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Multi-scale Features in Recent Development of Enzymic Biocatalyst Systems

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Abstract Functional relation among elements of different size scales in a system is probably a main challenge across the areas of the science of engineering ever since their emergence. Multi-scale time and size correlation for description and prediction of complex systems, however, has been systematically examined only recently with the aid of new computational tools. In the pursuit of efficient and sustainable chemical processing technologies, people have seen a growing emphasis on synthetic biotechnology in recent R&D efforts. In particular, industrial enzyme technologies are attracting enormous attention. Having been traditionally developed for food and detergent applications, industrial enzyme technologies are being re-examined and tested to their limits to keep abreast of the challenges in drug, biochemical, and the emerging biorenewable energy industries. Toward that, enzymes are required to function in non-conventional conditions, such as organic solvents, extreme pH, and temperatures; they also have to compete against alternative chemical technologies in terms of costs and efficiency. Accordingly, enzymic biocatalyst systems are being tackled dynamically at all size levels through efforts ranging from molecular level protein engineering and modification, nanoscale structure fabrication, and microenvironment manipulation to the construction of microchip devices and macroscopic industrial bioreactors and devices. These efforts are probably still on a case-to-case trial basis without much consideration of cross-scale correlations. Discovering, understanding, and controlling of the common features that relate functions of biocatalysts at different size scales may eventually be realized in future.

Keywords Synthetic biotechnology · Enzyme · Nanoscale science and engineering · Multi-scale design · Complex systems

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Introduction

Bio-based resources for energy and raw materials are pursued worldwide as probably the only sustainable solution to the concerned shortage of petrochemicals. This endeavor is bringing up a revolution that will not only reshape the structure and appearance of our industries but also alter the way our society to function fundamentally. To much extent, this revolution is about efficiency. The efficiency of transforming bio-based resources into usable energy and materials and the efficiency of these products serve people's needs. Nanoscale science and engineering, which deal with size-dependent properties and phenomenon at nanometer scale, are unveiling new mechanisms that people have to rely on heavily nowadays to achieve such efficiencies. To capitalize the power of nanoscale technologies, it is becoming a common need to understand and design complex systems of multi-scale structures.

The conversion of raw biomaterials to end-use products and energy can be achieved through either thermo-chemical or bioprocessing technologies. From many aspects, biotransformation is appealing. Compared to chemical technologies, biotransformation produces less by-products, consumes less energy, and generates less pollution to the environment and products. Living biological systems use enzymes to perform transformations of materials. Since 1960s, isolated and immobilized enzymes have been used in large-scale industrial reactors. In most cases, natively evolved biocatalysts, either in form of enzymes or microbes, are not optimized for use in industrial reactors. Recent advances in genetic engineering have made it possible to design and produce more efficient industrial biocatalysts. The use of isolated enzymes provides the chance to further improve the performance of biocatalysts through various after-isolation chemical and physical manipulations. Immobilization is a method widely applied in the production of industrial enzyme catalysts. Substantial R&D efforts have been conducted to optimize the carrier materials' structure for better catalytic efficiency. In this regard, nanostructured materials provide desirable features in balancing the key factors that determine the efficiency of biocatalysts, including specific surface area, mass transfer resistance, and effective enzyme loading [1, 2]. Various nanomaterials such as nanoparticles, nanofibers, nanotubes, and nanoporous matrices have demonstrated promising potentials in revolutionizing the preparation and use of biocatalysts. Extending from there, people are exploring broadly enzyme-based assemblies, microscale structures, and new types of bioreactors for biotransformation and biosynthesis. It is probably reasonable to say that synthetic biotechnology is entering a new phase of development that integrates biocatalysts' structural and functional features that exhibit at different size scale, ranging from atomic-molecular level, nanometer structure scale, to a degree of micro- and macrosize bioreactors. It is the intent of this paper to review this multi-scale trend in recent advances of enzyme-based synthetic biotechnology.

Molecular Level Design and Manipulation of Enzymes

Enzymes refer to a group of proteins that regulate and catalyze biological reactions. When enzymes are isolated from their native biological hosts and used in a man-made reaction environment, their native structures that have evolved through millions-of-years conditioning with natural environment may not best fit to the desired tasks. Their surface properties, 3-D conformation, size, and non-protein groups may all need to be redesigned, adjusted, or optimized to better capitalize the catalytic power of the enzymes.

We may classify the ways of manipulating enzyme molecules into three categories, i.e., physical fabrication, chemical modification, and genetic alteration. Physical and chemical manipulations are performed after enzymes get isolated from their biological hosts, while genetic tailoring regulates the primary structures of the enzymes *in situ* in their biological sources where the enzymes are produced.

Ion-pairing with charged surfactants is a typical method of physical fabrication of enzyme molecules (Fig. 1). People have long discovered the formation of microemulsion as an interesting physico-chemical phenomenon by using surfactants. Starting from 1980s, reversed micelles were found very useful for protein extraction [3] and were tested later for biocatalytic applications [4, 5]. Reversed micelles are water droplets surrounded by surfactant molecules that are dispersed in an continuous oil phase. Protein molecules can be contained within the water droplets and thus get extracted from their aqueous solutions into an organic phase. Although reversed micelles can be used to disperse enzymes into organic media, we may not classify this as a molecular manipulation of enzymes, as the micelles may enclose many free molecules. Later in 1990s, it was found that enzymes could be extracted into organic solvents with the assistance of the same surfactants but without the formation of reversed micelles [6, 7]. Several or tens of surfactant molecules can associate with one oppositely charged enzyme molecule through electrostatic interactions and thus alter the surface property of the protein to become hydrophobic enough to be soluble in organic solvents (Fig. 1). Surfactant-enzyme ion pairs have been applied for non-aqueous biocatalysis [7 9] and preparation of homogenous bioactive plastics [10]. Enzymes can also form molecular complex with larger molecules, such as polyelectrolytes (Fig. 1). It was reported that poly(ethyleneimine) and enzymes can form complexes through electrostatic interactions and thus increase the size of enzyme molecules to have them better retained in membrane reactors [11, 12].

Chemical modifications of enzyme molecules may be achieved through either addition or removal of chemical groups. For example, deglycosylation of glycoenzymes has been applied to generate organic-soluble enzyme derivatives [13]. As a reverse process, chemical groups were also attached to enzyme molecules by forming covalent bonds with side groups of polypeptide chains of enzymes. Poly(ethylene glycol) (PEG), an amphiphilic polymer, was probably the most widely used enzyme modifier [14]. PEGylation was first developed for biomedical proteins and has hitherto been applied as a versatile protein modification technology [15]. Most PEGylated enzymes have organic solubilities in the

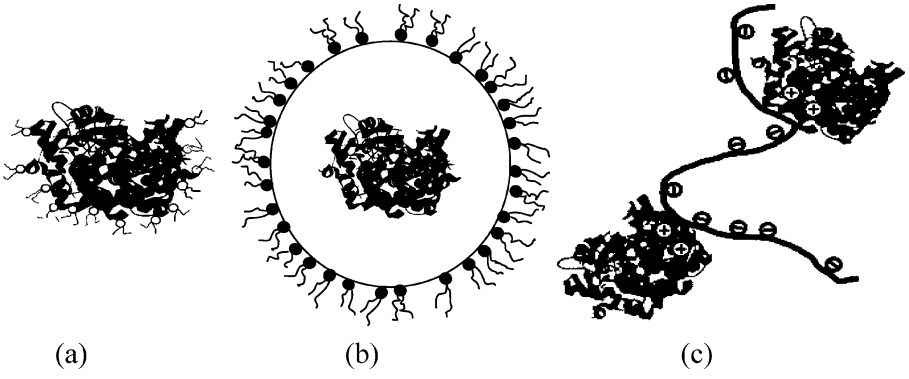

Fig. 1 Physical modification of enzymes. **a** Ion-paired enzyme; **b** reversed micelle contained enzyme; **c** polyelectrolyte–enzyme complex

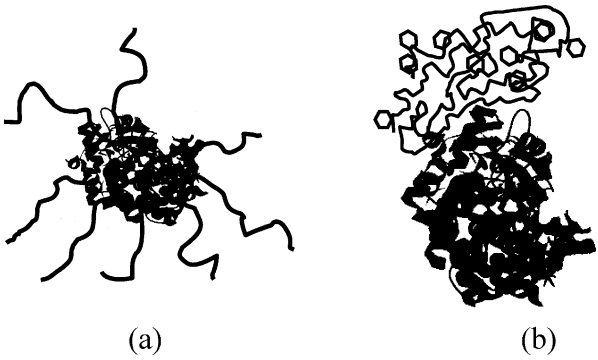

Fig. 2 Chemical modification of enzymes. **a** Multiple attachment of chemical modifiers for improved hydrophobicity; **b** polymer–enzyme conjugate for interface-specific activity

order of 1 mg/ml or lower [16, 17]. Industrial biosynthesis usually prefers more concentrated catalyst in a reactor for better reactor productivity. Recently, it was found that modification with decanoyl chloride can form enzymes that were soluble up to 44 mg/ml in organic solvents [18]. Chemical modification was also used to derive "smart" enzymes that self-target organic chemicals. Chemical modification examples discussed above usually lead to several modifier groups attached to one enzyme molecule. On-to-one attachment can form enzymes with very different properties. It was reported that enzymes conjugated with hydrophobic polymer groups could self-assemble and catalyze reactions at oil–water interfaces [19, 20]. Both the polymer coils and protein moieties have a size in nanometer scale, making the overall size of the conjugate small enough to be driven by affinity forces to the oil/water interface and thus forming unique biocatalysts that can selectively target interfaces for reactions (Fig. 2).

Genetic engineering has been explored extensively for production of enzymes with desired properties. One effort in this area was the design and production of thermo-stable enzymes by methods such as introducing genes from thermophile microorganisms. Only moderate success has been reported so far in this effort due to the lack of knowledge of structural regions governing the thermal stability of enzymes [21, 22]. However, gene mutation has been successfully applied in deriving enzymes with other specific properties. Substrate selectivity of enzymes, such as those of monooxygenases and hydrolases, could be shifted and altered significantly through genetic engineering [23]. Enzymes can also be tailored with specific binding properties. Rational design of enzymes through computational protein chemistry and genetic translation into biological hosts may eventually be realized in future; however, we are probably only at the starting point in that direction. At the present time, directed evolution and shuffling of homologous genes are being used as a quick and practical way to modify and improve important biocatalysts [24].

Bioactive Nanostructures

Molecules of enzymes are in the order of several nanometers. That makes it possible for enzymes to benefit from the nanometer-scale manipulations in their surrounding environments. On the contrast, living microbial cells are of micrometer sizes, and in most cases, they are generally not sensitive to nanometer-scale changes in their environments. The use of nanoparticles to attach enzymes has been reported in late 1980s. Since then, materials of various compositions, shapes, structures, and modified surfaces have been used to support

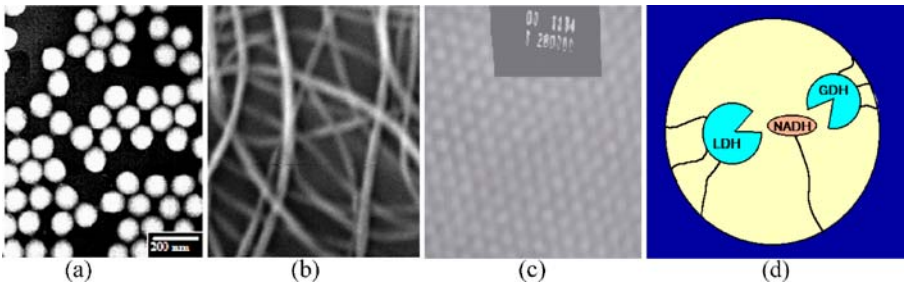

Fig. 3 Nanostructured biocatalysts. **a** TEM Image of nanoscale particles; **b** SEM image of nanofibers; **c** TEM image of nanoporous silica glass; **d** scheme of multienzyme system contained in a nanopore

biocatalysts (Fig. 3). Nanoparticles made of silica, magnetite, and gold are the first group of nanomaterials applied for biocatalysis. The attachment of nanoparticles can introduce various functionalities to enzymes. Magnetic nanoparticles for easy handling and reaction controlling, surface modification for better dispersion or assembling properties [25], and fabrication of multi-enzyme systems have all been reported [26]. Very interestingly, carbon nanotubes, as well as several types of nanoparticles carrying enzymes, were also found interface-assembling in the presence of adequate surfactant [27].

Cross-scale correlation for the prediction of catalysis of nanobiocatalysts may be achieved by combining the governing properties of nanostructures and the catalytic properties of free enzymes. This has been demonstrated at least for the simplest case, nanoparticle-attached enzymes [28]. Unlike large-size solid materials, nanoparticles dispersed in a solution are mobile in form of Brownian motion. According to Stokes–Einstein equation, the mobility or diffusivity of the nanoparticles has to be smaller than those of native free enzymes due to their relatively larger sizes. The mobility–activity relationship was examined through experimental measurements and theoretical modeling in a recent work [28]. The results suggested that the mobility of the catalysts is an important factor in determining their activities and thus providing an explanation, among other considerations, to the high activities usually observed for enzymes attached to nanoparticles. A model developed-based collision theory predicted the size-dependence of the nanoparticle enzymes. Enzymes associated with nanomaterials of more complicated properties, such as those attached to carbon nanotubes [29, 30], may be described through the same strategy, but better understanding of the responses of the nanomaterials to the reaction environment has to be realized first.

Nanocapsulation gives another important form of nanobiocatalysts. Through careful synthetic routes, enzymes can be entrapped into cores of discrete polymeric [31] and silica [32] particles, forming highly stable biocatalysts. In most cases, time-dependent activity loss of enzymes is caused by conformational changes of the enzymes. It seems reasonable to imagine that confining an enzyme molecule into a space of comparable size may limit the space available for the enzyme to undergo unfolding, thus providing a mechanism of enzyme stabilization. Polyacrylamide hydrogel granules with diameters less than 100 nm were reported first. A more recent study demonstrated that enzymes can be significantly stabilized when they were entrapped into discrete silica nanoparticles [32]. It was estimated that the half-life of such entrapped α-chymotrypsin (CT) could reach up to 143 days, a big improvement from the one-day half-life of free CT. Entrapping enzymes into nanoporous glass matrices either physically [33] or covalently [34] also stabilized the enzyme greatly. In particular, covalently bound CT in nanoporous silica gel glass showed a half-life that is

1,000 times higher than that of native CT against organic solvents [34]. Detailed theoretical description of such enzyme stabilization effect of nanostructures awaits further exploration.

When more than one enzyme are contained in a nanoscale pore, they may form an active molecular machine system that can catalyze multiple reactions, mimicking certain biological transformations. This concept was demonstrated recently [26]. In that work, two enzymes along with one cofactor, nicotinamide adenine dinucleotide [NAD(H)], were incorporated into glass nanoporous materials with pores of 30 and 100 nm in diameter (Fig. 3). Provided that the tethers were flexible, NAD(H) could shuttle back and forth between these two enzymes, thus achieving multiple reactions with cofactor regeneration.

Multi-scale Structured Biocatalysts

Micrometer scale structures are close to the visibility of human eyes. Fluid behaviors within micrometer-spaces can be manipulated, controlled, detected, and analyzed routinely in laboratories nowadays. That is not what is available for nanometer scale structures. Many of the molecular and nanoscale properties have to be verified through microscale or macroscale observations. From many aspects, the understanding and description of the functions of nanoscale structures provide transitional bridges correlating molecular behaviors of biocatalysts to micrometer scale observations. Theoretical multi-scale correlation of biocatalysis is a challenge that has not been well addressed yet. Breakthroughs may come first from biocatalyst systems that have well-defined multi-scale structures, such as the examples to be discussed in the following.

From a purely structural view point, 1-D nanomaterials offer an interesting case of multi-scale structures. One well-known example is carbon nanotubes. Their diameter is in the range of nanometers, while their length can be extended to several hundreds of micrometers. They have been examined for use as supports of enzymes for biocatalysis, among many other applications. Such nanotube-attached enzymes have shown interesting properties when they were used for composite materials and at the oil–water biphasic media [27, 35]. Electrospun polymeric nanofibers represent an extreme case of such kind of 1-D nanomaterials in that the length of the fibers can be unlimited. The surface area-to-volume ratio of nanofibers is also high, representing two thirds of that of nanoparticles of the same diameter and same amount of materials. Nanofibers, however, can be handled in form of macroscale materials. They can be applied in forms of coils, sheets, and dispersed fibers and can also be surface-attached to or blended with other materials, thus offering very flexible design for reactors [36].

2-D assemblies of nanoscale biocatalysts give another type of cross-scale structures. As mentioned previously, enzymes conjugated with hydrophobic polymer groups self-assemble at oil–water interfaces that can be extended throughout a biphasic reactor [19, 20]. Very interestingly, carbon nanotubes and several types of nanoparticles were also found interface-assembling when they were conjugated with enzymes [27]. All of these studies showed that better enzyme activities were realized when thinner assemblies were formed. Assemblies of nanoparticles carrying enzymes on surfaces of solids have been explored for development of bioactive materials [37, 38]. Pattern-controlled 2-D assembly of silica nanoparticles with covalently attached enzymes on surface of silica plates was also prepared [38]. It was expected that such assemblies may found applications in developing biosensors and biochips. Multiple layers of nanoparticles can also be formed, and thus, unique optical or electrical properties can be introduced to the surface along with the bioactivity of the enzymes [37].

Hierarchical structures that integrate nano- and micrometer scale structures are emerging as a very attractive area. Enzymes entrapped inside nanoporous silica gel glass via both physical adsorption [33] and chemical binding [34] has the potentials to be applied in form of macrosize membranes and particles. More sophisticated structures, such as porous materials hosting enzyme-carrying nanoparticles [39] and cross-linked enzyme aggregates [40], have been developed by applying enzyme modification and fabrication procedures. In the later case, hierarchically ordered mesocellular mesoporous silica particles (200–500 nm), which have large mesocellular pores (37 nm) connected by mesoporous channels (13 nm), was used for the entrapment of cross-linked enzymes. Such treated enzymes showed excellent stability, such that activity loss was not observed in a rigorously shaking condition for more than a month.

In a study of "artificial cells," nanoparticles carrying enzymes and cofactor were encapsulated into polymeric capsules of diameter of about 100 μm [41]. The membrane of the capsules was porous with pore size controlled to be in nanometer scale to allow substrates and products to transfer through while retain the capsulated particle-attached enzymes and cofactor (Fig. 4). Because the nanoparticles were mobile inside the capsules when they were filled with water, collision between particles could enable interactions among the enzymes and cofactor, thus allowing multiple reactions to take place inside the capsules.

Macroscale Bioreactors

Enzymes, both native and genetically engineered, have been used at large-scale industrial processes without much consideration of multi-scale structural properties of the catalysts. The performance of macroscale reactors is subject to the impact of numerous factors such as fluid dynamics, mass transfer, heat exchange, and concentration distribution. All of these factors, in concert with the governing factors at molecular and nanometer scale, may substantially complicate the catalytic behaviors of the catalysts. Bioprocessing technologies that reflect the power of multi-scale design have been thus far mostly reported and demonstrated for lab-scale demonstrations and tests. These results, however, have demonstrated the great potentials of the multi-scale design for tomorrow's bioprocessing technology. Some simple systems are actually ready for scale-up without imparting too much complication. Reaction kinetics of nanoparticle-carried enzymes, ion-paired enzymes,

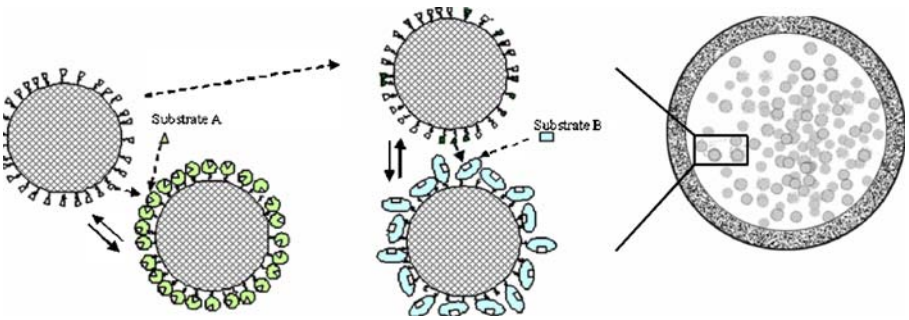

Fig. 4 Nanoparticle biocatalyst systems resided in microcapsules. Micrometer-scale capsules provide easy handling and flexible reactor design, while nanoparticles are mobile and can drive multiple reactions inside the capsules

or other forms of nanobiocatalysts suspended in reaction media may be irrelevant to the size of the reactors, as the dispersed nanostructures behavior are very much like free molecules in homogenous solutions and are insensitive to micro- and macroscale fluid dynamics. Similarly, 2-D interfacial assemblies of enzymes developed at bench-scale may be expected to catalyze reactions of larger-scale with the same reaction kinetics. The use and understanding of more complicated heterogeneous bioreactors with biocatalysts that are detail-designed at all structure scales are still greatly challenging.

Conclusion

Efficient bioprocessing technologies are critical to capitalize the potentials of biorenewable alternatives to petrochemicals. Driven by the recent advances in molecular biotechnology and nanoscale science and engineering, development of biocatalysts is showing a multi-scale trend that includes the optimization of protein molecular structures, manipulation of nanoscale environments of the enzymes, microscale properties of materials and fluids, and operational factors of macroscale reactors. How to translate molecular level catalytic properties of enzymes into performance of macroscopic bioreactors through relations across all the size levels of the structures is still challenging. The science and engineering of nanoscale matters, which represent a transitional region between molecules and macroscale structures, play a key role in achieving such correlation and analysis. Traditional catalytic technologies typically lacked the design and consideration of the nanoscale structural details. As nanoscale science grows into maturity, theoretical correlation and rational design of the multi-scale structured biocatalysts will be eventually realized and generate substantial impact on future industrial biotechnologies.

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