Chinese Journal of Chemical Engineering 28 (2020) 2028–2036

Contents lists available at ScienceDirect

Chinese Journal of Chemical Engineering

journal homepage: www.elsevier.com/locate/CJChE

Review

Microenvironmental engineering: An effective strategy for tailoring enzymatic activities

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ARTICLE INFO

Article history:
Received 25 January 2020
Received in revised form 7 April 2020
Accepted 19 April 2020
Available online 1 May 2020

Keywords:
Biocatalysis
Enzyme
Microenvironment
Activity enhancement

ABSTRACT

Rationally, engineering a favorable physicochemical microenvironment for enzymes has recently emerged as an effective strategy to improve their catalytic performance. In this review, we discuss four microenvironmental effects according to the mechanism of action: localizing and excluding reactants and regulators, regulating microenvironmental pH, creating a water-like microenvironment, and increasing the local temperature. These mechanisms are enzyme-independent and can in principle be used in combination to tailor enzyme behaviors, offering new approaches to enabling, enhancing, and regulating enzyme catalysis in diverse applications without the need for genetic engineering.

1. Introduction

Enzymes are versatile catalysts that have begun to reshape conventional chemical processes in a more efficient and eco-friendly manner [1–4]. While genetic approaches enable the discovery [5], mutation [6], and *de novo* design [7] of enzymes with better activity and stability, immobilization or chemical modification is equally important to endow enzymatic catalysts with features important for practical applications, including mechanical strength, reusability, stability, and even improved catalytic performance [8–11]. A key aspect of immobilization or chemical modification is that it provides a distinct microenvironment for the enzymes of interest.

Recent studies have shown that engineering a favorable microenvironment for enzymes is an effective approach to optimizing enzymatic reactions [12]. We define the microenvironment as the nano- and microscale physicochemical local environment around an enzyme or a group of enzymes catalyzing chemical reactions at their active sites. The physicochemical conditions (such as concentrations of reactants, pH and temperature) in the microenvironment can be significantly different from the bulk conditions due to immobilization on carriers, conjugation with polymers, encapsulation in nano- or microcompartments, aggregation into complexes, presence of additives and so forth. The construction of a microenvironment can stabilize an enzyme by sheltering it from an unfavorable bulk condition, regulate the enzymatic activity by concentrating or excluding substrates and cofactors, govern the reaction kinetics by influencing molecular diffusion, improve the efficiency of cascade reactions by compartmentalization, and even accelerate reactions by increasing the local temperature. These improvements can be additive and synergistic with improvements achieved by genetic protein engineering.

Although the importance of the microenvironment of enzymes has been well-accepted throughout the development of enzymatic catalysis, a comprehensive review of recent developments is timely. Past reviews are limited to specific aspects, such as the local pH effect of polyelectrolytes [13] and the enzyme hydration in organic solvents [14], which are known for more than 50 years and 30 years, respectively. A tutorial review by Lancaster *et al.* [12] published in 2018 introduced the advantages of microenvironment engineering in terms of enhancing enzyme reaction kinetics, tuning substrate specificity and shifting the pH optimum. However, the rapid progress in the field has already yielded new and significant developments.

Here, we aim to provide a comprehensive discussion of the microenvironmental engineering of enzymes. We particularly focus on the microenvironmental effects that are generally applicable independent of the enzyme species. We highlight four mechanisms: localizing and excluding reactants and regulators, regulating microenvironmental pH, creating a water-like microenvironment, and increasing the local temperature. These effects can be exploited to regulate the apparent enzyme performance without requiring an engineering of the intrinsic enzyme properties.

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https://doi.org/10.1016/j.cjche.2020.04.010

We do not discuss effects relying on specific features of certain enzymes, such as the interfacial activation of certain types of lipases upon environmental hydrophobicity [15], stabilization and/or activation of enzymes by nanoparticles [16], ions [17], ionic liquids [18] and so on. We also do not discuss conformational changes during enzyme immobilization or modification which often lead to changes in the activity, stability, pH or temperature optima, because the mechanisms are often ambiguous and the effects are unpredictable [19]. Similarly, the presence of crowding agents simulating a crowded cellular environment was reported to influence enzyme catalysis by factors including but not limited to volume exclusion [20], molecular diffusion [21], protein-folding dynamics [22], protein association/dissociation [23], stabilization [24], resulting in a decrease or increase in catalytic efficiency varying from case to case [25]. Due to this complexity, a complete understanding of the crowding effect is still lacking and it is not discussed here.

2. Localization and Exclusion of Reactants and Regulators

2.1. Single-enzyme catalysis

Past research has proven that a well-designed microenvironment can attract substrate molecules in the vicinity of an enzyme through electrostatic or hydrophobic interactions [26–28]. The increase in local substrate concentration promotes enzymatic activity showing a characteristic reduction in the apparent Michaelis–Menten constant, K_m. For example, trypsin covalently immobilized on an insoluble polyanionic polymer had a thirty-fold lower K_m value than the free enzyme toward its positively charged substrate benzoyl-L-arginine amide [29]. A similar reduction in K_m was also observed in the cases of lysozyme [30] and α-chymotrypsin [31] assembled with different polyelectrolytes when the polyelectrolytes were oppositely charged to their substrates. Additionally, the activity of α-chymotrypsin for the cationic substrates was inhibited in the presence of a cationic polymer, indicating a partitioning effect of the polymer on the substrates in the enzyme microenvironment based on electrostatic interactions [31].

Although the above examples demonstrated the effectiveness of the partitioning effect, a quantitative understanding of the attraction-induced partitioning is difficult in these experiments due to the complex composition, uncontrolled structural morphology, and random enzyme distribution. Enzymes on DNA scaffolds with well-defined structures can serve as ideal model systems. Wheeldon and colleagues [32] suggested that the activity of an enzyme could be altered by introducing substrate-DNA interactions outside its active site, which capture substrate molecules and direct them to the active site (Fig. 1A). By conjugating horseradish peroxidase (HRP) with a short double-stranded DNA which is binding the substrate tetramethylbenzidine (TMB) with a K_d of 11 μmol·L^{-1}, the K_m of HRP for TMB was 2.6-fold reduced while the k_{cat} was unchanged. The DNA sequence-dependent binding strength and the ionic strength-dependent K_m reduction indicated that the DNA-TMB binding involved both short-range interactions and electrostatic interactions. In contrast, the kinetics of HRP for the substrate ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was unaffected by DNA conjugation because ABTS does not interact with DNA ($K_d \gg 1$ mmol·L^{-1}). Inhibitors with binding affinities for DNA in a range of 69 to 151 μmol·L^{-1} can also be accumulated in the proximity of the HRP-DNA conjugate, causing stronger inhibition [32]. In addition,

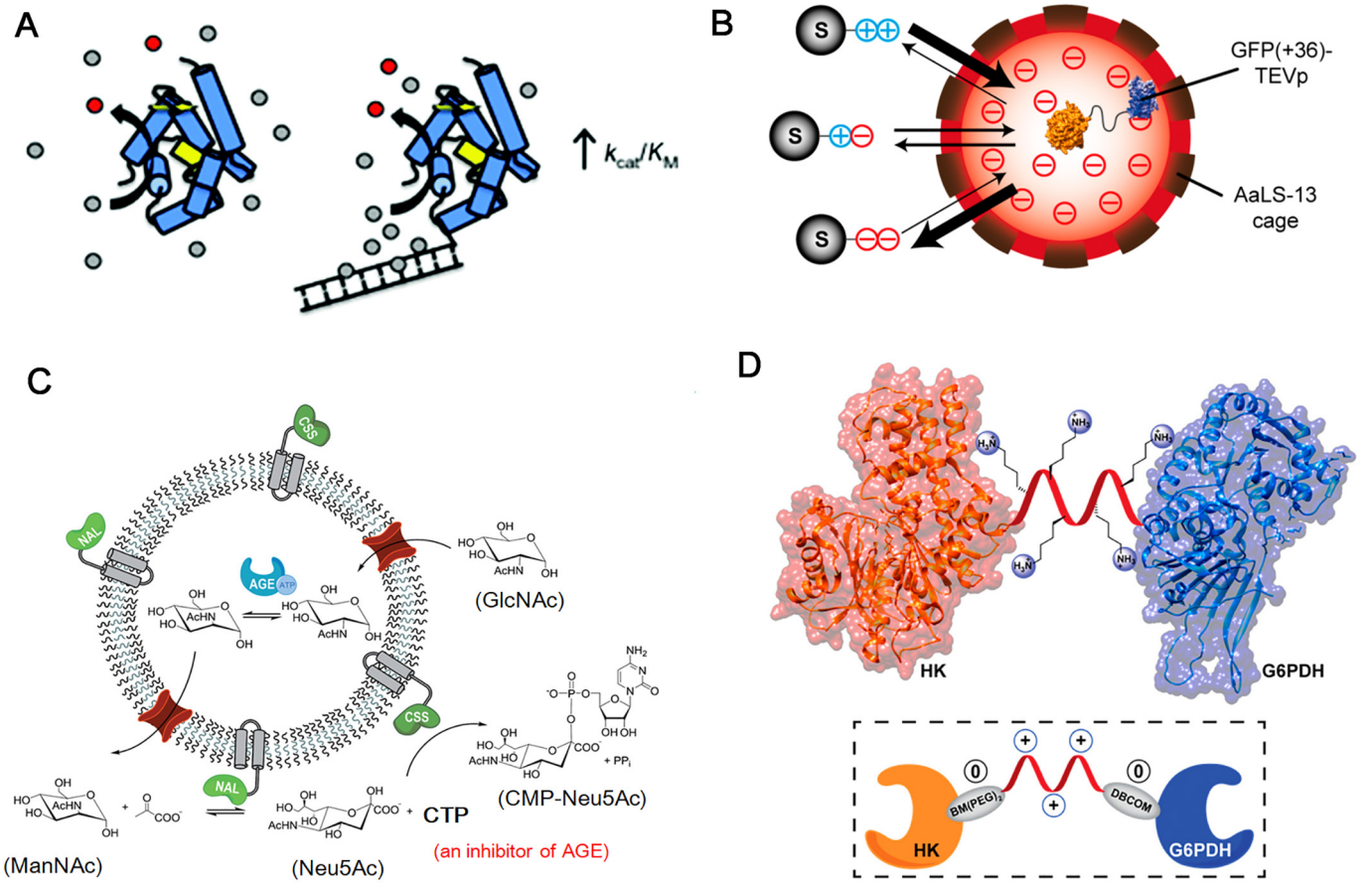

Fig. 1. Schematic illustrations of the partitioning effect of microenvironments. (A) Substrate-DNA interactions increase the local concentration of substrates around an enzyme. The gray circles represent substrate molecules and the red ones represent products. (B) A supercharged nanoreactor regulates the substrate specificity through electrostatic interactions. (C) Encapsulation of the enzyme *N*-acyl-$_D$-glucosamine 2-epimerase (AGE) prevents the cross-inhibition by cytidine triphosphate (CTP). (D) A peptide bridge enables the intermediate channeling in the HK-G6PDH cascade. Reprinted from Ref. [32], Ref. [33], Ref. [34], Ref. [35] with permission from the American Chemical Society.

the strong interactions between substrates and scaffolds also hinder the enzymatic activity [33-36]. Such regulation in activity resembles the Sabatier principle, that is, only moderate interactions between substrates/inhibitors/cofactors and scaffolds effectively tune the enzymatic kinetics; neither too weak nor too strong binding is desirable. By testing the activity of HRP-DNA conjugate toward varied substrates, Lin *et al.* demonstrated that the effective activity enhancement of HRP occurred for the substrates that interact with DNA structures with K_d values of several hundred micromoles [36].

Compartmentalization is also an effective way to localize and/or exclude reactants and regulators. Numerous compartments have been developed as biocatalytic nanoreactors, such as polyionic complex micelles [37], layer-by-layer-assembled capsules [38], viral capsids [39], liposomes [40], polymersomes [41], *etc.*, and each compartment shows a different partitioning efficiency and permeation selectivity. A summary of enzymatic reactions in confinement can be found in these comprehensive reviews [42–44]. An ideal partitioning effect requires compartmentalization which allows only specific molecules to diffuse in and out. Einfalt *et al.* [45] prepared an HRP-encapsulated polymersome self-assembled from an amphiphilic block copolymer inserted with a cysteine double mutant of outer membrane protein F porins (OmpF-M). Located in the pore of OmpF-M, these two cysteine residues can serve as a gate by forming/cleaving disulfide bonds with other thiol compounds controlled by the environmental redox potential. Such a construction controls the enzymatic reaction in terms of both substrate molecular weight and redox stimulus. The addition of glutathione can restore the catalytic activity of HRP-loaded polymersomes containing OmpF-M with a molecular cap. Azuma *et al.* [33] demonstrated that electrostatic interactions were also effective in improving the substrate specificity of enzymes in charged nanoreactors. They used a negatively supercharged protein cage made of *Aquifex aeolicus* lumazine synthase (AaLS-13) which can spontaneously encapsulate a protease from Tobacco Etch virus (TEV) fused to a supercharged green fluorescent protein, GFP(+36)-TEVp.(Fig. 1B). They called the proteins "supercharged" as these proteins are mutations that possess considerably more charges than their wild types. The encapsulated protease preferentially takes up and hydrolyzes cationic peptides over the anionic and zwitterionic peptides. The cationic peptides are supposed to be localized in the negatively charged lumen of the nanoreactor, leading to a 14-fold decrease in K_m and 5–6 fold increase in k_{cat}/K_m compared with the unencapsulated protease.

2.2. Enzyme cascades

The microenvironmental partitioning also plays an important role in enhancing enzyme cascade throughput by accumulating substrates, saving unstable intermediates and excluding toxic reactants. For a compartment with weak permeation selectivity, preventing the loss of intermediates also means an increased resistance for substrates to diffuse in and products to diffuse out. Therefore, enhancing cascade throughput requires optimization of the diffusive resistance of the compartment for the transport of substrate, intermediate and product molecules, as emphasized by Tsitkov and Hess [46]. Moreover, their modeling pointed out that the compartmentalized cascade is only more productive than the non-compartmentalized cascade if the elimination rate of intermediate in the bulk exceeds a critical rate that is determined by the turnover rates of the encapsulated enzymes [46].

Compartmentalization with selective permeability enables enzyme cascades that suffer from intrinsically incompatible reactions to work at a higher efficiency [47]. For example, a cascade composed of *N*-acyl-D-glucosamine 2-epimerase (AGE), *N*-acetylneuraminate lyase (NAL), and CMP-sialic acid synthetase (CSS) for the synthesis of CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac) is hampered by strong inhibition of the first enzyme AGE by the substrate, cytidine triphosphate (CTP), of the third enzyme CSS. Klermund *et al.* [34] segregated AGE inside a polymersome that incorporated with a selective channel protein capable of blocking CTP outside, while anchoring the other two enzymes NAL and CSS on the outer surface (Fig. 1C). This construction protects the activity of AGE in a microenvironment by avoiding cross-inhibition in cascade reactions, resulting in 2-fold increased production of CMP-Neu5Ac.

Without a physical boundary, moderate interactions between intermediate substrates and scaffolds/modifiers prevent the loss of intermediate substrates to some extent. Barton and coworkers [35] linked two cascade enzymes, hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PDH), through a cationic poly(lysine) peptide and found that the experimental lag time for reaching the steady-state activity shortened (Fig. 1D). They attributed this effect to the electrostatic channeling of anionic intermediates, oxalate and glucose-6-phosphate, *via* the peptide bridge. The channeling process has been studied in detail using computational approaches. Molecular dynamics simulations have revealed a rapid adsorption and a contacting surface diffusion of substrates on the peptide bridge [35], and described the transport probability between the peptide bridge and G6PDH binding pocket [48]. Integrated with MD trajectories, a Markov-state model was applied to map and visualize the two-step channeling process, first from the active pocket of HK to the peptide bridge and then to the active pocket of G6PDH [49].

The above investigations demonstrate versatile strategies for partitioning substrate/product/regulators to control over the activity of single and cascade enzymes. We expect that these design strategies will promote the developments of high-efficiency enzyme catalysts and enzyme-replacement therapy products in practical applications.

2.3. Stabilization

Entrapment or compartmentalization of enzymes in a protective microenvironment capable of blocking proteolytic agents is of particular interest in protein drug delivery. For example, enzymes encapsulated in liposomes [50], micelles [51], metal–organic frameworks [52], DNA origami cages [53], nanogels [54], *etc.* all showed significantly enhanced stability against protease digestion and *in vivo* delivery trials have been conducted by different groups [54,55]. As the benefit is readily comprehensible, we do not discuss this effect here in detail.

3. Regulating Microenvironmental pH

The catalytic activity of most enzymes is highly pH-dependent. The intrinsic pH dependence of an enzyme is usually dominated by the protonation/deprotonation of key residues at the active site at different pH [56]. Site-specific mutagenesis at the substrate binding pocket can alter the pH dependence of a particular enzyme, but also leads with a high likelihood to a significant decrease in the catalytic efficiency [57,58]. On the other hand, adjusting buffer pH for a specific enzyme is always much easier than mutagenesis. Therefore, the intentional manipulation of the pH dependence of an enzyme rarely became a practical concern. However, it is important for one-pot multienzyme catalysis that employs enzymes with varying pH optima since the maximal throughput cannot be achieved in the same buffer.

Immobilization provides an alternative approach to tuning the operating pH conditions of an enzyme, because the pH-activity profile of an enzyme often shifts after immobilization. The shift is significant and predictable if the enzymes are immobilized on polyelectrolyte carriers, as demonstrated by Goldstein and colleagues five decades ago [13,26,29]. They found that the pH-activity profiles of various enzymes, such as trypsin, chymotrypsin, papain and subtilopeptidase A, shifted toward more alkaline pH ranges if they were bound to polyanionic carriers, and shifted toward more acidic pH ranges if they were bound to polycationic carriers, compared to the native enzymes [13,26,29,59]. This effect was weakened at high ionic strength. They concluded that the highly charged matrix maintained a more acidic (in polyanionic matrices) or a more alkaline (in polycationic matrices) microenvironment

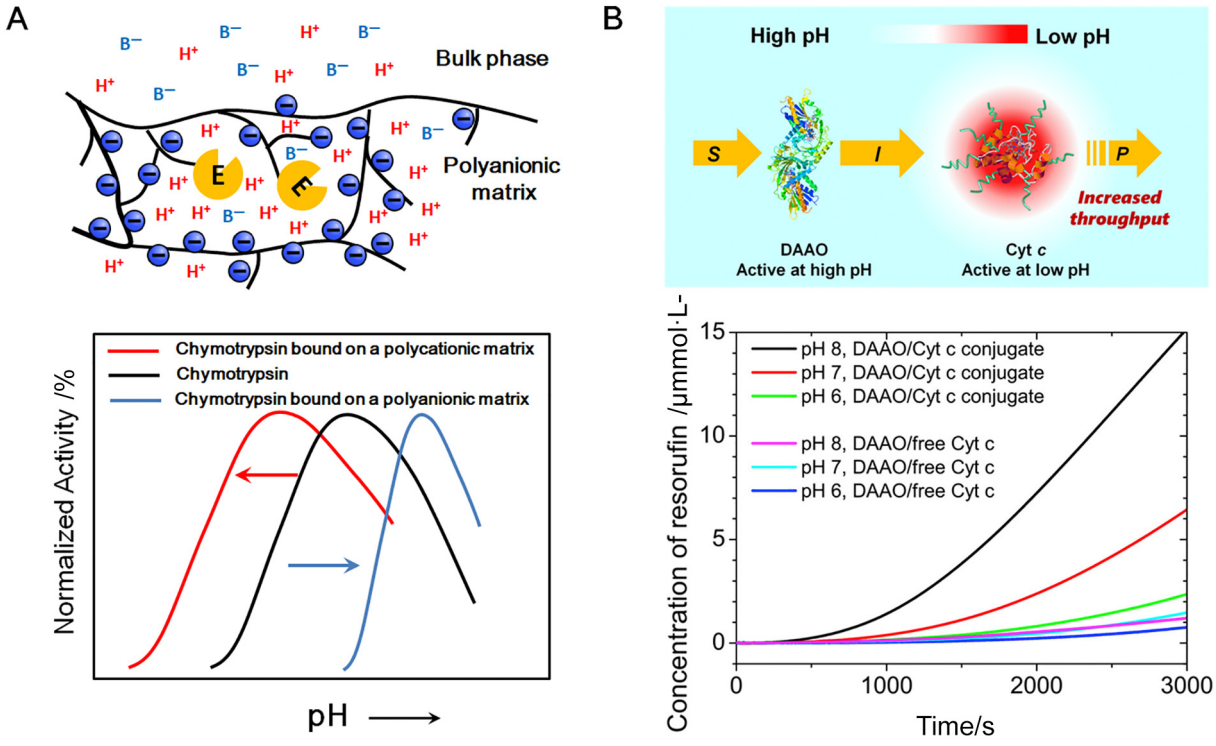

Fig. 2. Microenvironmental pH effect on single enzymes and cascade enzymes. (A) Schematic representation of the proton distribution between a polyanionic matrix and the bulk solution (upper left panel), and an illustration of the shifts in the pH-activity profile of chymotrypsin bound on a polyanionic or polycationic matrices (lower left panel). Adapted with permission from Ref. [59]. Copyright 1972, American Chemical Society. (B) Activity enhancement in the DAAO-Cyt *c* cascade by creating a more acidic pH microenvironment for Cyt *c*. Reprinted with permission from Ref. [60]. Copyright 2017, American Chemical Society.

around the immobilized enzymes relative to the bulk solution (Fig. 2A). This effect has been verified by different groups with various enzymes and polymers [27,61–64], therefore, immobilization of enzymes onto polyelectrolyte carriers can be considered as a general approach to manipulating the pH dependence of enzymes.

The microenvironmental pH effect regained interest when DNA nanotechnology met enzyme catalysis [65,66]. DNA nanostructures can be programmed with respect to shapes and conjugation sites and are capable of arranging multiple enzymes in space with nanometer precision. Many investigations in the last decade reported that cascade enzymes tethered on DNA scaffolds exhibited several-fold enhancements in overall activity compared to the untethered counterparts [56,66–68]. Such enhancements were also found when cascade enzymes were colocalized on protein scaffolds [69,70]. The enhancement was often attributed to the proximity channeling that facilitated the transfer of intermediate substrates from the first enzyme to the next. Although simulations suggested that the concentration of intermediate substrate was initially higher in the vicinity of the first enzyme, it would quickly equilibrate with the bulk solution within several milliseconds due to the high diffusivity of the small molecular intermediates [71]. Therefore, proximity cannot be responsible for the observed activity enhancement of an enzyme cascade reaction on a scaffold. This has been directly demonstrated for a model cascade system composed of glucose oxidase (GOx) and HRP [72]. When these two enzymes were conjugated with a short molecular linker, the conjugated enzymes did not show any enhanced activity compared with the freely diffusing enzymes. Moreover, adding a competing enzyme to the bulk solution for eliminating the intermediate substrate can completely suppress the production, indicating that the direct channeling within the conjugate is negligible [72]. More detailed discussions on proximity channeling can be found in a series of recent reviews by Zhang and Hess [73], Rabe *et al.* [74], Sweetlove and Fernie [75], and research reports by Eun *et al.* [76], Kuzmak *et al.* [77], *etc.* It is worth noting that the steady state of a cascade reaction does not depend on channeling [78–80], thus a steady-state enhancement of the overall production requires an increased activity of the rate-limiting enzymes. The observed steady-state enhancement thus implies that scaffolds have an effect on the activity of immobilized enzymes rather than the intermediate transport. We hypothesized that, particularly for the DNA-scaffolded enzymes, the highly negatively charged DNA nanostructures can create a relatively more acidic microenvironment for the enzymes than the bulk solution [72], resembling the effect of polyelectrolyte carriers on immobilized enzymes described above.

In fact, the activity changes of enzymes on DNA scaffolds were previously observed although they had not been considered as the origin of activity enhancement. For example, Linko *et al.* [81] found that the activity of GOx and HRP increased by 3-fold and 5-fold respectively when they were separately tethered in a tubular DNA origami nanoreactor. Zhao *et al.* [53] reported that enzymes such as GOx, HRP, G6PDH, lactate dehydrogenase, and malate dehydrogenase encapsulated in DNA nanocages all displayed 4–10 times higher activity than the free enzymes, indicating a significant effect of scaffolds. The apparent activity of enzymes is not only affected by DNA origami nanoreactors but also by long DNA strands. Collins *et al.* [82] confirmed activity enhancement of HRP conjugated with long DNA duplexes and the enhancements increased as more and longer DNA duplexes were associated with the enzyme.

The effect of polyelectrolytes on the pH dependence of the immobilized enzyme inspires an elegant method to overlap the operating pH of multienzyme systems composed of enzymes with distinct pH optima. A proof of concept was demonstrated with a cascade reaction that is catalyzed by cytochrome c (Cyt *c*) and D-amino acid oxidase (DAAO) [60]. Although Cyt *c* is more active at acidic pH while DAAO is more active at alkaline pH, conjugating negatively charged poly(methacrylic acid) with Cyt *c* can effectively shift its pH optimum to natural and alkaline conditions, thus enabling both enzymes to work ten times more efficiently at pH 8 than the mixture

of free enzymes (Fig. 2B). Looking to the future, cell-free biosynthesis processes that systematically integrate multiple enzymes to produce high-value-added chemicals will be an important trend in biomanufacturing. Rationally engineering the microenvironmental pH for the involving enzymes will greatly enhance the production of the cell-free multienzyme processes.

4. Creating a Water-like Microenvironment for Non-aqueous Enzymatic Reactions

It is generally accepted that water plays an essential role in the structure and function of enzymes, while a long-standing question remains as to what the minimal hydration level is required for catalysis to occur. The past three decades have proven that enzymes are catalytically active in non-aqueous media including organic liquids [83], supercritical fluids [84], and even gas phases [85], expanding the application scenarios of enzymes to reactions which are unfavorable in an aqueous system. However, the apparent enzyme activity in these media is highly dependent on the water activity in the media and is often several orders of magnitude lower than the activity of their counterparts in an aqueous environment. The significant reduction in activity can be mainly ascribed to the inflexibility of enzyme conformations, the intolerance to solvents, and the inaccessibility of substrates. Although there are limitations arising from the utilization of non-aqueous bulk media, achieving enzyme activity comparable to that in the aqueous phase is possible if optimal conditions are selected [86]. Engineering a favorable microenvironment is one of the most general and effective approaches to improving the activity and the stability of enzymes. In addition to the practical benefits, it sheds light on the mechanism of enzyme hydration upon protein folding and function.

4.1. Improving enzyme catalysis in organic solvents

Klibanov and coworkers discovered that enzymes can perform functions in numerous nearly anhydrous organic solvents 30 years ago [87], opening a new chapter of enzyme catalysis dealing with compounds poorly soluble or reactions unfavorable in water. The catalysis of an enzyme (usually in a lyophilized suspension) was thought to require a certain level of hydration to maintain its native conformation and conformational mobility [88,89]. Increasing the water content (often described as water activity) can dramatically increase the enzyme activity in organic solvents, and the steep rise often occurs at a water activity around 0.4 [90]. The nature of solvents also affects the hydration of enzymes. Nonpolar solvents, in which the water activity is commonly low, can promote the formation of water clusters bound to the enzyme surface, while the poor solvation of the enzyme molecule leads to a rigid conformation and consequently low activity but enhanced thermostability. In contrast, polar solvents can strip off and replace the essential bound water molecules from the enzyme surface, resulting in a severe decrease in activity and stability [91,92]. Although organic solvent tolerance varies from enzyme to enzyme, a common suggestion is that nonpolar organic solvents are preferred to polar solvents since the former ones ensure a more hydrated microenvironment [88,93]. The control of hydration to balance the structural flexibility and stability is key to achieving high catalytic efficiency of an enzyme in organic solvents.

Early investigations suggested that the addition of inorganic salts, polyols, polyethylene glycol, sugars, polysaccharides, *etc.* to an enzyme solution before lyophilization could improve the enzymatic performance in organic solvents. For example, Dai and Klibanov found that four oxidative enzymes colyophilized with lyoprotectants such as polyols and polyethylene glycol could exhibit ten- to hundred-fold higher activity in harsh solvents like 97% acetone [94]. These hydrophilic additives were supposed to be able to protect the overall native enzyme conformation by replacing the surface-bound water molecules, whose loss during lyophilization without additives leads to enzyme inactivation.

The effect of additives supports the feasibility and effectiveness of engineering a water-like microenvironment for an enzyme, but the additives are usually small molecules that are easy to lose to reaction solvents during catalytic processes and are difficult to remove from the products after reactions. Therefore, immobilization or encapsulation of enzymes in a matrix providing a protective microenvironment has practical advantages. Bruns and Tiller [95] presented a nanophase-separated amphiphilic network with sponge-like microstructures that can entrap enzymes in its hydrophilic domains while allowing the diffusion of substrates into its hydrophobic domains. The entrapped HRP and chloroperoxidase exhibited ten- to hundred-fold higher activity and also improved stability compared to the native enzyme suspension in *n*-heptane. Liu and colleagues [96] showed that confining enzyme in a hydrophilic polyacrylamide nanogel through *in-situ* polymerization could greatly improve the thermal stability and solvent tolerance in polar organic solvents (Fig. 3A). For example, the nanogel encapsulated HRP retained 80% of its initial activity in the mixed aqueous-organic solutions (such as 15 vol% methanol, 15 vol% tetrahydrofuran, and 15 vol%

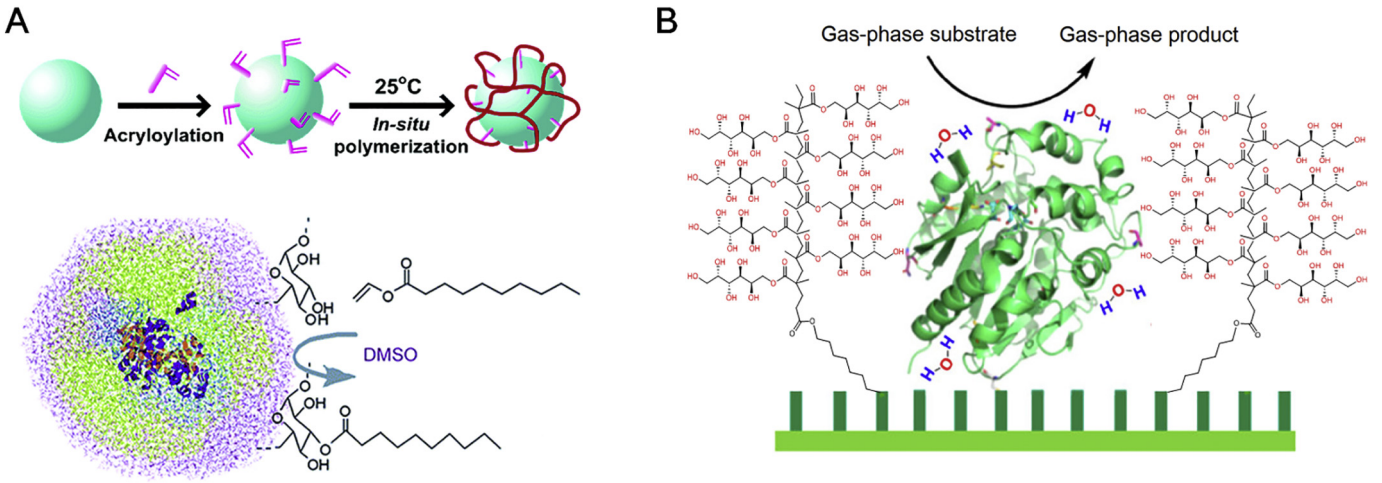

Fig. 3. Mimicking a water-like environment for non-aqueous enzymatic reactions. (A) Schematic illustrations of enzyme encapsulation in a polyacrylamide nanogel (upper left panel) and the hydrophilic polymeric network (green) around a lipase enzyme in the presence of DMSO (purple) (lower left panel). (B) Schematic representation of co-immobilized haloalkane dehalogenase on a surface displaying poly(sorbitol methacrylate) chains.
Reproduced with permission from Ref. [96], Ref. [97] and Ref. [98]. Copyright 2006, 2009 and 2017, American Chemical Society.

dioxane) at 60 °C for 10 min, whereas the free HRP completely lost its activity. Even in anhydrous DMSO, the *Candida rugosa* lipase nanogel maintained its initial transesterification activity at 60 °C for 10 days (Fig. 3A) [97]. Molecular Dynamics simulations indicated that the hydrophilic groups from the hydrophilic polymeric network near the enzyme prevented the stripping of essential water molecules from the enzyme surface by reducing the local content of polar organic solvents [99]. Such a mechanism may also play a role when enzymes are crosslinked into enzyme aggregates, conjugated with hydrophilic polymers, or immobilized on carriers.

4.2. Activating enzymes in the gas phase

Enzymes can also catalyze reactions in the gas phase. Yagi *et al.* introduced in 1969 the first gas-phase conversion of *ortho*-H_2 to *para*-H_2 catalyzed by lyophilized hydrogenase treated with $Na_2S_2O_4$ [100]. The investigation by Barzana, Klibanov and Karel in 1987 showed that dehydrated alcohol oxidase adsorbed on DEAE-cellulose could catalyze the oxidation of ethanol vapors with oxygen [85]. These dry enzyme preparations often show greatly enhanced thermal stability and hydration dependent activity, resembling the effects in organic solvents. Although some investigations suggested a degree of hydration threshold (*ca.* 0.2 g water per gram of protein) for enzyme to function in the gas phase, Dunn and Daniel disputed this notion by pointing out that many enzymes operate at very low degree of hydration (*e.g.*, porcine pancreatic lipase at a hydration degree of 0.002 g water g^{-1} protein), although the activity is also low [101]. Such a low hydration indicates a very limited surface coverage of water on protein and is inadequate to be structurally essential. Dunn and Daniel therefore suggested that the hydration activation may originate from an optimized structure or improved flexibility of enzymes in the presence of high humidity, but hydration itself was not required for enzymes to function [102]. Similar to enzymatic catalysis in the organic phase, the presence of additives such as glycerol and sucrose can enhance the activity and stability of enzymes acting on gaseous substrates [103,104]. Mann and colleagues [105,106] reported an intriguing solvent-free protein liquid prepared by lyophilization of a cationized protein-anionic polymer solution followed by annealing at 50 °C. The obtained protein liquid was highly viscous at room temperature. It had a very low content of water (*ca.* 0.1%), which was insufficient to generate a solvation shell for the protein. Instead, the protein is surrounded by a monolayer of polymer surfactant [106]. Interestingly, the solvent-free myoglobin liquid was still capable of reversibly binding oxygen, and the protein underwent unfolding and refolding upon temperature changes in the absence of water [107].

The above discussion implies that "essential water" may not be essential for catalysis and that it can possibly be replaced by other chemical agents capable of providing similar interactions with the protein. A recent investigation by Badieyan *et al.* [98] lends support to this hypothesis. They covalently immobilized haloalkane dehalogenase on a surface displaying poly(sorbitol methacrylate) chains, which provides a hydrophilic microenvironment for the enzyme (Fig. 3B). In catalyzing the gas-phase dehalogenation of 1-bromopropane, the activity of the immobilized haloalkane dehalogenase at ambient humidity was as high as 25% of its activity in buffer, 40-fold higher than the activity of the lyophilized enzyme at the same condition. Circular dichroism spectra revealed that the immobilized enzyme in the dry state kept its secondary structure almost independent of the relative humidity in the range from 26% to 80%. They concluded that poly(sorbitol methacrylate) could replace the water-protein interaction in an environment of low water activity. Inspired by this work, Xu *et al.* [108] prepared an aerogel from *Candida Antarctica* lipase B (CALB) and hydroxyl-rich graphene oxide (GO) through electrostatic gelation and subsequent lyophilization. Although the obtained CALB-GO aerogel displays its highest transesterification activity at a water activity of 0.34, it retains 67% of the activity maximum in an almost anhydrous condition. Molecular Dynamics simulations confirmed that the formation of hydrogen bonds between CALB and GO reduced the number of bound water molecules which were essential to maintain the active enzyme conformation [109]. B-factor analysis [110] revealed that the protein flexibility near the active site could be improved by interacting with GO sheets with higher oxygen content. These results not only inspire the construction of microenvironments for better enzyme catalysts used in non-aqueous systems, but also offer a new perspective on the previously stipulated essential role of water in enzyme catalysis.

The above demonstrations have shown the great promise of fully exploiting the catalytic ability of enzymes in non-aqueous systems, while the practical applications are still rare in industrial manufacturing. Future efforts should focus on developing alternative enzymatic approaches to replace conventional organochemical processes, and also improving the industrial applicability of the engineered enzymes in terms of the ease of preparation, stability, reusability and mechanical strength.

5. Locally Heating the Microenvironment of Enzymes

Temperature affects the rate of any chemical reaction. Similar to most chemical reactions, a 10 K increase in temperature can boost the reaction rates of most enzymes by 50% to 100%. On the other hand, the folding state of an enzyme is also highly sensitive to the surrounding temperature. A relatively high temperature (*e.g.* above 40 °C for most animal enzymes) can lead to inactivation and even denaturation of enzymes [111]. Therefore, the temperature dependence of enzyme activity is usually a bell-shaped curve, and maintaining the enzymatic reactions at the temperature optima is often recommended. However, heating the entire reaction system is energetically inefficient due to the high specific heat capacity of water. Thus, locally increasing the temperature near the enzymes becomes appealing for enhancing and controlling the activity of enzymes.

Considering the size of enzymes, plasmonic nanoparticles with photothermal effects are ideal nano-heaters for this task. Blankschien

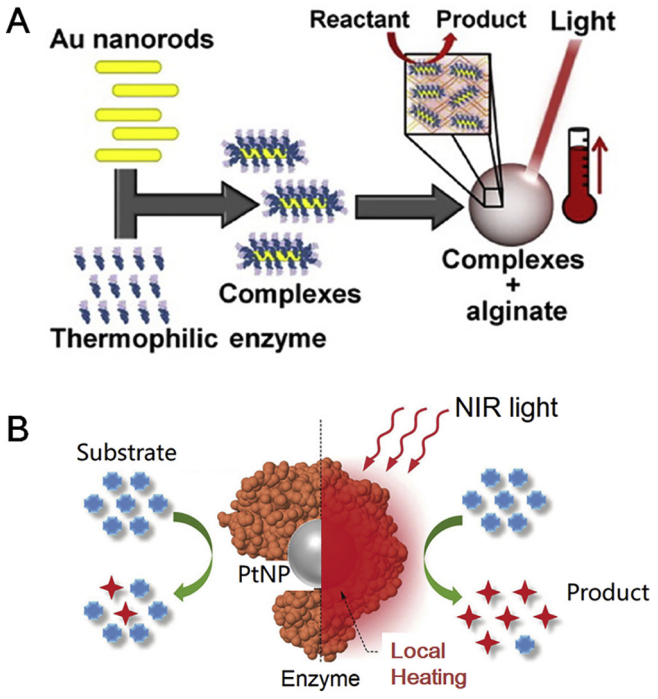

Fig. 4. Two proposed strategies to increase the local temperature around enzymes. (A) Immobilization of enzymes on gold nanorods followed by encapsulation in Ca-alginate beads. Reproduced with permission from Ref. [112].
Copyright 2013, American Chemical Society. (B) Incorporation of platinum nanoparticles inside enzymes. Reproduced with permission from Ref. [113]. Copyright 2017, Wiley.

et al. [112] demonstrated the first example of locally heating the microenvironment of enzymes through this strategy. They immobilized thermophilic *A. pernix* glucokinase on gold nanorods (33 nm × 10.4 nm) through the thiol–gold interaction and then encapsulated the obtained enzyme-AuNR complexes in Ca-alginate beads with a diameter of 2 mm (Fig. 4A). The specific activity of the Ca-alginate beads encapsulated enzyme-AuNRs complexes increased by 60% upon continuous irradiation with 11 $W\cdot cm^{-2}$ of 800 nm near-infrared light, which was interpreted to result from a 20 °C temperature increase inside the beads whereas the temperature in the bulk solution only increased 2 °C after irradiation. Researchers have also attempted to achieve a nanoscale temperature gradient around the bare enzymes. Wang *et al.* [113] synthesized platinum nanoparticles with diameters of 2–3 nm and anchored them inside enzymes (Fig. 4B). The platinum nanoparticles (Pt-NPs) are efficient thermoplasmonic light-to-heat converter that can generate a locally high temperature upon near-infrared irradiation. The enzymes (including glucoamylase, glucose oxidase, catalase, and proteinase K) embedded with Pt-NPs exhibited a two-fold activity enhancement after receiving pulsed near-infrared irradiation of 0.2 $W\cdot cm^{-2}$ for 10 min. During the irradiation, the bulk temperature in solutions remained stable. As a control, the mixture of enzymes and Pt-NPs did not show significant changes in activity in the presence and absence of light exposure, implying that the activity enhancement was from a local thermal effect near the enzymes rather than a bulk heating effect.

In addition to the temperature-induced activity increase, the remote manipulation of the local temperature near the enzyme can potentially switch the enzymatic activity on and off by taking advantage of thermoresponsive materials. Zhang *et al.* [114] presented a strategy for tuning the accessibility of macromolecular substrates toward the corresponding enzymes. The enzymes with 2–3 nm platinum nanoparticles embedded inside were conjugated with a temperature-responsive copolymer of acrylamide and acrylonitrile [poly(AAm-co-AN)], which formed microscale aggregates at a temperature below the upper critical solution temperature (UCST). Upon near-infrared irradiation, the photothermal heating by platinum nanoparticles increased the local temperature above the UCST and then the aggregates became soluble, exposing enzymes to their macromolecular substrates. The effectiveness of this strategy has been verified for glucoamylase toward starch, proteinase K toward casein, and deoxyribonuclease toward plasmid, showing 20 to 50 times higher activities in the on-state.

Although in the above investigations the plasmonic photothermal nanoparticles were all directly linked with the enzymes, the heating effect cannot be constrained to the vicinity of enzymes. As the rate of heat production *via* light absorption is much slower than thermal diffusion, the temperature in the bulk solution has also to increase during the light irradiation. Simulations suggested that the dissipation of heat from a 2 nm silver nanoparticle initially at 400 K to the surrounding 300 K water took only several picoseconds [115]. In other words, it is extremely difficult to maintain a stable temperature gradient across a few nanometers [116]. The proposed nanoscale temperature gradients were not confirmed by direct measurements. In some cases, such as the microscale aggregates incorporated with multiple nanoparticles, the hybrid and hierarchical structures may introduce additional heat transfer resistance and may thus contribute to a relatively higher temperature in the vicinity of heat sources [117], but a rigorous analysis is desired. Future investigations should profile the temperature distribution and the dynamics of heat transfer in the microenvironment of the enzyme-photothermal nanoparticle complexes, providing design principles for the construction of microstructures that can prevent the generated heat from rapid dissipating to the surroundings.

6. Conclusions

Enhancing and precisely tuning the activity of enzymes is remarkably beneficial for a variety of applications including pharmaceutical synthesis, biofuel production and clinical diagnostics. Microenvironmental engineering has been proven to be an effective strategy to improve and control the catalytic performance of enzymes without interfering with their intrinsic properties. In this review, we highlight the effects and mechanisms affecting the microenvironment such as partitioning reactants and regulators, altering local pH and temperature, and sheltering enzymes in a water-like milieu. These effects can be imposed on enzymes of interest either separately or synergistically, offering a tool chest to upgrade the functionality and controllability of enzymatic catalysts. Together with protein engineering, we believe that microenvironmental engineering will promote practical applications of enzymes in harsh conditions and unnatural scenarios.

Acknowledgements

This work was supported by the National Science Foundation of USA under Award Number NSF-ENG 1844149.

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