Nano on micro: tuning microbial metabolisms by nano-based artificial mediators to enhance and expand production of biochemicals

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Abstract

Advances in synthetic biology and metabolic engineering across the past few decades have enabled the successful production of many novel chemicals. However, bioproduction of such chemicals is often limited by low yield and titer due to disrupted metabolic homeostasis. Finely tuning cellular metabolism to restore robust metabolic functions entails various genetic modifications, which is often not practical. Alternatively, artificial mediators capable of tailoring microbial metabolisms open a new avenue for restoring physiological functions. In this context, nanoparticle-based artificial mediators have been pursued to tune cellular metabolisms. They can not only enhance production of molecules from endogenous metabolism, but also expand bioproducts spectrum. Here, we reviewed recent advances toward the employment of nano-based artificial mediators for the tuning of cellular metabolism, with a focus on their positive effects on electron transfer and pathway flux. Perspectives for potential applications of artificial mediators for mediating microbial metabolisms in the future were also provided.

Introduction

The depletion of fossil fuels and the adverse effects incurred by fossil fuels consumption and overexploitation have driven the exploration for alternative pathways for chemicals production from renewable resources. The great advancements in both synthetic biology and metabolic engineering have enabled the biological production of numerous renewable chemicals that are previously exclusively derived from petroleum resources. In addition, through engineered biological pathways, some innovative biochemicals that were unachievable even through petro-chemical routes can be produced. In particular, the advent of advanced genome editing technologies, such as the CRISPR-based methods, significantly expediated the development of microbial cell factories with enhanced performance for biochemical production. For example, by harnessing the native CRISPR-Cas system in *Clostridium tyrobutyricum*, a strain originally used for butyrate production was successfully transformed into a hyper-butanol producer, with a unprecedented titer of 26.2 g/L attained in a batch fermentation [1]. Recently, Liang et al. successfully achieved the production of styrene (a valuable precursor for polymers and copolymers) at a titer of 3.15 g/L directly from glucose using an engineered *E. coli* strain by taking advantage of iterative CRISPR enabled trackable genome engineering [2]. Despite such exciting progresses toward biochemical production via novel biological routes, in most cases the biological process is still not competitive to the chemical process in terms of productivity, yield and titer.

One would expect that such disadvantages of biological routes for chemical production could be eliminated in a slow yet progressive manner via extensive metabolic engineering of host cells. However, often multiplex genome editing and assembling different modules into one microorganism may overburden host strains, causing imbalanced metabolism--such as disrupted redox balance [3,4], and accumulation of toxic intermediates [5]. In fact, restoring metabolic homeostasis is intricate and requires amending various

inconspicuous physiological networks, and may turn out to be tedious and unpractical [6]. Instead of addressing such constraints through mediation of the endogenous cellular metabolism, artificial extraneous mediators could be utilized for the same purposes. In particular, nanoparticle-based mediators could be employed for mediating microbial metabolisms effectively. The nanoparticles featuring large surface area-to-volume ratios can interact with microorganisms in a subtle and beneficial manner, and in turn, conform the microbial metabolism for enhanced biochemical production [7-12]. For example, the photoexcited electrons from nanoparticles could be transferred to the membrane-bound electron acceptors in microbes, and further channeled to pathways for end products synthesis [13]. The electrons could also be transferred to membrane-bound hydrogenase, which uses H_2 as intermediates to generate reducing equivalents for the central metabolisms [14].

Recent advances in metabolic engineering for biochemical production have been extensively reviewed by various researchers, and interested readers are referred to several recent excellent reviews [15-17]. In this review, we synopsized recent advances in the application of nanoparticle-based artificial mediators for tuning the microbial metabolisms for enhanced biochemical production. Perspectives for potential applications of these artificial mediators for mediating microbial metabolisms in the future were also provided.

Mediation of electron supply and transfer using nanoparticles

Hydrogenation of the C=C or aldehydes/ketone groups in biogenic precursors is a canonical and important reaction for producing both bulk chemicals (e.g., ethanol and butanol) and fine chemicals (e.g., shikimate), with hydrion provided by NAD(P)H. The shikimate pathway is an important route for producing aromatic amino acids, and can be reconstructed for producing platform chemicals, such as shikimate and muconic acid. This pathway has been extensively explored for bioproduction using *Saccharomyces cerevisiae* as the host [18].

In *S. cerevisiae*, NADPH is primarily produced from the pentose phosphate pathway (PPP), but this lowers the carbon efficiency since two equivalents of CO_2 are released [11]. Eliminating the major NADPH production pathway (i.e., PPP) is expected to push more carbon flux toward the desired product (e.g, shikimate), but turned out to be impractical due to inadequate supply of NADPH. To sustain NADPH, indium phosphide (InP) nanoparticles were assembled with engineered *S. cerevisiae* designed for shikimate production, and the NADPH regeneration was restored by exploiting the photoexcited electrons, which enabled shikimate production in a higher yield than its control [11]. In a similar manner, Liu et al. [13] reported that the g-C_3N_4 nanoparticles could enhance the polyhydroxybutyrate (PHB) synthesis in *Ralstonia eutropha* by availing NADPH regeneration by photoexcited electrons.

Muconic acid is a valuable precursor for biopolymers. The hydrogenation of the two C=C bonds in muconic acid to yield adipic acid is necessary for its application for bioplastic production. By introducing enoate reductase into the muconic acid synthesis pathway, direct production of adipic acid was enabled, but the titer was rather low due in part to the insufficient NAD(P)H supply [19]. Indeed, in another study, by introducing the heterologous formate dehydrogenase into the pathway to increase NADH supply, the reduction of citraconate into 2-methyl succinic acid catalyzed by enoate reductase was enhanced [20]. However, the carbon yield would be compromised due to the requirement of supplementing formate during the fermentation. Alternatively, the hydrogenation of C=C bonds by enoate reductase coupled with nanometric photocatalyst has been proved to be feasible by several studies [21,22]. In a hybrid system integrating carbon nanodots and enoate reductase, the electrons derived from light exicitation could be used to regenerate NADH, driving the hydrogenation reaction forward [22]. Likewise, Brown et al. demonstrated that the electrons excited from CdSe quantum dots by light could be captured by $NADP^+$ to regenerate NADPH, which is then utilized for the reduction of aldehydes to alcohols catalyzed by a NADPH-

106 dependent alcohol dehydrogenase (ADH) [23]. Although thus far it has not been
107 demonstrated that the nano-photocatalysts could be used to couple with microorganisms in
108 order to hydrogenate bioprecursors containing C=C bonds or aldehyde groups to
109 achieve/improve the production of the target bioproducts, it could be envisioned that such a
110 strategy is highly feasible given the success of other similar hybrid systems as described
111 above (**Figure 1**).

112 In addition to being utilized in the bioproduction through heterotrophic processes,
113 nanometric photocatalysts have also been employed to improve biochemical production via
114 autotrophic routes. Compared with the biological photosystems, abiotic photosystems are
115 much more efficient in generating chemical energy from solar energy [7,12]. By leveraging
116 the highly efficient abiotic photosystem for garnering light and providing reducing
117 equivalents for CO_2 reduction in autotrophic metabolisms, enhanced performance has been
118 achieved for the production of a variety of bioproducts [24]. By deploying a biocompatible
119 nanowire array around *Sporomusa ovata*, an acetogenic bacterium that can convert hydrogen
120 and CO_2 into acetate, the electrons generated by the photocatalyst could be used for direct
121 reduction of CO_2 to acetate in the absence of H_2 [25]. To expand the spectrum of products
122 from such an hybrid system, acetate was further utilized by engineered *E. coli* for producing
123 various valuable products including *n*-butanol, PHB, and other natural products [25].

124 While photocatalysts as described above can be used to enhance biochemical
125 synthesis through directly supplying electrons under light illumination, other non-
126 photocatalysts can also be employed to mediate electron transfer. For example, it has been
127 demonstrated that H_2 production by *C. butyricum* could be enhanced by 30-60% by
128 supplemented metallic or metallic oxide nanoparticles owing to the enhanced rate of electron
129 transfer [8,9,26].

Overall, the photo-induced electrons from nano-photocatalysts can be captured by membrane-bound acceptors or enzymes, and then redirected to product synthesis pathways. Other nanoparticles, such as those used for enhancing H_2 production, could mediate enzyme activity and electron transfer to improve product formation. Depending on the host strains and target products, different types of nanoparticles can be used to mediate electron transfer and facilitate the production of both bulk and fine chemicals. Especially, such hybrid systems have great potentials to be redesigned for enhancing biochemical production via metabolic pathways that have high demand for reducing equivalents.

Mediation of metabolic flux using nanoreactors

The tremendous advancements in metabolic engineering and synthetic biology have enabled the enhanced production of broad spectrum of biochemicals from small molecules (e.g., ethanol, acetic acid, lactic acid, succinic acid) to larger molecules (e.g., medium chain fatty alcohols, fatty acids, and hydrocarbons) through biological fermentation processes [27]. However, many of these biobased molecules (especially the large molecules) are usually produced at very low levels due to their inherent toxicity to the host cells. To mitigate the toxicity and drive the flux toward target products, *in situ* product recovery (ISPR) is one common strategy. Currently, most ISPR methods solely focus on the isolation of the products from the fermentation process. However, if further upgrading (of the bioproducts) along with the separation could be synchronized into one pot, the metabolic flux may be further enhanced while novel products which might be unachievable via biological route alone could be obtained [28,29]. In a recent study, Wallace and Balskus [29] developed a micelle based system integrating biocatalyst and chemocatalyst for production, extraction and chemical conversion of styrene in a single vessel. Micelles, averaging ca. 50-100 nm in size, are formed by spontaneous aggregation of surfactant monomers in water when the surfactant is

present above the critical micelle concentration [30,31]. The hydrophobic core in nanometric micelles can serve as a reservoir for hydrophobic products. As a result, in the micelle system developed by Wallace and Balskus [29], a three-fold increase of styrene production was achieved. More strikingly, cyclopropanation of styrene by a chemo-catalyst could be also conducted in the same pot, yielding cyclopropanated styrene with a yield over 90% [29]. Compared to ISPR with solvent overlay, micelles based recovery strategy requires much less organic solvent (surfactant), but can achieve much higher titers and yields for the targeted bioproduction [29].

For those less hydrophobic (or even hydrophilic) yet toxic bioproducts, the ISPR may not be effective due to their low partition rate into hydrophobic solvents. Alternatively, the derivatization of them into more hydrophobic products via condensation (e.g., aldol condensation and esterification) could facilitate their recovery. One particular example is the esterification of alcohols and carboxylic acids into fatty acid esters to enhance the metabolic flux. Previous studies demonstrated that esterification of either octanol and acetate or butanol and butyrate during fermentation enhances the flux toward alcohols (i.e., octanol or butanol) production, with concomitant increase of product yield and productivity [28,32]. Compared to alcohols or carboxylic acids, the derived esters are usually less polar and more hydrophobic, which improves product migration into hydrophobic solvents and drives the flux accordingly. However, most condensation reactions perform poorly in aqueous solutions due to either the equilibrium reactions involving water (e.g., esterification) or the deactivation of catalysts by water. Micelles based nanoreactors can not only provide the reservoir for hydrophobic products, but also accelerate many catalytic reactions that perform poorly in the water [33-35]. Interestingly, micelles are also capable of maintaining the enzyme activity and decreasing noncompetitive enzyme inhibition [36]. Fatemeh Rajabi and Rafael Luque even reported that the glucose derived surfactant N-dodecanoyl-N-methyl-1-glucamine (C12MG)

180 can work as a catalyst for esterification of various carboxylic acids and ethanol with
181 remarkable yield obtained in micelles [34]. Possibly, the marriage of microbial fermentation
182 and "catalytic" surfactant enabled micelles system could enhance the flux and make the ester
183 production more efficiently with minimum amount of lipase.

184 In addition to the esterification strategy for tuning metabolic flux, there are other
185 potential reactions that can be mediated by micelles, but remain underexploited (**Figure 2**).
186 Aldehydes (e.g., butyraldehyde) and ketones (e.g., acetone, methyl ketone) are valuable
187 platform chemicals that can be biologically produced [37-39], but most of them can only be
188 produced at low titer due to their inhibition to microbes [39]. The self-condensation of
189 aldehyde or the cross condensation of aldehydes and ketone is an effective way for increasing
190 carbon-chain length, yielding condensed products with higher hydrophobicity. Similar to the
191 case of enhanced metabolic flux by esterification, the derivation of aldehydes and ketones via
192 aldol condensation may enhance metabolic flux by promoting products distribution into
193 hydrophobic solvents. However, condensation reactions are pretty sluggish in aqueous media,
194 especially when aldehydes/ketones are present at low concentrations [40]. Micelles acting as
195 nanoreactors can dramatically accelerate aldol condensations in aqueous media [41], and
196 hydrophobic pocket afforded by micelles could accommodate the condensed hydrophobic
197 products to relieve inhibition. Eventually, the flux toward aldehyde and ketone production
198 could be enhanced. Fatty acids are appealing biochemicals, and biological production of fatty
199 acids from renewable substrates have been reported by various studies [42-45]. Especially,
200 secretion of bio-produced free fatty acids from microbes represents a promising process for
201 their production, and has been reported by several studies [46,47]. However, fatty acids are
202 known to be toxic to microbes, and *in-situ* removal of them is necessary to enhance its
203 production [47]. Due to the relatively hydrophobic nature of fatty acids, micelles could be
204 used for enhancing flux toward fatty acids in a way similar to the micelle-enhanced styrene

205 production as discussed above. Furthermore, the fatty acids production could be integrated
206 with chemical conversions in the presence of micelles, yielding novel biochemicals. Wu et al.
207 reported the synthesis of cycloalkene from sebacic acid and other fatty acids via a cascade
208 bio- and chemo-catalytic reactions in micelles [48]. In their system, *E. coli* harboring UndB
209 (desaturase-like enzyme from *Pseudomonas*) enabled the decarboxylation of sebacic acid to
210 the corresponding α,ω-dienes, which was then catalytically converted to cyclohexene by a
211 ruthenium catalyst. The addition of the surfactant to form micelles improved the cyclohexene
212 conversion from 20% to 40%. This is because the diene derived from decarboxylation of
213 sebacic acid is highly hydrophobic. The formed micelle could serve as the reservoir for the
214 hydrophobic intermediates (which serve as the substrates for producing cyclohexene) and
215 endproducts, and in turn, enhance the transformation of dienes into cycloalkenes. Thus,
216 similar micelle-based strategies could be further integrated with the biocatalytic system
217 designed for fatty acids production, and thus the production of different cycloalkenes
218 accordingly could be realized. More importantly, the conversion of solubilized fatty acids
219 into highly hydrophobic cycloalkenes favors product partition into hydrophobic pockets of
220 micelles, and may enhance the metabolic flux as a result.

221 The nanometric nature of micelles enables them to be well dispersed in the aqueous
222 media, which enhances their contact and interaction with both cells and products. Such
223 interactions is believed to facilitate the secretion of hydrophobic products (e.g, styrene and
224 fatty acids) from microbial cells [29]. The hydrophobic pockets in micelles can reserve the
225 hydrophobic products, further driving the metabolic flux. Besides, the 'nano-environment'
226 created by micelles allows many water-sensitive reactions to proceed smoothly in nano-
227 reactors, while at the same time alleviates product inhibition. When properly designed, such
228 nanoreactors could be integrated with other catalytic systems, therefore, expanding the realm
229 of derivatives from biological routes.

Interactions between microorganisms and nanoparticles

Nanoparticles should interact with microorganisms intimately in order for them to ameliorate cellular metabolisms efficiently. Guo et al. demonstrated that only the assembled cells and InP nanoparticles could enhance the NADPH regeneration, while the dispersed ones did not show much activity [11]. Wallace and Balskus also showed that the interaction between micelles and cell membranes facilitated styrene excretion and subsequent partitioning into the hydrophobic core of micelles [29]. These studies highlight the importance of the contact between cell membrane and nanoparticles. To ensure immobilization of nanoparticles onto cell membranes, Wang X et al. engineered an *E. coli* strain to excrete a myloid monomers capable of self-assemble, to anchor nanoparticles in a spatially precise manner [49]. Similarly, Wei et al. demonstrated that the display of a lead-specific binding protein PbrR can effectively capture the CdS nanoparticles on cell membrane [50]. Due to the active binding of nanoparticles onto the cell membrane in these two cases, the H_2 production by these engineered strains under illumination could be significantly enhanced. Moreover, the active immobilization of nanoparticles on cell membrane may also benefit their recyclability. Despite the benefits offered by nanoparticles on cellular metabolism, the toxicity of nanoparticles on microbes could not be ignored. The toxicity of nanoparticles could be affected by their properties, and therefore, judicious selection of nanoparticles based on properties and applications is critical. For example, the positively charged fullerene-derived nanoparticles are toxic to *E. coli* W3110 and *Shewanella oneidensis* MR-1, while the neutrally charged and negatively charged ones show mild or no inhibition [51]. In most previous studies, the nano-based artificial mediators were selected based on 'trial-and-error'. The better understanding concerning how nanoparticles interact with microorganisms at molecular level is of significant importance in designing low-cost

and highly efficient artificial mediators [10].

Conclusions

Nano-based artificial mediators have emerged as innovative strategies to ameliorate cellular metabolisms, and enhance carbon flux toward target products. However, many issues, such as the recyclability of the nanoparticles, economics, and the environmental concerns remain to be addressed. Albeit the successful demonstration of harnessing nanoparticles to tune cellular metabolisms, in many cases the interaction between artificial mediators and microbial cell factories at molecular level remains elusive. Moreover, the artificial-mediator-assisted upgrading reactions for biochemical production are only limited to few applications, and should be further expanded. Recently, the US government is interested to promote future manufacturing, and encouraged researches in understanding and developing nano materials/machines capable of working at nano/bio interface [52], which would help stimulate the development of this interdisciplinary area. Through the concerted efforts in the metabolic engineering of microbial cell factories and the rational designing of nanoparticles capable of fitting various configurations considering all aspects as discussed above, efficient production of a broad spectrum of renewable biochemicals could be envisioned.

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● of special interest

●● of outstanding interest

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502 **Figure captions**

503 **Figure 1** Various example metabolic reactions mediated by nanometric artificial mediators.
504 VB: valence band; CB: to the conduction band; ADH: alcohol dehydrogenase; ER: enoate
505 reductase; ARO1: pentafunctional aromatic enzyme

506 **Figure 2** Micelles mediated conversion of biogenic products to other valuable biochemicals

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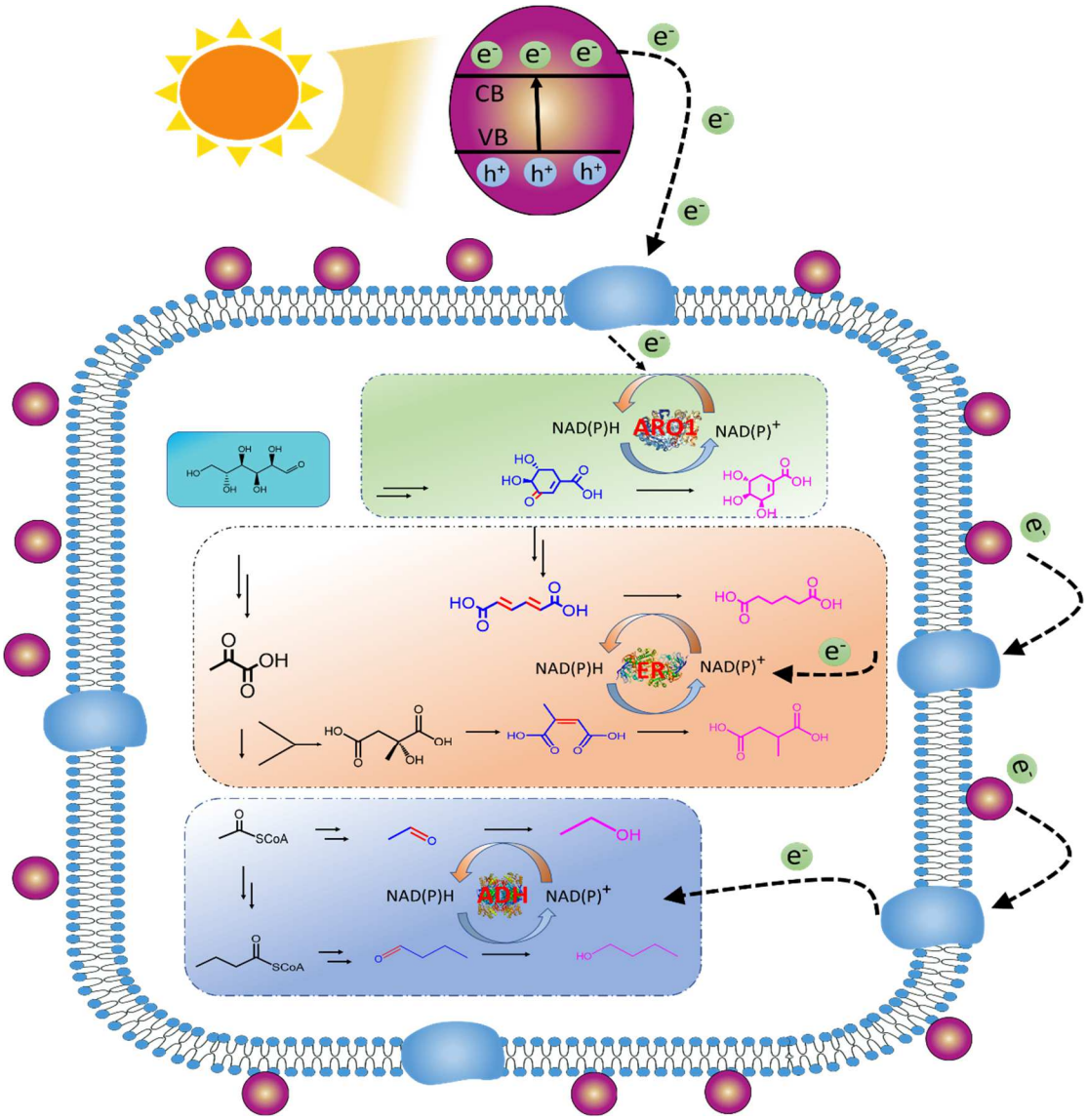

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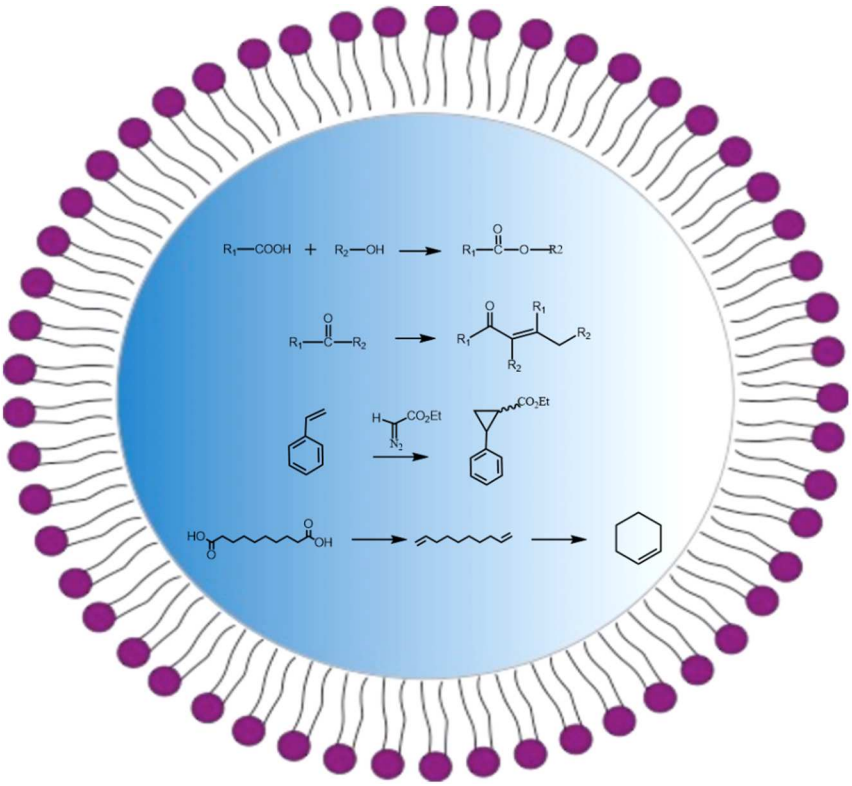

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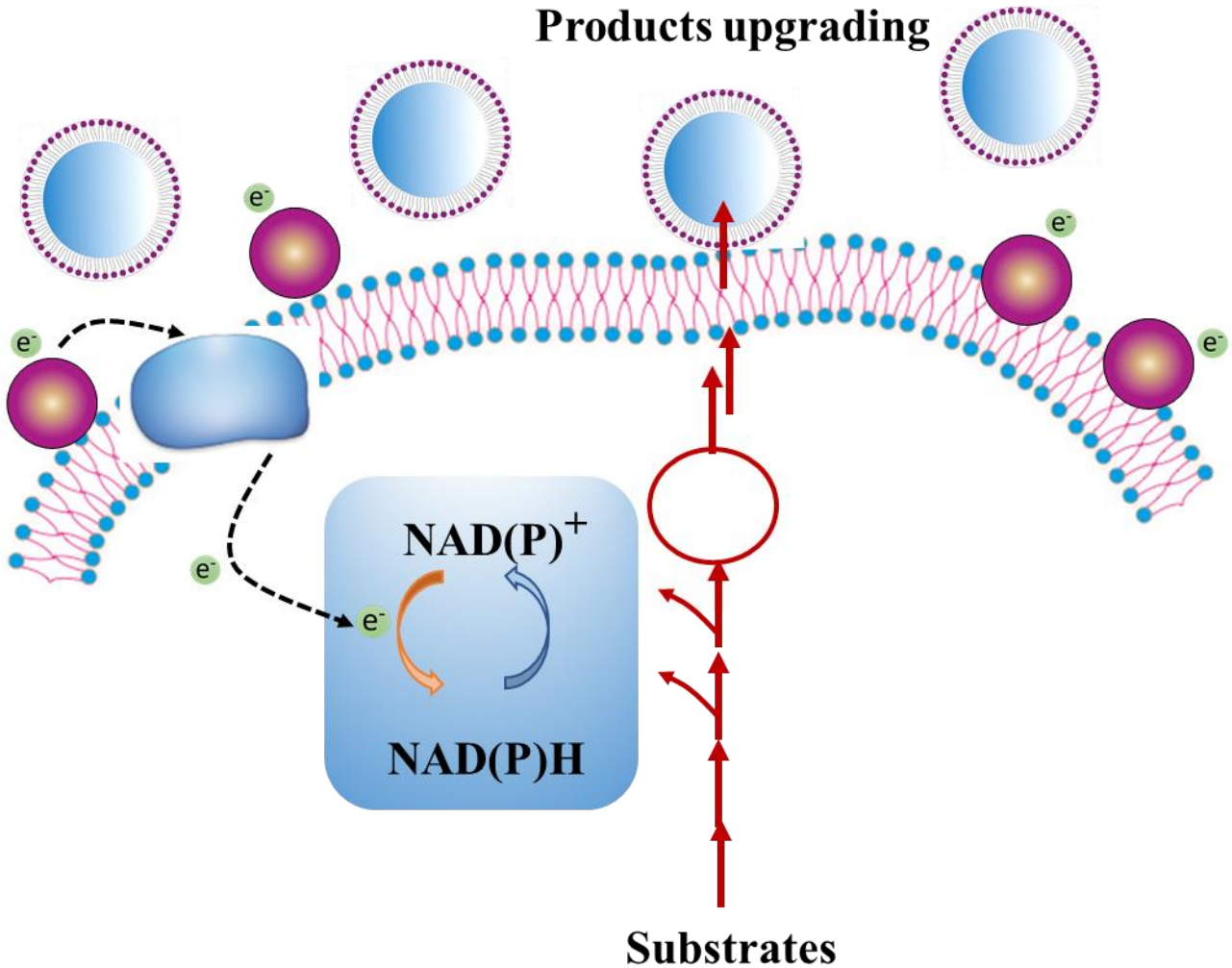
Products upgrading
e-
e-
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e-
NAD(P)+
NAD(P)H
Substrates