Designer metalloenzymes for synthetic biology: Enzyme hybrids for catalysis

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Abstract:

Combining organometallics and biology has generated broad interest from scientists working on applications from *in situ* drug release to biocatalysis. Engineered enzymes and biohybrid catalysts (also referred to as artificial enzymes) have introduced a wide range of abiotic chemistry into biocatalysis. Predominantly, this work has concentrated on using these catalysts for single step *in vitro* reactions. However, the promise of using these hybrid catalysts *in vivo* and combining them with synthetic biology and metabolic engineering is vast. This report will briefly review recent advances in artificial metalloenzyme design, followed by summarising recent studies that have looked at the use of these hybrid catalysts *in vivo* and in enzymatic cascades, therefore exploring their potential for synthetic biology.

1.1 Engineered metalloenzymes and enzyme hybrids for catalysis

1.1.1 Recent Advances

Over the last 20 years there have been two main approaches to designing novel metalloenzymes, a) the engineering of natural metalloenzymes to perform new-to-nature reactions or b) introducing synthetic metal complexes into protein scaffolds to create new abiotic active sites, often referred to as biohybrid catalysts or artificial metalloenzymes (ArMs). Several reviews have been recently published on both engineered metalloenzymes and artificial metalloenzymes covering their design and applications [1-4], therefore the examples below focus on reports from the last 3 years.

Metalloenzymes are nature's organometallic reagents, using metal cofactors to control reactions and expand the range of chemistry that the 20 canonical amino acids can perform. By taking advantage of the small amounts of promiscuity that some metalloenzymes possess, chemists have engineered these enzymes to perform new-to-nature reactions. The majority of work has focused on using directed evolution to expand the chemistry of heme enzymes including P450s, cytochrome cs, and myoglobins (Mb) to utilise metal carbene and nitrene intermediates, making a whole range of molecules from bicyclobutanes to organosilanes [1,5]. Recent work has pushed this further adding borylation [6-8] to S-H, N-H and Si-H insertions, as well as broadening the scope of carbene [9-11] and nitrene insertions [12,13]. Following the success of engineered heme enzymes in abiotic reactions, non-heme enzymes have been investigated as a starting platform. One of the benefits of non-heme enzymes is that they have multiple open coordination sites available for substrates, which unleashes the possibility of catalysing transition metal reactions that utilise mechanisms with two metal bound substrates e.g. oxidative addition/reductive elimination processes. Arnold and co-workers showed that the α-ketoglutarate (αKG)-dependent iron enzyme *Pseudomonas savastanoi* ethylene-forming enzyme (*Ps*EFE) can catalyse nitrene azridination and C-H amination using sulfonyl azides as nitrene precursors [14]. Initial studies on halogenases also show they have promise as scaffolds for evolution, having been shown to accept not just Cl^- ions, but also Br^-, N_3^- and NO_2^- ions [15,16].

Alongside genetic engineering of natural proteins, chemical optimisation by replacing the natural metal cofactor with an alternative metal has been explored, e.g. replacing heme in Mb or P450 with Ir(Me)(PIX) or Co(PIX) (PIX: protoporphyrin IX)[17,18] or copper with osmium in a thermostable cupin [19]. Initial results with the Ir-reconstituted heme proteins showed they catalysed more challenging carbene and nitrene transfer reactions than engineered hemoproteins [17]. However, further evolution of P450s has since meant that Fe-heme enzymes have caught up with these artificial constructs, highlighting the extraordinary power of directed evolution [20].

The engineering of natural metalloenzymes for abiotic reactions is limited to reactions occurring by similar mechanisms to those observed in the natural enzyme. Artificial metalloenzymes in contrast allow numerous reactions with no natural precedent to be introduced into the biocatalytic toolbox.[i] The most popular reactions; Diels-Alder and Friedel-Crafts reactions (Figure 1A), asymmetric hydrogenation (Figure 1B), and alkene metathesis; have become benchmark reactions for the screening of new ArMs and still dominate the literature [2, 21, 22]. Nevertheless, many different reactions have been targeted by chemists looking to develop more sustainable catalysts that utilise water as a solvent, low temperatures and allow metal recycling (see Figure 1C-E for examples) [23-27]. ArMs have also begun to provide solutions for reactions where small molecule catalysts have not achieved the desired selectivity [28,29].

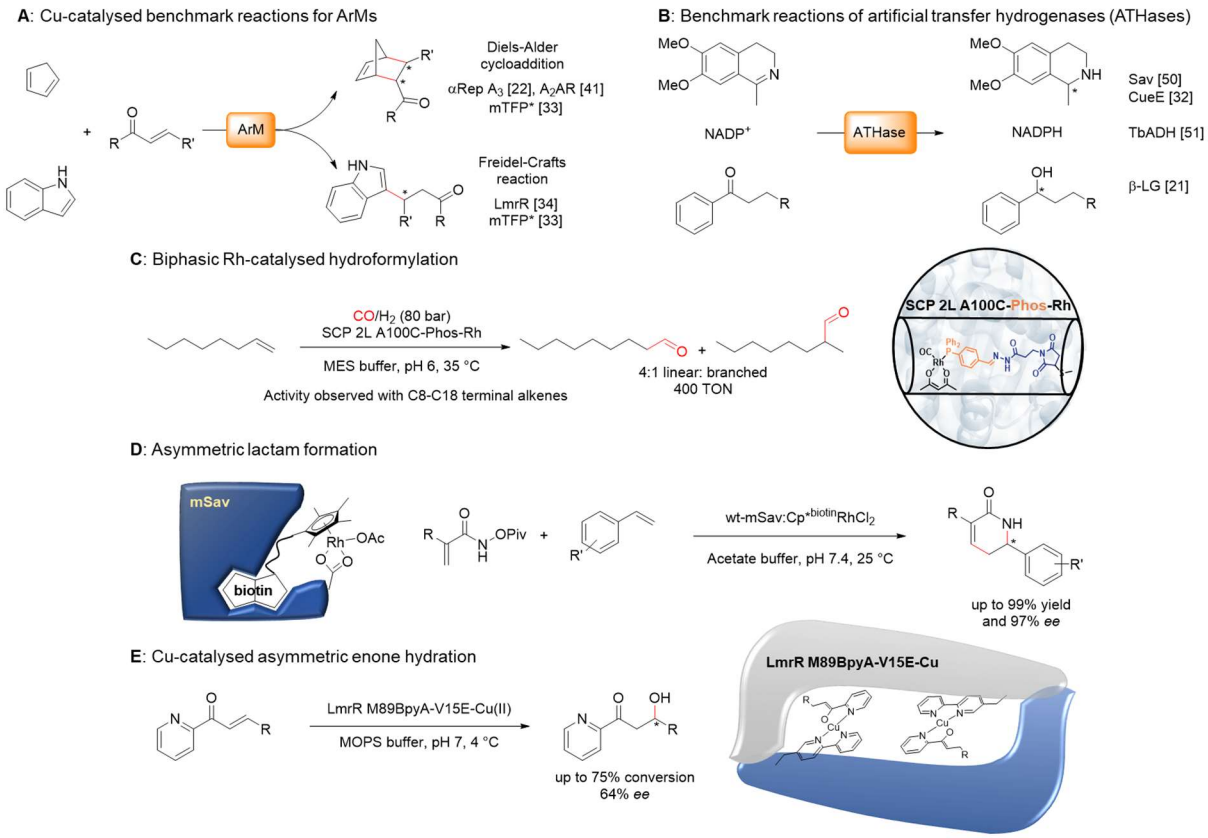

Figure 1: **A** and **B**: Common benchmark reactions for ArMs listing the different protein scaffolds used in these reactions. Examples of reactions catalysed by ArMs: **C**: Biphasic Rh-catalysed hydroformylation [24], **D**: Asymmetric lactam formation [26], **E**: Cu-catalysed asymmetric enone hydration [28].

[i] A useful resource for those looking to search for reported ArMs is the database of ArMs published and updated by the Ward group (URL: https://amp.ward-lab.ch/), see ref. [2].

1.1.2 Designing artificial metalloenzymes

The basic design principles of ArMs are the choice of a metal centre for a target reaction, and a protein scaffold to control selectivity. Numerous different scaffolds have been chosen over the years for the design of ArMs (see Figure 1 for examples), and successful scaffolds are often applied in many different reactions highlighting their versatility and generality. Of note is the lactococcal multidrug resistance regulator (LmrR) scaffold, which has been modified by several different binding methods including covalent binding through cysteine, supramolecular ligand binding, and by introducing unnatural amino acids [30,31]. This versatility, in combination with its application for multiple chemical reactions, suggests LmrR can be viewed as a 'privileged protein scaffold'. The most recent additions to the scaffolds applied in ArM design were chosen for their specific biological features e.g. CeuE, an iron-siderophore periplasmic binding protein, was utilised by the Duhme-Klair group to introduce a redox switchable anchor for metal complex recovery [32]. Whilst Arold, Groll, Eppinger and co-workers mutated monomeric *Clavularia* cyan fluorescent protein (mTFP*) to allow the quantification of metal binding using tmFRET titration experiments [33].

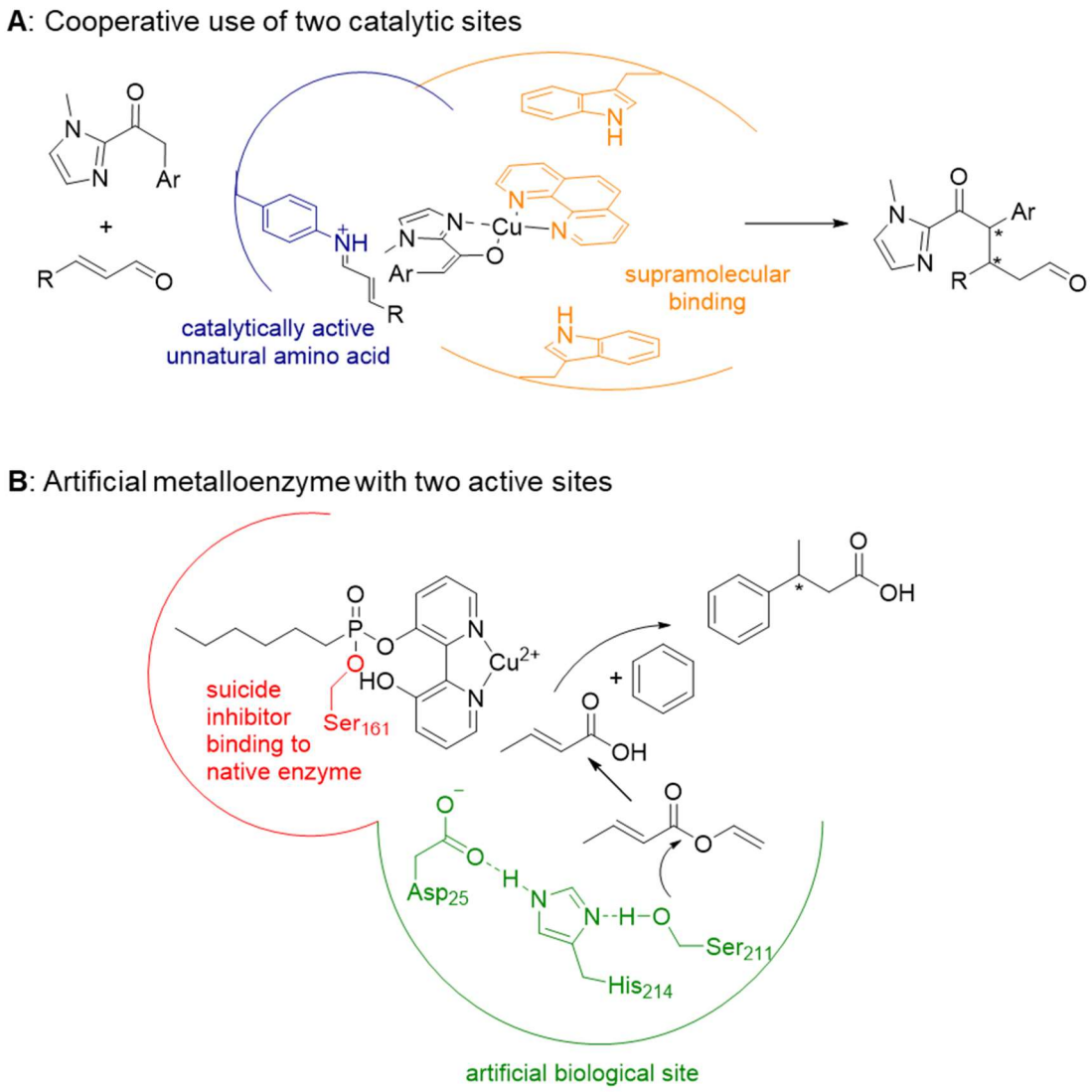

Figure 2: **A**: Organocatalytic and metal-catalysed cooperative sites in a designed ArM [34], **B**: An ArM containing two active sites for sequential catalysis [35].

The power of ArM design was showcased in recent reports that introduced two active sites to an ArM either to bind and activate a substrate or to enable a cascade reaction. In 2020, Roelfes and Zhou reported an ArM that combined both the supramolecular binding of a catalytically active Cu-phen complex with the addition of an unnatural amino acid *p*-aminophenyl alanine (*p*AF) (Figure 2A) [34]. The *p*AF was incorporated to bind and activate the enal substrate towards nucleophilic attack of the copper-bound enolate. Ferrer, Guallar, Sanz-Aparicio and co-workers described the design of an ArM containing two active sites, Figure 2B [35]. The natural biocatalyst $EH1_{A1}$ was converted into an ArM by using a metal complex attached to a phosphonate suicide inhibitor. Protein Energy Landscape Exploration (PELE) software was then applied to introduce a second binding pocket with a biologically active site (an ester hydrolase site). The resulting dual site ArM, $EH1_{AB1}$, catalysed ester hydrolysis followed by either a Cu-catalysed oxidation or a Friedel-Crafts alkylation with excellent conversions and enantioselectivities.

One of the major goals for the protein design community is the *de novo* design of functional proteins. Obtaining functional metalloproteins from first principles is challenging. Initial catalytic reports were limited to reactions catalysed by native metalloenzymes (oxidation, hydrolysis) and used substrates of limited synthetic utility [36-38]. A recent report by Anderson and co-workers showed that *de novo* metalloproteins can catalyse abiotic reactions of synthetically attractive substrates. They used a thermostable heme maquette C45 to catalyse a range of carbene transfer reactions [39]. Taken together these recent reports highlight an increasing ability to rationally design metalloenzymes for catalysis.

1.2 Merging synthetic biology and metalloenzymes for catalysis

1.2.1 Moving from in vitro to in vivo catalysis

Metalloenzymes engineered from natural metalloenzymes and *de novo* designed proteins that use the dative binding of metals to amino acids or natural metal complexes, such as heme, are inherently able to be used *in vivo*. This has enabled them to be optimised quickly using methods such as directed evolution leading to highly active catalysts. Additionally, whole cells can act as compartments which isolate metalloenzymes from starting materials, providing opportunities for the synthesis of reactive reagents *in situ* [9]. For these reasons, and to enable the use of ArMs in biosynthetic pathways, it is desirable to design ArMs that can be used *in vivo*.

The recent advances in molecular biology and synthetic biology have provided many different tools to design catalysts for use within cells. For example, the promiscuous ChuA transport protein was shown to transport Ir(Me)(DPIX) (DPIX: deuteroporphyrin IX) into cells. The heme-binding pocket of a P450 was engineered so it would preferentially accept Ir(Me)(DPIX) over heme. This enabled Ir(Me)-containing P450 variants to be directly expressed *in vivo* [40]. In comparison ArMs created using the covalent modification of cysteine with a metal complex are more complicated to move to *in vivo* systems. Challenges to overcome include the need to purify the protein before modification thus removing competitive thiols and the avoidance of catalyst deactivation by cellular metabolites (especially before conjugation with the protective protein scaffold). Protein engineering has been used to begin to overcome these challenges by locating the ArM either on the cell surface or in the periplasm, both of which have lower levels of thiols compared to the cytoplasm.

The cell surface is the most versatile location as it can be used with an ArM formed by either covalent or supramolecular attachment of metal complexes (Figure 3B). Mahy and Ghattas used the affinity of natural cell surface receptors, A_{2A} adenosine receptors (A_2 AR), for their antagonists to locate metal complexes on the surface of human embryonic cells (HEK) [41]. The resulting surface

attached ArM showed moderate activity in Cu-catalysed Diels-Alder reactions (Figure 3C). Ward and co-workers fused an Lpp-OmpA (Lpp: truncated lipoprotein, OmpA: the outer membrane protein A) fragment to both Streptavidin (Sav) and carbonic anhydrase II (CAII) so they were expressed on the cell surface of *E.coli*. Subsequent metal complex binding and use in fluorescent assays allowed for the genetic and chemical optimisation of the ArMs (Figure 3B) [42,43]. The Schwaneberg group used a different transporter, the esterase autotransporter (EstA), to locate nitrobindin (Nb) on the surface of *E.coli* which was then modified *in vivo* with a rhodium-maleimide complex to give a covalently bound ArM (Figure 3B) [44].

ArMs can be localised to the periplasm using either protein scaffolds that naturally locate to the periplasm (Figure 3D) or by the fusion of a leader sequence for periplasm localisation to the ArM protein scaffold gene (Figure 3E). Tezcan and co-workers used the former approach to optimise a *de novo* Zn-hydrolase by coupling the *in vivo* expression to the hydrolysis of the antibiotic ampicillin creating a live/death cell assay [45]. The Ward group have also constructed gene fusions using the *N*-terminal signal peptide from OmpA to locate both Sav and CAII in the periplasm [43,46]. Addition of a metal complex containing a complementary supramolecular binding motif gave rise to ArMs. Using pro-fluorescent substrates, it was possible to optimise transfer hydrogenases.

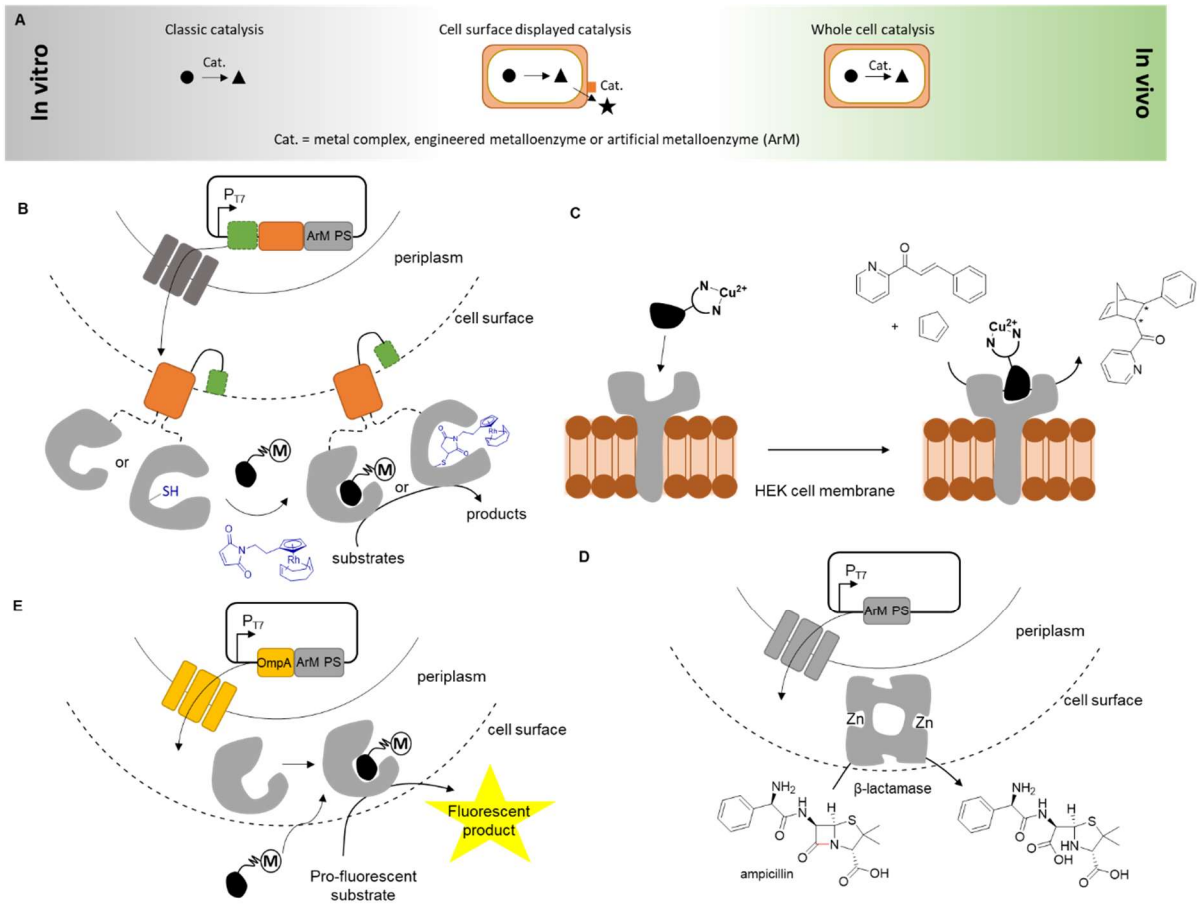

Figure 3: **A**: The progression from *in vitro* catalysis to whole cell catalysis, **B**: Bacterial cell surface display of both covalent and supramolecular ArMs [42-44], **C**: ArMs formed using A_2 AR receptors on the surface of human cells[41],**D**: *De novo* ArMs for periplasmic expression 44],**E**: Periplasmic location of supramolecular ArMs [43,46]. (ArM PS: ArM protein scaffold gene)

1.2.2 Genetic code expansion and the artificial metalloenzyme

Genetic code expansion (GCE) has long been of interest to biologists, but it is only in the last few years that chemists have applied GCE to ArM design (Figure 1E and 2B). Lewis and co-workers used *p*-azidophenylalanine (*p*AzF) to increase the throughput of the genetic evolution of a covalently bound dirhodium ArM [47]. The bioorthogonal reaction of the azide with a strained alkyne-tethered dirhodium complex allowed for efficient evolution of the ArM in cell lysate *via* random mutagenesis. A promising and exciting use of unnatural amino acids (UAAs), is the introduction of metal-binding UAAs directly into protein scaffolds, allowing the genetic incorporation of completely new abiotic activities into proteins. ArMs using UAAs can be directly expressed without the need to add synthetic metal complexes, providing an attractive approach towards ArMs for *in vivo* applications. Green, Hilvert and co-workers used amber stop codon suppression to introduce an *N*-methyl histidine into the proximal position in Mb [48]. This enabled them to convert Mb into a peroxidase. Following both genetic code expansion and directed evolution they obtained a highly active metalloenzyme that almost matched the peroxidase activity of Horse Radish Peroxidase (HRP). The Fasan group replaced the proximal histidine in their Mb-based carbene transferases with a range of UAAs known to bind metals as small molecules. However, none of the UAAs they used improved catalysis [49]. Roelfes and co-workers introduced bipyridyl alanine (BpyAla) into LmrR and upon copper binding, the resulting ArM was shown to catalyse the hydration of enones and Friedel-Crafts reactions (Figure 1E) [28]. Bipyridine is a common bidentate ligand in inorganic chemistry and transition metal catalysis therefore there are many more reactions that can be explored. This work opens the door to further research and the exploration of other metal-binding UAAs for the generation of ArMs. One challenge that needs to be overcome is how to bind the required metal *in vivo* without the purification of the protein. To successfully solve this challenge chemists and synthetic biologists will need to work together to understand cellular metal transport, particularly if non-biological metals are to be utilised.

1.2.3 Cascade reactions for chemical synthesis

As mentioned above one of the long-term goals in the field of ArMs is to use them in biosynthetic pathways (Figure 4A). A first step to show if engineered enzymes and ArMs can potentially be used in biosynthetic pathways is to explore them in *in vitro* cascades to ensure they are compatible with natural enzymes. The majority of reported cascades have focused on cofactor regeneration and/or kinetic resolution. NADPH-dependent artificial transfer hydrogenases (ATHases) have been used in imine reductions and coupled with multiple enzymes both to regenerate the NADPH and to improve the enantioselectivity of the amine product using kinetic resolution [50]. ATHases have also been used for the regeneration of $NADP^+$ following its production by a NADPH-dependent enzyme [51].

One of the hallmarks of biosynthetic pathways in cells is the exquisite control mechanisms used by the cell to control the spatial and temporal separation of incompatible catalysts/reagents/products. The extracellular location of transition metal catalysts has been successfully combined with biosynthetic pathways to catalyse the final step in several biosynthetic cascades (Figure 4). This approach has been used to make feedstock chemicals (Figure 4B) [52], small-molecule intermediates (Figure 4C) [53] as well as potential antibiotics (Figure 4D) [54]. Interestingly, as far as the author is aware no engineered metalloenzyme or ArM catalysing an abiotic reaction have been included in a biosynthetic pathway, even though their use *in vivo* has been described for both ring-closing metathesis and cyclopropanation, as featured in Figure 4B and C.

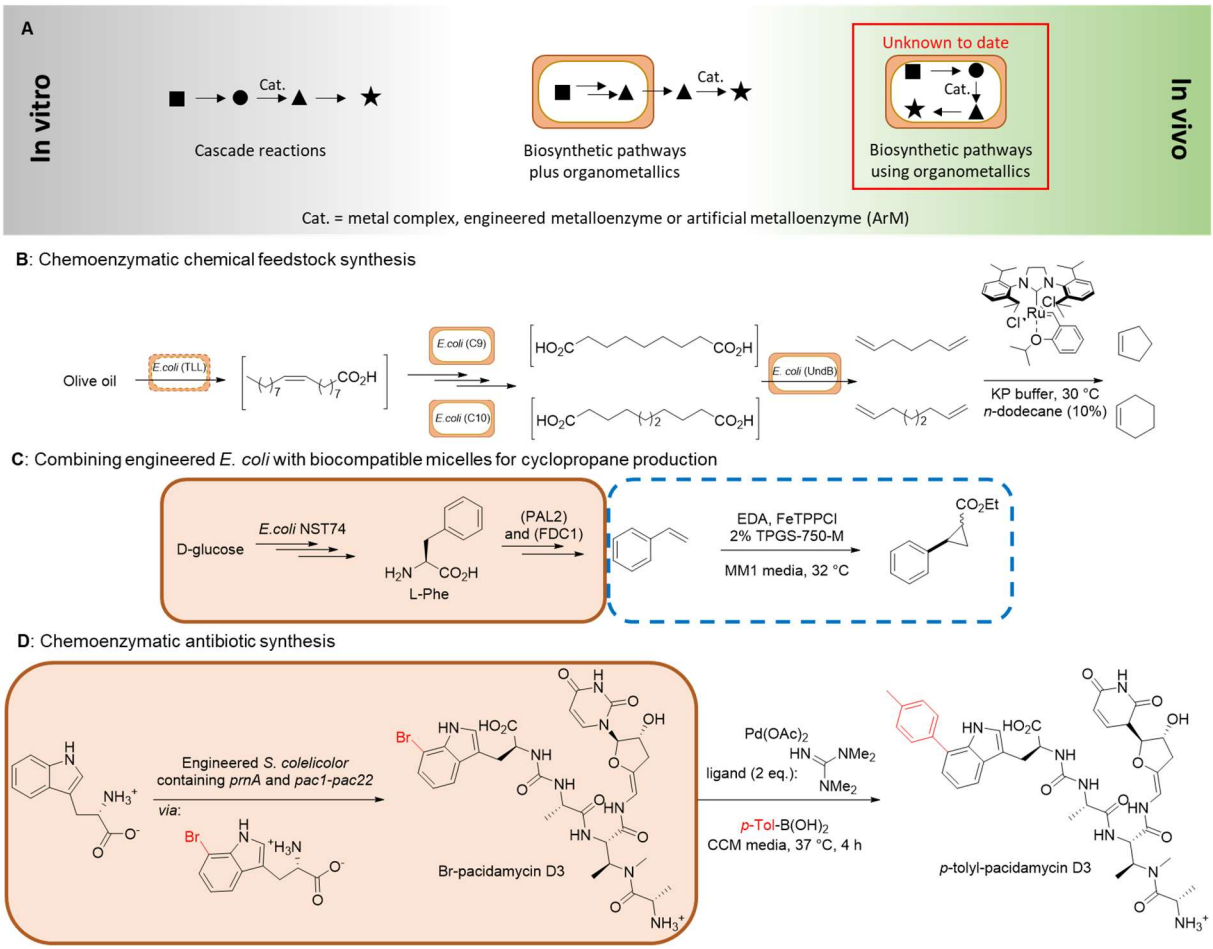

Figure 4: **A**: The progression from *in vitro* cascades to biosynthetic pathways, **B**: Chemoenzymatic chemical feedstock synthesis [52], **C**: Combining engineered *E. coli* with biocompatible micelles for cyclopropane production [53], **D**: Chemoenzymatic synthesis of antibiotics using an engineered *S. colelicolor* [54].

Whilst ArMs have not yet been used in *in vivo* biosynthetic pathways for chemical synthesis, their use in biologically relevant cascades has been explored. Matile, Fussengger, Ward, and co-workers showed that a cell-penetrating Sav based ArM could be used to regulate a T_3-responsive gene switch in HEK-293T cells by uncaging allylcarbamate-protected triiodothyronine (AT_3) to give the thyroid hormone, T_3 [55].

Note: Whilst this paper was under review a pre-print from Hartwig and co-workers was published in ChemRxiv describing the use of an iridium containing P450 in the biosynthesis of an unnatural terpenoid. The ArM was used to cyclopropanate biosynthetically derived limonene. (Huang, J. et al. ChemRxiv. DOI: 10.26434/chemrxiv.11955174.v1)

1.3 Perspectives

The engineering of natural metalloenzymes and design of ArMs has provided the chemical and biological communities with new catalysts for abiotic chemical reactions. By combining this technology with advances in synthetic biology, applications beyond chemical production are now being tackled. These include targeted drug activation [56] and small molecule biosensors [57]. Biological tools such as cell surface display have allowed the genetic evolution of ArMs, which in turn has improved their activity and selectivity. However, in most cases ArMs are still far less active than

engineered metalloenzymes hindering their widespread use. To overcome this challenge the ArM community needs to improve its understanding of ArM structure and catalytic mechanisms.

To date only a small subset of ArMs have been utilised for *in vivo* chemical synthesis. Potential roadblocks in the use of ArMs in biosynthetic pathways are their incomplete genetic encodability and the lack of regulatory components available for ArMs that are needed to control the flow of substrates through a pathway. The use of transporters to carry metal complexes into the cell and the utilisation of metal-binding UAAs in ArM design provide stepping stones that may widen ArM use *in vivo*. In addition, several groups have started to design methods to regulate ArM activity [32, 55, 58] and to control metal concentration and binding in vivo [59]. As illustrated in this review the field of ArMs is a fast-moving and exciting area to work in. Combining the knowledge of synthetic biologists and biochemists with that of the chemists in the field will be vital for ensuring that ArMs reach their potential.

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Declaration of interest: None

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Computational metalloenzyme design was used to design a genetically encoded ArM using bipyridyl alanine to create a copper active site. This ArM was designed to catalyse the asymmetric hydration of enones, a reaction that is challenging with small molecules due to the small size of the water nucleophile and the low reactivity at neutral pH.

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This study showcases an ArM that combines a supramolecular metal binding site with the catalytically active unnatural amino acid, *p*AF. These sites work synergistically to activate the substrates and control the substrate orientation, resulting in effective catalysis with excellent enatioselectivities.

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