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Divya Dheer, Julien Nicolas, Ravi Shankar. Cathepsin-Sensitive Nanoscale Drug Delivery Systems for Cancer Therapy and other Diseases. *Advanced Drug Delivery Reviews*, 2019, 151-152, pp.130-151. 10.1016/j.addr.2019.01.010 . hal-02323749

HAL Id: hal-02323749

<https://hal.science/hal-02323749>

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Cathepsin-Sensitive Nanoscale Drug Delivery Systems for Cancer Therapy and other Diseases

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Abstract

Cathepsins are an important category of enzymes that have attracted great attention for the delivery of drugs to improve the therapeutic outcome of a broad range of nanoscale drug delivery systems. These proteases can be utilized for instance through actuation of polymer-drug conjugates (e.g., triggering the drug release) to bypass limitations of many drug candidates. A substantial amount of work has been witnessed in the design and the evaluation of Cathepsin-sensitive drug delivery systems, especially based on the tetra-peptide sequence (Gly-Phe-Leu-Gly, GFLG) which has been extensively used as a spacer that can be cleaved in the presence of Cathepsin B. This Review Article will give an in-depth overview of the design and the biological evaluation of Cathepsin-sensitive drug delivery systems and their application in different pathologies including cancer before discussing Cathepsin B-cleavable prodrugs under clinical trials.

Keywords

Cathepsins, enzymes, cancer, drug delivery, optical imaging.

1. Introduction

Cathepsins are widely known proteolytic enzymes whose main function is to degrade proteins or peptides [1]. Nevertheless, this perception has changed over the past many years as they are being considered as important signaling molecules playing different crucial roles [2, 3]. There are dozens of Cathepsins which are classified according to their structure, catalytic mechanism and substrate. Based on the human genome draft sequence, the main Cathepsin categories are serine (Cathepsin A and G), aspartic (Cathepsin D and E) and lysosomal cysteine proteases (Cathepsin B,C,F,H,K,L1,L2/V,O,S,W,X/Z) [4, 5]. They have multiple functions, as one finds digestive proteases (present in saliva, stomach and intestines) for food processing inside the gastrointestinal tract (GIT), lysosomal proteases for intracellular housekeeping or caspases for transduction of one-way signal in apoptosis [6-8]. Interestingly, lysosomal Cathepsins (i.e., intracellular enzymes) have been widely involved in drug targeting as they require a slightly acidic environment to exhibit optimal enzymatic activity [9-11]. Given the features of disease-associated proteolysis (i.e., cleavage of amide bond), different types of prodrugs, nanocarriers, biomaterials or probes, have been designed and synthesized to exert their activity in endosomal/lysosomal compartments [12-14]. For instance, Cathepsins can induce the release of active ingredients from nanocarriers, chemically or physically, leading to enhanced therapeutic activity or *in situ* imaging sensitivity [15]. Kopecek, Duncan and others have shown the importance of protease-cleavable linkers, especially those sensitive to Cathepsin B, in polymer-based, nanoscale drug delivery constructs for enhancing the *in vivo* delivery of drugs to tumor tissues [16-18].

Cysteine cathepsins and their substrate interaction have been well-identified on the basis of papain (*Carica papaya*) used as a model of lysosomal proteases, as first introduced by Schechter and Berger [19]. In this model, the substrate residues (P) as well as the subsites (S) were given nomenclature based on their position bonded to the protease surface. Later, this model was revisited by Turk *et al.* [20] who showed that the subsites were positioned on the left-hand side (i.e., S2', S1 and S3) along with right-hand side of the active site (i.e., S1' and S2), and further composed of two L-domain loops consisting of Gln-19–Cys-25 as well as Arg-59–Tyr-67

residues and two R-domain loops consisting of Leu-134–His-159 as well as Asn-175–Ser-205 residues (Figure 1) [5].

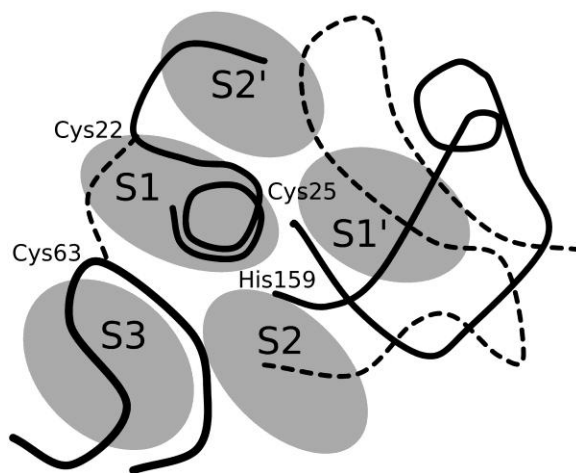
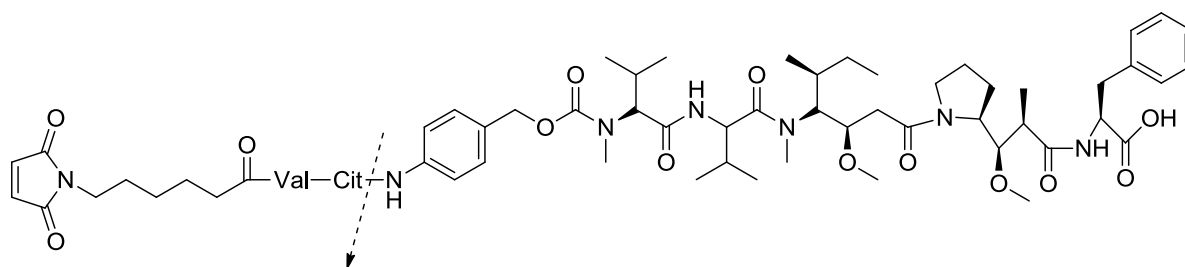
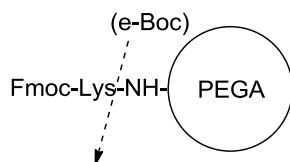


Figure 1. Schematic representation of the revised papain model showing different subsites based on substrate-mimicking inhibitors bonded to the active-site cleft. Reproduced with permission from Ref. [5].

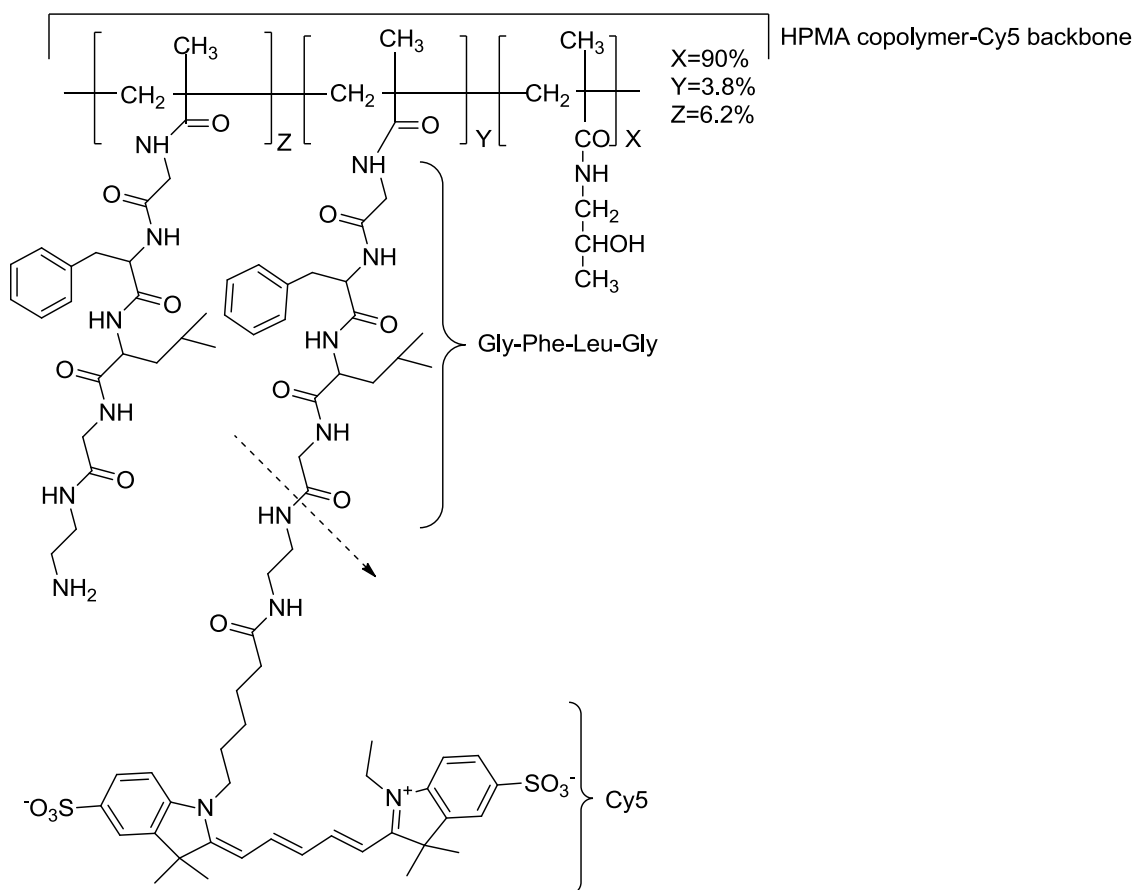
Among the different pathologies, Cathepsins have been largely employed as leverage to treat cancer from various Cathepsin-sensitive drug delivery systems because of its overexpression at the tumor sites. For instance, cysteine proteases have increased activity as well as aberrant localization within the tumour microenvironment, which contributes to cancer progression, proliferation and metastasis [21]. Such findings led to the development of the glycyl-penylalanyl-leucyl-glycine (GFLG) sequence that is hydrolyzed by Cathepsin B. In this area, poly(*N*-(2-hydroxypropyl)methacrylamide-doxorubicin (PHPMA-Dox, also called PK1) were the first clinically investigated conjugates for anticancer therapy that comprised Cathepsin-sensitive degradable GFLG sequences [22]. Since PK1, several PHPMA-drug conjugates have entered clinical trials [23, 24], which confirmed the great potential of these systems. Some of the structures of the different drug delivery conjugates are gathered in Figure 2 along with their cathepsin cleavable sites for better understanding [25-27].



1. N-terminus linked monomethylauristatin F derivative



2. Bis(2-acrylamido-1-yl)poly(ethylene glycol) based conjugate



3. HPMA copolymer-Cy5 cathepsin-B cleavable bioconjugate

Figure 2. Structure of different cathepsin-sensitive drug-linker bioconjugates along with their indicated cleavable sites.

Whereas several reviews have already been published on targeted polymer-based drug delivery systems [28-31], cysteine Cathepsins as imaging probes [32-35], ageing and neurodegeneration [36], disease management [37] and other protease functions [38, 39], the dynamic involvement of Cathepsins in targeted drug delivery systems including their role in various diseased states and their clinical prospects have never been covered in a single Review Article.

2. Cathepsin-sensitive drug delivery systems

2.1 Anticancer drug delivery systems

In the past few decades, anticancer drug delivery has attracted extensive interest from both academia and industry. A considerable effort is being spent on the design of nanoscale systems having suitable properties for drug delivery purposes such as stealthiness, non-immunogenicity, biocompatibility as well as biodegradability. The fate of stealth nanoscale systems is governed, at least in part, by the enhanced permeability and retention (EPR) effect (also called passive targeting). It allows for their preferential accumulation at the tumor site because of leaky vasculatures and lack of lymphatic drainage [40, 41]. Interestingly, a variety of different Cathepsins have been reported to be overexpressed in many types of cancers; mostly found in cancer cells but also in cancer-associated leukocytes, fibroblasts, osteoclasts, myoepithelial cells as well as endothelial cells [42]. The list of cancer overexpressing Cathepsins is given below (Table 1). Hence, the intimate relationships between Cathepsins and cancer stimulated the conception of (macro)molecules sensitive to the presence of Cathepsins for enhanced therapeutic effect.

Table 1. List of Cathepsin Overexpressing Cancer Types.

| Family | Cathepsin | Location | Tumour site | Reference |
|-----------------------|-------------|--------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|-------------|
| Cysteine Proteases | General | Intracellular, lysosomes | Most | [42-44] |
| | Cathepsin K | Extracellular | Breast, bone | [45-49] |
| | Cathepsin B | Extracellular and pericellular under pathological conditions | Breast, cervix, colon, colorectal, gastric, head and neck, liver, lung, melanoma, ovarian, pancreatic, prostate, thyroid | [50-61] |
| | Cathepsin L | | Breast, colorectal | [62-65] |
| Aspartic Proteases | Cathepsin E | Endosomal structures, ER, Golgi bodies | Cervical, gastric, lung, pancreas adenocarcinomas | [61, 66-70] |
| | Cathepsin D | Lysosomes | Breast, colorectal, ovarian | [71-77] |

In the following, we have covered Cathepsin-sensitive drug delivery systems for anticancer therapy, by distinguishing five different types of systems: (i) polymeric; (ii) inorganic; (iii) dendritic/comb-like; (iv) lipidic and (v) protein-based/peptidic.

2.1.1 Polymeric systems

Different types of polymeric systems have been utilized to develop drug-polymer conjugates for anticancer drug delivery [28, 78-85]. Given Cathepsin B is a lysosomal cysteine protease overexpressed in the microenvironment of advanced tumors [86], this feature has been widely exploited in cancer therapy using polymer-based drug delivery systems bearing the Cathepsin B-sensitive GFLG sequence [87]. This area was pioneered by Kopeck who developed PHPMA-based drug conjugates containing GFLG sequences on the polymer backbone as well as on the side-chains, giving enhanced therapeutic efficacy while still maintaining their biocompatibility.

This system was further extended to a two-drug combination approach using gemcitabine (Gem, unstable *in vivo*) and paclitaxel (Ptx, poorly water soluble) linked to either diblock, tetrablock or hexablock PHPMA copolymers obtained by a combining RAFT polymerization and “click” chemistry (Figure 3). The diblock copolymer ($M_n \sim 100$ kDa) was found to be the most efficient one *in vivo* on A2780 human ovarian carcinoma xenografts in nude mice. It indeed showed a more pronounced synergistic antitumor effect compared to other structures, thus overcoming the limitations of the free drug.

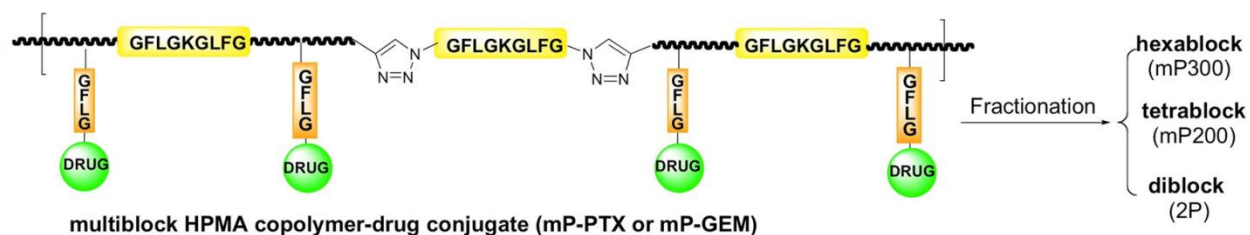


Figure 3. Illustration of GFLG-containing PHPMA prodrugs. Adapted with permission from ref. [87].

The strongest synergistic interactions in acute myeloid leukemia (AML) was also observed as assessed in HL-60 human AML cells when cytarabine and GDC-0980 were linked to similar GFLG-bearing PHPMA copolymers, conversely to daunorubicin or JS-K [88]. Similarly, another study reported on the combination of GDC-0980 (P13K/mTOR inhibitor) and docetaxel against prostate cancer and showed promising results (Figure 4) [89]. Several other combinations directed against cancer have also been explored from PHPMA copolymer bearing GFLG sequences [90-94].

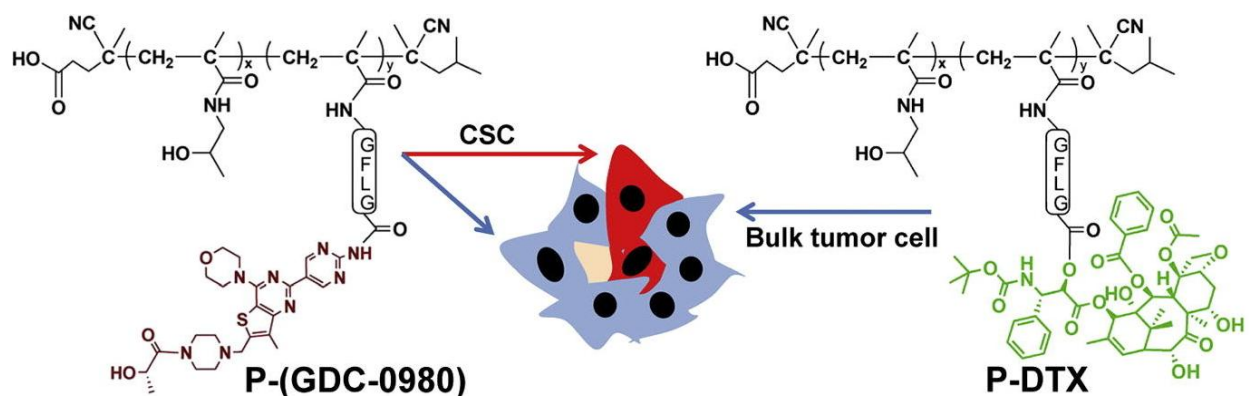


Figure4. GFLG-containing PHPMA prodrugs for combination therapy (GDC-0980 and docetaxel) for prostate cancer exhibiting effective anti-Cancer Stem Cell (CSC) effect and *in vitro* increased anti-bulk tumor effect. Adapted with permission from Ref. [89].

In a more mechanistic study, two PHPMA-based multiblock S-CMP (small copolymer block size) and L-CMP (long copolymer block size) have been synthesized [95]. Both the copolymer blocks and the peptide linkers were tagged with ^{125}I and ^{177}Lu , respectively (Figure 5). S-CMP showed increased cleavage rates by Cathepsin S compared to L-CMP resulting from the lower steric hindrance as assessed by *in vitro* studies. The cleavage and clearance of the different blocks were both greater inside the tumor and the liver, as observed from radioisotopic ratios.

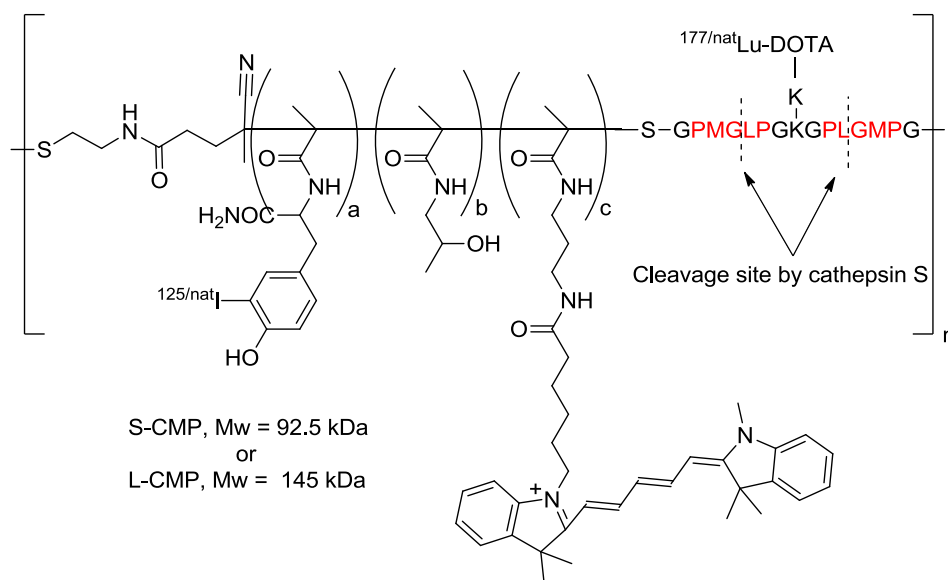


Figure5. Chemical structure of PHPMA-based, dual-labeled small copolymer block size (S-CMP) and long copolymer block size (L-CMP) showing cleavage sites for CathepsinS [95].

Dox has been conjugated to different polymeric architectures via Cathepsin-sensitive linkers. For instance, Dox was linked to an octa-guanidine-based peptide sequence (Phe-Lys) via 4-aminobenzyloxy carbonyl (PABC) as a self-immolative linker, resulting in a G8-PP1-FK-PABC-Doxprodrug. It was able to be cleaved by lysosomal Cathepsin B and inducing selective toxicity against HeLa cells without affecting healthy cells [96]. On the contrary, small-molecule ($\text{MW} < 500 \text{ g.mol}^{-1}$) self-assemblies have also been utilized to develop a generic cross-linked micellar drug delivery system based on gemcitabine (Gem) prodrugs (Figure 6a). This system proved to be advantageous as compared to well-known polymeric micellar systems in terms of composition, colloidal stability, drug payload ($\sim 58 \text{ wt.}\%$), biosafety, as well as ease of synthesis, functionalization and *in vitro/in vivo* anticancer activity [97-99]. Infact, nearly 60% of the drug was released from the micelles by Cathepsin B in phosphate buffer saline (PBS) at pH 5.5 for 240 h conversely to $< 7\%$ without Cathepsin B because of the amide bond in between the drug and the promoiety (Figure 6b) [100].

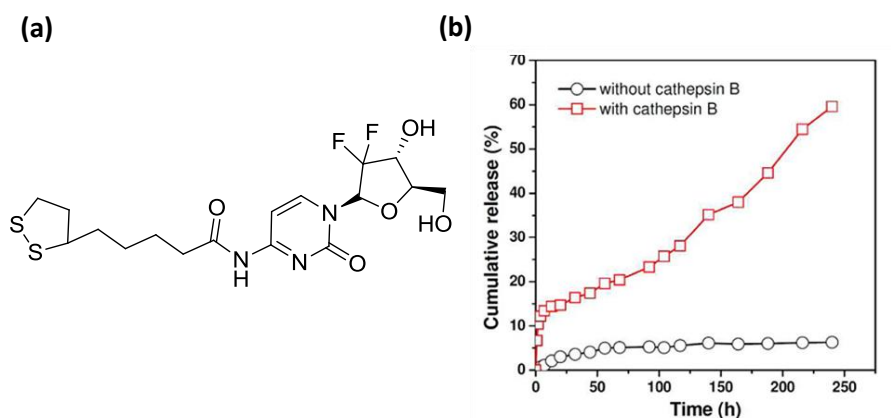


Figure 6. Chemical structure of the gemcitabine prodrug (a) and *in vitro* drug release profile from the gemcitabine prodrug micellar system (b). Adapted with permission from Ref. [100].

Another report focused on the construction of PEGylated, enzyme-sensitive, macrocyclic pillar[5]arene amphiphiles which self-assembled in water into micelles with high Dox loading capacity [101]. The micelles had enzyme-cleavable amide bonds that were cleaved by L-asparaginase (L-ASP) used here as a mimic of intracellular Cathepsin B because it can catalyze the hydrolysis of asparagine to aspartic acid (Figure 7). The Dox-loaded micelles led to significant cytotoxicity on MCF-7 and multidrug-resistant MCF-7/ADR cells, comparatively to drug-free micelles.

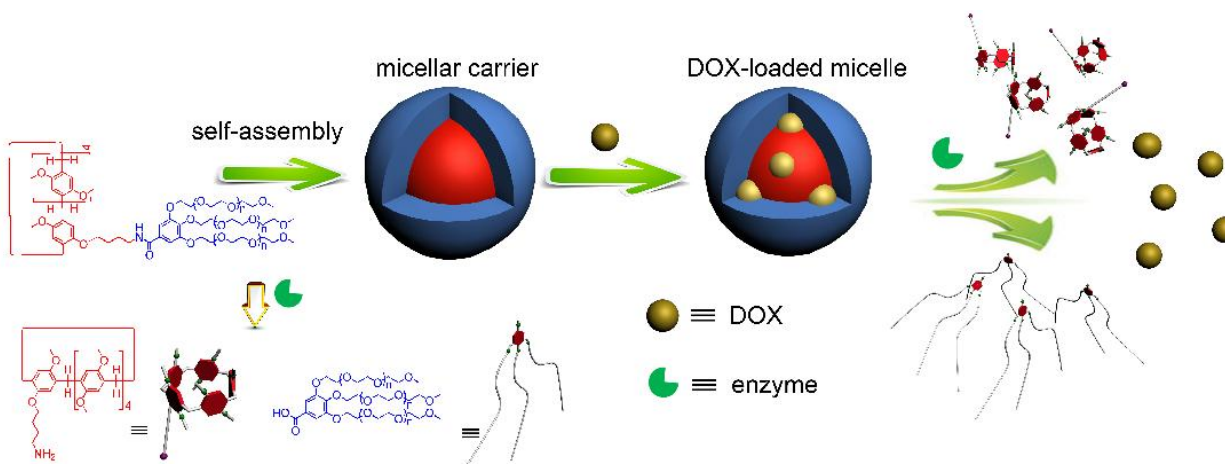


Figure 7. Structure of Dox-loaded, PEGylated, enzyme-sensitive, macrocyclic pillar[5]arene amphiphiles and their self-assembly into micelles. Adapted with permission from Ref. [101].

Folic acid (FA) surface-functionalized, biodegradable poly(ethylene oxide)-*b*-poly(L-glutamic acid) (FA-PEG-*b*-PLG) block copolymer vesicles loaded with cisplatin were also reported [102]. The drug was released intracellularly from the rigid block due to overexpressed Cathepsin B which cleaved the nanostructure because of the increased activity of this proteolytic enzyme in metabolizing PLG acid residues. The enzyme was also responsible for the higher activity in metabolizing polyglutamate (PGA) residues. The nanovesicles exhibited surface-positioned FA moieties for active targeting *via* selective cell binding and led to enhanced cytotoxicity towards HeLa cells.

PGA was also used as a polymer scaffold to link both Ptx and an integrin-targeted ligand (E-[c(RGDfK)₂]) on the side chains, to give PGA-Ptx-E-[c(RGDfK)₂]. The resulting conjugate gave significant enhancement in anticancer activity compared to free Ptx [103]. As assessed by the *in vitro* drug release profile, Ptx was released in the presence of Cathepsin B but PGA-Ptx-E-[c(RGDfK)₂] was found to be stable in plasma. Interestingly, incorporation of a targeting ligand towards integrin expressing cells led to anti-angiogenic mechanism to overcome multi-drug resistance.

Another targeted drug delivery system was reported and consisted in a heterobifunctional oligomeric PEG chains embedding octreotide as a ligand for the targeting of somatostatin receptors and either an anticancer drug (Dox) tethered *via* a dipeptidic substrate for Cathepsin B, or a fluorescent dye [104]. This oligomeric prodrug system was suitable for tumor cell imaging expressing both Cathepsin B and somatostatin receptors and led to selective cytotoxicity towards cancer cells.

2.1.2 Inorganic systems

Inorganic materials (e.g., silica, gold, iron oxide, quantum dots, etc.) is also an attractive family of materials that have been extensively investigated for anticancer drug delivery [105-111]. In this area, a Cathepsin B-induced tumor targeted drug delivery system loaded with Dox was developed by immobilizing cleavable rotaxanes onto mesoporous silica nanoparticles (MSNs) [112]. Nano-constructs comprising a rotaxane moiety and a GFLG sequence linked to the RGDS peptide were used as Cathepsin B-cleavable stoppers for the cyclodextrin valves by means of “click” chemistry (Figure 8). Thanks to the targeting ligand displayed at its surface, such system

demonstrated efficient receptor-mediated tumor cell uptake and selective enzymatic digestion of GFLG peptide.

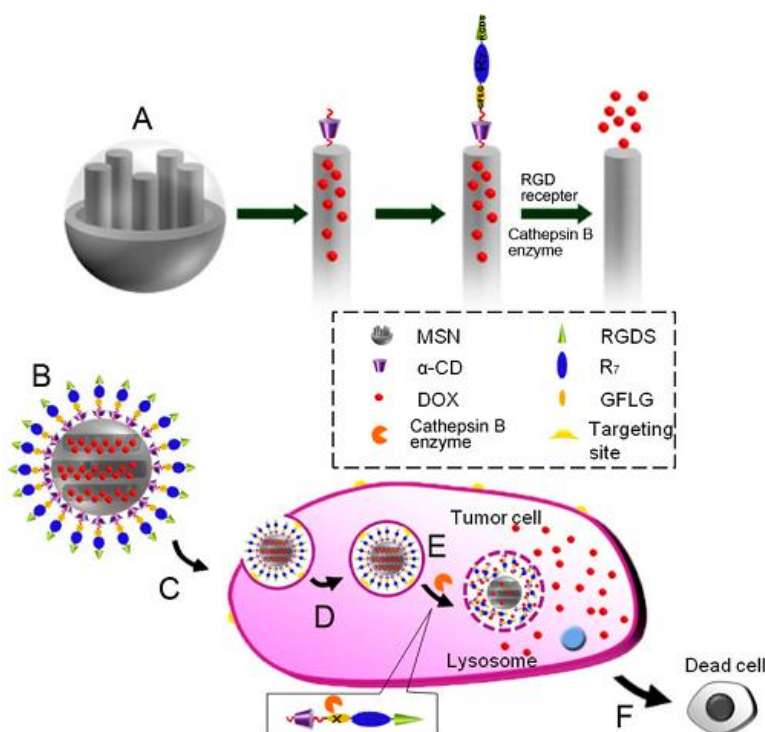


Figure 8. (A) Mesoporous silica nanoparticle (MSN) functionalization. (B) Cathepsin-sensitive Dox-loaded MSNs. (C) Cell integrin receptor-mediated targeting by RGDS (Arg-Gly-Asp-Ser). (D) Endocytosis. (E) Drug release mediated by Cathepsin B. (F) Tumor cell death. Adapted with permission from Ref. [112].

MSNs were also coated with Cathepsin B-sensitive peptide sequences (alkynyl-GIVRAKEAEGIVRAK-OH) through triazole rings and led to efficient Dox release (Figure 9). The study also proved that this peptide sequence was selectively cleaved by Cathepsin B as assessed by *in vitro* experiments [113].

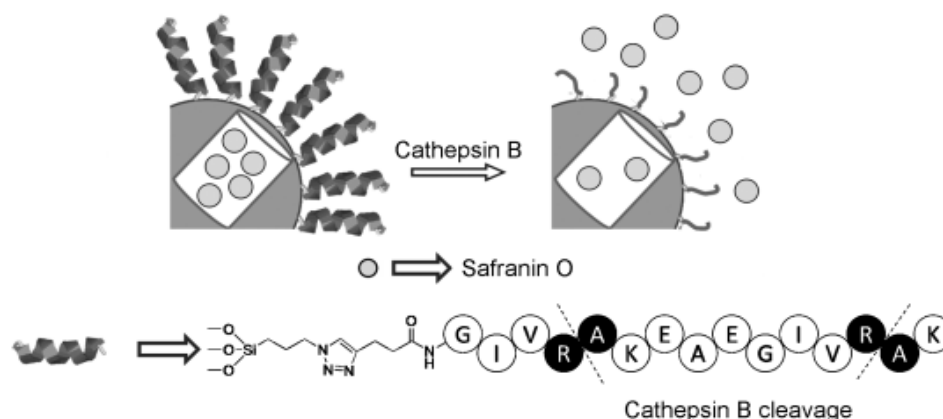


Figure 9. Synthesis of gatekeeper-supported functionalization with 3-(azidopropyl)triethoxysilane capped with peptide sequence and further delivery of Dox by action of Cathepsin B. Adapted with permission from Ref. [113].

This peptide sequence was also anchored onto silica supports to develop nanoparticles with prevented release the loaded $[\text{Ru}(\text{bipy})_3]^{2+}$ dye unless specific proteases are present [114]. In another study, an enzyme-cleavable peptide precursor conjugated to Dox was further linked onto the surface of silica-coated magnetic nanoparticles by using “click” chemistry (Figure 10) [115]. The nanocarriers exhibited efficient Dox release and selective intracellular Dox delivery into tumors with high Cathepsin B expression together with imaging of cancer cells.

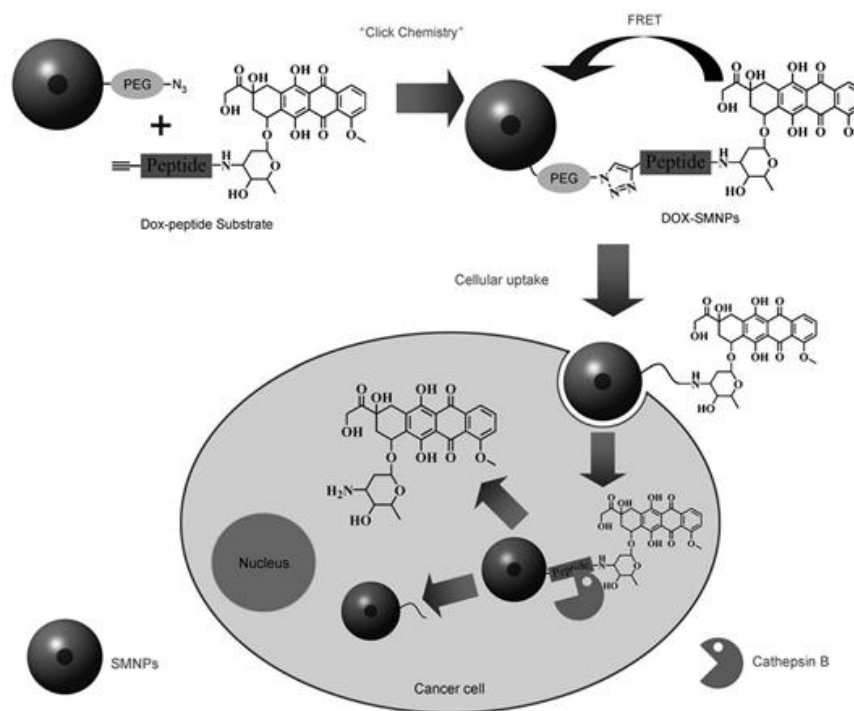


Figure10. Synthesis of Dox-peptide-coated, magnetic silica nanoparticles cleaved by Cathepsin B for Dox release inside cancer cells. Adapted with permission from Ref. [115].

A dual enzymatic responsive nanoconstruct for pancreatic cancer therapy was engineered and relied on surface functionalization of CdSe/ZnS quantum dots (QDs) by an amphiphilic PEG-GGPLGVRGK-NH₂ polymer sensitive to matrix metalloproteinases (MMP-9), and by Gem *via* a GELG Cathepsin B substrate sequence [116]. Some of the PEG chains were also functionalized by cyclo-RGD as a tumor-homing ligand. The nanocarrier exhibited long circulating features and increased drug accumulation at tumor sites, resulting in successful delivery of Gem in BxPD-3 cells because of their inherently elevated concentrations of Cathepsin B (Figure 11).

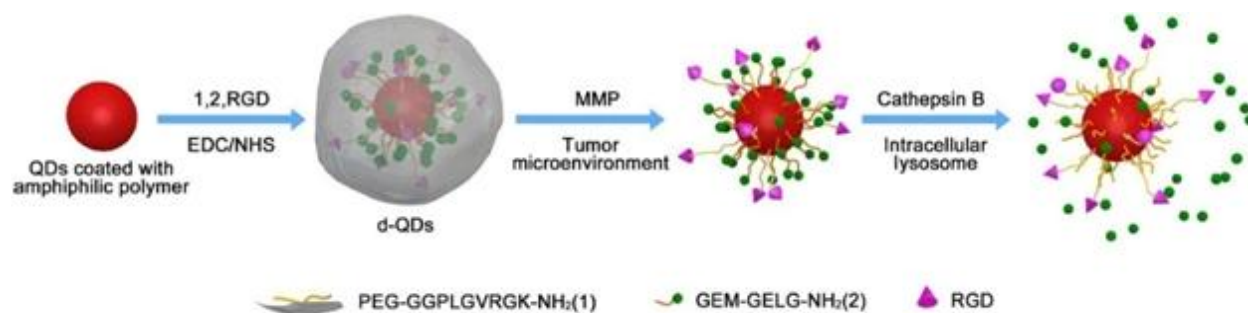


Figure11. Synthesis of Gem-loaded, decorated QDs and their dual enzymatic behavior. Adapted with permission from Ref. [116].

2.1.3 Dendrimeric/comb-like systems

Dendrimers, which are perfectly monodisperse and highly branched 3D macromolecules, have been the topic of great attention especially as drug carriers [117-119]. For example, peptide dendrimers surface-functionalized by methoxy polyethylene glycol (mPEG) and Dox through the GFLG sequence have been designed [120]. The resulting enzyme-responsive dendrimer-GFLG-Dox nanocarrier gave greater accumulation and retention in ovarian tumor cells (SKOV-3), leading to improved anticancer effect and no obvious systemic toxicity. Similarly, mPEG-PAMAM dendrimers of different chain lengths for the formation of Dox-loaded magnetite nanoparticles have also been reported [121]. In this system, Cathepsin B was used to selectively degrade the dendritic shell to trigger sustained Dox release near the tumor cells. The concept of enzymatic breakdown of the nanocarrier may represent a new approach for controlled drug delivery systems. Also, Cathepsin B-responsive and amphiphilic PEGylated dendritic polymer-drug conjugates (PEGylated dendron-GFLG-Dox) were obtained by “click” chemistry and led to enhanced antitumor efficacy (Figure 12).

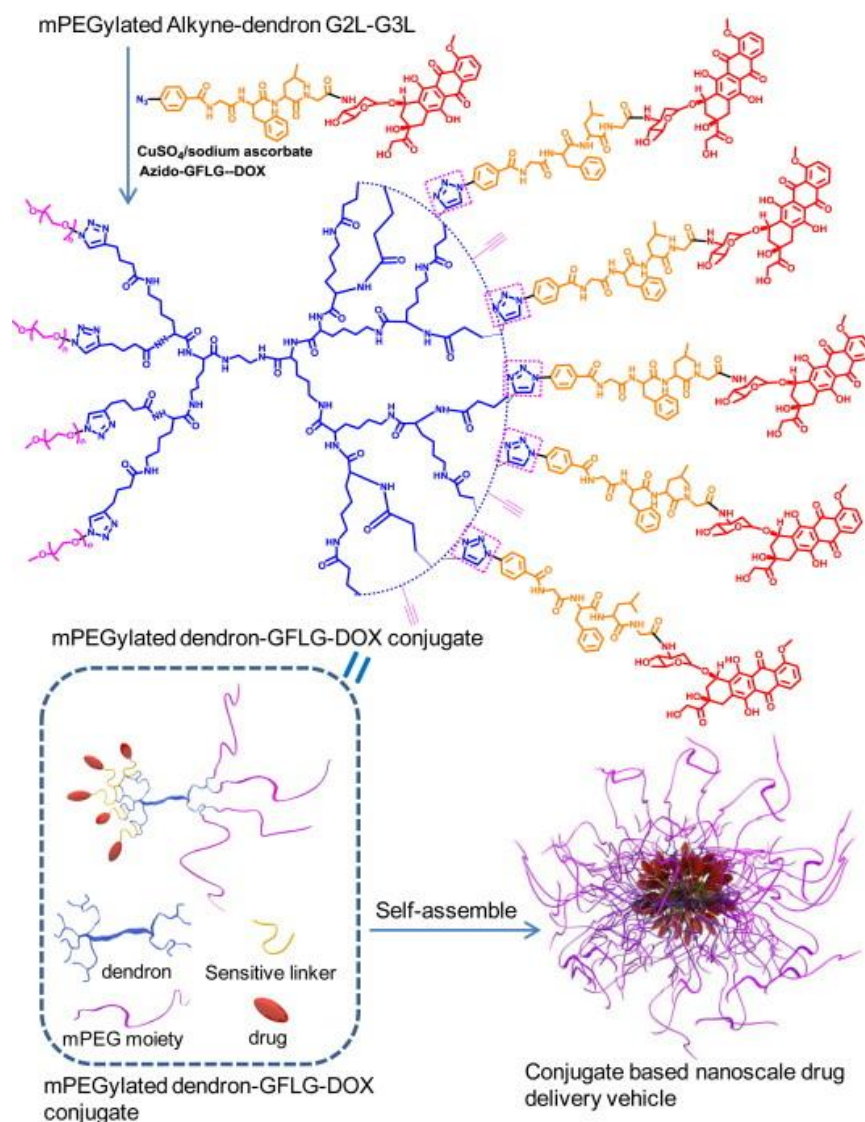


Figure 12. Synthesis of amphiphilic PEGylated dendron-GFLG-Dox conjugate followed by its self-assembly into NPs. Adapted with permission from Ref. [121].

Another study reported on the combination of undecapeptide KKLFFKKILKKL-NH₂ with the GFLG sequence for the delivery of chlorambucil (CLB) [122]. The free drug was inactive (IC₅₀ = 73.7 to >100 μM) conversely to its prodrug (IC₅₀ = 3.6 – 16.2 μM) on various cancer cell lines including MCF-7, PC-3, CAPAN-1, 1BR3G and SKMEL-28. CLB-Gly-OH was indeed released when Cathepsin B was present as evidenced by Cathepsin B enzymatic assays. Also, these studies supported the fact that CLB would be released in the lysosomal compartment. A

comparative study was reported between dendrimers based on mPEG conjugated to Dox via a Cathepsin B-cleavable Gly-Phe-Leu-Gly sequence and GFLG-free dendrimers [123]. The GFLG sequence-bearing nanoconstructs were formulated into nanoparticles exhibiting Cathepsin B-sensitive drug delivery properties. The enhanced anticancer activity compared to that of free Dox was validated *in vivo* in a CT26 tumor xenograft mouse model.

Asorbitol scaffold functionalized by octa-guanidine moieties and conjugated to Dox *via* a GLPG sequence, another peptidic substrate of Cathepsin B, was produced (Figure 13) [124]. This conjugate was efficiently taken up by the cells via electrostatic interaction between guanidine moieties and negatively-charged phospholipids/sulphates exposed at the surface of the cells. Dox was then released into lysosomes via selective cleavage by Cathepsin B. Enhanced cytotoxicity compared to that of free Dox was obtained on HeLa cells that are known to express Cathepsin B.

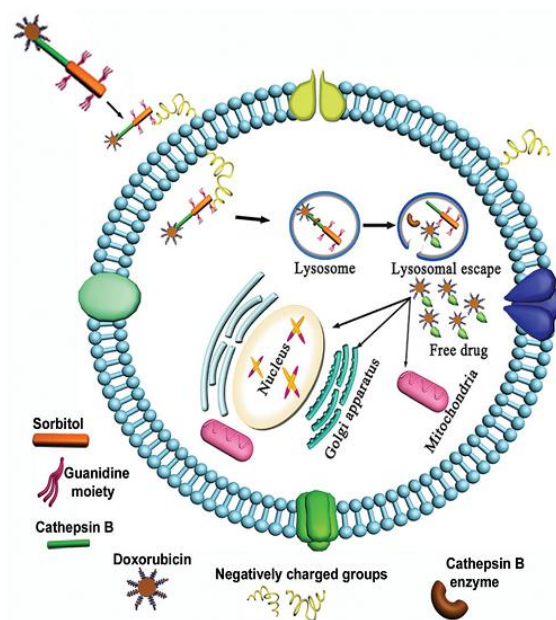


Figure13. Schematic representation and proposed action mechanism of a sorbitol scaffold functionalized by octa-guanidine moieties and conjugated to Dox. Adapted with permission from Ref. [124].

2.1.4 Lipidic systems

A great amount of work is also currently been carried out to design lipid-based drug delivery systems either as drug-loaded lipidic nanocarriers or lipidic prodrug nanocarriers [125, 126]. However, examples of Cathepsin-sensitive lipidic drug delivery systems are rather scarce. For instance, when a lipidated Cathepsin B inhibitor (NS-629) was anchored into a liposome bilayer (Figure 14), its selective targeting and internalization into tumors and stromal cells was shown *ex vivo* and *in vivo*, confirming that using Cathepsin B as an efficient leverage for cancer diagnosis and treatment [127].

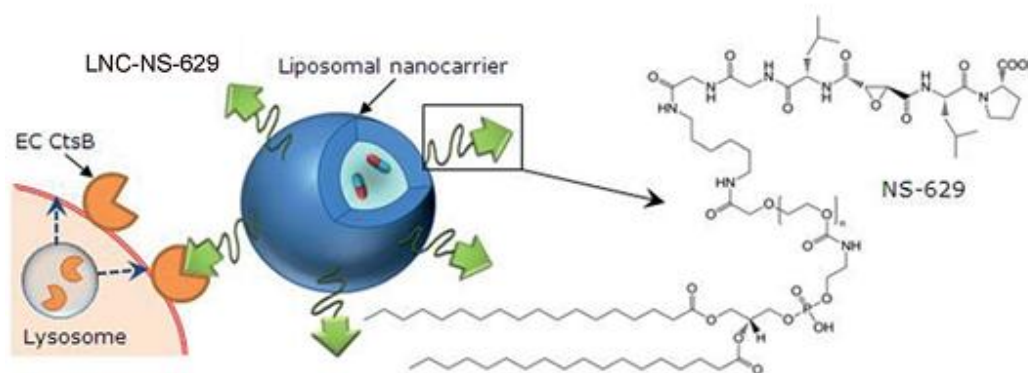


Figure 14. Schematic representation showing conjugation of the lipidated Cathepsin B inhibitor (NS-629) at the surface of a liposome to target extracellular (EC) Cathepsin B. Adapted with permission from Ref. [127].

Combination therapy, that relies on the simultaneous administration of at least two different drugs, is increasingly used to treat various diseases, including cancer [128, 129]. Combination therapy from cathepsin-sensitive lipidic systems was illustrated by the conception of methotrexate-methoxypoly(ethylene glycol)-1,2-distearoyl-snglycero-3-phosphoethanolamine (Mtx-MePEG-DSPE) prodrug micelles loaded with mitomycin C-soybean phosphatidylcholine (SPC-MMC) prodrugs [130]. This micellar system exhibited synergistic anticancer activity in presence of Cathepsin B because of the amide linker in between the polymer and the drugs, as opposed to the action of individual drugs.

2.1.5 *Protein-based/peptidic systems*

Drug delivery systems based on proteins or peptides represent an appealing class of materials especially because of their biocompatibility [131-135]. For instance, proteinticles, which are proteins that can self-assemble inside cells into nanoscale particles, can be employed in many different biomedical applications owing to their enhanced biocompatibility, conversely to synthetic nanomaterials [136]. Conferring cathepsin-sensitivity to such systems have also been reported, especially for small interfering RNA (siRNA) delivery where it showed great potential against various cancers. For instance, proteinticles based on human ferritin were genetically engineered to display at their surface different functional peptides in a simultaneous manner, such as cationic peptides for self-assembling siRNA, cancer cell-targeting or cell penetrating peptide [137]. They led to enhanced siRNA capture, cancer cell targeting together with enhanced penetration into the cytoplasm of tumor cells. They were eventually cleaved by Cathepsin B for intracellular release of siRNA inside tumor cells, leading to efficient gene silencing. One of the greatest advantages of proteinticles is that such functional peptides of different nature can be evenly placed on their surface, depending on the tumor cell type through a simple genetic modification, thus making it a very versatile system for targeted siRNA delivery. Another study revealed the development of a polyglutamate amine (APA) nanocarriers containing miRNA and siRNA polyplexes which showed great accumulation into pancreatic tumor cells [138]. It was also shown that the release of miRNA occurred from APA-containing polyplex in the presence of Cathepsin B.

Given the poor water-solubility of many anticancer drugs, a considerable amount of research has been done to improve their hydrophilicity by conjugation to hydrophilic moieties via Cathepsin-sensitive linkers. For instance, Ptx has been conjugated to a highly water-soluble nucleolin aptamer (NucA) for the targeting of ovarian cancer with reduced off-site toxicity [139]. The resulting bioconjugate proved to be biologically stable as assessed by fluorescence resonance energy transfer (FRET) (Figure 15) and also inactive in the blood circulation. NucA was conjugated to the hydroxyl group at position 2' of the drug via a dipeptide bond sensitive to Cathepsin B, which then got cleaved once inside the cells by Cathepsin B, thus triggering the anticancer mechanism.

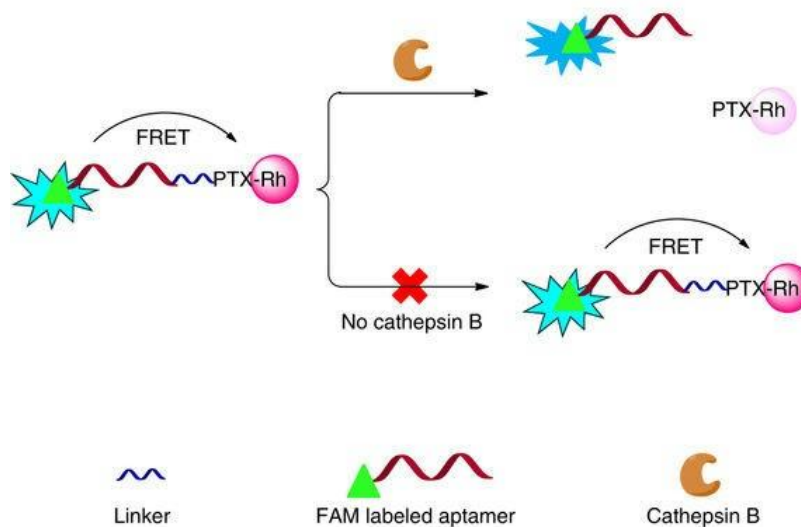


Figure 15. Schematic illustration of the *in vivo* tracking of the degraded Cathepsin B-labile dipeptide bond linker exploiting FRET with fluorescein amidate (FAM) and dual-labeled rhodamine B (Rh) NucA-Ptx bioconjugate. Adapted with permission from Ref. [139].

The GFLG sequence was also embedded into a star-shaped peptidic prodrug structures that can be cleaved by Cathepsin B. This feature has been used to develop drug delivery vehicles for 2-methoxyestradiol (2ME) which is a natural metabolite of estradiol with antiproliferative and anti-angiogenic activities (Figure 16) [140].

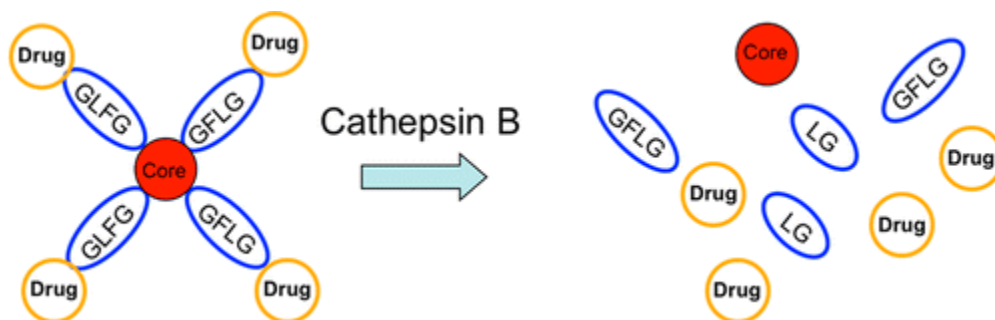


Figure16. Representation of the degradation of star-shaped peptidic prodrug structures that can be cleaved by Cathepsin B. Adapted with permission from Ref. [140].

In the context of combination therapy, a dual-functionalized linker bearing Dox and Ptx, and comprising a maleimide moiety for its subsequent coupling to albumin through its cysteine-34 position, was designed [141]. Each drug was linked by a self-immolative para-aminobenzyloxy carbonyl linker and a cleavable dipeptide (Phe-Lys) sensitive to Cathepsin B, leading to drug release at the tumor site (Figure 17). A similar approach combining a polymer prodrug and a polymer-enzyme bioconjugate was used to selectively and rapidly deliver a cytotoxic drug to the target site [142].

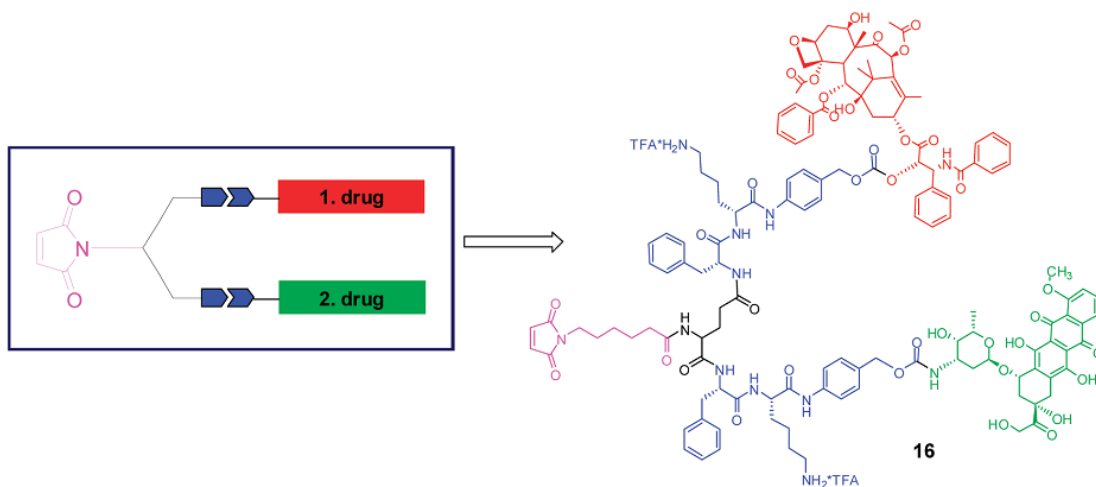


Figure 17. Structure of Cathepsin B-sensitive, dual-functionalized linker bearing Dox and Ptx, and comprising a maleimide moiety for its coupling to albumin. Adapted with permission from Ref. [141].

Pep42, which is a cyclic 13-mer oligopeptide, specifically binds to glucose-regulated protein 78 and translocates into the lysosomal compartment [143, 144]. In this context, Pep42 was advantageously used to efficiently deliver Ptx and Dox into cancer cells for enhanced cytotoxicity [145]. More specifically, Pep42-prodrug bioconjugates containing a Cathepsin B-sensitive linker were synthesized and facilitated the uptake of both cytotoxic agents for their delivery into cancer cells.

Nanoconstructs with methotrexate (Mtx) linked to a tuftsin-like peptide carrier via a GFLG spacer and several copies of a chemotactic targeting agent were designed [146]. These conjugates led to greater cytotoxic effect than free Mtx and represented potential candidates for

the specific targeting of cancer cells. Similarly, Dox-based dipeptide conjugates were designed and tethered to monoclonal antibodies (mAbs) recognizing tumor associated antigens on renal cell carcinoma and anaplastic large cell lymphoma [147]. The dipeptides were substrates for Cathepsin B and got cleaved with comparable kinetics. Importantly, both prodrugs were 70-fold more potent than free Dox.

In another study, cytotoxic drug-carrying filamentous bacteriophages were chemically modified to tune different key parameters (e.g., pharmacokinetics, biodistribution, immunogenicity) and compared to bare phages [148]. Anti-ErbB2 and anti-ERGR antibodies were used as targeting entities, whereas Dox was tethered to phages through an amide linkage and also to genetically-engineered Cathepsin-B (Figure 18). *In vitro* studies explained the good penetration into tumors cells by their needle-like structure. This conjugate can be seen as a novel drug-delivery platform which might solve many issues related to the hydrophobicity of drugs at the target specific sites.

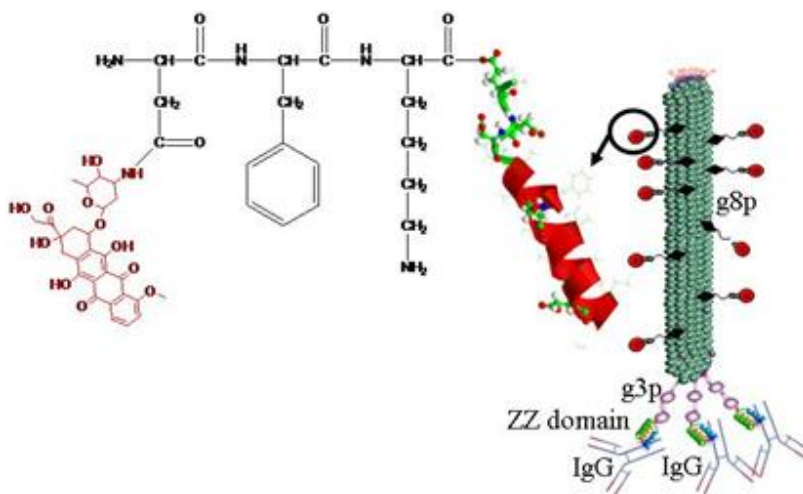


Figure18. Genetically engineered Cathepsin B-modulated bacteriophage conjugated to Dox. Adapted with permission from Ref. [148].

2.2 Bone-targeting drug delivery systems

The most common skeleton disorders are arthritis, osteoporosis, osteomyelitis, osteosarcoma as well as metastatic bone cancer [37, 149]. Bone metastasis is one of the most devastating stages of cancer [150]. In addition, there are several limitations associated with the systemic administration of drugs for bone treatment and bone-related diseases such as poor drug uptake at the target site, potential systemic toxicity as well as suboptimal efficacy [149]. Interestingly, there are examples in the literature describing Cathepsin-sensitive polymer conjugates for bone targeting purposes [151-154]. Therefore, drug delivery systems targeted towards bones can be adapted to bone diseases where the drug can be selectively delivered with minimal side effects [155].

In a similar fashion to what has been reported for anticancer therapy, HPMA was conjugated to prostaglandin E₁ (PGE₁) *via* a spacer sensitive to Cathepsin K, which is an enzyme overexpressed in osteoclasts [156]. The Cathepsin K-sensitive spacer comprised Gly-Gly-Pro-Nle as the tetrapeptide sequence and a self-eliminating 4-aminobenzyl alcohol moiety. Copolymerization of the resulting PGE₁-containing HPMA macromonomer with HPMA yielded the desired PHPMA-PGE₁ conjugates, that released unmodified PGE₁ after incubation with Cathepsin K. PHPMA was also post-functionalized by a D-aspartic acid octapeptide targeting ligand. Therefore, this new drug delivery system might be a solution to treat osteoporosis and other bone-related pathologies.

Targeting inflammatory joints in rheumatoid arthritis (RA) was achieved by AWO54, a new prodrug that binds to endogenous albumin and was composed of Mtx, a spacer based on lysine and an enzyme-sensitive peptide linker linked to a maleimide moiety for further linkage to albumin [157]. The prodrug was cleaved by two enzymes, Cathepsin B and plasmin, that exist in high concentrations in synovial effusion under RA condition, thus releasing Mtx lysine derivatives. The *in situ* coupling of endogenous albumin, AWO54 was found to be better in terms of dosage and efficacy than administration of the parent drug for treating collagen-induced arthritis.

2.3 Immune cell-targeting drug delivery systems

Lysosomal peptidases are part of innate and adaptive immune responses [158-160]. Hence, modulation of such responses with Cathepsin-sensitive prodrugs can further enhance the immunological action and regulate cytotoxicity issues related to NK and T cells. For instance, influence of superparamagnetic iron oxide nanoparticles (SPIONs) from both a physiological and immunological point of view was investigated on cell function and their interaction with oxysterol laden cells [161]. Iron-loaded nanoparticles upregulated Cathepsin, membranous ferroportin (cellular efflux channel for iron) and ferritin degradation, which further altered cellular immune functions, resulting in secretion of pro- (TNF- α) and anti-inflammatory (IL-10) cytokines and ferritin. Importantly, this study highlighted a specific relationship between SPION metabolism and atheroma cellfunction that might conduct to innovative approaches to treat atherosclerotic plaques.

Immunoconjugates were also prepared from cytotoxic agents using a valine-alanine-p-aminobenzyl-amine linker which was well-adapted for the bioconjugation to monoclonal antibody and further specific cleavage by proteases [162]. The linker efficiently released aminogeldanamycin and streptonigrin upon protease-mediated hydrolysis, emphasizing the activity and specificity of the conjugates *in vitro* and *in vivo*. In another study, different immunoconjugates comprising lysosomally cleavable peptides (i.e., Phe-Lys and Val-Cit), were synthesized [163]. The monoclonal antibody BR96 that is known to bind to Lewis^y-related tumor-associated antigen expressed at the surface of cancer cells was linked to Dox *via* a *p*-aminobenzyloxycarbonyl (PABC) spacer. Interestingly, the conjugates bearing the Phe-Lys sequence exhibited a 30-fold greater drug release kinetics in the presence of Cathepsin B than its counterpart with the Val-Cit linker.

2.4 Cathepsins as probes for imaging and theranostic

Different types of enzymes (e.g., caspases, secretases, furinases, phosphatases, etc.) have been exploited for cancer diagnosis. Furthermore, imaging probes utilizing these proteases have rapidly evolved [164-167]. It has been shown that the monitoring of protease activity was closely related to cancer progression especially in case of Cathepsin B [168]. Among the numerous studies on proteases for such a purpose, hollow mesoporous silica nanoparticles loaded with Dox and conferred with a dual-enzyme sensitivity were conceived for the *in situ* imaging of Cathepsin B and the release of Dox mediated by proteases (Figure 19) [169]. The peptide-based satellite/shell structures secured Dox inside the nanoparticles thus acting as three-dimensional gatekeepers and Dox release subsequently occurred upon incubation with Cathepsin B.

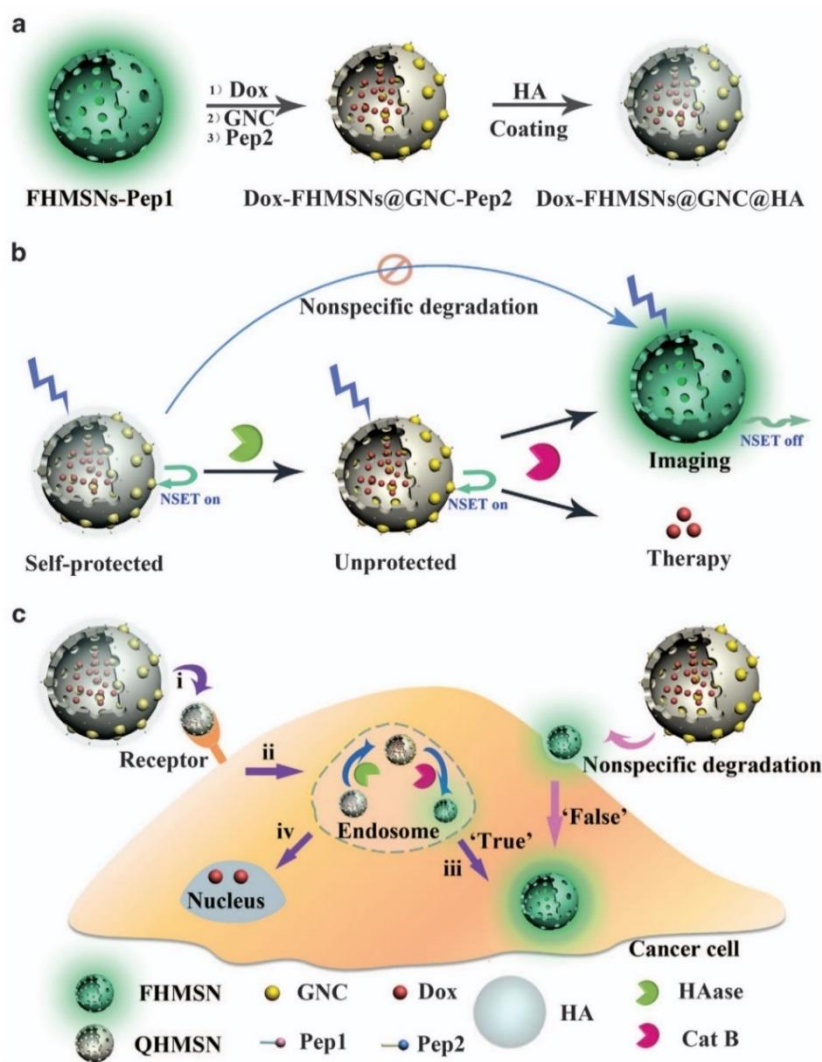


Figure 19. Illustration of Dox-loaded, hollow mesoporous silica nanoparticles for *in situ* imaging of Cathepsin B and protease-mediated Dox release. (a) Nanoparticle synthesis. (b) Nanoparticle

disassembly mediated by enzyme cascade reactions with acid hyaluronidase (HAase) and Cathepsin B (Cat B). (c) Specific delivery, controlled Dox release and intracellular imaging: (i) specific uptake via receptor-mediated endocytosis; (ii) accumulation in endosomes; (iii) endosomal escape and intracellular imaging of Cat B; (iv) Dox release triggered by enzymes. Adapted with permission from Ref. [169].

It was also recently discovered that indocyanine green (ICG)–containing PGA nanoparticles can be digested by Cathepsin B and induce a sensitization of the endo-lysosomal membrane mediated by the NIR properties of the released ICG (Figure 20) [170]. The system was combined with a ribosome-inactivating protein (saporin) which showed synergistic cytotoxicity because of the photo-induced release of saporin from endosomes or lysosomes.

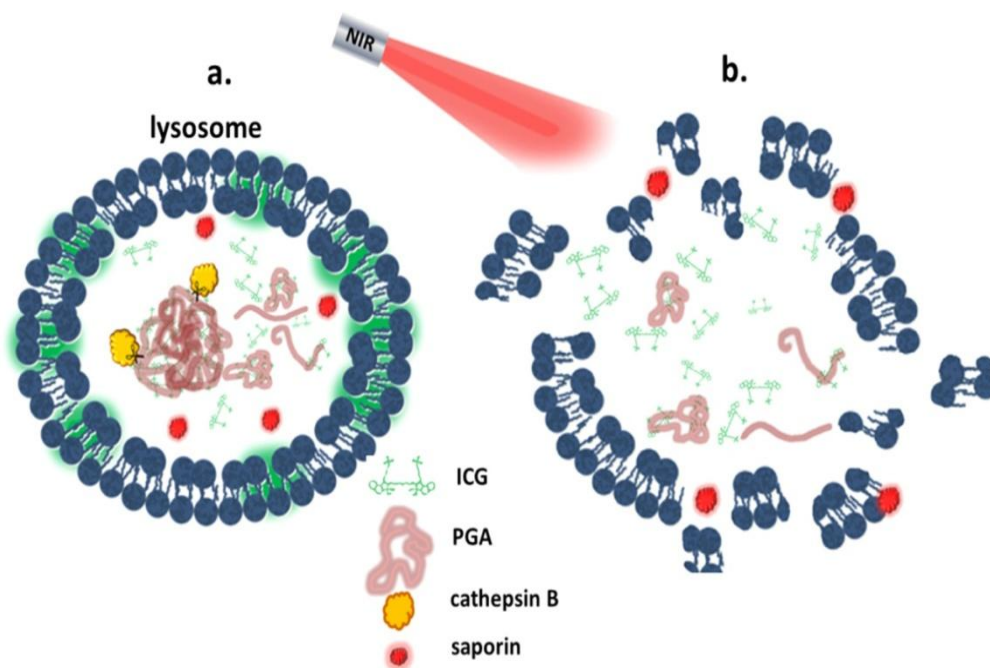


Figure 20. (a) Sensitization of endo-lysosomal membrane in the presence of dye released by enzymatic digestion of the nanoparticles. (b) Endo-lysosomal disruption by NIR laser leading to saporin release. Adapted with permission from Ref. [170].

An enzymatically dependent FRET process was also used to monitor the payload release from PHPMA prodrug nanocarriers [171]. PHPMA was functionalized with donor Cy5 and acceptor Cy7, thus inducing FRET. However, since only Cy7 was linked to the polymer via the GFLG

sequence, presence of Cathepsin B was accurately measured because of the change in the FRET signal during the Cathepsin B-mediated Cy7 release (Figure 21). The *in vitro* results showed that the high level of expression of Cathepsin B in cancer cells induced effective release of the dye while *in vivo* observations resulted in a faster release in the ovarian tumor as compared to normal tissues.

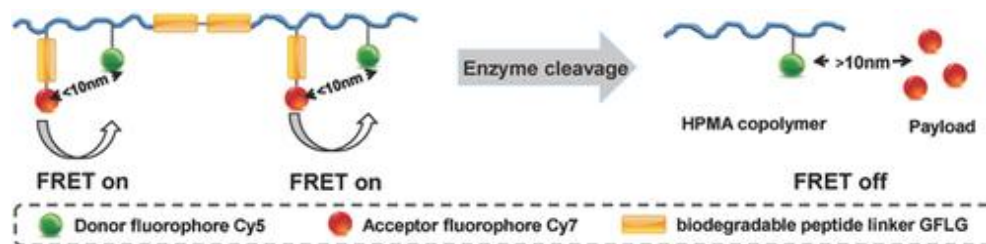


Figure 21. Structure of dual-functionalized PHPMA nanocarriers with Cy5 and Cy7 dyes for further Cathepsin B-mediated release of Cy7. Adapted with permission from Ref. [171].

Similarly, PHPMA was functionalized with Cy5 (acceptor fluorophore) and Cy3 (donor fluorophore) or epirubicin (EPI) through a GFLG linker and evaluated by FRET during cell uptake and intracellular drug delivery experiments (Figure 22) [172]. Thanks to the Cathepsin B-sensitive linker, the conjugates bearing EPI (2P-EPI) led to a fourfold terminal half-life compared to first generation (P-EPI) conjugate and complete tumor remission with ~100 days inhibition of tumorigenesis in mice.

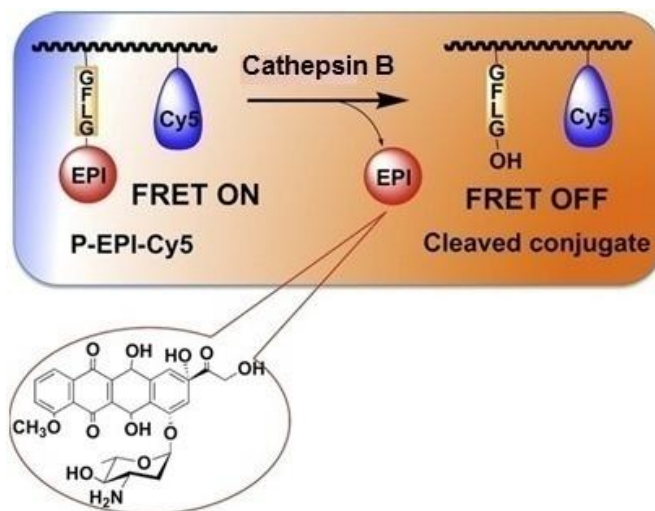


Figure22. Schematic structure of PHPMA functionalized by Cy5 and EPI for further FRET-monitoring of the EPI release mediated by Cathepsin B. Adapted with permission from Ref. [172].

The specific presence of cysteine Cathepsins has also been exploited to perform radiolabeled drug delivery for nanoscale conjugates with the aim of inducing enhanced diagnostic and radiotherapeutic efficacy. For instance, PHPMA was radiolabeled with Lutetium-117 (^{117}Lu) via a peptide sequence made of two consecutive metabolically active linkers (MALs) sensitive to Cathepsin B and S, that are overexpressed in the liver and the spleen [173]. The MALs were shown to be metabolized by enzymes into single metabolites. The ^{117}Lu -peptide-PHPMA conjugate showed a substantial retention decrease in the long run in the liver and the spleen, compared to non-cleavable counterparts on human pancreatic adenocarcinoma xenograft mouse model. In another study, the Garrison's group developed the synthesis of cathepsin S-susceptible ^{177}Lu -labeled or FRET-capable multiblock PHPMA copolymers, which resulted into fast *in vitro* cleavage of both copolymers. Quicker clearance and lower non-target retention without reducing tumor targeting was also shown on pancreatic ductal adenocarcinoma mouse model [174]. This study therefore took benefit of the presence of Cathepsin S in MPS tissues to lower non-target accumulation.

A targeted, theranostic prodrug relying on Cathepsin-B-sensitive Gem release and activation of a caspase-3 specific probe was designed (Figure 23) [175]. The targeting relied on

the RGD peptide for accumulation into pancreatic cancer cells with overexpressed $\alpha_v\beta_3$ integrin. The GFLG peptide was then hydrolyzed by Cathepsin B leading to Gem release as well as the apoptotic probe. This system showed promising properties as a platform for both pancreatic cancer cell targeting and real-time, non-invasive imaging.

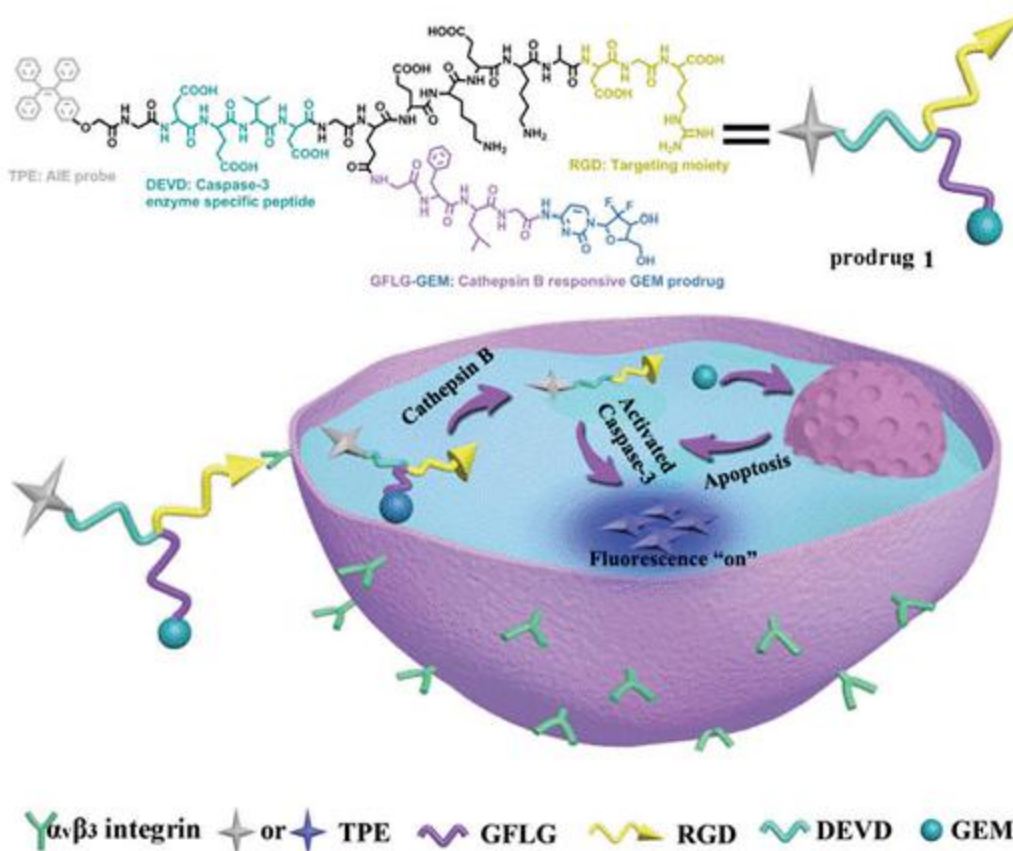


Figure23. Schematic structure of consecutive enzymatic reaction using a gemcitabine-based prodrug along with apoptotic probe for the killing and monitoring of pancreatic cancer cells. Adapted with permission from Ref. [175].

In tumor imaging, many proteases can be used for the activation of fluorescent probes including near-infrared emitting dyes. Therefore, *in vivo* molecular profiling of protease activity can be performed with such probes in endoscopy or tomographic optical imaging [176]. For instance, it has been reported the design of quenched activity-based probe (qABP) mediated by Cathepsin S

[177]. It showed high tumor-specific fluorescence in a syngeneic breast cancer model. Other activity-based probes targeting Cathepsin X have been designed [178]. Cathepsin X is involved in a many different biological mechanisms, such as aging, cancer, neurodegenerative disorders, inflammation, etc. [179-181]. These probes were successfully used for the selective labeling and imaging of Cathepsin X *in vitro* and *in vivo*, thus making them a valuable tool for examining protease activity and functions.

Malarial parasites are known to generate significant concentrations of mobile ferrous iron [182]. In this context, parasite-specific, Fe^{II}-sensitive delivery of a potent dipeptidyl aminopeptidase inhibitor through Cathepsin C was demonstrated by using activity-based probes [183]. Production of Fe^{II} was triggered in the presence of 1,2,4-trioxolone moiety leading to instant drug release prior to the fragmentation of the aforesaid moiety. Further *in vivo* evaluation was performed using *Plasmodium berghei* model of murine malaria which showed selective drug targeting in parasitic infections.

Cathepsin D-conjugated peptides were self-assembled into nanoparticles with the help of gelatin to bypass early nonspecific dissolution as well as off-target Dox release and is useful for optical imaging in animal models [184]. Cathepsin D is an enzyme for breast cancer cell secretion, which got triggered by degrading the nanoparticles coated with peptide strands through hydrolytic cleavage, thus releasing Dox. The nanoparticles were evaluated under ultrasound imaging both *in vitro* and *in vivo*, and were found to be localized in the bladder and the tumors of mice as a result of the fluorescent profile of Dox. Synthesis of Cathepsin B-sensitive, near-infrared fluorescent probe was also carried out (Figure 24) [185]. The probe was found to be water-soluble but still self-assembled into nanoparticles having potential for tumor-targeted imaging. A fluorescent molecule, DCPO (dicyanomethylene-4H-pyran), was released by Cathepsin B, leading to *in vitro* imaging Cathepsins of various tumor cells during incubation with different cell lines.

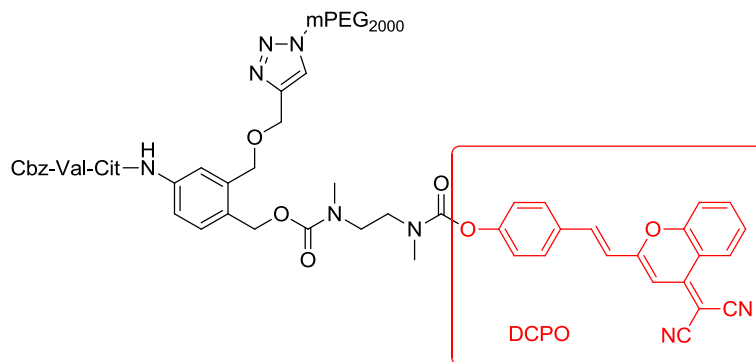


Figure24. Chemical structure of NIR fluorescent probe sensitive to CathepsinB [185].

Similarly, a Cathepsin B-sensitive nanoparticulate probe comprising a Cathepsin B substrate peptidic probe linked to chitosan nanoparticles was reported [186]. According to the study, this probe was successfully delivered into tumor cells after nanoparticle accumulation and exhibited fluorescent signals inside the cytosol in presence of Cathepsin B. It thus showed increased potential for the optical detection of biological activities especially related to tumor growth or metastasis (Figure 25).

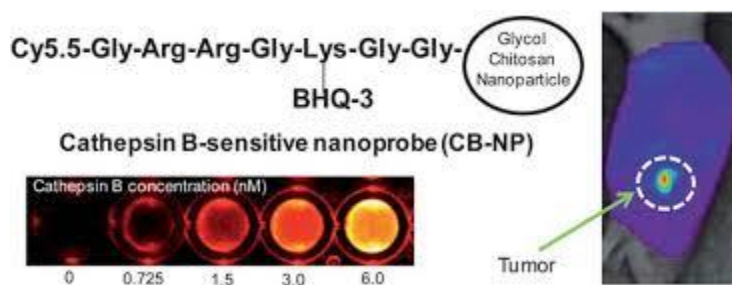


Figure25. Cathepsin B-sensitive nanoparticulate probes and tumor diagnosis *in vivo*. Adapted with permission from Ref. [186].

Recently, another strategy was used for Cathepsin imaging in breast cancer. It relied on a selective fluorogenic substrate and activity-based probe for the specific imaging of Cathepsin L

[63]. This approach enabled to differentiate Cathepsin L activity from that of other Cathepsins such as Cathepsin B.

2.5 Miscellaneous

As shown in the previous sections, Cathepsins have been exploited in targeted drug delivery systems and imaging. However, Cathepsin inhibitors can also be exploited in regard to their role in numerous diseased conditions, mainly cardiovascular diseases such as myocardial infarction, atherosclerosis, cardiac hypertrophy, cardiomyopathy and hypertension based on animal models [187]. Cathepsin inhibitors are also used against immune responses, osteoporosis, arthritis, inflammation and neurodegenerative disorders [36, 188-190]. A selection of representative examples is discussed below.

Interestingly, peptide-based pseudosubstrates for Cathepsin G and elastase were developed. These substrates can decrease the activated interleukin-36 (IL-36) family cytokines especially in case of inflammatory diseases(e.g., psoriasis, arthritis)because such cytokines are proteolytically processed in the presence of Cathepsin G and other proteases [191]. In another study, it was proven that amodiaquine, an antimalarial drug, inhibited host Cathepsin B to protect host cells against infection with multiple toxins or viruses [192]. Cathepsin K has also been involved in diabetes-associated cardiac abnormalities. Wild-type as well as Cathepsin K knockout mice-induced diabetes exhibited severe cardiac dysfunctions in the form of dampened calcium handling intracellularly, cardiac morphology alterations and also increase in cardiomyocyte apoptosis [193, 194]. Hence, Cathepsin K may be a suitable target in the aforementioned conditions. One study also investigated cysteine Cathepsin inhibitors such as GB111-NH₂ (that blocks the activity of Cathepsin B, L and S) as trigger in macrophage cell death especially in case of tumor-associated macrophages (TAMs) [195].

3. Clinical data on cathepsins

As seen throughout this review article, PHPMA has been extensively used for the design of Cathepsin-sensitive nanocarriers. This is explained by the favorable properties of PHPMA

regarding biomedical applications. PHPMA is indeed hydrophilic, non-immunogenic, chemically inert, non-toxic (even at the dose of 30 g/kg rat), biocompatible and exhibits relatively long circulation time which is dependent on its molar mass [196]. Among the different conjugates based on PHPMA that have been synthesized and evaluated so far, some of them entered clinical trials (Table 2). In particular, PK1 (Prague-Keele-1) has shown very promising results in oncology [197] and reached phase II trials in 2002 but clinical studies for both PK1 and PK2 were discontinued in 2008 because of lack of efficacy [198]. Other polymeric systems also entered clinical trials (Table 2). For instance, XYOTAX (based on polyglutamate) has shown encouraging results in phase III trials in women with non-small-cell lung cancer [199-205]. Among the different PHPMA clinical candidates, PNU166945 is based on Ptx for the treatment of advanced breast cancer and PNU166148 is based on camptothecin for the treatment of metastatic solid tumors but both were stopped in Phase I trials because of severe neurotoxicity and lack of anticancer action, respectively [206, 207]. Also, AP5280 prodrug has been introduced for PHPMA-carboplatinate and entered clinical phase II trial whereas PHPMA-oxiplatinate (ProLindac, also named AP5346) was in clinical phase II trial against ovarian cancer [208-210]. PHPMA polymer conjugated to Dox using GFLG linker has also been attempted with protein-bound Ptx (abraxane), carbohydrate residues such as galactosamine, lactose and also amino acids like phenylalanine-lysine (Phe-Lys), and are currently in preclinical studies [211-214]. On the contrary, high molar mass PHPMA was investigated to enhance the anticancer efficacy which also reached preclinical settings [215]. A second series of blockbuster polymers that entered clinical trials are PEG-based conjugates, namely, EZ-246 conjugated to camptothecin but its phase II trials has been stopped due to lack of efficacy. However, NK911 in the form of PEGylated micelles with aspartic acid and Dox had showed promising efficacy against various solid malignancies likely thanks to the EPR effect and is currently in phase II [216-219]. Carboxymethyldextrans another synthetic polymer forming prodrugs with exatecan and camptothecin [220], and which entered Phase I clinical trials, showed prominent results especially against colon cancer [221, 222]. Polyglutamate has been conjugated using Ptx by Cell Therapeutics Inc. company and is currently in Phase II trials [199, 200]. Other Cathepsin-sensitive drug delivery systems are under preclinical investigations, including polymeric dendritic systems containing Dox (KTB Tumorforschungs GmbH company) [223, 224].

Table 2.List of Cathepsin B-Cleavable Prodrugs Evaluated in Clinical Trials.

| Entry | Name | Composition | Spacer | Linker | Clinical Trial status | Company | Refs |
|-------|------------------------------------------|---------------------------------------|------------------------------------------------|---------------|---------------------------|-------------------------------------------|----------------|
| 1 | PK1; FCE28068 | PHPMA copolymer-Doxorubicine | Gly-Phe-Leu-Gly | Amide | Phase II (discontinued) | Pfizer Inc., Cancer Research Campaign, UK | [196, 225] |
| 2 | PK2; FCE28069 | PHPMA-Dox-Galatosamine | Gly-Phe-Leu-Gly | Amide | Phase I/II (discontinued) | Pfizer Inc., Cancer Research Campaign, UK | [226, 227] |
| 3 | PNU166945/H PMA-Ptx | PHPMA copolymer-Paclitaxel | Gly-Phe-Leu-Gly | Ester | Phase I (discontinued) | Pharmacia | [206] |
| 4 | PNU166148/H PMA-CPT/MAG-CPT | PHPMA copolymer-Camptothecin | Glycine residue/ Glycylaminohexanoyl spacer | Ester | Phase I (discontinued) | Pharmacia | [207] |
| 5 | CT-2103/PGA-Ptx-XYOTAXTM/OPAXIO® | Polyglutamate-Paclitaxel | L-glutamic acid | Ester | Phase II/III | Cell Therapeutics Inc. | [199-205] |
| 6 | CT-2106/PGA-CPT | Polyglutamate-Camptothecin | L-glutamic acid | Ester | Phase II discontinued | Cell Therapeutics Inc. | [228-230] |
| 7 | AP5280 | PHPMA copolymer-Carboplatinate | Gly-Phe-Leu-Gly | Aminomalonate | Phase I | Access Pharmaceuticals Inc. | [208, 209] |
| 8 | AP5346/ ProlindacTM | PHPMA copolymer-DACH Oxiplatinate | GGG-carboxylate-Pt coordination | Aminomalonate | Phase II | Access Pharmaceuticals Inc. | [210] |
| 9 | EZ-246/PEG-CPT/Pegamotectan/ProthectanTM | PEG-Camptothecin | Glycine | Ester | Phase II discontinued | Enzon Pharmaceuticals, Inc. | [216, 217] |
| 10 | NK911 | PEG-aspartic acid-Doxorubicin micelle | Aspartic acid | Amide | Phase II | National Cancer Institute Japan | [97, 218, 219] |
| 11 | P-Dox | PHPMA copolymer- | Gly-Phe-Leu-Gly | Amide | Preclinical | - | [211, 212] |

| | | Dox | | | | | |
|-----------|-------------------------------|----------------------------------------------|-----------------|-----------|-------------|---------------------------------|-------|
| 12 | P-(GFLG)-Dox-Ab | PHPMA copolymer-Dox-abraxane | Gly-Phe-Leu-Gly | Amide | Preclinical | - | [213] |
| 13 | P-(GFLG-Dox)-Ga IN | PHPMA copolymer-Dox-N-acylated galactosamine | Gly-Phe-Leu-Gly | Amide | Preclinical | - | [214] |
| 14 | P-(GFLG-Dox)-Lac | PHPMA copolymer-Dox-Lactose | Gly-Phe-Leu-Gly | Amide | Preclinical | | [214] |
| 15 | HMW1D | PHPMA copolymer-Dox (high molecular weight) | Gly-Phe-Leu-Gly | Amide | Preclinical | - | [215] |
| 16 | TET1D | PHPMA copolymer-Dox (non-targeted) | Gly-Phe-Leu-Gly | Amide | Preclinical | - | [215] |
| 17 | DOXO-EMCH (INNO-206) | EMC-Arg-Arg-Ala-Leu-Ala-Leu-Dox | Ala-Leu-Ala-Leu | Maleimide | Preclinical | CytRx Corporation | [231] |
| 18 | EMC-Phe-Lys-PABC-Dox | PHPMA copolymer-Phe-Lys-PABC-Dox | Phe-Lys | Amide | Preclinical | KTB Tumorforschungs GmbH | [223] |
| 19 | PG-Phe-Lys-Dox | Hyperbranched polyglycerol-Phe-Lys-Dox | Phe-Lys | Amide | Preclinical | KTB Tumorforschungs GmbH | [224] |
| 20 | DE-310 | Carboxymethyl dextran-exatecan | Gly-Phe-Leu-Gly | Amide | Phase I | Daiichi Pharmaceutical Co. Ltd. | [221] |
| 21 | Delimotecan (MEN 4901/T-0128) | Carboxymethyl dextran-camptothecin | Triglycine | Ester | Phase I | Mitsubishi Tanabe/Menarini | [222] |

4. Conclusion

As shown in this Review Article, Cathepsins could be very efficient tools, leverages or even actuators for the design of advanced drug delivery systems. It has been shown that these systems were sensitive to the presence of a broad spectrum of different Cathepsins, leading to enhanced therapeutic benefit and imaging capabilities. Among the numerous Cathepsin-sensitive conjugates reported so far, some of them have shown promising results and even reached advanced clinical trials. However, a great deal of work remains especially regarding the lack of site specificity and the still limited understanding of the biological roles of some proteases. These limitations must be resolved to develop optimized conjugates and offer more prominent clinical candidates.

Also, it has been demonstrated that cysteine Cathepsin proteases can act as regulators for cancer progression as well as therapeutic response [232]. It means that they can either promote tumor growth or suppress tumor depending upon the environment. However, more clinical investigations must be performed to have a complete and accurate picture of the situation *in vivo*.

5. Conflict of interest

The authors confirm that there are no known conflicts of interest associated with the content of this article.

6. Acknowledgement

D.D. thank to Indo French Centre for Promotion of Advanced Research (IFCPAR/CEFIPRA) for Raman Charpak Fellowship 2017 and CSIR-New Delhi, India for her research fellowship. This research work was financially supported by DST-EMR/2016/002304 and DST-EE2/2016/000102 and IIM communication no. IIM/2244/2018. CNRS and Université Paris-Sud are also acknowledged for financial support.

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