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Biomimetic cofactors and methods for their recycling

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Nicotinamide cofactor biomimetics (NCBs) belong to a class of compounds that, as the name suggests, mimic the structures and functions of natural nicotinamide cofactors, namely nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate and their corresponding reduced forms. The first set of NCBs was discovered in the 1930s; these were initially used to study the chemical properties of this class of cofactors as well as understand nicotinamide binding of oxidoreductases. Since then, various NCBs, enzymes, and recycling systems have evolved and lately, new NCBs have been developed and used to run biocatalytic reactions.

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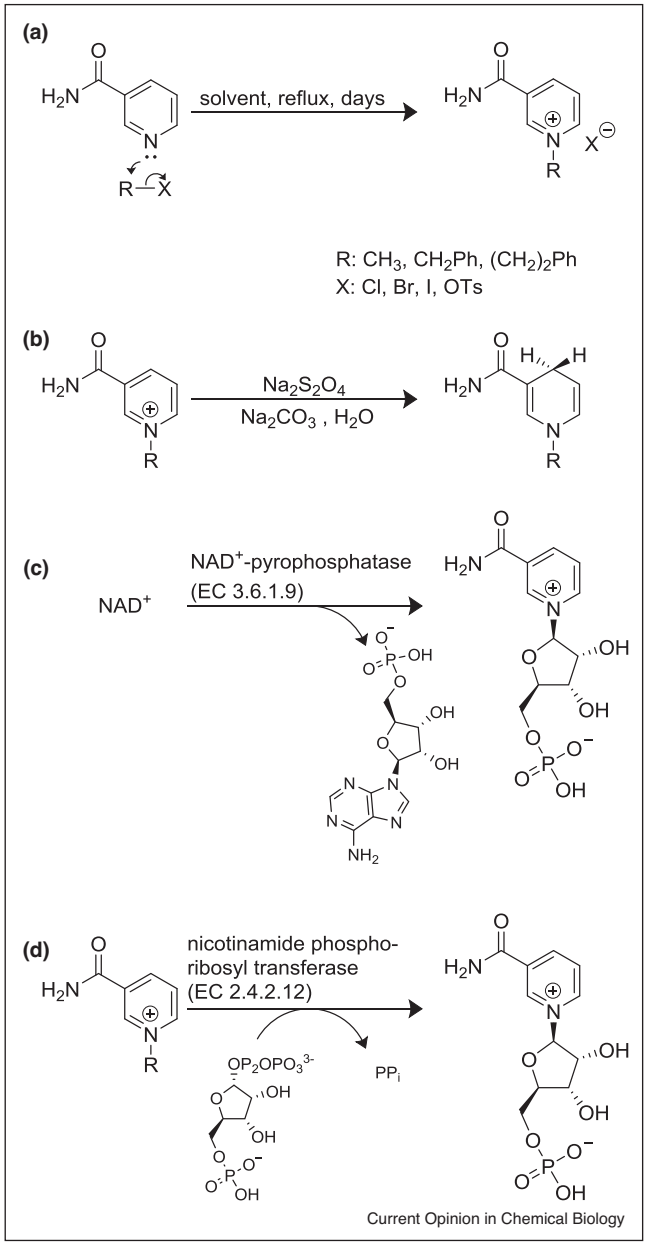
Introduction

Academic and industrial research and development in the field of biocatalysis faces enormous growth and has become extremely popular, for example, for developing renewable alternatives to petroleum-based products [1,2]. As we are all aware, many enzymes can perform a huge variety of reactions with remarkably high stereoselectivity and regioselectivity. Others show significant substrate promiscuity, which may be desirable depending on the overall biocatalytic process [3]. In contrast to traditional and often well-established *in vivo* approaches, a current trend in biotechnology is the design of cell-free reaction platforms and multi-step enzymatic reactions, which give easy access to plug-and-play assembly and versatile reaction conditions [4,5]. Many known and applied enzymes belong to the class of oxidoreductases [6], which catalyze controlled redox reactions (EC.1.X.X.X). A key challenge in cell-free biocatalysis for many of these enzymes is the need for stoichiometric amounts of the redox-equivalent nicotinamide adenine dinucleotide (phosphate) NAD(P)/H. The combination of high costs and low stability [7,8] under many conditions makes the natural cofactor the major limitation in many applications aiming for medium- or even low-cost products [9]. To overcome this difficulty, research has created options for reliable *in situ* regeneration and redox neutral cascade systems, leading to the regular use of oxidoreductases on an industrial scale [10,11]. However, more efficient, economical, and biorthogonal cofactor regeneration methods are desirable. To achieve this, it is necessary to simultaneously decrease the price and increase cofactor stability. Hence, for several years, much effort has been devoted to studying and enhancing synthetic nicotinamide cofactor biomimetics (NCBs, elsewhere mNADHs). In 1936, Karrer *et al.* first synthesized a set of totally synthetic nicotinamide analogues to obtain a better understanding of the chemical properties of cofactors and their interactions with enzymes and hydride (one proton, two electrons) transfer reactions [12]. The oxidized form can be prepared reasonably easily from nicotinamide and the appropriate alkyl-phenyl halide (e.g. benzyl chloride), followed by reduction with sodium dithionite to obtain the reduced biomimetic cofactor (Scheme 1), thus representing a low-cost one-step or two-step synthesis. Within the group of NCBs (Figure 1) we differentiate between two forms: The generally small totally synthetic biomimetic cofactors (e.g. *N*-benzyl nicotinamide (BNA)), most of which retain only a related nicotinamide group, and the semi-synthetic biomimetic cofactors, which are structurally more similar to natural nicotinamide cofactors. The latter forms are mostly truncated versions of the natural cofactor or include structural modifications through attachment of additional groups.

The greatest hurdle concerning their efficient utilization, enzymatic conversion, and recycling is that most natural enzymes barely accept any of the described NCBs. Although this provides an interesting opportunity for establishing biorthogonal systems, the drawbacks are serious. This might be due to the altered redox potential of the NCBs as well as improper positioning in the catalytic center of the enzyme. In fact, for many biocatalysts, cofactor-binding, including the corresponding hydrogen bond network and van der Waals interactions, often induces structural changes in enzymes that facilitate

Scheme 1



Frequently used synthesis of fully synthetic cofactors starting from **(a)** nicotinamide and an alkyl-phenyl halide, followed by **(b)** reduction with sodium dithionite to obtain the reduced form of the biomimetic cofactors. **(c)** Possible routes to produce the semi-synthetic cofactor analogue β-nicotinamide mononucleotide (NMN) stating from NAD^+ using a pyrophosphatase or from **(d)** nicotinamide and 5-phosphoribosyl-1-pyrophosphate with a nicotinamide phosphoribosyl transferase.

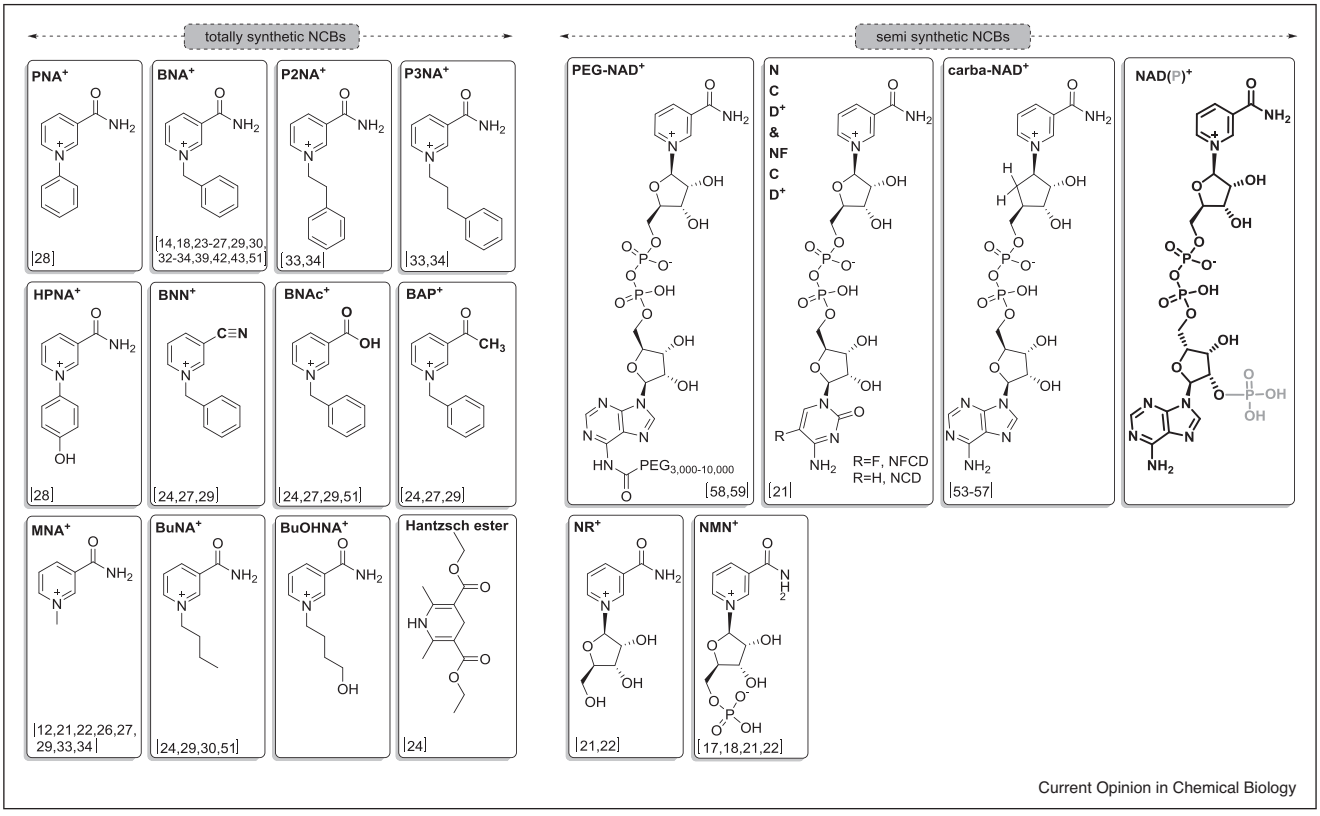
catalysis [13,14]. Thus, even a change in the phosphorylation state of the natural cofactor can tremendously impact the catalytic properties, and protein engineering is required to restore the activity [15]. The small non-natural and even the semi-synthetic NAD(P)/H analogues lack most of the essential and conserved bonding interactions, resulting in increased K_m values and reduced reaction rates [16–18]. However, looking at the current state of the art, several processes involving biomimetic nicotinamide cofactors have been developed for biocatalysis applications to reduce process costs, increase cofactor stability toward hydrolysis (in acids and bases), and synthesize readily available compounds as well as to show novel enzyme activity [10,19,20]. In this review, we focus on the most recent applications of synthetic cofactors replacing of NAD(P)/H in (chemo-) enzymatic catalysis, including regeneration systems.

Enzymatic systems using artificial cofactors

Application of NCBs in reductive reactions using flavin-dependent enzymes has proven to be extremely successful during the last few years. Initial attempts were already made in the 1990s by Friedlos and Knox using two flavin mononucleotide (FMN)-dependent enzymes, DT diaphorase and nitroreductase from *Escherichia coli* to convert semi-synthetic analogues as well as methyl-1,4-dihydronicotinamide (MNAH) with reasonable catalytic efficiency [21,22]. Within the last decade, a remarkably high number of reports indicates the extensive effort, which was devoted to investigating these exciting yellow enzymes in combination with NCBs [19,23–26,27••,28•,29–31,32•,33••,34•]. Currently, within the class of flavoenzymes, special attention is given to ene-reductases (EREDs), or formerly old yellow enzymes (OYEs) [35–37], which not only catalyze the asymmetric reduction of activated C=C double bonds (Scheme 2), but also perform further highly interesting reactions including asymmetric reductive carbocyclizations [38]. Paul *et al.* reported a whole set of NCBs, which were effective alternatives to the established natural nicotinamide cofactors for promoting ERED-catalyzed reduction, reactions and applied them on a preparative scale, resulting in excellent conversion and optical purities of the products (>99%) with acceptable isolated yields using stoichiometric reductants [24].

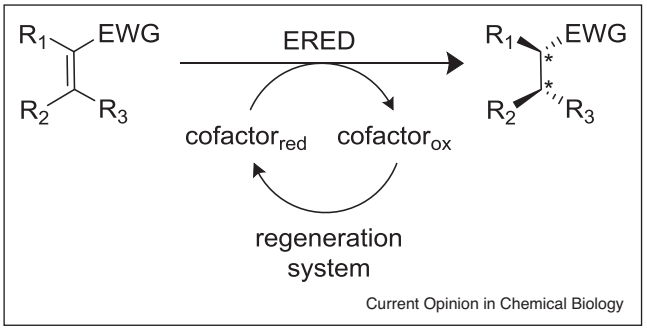
A similar approach was published in 2016 by Löw and coworkers. They used four different reductases in combination with two newly synthesized cofactor analogues, specifically 1-(4-hydroxyphenyl)-1,4-dihydronicotinamide (HPNA) and 1-phenyl-1,4-dihydronicotinamide (PNAH). The best results were achieved using an NAD(P)H-dependent 2-cyclohexen-1-one reductase from *Zymomonas mobilis* (*Zm*NCR) and the novel biomimetic HPNAH. With this mimic, the overall reductase activity using *Z*-citral as substrate was enhanced six-fold. Here, the maximum rate (v_{max}) increased to 3.12 μmol min^{-1} compared to 0.45 μmol min^{-1} for NADH. Cyclic voltammetry measurements confirmed the lowered oxidation potential of HPNAH, indicating a higher hydride donation ability. It is worth mentioning that when using a different ERED, for example OYE3 from *Saccharomyces*

Figure 1



Artificial and natural nicotinamide cofactors in their oxidized forms. Totally synthetic NCBs: PNA^+ (1-phenylnicotinamide), BNA^+ (1-benzylnicotinamide), $P2NA^+$ (1-phenylethylnicotinamide), $P3NA^+$ (1-(3-phenylpropyl)nicotinamide), $HPNA^+$ (*p*-hydroxyphenylnicotinamide), BNN^+ (1-benzylnicotinonitrile), $BNAc^+$ (1-benzyl nicotinic acid), BAP^+(1-benzyl 3-acetylpyridine), MNA^+ (1-methylnicotinamide), $BuNA^+$ (1-butylnicotinamide), $BuOHNA^+$ (1-(4-hydroxybutyl)nicotinamide), Hantzsch ester. Semi synthetic NCBs: NR^+ (nicotinamide riboside), NMN^+ (nicotinamide mononucleotide), $NFCD^+$ (nicotinamide flucytosine dinucleotide), NCD^+ (nicotinamide cytosine dinucleotide), PEG (polyethylene glycosylated)-NAD^+, carba-NAD^+ and the natural cofactors: $NAD(P)^+$(nicotinamide adenine dinucleotide (phosphate)).

Scheme 2



Ene-reductase (ERED) catalyzed reduction of activated C=C double bonds. Activation is possible by an EWG: electron withdrawing group (aldehyde, ketone, carboxylic acid, ester, cyclic imide, lactone, nitrile, nitro, etc.). Reduced cofactor can be renewed by an appropriate regeneration system.

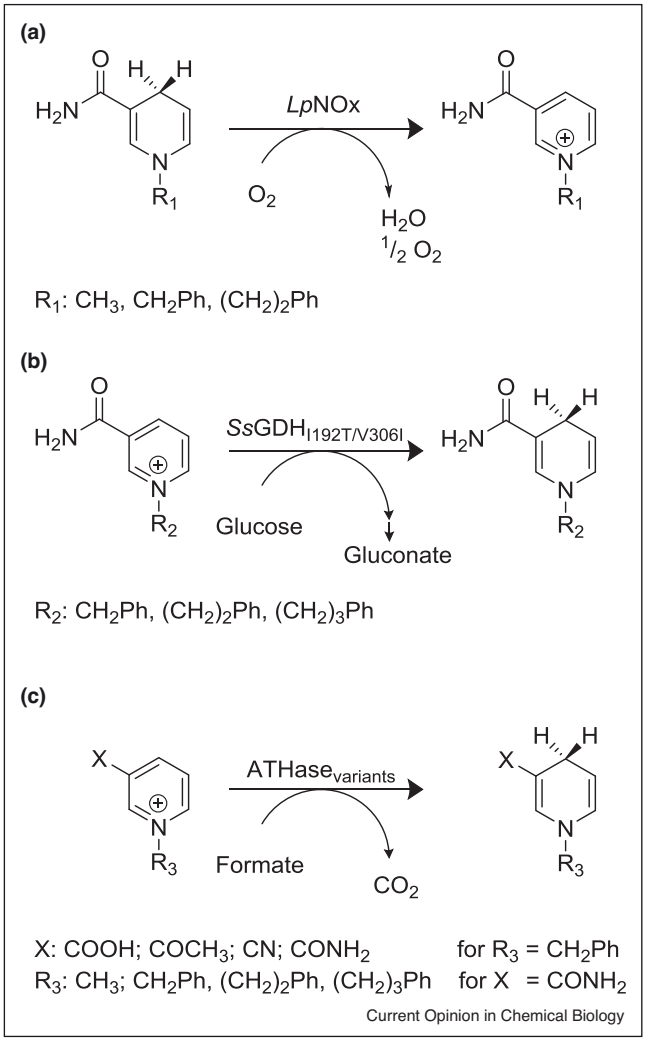
pastorianus, the highest activities were observed with BNAH. Interestingly, for *Zm*NCR, the synthetic cofactors did not display significantly lower activity with any of the substrates, whereas when using NADH, smaller substrates were preferred [28•]. This shows that the substrate scope can strongly depend on the cofactor choice, since both substrate and cofactor are competing for the same active site. In 2017, Scholtissek *et al.* observed and summarized that Class I EREDs can accept NCBs better than the natural cofactor, whereas closely related Class II EREDs do not accept NCBs. Class III also accepts NCBs and, furthermore, converts carboxy-(BNAcH) and nitrile substituted NCBs (BNNH) the most effectively of all the classes [31]. An azoreductase also clearly outperformed the natural system (123% activity of NADH) and in addition significantly affected the optimum pH of the reaction [32•]. By co-crystallization of xenobiotic reductase A (XenA) of *Pseudomonas putida*, with the natural cofactor (PDB: 5CPM) as well as various biomimetics (PDB: 5CPL, 5CPO, 5CPN). Knaus and co-workers discovered that the amino acid residue of W302 adopts

a different conformation depending on the size of the cofactor. Thus, for smaller biomimetics, the volume of the active site is reduced [29]. Understanding the enzymatic mechanisms of NCB hydride transfer is fundamental to developing and improving the performance of nicotinamide coenzyme-dependent biocatalysts. Recent results further imply that faster reactions with NCBs are associated with enhanced donor–acceptor distance sampling [30]. In this manner, NCBs typically appear to have a decreased activation enthalpy compared to NAD(P)H. But, this is at the expense of elevated activation entropies, suggesting that enzyme-NCB complexes are more disordered than the natural enzyme–coenzyme complex [30]. For example, in contrast to most EREDs, the flavin dependent P450-BM3 did not accept NCBs in initial studies. After introducing the two mutations, W1064S and R966D, excellent activities turned up [23]. That may suggest that not all flavin dependent enzymes will accept NCBs *per se*, even though FAD/FMN might facilitate hydride transfer.

The scope of flavoenzymes known to accept NCBs was further broadened by a styrene monooxygenase from *Rhodococcus opacus* 1CP (StyA). Since BNAH *in situ* regeneration systems are limited, the authors decided to use stoichiometric amounts of reducing agent [25]. The direct non-enzymatic regeneration of the reduced flavins was applied earlier for *in situ* formation of H_2O_2 using BNAH as sacrificial electron donor and O_2 as oxidant to promote P450 peroxygenase-catalyzed oxyfunctionalization of fatty acid hydroxylases [39]. NADH oxidase (NOx) is a popular regeneration system. Since the flavoenzyme requires only oxygen and produces no more than hydrogen peroxide or water, the use of additional reactants can be avoided. A current study shows the conversion of some NCBs (e.g. MNAH, BNAH, P2NAH) using a water-forming NOx from *Lactobacillus pentosus* (*Lp*NOx) with k_{cat} values between 100×10^{-3} s^{-1} and 200×10^{-3} s^{-1} (Scheme 3a) [26]. Despite this, only a few enzymes are known that utilize the oxidized NCBs.

Cofactor reduction proves to be rather difficult due to the altered redox potential of the artificial mimics. Nevertheless, there are several examples of *in situ* reduction of NCBs. One of the most frequent and established regeneration systems in many bioconversions is the use of a glucose dehydrogenase. In 2017, a promising study reported that a few NCBs (e.g. BNA, P2NA and P3NA) were converted by a modified glucose dehydrogenase (*Ss*GDH$_{I192T/V306I}$) from the thermophilic organism *Sulfolobus solfataricus* (Scheme 3b). The double mutant exhibits a ten-fold activity increase over the wild-type enzyme, reaching a k_{cat} of 42×10^{-3} s^{-1} [33••]. Many other common commercially available recycling enzymes (e.g. formate dehydrogenase from *Candida boidinii*, glucose-6-phosphate dehydrogenase from *Saccharomyces cerevisiae*, glucose dehydrogenases from

Scheme 3



Enzyme-based cofactor recycling methods using **(a)** a water-forming NAD oxidase from *L. pentosus* (*Lp*NOx) for the oxidation of NCBs using O_2 [26]. **(b)** For biomimetic cofactor reduction an engineered glucose dehydrogenase from *S. solfataricus (*SsGDH) is used with glucose as sacrificial substrate [33••]. **(c)** Twelve different variants of an artificial transfer hydrogenase (ATHase) based on an abiotic biotinylated iridium-piano-stool cofactor and streptavidin were shown to convert a broad selection of NCBs [27••].

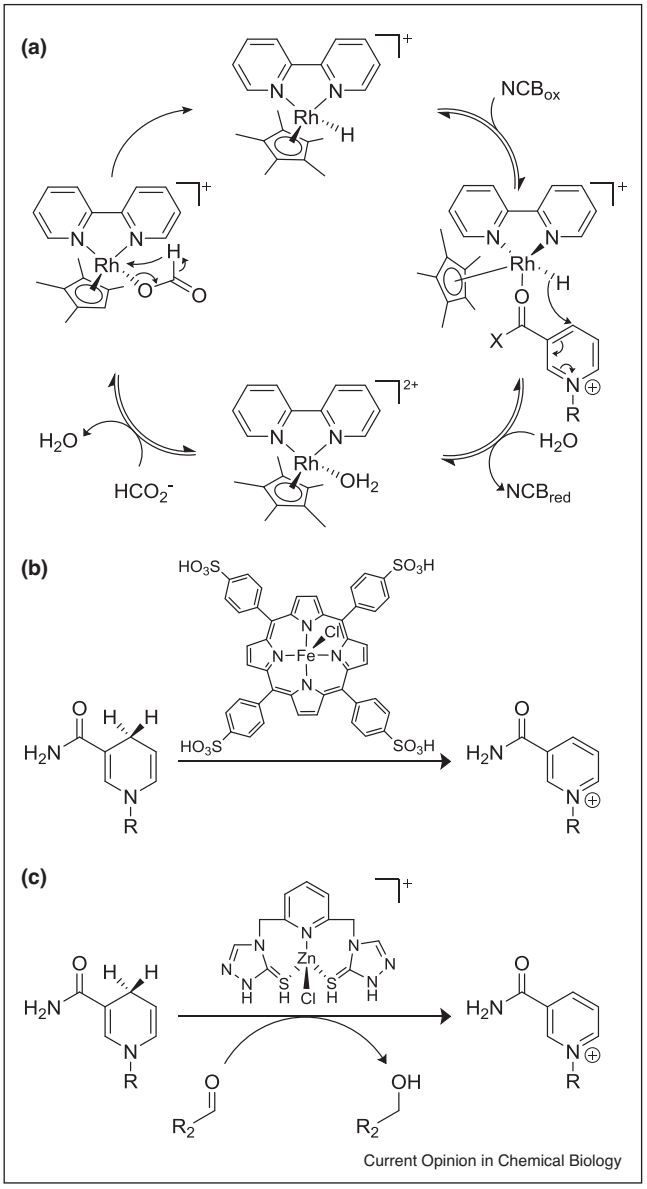
Thermoplasma acidophilium as well as from *Bacillus megaterium*, and the E170K/Q252L mutant from *Bacillus subtilis*) exhibited no significant activity. The acceptance by *Ss*GDH was explained by two phenylalanine residues in the nucleotide binding site. The resulting π–π-stacking interactions may assist NCB binding and stabilization in the active site. Enzyme engineering was also necessary to create a biorthogonal regeneration system for the oxidative decarboxylation of L-malate. Mutant libraries of malic enzyme from *E. coli* were screened with a biomimetic, in which the adenine of NAD/H is replaced by flucytosine (NFCD) or with a defluorinated version

(NCD) of that cofactor. Enzyme engineering produced a double mutant that showed higher activity with the synthetic cofactors compared to the wild-type enzyme and reduced NAD/H acceptance [40]. Another sophisticated method regenerating reduced NCBs was achieved by Okamoto *et al.* by developing artificial metalloenzymes (Scheme 3c). In their work, it was shown that artificial transfer hydrogenases (ATHases) based on an abiotic biotinylated Ir-piano-stool cofactor and streptavidin variants provide an attractive solution to the incompatibility of synthetic transition-metal catalysts and enzymes, and the combination was demonstrated with a range of EREDs [27••]. The catalytic NCB regeneration performance in terms of initial rates and total turnover numbers (approx. 2000, with respect to the Ir cofactor) for this system is one of the best reported so far.

Chemical systems using NCBs

Combining the fields of chemocatalysis and biocatalysis is a modern and attractive concept in organic synthesis. A range of combinations of biotransformations with organocatalysts as well as metal catalysts in aqueous reaction media were successfully demonstrated [41]. A recent study from 2017 shows the first cationic imine reduction using stoichiometric amounts of the biomimetic cofactor BNAH and a synthetic organometallic (η5-pentamethylcyclopentadienyl) iridium complex with excellent yields for cyclic imines (40%–99%) in neutral buffered aqueous medium [42]. For both NAD(P)H and NCB regeneration iridium, ruthenium and rhodium complexes such as $[Cp{\cdot}Rh(bpy)(H_2O)]^{2+}$ are the most frequently used catalysts [18,29,43•,44]. At the expense of formate or hydrogen [45], they perform regioselective reduction of NCBs (Scheme 4). In 2002, this system was used by Lo and coworkers to study the catalysis of horse liver alcohol dehydrogenase (HLADH). However, the latest research questioned these findings as the conversion of BNAH with HLADH could not be reproduced [19]. Presumably, the activity was initially caused by impurities in the enzymatic preparations with natural cofactors. Conversely, a study from 2016 on HLADH by Sunderland *et al.* compared commercial HLADH with a synthetic Zn complex, which models the enzyme active site with BNAH. Again, HLADH appears to show activity here, compared to negative controls. Additionally, they used five substrates that exhibited different inductive substituent effects (the ability to donate or withdraw electron density away from the carbonyl functional group). While in the presence of BNAH, there is a clear correlation between the substrate electronic environment and the measured catalytic rate, this is absent when NADH is used as cofactor [14]. The question of whether ADH converts NCBs is at least discussed contradictory in the literature. A clear explanation for these inconsistent reports can only be solved by proper and smart controls, which are crucial and indispensable for distinguishing between correlation and causality. Another chemical

Scheme 4



Metal-complex-based cofactor recycling method using **(a)** $[Cp{\cdot}Rh(bpy)(H_2O)]^{2+}$ and a proposed mechanism for the reduction of the biomimetic cofactor (NCB_{ox}) at the expense of formate. **(b)** Recycling of NCBs using TSPP-Fe(III)Cl. **(c)** A synthetic zinc model complex in the presence of BNAH for hydride transfer to aldehydes.

method for regenerating the oxidized cofactors is the use of an iron porphyrin catalyst (5,10,15,20-tetrakis-(4-sulfonatophenyl)-porphyrin-Fe^{III} chloride). For MNA^+, BNA^+, $P2NA^+$ and $P3NA^+$ regeneration, turnover numbers (TONs) were achieved with a factor of 0.5–10 less than for the earlier cited *Lp*NOX system [34•].

Photobiochemical systems using NCBs

An upcoming field in biocatalysis is indicated by a broad collection of new photobiochemical approaches to driving

redox enzymes using light energy [46–50]. With respect to NCBs, a current work by Kim *et al.* used N-doped carbon nanodots (N-CDs) as photocatalysts for regenerating NADH and its analogues (BNAH, BuNAH, and BNAcH), suggesting that photochemical regeneration of NCBs can provide a platform for improving biocatalytic efficiency using light energy. Photoexcited electrons generated by N-CDs reduced an oxidized rhodium-based complex that regioselectively reduces NCBs. Among the cofactors, the photochemically regenerated BNAH enabled the best catalytic activity for the reduction of the substrates 2-methyl-2-cyclohexen-1-one and *trans*-cinnamaldehyde using *Thermus scotoductus* OYE [51].

Stability of NCBs

Molecular stability must not be neglected when considering NCB development and enhancement, as this differ considerably depending on the solvent and system. Okamoto in 2016 and in Nowak *et al.* in 2017 showed that the half-lives of BNAH, P2NAH, and P3NAH were similar to those of the natural cofactors [27••,34•]. Although fully synthetic NCBs are relatively stable in organic solvents, they are sensitive toward certain substrates, like formate [27••], which is used in the ATHase regeneration system, as well as pH [34•] and temperature [27••,34•] in buffered reaction solutions. Indeed, most fully synthetic NCBs can be produced rather inexpensively, and the use of stoichiometric amounts or even excess cofactor is sometimes possible. But then again, low solubilities especially those of the reduced synthetic analogues, make industrially relevant concentrations and space-time-yields challenging to achieve. Moreover, there is a demand in biocatalysis for the development and design of more sophisticated fully synthetic NCBs. Novel high-throughput screening methods for cofactors could certainly facilitate this process. The semi-synthetic carbanicotinamide adenine dinucleotide (carba-NAD) synthesized by Slama *et al.* in 1988 is expensive to synthesize but has excellent stability. The ribose oxygen was replaced in this mimic by a methylene group to determine the relationship between the ribonucleotide part and the enzymatic activity [52]. NAD(P)H is prone to hydrolysis and subsequent decomposition [8]. The carbocyclic sugar in carba-NAD clearly disfavors hydrolysis of the pyridinium-*N*-glycosidic bond, making it more stable [53]. *Vice versa* some enzymes can be stabilized by storing the biocatalyst in the presence of a stabilized coenzyme such as carba-NAD, for example, for developing diagnostic test elements [54]. As mentioned earlier, the production cost of carba-NAD will be much higher than that of the small counterparts, but since the structure is more closely related to the native cofactor, the probability of achieving satisfactory enzyme properties is higher as well. Surprisingly, this is not always true and the small change in structure was shown to inhibit certain enzymes, which is not the case for the natural cofactor [55,56]. A further concept worth mentioning here as well is the PEGylation of the natural cofactor (PEG-NAD(H)) with different chain lengths to facilitate separation of reaction products and recover the cofactor in enzymatic bioprocesses [57–59].

Summary

Research on NCBs for biocatalytic purposes is still in its infancy and offers many promising opportunities. In summary, in the last few years, increasing numbers of enzymes have been identified that accept biomimetic nicotinamide cofactors. Interestingly, most of these include a flavin cofactor, which in combination with the higher reduction potential of many NCBs may facilitate hydride transfer. However, there is still no universal solution for the enzymatic use of biomimetics, as correct positioning and distances of the different cofactors within an active site are crucial. Besides many ene-reductases, attention-grabbing enzymes were found, which are frequently used as regeneration systems. Firstly, the NADH oxidase for the regeneration of the oxidized NCBs was described. Secondly, for the first time, a commonly used glucose

dehydrogenase system was shown to enzymatically reduce NCBs, and this activity was enhanced using protein engineering.

A further option for NCB reduction is an artificial metalloenzyme incorporating an inorganic iridium complex in a protein scaffold. From a (photo)chemical viewpoint, the use of complexed platinum-group metals (Ru, Rh, Ir and Pt) is the most frequently used method for selective cofactor reduction. Although it appears reasonable to add stoichiometric amounts of inexpensive mimics, cofactor regeneration should still be considered when creating environmentally friendly processes. The catalogue of newly synthesized NCBs is growing (e.g. P2NA, P3NA PNAH, HPNA, PEG-NAD, NCD, NFCD) and new structures with better properties in terms of stability, solubility and enzymatic acceptance are highly desirable. Equally important, however, are appropriate redox properties to allow the electrons to flow at the desired potential.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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