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Combining enzymes and organometallic complexes: novel artificial metalloenzymes and hybrid systems for C-H activation chemistry

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Abstract

This review describes the recent advances in the design of novel artificial metalloenzymes and their application in C-H activation reactions. The combination of enzymes and metal or organometallic complexes for the creation of new artificial metalloenzymes has represented a very exciting research line. In particular, the development of proteins with the ability to perform C-H functionalization presents a significant challenge. Here we discuss the development of these processes on natural metalloenzymes by using directed evolution, biotin-(strept)avidin technologies, photocatalytic hybrids or reconstitution of heme-proteins technology.
1. Introduction

In the last years, the creation of new artificial metalloenzymes by the combination of metal or complex organometallic systems and enzymes has represented a very exciting line of research. This field is expanding but not only in its applications but also in the design.\textsuperscript{1–8} The success of this kind of hybrids is based on the advantages offered by the combination of these two worlds (Figure 1). The enzyme provides a tridimensional structure, excellent for generating a coordination sphere, that has shown high selectivity in different processes. In addition, the use of metal catalysts (by using a coordinated metal or an organometallic structure) offers a wide range of catalytic possibilities. Therefore, this concept has allowed obtaining improved activity and selectivity of natural metalloenzymes and metalloenzymes with catalytic activities not existing in nature.\textsuperscript{1–8}

\textbf{Figure 1.} Concept on the preparation of artificial metalloenzymes.
Different approaches have been described for the preparation of artificial metalloenzymes (Figure 2).\textsuperscript{9} Mainly, we have emphasized three different strategies: I) creation of metalloenzymes with new catalytic activities or improved activity from a natural one. The application of directed evolution technique to modify the metal-coordinated aminoacids environment in natural metalloenzymes has been applied to produce enzymes with tuned or enhanced reactivity and selectivity towards non-natural substrates.\textsuperscript{9-10}

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure2}
\caption{Different approaches for artificial metalloenzymes synthesis. Square, circle and pentagon represent organometallic complexes.}
\end{figure}

A second approach (II, Figure 2) has been focused on the creation of a completely new metalloenzyme by insertion strategies of an organometallic complex in a specific area of three-dimensional space within the protein cavity \textsuperscript{6,9,11-12} with the potential to confer
selectivity to chemical reactions or completely new reactivities. This selectivity would be difficult to achieve using metal catalysts alone.

The third strategy consists of the replacement of the existing metal ion or organometallic complex inside the natural metalloenzyme with an alternative one (III, Figure 2), mainly using engineered heme-containing proteins.

The application of these biocatalysts in organic chemistry has been a major challenge in the last few years. In particular, the C-C bond formation represents one of the most useful synthetic tools, since the generation of this bond allows the obtaining of innumerable "building blocks", being a necessary synthetic step in most of the pharmaceutical compounds. This type of bond can be obtained in several ways. One of the most advantageous is the C-H bond activation reaction since it uses molecules not previously activated, which is important for the atom economy and the synthesis process. This is, however, a complex reaction, much more difficult than other C-C bond formation such as cycloaddition, Suzuki-Miyaura or Heck reactions. Therefore, the creation of active and selective artificial metalloenzymes in this C-H functionalization process, is a challenge necessary to move towards green chemistry.

This mini-review focuses on describing the few examples that up to now exist in the literature about the creation of artificial metalloenzymes with C-H activation activity.
2. New metalloenzymes by directed evolution

The application of enzymes and microorganisms in synthetic chemistry is the main focus of biocatalysis. The main reason is based on the high specificity and selectivity of enzymes in a particular process. However, these good properties normally correspond to the natural substrate and normally they show very poor or no reactivity or selectivity towards non-natural compounds. Thus, innovation is mandatory to exploit its industrial capacity. Currently, DNA sequencing and gene synthesis together with bioinformatics are key for engineering proteins. The modification of biocatalysts using *in vitro* Darwinian evolution is commonly called directed evolution. This technique requires a method for generating variations in a protein, followed by screening and selection of variants with improved characteristics. Pim Stemmer and Frances Arnold are the pioneers in this field, which they started exploring in the 1990s. Arnold efforts in this field awarded her the Nobel Prize in Chemistry (2018).

The Arnold group has used directed evolution to improve biocatalysis, in particular creating enzymes for reactions that neither synthetic chemistry nor biology has exploited. Regarding C-H bond activation, they have devoted to engineering iron-containing proteins, making iron a suitable catalyst for these reactions. This strategy consisted in altering the coordination of the iron metal in the catalytic core of the enzyme by modifying the protein aminoacids (type I class of metalloenzymes, Figure 2).

Thus, highly enantioselective intramolecular amination of benzylic C-H bonds was obtained using an engineered modified cytochrome P450 monooxygenase (Cytocrome P411<sub>CHA</sub>). Particular mutations were located in the active site (Figure 3A) impacting positively on the selectivity and activity when compared with another variant that does not have them. This variant showed excellent results in the enantioselective amination of
alkane 1 (Figure 3B) obtaining, in a particular set of conditions, the benzylic amine 2 at >99% enantiomeric excess (e.e.).

![Figure 3. A) Active site of the cytochrome P411 mutant (P-4 A82L A78V F263L). The haem group is presented in white and in orange the iron atom. Key active site residues are shown as sticks in blue and labelled. B) Cytochrome P411-catalysed C-H amination proposed mechanism. Nitrene transference is achieved thanks to the haem group reduction. Ts: p-toluenesulfonyl; Ser: serine. Figure adapted from ref. 22.](Image)

The mechanism proposed for the intermolecular amination catalyzed by the enzyme consists, first of all, in the reduction of the ferric state of the haem cofactor ((a), Figure 3b), with electrons derived from NADPH, gives the ferrous state (b). The reaction with tosyl azide (TsN3), a source of nitrene, provides the iron nitrenoid (c) (Figure 3b). Subsequent reaction of this intermediate with 4-ethylanisole (1) would yield the amination product C-H 2 and regenerate the ferrous state of the catalyst (b).

Indeed, using 10 mM of both substrates (1 and TsN3), P411CHA artificial enzyme can support up to 1,300 turnovers, far exceeding the highest turnover number (TON) reported
with any chiral transition metal complex for intermolecular enantioselective C–H amination (the best reported is 85 TON using a chiral manganese porphyrin).\textsuperscript{23}

Optimization of the reaction allowed the synthesis of the product 2 with a yield of 86%, with >99% e.e. and 670 turnovers in water at room temperature in contrast to the chemical protocols reported (Figure 4).

Substrate range studies revealed that this artificial metalloenzyme recognized a wide variety of alkanes (Figure 4), which makes this iron-based catalyst suitable for synthetic chemistry. The enantiomeric excess for all substrates was excellent in most cases (>95% ee) (Figure 4). The yields, on the other hand, depended on the substituents and the position in the aromatic ring, e.g. with the methyl substituent in \textit{para} or \textit{meta} (4a-4b), more than 50% yield was obtained (Figure 4) while less than 15% if the group was in \textit{ortho} position (4c).\textsuperscript{22}
The intramolecular activation of C-H produces good overall yields (>70%) (4f-4i) (Figure 4).

A very recent work by the Arnold group also showed an outstanding application of another version of evolved cytochrome P450 enzyme (Cytochrome P411\textsubscript{CHF}) in catalytic enantio-, regio- and chemoselective intermolecular alkylation of sp\textsuperscript{3} C–H bonds through carbene C–H insertion with high turnover and excellent selectivity (Figure 5).\textsuperscript{24}

They created more than 70 different haem proteins tested in the C-H alkylation reaction at room temperature and anaerobic conditions using p-methoxybenzyl methyl ether (5) and ethyl diazoacetate (6) (Figure 6).\textsuperscript{24} Product 7 was synthesized using the cytochrome P411\textsubscript{CHF} variant with excellent stereoselectivity 96.7:3.3 enantiomeric ratio (e.r.) and 2,020 total turnover number (TTN). Even, the enzymatic C-H activation was successfully extended using 1.0 mmol 5, obtaining an isolated product yield of 82\%, 1,060 TTN, and 98.0: 2.0 e.r.\textsuperscript{24}

Finally, the authors demonstrated the applicability of this methodology to extend the enzymatic C-H alkylation in different benzylic, allylic, propargylic, alkylamine and diazo
compounds, being possible to successfully synthesize up to 30 different compounds (Figure 6B).24

Figure 6. C-H alkylation catalyzed by P411\textsubscript{CHF} enzyme variant. A) Alkylation of 5. B) Selected examples of synthesized products by this strategy.

C-H alkylation represents a very interesting chemical reaction. In this term, recently, the Fasan group has developed different evolved Myoglobin variants for site-specific C-H cyclopropanation\textsuperscript{25-27} or functionalization of unprotected indoles.\textsuperscript{28} Functionalized indoles are very interesting molecules which have received significant attention.\textsuperscript{29} They are structural motifs found in many biologically active molecules.\textsuperscript{30} Initially, the Fasan group prepared a set of different variants of sperm whale myoglobin (Mb) by amino acid modifications at the active site.\textsuperscript{28} Then, they performed the first test
of C3-functionlization directly on the reaction of an unprotected indole 14 and ethyl α-diazoacetate (6) to synthesize 15 (Figure 7). They compared the new variants with the native Mb and the best result was found at pH 9, where 85% conversion of a completely C-H activation product was found using the Mb variant (H64V,V68A). The catalytic efficiency of this enzyme was more than 3 times higher than that reported using metal based catalysts for the C3 functionalization of N-protected indoles.\(^{31}\)

\[
\begin{align*}
14 & \xrightarrow{\text{Mb aqueous media \text{r.t}}} 15 \\
\text{Mb(H64V,V68A) protein 85\% (pH 9)} & \quad \text{cell \textgreater99\% (pH 7)}
\end{align*}
\]

**Figure 7.** C-H functionalization of indoles alkylation with ethyl α-diazoacetate catalyzed by Mb(H64V,V68A) variant.

However, they obtained a complete conversion in the process by using a whole cell biotransformation, using *E. coli* cells expressing Mb(H64V,V68A) variant (Figure 7). The scope of the reaction was carried out using whole cells as a catalyst using different substituted-indole substrates with excellent results in many cases (Figure 7).
Engineered variants of cytochrome P450\textsuperscript{32} or myoglobin\textsuperscript{33} have also been created for synthetic C-H oxidation processes, with a clear application in the total enantioselective synthesis of the norditerpenoid alkaloid nigelladine A.\textsuperscript{32}

The expansion in the C-H reactivity of evolved cytochrome P450 has been recently demonstrated in the tunable enantioselective C-H intramolecular amidation reactions of divergently construct β-, γ- and δ-lactams.\textsuperscript{34}

In this case, directed evolution was used to obtain the LS\textsubscript{sp3} variant that catalyzed the amidation reaction of \textbf{20} with a TTN (total turnover number) value >200,000 with an enantiomeric excess (ee) of 96% (Figure 8). The application of this variant to other substrates once again demonstrated the potential of this technique.\textsuperscript{34}

![Figure 8. Synthesis of β-lactams by enantioselective C-H reaction catalyzed by P411 variants.\textsuperscript{34}](image)

\textbf{3. Artificial metalloprotein hybrids}

A second approach to produce artificial metalloenzymes is based on anchoring a metal cofactor within a protein, combining characteristics that typically are from homogenous catalysts with those from enzymes (II, Figure 2).\textsuperscript{14,17} There are different strategies to achieve this. They differ in how the metal is incorporated into the protein and in how the
purpose of the protein was redesigned. Artificial metalloenzymes can be synthesized by modifying the abiotic cofactor that is inserted or by genetic engineering of the protein.

This extends its possibilities since chemistry and biotechnology can work together to improve the catalytic properties.

In this area, a successful strategy has been developed in recent years in the Ward group, using the biotin-(strept)avidin technology to locate biotinylated metal derivatives within (strept)avidin. This methodology has allowed the synthesis of different artificial metalloenzymes with very efficient catalytic properties in different reactions such as reduction, oxidation and C-C bond formation.

In particular, one of the most successful complex applications of this technology has been the activation of C-H bonds. In this term, the conjugation of streptavidin with biotin site-specifically modified with organometallic rhodium(III) molecule generated a highly active and selective catalyst for an asymmetric C-H activation (Figure 9).
Interesting results were found depending on the substituent on the aromatic ring in the pivaloyl-protected benzhydroxymic acid (22-24) or on the alkene moiety (25a-d) (Figure 9b). In particular, isoquinolone 26d was synthesized using this artificial metalloenzyme with a very high enantiopreference and enantioselectivity with >85% enantiomeric excess (ee), showing a 100-fold rate acceleration compared to the isolated Rh(III) complex.38

Following this previous approach, the Rovis group has recently performed the asymmetric synthesis of α,β-unsaturated-δ-lactams by a tandem C–H activation and [4+2] annulation reaction using a monomeric streptavidin-Rh(III)-complex hybrid.39
In this case the Rh cyclopentadienyl embedded on monomeric streptavidin, in comparison with the previous group where tetrameric protein was employed, was an excellent catalyst for the preparation of different δ-lactams, via a direct enantioselective coupling of acrylamide hydroxamate esters and styrenes, with an excellent yield (99%) and enantiomeric excess (>96%) under aqueous conditions at room temperature.\textsuperscript{39}

A different approach has focused on the creation of an artificial Ru-P450 hybrid for a photocatalytic C-H activation (Figure 10).\textsuperscript{40-41}

A set of different Ru\textsuperscript{(ii)} complex as photosensitizers were covalently attached near the heme active site of P450 BM3 enzyme, in order to evaluate the effect on the substituent in photocatalytic applications. The mechanism of action of the new hybrid system is based on, upon light activation, the Ru(II) photosensitizer is quenched by sodium diethyldithiocarbamate (DTC), generating highly reductive species capable of rapidly delivering electrons to the heme active site to initiate the C-H activation reaction.

One of this hybrid catalyst was successfully applied in the C-H activation under visible light by selective monohydroxylation of 10-undecenoic acid (27) producing the enantiomer R-28 in 85% ee. Hydroxylation occurs exclusively in the allylic position (Figure 10).\textsuperscript{41}

This result opens a future line of research into the generation of hybrid enzymes activated by light for photocatalytic C-C bond reactions.
Figure 10. Representation of a photocatalytic C-H activation by a novel Ru-P450 hybrid enzyme. Reprinted with permission from ref 41. Copyright 2014 Elsevier.

4. Heme-reconstituted artificial metalloenzymes

Artificial metalloenzymes obtained through the replacement of a naturally existing metal ion or organometallic complex inside the enzyme with a different one (III, Figure 2) has
been described as a very useful strategy for creating new abiological catalysts, expanding the applicability to a particular enzyme for a wide range of non-natural reactions.\textsuperscript{42-45}

In this case, the incorporation of the metal by this strategy could overcome some limitations that could exist in terms of spatial arrangement of the metal complex in the protein active sites in the case of the preparation of artificial metalloproteins from unnatural metalloenzymes.

The methodology has been clearly demonstrated in oxidation reactions, and C-C bond processes\textsuperscript{46} and, recently, some examples has confirmed the applicability in the activation of C-H in water.\textsuperscript{42-45}

The Hartwig group has pioneered the development of new artificial enzymes with new reactivities by formally replacing iron in Fe-porphyrin IX (Fe-PIX) proteins with non-biological metals (Figure 11), with a particular focus in C-H activation enzymes.\textsuperscript{42-44} They selected cytochrome P450 enzymes (P450s) that are native metalloproteins containing Fe-protoporphyrin IX (Fe-PIX) as a model for the creation of new reconstituted enzymes. This was a good example because, in principle, the native enzymes could catalyze some C–H oxidation and halogenation\textsuperscript{47} but not less reactive C-H bond functionalization.

In the way of the creation of robust artificial metalloenzymes, they designed an artificial reconstituted enzyme from an engineered P450 enzyme variants (CYP119)\textsuperscript{44} (Figure 11), a thermally stable P450 from the archaeon \textit{Sulfolobus solfataricus}. 
This CYP119 P450 enzyme variant was used for the creation of a reconstituted metalloenzyme in which the heme was exchanged for an iridium porphyrin cofactor (Ir(Me)-PIX) that successfully catalyzed the enantioselective intramolecular C–H bond amination reaction of sulfonyl azides (Figure 12).  

Initially, the previous artificial enzyme Ir(Me)-PIX CYP119, with CYP119 variant containing five mutations (C317G, L69V, T213G, V254L, L155G), showed excellent catalytic performance in the C-H reaction to transform sulfonyl azide 29 in the sulfonamide 30 with a yield of 98% with an enantiomer ratio of 63:37 (Figure 12a).  

Different variants of CYP119 were created, in which 3 to 5 aminoacids were modified from those mutated in the active site by means of molecular biology techniques. These were used to create the new Ir-artificial enzymes. By changing the mutations in the variant, this artificial metalloenzyme showed excellent results in the C-H activation, for example with 29 improving the enantiomeric ratio (84:16) or using different substrates that produce different sulfonamides 31-32, particularly 32 at almost quantitative yield with an excellent enantioselectivity (95:5 e.r.) and chemoselectivity (20:1) (Figure 12b).
In addition, the preparation of Ir-CYP119 artificial enzymes, where the protein contained three mutations, showed excellent results in the enantioselective C-H amination of aryloxysulfonyl azides 33-34, especially in 33 (with azide instead of amino group), where >80% yield of product 35 was obtained with a high enantiomeric ratio (90:10) (Figure 12c). This result represents a challenging C-H amination reaction, because no known enzyme catalyzes the transformation of compound 33 to 35, and forms the product with low yield and enantioselectivity with rhodium catalysts.

Myoglobins are another type of hemoproteins used as a starting point for the creation of artificial metalloenzymes through this reconstituted strategy.  

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**Figure 12.** Chemoselective C-H bond amination to the formation of benzo-fused sulfamates using different artificial Ir(Me)-PIX CYP119 variant enzymes. A) Reaction of sulfonyl azide 29. B) Different sulphonamides synthesized (30-32) by using different artificial enzyme variants as catalysts. C) C-H amination of aryloxysulfonyl azides 33-34.
In this case, the Hartwig group applied similar strategy discussed earlier in a previous section to create a number of novel artificial enzymes by adding abiological metal complexes such as the metal-porphyrin complexes of Ru, Rh, Ir, Co, Cu, Mn and Ag to apo-PIX-proteins. These artificial [M]-myoglobins, which contain various metals and axial ligands, were tested in the C-H functionalization reaction of diazoester 36 to form chiral dihydrobenzofuran 37 (Figure 13). All native myoglobins (containing iron complex) were completely inactive in the reaction, whereas the new artificial myoglobins containing different metal complexes (Co(Cl), Rh or Ir(Cl), among others) were active catalysts in this C-H activation process.

![Image]

**Figure 13.** Evaluation of the artificial Myo catalysts for the insertion of carbenes into C-H bonds. TON: turnover number. Figure adapted from ref.36.

Differences were also observed with the same metal complex introduced in Myo-H93X, each with a different mutation to the axial ligand position (H93X) (Figure 13). However, the best catalyst for the C-H functionalization was the variant containing Ir(Me)-PIX.
synthesised incorporating both a non-native metal (Ir) and an axial ligand (-CH$_3$) that cannot be incorporated by standard mutagenesis techniques. In this case, good results were obtained in almost all cases, independently of the mutation at position 93 (50% yield without any mutation) (Figure 13).

Directed evolution strategy was also used in the enzyme to find the optimal Ir(Me)PIX-Myo artificial enzymes able to perform the C-H activation on a variety of substrates.$^{42}$ After four round and more than 20 mutants, the variant containing mutations in 93G, 64L, 43L and 99F (Figure 14) was the optimal Ir-PIX-Myo enzyme that catalyzes the production of 38 with excellent enantioselectivity, with an enantiomeric ratio of 98:2 (TON of 92)$^{42}$ instead of the non-selective results using the starting non-evolved enzyme (Figure 14), demonstrating that the combination of chemical and molecular biological strategies allows the expansion and scope of the most difficult C-H functionalization of these new artificial enzymes.

**Figure 14.** Strategy using directed evolution on Myoglobin to produce new enantioselective Ir-Myo-mutants. A. Image of the active site and its surrounding produced in Chimera from PDB 1MBN. B. Directed evolution (4-rounds) to produce the best final mutant to obtain the most enantioselective artificial enzyme in the synthesis of 39. Figure adapted from ref.42.
5. Conclusions

C-H bond activation is one of the main challenges facing organic chemistry. In this review, we have compiled recent advances in the design of biocatalysts for C-H bond functionalization, using combinations of proteins/enzymes and metals or organometallic complexes for the creation of new artificial metalloenzymes. This is an important area, the chemistry of C-H activation in mild conditions is still in its infancy and new research studies are still necessary to improve this technology for industrial applications.

The use of genetic tools, especially directed evolution, has allowed the transformation of natural enzymes with specific activity to synthetic enzymes, many of them non existing in Nature. Direct insertion of organometallic complexes into specific regions of a protein has proved to be a solid tool for performing C-H activation reactions, also for a photocatalytic performance.

In addition, the search for new alternatives in the development of this type of reactions with biohybrids is a safe bet. One of the examples in which biocatalysis has a wide field to explore is the use of earth-abundant metals such as Fe, Cu, Co or Mn as catalysts in C-H reactions. This can be achieved in some cases by inserting a metallic complex in a protein structure, but research is still needed to expand this field.

The development of methodologies that allow the generation of alternative active sites in a specific enzyme, generating enzymatic and metallic activities, has been the key for the success of biocatalysis. Future advances in molecular genetics, protein engineering, bioinformatics or nanotechnology will allow further research. The development of artificial metalloenzymes that combine multiple catalytic activities in a single structure, will reduce the steps necessary to obtain a final product. This is really interesting from an
economic point of view, due to its possible impact in chemical or pharmaceutical industries, for example.

**Conflicts of interest**

There are no conflicts to declare.

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References


(9) Artificial Metalloenzymes and MetalloDNAzymes in Catalysis; Diéguez, M.,


(16) K. Oohora, Y. Kihira, E. Mizohata, T. Inoue and T. Hayashi, C(Sp 3 )–H Bond


(33) K. Oohora, H. Meichin, Y. Kihira, H. Sugimoto, Y. Shiro and T. Hayashi,


(47) P. R. Ortiz de Montellano, Hydrocarbon Hydroxylation by Cytochrome P450