In vivo catalyzed new-to-nature reactions

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Abstract

Bioorthogonal chemistry largely relies on the use of abiotic metals to catalyze new-to-nature reactions in living systems. Over the past decade, metal complexes and metal-encapsulated systems such as nanoparticles have been developed to unravel the reactivity of transition metals, including ruthenium, palladium, iridium, copper, iron, and gold in biological systems. Thanks to these remarkable achievements, abiotic catalysts are able to fluorescently label cells, uncage or form cytotoxic drugs and activate enzymes *in cellulo/vivo.* Recently, strategies for the delivery of such catalysts to specific cell types, cell compartments or proteins were established. These studies reveal the enormous potential of this emerging field and its application in both medicinal chemistry and in synthetic biology.

Introduction

Over the past decade, abiotic transition metal-catalyzed reactions have successfully been introduced into living cells and organisms. With *in vivo* applications in mind, several challenges were identified and addressed: i) overcome the inherent cytotoxicity of the abiotic catalyst, ii) ensure the efficient metal uptake by the cell and iii) circumvent the deactivation of the catalyst by thiols, proteins and other cell components [1]. To address these issues, several strategies were developed: i) metals were incorporated into nanoparticles or embedded in microspheres and resins (see sections: "Palladium nanoparticles" and "Copper and gold nanoparticles"; the catalyzed reactions are summarized in Table 1) or ii) used as homogeneous metal complexes (see sections: "Homogenous ruthenium catalysts", "Homogenous palladium catalysts" and "Homogenous iron, iridium and gold catalysts"; the catalyzed reactions and complexes are collected in Table 2). Most reactions developed to date are based on either a cleavage, or a cross-coupling reaction. As a result, a variety of *in vivo* functions were established that are schematically presented in Figure 1 including: i) fluorescent labeling of cells, cell compartments and proteins, ii) synthesis of cytotoxic agents and iii) enzyme rescue. Several excellent reviews cover bioorthogonal reactions [2-7] or bioorthogonal protein labeling and protein chemistry [8-12]. This review focuses on bioorthogonal transition metal-catalyzed reactions *in cellulo* (inside single cells in a cell culture) and *in vivo* (inside living organisms), highlighting recent developments towards therapeutic applications.
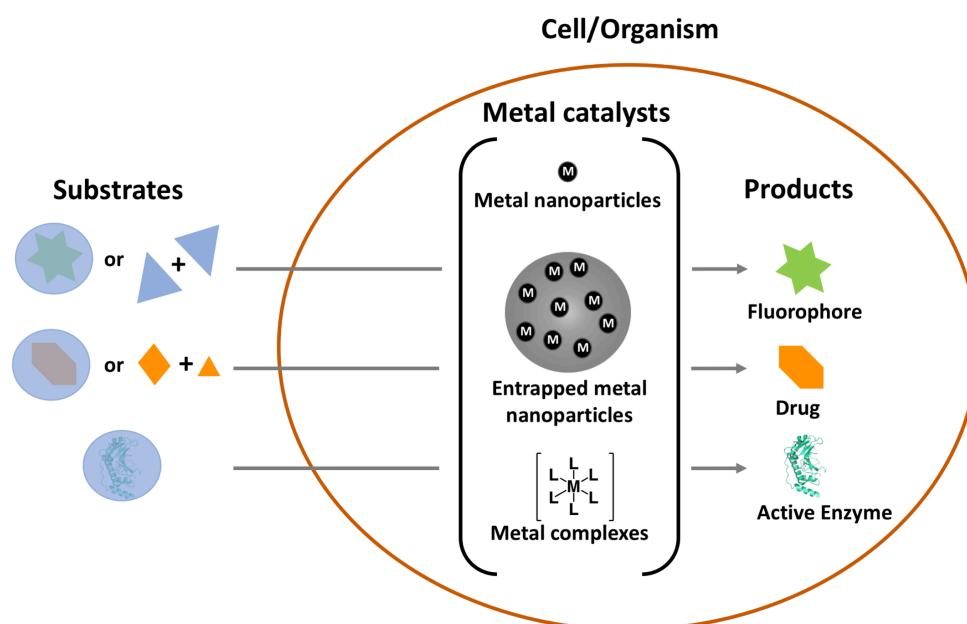
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Figure 1. Strategies for the introduction of metal-catalyzed bioorthogonal reactions *in cellulo/vivo*. Biocompatible transition metal catalysts including metal-nanoparticles (NPs), NPs entrapped in microspheres or resins, and metal complexes are taken-up by cells or introduced into organisms. These metal-platforms convert *in cellulo/vivo* various substrates through coupling or uncaging reactions into fluorophores, drugs and active enzymes. Recent progress allows to direct metal complexes to specific cell-types or cell compartments such as organelles.

Palladium nanoparticles.

The use of metal nanoparticles (NPs) for *in cellulo* catalysis was first demonstrated by the Bradley group [13]. They exploited the biocompatibility of polystyrene microspheres [14] by entrapping Pd-NPs, creating fluorescently-labelled Pd^0-microspheres [13,15]. These Pd^0-catalysts cleave allyloxycarbonyl (alloc)-groups, leading to the uncaging of fluorescent rhodamine 110 (R110) within the cytoplasm of HeLa cells (Table 1, entry 1) [13]. Moreover, these Pd^0-loaded microspheres also catalyze the Suzuki-Miyaura cross-coupling inside HeLa cells, leading to the accumulation of a rhodamine-fluorophore within mitochondria (Table 1, entry 9) [13].

These findings suggest that such Pd-NPs may find applications, both, in labeling studies as well as in medical applications whereby one can specifically target an organelle. With this goal in mind, Broceta and Bradley *et al.* implemented a Pd-NP catalyzed drug release. Pd^0-NPs were linked to polyethylene glycol (PEG)-polystyrene resins forming micrometer-sized beads (~150 μm) with a remarkable biocompatibility [16]. A single resin bead was implanted into the yolk sac of zebrafish embryos. The embryos developed normally without any signs of toxicity. To prove the *in vivo* activity of the Pd^0-resin, the uncaging of a propargyloxycarbonyl (poc)-protected R110 was assayed within the yolk sac (Table 1, entry 3). This Pd^0 resin was also able to extracellularly uncage 5-fluoro-1-propargyl (pro)-uracil (pro-5FU). This lead to the release of the cytotoxic drug 5-flurouracil (5FU) in a cancer cell culture of either HCT116 or BxPC-3 cells (Table 1, entry 6) [16].

This therapeutic strategy was refined by combining a dual drug synthesis strategy with tumor specificity [17]. To achieve cell specificity, fluorescent Pd-microspheres were decorated with the cancer targeting motif cyclic-RGD (cRGD). cRGD is an antagonist of the $\alpha_v\beta_3$ receptor, an angiogenesis factor overexpressed in many tumors and tumor vasculature.

These tumor-targeting microspheres were used for the activation of two prodrugs inside U87-MG glioblastoma cells. Besides uncaging pro-5FU, the cytotoxic agent PP-121 was produced via a Suzuki-Miyaura cross coupling reaction leading to increased cell death, compared to the synthesis of either 5FU or PP-121 alone (Table 1, entry 6 and 10) [17]. This approach illustrates the potential of metal-NPs for targeted drug delivery by converting innocuous substrates into active drugs inside tumor cells.

Subsequently, Miller *et al.* explored a Pd-NP based drug synthesis strategy in mice [18]. For this purpose, the palladium complex bis[tri(2-furyl)phosphine]palladium(II)-dichloride ($PdCl_2(TFP)_2$) was encapsulated into poly(lactic-co-glycolic acid)-polyethyleneglycol (PLGA-PEG)-NPs, a material which is in clinical trials for drug administration [19]. The authors thoroughly studied the effect of the nano-encapsulation, observing an improved solubility, bioavailability, biocompatibility and a low cytotoxicity (IC_{50} of 70-130 µM). Furthermore, Pd-NPs were enriched in tumor tissue of cancer mouse models compared to other organs such as brain, skin, lung, bone marrow and heart. Interestingly, the highest catalytic activity of Pd-NPs for the release of either fluorescent R110 or the chemotherapeutic drug doxorubicin was observed in tumor tissue (Table 1, entry 1 and 2). Therefore, combining nano-encapsulated Pd-NPs and caged doxorubicin helped to minimize systemic exposure and toxicity, thus increasing the maximum-tolerated dose by 300 %, resulting in the halt of tumor growth and extending the survival of tumor-bearing mice [18].

Copper and gold nanoparticles.

The range of metal-NPs was recently broadened to include Cu [20,21] and Au [22,23], thus expanding the *in vivo* catalytic repertoire. Although Cu^I-catalyzed azide-alkyne cycloaddition (CuAAC) has been known since 2002 [24,25], it was hardly used *in vivo* due to its marked cytotoxicity [26]. The incorporation of Cu into aspartate-containing polyolefins [20] or entrapping Cu in TentaGel resin [21], contributed to overcoming these biocompatibility issues. On the one hand, Cu-based NPs convert coumarin-derivatives via CuAAC into fluorophores inside NCI-H460 and MDA-MB-231 cancer cells (Table 1, entry 12) [20]. On the other hand, Cu-particles produce *in cellulo* cytotoxic agents, decreasing the cell viability of *Escherichia coli* (Table 1, entry 14) [20]. Furthermore, the implantation of the Cu-resin into the yolk sac of zebrafish embryos did not exhibit any toxicity but catalyzed the release of a coumarin-fluorophore (Table 1, entry 13) [21].

Besides Cu and Pd, gold was immobilized on a solid support, a polyethylene glycol (PEG)-grafted low-cross-linked polystyrene matrix [22]. This Au-resin was employed for the local release of R110 in the brain of zebrafish (Table 1, entry 3) as well as for the activation of structurally diverse prodrugs inside A549 cancer cells (Table 1, entry 5, 7 and 8). This demonstrates the potential application of Au-resin for *in vivo* drug-release [22]. Furthermore, Tonga *et al.* created controllable, Au-NPs by blocking the access to the Au-encapsulated, catalytic Ru- or Pd-complexes [23]. This 'gate-keeping' was achieved through steric hindrance by supramolecular binding of cucurbit[7]uril onto the surface. The NPs were activated through the addition of a competitor, 1-adamantylamine to unmask alloc-R110 (by Ru- and Pd-loaded NPs) and the prodrug pro-5FU (by Pd-loaded NPs) inside live HeLa cells (Table 1, entry 1 and 6) [23]. These gated NPs reduce the interference with cell components, protect the metal from poisoning and increase the biocompatibility. Moreover, the introduction of a switch into these NPs allows a more controlled drug treatment and suggests their use for restoring malfunctioning pathways in chronic disease.

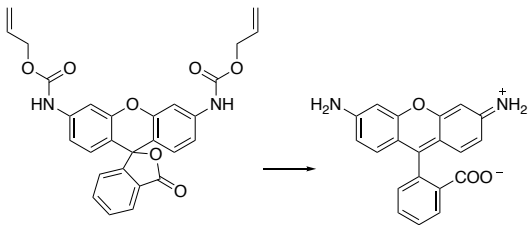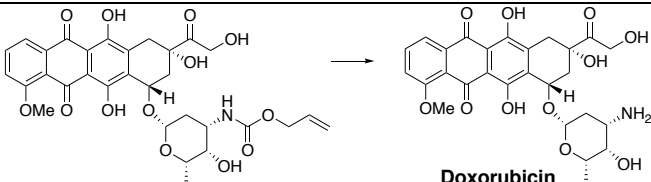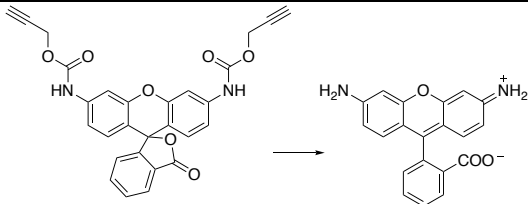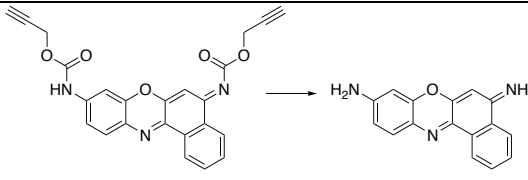**Table 1: Reactions catalyzed by metal loaded nanoparticles**
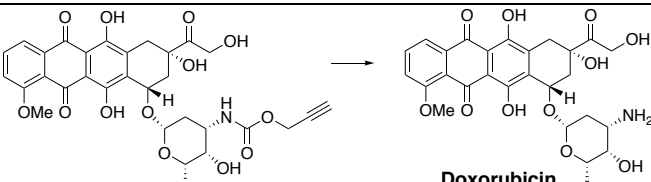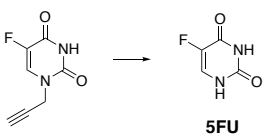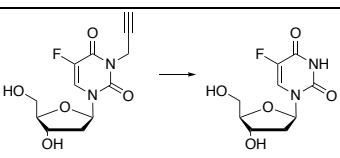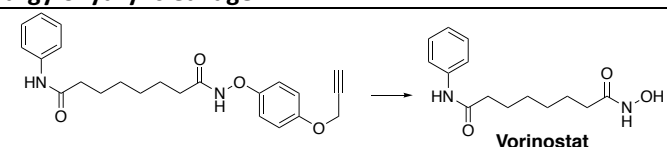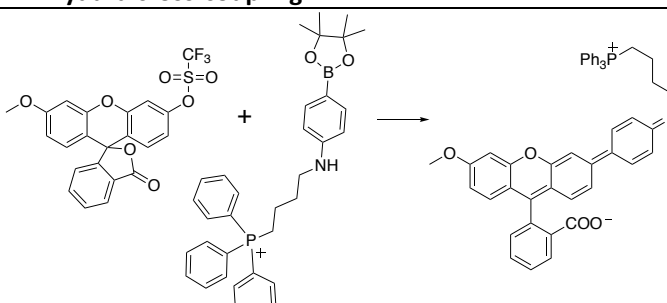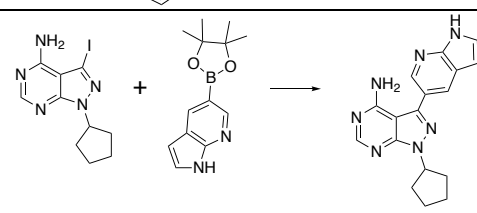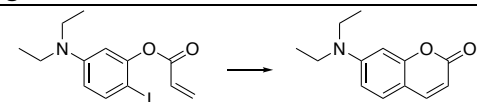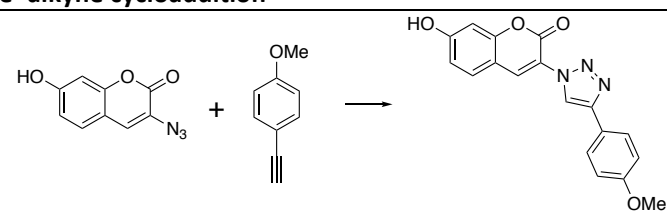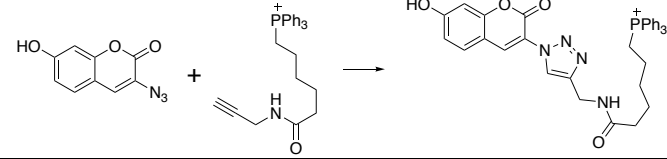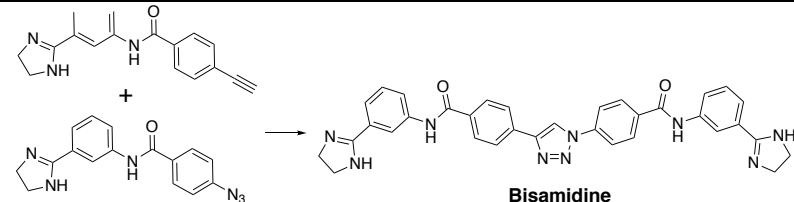
Entry	Reaction	Catalyst	Organism	Ref.
	Allyloxycarbonyl cleavage			
1	Rhodamine 110	Entrapped Pd-NPs	HeLa cells	[13,15]
		Free Pd-NPs	HT1080, ID8, A2780CP cells; mouse model;	[18]
		Ru/Pd-complex entrapped in Au-NPs	HeLa cells	[23]
2	Doxorubicin	Free Pd-NPs	Mouse model	[18]
	Propargyloxycarbonyl cleavage			
3	Rhodamine 110	Entrapped Pd-NPs	Zebrafish	[16]
		Entrapped Au-NPs	Zebrafish	[22]
4	Cresyl violet	Entrapped Pd-NPs	U87-MG cells	[17]
5	Doxorubicin	Entrapped Au-NPs	A549 cells	[22]
	Propargyl cleavage			
6	5FU	Entrapped Pd-NPs	HCT116, BxPC-3 cells; U87-MG cells; HeLa cells	[16] [17]
		Pd-complex entrapped in Au-NPs		[23]
7	FUdR	Entrapped Au-NPs	A549 cells	[22]

Table 1 (continued)

Entry	Reaction	Catalyst	Organsim	Ref.
	Propargyloxyaryl cleavage			
8	Vorinostat	Entrapped Au-NPs	A549 cells	[22]
	Suzuki-Miyaura cross-coupling			
9		Entrapped Pd-NPs	HeLa cells	[13,15]
10	PP-127	Entrapped Pd-NPs	U87-MG cells	[17]
	Heck Coupling			
11		Free Pd-NPs	HT80 cells	[18]
	Azide–alkyne cycloaddition			
12		Free Cu-NPs	NCI-H460, MDA-MB-231 cells;	[20]
13		Entrapped Cu-NPs	Zebrafish	[21]
14	Bisamidine	Free Cu-NPs	*E. coli*	[20]

Homogenous ruthenium catalysts

Streu's and Meggers' seminal work on *in cellulo* catalyzed Ru-reactions laid the foundation of bioorthogonal organometallic chemistry [27]. In 2006, they suggested that the complex [Cp*Ru(cod)Cl] **Ru1** (Cp*=pentamethylcyclopentadienyl, cod=1,5-cyclooctadiene, Table 2, entry 1) catalyzes the uncaging of alloc-protected R110 inside HeLa cells, while not affecting the cell viability [27]. In 2014 and 2017, they reported on significantly more active Ru-pianostool complexes **Ru2**-**Ru6** (Table 2, entry 1 and 4) catalyzing alloc-unmasking [28,29]. Moreover, alloc-protecting groups were cleaved off from DNA-binding agents such as 4',6-diamidino-2-phenylindole (DAPI) and ethidium bromide inside chicken embryo fibroblasts (CEF) and the kidney epithelial cell line Vero (Table 2, entry 2 and 3) [30].

Subsequently, the quinolone based Ru-complex was improved by reducing the π-backbonding of the bidentate ligand (**Ru2**-**Ru4**) and improving the *in vivo* R110 release by 130-fold, comparing **Ru4** to **Ru1** [28]. Furthermore, **Ru4** unmasked efficiently alloc-doxorubicin, triggering the apoptosis of HeLa cells [28]. Very recently, the activity of the Ru-complex was further enhanced tenfold by replacing the quinoline of **Ru4** with an 8-hydoxiquinolate motif (**Ru5** and **Ru6**) [29]. **Ru6** decages almost quantitatively alloc-doxorubicin decreasing the IC_{50} concentration of alloc-doxorubicin from 15 µM to 2 µM in the presence of 1 µM **Ru4** or **Ru6**, respectively [29]. ICP-MS studies reported by Wender *et al.* however suggest that the Ru-complex **Ru3** does not penetrate into the cytoplasm of 4T1 cells, indicating that the reaction occurs extracellularly [31]. To overcome this shortcoming, Mascareñas *et al.* appended a cation triphenylphosphonium (TPP) moiety to **Ru2** to afford **Ru7** (Table 2, entry 1) [32]. Such lipophilic cations are taken up by mitochondria [33,34]. After treading HeLa cells with **Ru7** and caged-R110, fluorescence was measured within mitochondria. This suggests that **Ru7** is present in the mitochondria, thanks to the TPP targeting moiety [32]. To improve the traceability of **Ru7**, a pyrene derivative was added to afford **Ru8** (Table 2, entry 1), enabling fluorescent monitoring of the complex itself. As anticipated, both the catalyst **Ru8** and R110 were localized inside the mitochondria. The authors specifically depolarized mitochondria by deprotecting the proton ionophore 2,4-dinitrophenol (Table 2, entry 5) [35,36], demonstrating the potential of organelle-specific complexes for disturbing and modifying biological systems [32].

A different approach to target cell compartments and proteins was pursued by the Winssinger lab [37-39]. A Ru-complex (**Ru10**, Table 2, entry 6) was linked to a peptide nucleic acid (PNA)-probe which hybridizes with tissue specific miRNAs [39]. The **Ru10**-PNA and a fluorogenic rhodamine-PNA were injected into a one-cell zebrafish embryo. Using PNA-recognition sequences on both the catalyst and the azide-bearing substrate ensures the photocatalytic reduction of the azide upon docking of the substrate and catalyst to the target miRNA. This yields fluorescence in the specific tissues of live zebrafish. The same Ru-complex conjugated to an epidermal growth factor receptor (EGFR)-inhibitor, **Ru11**, and an estrogen agonist (raloxifene), **Ru12**, (Table 2, entry 7) targeted the receptor proteins and provided a spatial and temporal unmasking of the fluorescent dye in live HEK293T and MCF-7 cells [38].

By targeting a biotinylated Ru-cofactor **Ru13** to streptavidin (Sav) in *E. coli*, The Ward group could evolve an artificial metalloenzyme *in cellulo* (Table 2, entry 8) [40]. Fusing the signal peptide of the outer membrane protein A (OmpA) to Sav lead to the efficient secretion of the overexpressed Sav into the periplasm of *E. coli.* Addition of **Ru13** to these cells allowed the assembly of an artificial metalloenzyme **Ru13**·Sav within the periplasm of *E. coli*. The resulting strain catalyzed the ring closing metathesis (RCM) of an umbelliferone precursor in

the periplasm of *E. coli*. The catalytic performance of **Ru13**·Sav was improved by directed evolution relying on the fluorescence of the formed umbelliferone. The evolved strain was shown to catalyze the RCM of typical diolefin substrates, leading to 660 TONs, outperforming the standard homogeneous RCM catalysts [40].

Homogeneous palladium catalysts

To the best of our knowledge, the first homogeneous Pd-catalyzed reaction *in cellulo* was reported by Lin *et al.* [41]. A copper-free Sonogashira cross-coupling reaction was used to selectively label homopropargylglycine *E. coli* cells. Fluorescein iodide was coupled to the metabolically incorporated homopropargyglycine, by a newly discovered Pd-complex **Pd1** (Table 2, entry 9) [41].

Subsequently, unnatural amino acids (UAAs) were genetically encoded by amber stop codon suppression [42,43] and *p*-iodophenylalanine was incorporated on the external loops of the bacterial pore protein OmpC [44]. The palladium complex **Pd1** coupled a fluorescent boronic acid to the *p*-iodophenylalanine of the OmpC-mutants via Suzuki-Miyaura cross-coupling on the surface of live *E. colis* (Table 2, entry 12) [44]. Furthermore, proteins carrying an encoded alkyne were cross-coupled via a Sonogashira reaction with a fluorophore bearing a iodophenyl group (Iph-FL-525) using [Pd(NO$_3$)$_2$] inside *E. coli* and *Shigella* (Table 2, entry 10) [45].

The Pd-catalyzed fluorescent labeling of UAAs was also expanded to mammalian cells [46]. N$^\epsilon$-butynyloxycarbonyllysine encoded in the EGFR-protein was cross-coupled to biotin-phenyl iodide using catalyst **Pd1** (Table 2, entry 11). The biotinylated EGFR was monitored by fluorescence through addition of Sav conjugated to Alexa Fluor® 568 [46].

Besides labeling specific proteins *in vivo,* Chen and coworkers were the first to employ Pd-complexes for a gain-of-function study, using a deprotection-strategy [47]. The phosphothreonine lyase OspF was blocked by masking the catalytic Lys134 with a poc-group. The catalyst [Pd(η3-allyl)Cl]$_2$ **Pd3** (Table 2, entry 14) cleaved the poc-OspF and allowed the *in vivo* monitoring its substrate, the Erk kinase, in HeLa cells [47]. This gain-of-function approach was extended by incorporating the UAA allenyl-tyrosine that can be uncaged thanks to homogeneous Pd-catalysts **Pd3**-**Pd6** (Table 2, entry 15) [48]. The following enzymes were rescued by allenyl-removal through **Pd3**-**Pd6** in HEK293T cells: *Taq* DNA polymerase, Src kinase and the anthrax lethal factor endopeptidase [48].

Recently, cell-penetrating peptides were linked to a Pd-complex **Pd7** (Table 2, entry 13), improving its internalization (Table 2, entry 13) [49]. The uncaging of poc-R110 in prostate adenocarcinoma (PC-3) cells was demonstrated [49]. The peptide-based platform facilitates the delivery of metal-complexes to specific cell-types, thus opening the possibility of targeted drug delivery [50,51].

Homogenous iron, iridium and gold catalysts

Besides Ru- and Pd-complexes, other metals have been employed for intracellular labeling: An iron(III) meso-tetraarylporphin **Fe1** (Table 2, entry 16) was shown to catalyze azide reduction leading to the release of R110 in HeLa cells [52]. The Ir-complex **Ir1** (Table 2, entry 17) reduces an aldehyde leading to the release of a fluorescent Bodipy-OH inside NIH-3T3 cells [53]. A more sophisticated Au-catalyzed approach was developed by Tanaka *et al.* [54]. They relied on an Au-complex linked to coumarin, **Au1** (Table 2, entry 18 and 19) which has marked affinity for albumin. These albumins were coated with glycans through the "RIKEN click" reaction, influencing the trafficking pathways and resulting in an accumulation either

on the liver or the intestine by using (2-6)-disialoglycoalbumin or galactosylglycoalbumin, respectively. These organ-targeted Au-complexes catalyzed an amide bond formation between a propargyl-ester of fluorescent probes (TAMRA-O-pro, Cy7.5-O-pro) and surface-exposed amines of nearby proteins leading to an organ-specific labeling inside living mice [54].

Conclusions

Since 2014, the field of metal-catalyzed bioorthogonal reactions has made significant progress. Initially, it was demonstrated that metal-NPs and metal complexes can uncage or synthesize drugs *in cellulo/vivo.* Subsequently, the specificity of these metal-based platforms was considerably improved. Now, it is possible to target specific organs, cells, cell compartments and certain proteins. Thanks to these efforts, one can envision the possibility of targeting and killing cells as well as gaining abiotic cellular functions.

The exploitation of novel targeting and packaging strategies should further contribute to decreasing the toxicity of the metal catalysts and increasing their specificity. This extends the safety window of metal catalysts *in vivo*, hopefully resulting in the development of new therapies. Beyond clinical applications, one can envisage to design bioorthogonal metabolic pathways *in cellulo/vivo* for the production of high-value chemicals.

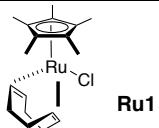**Table 2: Reactions catalyzed by metal- complexes in a biological environment**
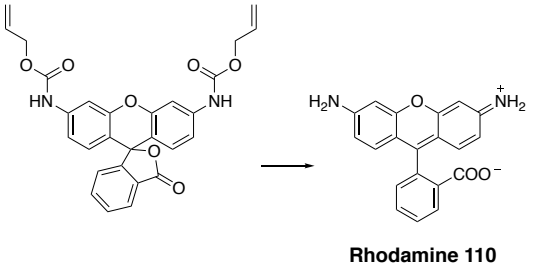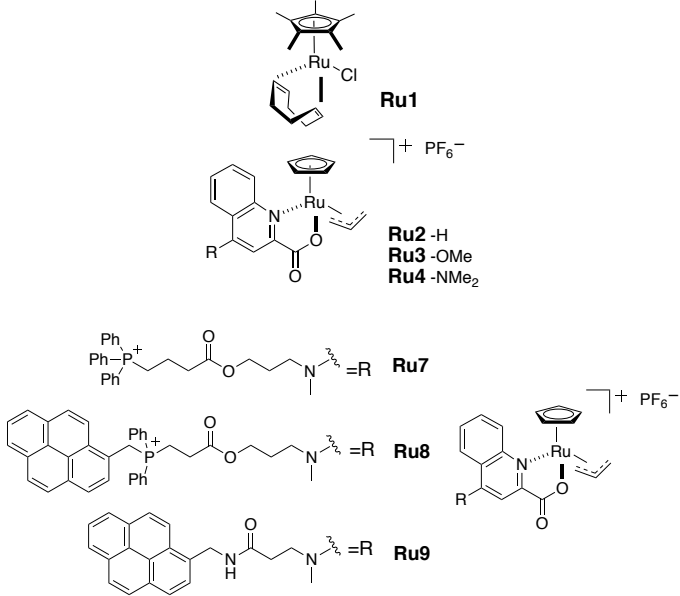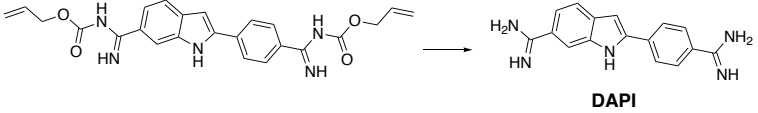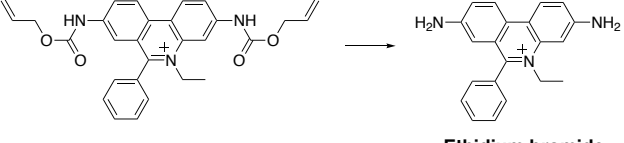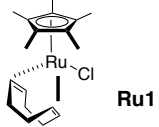
Entry	Reaction	Catalyst	Organism	Ref.
	Allyloxycarbonyl cleavage	**Ruthenium**		
1		**Ru1**	HeLa cells	[27]
		Ru2 -H **Ru3** -OMe **Ru4** -NMe$_2$	HeLa cells	[28]
	Rhodamine 110	**Ru7** **Ru8** **Ru9**	HeLa cells and mitochondria of HeLa cells	[32]
2	DAPI	**Ru1**	CEF, Vero cells	[30]
3	Ethidium bromide	**Ru1**	CEF cells	[30]

Table 2 (continued)
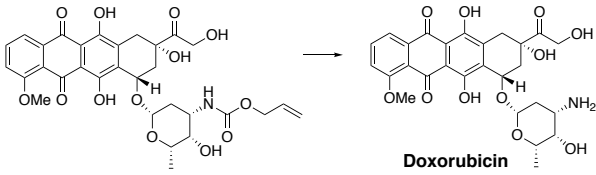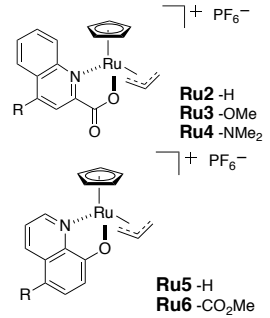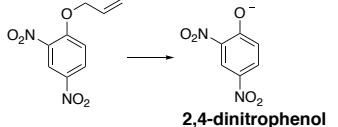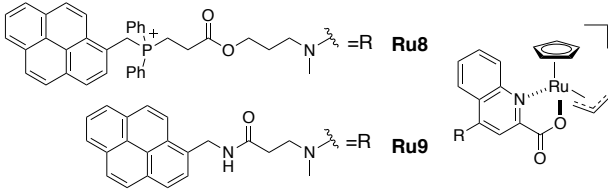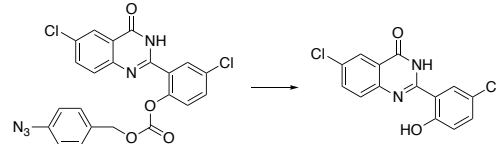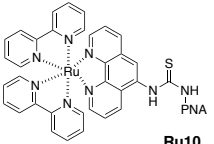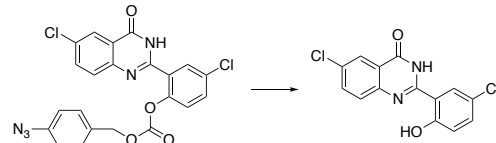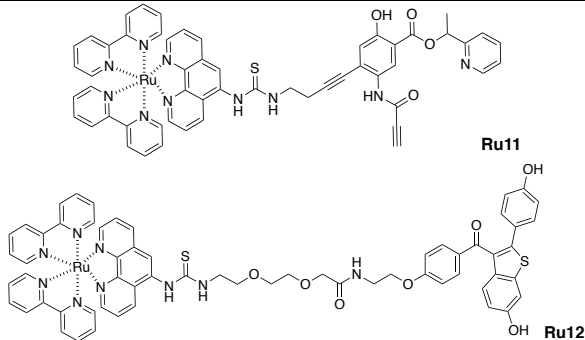
Entry	Reaction	Catalyst	Organism	Ref.
	Allyloxycarbonyl cleavage	**Ruthenium**		
4	**Doxorubicin**	**Ru2** -H **Ru3** -OMe **Ru4** -NMe₂	HeLa cells	[28]
		Ru5 -H **Ru6** -CO₂Me	HeLa cells	[29]
	Allyl cleavage	**Ruthenium**		
5	**2,4-dinitrophenol**	**Ru8** **Ru9**	HeLa cells and mitochondria of HeLa cells	[32]
	Photocatalyzed azide reduction	**Ruthenium**		
7		**Ru10**	Zebrafish	[39]
6		**Ru11** **Ru12**	HEK293T, MCF-7 cells	[38]

Table 2 (continued)
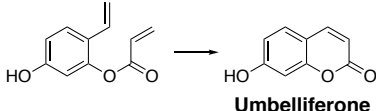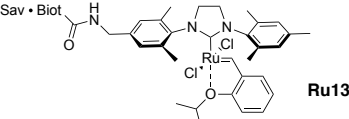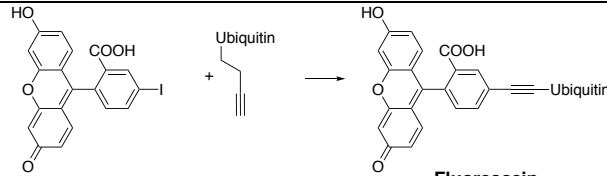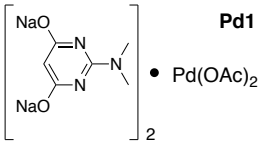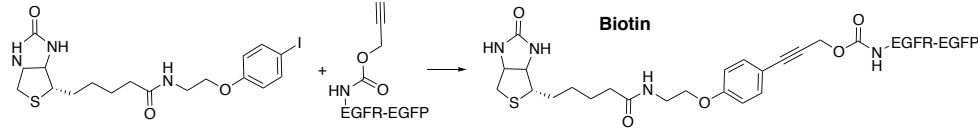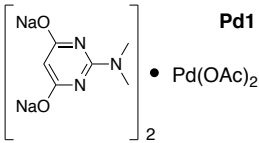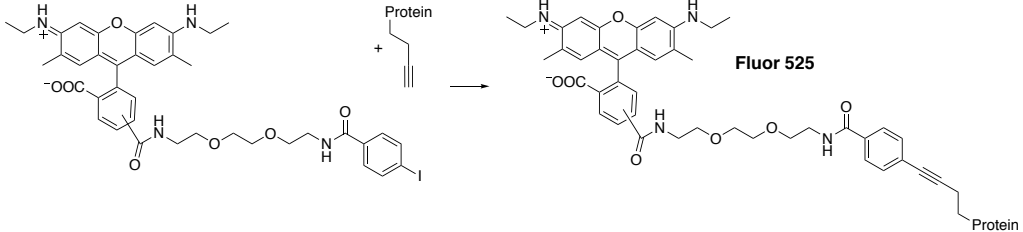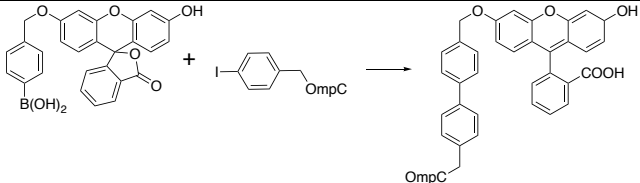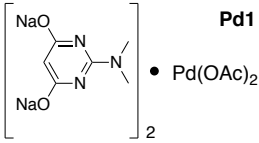
Entry	Reaction	Catalyst	Organism	Ref.
	Ring closing metathesis	**Ruthenium**		
8	Umbelliferone	**Ru13**	Periplasm of *E. coli*	[40]
	Copper-free Sonogashira cross-coupling	**Palladium**		
9	Fluorescein	**Pd1**	*E. coli*	[41]
10	Biotin	**Pd1**	HEK293	[46]
11	Fluor 525	Pd(NO$_3$)$_2$ (**Pd2**)	*E. coli* and *Shigella*	[45]
	Suzuki-Miyaura cross-coupling	**Palladium**		
12		**Pd1**	*E. coli*	[40,44]

11

Table 2 (continued)
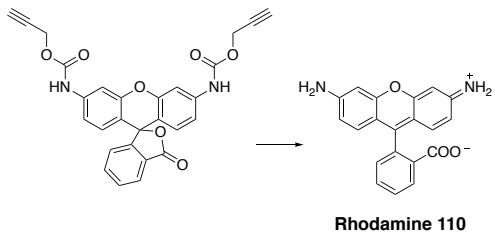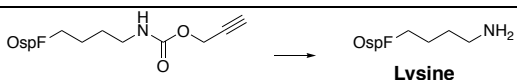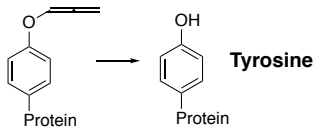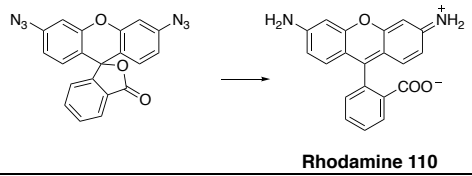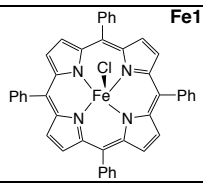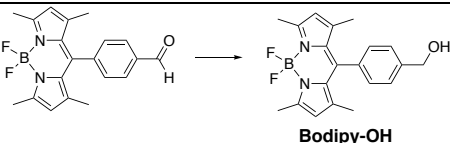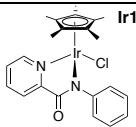
Entry	Reaction	Catalyst	Organism	Ref.
	Propargyloxycarbonyl cleavage	**Palladium**		
13	**Rhodamine 110**	[Pd(η3-allyl)Cl]₂ (**Pd3**) Pd(dba)₂ (**Pd4**) **Pd7**	HEK293T, NIH-3T3, A549, HeLa, Caco-2, CHO cells PC-3 cells	[47] [41] [49]
14	**Lysine**	[Pd(η3-allyl)Cl]₂ (**Pd3**) Pd(dba)₂ (**Pd4**)	HeLa cells	[47]
	Allenyl cleavage	**Palladium**		
15	**Tyrosine**	[Pd(η3-allyl)Cl]₂ (**Pd3**) Pd(dba)₂ (**Pd4**) Pd(TAPAd)₂ (**Pd5**) Pd(TPPTS)₄ (**Pd6**)	HEK293T cells	[48]
	Bisazide reduction	**Iron**		
16	**Rhodamine 110**	**Fe1**	HeLa cells	[40,52]
	Transfer Hydrogenation	**Irridium**		
17	**Bodipy-OH**	**Ir1**	NIH-3T3 cells	[41,53]

Table 2 (continued)

Entry	Reaction	Catalyst	Organism	Ref.
	Amide bond formation	**Gold**		
18		**Au1**	Mouse liver and intestine	[54]
19		**Au1**	Mouse liver and intestine	[54]

13

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    as:
• of special interest
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