Bioorthogonal nanozymes: progress towards therapeutic applications

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Abstract:

Bioorthogonal nanocatalysts in the form of 'nanozymes', are promising tools for generating imaging and therapeutic molecules in living systems. These systems use transformations developed by synthetic chemists to effect transformations that cannot be performed by cellular machinery. This emerging platform is rapidly evolving towards the creation of smart nanodevices featuring the capabilities of their enzyme prototypes, modulating catalytic activity through structure as well as chemical and physical signals. Here we describe different strategies to fabricate these nanocatalysts and their potential in diagnostic and therapeutic applications.

Bioorthogonal chemistry and nanozymes

Bioorthogonal chemistry [1-4] offers a strategy for monitoring [5-8] and modulating bioprocesses [9-11] through reactions that are complementary to enzymatic mechanisms [1,4,11,12]. Bioorthogonal reactions using transition metal catalysts (TMCs) offer a promising direction [13,14], using the diversity of transformations developed by organic chemists to enable chemical transformations in biosystems for *in situ* generation of imaging and therapeutic agents [15-20]. However, the direct application of TMC-mediated reactions in living systems is challenging due to

limitations in biocompatibility, poor water solubility/stability, and rapid efflux from living cells for these high molecular weight and often hydrophobic catalysts [14].

The incorporation of TMCs into engineered nanomaterial scaffolds enhances water solubility and provides a protective environment for metal complexes [21-23] that mimics the isolation provided by enzyme active sites. In addition, these 'nanozymes' can be conveniently engineered for – (i) their localization in targeted tissues [24] and cellular environments [25,26], and/or (ii) to modulate their catalytic properties with chemical [27] and physical signals [28]. These properties provide new directions for systems with spatiotemporal control of molecular activation for therapeutics and or diagnostics [28]. In the present review we describe nanozyme design as well as the opportunities and challenges of this emerging platform towards in biomedicine.

Strategies to fabricate bioorthogonal nanozymes

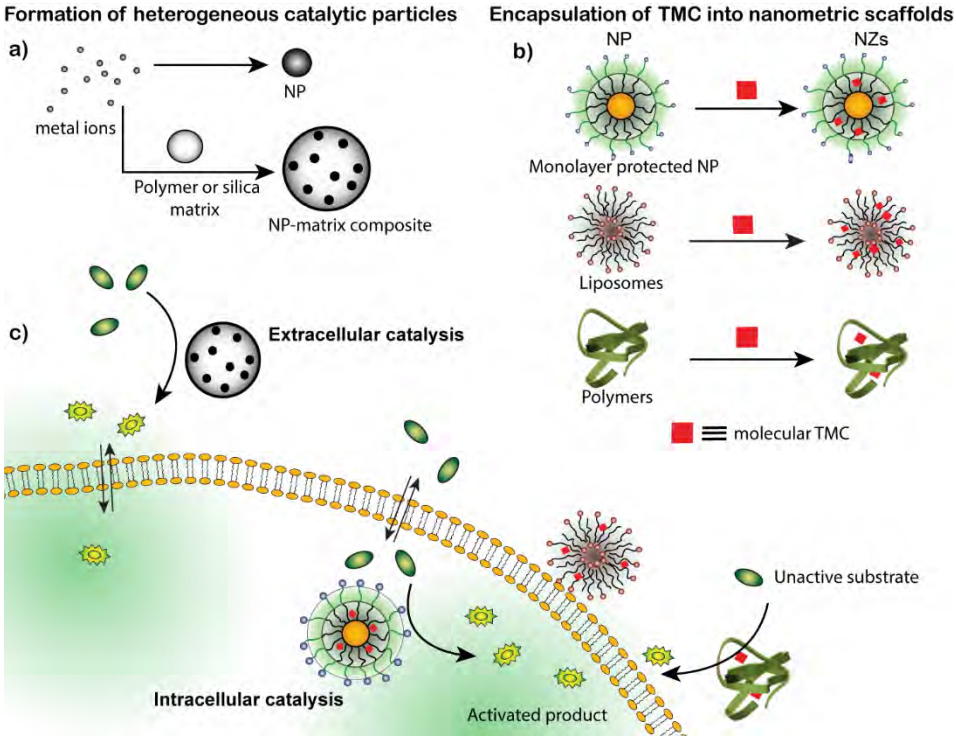


Figure 1. Fabrication of bioorthogonal nanozymes. a) Formation of nanoparticles for heterogeneous bioorthogonal catalysis. b) Fabrication of nanozymes by including molecular TMC into nanometric scaffolds. c) Schematic representation of bioorthogonal activation of substrates intra- and extracellularly.

Two primary strategies have been developed to fabricate nanozymes according to the nature of the catalyst used (Figure 1, Key Figure). Bradley and Unciti-Broceta pioneered on the use of nanoparticles as heterogeneous bioorthogonal nanocatalysts [14,24,25]. This strategy can be applied for the case of palladium and gold-mediated chemistries where the catalyst can be used in its reduced form [25]. Alternatively,

Rotello and coworkers fabricated nanozymes by the encapsulation of molecular TMCs in the monolayers of nanoparticle scaffolds [26-28]. This strategy works well for nanozymes using TMCs that require specific coordinating ligands to perform their catalytic function [17-20,29-31]. The potential of both strategies is described below.

Heterogeneous bioorthogonal nanozymes

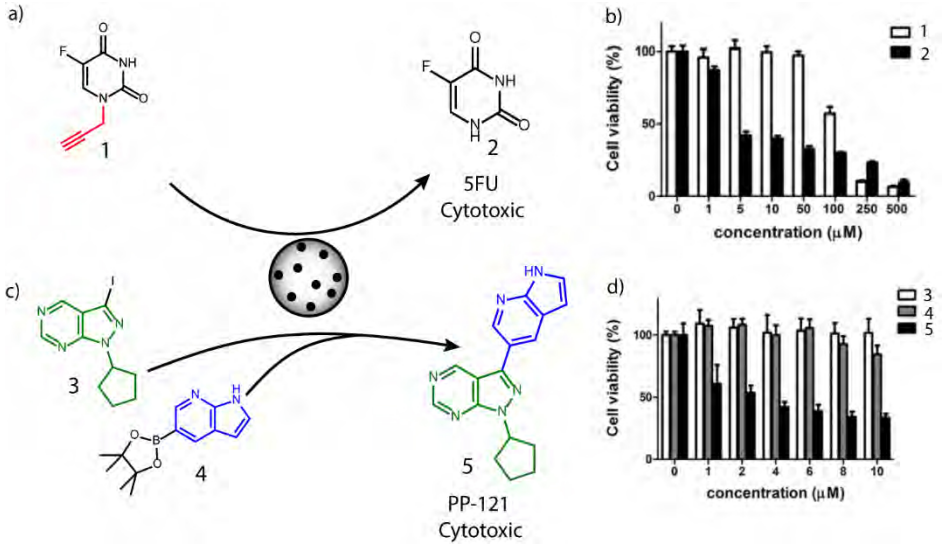


Figure 2. Palladium nanoparticle (PdNP) mediated bioorthogonal reactions for generating therapeutic molecules. PdNPs (2-3 nm) were synthesized inside a micrometric polystyrene particle (gray sphere with black dots). a) Bioorthogonal uncaging of 5FU-prodrugs (cleavable units highlighted in red) by polystyrene-palladium composite. b) Viability of cells cultured with propargylated 5-FU (1) and a mixture of 1 with polystyrene-PdNP to produce 5-FU (2). c) Suzuki-Miyaura cross-coupling reaction generating of PP-121 anticancer agent (coupling units highlighted in blue and green). d) Viability of cells cultured with polystyrene-PdNP composite in the presence of 3, 4, and a mixture of 5 and 6 precursors to form 5. Adapted from reference 33.

Bradley, Unciti-Broceta and coworkers utilized the properties of Pd catalysts to develop nanozymes that can perform reactions in complex biological systems. For this, a family composites comprising 3 nm palladium nanoparticles entrapped in polystyrene matrixes [14,24,25,32]. These catalysts were able to perform two types of reactions in biosystems: the deprotection of caged fluorophores, and C-C bond formation through Suzuki-Miyaura reaction. In addition, the authors were able to localize the bioorthogonal reactions intra- [14,32,33] and extracellularly [24] by controlling the size of the PdNP-styrene composites. For extracellular activation of substrates, authors used nanoparticles of 150 μm in diameter which are larger than human cells [24]. These particles were microinjected in zebra fish embryos' compartments and observed to perform localized uncaging of allyl carbamate-protected profluorophores [24].

However, for intracellular catalysis much smaller nanoparticles (<500 nm) were used to facilitate particle internalization [32]. The conjugation of these smaller Pd-styrene particles to targeting elements such as cRGDfE cyclopeptide enhanced their accumulation in human $\alpha_v\beta_3$ receptors overexpressing cell lines including U87-MG tumor cells [33].

The catalytic properties of PdNPs were also used for the activation of therapeutic agents in tumor cell cultures (Figure 2a). Deactivated 5-fluoro-uracil (5FU)-based prodrugs (with benzyl, allyl, and propargyl protecting groups at the N1 position) were uncaged by PdNP-Polystyrene particles in cell cultures, eliciting a therapeutic effect [24]. Unciti-Broceta and coworkers,in further reports developed a variety of prodrugs including gemcitabine [34], amsacrine [35], doxorubicin, and floxuridine [36] that were activatable with palladium deprotection reactions.

In a more recent report, Bradley and coworkers used a Suzuki-Miyaura reaction to intracellularly generate PP-121, a kinase inhibitor (Figure 2c) [33]. In this study, tumor cells only showed reduction in viability in the presence of all the components, the Pd-styrene particles, and each of the precursors (compound **5** and **6**, figure 2d). The nature of this strategy can, in principle, be effective to generate therapeutic drugs at the site of interest, with minimum damage to untargeted tissues and organs. However, the complexity of these reactions makes generalization to a large variety of therapeutic drugs challenging. For these reasons, the deprotection of masked prodrugs is currently more common in the use of bioorthogonal catalysis for therapy.

Very recently Unciti-Broceta employed this strategy to perform gold-mediated catalysis in biosystems. Gold nanoparticles (AuNPs) were generated inside of a polystyrene resin and demonstrated catalytic capabilities in uncaging propargylated substrates, including profluorophores and prodrugs. In this system the polystyrene scaffold provided protection against passivation of AuNPs by endogenous glutathione [25].

Nanozymes through encapsulation of TMC in nanoparticle monolayers

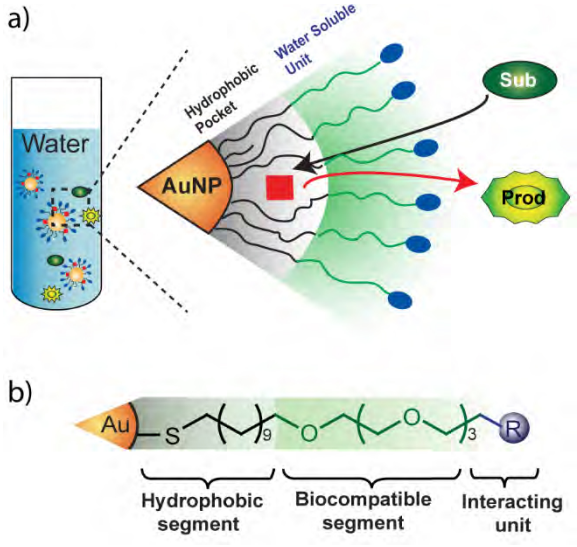


Figure 3. Structure of nanozymes based on encapsulating molecular TMC in gold nanoparticles. a) Localization of molecular TMCs in hydrophobic pockets of nanozymes b) Structure of ligands capping the AuNP scaffold.

Rotello and coworkers developed a strategy to fabricate nanozymes based on the encapsulation of molecular TMCs in nanoparticle scaffolds. In their studies, they used 2 nm gold nanoparticles (AuNPs) functionalized with specially designed ligands to generate structures with an overall size of ~10 nm [27] (Figure 3a). The ligands of these AuNPs contain three main sections: (i) an alkane chain to provide a hydrophobic inner shell to encapsulate TMCs, (ii) tetraethylene glycol to provide with a hydrophilic spacer and (iii) a terminal interacting unit that can be engineered to provide different functions including the modulation of the enzymatic behavior of nanozymes [36], and their interaction with cells [37-39] (Figure 3b). A variety of hydrophobic TMCs can be incorporated into the monolayer of these scaffolds to develop nanozymes able to perform different functions in aqueous environments [27].

The surface functionality of the monlayer-protected nanozymes plays a key role in defining their chemical properties, including their kinetic behavior [36]. Rotello and coworkers developed a family of nanozymes with positively charged surface functionalities differing in hydrophobicity. Nanozymes with hydrophobic groups displayed classical Michaelis-Menten kinetic behavior. In contrast, nanoparticles with more hydrophilic surface functionalities presented more complex kinetic behavior, including substrate inhibition effect [36].

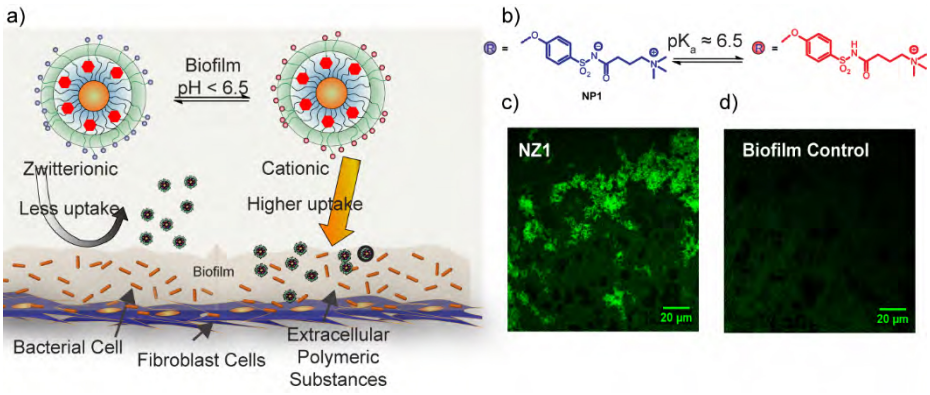


Figure 4. Engineered nanozymes surface functionalities for selective staining of biofilms a) The schematic mechanism of charge switchable NZ in biofilm imaging. **b)** Structure of pH switchable surface functionalities of nanozymes. **c)** Confocal image of biofilms stained with activated rhodamine using charge switchable NZs. d) Confocal image of biofilms only treated with prorhodamine (control). Adapted from reference 26.

The surface functionality of nanozymes also modulates their interaction with biological systems. As an example, zwitterionic nanoparticles have been observed to weakly interact with cells [40,41], while cationic nanoparticles are readily adsorb to both bacterial and mammalian cells [42-44]. With that concept in mind, Rotello and coworkers developed a family of Ru-containing nanozymes functionalized with zwitterionic pH switchable chemical groups to target acid microenvironments of biofilms (Figure 4a). Nanozymes featuring alkoxyphenyl acylsulfonamide (pKa ~ 6.5) (Figure 4b) were administered to co-culture samples containing bacterial biofilms and mammalian cells. The acidic conditions of biofilms were able to protonate this nanozymes, resulting in with a net positive charge and high localization in the bacterial biofilms domains. These nanozymes were then used to generate a rhodamine dye that provided specific staining of the bacterial domains within the co-culture (Figure 4c, d). These results show the potential of nanozymes towards selective imaging of biofilm infections, which can be beneficial for their selective treatment [26].

Proteins can be also used as nanometric scaffolds to encapsulate TMCs [45,46]. Ward and coworkers conjugated ruthenium catalysts to biotin to allow its association to an engineered streptavidin [47]. The protein-ruthenium construct was uptaken by mammalian cells and performed the catalytic uncaging of a hormone from endosomes. As a result, treated cells were able to upregulate the activity of secreted nanoluc, bioluminescence reporter [45]. In a similar way, Hoveyda–Grubbs catalyst was incorporated into an engineered streptavidin scaffold to fulfill olefin metathesis. The metathesis reaction was also demonstrated in living bacteria, evidencing the protective effect that this proteic scaffolds can provide to encapsulated TMCs [46].

Synthetic polymers are also an alternative scaffold to fabricate nanozymes. Palmans and Albertazzi covalently attached palladium bipyridine and copper phenantroline complexes to single-chain polymeric particles. The obtained nanozymes were delivered to cells to provide imaging tools [48].

Stimuli-responsive bioorthogonal nanozymes

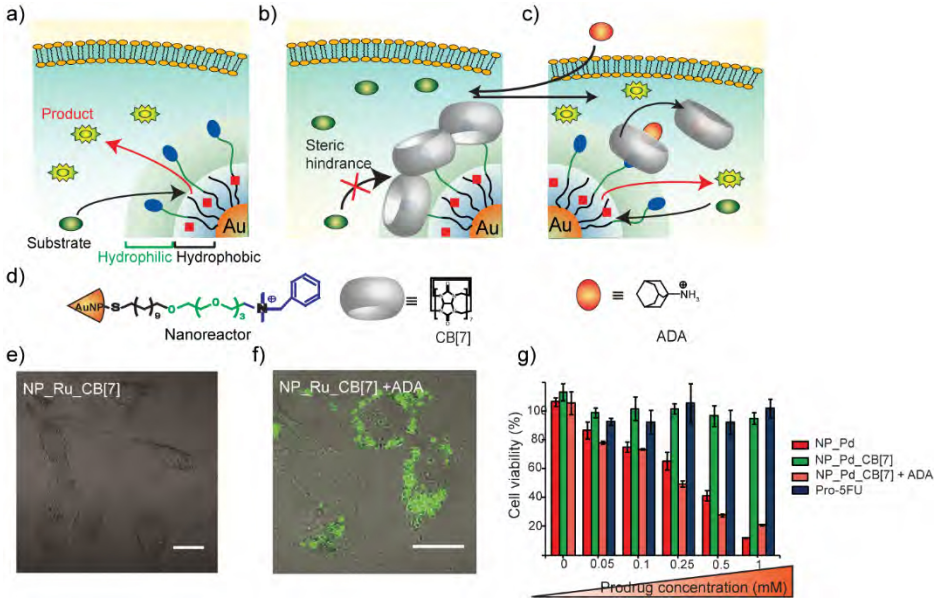


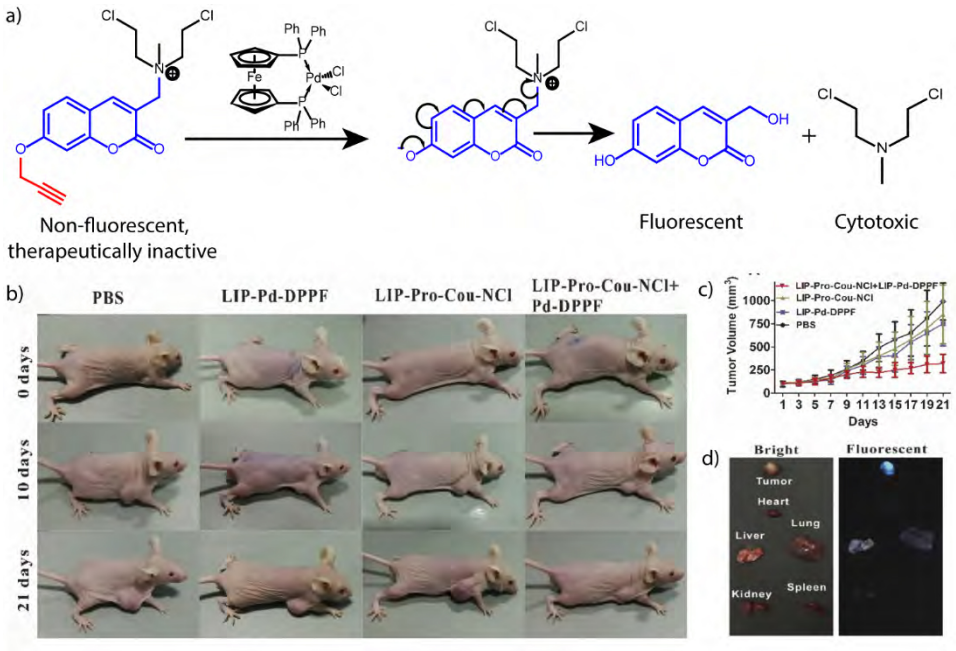
Figure 5. A stimuli responsive nanozyme a) Intracellular catalysis by NZ. **b)** Catalysis inhibition by complexation of NZ's surface benzylammonium moieties with CB[7] **c)** Reactivation of NZ's catalytic activity by adding ADA. **d)** Structure of NZ, CB[7] and ADA. **e)** Inhibited intracellular activation of prorhodamine by complexation of NZ with CB[7] **f)** Reactivation catalytic properties of NZ-CB[7] by addition of ADA. Scale bar= 20 μm. **j)** Intracellular prodrug activation using gated catalysis. NP_Pd and NP_Pd_CB[7] + ADA showed increased toxicity with increased concentration of prodrug. NP_Pd_CB[7] showed no toxicity at all prodrug concentrations tested. Adapted from reference 28.

Programable nanozymes with the ability to regulate catalytic activity through chemical and physical signals would enable dynamic control of bioorthogonal reactions. Rotello and coworkers demonstrated that AuNP nanozymes encapsulating different TMCs ([Cp*RuCl(cod)), and ferrocene diphosphine palladium chloride) can be further engineered to impart allosteric control on their catalytic activity through supramolecular interactions [28] (Figure 5). In this design, AuNPs bearing benzylammonium surface functionalities bind with curcubit[7]uril (CB[7]) to form stable host-guest complexes. Complexation of nanozymes with CB[7] blocks the access of substrates to active centers (Figure 5b), inducing complete cessation of catalytic activity. However, in the presence of a competing guest, 1-adamantylamine (ADA), CB[7] was removed from

nanozymes, allowing substrate access to catalytic sites (Figure 5c). The efficacy of this supramolecular gated-activation system was demonstrated in cells by the activation of allylcarbamate-caged pro-Rhodamine (Figure 5e, f) and propargyl-masked prodrug (Figure 5g). Activation of these substrates in cells incubated with nanozymes complexed with CB[7], was only observed after the addition of the competing guest ADA. This study constitutes an example of controlling the catalytic activity of bioorthogonal nanozymes by means of abiotic chemical signals [28].

Light provides another external tool for regulating nanozyme behavior. Qu and coworkers reported a light-controlled PdNP mesoporous silica-based nanozyme. In their system, the supramolecular interactions between α-cyclodextrin (CD) and an azobenzene ligand was used to gate the access of substrates to Pd, encapsulated within mesoporous silica particles. Irradiating with UV light, causes switching of azobenzene from *trans* to *cis* isomer, leading to the release of CD thereby activating catalysis. This system showed programable intracellular catalytic properties inducing the cleavage of allylcarbamate profluorophores and Suzuki-Miyaura coupling reaction, after irradiation with UV light [29].

In vivo bioorthogonal catalysis using nanozymes



6. Palladium-mediated bioorthogonal activation of drugs in vivo. a) Propargylated probe containing a profluorophore and a cytotoxic moiety. Uncaging the probe using palladium TMC encapsulated within liposomes induces the fragmentation of the probe releasing a fluorescent coumarin and cytotoxic drug. b) Images over time of mice treated with intratumoral injection of PBS, NCl, LIP-Pro-Cou-NCl, LIP-Pd-DPPF or LIP-Pro-Cou-NCl/LIP-Pd-DPPF. c) The tumor inhibitory rate (TIR) for three groups upon treatment with different formulations. d) Images of dissected organs of the mice treated with LIP-Pro-Cou-NCl/LIP-Pd-DPPF for 24 h. Adapted from reference 49.

Recently, palladium-mediated bioorthogonal activation of prodrugs was tested for treating solid tumors in animal models [49-51]. The first study was reported by Zeng, Wu and coworkers, where ferrocene diphosphine palladium complexes encapsulated in liposomes (LIP-Pd-DPPF) were utilized. In their study they designed a molecular probe containing a propargylated profluorophore and a cytotoxic moiety. Activation of the propargylated probe through LIP-Pd_DPPF releases a fluorescent coumarin and a cytotoxic nitrogen mustard (HN1) (Fig. 6). Animal models were generated by implanting HeLa cells in the right flank of nude mice. LIP-Pd-DPPF followed by the specially designed probe were intratumorally administered. Only mice treated with both encapsulated palladium complex and the prodrug, exhibited inhibition of tumor growth (Figure 6c). A fluorescence study illustrated that most of the activated molecules were retained at the site of injection (Figure 6d) [49].

Weissleder and coworkers, utilized palladium allyl carbamate deprotection reactions for site selective generation of doxorubicin in tumor bearing mice. In their study

nanozymes were fabricated by encapsulating a bisphosphine palladium(II)dichloride complex into a biodegradable polylactic acid-polyethyleneglycol (PLGA-PEG) copolymers. Both, nanozymes and proDox, were administered intratumorally to mice to contain their tumor growth. Interestingly, although proDox was observed to spread among several organs of treated mice, only activated Dox was observed in tumorous tissues where their nanozymes were also present [50].

Similarly, Hoop *et al.* synthesized bimetallic Fe-Pd magnetic nanowires for the heterogeneous catalytic activation of pro-5FU in tumor-bearing mice. In this study authors injected Fe-Pd intratumorally and pro-5FU intravenously. Significant reduction of tumor growth and vascular invasion was obtained for specimens that were treated with the combination of the prodrug and Fe-Pd bimetallic nanoparticle [51].

Concluding remarks and future perspectives

Bioorthogonal catalysis is rapidly evolving as a practical tool for the delivery of therapeutic and imaging molecules into biosystems. *In vivo* studies have demonstrated selective activation of drugs in tissues where nanozymes have been implanted. In addition, these studies have shown promising results in the development enhanced chemotherapies that require repeated generation of drugs.

It is important to highlight that most chemotherapeutics are administered to organisms systemically, As such, further studies need to be carried to elucidate pharmacokinetic/pharmacodynamic phenomena of nanozymes, including i- the natural biodistribution of nanozymes systemically administered to organisms, ii- the degradation and clearance of nanozyme components from biosystems, and iii- the active accumulation in desired tissues/organs of nanozymes conjugated to targeting elements. Data collected from these studies will provide valuable information to develop methods to administrate nanozymes and prodrugs to organisms, as well as for designing proper therapies in general.

Finally, we believe that in the specific case of programable nanozymes, new designs should be able to reversibly turn on and off their catalytic properties upon changes in environmental conditions. Such smart devices could be used to enhance the spatiotemporal controlled activation of drugs into targeted tissues. Further designs can include elements that allow to self-modulate their catalytic activity with endogenous signals and reach homeostatic states.

Acknowledgements

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