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Expanding the boundary of biocatalysis: design and optimization of in vitro tandem catalytic reactions for biochemical production

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ABSTRACT

Biocatalysts have been increasingly used in the synthesis of fine chemicals and medicinal compounds due to significant advances in enzyme discovery and engineering. To mimic the synergistic effects of cascade reactions catalyzed by multiple enzymes in nature, researchers have been developing artificial tandem enzymatic reactions in vivo by harnessing synthetic biology and metabolic engineering tools. There is also growing interest in the development of one-pot tandem enzymatic or chemo-enzymatic processes in vitro due to their neat and concise catalytic systems and product purification procedures. In this review, we will briefly summarize the strategies of designing and optimizing in vitro tandem catalytic reactions, highlight a few representative examples, and discuss the future trend in this field.

Introduction

In living organisms, an individual enzyme typically catalyzes a simple reaction, but the cooperative action of a series of enzymes builds up complex molecules from simple molecules to support the living functions such as generation or storage of energy, as well as environmental sensing and communication. The cascade or concurrent reactions catalyzed by multiple enzymes demonstrate the strongest synthetic power in nature, which makes an irreversible process reversible, eliminates inhibition problems caused by excess products, or circumvents the lack of substrates scattered in bulk solution. The synergistic effect of natural synthetic networks inspires chemists to develop artificial multi-step tandem reactions for selective synthesis of complex molecules (Clomburg et al. 2017). To date, tandem reactions by using whole microorganisms (in vivo) have been extensively studied in the synthetic biology and metabolic engineering fields (Nielsen and Keasling 2016). There are growing interests to build cooperative tandem reactions by coupling multiple enzymes or combining organometallic catalysts with biocatalysts in vitro since the resulting catalytic systems are simpler for optimization, generate less side products and waste and allow for easy product purification (Kohler and Turner 2015).

To date, in vitro tandem enzymatic reactions have been well developed for the preparation of alcohols, acids, acid derivatives, amino acids, amines, nucleosides/nucleotides, nucleosides/nucleotides derivatives, oligosaccharides and glycoconjugates (Bornscheuer et al. 2012). The field of tandem chemo-enzymatic reaction is still in its infancy (Wang and Zhao 2016). Recently, lipase has been successfully coupled with various transition metal complexes for readily deracemizing secondary alcohols and primary amines (Verho and Bäckvall 2015). This accomplishment clearly shows the advantages of multistep one-pot processes, so-called tandem reactions. Compared with stepwise synthesis, one-pot tandem reactions improve overall synthetic efficiency and reduce waste generation by avoiding the intermediate purification steps. Additionally, tandem reactions improve the overall asymmetric synthesis efficiency by converting achiral substrates to single enantiopure product containing a single enantiomer. Last but not least, the cooperative effect among different catalytic steps enhances the selectivity and activity by an equilibrium process.

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However, combining multiple enzymes or combining enzymes with chemical catalysts in a tandem process is not simply replicating the catalytic behavior of each catalyst. For multienzymatic reactions, several strategies such as cell free metabolic engineering (CFME) and modeling (Dudley et al. 2015) have been developed to balance the reaction rate of each step and maintain the enzymatic activity under reaction conditions. Protein engineering methods have been applied, such as protein immobilization and fusion protein construction, to improve the overall efficiency of multi-enzymatic reactions (Ji et al. 2016; Jia et al. 2014). Specifically, it is more challenging to find conditions to enable catalytic activity of organometallic catalysts and biocatalysts in one-pot due to incompatibility and mutual inactivation. To overcome those difficulties, several novel strategies have been developed, including supramolecular assembly and compartmentalization. In this review, we will summarize the major tandem enzymatic and chemoenzymatic reactions developed so far for selective synthesis and discuss the design and engineering strategies for system optimization.

Tandem enzymatic reactions

Tandem enzymatic reactions: simple systems

From aerobic respiration to photosynthesis, millions of compounds are synthesized through enzymatic reactions during the lifespan of all living organisms. These compounds, even those with very complex structures, are converted from simple chemical resources such as glucose, through the metabolic network in the cell. Tandem enzymatic reactions, or pathways in other words, can be considered as subunits of the whole metabolic network. Although enzymatic reactions have long been used for chemical synthesis due to many of their excellent features, particularly the high selectivity and specificity, multiple tandem enzymatic reaction systems developed by chemists recently are not simple combinations of enzymatic reactions catalyzed by individual enzymes. Besides all the advantageous properties of enzymatic reactions, tandem enzymatic reactions can be much more cost-effective by using inexpensive substrates, potentially making them economically feasible. Moreover, one-pot tandem enzymatic reactions simply avoid the purification of the intermediates, which makes the process more efficient than step-wise enzymatic reactions.

While such tandem enzymatic reactions can be accomplished in vivo, as widely used in today’s fermentation industry, there are many advantages to use in vitro systems. First, as no competing pathways exist in the in vitro system and no metabolic burden or toxicity issues need to be considered, the yield can be much closer to the theoretical yield than the in vivo system. Second, due to its simplicity, it would be more practical and effective to optimize the system through modeling and adjusting the enzymes’ concentrations. Moreover, artificial tandem enzymatic reactions which can produce non-natural compounds are readily set up without potential toxicity issues brought by the product. In the past decade, many tandem enzymatic reaction systems have been developed to produce chemicals which were comprehensively reviewed elsewhere (Oroz-Guinea and Garcia-Junceda 2013). Here we will focus on a few recent examples.

Although wild type enzymes are used in most cases, variants with novel activities which are usually created by protein engineering can also be used in the tandem reaction systems (Birmingham et al. 2014; Tavanti et al. 2017). For example, through computational design, researchers have engineered the promiscuous aldolase RA95.5–8 to catalyze asymmetric Michael additions and Knoevenagel reactions via Schiff base intermediates (Garrabou et al. 2015, 2016). Recently Hilvert’s group discovered novel activities of those aldolase variants. The variant RA95.5–8F can catalyze the synthesis of (R)-methodol from acetone and 6-methoxy-2-naphthaldehyde, while the variant T53L/K210H RA95.5–8 can catalyze the dehydrated (R)-methodol to γ-nitroketones (Garrabou et al. 2017; Obexer et al. 2017). By combining those two aldolase variants, they achieved similar yield and stereoselectivity in one-pot as the reaction using the purified intermediate (Figure 1).

Tandem enzymatic reactions: complex systems

Although in vitro tandem enzymatic reactions have a number of advantages over in vivo tandem enzymatic reactions, their weaknesses are also obvious, with the main weakness being the decreased flexibility.

Figure 1. Synthesis of γ-nitroketones by aldolase variants (Garrabou et al. 2017).
For example, most of the tandem reactions only include two or three enzymes. As a result, the substrates that can be used as well as the products that can be generated are largely limited. However, an ideal in vitro tandem reaction system should be sufficiently flexible to generate a broader product scope while using the low-cost substrates. Therefore, in vitro tandem reaction systems with higher complexity for synthesizing chemicals, such as carbohydrates, fuels, and fine chemicals are desired (Fessner, 2015; Guterl et al. 2012; Khattak et al. 2014; Schoevaart et al. 2000; Wagner et al. 2015). In recent years, great efforts were spent on the emerging CFME field to address such a challenge (Dudley et al. 2015). A CFME system usually involves four or more enzymes and uses inexpensive substrates if possible, as the cost-effectiveness is a required design criteria (Caschera and Noireaux 2015; Zhang 2015). Ideally, it should combine catabolic pathways and anabolic pathways, so that both the building blocks and the energy for synthesizing the target molecules can be generated from simple resources, such as glucose. However, the design and test of such a system would be very challenging as tens of enzymes might be involved (Karzbrun et al. 2011). Recently, Bowie and coworkers successfully combined the Embden-Meyerhof-Parnas glycolytic pathway and the mevalonate pathway to produce monoterpenes (Korman et al. 2017). A super-complex system with 27 enzymes was reconstituted in one-pot which enabled the system to synthesize monoterpenes by using glucose as the only substrate. While specific designs, such as the enzymatic purge valve nodes, helped the catabolic pathway and the anabolic pathway work harmonically in the system (Opgenorth et al. 2014). By switching the monoterpene synthases in the system, different types of monoterpenes such as limonene, pinene and sabinene can be produced. This system can produce monoterpenes with titers an order of magnitude higher than the cellular toxicity limit (15 g/L) with an almost theoretical yield (>95%), which demonstrates the great advantage of using enzymatic tandem reactions in vitro.

In addition to transferring the existing pathways from in vivo to in vitro, scientists are also able to design de novo complex pathways, which can accomplish difficult biosynthetic tasks. In 2017, the crotonyl--coenzyme A (CoA)/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle was constructed by Erb and coworkers, which provides a seventh CO2 fixation pathway besides the six naturally evolved ones (Schwander et al. 2016) (Figure 2). Instead of being restricted to known enzymes, the researchers sought for all reactions that are biochemically feasible. Multiple cycles were designed from scratch and their thermodynamic feasibilities were evaluated. Then, enzymes that can catalyze the reactions in the CETCH cycle were identified from bioinformatics databases and characterized individually in vitro. After combining the auxiliary proofreading and cofactor regeneration processes, the CETCH cycle involves in total of 17 enzymes from nine different organisms from all three domains of life. Through enzyme engineering and metabolic proofreading, the CETCH cycle can convert CO2 into organic molecules at a rate of 5 nanomoles of CO2 per minute per milligram of protein.

**Optimization of the tandem enzymatic reactions**

In order to optimize tandem enzymatic reaction systems, especially systems with high complexity, modeling is an indispensable strategy (Chen et al. 2013; Rollin et al. 2015). Although the modeling of stoichiometry seems to be easy, to set up a dynamic model is much harder due to not only the non-linear behavior of enzymes, but also the unpredictable interactions between chemicals and enzymes in the system. Even though plenty of studies have shown the possibility to set up dynamic models for the in vitro systems, most of them dealt with relatively simple systems (Findrik and Vasic-Racki 2007; Ishii et al. 2007; Van Hecke et al. 2009; Ardao and Zeng 2013; Dvorak et al. 2014). Moreover, as the dynamic parameters of enzymes vary a lot under different conditions, such as pH and buffer components, the modeling strategies depend on the parameters which are obtained from specific condition and can hardly be accurate. To overcome this limitation, Panke and coworkers applied an approach based on classical engineering systems theory for optimization of their dihydroxyacetone phosphate (DHAP) producing system (Hold et al. 2016). By characterizing this 10-enzyme system in a continuous stirred tank reactor (CSTR) coupled by an online electrospray ionization mass spectrometry, a sufficiently detailed response of the system to the dynamic challenges was recorded. The successful parameterization of this system enabled the researchers to perform forward design. Although this work was done in CSTR, the obtained model should also be readily used to design batch reactions.

In addition to modeling, inspired by nature where tandem reactions are catalyzed by multi-enzyme complexes (MECs), the immobilization of the enzymes involved in a tandem reaction system serves as another promising way to improve the reaction efficiency (Jia et al. 2014; Ji et al. 2016). Since the enzymes form an artificial complex, the intermediates involved in the tandem reaction can be transported between enzymes more efficiently. As high local concentrations of the
intermediates are maintained, the formation of the final product should be more dynamically favorable. Moreover, diffusion losses, especially of the highly reactive intermediates, can be largely avoided (Ogawa et al. 2002). The immobilization of enzymes can offer other advantages, such as the improvement of the stability of enzymes, though the conformation of the enzyme might get affected, resulting in the activity loss to some extent. To date, multiple methods for the attachment of enzymes to inorganic surfaces have been developed. However, in most cases, only 3 or fewer enzymes can be co-localized due to various challenges, such as the different responses of enzymes to different attachment methods (You and Zhang 2013, 2014). But recently Travis and coworkers demonstrated the possibility to create a 10-step enzymatic reaction on nanoparticles (Mukai et al. 2017). Although the activity of individual enzyme was higher in solution, the conversion of glucose to lactate was much higher when all the enzymes were tethered. In addition to the inorganic surfaces, DNA as a programable polymer offers chemists another option for enzyme immobilization (Rajendran et al. 2017). The ability of DNA to form various nanostructures makes it possible to control the spatial organization of the enzymes. Besides the assembly of enzymes involved in the tandem reaction, the cofactors involved in the tandem reaction could also be readily controlled (Fu et al. 2014).

As an alternative to the enzyme immobilization strategy, another approach is to directly engineer the enzymes to either form fusion proteins or induce self-assembly. In order to form fused proteins, linkers between enzymes involved in the tandem reaction were frequently used (Yu et al. 2015). Similar to the enzyme immobilization methods, the conformation of enzyme might be affected by the linkers or its fusion partners, which may lower or even completely abolish the activity. However, as the fusion protein can be prepared as a single protein, and no further chemical modification is needed, such an approach facilitates researchers to prepare enzyme complexes for tandem reactions. Besides the application for the assembly of enzymes that are directly involved in the product synthesis, another application is to fuse the enzymes with...
their redox partners, such as the widely used fusion protein of P450 with the NAD(P)H reductase (Andre et al. 2013; Peters et al. 2017). In addition to using linkers to form enzyme complex, adding peptide/protein tags to the enzymes can serve as an alternative approach (Proschel et al. 2015; Han et al. 2017; Liu et al. 2017).

Tandem chemo-enzymatic reactions

Organometallic catalysis and biocatalysis are two different disciplines in terms of reaction categories, catalytic conditions, substrates scope and selectivities. Organometallic catalysts have wide substrates scope and high productivity, and are still the key for bulk chemical manufacturing. Biocatalysis is becoming more widely used in the pharmaceutical industry due to its high regio-, stereo- and enantioselectivity, and growing advances in enzyme discovery and engineering. To access the advantages of both catalytic disciplines, there is a growing interest to combine organometallic catalysts in tandem with enzymes to realize synthetic power that cannot be achieved by either of them alone (Denard et al. 2013; Wang and Zhao 2016).

Unlike developing tandem enzymatic reactions, it is more challenging to combine organometallic catalysts and biocatalysts due to incompatibility and mutual inactivation of metal complexes and enzymes. Up to date, only lipases and serine proteases could maintain high catalytic activities in organic solvents and at high temperature. Similarly, most transition-metal complexes are inhibited in aqueous solution with or without cellular components. In this section, we will summarize the major tandem chemo-enzymatic reactions developed in organic solvent and aqueous buffer solutions, and discuss the novel strategies used to combine organometallic catalysts with biocatalysts in one-pot.

One-pot tandem chemo-enzymatic reactions in organic solvents

Dynamic kinetic resolution (DKR) of alcohols and amines based on transition metal-catalyzed racemization and lipase-catalyzed resolution in pure organic solvents has been developed towards a mature technology for preparation of enantiopure secondary alcohols and primary amines (Scheme 1). A lipase deracemizes a mixture of enantiomers by selectively acylating the desired enantiomer. The drawback of such enzymatic resolution is that a maximum yield of only 50% can be obtained. However, it can be resolved by using a racemization catalyst to replenish the consumed enantiomer and drive the resolution up to 100% yield of the desired enantiomer. Since the pioneering demonstration of the DKR concept by the group of Williams (Allen and Williams 1996), Bäckvall (Pamies and Backvall 2003) and Kim (Kim et al. 2003), intense studies have been conducted to develop efficient racemization catalysts, improve enzyme stability and expand substrate scope (Verho and Bäckvall 2015; Wang and Zhao 2016). To date, immobilized Candida antarctica lipase B (CALB) and C. antarctica lipase A (CALA) are the best enzymes of choice due to their robustness and activities in pure organic solvent at elevated temperature up to 100 °C (Martin-Matute and Backvall 2007).

Ruthenium, iridium, alumina and vanadium complexes are the most well-developed catalysts for alcohol racemization. They were used in tandem with various lipases to deracemize a wide range of functionalized secondary alcohols, including aliphatic alcohols, allylic alcohols, chlorohydrins, diols, homallylic alcohols, N-heterocyclic 1,2-aminos alcohols, and α-hydroxyl ketones with excellent yields and enantiomeric excess (ee) (Verho and Bäckvall 2015). Besides the above-mentioned homogeneous catalysts, heterogeneous acids, zeolites (Magadum and Yadav 2017; Xu et al. 2017) and vanadium based mesoporous silica (Sugiyama et al. 2016) were also reported as racemization catalysts of secondary alcohols. The DKR of amines is more challenging due to the lack of efficient racemization catalysts. To date, coupling ruthenium complexes, analogs of Shvo catalyst, with CALB is the most practical approach for DKR of aliphatic or benzylic primary amines at 90 °C (Verho and Bäckvall 2015). In recent years, Pd nanoparticle-based heterogeneous catalysts were employed for racemization of benzylic primary amines under mild conditions. However, they did not work well for aliphatic amines (Zhang et al. 2017).

Scheme 1. Dynamic kinetic resolution of amines or alcohols by coupling CALB or CALA in tandem with racemization catalysts.
table 1. Typical one-pot tandem chemo-enzymatic reactions in aqueous solution.

<table>
<thead>
<tr>
<th>Organometal catalysts</th>
<th>Comments</th>
<th>Enzymatic reaction</th>
<th>Reaction type</th>
<th>Product</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt (hydrogenation)</td>
<td>HE/Whole cell</td>
<td>D-glucose isomerase/monoa mine oxidase</td>
<td>concurrent/cooperative</td>
<td>D-mannitol/Chiral quinoline</td>
<td>(Makkee et al. 1980; Makkee et al. 1985; Foulkes et al. 2011)</td>
</tr>
<tr>
<td>Rh (hydrogenation)</td>
<td>HE</td>
<td>hydrolase</td>
<td>sequential</td>
<td>L-alanine</td>
<td>(Simons et al. 2007)</td>
</tr>
<tr>
<td>Pt (racemization)</td>
<td>HO</td>
<td>hydrolase</td>
<td>DKR</td>
<td>Enantiopure allyl alcohol</td>
<td>(Allen and Williams 1996)</td>
</tr>
<tr>
<td>Pyridoxal 5-phosphate</td>
<td>HO</td>
<td>hydrolase</td>
<td>DKR</td>
<td>L-amino acid</td>
<td>(Chen et al. 1994)</td>
</tr>
<tr>
<td>Fe (Hydrogenation-racemization)</td>
<td>HO</td>
<td>lipase (acylation)</td>
<td>cooperative</td>
<td>Enantiopure secondary alcohol</td>
<td>(El-Sepelgy et al. 2017)</td>
</tr>
<tr>
<td>Pd/Cu (Wacker oxidation)</td>
<td>HO/Compartment</td>
<td>(R) - ADH (ketone reduction)</td>
<td>sequential/concurrent</td>
<td>Chiral alcohols</td>
<td>(Schnapperelle et al. 2012; Sato et al. 2015)</td>
</tr>
<tr>
<td>Pd/Cu (Wacker oxidation)</td>
<td>HO/Compartment</td>
<td>transaminase (asymmetric reductive amination)</td>
<td>concurrent</td>
<td>Chiral amines</td>
<td>(Uthoff et al. 2017)</td>
</tr>
<tr>
<td>Ru (metathesis)</td>
<td>HO/Compartment</td>
<td>decarboxylase (Decarboxylation)</td>
<td>concurrent</td>
<td>Antioxidant 4,4’-dihydroxyylstilbene derivatives</td>
<td>(Gómez Baraibar et al. 2016)</td>
</tr>
<tr>
<td>Ru (metathesis)</td>
<td>HO</td>
<td>esterase (Hydrolysis)</td>
<td>sequential</td>
<td>Cyclic malonic acid monoesters</td>
<td>(Tenbrink et al. 2011)</td>
</tr>
<tr>
<td>Ru (metathesis)</td>
<td>HO/biphasic</td>
<td>P450 (epoxidation)</td>
<td>cooperative/sequential</td>
<td>Epoxide</td>
<td>(Denard et al. 2014; Denard et al. 2015)</td>
</tr>
<tr>
<td>Ru (metathesis)</td>
<td>HC</td>
<td>MAO-N (Aromatizing)</td>
<td>ADH (Ketone reduction)</td>
<td>Pyrrolines</td>
<td>(Scalacci et al. 2017)</td>
</tr>
<tr>
<td>Ir (Barbier-type coupling)</td>
<td>HO</td>
<td>galactose (alcohol oxidation)</td>
<td>sequential</td>
<td>Homoallylic sec-alcohols</td>
<td>(Burda et al. 2008; Gauchot et al. 2010)</td>
</tr>
<tr>
<td>Pd (Heck coupling)</td>
<td>HE</td>
<td>(R)-ADH (Ketone reduction)</td>
<td>sequential</td>
<td>4-[4-(4-Methoxyphenyl)-butan-2-one</td>
<td>(Fuchs et al. 2011)</td>
</tr>
<tr>
<td>Rh (diazo-coupling)</td>
<td>HO</td>
<td>ene-reductase (reduction)</td>
<td>sequential</td>
<td>Chiral 2-aryl-substituted succinate derivatives</td>
<td>(Wang et al. 2017)</td>
</tr>
<tr>
<td>Au (cycloisomerization)</td>
<td>HO</td>
<td>lipase (hydrolysis)</td>
<td>sequential</td>
<td>2,5-dihydrofurans</td>
<td>(Asikainen and Krause 2009)</td>
</tr>
<tr>
<td>Au (hydroalkylation)</td>
<td>Supramol.</td>
<td>esterase/lipase/ADH</td>
<td>concurrent</td>
<td>Substituted tetrahydrofuran /propanal</td>
<td>(Wang et al. 2013)</td>
</tr>
<tr>
<td>Organocatalytic aldol reaction</td>
<td>HO</td>
<td>ADH</td>
<td>concurrent</td>
<td>1,3-Diols</td>
<td>(Rulli et al. 2017)</td>
</tr>
<tr>
<td>Potassium phosphate (cyclization)</td>
<td>HO</td>
<td>carboligase/transaminase</td>
<td>sequential</td>
<td>Trisubstituted tetrahydroisoquinolines</td>
<td>(Erdmann et al. 2017)</td>
</tr>
</tbody>
</table>

Compat: compartmentalization; HC: whole cell; HE: heterogeneous catalysts; HO: homogeneous catalysts; Supramol: supramolecular encapsulation.

One-pot tandem chemo-enzymatic reactions in aqueous solutions

Except for lipases and serine proteases, most biocatalysts have poor stability in organic solvents and at elevated temperature. In the past 20 years, there has been a growing interest to develop tandem reactions in aqueous solutions to combine unique transformations catalyzed by organometallic catalysts and biocatalysts (Wang and Zhao 2016). The major motivation to associate biocatalysts with organometallic catalysts in one pot is to utilize the synthetic power from each discipline for specific transformations that could not be realized by either of them alone. In nature, enzymes have specific substrate binding sites for highly regio- stereo- and enantioselective synthesis. Accordingly, organometallic catalysts could access some reactions that enzymatic processes do not exist or work only on limited substrate scope, for example, hydrogenation, cross-coupling reaction, Wacker-oxidation, olefin metathesis, etc.

Unlike the DKR system in organic solvents, the development of tandem chemo-enzymatic reactions in aqueous solutions is still in its infancy. Only a small portion of catalytic reactions in organometallic chemistry and synthetic biology is amenable to these processes. Table 1 summarizes the representative works of one-pot chemo-enzymatic reactions that can occur in aqueous-based solutions. They are metal-catalyzed hydrogenation in tandem with isomerase or hydrolase catalyzed reactions; organometallic-catalyzed racemization in tandem with enantioselective hydrolysis; Pd/Cu catalyzed Wacker oxidation in tandem with enzymatic catalyzed asymmetric reduction or reductive amination; metal-catalyzed metathesis in tandem with enzymatic decarboxylation, ester hydrolysis or P450-catalyzed epoxidation; metal-catalyzed cross coupling in tandem with enzymatic reduction, oxidation and hydrolysis; Au catalyzed isomerization in tandem with enzymatic hydrolysis.

Those reactions were either performed in an one-pot sequential manners by avoiding the purification of intermediates when different reaction conditions are required for the organometallic and enzymatic steps,
or in a concurrent manner when two catalytic steps are compatible with each other. Enlighteningly, a few cooperative concurrent reactions were reported to reveal the significance of tandem reactions compared with the sequential transformations. For example, Zhao and Hartwig groups jointly reported an example of Ru-catalyzed olefin metathesis coupled with a P450-monooxygenase that selectively oxidized one of crossmetathesis products against others. The overall yield of concurrent reactions is 1.5 times higher than the sequential one (Figure 3) (Denard et al. 2014).

**Novel strategies to combine transition metals and enzymes in one-pot tandem reactions**

The incompatibility of the individual reaction steps with one another is the major challenge to combining transition metal catalysts with biocatalysts in one-pot. Besides immobilized lipases and serine proteases, the majority of biocatalysts have poor stability in organic solvents. Most of the transition metal catalysts are not active in aqueous solutions. The mutual inactivation also occurs frequently due to the undesired coordination of the transitional metal complex to the protein catalyst. In this section, we will discuss how transition metals are combined with enzymes in one-pot by using some novel strategies including supramolecular assemblies, artificial metalloenzymes, compartmentalization, whole cells, and co-immobilization (Table 2).

Incorporation of transition metal complexes within supramolecular hosts is a valuable way to solve those problems. A long-term goal of supramolecular chemistry is to construct assemblies that mimic the desirable qualities of protein catalysts (Wiester et al. 2011). It has been found that supramolecular assemblies could stabilize reactive metal complexes and increase their turn over numbers (Brown et al. 2011). Additionally, supramolecular complexes enable the catalytic activity of several transition metal catalysts in water by providing bio-inspired hydrophobic cavities (Pluth et al. 2007; Bistri and Reinaud 2015; Kaphan et al. 2015). Importantly, supramolecular cages prevent the direct interaction of the transition metal with the enzymes, thereby averting their mutual inactivation (Kohler et al. 2013; Wang et al. 2013). Lastly, supramolecular assemblies provide extra stereo-, regio- and enantio-selectivity by providing a well-defined cavity for the reaction compared with the reaction performed in bulk solvent (Brown et al. 2009; Yoshizawa et al. 2006). Using a supramolecular approach, Toste and coworkers created various tandem reactions employing esterases, lipases or alcohol dehydrogenase with gold (I) or ruthenium (II) complexes encapsulated in a Ga4L6 tetrahedral supramolecular cluster without mutual inactivation (Figure 4) (Wang et al. 2013). Their findings suggested that supramolecular assemblies may provide a general strategy for carrying out classic organic reactions in tandem with enzymatic reactions in aqueous solution.

An artificial metalloenzyme resulting from encapsulation of a transition metal catalyst within a protein scaffold is a special form of supramolecular host-assemblies. Ward and coworkers developed an artificial (R)-selective transfer-hydrogenase (ATHase) by incorporation of a biotinylated [Cp*Ir(Biot-p-L)Cl] complex within streptavidin (Sav). Sav worked as a neutral shield for the Ir complex to keep it from interacting with other biocatalysts.
In that way, they successfully combined ATHase with various NADH-, FAD- and haem-dependent enzymes resulting in orthogonal redox cascades that could not be achieved when the free Ir-complex was used (Kohler et al. 2013), including a double stereoselective amine deracemization and production of L-pipecolic acid from L-lysine (Figure 5).

Compartmentalization is another strategy to couple chemical catalysts with biocatalysts in one-pot by protecting enzymes from organic solvents or metal catalysts from cellular components; as well as shielding catalytic centers from one another to avoid mutual inactivation. Besides the traditional biphasic system (Marr and Liu 2011), more novel methods have been developed for the compartmentalization purpose including hydrogel immobilization, membrane filtration and whole-cell isolation. Gröger and coworkers realized enzymatic decarboxylation (Gómez Baraibar et al. 2016) and alcohol dehydrogenation (Heidlinemann et al. 2014) in organic solvent by encapsulating the enzymes in poly(vinyl alcohol)/poly(ethylene glycol) cryogels or acrylate-based superabsorbent polymers respectively.

Table 2. The novel strategies to couple transition metals (or chemical catalysts) with enzymes in one-pot tandem reactions.

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Advantages</th>
<th>Tandem reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supramolecular assembly</td>
<td>• realize catalytic activity of metal catalysts in aqueous solution</td>
<td>• Au or Ru in tandem with esterase, lipase, ADH</td>
</tr>
<tr>
<td>(Artificial metalloenzyme)</td>
<td>• stabilize metal complexes and improve turn over number (TON)</td>
<td>• Ir in tandem with oxidoreductases</td>
</tr>
<tr>
<td></td>
<td>• prevent interaction of metal complexes with enzymes</td>
<td>• Ru in tandem with decarboxylase</td>
</tr>
<tr>
<td></td>
<td>• provide extra selectivity of metal catalysts</td>
<td>• proline in tandem with ADH</td>
</tr>
<tr>
<td></td>
<td>• protect enzyme from organic solvent</td>
<td>• Wacker oxidation in tandem with enzymatic</td>
</tr>
<tr>
<td></td>
<td>• protect metal catalysts from aqueous solution</td>
<td>reduction or hydroamination</td>
</tr>
<tr>
<td></td>
<td>• prevent mutual inactivation</td>
<td>• Pd(0)-catalyzed reduction in tandem with mono-</td>
</tr>
<tr>
<td></td>
<td>• stabilize metal catalysts</td>
<td>amine oxidation</td>
</tr>
<tr>
<td></td>
<td>• improve synergic effect</td>
<td>• CALB and Pd nanoparticles co-immobilized into</td>
</tr>
<tr>
<td></td>
<td>• enable catalyst regeneration</td>
<td>the mesoporous silica for DKR of amine</td>
</tr>
<tr>
<td>Compartmentalization</td>
<td>• protect enzyme from organic solvent</td>
<td>• Ru in tandem with decarboxylase</td>
</tr>
<tr>
<td></td>
<td>• protect metal catalysts from aqueous solution</td>
<td>• proline in tandem with ADH</td>
</tr>
<tr>
<td>Whole-cell</td>
<td>• prevent interaction of metal with cellular components</td>
<td>• Wacker oxidation with enzymatic reduction or</td>
</tr>
<tr>
<td></td>
<td>• avoid protein purification</td>
<td>hydroamination</td>
</tr>
<tr>
<td>Co-immobilization</td>
<td>• stabilize metal catalysts</td>
<td>• Pd(0)-catalyzed reduction in tandem with mono-</td>
</tr>
<tr>
<td></td>
<td>• improve synergic effect</td>
<td>amine oxidation</td>
</tr>
<tr>
<td></td>
<td>• enable catalyst regeneration</td>
<td>• CALB and Pd nanoparticles co-immobilized into</td>
</tr>
<tr>
<td></td>
<td></td>
<td>the mesoporous silica for DKR of amine</td>
</tr>
</tbody>
</table>

Figure 4. (A) Esterase- or lipase-mediated acetate hydrolysis followed by Au (I)-catalyzed hydroalkoxylation. (B) Ru(II)-mediated olefin isomerization followed by ADH-catalyzed reduction (Wang et al. 2013).
They coupled these two enzymes with ruthenium-catalyzed metathesis and a proline-derivative-catalyzed aldol reaction for efficient preparation of antioxidants 4,4’-dihydroxystilbene and 1,3-diols separately. In another study, they combined Wacker oxidation with enzymatic reduction or hydroamination in a one-pot process in aqueous solution by withholding the detrimental effect of Cu ions from biotransformation in the interior of a polydimethylsiloxane thimble (PDMS) that only enabled the free exchange of organic chemical (Figure 6) (Sato et al. 2015; Uthoff et al. 2017).

Although supramolecular strategies have enabled the catalytic activity of several transition metal catalysts in aqueous environment, many metal cofactors are still inhibited by the cellular components and therefore purified proteins are required for tandem reactions in most cases. Using cellular membrane as a barrier to protect metal catalysts from cellular inhibitors is an attractive strategy. Recently, Ward and coworkers created an E. coli strain for periplasmic expression of Sav with a biotinylated Hoveyda-Grubbs catalyst for olefin metathesis in vivo (Jeschek et al. 2016). The periplasm provided an environment with low concentrations of inhibitors for metathesis and thus enabled the high-throughput directed evolution of artificial metalloenzymes in vivo. Additionally, the use of whole cell provides a natural protection for biocatalysts. Lloyd and coworkers prepared (R)-1-methyltetrahydroisoquinoline (MTQ) with 96% ee by a cooperative tandem reaction consisting of enantioselective monoamine oxidation catalyzed by an engineered E. coli strain and nonselective reduction catalyzed by nanoscale Pd (0) coated on the cell surface (Foulkes et al. 2011) (Figure 7).
Another interesting strategy to enable chemoenzymatic reactions in one pot is to create hybrid enzyme-metal nanoparticles that bear orthogonal but cooperative catalytic centers. This design straddles the disciplines of supramolecular and compartmentalization. The peptide is a robust biomaterial and can stabilize the performance of inorganic catalysts (San et al. 2011). The resulting enzyme-metal complex could work as a metalloenzyme with the catalytic activity originated from the introduced metal. Significantly, the surrounding proteins not only serve as protecting cages but also work as catalysts for the distinct bioactivity (Engström et al. 2013; Filice et al. 2013; San et al. 2011).

For example, Backvall and coworkers created a hybrid catalyst consisting of CALB and Pd nanoparticles co-immobilized into the compartments of mesoporous silica for DKR of 1-phenylethylamine (Engström et al. 2013). The co-immobilization resulted in a more efficient cooperation between two catalysts and recyclability of both Pd nanoparticles and CALB at the same time (Figure 8).

**Future perspectives and conclusions**

Unlike multistep enzymatic transformations occurring in cells that generate complex molecules, tandem reactions in vitro generally consist of two to three steps and the reaction categories only represent a small portion of bio- or chemo-transformation available in synthetic biology or organometallic chemistry. The development of in vitro tandem processes is challenged by the limited number of biocatalysts and the strategies to balance the activity of each step. The combination of chemocatalysts and biocatalysts in one-pot further illustrates the issues of incompatibility and mutual inactivation between individual catalysts. To address these problems, it is essential to take advantages of several research areas including but not limited to protein engineering, artificial metalloenzymes, system modeling, chemical catalysts design and development, supramolecular assembly, and compartmentalization.

Protein engineering strategies can be used to broaden substrate scopes and enhance regio-, stereo-, enantio-selectivities and the stability of current biocatalysts, and these properties are important for developing both multi-enzymatic and tandem chemo-enzymatic reactions. It is especially important to enable enzymatic function in organic solvents or at high temperature to realize more possibilities of cooperative metal-enzymatic transformations. There are some well-developed methods to improve enzyme performance in harsh condition, such as immobilization of enzymes on heterogeneous support (Sheldon and van Pelt 2013); preparation of cross linked enzyme aggregates (Velasco-Lozano et al. 2015), surfactant treatment (Kim et al. 2011),
creation of protein mutants via rational design and directed evolution (Bornscheuer et al. 2012).

Artificial metalloenzyme engineering is a growing field to expand the natural enzymatic repertoire with abiotic reactions (Hyster and Ward 2016). Artificial metalloenzymes combine the versatile reaction scope of transition metal catalysts with the beneficial effects of enzymes, such as high selectivity over both substrates and products. So far, a few well-developed strategies are available for developing new artificial metalloenzymes, for example, engineering natural metalloenzymes for abiotic transformations (Kan et al. 2016); computational redesign (Khare et al. 2012); incorporating noble-metal complexes with protein scaffold (Srivastava et al. 2015) and replacing the native metal of a metalloenzyme with a noble metal complex (Key et al. 2016). Importantly, artificial metalloenzymes could improve the performance of metal catalysts in aqueous solution and prevent the mutual inactivation of metal catalysts with other biocomponents (Cangelosi et al. 2014), which enables the coupling of more metal catalysts in tandem with multi-step enzymatic reactions for selective synthesis. Last but not least, advanced protein engineering tools such as directed evolution can be directly applied to an artificial metalloenzyme to improve its catalytic performance. Artificial metalloenzymes undoubtfully would contribute more to in vitro tandem reactions in near future.

Although there are countless enzymatic reactions in nature, only few of them can serve as useful catalysts for either tandem enzymatic or chemoenzymatic reaction systems. One of the major challenges is to obtain functional enzymes in reasonable amount and purity. *Escherichia coli* has been widely used for the heterologous expression of proteins, but most of the enzymes, especially those from eukaryotes, can get misfolded when expressed in *E. coli*, which abolishes their activities. Moreover, the expression of enzymes which require post-transcriptional or post-translational modifications, or enzymes which have very high molecular weights can be even more challenging. Therefore, the development of hosts and processes for more efficient protein expression and purification is always desirable. In addition, as the tandem enzymatic reaction systems tend to be more complex, dynamic modeling of such systems can not only be an effective way for optimization, but also one of the main approaches for the system design. As the activities of enzymes can vary significantly from one system to another, how to model the reaction system with high accuracy can be a major problem. Besides making more detailed dynamic data-set for each enzyme, developing methods for real time monitoring of the components in the reaction system is another possible solution.

The combination of chemocatalysis and biocatalysis in one-pot for concurrent transformations is still in...
its infancy. The development of this field is relatively slow mainly due to the incompatibility and mutual inactivation of metal complexes and enzymes. To expand the spectrum of chemical catalysts in buffer systems, chemists have made efforts on developing aqueously effective transition metal catalysts. The achievements made so far have enabled several metal-catalyzed reactions in aqueous solutions, including olefin metathesis (Vougioukalakis 2016), hydrogenation, and C-C coupling reactions (Wang and Zhao 2016). Importantly, a few metal catalysts such as Cu, Pd and Ru have been integrated into living cells for bioorthogonal protein chemistry (Li et al. 2014; Yang et al. 2014). Although there are very few examples to integrate bio-orthogonal reactions into chemical synthetic pathways in living cells, there is an opportunity to create new cell factories for distinct chemical production by harnessing the synthetic power of transition metal catalysts. Additionally, as discussed in the previous section supramolecular assemblies and compartmentalization could provide alternative strategies to enable the function of water-insoluble metal complexes in buffer solutions and avoid mutual inactivation of chemical catalysts and biocatalysts.

In summary, the development of in vitro tandem enzymatic and chemo-enzymatic processes continues to grow. With the development of protein engineering, artificial metalloenzyme and system design and modeling, there is an immense opportunity to develop sophisticated tandem processes mimicking biosynthetic pathways for complex synthesis. Additionally, with the aid of organic synthesis, supramolecular assemblies and compartmentalization, more organometallic catalysts can be utilized in aqueous solutions and integrated with enzymatic steps for selective synthesis. Based on these achievements, the field will continue to expand in the future.

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