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Review Article

Cathepsin B-responsive prodrugs for cancer-targeted therapy: Recent advances and progress for clinical translation

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ABSTRACT

The cathepsin B-responsive prodrugs are promising strategies to reduce the serious adverse effects of anticancer drugs by improving the cancer selectivity that can be specifically activated by overexpressed cathepsin B in targeted cancer cells. However, clinical translation of such therapeutic approaches has been restricted by low antitumor efficacy that is mainly attributable to undesirable pharmacokinetic profiles and inefficient tumor-targeting of cathepsin B-responsive prodrugs, due to their small-molecule structure. In recent decades, many researchers have widely investigated the drug delivery system (DDS) to improve the *in vivo* pharmacokinetic profiles and tumor-targeting efficiency of cathepsin B-responsive prodrugs via the application of polymers, dendrimers, antibodies, lipids, and inorganic nanoparticles as drug carriers. In addition, the potential therapeutic efficacy of DDS for cathepsin B-responsive prodrugs is demonstrated in multiple studies and combinatorial treatment with typical therapeutic modalities can effectively overcome the challenges of tumor heterogeneity and multidrug resistance. In this review, recent advances and progress of new DDS for cathepsin B-responsive prodrugs are outlined, and their clinical trials are discussed. Besides, potential challenges and the outlooks for clinical translation of cathepsin B-responsive prodrugs are highlighted.

KEYWORDS

cathepsin B, prodrug, chemotherapy, drug delivery system, targeted cancer therapy

1 Introduction

Chemotherapy is still the main treatment approach to combat a variety of cancers. Conventional anticancer drugs induce cytotoxicity in cancer cells characterized by rapid proliferation [1]. For instance, doxorubicin (DOX) is DNA intercalating agent to inhibit topoisomerase II, resulting in apoptosis by DNA damage [2]. This mode of action (MOA) of doxorubicin necessarily more affects rapidly dividing or growing cancer cells. Unfortunately, this is the reason that conventional anticancer drugs have serious side effects because normal cells in the digestive tract, bone marrow, and lymphatic system also indicate the same trait [3]. One of the recent progress to address these limitations of chemotherapy, prodrug is bioreversible medication that undergoes enzymatic or chemical transformation to release the active drug, which can exert desired pharmacological effect [4, 5]. This concept is now well established as a strategy to reduce serious side effects of anticancer drugs by improving the cancer selectivity that can be specifically activated in targeted cancer cells or tumor microenvironment [6–9]. Selective activation of prodrugs can be achieved by intrinsic differences between tumor and normal tissues, especially diverse enzymes that are overexpressed in cancer cells compared to normal cells [10–12].

Lysosomal cysteine protease, cathepsin B, is an important category of the enzyme that is considered a promising target for cancer-targeted diagnosis and treatment [13–15]. This is because cathepsin B plays a prominent role in extra- and intracellular proteolysis and its pericellular activity is critical in extracellular matrix proteolysis and tumor invasion [16]. Thus, cathepsin B is highly upregulated in malignant tumors at the mRNA and protein levels and involved in tumor progression and metastasis [17]. For this reason, many prodrugs have been proposed, which are composed of anticancer drugs with cathepsin B-cleavable peptide linkers, such as *Leu* (L), *Arg-Arg* (RR), *Ala-Leu* (AL), *Phe-Arg* (FR), *Phe-Lys* (FK), *Val-Ala* (VA), *Val-Cit* (VC), *Ala-Phe-Lys* (AFK), *Gly-Leu-Phe-Gly* (GLFG), *Gly-Phe-Leu-Gly* (GFLG), *Ala-Leu-Ala-Leu* (ALAL), and *Phe-Arg-Arg-Gly* (FRRG) [18–21]. Zhong et al. developed the Ac-*Phe-Lys*-p-aminobenzyl carbamate (PABC)-DOX (PDOX) containing cathepsin B-cleavable linker (*Phe-Lys*; FX), self-immolative spacer (PABC), and DOX [18]. The *in vivo* study of PDOX in gastric tumor models with peritoneal carcinomatosis indicated the superior anticancer efficacy with reduced liver, kidney, and heart toxicities, compared to free DOX [22]. In addition, Groot et al. and Dubowchik et al. also developed a series of other cathepsin B-responsive prodrugs, including *Gly-Phe-Lys*-PABC-DOX, *Phe-Phe-Lys*-PABC-DOX, and *Val-Cit*-PABC-DOX, and their high cathepsin B-specificity was clearly evaluated [23, 24]. However, clinical translation of these cathepsin B-responsive prodrugs has been restricted by low antitumor efficacy that is mainly attributable to undesirable pharmacokinetic profiles and inefficient tumor-targeting, due to their small-molecule structure [25].

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Recently, a considerable effort is being spent on the design of a drug delivery system (DDS) for cathepsin B-responsive prodrugs via the application of polymers, dendrimers, antibodies, lipids, and inorganic nanoparticles as drug carriers (Fig. 1). The carrier-based prodrugs achieve the high tumor-targeting of payloads to the cancers in a spatially controlled manner via passive accumulation and active targeting [26, 27]. Consequentially, cathepsin B-responsive prodrugs with new DDS effectively maintain the drug concentration in targeted cancers with minimal dosage frequency, which offer fascinating properties and broad insights for improving antitumor efficacy, decreasing drug concentration fluctuation, and further reducing the drug toxicities of cathepsin B-responsive prodrugs [28]. In addition, their combinatorial treatment with typical therapeutic modalities can effectively overcome the challenges of tumor heterogeneity and multidrug resistance (MDR). In this review, we classified the recent advances and progress of cathepsin B-responsive prodrugs with new DDS according to drug carriers, and their clinical trials are outlined. Furthermore, potential challenges and the outlooks for clinical translation of cathepsin B-responsive prodrugs are discussed.

2 New DDS for cathepsin B-responsive prodrugs

To compensate for the drawbacks of small-molecule cathepsin B-responsive prodrugs, a variety of research has been conducted for the exploration of carrier-based prodrugs. Various types of cathepsin B-cleavable linkers and carriers used for the development of carrier-based cathepsin B-responsive prodrugs are overviewed in Table 1. These carriers are generally hydrophilic and have much bulkier structures than drugs so the carrier-based cathepsin B-responsive prodrug is a good strategy to modulate the hydrophobicity of small molecular drugs and to delay their rapid clearance in blood circulation. The bulkiness of carriers also increases the tumor-specific accumulation of prodrugs via passive targeting due to the distinct tumor microenvironment, which has highly permeable vasculature and is lack lymphatic drainage. Some carriers possess ligands for tumor-overexpressed receptors, enhancing their active targetability at the same time. After being delivered to the tumor site, the carriers release drugs as active forms through the cleavage of linkers between carriers and drugs under the presence of cathepsin B. The detailed explanations about each carrier for cathepsin B-responsive prodrugs are provided in the following chapters.

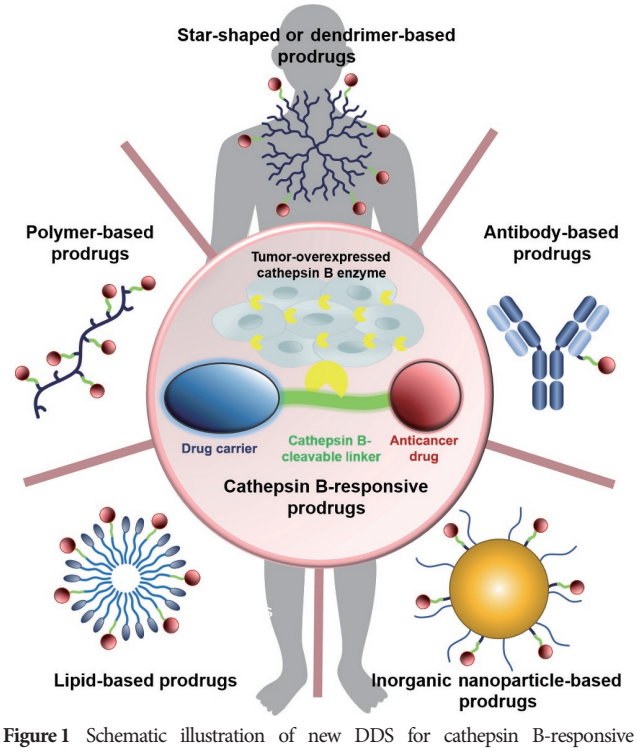


Figure 1 Schematic illustration of new DDS for cathepsin B-responsive prodrugs.

2.1 Cathepsin B-responsive prodrug conjugated polymers

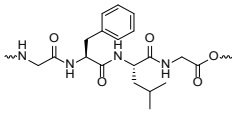
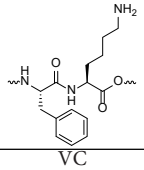
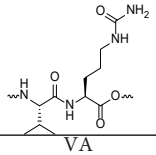
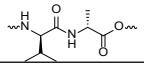
The polymer-prodrug conjugates (PDCs), which refer to compounds with macromolecular substances and covalently bound drugs to them, are one of the classical approaches for prodrug delivery at targeted tumor tissues. Through the chemical conjugation of anticancer drugs to polymer backbones via cathepsin B-cleavable linkers, the solubility and systemic toxicity of drugs can be extensively modulated, improving their pharmacokinetic properties as well. Moreover, PDC-based prodrugs have advantages over delivery systems in which free drugs are physically entrapped, with regard to their stability, prevention of undesirable drug release, and reproducibility control [29, 30]. There are some requirements to the polymers used for PDC-based prodrugs, which narrow their candidates for applications. They must not have any toxicity or immunogenicity themselves and should be biodegradable or have limited molecular weights that can be excreted from the body [31]. In addition, their chemical modification should be simple for the smooth introduction of drugs and cathepsin B-cleavable linkers. Chemical structures of cathepsin B-responsive prodrug conjugated polymers most widely studied were depicted in Fig. 2.

2.1.1 Cathepsin B-responsive prodrug conjugated acrylamide copolymers

The first cathepsin B-responsive prodrug conjugated polymer was investigated using the N-(2-hydroxypropyl)methacrylamide copolymer (polyHPMA) [32]. Since it was initially developed as a plasma expander by Kopecek and coworkers, polyHPMA has been widely studied for a prodrug carrier due to its water solubility, biocompatibility, and non-immunogenicity [33]. The basic structures of polyHPMA-based prodrugs contained linear polyHPMA backbones and anticancer drugs conjugated to side groups of the polymer via cathepsin B-cleavable peptide linker, GFLG. DOX-conjugated polyHPMA with cathepsin B-cleavable peptide linker, named PK1, was the first cathepsin B-responsive prodrug conjugated polymers that entered clinical trials in 1994 [34, 35]. The molecular weight of polyHPMA used in PK1 was about 28 kDa and it contained ~ 8.8% (w/w) of DOX. The GFLG linker in PK1 was rarely degraded during its circulation and specifically released DOX by cathepsin B in cancer cell lysosomes, showing reduced toxicities to normal tissues [36]. PK2, a derivative of PK1, has also been in clinical trials. The molar mass or DOX content of PK2 was not far different from that of PK1 but introduced additional galactosamine groups for the active targeting of the asialoglycoprotein receptor [37]. With the successful results in preclinical tests of PK1 and PK2, several different anticancer drugs such as paclitaxel (PTX) and carboplatinum were also introduced to the polyHPMA-based prodrugs, and their promising therapeutic efficacies were evaluated [38, 39]. Exploiting GFLG sequences as linkers, their general properties and mechanisms of action were analogous to those of PK1 and PK2.

Since polyHPMA is not biodegradable, the molecular weight of polyHPMA in PK1 and PK2 was precisely controlled to ~ 30 kDa for their complete renal excretion [35]. However, polyHPMAs with limited molecular weights have a short blood half-life,

Table 1 Types of cathepsin B-cleavable peptide linkers and carriers explored in related researches

Chemical structure of linker	Carrier type	Payload	Advantages of carrier	References
GFLG 	PolyHPMA (1st generation)	DOX PTX	High water solubility Biocompatibility Non-immunogenicity Multiple functionalities	[33–35, 38, 39]
	PolyHPMA (2nd generation)	DOX GEM PTX DTX	Long blood circulation Passive tumor targeting Renal clearance	[42–46]
	Polysaccharide (chitosan)	PTX	Biocompatibility Biodegradability	[87]
	Dendrimer (PAMAM)	PTX	Narrow size distribution Globular structure Multiple functionalities	[92]
	Inorganic NP (Au)	AuNP	Radiosensitivity Biocompatibility	[136]
FK 	Dendrimer (polyglycerol)	MTX DOX	High water solubility Globular structure Multiple functionalities	[95]
	Antibody (anti-CD30)	MMAE	Active tumor targeting Biocompatibility	[162]
	Inorganic NP (iron oxide)	DOX	MR imaging contrast Biocompatibility	[138]
VC 	PEG	PTX TNF	High water solubility Biocompatibility Antifouling effect	[70]
	Antibody (anti-CD33)	PBD	Active tumor targeting Biocompatibility	[100]
VA 	Antibody (anti-LIV1)	MMAE	Active tumor targeting Biocompatibility	[163]
Simple amide (–NHCO–)	PEG	IND	High water solubility Biocompatibility Antifouling effect	[71]
	Lipid	GEM	Biocompatibility Self-assembly	[128, 133]

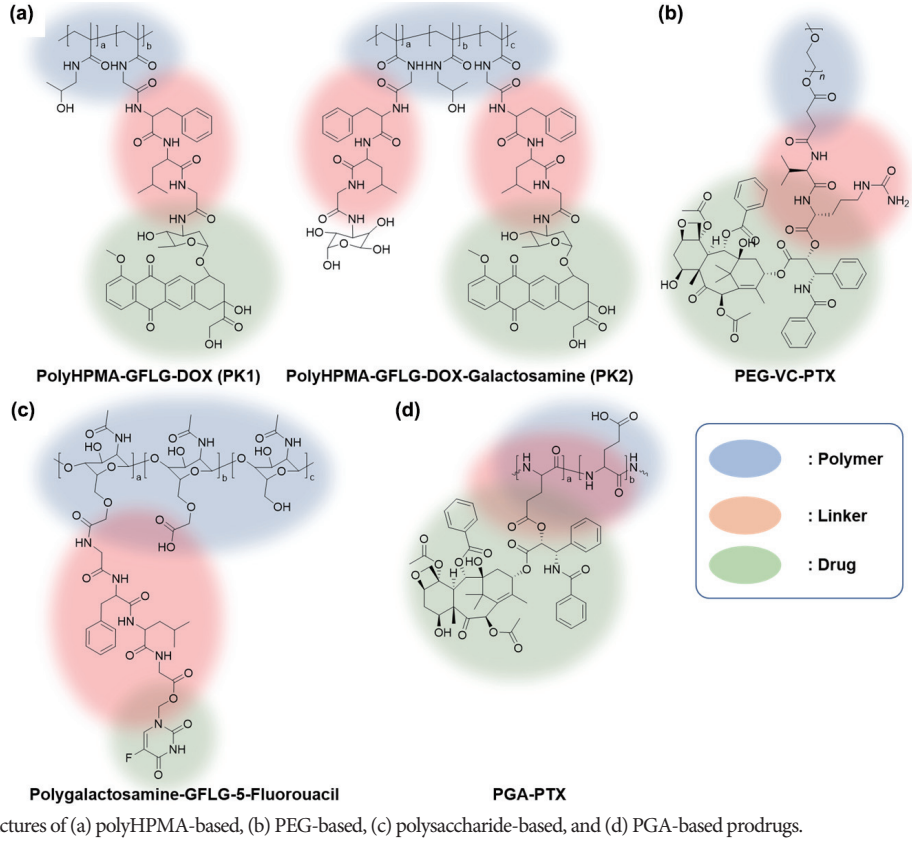


Figure 2 Chemical structures of (a) polyHPMA-based, (b) PEG-based, (c) polysaccharide-based, and (d) PGA-based prodrugs.

resulting in their insufficient accumulation in tumor tissues and diminished clinical efficacy of PK1 and PK2 [40, 41]. For this reason, backbone-degradable polyHPMA with large molar mass has been developed to extend their circulation time [42, 43]. By

linearly linking short-chain polyHPMA with each other through the GFLG sequence, it was designed to be cleaved under the presence of cathepsin B. Compared to the previous polymer used for PK1 and PK2 (1st generation polyHPMA conjugate), this multiblock polyHPMA was named as 2nd generation polyHPMA conjugate, which exhibited prolonged intravascular half-life as well as augmented renal clearance after its degradation [34]. Each linear block of 2nd generation polyHPMA conjugate was polymerized via the reversible addition-fragmentation chain transfer (RAFT) method to secure its reproducibility and control the polydispersity [44, 45]. GFLG-containing telechelic polyHPMA blocks with narrow molecular weight distribution could be obtained through the RAFT polymerization, and synthesized polymers were then reacted with difunctional cathepsin B-cleavable sequences, GFLG-Lys-GLFG (GFLGKGLFG), producing multiblock polymers. The multiblock polyHPMA-based prodrugs expressed enhanced antitumor effects over the traditional polyHPMA-based ones, and their optimal molecular weight was found to be about 94 kDa (three polyHPMA blocks and 20 kDa for each segment) [46].

Similar to the case of 2nd generation polyHPMA development, the particulation of polyHPMA also improves its blood circulation time and enlarges its intratumoral concentration via the enhanced permeation and retention (EPR) effect. The introduction of hydrophobic moieties to the side groups of polyHPMA induces the self-assembly of the polymer chain, which is a simple method to form polyHPMA nanoparticles [47]. Y. Yang et al. chemically modified the side groups of methacrylate (MA) monomers with GFLG-DOXs and co-polymerized them with HPMA monomers to obtain triblock copolymers [48]. Due to their amphiphilic structures, synthesized triblock copolymers self-assembled into uniform and spherical nanoparticles about 100 nm in diameter, with excellent stability during *in vivo* circulation and selective accumulation to tumor tissues. Chemical crosslinking of polyHPMA chains with cathepsin B-cleavable linkers is another strategy to construct polymeric nanoparticles. The covalent bonding between polyHPMA chains can further improve the particle stability in plasma when compared to the self-assembled ones whose driving force is a weak physical interaction [49]. Through the co-polymerization of HPMA with PTX-conjugated GFLG-MA and GFLG-containing di-methacrylate monomers, polyHPMA nanoparticle-based prodrugs could be prepared [50]. The chemically crosslinked nanoparticles were degraded into renal-clearable fragments as well as released the PTX in the cathepsin B-overexpressed tumor microenvironment.

Besides its high water-solubility and biocompatibility, polyHPMA has the advantage that it has sufficient side groups for additional functionalization. Incorporating other therapeutic agents, imaging modalities, or active targeting moieties, polyHPMA-based prodrugs are greatly applicable for combination therapies or theranostics. A sort of study concerning the combination therapy using the 2nd generation polyHPMA has been carried out mainly by Kopecek and coworkers. Several anticancer drug pairs including gemcitabine (GEM)/PTX [51, 52], GEM/platinum agent [53], docetaxel/GDC-0980 (PI3K/mTOR inhibitor) [54], and docetaxel/cyclopamine [55] have been conjugated to polyHPMA via GFLG linkers and was delivered together, demonstrating their synergistic effects for the effective treatment (Fig. 3). The most powerful point of the PDC-mediated combination therapy is that the pharmacokinetics of small molecular drugs can be significantly altered, thereby their cytotoxicity to normal tissues and clinical efficacies are adjusted.

The combination of chemotherapy with photodynamic therapy (PDT) has shown another considerable promise in cancer treatment. PDT utilizes the photosensitizers (PSs) which generate cytotoxic reactive oxygen species (ROS) under the irradiation of laser with a certain wavelength, eradicating tumors in a non-invasive and loco-specific manner [56]. The combination of PDT and chemotherapy facilitates mainly to overcome the MDR which is a chronic obstacle of the single chemotherapy [57, 58]. N. L. Krinick et al. synthesized polyHPMA-based prodrugs that contain GFLG-DOX and GFLG-meso-Chlorin e_6 (Mce_6), respectively, and examined their synergistic effects on cancer therapy [59]. Mce_6 was bound to polyHPMA with cleavable linkers to reduce their systemic toxicity and efficiently released in tumor tissues. The combination therapy of polyHPMA-DOX and polyHPMA-Mce_6 indicated a synergistic potency in neuroblastoma models, and even better therapeutic results were observed against the human ovarian carcinoma-bearing mice [60]. In the following study, the cathepsin B-responsive prodrug conjugated polymers were additionally functionalized with OV-TL, a monoclonal antibody [61]. The incorporation of antibodies to the prodrugs dramatically increased their localization to tumors.

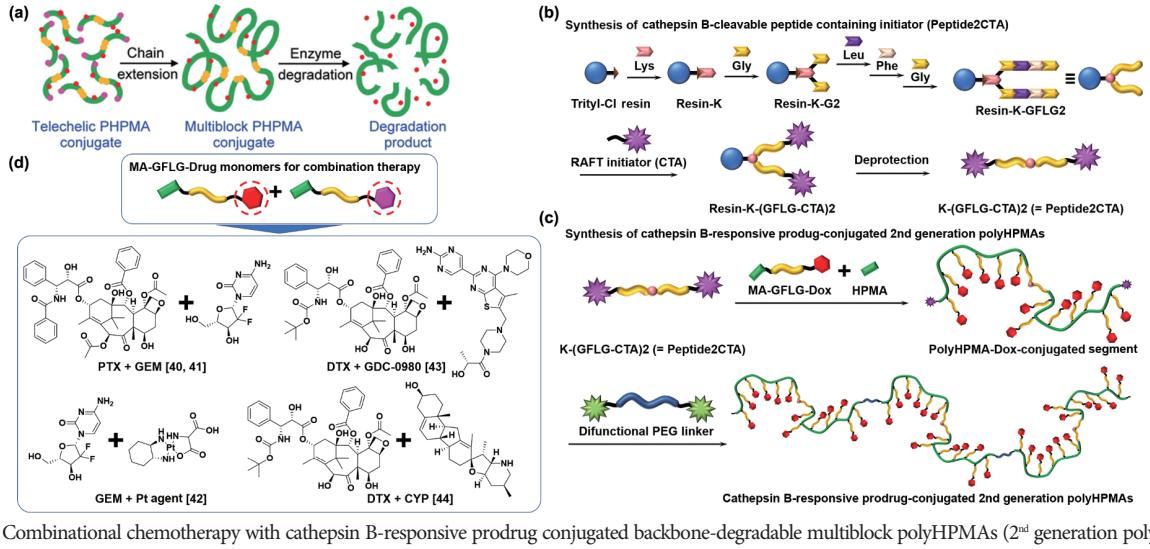


Figure 3 Combinational chemotherapy with cathepsin B-responsive prodrug conjugated backbone-degradable multiblock polyHPMAs (2nd generation polyHPMAs). (a) Schematic illustration for the mechanism of action of cathepsin B-responsive prodrug conjugated 2nd generation polyHPMAs. (b) Synthetic scheme of cathepsin B-cleavable peptide-containing initiator. (c) General synthetic scheme for cathepsin B-responsive prodrug conjugated 2nd generation polyHPMAs. PolyHPMA blocks were synthesized via the RAFT method using cathepsin B-cleavable peptide-containing initiators. Polymerized blocks were linked to each other using telechelic oligomers. (d) Cathepsin B-responsive prodrug-conjugated monomer pairs examined for combination chemotherapies using cathepsin B-responsive prodrug conjugated 2nd generation polyHPMAs. Reproduced with permission from Ref. [45], © American Chemical Society 2010.

Polymer-directed enzyme prodrug therapy (PDEPT) is a therapeutic method where polymer-conjugated enzymes are successively delivered with enzyme-specific polymeric prodrugs. The concept for the combinatorial delivery of enzymes and their corresponding prodrugs was originated from the antibody-directed enzyme prodrug therapy (ADEPT), in which enzymes that have reached the tumor site by antibodies trigger the activation of prodrugs [62]. The optimal procedure for PDEPT involves the administration of polymeric prodrugs for boosting the tumor targeting, followed by the subsequent treatment with the polymer-enzyme conjugate. The polymer-enzyme conjugates arrive at tumor tissue via the EPR effect and promote the release of active drugs from the polymeric prodrugs. PDEPT is a complementary strategy to overcome tumor heterogeneity by managing the pharmacokinetics of prodrugs. The first PDEPT system was established by R. Satchi and R. Duncan, using the polyHPMA-conjugated cathepsin B and PK1 [63, 64]. The tumor accumulation of polyHPMA-conjugated cathepsin B increased 4.2 times compared to free enzyme and accelerated the release of DOX from PK1 by 3.2 times. Various polymer-based enzyme/prodrug combinations for PDEPT have been developed in subsequent studies, but the first system by R. Duncan's group is the only case of using cathepsin B as a target enzyme.

Recently, traditional chemotherapy is newly emerging as a promising treatment to initiate antitumor immune responses by inducing immunogenic cell death (ICD) [65]. Some anticancer drugs, such as anthracyclines, mitoxantrone, oxaliplatin, and paclitaxel, induce the expression of damage-associated molecular patterns (DAMPs) from cancer cells to promote the cross-presentation of tumor-associated antigen to T cells [66]. This series of ICD events could recruit a large number of immune cells in the tumor microenvironment to reverse immunosuppressive "cold tumor" into immune-responsive "hot tumor" and thus improve the immunotherapy efficiency [67]. By these mechanisms, polymeric prodrugs have been investigated for effective and safe cancer immunotherapy. L. Li et al. proposed polyHPMAs containing epirubicin-GFLG (KT-1) for combinatorial treatment with immune checkpoint blockade [68]. As depicted in Fig. 4, the treatment of KT-1 elicited an effective ICD of cancer cells via efficient delivery by polymer and activated the CD8+ T cell responses to tumors, but they also resulted in upregulation of PD-L1, an immune checkpoint on cancer cells. The MPPA, PD-L1 antagonist peptide-conjugated polyHPMAs, were additionally delivered to tumor tissues for blocking the PD-L1 and thus disrupting the immune-suppressing pathways of cancer cells. The anticancer efficacy of this polymer-based combinational immunotherapy was reassured in transgenic mouse mammary tumor virus-polyoma middle tumor-antigen (MMTV-PyMT) models, which mimics many aspects and complexities of the clinical breast carcinomatosis [69].

2.1.2 Cathepsin B-responsive prodrug conjugated poly(ethylene glycol)s (PEGs)

PEG is the most frequently used polymeric biomaterial in a clinic, which has some unique properties which differentiate it from other biocompatible polymers. Although it does not contain any side group in its repeating unit, the backbone of PEG is highly water-soluble itself. When hydrophobic drug molecules are linked at the ends of PEG chains to produce cathepsin B-responsive prodrug conjugated PEGs, their solubility in water can be remarkably modulated and they occasionally self-assemble into nanostructures (Fig. 5). For example, the hydrophobic PTXs conjugated with 5 kDa PEGs via valine-citrulline-PABCs (VC-PABCs), a cathepsin B-sensitive peptide and self-immolative linkers, formed monodisperse nanoparticles with a size of 120 nm [70]. This nano-particulated cathepsin B-responsive prodrug conjugated PEGs showed much higher water solubility, lower systemic toxicity, and a better antitumor effect than free PTX or taxol formulation. Similarly, PDCs composed of 3 kDa linear PEG and indomethacin (IND) conjugates at both ends of the polymers with amide bonds were reported to form nanoparticles due to their amphiphilic properties and be selectively degraded by cathepsin B [71]. The PEGylation of IND and thus its nano-formulation effectively improved the aqueous

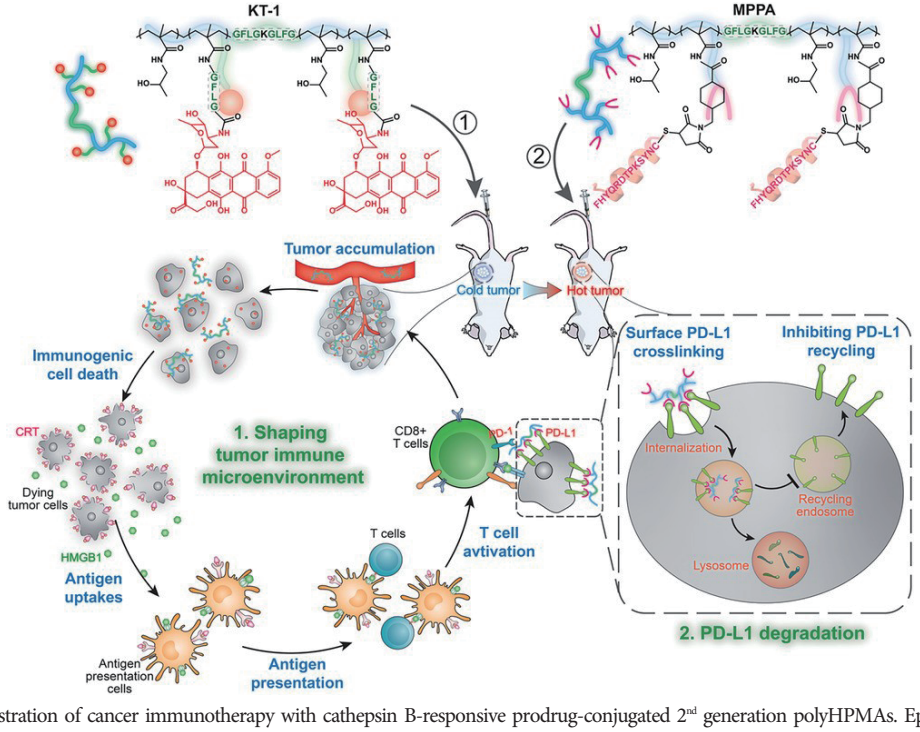


Figure 4 Schematic illustration of cancer immunotherapy with cathepsin B-responsive prodrug-conjugated 2nd generation polyHPMAs. Epirubicin-conjugated and PD-L1 antagonist-conjugated 2nd generation polyHPMAs were co-delivered to tumor tissues, exhibiting ICD with PD-L1 blockade. Reproduced with permission from Ref. [68], © WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 2020.

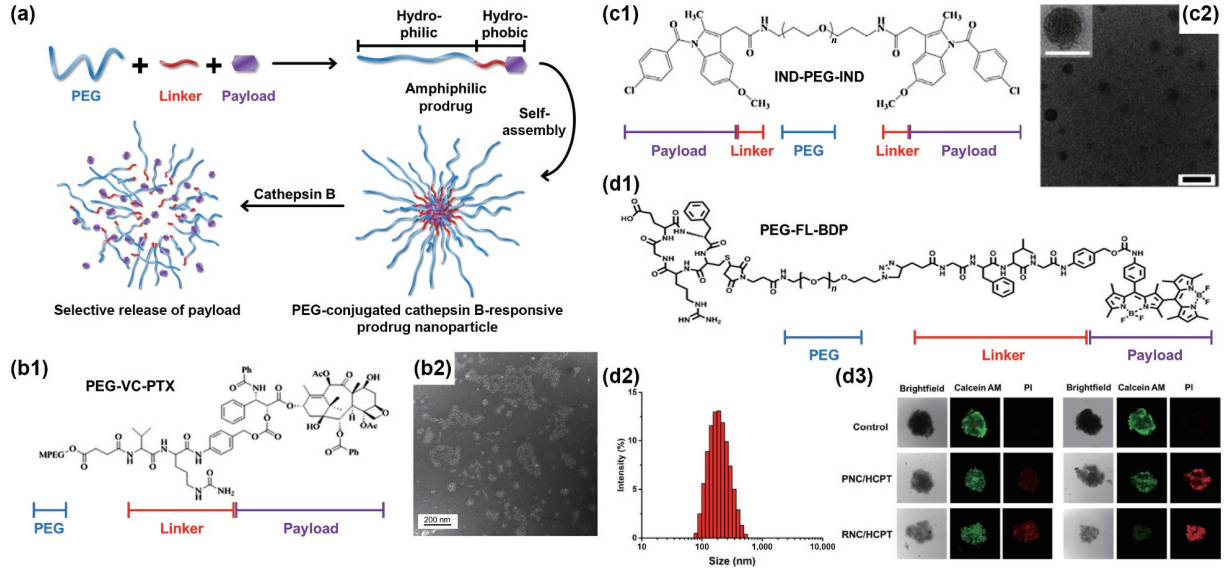


Figure 5 (a) Schematic illustration for the synthesis of cathepsin B-responsive prodrug conjugated PEGs and preparation of their self-assembled nanoparticles. (b1) A chemical structure of the PEG-VC-PTX prodrug and (b2) TEM image of their self-assembled nanoparticles (scale bar = 200 nm). (c1) A chemical structure of the IND-PEG-IND prodrug and (c2) their TEM image (scale bar = 100 nm). (d1) A chemical structure of PEG-FL-BDP and (d2) their DLS analysis. (d3) The treatment of PEG-FL-BDP nanoparticles induced highly selective therapeutic effects against 4T1 tumor spheroids under laser irradiation. (b2) Reproduced with permission from Ref. [70], © Elsevier B.V. 2012. (c2) Reproduced with permission from Ref. [71], © Elsevier B.V. 2017. (d2) and (d3) Reproduced with permission from Ref. [73], © American Chemical Society 2020.

solubility and bioavailability of the hydrophobic drug. Camptothecin [72] or boron dipyrromethene chromophore [73] have also been exploited as a payload of nanoparticles of cathepsin B-responsive prodrug conjugated PEGs.

Unique characteristics of the PEGs such as the total electrical neutrality, high hydrophilicity, and fully stretched conformations in aqueous conditions endow the antifouling effect to prodrugs, reducing the approach of bio-substances related to the *in vivo* drug clearance. The PEGylation of cathepsin B-responsive prodrug can therefore efficiently prolong their circulation time and is a widely used strategy, especially for protein or antibody delivery [74, 75]. C. Y. Dai et al. modified the tumor necrosis factor-α (TNF-α) with VC dipeptide linker-attached 40 kDa PEGs (PEG-vcTNF-α) [76, 77]. The half-life of TNF-α was increased to 21.22 h after the binding of PEG-vc, 65-fold longer than before the modification. The activity of PEG-vcTNF-α was rapidly recovered in tumor tissues by the detachment of PEG chains through cathepsin B enzymatic reaction, showing 10-fold higher potent than the PEGylated TNF-α without VC linker against a murine fibrosarcoma.

One minor drawback of cathepsin B-responsive prodrug conjugated PEGs is that the PEG chains have a limited number of points where drugs and stimuli-responsive linkers are introduced, which reduces its drug/polymer ratio. The shortage of drug junction points in the PEGs is originated from the absence of side functional groups in its backbone. To ease this complication, PEGs are often grafted to other multifunctional polymers [78, 79] or peptides [80] which are able to carry several moieties together. Another strategy is to tailor the molecular architecture of PEGs to a brush, star, hyper-branched, or dendritic shape, which will be dealt with in Section 2.2.

2.1.3 Cathepsin B-responsive prodrug conjugated polysaccharides and polypeptides

Biopolymers (e.g., polysaccharides or polypeptides) are attractive modalities for cathepsin B-responsive prodrug delivery systems [81]. Composed of carbohydrates or amino acids, main chains are enzymatically degradable so that their applications to PDCs are less limited by molecular weights, which is a significant difference from those of polyHPMAs or PEGs. Because of their repeating units, biopolymers are usually biocompatible even after their degradation and contain functional groups where drugs can be introduced. Among the polysaccharide derivatives, dextran [82–84], polygalactosamine [85, 86], and chitosan [87] were adopted as prodrug carriers. Anticancer drugs were coupled to polymers via cathepsin B-cleavable peptide linkers and main chains in polysaccharide-based prodrugs were successfully degraded under lysosomal conditions. X. Zhang et al. further modified the chitosan PTX prodrug nanoparticles with hyaluronic acid-PEGs (HA-PEGs) to target the CD44 receptor for enhancing the receptor-mediated endocytosis via active targeting [88]. The HA-PEG modification of prodrug nanoparticles increased their tumoral uptake and degraded by hyaluronidase after the cellular internalization. Poly(glutamic acid) (PGA) is a type of polypeptide containing carboxylic acids in side groups. The intrinsic feature of PGA is that the cathepsin B can hydrolyze its backbone, thereby no additional linkers are required for the formulation of PGA-based prodrugs. Cell Therapeutics, Inc. developed the PTX-conjugated PGA (PTX-PGA) to improve the PTX solubility [89]. PTXs were covalently bound to the carboxylic acids in PGA with ester bonds, and their conjugation ratio was controlled to approximately 10%. PTX-PGA was found to be highly soluble without using Cremophor or alcohol, conventional solubilizing agents for PTX, and underwent the enzymatic proteolysis and subsequent hydrolysis to release free PTXs. Determining its biocompatibility through a series of preclinical toxicology tests, the PTX-PGA (named CT-2103; OPACIO™) has entered clinical trials in 2003 [90].

2.2 Cathepsin B-responsive prodrug conjugated dendrimers or star-shaped polymers

One of the main hurdles that block cathepsin B-responsive prodrug conjugated polymers from clinical translation is their broad and uncontrollable molecular weight distribution, thereby hindering reproducibility [91]. Several studies have described dendrimers, hyperbranched polymers whose architectures and molecular weights are precisely controlled, as alternatives to linear polymers. They are generally highly soluble in water and form

globular shapes in the aqueous media, which allows each of their molecules to exist as an independent nanoparticle. In addition, dendrimers have abundant peripheral functional groups to attach anticancer drugs, indicating suitable characteristics as prodrug carriers. J. L. Ong et al. exploited the dendritic polyamidoamine (PAMAM), a representative dendrimer for biomedical applications, to enhance the bioavailability of PTX [92]. PTXs were introduced on the surfaces of generation 4 (G4) PAMAM dendrimers through GFLG sequences with approximately 7 drug/polymer ratios. The PAMAM dendrimer-based cathepsin B-responsive prodrugs showed an acceptable water solubility even after the PTX conjugation, and they induced 48% better tumor reduction than free PTX. In the following study, the authors loaded PTX-conjugated PAMAM dendrimers in folate-functionalized liposomes for an active tumor targeting, which further improved the antitumor effects [93]. M. Calderon et al. prepared a different type of dendrimer-based prodrug via polyglycerol dendrimer [94]. The polyglycerol dendrimer contained lots of hydroxyl groups not only on its periphery but also in its core structure, thus it was extremely hydrophilic. Methotrexates (MTXs) and DOXs were attached to polyglycerol dendrimers with FK-PABC linkers, respectively, and their superior antiproliferation effects were examined against human tumor cell lines.

The conjugation of drugs, particularly those poorly soluble in water, onto dendrimers can efficiently alter their physicochemical properties and physiological stabilities [95]. The star-shaped polymers, which can be generated by grafting the hydrophilic linear polymers to the peripheral groups of dendrimers, are therefore adopted to alleviate the drawback of dendrimers. T. Etrych et al. designed star-like polyHPMA-based prodrugs through grafting the semi-telechelic linear polyHPMAs to generation 2 (G2) PAMAM dendrimers [96]. Cathepsin B-cleavable peptide linker was inserted between the polyHPMA and PAMAM dendrimer for the renal clearance of the star-like polymer, and GFLG-DOXs were conjugated to the side groups of polyHPMA rather than to dendrimers. Under the lysosomal condition, the polyHPMA arms were detached from PAMAM dendrimers and free DOX was released simultaneously.

PEG is another water-soluble polymer that can be grafted onto dendrimers for star-like morphologies. S. J. Lee et al. functionalized the small molecular dendrimer-like compound (NTN1956) which has 12 carboxylic acid groups at the end with GFLG-PEG and GFLG-DOX to prepare star-shaped PEG-based prodrugs [97]. Unlike star-polymers with polyHPMA arms, GFLG-DOXs were directly linked to the dendrimer since the PEG arm does not contain any side group. The star-shaped PEG-based prodrugs exhibited cathepsin B-responsive DOX release and potent anticancer activity. Z. Gu's group also developed another PEG-grafted star-shape polymer as a prodrug carrier [98, 99]. Using the polylysine dendrimers for the core of star-polymers, GFLG-DOX and GFLG-GEM were introduced with GFLG-PEG to the dendrimer. To overcome the steric hindrance by PEG and improve the reaction efficiency, the covalent conjugation between the dendrimer and GFLG linker was achieved via the copper-catalyzed alkyne-azide click chemistry. The PEG graft effectively improved the rapid *in vivo* clearance of drug-conjugated dendrimers by enlarging their hydrodynamic volumes. In further studies by Z. Gu and coworkers, the PEGylated Janus-type dendrimer was newly proposed as a DDS for the prodrug [100, 101]. The Janus dendrimers were composed of polylysine cores, PEGylated hemispheres, and the other half parts functionalized with GFLG-drugs (Fig. 6). Janus dendrimers had amphiphilic properties and were self-assembled into nanoparticles with sizes of ~ 90 nm, resulting in their prolonged circulation time and increased tumor accumulation.

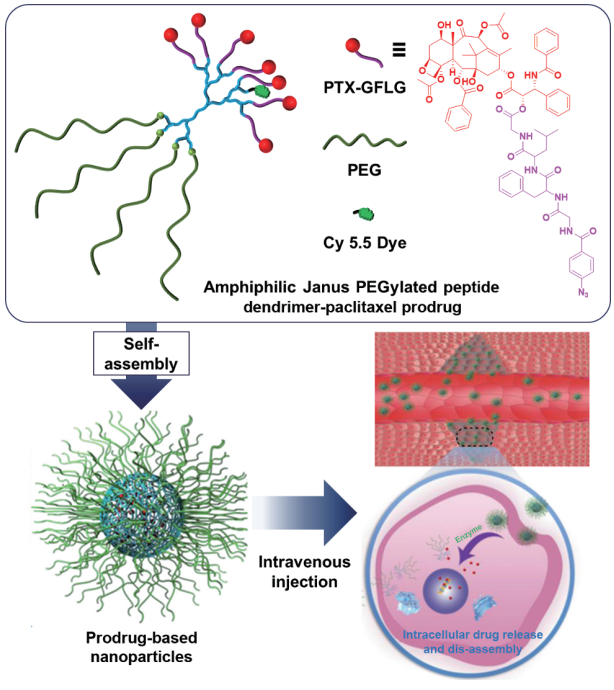


Figure 6 A chemical structure of the cathepsin B-responsive prodrug conjugated Janus-type dendrimer and description of their self-assembled nanoparticles. GFLG-DOX was conjugated to hemispheres of polylysine dendrimers via the copper-catalyzed click chemistry. With the cleavage of GFLG linkers, dendrimer-based nanoparticles were degraded and released free DOXs in tumor cells. Reproduced with permission from Ref. [101], © American Chemical Society 2017.

2.3 Cathepsin B-responsive prodrug conjugated antibodies

Over the past few decades, antibody-mediated DDS has emerged as a promising technique for cancer treatment with notable potency. By recognizing and specifically binding the tumor-associated biomarkers, anticancer payloads conjugated to antibodies can be localized to the targeted tumors, relieving their systemic toxicities and improving their therapeutic effects. This therapeutic system is known as an antibody-drug conjugate (ADC) and is a major alternative for small molecular anticancer drugs due to its plasma stability, tumor targeting, and biocompatibility. About 30 kinds of different monoclonal antibodies have been approved by the US Food and Drug Administration (FDA), accordingly, over 100 ADCs targeting various types of diseases are also under clinical evaluation [102].

ADCs are typically composed of antibodies, therapeutic agents, and linkers between them. Shown in Fig. 7, conventional ADCs include the conjugates in which linker is not cleavable as well, where the degradation of entire antibodies must occur for the discharge of active drugs [103, 104]. In recent studies, ADCs with stimuli-sensitive linkers have been more preferentially explored since their mechanism of drug release is much simpler than that of non-cleavable ADCs. There are some requirements for the linker for the development of cathepsin B-responsive prodrug-conjugated antibodies. The typical cathepsin B-cleavable tetrapeptidic linkers (e.g., GFLG or ALAL) cannot be used for the synthesis of ADC since their structure is too complex and the cleavage rate is to be slow [105]. Rather, dipeptidic linkers have been preferentially investigated for ADCs. VC and FK are representative sequences for ADC linkers showing high systemic stability and rapid hydrolysis under the presence of cathepsin B [103, 106]. For the drugs with bulky structure, self-immolative spacers such as PABC can be added to the linkers to prevent the reduction of enzymatic activity by steric hindrance [107]. There

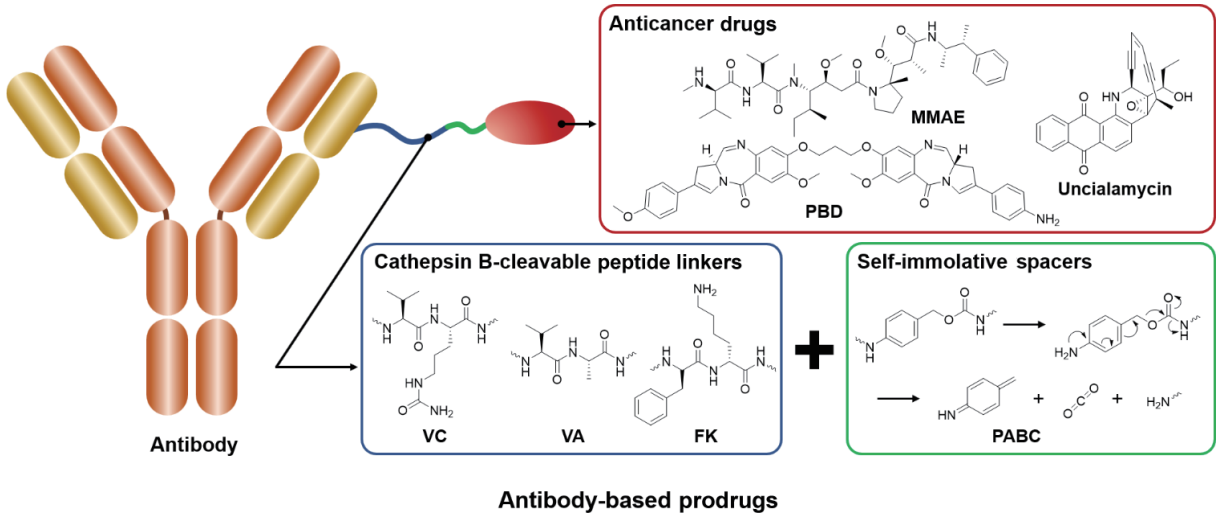


Figure 7 Schematic illustration of cathepsin B-responsive prodrug conjugated antibodies.

are mainly two sites on the antibodies for the introduction of linkers, lysine and cysteine residues [108]. Incorporating the N-hydroxylsuccinimide (NHS) ester or maleimides, cathepsin B-cleavable linkers can be conjugated to primary amines or thiols on the antibodies, respectively. It was reported that the cleavage of linkers by cathepsin B is not dependent on the antibody species or the location of linkers on antibodies [109]. Nonetheless, the binding site of linkers should be precisely controlled because the non-specific attachment of linkers can impact the stability and pharmacokinetics of ADCs [110].

Through the chemical modification of cathepsin B-cleavable linkers, the stability and degradation rate of ADC-based prodrugs can be improved. Despite high enzymatic selectivity and systemic stability, the VC-PABC linker was found to be vulnerable to mouse serum, which made the preclinical assessment with the mouse tumor model difficult. Y. B. Poudel et al. determined that the use of *m*-amide *p*-aminobenzyl carbamate (MA-PABC) instead of PABC remarkably improved the stability of linkers in mouse serum [111]. The ADCs with MA-PABC spacers released out only 6% of the conjugated drug after 24 h in the mouse serum, whereas 80% of those with normal PABC were degraded at the same condition. The use of a peptidomimetic linker can also be an effective approach to improve the cathepsin B sensitivity of ADC-based prodrugs. B. Wei et al. modified the VC linkers by substituting valines to cyclobutane-1,1-dicarboxamide (cBu) groups [112]. When the cathepsin B inhibitor was treated, the cleavage of the cBu-Cit linker was drastically suppressed to 25% whereas that of the VC linker hardly changed. The antitumor effect and *in vivo* stability of cBu-Cit linker-containing ADCs were preserved to an equivalent level to those of ADCs with VC linkers. In another study, the carbamate bond in the VC-PABC linker was replaced with phosphate groups [113]. The VC linker with phosphate group indicated higher water solubility and plasma stability than the VC-PABC linker. The phosphate bridge in the linker was enzymatically cleaved, releasing the free drugs.

The selection of adequate payloads for ADCs is a critical key factor that determines their therapeutic achievements. When the anticancer drugs are conjugated to antibodies, they must be inactive to prevent any severe adverse reactions and maintain their stabilities during circulation. After anticancer drugs are cleaved from antibodies under cathepsin B-overexpressed tumor microenvironment, they should immediately restore potencies. Payloads with extreme potencies are often more favorable for ADC applications than traditional chemotherapeutics since ADCs have limitations not only in their drug/antibody ratio (DAR) but also in their biodistribution and tumoral accumulation [114, 115]. Focusing on the screening of novel drugs, derivatives of enediynes [116, 117], liver X receptor agonists [118], pyrrolobenzodiazepines (PBDs) [119, 120], and isoquinolidinobenzodiazepine [121] have been assessed to develop cathepsin B-responsive ADCs. Those drugs exhibited excessively low IC_{50} values in their free forms, were successfully conjugated to antibodies via VC or FK linkers, and promptly recovered their cytotoxicities in tumors.

In general, ADC is not a good platform for the co-delivery of multiple payloads or imaging modalities because there is only a restricted number of binding sites on the antibodies. The administration of ADC-based prodrugs with subsequent chemotherapies is rather a plausible approach to combination therapy. E. Oflazoglu et al. combined the ADCs (SGN-35) with anticancer drugs such as ABVD (DOX, bleomycin, vinblastine, and dacarbazine) or GEM for the treatment of Hodgkin lymphoma (HL) [122]. SGN-35 was composed of monomethyl auristatin E (MMAE; tubulin inhibitor), cAC10 (chimeric anti-CD30 monoclonal antibody), and VC linker between them. The combination of SGN-35 with ABVD or GEM significantly increased the antitumor responses when treated to HL-bearing mice. Another preliminary test was conducted using the trastuzumab-based ADCs and free geldanamycin as combinants [123]. The VC-MMAE-conjugated trastuzumab (T-MMAE) was prepared and co-treated to human epidermal growth factor (HER2) overexpressing cancer with geldanamycin (Fig. 8). The co-delivered geldanamycin effectively increased the lysosomal accumulation of T-MMAE by inhibiting the endosomal recycling of HER2, resulting in enhanced cytotoxicity to tumor. D. Xiao et al. designed a special self-immolative spacer to harness ADCs to theranostic purposes [124]. Utilizing the 7-amino-3-hydroxyethyl-coumarin (7-AHC) as a spacer, a novel ADC comprising MMAE, anti-HER2 antibody, and VA linker was synthesized. The 7-AHC maintained fluorescence quenching until the VA linker was cleaved. With the enzymatic action of cathepsin B, both MMAE payload and 7-AHC spacer were released out, regaining the fluorescence signal. This technique is quite remarkable as it is one of the rare cases that endowed multiple functionalities into one antibody.

2.4 Cathepsin B-responsive prodrug conjugated lipids

Lipids are hydrophobic or slightly amphiphilic biomolecules including fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides [125]. When lipids are conjugated to drug molecules, called lipid-drug conjugates (LDCs), the pharmacological

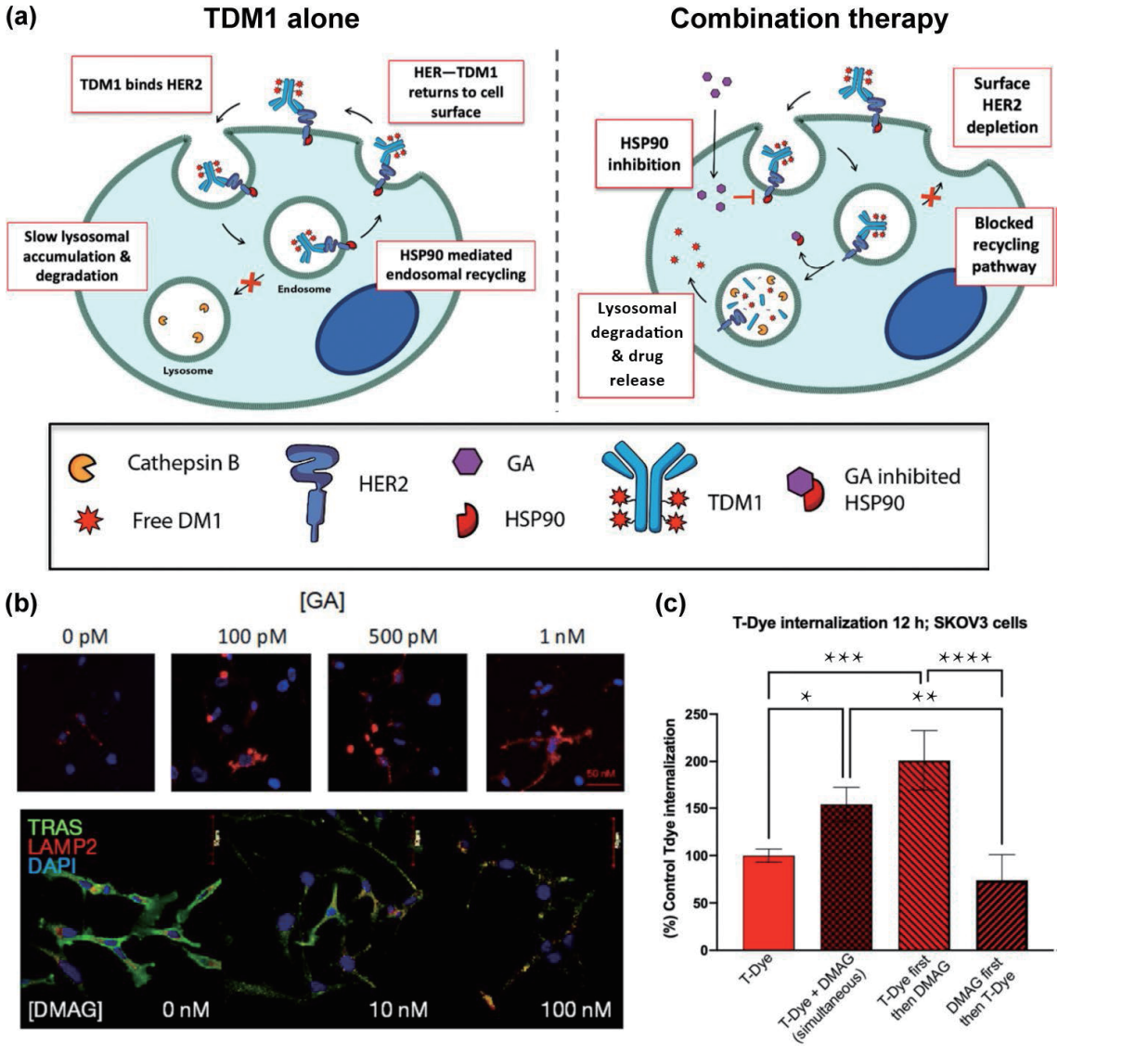


Figure 8 Combination therapy with cathepsin B-cleavable prodrug conjugated antibodies (TDM1) and small molecular chemodrugs (geldanamycin; GA). (a) Schematic description for the mechanism of action of the TDM1/GA combination therapy. The co-administration of GA inhibited the re-expression of HER2 proteins. (b) Fluorescence images showing the TDM1 internalization with different amounts of GA and its analog drug, DMAG. (c) The treatment of GA or DMAG enhanced the internalization of TDM1. Reproduced with permission from Ref. [123], © Elsevier B.V. 2021.

properties of drugs are greatly modified. LDCs can be more efficiently loaded in the drug carriers such as liposomal vehicles due to the changes in their water solubility, and sometimes self-assemble themselves and form various shapes of particles depending on their molecular morphologies and compositions [126]. The encapsulation of LDCs in liposomes or their spontaneous assembly into nanostructures promotes desirable effects on the pharmacokinetics and systemic availabilities of drugs. LDCs also have some advantages over synthetic polymer-based prodrugs in terms of biocompatibility and clearance, since they are originally bioderived materials and have a small molecular weight [127].

The LDCs with amide bonds between lipids and drug molecules have been reported to be sensitive to cathepsin B [128]. 4 kinds of saturated fatty acids, valeric, heptanoic, lauric, and stearic acid, were directly linked to GEM via amide bonds and loaded to the shell of liposomes composed of 1,2-distearoyl-phosphatidylcholine and 1,2-distearoyl-phosphatidylglycerol [129]. The loading efficiency of the lipid-GEM prodrug was closely related to the carbon length of lipids, showing 99% of loading efficiency when stearic acids were conjugated. Through the conjugation of fatty acid and encapsulation in a liposome, the prodrugs exhibited 3.5 times longer plasma half-life and 50-fold higher AUC values compared to free drugs. The amide bonds between the drug and fatty acid were successfully degraded in presence of cathepsin B, resulting in controlled release of free drugs at intracellular condition.

Since GEM is one of the hydrophilic drugs, the lipid-GEM prodrugs show amphiphilic natures and can spontaneously assemble to form nanoparticles themselves (Fig. 9) [130, 131]. The physicochemical properties of lipid-GEM prodrug nanoparticles depend on the structure of lipid and thereby the hydrophilic-lipophilic balance (HLB) [132]. If the HLB is lower than 8.34, lipid-GEM prodrugs could form stable nanoparticles without using additional excipients. In another study, the lipid-GEM prodrug nanoparticles were also functionalized with active targeting moieties and their therapeutic effect was assessed against pancreatic ductal adenocarcinoma [133]. Linoleic acid was conjugated to GEM via an amide linkage, mixed with plectin-1 targeting peptide (PTP)-bound 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol) 2000] (DSPE-PEG_{2k}), and allowed to form nanoparticles. The lipid-GEM prodrug and DSPE-PEG_{2k}-PTP mixtures formed stable nanoparticles with sub-100 nm size, which successfully delayed the

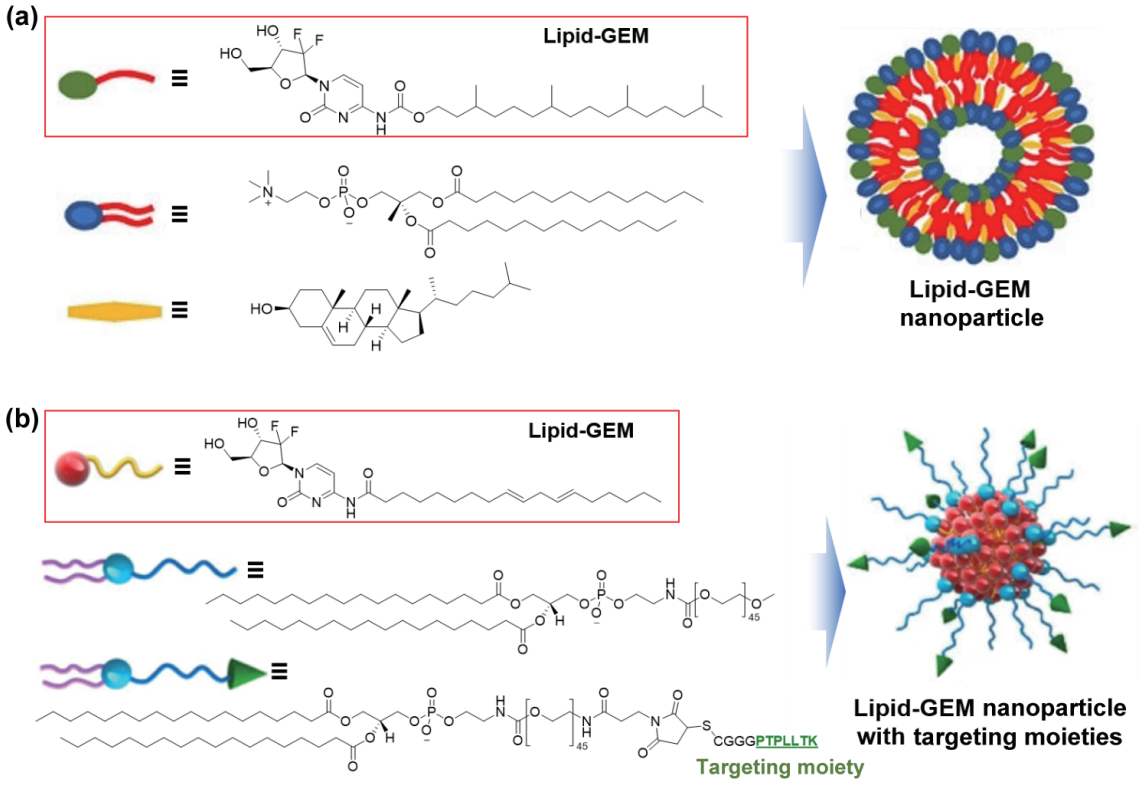


Figure 9 Schematic description of prodrug nanoparticle formation using the lipid-conjugated GEM (Lipid-GEM). (a) Lipid-GEM is spontaneously self-assemble into nanoparticles with a size of 136 nm. The addition of phospholipid and cholesterol enhanced the stability of GEM-Lipid nanoparticles. (b) The use of tumor-targeting peptide-conjugated phospholipid furtherly endows active targeting properties to Lipid-GEM-based prodrug nanoparticles. (a) Reproduced with permission from Ref. [130], © Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim 2020. (b) Reproduced with permission from Ref. [133], © American Chemical Society 2019.

undesirable metabolism of GEM during systemic circulation. The cathepsin B-responsive prodrug nanoparticles also showed specific accumulation in the tumor site via the EPR effect and the active targeting of PTPs. After being internalized to target cancer cells, cathepsin B-responsive prodrug nanoparticles were degraded by lysosomal cathepsin B and exhibited high therapeutic efficacy compared to the free GEM.

LDC prodrugs are greatly adaptable for combination therapy because they are able to form nanoparticles and easily encapsulate other drugs inside the core. Y. Li et al. synthesized lipid-conjugated mitomycin C (MMC) and MTX, and developed prodrug micelles for the combined anticancer therapy [134]. MTX-bound DSPE-PEG_{3k} and MMC-bound soybean phosphatidylcholine were prepared respectively, and they were mixed to form micelle with ~ 40 nm in size. The cathepsin B-responsive prodrug micelles were stable at common physiological conditions and rapidly released drugs under the presence of cathepsin B. MMCs on the surfaces of the prodrug micelles not only acted as active targeting moieties but also showed synergistic anticancer effect together with MTX in tumor tissues. The co-delivery of GEM and isocombretastatin A-4 (isoCA-4; a vascular disrupting agent) via LDC displayed favorable outcomes against colon cancer as well [135]. The squalene-conjugated GEM (SQ-GEM) prodrug constructed the nanoparticle without additional materials and the hydrophobic isoCA-4 molecules were embedded. The isoCA-4-loaded SQ-GEM nanoparticle demonstrated its high antiangiogenic and antiproliferative activities, showing a 1.3-fold higher tumor suppression effect than SQ-GEM alone.

Unfortunately, only a few cases of the cathepsin B-responsive LDC prodrugs have been reported even if there are various studies conducted with LDCs. Moreover, the drugs and lipids in LDC are conjugated with simple amide bonds, not with oligopeptide sequences, which are cleaved under lysosomal conditions. In other words, the LDC prodrugs are lack enzyme specificity, being cleaved even with other enzymes such as cathepsin D. Further investigation into the discovery of novel linkers specific for cathepsin B and applicable to the LDC system is required.

2.5 Cathepsin B-responsive prodrug conjugated inorganic nanoparticles

Inorganic nanoparticles have been widely used for biomedical applications due to their distinct characteristics which are difficult to implement with organic biomaterials, in regard to thermogenicity, electromagnetic property, or radiosensitivity. Their features endow the inorganic nanoparticle-based prodrugs with additional functionalities, making them appropriate for combination therapies or theranostic purposes. Cathepsin B-responsive prodrug conjugated inorganic nanoparticles can be prepared by conjugating drugs on the nanoparticle surfaces with cathepsin B-cleavable linkers, and the strategies for the surface functionalization are fully established depending on the kind of nanoparticles. Y. Ding et al. prepared ultra-small gold nanoparticles (AuNPs) and covered their surfaces with triblock peptides via Au–S bond for their application to cancer radiotherapy [136]. Triblock peptides consisted of cell-penetrating peptides (CPPs; VEKEKEKEKEK), cathepsin B-cleavable linkers (GFLG), and zwitterionic peptides which has an antifouling property. AuNPs were internalized to cancer cells through the action of CPPs, the linker was cleaved with cathepsin B, and bare AuNPs reached the nucleus. Under the X-ray irradiation, AuNPs exhibited radiosensitivity and damaged the DNA in the cancer nucleus. After the radiotherapy, the AuNPs underwent renal excretion due to their small sizes (~ 3.9 nm).

To prolong the stability of inorganic nanoparticles in the physiological condition, they are often coated with biocompatible polymers where drug molecules can be attached [137]. Y. Yang et al. modified the surfaces of silica-coated iron oxide magnetic

nanoparticles with linear PEGs and conjugated FK-PABC-DOXs to the distal point of PEGs via click chemistry [138]. Magnetic nanoparticles released free DOX under the presence of cathepsin B and simultaneously performed as contrast agents for magnetic resonance imaging (MRI) as well, indicating theranostic properties. In another study, quantum dots (QDs) were functionalized on their surfaces with amphiphilic polymers which were linked with GFLG-GEM prodrugs (Fig. 10) [139]. QD surfaces were further modified with PEGylated matrix metalloproteinases (MMP)-sensitive peptide and RGD sequences, becoming dual-enzyme sensitive. The QDs selectively accumulated to tumor tissues via the EPR effect and their cellular internalization was accelerated due to the active targeting by RGDs. The lysosomal cathepsin B cleaved the GFLG peptide on them, inducing the specific delivery of GEM to cancer cells.

Throughout the studies in the majority, inorganic nanoparticles have been exploited as carriers in which free therapeutic agents were entrapped via physical interactions. Cathepsin B-responsive prodrug conjugated inorganic nanoparticles have rarely been investigated because the direct conjugation of drug molecules on nanoparticles is quite inefficient in terms of their loading amounts, and an excessive amount of drugs on the surface of nanoparticles can substantially impair its stability. There are various studies about inorganic drug carriers which can release entrapped drugs in response to the cathepsin B [140–143], which is, in strict, hard to be defined as prodrugs since therapeutic agents loaded in nanoparticles are not in their inactive states. Certain types of inorganic nanoparticles frequently studied (e.g., AuNP and QD) are not biodegradable, which is another challenge for the prodrug design. The low drug capacity and poor biodegradability of inorganic nanoparticles necessitate the further optimization of their substances and morphologies for the successful application to prodrug carriers.

3 Clinical application and future perspectives

Inspired by encouraging results in their preliminary tests, diverse carrier-based cathepsin B-responsive prodrugs have been transferred to clinical trials. Table 2 describes the basic information and recent status of carrier-based cathepsin B-responsive prodrugs in clinical trials so far. PolyHPMA has been most frequently explored for polymeric prodrug development, some of which have passed the preclinical stages and progressed into phase I assessment. PK1 is the first cathepsin B-cleavable polyHPMA-based prodrug to be evaluated in clinical trials. In its phase I trial, PK1 was intravenously infused to patients with solid tumors every 3 weeks to determine its maximum tolerated dose (MTX), pharmacokinetics, and toxicity. It was demonstrated that PK1 had an MTD of 320 mg/m^2 (DOX equiv.) and a distribution half-life ($T_{1/2}$) of 1.8 h, which were 5-fold and 15-fold higher than those of free DOX, respectively [144]. The polyHPMA did not induce any toxicity or immunogenicity, and no DOX-related cardiotoxicity was observed at its cumulative doses up to 1,680 mg/m^2 (DOX equiv.). Its toxicity to non-specific tissues was effectively reduced compared to free DOX, and the adverse effects were also not significant. According to the outcome, the optimal dose of PK1 for phase II analysis was set to 280 mg/m^2 of intravenous infusion every 3 weeks. Phase II trial of PK1 showed that 6/62 of patients with breast cancer, colorectal cancer, and non-small cell lung cancer (NSCLC) had partially responded to the repeated dose of PK1 [36]. As a derivative of PK1, PK2 has also been in clinical evaluations. The target disease for PK2 was hepatocellular carcinoma, as it was modified with galactosamine groups for the active targeting of the asialoglycoprotein receptor expressed on liver tumor cells [37]. Phase I studies using PK2 revealed its MTD of 160 mg/m^2 and $t_{1/2}$α of 78 min, somewhat lower than those of PK1 [145]. Patients with PK2 administration

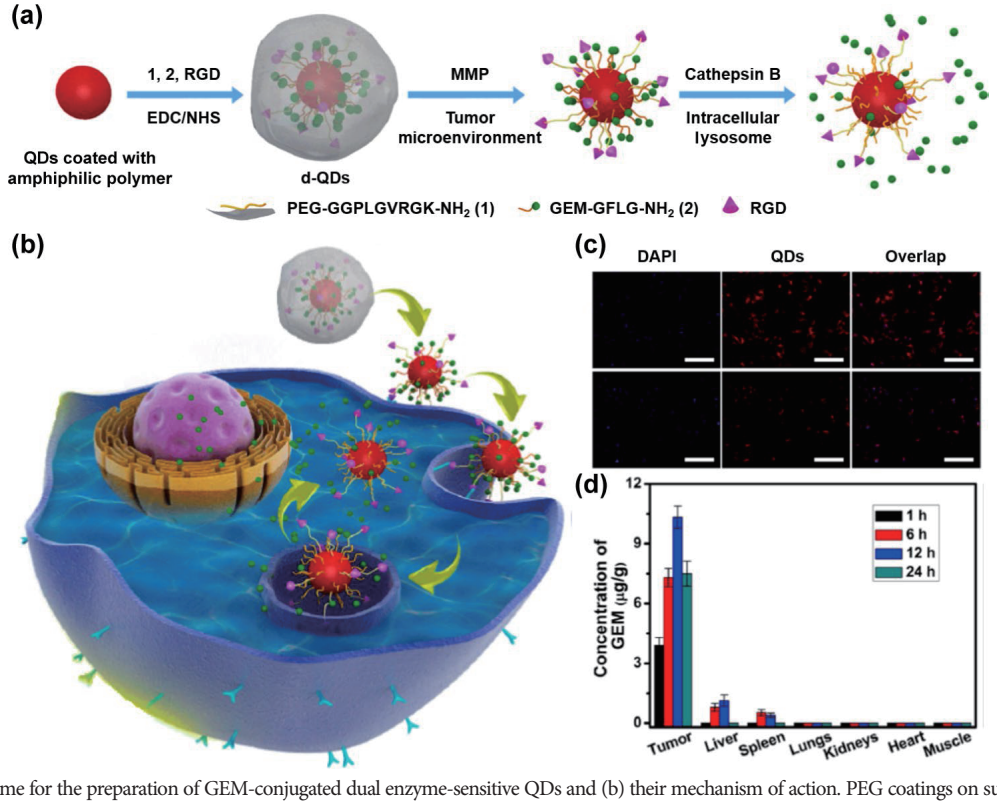


Figure 10 (a) Scheme for the preparation of GEM-conjugated dual enzyme-sensitive QDs and (b) their mechanism of action. PEG coatings on surfaces of QDs could be detached by MMP activity, improving the cellular uptake of QDs. After their internalization, endosomal cathepsin B catalyzed the release of free GEMs from QDs. (c) Fluorescence images of internalized QDs under the absence and presence of MMP inhibitor. The inhibition of MMP activity significantly blocked the endocytosis of QDs. (d) The dual enzyme-sensitive QDs efficiently delivered free GEMs to tumor tissues with high selectivity. Reproduced with permission from Ref. [139], © American Chemical Society 2017.

Table 2 Carrier-based cathepsin B-responsive prodrugs in clinical trials

Carrier type	Name	Carrier specification	Linker	Payload	Clinical status	References
Polymer	PK1 (FCE 28068)	PolyHPMA	GFLG	DOX	Phase I/II (discontinued)	[36]
	PK2 (FCE 28069)	PolyHPMA	GFLG	DOX	Phase I (discontinued)	[145]
	PNU 166945	PolyHPMA	GFLG	PTX	Phase I (discontinued)	[38]
	AP 5280	PolyHPMA	GFLG	Diammineplatium (II)	Phase I	[146, 164]
	AP 5286	PolyHPMA	GFLG	Diaminocyclohexaneplatinum (II)	Phase I	[165]
	OPAXIO™ (CT-2103)	PGA	None (degradable polymer)	PTX	Phase III	[166]
Antibody	Brentuximab vedotin (Adcetris)	Anti-CD30 (chimeric IgG1)	VC	MMAE	Approved	[102]
	Polatuzumab vedotin-piiq (Polivy)	Anti-C79b (humanized IgG1)	VC	MMAE	Approved	[152]
	Enfortumab vedotin (Padcev)	Anti-Nectin-4 (human IgG1)	VC	MMAE	Approved	[155]
	Tisotumab vedotin (Humax®-TF-ADC)	Anti-TF (human IgG1)	VC	MMAE	Approved	[167]
	Trastuzumab deruxtecan (Enhertu)	Anti-HER2 (humanized IgG1)	GGFG	Deruxtecan	Approved	[168]
	[vic]-Trastuzumab duocarmazine (SYD 985)	Anti-HER2 (humanized IgG1)	VC	Duocarmycin	Phase III	[169]
	Glembatumumab vedotin (CDX-011)	Anti-gpNMB (human IgG2)	VC	MMAE	Phase III	[170]
	Rovalpituzumab tesirine (Rova-T)	Anti-DLL3 (humanized IgG1)	VA	PBD	Phase III (discontinued)	[171]
	Vadastuximab talirine (SGN-CD33A)	Anti-CD33 (chimeric IgG1)	VC	PBD	Phase III (discontinued)	[100]
	PSMA ADC	PSMA (human IgG1)	VC	MMAE	Phase II	[172]
	Telisotuzumab vedotin (Teliso-V)	Anti-c-MET (humanized IgG1)	VC	MMAE	Phase II	[173]
	Ladiratuzumab vedotin (SGN-LIV1A)	Anti-LIV1 (humanized IgG1)	VA	MMAE	Phase II	[174]
	Lifastuzumab vedotin (RG-7599)	Anti-NaPi2b (humanized TAT211)	VC	MMAE	Phase II (discontinued)	[163]
	Indusatumab vedotin (MLN-0264)	Anti-GCC (human IgG1)	VC	MMAE	Phase II (discontinued)	[175]
	Pinatuzumab vedotin (RG-7593)	Anti-CD22 (humanized IgG1)	VC	MMAE	Phase II (discontinued)	[176]
	AGS67E	Anti-CD37 (human IgG2)	VC	MMAE	Phase I	[177]
	Sirtratumab vedotin (ASG-15ME)	Anti-SLTRK6 (human IgG2)	VC	MMAE	Phase I	[178]
	MDX-1203 (BMS936561)	Anti-CD70 (human IgG1)	VC	Duocarmycin	Phase I (discontinued)	[179]
	Vandortuzumab vedotin (RG7450)	Anti-STEAP1 (humanized IgG1)	VC	MMAE	Phase I (discontinued)	[180]
	Sofituzumab vedotin (RG7458)	Anti-Mucin 16 (humanized IgG1)	VC	MMAE	Phase I (discontinued)	[181]
	SGN-CD70A	Anti-CD70 (humanized IgG1)	VA	PBD	Phase I (discontinued)	[182]

experienced some systemic symptoms such as mucositis, fatigue, and neutropenia as dose-limiting toxicities, which limited its dose for phase II study to 120 mg/m^2. PK2 was found to be 4-fold more concentrated in normal liver tissues than in tumoral tissues, but still provided enhanced tumor accumulation compared to free DOX.

Other polyHPMA-based prodrugs conjugated with PTX (PNU 166945) or platinum agents (AP 5280 and AP 5286) have also been translated to clinical practices. PNU 166945 is a polyHPMA-based prodrug with GFLG-PTX side groups, developed for the mitigation of the poor solubility of PTX [38]. PNU 166945 contained ~ 5% (w/w) of PTXs and could be dissolved in water at concentrations of over 2 mg/mL (PTX equiv.), which was 20,000-fold higher than that of free PTX. Its phase I study was conducted in breast cancer patients, showing antitumor activity even at a low prodrug dose (100 mg/m^2, PTX equiv.). The toxicological results of PNU 166945 indicated that the systemic toxicity of free PTX could not be relieved by the prodrug, consistently inducing nausea and neuropathy [35]. AP 5280 and AP 5286 (an analog of AP 5286) are the latest works to enter clinical trials in a series of polyHPMA-based prodrug productions. The initial aim of their formation was to diminish the systemic toxicities of platinum agents which are notorious for side effects. AP 5280 included ~ 8.5% (w/w) of platinum agents linked to polyHPMA via GFLG peptides, and the platinates bound to polyHPMA were confirmed to be totally inactive [146]. In the clinical test, AP 5280 was intravenously infused to patients with solid tumors every 3 weeks. The MTD and termination half-life of AP 5280 were examined to be 4,500 mg/m^2 (Pt equiv.) and 116 h, respectively. The renal damage and myelosuppression, typical complications of platinum agents, were negligible, and only Common Toxicity Criteria (CTC) grade 3 vomit was observed, proving that the systemic toxicity of platinum agent became minimal.

Among cathepsin B-responsive polymeric prodrugs under clinical evaluations, Opaxio™, a PTX-conjugated PGA prodrug, is the sole product that has reached clinical studies by forming a

prodrug using a polymer other than polyHPMA. Opaxio™ consists of PTXs directly bonded to PGA chains without using peptidic linkers, hence its drug content is relatively higher (~ 37%, w/w) than that of PNU 166945. The phase I test of Opaxio™ was carried out on 19 patients diagnosed with a solid tumor, demonstrating its MTD of 177 mg/m^2 (PTX equiv.) and $t_{1/2}\beta$ over 100 h, respectively [147]. Opaxio™ showed considerably reduced adverse effects and higher convenience in its administration than free PTXs [148]. The final clinical status of Opaxio™ is reported to be phase III.

Despite active investigations over the decades, there are no polymer-based cathepsin B-responsive prodrugs that have been approved in the clinic yet. PK1 and PK2 had been discontinued after phase I/II trials due to their marginal anticancer efficacy [149]. For the higher accumulation in tumor and better tumor eradication effect of prodrugs, polyHPMAs in PK1 and PK2 should be much longer, which cannot be achieved with non-biodegradable polymers due to issues in their renal clearance [34]. PNU 166945 was failed in phase I before determining its dose-limiting toxicity since the polymer conjugation could not reduce the systemic toxicity of PTX enough, exerting the neurotoxicity [150]. The most critical complication of polymeric prodrugs that hinders their translation to clinics is the heterogeneity of themselves [35]. Each chain of polymeric prodrugs has a different molecular weight and DPR from one another, which significantly affects their pharmacokinetics. The molecular weight distribution of polymeric prodrugs makes it difficult to predict their therapeutic or adverse effects and to control their qualities in bulk manufacturing. There are also limitations in drug contents of polymeric prodrugs since the excessive drug conjugation on polymer chains significantly deteriorates their pharmacokinetic properties. The low drug content in polymeric prodrugs weakens their clinical efficacy and disturbs their application.

Antibody is an exclusive carrier type that has been approved by FDA for the cathepsin B-responsive prodrug delivery. There are four antibody-based cathepsin B-responsive prodrugs recently available in the clinic. All of them are comprised of MMAEs as payloads and VC peptides as cathepsin B-cleavable linkers in common, and their only structural difference is the species of antibodies. Brief explanations about the four antibody-based prodrugs were noted below.

Brentuximab vedotin (Adcetris) is a CD-30 targeting ADC for the treatment of Hodgkin's lymphoma and anaplastic large cell lymphoma (ALCL). It was developed by Seattle Genetics (Bothell, WA, USA) and FDA-approved in August 2011 [151]. Its overall response rate was measured to be 86% (57% of complete response and 29% of partial response). *Polatuzumab vedotin-piiq (Polivy)* was developed by Genentech (South San Francisco, CA, USA) and approved by FDA in June 2019 [152]. It contains a humanized immunoglobulin G1 (IgG1) antibody targeting the CD79b on the B-cell surface. It is limitedly used for the treatment of diffuse large B-cell lymphoma (DLBCL), especially in combination with bendamustine and rituximab. The combination therapy with bendamustine and rituximab showed 40% of complete response [153]. *Enfortumab vedotin (Padcev)* is an ADC containing Nectin-4-directed Human IgG1 antibody and is indicated for patients with metastatic urothelial cancer [154]. Being produced by Astellas Pharma US, Inc. (Northbrook, IL, USA), the enfortumab vedotin has received FDA approval in December 2019. Clinical studies have proven its anticancer efficacy with a 44% of response rate (12% for complete response) [155]. *Tisotumab vedotin (Humax®-TF-ADC)* has attained FDA approval in September 2021 for the treatment of recurrent or metastatic cervical cancer. Composed of human IgG1 antibody, it is directed to tissue factors (TFs) on cancer cells. The confirmed overall response rate of Tisotumab vedotin in the phase II study was 24%, with 7% of complete response [156]. Besides these products, numerous antibody-based prodrugs are undergoing clinical evaluations as illustrated in Table 2.

Although the clinical results have guaranteed the safety and versatility of antibodies as prodrug carriers, they still encounter challenges in their therapeutic efficacy, drug loading capacity, drug price, and productivity. For the antibody-based prodrugs, it was reported that the amount of drugs that are internalized to target tumor tissues are cleaved from antibodies, and finally express their therapeutic effects is only about 1.56% of the initial dose [157]. Considering the average molecular weight of antibodies (~ 150 kDa) and the limitation in DAR (< 10) of ADCs, their drug loading amount is very hard to exceed 7% (w/w). The low delivery efficiency and drug loading capacity of antibody-based drugs severely restrict their anticancer effects. Moreover, antibodies are inevitably produced via biosynthetic methods, which desperately increases the price of antibody-based prodrugs and makes their bulk manufacturing almost impossible. The problems mentioned above are not merely limited to polymer- or antibody-based prodrugs but are inevitably accompanied when using carriers with bulky structures for the prodrug design.

To address the above concerns, carrier-free prodrug nanoparticles have indicated a great potential to develop a simple and effective drug delivery system. In recent years, many researchers reported that rationally designed small-molecule prodrugs to promote the intermolecular interactions via balanced hydrophobic-hydrophilic structures could self-assemble into nanoparticles themselves without any additional carriers. These prodrug nanoparticles could achieve ultrahigh and accurate drug loading and reduce the adverse effects of carrier materials. In addition, their simple synthetic protocol allows the reproducible and scale-up industrial production for clinical translation, which overcome the shortcomings of carrier-based drug delivery. Recently, we proposed the carrier-free cathepsin B-responsive prodrug nanoparticles, fabricated by conjugation of cathepsin B-cleavable peptide FRRG with DOX. Importantly, FRRG-DOX molecules were self-assembled into spherical nanoparticles by intermolecular π–π stacking interactions among the planar aromatic rings of DOX and hydrophobic interactions of phenylalanine (F) in the peptide (Fig. 11) [19]. It was also demonstrated the precise design of FRRG-DOX by confirming that RRG-DOX in absence of hydrophobic F sequence did not form nanoparticles. As a result, FRRG-DOX nanoparticles indicated approximately 50% DOX loading and were easy to prepare large-scale samples with a simple one-step synthetic protocol. With integrated the advantages of both nanomedicine and prodrug, these prodrug nanoparticles were efficiently localized at tumor tissues via the EPR effect, inducing a potent antitumor efficacy. Concurrently, they greatly minimized the adverse effects during treatments by selective DOX release in tumors via high cathepsin B-specificity. This was our first study reporting the concept of carrier-free cathepsin B-responsive prodrug nanoparticles and their preclinical study is now underway. We successfully established the process for multi-hundred gram scaled mass production, and also the great therapeutic efficacy and single-/multi-dose toxicity *in vivo* were confirmed. Through a follow-up study, FRRG-DOX nanoparticles were further used for combinatorial treatment with drug resistance inhibitors or immune checkpoint inhibitors to treat the advanced cancers with high heterogeneity and MDR [158, 159]. Recently, we are now developing many carrier-free cathepsin B-responsive prodrug nanoparticles containing two different therapeutic modalities as one system (Fig. 12). By additionally conjugating the drug resistance inhibitor, immune checkpoint inhibitor, or

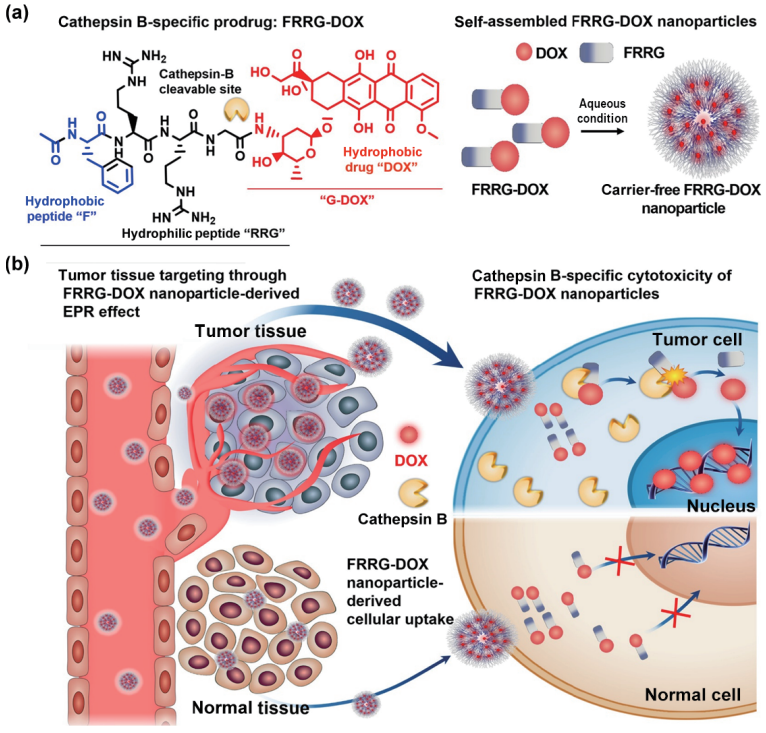


Figure 11 Scheme to explain the action of cathepsin B-responsive prodrug nanoparticles, FRRG-DOX, for cancer-targeted treatment. (a) The FRRG-DOX molecules, composed of cathepsin B-cleavable peptide linker FRRG and DOX, are spontaneously self-assemble into nanoparticles by intermolecular interactions. (b) FRRG-DOX nanoparticles efficiently accumulated at tumor tissues via the EPR effect, inducing a potent antitumor efficacy by DOX release. Concurrently, non-specifically localized FRRG-DOX nanoparticles at normal tissues maintain a non-toxic inactive state due to their innately low cathepsin B expression, thereby reducing adverse effects during treatment. Reproduced with permission from Ref. [19], © Elsevier B.V. 2018.

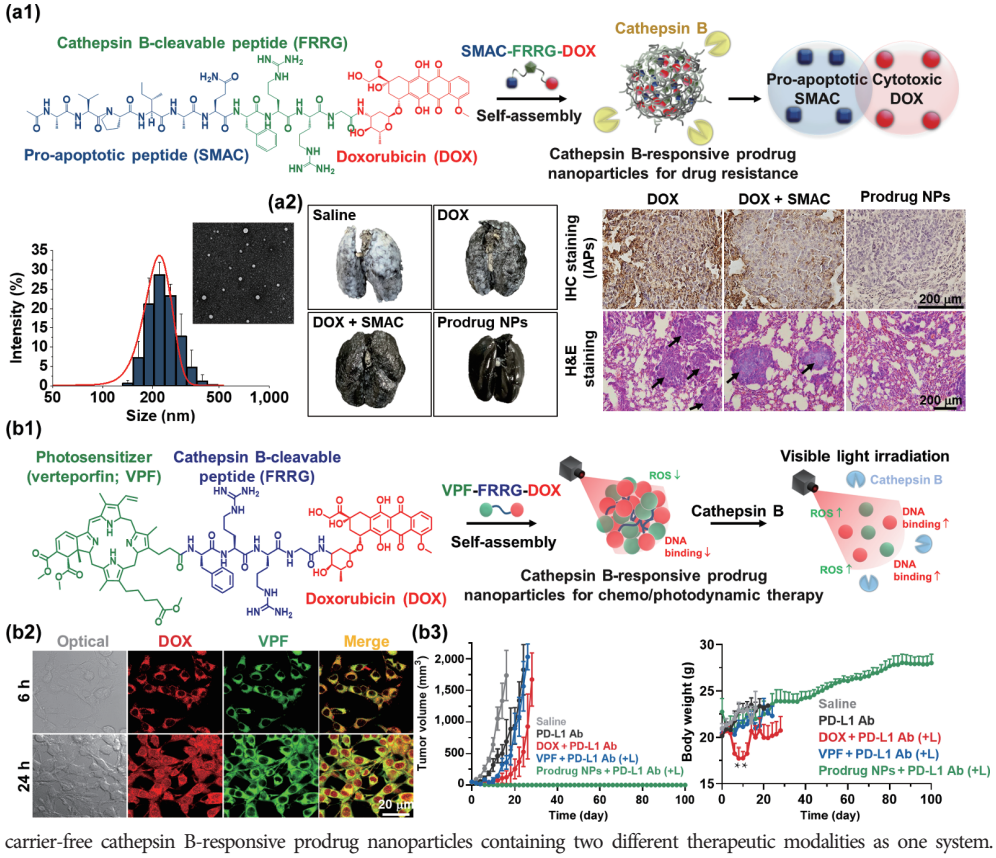


Figure 12 Various carrier-free cathepsin B-responsive prodrug nanoparticles containing two different therapeutic modalities as one system. (a1) For the drug-resistant cancer treatment, drug resistance-inhibitory peptide SMAC (AVPIAQ) is conjugated with DOX via cathepsin B-cleavable peptide FRRG, resulting in prodrug nanoparticles without any additional carriers. (a2) Prodrug nanoparticles efficiently inhibit the pulmonary metastatic tumor growth by inhibiting drug resistance-inducing proteins (IAPs; inhibitors of apoptosis proteins). Reproduced with permission from Ref. [160], © Elsevier Ltd. 2020. (b1) For the heterogeneous cancer treatment, a photosensitizer (verteporfin; VPF) and DOX are conjugated through FRRG peptide. The VPF-FRRG-DOX are spontaneously self-assembled into prodrug nanoparticles and induce chemo/photodynamic therapy under visible light irradiation. (b2) Prodrug nanoparticles deliver VPF and DOX in cancer cells. (b3) Under visible light irradiation, they exhibited 100% tumor regression when combined with immune checkpoint blockade and efficiently minimized the side effects during treatment. Reproduced with permission from Ref. [161], © American Chemical Society 2021.

photosensitizer at the opposite side to DOX in FRRG-DOX, multiple prodrug nanoparticles that can accurately deliver different two drugs have been designed for heterogeneous cancer treatment [160, 161]. Not only our study, but many researchers have also proposed the novel prodrugs that are spontaneously self-assembled into nanoparticles and their promising results have been being reported, which opened new avenues for advanced prodrug design.

As the carrier-free prodrug nanoparticle is a relatively brand new concept that has been about a decade since its development, it still has many challenges to go through for its practical use. The FRRG-DOX nanoparticle, which is the prototype of carrier-free cathepsin B-responsive prodrug nanoparticles, has just entered the preclinical stage, and the others have not been reported for their clinical investigation yet. Nonetheless, its outstanding performances that overcome the unavoidable limitations of carrier-based prodrugs make itself to be optimistic for its future prospects. As mentioned above, there is an obvious ceiling on the improvement in tumor accumulation efficiency of nanomedicines, therefore, the critical issues in current DDS development are focused on the way to increase the drug capacity of each particle and to minimize its off-target toxicity. In this circumstance, the carrier-free cathepsin B-responsive prodrug nanoparticle can effectively reduce systemic adverse effects both by tumor-selective activation of drugs and by basically excluding the possible toxicities and immunogenicity from the carrier itself. Moreover, its simple and small molecular structure enables it to capacitate a large amount of various therapeutic agents, not just limited in chemodrugs, in addition to endowing its industrial producibility and simplicity in QC. The carrier-free prodrug nanoparticle is expected to provide a new direction for the development of nanomedicine.

4 Conclusion

The research and development process for cathepsin B-responsive prodrugs were summarized from the past to the present. In this review, we classified the application of drug carriers for improving the properties of cathepsin B-responsive prodrugs according to the characteristics of materials. The pros and cons of each material as a drug carrier were then discussed in detail. Although we pointed out the critical disadvantages of a carrier-based prodrug delivery system and there is a desperate need for a new approach, numerous of them are used in practice and the mass clinical result from multiple failure cases could help to develop the advanced prodrug. Continued efforts to understand recent advances and progress for cathepsin B-responsive prodrugs will provide important insights into designing more suitable for their clinical translation.

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