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Bioorthogonal strategies for the *in vivo* synthesis or release of drugs

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ABSTRACT

The site-specific delivery of antitumor agents is a rapidly developing field that relies on prodrug activation and uncaging strategies. For this purpose, a wide range of homogeneous and heterogeneous biocompatible activators/catalysts have been developed to convert caged drugs with low toxicity and high stability in physiological settings into active substances in a bioorthogonal manner. The current methods allow for the site-specific delivery of activators and prodrugs to organelles, target cells, or tumors in living organisms. Here, we present an overview of the latest advances in catalytic drugs, highlighting the expanding toolbox of bioorthogonal activation strategies made possible by transition metals acting as activators or catalysts.

1. Introduction

Until recently, the spectrum of *in-vivo* cleavable protecting groups amenable to minimize the toxicity of drugs (anti-inflammatory, antitumor, and others) was strictly limited by the repertoire of natural enzymes. –The term *in vivo* will be used throughout to describe catalytic systems performed in the presence of living cells (e.g. cell cultures). Whether the catalytic event occurs "inside" or "outside" of the cells will be specified in each case.– For example, native hydrolases and oxidoreductases, among others, can be used to uncage a drug *in vivo*. Since such enzymes are widely distributed in most tissues in the body, it is challenging to site-specifically uncage a bioactive molecule where its therapeutic action is most desirable (see Fig 1).

The pronounced toxicity of many antitumor drugs, both for diseased and healthy cells, calls for strategies to minimize these severe side effects where the drug is not needed. The term *bioorthogonality* was introduced by Hang et al. in 2003 and is used to describe "chemical reactions that neither interact with nor interfere with a biological system".[1] The presence of a large number of functionalities in cells and intercellular space complicates the identification of such reactions and reagents that do not interact with the native biological environment.

In the early 2000s, there were very few reactions displaying low reactivity in biological systems and high specificity of binding to the target substrate. Among these, the Staudinger ligation, developed in 2003,[1] and the metal-free click reaction, reported in 2007,[2] occupies a prominent place. Capitalizing on these tools, it proved possible to label cells, tissues and microorganisms using fluorescent probes via reaction of organic azides with esters and triple bonds, respectively.

Despite their high specificity, these approaches are strictly stoichiometric and therefore cannot be used to generate high concentrations of free fluorophores or potentially bioactive molecules.

In 2006, Streu and Meggers[3] reported an important milestone in metal-catalyzed bioorthogonal reactions: the cleavage of allylcarbamates by [Cp*Ru(cod)Cl] (Cp*=pentamethylcyclopentadienyl, cod = 1,5-cyclooctadiene) (Fig. 2a). This uncaging reaction was demonstrated for the first time in HeLa cells. This achievement marked the starting point in the development of bioorthogonal organometallic catalysis as tool for new-to-nature reactions performed *in vivo*.

From this point on, the number of publications dedicated to bioorthogonal transformations in cells, tissues, and organisms has grown significantly (Fig. 3).

This area of research has developed primarily along two avenues: i) metal-free click-chemistry and ii) metal-catalyzed uncaging of drugs. The first approach allows for the site-specific delivery of activators and prodrugs to organelles and tissues. For this purpose, antibody-drug conjugates (ADCs) have been widely used. The metal-free click reaction has been expanded to a click-and-release strategy, whereby an inverse-electron-demand Diels-Alder reaction generates an intermediate that spontaneously undergoes tautomerization to release its cargo. This elegant strategy was developed by Robillard and coworkers in 2013[4] and demonstrated in a mouse model by Wu and coworkers,[5] wherein they developed a system that simultaneously releases the anticancer agent camptothecin and a NIR-fluorophore, allowing real-time monitoring of the drug release event. In another study, Xie and coworkers[6] used pH-responsive polymers to target the delivery of the prodrug doxorubicin to tumor areas. Strictly speaking, the click-and-release

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approach is not catalytic as it requires stoichiometric amounts of diene and alkene (or alkyne). The advantages and disadvantages of recent advances in this area are discussed in detail in the reviews by Wang and coworkers[7] and together with catalytic systems by many other researchers.[8–13]

From a synthetic organic chemistry perspective, the most versatile means of carrying out chemical reactions *in vivo* is catalysis by transition metal complexes. The biocompatibility and bioorthogonality of these complexes have led to various applications in a cellular environment for the production or release of fluorophores and, more importantly, drugs. In this arena, an important milestone was achieved by the Bradley group.[14–15] They reported that palladium nanoparticles anchored to the surface of polystyrene microspheres (approx. 0.5 μm) are effective for allylcarbamate cleavage and Suzuki–Miyaura coupling. These beads can penetrate the cell membrane and maintain their bioorthogonal activity in the cytoplasm. The Pd-nanodevices were used to uncage rhodamine 110 and separately synthesize a mitochondria-specific fluorophore.[14] This approach was adapted by the Unciti-Broceta group by applying these Pd nanoparticles to uncage antitumor drugs.[16–17] They showed that both caged 5-fluorouracil (5FU)[8] and gemcitabine[9] could be depropargylated in PBS in the presence of HCT116 or BxPC-3 and BxPC-3 or Mia PaCa-2 cells, respectively. Importantly, the propargylated and allylated gemcitabine was found to be 23–67 times less active than the free drug and pro-5FU showed no toxicity at all (EC50 > 1 mM) for various cell lines. In each case, the prodrug–Pd nanoparticles combination had similar cytotoxicity to the unmodified drug. Additionally, zebrafish embryos injected with PEGylated Pd nanoparticles grew and developed without any noticeable negative effect.[8]

An alternative to nanoparticle catalysis is the use of transition metal complexes to carry out homogeneous catalysis *in vivo*, which does not require the implantation of solid particles into the body. Sánchez et al. reported on the unmasking of DNA-intercalating agents (DAPI and ethidium bromide) using [Cp*Ru(cod)Cl] in the presence of an excess of the nucleophile.[18] Building on the original work of Meggers and coworkers,[2] the same group developed new ruthenium catalysts that are competent for uncaging of allyl carbamate-protected amines with turnover numbers (TONs) as high as 270, representing more than a 33-fold increase over the parent [Cp*Ru(cod)Cl] (i.e. 8 TON).[19] In particular, using glutathione as a nucleophile, they demonstrated that the complex [CpRu(QA-NMe_2)(η^3-allyl)]PF_6 (QA-NMe_2 = 4-(*N*,*N*-dimethylamino)-2-quinolinecarboxylate) (Fig. 2b) is significantly more active than [Cp*Ru(cod)Cl] in releasing doxorubicin from its allyloxycarbonyl-protected form.

In subsequent years, the list of bioorthogonal reactions has expanded significantly as a result of the work of Weissleder,[20] Rotello,[21–22] Bernardes,[23] Qu,[24] Chen,[25] Unciti-Broceta,[26–29] Ward,[30–32] Mascareñas,[33–35] Tanaka,[36–38] and others.[39–40] The development of such a diverse repertoire of bioorthogonal catalyst- and substrate-pairs has made it possible to expand the class of biocompatible protective groups for *in vivo* applications (Scheme 1) (see Scheme 2).

In addition to the above-mentioned drugs, the field of biorthogonal chemistry has been applied to the uncaging/synthesis of ciprofloxacin,[21] endoxifen,[37] monomethyl auristatin E (MMAE)[23,41] SN-38 (the active metabolite of irinotecan),[42] paclitaxel,[27] floxuridine,[43] resatorvid,[44] cisplatin,[39] panabinostat,[29] etoposide,[45] and others.

Here, we present a review of *catalytic* release of bioactive molecules *in cellulo* and *in vivo* and the potential prospects for this area of research from the perspective of heterogeneous and homogeneous catalysis.

2. Heterogeneous transition metal catalysts

2.1. Palladium-based heterogeneous in vivo catalysts

As mentioned above, one of the key breakthroughs in the field of bioorthogonal catalysis in living systems was the development of Pd^0 nanoparticles embedded in polystyrene beads. First described in 2011 for allyl and allyloxycarbonyl cleavage,[14–15] this approach has been expanded by Unciti-Broceta group to the cleavage of propargyl, propargyloxycarbonyl and propargyloxyaryl groups.[29,42,46] For example, propargyloxyaryl-protected SN-38 (Table 1, Entry 1) (an analogue of topoisomerase I inhibitor) and the previously known 1-propargyl-5-fluorouracil (Table 1, Entry 5)[17,47] were uncaged using entrapped Pd-nanodevices in HCT116 and U-87 cancer cells.[42] This work was the first example of the simultaneous release of a combination of therapeutic agents from their caged forms using bioorthogonal reactions. The TON could not determined in this and subsequent studies, since the exact amount of catalyst is not known, and the amount of products formed can only be roughly estimated using fluorescence analysis for dyes/prodyes or cell viability for drugs/prodrugs.

Unciti-Broceta and coworkers used this approach for the catalytic release of doxorubicin protected by *para* and *ortho* propargyloxybenzyl groups (Table 1, Entry 2).[46] The novel caged drug featuring the *ortho* group afforded the greatest therapeutic window (150–350 times less toxic than free doxorubicin for various cell lines) and was not cardiotoxic to zebrafish larvae, even at concentrations much higher than would be achieved with maximum drug dosage. In addition to biocompatibility, the functionality of the system was also investigated. Nanoparticles of different sizes (10 μm-110 μm) were tested under the conditions of catalytic depropargylation of rhodamine. The particles with 110 μm and 30 μm sizes displayed the highest activity and stability (>20 months when stored at 4 °C). The catalytic activity of size-optimized Pd-particles was studied on U87 glioblastoma cells and DU145 prostate cancer cells in mice. The nanoparticles retained their activity three weeks after implantation into the body. The initial concept

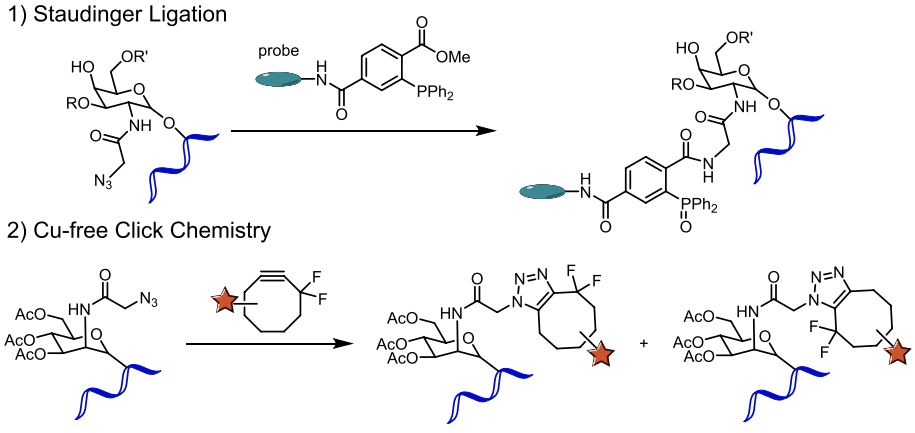


Fig. 1. Pioneering reactions applicable in the context of bioorthogonal chemistry.

laid on a solid proof: experiments on zebrafish have become the first clinically-relevant result for the release of a drug (doxorubicin) in a bioorthogonal manner in an animal model. Experiments on mice have confirmed the possibility of precise injection of Pd-nanodevices directly into the tumor area. All this opens up broad prospects for the use of bioorthogonal systems for cancer therapy.

Recently, Unciti-Broceta and coworkers devised a creative "Trojan horse" approach to enable targeted delivery of palladium catalyst into A549 and U-87 cells.[29] They succeeded in growing palladium nanosheets inside the respective cell exosomes, and showed that these hybrid systems selectively penetrate into their parent cells to deliver catalyst with high activity for the uncaging of the drug panobinostat (Table 1, Entry 4). Unciti-Broceta and coworkers[27] capitalized on the above work and designed palladium nanosheets encapsulated in biocompatible hydrogel networks that are highly effective for the depropargylation of a caged paclitaxel prodrug, as demonstrated from treating A549 and U87 cells (Table 1, Entry 12).

Numerous other groups have continued to work towards palladium-catalyzed reactions *in vivo*. For example, Wang et al.[24] developed photosensitive Pd-loaded microporous silica nanoparticles functionalized with azobenzene and capped with cyclodextrin. Upon exposure to UV light, the *trans* to *cis* azobenzene photoisomerization results in the release of cyclodextrin from the surface of the NPs and thus catalyst activation. This process is reversible upon irradiation with visible light. As demonstrated in HeLa cells, this system is able to cleave allyloxycarbonyl-protected rhodamine 110, catalyze bimolecular Suzuki-Miyaura cross-coupling to generate a mitochondria-specific probe, and convert 5-fluoro-1-propargyl-uracil into 5-fluorouracil. Such light-gated heterogeneous transition metal catalysis could be broadly applied to the selective release/synthesis of anticancer drugs using UV light irradiation (Table 1, entries 16, 19).

Gu and coworkers developed new devices based on titanium oxide coated with palladium nanoparticles.[48] Biocompatible and easy-to-use patches easily penetrate the area of a tumor and release the active compound with high efficiency and precise localization (without leakage of toxic agents into healthy tissues). This model has been tested on B16-F10 tumor in mice treated with the allyloxycarbonyl-DOX (Table 1, Entry 17) and has shown the potential of such bioorthogonal systems in cancer-targeted therapy compared to passive drug-releasing systems.

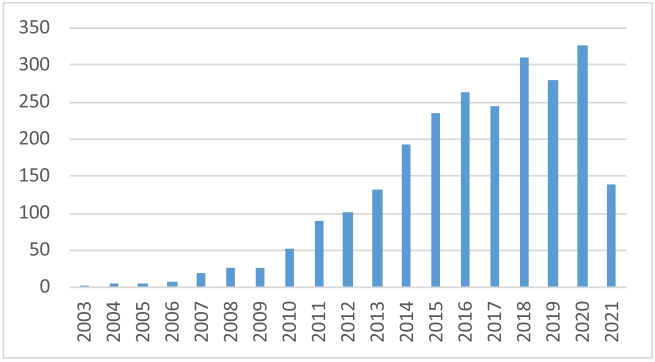


Fig. 3. Number of publications that include the topic *bioorthogonality*.

Qu and coworkers[49] designed novel catalysts capable of the targeted synthesis of chiral drugs in inflamed tissue. These catalysts consist of neutrophil-coated mesoporous silica nanoparticles embedded with palladium and chiral alkaloids (cinchonidine, cinchonine, quinidine, and quinine). The cinchonidine variant was shown to catalyze the enantioselective reduction of 2-(4-isobutylphenyl)acrylic acid to (*S*)-ibuprofen with an enantiomeric excess (*ee*) of 78% (Table 1, Entry 22). These catalysts were shown to be effective in reducing inflammation in RAW264.7 cells and mouse paw tissue.

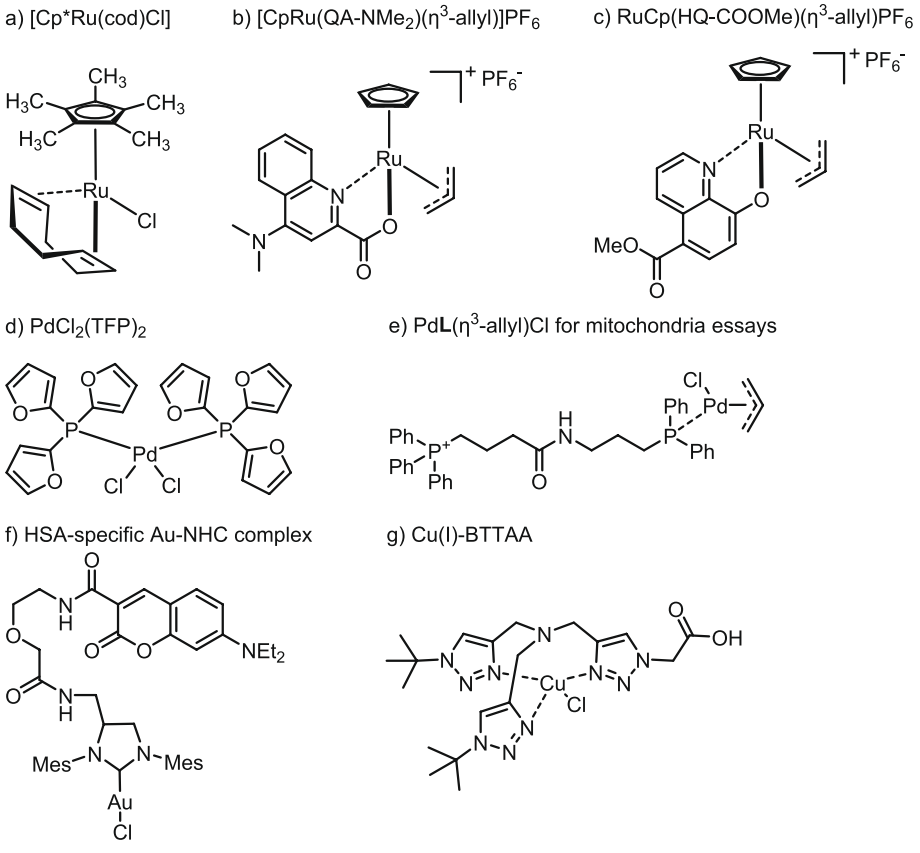
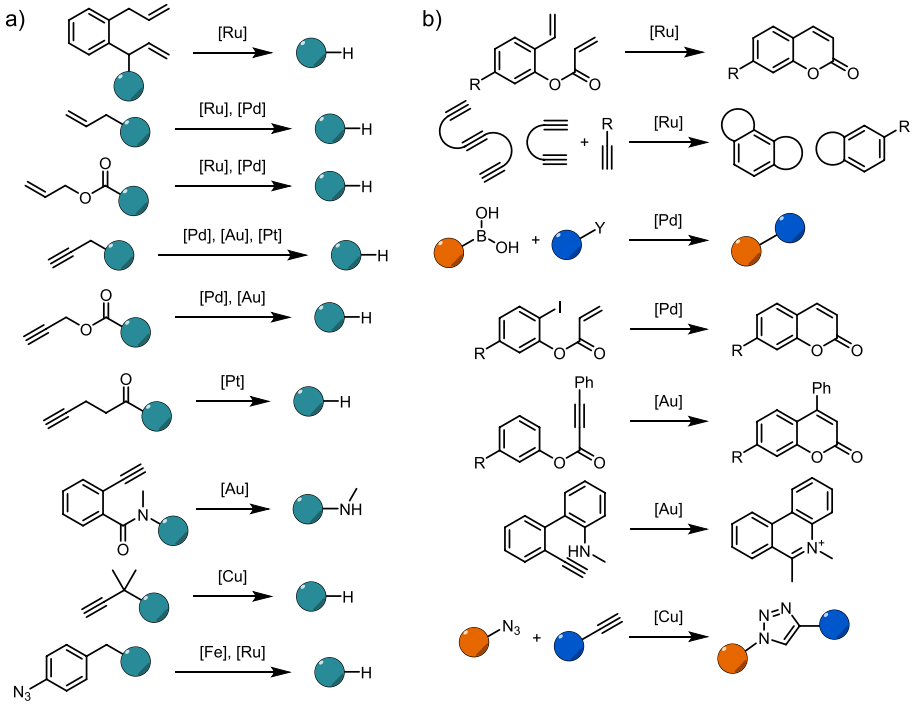
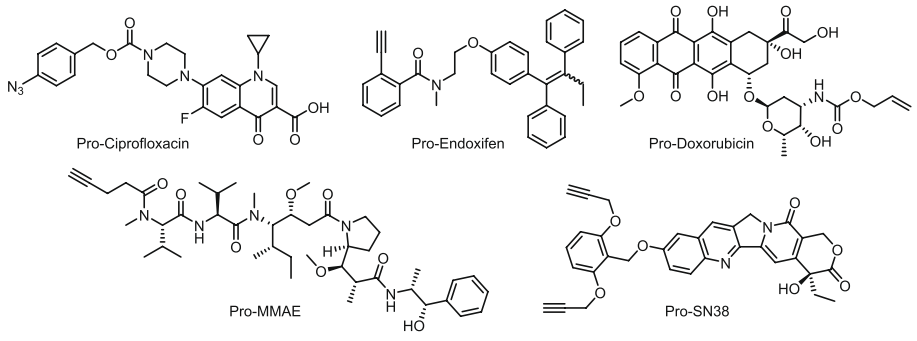


Fig. 2. Structures of selected organometallic catalysts used in bioorthogonal chemistry.



Scheme 1. Bioorthogonal reactions used *in vivo* for a) the uncaging of a product (blue-green sphere); b) the synthesis of bioactive molecules or fluorophores.



Scheme 2. Selection of caged drugs.

TentaGel resins, known for their biocompatibility and metal retention, are widely used in biomedical applications. Kane and coworkers[44] used palladium nanoparticles immobilized on TentaGel resins in a TLR4 reporter cell assay to release propagyl- and propargyloxycarbonyl-protected resatorvid (Table 1, Entries 7, 13).

Weissleder and coworkers[20] created a biocompatible catalyst featuring $PdCl_2(TFP)_2$ (TFP = tri(2-furyl)phosphine) (Fig. 2d) encapsulated in poly(lactic-*co*-glycolic acid)-polyethyleneglycol nanoparticles. Under physiological conditions, the encapsulated complex is reduced to Pd^0, and this species was used to synthesize fluorescent 7-diethylaminocoumarin via an intramolecular Heck reaction, carried out *in vivo* for the first time (Table 1, Entry 21). To date, the reaction has not found use in the synthesis and release of a drug, however it shows another unique reactivity achievable via Pd-catalysis. Weissleder and coworkers[33] has shown this catalytic micelle system to be effective in deallylation reactions leading to the release of rhodamine 110 and doxorubicin into HT1080 fibrosarcoma cells in mice (Table 1, Entry 17), as well as the delivery and activation of MMAE and doxorubicin prodrugs. Notably, the protective group reduces the toxicity of MMAE by more than four orders of magnitude (Table 1, Entry 18).

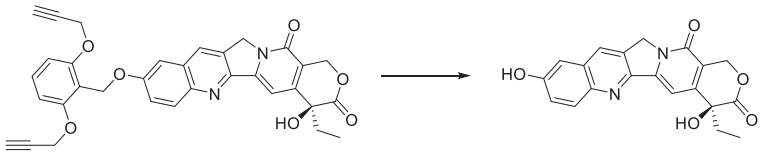
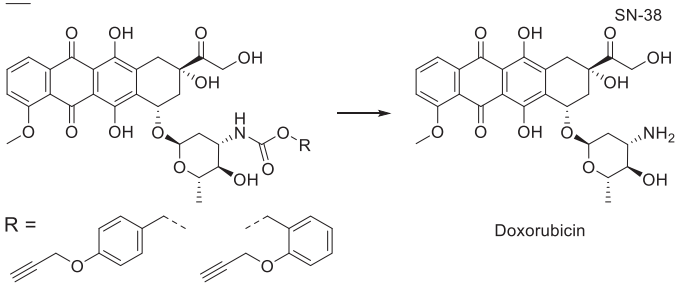
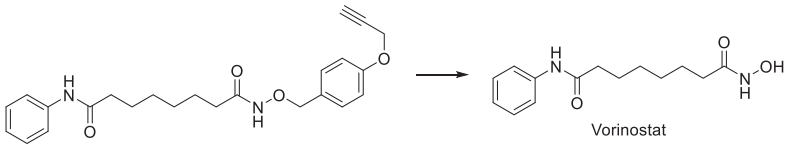
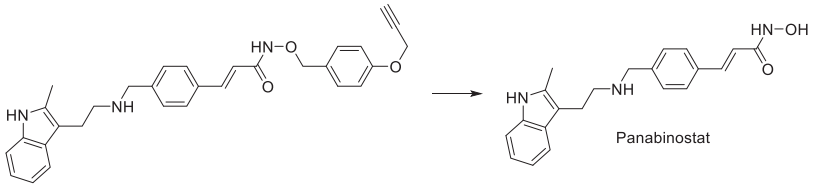
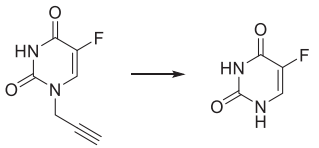
The use of polymers for encapsulating palladium has been pursued Palmans and coworkers.[50] They developed self-assembling micelles, formed by block-copolymers carrying ligands for binding to Pd(II) and copper(I) (see below), which were used for depropargylation and deallylation of caged-fluorophores in HeLa cells (Table 1, Entry 14).

Significant contributions to the use of scaffold structures for the delivery of palladium into the cellular environment were made by Mascareñas group,[35,51] who used Pd-loaded mesoporous hollow SiO_2 spheres and core–shell Pd/metal–organic framework nanoreactors to depropargylate caged-fluorophores in HeLa cells (Table 1, Entries 6, 11, 20).

Purely inorganic catalytic systems for *in vivo* bioorthogonal transformations have been developed by Pané and coworkers.[52] They synthesized iron-palladium-nanowires via a template-assisted electrode position, which showed high biocompatibility and moderate efficiency for the activation of 5-fluoro-1-propargyl-uracil in MDA-MB-231 cells in mice (Table 1, Entry 5).

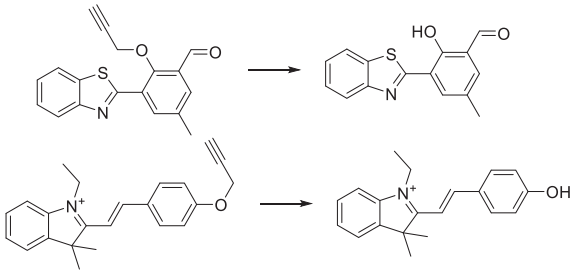
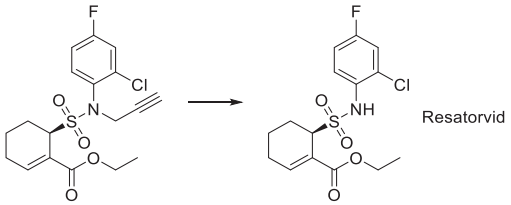
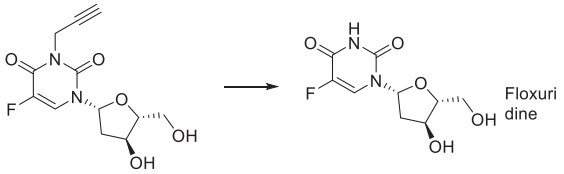
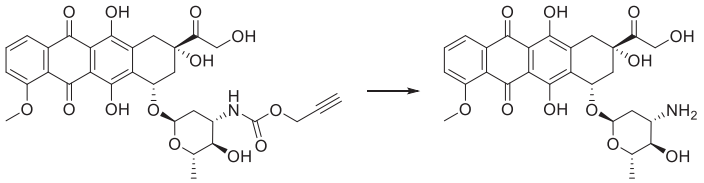
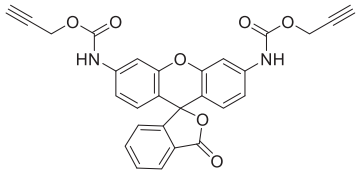
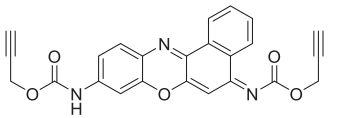
Rubio-Ruiz and coworkers[26] developed a palladium-coated titanium catalyst and tested its activity in propargyl and propargyloxyaryl cleavage. These biocompatible and reusable devices were shown to be highly efficient in the release of rhodamine 110 and the anticancer drug Vorinostat (Table 1, Entries 3, 10), illustrating the promise of metal-based devices for clinical therapy.

Table 1

Entry	Reaction	Catalyst	Organism/organelle	Ref.
	Propargyloxyaryl cleavage			
1		Pd-devices	HCT116 U-87 U-251 In combination with 5FU	42
2		Pd-devices 10–110 um Au-resins	Zebrafish U87 DU145 prostate cancer in mice A549 cells	46 43
3		Au-resins (Pd) loaded titanium (Ti) devices	A549 cells A549 cells	43 26
4		Cancer-derived exosomes Pd-Exo^{A549}	A549 and U87 cells	29
	Propargyl cleavage			
5		Pd-devices FePd-nanowires Au-PINER	HCT116 U-87 U-251 In combination with SN-38 MDA-MB-231 cells in mice LNCaP, MDA-MB-231 cells	42 52 40

(*continued on next page*)

Table 1 (*continued*)

Entry	Reaction	Catalyst	Organism/organelle	Ref.
6		Pd-loaded mesoporous hollow SiO_2 sphere Core-shell Pd/MOF Nanoreactor	HeLa cells HeLa cells	61 51
7		Pd-NPs immobilized on TentaGel resins	TLR4 reporter cell assay	44
8	 Propargyloxycarbonyl cleavage	Au-resins	A549 cells	43
9	 Doxorubicin	Pd-devices 10–110 um	Zebrafish U87 DU145 prostate cancer in mice	46
10		(Pd) loaded titanium (Ti) devices Pd-PINER Au-resins	A549 cells LNCaP, MDA-MB-231 cells A549 cells	26 40 43
11		Core-shell Pd/MOF Nanoreactor	HeLa cells	51
12		Pd-nanosheet-hydrogel network	A549, U87 cells and HBVPs	27

(*continued on next page*)

Table 1 (*continued*)

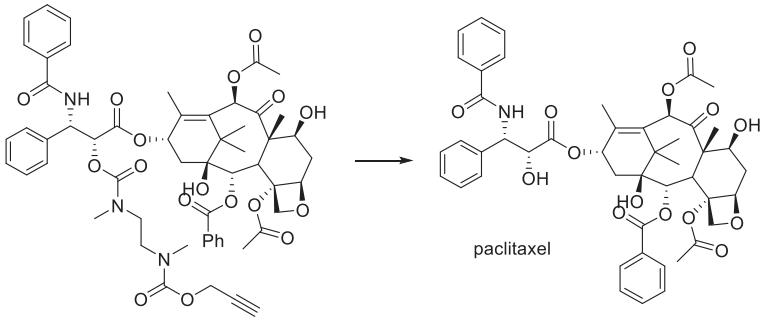
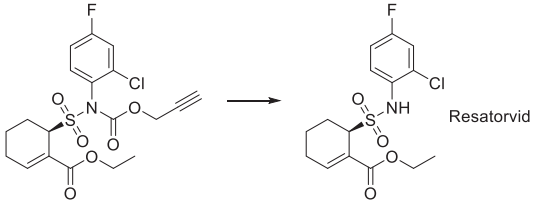
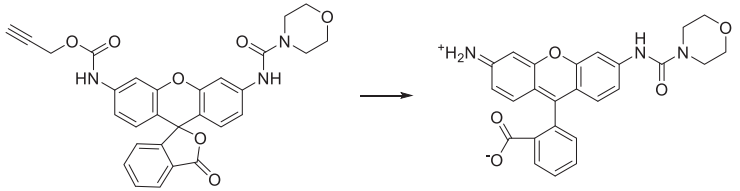
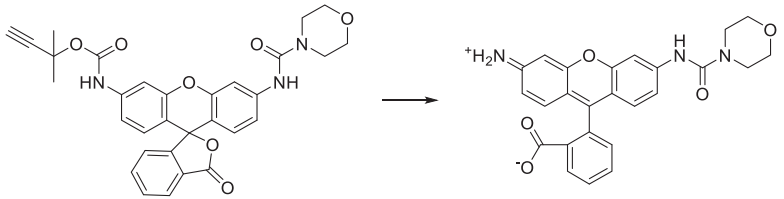
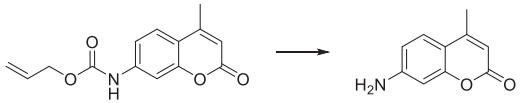
Entry	Reaction	Catalyst	Organism/organelle	Ref.
				
13		Pd-NPs immobilized on TentaGel resins	TLR4 reporter cell assay	[44]
14	 Dialkylpropargyl cleavage	Pd(II) SCPNs	HeLa cells	[50]
15	 Allyloxycarbonyl cleavage	Cu(I) SCPNs	HeLa cells	[50]
16		Microporous silica nanoparticles (DMSN) +Photosensitizer + Pd + cyclodextrin	HeLa Mitochondria of HeLa cells	[24]
17		Pd-NP ($PdCl_2TFP_2$) + PLGA-PEG micelles	HT1080 fibrosarcoma cells in mice	[20]
		Pd-NPs on TiO_2 nanosheets	B16-F10 in mice	[48]

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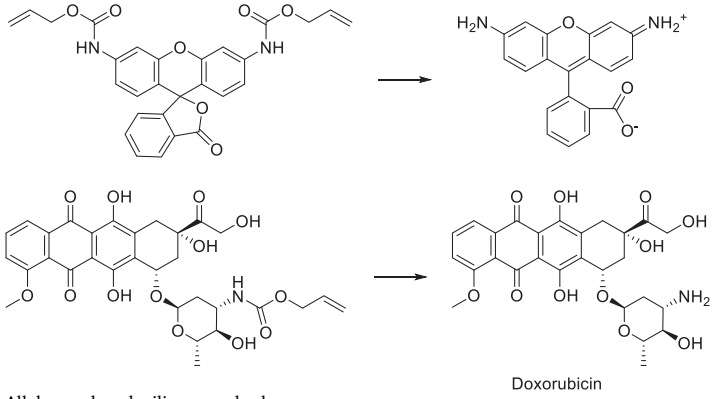
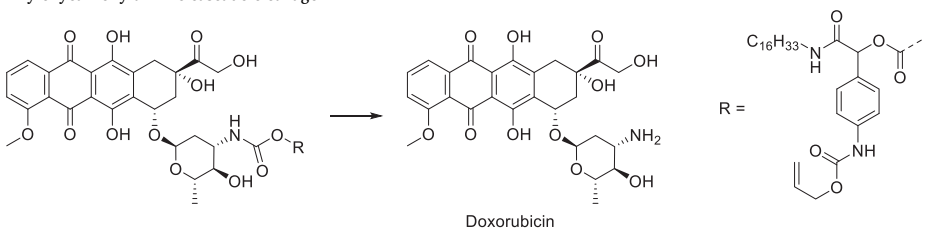
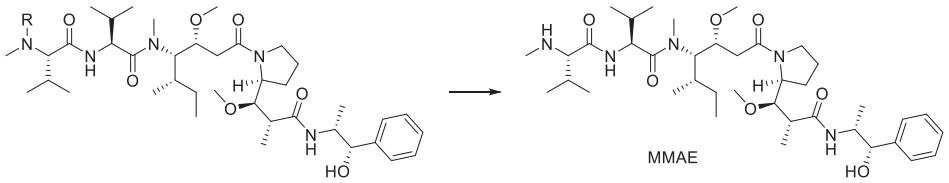
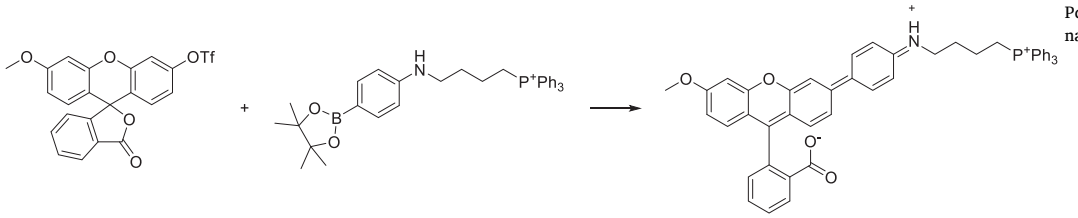
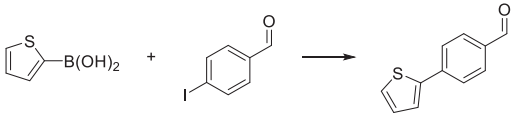
Table 1 (*continued*)

Entry	Reaction	Catalyst	Organism/organelle	Ref.
	 Allyloxycarbonylaniline cascade cleavage			
18		Pd-NP ($PdCl_2TFP_2$) + PLGA-PEG micelles	HT1080 fibrosarcoma cells in mice	[41]
	 Suzuki-Miyaura cross-coupling			
19		Pd-loaded photosensitive microporous silica nanoparticles (DMSN)	HeLa Mitochondria of HeLa cells	[24]
20	 Heck cross-coupling	Pd-loaded mesoporous hollow SiO_2 sphere	HeLa cells	[35]
21		Pd-NP ($PdCl_2TFP_2$) + PLGA-PEG micelles	HT1080 fibrosarcoma cells in mice	[20]

(*continued on next page*)

Table 1 (*continued*)

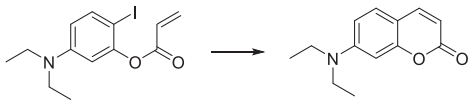
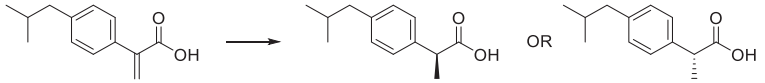
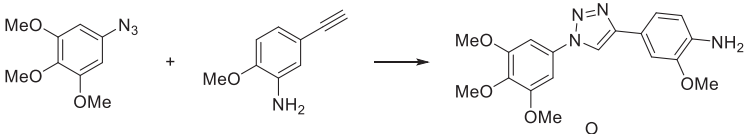
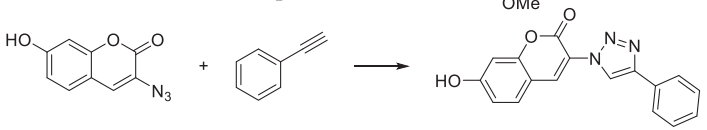
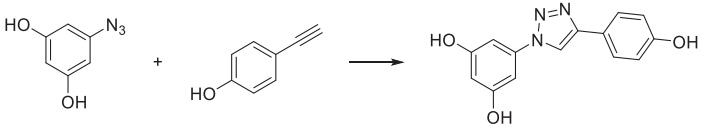
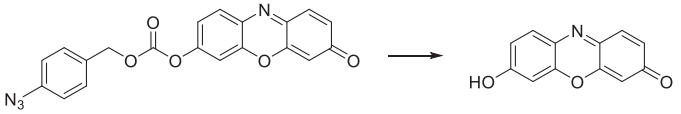
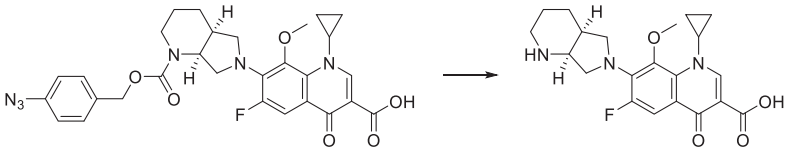
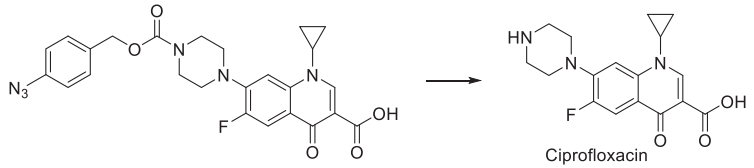
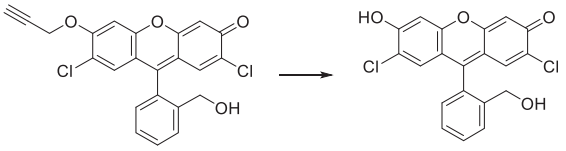
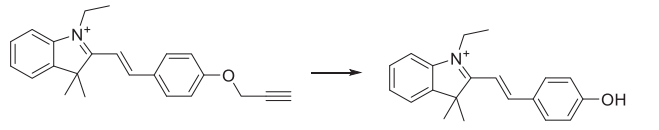
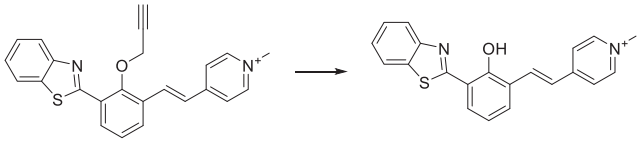
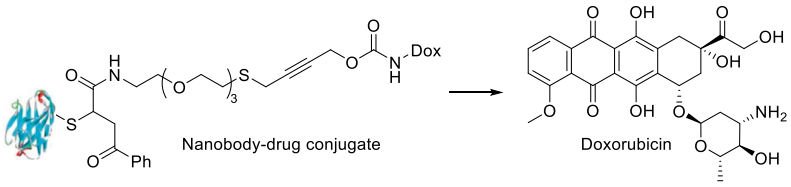
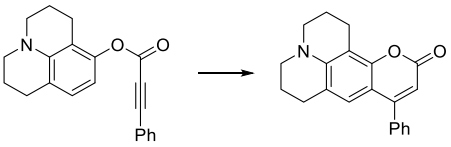
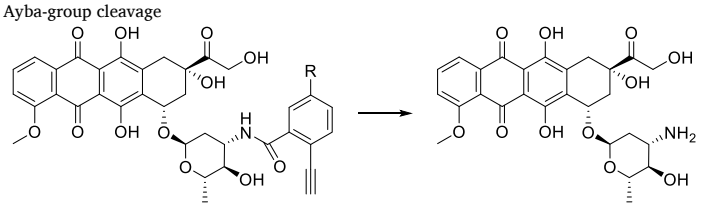
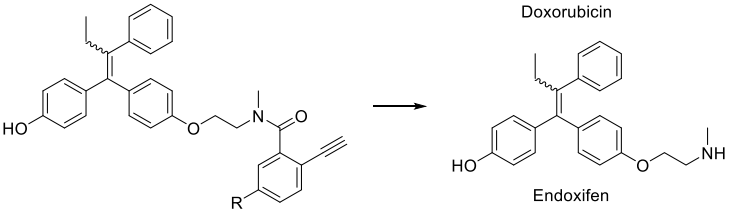
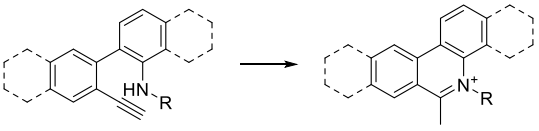
Entry	Reaction	Catalyst	Organism/organelle	Ref.
	 Reduction			
22	 [3 + 2]-Cycloaddition	Neutrophil- membrane-coated chiral Pd catalysts	RAW264.7 cells	49
23		Cu cross-linked lipoic acid NPs MOF-Cu catalyst	HeLa cells C. elegans MCF-7 cells Mitochondria	59 60
				
24	 Azide reduction	MOF-Cu catalyst	C. elegans MCF-7 cells	60
25		Fe-polyzymes	Bacterial biofilms (E. coli)	22
26	 Moxifloxacin  Ciprofloxacin	Fe-polyzymes	E. coli and P. aeruginosa biofilms NIH 3T3 fibroblast cells	21

Table 2

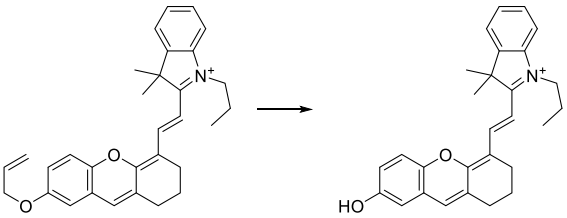
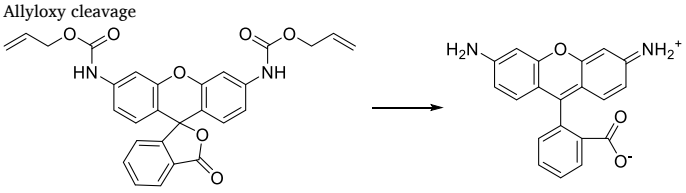
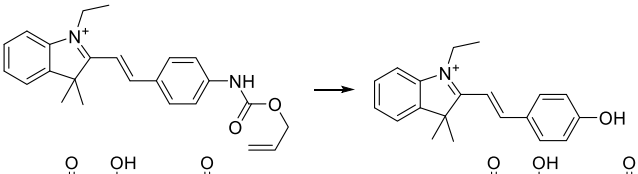
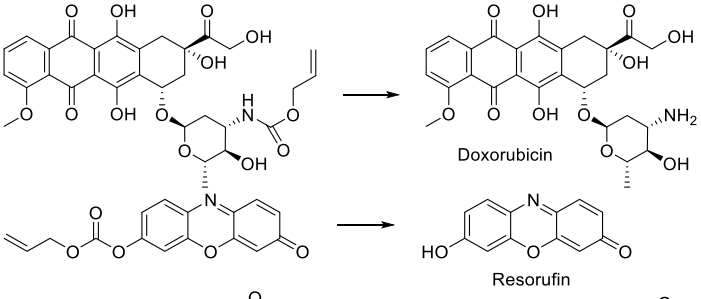
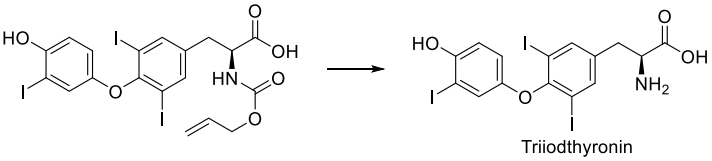
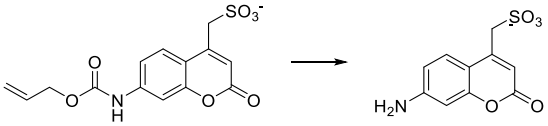
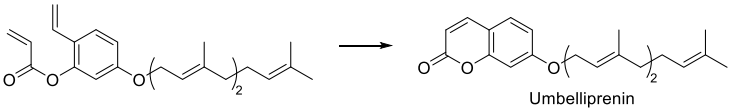
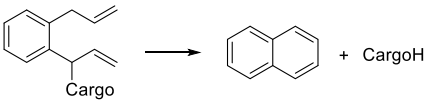
Entry	Reaction	Catalyst	Organism/ organelle	Ref.
	Propargyl cleavage			
1		Biocompatible NHC-Pd catalysts	MCF-7 cells	68
		K_2PtCl_4, cisplatin	HeLa cells Zebrafish	23
2		Biocompatible NHC-Pd catalysts	MCF-7 cells	68
3		 -labeled for mitochondria essays	Cell lysate Vero cells	69
		$PdCl_2brHis_2$	HeLa cells	70
	Propargyloxycarbonyl cleavage			
4		$Pd(COD)Cl_2$	HEK293 and MCF-7 cells	71
	Au-activated C—C—bond formation			
5		Water-soluble Au (I)-catalysts	HeLa cells	73
	Ayba-group cleavage			
6		Au-NHC complexes HSA-based Gold ArMs	PBS buffer	37
				
	Hydroamination			
7		$NaAuCl_4$, Au-NHC complexes HSA-based Gold ArMs	A549 cells	38

(*continued on next page*)

2.2. Heterogeneous gold, copper, and iron catalysts

The use of heterogeneous catalysts is not limited to palladium or its compounds. Indeed, in principle, any metals that are biocompatible and stable under physiological conditions may be promising as bio-orthogonal catalysts. After palladium, gold was the next metal to feature prominently in the catalysis of new-to-nature reactions. Until 2017, gold nanoparticles were used as a basis for the immobilization of chemically-active agents. Thus, Rotello and coworkers showed that ([Cp*Ru(cod)Cl]), entrapped on the surface of gold nanoparticles in a HeLa cell culture medium, catalyzes deallylation of pro-rhodamine 110, and Pd $(dppf)Cl_2$ – depropargylation of pro-5FU.[53] Unciti-Broceta and

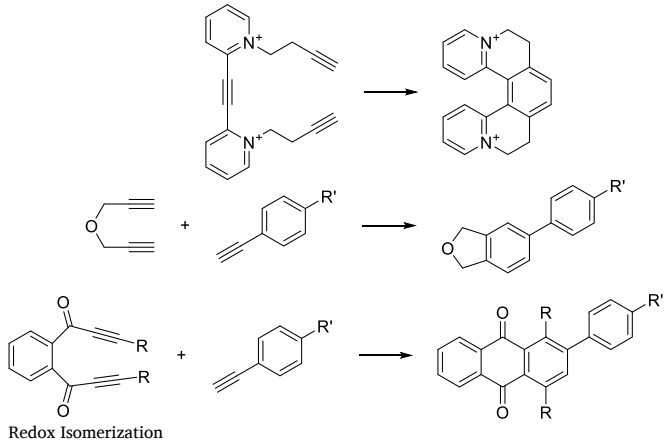
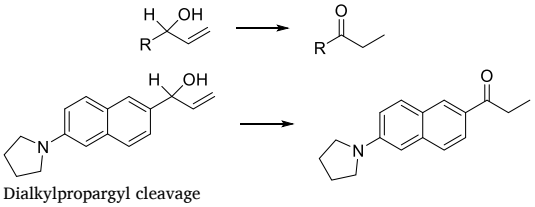
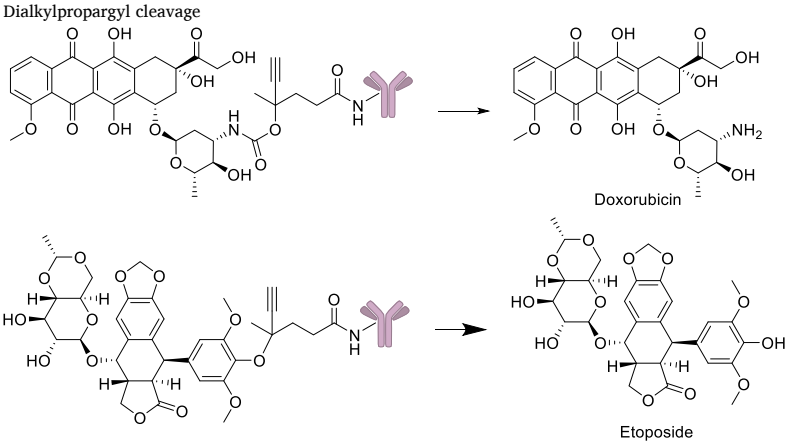
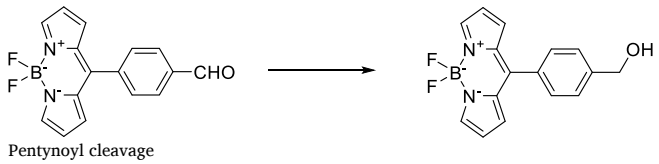
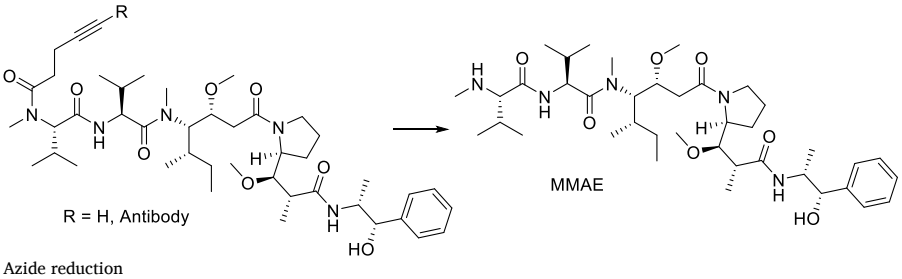
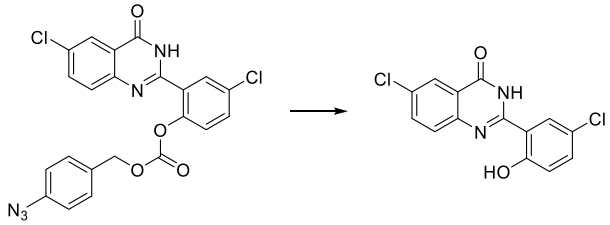
Table 2 (*continued*)

Entry	Reaction	Catalyst	Organism/ organelle	Ref.
	Allyl cleavage			
8		CpRu(QA)(η^3-allyl)-complex	HeLa cells	73
	Allyloxy cleavage			
9		CpRu(QA)(η^3-allyl)-complex	HeLa cells	19,34,74
		 -labeled for mitochondria essays	HeLa and Vero cells mitochondria	69
		[RuCp*Cl(COD)]-based nanozymes	HeLa and Raw 264.7 cells	85
		Ru-based nanozymes w/ or w/o protein corona	Serum HeLa cells	86
10			Cell lysate	69
11		CpRu(QA)(η^3-allyl)-complex	HeLa cells	19,74
		[RuCp*Cl(COD)]-based nanozymes	HeLa and Raw 264.7 cells	85
12		Artificial deallylase based on SAV and Ru cofactor	HEK-293 T cells	75
13		Artificial deallylase based on SAV and Ru cofactor	C. reinhardtii	32
	Metathesis			
14		Artificial metathesase based on HSA and Ru cofactor	SW620, A549, and HeLa cells	36
15		Ru Hoveyda-Grubbs 2nd generation catalyst	HeLa cells, E. coli	31
	(2 + 2 + 2)-Cycloaddition			

(*continued on next page*)

coworkers[43] developed cell-penetrating Au-resins that can release doxorubicin, floxuridine, and vorinostat within A549 cancer cells and significantly reduce cell viability (Table 1, Entries 2, 3, 8) via catalytic depropargylation and depropargyloxyarylation. Gold nanoparticles implanted in the brain of zebrafish have been shown to be effective for the release of the fluorescent rhodamine 110 (Table 1, Entry 10). Lee and coworkers[40] synthesized a family of new plasmonically integrated nanoreactors (PINERs) that can be doped with gold, as well as palladium (Table 1, Entry 10) and platinum. These catalysts are activated upon near IR irradiation, and effectively release 5FU and rhodamine 110 upon

Table 2 (*continued*)

Entry	Reaction	Catalyst	Organism/ organelle	Ref.
16	 Redox Isomerization	[RuCp*Cl(COD)], RuCp(QA)(η^3-allyl)	HeLa cells	[76]
17	 Dialkylpropargyl cleavage	[Ru(η^3:η^3-$C_{10}H_{16}$)Cl (μ^2-O,O-O_2CMe)]	HeLa, Vero and A549 cells	[33]
18	 Transfer hydrogenation	Cu(I)-BTTAA	HeLa and SKBR-3 cells	[45]
19	 Pentynoyl cleavage	Cp*Ir(N,N')-complexes	NIH-3T3 cells	[80]
20	 Azide reduction	K_2PtCl_4, cisplatin	HeLa cells Zebrafish	[23]
21		Ru(bpy)$_2$(phen)-derivatives Ru(bpy)$_2$(phen)-derivatives	HEK293T, MCF-7 cells Zebrafish	[77] [78]

propargyl cleavage in LNCaP and MDA-MB-231 cells (Table 1, Entry 5).

The diversity of bioorthogonal chemistry that can be carried out using copper makes it possible to break bonds and remove protective groups and also to create key fragments of fluorophores and cytotoxic agents.[54–57] Additionally, it has been shown that the incorporation of copper compounds into polymer shells reduces the intrinsic toxicity of copper compounds.[58]

Copper bound by single-chain polymeric nanoparticles[50] removes 1,1-dialkylpropargyl protecting groups catalytically. Palladium, which is effective in propargyl cleavage, is significantly less active in 1,1-dialkylpropargyl cleavage than copper, highlighting the relative orthogonality in the reactivity of the two metals. For example Cu-based nanoparticles were shown to uncage rhodamine 110 in HeLa cells (Table 1, Entry 15).

Zhang and coworkers[59] used nanocopper-doped cross-linked lipoic acid nanoparticles (Cu@cLANP) for the copper-catalyzed azide-alkyne cycloaddition. A potent coumarin-based fluorophore and tubulin polymerization inhibitor (IC50 15 μM) displayed in Table 1, Entry 23 was synthesized in HeLa cells using Cu@cLANP via an intermolecular [3 + 2] cycloaddition.

Qu and coworkers[60] developed a Cu-loaded metal–organic framework for targeted azide-alkyne cycloaddition in mitochondria. The corresponding products are displayed in Table 1, Entries 23 and 24. The resveratrol analogue (Table 1, Entry 23) significantly decreased the viability of MCF-7 cells, and the catalyst was also shown to be biocompatible with *C. elegans*.

Another metal of interest in the context of bioorthogonality is iron. Rotello and coworkers[21–22] used self-assembling polyzymes based on a quaternary ammonium polymer possessing hydrophobic alkyl side chains and [Fe(TPP)]Cl for the catalytic reduction of aryl azides to anilines, followed by 1,6-elimination of fluorophores and cytotoxic agents. The activity of the polyzymes was established during experiments on NIH 3T3 fibroblast cells, as well as *E. coli* and *P. aeruginosa* biofilms. The polyzymes had no adverse effects on cells, and researchers showed that these catalysts can be used to release moxifloxacin and ciprofloxacin, reducing cell viability with $IC_{50} \sim 10$ μM (Table 1, Entries 25, 26).

3. Homogeneous transition metal catalysts

3.1. Homogeneous palladium catalysts

In light of its versatility in homogeneous catalysis, Pd occupies a place of choice in bioorthogonal chemistry. Lin and coworkers reported a water soluble catalyst for *in cellulo* cross-coupling reactions.[62] The Lin, Chen and Davis groups used this catalyst (as well as other water-soluble Pd(II) salts) for Sonogashira and Suzuki-Miyaura cross-couplings for labeling *E. coli*, *Shigella*, and HEK293 cells.[63–65]

Following this work, research shifted toward the development of complexes for protection/deprotection reactions.[25,62,66–67] Biocompatible *N*-heterocyclic carbene (NHC)-Pd catalysts developed by the Bradley group are effective for propargyl and propargyloxycarbonyl cleavage.[67–68] These catalysts were used to release rhodamine 110 in PC-3 cells and 2,7-dichlorofluorescein and 5-fluorouracil in MCF-7 cells (Table 2, Entries 1, 2).[68]

Mascareñas and coworkers synthesized a family of Pd**L**(η^3-allyl)Cl complexes (**L** = pyridine, triphenylphosphine, and phosphonium ions, etc., Fig. 2e) and studied their activity in deprotection reactions in PBS, cell culture media, and *in vivo*.[69] Depending on the identity of **L** and the reaction environment, the catalyst's performance varied significantly. Complexes featuring phosphine or phosphonium-ion ligands were found to localize preferentially in the mitochondria of HeLa and Vero cells, and catalyzed depropargylation and deallylation reactions, leading to the formation and accumulation of fluorophores in the cytoplasm and on the mitochondrial surface (Table 2, Entries 3, 9, 10).

Mascareñas and coworkers also described the use of metallopeptides containing palladium coordinated to two histidine residues.[70] This example is the first example of a so-called "bottom-up" strategy to generate metalloproteins displaying a synergistic effect. The histidine residues coordinate to Pd and increase the catalyst's activity, while the short peptide plays a protective role, preventing the degradation of the catalytic activity. Experiments on HeLa cells highlighted the efficiency for the uncaging of dyes which were unreactive using other palladium (II) catalysts (Table 2, Entry 3).

The use of antibody-drug conjugates for the delivery of prodrugs in the vicinity of target tissues/cells/organelles offers an alternative to targeted-catalyst delivery. In this case, the catalyst can circulate freely in the body or cellular environment. Bernardes and coworkers[71] demonstrated this approach for the first time. For this purpose, they relied on a bifunctional PEGylated caged doxorubicin, featuring a thioether group for directing palladium-mediated depropargylation and a carbonylacrylic group that reacts with cysteine to form a nanobody-drug conjugate. In the presence of non-toxic amounts of $Pd(cod)Cl_2$, the caged doxorubicin combined with the Pd-catalyst proved as effective at killing MCF-7 cells as doxorubicin (Table 2, Entry 4). As the authors note, new palladium catalysts will need to be developed to optimize cell selectivity and minimize toxicity.

3.2. Homogeneous gold catalysts

Until recently, the use of water-soluble gold complexes in bioorthogonal chemistry was limited to the formation of amide bonds and was used only for attaching fluorescent labels to proteins.[72] However, in 2018, Mascareñas and coworkers[73] reported that C—C-bond formation can be catalyzed by a water-soluble Au(I) complex in HeLa cells. In particular, a phenylpropiolic acid ester was converted to a highly fluorescent 7-aminocoumarin derivative in the presence of complexes with phosphine and phenanthroline ligands as well as either $NaAuCl_4$ or $HAuCl_4$ (Table 2, Entry 5).

Recently, Tanaka and coworkers developed the new protecting group 2-alkynylbenzamide (Ayba) for uncaging prodrugs via Au-triggered biorthogonal chemistry.[37] They showed that gold(I)–NHC complexes are effective for converting pro-endoxifen and pro-doxorubicin, protected with Ayba (Table 2, Entry 6) to the active molecules in different cancer cell lines. Although the uncaging reactions do not display multiple turnovers, they were highly specific and unreactive to either palladium or ruthenium catalysts.

The same group subsequently reported the use of $Na[AuCl_4]$ and Au-NHC complexes as catalysts in the hydroamination of 2′-alkynyl-*N*-methyl-2-aminobiphenyls to form phenanthridinium derivatives (Table 2, Entry 7).[38] Using an Au-NHC (Fig. 2f), they created new human serum albumin (HSA)-based gold artificial metalloenzyme (ArM) that results from the incorporation of an NHC-gold cofactor bearing an aminocoumarin moiety enabling its anchoring in HSA. The resulting ArM exhibits high efficiency (up to 211 TON) even in the presence of highly reactive cell components, such as glutathione, lysine, and arginine.

3.3. Homogeneous ruthenium catalysts

Thanks to its biocompatibility and chemical versatility, ruthenium is a valuable metal in the context of biorthogonal chemistry. It gained attention following the proof-of-principle report on catalytic allylcarbamate cleavage by [Cp*Ru(cod)Cl] in the presence of strong nucleophiles.[3,18] Subsequent work described the use of RuCp(QA-R)(η^3-allyl) (QA = 2-quinolinecarboxylate) and RuCp(HQ-R)(η^3-allyl) (HQ = 8-hydroxyquinolinate) (Fig. 2c) as catalyst for the deallylation of a wide range of substrates *in vivo* (Table 2, Entries 9, 11).[19,34,74]

Mascareñas and coworkers[73] demonstrated that deallylation and hydroarylation of different substrates can be carried out concurrently and orthogonally in HeLa cells, using [RuCp(QA-R)(η^3-allyl)]PF_6 (Table 2, Entry 8) and a gold-phosphine catalyst, respectively.

Ward and coworkers[75] designed a cell-penetrating artificial

deallylase based on streptavidin (SAV) and biotinylated Ru-cofactor for the catalytic deallylation of allyloxycarbonyl-triiodthyronine in HEK-293 T cells (Table 2, Entry 12). The efficacy of the thyroid hormone release was optimized through directed mutagenesis, and the consumption of the released triiodothyronine was monitored by furimaznine luminescence. A similar catalytic system was used by Gademann and coworkers.[32] They developed a SAV-based Ru-deallylase that was shown to convert allyloxycarbonyl-protected 7-aminocoumarin to a fluorescent product on *C. reinhardtii* cells (Table 2, Entry 13).

A significant advance in the use of the ruthenium to catalyze bioorthogonal reactions was pioneered by Ward and coworkers.[30] They developed and evolved an ArM that catalyzes olefin metathesis *in vivo*. This area of research has since been expanded over by a number of groups. Tanaka and coworkers[36] used ring-closing metathesis to synthesize the anticancer drug umbelliprenin (Table 2, Entry 14). Glycosylated HSA was used to shield the ruthenium metathesis cofactor from glutathione. The activation of umbelliprenin *in vivo* reduced the viability of SW620, A549, and HeLa cells.

Ward and coworkers[31] used Ru-catalyzed ring-closing metathesis to release caged molecules from diolefins-containing substrates with TONs up to 260. This method introduces the prospect of efficient and bioorthogonal release of fluorophores and drugs (Table 2, Entry 15).

The deallylation catalysts of the type RuCp(QA-R)(η^3-allyl) and [Cp*Ru(cod)Cl] also promote [2 + 2 + 2]-cycloadditions *in vivo*, as reported by Mascareñas and coworkers (Table 2, Entry 16).[76] The approach is highly versatile, facilitating intra- and intermolecular reactivity and the *in vivo* generation of anthraquinones that exhibit aggregate-induced emission, which cannot otherwise be introduced into cells directly.

Finally, Ru(IV) bis-allyl complexes catalyze the isomerization of allyl alcohols to ethyl ketones.[33] This approach has been applied for the synthesis of fluorescent molecules in HeLa, Vero, and A549 cells (Table 2, Entry 17).

Luminescent ruthenium complexes synthesized by Winssinger and coworkers have also been used to photocatalyze azide-reductions (Table 2, Entry 21) in HEK293T and MCF-7 cells[77] and in zebrafish,[78] similarly to the above-mentioned Fe-polyzymes reductions. However, this approach has not been significantly developed and applied recently.

3.4. Homogeneous copper, platinum and iridium catalysts

The rest of the biocompatible metals are poorly represented in the topic of homogeneous bioorthogonality. For example, since 2017, only one article has been published in which a water-soluble copper compound is used for bioorthogonal deprotection/drug conversion. Chen and coworkers[45] used Cu(I)-BTTAA (BTTAA[79] = 2-[4-{(bis[(1-*tert-butyl*-1*H*-1,2,3-*triazol*-4-*yl*)*methyl*]amino)methyl}-1*H*-1,2,3-triazol-1-yl]acetic acid, Fig. 2g) for dialkylpropargyl cleavage of doxorubicin and etoposide from antibody-drug conjugates (Table 2, Entry 18). The released drugs are 120 times more toxic than the ADCs, making this a promising approach to drug delivery and release.

As of this writing, the last report on the use of iridium in the field of new-to-nature reactions *in vivo* was published in 2017. In that study, Do and coworkers[80] reported that aldehydes can be converted to alcohols using unprotected transfer hydrogenation catalysts [Cp*Ir(*N*-phenyl-2-pyridinecarboxamidate)Cl], [Cp*Ir(2,2′-bipyridine)Cl], and [Cp*IrCl$_2$]$_2$, reactivity that was monitored in NIH-3T3 cells using a fluorogenic Bodipy substrate (Table 2, Entry 19). In a series of elegant studies,[81–82] Sadler and coworkers have investigated the use of d6-pianostool complexes Ru(II), Os(II), Ir(III) *in vivo* to perturb the redox status of cells. Excellent reviews have recently been published on the topic.[83–84]

In 2020, Bernardes and coworkers showed that K_2PtCl_4 and cisplatin are effective catalysts for pentynoyl and propargyl cleavage.[23] The biocompatibility of these complexes was studied using HeLa cells and zebrafish. Despite its toxicity, cisplatin selectively deprotected 5-fluorouracil and MMAE (Table 2, Entries 1 and 20, respectively).

4. Conclusions

Since 2017, there has been substantial progress in the development of bioorthogonal reactions catalyzed by transition metals. Homogeneous and heterogeneous catalysts have become significantly more diverse and effective. The ability of solid nanoparticles to cross the membrane of cancer cells has been enhanced by the use of cancer cell-derived markers. New polymeric self-assembling nanoparticles with zero cytotoxicity and high specificity have been shown to be effective for the release of anticancer drugs. Homogeneous catalysts, the use of which was limited by the presence of a large number of reactive components in cells, significantly expanded the field of application. For example, scaffolds of native proteins and their mutants play the role of shells that protect the metal cofactor, increase the penetrating ability and, at the same time, improve the selectivity of catalysis. The number of new-to-nature reactions in biological media has increased significantly. The development of new anticancer drugs and their prodrugs (caged drugs, antibody-drug conjugates, precursors for *in vivo* synthesis) expand the tools for targeted delivery of bioactive molecules to the area of action. In tandem with the increasing progress in the development of catalysts with reduced cytotoxicity and increased efficacy, the wealth of research within recent years opens up exciting prospects for new therapies for cancer and other diseases.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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