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Large, Benjamin, Baranska, Natalia orcid.org/0000-0002-4193-6150, Booth, Rosalind et al. (2 more authors) (2021) Artificial metalloenzymes:the powerful alliance between protein scaffolds and organometallic catalysts. Current Opinion in Green and Sustainable Chemistry. 100420. ISSN 2452-2236

https://doi.org/10.1016/j.cogsc.2020.100420

Artificial metalloenzymes: the powerful alliance between protein scaffolds and organometallic catalysts

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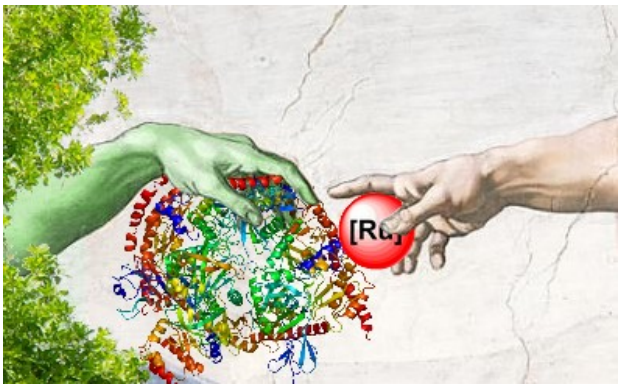
Abstract

In recent years, substantial advances have been made in the development of artificial metalloenzymes. These hybrid bioinorganic catalysts have seen their selectivity improved through techniques including site-directed and site-saturation mutagenesis and directed evolution. Their scope has been expanded by the introduction of various abiological metal catalysts, the development of new anchoring strategies and new designs to enhance their reusability by conceiving modular and reversible assemblies. This review highlights a selection of the most significant advances made in the last two years and their contribution to the development of a more sustainable chemistry.



Keywords: Artificial metalloenzyme, organometallic catalysis, biocatalysis

Introduction

Whilst a few years ago a catalyst was only required to work fast and selectively to be considered as effective, growing concerns over climate change and its heavy impact on our planet have led to a different viewpoint. Catalysts now need to be not only fast and selective, but also environmentally compatible, *i.e.* to consume a minimum amount of or even no precious metals, to function in green solvents and to be reusable. In summary, they need to be more similar to enzymes. While natural enzymes fulfil these criteria, and enzyme-catalysed reactions often conform to 10 out of the 12 green chemistry principles [1], their applications are limited by their biological reaction scope.

To address these challenges, chemists and biologists have combined natural proteins, known to function in water in a most selective manner whilst achieving outstanding turnover numbers, with synthetic catalysts able to perform a broad range of reactions, many of which do not occur in living organisms, to build artificial metalloenzymes (ArMs) [2–4]. ArMs aim to combine the best of both worlds, outperforming synthetic metallic complexes in both yield and selectivity, while being able to perform multiple reactions with a high turnover under aqueous conditions. Various applications and synthetic approaches to create these architectures have been developed. This review aims to highlight the road covered by ArMs since 2018, from natural enzymes to synthetic catalysts, and their recent contributions to green chemistry.

Mutagenesis and haem proteins

Frances H. Arnold's work (Nobel Prize in Chemistry 2018) on the development of directed evolution paved the way for the further development of ArMs. Taking advantage of small mutations allowed the optimisation of their catalytic properties, and

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enabled them to perform reactions previously not catalysed by any known natural enzyme [5]. Cytochromes bind a modified haem cofactor and provide an example of a design where recent advances have led to highly efficient catalysts. Haem proteins include monooxygenases, the oxygen-binding protein myoglobin, and Rma-cytC proteins, small haemoproteins found loosely associated with the inner membrane of the mitochondrion that play a major role in cell apoptosis.

Thanks to their reactive iron centre, variants of cytochrome P450BM and P411 are known to perform efficient carbene transfer and, as such, cyclopropane formation. Arnold *et al.* described pathways leading to various cyclopropanes with outstanding selectivities and turnover numbers (Figure 1A) [6–10].

Following their recent research on stereoselective C-H sulfonamidation, which led to cyclic sulfonamides with enantiomeric excess (*e.e.*) up to 99%, yields up to 86% and turnover numbers up to 62,000 [11], they recently reported an iron-based P411 variant able to catalyse a direct stereoselective C-H amination on the benzylic site or the allylic site of the substrate (Figure 1B). This engineered enzymatic transformation constitutes an attractive alternative to conventional and often low-yielding transition metal catalysis. By using metalloenzymes, the quantity of transition metals needed to perform this transformation is significantly lowered and the selectivity greatly improved. This demonstrated the great potential of metalloenzymes in green chemistry, and their ability to catalyse complex enantioselective and abiological reactions using low-cost and abundant metals, such as iron [12].

Moving further away from natural enzymes, the haem cofactor of haemoproteins can be removed, optimised and then re-introduced into its biological scaffold. This strategy allows the independent optimisation of the protein, through directed evolution, and its cofactor, through chemical processes, efficiently reducing both the costs and time required.

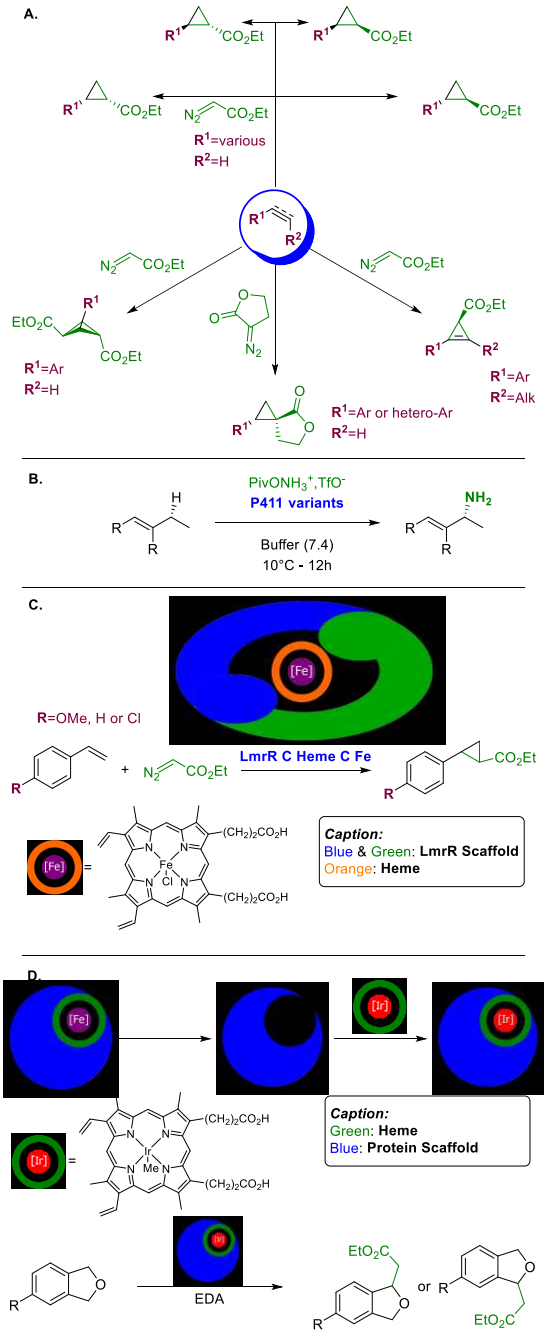

*Figure 1 – **A.** Cyclopropanation reactions catalysed by mutated P411s. Through different mutations, cyclopropanation reactions were performed with high yields and selectivities - **B.** ArM-catalysed allylic C-H amination. An amine function can be selectively introduced on the allylic position using an ArM consisting of a mutated P411 as the catalyst. The reaction was also reported on the benzylic position - **C.** By introducing a haem into a LmrR scaffold, an efficient catalyst of cyclopropanation was obtained – **D.** Regio- & chemo-selective C-H functionalisation of phtalans reported by Hartwig et al. using a haem-containing protein that accommodate iridium.*

This strategy was successfully applied to proteins that do not naturally contain haem, with the insertion of a haem moiety into a transcription protein, a multidrug resistance regulator (LmrR) that has been used as a scaffold for the design of various ArMs [13]. This artificial haem-containing enzyme was applied to catalyse cyclopropanation reactions with moderate yield and turnover number (45% - 445 TTN) (Figure 1C) [14].

Using a similar strategy, Hartwig & co-workers [15] reported a P450 scaffold, in which the haem cofactor was replaced by an iridium complex, giving it the ability to catalyse a regioselective C(sp^3)–H functionalisation of 4-substituted phthalans (Figure 1D). While both possible product isomers were obtained with only a moderate selectivity and turnover number, this study showed that even metals that are not found in natural enzymes can be docked into a protein to engineer new metalloenzymes, building a bridge between organo-metallic catalysts and natural enzymes.

A further development was revealed in a recent preprint that describes the incorporation of an iridium-based haem ArM into an engineered biochemical reaction cascade, thereby enabling the production of chemically-modified natural products, simply from glucose, inside living *Escherichia coli* cells [16]. In this approach, the enzymes do not have to be isolated and purified; instead the metabolism of the cell is exploited to support chemical transformations *in vivo,* under mild conditions and in aqueous solution.

Anchoring strategies

Most synthetic catalysts cannot directly fit into the active sites of native enzymes. Hence, to broaden the scope of ArM design, various types of anchors and linkers have been developed with the aim of docking a greater variety of catalysts into a biological scaffold, in order to better exploit the advantages that the protein scaffold can confer.

Some proteins have a particularly high affinity for specific ligands. Taking advantage of these strong affinity pairs is a powerful route to designing new anchors. For example, the strong interaction between biotin (Biot) and streptavidin (Sav) (Figure 2A) was used over the last decade to construct assemblies with various synthetic catalysts (Figure 2B) [17].

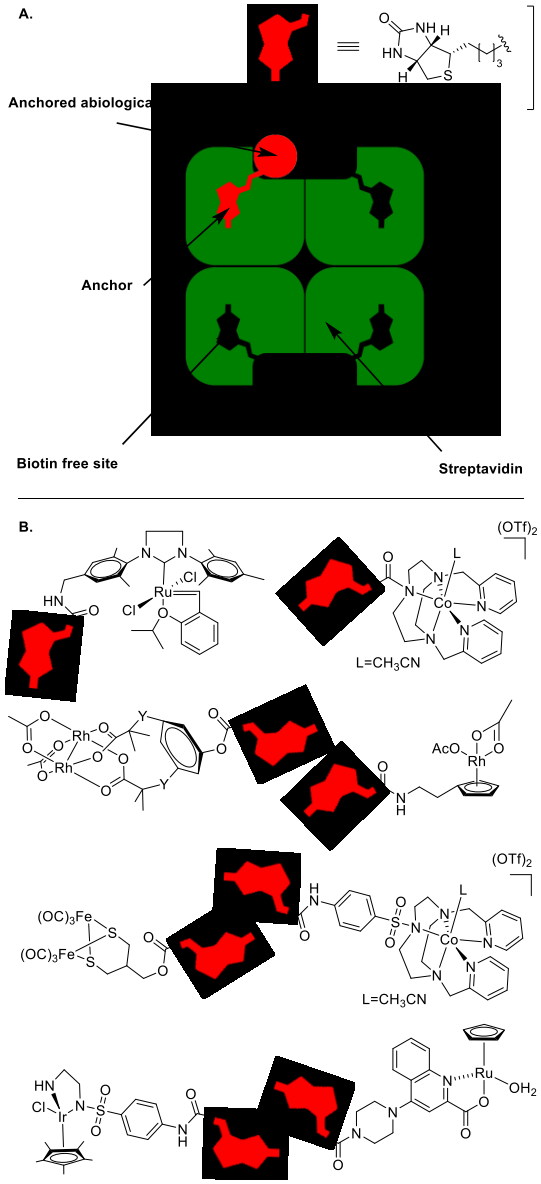

*Figure 2 – **A.** Biotin-Streptavidin (Biot-Sav) anchoring strategy. This strong affinity pair can be exploited to assemble a scaffold and a catalyst - **B.** Selected catalysts that have been anchored to streptavidin using biotin.*

A range of reactions has been reported using this anchoring strategy, including asymmetric transfer hydrogenation, ring-closing metathesis, tandem C–H activation and [4+2] annulation, and photocatalytic water reduction.

In addition to the numerous Sav-based ArMs already reported [18], Ward & co-workers have extended the scope of iridium-based ArMs, for example through the use of a Sav-based artificial transfer hydrogenase (ATHase) in the periplasm of *E. coli* [19]. In this study, the screening was greatly improved by the use of a self-immolative substrate, able to release a fluorophore upon reduction of its iminium moiety (Figure 3A). This substrate may now be used to facilitate the screening process of future transfer hydrogenation ArMs. A similar self-immolative substrate was used recently in the optimisation of a ring-closing metathesis (RCM) catalysing ArM that was shown to function inside synthetic protocells [20].

However, relying only on natural affinity pairs can be limiting. To design completely new ArMs, access is required to a broad range of proteins, not just those that show high affinity for a single specific biological ligand. Hence new anchoring strategies are being researched. To prevent a metal catalyst from leaching out from the protein scaffold, it can be advantageous to covalently link it to the scaffold. Some amino acids can be exploited to construct such bonds: for example, the nucleophilic sulphur atom of a cysteine has been extensively used to build covalent links between proteins and maleimide moieties [21]. In 2018, Doble *et al.* [22] used this strategy to design an ArM for the oxidation of lignin as a first step in its degradation. Various iron catalysts were anchored to a cysteine residue in a SCP-2L (Steroid Carrier Protein 2L) scaffold through maleimide groups. When fuelled with H_2O_2, these assemblies promoted the oxidation of various β-O-4 model compounds (Figure 3B) with good yields, providing a significant advance in the selective depolymerisation of lignin.

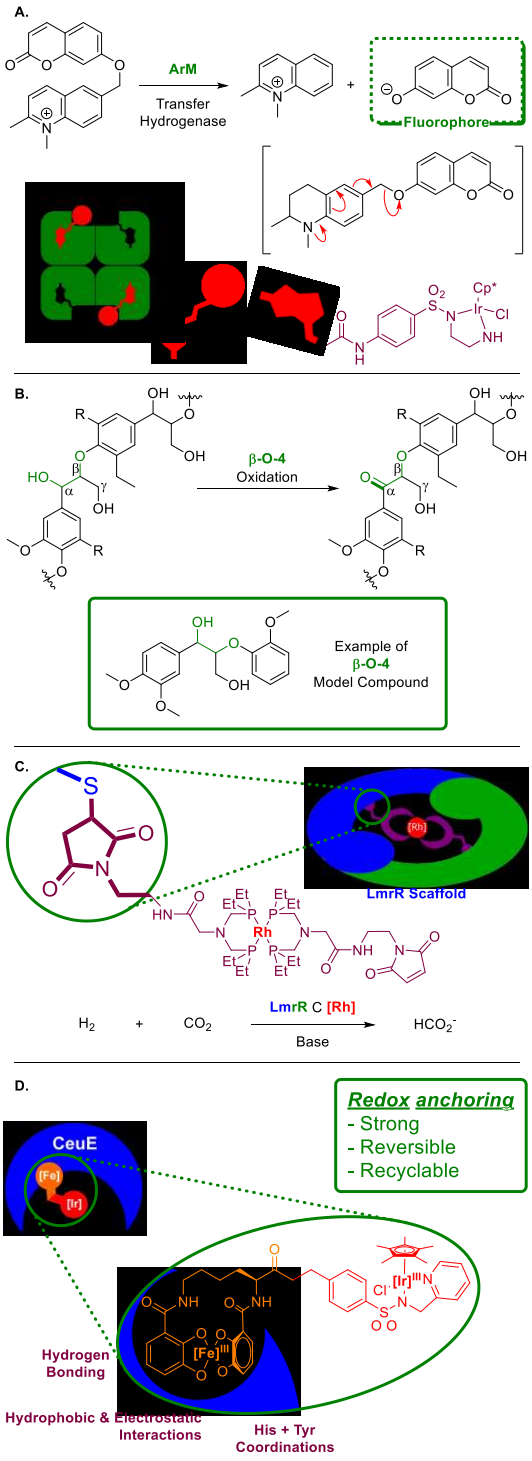

*Figure 3 – **A.** Self-immolative substrate for the development of artificial transfer hydrogenases. Upon reduction, the unstable intermediate decomposes into a fluorophore, allowing efficient screening. **B.** The β-O-4 linkage is the most abundant in lignin. The oxidation of this moiety leads to Lignin$^{\alpha\text{-}Ox}$, a common intermediate in the complete breakdown of lignin. **C.** Artificial hydroformylase based on a cysteine-maleimide anchoring strategy. The LmrR scaffold activates the rhodium catalyst, which is unreactive on its own. **D.** A redox-reversible anchoring strategy based on iron-siderophore (shown in orange) binding interactions.*

The cysteine-maleimide anchoring strategy was similarly exploited by Laureanti *et al.* [23] to anchor a rhodium complex to an LmrR scaffold, a transcriptional repressor of expression of the multidrug ABC transporter LmrCD in *Lactococcus lactis*, and build a catalyst for CO_2 hydrogenation from an inactive rhodium complex (Figure 3C).

Schwaneberg & co-workers [24,25] used the cysteine-maleimide anchoring strategy to explore nitrobindin as a scaffold for ArMs. They described the anchoring of multiple rhodium catalysts on the outer surface of *E. coli* cells and used these assemblies to catalyse the *trans*-selective polymerisation of phenylacetylene. This approach represents a promising step towards the industrial use of ArMs for the production of high-quality materials.

A novel redox-reversible anchoring strategy has been recently reported [26,27]. The reversible nature of the resulting assembly allows, after reduction of the iron centre, the reclaiming of valuable components of the ArM, and potentially the repurposing of the protein scaffold with a different catalyst bearing the same reversible anchor that exploits the Fe^{II}/Fe^{III} redox couple. It is anticipated that this development could have future applications in asymmetric catalysis, given that the L-lysine backbone and interactions with the chiral protein binding pocket could positively affect the diastereomeric ratio of the iron and iridium centres (Figure 3D).

Cascade & multisite ArMs

Cascade reactions

Because they are protein-based, ArMs can take advantage of enzymatic cascade reactions, which can be used to suppress the need for expensive or unsustainable cofactors required to keep ArMs running, such as NADPH, H_2O_2, metal salts or complex vitamins. For instance, an enzymatic cascade, benefiting from the Sav-Biot affinity pair, was reported for the benzylic hydroxylation of tetralin, followed by the kinetic resolution of the two enantiomers formed (Figure 4A) [28]. This cascade relies on a glucose oxidase to generate hydrogen peroxide *in situ* from oxygen which is then used by a [Fe]-Sav complex for the hydroxylation and the resolution, eliminating the need to feed it into the reaction. This enables the use of readily available and safe compounds, oxygen and D-glucose, instead of stoichiometric amounts of H_2O_2, which is hazardous and unstable at high concentration.

Some natural enzymes require expensive fuel: alcohol dehydrogenases (ADH – enzymes that facilitate the interconversion between alcohols and aldehydes) require NAD(P)H, an expensive and poorly stable cofactor, to function. To address this issue, Morra & Pordea [29] introduced a rhodium centre into an ADH to enable it to recycle NADPH *in situ* using a more readily available hydride source: formate.

This chemo-enzymatic cascade was reported to be compatible with elevated temperatures. The enzymatic scaffold efficiently shields the rhodium catalyst from deactivation, and prevents the ADH from losing half its activity after less than 1h, lengthening its lifetime to more than 24h (Figure 4B).

Multisite artificial enzymatic assemblies

Since both catalysts of the preceding cascades are enzyme-based, it should be possible to merge them into a single enzymatic scaffold, bearing multiple catalytic sites to catalyse different reactions sequentially. Such a catalytic platform has been reported by Sanz-Aparicio, Guallar, Ferrert *et al.* After genetically engineering an additional biologically-active site in a serine ester hydrolase, they reconfigured one site into a copper-based artificial cofactor. This two-site ArM was used to perform a two-step sequence: the deprotection of naphthyl acetate, followed by its oxidation to naphthoquinone (Figure 4C). Despite a rather limited range of compatible substrates, this study paved the way for the development of artificial multi-site enzymatic platforms able to catalyse one-pot multistep syntheses [30].

Taking advantage of π-stacking, Roelfes *et al.* introduced two abiological catalytic sites into an enzymatic scaffold by non-covalently

anchoring a copper-based catalyst inside the large hydrophobic cavity of LmrR using π-π interactions with tryptophan residues and an organic catalyst, by introducing *p*-amino phenylalanine, an unnatural amino acid, into the protein. This multi-catalytic platform was used to activate both substrates of a stereo-selective Michael addition, leading to the desired products with high yield and selectivity (Figure 4D) [31]. This flexible design should allow the design of more efficient ArMs, displaying multiple catalytic sites able to drastically reduce the energy barrier of a range of reactions in various media. In addition, this scaffold may be compatible with whole-cell biocatalysis, as described in a recent preprint article [32].

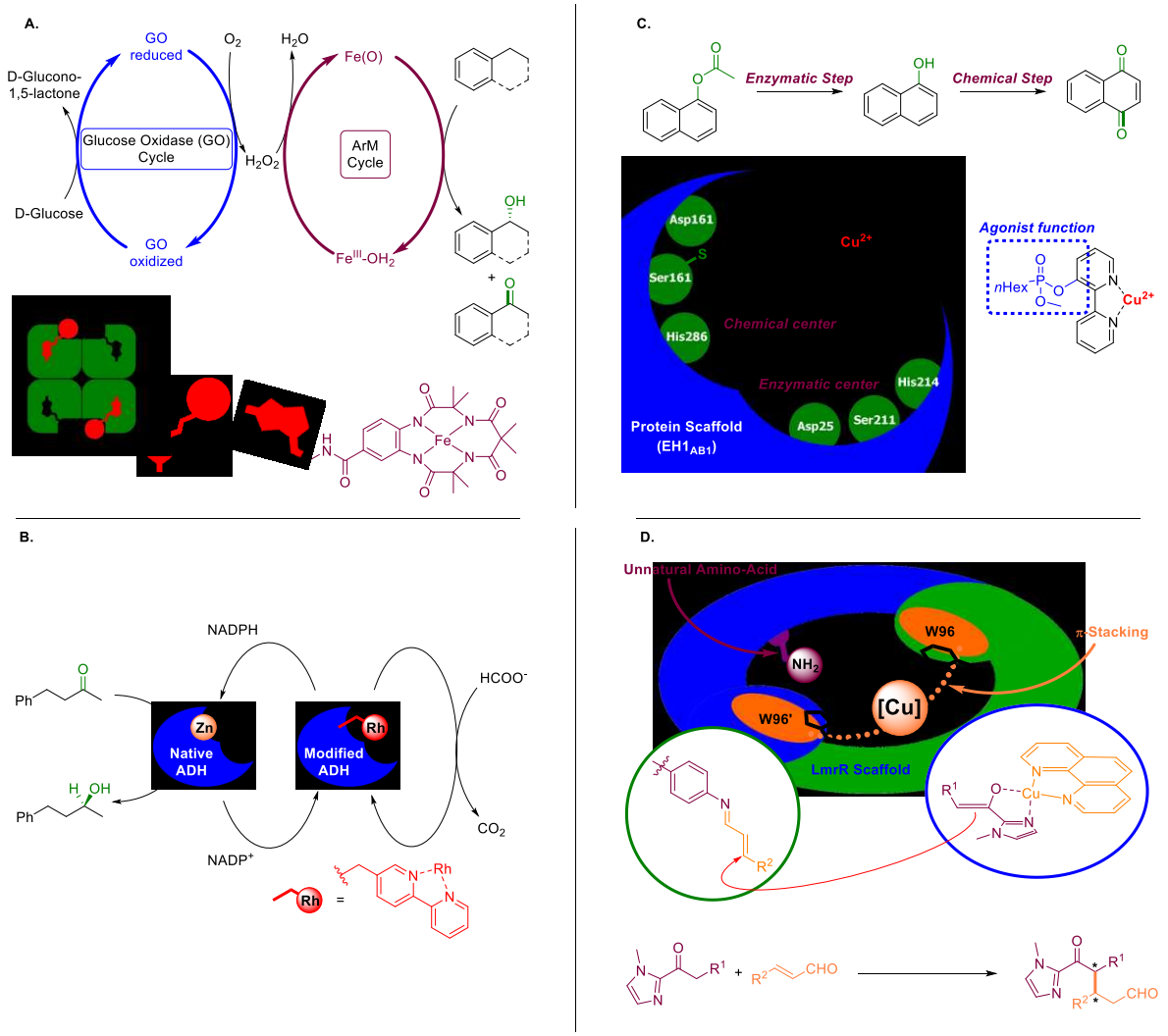

*Figure 4 – **A.** Benzylic hydroxylation & kinetic resolution. The ArM is reported to be biocompatible and the catalytic cycle is fuelled by a glucose oxidase, removing the need to use H_2O_2. **B.** Chemo-enzymatic cascade. ArMs can be used to fuel natural enzymes with cheap and readily available hydride sources, such as formate, instead of NADPH. **C.** Cascade merging metallo- and biocatalysis inside a single scaffold. Several catalytic sites were designed into this protein scaffold to build an ArM able to perform multistep synthesis. **D.** Multisite ArM. This 2-site ArM performs a single reaction step, but activates both substrate of the reaction in a selective way, thereby efficiently reducing the energy requirement of this transformation.*

Conclusions

Artificial metalloenzymes have come a long way since researchers first took natural protein scaffolds and inserted non-natural metal cations into pre-existing or engineered metal-binding sites. An ever-increasing number of reactions have been catalysed by ArMs utilising a variety of enzyme scaffolds, anchoring strategies and synthetic catalysts. Many of these are 'bio-orthogonal' reactions that cannot be catalysed by natural enzymes. Promising future directions have emerged

through systems displaying cell compatibility, multiple different catalytic sites and recyclability. In some cases, the design of cascade reactions has eliminated the need for environmentally unfriendly cofactors.

While large-scale applications of ArMs remain impractical at present due to the challenges associated with their production and optimisation, their enormous potential is clear: the steric constraints and chirality of the protein environment can render an inserted chemical catalyst selective. Recent advances in both rational protein engineering and the directed evolution of ArMs, coupled with progress in synthetic biology, have prompted researchers to seek ways of overcoming these limitations.

The growing interest from the scientific community for these hybrid systems will undoubtedly lead to the development of greener and more sustainable ways to perform catalysis, and it can be expected that many more results will soon be reported on recyclable modular enzymes and immobilised artificial hybrid catalysts.

Conflict of interest statement

None

Acknowledgements

We thank the Engineering and Physical Sciences Research Council (EPSRC) for financial support: grant EP/T007338/1 (A.-K.D.-K., K.S.W. and B.L.) and studentship EP/N509802/1 (R.L.B.) and the University of York for a teaching studentship (N.G.B.).

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