Artificial metalloenzymes have emerged over the last decades as an attractive approach towards combining homogeneous 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. These include novel designs, novel catalytic reactions, some of which have no equivalent in both homogenous catalysis and biocatalysis and the incorporation of artificial metalloenzymes in chemoenzymatic cascades. Some of these developments represent promising steps towards integrating artificial metalloenzymes in biological systems. This review will focus on advances in this field and perspectives discussed.

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Introduction
The concept of artificial metalloenzymes, introduced by Wilson and Whitesides in their seminal paper in 1978 [1], aims to combine the attractive features of transition metal and bio-catalysis. It was long considered a curiosity but since its revival in the early 2000s, artificial metalloenzymes have become a very active and vibrant field. In the past decade the proof of concept has been firmly established and a variety of artificial metalloenzymes catalyzing enantioselective reactions have been reported. These developments have been described comprehensively in several recent reviews [2,3]. The field is now slowly transforming to take on new challenges such as novel catalytic reactions, cascade reactions and, ultimately, chemistry in vivo.

Here, first an overview of developments in the field of artificial metalloenzymes in the past 2 years, with a particular focus on enantioselective catalysis, will be given. Then the recent trends and future perspectives will be discussed.

New designs
The key parameter in artificial metalloenzyme design is the second coordination sphere provided by the biomolecular scaffold, that is proteins, peptides, DNA, etc., which provides the supramolecular interactions that are expected to contribute to achieving enzyme-like rate accelerations and selectivities. Hence, design parameters such as the choice of biomolecular scaffold and the position and mode of anchoring of the transition metal complex are of the utmost importance. Many of the examples of artificial metalloenzymes described in literature to date rely on a limited number of protein scaffolds, such as streptavidin [4], bovine serum albumin (BSA), and apo-myoglobin [5]. 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. Recently, a number of new scaffolds and design approaches have been introduced.

The Roelfes group introduced a novel artificial metalloenzyme design, which involves the creation of an active site on the dimer interface of the transcription factor Lactococcal multidrug resistance Regulator (LmrR) by rational design (Figure 1a) [6]. On the basis of crystal structures of LmrR, a copper(II) phenantroline complex was anchored in the hydrophobic pocket in the protein using a cysteine conjugation strategy. The resulting artificial metalloenzyme catalyzed the Diels-Alder reaction with up to 97% ee. Interestingly, a positive correlation was observed between the enantioselectivity and the conversion, showing that selectivity and activity are coupled. In a follow-up publication the first enantioselective artificial metallo-hydrolase based on this design was reported [7]. Chiral β-hydroxy ketones were obtained by hydration of enones, with ee’s up to 84%. A mutagenesis study showed that two residues inside the pocket, Asp100 and Phe93, were very important for catalysis, confirming that the reaction indeed occurs in this novel active site. This suggests that further mutagenesis may give rise to improved catalysis.

A new method for the construction of an artificial metalloenzyme by focussing on the binding mode of substrates was reported by Ménage and coworkers (Figure 1b) [8]. The periplasmic nickel-binding protein NiK₂ was used as a host for iron catalyzed sulfoxidation reactions. On the basis of the crystal structures of a hybrid of the NiK₂ protein with a bound iron complex of an N₅P₂ ligand, substrate molecules containing a C₆H₅–CH₂–X motif
New scaffolds and design approaches of artificial metalloenzymes. (a) A novel design of an artificial metalloenzyme, which involves the creation of an active site on the dimer interface of the transcription factor Lactococcal multi drug resistance Regulator (LmrR) \[6,7\]. Highly enantioselective Diels-Alder reactions and enantioselective hydrations of enones could be performed. (b) Design of an artificial metalloenzyme based on the binding mode of substrates. On the basis of X-ray data of the nickel-binding protein NiKa, sulfides were screened by molecular docking. High turnover numbers in the sulfoxidation reaction were observed \[8\]. (c) A scandium binding site was created on the tubular protein [(gp5]pfh\[2\] \[9\]. Tetradentate coordination of a Sc\(^{3+}\) ion was achieved by dative interactions with tyrosine groups and by a introduced bipyridine. The resulting artificial metalloenzyme was used in the epoxide ring opening reaction of cis-stilbene oxide with aniline.
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–CH2–CONH–Ph motif were identified. Several members of this family were converted with high turnover numbers in the sulfoxidation reaction and no overoxidation was observed. The experimental data was 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 Sc3+ binding site was constructed on the rigid tubular protein [{(gpSβ)3}] by Ueno et al. [9]. The binding site on [{(gpSβ)3}] was created by a combination of a conjugated synthetic ligand and dative interactions with the amino acids of the scaffold (Figure 1c). Tetradentate coordination of a Sc3+ ion was achieved by positioning a bipyridine ligand at appropriate distance, to 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 threefold rate enhancement compared to a metal complex alone. A small enantioselective excess was observed using this design, that is up to 17% ee.

Peptides

Peptides are small compared to proteins, but potentially still have enough functional diversity to generate a defined 2nd coordination sphere. Much effort has been devoted to the design of metallopeptides as functional mimics for metalloenzymes [10]. However, here we will focus only on metallopeptides used for enantioselective catalysis.

Ball et al. have developed a de novo designed peptide based on di-rhodium complexes by employing as ligands the carboxylate groups of aspartic acid residues in a dimeric nonapeptide in a DxxxD motif [11]. These were used before in asymmetric Si–H insertion reactions with excellent ee’s [12]. However, obtaining the opposite enantiomer of the product using these metallopeptides, without turning to using l amino acids, often needs rigorous editing of the active site and is difficult to predict. Especially 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 peptides complexes are also selective in the dimer-complex form, as was suggested in previous studies [13,14]. 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 catalyzed transfer hydrogenation reactions was performed using simple tripeptide Gly-Gly-Phe based iridium catalyst in aqueous media by rational design [15]. These showed high turnover frequencies for the transfer hydrogenation of a variety of aldehydes, ketones and imines (up to 391 hour−1). Additionally, the biologically important regeneration of NADH was demonstrated using this catalyst. It was suggested that the tripeptides act as a Noyori-type catalyst, in which iridium binds the N-terminal amine and the adjacent amide group.

A tetrapeptide containing a double methylated histidine was used to form a N-heterocyclic carbene that acted as a ligand for Rhodium [16,17]. 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 silyl ether compared to the silyleneolether (up to 83%), but no enantioselectivities were obtained.

The natural cyclic decapptide gramicidin S served as chiral host for peptide-based bisphosphine ligands for rhodium and palladium catalysis in organic media [18]. Modelling studies of the peptide were used to determine the positions for the bisphosphine ligands and the resulting catalysts were able to catalyze rhodium based transfer hydrogenation reactions up to 52% ee and asymmetric palladium catalyzed allylic alkylations up to 15% ee.

DNA

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 [19] and applied it successfully to several Cu(II) catalyzed reactions [20].

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, that is bipyridine versus terpyridine ligands, the opposite enantiomers of Diels-Alder and Friedel-Crafts alkylation reaction products were obtained [21]. In a study by Smietana and Arseniyadis, ds-DNA made from d-nucleic acids instead of the natural occurring l-nucleic acids was used as 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 [22].

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 [23].

In addition to doubled stranded DNA also G-quadruplex DNA has been investigated as scaffold for DNA-based catalysis. Human telomeric G-quadruplex DNA, in combination with Cu²⁺ ions was found to catalyze the enantioselective Friedel-Crafts alkylation and Diels-Alder reactions with good enantioselectivities, that is up to 75% and up to 74% respectively [24,25]. 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 the catalysis were identified [26]. Finally, Cu(II) phenantroline 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%) [27].

Reactions
The next section will describe catalytic reactions reported using artificial metalloenzymes that were created using the previously established designs, including reaction types that have no equivalent in homogenous or enzyme catalysis (Scheme 1).

Asymmetric transfer hydrogenation (ATH)
Salmain and co-workers reported on two new ATHase metalloenzymes. Ru(II) and Rh(III) ‘-piano stool complexes were bound to Papain via covalently attached 2,2’-dipyridylamine ligands [28]. 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 [29].

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 [30]. Moderate ee’s were obtained in the transfer hydrogenation of TFAP using formate. On the basis of 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 [31].

The most successful examples to date of artificial ATHases have been reported using the streptavidin/biotin systems. On the basis of their experience with the ATH of imines [33]. A 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-dihydrisoquinoline) could be obtained by a single point mutation on S112 of 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. On the basis of X-ray data, Lys121 was identified as playing a role in the protonation step and it was that both the ketone and imine reduction proceeds through the same mechanism.

Next, the activity of the artificial ATHase was further improved [34]. The introduction of lipophilic residues (R84A-S112A-K121A) in the active site led to an 8-fold increase of 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 introduced in the streptavidin scaffold at positions 112 and 121, respectively, to activate and localize the metal-complex by the formation of an additional dative bond with the metal [35]. 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-enantiomer and R-enantiomer, respectively, depending on the position of the histidine residue. Moreover, the new artificial metalloenzyme displayed a sixfold increase in turnover frequencies compared to wild type streptavidin.

It was demonstrated that the ATHase, consisting of a biotinylated [Cp*Ir(Biot-p-L)Cl] combined with streptavidin, was still active when encapsulated in biocompatible polymersomes [36]. This system remained active and proved to be stable under physiologically relevant conditions for several months, suggesting the potential for applications in cells.

Dative anchoring of an IrCp* moiety in a genetically optimized Human carbonic anhydrase II (hCAII) resulted in an artificial metalloenzyme capable of transfer hydrogenation of salolidine with good activity and enantioslectivities up to 68% ee [37].

Olefins 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 et al. attached the Grubbs-Hoveyda
Reactions catalyzed by artificial metalloenzymes.
catalyst covalently to the heat shock protein from *M. jannaschii* [38], whereas Ward used the non-covalent strategy of biotin–(strept)avidin [39] and Matsuo introduced the catalyst covalently to α-chymotrypsin through the intrinsic inhibition mechanism of α-chymotrypsin [40]. While proof of concept was established, in all cases the catalytic activity was not improved compared to the metal complex alone.

**C–H activation**
Cyclopentadienylrhodium complexes such as [Cp*RhCl₂]₂ are versatile catalysts for electrophilic aromatic C–H activation reactions. For example, dihydroisoquinolones can be prepared by the benzannulation reaction in good yields, but no enantioselective version of this reaction exists [41]. The problem lies in the fact that there is a negligible barrier for rotation of the Cp ligand, and the use of chiral Cp ligand would generate different conformations of almost the same energy. Ward and Rovis reported a biotinylated [Cp*RhCl₂]₂ bound in the chiral environment of streptavidin [42*]. On the basis of inspection of an auto-Dock model of biotinylated [Cp*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 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.

**Miscellaneous**
DNA-based catalysis, that is, the supramolecular anchoring of a Cu(II) complex in DNA as scaffold, was used to for the enantioselective oxo-Michael addition of alcohols to enones [43]. 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, also the intramolecular cyclopropanation of α-diazo-β-keto sulfones was reported [44]. Up to 84% ee was achieved using a hybrid of salmon testes DNA and an achiral Cu(II) 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 KHSO₃ as oxidant [45]. Electron donation groups on the styrene, like the 4-methoxy substituent, resulted in lower chemoselectivities, that is 32% towards the epoxide, 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 cylindrical shaped hydrophobic cavity of the sterol carrier protein type 2 like domain (SCP-2L) was used by Kamer et al. to attach various nitrogen donor ligands covalently [46]. Using phenanthroline conjugate, a moderate ee of 25% was obtained in the catalyzed Diels-Alder reaction.

**Cascade reactions**
Combined chemo and biocatalytic cascade reactions are highly desirable. However combining chemical and biocatalysts is often complicated by mutual inactivation. Nature solves this by compartmentalizing and thus spatially separating incompatible process. Inspired by nature, Hollmann, Turner and Ward compartmentalized an iridium p-piano stool complex within streptavidin, to generate an artificial transfer hydrogenase (ATHase) (Figure 2a) [47]. 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 enantioselectivities. In addition, the ATHase could act as a redox mediator to regenerate NADPH. In all cases, mutual inactivation of the metal catalysts and 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 (Figure 2b) [48*]. 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 showed to have an enhanced efficiency in the dynamic kinetic resolution of an amine compared to the simple combination of the two components.

**Perspectives**
On the basis of the recent developments in the field, several trends can be discerned.

The design of artificial metalloenzymes is moving away from the trial and error approach. The design of an increasing number of artificial metalloenzymes is now, at least in part, based on X-ray structural information supplemented with computational studies. For example, additional dative interactions to a bound catalytic metal complex are now increasingly taken into account in the design process. This should give rise to a more rational
approach to the design and optimization of the second coordination sphere.

While most artificial metalloenzymes are still employed in model reactions, there now are several examples of real synthetic challenges, for which there are no alternatives using conventional approaches, that have been addressed by taking advantage of the 2nd coordination sphere interactions [7*,42*]. This is an important development for the field to become a viable alternative to chemical or bio-catalysis.

Additionally, emphasis has been placed on the development of artificial ATHases. In addition to their importance for synthetic chemistry, also application for, for example, the regeneration of NADPH, are being explored. Due to its biocompatibility, this reaction is one of the prime candidates to realize the integration of artificial metalloenzymes in biological systems. While this is a very challenging prospect, the feasibility of performing abiotic reactions in vivo is suggested by the impressive recent demonstration by Arnold and Brustad that a promiscuous P450 enzyme
can be used for catalytic asymmetric carbene reactions in living cells [49].

Finally, artificial metalloenzymes can be used to create combined chemical/enzymatic cascade processes. In this case, the artificial metalloenzyme is not primarily used for generating enantioselectivity, but to compartmentalize chemical catalysts and avoid mutual inactivation of transition metal species and enzymes.

In conclusion, the field of artificial metalloenzymes is rapidly becoming a mature discipline, with many exciting new developments that suggest the potential of this concept for applications in asymmetric catalytic synthesis and beyond.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


A library of new rhodium metallopeptides where developed and screened on beads, generating catalysts producing both enantiomers in the asymmetric cyclopropanation reactions.


Highly enantioselective asymmetric C–H activation reaction using streptavidin coupled with a rhodium complex was performed. No obvious alternative conventional approach exists for this type of reaction.


It was shown that an artificial metalloenzyme could work in concert with natural enzymes in chemo-biocatalytic cascade processes. In contrast, mutual inactivation existed between the transition metal complex alone and the natural enzymes. Using artificial metalloenzymes to compartmentalize transition metal catalysts represents a new and powerful application of this concept.


This article demonstrated that the natural lipase CalB can work in concert with palladium nanoparticles when they are co-immobilized in siliceous mesoscellular foams.