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Artificial Biocatalysis: Quo Vadis?

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Astonishing progress has been achieved in unlocking new-to-nature biocatalysis in the past decades. The progress in protein engineering enabled research to efficiently incorporate artificial structural elements into enzyme design. Recent trends include cofactor mimetics, artificial metalloenzymes and non-canonical amino acids. In this perspective article, we present the state-of-the-art, discuss recent examples and our view on what we call artificial biocatalysis. Although these artificial systems undoubtedly increase the scope of biocatalysis, their applicability remains challenging. Fundamental questions regarding the impact of this research field are addressed in this perspective.

Biocatalysis Making Waves

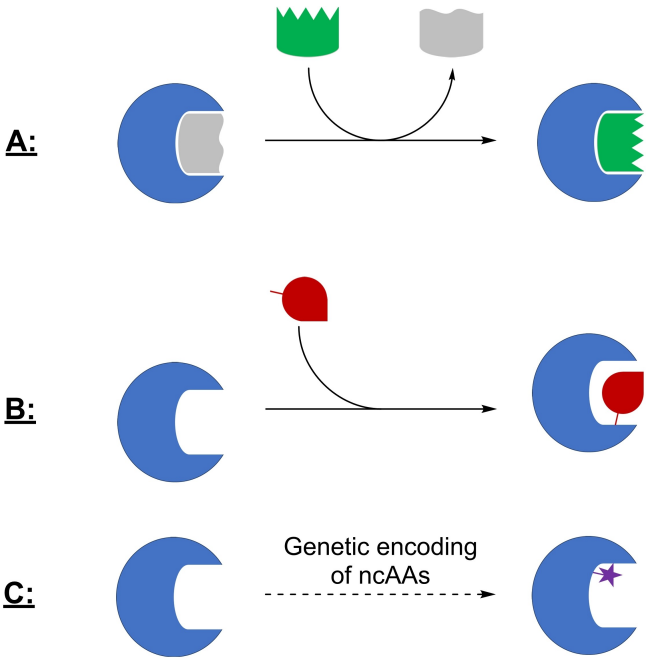
Over the past decades, biocatalysis has developed into a valuable tool in organic chemistry.[1] The expanding of the genetic toolbox ultimately leading to the principle of directed evolution opened the path for tailored design of enzymes.[2] This progress was defined as the third wave of biocatalysis in a review by Bornscheuer et al. receiving a lot of attention.[3] Despite remarkable progress in the past decades, integration of biocatalysis into industry is still not as simple as it appears at first glance.[4] Nevertheless, many researchers consider biocatalysis as an importable technique for the sustainable transformation of the chemical industry.[5] Recently, several researchers independently suggested that a fourth wave of biocatalysis is already upcoming or has already started.[6–7] Highly anticipated topics are the integration of biocatalysis into biosynthetic pathways[6] and the introduction of new-to-nature reactions in the enzyme repertoire.[7] In this perspective article from a young researcher's view, we would like to share our opinion on what we call artificial biocatalysis, a cutting-edge approach to modify enzymes for new-to-nature transformations.

Beyond Natural Biocatalysis

The reaction space and thus the potential of enzymes is not even close to be fully explored. There are still countless novel enzymes and enzymatic functions to be unraveled.[8] Some of these can be found in nature, for example by screening metagenomic libraries. Others are not even existing yet but can be designed by humans.

As the chemical variety of the canonical amino acids is limited, cofactors play a crucial role in enzyme catalysis which is demonstrated by the fact that almost half of all natural enzymatic reactions require a cofactor of any kind.[9] A cofactor is considered as a non-protein chemical compound or metallic ion that is required for the protein's biological activity.

When aiming for new-to-nature reactivities, natural protein scaffolds can be repurposed by three different approaches: a) replacement of native cofactors by artificial moieties, b) generation of new binding sites for artificial moieties and c) introduction of non-canonical amino acids (ncAAs)[10] (Scheme 1). Alternatively, these scaffolds can be designed computationally by de novo protein design.[11] Artificial moieties can be mimetics or derivatives of native cofactors, metal ions (in non-native surroundings), organic and organometallic compounds. When metals are contained in the moieties, these



Scheme 1. Different strategies for the preparation of artificial biocatalysts. A) Replacement of native cofactors with artificial ones, B) Generation of new binding site for artificial moieties, C) Introduction of ncAAs.

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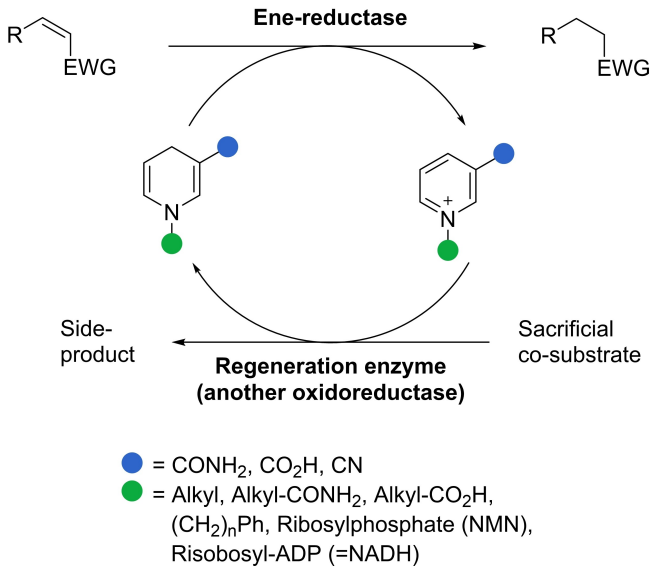
constructs are typically referred to as artificial metalloenzymes (ArMs).[12] Under the term "artificial biocatalysis", we aim to link the emerging research fields of cofactor mimetics, ArMs and ncAAs under one hat. Supplying enzymes with artificial moieties can be considered as an additional dimension of modulating enzymatic activities expanding the classical toolbox of protein engineering.

Cofactor Mimetics

Historically, learning from nature has been the most obvious, nonetheless a very successful approach to engineer enzymes. Chemists also took up this approach for designing catalyst based on native enzymes and cofactors.[13] Albeit not necessarily representing a novel reactivity, we consider the approach of employing cofactor mimetics in biocatalysis worth mentioning here. Different types of action can be observed splitting cofactors in two categories. Those which act as electron donor/acceptors such as nicotinamides and flavins[14] and those which directly interact with the substrate such as metal ions.[15–16] Especially the cofactors from the first category are not necessarily continuously present in the enzyme.

There is a broad variety of different enzyme classes which were reported to utilize synthetic nicotinamide mimetics as electron source (Scheme 2). The synthetic mimetics overcome the limitation of expensive fermentative production of nicotinamide nucleotides, allow tuning of the reduction potential and can also boost stability issues occurring with native cofactors under process conditions.[9,14] A drawback is so far their recyclability due to poor affinity of most oxidoreductases towards the oxidized form of these analogues.[17–18] Prospectively, these cofactors could allow orthogonal control of electron transfer in fermentative processes.[19] Functionalized derivatives thereof could even allow their utilization in new-to-nature reactions such as diverse photocatalytic reactions.

Many metal ions or complexes fill into the other category of cofactors. Manifold native proteins are containing metalloporphyrins as structural motifs with the most prominent representative to be probably heme enzymes.[20] The native moiety in hemoproteins itself proved to be a viable target for directed evolution studies realizing new-to-nature reactivities such as carbene[21] and nitrene transfer,[22] formation of carbon-heteroatom bonds[23] and C–H functionalization.[24—25] The boundaries between these engineered reactivities and artificial biocatalysis are transcendent. Both (chemical) modification of the porphyrin ligand backbone[26] and metal exchange are viable approaches to further tune reactivity and selectivity of natural hemoproteins as well as for the design of artificial biocatalysts from catalytically non-active proteins.[15—16,27] In certain cases, the exchange of the heme iron to noble metals can increase the repertoire of reactivities which are challenging to access with native iron-containing hemoproteins such as the intramolecular C–H insertion catalyzed by iridium(methyl)-substituted myoglobin.[28]



Scheme 2. Biocatalytic application of nicotinamide cofactor mimetics illustrated exemplarily for the reduction of double bonds by ene reductases. Reported activities for the oxidative direction are comparably low.

Engineering ArMs for Non-Biological Reactions and In Vivo Applications

The introduction of artificial moieties is not limited to analogues of native cofactors, but nearly all types of molecules can be

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Aaron A. Ingram completed his bachelor and master in chemistry with Prof. Harald Gröger at Bielefeld University (Germany) where he got fascinated by the wonderful research topic of biocatalysis. He joined the lab of Prof. Jun Okuda at RWTH Aachen University (Germany) for his doctoral studies on artificial metalloenzymes for olefin metathesis. He recently joined the Biocatalysis group in Delft working with Dr. Caroline E. Paul on the engineering of oxidoreductases towards selectivity for non-natural cofactor mimetics.

introduced by means of covalent (e.g. functionalization of cysteine residues), supramolecular (e.g. the biotin-streptavidin interaction[29]), dative anchoring (e.g. interaction histidine-metal), and assembly of protein complexes.[30] Although examples for introduction of organocatalytic functionalities into proteins are reported, they are typically limited to nature-known reactions such as imine and enamine catalysis.[31] The introduction of metal-containing functionalities forming ArMs significantly broadens the reaction space accessible. This approach represents a merge of homogeneous and enzyme catalysis. From a chemical perspective, the protein environment provides a defined first and second coordination sphere[32] and beyond,[33] thus tuning the catalytic properties. From the biological counterpart, new-to-nature reactivities can be introduced into proteins. To date, numerous reaction types ranging from oxygen insertion, reduction, C–C bond formation and hydration reactions have been realized with ArMs in part with very high selectivity close to natural enzymes (Figure 1).

This astonishing development was not anticipated when Yamamura and Kaiser[34] as well as Wilson and Whitesides[35] reported the first construction of ArMs. Without the significant progress in molecular biology and microbiology regarding recombinant protein expression, mutagenesis and the concomitant development of directed evolution, this advancement would not have been possible. In the 2000s, Manfred Reetz pioneered the engineering of ArMs laying the foundation for current research.[36] Protein engineering and directed evolution significantly contributed to increased performance of ArMs both regarding reactivity and selectivity – but it is by far not trivial and more complicated compared to classic directed evolution. A bottleneck in the development and engineering of ArMs is the often required necessity of in vitro assemblies.[37] Especially if the artificial moieties do not bind with high selectively to the scaffold, intermediate purification steps are required which tremendously reduce the throughput (Figure 2). Successful protein engineering studies rely on selective enzyme immobilization e.g. via His-tag on Ni-functionalized beads[38] or via interaction of starch with a maltose-binding protein fused to the ArM.[39]

In vivo assembly is by far more favorable, however this approach is limited to certain scaffold and catalysts. Ward and coworkers developed a fluorescence-based high-throughput screening assay monitoring the formation of umbelliferon for ring-closing metathesis active ArMs by assembling them in the periplasm of *Escherichia coli* cells.[40] While originally being developed for the polymerization of phenylacetylene by rhodium-based ArMs,[41] cell-surface display of ArMs has been proven as a useful tool to facilitate directed evolution studies with ArMs.[42–43] For metalloporphyrins, the in vivo assembly is less challenging when making use of heme-permeable *E. coli* strains such as RP523, the co-expression of (promiscuous) heme transporters[44] or using the strain Nissle 1917 with a natural metalloporphyrin uptake system.[45] These developments point towards the real strengths of ArMs which are unfolded when switching to in vivo reactions.[46] They display a challenge for common homogeneous catalysis of which the vast majority is not compatible for in vivo application.[47] The designed systems for in vivo assembly-based engineering of ArMs also serve as feasible strategies to enable novel biosynthetic pathways.[48] Although a proof-of-principal for the integration of an ArMs in the limonene biosynthesis pathway yielding an unnatural terpenoid was recently reported,[49] there are still several open challenges which are well discussed in two recent perspective articles.[48,50]

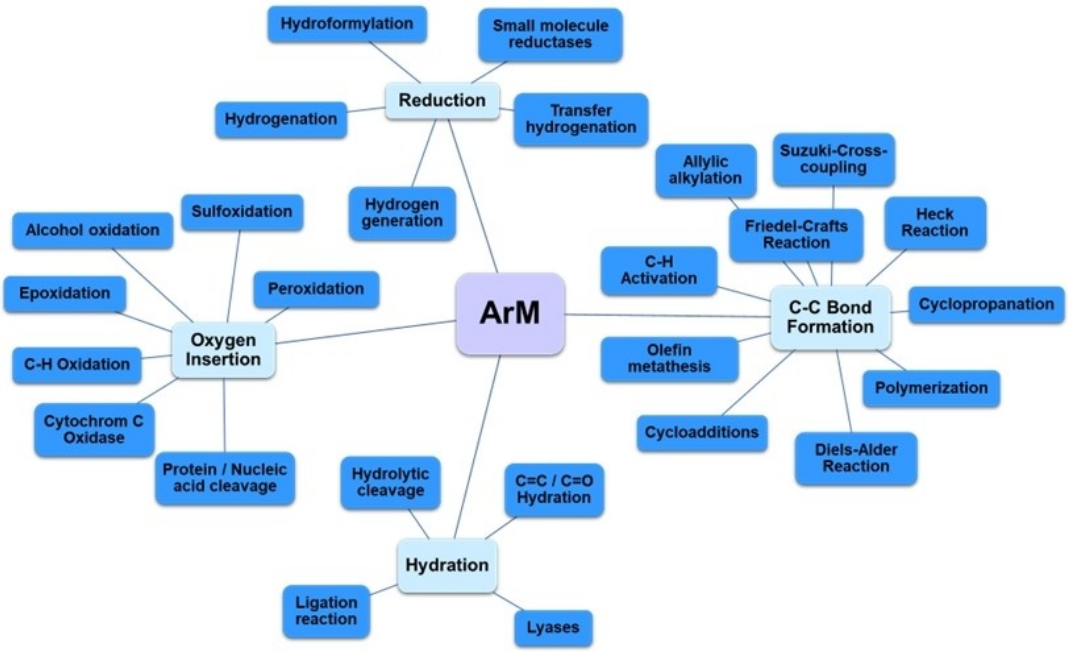


Figure 1. Reaction scope of ArMs.

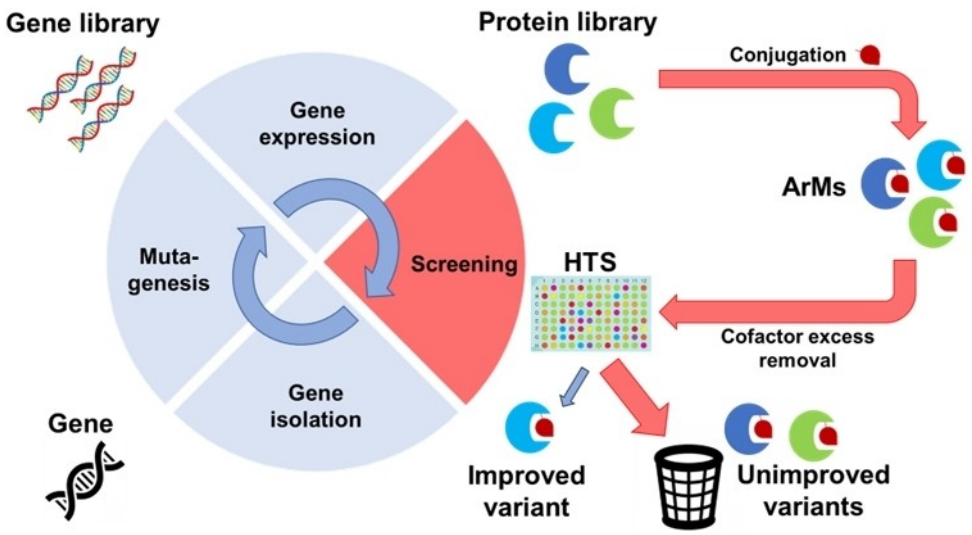


Figure 2. Principal workflow of directed evolution studies on ArMs. The blue arrows represent standard directed evolution methods and the red arrows point out additional steps necessary for ArMs.[37]

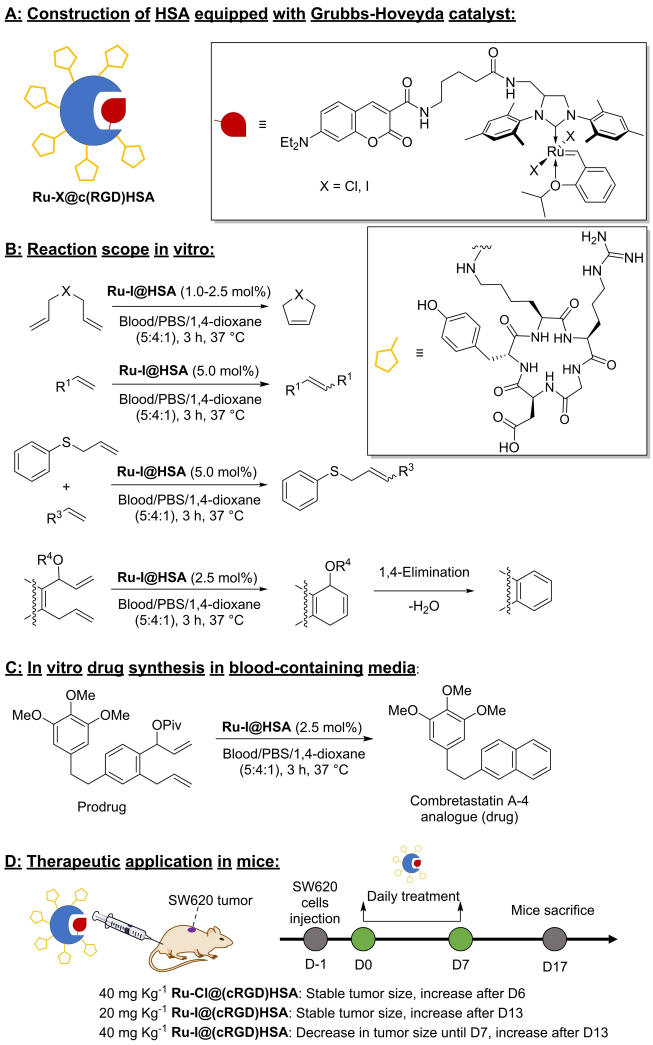
Case Study 1: An ArM for Olefin Metathesis with Therapeutic Application

Another direction for in vivo application of ArMs are therapeutic purposes.[51] ArMs are particularly fascinating tools for cancer therapy given the bioorthogonality and chemoselectivity of certain metals.[52] The ArMs scaffold can be functionalized (e.g. by genetic fusion to antibodies, glycosylation, etc.) allowing specific targeting of certain cell types.[53] This approach makes it possible to release certain drugs in biorthogonal reactions spatially guided in vivo. To this end, Tanaka et al. developed an ArM for olefin metathesis by conjugating a Grubbs-Hoveyda type ruthenium complex equipped with a coumarin moiety into the hydrophobic pocket of human serum albumin (HSA). A glycosylated derivative of this ArM was successfully used for tumor treatment of mice by in vivo drug synthesis, albeit at a high dose of 116 mg kg^{-1}.[53] Recently, they reported an optimization of this system by exchange of the chloride ligands with iodide (**Ru-I@HSA**, Scheme 3). This displays an optimization of the first coordination sphere which improved the catalyst performance in ring-closing metathesis and cross-metathesis reactions approximately by 3-fold and notably increased its stability.[54] This is an impressive result taking into account that the reaction proceeds in blood as reaction medium, conditions typically poisoning organometallic complexes.

The screening of diverse reactions showed that the cyclization of 1,4,7-triene-3-ols by olefin metathesis followed by spontaneous dehydrative aromatization was feasible at catalyst loadings of 2.5 mol%. This reaction type gives access to an analogue of the anti-cancer drug Combretastin A-4, which was proven with a cyclopeptide (Arg-Gly-Asp-D-Tyr-Lys; here shortened to c(RGD)) functionalized ArM (**Ru-I@c(RGD)HSA**). While 40% conversion were achieved with the iodide-containing ArM, only 8% conversion were reported with the chloride parent ArM (**Ru-Cl@c(RGD)HSA**). The c(RGD) functionalization allows specific targeting of the cancer cells. In vivo animal experiments showed that a dose of 40 mg kg^{-1} **Ru-I@c(RGD)HSA** given intravenously together with a precursor of the Combretastin A-4 analogue was sufficient to decrease SW620 tumor growth in xenografted mice for seven consecutive days. The same dose of **Ru-Cl@c(RGD)HSA** could not stop tumor growth.[54] This publication not only highlights the significant potential of ArMs for future therapeutic application, but also displays the importance of optimizing interactions between host protein and the metal coordination sphere. In total view, it is to notice that the field of therapeutic application of ArMs is still in an early stage.[52] Regulatory issues with heavy metals might further impede the their introduction in drug development.

Artificial Enzymes Using Non-Canonical Amino Acids

The 20 canonical amino acids limit the reaction space of proteins to electrophile/nucleophile and acid-base catalysis. The possibilities to integrate novel chemical motifs into protein are not limited to mimetics of native cofactors and metals, although their versatility enables most reactions. Nature developed the strategy of post-translational functionalization to tune certain activities, which can be to some extent considered as the analogue to ncAAs. In contrast, ncAAs are introduced during the protein biosynthesis. This can be achieved via two methods: a) selective pressure incorporation (SPI) and b) stop codon suppression (SCS).[55] SPI leads to global exchange of one amino acid to an ncAA which can be problematic in regard of protein stability. This approach is also limited to the introduction of structurally analogue derivatives of natural amino acids. SCS typically utilizes the least used stop codon of *E. coli* (UAG) to



Scheme 3. Development of a therapeutic application of **Ru-I@c(RGD)HSA**. A) Schematic structure of the components of **Ru-I@c(RGD)HSA**, B) Reaction scope of **Ru-I@HSA** in blood containing reaction media, C) In vitro drug synthesis of a Combretastin A-4 analogue, D) Animal experiments with SW620.

encode a ncAA in a selected position. Therefore, a set of orthogonal aminoacyl-tRNA synthetase in combination with an orthogonal tRNA specifically engineered for a given ncAA is required.[10]

Although biocatalysis serves as a very useful tool for the preparation of ncAAs,[56] they have been less prominent as structural elements in enzyme catalysis itself.[57] In contrast, they are widespread in proteomics and biotechnology research[58] often in relation to metal binding properties of certain ncAAs.[59] For example, (2,2-bipyridin-5-yl)alanine can datively anchor copper(II) ions leading to ArMs active for Friedel-Crafts reactions and hydrations. Also organocatalytic moieties such as 4-amino-phenylalanine mediating iminium catalysis can be introduced. While the use of ncAAs undoubtedly increases the sequence space of enzymes and thus their versatility in catalysis, their introduction is not yet economical.[10] Directed evolution approaches are so far limited to global exchange by SPI limiting the positions targetable.[60] With SCS, in principle all positions can be targeted, however the introduction is limited mostly to one ncAA per screening round. Regarding chemical versatility, it might seem demotivating that the best results in protein engineering studies are often achieved with analogues of canonical amino acids.[55] The design of artificial biocatalysts employing ncAAs as catalytic moieties is so far limited by their synthetic availability (Figure 3). To integrate structurally new ncAAs, new aminoacyl-tRNA synthetases need to be identified.[10] Although tools such as molecular modelling and directed evolution principles can also be applied to biocatalysts containing ncAAs, generalized rational design options remain yet limited, thus it requires quite high efforts to generate catalytically active enzymes.

Case Study 2: Construction of Photoenzymes by Introduction of an ncAA

Photobiocatalysis is another uprising research topic also finding its way in biocatalysis (so called photoenzymes), however their artificial pendants remain underrepresented so far. Approaching this direction, Wu and coworkers[61] and Green and coworkers[62] recently developed artificial photoenzymes for [2+2] cycloadditions. These are thermally forbidden reactions due to incompatible orbital symmetries in the ground state. Therefore, 4-benzoylphenylalanine (BpA) as photosensitizer was introduced into the sequence of the lactococcal multidrug resistance regulator (LmrR) and the artificially designed Diels-Alderase DA_20_00 yielding TPe (LmrR-based)[61] and EnT (DA_20_00-based),[62] respectively (Scheme 4). The structural analogue

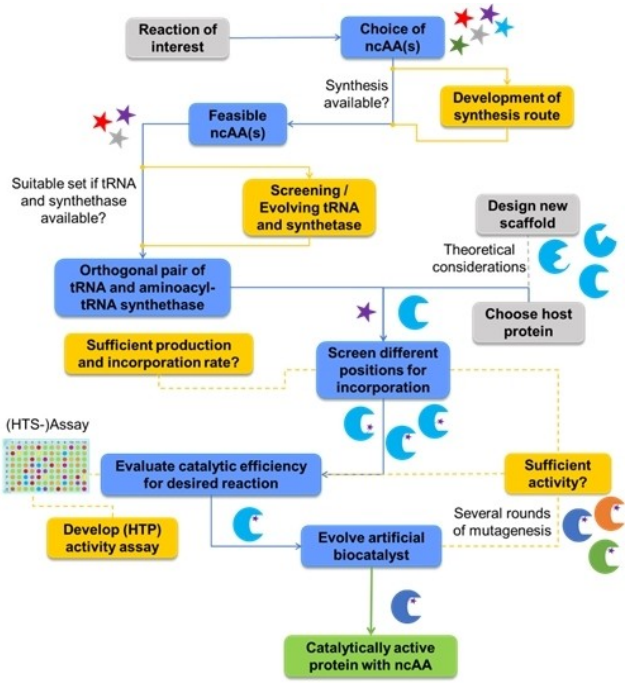
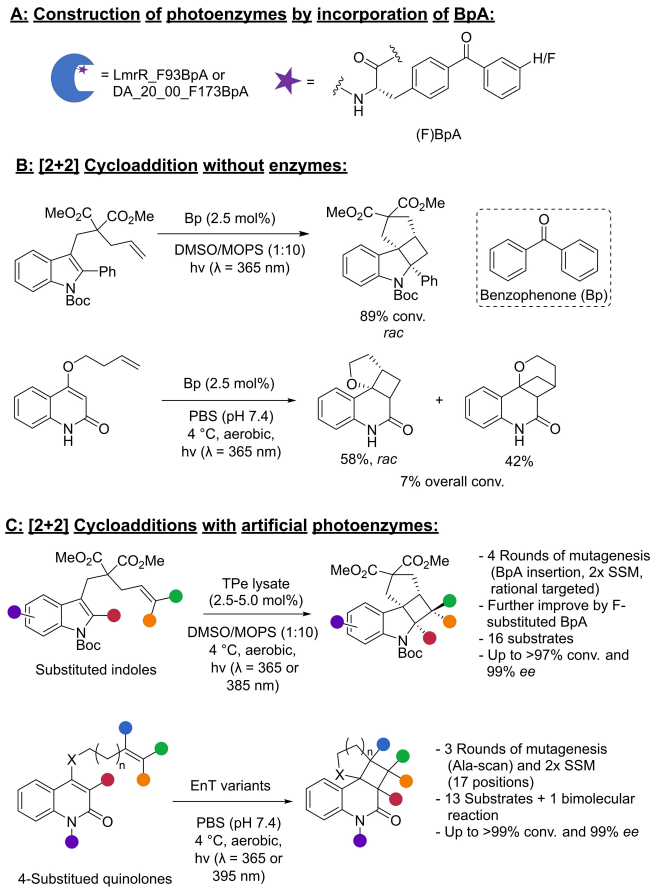


Figure 3. Typical workflow and bottlenecks in the introduction of catalytically active ncAAs into protein scaffolds.



Scheme 4. Development of artificial photoenzymes. A) Schematic design of artificial photoenzymes employing the ncAA BpA, B) Protein-free [2+2] cycloadditions catalyzed by benzophenone, C) Enantioselective [2+2] cycloadditions catalyzed by photoenzymes TPe and EnT.

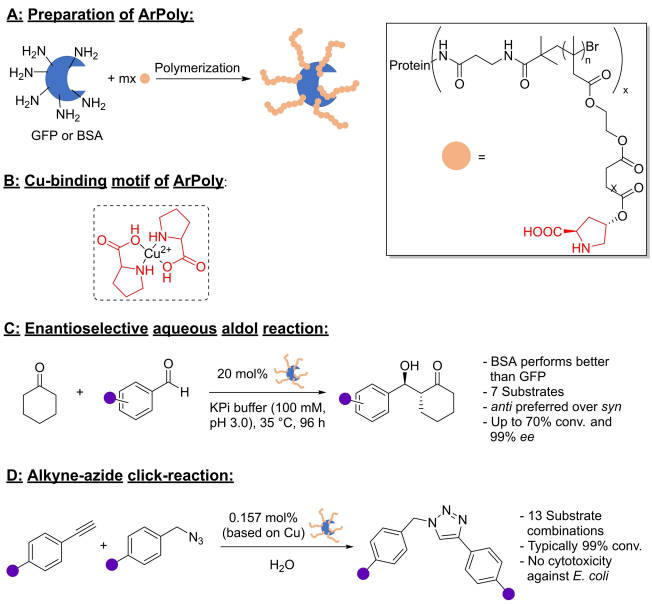
benzophenone can also promote [2+2] cycloadditions of substituted indoles and 4-substituted quinolones by promoting the light-induced formation of a relatively stable triplet excited state. However, these reactions form exclusively racemic cycloaddition products and can suffer from oxygen sensitivity (for the quinolones).[61–62]

In case of the photoenzymes, the enzyme scaffold binding the substrate serves as a chiral environment enabling enantioselective formation of the cycloaddition product. In addition, the oxygen sensitivity of the reaction was significantly decreased. In both studies, the artificial photobiocatalysts were evolved over several rounds of site-saturation mutagenesis (SSM). Analysis was typically performed with UPLC employing cell lysates facilitating the screening and increasing the throughput, thus >15 saturated positions were screened in both studies. This displays an advantage over the tedious directed evolution of ArM often requiring intermediate enzyme purification steps. The exchange of BpA with 3-fluoro-4-benzoylphenylalanine (F-BpA) further increased the performance of the evolved TPe.[61] Both artificial photoenzymes could selectively convert a variety of different substituted indoles (TPe) and 4-substituted quinolones (EnT) with high conversions >90%. Even in small scale, high yields of >90% cycloaddition product could be isolated in preparative scale experiments.[61–62]

These studies point towards potential uses of ncAAs. While it may not be the best choice to introduce ncAAs by means of directed evolution to improve the properties of a biocatalysts (yet), the design of artificial biocatalysts *around* ncAAs aiming for new-to-nature reactions seems feasible. In this case, the design can profit from established protocols for protein engineering and could represent as an alternative to ArM. However, the intrinsic limitations such as ncAA preparation, issues regarding incorporation and chemical variety of ncAAs remain to be addressed adequately.

Case Study 3: Artificial Polyenzymes

Apart from cofactor mimetics, ArMs and ncAAs, another artificial biocatalytic system was recently reported. Proteins can also be equipped with functional, catalytically active groups yielding hybrid systems of protein and polymers, so called artificial polyenzymes (ArPoly, Scheme 5).[63] It shares certain aspects with enzyme immobilization on polymeric particles, however with a different aim. This principle is a very recent development (first published in 2022) with two catalytic applications so far. This approach combines benefits from polymer catalysis such as modular structural design and macromolecular effects for catalysis regulation with those of proteins. Notable are here especially the water solubility and chirality. The first ArPoly was generated by grafting multiple L-proline organocatalysts from proteins scaffolds, in particular green fluorescent protein (GFP) and bovine serum albumin (BSA), via atom-transfer radical polymerization.[63]



Scheme 5. Development and application of ArPolys. A) Schematic structure of the components of an L-proline based ArPoly, B) Copper(II)-binding by coordination of two L-proline moieties, C) Enantioselective ArPoly-catalyzed aldol reaction in water, D: ArPoly-catalyzed alkyne-azide click reaction.

While aqueous aldol reactions catalyzed by L-proline do not proceed well and with poor selectivity, the first ArPolys with GFP as scaffold yielded the aldol product of cyclohexanone with 4-nitrobenzaldehyde with conversions between 56% and 82% at 22–26% *ee* in correlation with the polymer chain length. Under optimized conditions (pH 3.0; 35 °C), the selectivity could be improved to 94% *ee* also outperforming free L-proline polymer, although with a drawback of lower conversion. The change of the protein scaffold to BSA improved the performance of the ArPoly and up to 65% conversion and 99% *ee* were achieved with different arylaldehyde aldol acceptors.[63] The ArPoly platform allows further tuning of the polymer chain, e.g. by introduction of hydrophobic moieties leading to increased reactivity and selectivity.[64] In addition, the L-proline moiety in the polymer side chain can coordinate copper(II) ions leading to an ArPoly generating an artificial clickase for alkyne-azide coupling. This clickase converted several alkynes and azides completely to the corresponding triazols at a low catalyst loading of 0.157 mol% (referring to the copper content). While free copper(II) complexes possess cytotoxic properties, the Cu(II)-loaded ArPoly did not effected the growth of *E. coli* cell cultures.[65]

The further development of this new research field is yet difficult to predict. On the one hand, the high loadings for organocatalysis and preparation efforts seem like a bottleneck from the synthetic perspective. On the other hand, cross-interactions between engineered (or designed) protein scaffolds might prove beneficial for certain reaction types. This could be especially interesting when considering the design of bifunctional ArPoly comprising of catalytically active polymers and enzymes. The development of cascade-reactions could benefit from this design in terms of mass transfer but also considering incompatibility issues often occurring when combining biocatalysis with organometallic catalysis.

Current Limitations and Possible Solutions

From an application perspective in preparative biocatalysis, the competition for artificial biocatalysis is quite harsh. The dedication of organic and organometallic chemists towards optimization of classical catalysts should not be underestimated (as also stated by M. Reetz).[36] Intelligent ligand design can serve as an powerful tool to optimize catalyst performance. Naturally, they are examples where artificial biocatalytic systems can prospectively outperform common homogeneous catalysts. At the same time, there are certain reactions which no chemocatalyst can perform efficiently, opening a perspective for artificial systems. Nevertheless, it should not be neglected that the preparation of most artificial systems is quite tedious and resource consuming. Is it worth it? If so, what advantage do these artificial systems offer in comparison with common homogeneous catalysts, and do they justify the additional efforts? They offer the ability to switch to in vivo systems, a challenge for the vast majority of (metallo)organic catalysis and even metal ions which are not compatible for in vivo application.[47]

Cofactor mimetics allow in theory the orthogonal programming of microorganisms, however their development towards in vivo application is yet to be explored. The same should be stated regarding the use of ncAAs. In vivo synthesis of several ncAAs is possible, but combined approaches of biosynthesis and in vivo assembly were not undertaken so far.[66] In addition, ncAAs are promising tools for enabling the implementation of organo- and photocatalysis in protein scaffolds, however the overall progress in this area remains limited. Further improvement of genetic tools and protein engineering will help to boost these two research fields. At the same time, researchers remain creative and started developing new artificial biocatalytic systems such as ArPolys.

The research field of ArMs gained by far the most attention amongst the four discussed areas in this article. A dilemma in design of ArMs is the instability of many reactive metal complexes employed in ArMs in aqueous or biological systems.[47] The current focus on application of directed evolution of ArMs tackles the second coordination sphere of metals.[32] These have an impact on selectivity, but not necessarily on stability. Tuning of this factor on the other hand can quite simply be achieved by chemically designing the first coordination sphere as also recently proven by Tanaka and coworkers.[54] Combined chemo-genetic approaches investigating cross-relationships between first coordination sphere targeted ligand design and remain rare. We believe that an increased engagement in the design of water compatible catalysts in the homogeneous catalysis community would contribute massively towards the performance of ArMs. In addition, the implementation of ncAAs can serve as an additional tool even for first ligand-sphere optimization by introducing interactions not possible with the canonical amino acids. However, neither rational functionally guided introduction of ncAAs nor their introduction by directed evolution methods is yet part of the chemist's toolbox. This is mostly attributed to the challenges upcoming with the introduction of ncAAs. Another intrinsic limitation of not a few ArMs is their dependency on expensive noble metals. Here we can also learn from nature. There is a growing interest in investigating native proteins containing heavy metals such as lanthanides. Benefiting from substantial progress in this area will become valuable for designing ArMs of the future.

Future Perspective

Coming back to the challenges coming up even with classical biocatalysis methods mentioned by Hauer,[4] one might ask if artificial biocatalytic systems will ever be applied in industry or are they rather an academic phenomenon? Again, it comes down to what advantage does these artificial systems offer compared to common homogeneous catalysts and biocatalysis. An emerging question is: should we develop artificial systems just because we can as Drienovska and Roelfes critically asked in their perspective regarding ncAAs?[10]

Nature itself made a pretty good job in designing enzymes as well as chemists have done for homogeneous catalysts.

Especially regarding selectivity, nature or its man-made evolution processes outperform most chemical methods/ArMs *if* they represent nature-known reactions. Nonetheless, considering the, to some degree unexpected, astonishing progress this field has undertaken in the past decades, we believe in a quite prosperous future for this research. Artificial biocatalysis will add new dimensions to the repertoire, we believe this will be possible also for "new-to-nature" reactions, however this is not trivial to achieve. On this way, we can learn a lot more about structure-function relationships in enzymes. Thus, researchers should continuously be encouraged to be creative. This issue further underlines the importance of studies focusing on the characterization (also reporting negative results) to gain understanding to the highest degree possible. This can serve as basis for the design of new artificial biocatalytic systems in the future.

In a dream world, we will be able to develop novel biosynthesis routes with artificial systems generated in vivo. These developments should be appealed *in synergy* and not *in concurrence* to classic biosynthetic routes. Maybe this dream becomes true much earlier than we anticipate.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Keywords: Biocatalysis · Artificial metalloenzymes · Directed evolution · Protein engineering · non-canonical amino acids

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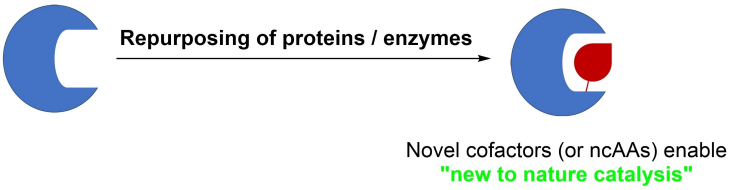

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Proteins and enzymes can be repurposed by the introduction of artificial cofactors or non-canonical amino acids (ncAAs). These artificial biocatalytic constructs turned into valuable tools to perform new-to-nature reactions with biocatalysts increasing their scope. This perspective focuses on the limitations and future application for in vivo biosynthetic pathways.