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Engineering Spatially Organized Multienzyme Assemblies for Complex Chemical Transformation

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INTRODUCTION

Over the past two decades, enzyme catalysis has emerged as an economical and environmentally friendly alternative to conventional catalysis with numerous applications in the pharmaceutical,[1] food,[2] and cosmetic[3] industries. Among their advantages, many enzymes exhibit significantly greater activity, enantioselectivity, and specificity than their metal counterparts,[4] improving process efficiency and increasing product yields. In addition, enzymes tend to exhibit optimal activities at relatively mild temperatures and pressures, reducing energy consumption and thereby lowering operating costs. The majority of industrial enzymes are used in single-step reactions; however, the potential applications of enzyme catalysis can be greatly expanded by using multiple enzymes to catalyze complex chemical transformations.

Complex chemical transformations involve two or more enzyme-catalyzed reactions, which are related through sequential, coupled, divergent, and/or convergent reaction steps[5−7] (Figure 1). Complex chemical transformations are common in biological processes ranging from protein synthesis to cellular metabolism. In nature, microorganisms have evolved features to maximize the catalytic efficiency of some complex chemical transformations by colocalizing functionally related enzymes.[8−11] In recent years, researchers have drawn inspiration from natural enzyme colocalization and applied advances in protein engineering to create novel multienzyme assemblies (MEAs) that provide extraordinary control over the molecular ratio and spatial organization of the participating enzymes. Several strategies have been employed to achieve control over these parameters including DNA scaffolds,[12] protein scaffolds,[13] polymeric particle-based assemblies,[14] metal−organic frameworks,[15] and cross-linked enzyme aggregates.[16] While structurally and mechanistically diverse, the ultimate goal of MEAs is to accelerate reaction rates and increase reaction efficiency.

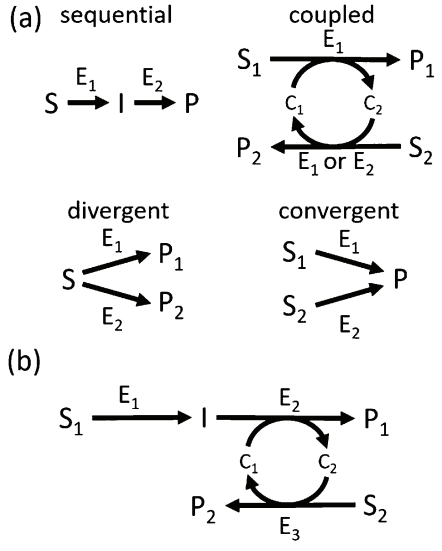

Figure 1. Complex chemical transformations consist of two or more enzymatic reactions. (a) Enzymatic reactions can be linked to form a complex chemical transformation through four basic relationships: sequential, coupled, divergent, and convergent. (b) A hypothetical example of a three-enzyme complex chemical transformation that includes sequential and coupled reactions. E = Enzyme, S = Substrate, I = Intermediate, P = Product, C = Cofactor.

In our view, MEAs will become an increasingly attractive alternative to conventional catalytic processes for the production of complex molecules in a variety of fields. Here we highlight promising advances in MEA engineering and summarize technical challenges that must be overcome for these systems to be used in commercial production processes.

MULTIENZYME ASSEMBLY SYNTHESIS STRATEGIES

MEAs can be synthesized using a variety of chemical and biomolecular strategies. While some of these chemistries have been widely used for single enzyme immobilization,[17−19] most strategies are enabled by recent advances in molecular biology.[20−22] Each strategy is associated with its own advantages and limitations (Figure 2), and the suitability of any one strategy to catalyze a complex chemical transformation is determined by the specific demands of that chemical reaction such as harsh reaction conditions, competing side reactions, enzyme activities, and whether the reaction takes place in vitro or intracellularly. Here, we will briefly introduce common strategies for synthesizing MEAs to provide some context for the sections below.

Stochastic Colocalization. The simplest method to synthesize MEAs is the stochastic colocalization of enzymes, with the most common example being cross-linked enzyme aggregates (CLEAs, Figure 2a). CLEAs are formed by using salts, polymers, or organic solvents to precipitate enzymes from an aqueous buffer and cross-linking the resulting enzyme aggregates (5 to 50 μm)[23] with a nonspecific fixative such as glutaraldehyde.[16] CLEA synthesis is simple, amenable to rapid optimization, and applicable to a wide range of enzymes.[24] As a result, CLEAs and CLEA variants have been used as industrial biocatalysts since the early 1990s.[25]

Another strategy for the stochastic colocalization of enzymes is immobilizing them on or encapsulating them within metal

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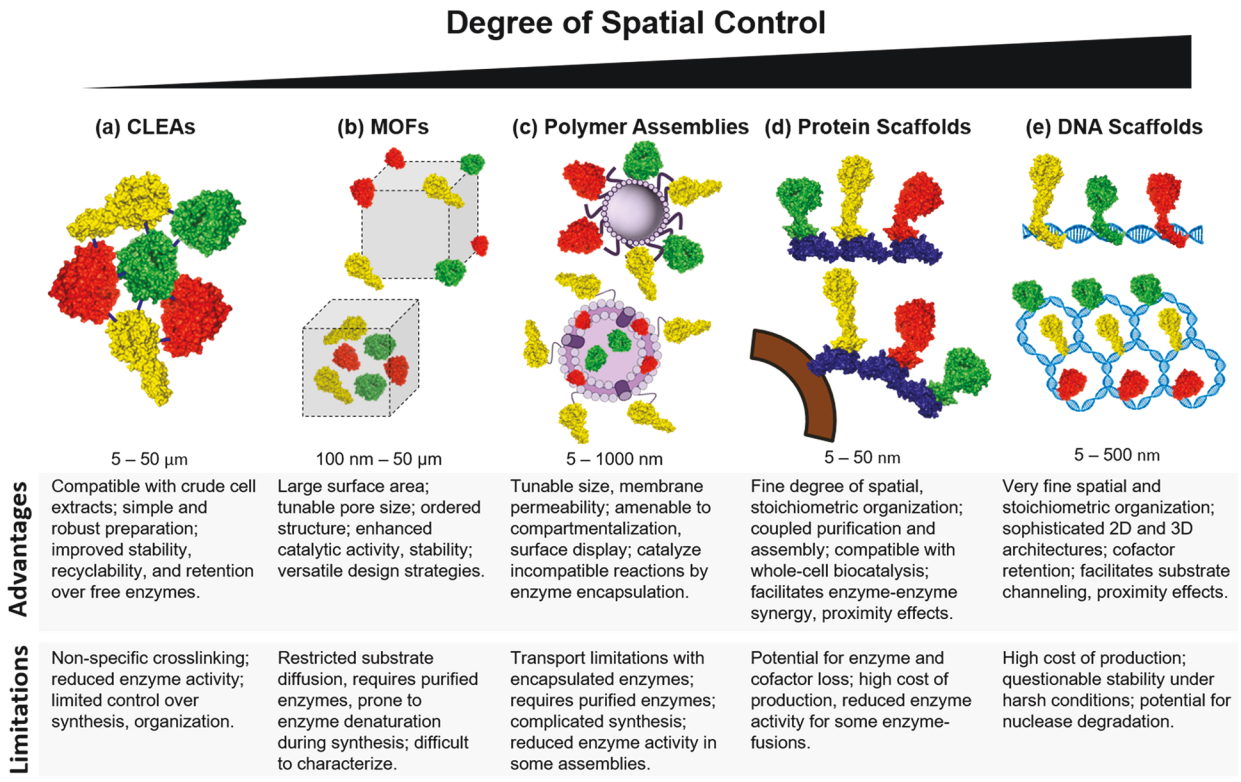

Figure 2. Overview of multienzyme assembly (MEA) synthesis strategies. (a) Cross-linked enzyme aggregates (CLEAs). (b) Metal−organic frameworks (MOFs). (c) Polymer assemblies. (d) Protein scaffolds. (e) DNA scaffolds.

organic frameworks (MOFs, Figure 2b). MOFs are highly porous (typically 100 to 6000 m^2/g),[26] crystalline structures (0.1 to 50 μm) composed of inorganic nodes and organic linkers that can be tuned to adsorb specific molecules.[26−28] In addition to providing a high-surface-area support, enzymes organized on MOFs can benefit from the intrinsic affinity of MOFs for a substrate and thereby concentrate the substrate near the MEAs.[29] While the MOF MEAs are traditionally assembled through stochastic adsorption or encapsulation, a recent study reported synthesis of a MOF that separated two enzymes into distinct layers within the same MOF, offering some spatial control over the enzyme assembly.[15]

Alternatively, enzymes can be randomly immobilized on the surface of polymeric particles such as micelles[30] (Figure 2c, top) or polymersomes[31] (Figure 2c, bottom). Polymersomes can also encapsulate enzymes within the polymer bilayer (Figure 2c, bottom),[31] allowing additional enzymes to be incorporated into the MEA and avoiding immobilization (e.g., adsorption, cross-linking, etc.), which often reduces enzyme activity. In order to allow reactants to access the encapsulated enzymes, pores or channel proteins can be added during bilayered polymersome synthesis.[32] Another advantage of bilayered polymersomes is that their size can be easily tuned, with diameters ranging from 100 to 1000 nm.[33] This flexibility provides a wide range of design possibilities and significant control over enzyme density within or on the surface of spherical polymersomes.

While the features of each particular strategy vary, the primary advantage of using stochastic assembly to synthesize MEAs is the simplicity of the process because no prior genetic or chemical modifications of the enzymes are needed. However, in addition to offering little to no control over the spatial organization of enzymes, stochastically assembled MEAs also provide limited control over the stoichiometric ratio of enzymes within the MEA. For example, it is more difficult to encapsulate large enzymes than small enzymes,[34] and not all enzymes will nonspecifically adsorb or bind to sites on a surface at the same rate. Nevertheless, stochastically assembled MEAs have proven to be useful for complex chemical transformations involving the synthesis of pharmaceuticals, the production of bioethanol, and the manufacturing of food and textiles.

Directed Assembly. In contrast to stochastic colocalization, highly spatially organized MEAs can be synthesized by directed assembly using high affinity, specific biomolecular interactions involving proteins (Figure 2d) and nucleic acids (DNA or RNA) (Figure 2e). One of the most studied MEAs found in nature is the cellulosome,[8] which is formed by the protein-scaffold directed assembly of cellulolytic enzymes. Cellulosome assembly relies on the interaction between the cohesin domains in the protein scaffold and the dockerin domains of enzymes. Cellulosome assembly has been applied to a diverse range of biological problems[35,36] and has inspired a number of protein-scaffold directed MEA designs (Figure 2d).[37−42] The cohesin−dockerin interaction is among the strongest protein−protein interactions found in nature, with recorded binding affinities as low as 10 pM.[43] Other protein binding pairs with strong binding affinities, such as affibodies (antibody mimetics with K_d ∼ 50 to 900 nM)[44] and binding domains involved in cellular signal processing (K_d ∼ 100 to 10 000 nM)[13,45,46] are also commonly used in protein-scaffold directed MEAs with MEA sizes on the order of ∼5 to 50 nm.[38,45]

DNA-scaffold directed MEA synthesis is generally performed by genetically fusing each enzyme to a DNA binding protein[47−49] or using a cross-linker to conjugate amine groups

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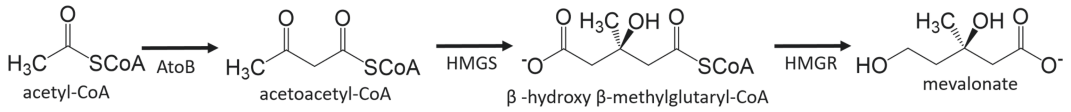
Scheme 1. Mevalonate Synthesis Pathway



in the enzyme to a thiol group added to a short single-stranded DNA sequence.[12,50,51] This enables the enzymes to interact with DNA scaffolds with defined sequences at nanomolar affinities (Figure 2e).[12,52,53] This interaction combined with DNA origami techniques enables DNA scaffolds as large as hundreds of nanometers[54] to direct the assembly of enzymes with nanometer scale precision.[12,51] However, producing the amount of DNA scaffolds needed for industrial scale processes is expensive.[37,55] RNA can also direct the synthesis of MEAs in a manner very similar to DNA but is not well suited for industrial application because it is highly unstable.[56]

Given the extraordinary control over spatial organization of MEAs offered by directed assembly strategies, these methods can bring enzymes in close proximity to direct intermediates between their active sites and are especially well suited for complex reactions involving competing side reactions or situations in which control over enzyme ratios is important. Furthermore, directed assembly strategies can be used in whole-cell biocatalysts in addition to in vitro applications, making them more versatile than stochastic assembly strategies which are limited to in vitro applications.

■ ADVANCES IN MULTIENZYME ASSEMBLY ENGINEERING

Promoting Enzyme Proximity Effects. By colocalizing enzymes in a single assembly, MEAs tend to reduce the distance between enzyme active sites, and thereby enhance catalytic activity by promoting advantageous "enzyme proximity effects".[57,58] These proximity effects often increase reaction rates and/or product yields by increasing the local intermediate concentration and reducing intermediate loss through diffusion. Proximity effects have been observed in reactions catalyzed by enzymes colocalized via CLEAs,[59−62] MOFs,[15] protein scaffolds,[37,63,64] and DNA scaffolds[65] with activity enhancements as high as a 33-fold increase in the catalytic efficiency.[37] Proximity effects have been further characterized beyond simple colocalization. The precise spatial control offered by DNA-scaffold directed MEAs has been used to demonstrate an inverse relationship between interenzyme distance and cascade activity.[12,51] Specifically, in a two-enzyme cascade reaction, a 20% activity increase was observed as the interenzyme distance was reduced from ∼26 to ∼6 nm.[12]

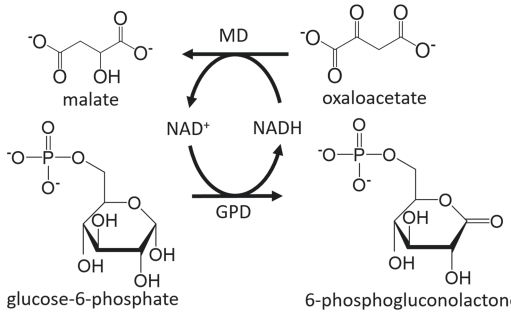
MEAs can further leverage enzyme proximity effects to enhance overall catalytic activity by controlling the stoichiometric ratio of enzymes within a MEA to optimize pathway flux. Modulating the ratio of enzymes within a MEA allows rate-limiting steps to be accelerated by overrepresenting the slowest enzyme. As a result, MEAs have the ability to optimize multistep pathway flux both in vitro[46,66] and intracellularly.[13,45,49] For example, when a three-enzyme pathway was heterologously expressed in *E. coli* to produce mevalonate (Scheme 1), a precursor to the antimalarial drug artemisinin, a bottleneck formed causing an accumulation of the toxic intermediate *β*-hydroxy *β*-methylglutaryl-CoA (HMG-CoA). To overcome this bottleneck, a protein-scaffold directed MEA was synthesized to optimize enzyme ratios, culminating in a 77-fold increase of the mevalonate yield compared to cells without the protein scaffold.[13] Intracellular protein-scaffold directed MEAs have similarly been shown to increase product yields in other studies;[45,46] however, their application can be complicated by protein-scaffold aggregation and/or misfolding.[67] Under such conditions, plasmid DNA[49] and RNA[54] scaffolds can provide an alternative assembly strategy for MEA synthesis, although DNA supercoiling[49] and RNA degradation[56] can also adversely affect MEA formation.

While effective, proximity effects rely on the random diffusion of intermediates from the active site of one enzyme to another. To further increase the efficiency of the transport of intermediates between enzymes in a MEA, bioengineers have looked to designs that enable substrate channeling.

Harnessing Substrate Channeling. The most efficient form of proximity effects occurs when the intermediate is directly transferred from the active site of one enzyme to that of another.[10,57,68] This phenomenon, termed substrate channeling, has been extensively reviewed elsewhere[9,10,68,69] and results in little or no loss of reaction intermediates to the bulk solution, increasing reaction rates, and product yield. Incredibly efficient substrate channeling mechanisms have evolved in nature including shuttling intermediates through molecular tunnels,[9] transferring intermediates via swinging arms,[70] and transporting intermediates across enzyme surfaces via electrostatic interactions.[71,72] These examples of substrate channeling have provided inspiration for designing synthetic MEAs with specific structural components that direct intermediates between active sites of different enzymes.

The modularity and spatial precision offered by DNA-scaffold directed MEAs make this strategy particularly well suited to realize substrate channeling between individual enzymes. Glucose-6-phosphate dehydrogenase (GPD) and malic dehydrogenase (MD) (Scheme 2) were arranged on a

Scheme 2. Cofactor Coupling between Malate Dehydrogenase (MD) and Glucose-6-Phosphate Dehydrogenase (GPD)



DNA scaffold with the NADH cofactor tethered between the two enzymes by flexible single-stranded DNA functioning as a swinging arm.[73] Compared with soluble NADH at the same total concentration, the swinging arm increased the overall cascade activity by ∼90 fold. This is an excellent example of a synthetic MEA that achieves substrate channeling, representing

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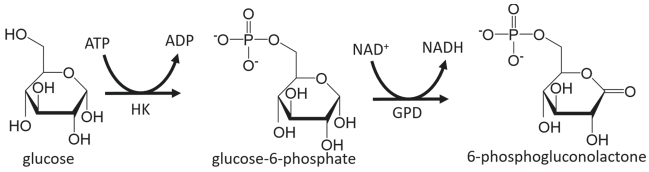
the first step toward developing more complex swinging arm mechanisms.

In addition to emulating natural swinging-arm mechanisms to promote substrate channeling between enzymes, a synthetic MEA has been constructed to take advantage of electrostatic channeling. Click chemistry was used to build a positively charged poly(lysine) peptide bridge between cysteine residues on the hexokinase (HK) and GPD enzymes (Scheme 3) to

Scheme 3. Conversion of Glucose to 6-Phosphogluconolactone through a Charged Intermediate



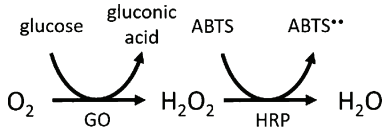
guide the transport of glucose-6-phosphate.[74] The charged bridge reduced the time it took the system to reach steady state (i.e., the transient time) to 70 s, a ∼30% reduction when compared with a solution of the free enzymes or compared to a MEA design with a neutral glycine bridge. A transient time of ∼0 s is characteristic of substrate channeling, so the reduced but measurable transient time in this study indicates leaky substrate channeling.

Substrate channeling via molecular tunnels has not been implemented in synthetic MEAs, probably due to the unique challenges associated with constructing a functional molecular tunnel connecting the active sites of two enzymes through genetic fusion or chemical conjugation. First, while the active sites of some enzymes are positioned on the surface,[75,76] for many enzymes, the active sites are buried within the enzyme and only accessible through a cleft in the enzyme[77,78] or conformational changes (such as the repositioning of side chains[79] or flap domains[80,81]), making it difficult to identify an accessible passage to build the molecular tunnel. Additionally, residues near the enzyme active site often play important roles in catalysis,[82] and their modification may have detrimental effects on enzyme activity.[83−85] The final, and most difficult, challenge is directing the intermediate through the molecular tunnel toward the second active site. To address this challenge, natural MEAs employ multiple conformational changes.[9,10,86] For example, the reactant of tryptophan synthase enters the first active site through a "front door". A protein loop then moves to close the "door", and the tunnel becomes more accessible, forcing the transport of the intermediate toward the second active site. These complex structural changes likely require the coevolution of all residues around both active sites, which is corroborated by the fact that molecular tunnels found in nature are situated between enzyme subunits interfaced with large surface contact areas.[86] These complex conformational changes are extremely challenging to achieve by simply building an external molecular tunnel between two individually evolved enzymes in a MEA.

Using Microenvironment Effects. Bringing multiple enzymes together to form a MEA changes the microenvironment surrounding the individual enzymes, which may affect their activities. These microenvironment effects are not typically associated with homogeneous mixtures of soluble enzymes and are instead caused by noncovalent or covalent interactions among the MEA components. Almost all MEA synthesis strategies require covalent modifications of enzymes including cross-linking in CLEAs, chemical conjugation for polymeric assemblies, and fusion to proteins or conjugation to DNA for directed assembly. These modifications can alter the chemistry of the covalently modified residues or restrict the conformation of enzymes within a MEA. The microenvironment effects in CLEAs often reduce enzyme activities but prevent enzyme unfolding to increase their stabilities at high temperatures,[87−89] at extreme pH values,[88] and in organic solvents.[62] However, in most cases, it is difficult to predict how a given enzyme will respond to covalent modifications such as protein fusions for assembly on a protein scaffold. In our own work, we have observed that some enzymes—for example, xylose reductase—are incredibly sensitive to both the size and positioning of larger proteins or even peptide tag fusions.

Potential noncovalent interactions that could affect the activities of enzymes in a MEA include van der Waals forces, electrostatic interactions, hydrogen bonding, hydrophobic interactions, and steric clashes. For example, the small pore spaces within MOFs prevent enzymes from unfolding. Similar to CLEAs, this increases enzyme stabilities under a variety of harsh conditions, but MOFs lack the rigid covalent restrictions of CLEAs and typically do not significantly reduce enzyme activities.[15,26−28,90] The noncovalent interactions between MEA components and the solution can also cause changes in the microenvironment. A modeling study has shown that the negative charge of DNA could reduce the pH of its microenvironment by a full unit within ∼2 nm.[91] If the enzymes comprising a DNA-scaffold directed MEA exhibit greater activities in more acidic environments (e.g., glucose oxidase (GO) and horseradish peroxidase (HRP) in Scheme 4), the reduced pH around the DNA scaffold will enhance the overall catalytic activity of the MEA.

Scheme 4. Conversion of Molecular Oxygen to Water through the Unstable Hydrogen Peroxide Intermediate



Although these microenvironment effects have been observed in many studies, it is difficult to predict how a particular noncovalent or covalent modification affects the activity of the enzyme in a MEA. For example, assembling five different enzymes on separate poly(acrylic acid) scaffolds by cross-linking lysine amino groups to carboxyl groups on the polymer caused changes in enzyme activities ranging from a reduction of 60% to an increase of 500% compared to the free enzymes.[92] Ideally, to maximize the catalytic performance of a MEA, synthesis strategies should be carefully chosen to avoid reducing individual enzyme activities. Ultimately, cost-benefit analysis is required to ensure that the synthesis cost of a MEA is justified by its improved cascade activity for an industrial application.

INDUSTRIAL PERSPECTIVE

The global market for industrial enzymes reached $4.9 billion in 2015 and is expected to grow at a compound annual rate of 4.7% to $6.3 billion by 2021.[93] The accelerating adoption of enzyme-based processes in industry has been spurred by a

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growing interest in renewable "green" technologies[94] as well as breakthroughs in protein engineering that allow researchers to tailor the properties of enzymes to meet the demands of industry.[95,96] However, the vast majority of enzyme-catalyzed industrial processes currently use single enzymes,[81] and several important challenges must be overcome if MEAs are to reach their full commercial potential.

The single greatest challenge associated with translating the success of MEAs in research laboratories to industrial processes is the cost associated with their purification, assembly, and immobilization on a biocatalyst carrier. In sharp contrast to whole-cell biocatalysts and crude enzymes, which generally cost ∼$120/kg and ∼$240/kg, respectively, purified enzymes can cost as much as $1200/kg.[97] Moreover, most MEAs also need to be immobilized on a biocatalyst carrier, which reduces enzyme contamination of the product stream while allowing MEAs to be reused for multiple reaction cycles. A number of strategies have already been developed to immobilize enzymes on biocatalyst carriers, including adsorption,[98] cross-linking,[98] and whole-cell entrapment,[99] and these strategies should be directly transferable to immobilizing MEAs. Finally, while MEA immobilization reduces enzyme and operating costs by obviating the need to separate enzyme from the product stream, the additional immobilization steps generally increase specific enzyme cost by a factor of 4 due to higher material, labor, and equipment costs.[97]

The cost of using MEAs can be reduced by coupling biocatalyst preparation processes into fewer steps. One major advantage of many of the directed assembly strategies described earlier is the high specificity and affinity of enzyme loading on the scaffold. Using such a strategy, scaffolds could be immobilized on a biocatalyst carrier to allow the direct assembly of highly ordered MEAs on the carrier.[40,100] Nevertheless, given the high costs associated with enzyme purification and MEA assembly and immobilization, MEAs are generally better suited for the production of high-value products (e.g., pharmaceuticals and fine-chemicals). As a result, MEAs are unlikely to displace conventional catalytic systems in the production of bulk commodities in the near future.

The stability of the biomolecular interactions that hold a particular MEA together must also be evaluated before using MEAs in an industrial setting. The biomolecular interactions that facilitate protein-scaffold- and DNA-scaffold-directed assembly are reversible, and thus, MEAs engineered using these strategies are prone to enzyme dissociation. Enzyme loss through dissociation will not only result in unacceptably high material costs but also lead to greater operating costs, as additional processing steps are required to separate dissociated enzymes from the product stream. Further, enzyme dissociation from the engineered MEA will likely affect the overall catalytic efficiency, complicating process modeling.[101,102] It is also worth noting that the biocatalyst carrier supporting the MEA (e.g., silica-based carriers, acrylic resins, synthetic polymers, active membranes, and exchange resins),[103] can cost more than the enzymes themselves, depending on its material properties.[97] Under these circumstances, reversible immobilization of MEAs on the biocatalyst carrier may be desirable, and a balance must be struck between reuse of the biocatalyst carrier and the reuse of the enzymes. No one-size fits all approach exists for designing MEAs for biocatalysis, and the optimum purification, assembly, and immobilization strategies will vary depending on the specific process.

Another phenomenon that complicates the commercial application of MEAs is the recycle of cofactors, the most common of which are nicotinamides (NAD(P)H). Ideally, the enzymes comprising a MEA would either not require cofactors or be cofactor neutral. Cofactor-independent and cofactor-neutral reactions are desirable because most cofactors are far too expensive for single-use in large-scale in vitro industrial processes;[104] for example, NADP and NAD cost approximately $4500/kg and $1500/kg, respectively.[94] When cofactor-dependent enzymes cannot be avoided, cofactors must be regenerated in situ for the process to be economically feasible. Several regeneration strategies have been attempted including electrochemical[105] and chemical[106] regeneration systems, cofactor immobilization,[107] and supplementing the reaction with additional enzymes for enzymatic cofactor regeneration.[108−110] Of all these strategies, enzymatic cofactor regeneration schemes have been the most-studied, and several types of enzymes have been used to regenerate nicotinamide cofactors. For example, NAD(P)H can be regenerated from NAD(P) by adding alcohol dehydrogenase,[111] formate dehydrogenase,[111] or glucose dehydrogenase[112] along with its respective sacrificial substrate to a reaction in which NAD(P)H is oxidized. Similarly, NAD(P) can be regenerated from NAD(P)H by adding glutamate dehydrogenase or NADH oxidase.[108,110,113]

Despite advances in cofactor regeneration systems, acellular cofactor-dependent multienzyme reactions remain prohibitively expensive on an industrial scale, as cofactor loss and susceptibility to deactivation continue to be significant obstacles. To ameliorate the high material costs associated with cofactor-dependent reactions, researchers have pursued low-cost biomimetic cofactor analogs.[114] One particularly promising study demonstrated that a synthetic analogue of NADH used in concert with a modified glucose dehydrogenase enzyme could not only be regenerated, but the modified enzyme also exhibited a 10-fold activity increase over the wild-type enzyme.[115] Although such results are encouraging, biomimetic cofactors generally require enzyme modification and have yet to be applied to industrial biocatalysis.

Like all chemical production operations, multiscale modeling of industrial biocatalytic processes is crucial to ensuring the reliable production of the target molecule when operating conditions (i.e., temperature, pH, reactant concentration, etc.) are perturbed. However, modeling production processes involving reactions catalyzed by MEAs is uniquely challenging because of the significance of parameters that are only observed at nanoscales. In contrast to less-complex reactions catalyzed by well-mixed soluble enzymes, assumptions related to local reactant concentration and mixing are complicated by the aforementioned proximity effects and the difficult-to-monitor microenvironment of MEAs.[94] Moreover, while a change in process conditions (e.g., temperature or pH) will affect single-enzyme reactions, the effect on the reaction kinetics will be more difficult to predict for MEAs in which the constituent enzymes have different optimum operating conditions.[109] In addition to the complexity of reaction kinetics, variation in MEA assembly efficiency and stability are also important parameters in modeling the overall reaction rate. We have observed that complete assembly efficiency of MEAs on the surface of a cellulolytic yeast whole-cell biocatalyst cannot be taken for granted, and models assuming

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100% MEA assembly efficiency will overestimate the specific enzymatic activity of the biocatalyst. Although modeling chemical transformations mediated by MEAs is challenging, it is necessary for any commercial production process, as changes in reaction environment will have consequences in downstream operations such as product separation and quality control.

CONCLUSIONS

MEAs have the potential to significantly improve the efficiency and yield of many industrial production processes by catalyzing complex, multistep reactions under mild process conditions. However, long-standing and significant technical challenges such as cofactor usage, immobilization cost, the lack of predictable results, and the lack of universal approaches must be addressed if MEAs are to become competitive with conventional catalysts in industrial processes. Nevertheless, given the importance of energy costs, a growing environmental consciousness, and recent advancements in MEA engineering, we believe industries will continue to turn toward more advanced and renewable multistep enzyme-catalyzed processes to augment and eventually replace less efficient ones. The use of MEAs in industry will likely begin with processes that produce highly pure or valuable products due to the cost of MEA synthesis and the ability of MEAs to reduce side products and increase yields. This shift toward enzyme-based processes for complex chemical transformations will be catalyzed by reducing the costs associated with MEA synthesis and incentivized by the development of ever more efficient multienzymatic reaction systems.

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Notes

The authors declare no competing financial interest.

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