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FEATURE ARTICLE

Toward low-cost biomanufacturing through *in vitro* synthetic biology: bottom-up design

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While most *in vitro* synthetic biology projects are usually used for the purposes of basic science research or the formation of high-value products, cell-free synthetic pathway biotransformation (SyPaB), which can implement complicated biochemical reactions by the *in vitro* assembly of numerous enzymes and coenzymes, would be used for low-cost biomanufacturing. In this article, we present bottom-up design principles for SyPaB from basic building blocks (enzymes and/or immobilized enzymes) to basic modules, such as NAD(P)H regeneration, NAD(P)H consumption, ATP regeneration, and extra ATP removal. A combination of thermostable enzymes (called thermoenzymes) with immobilization on solid supports, especially nano-materials and/or electrodes, would greatly prolong enzyme lifetime, enhance mass transfer, and facilitate product/biocatalyst separation. With developments in stable building blocks and modules (called biocatalytic modules), SyPaB has the potential to become a low-cost biomanufacturing platform for biofuels production and even biological CO_2 fixation.

1. Introduction

Synthetic biology is an emerging interdisciplinary area that combines biology, chemistry, and engineering for designing and building novel biological functions and systems that function unnaturally or function much better than their natural counterparts.[1–4] This field is also described as the engineering-driven construction of increasingly complicated biological

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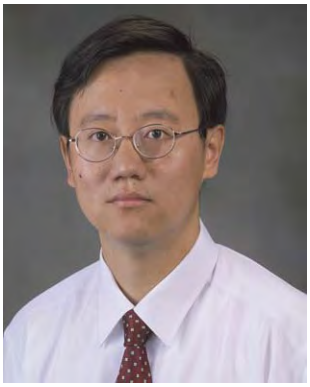

Y.-H. Percival Zhang

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entities (*e.g.*, parts, devices, and systems) from simple and basic building blocks.[4,5] Synthetic biology can be used as a research tool for understanding the complexity of biological systems and for a wide variety of applications: constructing programmable biodevices and control logic, nano-material assembly, molecular devices, computation and signal processing, biosensors, *etc.* Its most important application may be the low-cost production of new drugs, materials, and energy.[2–4,6]

Synthetic biology can be divided into two directions: *in vivo* and *in vitro*.[2,3,7] Although most synthetic biology projects are based on living entities, the state of the art in *in vitro* synthetic biology has advanced greatly and is moving forward rapidly. This fast progress is due to several important features of *in vitro* synthetic biology platforms, including (i) great engineering flexibility without cellular viability, system complexity, physiology, and membrane/wall, (ii) very high product yields without cell duplication or other side products, (iii) fast reaction rates due to high enzyme loadings and no cellular membrane, (iv) broad reaction conditions (*e.g.*, high temperature, presence of organic solvents or microbe-toxic compounds), and (v) industrial scale-up potential.[2,3,8,9]

Biocatalysis or biotransformation mediated by biocatalysts (*e.g.*, enzymes or whole cells) can be divided into four classes, based on an increasing order of biocatalyst complexity: (i) single enzyme, (ii) multi-enzyme one pot, (iii) cell-free biotransformation, and (iv) whole-cell fermentation (Fig. 1). A single enzyme can only catalyze a simple chemical reaction; approximately 4800 enzyme entries have been documented in the Brenda database.[10]

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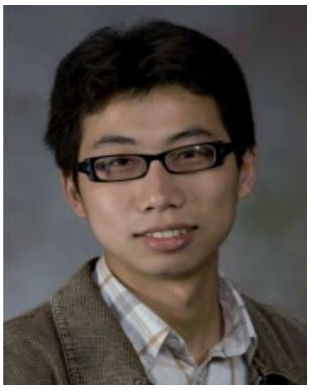

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Relatively complicated chemical reactions can be mediated by multi-enzyme one pot, for example, cellulose hydrolysis by a synergetic action of endoglucanase, cellobiohydrolase, and beta-glucosidase,[11] synthesis of chiral alcohols with NAD(P)H regeneration,[12,13] and biosynthesis of polymers.[14] However, one or a few enzymes cannot implement very complicated reactions, such as ethanol production from glucose and biological CO_2 fixation. Whole cell biocatalysts or/and their cell lysates containing numerous enzymes can implement very complicated reactions. The difference between living cell fermentation and cell-free enzyme mixture biotransformation seems to be very clear: living cells can duplicate themselves while enzyme mixtures cannot (Fig. 1). For living entities, a significant fraction of resources (*e.g.*, energy and carbon sources) have to be consumed for self-duplication, maintenance, and many other functions, resulting in relatively low product yields. Until now human beings have not created any living or self-duplicating entities from scratch (*i.e.*, known composition protein mixtures and DNA sequences) but have created a myriad of non-living machines specifically for unique applications. Self-duplicating machines usually mean low production efficiencies. Cell-free biotransformation by using highly stable enzymatic components can mitigate the yield, rate, and efficiency limits inherent in living or other self-duplicating systems. Therefore, developing non-living biomanufacturing systems featuring high product yields is extremely important when product yield (or energy efficiency) is the most important criterion of the desired industrial application, for example, biofuels production.[2,4,8,15]

In this feature articale, we present an important direction of synthetic biology—cell-free synthetic pathway biotransformation (SyPaB), including its brief history, latest advances, and future applications; we discuss bottom-up design principles for SyPaB; and we highlight its challenges and opportunities for material chemists.

2. Cell-free synthetic pathway biotransformation (SyPaB)

Cell-free fermentation/biotransformation originated from cell-free ethanol fermentation, discovered by Eduard Buchner in

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1897.[2] As a result, he won the 1907 Nobel Chemistry Prize for *"his biochemical researches and his discovery of cell-free fermentation"*. From then on *in vitro* assembly of numerous purified enzymes, mimicking natural pathways, has been widely used for the study of numerous natural metabolisms.[16,17] Recently, *in vitro* assembly of both natural and non-natural pathways has been used to synthesize high-value products, such as labeled purine nucleotides, carbohydrates, circular DNA, modified RNA, liposomes, and proteins.[7,18–24]

Cell-free synthetic pathway biotransformation (SyPaB) is the implementation of complicated biochemical reactions by the *in vitro* assembly of numerous enzymes and co-enzymes.[2,3] Different from cell-free biotransformations that produce high-value products on a small scale, SyPaB is designed to produce low-value biocommodities, such as biofuels, organic acids, and starch, on a large scale. Since large volume biocommodities have low selling prices, ranging from less than one to several US dollars per kg, raw material costs (*e.g.*, carbohydrate) or energy inputs often account for ~30–70% of final product's selling prices.[2,25] Therefore, the design principles of SyPaB are completely different from the other cell-free fermentation/biotransformation. These include the careful consideration of substrate costs and final product values, biocatalyst costs, product separation costs, and so on. It is economically prohibitive to produce low-value products by consuming costly substrates (*e.g.*, ATP or NADH) or by using unstable biocatalysts.

SyPaB can be regarded as an integrated platform based on three elements—pathway reconstruction, enzyme engineering, and reactor engineering. Synthetic pathways can be assembled based on natural pathways or their components. Unlike living systems, which can self-balance coenzymes and generate ATP for the synthesis of numerous biomolecules, SyPaB must have balanced coenzymes and ATP *in vitro*. In addition, thermodynamics analysis must be analyzed to ensure designed processes to take place as expected. Enzyme engineering is a relatively mature field after nearly a half century of development as a sub-direction of biochemical engineering. It involves enzyme discovery; bulk enzyme production; protein engineering by rational design, directed evolution, or their combination; and enzyme immobilization. Reactor engineering includes bioreactor design (*e.g.*, membrane reactor, flow-through reactor, continuous stirred-tank reactor, *in situ* product separation) and arrangement—one reactor or several ones in series.

A good example of SyPaB is the high-yield production of hydrogen from starch and water by assembling 13 enzymes from five different sources (*e.g.*, bacterium, yeast, plant, animal, and archaea)[26] as below

$$C_6H_{10}O_5\ (aq) + 7\ H_2O\ (l) \rightarrow 12\ H_2\ (g) + 6\ CO_2\ (g) \quad (1)$$

This non-natural pathway can produce 12 moles of hydrogen per mole of glucose, three times of the theoretical yield of natural hydrogen-producing microorganisms—called the Thauer limit.[27] This non-natural synthetic catabolic pathway is comprised of 13 enzymes in one bioreactor. The pathway contains four biocatalytic modules: (i) a chain-shortening phosphorylation reaction in the presence of phosphate ions (P_i), which produces glucose-1-phosphate (G-1-P), catalyzed by starch phosphorylase (eqn (2)); (ii) generation of glucose-6-phosphate (G-6-P) from G-1-P, catalyzed by phosphoglucomutase (eqn (3)); (iii) generation of 12 NADPH from G-6-P through the pentose phosphate pathway and some glycolysis/gluconeogenesis enzymes (eqn (4)); and (iv) generation of hydrogen from NADPH, catalyzed by a NADP-dependent hydrogenase (eqn (5)).

$$(C_6H_{10}O_5)_n + P_i \rightleftharpoons (C_6H_{10}O_5)_{n-1} + \text{G-1-P} \quad (2)$$

$$\text{G-1-P} \rightleftharpoons \text{G-6-P} \quad (3)$$

$$\text{G-6-P} + 12NADP^+ + 7\ H_2O \rightleftharpoons 12NADPH + 12H^+ + 6CO_2 + P_i \quad (4)$$

$$12NADPH + 12H^+ \rightleftharpoons 12H_2 + 12NADP^+ \quad (5)$$

The overall reaction (eqn (1)) from starch and water is spontaneous and endothermic (*i.e.*, $\Delta G^\circ = \sim -50$ kJ mol^{-1} and $\Delta H^\circ = +598$ kJ mol^{-1}).[26] This entropy-driven reaction can generate the chemical energy output in the form of hydrogen more than the chemical energy input as polysaccharides by absorbing ambient temperature waste heat. This reaction is possible due to the phase changes that occur over the course of this reaction, taking the system from more orderly to less orderly, resulting in a large positive ΔS.[26,28] Such catabolism does not occur in microorganisms because it cannot produce any energy currency (*i.e.*, ATP) for microorganism growth and duplication. Later, a similar non-natural pathway has been designed to produce high-yield hydrogen from cellulosic materials and water.[28]

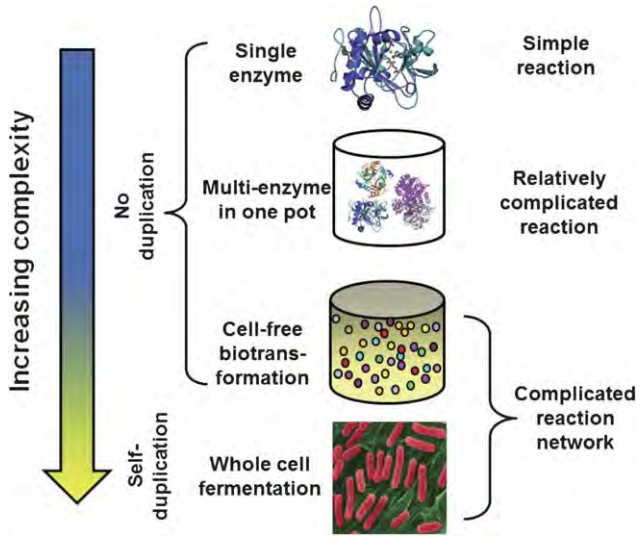

Fig. 1 Comparison of biocatalysis mediated by a single enzyme, multiple enzymes, cell-free synthetic enzymatic pathway, and whole cells, in an order of increasing complexity.

3. Basic *in vitro* building blocks and modules

Bottom-up design of SyPaB starts from basic building blocks (*i.e.*, thermoenzymes and immobilized enzymes) to building modules made of several enzymes with defined functions (*e.g.*, NADH regeneration, ATP generation) to complicated synthetic pathways for special applications (*e.g.*, sugary hydrogen production). SyPaB must have balanced cofactors and ATP

in vitro and substrates must be less costly than products.[2,4] Therefore, it is vital to design *in vitro* synthetic enzymatic pathways by combining basic blocks and modules with balanced cofactors and ATP or low-cost ATP generation systems. BioBrick™ DNA sequences are standard biological parts in *in vivo* synthetic biology projects, while basic building blocks of SyPaB are enzymes. Numerous enzymes are used in several different industries to replace chemical catalysts due to their high specificity and high efficiency under mild and environmentally-friendly conditions, while more enzymes are utilized as molecular biology tools or are research targets in life science studies. The most widely-known thermostable enzyme (called thermoenzyme) in life sciences is the Taq polymerase, which was isolated from the thermophile *Thermus aquaticus*.[29] Now this recombinant enzyme or similar enzymes produced in *E. coli* is widely used to catalyze the polymerase chain reaction (PCR) with an annual market of a half billion US dollars. This enzyme has an optimal temperature of 75–80 °C. Two other widely-known thermoenzymes in the food industry are amylase, which works at ~100 °C, and immobilized glucose isomerase, which works at ~55 °C for up to two years. Discovery and utilization of thermoenzymes greatly simplifies numerous biotechnological processes and decreases processing costs.

3.1. Immobilized enzymes

First generation biochemical engineers developed enzyme immobilization technology by using available mesophilic enzymes, not only to prolong their life time and selectivity but also to facilitate soluble product/solid biocatalyst separation and recycling.[30–33] Immobilized enzymes on insoluble supports have been widely applied in the food industry, in the biofuels production, in fine chemical synthesis, and for environmental purposes.[34–36] Following the discovery and utilization of thermostable enzymes from thermophiles and the invention of protein engineering, a hybrid of stable enzymes and enzyme immobilization has enabled enzyme technology to replace more traditional chemical catalysis and simple microbial biotransformations.[31,37–40]

During the past several decades, numerous enzyme immobilization technologies have been established, including physical adsorption, covalent bonding, encapsulation/entrapment on insoluble supports, and cross-linking between enzyme molecules[34–36,41] (Fig. 2). Support materials and methods of immobilization are greatly influenced by numerous aspects, such as physical and chemical characteristics of enzymes and supports.[32] Combinations of two or more immobilization methods are continually being developed to improve the performance of immobilized enzymes. The best ideal immobilization may have zero influence on enzyme activity. There is not a single method or material suitable for all enzymes and their applications. Among these methods, adsorption of an enzyme to an insoluble support is attractive because it is simple, quick, cheap, and causes no or little damage to the enzyme. It may have some disadvantages, such as leakage of the enzyme from the support, nonspecific binding, and steric hindrance by the support.[31] Since it is easy to produce recombinant proteins containing a specific affinity tag, one-step protein purification and immobilization has been developed to avoid costly protein purification.[39,42,43] For instance, the cellulose-binding module, a key component of cellulase, is responsible for specifically binding to low-cost, biodegradable, inert cellulose.[42] A one-step protein purification and immobilization method has been developed by using low-cost, ultra-high adsorption capacity RAC to adsorb CBM-tagged *C. thermocellum* phosphoglucose isomerase (PGI).[31] The resulting immobilized PGI is highly active and ultra-stable, with a total turn-over number (TTN) of more than 10^9 mol of product per mol of enzyme at 60 °C.[31]

Cross-linked enzyme aggregation (CLEA) is a simple and low-cost carrier-free immobilization technology that allows high volumetric enzyme loading within a short preparation time.[30,44,45] The preparation of CLEAs requires two steps: physical aggregation, where an enzyme is precipitated by the addition of salts, organic solvents, non-ionic solvents, or polymers, and cross-linking by the addition of a cross-linker, such as bi-functional reagents.[46] Recently, cascade enzyme CLEAs have been developed, called "Combi-CLEAs".[47,48] Combi-CLEA can catalyze cascade enzymatic processes in one pot, providing numerous potential benefits: few unit operations, less reactor volume, higher volumetric and space-time yield, shorter cycle time, and less waste generation.[2,49]

Nano-materials have attracted much attention for enzyme immobilization because of their large surface areas. Examples

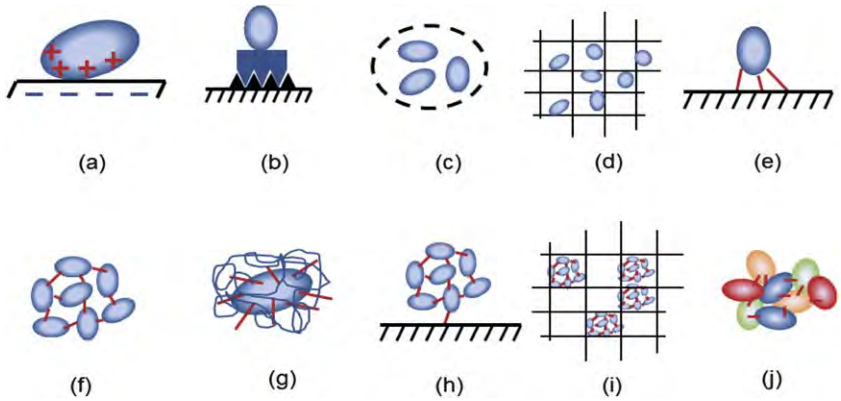

Fig. 2 General and combined methods of enzyme immobilization: ionic physical adsorption (a), high-affinity physical adsorption (b), encapsulation (c), entrapment (d), covalent binding on an insoluble support (e), cross-linked enzymes (f), a single nano-gel enzyme (g), a combination of e and f (h), a combination of d and f (i), and combi-CLEAs (j).

include nano-molecular sieves,[50,51] nano-fibers,[52–54] carbon nanotubes,[55] nano-particles[56,57] and graphene-based materials.[58,59] Mesoporous molecular sieves have been applied in a diverse range of fields, such as biosensors,[60] biocatalysis[61] and biomolecule separation systems,[62] because of their high surface areas, controlled porosity, and relatively simple adsorption and desorption.[51,63] The stability and leakage of adsorbed enzymes in mesoporous molecular sieves are strongly influenced by the structure, pore size, and charge interaction between support and enzyme.[41,50,51,60] For example, loading amounts of cytochrome *c* into various molecular sieves (MCM-48, SBA-15, and Nb-TMS4) have been investigated carefully.[60] The three dimensional MCM-48 has higher protein loading capacity than the others (one-dimensional hexagonal SBA-15 and two-dimensional layered Nb-TMS4).[60] For mesoporous materials, the adsorption of enzyme onto nano-porous carbon molecular sieves and mesoporous silica with various pore diameters suggests that the proper pore size for enzyme adsorption should be similar or larger than the size of the enzyme.[50,51] Charge interaction is also important for successful enzyme immobilization, as having opposite charges between porous materials and enzymes.[50,62] However, one of the major obstructions for the wide application of this method is the leakage of enzymes from mesoporous materials because of a lack of strong interaction or bonding between enzymes and supports. A combination of adsorption and covalent bonding between an enzyme and the inside surface of the pore can be used to prevent the enzyme leakage.[41] Because of the addition of covalent bonding, the stability of the immobilized enzyme can be improved compared to the native enzyme. Multi-enzyme reactions integrating the co-immobilized system in nanoporous materials with covalent bonding has also been studied.[64] In this setup, the catalytic efficiency of the system is improved due to enhanced molecular interactions among immobilized enzymes and cofactors in the nanoporous structure of the support. This enhancement can be increased by more flexibility of the spacers between the enzymes (or cofactors) and surface in 30 nm pores. In another study, the reaction rates between two cascade enzymes were found to be inversely correlated with the distance of two cascade enzymes[65] due to substrate channeling among cascade enzymes.[66]

3.2. NAD(P)H balance

While living organisms can continuously adjust NAD(P)H balance through anabolism and catabolism, low-cost SyPaB must have NAD(P)H balanced in its pathway design.[2] When energy-intensive compounds are synthesized, extra NAD(P)H is supplied. NAD(P)H can be usually generated by using a hydrogen-donor substrate and one of the following: a single enzyme, cascade enzymes, and electro-enzymes (Fig. 3). Single-enzyme systems include alcohol/alcohol dehydrogenase,[13] formate/formate dehydrogenase,[67] glucose/glucose dehydrogenase,[68] G-6-P/G-6-P dehydrogenase,[69] dihydrogen/hydrogenase,[70,71] and phosphite/phosphite dehydrogenase.[72,73] Single-enzyme NADPH regeneration systems have been widely used in the synthesis of high-value of chiral compounds in the pharmaceutical industry. Three representative single-enzyme substrates to regenerate NAD(P)H are the dehydrogenation of propanol, formate, and hydrogen (Fig. 3a-c). As an example multi-enzyme process, three enzymes—formate dehydrogenase, formaldehyde dehydrogenase, and alcohol dehydrogenase—can completely oxidize methanol to generate three NADH (Fig. 3d). A 12-enzyme system has recently been shown to produce nearly 12 NAD(P)H from one glucose unit of cellobiose.[8] Among all hydrogen-donor compounds, renewable sugars have the lowest substrate costs, but they require more enzymes and increased system complexity. Utilization of electrochemistry to generate reduced cofactors is low-cost and clean, but the instability of NAD(P)H under high over-potential must be solved before this technique becomes industrially feasible.[74,75]

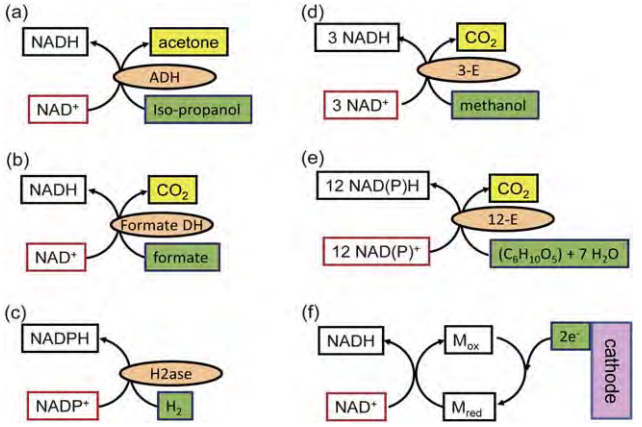

Fig. 3 NAD(P)H regeneration by a single enzyme (a-c), multiple enzymes containing three enzymes (d), a synthetic pathway containing 12 enzymes (e), and electrochemistry based on an enzyme (f). DH, dehydrogenase; ADH, alcohol dehydrogenase; H2ase, hydrogenase; and M_{ox} or M_{red}, oxidized or reduced mediator.

Sometimes designed products have a lower degree of reduction than that of the substrates, for example, the production of 1,3-butanediol and fatty acid ethyl esters from glucose.[15] That is, extra NAD(P)H is generated through such biotransformations. Unlike microbial fermentation, which can consume NAD(P)H through oxidation or cell mass synthesis, it is vital to remove extra NAD(P)H in SyPaB. Fig. 4 presents two different ways to remove extra NAD(P)H: enzymatic (a) and electrochemical (b&c). Extra NADPH can be removed by addition of a hydrogenase under low hydrogen partial pressure, in a manner similar to sugar-to-hydrogen experiments.[26,28] It is noted that this operation must be conducted in the absence of oxygen. The other way is to generate electrons from NADH, as occurs in enzymatic fuel cells through a single mediator (Fig. 4b) [76] or a diaphorase and a mediator (Fig. 4c).[77,78]

In the end of this section we would like to note that NAD and NADP may be regarded as biologically equivalent. In fact, most redox enzymes prefer one to the other.[8,79] If necessary, transhydrogenase may be added to enable NAD-preferred and NADP-preferred redox enzymes to work together. For generating reduced coenzymes by electricity or oxidizing reduced coenzymes to electrons, NAD is always preferred to NADP.

3.3. Enzyme immobilization on electrodes

Bioelectrochemistry is a well-established and reliable field for analytical purposes, for example, the determination of glucose concentration in the blood of diabetes patients. As previously discussed, the combination of electrochemistry and enzymology

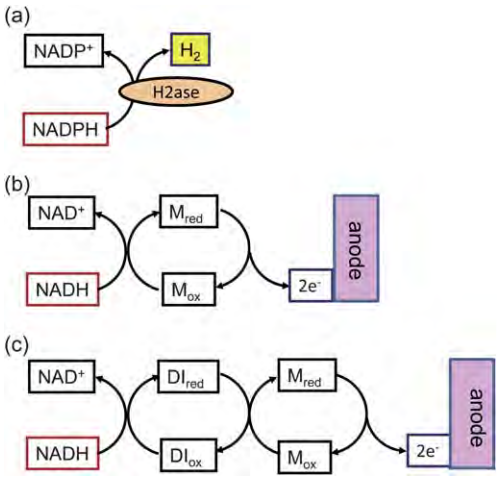

Fig. 4 NAD(P)H removal by a hydrogenase (a), and electrochemistry through a mediator (b) and a diaphorase and a mediator (c). M_{ox} or M_{red}, oxidized or reduced mediator; and DI, diaphorase.

can be used to keep *in vitro* NAD(P) balanced, where electrons instead of chemical compounds serve as redox equivalents for enzymatic redox reactions (Fig. 3f and 4b and c). For this purpose, redox enzymes immobilized on electrodes facilitate electron transfer between enzymes and electrodes and increase enzyme stability.[80] Methods for immobilizing enzymes on the electrode surface can be characterized as physical adsorption, entrapment, or chemical cross-linking.[41,78] Physical adsorption is mainly based on physical forces, including adsorption on conductive particles, such as carbon black or graphite powder, and more popularly, layer by layer adsorption using electrostatic forces (Fig. 5a). In layer-by-layer adsorption, enzymes are sandwiched between two layers of opposite charges of poly-ions.[81] This method has been shown to produce very high power in an enzymatic fuel cell.[77] However, physical adsorption may suffer from enzyme leakage. Enzyme entrapment in conductive polymers (Fig. 5b) is a very effective approach for addressing this issue. Minteer and her coworkers have successfully entrapped redox enzymes on electrodes by using a Nafion solution-cast membrane[82] or by using hydrophobically modified chitosan plus Nafion polymer.[83] However, slow mass transfer in these systems may result in low power outputs. Chemical bond cross-linking techniques (Fig. 5c) have also been used for wiring enzymes onto the surface of electrodes and binding enzymes with mediators.[84] Such cross-linking of enzymes usually increases enzyme stability at the expense of a decreased activity due to disruption of the redox centers of some enzymes.[85] Thanks to recent interest in nanotechnology, large surface area, highly conductive carbon nano-tubes (CNTs) have been used in enzyme immobilization on electrodes to improve the performance of EFCs and biosensors (Fig. 5d).[86,87] The positive effects of CNT are mainly due to its large surface area that adsorbs more enzymes, its tiny size, and its high conductivity, which speeds up electron shuttling from enzyme redox centers to electrodes. In addition of CNT, several large-surface area and high-conductivity electrode materials, such as mesoporous carbon CMK and grapheme, have been used for redox enzyme immobilization.[58,59,88] The combination of electrochemistry and enzymology would have great potential in the SyPaB platform.

3.4. ATP regeneration and dissipation

ATP, an energy currency of all living entities, is produced by photophosphorylation and cellular respiration. It is used for enzymes and structural proteins in many cellular processes, including biosynthetic reactions, mobility, and cell division. Different from living entities that can constantly regenerate ATP, some enzymes in cell-free systems may require ATP for some special enzymatic reactions. Low-cost regeneration of ATP is one of the most critical obstacles to some cell-free systems. (Note: high-yield hydrogen generation in Section 2 is a ATP-free

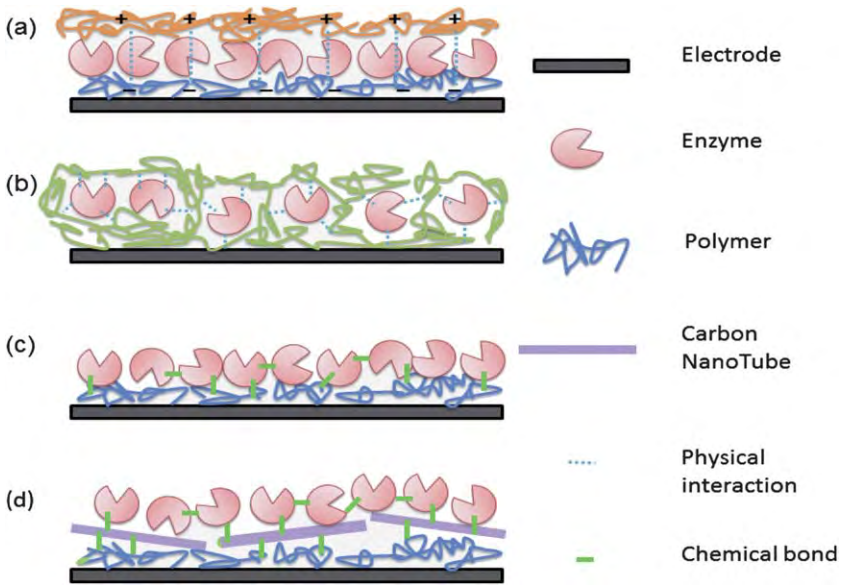

Fig. 5 Schematic layout of four immobilization methods for redox enzymes on anodes: electrostatic layer-by-layer adsorption (a), entrapment in a matrix (b), chemical cross-linking (c), and chemical cross-linking with CNT (d). The various components are not drawn to scale.

process[26,28]). *In vitro* ATP regeneration reactions can be classified as substrate-level phosphorylation,[21,89] oxidative phosphorylation of NADPH,[9] and photo-induced ATP synthesis.[90,91]

Most *in vitro* ATP regeneration technologies are based on substrate-level phosphorylation, where phosphorylated intermediates transfer a phosphoryl group to adenosine diphosphate (ADP) or adenosine monophosphate (AMP). The common phosphoryl donors include acetyl phosphate, phosphoenolpyruvate, methoxycarbonyl phosphate, dihydroxyacetone phosphate, 5-phospho-α-D-ribosyl pyrophosphate, uridine-5′-diphosphoglucose, phosphoenolpyruvate, acetyl phosphate, and creatine phosphate.[21] The utilization of these phosphate-containing energy sources inevitably causes the accumulation of inorganic phosphate, which inhibits the activity of ATP-dependent enzymes, changes pH, and precipitates Mg^{2+}.[21] Also, high substrate costs prohibit scale-up applications in biocommodity production. The use of non-phosphate-containing compounds can avoid problems associated with phosphate accumulation. By utilization of the natural glycolysis pathway, glucose and pyruvate have been used to generate ATP for cell-free protein synthesis.[89,92] Recently, maltodextrin has been used to generate ATP through substrate phosphorylation mediated by maltodextrin phosphorylase and phosphoglucomutase, followed by the glycolytic pathway.[21] Compared to glucose, maltodextrin can generate one more ATP per glucose equivalent and slowly release the energy compound (G-6-P) for better protein synthesis.[21]

Low-cost and high-stability polyphosphate is an attractive phosphoryl donor for ATP generation.[93–95] Recombinant *Thermus* polyphosphate kinase (PPK) produced by *E. coli* has been shown to regenerate ATP by using exogenous polyphosphate and ADP.[94] Alternatively, one ATP can be generated from two ADP mediated by polyphosphate-independent adenylate kinase (ADK), yielding one AMP (Fig. 6). AMP can be converted to ADP from polyphosphate mediated by polyphosphate:AMP phosphotransferase (PPT). The PPT/ADK system provides an alternative to existing enzymatic ATP regeneration systems and has the advantage that AMP and polyphosphate are both stable, inexpensive substrates.[95]

Inspired by the natural generation of ATP through the oxidation of reduced coenzymes (NADH and $FADH_2$), Swartz and his coworkers attempted to re-package inner membrane vesicles (IMVs) that retained their *in vivo* oxidation phosphorylation ability.[9] By combining the glycolytic pathway and critic acid cycle, the transmembrane F_1F_0-ATP synthases in IMVs make ATP from ADP through the proton gradient generated by oxidation of reducing equivalents. However, this technology might be difficult to scale up due to the short lifetime of labile membranes and small volume of IMVs.

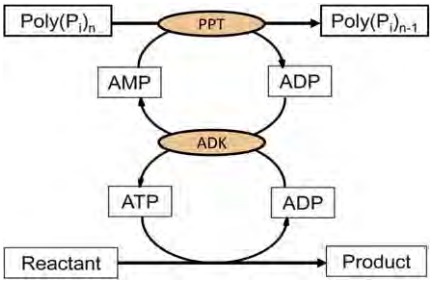

Fig. 6 Scheme of ATP regeneration from polyphosphate—$Poly(P_i)_n$—mediated by polyphosphate:AMP phosphotransferase (PPT) and adenylate kinase (ADK).

Bacteriorhodopsin (BR) can capture light energy and use it as energy to move protons across the membrane for generating proton gradients. When BR and F_1F_0-ATP synthase are coupled across the membrane of some vesicles, the resultant polymersome can produce ATP from ADP using a photo-induced proton gradient.[90,91] In a similar manner to the previous case, this membrane-based technology might be difficult to scale up due to poor photon utilization efficiency of BR and high costs and labile nature of cellular membrane.

Unlike living entities that consume ATP for synthesizing biomacromolecules, it is important for cell-free systems to remove extra ATP if necessary. For example, cell-free ethanol fermentation by using yeast lysate cannot produce ethanol continuously because of accumulation of ATP. Several technologies can be used to remove extra ATP. First, the addition of transmembrane ATPase can catalyze the decomposition of ATP to ADP and a free phosphate ion.[96] Second, some phosphatases can cleave phosphate bonds of phosphate-containing metabolites.[21] Third, addition of a suitable amount of arsenate dissipates high-energy phosphate bonds of some metabolites; rapid decomposition occurs by spontaneous hydrolysis due to the instability of arsenate-containing organic compounds.[3,97]

4. Economic analysis of SyPaB

SyPaB, a biomanufacturing alternative, might compete with microbial fermentation, especially in biocommodity production, due to very high product yields - the most important criterion.[2,8] Since products must be more valuable than feedstock in any scalable industrial manufacturing, it is economically prohibitive to produce low-value products from expensive feedstocks.

Taking the sugar-to-hydrogen conversion as an example, we conducted an economical analysis for elucidating the potential of low-cost SyPaB biomanufacturing. Hydrogen production costs mainly include five parts: (i) feedstock (*i.e.*, sugar), (ii) biocatalysts – enzymes, (iii) sugar consumption used for enzyme synthesis, (iv) coenzymes, and (v) product (hydrogen) separation. We briefly discuss them one by one.

First, substrates are less costly than products, where current sugar costs are \$0.20/kg (*i.e.*, \$11.76/giga joule) and hydrogen prices are approximately \$2.00/kg (*i.e.*, \$13.99/giga joule). It may be economically feasible to produce hydrogen from sugars.

Second, enzyme costs may be minimal for SyPaB[3,98] when low-cost enzyme production costs, low-cost enzyme separation and immobilization costs are available and enzyme stability (*i.e.*, total turn-over number (TTN) represents enzyme's biocatalytic ability before its death in terms of mol of product per mol of enzyme) is enhanced greatly. Bulk industrial enzymes can be produced and obtained at very low costs, for example, US\$∼5 per kg of crude protease produced by *Bacillus subtilis*, US\$5–10 per kg of cellulase produced by *Trichoderma* spp., and tens of US dollars per kg of recombinant proteins produced in *E. coli*.[99–102] The DOE Biomass Program anticipated industrial enzyme production costs as low as \$0.7 per kg of dry enzyme in the future, equaling to that of soy bean protein. For industrial

enzyme manufacturers, high-cell density fermentations are usually conducted. Systematic efforts can be conducted to increase recombinant protein expression levels. For example, recombinant expression of a hyperthermophilic *Thermotoga maritima* 6-phosphogluconate dehydrogenase in *E. coli* has been increased by ~500-fold by optimization of codon usage, expression plasmid and host, inducer type, concentration, addition time, and so on.[79] We estimate that the enzyme costs would be very small when every enzyme in the SyPaB cocktails has TTN values of more than 10^7–10^8 mol of product per mol of enzyme.[2,3,8] In reality, it is relatively easy to obtain thermoenzymes meeting this TTN requirement. A few examples include *Clostridium themocellum* phosphoglucomutase (PGM),[103] *C. themocellum* phosphoglucose isomerase (PGI),[31] *T. martima* 6-phosphogluconate dehydrogenase (6PGDH),[79] *T. martima* fructose bisphosphatase (FBP),[104] and immobilized glucose isomerase.[105] TTN values of enzymes can be enhanced using several approaches: (1) discovery and utilization of thermoenzymes, such as, Taq polymerase and amylase;[79,106] (2) protein engineering by rational design, directed evolution, and their combination;[100,107,108] and (3) enzyme immobilization.[31] A good example, thermophilic glucose isomerase plus immobilization has a life time of more than two years at 55 °C before it must be replaced.[105] Several low-cost, scalable protein purification approaches are available, for example, simple centrifugation for secretory enzymes, adsorption/desorption on low-cost cellulosic materials,[42,43] heat precipitation for thermostable enzymes,[79,109] and ammonia precipitation.[98,110] Therefore, purification costs for bulk recombinant enzymes could become relatively minor. Low-cost enzyme immobilization technologies are becoming widely-adopted, for example, CLEA[30,111,112] and CBM-tagged protein immobilization.[43,113,114] One-step CBM-tagged enzyme purification and immobilization can greatly decrease processing costs and operational complexity.[31]

Third, carbohydrate (substrate) consumption for the synthesis of enzyme cocktails would be very low where the carbohydrate allocation to enzyme synthesis decreases hydrogen yields.[3,8] Typical recombinant protein yield ($Y_{P/S}$) values by microbial fermentation range from ~0.01 to 0.2 g of protein per g of sugar consumed.[3,8,15] When each enzyme in the SyPaB system has TTN values of $\geq 3 \times 10^7$ mol of product per mol of enzyme and a $Y_{P/S}$ value of ~0.05 [note: a conservative value for recombinant protein production in *E. coli*, as compared to that of fungal cellulase (*e.g.*, 0.24)], the carbohydrate allocation to the production of the enzyme mixtures is < 0.01, *i.e.*, > 99% of carbohydrate would be used to produce hydrogen. The calculation of carbohydrate allocation to the enzyme mixture is presented below. When $Y_{P/S}$ is 0.05 g of protein/g of glucose, one kg of glucose can produce 0.05 kg of enzyme (*i.e.*, 1×10^{-3} mol of enzyme, molecular weight = 50 000). So 48 kg of glucose is needed to produce 48×10^{-3} mol of the enzyme mixture, where 48 is the lumped stoichiometric coefficient for the 14-E system. When each enzyme has TTN values of more than 3×10^7, a 14-E cocktail made from 48 kg of glucose can produce 720 kg of H equivalent = 24 H equivalent $\times$ 3×10^7 mol reaction/mol enzyme $\times$ 1×10^{-3} mol enzyme $\times$ 10^{-3} kg mol^{-1} H equivalent. So, 720 kg of H equivalent is made from 5400 kg of glucose equivalent. Therefore, the allocation of carbohydrate to the enzyme cocktail is only 48/(5400 + 48) $\times$ 100% = 0.88%. The higher $Y_{P/S}$ and TTN values are, the lower allocation of carbohydrate to the synthesis of the enzyme cocktails and the more hydrogen produced. The above analysis clearly suggests that obtaining nearly 100% theoretical yield is feasible including sugar consumption for recombinant protein production through microbial fermentation.

Fourth, high-efficiency recycling of NAD or the use of low-cost biomimetic cofactors would decrease cofactor costs to minimal levels. For example, TTN values of NAD should be higher than 10^6 for the economically viable production of biocommodities.[2] Efficient NAD(P)H recycling through immobilization of the coenzyme linked to polymers has been demonstrated.[115] The highest TTN value for NAD recycling reported in the literature is more than10^6.[116] Alternatively, the labile cofactor issue can be addressed by the use of low-cost, more stable NAD biomimics.[2,98] This research area is in its infancy.[117,118] However, great potential markets for SyPaB would motivate the development of redox enzymes that can work on biomimetic cofactors.[4] For example, several redox enzymes have been engineered for better performance on biomimetic coenzymes.[119–121]

Fifth, hydrogen separation costs from the aqueous enzymatic solution are very low. Hydrogen produced by SyPaB contains 66.7% H_2 and 33.3% CO_2.[26,28] Hydrogen and CO_2 can be separated by membrane technology, pressure swing adsorption (PSA), or a hybrid of both.[122] Since the hydrogen produced is high purity, mixed with an inert gas CO_2, this hydrogen/CO_2 mixture can be directly used by PEM fuel cells with only a slight loss in fuel cell efficiency (*ca.* 1%), estimated by thermodynamics calculations.[15]

The above analysis clearly suggests that it would be economically feasible to produce low-value hydrogen from sugars and water through SyPaB. Ultimate hydrogen production costs might be as low as $2.00/kg of hydrogen, where sugar accounts for approximately 95% of the costs, while enzymes and coenzymes account for the other 5%. Similar economical analyses can be conducted for the production of other biocommodities through SyPaB. In short, *in vitro* assembly of purified, stabilized enzymes plus biomimetic coenzymes would have much lower production costs than microbial fermentations, because the former can work much longer and achieve much higher product yields.

5. Conclusions

Cell-free SyPaB, which originated from cell-free ethanol fermentation, may become a new low-cost biomanufacturing platform due to high product yields, fast reaction rates, broad reaction conditions, as well as easy process control and regulation. *In vitro* synthetic pathways are made of building blocks (*e.g.*, enzymes) and building modules with specific functions. Thermoenzyme discovery and utilization, protein engineering, and enzyme immobilization on a variety of (nano-)materials would result in ultra-stable enzymes – basic building blocks of SyPaB projects. The assembly of building blocks and building modules (*e.g.*, several-enzyme complexes—biocatalyst modules) would make low-cost production of numerous biocommodities feasible. The SyPaB technology has successfully achieved some breakthroughs that neither microbes nor chemical catalysis can

implement, such as the production of high-yield hydrogen from carbohydrates and water,[26,28] regeneration of ultra-high-yield NAD(P)H based on a biomass sugar and water,[8] in-depth oxidation of ethanol and pyruvate to electrons in enzymatic fuel cells,[76,123] enzymatic CO_2 fixation.[124] Although SyPaB is on its early stage, we believe that its unique feature—high product yields—will allow it to play a large role in several important fields, such as biofuels production and CO_2 fixation.

Abbreviation

6PGDH	6-phosphogluconate dehydrogenase
ADK	adenylate kinase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BR	bacteriorhodopsin
CBM	carbohydrate-binding module
CLEA	cross-linked enzyme aggregation
CNT	carbon nano-tube
FBP	fructose bisphosphatase
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
IMV	inner membrane vesicle
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
Nafion	sulfonated tetrafluoroethylene-based fluoropolymer-copolymer products
PGI	phosphoglucose isomerase
PPK	polyphosphate kinase
PPT	polyphosphate:AMP phosphotransferase
SyPaB	synthetic pathway biotransformation
TTN	total turn-over number (of enzyme or coenzyme), mol product per mol enzyme or coenzyme
$Y_{P/S}$	protein yield based on substrate (g protein/g substrate)

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