradiation improved the adsorption immobilization of enzyme in mesocellular siliceous foams.

As for covalent immobilization under microwave irradiation, macromolecules crowding was combined with small molecular quenching to perfect microwave-assisted covalent immobilization (Wang et al., 2009a). The maximum specific activities of PA assembled with Dextran 10000 (Dex 10) and BSA in MCFs under microwave irradiation, 85.3 and 112.7 U/mg, were 1.73 and 1.31 times of that of PA solely immobilized with conventional method. PA co-immobilized with Dex 10 in mesopores retained 88% of its initial catalytic activity after heating at 50ºC for 6 h; this resulted from glycine quenching excessive activated groups. This biomolecule enhanced the thermo-stability of this enzyme preparation by more than 2 times. Crowding environment, resembling cell that resulted from macromolecular reagents would be suitable for stabilizing the structure of penicillin acylase and improving its catalytic activity. Glycine, biocompatible small molecule, quenched the excessive active groups and modified the surface chemical properties of mesoporous support, which would further favor the stability of penicillin acylase at higher temperature (Figure 4).

Single enzyme nanoparticles

Industrial biotechnology is consistently seeking improved

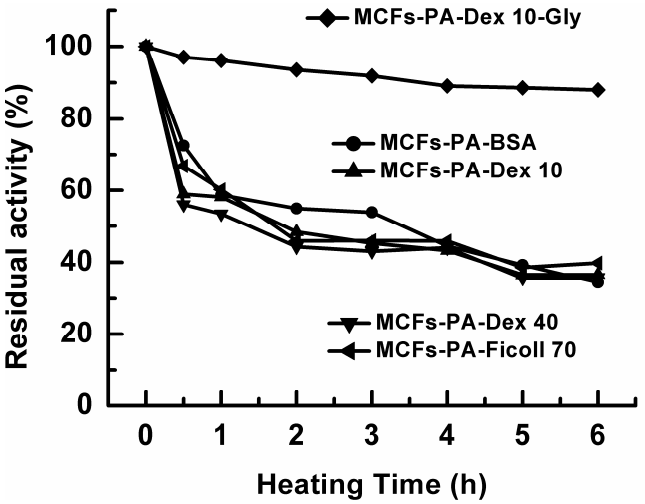

Figure 4. Thermostability of PA preparations at 50°C. MCFs–PA, PA covalently assembled in MCFs; MCFs–PA–X, PA covalently assembled with X in MCFs; BSA, bovine serum albumin; Dex 10, dextran 10000; Dex 40, dextran 40000; Ficoll 70, Ficoll 70000.

enzyme performance and stability under harsh conditions found in most industrial processes to decrease cost. As an innovative way of enzyme stabilization, "single-enzyme nanoparticles (SENs)" technology was rather attractive because enzyme in the nanoparticle exhibited very good stability under harsh conditions (Hegedus and Nagy, 2009; Yan et al., 2006) (Figure 5). Kim and Grate (2003) have developed armored single-enzyme nanoparticles (SENs) (Kim and Grate, 2003) that surround each enzyme molecule with a porous composite organic/inorganic network of less than a few nanometers thick. Their approach has significantly stabilized 2 proteases (chymotrypsin, CT and trypsin, TR) and the armor network around CT is so thin and porous that a large mass-transfer limitation on the substrate could not take place. To simplify the versatile preparation that yields a single enzyme capsule with enhanced stability, high activity and uniformed size, Yan et al. (2006) have presented a 2-step procedure including surface acryloylation and *in situ* aqueous polymerization to encapsulate a single enzyme in nanogel to provide robust enzymes for industrial biocatalysis. Compared with the free horseradish peroxidase (HRP), the HRP nanogels exhibit similar biocatalytic behavior. This is evidenced from their similar Km and kcat, and significantly improved stability at high temperature in the presence of polar organic solvent.

In contrast with the above procedure, with enzyme in the inner particles, Ho et al. (2008) have prepared core-shell nanoenzyme particles consisting of well-defined poly (methyl methacrylate) cores and cellulase shells. Particle sizes in the range between 80 and 124 nm (volume average diameter) could be tailored by a variation of cellulase concentration. The activity of immobilized cellulase on the nanoparticles was 41% less than that of the native cellulose. After the polymerization, the immobilized cellulase showed better thermal stability. For thermally stable enzyme, the route may be good immobilization strategy because enzymes load on the outer surface and inner diffusion limitation of substrate and product could be avoided.

Photoimmobilization technology

When photoreactive polymer and horseradish peroxidase or glucose oxidase are exposed to ultraviolet (UV) light at 365 nm, the reactive nitrene immobilizes the protein molecules in 10 to 20 min through covalent bonding (Naqvi and Nahar, 2004). As nitrene has a property of inserting into C-H bond, the method may find potential applications for immobilization of biomolecules irrespective of their functional groups. Nahar and Kumar (2007) have also immobilized horseradish peroxidase (HRP) and glucose oxidase (GOD) onto the photoreactive cellulose membrane by sunlight (Kumar and Nahar, 2007). They found that sunlight intensity required for optimum immobilization was 21,625 lux beyond which no appreciable increase in immobilization was observed. Moreover, sunlight exposure gave better immobilization compared to 365 nm UV light. The method does not require any equipment and is suitable for large scale as well as small-scale immobilization.

To prepare the urease thin films with accurate thickness control, the targets were exposed to laser radiation which consisted of frozen composites made by dissolving or suspending biomaterials in distilled water, to minimize photochemical damage. The evaporated water molecules thus protected/shielded the biomolecules through collisions and transferred them to the substrate surface that laid plan parallel in front of the frozen target (Gyorgy et al., 2009). At the same time, matrix-assisted pulsed laser evaporation (MAPLE) was used and laser pulse intensities were cut down by an order of magnitude, compared with their common level on irradiating other type of materials, e.g. inorganics to give an additional precaution.

Enzymatic immobilization of enzyme

To avoid the harsh immobilization process and partial denaturation of enzyme protein, enzyme mediating immobilization of enzyme has been emerging as a novel technology to fabricate solid protein formulations (Tanaka et al., 2007; Wong et al., 2008). As model proteins, enhanced green fluorescent protein (EGFP) and glutathione S-transferase (GST) were tagged with a neutral Gln-donor substrate peptide for MTG (Leu-Leu-Gln-Gly, LLQG-tag) at their C-terminus and immobilized onto the casein-coated polystyrene surface (Tanaka et al., 2007) (Figure 6). Only in the presence of active MTG they could be attached on the support surface, which indicated that those proteins were enzymatically immobilized to the

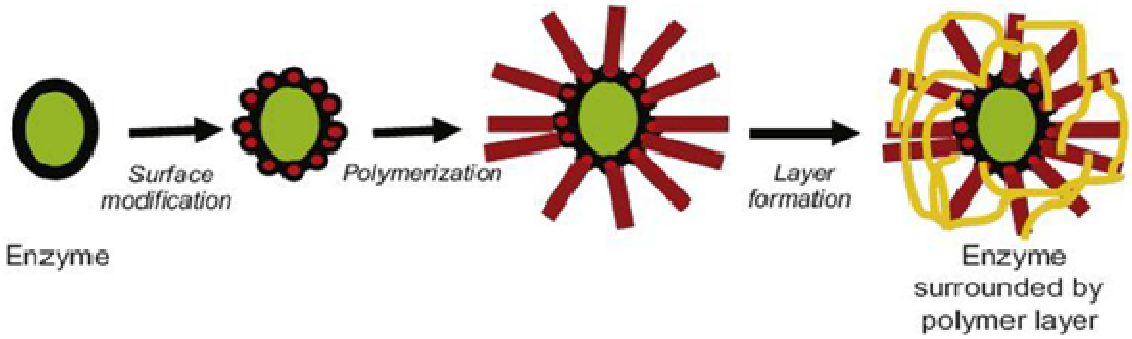

Figure 5. Synthesis steps of single enzyme nanoparticles.

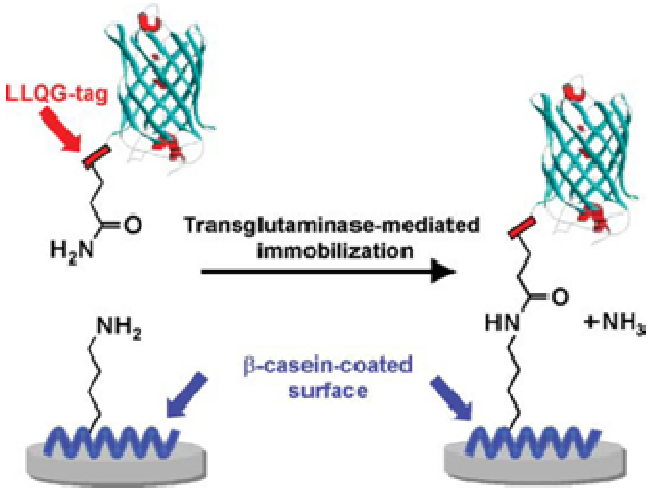

Figure 6. Covalent and site-specific immobilization of proteins onto a casein-coated surface through MTG-mediated cross linking reaction.

surface. In another case of phosphopantetheinyl transferase Sfp mediating site-specific covalent immobilization of recombinant proteins, it was shown that the Sfp catalyzes immobilization of a model acyl carrier protein (ACP) onto CoA-derivatized PEGA resin beads through specific covalent bond formation, using mass spectrometry (Wong et al., 2008). Luciferase (Luc) and glutathione-S-transferase (GST) ybbR-fusion proteins were similarly immobilized onto PEGA resin retaining high levels of enzyme activity. Wong et al. (2008) have also applied this strategy to the immobilization of the ACP, as well as ybbR-Luc, -GST and -thioredoxin fusion proteins, on hydrogel microarray slides. Overall, the Sfp-catalyzed surface ligation is mild, quantitative and rapid, occurring in a single step without prior chemical modification of the target protein. However, further improvements must be achieved, especially in terms of immobilization yields, if the technology will be used in the industrial biocatalysis.

Click-chemistry protocol has attracted great attention in the past several years, and it has been applied in organic synthesis, medicinal chemistry, polymer and material sciences, bioconjugate and peptide chemistry (Lewis et al., 2002). The required process characteristics include simple reaction conditions (ideally, the process should be insensitive to oxygen and water), readily available starting materials and reagents, the use of no solvent or a solvent that is benign (such as water) or easily removed and simple product isolation. A simple and versatile method using “click chemistry” has been developed to prepare the functional enzyme-gold nanoparticle conjugates (Brennan et al., 2006). In a copper-catalyzed 1, 2, 3-triazole cycloaddition, an acetylene-functionalized Thermomyces lanuginosus lipase has been attached to azide-functionalized water-soluble gold nanoparticles under retention of enzymatic activity. The coupling chemistry used here is very general and independent of the chemical nature of the nanoscopic subunits (Figure 6).

Click chemistry technology using Huisgen [2+3] cycloaddition reaction has also been used to construct a novel lipase–BSA heterodimer, in which the latter protein acts as a foot enabling the anchoring of the enzyme onto the surface for single enzyme studies (Hatzakis et al., 2006). The resulting TLL–BSA hetero-dimer was shown to possess a higher activity than TLL, which is tentatively attributed to the ‘‘interfacial activation’’ of the lipase caused by the presence of the hydrophobic BSA. More over, all studied dimers exhibited good activity and stability.

Multi-step immobilization

Multi-step immobilization is one of the technologies to enhance enzyme immobilization, especially covalent immobilization. The intermolecular reaction between functional group on support surface with that of enzyme, such as epoxy groups and soluble enzymes, is extremely slow (van Langen et al., 2002). To solve this problem, Fernandez-Lafuente (???) and his co-workers designed ‘‘tailor-made’’ heterofunctional epoxy supports and immobilized enzymes via a two-step process (Mateo et al., 2007). This process included:

(i) An initial physical or chemical intermolecular

interaction of the enzyme surface with the new functional groups introduced on the support surface and
(ii) A subsequent intense intramolecular multipoint covalent reaction between the nucleophiles of the already immobilized enzyme and the epoxy groups of the supports.

Even though it may not be current to immobilize many native proteins, the immobilization protocol with the ‘‘tailor-made’’ heterofunctional epoxy supports was a powerful tool to get a site-directed rigidification of proteins and prepare high-stability immobilized enzyme for Industrial biotechnology. For the differences between primary structures of many native proteins, the functional supports used in the above method may not be able to immobilize many native enzymes.

In the future, information derived from protein sequences, 3D-structures and reaction mechanism should be further combined with the properties of carriers (functional groups, hydrophobicity and magnetic properties) and physical/chemical methods in order to produce the immobilized enzyme with even more stability and higher catalytic activity. In addition, with the growing attention paid to the cascade enzymatic reaction (Dalal et al., 2007; Schoevaart et al., 2000) and i*n vitro* synthetic biology (Forster and Church, 2007; Gore and van Oudenaarden, 2009), multi-enzyme immobilization will be one of next goals.

ACKNOWLEDGEMENTS

This work was financially supported by the National Natural Science Foundation of China (20906016), Major Science and Technology Project of Zhejiang Province (2007C01004-2), Technology Research and Development Program of Hangzhou (20090331N03) and Special Foundation for New Researcher in Hangzhou Normal University (YS05203141).

REFERENCES

Albayrak N, Yang ST (2002). Immobilization of Aspergillus oryzae beta-galactosidase on tosylated cotton cloth. Enzyme Microb. Technol. 31(4): 371-383.

Bayramoglu G, Kiralp S, Yilmaz M, Toppare L, Arica MY (2008). Covalent immobilization of chloroperoxidase onto magnetic beads: Catalytic properties and stability. Biochem. Eng. J. 38(2): 180-188.

Bevan MW, Franssen MCR (2006). Investing in green and white biotech. Nat. Biotechnol. 24(7): 765-767.

Blanco RM, Terreros P, Munoz N, Serra E (2007). Ethanol improves lipase immobilization on a hydrophobic support. J. Mol. Catal. B Enzym. 47(1-2): 13-20.

Boller T, Meier C, Menzler S (2002). Eupergit oxirane acrylic beads: How to make enzymes fit for biocatalysis. Org. Process Res. Dev. 6(4): 509-519.

Bornscheuer UT (2003). Immobilizing enzymes: How to create more suitable biocatalysts. Angew. Chem. Int. Edit. 42(29): 3336-3337.

Brennan JL, Hatzakis NS, Tshikhudo TR, Dirvianskyte N, Razumas V, Patkar S, Vind J, Svendsen A, Nolte RJM, Rowan AE (2006). Bionanoconjugation via click chemistry: The creation of functional hybrids of lipases and gold nanoparticles. Bioconjug. Chem. 17(6): 1373-1375.

Buchholz K (1979). Non uniform enzyme distribution inside carriers. Biotechnol. Lett. 1(11): 451-456.

Burton SG, Cowan DA, Woodley JM (2002). The search for the ideal biocatalyst. Nat. Biotechnol. 20(1): 37-45.

Cao L, Schmid RD (2005). Carrier-bound Immobilized Enzymes: Principles, Application and Design. WILEY-VCH Verlag GmbH & Co. Weinheim.

Cao LQ (2005). Immobilised enzymes: science or art? Curr. Opin. Chem. Biol. 9: 217-226.

Cao LQ, Van Langen L, Sheldon RA (2003). Immobilised enzymes: carrier-bound or carrier-free? Curr. Opin. Biotechnol. 14(4): 387-394.

Cao LQ, van Rantwijk F, Sheldon RA (2000). Cross-linked enzyme aggregates: A simple and effective method for the immobilization of penicillin acylase. Org. Lett. 2(10): 1361-1364.

Chen B, Miller EM, Miller L, Maikner JJ, Gross RA (2007a). Effects of macroporous resin size on Candida antarctica lipase B adsorption, fraction of active molecules, and catalytic activity for polyester synthesis. Langmuir, 23: 1381-1387.

Chen B, Miller ME, Gross RA (2007b). Effects of porous polystyrene resin parameters on Candida antarctica lipase B adsorption, distribution, and polyester synthesis activity. Langmuir, 23(11): 6467-6474.

Chen RJ, Zhang YG, Wang DW, Dai HJ (2001). Noncovalent sidewall functionalization of single-walled carbon nanotubes for protein immobilization. J. Am. Chem. Soc. 123(16): 3838-3839.

Cheung MS, Thirumalai D (2006). Nanopore-protein interactions dramatically alter stability and yield of the native state in restricted spaces. J. Mol. Biol. 357: 632-643.

Chong ASM, Zhao XS (2004). Design of large-pore mesoporous materials for immobilization of penicillin G acylase biocatalyst. Catal. Today, 93-95: 293-299.

Dalal S, Kapoor M, Gupta MN (2007). Preparation and characterization of combi-CLEAs catalyzing multiple non-cascade reactions. J. Mol. Catal. B Enzym. 44(3-4): 128-132.

Dyal A, Loos K, Noto M, Chang SW, Spagnoli C, Shafi K, Ulman A, Cowman M, Gross RA (2003). Activity of Candida rugosa lipase immobilized on gamma-Fe2O3 magnetic nanoparticles. J. Am. Chem. Soc. 125(7): 1684-1685.

Forster AC, Church GM (2007). Synthetic biology projects in vitro. Genome Res. 17(1): 1-6.

Gemeiner P (1992). Materials for enzyme engineering. In Enzyme Engineering-Immobilised Biosystems. England: Ellis Horwood Limited, pp. 13-119.

Gore J, van Oudenaarden A (2009). Synthetic biology: The yin and yang of nature. Nature, 457(7227): 271-272.

Gutowski KE, Broker GA, Willauer HD, Huddleston JG, Swatloski RP, Holbrey JD, Rogers RD (2003). Controlling the aqueous miscibility of ionic liquids: Aqueous biphasic systems of water-miscible ionic liquids and water-structuring salts for recycle, metathesis, and separations. J. Am. Chem. Soc. 125(22): 6632-6633.

Gyorgy E, Sima F, Mihailescu IN, Smausz T, Megyeri G, Kekesi R, Hopp B, Zdrentu L, Petrescu SM (2009). Immobilization of urease by laser techniques: Synthesis and application to urea biosensors. J. Biomed. Mater. Res. Part A, 89A(1): 186-191.

Hatzakis NS, Engelkamp H, Velonia K, Hofkens J, Christianen PCM, Svendsen A, Patkar SA, Vind J, Maan JC, Rowan AE (2006). Synthesis and single enzyme activity of a clicked lipase-BSA heterodimer. Chem. Commun. (19): 2012-2014.

Hegedus I, Nagy E (2009). Improvement of chymotrypsin enzyme stability as single enzyme nanoparticles. Chem. Eng. Sci. 64(5): 1053-1060.

Hideno A, Ogbonna JC, Aoyagi H, Tanaka H (2007). Acetylation of loofa (Luffa cylindrica) sponge as immobilization carrier for bioprocesses involving cellulase. J. Biosci. Bioeng. 103(4): 311-317.

Ho KM, Mao XP, Gu LQ, Li P (2008). Facile route to enzyme immobilization: Core-shell nanoenzyme particles consisting of well-defined poly(methyl methacrylate) cores and cellulase shells. Langmuir, 24(19): 11036-11042.

Isgrove FH, Williams RJH, Niven GW, Andrews AT (2001). Enzyme immobilization on nylon-optimization and the steps used to prevent

enzyme leakage from the support. Enzyme Microb. Technol. 28(2-3): 225-232.
Kim J, Grate JW (2003). Single-enzyme nanoparticles armored by a nanometer-scale organic/inorganic network. Nano Lett. 3(9): 1219-1222.
Kim J, Lee J, Na HB, Kim BC, Youn JK, Kwak JH, Moon K, Lee E, Park J, Dohnalkova A (2005). A magnetically separable, highly stable enzyme system based on nanocomposites of enzymes and magnetic nanoparticles shipped in hierarchically ordered, mesocellular, mesoporous silica. Small, 1(12): 1203-1207.
Kim MI, Kim J, Lee J, Jia H, Bin Na H, Youn JK, Kwak JH, Dohnalkova A, Grate JW, Wang P (2007). Crosslinked enzyme aggregates in hierarchically-ordered mesoporous silica: A simple and effective method for enzyme stabilization. Biotechnol. Bioeng. 96: 210-218.
Klibanov AM (1983). Immobilized enzymes and cells as practical catalysts. Science, 219(4585): 722-727.
Ku BS, Cha JH, Srinivasan A, Kwon SJ, Jeong JC, Sherman DH, Dordick JS (2006). Chip-Based Polyketide Biosynthesis and Functionalization. Biotechnol. Prog. 22: 1102-1107.
Kumar S, Nahar P (2007). Sunlight-induced covalent immobilization of proteins. Talanta, 71(3): 1438-1440.
Lalonde JJ, Govardhan C, Khalaf N, Martinez AG, Visuri K, Margolin AL (1995). Cross-linked crystals of Candida-rugosa lipase-highly efficient catalysts for the resolution of chiral esters. J. Am. Chem. Soc. 117(26): 6845-6852.
Lee MY, Srinivasan A, Ku B, Dordick JS (2003). Multienzyme catalysis in microfluidic biochips. Biotechnol. Bioeng. 83(1): 20-28.
Lei CH, Shin YS, Liu J, Ackerman EJ (2002). Entrapping enzyme in a functionalized nanoporous support. J. Am. Chem. Soc. 124(38): 11242-11243.
LeJeune KE, Russell AJ (1996). Covalent binding of a nerve agent hydrolyzing enzyme within polyurethane foams. Biotechnol. Bioeng. 51(4): 450-457.
Lewis WG, Green LG, Grynszpan F, Radic Z, Carlier PR, Taylor P, G FM, Sharpless KB (2002). Click Chemistry In Situ: Acetylcholinesterase as a Reaction Vessel for the Selective Assembly of a Femtomolar Inhibitor from an Array of Building Blocks. Angew. Chem. Int. Ed. 41(6): 1053-1058.
Li FY, Xing YJ, Ding X (2007). Immobilization of papain on cotton fabric by sol-gel method. Enzyme Microb. Technol. 40(7): 1692-1697.
Liu XQ, Guan YP, Shen R, Liu HZ (2005). Immobilization of lipase onto micron-size magnetic beads. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 822(1-2): 91-97.
Luckarift HR, Ku BS, Dordick JS, Spain JC (2007). Silica-immobilized enzymes for multi-step synthesis in microfluidic devices. Biotechnol. Bioeng. 98(3): 701-705.
Luckarift HR, Spain JC, Naik RR, Stone MO (2004). Enzyme immobilization in a biomimetic silica support. Nat. Biotechnol. 22(2): 211-213.
Mateo C, Grazu V, Palomo JM, Lopez-Gallego F, Fernandez-Lafuente R, Guisan JM (2007). Immobilization of enzymes on heterofunctional epoxy supports. Nat. Protoc. 2(5): 1022-1033.
Naqvi A, Nahar P (2004). Photochemical immobilization of proteins on microwave-synthesized photoreactive polymers. Anal. Biochem. 327(1): 68-73.
Noritomi H, Sasanuma A, Kato S, Nagahama K (2007). Catalytic properties of cross-linked enzyme crystals in organic media. Biochem. Eng. J. 33(3): 228-231.
Parmar A, Kumar H, Marwaha S, Kennedy JF (2000). Advances in enzymatic transformation of penicillins to 6-aminopenicillanic acid (6-APA). Biotechnol. Adv. 18(4): 289-301.
Quiocho FA, Richards FM (1964). Intermolecular cross linking of a protein in the crystalline state: carboxypeptidase-A. Proc. Natl. Acad. Sci. USA, 52(3): 833-839.
Rogers RD, Seddon KR (2003). Ionic liquids-Solvents of the future? Science, 302(5646): 792-793.
Rosales-Hernandez MC, Mendieta-Wejebe JE, Correa-Basurto J, Vazquez-Alcantara JI, Terres-Rojas E, Trujillo-Ferrara J (2007). Catalytic activity of acetylcholinesterase immobilized on mesoporous molecular sieves. Int. J. Biol. Macromol. 40(5): 444-448.
Roy JJ, Abraham TE (2004). Strategies in making cross-linked enzyme crystals. Chem. Rev. 104(9): 3705-3721.
Rumbau V, Marcilla R, Ochoteco E, Pomposo JA, Mecerreyes D (2006). Ionic liquid immobilized enzyme for biocatalytic synthesis of conducting polyaniline. Macromolecules, 39(25): 8547-8549.
Sadasivan S, Sukhorukov GB (2006). Fabrication of hollow multifunctional spheres containing MCM-41 nanoparticles and magnetite nanoparticles using layer-by-layer method. J. Colloid. Interface Sci. 304(2): 437-441.
Safavi A, Maleki N, Moradlou O, Sorouri M (2008). Direct electrochemistry of hemoglobin and its electrocatalytic effect based on its direct immobilization on carbon ionic liquid electrode. Electrochem. commun. 10(3): 420-423.
Schoevaart R, van Rantwijk F, Sheldon RA (2000). A four-step enzymatic cascade for the one-pot synthesis of non-natural carbohydrates from glycerol. J. Org. Chem. 65(21): 6940-6943.
Sheldon RA (2007). Enzyme immobilization: The quest for optimum performance. Adv. Synth. Catal. 349(8-9): 1289-1307.
Sheldon RA, Lau RM, Sorgedrager MJ, Van Rantwijk F, Seddon KR (2002). Biocatalysis in ionic liquids. Green Chem. 4(2): 147-151.
Soetan KO (2008). The dynamic and ubiquitous nature of biotechnology. Afr. J. Biotechnol. 7(16): 2768-2772.
Stclair NL, Navia MA (1992). Cross-linked enzyme crystals as robust biocatalysts J. Am. Chem. Soc. 114(18): 7314-7316.
Sun JM, Zhang H, Tian RJ, Ma D, Bao XH, Su DS, Zou HF (2006). Ultrafast enzyme immobilization over large-pore nanoscale mesoporous silica particles. Chem. Commun. (12): 1322-1324.
Szamocki R, Velichko A, Mucklich F, Reculusa S, Ravaine S, Neugebauer S, Schuhmann W, Hempelmann R, Kuhn A (2007). Improved enzyme immobilization for enhanced bioelectrocatalytic activity of porous electrodes. Electrochem. commun. 9(8): 2121-2127.
Tanaka Y, Tsuruda Y, Nishi M, Kamiya N, Goto M (2007). Exploring enzymatic catalysis at a solid surface: a case study with transglutaminase-mediated protein immobilization. Org. Biomol. Chem. 5(11): 1764-1770.
Van Langen LM, Janssen MHA, Oosthoek NHP, Pereira SRM, Svedas VK, van Rantwijk F, Sheldon RA (2002). Active site titration as a tool for the evaluation of immobilization procedures of penicillin acylase. Biotechnol. Bioeng. 79: 224-228.
Van Rantwijk F, Lau RM, Sheldon RA (2003). Biocatalytic transformations in ionic liquids. Trends Biotechnol. 21(3): 131-138.
Wan LS, Ke BB, Xu ZK (2008). Electrospun nanofibrous membranes filled with carbon nanotubes for redox enzyme immobilization. Enzyme Microb. Technol. 42(4): 332-339.
Wang A, Wang H, Zhu S, Zhou C, Du Z, Shen S (2008a). An efficient immobilizing technique of penicillin acylase with combining mesocellular silica foams support and p-benzoquinone cross linker. Bioprocess Biosyst. Eng. 31(5): 509-517.
Wang AM, Liu MQ, Wang H, Zhou C, Du ZQ, Zhu SM, Shen SB, Ouyang PK (2008b). Improving enzyme immobilization in mesocellular siliceous foams by microwave irradiation. J. Biosci. Bioeng. 106(3): 286-291.
Wang AM, Wang H, Zhou C, Du ZQ, Zhu SM, Shen SB (2008c). Ag-induced efficient immobilization of papain on silica spheres. Chin. J. Chem. Eng. 16(4): 612-619.
Wang AM, Zhou C, Liu MQ, Du ZQ, Zhu SM, Shen SB, Ouyang PK (2009a). Enhancement of microwave-assisted covalent immobilization of penicillin acylase using macromolecular crowding and glycine quenching. J. Biosci. Bioeng. 107: 219-224.
Wang AM, Zhou C, Wang H, Shen SB, Xue JY, Ouyang PK (2007). Covalent Assembly of Penicillin Acylase in Mesoporous Silica Based on Macromolecular Crowding Theory. Chin. J. Chem. Eng. 15(6): 788-790.
Wang P (2006). Nanoscale biocatalyst systems. Curr. Opin. Biotechnol. 17(6): 574-579.
Wang Q, Li CX, Fan XR, Wang P, Cui L. (2008d). Immobilization of catalase on cotton fabric oxidized by sodium periodate. Biocatal. Biotransformation, 26(5): 437-443.
Wang ZG, Wan LS, Liu ZM, Huang XJ, Xu ZK. (2009b). Enzyme immobilization on electrospun polymer nanofibers: An overview. J. Mol. Catal. B Enzyme, 56(4): 189-195.
Wong LS, Thirlway J, Micklefield J (2008). Direct site-selective covalent protein immobilization catalyzed by a phosphopantetheinyl trans-

ferase. J. Am. Chem. Soc. 130(37): 12456-12464.
Yan M, Ge J, Liu Z, Ouyang PK (2006). Encapsulation of single enzyme in nanogel with enhanced biocatalytic activity and stability. J. Am. Chem. Soc. 128(34): 11008-11009.
Zhou J (2009). Immobilization of alliinase and its application: Flowinjection enzymatic analysis for alliin. Afr. J. Biotechnol. 8(7): 1337-1342.