Artificial control of protein activity

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Chapter 7

Conclusion and perspectives

This chapter provides a brief overview of the research presented in this thesis and discusses possible future developments in the topics described.

7.1 Introduction

The correct functioning of the cellular machinery requires tight spatiotemporal control of protein and enzyme activity. Cells have developed a number of fine-tuned regulation mechanisms to achieve this strict control over protein function.[1,2] Over the years extensive efforts have been devoted to study natural regulation mechanisms and to engineer artificial regulation in natural proteins with diverse purposes, from gaining a better understanding of natural metabolic pathways and signaling networks, to design artificial proteins or enzymes for a number of applications. Advances in protein engineering have allowed the development of artificially regulated proteins and enzymes that proved to be useful in synthetic biology, drug design and delivery as well as biosensing.[3–5] More specifically, engineering of allosteric regulation – that is the control of protein activity at a site distinct from the active site – allowed the design of proteins or enzymes whose activity is dependent on external stimuli and/or changes in the environment.[3,5]

A distinct aspect of controlling protein function is the introduction of non-natural reactivity into biological scaffolds. The field of biocatalysis has evolved from the study of natural enzymes and methods to enhance their activities and broaden their substrate scope, to creating entirely new enzymes with non-natural reactivities. A number of designer enzymes, obtained by *de novo* enzyme design or by redesign of existing proteins, for new-to-nature chemical reactivity have been presented in literature.[6–8] The introduction of non-natural chemistry into proteins has exploited the reactivity of natural and unnatural amino acids and the installation of natural or artificial cofactors. Embedding transition metal complexes into protein scaffolds proved to be a viable strategy to broaden the repertoire of chemical reactions catalyzed by enzymes. Specifically, artificial metalloenzymes aim to combine the broad substrate and catalytic scope of transition metal catalysis with the high activity and selectivity typical of natural enzymes.[9,10]

The research presented in this thesis focuses on both of these concepts for controlling protein activity. It spans from engineering artificial regulation mechanisms for natural and artificial enzymes, to introducing catalytic potential in proteins that do not show any native activity. This chapter provides a short overview of the research presented in this thesis and discusses possible developments in the research fields addressed.

7.2 Research overview

The research presented here describes our work in the field of artificial regulation of protein activity. The first part of the thesis (Chapters 2-4) focused on engineering regulation mechanisms for natural (Chapters 2 and 3) and artificial (Chapter 4) enzymes, while in the second part the design and characterization of new artificial metalloenzymes is described (Chapters 5 and 6).

Chapters 2 and Chapter 3 presented efforts to introduce artificial regulation into the natural enzyme murine dihydrofolate reductase (mDHFR). mDHFR is a widely studied split protein that has been used to study protein-protein interactions[11,12] or to detect DNA sequences.[13]

In **Chapter 2** a modular approach for the reassembly of the split enzyme mediated by small molecules was described. In this split enzyme mDHFR fragments were first conjugated to short oligonucleotides and sequences complementary to these oligonucleotides were functionalized with receptor moieties, specifically cyclodextrins. Hybridization between the receptor-functionalized oligonucleotides and those covalently appended to the mDHFR fragments resulted in each mDHFR fragments linked with a receptor moiety. Ultimately, simultaneous binding of a guest molecule to both receptors was envisioned to facilitate reassembly of the split enzyme. The preparation and characterization of the different modules of the split enzyme were described: (1) cyclodextrin-DNA conjugates were successfully obtained via CuAAC from 6-azido-β-cyclodextrin and alkyne modified oligonucleotides and (2) mDHFR fragments-DNA conjugates were also synthesized by applying CuAAC from alkyne-modified mDHFR fragments and azide-functionalized oligonucleotides. This conjugation method led to notable improvements in the preparation of the protein-DNA conjugates when compared to a previously reported strategy.[13] All the conjugates were obtained in high purity and conversions were reproducible. Nevertheless, this procedure would benefit from further optimization. For example, introducing the alkyne (or azide) functionality into the protein fragments *in vivo* via expanded genetic code methodology would avoid the need for a post-translational modification step and could significantly improve and simplify the experimental protocol toward the protein-DNA conjugates. Lastly, preliminary experiments on the reassembly of the split enzyme in presence of a target molecule were described, but further studies are necessary in order to achieve small molecule-mediated reassembly of this split enzyme. Determination of the binding affinity of the guest molecule for the cyclodextrins in the envisioned design is required.

Chapter 3 presented an alternative approach to enable the reassembly of the split mDHFR enzyme. In this design coordination of a metal ion to two ligands, each one linked to an mDHFR fragment, was proposed to facilitate the reassembly of the split enzyme. This design would provide an example of metal-directed protein self-assembly that could have potential applications in metal ion sensing. The preparation of mDHFR fragments containing a metal binding moiety was described using two different strategies: (1) genetic incorporation of a metal binding unnatural amino acid and (2) introduction of ligands via post-translational protein modification. Both strategies have advantages and disadvantages and different applications might be envisioned. Incorporation of metal binding moieties by *in vivo* incorporation of the unnatural metal binding amino acid (2,2-bipyridin-5yl)alanine (BpyA) into each fragment of mDHFR did not allow precise control over metal coordination. Protein fragments were, indeed, isolated from bacterial cultures with Fe^{2+} ions bound to the BpyA and, to date, it was not possible to remove these metal ions and study metal ion coordination *in vitro*. The possibility for protein containing metal binding unnatural amino acids to bind metal ions during bacterial growth, specifically Fe^{2+}, has already been reported[14,15] and discussed in this thesis and it is a general complication related with the genetic incorporation of unnatural amino acids. Usually, binding of metal ions to the unnatural amino acid happens when the metal binding moiety is located in solvent exposed positions. Unfortunately, when working with protein fragments a disordered structure of the protein is usually unavoidable, therefore binding of metal ions *in vivo* was not unexpected. Optimization of the expression protocol in media containing minimal amounts of metal ions or the use of a different metal binding amino acid with a lower affinity for Fe^{2+} can be considered to pursue this approach towards a metal-mediated reassembly of the split enzyme. The introduction of the metal binding moiety via alkylation of cysteine variants of mDHFR fragments was achieved with mDHFR fragments fused to maltose binding proteins (MBP). Fusion to MBP was necessary in order to achieve bacterial expression of the protein fragments and allowed to perform the purification steps in native conditions thanks to the increased solubility of the fragments. MBP_mDHFR fragments bearing a bipyridine, phenanthroline and terpyridine moiety were prepared and characterized and the binding of a number of divalent transition metal ions (Fe^{2+}, Zn^{2+}, Cu^{2+}) was studied by UV-visible titrations. The reassembly of these conjugates was tested in presence of the aforementioned metal ions, but unfortunately no functional enzyme could yet be obtained. Redesign of the fragments as fusion proteins with other solubility tags was envisioned as a possible solution to achieve the metal-mediated reassembly of the split enzyme.

In **Chapter 4** the possibility of introducing regulation mechanisms to control the activity of a designer enzyme with new-to-nature activity was investigated. Specifically, the design, synthesis and characterization of an LmrR-based metal ion regulated artificial metalloenzyme were presented. A previously described supramolecularly assembled LmrR-based artificial metalloenzyme was used for this study.[16] In this design, recruiting a catalytically active Cu^{2+} phenanthroline complex to the hydrophobic binding pocket of LmrR resulted in a functional enzyme that displayed good activity and selectivity in the vinylogous Friedel–Crafts alkylation of indoles with α-β unsaturated imidazoles. To achieve metal ion-mediated control over the activity of this hybrid enzyme, a regulatory site was introduced within the LmrR scaffold by conjugation of bipyridine ligands in specific positions. These ligands allowed the formation of chelate metal complexes upon coordination of divalent transition metal ions (Fe^{2+} and Zn^{2+}). This combination allowed us to obtain an artificial metalloenzyme whose activity was regulated by metal ion coordination. Notably, activation of the metalloenzyme was possible upon coordination of Fe^{2+}, but only marginally in presence of Zn^{2+} ions. This metal-mediated regulation can be considered reminiscent of natural allosteric mechanisms to control protein activity. The synthesis and characterization of LmrR_bipyridine conjugates and their complexes with Fe^{2+} and Zn^{2+} were described, as well as the performance of the regulated hybrid enzymes in enantioselective catalysis.

Chapter 5 introduces Multidrug Resistance Regulators (MDRs)[17] as a novel class of protein scaffolds for the design of artificial metalloenzymes. Three proteins belonging to the TetR family of MDRs – QacR, CgmR and RamR – were investigated. Two different anchoring strategies for the introduction of the active transition metal complex were explored: the supramolecular approach and the genetic incorporation of an unnatural metal binding amino acid. The reactivity of the resulting artificial metalloenzymes was evaluated for two different Lewis acid catalyzed reactions: the vinylogous Friedel–Crafts alkylation of indoles and the tandem Friedel–Crafts conjugate addition/enantioselective protonation. Compared to the supramolecular approach with $[Cu(phen)(NO_3)_2]$, *in vivo* incorporation of the unnatural amino acid (2,2-bipyridin-5yl)alanine (BpyA) proved to be a superior strategy to create active metalloenzymes from the protein scaffolds explored. The results presented in this chapter suggested that the hydrophobic pockets of this family of MDRs are too small to simultaneously accommodate both the Cu^{2+} phenanthroline complex and the substrates for the reactions. Further studies are needed to confirm this hypothesis, a crystal structure, for example, would provide valuable information regarding the positioning of the Cu^{2+} complex inside these hydrophobic pockets. Finally, several Cu^{2+}-based metalloenzymes were prepared

and characterized by genetic incorporation of BpyA and the resulting hybrid catalysts showed distinct reactivities in the two reactions studied. Among these new metalloenzymes one QacR variant, QacR Y123BpyA, displayed excellent reactivity in the Cu^{2+}-catalyzed vinylogous Friedel–Crafts alkylation. This designer enzyme afforded the best levels of enantioselectivity for any metalloenzyme created by *in vivo* incorporation of an unnatural amino acid and showed preference for the opposite enantiomer of the reaction product compared to other QacR- and LmrR-based metalloenzymes.[15,16]

The properties and reactivity of this QacR variant were further investigated in **Chapter 6**. QacR Y123BpyA was purified from bacterial cultures with metal ions bound to its scaffold and presented an intrinsic catalytic activity in the vinylogous Friedel–Crafts alkylation of indoles without the need for supplementing any additional metal ion. Spectroscopic and catalytic studies were combined to elucidate the nature of the bound metal ion(s) responsible for this inherent catalytic activity. The data collected thus far suggested that Zn^{2+} binds to the BpyA in position 123 giving rise to the intrinsic catalytic activity. Preliminary studies for the potential application of this artificial metalloenzyme for *in vivo* catalysis were also described. From these initial results it was concluded that performing catalysis with this metalloenzyme in absence of any additional metal ions in *E. coli* cells was not yet possible as an insufficient amount of active enzyme is present in the cells. Optimization of the protocol might lead to improved conversions. Conversely, addition of Cu^{2+} to all BpyA-containing QacR variants allowed the detection of appreciable levels of product formation. Although, the conditions for this type of *in vivo* catalysis have not been optimized yet, these results represent a significant advance toward the use of artificial metalloenzymes bearing metal binding unnatural amino acids inside cells.

7.3 Research perspective

7.3.1 Split protein reporters

Split protein reporters not only have numerous applications in the study, sensing and visualization of protein-protein interactions and signaling networks, but are also useful for discovering and evaluating protein inhibitors, detecting unmodified protein targets and controlling the amount of a functional protein in the cell. Moreover, split proteins have been applied for the detection of DNA and RNA sequences.[18,19] In this thesis we focused on the design of split enzymes for small molecules and metal ions recognition. The creation of small molecules-dependent proteins whose function depends on the concentration of a target molecule is particularly interesting, as it allows the design of dose-dependent artificial

switches. Although a number of successful split protein reporters whose reassembly is mediated by small molecules have been reported, most of them are based on chemical inducers of dimerization.[18] Moreover, the ligand-binding domains of these reporter proteins are typically limited to domains derived from natural proteins. As a consequence, the choice of the effector molecule is limited to molecules that interact with specific binding domains. The design of artificial split protein reporters that are responsive to a number of chemically unrelated small molecules would significantly expand the versatility of small molecule-dependent proteins. The combination of synthetic receptors with biomolecules is promising toward this goal as it allows recognition of different small molecules. Moreover, the use of synthetic host-guest architectures allows the formation of well-defined, reversible and tunable assemblies.[20] One example of this approach applied to a split protein is the work of Sakamoto *et al.*, in which incorporation of a synthetic host-guest couple rendered a fragmented GFP responsive to an exogenous guest molecule (Chapter 2, Figure 2b).[21] In the context of engineering responsiveness for different analytes this work is promising as the receptor moiety conjugated to the N-terminal GFP fragment can be readily modified. However, generalization of this approach is not straightforward. The N-terminal GFP fragment was obtained by peptide synthesis and required a double modification of the fragment (one for the receptor moiety and another one for the binding agonist). This protocol is synthetically challenging and not applicable to other split proteins whose fragments cannot be achieved by synthetic procedures. Moreover, the formation of the intramolecular auto-inhibited complex worked for this GFP design, but might not be successful when expanded to other split proteins. Finally, the use of a split protein presenting catalytic activity would assure signal amplification upon substrate binding, which is advantageous when applying a split allosteric system as a sensor for small molecules.

The work described in Chapter 2 aimed to expand the concept of small molecule-dependent proteins and create a general and modular approach for the supramolecular reassembly of split enzymes. Ideally, once a proof-of-concept is established, this modular system could be easily adapted and optimized for the detection of structurally unrelated small molecules: simply by exchanging receptor moieties conjugated to the oligonucleotides a library of artificial allosteric systems responsive to different analytes could be obtained. This design might offer a general approach for the detection of a variety of small molecules for which a receptor moiety is known. Furthermore, it is conceivable that this strategy could be expanded to different split enzymes, offering different detection methods. Nevertheless, considering the design of the split enzyme, binding of the guest molecule to both receptors is required, therefore this approach is limited to ditopic

substrates that can bind simultaneously to two receptors. Based on the work described in this thesis, the design of a modular assembly of split enzymes for small molecule recognition is promising, but needs further optimization.

Finally, the use of aptamers could provide a different modular approach toward small molecule-dependent split enzymes. Aptamers are short single stranded oligonucleotidic sequences characterized by a specific three-dimensional structure that allows them to bind targets molecules (small molecules or large marcomolecules). Usually, upon binding to the target, aptamers undergo a structural change. By introducing an aptamer sequence in-between the oligonucleotides that are complementary to the protein-bound sequences, the split mDHFR fragments could be made responsive to an aptamer-binding molecule. Ideally, addition of the target small molecule would induce a conformational change in the aptamer sequence that facilitates the reassembly of the enzyme. Initially, this strategy could be applied to existing aptamers, but once established, new aptamers could be designed for the recognition of a small molecule of interest via the SELEX methodology.[22] This strategy does not require simultaneous binding of a target molecule to two receptor moieties and therefore can offer an additional approach to the detection of small molecule compared to the design described above.

Metal coordination has been widely applied to direct peptide and protein self-assembly creating novel structures for a number of applications. The metal ion-dependent split enzyme described here would represent an alternative approach toward Metal Directed Protein Self Assembly (MDPSA) and could find applications as a sensor for metal ions. Possibly, by tuning the metal binding moieties incorporated into the fragments, selectivity for different metal ions could also be achieved. Two strategies toward a metal-mediated split enzyme for distinct purposes were explored in this thesis. The introduction of ligands by genetic incorporation of unnatural metal binding amino acid would pave the way for *in vivo* applications of the split enzyme sensor taking advantage of the robust selection systems available for mDHFR. Nevertheless, as shown in Chapter 3, the use of this design finds complications related with the binding of metal ions by each protein fragment during protein production. Careful selection of the metal binding moiety and the use growth media containing minimal amount of metal ions are required for this type of application. On the other hand, the introduction of metal binding moieties via post-translational modification would impart higher versatility to the system and significantly expand the set of metal binding moieties that could be introduced into the protein fragments. In this modular design, it could be possible to induce selectivity for different metal ions by changing the appended ligands. However, this strategy would limit the applicability of the split enzyme to

in vitro assays. Thus far, a functional metal-mediated split enzyme could not be obtained, therefore future work should focus on redesigning the fragments to obtain a functional enzyme *in vitro*, which subsequently can be studied for further applications.

7.3.2 Artificial metalloenzymes

The field of artificial metalloenzymes has expanded greatly in the last few decades and has, in part, lived up to its promise to combine the broad reaction scope of homogeneous catalysis with the high selectivity, biorthogonality and mild conditions of enzymatic catalysis. Several examples of artificial metalloenzymes able to selectively perform new-to-nature reactions have been developed,[23,24] yet a number of challenges concerning their broad application and optimization remain. Some of them have been addressed in this thesis.

Recently, research efforts focused on combining the activity of metalloenzymes with natural enzymes toward the realization of abiological enzyme cascades have been reported.[25–28] Despite the number of *in vitro* studies that have been presented, these systems are far from incorporating the designer enzymes into artificial metabolic pathways. Two major challenges toward this goal need to be addressed: (1) the development of regulation mechanisms for the activity of artificial metalloenzymes and (2) the ability of these hybrid catalysts to function *in vivo*. As previously described in this thesis, in the cell the activity of proteins and enzymes is tightly controlled by several regulations mechanisms.[1,2] The introduction of artificial metalloenzymes in metabolic pathways cannot be foreseen unless mechanisms to modulate their activity have been developed. To date, examples of such regulatory mechanisms for artificial metalloenzymes are scarce. The only two examples reported so far come from the Ward group in which the activity of artificial asymmetric transfer hydrogenases were upregulated by proteolysis[29] or cross-regulated by combination with an enzyme that, upon an external stimulus, produces an inhibitor for the metalloenzyme itself.[30] A proof-of-concept study to engineer a regulated artificial metalloenzyme is presented in Chapter 4. Specifically, we achieved regulation of activity of an LmrR-based artificial metalloenzyme by binding of Fe^{2+} ions. Although this system is not suitable for application in a cellular environment yet, it offers a novel approach toward modulating the activity of artificial metalloenzymes. Future work toward a cell-compatible metal ion regulated artificial metalloenzyme should involve the introduction of the regulatory site via genetic incorporation of unnatural amino acids. Complication related with the formation of the chelate complexes, due to a shorter linker between the metal binding moiety and the protein backbone, or the binding of metal ions *in vivo*, need to be addressed.

The design of artificial metalloenzymes predominantly focuses on modifying existing proteins to introduce an abiotic cofactor and confer the chemical reactivity of interest. However, the number of scaffolds that have been applied to asymmetric catalysis is limited and no general protocol exists to select a specific bioscaffold for a particular reaction.[31] Several considerations can be made when choosing a protein scaffold, but most studies focused on very specific examples. In Chapter 5 we explored a new class of proteins – multidrug resistance regulators (MDRs) from the TetR family – as potential bioscaffolds for the design of artificial metalloenzymes. Based on our experience with the transcriptional regulator LmrR, we envisioned that other MDRs could be viable scaffolds for this application thanks to their general features. MDRs are indeed a large class of transcriptional regulators that possess structural diversity, but most of them contains a large hydrophobic pore that guarantees binding of structurally unrelated ligands. Considering these features MDRs could offer a platform of bioscaffolds that can be selected and applied to different reactions following similar protocols.

As mentioned above, the application of artificial metalloenzymes *in vivo* is important for synthetic biology applications such as developing cascade reactions and ultimately for their incorporation into artificial metabolic pathways. Moreover, the possibility to develop artificial metalloenzymes that function inside cells or cell lysates would be desirable to improve their catalytic activities by directed evolution protocols. To date, optimization of artificial metalloenzymes has largely been based on chemical intuition: starting from available structural information, residues are selected and a limited number of mutations is introduced. While, the implementation of computation tools for modeling and redesign of artificial metalloenzymes could further streamline this process, the use of high-throughput screening systems is desirable to identify improved enzyme variants in large scale. However, applying directed evolution protocols to artificial metalloenzymes remains challenging since purification of the host protein is typically required for the installation of the abiotic cofactor. Therefore, performing catalytic reactions with artificial metalloenzymes that can be assembled inside cell, or in cell-free extracts, would be highly advantageous for carrying out high-throughput screens. As mentioned in Chapter 6, for the application of artificial metalloenzymes *in vivo* several challenges need to be addressed, including the development of a cell-compatible anchoring strategy and the biocompatibility of the transition metal complex. Up to date only two examples of artificial metalloenzymes that function inside cells have been developed and both are limited to function in the periplams of *E. coli*.[32,33] Although still at its beginning, the work described in Chapter 6 paves the way for another example of artificial metalloenzymes functioning in a whole-cell setup.

Despite the genetic incorporation of unnatural amino acids for the design of artificial metalloenzymes proved to be successful and promising for possible *in vivo* studies, several limitations still need to be overcome. First, up to date only a limited number of metal binding amino acid is available for expanded genetic methodology: (2,2-bipyridin-5yl)alanine, (8-hydroxyquinolin-3-yl)alanine, 2-amino-3-[4-hydroxy -3-(1H-pyrazol-1-yl)phenyl] propanoic acid and 2-amino-3-(8-hydroxyquinolin-5-yl)propanoic acid.[34] Expanding the toolkit of unnatural amino acid available would increase metal selectivity and would open possibilities for new reactivities. Moreover, the synthesis of some unnatural metal binding amino acids is laborious and requires multistep synthesis that might limit their broad application and can be considered a drawback for high-throughput screening. Recently the group of Wang has presented efforts to reduce the number of synthetic and purification steps required for the synthesis of unnatural amino acids, also making use of enzymatic catalysis.[35,36] Finally, the low expression yields of proteins bearing unnatural amino acids might prevent their use *in vivo* catalysis (Chapter 6). Nevertheless, their ease of assembly is promising and research efforts should be focused on overcoming these limitations.

7.4 References

1 S. Nadaraia, G. J. Yohrling, G. C.-T. Jiang, J. M. Flanagan and K. E. Vrana, in *eLS*, John Wiley & Sons, Ltd, 2001.
2 G. A. Petsko and D. Ringe, *Protein Structure and Function*, New Science Press, 2004.
3 J. Fastrez, *ChemBioChem*, 2009, **10**, 2824–2835.
4 M. V. Golynskiy, M. S. Koay, J. L. Vinkenborg and M. Merkx, *ChemBioChem*, 2011, **12**, 353–361.
5 O. V. Makhlynets, E. A. Raymond and I. V. Korendovych, *Biochemistry*, 2015, **54**, 1444–1456.
6 U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore and K. Robins, *Nature*, 2012, **485**, 185–194.
7 H. Renata, Z. J. Wang and F. H. Arnold, *Angew. Chem. Int. Ed.*, 2015, **54**, 3351–3367.
8 M. T. Reetz, in *Directed Evolution of Selective Enzymes*, Wiley-VCH Verlag GmbH & Co. KGaA, 2016, pp. 237–266.
9 F. Rosati and G. Roelfes, *ChemCatChem*, 2011, **3**, 973–977.
10 J. Bos and G. Roelfes, *Curr. Opin. Chem. Biol.*, 2014, **19**, 135–143.
11 J. N. Pelletier, K. M. Arndt, A. Plückthun and S. W. Michnick, *Nat. Biotechnol.*, 1999, **17**, 683.
12 I. Remy and S. W. Michnick, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 5394–5399.
13 N. Sancho Oltra, J. Bos and G. Roelfes, *ChemBioChem*, 2010, **11**, 2255–2258.

14 J. H. Mills, S. D. Khare, J. M. Bolduc, F. Forouhar, V. K. Mulligan, S. Lew, J. Seetharaman, L. Tong, B. L. Stoddard and D. Baker, *J. Am. Chem. Soc.*, 2013, **135**, 13393–13399.
15 I. Drienovská, A. Rioz-Martínez, A. Draksharapu and G. Roelfes, *Chem. Sci.*, 2014, **6**, 770–776.
16 J. Bos, W. R. Browne, A. J. M. Driessen and G. Roelfes, *J. Am. Chem. Soc.*, 2015, **137**, 9796–9799.
17 H. Wade, *Curr. Opin. Struct. Biol.*, 2010, **20**, 489–496.
18 S. S. Shekhawat and I. Ghosh, *Curr. Opin. Chem. Biol.*, 2011, **15**, 789–797.
19 M. C. Wehr and M. J. Rossner, *Drug Discov. Today*, 2016, **21**, 415–429.
20 D. A. Uhlenheuer, K. Petkau and L. Brunsveld, *Chem. Soc. Rev.*, 2010, **39**, 2817–2826.
21 S. Sakamoto and K. Kudo, *J. Am. Chem. Soc.*, 2008, **130**, 9574–9582.
22 R. Stoltenburg, C. Reinemann and B. Strehlitz, *Biomol. Eng.*, 2007, **24**, 381–403.
23 J. Steinreiber and T. R. Ward, *Coord. Chem. Rev.*, 2008, **252**, 751–766.
24 O. Pàmies, M. Diéguez and J.-E. Bäckvall, *Adv. Synth. Catal.*, 2015, **357**, 1567–1586.
25 V. Köhler, Y. M. Wilson, M. Dürrenberger, D. Ghislieri, E. Churakova, T. Quinto, L. Knörr, D. Häussinger, F. Hollmann, N. J. Turner and T. R. Ward, *Nat. Chem.*, 2013, **5**, 93–99.
26 K. Engström, E. V. Johnston, O. Verho, K. P. J. Gustafson, M. Shakeri, C.-W. Tai and J.-E. Bäckvall, *Angew. Chem. Int. Ed.*, 2013, **52**, 14006–14010.
27 Y. Okamoto, V. Köhler, C. E. Paul, F. Hollmann and T. R. Ward, *ACS Catal.*, 2016, **6**, 3553–3557.
28 Y. Okamoto, V. Köhler and T. R. Ward, *J. Am. Chem. Soc.*, 2016, **138**, 5781–5784.
29 Z. Liu, V. Lebrun, T. Kitanosono, H. Mallin, V. Köhler, D. Häussinger, D. Hilvert, S. Kobayashi and T. R. Ward, *Angew. Chem. Int. Ed.*, 2016, **55**, 11587–11590.
30 Y. Okamoto and T. R. Ward, *Angew. Chem. Int. Ed.*, 2017, **56**, 10156–10160.
31 C. Trindler and T. R. Ward, in *Effects of Nanoconfinement on Catalysis*, ed. R. Poli, Springer International Publishing, 2017, pp. 49–82.
32 W. J. Song and F. A. Tezcan, *Science*, 2014, **346**, 1525–1528.
33 M. Jeschek, R. Reuter, T. Heinisch, C. Trindler, J. Klehr, S. Panke and T. R. Ward, *Nature*, 2016, **537**, 661–665.
34 C. Hu, S. I. Chan, E. B. Sawyer, Y. Yu and J. Wang, *Chem. Soc. Rev.*, 2014, **43**, 6498–6510.
35 X. Liu, J. Li, J. Dong, C. Hu, W. Gong and J. Wang, *Angew. Chem. Int. Ed.*, 2012, **51**, 10261–10265.
36 X. Liu, J. Li, C. Hu, Q. Zhou, W. Zhang, M. Hu, J. Zhou and J. Wang, *Angew. Chem. Int. Ed.*, 2013, **52**, 4805–4809.