Artificial metalloenzymes have emerged over the last decades as an attractive approach towards combining homogenous catalysis and biocatalysis. A wide variety of catalytic transformations have been established by artificial metalloenzymes, thus establishing proof of concept. The field is now slowly transforming to take on new challenges. This chapter describes design strategies of these artificial metalloenzymes and focusses on advances in this field over the last two years.

Part of this chapter is an adaptation from the original paper: J. Bos and G. Roelfes, *Curr. Opin. Chem. Biol.*, 2014, 19, 135-143.
1.1 Introduction

The concept of artificial metalloenzymes was introduced in 1978 by Wilson and Whitesides.\cite{1} In their seminal paper, a non-catalytically active protein was converted into an enzyme capable of performing an abiotic reaction. This was achieved by the introduction of a transition metal complex into the protein environment of avidin. In general, artificial metalloenzymes aim to combine the attractive features of transition metal catalysis and bio-catalysis, \textit{i.e.} the broad scope of reactions catalyzed by transition metals and the high selectivities (both regio- as enantioselective), bioorthogonality and mild conditions achieved by bio-catalysis. Essential for activity and high enantioselectivities obtained by artificial metalloenzymes is the second coordination sphere, which is provided by the bioscaffold. The second coordination sphere is defined as the combination of interactions that the bioscaffold provides to the substrate, the transition state, and metal complex, \textit{e.g.} hydrophobic, electrostatic and hydrogen bonding interactions (Figure 1). This is in contrast to conventional homogenous catalysis, in which selectivity and rate acceleration are the result of an interplay between the transition metal and the ligand, defined as the first coordination sphere.

The use of artificial metalloenzymes was long considered a curiosity, but after its revival in the early 2000’s, the field has become very active and vibrant. The proof of concept has been well established in the last decade and the field is currently slowly transforming to take on new challenges such as novel catalytic reactions, cascade reactions and, ultimately, chemistry in vivo. This chapter focuses on key examples.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Schematic representation of the concept of artificial metalloenzymes.}
\end{figure}
Design

Several key parameters have to be considered for the construction of an artificial metalloenzyme (Figure 1). First, depending on the reaction that has to be catalyzed, a transition metal capable of performing this desired transformation has to be selected. Second, a decision has to be made about the anchoring strategy of the transition metal (complex) to the bioscaffold. Finally, a bioscaffold which provides the second coordination sphere, has to be selected. This section will describe the latter two key parameters and will provide key examples to illustrate them.

1.2.1 Anchoring Strategies

The transition metal (complex) can be anchored to the bioscaffold via several anchoring strategies. Three major classes are distinguished in this chapter; covalent and non-covalent anchoring and the introduction of biosynthetically incorporated unnatural amino acids (UAA) capable of binding metals (Figure 2).

1.2.1.1 Covalent Anchoring

Covalent attachment of the metal complex is generally achieved by creation of a chemical bond between the bio-molecular scaffold and the ligand for the metal. For this, the bioscaffold needs to undergo a post-biosynthetic modification. This strategy requires a unique reactive residue in the bioscaffold, e.g. a nucleophile, and an equivalent partner in the ligand, e.g. an electrophile, that can react with each other in a bio-orthogonal fashion. As a consequence, both reaction partners need to be orthogonal to the bioscaffold to achieve selective modification. Several strategies that have been employed will be described below (Scheme 1).

The most common bioconjugation strategy used for the construction of artificial metalloenzymes entails alkylation of a cysteine residue. For this, a cysteine...
needs to be introduced into the bioscaffold by mutation at the appropriate location or a native cysteine can be used. As for its partner, a variety of reactive electrophilic groups on the ligand can be applied. For example, α-halogenated carbonyl compounds can react with the cysteine to accomplish the bioconjugation.\[5-7\] In an example, Distefano introduced an iodoacetamide functionalized phenanthroline into the adipocyte lipid binding protein (ALBP) via this strategy.\[5\] The resulting conjugate was used in the copper(II) catalyzed hydrolysis of amide bonds of several unactivated amino acid esters with \( ee \)'s up to 86%. Maleimide substituted ligands is another class of electrophiles that is often used for cysteine alkylation.\[8-10\] For example, Reetz and coworkers used this method to couple a maleimide functionalized phenanthroline to the thermostable enzyme tHisF.\[8\] The resulting artificial metalloenzyme was constructed as a platform for directed evolution, but no catalysis was performed. Lu and coworkers used a different approach to anchor a Mn(salen) complex to apomyoglobin.\[11,12\] Double anchoring of the complex was achieved by the reaction of cysteines, introduced into the scaffold by mutation, with methane thiosulfonate groups on the complex, forming a disulfide bridge between the complex and the protein. The resulting artificial metalloenzyme was used in the enantioselective and chemoselective sulfoxidation of thioanisole with \( ee \)'s up to 51% with no overoxidation of the product.\[11\] By changing the position of dual attachment of the metal complex in the scaffold, the ee increased up to 66%.\[12\] This result showed the importance of a correctly placed metal complex in a bioscaffold to achieve high selectivities. For a long time, these artificial metalloenzymes achieved the highest \( ee \)'s in catalysis of
all artificial metalloenzymes created by covalent anchoring. Kamer and coworkers developed a general two-step method to introduce phosphine ligands into a variety of bioscaffolds. In the first step, a cysteine of the protein was transformed into a hydrazine, and then reacted with the phosphine ligand containing an aldehyde as reactive handle. This two-step method enabled the introduction of phosphine ligands that otherwise would react non-specifically with a cysteine residue. No catalysis was reported with these conjugates.

Nucleophilic residues other than cysteines can be used for anchoring the metal complex as well. The native serine residue in serine hydrolases was used to introduce an organometallic NCN-pincer into the lipase cutinase. Known inhibitors of serine hydrolases, i.e. phosphonates bearing a good leaving group, were used to achieve the anchoring. Only the conjugation of the NCN-pincer was demonstrated and no catalysis was reported.

A disadvantage of the selective cysteine alkylation strategy is that no other cysteines can be present in the scaffold. Removal of the other cysteines by mutagenesis can circumvent the selectivity problem. However, the structure of the scaffold can be affected by this removal. Lewis and coworkers demonstrated the use of the unnatural amino acid p-azidophenylalanine to achieve unique selectivity of anchoring in the scaffold. The unnatural amino acid was introduced using the expanding genetic code concept and through strain-promoted azide–alkyne cycloaddition several ligands were introduced in the thermostable protein tHisF. The resulting artificial metalloenzymes catalyzed rhodium cyclopropanation reactions and Si-H insertion reactions. However, no enantioselectivity was observed and the activity of the artificial metalloenzyme was lower than that of the metal complex alone.

### 1.2.1.2 Non-covalent Anchoring

Non-covalent, or supramolecular, anchoring of the metal complex to the bioscaffold is based on a variety of supramolecular interactions, e.g. hydrophobic interactions, hydrogen bonds and electrostatic interactions. This strategy puts some restraints on the bioscaffold, as it should provide an environment in which binding can occur and still leave enough space for the reactants of the catalytic reaction. In addition, strong binding of the metal complex with the scaffold is required to avoid catalysis outside the scaffold, resulting in lower selectivities. On the other hand, no post-biosynthetic modification of the scaffold is required and the artificial metalloenzyme is created by self-assembly.

The most famous example of non-covalent anchoring is based on the tight binding of biotin to (strep)avidin. Whitesides successfully used a biotinylated Rh-bisphosphine complex bound in avidin as the first artificial metalloenzyme for enantioselective hydrogenation, albeit with moderate ee’s up to 41%. Ward continued this approach of introducing biotinylated metal complexes into the protein scaffold, but changed to streptavidin instead of avidin, as key innovation.
approaches improved the hybrid catalyst, \textit{i.e.} using a longer spacer between the Rh complex and biotin and by introducing a S112G mutation in streptavidin. As a result, 96\% \textit{ee} was obtained in the catalytic enantioselective hydrogenation reaction. Saturation mutagenesis of position S112 led to various artificial metalloenzymes which gave rise to both enantiomers of the product (<95\% \textit{ee}) of the hydrogenation reaction\cite{19}. Since then, more streptavidin-based artificial metalloenzymes have been constructed using biotinylated diamine-d6 transition metal piano stool complexes of Ru, Rh and Ir. These were applied successfully in several reaction types (see section 1.4 catalysis)\cite{20,21}. Another strategy for the supramolecular anchoring of a metal complex for the construction of an artificial metalloenzymes involves the replacement of a “natural” cofactor with a synthetical metal complex, thus using the existing natural active site. For example, myoglobin, a heme protein, has been used extensively. Watanabe demonstrated that Mn-salen, Cr-salen and Ru-phebox complexes could be inserted into apomyoglobin and then applied in catalysis, for example for catalytic sulfoxidations, albeit with low enantioselectivities\cite{22-25}.

The examples discussed so far used a defined binding pocket in the scaffold to bind the metal complex. Roelfes and coworkers have demonstrated that no pre-existing binding pocket is needed to construct an artificial metalloenzyme\cite{26,27}. Their approach is based on anchoring a metal complex in the structure of DNA. In the first generation DNA-based catalysts, a catalytically active Cu(II) complex was linked to an acridine moiety that intercalates into DNA. The DNA-based catalysts were applied in the enantioselective Diels-Alder reaction with moderate \textit{ee}'s up to 53\%\cite{26}. In a second generation, the Cu(II) metal complex, \textit{e.g.} based on bipyridine type ligands, did not contain a separate DNA binding moiety. These DNA-based catalysts were successfully applied in a variety of Lewis acid catalyzed enantioselective reactions (\textit{ee}'s up to 99\%), including Diels-Alder, Michael addition and Friedel-Crafts alkylation reactions.

\subsection*{1.2.1.3 Dative Anchoring}

Direct coordination of a metal ion to native residues of the bioscaffold that can act as ligands, \textit{i.e.} N, O, and S functional groups, is regarded as dative anchoring. Natural metal binding enzymes use this strategy to bind their catalytically active metal ion\cite{28}. Replacement of the native metal ion by a nonnative metal ion is a strategy that has been employed for the construction of artificial metalloenzymes. For example, Kazlauskas and coworkers have converted human carbonic anhydrase (hCA) into a peroxidase by substituting the three histidine ligated Zn(II) ion by a Mn(II) ion\cite{29}. Moderate enantioselectivities were obtained in the epoxidations of a variety of styrene substrates, \textit{i.e.} up to 67\% \textit{ee}. The use of naturally occurring metal binding sites can be a limiting factor in the type of metal ions to be used, since different metals need different coordination environments, thus limiting the catalytic scope. However, the engineering of metal binding sites in nonnative metal binding proteins...
can expand the scope of coordination environments. Reetz and coworkers have engineered a Cu(II) binding site into the thermostable protein tHisF by introducing a histidine-histidine-aspartic acid triad at an appropriate position and the resulting artificial metalloenzyme was used in the Cu(II) catalyzed Diels-Alder reaction, although moderate enantioselectivities were obtained, \(i.e\.\, up\to 46\% \text{ ee}\).\[30\]

### 1.2.1.4 Non-natural Amino Acids

The geometrically precise placement of residues to provide the correct coordination environment for the metal ions is quite challenging. This difficulty can be circumvented by the introduction of genetically encoded metal ligands using the expanding genetic code approach.\[17\] Schultz and coworkers introduced the metal binding ligand bipyridylalanine (BpyAla) into the Z-domain protein using this strategy.\[31\] An aminoacyl-tRNA synthetase pair from \textit{Methanococcus jannaschii} was evolved to incorporate BpyAla by biosynthetic means. This approach was used to construct an artificial metalloenzyme by the introduction of BpyAla in the Catabolite Activator Protein from \textit{E. coli} (CAP).\[32\] The resulting Cu(II) artificial metalloenzyme was capable of selectively cleaving DNA. In another study, the metal binding moiety 2-amino-3-(8-hydroxyquinolin-3-yl)propanoic acid (HQ-Ala) was introduced into a Z-domain protein.\[33\] When metalated with Zn\(^{2+}\) ions, the HQ-Ala residue in the protein scaffold acted as a fluorescent probe and as a heavy metal binding site for phasing in X-ray structures. However, artificial metalloenzymes constructed by this design have not been used in asymmetric catalysis to date.

### 1.2.2 Bioscaffolds

The 2nd coordination sphere plays a key factor in artificial metalloenzymes and is provided by the bioscaffold. Therefore, a few important factors needs be taken into consideration when choosing a bioscaffold. The bioscaffold should show stability over a desired temperature and pH range. Additionally, the scaffold has to show tolerance towards organic solvent, \(i.e\.\, reactants used in catalysis often need a particular amount of organic solvent to be dissolved. Another choice that has to be made is whether a scaffold has an existing active site or one has to be created.

Artificial metalloenzymes constructed from a scaffold possessing an existing active site has advantages. First, the stability, \(e.g.\, temperature and pH\) of the scaffold can be estimated from the apoform. Second, the 2nd coordination sphere is already in place. However, the binding site should be large enough to accommodate the metal complex and leave space for the reactants of the catalyzed reaction. Most binding sites of proteins bind the binding partner and/or reactant perfectly, but do not provide the extra space that is needed. Only a few bioscaffolds used successfully in enantioselective catalysis have such a binding site for the construction of artificial metalloenzymes, for example avidin/streptavidin\[^{1,34}\], bovine serum albumin (BSA) \[^{35}\] and (apo)myoglobin\[^{36}\]. The creation of a new active site in a bioscaffold offers
more freedom as the 2nd coordination sphere can be designed to fit the needs of the catalyzed reaction. Moreover, the number of potential scaffolds for the construction of artificial metalloenzymes can be greatly expanded. However, designing a whole protein scaffold from scratch in silico is still very challenging and requires a lot computer calculation power. This strategy has not been applied to the construction of an artificial metalloenzyme yet. However, redesigning existing sites using modeling approaches based on X-ray structural data has been applied for to construction of artificial metalloenzymes. For example, using computational design, Lu and coworkers constructed an artificial nitric oxide reductase. A non-heme iron binding site was created by the introduction of two additional histidines and a glutamate into the distal pocket of myoglobin containing an iron heme complex. The accuracy of the predicted model was confirmed by overlaying the X-ray structure of the predicted and experimental proteins.

The creation of a new active site in a non-existing pocket can also be accomplished without de novo design of the active site. Roelfes has reported the construction of an artificial metalloenzyme using DNA as bioscaffold. The active site was created in the chiral groove of DNA by supramolecular anchoring of a Cu(II) complex. The chiral information of the microenvironment created by the DNA was successfully transferred in several Lewis acid catalyzed reactions, as described in the previous section.

Peptides are small compared to proteins, but are easier to design de novo and potentially still have enough functional diversity to generate a defined 2nd coordination sphere. For example, Ball and coworkers have developed peptide based dirhodium complexes by employing as ligands the carboxylate groups of aspartic acid residues in a dimeric nonapeptide in a DxxxD motif. This metallopeptide was applied successfully in asymmetric Si-H insertion reactions with excellent ee’s, i.e. up to 92% ee (see more in section 1.3.2 Peptides as Bioscaffolds).

1.2.3 Optimization

Artificial metalloenzymes have the benefit that both the metal complex and the bioscaffold can be optimized independently. This optimization approach is referred to as the chemogenic approach.

The optimization of the metal complex is often achieved by rational design. In contrast to metal complexes used in homogenous catalysis, no chiral version of the metal complex is needed, which often simplifies the synthesis.

On the other hand, optimization of the scaffold can be achieved by several approaches. The empirical approach is based on visual inspection, often using an X-ray structure of the non-functionalized protein or based on homology models to introduce mutations. The theoretical approach uses computer algorithms to determine the effects of mutations on the active site. The semi-theoretical approach is a combination of the approaches mentioned above. However, these three approaches
already require some knowledge of the structure of the scaffold. Directed evolution of the bioscaffold is a non-biased approach for optimization.

Optimization of artificial metalloenzymes is often accomplished using a semi-theoretical approach. The resulting optimized model is checked by X-ray structures to refine the model. As an example, Ward and coworkers used this approach to optimize the streptavidin/biotin artificial metalloenzyme in an asymmetric transfer hydrogenation reaction (ATHse). Via computer modeling, a histidine residue was introduced into the active site that coordinated with either Ir or Rh.[46] A dual anchoring of the Ir or Rh metal with the ligand and the histidine residue improved catalytic efficiency (see more details in section 1.4.1 Asymmetric Transfer Hydrogenation).

Optimization by directed evolution, for example by error prone PCR, depends on random mutagenesis of the bioscaffold and screening of the resulting library for catalytic activity. High-throughput screening is then needed to find a positive hit. An artificial metalloenzyme constructed via supramolecular or dative anchoring speeds up the optimization as it does not require time consuming functionalization of the scaffold. However, random mutation of the bioscaffold needs a large library to include all possible mutations randomly distributed. The library expands greatly as a function of the size of the bioscaffold, and such a large library is difficult to screen. This is because screening for enantiomeric excess of the catalyzed reaction requires high throughput GC or HPLC analysis, since no color assay exists for these improvements. For that reason, a method was developed in which a more focused library was constructed. The Combinatorial Active-site Saturation Test (CAST) focuses on the active site residues and was applied successfully to improve a streptavidin/Rh-biotin artificial metalloenzyme from 23% ee to 65% ee in three rounds of mutagenesis.[47]

1.3 New Designs of Artificial Metalloenzymes

The key parameter in artificial metalloenzyme design is the second coordination sphere provided by the biomolecular scaffold, i.e. proteins, peptides, DNA, etc, which provides the supramolecular interactions that are expected to contribute to achieving enzyme-like rate accelerations and selectivities. Hence, the choice of biomolecular scaffold and the position and mode of anchoring of the transition metal complex are of the utmost importance, as described in the previous sections. Many of the examples of artificial metalloenzymes described in the literature to date rely on a limited number of protein scaffolds, such as streptavidin,[48] BSA, and apomyoglobin.[49] These have in common that they have a pocket that is large enough to accommodate the metal complex and leave enough space for the substrate. This section describes new scaffolds and design approaches that have been introduced in the last two years.
1.3.1 Proteins as Bioscaffolds

A new method for the construction of an artificial metalloenzyme by focusing on the binding mode of substrates was reported by Ménage and coworkers (Figure 3A).[^50] The periplasmic nickel-binding protein NiKa was used as a host for iron catalyzed sulfoxidation reactions. Based on the crystal structures of a hybrid of the NiKa protein with a bound iron complex of an N\textsubscript{2}Py\textsubscript{2} ligand, substrate molecules containing a C\textsubscript{6}H\textsubscript{5}-S-CH\textsubscript{2}-X motif were screened by molecular docking. By constraining the distance between the Fe of the complex and the S of the substrate, in accord with the suggested Fe-O···S transition state, a family of potential substrates with a Ph-S-CH\textsubscript{2}-CONH-Ph motif was identified. Several members of this family were converted with high turnover numbers in the sulfoxidation reaction and no overoxidation was observed. The experimental data were in agreement with the predicted relationship between the substrate and protein scaffold. However, only up to 10% ee was obtained.

Using rational design based on X-ray data, a Sc\textsuperscript{3+} binding site was constructed on the rigid tubular protein [(gp5βf)\textsubscript{3}]	extsubscript{2} by Ueno and coworkers[^51]. The binding site

---

Artificial Metalloenzymes

on [(gp5βf)3] was created by a combination of a conjugated synthetic ligand and dative interactions with the amino acids of the scaffold (Figure 3b). Tetradentate coordination of a Sc3+ ion was achieved by positioning a bipyridine ligand at an appropriate distance from the OH groups of Tyr pairs, as estimated from the crystal structure. This artificial metalloenzyme proved capable of catalyzing the epoxide ring opening reaction of cis-stilbene oxide with aniline derivatives with almost a 3 fold rate enhancement compared to a metal complex alone. A small enantiomeric excess was observed using this design, i.e. up to 17% ee.

1.3.2 Peptides as Bioscaffolds

Peptides are small compared to proteins, but potentially still have enough functional diversity to generate a defined 2\textsuperscript{nd} coordination sphere. Much effort has been devoted to the design of metallopeptides as functional mimics for metalloenzymes. However, here the focus will only be on metallopeptides used for enantioselective catalysis.

Ball and coworkers have developed de novo designed peptide-based dirhodium complexes by employing as ligands the carboxylate groups of aspartic acid residues in a dimeric nonapeptide in a DxxxD motif.\[42] These were used before in asymmetric Si-H insertion reactions with excellent ee’s.\[43] However, obtaining the opposite enantiomer of the product using these metallopeptides, without turning to using D-amino acids, often needs rigorous editing of the active site and is difficult to predict. In this specific case predictions are especially problematic since these bis-peptides complexes were formed as mixtures of parallel and anti-parallel isomers. Therefore a screening method was developed in which it was assumed that sequences optimized for monomeric peptide complexes are also selective in the dimer-complex form, as was suggested in previous studies.\[53,54] Hence, libraries of monomeric peptides where synthesized on beads and screened in a high-throughput fashion. Hits were identified and the corresponding bis-peptides prepared and tested in catalysis, resulting in the discovery of metallopeptides that gave up to 97% ee of the si product in the catalyzed cyclopropanation reaction. From the same library another metallopeptide was identified that gave 90% ee of the re product.

Iridium-catalysed transfer hydrogenation reactions were performed using simple tripeptide Gly-Gly-Phe based iridium catalyst in aqueous media by rational design.\[55] These showed high turnover frequencies for the transfer hydrogenation of a variety of aldehydes, ketones and imines (up to 391 h\textsuperscript{-1}). Additionally, the biologically important regeneration of NADH was demonstrated using this catalyst. It was suggested that the tripeptides act as Noyori-type catalysts, in which iridium binds the N-terminal amine and the adjacent amide group.

A tetrapeptide containing a double methylated histidine was used to form an N-heterocyclic carbene that acted as a ligand for rhodium.\[56,57] The sulphur atom of a methionine in the same peptide acted as chelator for the rhodium complex. The
resulting catalytic peptide was capable of hydrosilylation of 4’-fluoroacetophenone in organic solvents with high chemoselectivities towards the silylether compared to the silylenolether (up to 83%), but no enantioselectivities were obtained.

The natural cyclic decapeptide gramicidin S served as chiral host for peptide-based bisphosphine ligands for rhodium and palladium catalysis in organic media. Modeling studies of the peptide were used to determine the positions for the bisphosphine ligands and the resulting catalysts were able to catalyse rhodium based transfer hydrogenation reactions up to 52% ee and asymmetric palladium catalysed allylic alkylations up to 15% ee.

1.3.3 DNA as Bioscaffold

Similar to proteins and peptides, DNA can offer a defined chiral 2nd coordination sphere for the construction of an artificial metalloenzyme. Roelfes and Feringa have introduced the concept of DNA-based asymmetrical catalysis\cite{26} and applied it successfully to several Cu(II)-catalyzed reactions\cite{40}.

Two approaches to controlling the enantiomeric outcome of the catalyzed reaction have been reported. By changing the denticity of the ligand coordinated to the copper(II) ion, i.e. bipyridine versus terpyridine ligands, the opposite enantiomers of Diels-Alder and Friedel-Crafts alkylation reaction products were obtained.\cite{59}. In a study by Smietana and Arseniyadis, ds-DNA made from L-nucleic acids instead of the natural occurring D-nucleic acids was used as a scaffold. The resulting DNA-based catalyst gave rise to the mirror image products in the Cu(II)-catalyzed Friedel-Crafts alkylation and Michael addition reactions compared to using natural DNA.\cite{60}

A new DNA-based catalyst was created by covalent anchoring of a Cu(II) complex to double stranded DNA through a tethered cisplatin moiety. The resulting hybrid catalyst was used successfully in Diels-Alder reactions and Friedel-Crafts alkylation reactions and could be recycled up to 10 times without loss of activity and enantioselectivity.\cite{61}

In addition to doubled stranded DNA G-quadruplex DNA has also been investigated as scaffold for DNA-based catalysis. Human telomeric G-quadruplex DNA, in combination with Cu\textsuperscript{2+} ions was found to catalyze the enantioselective Friedel-Crafts alkylation and Diels-Alder reactions with good enantioselectivities, i.e. up to 75% and up to 74% respectively.\cite{62,63} The opposite enantiomer of the products could be obtained by switching from the antiparallel to the parallel conformation of the G-quadruplex. The hybrid of a Cu(II) porphyrin and G-quadruplex DNA resulted in a catalyst capable of performing Diels-Alder reactions (up to 69% ee) and residues which had an effect on catalysis were identified.\cite{64} Finally, Cu(II) phenanthroline based ligands in combination with G-quadruplex DNA could be used in the intramolecular Friedel-Crafts alkylation reaction, with moderate ee’s (up to 26%).\cite{65}
1.4 Catalysis

Initially artificial metalloenzymes were constructed out of curiosity and their catalytic capabilities were often tested with benchmark reactions. These included simple oxidation reactions, Lewis acid catalyzed Diels-Alder reactions, etc. that proved very effective in proof-of-principle studies.[49] For an in-depth overview a number of review articles are available.[3,49] The field is now slowly transforming and more challenging catalytic reactions can be performed, including reaction types that have no equivalent in homogeneous or enzyme catalysis. The next section will describe these advances for artificial metalloenzymes used in enantioselective catalytic transformations of the last two years (scheme 2).

1.4.1 Asymmetric Transfer Hydrogenation

Salmain and coworkers reported on two new asymmetric transfer hydrogenases (ATHase) metalloenzymes. Ru(II) and Rh(III) d₆-piano stool complexes were bound to papain via covalently attached 2,2’-dipyridylamine ligands. [66] These artificial metalloenzymes were employed in the transfer hydrogenation of trifluoracetophenone (TFAP), using formate as hydrogen source, resulting in high conversions but low ee’s. Additionally, these papain constructs where used as artificial formate dehydrogenase for NAD(P)H regeneration.[9]

Similar Ru(II) and Rh(III) complexes where anchored in a non-covalent fashion to bovine β-lactoglobulin (β-LG) by using a 2,2’-dipyridylamine ligand equipped with a long aliphatic chain that can be bound by β-LG.[67] Moderate ee’s were obtained in the transfer hydrogenation of TFAP using formate. Based on X-ray structural information, the observed enantioselectivities were explained by interactions of the complex with a loop in β-LG, which restricts the number of conformations.[68]

The most successful examples to date of artificial ATHases have been reported using the streptavidin/biotin systems. Based on their experience with the ATH of ketones,[69] the Ward group has focused on the ATH of imines.[70] Screening revealed [Cp’Ir(Biot-p-L)Cl] streptavidin as the most promising catalyst. Both enantiomers of the reduction of a prochiral imine (1-methyl-3,4-dihydroisoquinoline) could be obtained by a single point mutation of S112 in streptavidin. (R)-Selectivities up to 96% ee were obtained with a small amino acid at position 112 in the active site (glycine or alanine). In contrast, cationic residues (lysine or arginine) at this position resulted in (S)-selectivities, up to 78% ee. Based on X-ray data, Lys121 was identified as playing a role in the protonation step and it was proposed that both the ketone and imine reduction proceeds through the same mechanism.

Next, the activity of the artificial ATHase was further improved.[71] The introduction of lipophilic residues (R84A-S112A-K121A) in the active site led to an 8-fold increase in catalytic efficiency compared to wild-type streptavidin as host and a 2-fold increase compared to the Ir-complex alone. However, only moderate ee’s
were obtained.

In an alternative approach, based on computational studies, a histidine was

\[ \text{Scheme 2: Reactions catalyzed by artificial metalloenzymes.} \]

---

**Scheme 1:** Reactions catalyzed by artificial metalloenzymes.
Artificial Metalloenzymes

introduced into the streptavidin scaffold either at positions 112 and 121, to activate and localize the metal-complex by formation of an additional dative bond with the metal.\[46\] The modelled structures were confirmed by X-ray crystallography. Both the enantiomers of the hydrogenation reaction could be obtained with up to 55% \( ee \) and 79% \( ee \) of the S- and R-enantiomer, respectively, depending on the position of the histidine residue. Moreover, the new artificial metalloenzyme displayed a 6-fold increase in turnover frequency compared to wild type streptavidin.

It was demonstrated that the ATHase, consisting of a biotinylated \([\text{CP}^*\text{Ir(Biot-p-L)}\text{Cl}]\) combined with streptavidin, was still active when encapsulated in biocompatible polymersomes.\[72\] This system remained active and proved to be stable under physiologically relevant conditions for several months, indicating its potential for future applications in cells.

Dative anchoring of an \( \text{IrCp}^* \) moiety in an genetically optimized human carbonic anhydrase II (hCAII) resulted in an artificial metalloenzyme capable of transfer hydrogenation of salsolidine with good activity and enantioselectivities up to 68% \( ee \).\[73\]

1.4.2 Olefin Metathesis

Cross metathesis (CM), could become an important tool for protein modification, due to its bio-orthogonal nature. However, large excesses of Grubbs-Hoveyda type metathesis catalysts are typically needed to perform these reactions in an aqueous environment. Several artificial metalloenzymes capable of cross-metathesis reaction were reported. Hilvert and coworkers attached the Grubbs-Hoveyda catalyst covalently to the heat shock protein from \( M. \text{jannaschii} \)[74], whereas Ward and coworkers used the non-covalent strategy of biotin-(strept)avidin\[75\] and Matsuo and coworkers introduced the catalyst covalently to \( \alpha \)-chymotrypsin through the intrinsic inhibition mechanism of \( \alpha \)-chymotrypsin.\[76\] While proof of concept was established, in all cases the catalytic activity was not improved compared the metal complex alone.

1.4.3 C-H Activation

Cyclopentadienyl rhodium complexes such as \([\text{Cp}^*\text{RhCl}_2]_2\) are versatile catalysts for electrophilic aromatic C-H activation reactions. For example, dihydroisoquinolones can be prepared by the benzannulation reaction in good yield, but no enantionselective version of this reaction existed.\[77\] The problem lies in the fact that there is a negligible barrier for rotation of the \( \text{Cp} \) ligand, and the use of a chiral \( \text{Cp} \) ligand would generate different conformations of almost the same energy. Ward and Rovis reported a biotinylated \([\text{Cp}^*\text{RhX}_2]_2\) bound in the chiral environment of streptavidin.\[78\] Based on inspection of an auto-Dock model of biotinylated \([\text{Cp}^*\text{Rh(OAc)}_2]_2\), a carboxylate residue was introduced at position 112 which seemed crucial for high activity by acting as a general base. The artificial metalloenzyme
Chapter 1

catalyzed the coupling of benzamides with alkenes, resulting in dihydroisoquinolones in up to 86% ee. An up to 92-fold acceleration compared to isolated rhodium complexes was observed. This is a catalytic enantioselective reaction for which no obvious alternative “conventional” approach exists.

1.4.4 Miscellaneous

DNA-based catalysis, i.e. the supramolecular anchoring of a Cu(II) complex in DNA as scaffold, was used for the enantioselective oxa-Michael addition of alcohols to enones.[79] Using achiral copper(II) complexes in combination with salmon testes DNA, enantioselectivities up to 81% and 86 % ee were achieved for the addition of methanol and propanol, respectively, to enones in aqueous media.

Using the same strategy, the intramolecular cyclopropanation of α-diazo-β-keto sulfones was also reported.[80] Up to 84% ee was achieved using a hybrid of salmon testes DNA and an achiral Cu(I)complex. The O-H bond insertion in H2O was observed as a major side reaction. This represents the first example of DNA-based asymmetric organometallic catalysis.

The introduction of an anionic manganese porphyrin into xylanase 10A from *Streptomyces lividans* (Xln10A) resulted in a catalyst for the enantioselective epoxidation of styrene derivatives by KHSO5 as oxidant.[81] Electron donating groups on the styrene, like the methoxy group at the para-position, resulted in lower chemoselectivities, i.e. 32% towards the exopoxide, but with the highest ee reported to date (80% R-selectivity). Differences in enantioselectivities were rationalized by docking experiments, suggesting interactions of the substrate with residues in the active site.

The cylindrically shaped hydrophobic cavity of the sterol carrier protein type 2 like domain (SCP-2L) was used by Kamer and coworkers to attach various nitrogen donor ligands covalently.[10] Using a phenanthroline conjugate, a moderate ee of 25% was obtained in the catalyzed Diels-Alder reaction.

1.4.5 Cascade Reactions

Combined chemo and biocatalytic cascade reactions are highly desirable. However combining chemical and bio-catalysts is often complicated by mutual inactivation. Nature solves this problem by compartmentalizing and thus spatially separating incompatible process. Inspired by nature, Hollmann, Turner and Ward compartmentalized an iridium d6-piano stool complex within streptavidin, to generate an artificial transfer hydrogenase (ATHase).[82] The ATHase was successfully included in several cascade reactions. For example, a double stereoselective deracemization of amines was achieved resulting in up to 99% ee. Compatibility with other oxidases was demonstrated, in a cascade reaction resulting in the formation of L-pipeolic acid with high enantioselectivity. In addition, the ATHase could act as a redox mediator to regenerate NADPH. In all cases, mutual inactivation of the metal catalysts and
Artificial Metalloenzymes

enzymes was observed when the free Ir-complex was used, demonstrating the power of encapsulation.

In the Bäckvall laboratory a cascade reaction was performed by immobilizing two catalysts, namely the lipase CALB and palladium nanoparticles, in siliceous mesocellular foams. This artificial metalloenzyme was used in the dynamic kinetic resolution of primary amines affording the product in quantitative yields and 99% ee. This hybrid system was shown to have an enhanced efficiency in the dynamic kinetic resolution of an amine compared to the simple combination of the two components.

1.5 Thesis Aims

The aim of this thesis was to develop a new design concept for artificial metalloenzymes by creation of a novel active site at the subunit interface of dimeric proteins. This concept can greatly expand the number of scaffolds applicable to the construction of artificial metalloenzymes.

In a first approach, we attempted the creation of an artificial metalloenzyme using a dimeric hormone peptide as scaffold. Despite achieving good enantioselectivities using this metallo-peptide as a catalyst, the introduction of the transition-metal catalyst into the scaffold caused a significant disruption of the structure and loss of the dimerization affinity, i.e. the catalyst predominantly existed in the monomeric form. Therefore, the dimeric protein Lactococcal multidrug resistance Regulator (LmrR) from Lactococcus lactis, with a high dimerization affinity, was selected as a new scaffold.

LmrR was used for the construction of artificial metalloenzymes, using the three anchoring strategies outlined in this chapter, i.e. covalent and supramolecular attachment of a Cu(II) complex and dative anchoring of Cu(II) ions by genetically encoded non-natural amino acids. The resulting hybrid catalysts were used in several enantioselective Lewis acid catalyzed reactions, i.e. the Diels-Alder reaction, the Friedel-Crafts alkylation reaction, and the conjugate addition of water. Furthermore, the effect of the 2nd coordination sphere on the catalyzed reactions were probed by mutagenesis studies and important residues for catalysis were identified.

1.5.1 Overview

Chapter 2 will discuss the construction of an artificial metalloenzyme based on LmrR using the covalent anchoring approach and its application in the enantioselective Diels-Alder reaction. Chapter 3 will discuss the application of this LmrR based hybrid catalyst in the conjugate addition of water. A subsequent mutagenesis study identified residues in the scaffold important for catalysis. In chapter 4 a more comprehensive mutagenesis study is described, in which the catalysis results of the enantioselective Diels-Alder reaction and the conjugate addition of water reaction are compared. Chapter 5 describes the construction of
an artificial metalloenzyme based on LmrR using the supramolecular anchoring approach of a Cu(II) complex. The resulting hybrid was used in the enantioselective Friedel-Crafts alkylation reaction. Chapter 6 describes the preliminary results of the construction of an artificial metalloenzyme based on LmrR in which aza-tryptophan residues where introduced, i.e. a non-natural amino acid incorporated via a biosynthetic route. Dative anchoring of Cu(II) ions led to the formation of a hybrid catalyst which was applied in the enantioselective Diels Alder reaction. In Chapter 7 conclusions will be given, as well as perspectives for the field of artificial metalloenzymes.

1.6 Reference

Artificial Metalloenzymes


[68] Cherrier MV, Engilberge S, Amara P, Chevalley A, Salmain M, Fontecilla-Camps JC: *Structural basis for enantioselectivity in the transfer hydrogenation of a ketone catalyzed by an artificial*


