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Enhanced Activity of Immobilized or Chemically Modified Enzymes

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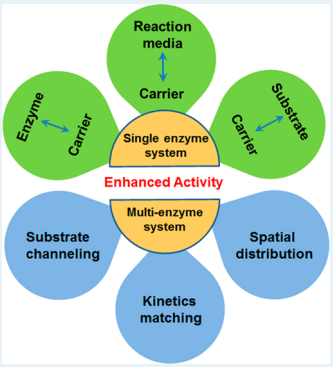
ABSTRACT: Re-engineering enzymes with high activities in the given environments different from the physiological one has been constantly pursued for application of enzymatic catalysis in industrial biocatalytic processes, pharmaceutical industry, biosensing, etc. Re-engineering enzyme catalysts by chemical approaches, including immobilization and chemical modification, represents a simple but effective route. The unusual phenomenon that immobilized or chemically modified enzymes display higher activities than native enzymes has been observed in both single- and multiple-enzyme systems. Recent achievements in enhancing enzymatic activities in both single-and multiple-enzyme systems by chemical approaches are summarized in this review. We propose that these enhanced enzymatic activities can be attributed to the well-designed specific interactions between immobilization carriers (or chemical modifiers) and enzymes, substrates, or reaction media. In addition to this mechanism, which is applicable for both single- and multiple-enzyme systems, other important factors responsible for enhanced activities of multiple-enzyme systems, including substrate channeling, kinetic matching, and an ordered spatial distribution of enzymes, are also discussed. Understanding the origin of enhanced activity in enzymatic catalysis may provide new insights and inspiration to design efficient enzyme catalysts for practical applications.



KEYWORDS: *immobilized enzyme, chemically modified enzyme, enzymatic catalysis, enhanced enzymatic activity, multienzyme system*

INTRODUCTION

Enzymatic catalysis has been extensively applied in as many fields as the synthesis of pharmaceuticals and fine chemicals, analysis of chemical and biological substances, food processing, biofuel production, etc. High activity and stability of enzymes are generally expected for enzymatic catalysis. The efforts toward engineering enzyme catalysts with high activity and stability can generally be divided into two groups: The first is protein engineering,[1] such as site-directed mutagenesis and direct evolution, and the second is a chemical approach, such as immobilization and chemical modification.[2] In addition, the combination of these two approaches is appealing for improving the catalytic properties of enzymes.[3]

Protein engineering offers a straightforward method to improve enzymatic activity, generally by changing the structure of the enzyme active site or the substrate and product channel by site-specific mutagenesis, fusion protein technologies, and directed evolution. The advancement of structural biology and computer modeling enables the quantification of interaction between amino acid residues at enzyme active site and substrates and thus helps the rational design of new enzymes and the screening of appropriate mutants for a given application. A series of achievements have been made in recent decades. For example, in 1988, Novozyme presented the world's first commercial genetically engineered lipase, Lipolase. Directed evolution led to a successful re-engineering of various oxygenases for biocatalysis in the early 2000s.[4] Nowadays, the de novo computational design can even enable the construction of novel enzymes capable of catalyzing unnaturally occurring reactions, such as the intermolecular Diels–Alder reaction.[5] Many cases of protein engineering have been well summarized in previous reviews.[6]

First reported in 1916[7] and commercialized in 1960s,[8] enzyme immobilization was developed as a chemical engineering approach to facilitate the recovery and reuse of enzyme catalysts. The high stability of an enzyme is often obtained via immobilization, which can also improve the process economy of industrial enzymatic catalysis. Over recent decades, continuous efforts in this category have yielded various immobilization strategies, such as immobilization on solid carriers, including bulk materials,[9] particles[10] and fibers (or tubes);[11] conjugation with polymers, such as polyethylene glycol (PEG)[12] and dextran;[13] encapsulation in nano- or microgels,[14] porous materials,[15] hollow, or yolk–shell structures;[16] and preparation of cross-linked enzyme crystals or aggregates.[17]Among these strategies, enzyme immobilization on solid surfaces through physical adsorption or through covalent links, constitutes one of the most common procedures. Generally for these immobilized enzymes, the multipoint attachment model can be used to describe the contribution of increased protein rigidity to enzyme activity and stability, in which the multiple covalent links between protein molecules and supports can significantly protect the protein configuration so as to maximally retain the enzymatic activity as well as stability.[18]

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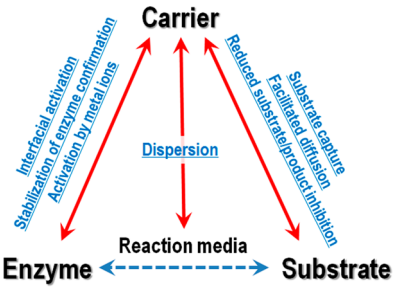
The immobilization of enzyme greatly enhances the properties of enzymes in terms of thermal stability, tolerance to extremely high pH and organic solvents, selectivity, and activity to meet the demands of practical uses.[19] Although in some specific cases, immobilized enzymes can appear to have higher activities than native enzymes under drastic conditions because of the enhanced stability, the apparent activity of conventional immobilized enzymes is usually lower than that of its native counterpart, mainly because of the hindered substrate accessing or unfavorable conformational transition of the enzyme within the matrix. In recent years, a growing number of examples have demonstrated that an immobilized or chemically modified enzyme can surprisingly display an enhanced apparent activity higher than that of a native enzyme in solution.[2a,19b,20] In this Review, we summarize these examples and extract the fundamental principles of the activity amplification of the immobilized or chemically modified enzymes. The activity amplification effects can be attributed to the well-designed specific interactions between the immobilization carriers (or chemical modifiers) and enzymes, substrates, or reaction media. For multienzyme catalysis, in addition to this general principle, the influences of other factors on overall enzymatic activities are also discussed, including substrate channeling, kinetic matching, and an ordered spatial distribution of enzymes.

SINGLE-ENZYME CATALYSIS

A typical enzymatic reaction generally consists of three steps: diffusion of substrate toward the active site, formation of the transition state that enables the catalysis, and release of product from the active site. Therefore, the enzymatic activity is influenced by the interactions between enzyme and substrates or products in the steps above. For immobilized or chemically modified enzymes, the chemical carriers or surroundings can interact with enzymes, substrates/products, and reaction media to improve the enzymatic catalysis and yield an enhanced apparent activity (Scheme 1). Different from many previous reviews focusing on the process description of immobilized enzymes,[2a,18e] we provide a molecular description based on the above-mentioned interactions to discuss why immobilized or chemically modified enzymes can have enhanced apparent activities compared with native enzymes. The discussion will be categorized into three aspects, as shown in Scheme 1, on the basis of interactions between carriers (or surroundings) and enzymes, carriers, and substrates and between carriers and reaction media.

Scheme 1. Interactions between the Well-Designed Chemical Carriers and Enzymes, Substrates, or Reaction Media Can Enhance the Enzymatic Activity by Influencing Enzyme Property and Mass Transport during the Catalytic Process



1. Enzyme Activation by the Interaction between Carriers (Or Surroundings) And Enzymes. *Interfacial Activation.* Enzymes with flexible configurations may change their conformations according to the surrounding microenvironments. In this case, an enhanced activity might be obtained via an optimized conformational transition upon the participation of chemically synthesized matrices. A frequently reported example in this category is the interfacial activation of lipase.[21] Most types of lipase have a peptide "lid" covering the active site. The lid shifts to an "open" conformation in hydrophobic environments. A lipase from *Rhizomucor miehei* (RmL), which has a lid (residues 83−94) located near the active site, shows a significant interfacial activation at the interface of water and lipid (Figure 1). The crystal structure[22] of RmL and molecular simulation[23] revealed that the lid covers the active site in water. Once meeting the hydrophobic solvent or hydrophobic interface, the lid rotates around two hinge regions, making the active site exposed to the hydrophobic phase.[24] The electrostatic interaction of Arg86 and Asp91 plays an important role in the displacement of the lid and stabilization of the open conformation.[23,25] Most of the lipase from other sources, including lipase from *Pseudomonas cepacia*, from *Candida rugosa*, from *Thermomyces lanuginosus*, and pancreatic lipase have the similar interfacial activation phenomenon.

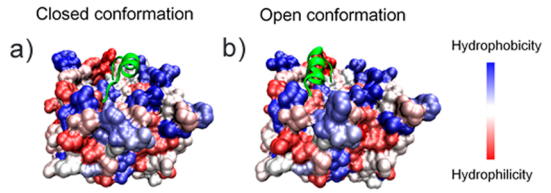


Figure 1. Illustration of the structure of lipase from RmL: (a) the closed conformation and (b) the open conformation. The green cartoon: the lid region (residues 83−94); the intensity of the blue or red indicates the hydrophobicity or hydrophilicity. The structure of RmL was from RCSB Protein Data Bank: 3TGL (closed) and 4GTL (open). The figure was generated with VMD (visual molecular dynamics).

The interfacial activation of lipase inspires the design of immobilized lipase with high enzymatic activity. Guisán and colleagues[26] first reported the hyperactivation of lipase via interfacial adsorption on hydrophobic supports. Because of the stabilization of its open form, lipase immobilized on the octyl-agarose gels displayed a 6−20-fold enhanced activity compared with the soluble one. In a very recent study, Fernandez-Lafuente and co-workers[27] further confirmed the involvement of the open form of lipase in this type of immobilization. Jin et al. immobilized lipase from *P. cepacia* (PCL) on siliceous mesocellular foams (MCFs) with different hydrophobicities.[28] Functional modifiers, including *N*-trimethoxysilylpropyl-*N*,*N*,*N*-trimethylammonium chloride (TMNCL), *n*-octyl triethoxysilane (C8), and phenethyltrimethoxysilane (Ph), were used to covalently graft onto the surface of MCF to control the hydrophobicity. The transesterification activity was greatly increased (maximal to 25 folds) with the increase in the surface hydrophobicity of the MCFs (Figure 2), due to the exposed active site of lipase on the hydrophobic surface, which was further confirmed by time-resolved fluorescence spectra.

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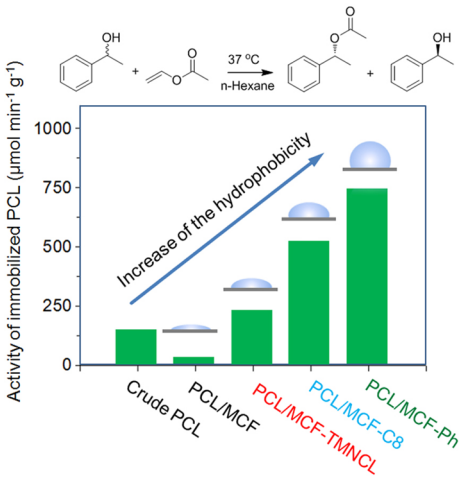

Figure 2. Specific activities of immobilized PCL on MCFs with different surface hydrophobicities. With an increase in the hydrophobicity (characterized by water contact angle), the specific activity of the immobilized lipase was dramatically enhanced. Reconstructed with permission from ref 28, Copyright (2011) American Chemical Society.

Examples of similar activation of immobilized lipase are also found by using other immobilization carriers, such as mesoporous silicas,[29] polymer nanogels,[30] and dendrimers.[31]

A similar activation effect from hydrophobic interaction has also been reported for cytochrome *c* (Cyt *c*) in the presence of cardiolipin,[32] SDS,[33] and other types of surfactants. For example, the presence of bovine heart cardiolipin led to a 95-fold enhancement of peroxidase-like activity of Cyt *c*.[32] This was attributed to the partial unfolding of Cyt *c* caused by the hydrophobic interaction. The partially unfolded configuration facilitated the access of H_2O_2 to the heme group of Cyt *c*. The immobilization of Cyt *c* on hydrophobic mesoporous silica materials[34] and the conjugation of Cyt *c* with a macromolecular surfactant Pluronic-F127[35] also significantly increased the activity of Cyt *c* by 1.7−5.7-fold, based on the same mechanism.

Interfacial activation has been widely used for the immobilization of enzymes such as lipase; however, most types of enzymes, including lipase, may undergo serious denaturing upon a very hydrophobic surface. Thus, a careful design of the immobilization carriers with balanced hydrophilic/hydrophobic property is required when preparing such immobilized or chemically modified enzymes.

Electrostatic Stabilization. Electrostatic interaction between enzymes and carriers can also affect the apparent enzymatic activity of immobilized or chemically modified enzymes. An acceleration of the enzymatic reaction of trypsin was observed when the enzyme was incorporated into water-soluble, self-assembled complexes made of poly(ethylene glycol)-*block*-poly(*α*,*β*-aspartic acid), PEG−PAA.[36] The trypsin−polymer complex showed a catalytic rate constant, k_{cat}, enhanced by 15-fold, but a similar K_m compared with free trypsin. The enhancement of enzymatic activity is mainly due to the stabilization of imidazolium ion (His residue at the active site) through the electrostatic interaction between PEG−PAA and trypsin.

Activation of enzymes by ionic liquids is particularly noteworthy. Itoh and co-workers studied the catalytic behaviors of ionic-liquid-coated lipase in organic solvents or in a series of ionic liquids and observed remarkable activations.[37] For instance, by coating with a well-designed ionic liquid additive, imidazolium PEG−alkyl sulfate, the lipase-catalyzed transesterification rate was greatly increased, by 10−1000-fold, compared with that of native lipase in diisopropyl ether.[38] The ionic liquid surroundings can not only enhance the catalytic activity but also alter the enatioselectivity, indicating that the ionic groups (e.g., imidazolium cations) have direct interactions with the active site of the enzyme to keep the enzyme in an active form.

Effect of Metal Ions. Activation of metalloenzymes such as laccase and carbonic anhydrase was observed when incorporated into $Cu_3(PO_4)_2{\cdot}3H_2O$ nanocrystals with flower-like structures (nanoflowers).[39] By adding Cu(II) ion to the phosphate buffered saline solution containing laccase at pH 7.4 and incubating the solution at 25 °C for 3 days, laccase can be incorporated into the precipitates made of protein and copper phosphate nanoflower. The enzymatic activity of the incorporated laccase was increased by 6-fold compared with that of free laccase in solution. The carbonic anhydrase in $Cu_3(PO_4)_2{\cdot}3H_2O$ nanoflowers displayed a 2.6-fold higher activity than that of free enzyme. Lipase without metal ions at its active site did not show any activation effect. Since laccase and carbonic anhydrase are metalloenzymes that contain copper and zinc ions, respectively, at their active sites, the authors proposed that the interaction of Cu^{2+} in crystals with amino acid residues of enzymes may cause the activation of enzymes. Such an activation effect of enzyme−inorganic hybrid nanoflowers has also been found in the horseradish peroxidase (HRP)−copper phosphate nanoflower (5-fold enhancement)[40] and *α*-amylase-$CaHPO_4$ hybrid nanoflowers (38-fold enhancement).[41] For all these reported enzyme−inorganic crystal nanoflowers, the enhanced activity can be found only in the cases of metalloenzymes precipitating with the appropriate metal ions. Because metal ions can have the effects of either activating or deactivating enzymes, depending on the properties of the enzymes and metal ions, it is necessary to choose the appropriate metal ions when preparing such enzyme nanoflowers.

2. Enhanced Activity by the Interaction between Carriers and Substrates/Products. *Enhanced Capture of Substrates.* Properly designed materials used in the immobilization or modification of enzymes can capture the substrates through hydrophobic or electrostatic interactions. The adsorption and desorption of substrates on supports is balanced dynamically, leading to a higher substrate concentration in the vicinity of the enzyme than that in the bulk solution. This enrichment of substrate, which is also known as the positive partition effect, apparently reduces the Michaelis constant, K_m, giving an increased apparent enzymatic activity. Goldstein et al.[42] studied the enzymatic kinetics behavior of trypsin covalently bounded with a water-insoluble polyanion, a copolymer of maleic acid and ethylene, and found that the Michaelis constant was decreased by ∼30 times compared with that of free trypsin. The activation of trypsin is attributed to the attraction of the positively charged substrate molecules by the negatively charged polyanions. Instead of covalent modification, polyelectrolytes can physically entrap oppositely charged enzymes by electrostatic attraction.[43] When lysozyme was entrapped into the core of polyion complex micelles (PIC micelles), the apparent enzymatic activity (using small-molecule substrate, *p*-nitrophenyl-penta-*N*-acetyl-*β*-chitopentaoside) was 2-fold higher than that of the free enzyme, accompanied by a decrease in the observed K_m. Moreover, the $1/K_m$ was linearly increased with the corona thickness of PIC micelles, indicating

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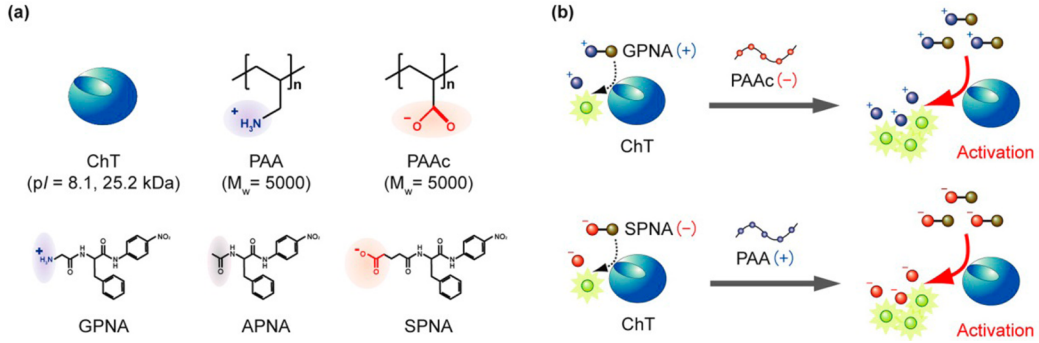

Figure 3. Hyperactivation of α-chymotrypsin (ChT) complexed with polyelectrolyte. (a) Enzyme ChT, cationic polymer PAA, anionic polymer PAAc, and three substrates with different charges. (b) ChT was activated in the presence of oppositely charged polyelectrolytes. Reprinted with permission from ref 44. Copyright (2014) American Chemical Society.

that the corona layer of PIC micelles can accumulate substrates, serving as a substrate "reservoir".[43a] A more detailed mechanism was demonstrated by the hyperactivation of α-chymotrypsin (ChT) in polymeric complexes (Figure 3).[44] The enzymatic activity of ChT toward cationic substrates was enhanced by 7-fold in the presence of anionic ploy (acrylic acid) (PAAc), while its activity toward anionic substrates, by 18-fold in the presence of cationic poly(allylamine) (PAA). However, the addition of polyelectrolytes has no influence on enzymatic activity toward a neutral substrate. The enzymatic kinetics analysis showed that the K_m value decreased in both cases when PAA and PAAc were added, indicating that the improvement in the affinities of ChT for its substrates were due to the substrate accumulation by electrostatic attraction.

Because of the high specific surface area and large amount of nanopores, nanoscaled carriers can facilitate the uptake of substrate and thus increase the apparent activity of immobilized enzymes. Lyu et al.[45] successfully incorporated cytochrome *c*, lipase, and horseradish peroxidase into metal−organic frameworks (MOFs) such as ZIF-8 and ZIF-10. The Cyt *c*/ZIF-8 composite displayed a 10-times higher apparent activity than free Cyt *c* in aqueous solution. The incorporated Cyt *c* showed a K_m value of 2 mM, whereas that of free Cyt *c* is 15 mM. The decreased K_m indicates a possible substrate enrichment effect of the MOF material that enhances the contact of Cyt *c* to H_2O_2, resulting in the significantly increased peroxidase-like activity of Cyt *c*.

However, when designing the immobilization carriers, although a high affinity of the carriers toward substrates may result in an enhanced capture of substrates by immobilized enzymes, too intensive adsorption of substrates on immobilization carriers may, on the other hand, hinder the enzymatic catalysis. Lin et al.[46] attached HRP onto a triangular DNA scaffold by self-assembly and measured the enzymatic activity by using a library of substrates having different substrate−scaffold interactions (Figure 4). It is demonstrated that the HRP attached on the DNA scaffold can display an enhanced enzymatic activity when the substrate has a "just right" interaction with the scaffold.

According to the above discussions, the interaction between carriers and substrates is an important factor that influences the apparent enzymatic activity. Furthermore, carefully regulating the attraction between the carriers and substrates can achieve not only an enhanced apparent activity but also a tunable activity.

Improved Diffusion of Substrates. The apparent activity of the immobilized enzyme is restricted to the internal and

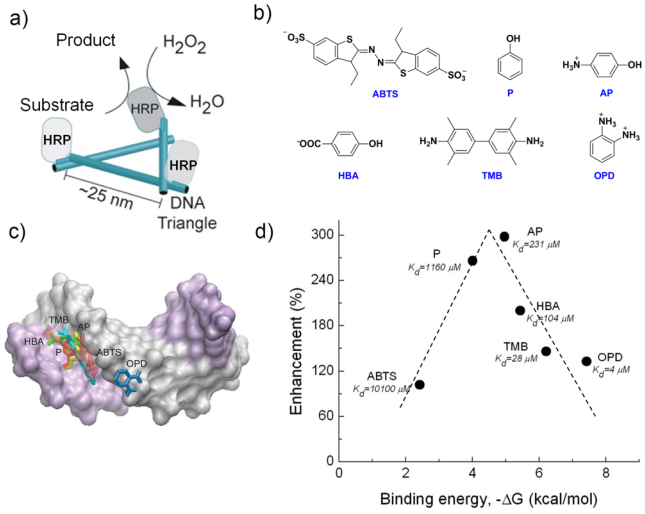

Figure 4. (a) Illustration of HRP3−DNA nanostructures and the enzymatic reaction. (b) Substrates for HRP activity assay. (c) Molecular models of putative binding of substrates on DNA helix. (d) Relationship between the enhancement of HRP activity and the predicted substrate−DNA binding energy, K_d, the calculated binding constant. Reprinted with permission from ref 46, Copyright (2013) American Chemical Society.

external diffusions.[47] Numerous examples show that the enzymatic kinetics parameters are strongly dependent on the structure and size of the carriers for enzyme immobilization or modification, which indicates that the internal diffusion within the carriers usually plays a dominant role rather than the external diffusion of the substrates in the bulk solution. A smaller size of carriers often gives a higher catalytic efficiency and lower apparent K_m value because of the reduced internal diffusion resistance.[48] Guisan et al.[49] reported the immobilization of micrococcal endonuclease on BrCN-activated agarose with two different enzyme distributions: (1) uniform distribution of enzyme molecules within the support and (2) preferentially bonding of enzyme molecules on the outer shell of the particles. It shown that the enzyme bound on the external part of the agarose beads displayed a higher apparent activity compared with the case of enzyme uniformly distributed in particles. In their study, a mixed-enzyme reaction−internal diffusion kinetics was proposed, which perfectly quantified the effect of internal diffusion on the apparent enzyme activity, and the Thiele modulus was an effective nondimensional number to describe the influence of

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the enzyme distribution, pore size, and shape of the carriers on the substrate diffusion.

Fabricating artificial channels or pores in the immobilization carriers is a reasonable and attractive method to improve the substrate diffusion and thus increase the apparent enzymatic activity. One method is substrate "imprinting". Wang et al.[50] prepared a type of substrate-imprinted lipase polymer nanogel with enhanced substrate internal diffusion. By lyophilizing the lipase polymer nanogel in the presence of palmitic acid, the obtained imprinted lipase nanogel showed a 2.9-fold increase in adsorption capacity for the substrate palmitic acid and, thus, a 2-fold transesterification activity increase in organic solvents compared with the native lipase. The enhancement of the apparent activity is due to the reduced substrate internal diffusion resistance in the polyacrylamide shell having imprinted molecular "cavities".

Reducing Substrate or Product Inhibition. Reducing the substrate or product inhibition in enzymatic catalysis by immobilization carriers may also increase the enzymatic activity. Dickensheets et al.[51] immobilized invertase from *Candida utiliz* on porous cellulose beads (activated with an ionic-guanidino group) via electrostatic adsorption. The enzymatic kinetics followed the Michaelis−Menten equation with uncompetitive inhibition by substrate, $V = V_{max}/(1 + (K_m/S) + (S/K_i))$, where V is the reaction velocity, V_{max} is the maximum reaction velocity, K_m is the Michaelis constant, S is the substrate concentration, and K_i is the substrate inhibition constant. Compared with free enzyme, the apparent K_i of the immobilized invertase was increased from 4884 to 8304 mM, indicating that the immobilization reduced the substrate inhibition effect. Therefore, the immobilized invertase resulted in a higher catalytic reaction rate than free enzyme when the concentration of substrate sucrose was higher than 60 mM. This strategy has also been proven as effective by the immobilization of nitrile hydratase (NHase) from *Geobacillus pallidus* RAPc8 on Eupergit C macroporous beads.[52] In the hydrolysis of lactose into the equivalent glucose and galactose, β-galactosidase immobilized on porous carriers showed only half of the product inhibition constant for galactose compared with the free enzyme at a wide range of temperatures (30−90 °C).[53] In addition, the inhibition by product glucose appears to be eliminated when β-galactosidase was immobilized on porous carriers, allowing the enzyme catalyst to be active in the presence of a high concentration of products to achieve a satisfied time−space yield. More interesting examples of improving enzyme activity via reduced inhibition can be found in other reviews.[2a,19a]

3. Enhanced Activity by the Interaction between Carriers and Reaction Media. Application of enzymatic catalysis for the synthesis of fine chemicals and therapeutic compounds in organic media is often limited by the extremely low apparent activity of enzymes that are insoluble in organic solvents.[54] The insoluble form of enzyme powders in organic media hinders the access of substrates toward enzyme active sites and the conformational transition of enzyme. Dispersing and even solubilizing enzymes in organic media, with the assistance of immobilization/modification carriers that have specific interactions with reaction media, is an effective way to achieve an enhanced apparent enzymatic activity in organic media.[55] For instance, Bruns and Tiller[56] entrapped horseradish oxidase and chloroperoxidase into a nanophase-separated amphiphilic network. The swelling/solubilization of the hydrophobic phase of the network in nonpolar solvents makes the substrates accessible to the well dispersed enzymes. The apparent activity of entrapped HRP was enhanced by 2 orders of magnitude compared with free HRP suspended in *n*-heptane (20−46 mU/μg for entrapped HRP and 0.44 ± 0.15 mU/μg for free HRP). Similarly, the entrapped chlorperoxidase showed a 10-fold activity enhancement compared with the native enzyme.

Enzymes can be dissolved in organic media with the help of surfactants. Paradkar and Dordick[57] reported a method for solubilizing enzymes in organic solvents via ion-pairing of the protein with surfactants. The organic solvent-soluble α-chymotrypsin was obtained from the extraction of enzyme in aqueous solution into an isooctane phase containing 2 mM Aerosol OT. Because of the very low ratio of AOT molecules to protein, the solubilization of the protein was attributed to the ion-pairing interaction rather than the formation of micelles. α-Chymotrypsin in such an enzyme-surfactant complex retained their native structure and exhibited a higher catalytic activity, ∼2400-fold enhancement, compared with the suspended enzyme in organic media. The effectiveness of this method was proven by using *Mucor javanicus* lipase,[58] subtilisin Carlsberg,[59] lipase B from *Candida antarctica*, and soybean peroxidase.[60] Although this method is very effective, the reuse of the organic solvent-soluble enzyme is still a challenge.

Zhu et al.[35] developed a type of organic solvent-soluble enzyme−Pluronic nanoconjugate with temperature responsiveness, which enables homogeneous catalysis and heterogeneous separation of the enzyme catalyst by a temperature-induced precipitation. The enzyme−Pluronic conjugate was synthesized by covalently linking Pluronic F127 with enzyme via the Schiff base reaction, followed by lyophilization. The generality of the synthesis was validated by conjugation of bovine serum albumin (BSA), *C. rugosa* lipase (CRL), *C. antarctica* lipase B (CALB), and cytochrome *c* (Cyt *c*) with Pluronic. The conjugated CALB and CRL exhibited increased esterification activities of 67-fold and 57-fold in toluene, respectively, compared with suspended native enzymes, and the peroxidase activity of the conjugated Cyt *c* was increased by 670-fold compared with that of the suspended native Cyt *c*. Moreover, in a practical application of using lipase in the chemoenzymatic synthesis of an anticancer drug, valrubicin, the conjugated CALB showed an 11-fold increase in the initial transesterification activity compared with native CALB.[61] The CALB−Pluronic conjugate also showed improved catalytic performance in other enzymatic catalyses, such as the enzymatic ammonolysis.[62]

In addition to the reasons described above, the enhanced activity of immobilized or chemically modified enzymes sometimes may be induced by other important factors, such as a partition of undesired components, a change of enzyme confirmations, and an enhancement of enzyme stability under certain conditions, which have been well summarized by other reviews.[2a,3a,18e] The distribution or organization of enzymes on immobilization carriers is also an important factor that influences the enzymatic activity. For instance, when attaching lipase onto polymer nanofibers, the lipase aggregates with different aggregation degrees showed different apparent activities and stabilities.[63] A high aggregation degree of enzymes on the nanofibers resulted in a high enzyme loading and more structural rigidity of enzymes. Similar phenomena can be found in the immobilization of a multimeric enzyme, glucose-6-phosphate dehydrogenase, suggesting that the aggregation of enzyme benefits the thermal stability and the activity recovery of immobilized enzyme.[18d] All of these issues need to be well

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Table 1. Some Examples of Substrate Channeling Effect in Multienzyme Complexes

enzyme	mechanism	type	remarks	ref
trytophan synthase	hydrophobic intramolecular tunnel	natural	25 Å long	71
carbamoyl-phosphate synthase	hydrophilic tunnel with few charged and hydrophobic residues	natural	96 Å long	72
dihydrofolate reductase-thymidylate synthase	electrostatic interaction for channeling substrates	natural		73
glutamine phosphoribosylpyrophosphate amidotransferase	transient hydrophobic channel	natural	20 Å long	74
β-galactosidase and galactose dehydrogenase	proximity effect	fusion protein	shorter transient time and higher activity	75
cytochrome p450, putidaredoxin and putidaredoxin reductase	proximity effect, coupled with electron transfer mediator	protein scaffold	up to 50-fold enhanced	67b, c
leucine dehydrogenase and formate dehydrogenase	proximity effect	protein scaffold	up to 2-fold	76
GOx and HRP	proximity effect	DNA scaffold	up to 2-fold enhanced	77
GOx, HRP and Inverase	proximity effect and restricted space	co-encapsulation	up to 30-fold enhanced	66b
glucose-6-phosphate dehydrogenase and malic dehydrogenase	NAD^+-modified swinging-arm is positioned halfway between the two enzymes	DNAscaffold	90−277-fold enhanced	78
acyl-ACP reductase and Aldehyde deformylating oxygenase	proximity effect	RNA scaffold	80% increased	79

considered in the preparation of highly efficient enzyme catalysts.

MULTIENZYME SYSTEM

Multienzyme catalysis that performs biochemical reactions in a cascade or coupled manner with promising applications in biocatalysis and biosensing has attracted increasing interest from academic and industrial communities.[64] Strategies such as a cell-free system,[65] coimmobilization,[66] scaffold-induced self-assembly,[67] and conjugation with polymers[68] have been employed to construct multienzyme systems. The activity enhancement phenomenon has been frequently observed in immobilized or chemically modified multienzyme systems. The general considerations discussed above in single-enzyme systems are also applicable to multienzyme systems. In addition to these, the multienzyme systems have some unique features that affect the overall activity, such as substrate channeling, kinetics matching, and spatial distribution of involved enzymes.

1. Substrate channeling. Substrate channeling, also known as metabolic channeling, is common in nature.[69] The direct substrate transporting among enzymes, without diffusing into bulk solution, is advantageous for enhanced overall activity through concentrating the intermediates, protecting unstable cofactors, circumventing unfavorable pathways, etc.[70] Recently, the concept of substrate channeling has been attempted in immobilized or chemically modified artificial multienzyme systems. Some selected examples[66b,67,71−79] of the substrate channeling effect reported in both natural enzymes and artificial multienzyme systems are listed in Table 1. When designing artificial multienzyme systems by immobilization or chemical modification, to achieve the substrate channeling effect and an enhanced overall activity, the distance between the involved enzymes, their orientations, and the incorporation of mediators are critical factors to be considered.

Distance between Enzymes. Numerous experiments have indicated that properly coimmobilized multiple enzymes can display an enhanced overall activity in comparison with the equivalent concentrations of free enzymes.[80] It is obvious that the distance between involved enzymes needs to be close enough for the realization of direct transport of intermediates. Many efforts have been made to investigate the effect of distance on the overall activity. Wilner et al.[81] attached glucose oxidase (GOx) and horseradish peroxidase (HRP) onto the topologically programmed DNA scaffold. The distances between the enzymes located on the two-hexagon and four-hexagon scaffold are controlled at ∼6 and 23 nm, respectively. The organized GOx−HRP−DNA scaffold complexes showed an over 10-fold increase in overall enzymatic activity than untethered enzymes. Moreover, a closer distance of enzymes (located on the two-hexagon scaffold) gave a higher overall activity, 1.2-fold higher than that located on the four-hexagon scaffold. Fu et al.[82] varied the distance between HRP and GOx from 10 to 65 nm with specific DNA origami tiles. They found all the coupled systems showed increased overall activities, and the largest enhancement (>15 times) was observed for the closest distance of enzymes (∼10 nm) (Figure 5). A similar

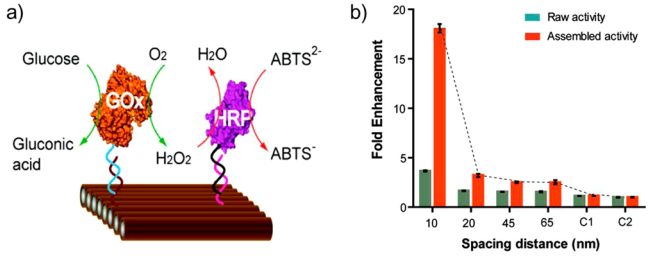

Figure 5. (a) Self-assembly of GOx and HRP enzymes on DNA origami tiles with controlled interenzyme distances. (b) The overall cascade enzymatic activity of assembled GOx/HRP pairs spacing from 10 to 65 nm, compared with free enzymes in solution with (C2) or without (C1) DNA scaffold. Reconstructed with permission from ref 82, Copyright (2012) American Chemical Society.

conclusion was also obtained by the investigation of a ternary system assembled on PCNA (proliferating cell nuclear antigen, a homotrimer with a ring-like structure, with an approximate diameter of 8 nm) scaffold.[67b]

On the other hand, the absence of such expected substrate channeling in artificial multienzyme systems was also reported.[68,83] Idan and Hess[84] developed a physical model that considered the diffusion and enzyme kinetics to challenge the concept of substrate channeling. In their calculation, the proximity of the involved enzymes can only increase the

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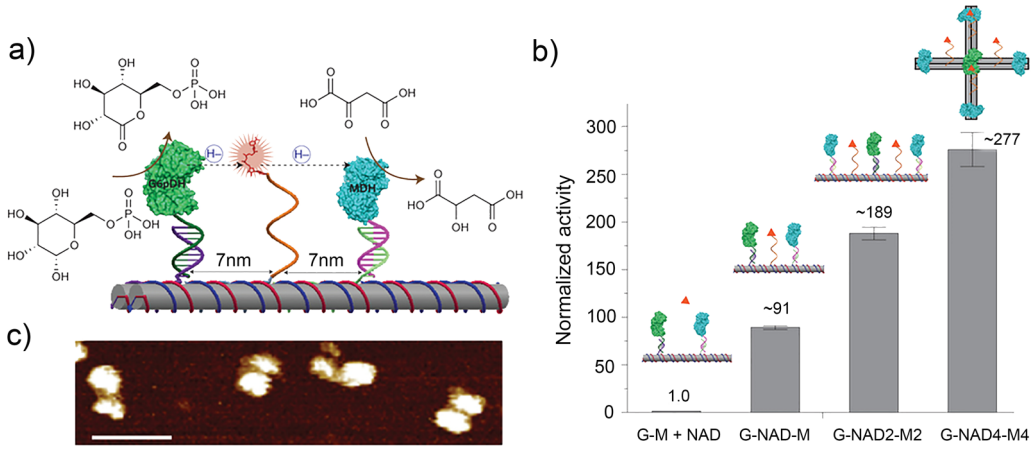

Figure 6. (a) Schematic of G6pDH and MDH bienzyme system organized on a nanostructured DNA scaffold. (b) Catalytic efficiency of different organized multienzyme complexes with/without NAD^+ arms: G6pDH−MDH assembly with free NAD^+; G6pDH−NAD^+−MDH swinging-arm structure; G6pDH−$NAD2^+$−MDH2 swinging-arm structure and G6pDH−$NAD4^+$−MDH4 structure. (c) AFM image of G6pDH−NAD^+−MDH swinging-arm structures. Reconstructed with permission from ref 78, Copyright (2014) Nature Publishing Group.

throughput of the cascade enzymatic reaction at very initial stage within milliseconds to seconds, which is negligible to influence the overall enzymatic activity. Instead, they attributed the magnification of overall activity of artificial multienzyme systems to the aggregation of enzymes on immobilization carriers (which provides multiple targets for the accessing of substrates and intermediates), the attraction between substrates and immobilization carriers, and the protection of unstable intermediates. Although the model described in the study cannot sufficiently represent the true situation of multienzyme catalysis, it provides new insights to investigate the substrate channeling effect in artificial multienzyme systems.

However, there are also simulations confirming the existence of the substrate channeling effect in artificial multienzyme systems. Bauler et al.[85] simulated the trajectory of the substrate diffusing between the active site zones of two cascade enzymes using Brownian dynamics and evaluated the approximate effect on the overall activity. It showed that the reaction probability was greater when the distance between the two enzymes reached 25 Å at an orientation with their active sites facing each other. Interestingly, too short a distance may decrease the overall activity because of the increased obstacle for the first enzymatic reaction. Simulation carried out by Buchner et al.[86] also confirmed the substrate channeling effect. It was revealed that placing enzyme 2 close to the center of enzyme 1 can dramatically increase the flux of intermediates. This channeling effect is highly dependent on the distance between two enzymes at the range of several nanometers. Therefore, the controversial observation of the substrate channeling effect in both simulations and experiments suggests that, in addition to the distance of the involved enzymes, other factors, such as the orientation, limitation of rate-determining enzymatic reaction, and even the spatial distribution of involved enzymes, also need to be considered for establishing a substrate channeling.

Orientation of the Involved Enzymes. A controlled orientation of the involved enzymes with their active sites facing each other ensures the direct transport of the intermediates. Early in 1980s, Mansson and co-workers[87] immobilized the lactate dehydrogenase (LDH) and alcohol dehydrogenase (ADH) on agarose beads with the aid of a bis-NAD analogue, followed by glutaraldehyde cross-linking to achieve an orientation of enzymes with their active sites facing each other. In the comparison with the site-to-site directed immobilization and randomly coupled manners, the face-to-face oriented complex showed a much higher activity for the oxidation of NADH, indicating a possible occurrence of the substrate channeling effect. Bauler et al.[85] studied the effect of orientation on the overall activity using Brownian dynamic simulation and found that the highest reaction probability occurred at 0° orientation (fixed at 10 Å distance), at which the two active zones face each other. When the orientation was more than 90°, the probability was down to a very low level. This evidence suggested the effect of enzyme orientation on the displaying of substrate channeling in artificial multienzyme systems.

Artificial Swinging Mediator for Intermediate Channeling. In the enzymatic reaction with cofactors involved, transportation of cofactors sometimes determines the overall activity. Very recently, Fu and colleagues[78] presented an ingenious multienzyme−DNA complex by employing a swinging arm modified with NAD^+ on its terminal to mimic the substrate channeling (Figure 6). The swinging arm placed between the glucose-6-phosphate dehydrogenase (G6pDH) and malic dehydrogenase (MDH) facilitated the channeling of NADH/NAD^+ between the two enzymes. The shortest distance between the NAD arm and each relevant enzyme (7 nm) resulted in the highest activity. The assembled G6pDH−NAD^+−MDH complex containing 100 nM armed NAD^+ displayed an activity comparable to the G6pDH−MDH complex with 20 μM free NAD^+. Further, by precise control of the topology and stoichiometry of enzymes and NAD arms, ∼90−277-fold enhancements of overall activity were observed in comparison with the freely diffusing NAD^+ system.

2. Kinetics Matching. The kinetics matching (optimization of ratio) of enzymes involved in multienzyme catalysis is a key factor to achieve the maximum overall activity. Major benefits from kinetics matching are recognized as achieving the maximum overall activity with minimum input of enzymes, preventing accumulation of toxic intermediates, reducing competitive side reactions, and strengthening the rate-limiting reaction step.

The effect of the optimization of enzyme ratios in immobilized or chemically modified multienzyme systems has been demonstrated in many cases, such as the fabrication of the

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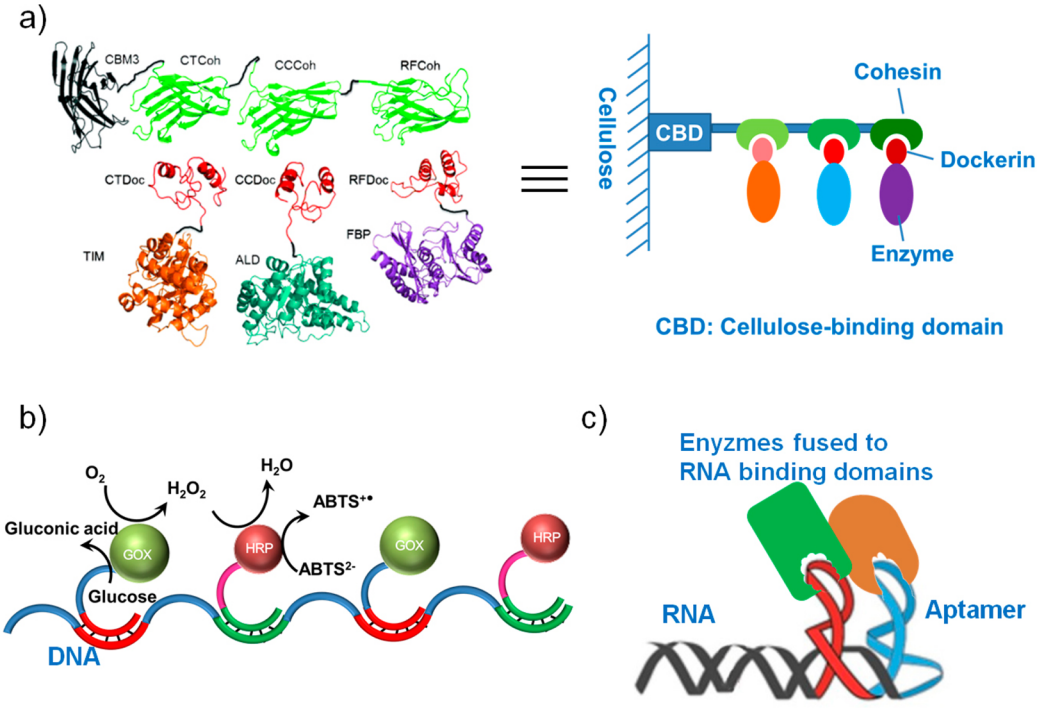

Figure 7. Representative approaches for constructing one-dimensional multienzyme systems. (a) Protein scaffolds such as the cohesin and dockerin modules. (b) DNA hybridization. (c) RNA−protein interactions. Reprinted with permission from ref 97, Copyright (2012) Wiley-VCH Verlag GmbH & Co. KGaA; ref 98, Copyright (2009) American Chemical Society; and ref 79, Copyright (2014) Oxford University Press, respectively.

HRP-GOx bienzyme electrode,[88] the coimmobilization of xylose isomerase, mutarotase, and glucose dehydrogenase for the analysis of D-xylose and D-xylulose,[89] and the pretreatment of biomass feedstocks with 16 involved enzymes.[90] Recently, Zhang et al.[91] presented a high-throughput method for the optimization of enzyme ratios by employing a color inkjet printer to construct an artificial multienzyme system on printing paper. Dvorak et al.[92] demonstrated a workflow to optimize the stoichiometry of a three-enzyme process by using kinetics modeling.

More recently, it has been demonstrated that the optimization of the stoichiometry of the involved enzymes can also be carried out in vivo. For example, Dueber et al.[93] described the stoichiometric optimization of relative enzymes in *Escherichia coli*. They constructed a synthetic protein scaffold to anchor together three enzymes involved in the mevalonate biosynthetic pathway with different stoichiometries. The three enzymes are acetoacetyl−CoA thiolase (AtoB), hydroxymethylglutaryl−CoA synthase (HMGS), and hydroxymethylglutaryl−CoA reductase (HMGR), all of which are recombined with ligands at the C/N terminus to attach the protein scaffold. A 77-fold improvement in product titer was achieved at a ratio of AtoB/HMGS/HMGR of 1:2:2. The enhancement of production capacity was attributed to the balanced metabolic fluxes and reduced metabolic load, which greatly prevented the accumulation of toxic intermediates.

3. Spatial Distribution. The spatial distribution of enzymes in immobilized or chemically modified multienzyme systems can affect the transport behavior of the substrates or the intermediates, the enzymatic kinetics, and the effective amount of enzymes, all of which can influence the overall enzymatic activity. The artificial multienzyme systems with controlled spatial distribution have been constructed through many different strategies, including protein[94] or DNA[77] scaffolds, layer-by-layer encapsulation,[95] multilayer adsorption,[96] etc.

The one-dimensional sequential localization of relative enzymes has been achieved by using protein−protein interactions,[94,97] DNA hybridization,[77,81,98] and RNA-protein interactions[79,99] at molecular levels (Figure 7). For example, You et al.[97] attached engineered triosephosphate isomerase, aldolase, and fructose 1, 6-bisphosphatase to a family 3 cellulose-binding module (CBM3) containing trifunctional cohesins, based on the high affinity between cohesin and dockerin. The initial activity of the three-enzyme complex was 33-fold higher than that of free enzymes mixture. Winler et al.[98] posited multiple copies of HRP and GOx on the sequence-specific programmed DNA (up to 30 μm), in which arrangement each GOx was surrounded by HRP molecules, and vice versa. The overall activity was effectively increased, while free enzymes without arrangement produced almost no product.

The three-dimensional position of enzymes also greatly affects the overall activity of immobilized or chemically modified multienzyme systems. Pescador et al.[100] assembled GOx and HRP together with polyelectrolyte layers on the surface of silica microparticles. By this approach, they achieved two types of spatial distributions of enzymes, with GOx and HRP in separate polymer layers (GOx in the inner layer, HRP in the outer layer) and in the same layer. Both the separated and random coimmobilization of enzymes showed an activity enhancement compared with free enzymes. The peroxidase activity of the coimmobilized GOx and HRP in the same layer was 2.5 times higher than that of the colocalized enzymes in separate layers (HRP in the outer layer). The lower activity of the separated colocalization manner compared with random colocalization may be attributed to the diffusion limitation of substrates and intermediates in the enzymatic cascade.

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Jia[101] colocalized HRP and GOx on polystyrene nanoparticles with different spatial distributions using a DNA hybridization method. The colocalized bienzyme showed a 2-fold increase in the overall activity, compared with the mixture of single-enzyme immobilized nanoparticles. Moreover, the immobilized bienzyme system with GOx distributed on the outer layer displayed the highest activity, which was ∼1.2 times higher than the immobilized bienzyme system with HRP on the outer layer. Similar effects of the spatial distribution of enzymes can be found in the microplate-immobilized DNA−GOx−HRP complexes,[77] malate dehydrogenase (MDH) and citrate synthase on Au nanoparticles,[96] and colocalization of GOx−HRP by protein−$Cu_3(PO_3)_2$ nanoflowers.[102] In these above studies, locating enzyme that catalyzes the first-step reaction in the outer layer usually leads to a higher overall enzymatic activity, mainly because of the reduced diffusion resistance of substrates and intermediates. A facilitated contact of GOx with dissolved oxygen in solution is also considered a main reason for the high overall activity when locating GOx in the outer layer. It is worth noting that the controlled distribution of different enzymes has an influence on the overall activity, but the distribution with the first enzyme outside would not guarantee the activity enhancement.

CONCLUSION AND OUTLOOK

The development of chemistry, materials science, and biological science offers opportunities to design and fabricate chemically re-engineered enzyme catalysts with enhanced activities over their native counterparts, although currently, the enhancement of enzymatic activity for immobilized or chemically modified enzymes is usually unpredictably discovered. The established practices described above have proved that general considerations on the interactions between carriers (or surroundings) and enzymes, substrates, or reaction media would probably lead to the success of preparing an efficient enzyme catalyst with enhanced activity. For constructing an immobilized or chemically modified multiple-enzyme system, extra attention needs to be paid to the substrate channeling, kinetic matching, and spatial distribution of enzymes to achieve a high overall activity. Investigation of the origin of enhanced activity of chemically re-engineered enzyme catalysts may provide new insights and inspirations for utilizing new chemical structures and novel materials to prepare highly efficient enzyme catalysts with ever-expanded applications.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Bornscheuer, U. T.; Huisman, G. W.; Kazlauskas, R. J.; Lutz, S.; Moore, J. C.; Robins, K. *Nature* **2012**, *485*, 185−194.
(2) (a) Rodrigues, R. C.; Ortiz, C.; Berenguer-Murcia, A.; Torres, R.; Fernandez-Lafuente, R. *Chem. Soc. Rev.* **2013**, *42*, 6290−6307. (b) Rodrigues, R. C.; Berenguer-Murcia, A.; Fernandez-Lafuente, R. *Adv. Synth. Catal.* **2011**, *353*, 2216−2238. (c) Singh, R. K.; Tiwari, M. K.; Singh, R.; Lee, J. K. *Int. J. Mol. Sci.* **2013**, *14*, 1232−1277.
(3) (a) Hernandez, K.; Fernandez-Lafuente, R. *Enzyme Microb. Technol.* **2011**, *48*, 107−122. (b) Rodrigues, R. C.; Barbosa, O.; Ortiz, C.; Berenguer-Murcia, Á.; Torres, R.; Fernandez-Lafuente, R. *RSC Adv.* **2014**, *4*, 38350−38374.
(4) Cirino, P. C.; Arnold, F. H. *Curr. Opin. Chem. Biol.* **2002**, *6*, 130−135.
(5) Siegel, J. B.; Zanghellini, A.; Lovick, H. M.; Kiss, G.; Lambert, A. R.; St. Clair, J. L.; Gallaher, J. L.; Hilvert, D.; Gelb, M. H.; Stoddard, B. L.; Houk, K. N.; Michael, F. E.; Baker, D. *Science* **2010**, *329*, 309−313.
(6) (a) Adamczak, M.; Krishna, S. H. *Food Technol. Biotechnol.* **2004**, *42*, 251−264. (b) Hibbert, E. G.; Dalby, P. A. *Microb. Cell Fact.* **2005**, *4*, 29. (c) Morley, K. L.; Kazlauskas, R. J. *Trends Biotechnol.* **2005**, *23*, 231−237. (d) Adrio, J. L.; Demain, A. L. *Biomolecules* **2014**, *4*, 117−139.
(7) Nelson, J. M.; Griffin, E. G. *J. Am. Chem. Soc.* **1916**, *38*, 1109−1115.
(8) Tosa, T.; Mori, T.; Fuse, N.; Chibata, I. *Biotechnol. Bioeng.* **1967**, *9*, 603−615.
(9) Sheldon, R. A. *Adv. Synth. Catal.* **2007**, *349*, 1289−1307.
(10) (a) Ansari, S. A.; Husain, Q. *Biotechnol. Adv.* **2012**, *30*, 512−523. (b) Petkova, G. A.; Zaruba, K.; Zvatora, P.; Kral, V. *Nanoscale Res. Lett.* **2012**, *7*, 287.
(11) (a) Wang, Y.; Hsieh, Y. *J. Polym. Sci., Part A: Polym. Chem.* **2004**, *42*, 4289−4299. (b) Wang, S. G.; Zhang, Q.; Wang, R.; Yoon, S. F.; Ahn, J.; Yang, D. J.; Tian, J. Z.; Li, J. Q.; Zhou, Q. *Electrochem. Commun.* **2003**, *5*, 800−803. (c) Chen, R. J.; Zhang, Y.; Wang, D.; Dai, H. *J. Am. Chem. Soc.* **2001**, *123*, 3838−3839.
(12) (a) Veronese, F. M. *Biomaterials* **2001**, *22*, 405−417. (b) Woodward, C. A.; Kaufman, E. N. *Biotechnol. Bioeng.* **1996**, *52*, 423−428.
(13) Marshall, J. J.; Rabinowitz, M. L. *Biotechnol. Bioeng.* **1976**, *18*, 1325−1329.
(14) (a) Schachschal, S.; Adler, H. J.; Pich, A.; Wetzel, S.; Matura, A.; van Pee, K. H. *Colloid Polym. Sci.* **2011**, *289*, 693−698. (b) Yan, M.; Ge, J.; Liu, Z.; Ouyang, P. *J. Am. Chem. Soc.* **2006**, *128*, 11008−11009.
(15) Wang, Y.; Caruso, F. *Chem. Mater.* **2005**, *17*, 953−961.
(16) (a) Ji, X.; Su, Z.; Wang, P.; Ma, G.; Zhang, S. *ACS Catal.* **2014**, *4*, 4548−4559. (b) Ji, X.; Wang, P.; Su, Z.; Ma, G.; Zhang, S. *J. Mater. Chem. B* **2014**, *2*, 181−190. (c) Zhao, Z. Y.; Liu, J.; Hahn, M.; Qiao, S.; Middelberg, A. P. J.; He, L. *RSC Adv.* **2013**, *3*, 22008−22013.
(17) (a) Haring, D.; Schreier, P. *Curr. Opin. Chem. Biol.* **1999**, *3*, 35−38. (b) Sheldon, R. A. *Org. Process Res. Dev.* **2011**, *15*, 213−223.
(18) (a) Fernandez-Lafuente, R. *Enzyme Microb. Technol.* **2009**, *45*, 405−418. (b) Cowan, D. A.; Fernandez-Lafuente, R. *Enzyme Microb. Technol.* **2011**, *49*, 326−346. (c) Aissaoui, N.; Landoulsi, J.; Bergaoui, L.; Boujday, S.; Lambert, J. F. *Enzyme Microb. Technol.* **2013**, *52*, 336−343. (d) Aissaoui, N.; Bergaoui, L.; Boujday, S.; Lambert, J. F.; Methivier, C.; Landoulsi, J. *Langmuir* **2014**, *30*, 4066−4077. (e) Garcia-Galan, C.; Berenguer-Murcia, A.; Fernandez-Lafuente, R.; Rodrigues, R. C. *Adv. Synth. Catal.* **2011**, *353*, 2885−2904.
(19) (a) Mateo, C.; Palomo, J. M.; Fernandez-Lorente, G.; Guisan, J. M.; Fernandez-Lafuente, R. *Enzyme Microb. Technol.* **2007**, *40*, 1451−1463. (b) Guzik, U.; Hupert-Kocurek, K.; Wojcieszynska, D. *Molecules* **2014**, *19*, 8995−9018. (c) Sheldon, R. A.; van Pelt, S. *Chem. Soc. Rev.* **2013**, *42*, 6223−6235.
(20) (a) Lei, C.; Soares, T. A.; Shin, Y.; Liu, J.; Ackerman, E. J. *Nanotechnology* **2008**, *19*, 125102. (b) Katchalski, E.; Silman, I.; Goldman, R. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1971**, *34*, 445−536. (c) Subrizi, F.; Crucianelli, M.; Grossi, V.; Passacantando, M.; Pesci, L.; Saladino, R. *ACS Catal.* **2014**, *4*, 810−822. (d) Lopez-Serrano, P.; Cao, L.; van Rantwijk, F.; Sheldon, R. A. *Biotechnol. Lett.* **2002**, *24*, 1379−1383.
(21) (a) Verger, R. *Trends Biotechnol.* **1997**, *15*, 32−38. (b) Colton, I. J.; Ahmed, S. N.; Kazlauskas, R. J. *J. Org. Chem.* **1995**, *60*, 212−217.

DOI: 10.1021/acscatal.5b00996
ACS Catal. 2015, 5, 4503−4513

(22) (a) Derewenda, Z. S.; Derewenda, U.; Dodson, G. G. *J. Mol. Biol.* **1992**, *227*, 818−839. (b) Derewenda, U.; Brzozowski, A. M.; Lawson, D. M.; Derewenda, Z. S. *Biochemistry* **1992**, *31*, 1532−1541.
(23) Peters, G. H.; Olsen, O. H.; Svendsen, A.; Wade, R. C. *Biophys. J.* **1996**, *71*, 119−129.
(24) Rehm, S.; Trodler, P.; Pleiss, J. *Protein Sci.* **2010**, *19*, 2122−2130.
(25) Jaaskelainen, S.; Verma, C. S.; Hubbard, R. E.; Lestko, P.; Caves, L. S. D. *Protein Sci.* **1998**, *7*, 1359−1367.
(26) Bastida, A.; Sabuquillo, P.; Armisen, P.; Fernández-Lafuente, R.; Huguet, J.; Guisán, J. M. *Biotechnol. Bioeng.* **1998**, *58*, 486−493. (b) Palomo, J. M.; Muñoz, G.; Fernández-Lorente, G.; Mateo, C.; Fernández-Lafuente, R.; Guisán, J. M. *J. Mol. Catal. B: Enzym.* **2002**, *19*−*20*, 279−286.
(27) Manoel, E. A.; dos Santos, J. C. S.; Freire, D. M. G.; Rueda, N.; Fernandez-Lafuente, R. *Enzyme Microb. Technol.* **2015**, *71*, 53−57.
(28) Jin, Q.; Jia, G.; Zhang, Y.; Yang, Q.; Li, C. *Langmuir* **2011**, *27*, 12016−12024.
(29) (a) He, J.; Xu, Y.; Ma, H.; Zhang, Q.; Evans, D. G.; Duan, X. *J. Colloid Interface Sci.* **2006**, *298*, 780−786. (b) Galarneau, A.; Mureseanu, M.; Atger, S.; Renard, G.; Fajula, F. *New J. Chem.* **2006**, *30*, 562−571.
(30) Xu, D.; Tonggu, L.; Bao, X.; Lu, D.; Liu, Z. *Soft Matter* **2012**, *8*, 2036−2042.
(31) Ge, J.; Yan, M.; Lu, D.; Zhang, M.; Liu, Z. *Biochem. Eng. J.* **2007**, *36*, 93−99.
(32) (a) Vladimirov, Y. A.; Proskurnina, E. V.; Izmailov, D. Y.; Novikov, A. A.; Brusnichkin, A. V.; Osipov, A. N.; Kagan, V. E. *Biochemistry (Moscow)* **2006**, *71*, 998−1005. (b) Belikova, N. A.; Vladimirov, Y. A.; Osipov, A. N.; Kapralov, A. A.; Tyurin, V. A.; Potapovich, M. V.; Basova, L. V.; Peterson, J.; Kurnikov, I. V.; Kagan, V. E. *Biochemistry* **2006**, *45*, 4998−5009.
(33) Gebicka, L.; Didik, J. *Acta Biochim. Polym.* **2005**, *52*, 551−555.
(34) Kato, K.; Suzuki, M.; Tanemura, M.; Saito, T. *J. Ceram. Soc. Jpn.* **2010**, *118*, 410−416.
(35) Zhu, J.; Zhang, Y.; Lu, D.; Zare, R. N.; Ge, J.; Liu, Z. *Chem. Commun.* **2013**, *49*, 6090−6092.
(36) Kawamura, A.; Yoshioka, Y.; Harada, A.; Kono, K. *Biomacromolecules* **2005**, *6*, 627−631.
(37) (a) Abe, Y.; Hirakawa, T.; Nakajima, S.; Okano, N.; Hayase, S.; Kawatsura, M.; Hirose, Y.; Itoh, T. *Adv. Synth. Catal.* **2008**, *350*, 1954−1958. (b) Abe, Y.; Yoshiyama, K.; Yagi, Y.; Hayase, S.; Kawatsura, M.; Itoh, T. *Green Chem.* **2010**, *12*, 1976−1980. (c) Itoh, T.; Han, S. H.; Matsushita, Y.; Hayase, S. *Green Chem.* **2004**, *6*, 437−439. (d) Abe, Y.; Yagi, Y.; Hayase, S.; Kawatsura, M.; Itoh, T. *Ind. Eng. Chem. Res.* **2012**, *51*, 9952−9958.
(38) Itoh, T.; Matsushita, Y.; Abe, Y.; Han, S. H.; Wada, S.; Hayase, S.; Kawatsura, M.; Takai, S.; Morimoto, M.; Hirose, Y. *Chem. - Eur. J.* **2006**, *12*, 9228−9237.
(39) Ge, J.; Lei, J.; Zare, R. N. *Nat. Nanotechnol.* **2012**, *7*, 428−432.
(40) Lin, Z.; Xiao, Y.; Yin, Y.; Hu, W.; Liu, W.; Yang, H. *ACS Appl. Mater. Interfaces* **2014**, *6*, 10775−10782.
(41) Wang, L. B.; Wang, Y. C.; He, R.; Zhuang, A.; Wang, X.; Zeng, J.; Hou, J. G. *J. Am. Chem. Soc.* **2013**, *135*, 1272−1275.
(42) Goldstein, L.; Levin, Y.; Katchalski, E. *Biochemistry* **1964**, *3*, 1913−1919.
(43) (a) Harada, A.; Kataoka, K. *J. Controlled Release* **2001**, *72*, 85−91. (b) Harada, A.; Kataoka, K. *Langmuir* **1999**, *15*, 4208−4212. (c) Harada, A.; Kataoka, K. *J. Am. Chem. Soc.* **1999**, *121*, 9241−9242. (d) Harada, A.; Kataoka, K. *Macromolecules* **1998**, *31*, 288−294.
(44) Kurinomaru, T.; Tomita, S.; Hagihara, Y.; Shiraki, K. *Langmuir* **2014**, *30*, 3826−3831.
(45) Lyu, F.; Zhang, Y.; Zare, R. N.; Ge, J.; Liu, Z. *Nano Lett.* **2014**, *14*, 5761−5765.
(46) Lin, J. L.; Wheeldon, I. *ACS Catal.* **2013**, *3*, 560−564.
(47) Engasser, J. M.; Horvath, C. *J. Theor. Biol.* **1973**, *42*, 137−155.
(48) Dey, G.; Singh, B.; Banerjee, R. *Braz. Arch. Biol. Technol.* **2003**, *46*, 167−176.
(49) Guisan, J. M.; Serrano, J.; Melo, F. V.; Ballesteros, A. *Appl. Biochem. Biotechnol.* **1987**, *14*, 49−72.
(50) Wang, R.; Zhang, Y.; Huang, J.; Lu, D.; Ge, J.; Liu, Z. *Green Chem.* **2013**, *15*, 1155−1158.
(51) Dickensheets, P. A.; Chen, L. F.; Tsao, G. T. *Biotechnol. Bioeng.* **1977**, *19*, 365−375.
(52) Chiyanzu, I.; Cowan, D. A.; Burton, S. G. *J. Mol. Catal. B: Enzym.* **2010**, *63*, 109−115.
(53) Ladero, M.; Perez, M. T.; Santos, A.; Garcia-Ochoa, F. *Biotechnol. Bioeng.* **2003**, *81*, 241−252.
(54) (a) Klibanov, A. M. *Nature* **2001**, *409*, 241−246. (b) Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 3194−3201.
(55) Ge, J.; Yang, C.; Zhu, J.; Lu, D.; Liu, Z. *Top. Catal.* **2012**, *55*, 1070−1080.
(56) Bruns, N.; Tiller, J. C. *Nano Lett.* **2005**, *5*, 45−48.
(57) Paradkar, V. M.; Dordick, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 5009−5010.
(58) Altreuter, D. H.; Dordick, J. S.; Clark, D. S. *Enzyme Microb. Technol.* **2002**, *31*, 10−19.
(59) Altreuter, D. H.; Dordick, J. S.; Clark, D. S. *J. Am. Chem. Soc.* **2002**, *124*, 1871−1876.
(60) Akbar, U.; Aschenbrenner, C. D.; Harper, M. R.; Johnson, H. R.; Dordick, J. S.; Clark, D. S. *Biotechnol. Bioeng.* **2007**, *96*, 1030−1039.
(61) Zhang, Y.; Dai, Y.; Hou, M.; Li, T.; Ge, J.; Liu, Z. *RSC Adv.* **2013**, *3*, 22963−22966.
(62) Wu, X.; Wang, R.; Zhang, Y.; Ge, J.; Liu, Z. *Catal. Lett.* **2014**, *144*, 1407−1410.
(63) An, H. J.; Lee, H. J.; Jun, S. H.; Hwang, S. Y.; Kim, B. C.; Kim, K.; Lee, K. M.; Oh, M. K.; Kim. *Bioprocess Biosyst. Eng.* **2011**, *34*, 841−847.
(64) Schoffelen, S.; van Hest, J. C.M. *Soft Matter* **2012**, *8*, 1736−1746.
(65) Ye, X.; Wang, Y.; Hopkins, R.; Adams, M. W.; Evans, B. R.; Mielenz, J. R.; Zhang, Y. H. *ChemSusChem* **2009**, *2*, 149−152.
(66) (a) Betancor, L.; Luckarift, H. *Biotechnol. Genet. Eng. Rev.* **2010**, *27*, 95−114. (b) Liu, Y.; Du, J.; Yan, M.; Lau, M. Y.; Hu, J.; Han, H.; Yang, O. O.; Liang, S.; Wei, W.; Wang, H.; Li, J.; Zhu, X.; Shi, L.; Chen, W.; Ji, C.; Lu, Y. *Nat. Nanotechnol.* **2013**, *8*, 187−192. (c) Rocha-Martin, J.; Velasco-Lozano, S.; Guisan, J. M.; Lopez-Gallego, F. *Green Chem.* **2014**, *16*, 303−311. (d) Rocha-Martin, J.; de las Rivas, B.; Munoz, R.; Guisan, J. M.; Lopez-Gallego, F. *ChemCatChem* **2012**, *4*, 1279−1288.
(67) (a) Niemeyer, C. M.; Koehler, J.; Wuerdemann, C. *ChemBioChem* **2002**, *3*, 242−245. (b) Hirakawa, H.; Nagamune, T. *ChemBioChem* **2010**, *11*, 1517−1520. (c) Haga, T.; Hirakawa, H.; Nagamune, T. *PLoS One* **2013**, *8*, e75114.
(68) Grotzky, A.; Nauser, T.; Erdogan, H.; Schluter, A. D.; Walde, P. *J. Am. Chem. Soc.* **2012**, *134*, 11392−11395.
(69) Miles, E. W.; Rhee, S.; Davies, D. R. *J. Biol. Chem.* **1999**, *274*, 12193−12196.
(70) Zhang, Y. H. *Biotechnol. Adv.* **2011**, *29*, 715−725.
(71) Pan, P.; Woehl, E.; Dunn, M. F. *Trends Biochem. Sci.* **1997**, *22*, 22−27.
(72) Thoden, J. B.; Holden, H. M.; Wesenberg, G.; Raushel, F. M.; Rayment, I. *Biochemistry* **1997**, *36*, 6305−6316.
(73) Knighton, D. R.; Kan, C. C.; Howland, E.; Janson, C. A.; Hostomska, Z.; Welsh, K. M.; Matthews, D. A. *Nat. Struct. Biol.* **1994**, *1*, 186−194.
(74) Krahn, J. M.; Kim, J. H.; Burns, M. R.; Parry, R. J.; Zalkin, H.; Smith, J. L. *Biochemistry* **1997**, *36*, 11061−11068.
(75) Ljungcrantz, P.; Carlsson, H.; Mansson, M. O.; Buckel, P.; Mosbach, K.; Buelow, L. *Biochemistry* **1989**, *28*, 8786−8792.
(76) Gao, X.; Yang, S.; Zhao, C.; Ren, Y.; Wei, D. *Angew. Chem., Int. Ed.* **2014**, *53*, 14027−14030.
(77) Muller, J.; Niemeyer, C. M. *Biochem. Biophys. Res. Commun.* **2008**, *377*, 62−67.
(78) Fu, J.; Yang, Y. R.; Johnson-Buck, A.; Liu, M.; Liu, Y.; Walter, N. G.; Woodbury, N. W.; Yan, H. *Nat. Nanotechnol.* **2014**, *9*, 531−536.

DOI: 10.1021/acscatal.5b00996
ACS Catal. 2015, 5, 4503−4513

(79) Sachdeva, G.; Garg, A.; Godding, D.; Way, J. C.; Silver, P. A. *Nucleic Acids Res.* **2014**, *42*, 9493−9503.
(80) (a) Jia, F.; Zhang, Y.; Narasimhan, B.; Mallapragada, S. K. *Langmuir* **2012**, *28*, 17389−17395. (b) Jia, F.; Narasimhan, B.; Mallapragada, S. K. *AIChE J.* **2013**, *59*, 355−360.
(81) Wilner, O. I.; Weizmann, Y.; Gill, R.; Lioubashevski, O.; Freeman, R.; Willner, I. *Nat. Nanotechnol.* **2009**, *4*, 249−254.
(82) Fu, J.; Liu, M.; Liu, Y.; Woodbury, N. W.; Yan, H. *J. Am. Chem. Soc.* **2012**, *134*, 5516−5519.
(83) (a) Pettersson, H.; Pettersson, G. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **2001**, *1549*, 155−160. (b) Pettersson, H.; Olsson, P.; Bulow, L.; Pettersson, G. *Eur. J. Biochem.* **2000**, *267*, 5041−5046.
(84) Idan, O.; Hess, H. *ACS Nano* **2013**, *7*, 8658−8665.
(85) Bauler, P.; Huber, G.; Leyh, T.; McCammon, J. A. *J. Phys. Chem. Lett.* **2010**, *1*, 1332−1335.
(86) Buchner, A.; Tostevin, F.; Gerland, U. *Phys. Rev. Lett.* **2013**, *110*, 208104.
(87) Mansson, M. O.; Siegbahn, N.; Mosbach, K. *Proc. Natl. Acad. Sci. U. S. A.* **1983**, *80*, 1487−1491.
(88) Mackey, D.; Killard, A. J.; Ambrosi, A.; Smyth, M. R. *Sens. Actuators, B* **2007**, *122*, 395−402.
(89) Dominguez, E.; Markovarga, G.; Hahnhagerdal, B.; Gorton, L. *Enzyme Microb. Technol.* **1994**, *16*, 216−222.
(90) Banerjee, G.; Car, S.; Scott-Craig, J. S.; Borrusch, M. S.; Walton, J. D. *Biotechnol. Biofuels* **2010**, *3*, 22.
(91) Zhang, Y.; Lyu, F.; Ge, J.; Liu, Z. *Chem. Commun.* **2014**, *50*, 12919−12922.
(92) Dvorak, P.; Kurumbang, N. P.; Bendl, J.; Brezovsky, J.; Prokop, Z.; Damborsky, J. *ChemBioChem* **2014**, *15*, 1891−1895.
(93) Dueber, J. E.; Wu, G. C.; Malmirchegini, G. R.; Moon, T. S.; Petzold, C. J.; Ullal, A. V.; Prather, K. L. J.; Keasling, J. D. *Nat. Biotechnol.* **2009**, *27*, 753−759.
(94) You, C.; Zhang, Y. H. *ACS Synth. Biol.* **2013**, *2*, 102−110.
(95) (a) Kreft, O.; Prevot, M.; Mohwald, H.; Sukhorukov, G. B. *Angew. Chem., Int. Ed.* **2007**, *46*, 5605−5608. (b) Baumler, H.; Georgieva, R. *Biomacromolecules* **2010**, *11*, 1480−1487.
(96) Keighron, J. D.; Keating, C. D. *Langmuir* **2010**, *26*, 18992−19000.
(97) You, C.; Myung, S.; Zhang, Y. H. *Angew. Chem., Int. Ed.* **2012**, *51*, 8787−8790.
(98) Wilner, O. I.; Shimron, S.; Weizmann, Y.; Wang, Z. G.; Willner, I. *Nano Lett.* **2009**, *9*, 2040−2043.
(99) Lunde, B. M.; Moore, C.; Varani, G. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 479−490.
(100) Pescador, P.; Toca-Herrera, J. L.; Donath, E.; Katakis, I. *Langmuir* **2008**, *24*, 14108−14114.
(101) Jia, F. *Graduate Theses and Dissertations, Paper 13378*; Iowa State University, 2013.
(102) Li, Z.; Zhang, Y.; Su, Y.; Ouyang, P.; Ge, J.; Liu, Z. *Chem. Commun.* **2014**, *50*, 12465−12468.

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ACS Catal. 2015, 5, 4503−4513