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Recent progress in lipase immobilization

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ABSTRACT

Lipases are versatile biocatalysts in research laboratories and various industries. Immobilized lipases have attracted tremendous attention due to their facile separation from the reaction system, improved stability towards harsh reaction conditions and recycled use. The different methods and carriers for lipase immobilization in progress are the topic of this review.

KEYWORDS

Lipase; Adsorption;
Covalent binding; Cross-linking;
Entrapment;
Cell surface display.

INTRODUCTION

Lipases (triacylglycerol acylhydrolase, E.C.3.1.1.3) constitute a versatile class of enzymes, which catalyze hydrolysis, esterification, interesterification, trans-esterification reactions. The multifaceted properties make lipases find applications in detergent, food, bioremediation, chiral resolution of pharmaceuticals, synthesis of fine chemicals, cosmetics and perfumery[1]. Although lipases can be sourced from various animals, plants and microorganisms, all the lipases belong to α/β hydrolase superfamily, which composed of a central, parallel or mixed β sheet surrounded by α helices[2]. Lipases with different origin do not share high homolog in the protein sequences. However, the activity of lipase depends on a catalytic triad composed by Ser, His and Asp residues, among which serine residue is included in the conserved pentapeptide Gly/Ala-Xaa-Ser-Xaa-Gly. There is a region homologous to the oxyanion hole region (-His-Gly-) of the *Pseudomonas glumae* lipase located 60-108 aa upstream of the active site in the lipases possessing the active-site consensus sequence[3]. Some lipases have a conserved signal peptide SS or SS+P located 10-40 aa upstream of oxyanion hole region (-His-Gly-)[4]. Except from three conserved sequences, various lipases with different origin have low similarities in primary sequences. Furthermore, most lipases have a unique property called interface activation. When lipases contact with the oil/water interface, the lid covering the active site moves and exposes hydrophobic pocket, in which catalysis occurs[5]. The phenomenon has a direct impact on lipase immobilization.

Until now, screening, optimization of fermentation conditions, purification and characterization, cloning and expression, molecular engineering, immobilization and application of lipase have been described in numerous publications[6-16]. Immobilized lipases have attracted tremendous attention due to their facile separation from the reaction system, improved stability towards harsh reaction conditions such as extreme pH, high temperature or the presence of organic solvents and recycled use. Generally, non-covalent adsorption and deposition, ionic interaction, covalent binding, cross-linking,

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entrapment and cell surface display are the frequently used techniques for lipase immobilization. Pros and cons of these methods are summarized in TABLE 1. The present review focuses on the recent progress in lipase immobilization.

TABLE 1 : Pros and cons of six methods for lipase immobilization

Methods	Benefits	Drawbacks
Non-covalent adsorption and deposition	Little loss of activity, simple method	Easy leakage of enzyme from carriers
Ionic interaction	High recovery of enzyme, easy preparation	Dependent on pH and ionic strength
Covalent binding	Tight binding of enzyme, good stability	High loss of activity
Cross-linking	Strong attachment of enzyme	Low recovery of activity
Entrapment	High recovery of activity	Only for immobilizing enzyme using small molecular as substrate
Cell surface display	Production of biocatalysts without the extraction and purification of enzymes, a useful tool for the engineering of lipase	Overwhelming numbers of false positives, a complicated method

NON-COVALENT ADSORPTION AND DEPOSITION

Non-covalent adsorption and deposition are simple methods for lipase immobilization. Van der Waals force, hydrophobic interaction and hydrogen bond play an important role in the technique. Inorganic supports such as silica gel, Celite, porous glass, hydroxylapatite and organic supports such as cellulose, porous acrylic resins and ceramic are generally used as carriers. The binding capacity of organic supports is usually higher than that of inorganic supports, and thus organic supports attract more interest among researchers. Some successful examples are showed in TABLE 2[17-29]. According to Yeþiloðlu *et al.*, lipase from *Candida rugosa* (CRL) was immobilized onto Al_2O_3. By immobilization, the lipase witnessed shifts in optimum pH from 7 to 8.5 and optimum temperature from 35 to 40°C, as compared with free enzymes[17]. Sun *et al.* were succeeded in the immobilized of *Candida antarctica* lipase B (CALB) on the macroporous resin by physical adsorption. The immobilized lipase exhibited a similar stability to Novozyme 435 in the synthesis of ethyl lactate[18]. With the efforts of katiyar *et al.*, a novel material called MCM-41 as a support for immobilization of *Candida rugosa* lipase by the physical adsorption technique. The immobilized lipase was employed as biocatalyst for biodiesel preparation with high conversion[19]. An eco-friendly support called poly [bis (*p*-methyl phenoxy)] phosphazene was tried for lipase immobilization by Cabrera- Padilla *et al*. The test for operational stability indicated the immobilized lipase retained 50% of original activity after 12 cycles of reuse[20]. In recent years, there was a rise in the study on the enzyme binding to nanomaterials including nanoparticles, nanotube and nanofiber. Generally speaking, enzyme molecules have a size of several nanometers, which make it possible to benefit from the nanometer-scale manipulations[21]. Mesoporous silica, one kind of nanoparticles, were frequently applied to immobilize lipase due to its uniform pore diameters (2-40 nm), very high surface areas (300-1500 m^2/g) and pore volume (*ca.* 1 ml/g)[22]. Gustafsson *et al.* synthesized three kinds of mesoporous silica, all of which had 9 nm pores but varied particle size (1000 nm, 300 nm and 40 nm). The prepared silica was used as carriers for immobilization of *Mucor miehei* and *Rhizopus oryzae* lipases, among which the 300 nm particles was the most suitable supports for both lipases in term of specific activity[23]. In a study by Du *et al*., lipase from *Pseudomonas cepacia* was immobilized onto nanoporous gold (NPG), a new kind of nanoporous material with tunable porosity and excellent biocompatibility. Lipase loading and catalytic activity of the immobilized lipase were closely dependent on the pore size of NPG and adsorption time. In addition, lipase-NPG complex with a pore size of 35 nm have exhibited good reusability without loss of activity after ten recycles[24]. Carbon nanotubes are one of one dimensional nanomaterials. Shah *et al.* reported the immobilization of *Candida rugosa* lipase (CRL) on multi-

walled carbon nanotubes by adsorption. The immobilized biocatalyst actually gave rise to considerable increase of transesterification activity and enantioselectivity in non-aqueous media[25]. Electrospun nanofibers represent an extreme example of one dimensional nanomaterials because fibers can be unlimited long[21]. Electrospun nanofibers also have attracted much attention as carriers for enzyme immobilization. Wang *et al.* investigated the feasibility of Poly[bis(*p*-methylphenoxy)] phosphazene nanofiber membrane for the immobilization of *Candida rugosa* lipase by physical adsorption due to biocompatibility, high surface/volume ratio, and large porosity. The immobilized lipase showed the adsorption capacity (20.4 ± 2.7 mg/g) and activity retention (63.7%)[26]. Immobilization of *Pseudomonas cepacia* lipase onto electrospun polyacrylonitrile fibers through physical adsorption was achieved by Sakai *et al.* The immobilized lipase retained 80% of activity in the first run after 10 reuse for conversion of (S)-glycidol with vinyl n-butyrate to glycidyl n-butyrate in isooctane[27]. Recently, lipases have been immobilized using a smart technique, by which enzyme molecules were attached to functionalized magnetically nanoparticles via hydrophobic interaction. Usually, the very small particles (micron- or nano-size) were difficult to separation by filtration and centrifugation. However, lipase bound to functionalized magnetically nanoparticles can be separated by magnetic field. *Pseudomonas fluorescens* lipase (PFL) was immobilized onto the magnetic Fe_3O_4 nanoparticles via hydrophobic interaction. The immobilized AKL was successfully used for resolution of 2-octanol, with the highest enantioselectivity (E=71.5±2.2) under the optimum conditions[28]. *Candida rugosa* lipase was immobilized onto magnetite nanoparticles coated with alkyl silanes of different alkyl chain lengths through hydrophobic interaction. The lipase immobilized on C18-Fe_3O_4 retained 65% of its original activity after 7 times recycle use[29].

TABLE 2 : Examples of non-covalent adsorption and deposition for lipase immobilization

Lipase origin	Carrier	References
Candida rugosa lipase	Al_2O_3	[17]
Candida antarctica lipase B	Macroporous resin	[18]
Candida rugosa lipase	MCM-41	[19]
Candida rugosa lipase	Poly(3-hydroxybutyrate-co-hydroxyvalerate)	[20]
Mucor miehei and Rhizopus oryzae lipase	Mesoporous silica	[23]
Pseudomonas cepacia lipase	Nanoporous gold	[24]
Candida rugosa lipase	Carbon nanotube	[25]
Candida rugosa lipase	Poly[bis(p-methylphenoxy)]phosphazene	[26]
Pseudomonas cepacia lipase	Polyacrylonitrile fibers	[27]
Pseudomonas fluorescens lipase	Magnetic nanoparticles	[28]
Candida rugosa lipase	Magnetite nanoparticles	[29]

IONIC INTERACTION

For most of enzymes, their surface may be positively or negatively charged, and thus they have ionic interaction with supports such as ion exchanger polymers. Ion exchanger polymers include DEAE-Sephadex, Amberlite IRA-93, DEAE-cellulose, CM-cellulose, Amberlite CG-50, Dowex-50 etc. Fuentes *et al.* successfully prepared novel tailor-made cationic exchange resins by covalently binding aspartic-dextran polymers (e.g. MW 15 000-20 000) to porous supports (aminated agarose and Sepabeads). Very high activity recovery and immobilization rates were achieved, with the immobilization of *Candida antarctica* B lipase onto these supports[30]. Agarose gels coated with a dense layer of polyethylenimine (anion exchanger) were used to adsorb *Candida antarctica* lipase. The resultant preparations were applied for the enantioselective hydrolysis of *R,S*-mandelic acid methyl ester. Interestingly, the activity and the enantioselectivity were dependent on the pH value and temperature in the immobilization process[31]. *Rhizomucor miehei* lipase (RML) was immobilized in the presence of sucrose laurate on different anion exchangers by multipoint anionic exchange through the region with the highest density of negative charges. RML immobilization based on ionic interaction was superior to that based on covalent binding in term of activity for hydrolysis of fish oils. In addition, the immobilized li-

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pases are fairly stable against heat and organic co-solvents[32]. Except ion exchanger polymers, Fe_3O_4 nanoparticles also used to bind lipase via ionic interaction. Mukherjee *et al.* described *Candida rugosa* lipase adsorbed on Fe_3O_4 nanoparticles coated with PEI via electrostatic interactions. The immobilized lipase exhibited 110 folds higher transesterification activity in low-water media[33].

COVALENT BINDING

Lipase can be bound to carriers by covalent coupling. The coupled amino acids in lipases can not play a role in the active sites of enzymes. Otherwise it would result in the complete loss of enzyme activity. There are some coupling reactions between lipases and carriers, which depend on the functional groups of supports and the side chain groups of enzyme. In normal case, carriers are not capable of reacting with enzyme directly, and thus the activation of supports is necessary, which makes them bearing functional groups. TABLE 3 describes some examples of covalent coupling for lipase immobilization[34-41]. In order to improve the performance of the enzyme, *Candida rugosa* lipase (Lipase AY-30) was immobilized on Poly (gamma-glutamic acid) by covalent binding, and then immobilization parameters were optimized by response surface methodology[34]. Three different covalent immobilization methods were applied for the immobilization of *Candida rugosa* lipase on Eupergit® C by Knezevic *et al.* Coupling *Candida rugosa* lipase via its carbohydrate moiety modified by periodate oxidation gave rise to the highest activity retention of 43.3% among the three methods. The immobilized lipase was 18-fold more stable than free lipase[35]. Pahujani *et al* were succeeded in the immobilization of an extracellular alkaline lipase of a thermo tolerant *Bacillus coagulans* BTS-3 onto glutaraldehyde activated Nylon-6 by covalent couping. The immobilized lipase only lost 12% of original activity at 55°C for 2h and also retained 85% of its original activity after eight cycles of hydrolysis of p-NPP[36]. Free hydroxyl groups in insoluble yeast beta-glucan were converted into activated epoxy groups using epichlorohydrin. The immobilization of *Candida rugosa* lipase onto the epoxy-activated IYG was carried out. There remained 51.05% of original activity after six repeated esterification cycles[37]. Lipases from *Candida rugosa* and porcine pancreas were covalently bound using glutaraldehyde as binding agent and silica amino as support. In continuous reactors, no significant loss of activity was detected along the period of 17 days for both immobilized PPL used in aqueous medium at 32 °C and immobilized CRL in organic medium at 40°C[38]. A novel support to bind enzyme by covalent coupling is stimulus-responsive polymer. Changes in temperature, pH and ionic strength result in the dramatic conformation change of these polymers[39]. For example, temperature-responsive polymers have a critical solution temperature (LCST). Above LCST, the polymer is insoluble, while below LCST the polymer becomes dissolved in the solution. The property makes it a useful support in enzyme immobilization. When enzymes bind to such polymer, below LCST the immobilized enzyme is soluble, thereby decreasing the diffusion resistance. Above LCST the immobilized enzyme is insoluble, thus facilitating its recovery. Matsukata *et al.* have synthesized poly (*N*-isopropylacryl-amide)[poly(IPAAm)] cooligomer with *N*, *N*-dimethylacrylamide (DMAAm), which exhibits a LCST at 37°C, and then lipase was covalently attached to the polymer. The immobilized lipase retained its native enzymatic activity and was readily separated from the aqueous reaction system induced by a small temperature change[40]. *Candida rugosa* lipase was covalently bound to a pH-sensitive support. After eight reuses, the immobilized lipase retained 46% of original activity and its enantioselectivity remained unchanged[41].

CROSS-LINKING

For carrier bound insoluble enzymes, the carriers increase the cost of enzyme immobilization and diffusion hindrance. A carrier-free immobilization is a robust method for biocatalysis. Enzyme molecules can be linked by bi- or multi-functional crosslinking agents such as glutaraldehyde. Two kinds of preparation, cross-linked enzyme crystals (CLECs) and Cross-linked enzyme aggregates (CLEAs) have been applied to the field of biocatalysis. Cross-linked enzyme crystals (CLECs) were obtained by adding a crossing agent to cross-link enzyme crystals, which were formed under optimum condition. Lipase from *Burkholderia cepacia* was

TABLE 3 : Examples of covalent binding for lipase immobilization

Lipase origin	Carrier	References
Candida rugosa lipase	Poly(gamma-glutamic acid)	[34]
Candida rugosa lipase	Eupergit® C	[35]
Bacillus coagulans lipase	Nylon-6	[36]
Candida rugosa lipase.	Insoluble yeast beta-glucan	[37]
Candida rugosa lipase/ Porcine pancreatic lipase	Silica	[38]
Crystallized lipase	Poly(N-isopropylacrylamide)	[40]
Candida rugosa lipase	A terpolymer of methacrylic acid, acrylamide and maleic anhydride	[41]

crystallized using ammonium sulfate and then cross-linked with glutaraldehyde. CLECs lipase had improved thermal and reuse stability[42]. A crystallized enzyme of *Candida antarctica* lipase B was prepared for enantioselective esterification of racemic 1-phenyl ethanol with vinyl acetate in supercritical carbon dioxide[43]. However, enzymes require a high purity in order to obtain crystals, which is a laborious process. Consequently, CLEA is an alternative to CLEC due to its simpler and less expensive preparation. These CLECs are prepared by adding a precipitant such as ammonium sulphate and then these aggregates are crosslinked by crossing agents. Pan *et al.* prepared crosslinked enzyme coaggregates of *Serratia marcescens* lipase, with polyethyleneimine as coprecipitant and glutaraldehyde as crosslinking reagent. The immobilized lipase exhibited excellent operational stability for asymmetric hydrolysis of *trans*-3-(42 -methoxyphenyl) glycidic acid methyl ester, without no significant activity loss after 10 cycles of repeated use[44]. *Pseudomonas* sp. Lipase was immobilized via cross-linked enzyme aggregates (CLEAs), using acetone as the optimal precipitant. The immobilized lipase not only exhibited excellent enantioselectivity (*E*-value > 100), but had higher activity and thermal stability as compare with the free lipase for the kinetic resolution of *N*-(2-ethyl-6-methylphenyl) alanine[45].

ENTRAPMENT

In the case of entrapment, monomers and enzyme solution are mixed, and then monomers are polymerlized, whereby enzyme molecules are entrapped in polymers. The technique also is applied for entrapping whole cell. Sol-gel is a kind of silica based material used for enzyme entrapment. In the sol-gel methodology, organic silane derivatives such as $Si(OCH_3)_4$ and $RSi(OCH_3)_3$ were added into enzyme solution, which are hydrolyzed to produce a sol of colloidal silicon dioxide particles by a catalyst. The sols undergo condensation to form a gel[46]. Additives such as isopropanol, polyvinyl alcohol, cyclodextrins, ionic liquids or surfactants increase the efficiency of sol-gel methodology, which is industrially viable owing to the low price of sol-gel entrapment, the excellent performance of immobilized enzyme, and the recycle ability for biocatalysis[47]. According to Yang *et al*., different silanizing agents including vinyltrimethoxy silane, octyl-trimethoxy silane, gamma-(methacryloxypropyl)- trimethoxy silane and tetraethoxysilane were selected as the precursors to produce gel for encapsulation of lipase from *Arthrobacter sp*.. Among all of silanizing agents, etraethoxysilane was the most suitable precursor, with the highest activity in both the hydrolysis of p-nitrophenyl palmitate and the asymmetric acylation of 4-hydroxy-3-methyl-2-(2-propenyl)-2-cyclopenten-1-one[48]. Lipase from *Candida antarctica* B was immobilized by sol-gel entrapment. Immobilization gave rise to an increase of stability in several organic solvents and excellent operational stability[49]. *Candida rugosa* lipase was encapsulated, using tetraethoxysilane and octyltriethoxysilane as silane precursors in the presence of β-cyclodextrin-based polymer. The β-cyclodextrin-based encapsulated lipases had higher conversion and enantioselectivity than covalently immobilized lipase for the enantioselective hydrolysis of racemic Naproxen methyl ester[50]. Except that polymers based on inorganic material, organic polymers such as temperature-responsive polymers are also applied to entrap enzymes. *Candida antarctica* lipase B (CALB) was immobilized within micron-sized thermosensitive p-NIPAM hydrogel particles. For the immobilization of the en-

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zyme, p-NIPAM particles and CALB were mixed at 25 °C, followed by heating the mixture to 50 °C overnight. Another solvent exchange was performed by the exchange of isopropanol against n-hexane. Specific activity of CALB in *n*-hexane increased after immobilization[51]. Another organic polymers to entrap is electrospinning fibers. Electrospinning constitutes a simple and versatile way to fabricate nanofibrous supports, which show many advantages for their high porosity and interconnectivity as compared with other nanostructured supports (e.g. mesoporous silica, nanoparticles). The encapsulation of enzymes in the nanofibers can be achieved by direct co-electrospinning of enzymes and other components (organic or inorganic materials)[52]. Sakai *et al.* entrapped lipase in electrospun poly(vinyl alcohol) fibers of approximately 1 μm in diameter for the synthesis of glycidyl *n*-butyrate with (*s*)-glycidol and vinyl *n*-butyrate as substrate. The initial transesterification rate of the immobilized lipase was 5.2-fold higher than that of free lipase[53].

DISPLAY LIPASE ON THE SURFACE OF CELLS

The display of enzyme on cell surfaces represents a novel immobilization technique. The method enables us to produce biocatalysts without the extraction and purification of enzymes, which helps to drive down the product cost and makes the enzymatic process economically viable. At first, the technology was hampered by the lack of a suitable anchoring motif[54]. In recent years, there are more and more successfully used anchoring motifs[55-64] (TABLE 4). Lee *et al.* used OprF, a major outer membrane protein of *Pseudomonas aeruginosa*, as an anchoring motif to display *Pseudomonas fluorescens* SIK W1 lipase on the surface of *Escherichia coli*. The E. coli whole cell catalyst was used for enantioselective resolution of racemic 1-phenylethanol in hexane, which gave rise to the enantiomeric excess of greater than 96% in 36 h of reaction[56]. A thermostable lipase (TliA) from *Pseudomonas fluorescens* was immobilized on the cell surface of a solvent-resistant bacterium, *Pseudomonas putida* GM730. The Whole cells exhibited potential for hydrolysis of olive oil, synthesis of triacylglycerol and chiral resolution[57]. *Yarrowia lipolytica* lipases Lip7 and Lip8 were attached to the cell surface of *Saccharomyces cerevisiae*, using small binding subunit Aga2 of a-agglutinin as an anchoring motif. The activities of surface-displayed Lip7 and Lip8 towards p-nitrophenyl caprylate were much higher than that using Flo1 as anchor protein in *Pichia pastoris*[61]. *Rhizomucor miehei* lipase (RML) variants were surface-displayed on *Pichia pastoris* by fusion to Flo1p as an anchor protein. The activities of four variants increased from 1.1- to 5-fold in an esterification reaction in heptane as compared with the activity of native lipase[62].

TABLE : 4 Display lipases on the surface of cells

Lipase	Host and anchoring motif	Reference
P. fluorescens SIK W1 lipase	*P. putida* KT2442 /OprF	[55]
P. fluorescens SIK W1 lipase	*E. coli*/OprF	[56]
TliA	*P. putida* GM730/INP	[57]
B. subtilis lip	*B. subtilis* /CWB_c	[58]
Lip A	*S. cerevisiae* /Pir4	[59]
Lip2	*S. cerevisiae* /Cwp2	[60]
Lip7 and Lip8	*S. cerevisiae* /Aga2 of a-agglutinin	[61]
RML variants	*P. pastoris*/Flo1p	[62]
CALB	*P. pastoris*/alpha-agglutinin and Flo1p	[63]
LipB52	*P. pastoris* KM71/ FLO	[64]

CONCLUDING REMARK

Currently, immobilized lipases are of special importance because they have found wide applications not only in the field of biotechnology, but also in pharmaceutical, environmental, food and biosensor industries. The technique and skill to prepare the immobilized lipase rendering an enzymatic process economically viable arouse considerable interests among researchers. Several novel type of carriers and methods have been tried to improve traditional lipase immobilization. New carriers include nanometer scale materials, magnetic particles, mesoporous support etc, while new methods are involved in cell surface display, sol-gel method, CLEC and CLEA, combinational use of immobilization methods[65-67]. More and more new methods and supports should be explored in order to enhance lipase

loading, activity and stability, thereby decreasing biocatalyst cost in various industries. It should be noted several new ways for immobilization have been applied to other enzyme. For instance, microwave irradiation technology was used to immobilize papain and penicillin acylase in the mesocellular siliceous foams (MCFs) to decrease immobilization time and increase enzyme loading[68,69]. Kumar and Nahar used photoimmobilization technology to bind horseradish peroxidase (HRP) andglucose oxidase (GOD) onto the photoreactive cellulose membrane by sunlight. In addition, sunlight exposure gave better immobilization results than 365 nm UV light[70]. Although lipase has its unique properties, fine-tuning of techniques for other enzymes to suit lipase will be fruitful in the future.

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