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Two-step polymer- and liposome- enzyme prodrug therapies for cancer: PDEPT and PELT concepts and future perspectives

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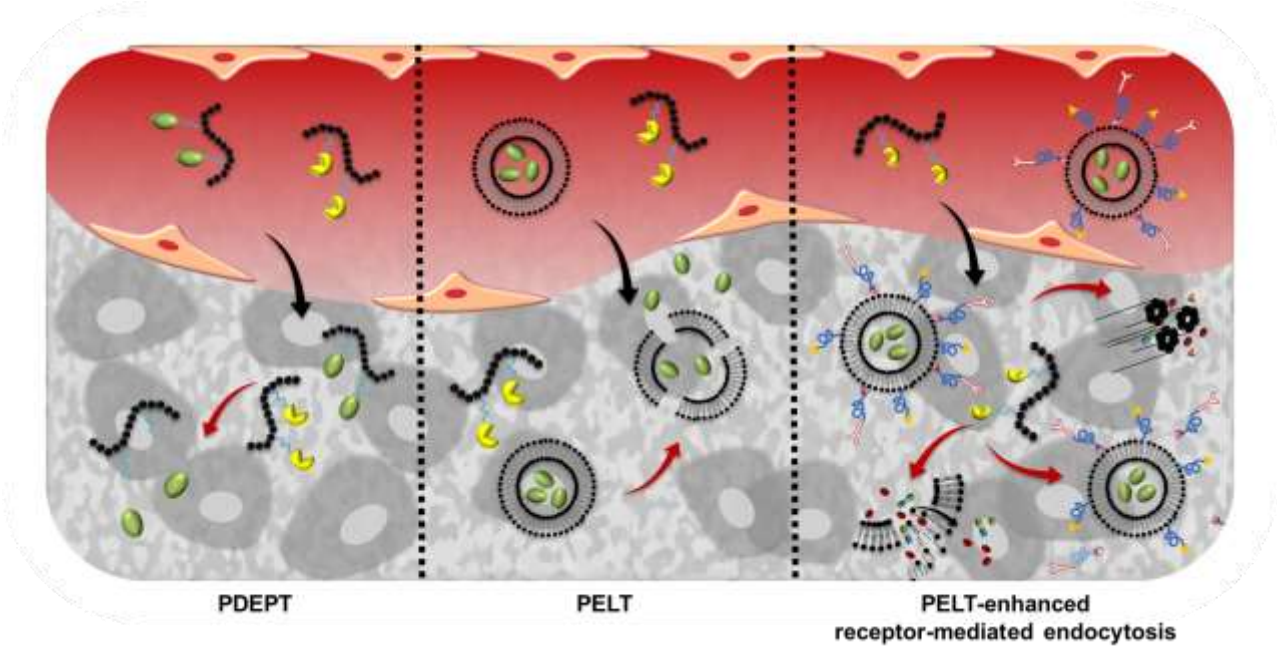
Two-step therapy, cancer therapy, nanomedicine, enzyme-sensitive polymers, enzyme-sensitive liposomes, polymer-enzyme conjugates, PDEPT, PELT, PUMPT.

ABSTRACT

Polymer-directed enzyme prodrug therapy (PDEPT) and polymer enzyme liposome therapy (PELT) are two-step therapies developed to provide anticancer drugs site-selective intratumoral accumulation and release. Nanomedicines, such as polymer-drug conjugates and liposomal drugs, accumulate in the tumor site due to extravasation-dependent mechanism (enhanced permeability and retention – EPR – effect), and further need to cross the cellular membrane and release their payload in the intracellular compartment. The subsequent administration of a polymer-enzyme conjugate able to accumulate in the tumor tissue and to trigger the extracellular release of the active drug showed promising preclinical results. The development of polymer-enzyme, polymer-drug conjugates and liposomal drugs had undergone a vast advancement over the past decades. Several examples of enzyme mimics for *in vivo* therapy can be found in the literature. Moreover, polymer therapeutics often present an enzyme-sensitive mechanism of drug release. These nanomedicines can thus be optimal substrates for PDEPT and this review aims to provide new insights and stimuli towards the future perspectives of this promising combination.

GRAPHICAL ABSTRACT



1. INTRODUCTION

The concept of using a specific trigger to convert a prodrug into an active moiety has been developed since the late 80's, with the work of Bagshawe and colleagues on antibody-directed enzyme prodrug therapy (ADEPT) [1] and Huber and colleagues on viral/gene-directed enzyme prodrug therapy (VGEDT) [2].

More than a decade after, Duncan and Satchi introduced a new stimuli-dependent prodrug activation mechanism, known as polymer-directed enzyme prodrug therapy (PDEPT) [3] **(Fig. 1)**, in which the trigger is a polymer-enzyme conjugate that promotes the release of an active drug from a polymeric backbone. The same idea was later applied also to liposomal formulations, developing the so-called polymer enzyme liposome therapy (PELT) **(Fig. 1)** [4]. PDEPT combined the knowledge on polymer-enzyme conjugation (to improve stability and reduce immune responses upon administration of proteins) and polymer-drug conjugation for tumor-targeted drug therapy. The development of polymer therapeutics in the early 2000's, with the first polymeric prodrugs (PK1) reaching clinical trials [5], highlighted the need for a targeted therapy to trigger the release of the active compound in a particular site of action. Relying solely on the enhanced permeability and retention (EPR) effect [6] for tumor accumulation is not sufficient to guarantee intracellular accumulation of an active drug. Tumor heterogeneity due to individual differences in tumor size, vasculature and stroma, as well as immune system components infiltration [7] can dramatically alter the biodistribution and accumulation of macromolecular drugs. For this reason, over the past decades, tumor-specific targeting agents were investigated to modulate the biodistribution and clearance of different classes of nanomedicines [8]. The receptor/ligand-mediated targeting can also affect the internalization pathway, promoting intracellular accumulation of the active moiety and carrier payload. Alternative approaches have been developed to improve cancer tissue targeting by exploiting specific physical and pathological conditions of the tumor microenvironment to trigger the release of the drug [4, 9, 10]. PDEPT and PELT can be considered further in this direction, not only by exploiting the conditions naturally present at the tumor site, but also by triggering the desired response via co-administration of the enzyme required to promote drug release.

In this review, we will describe the fundamental aspects for the rational design of PDEPT and PELT systems, with particular focus on future perspectives in this field, which potential has not yet been fully explored.

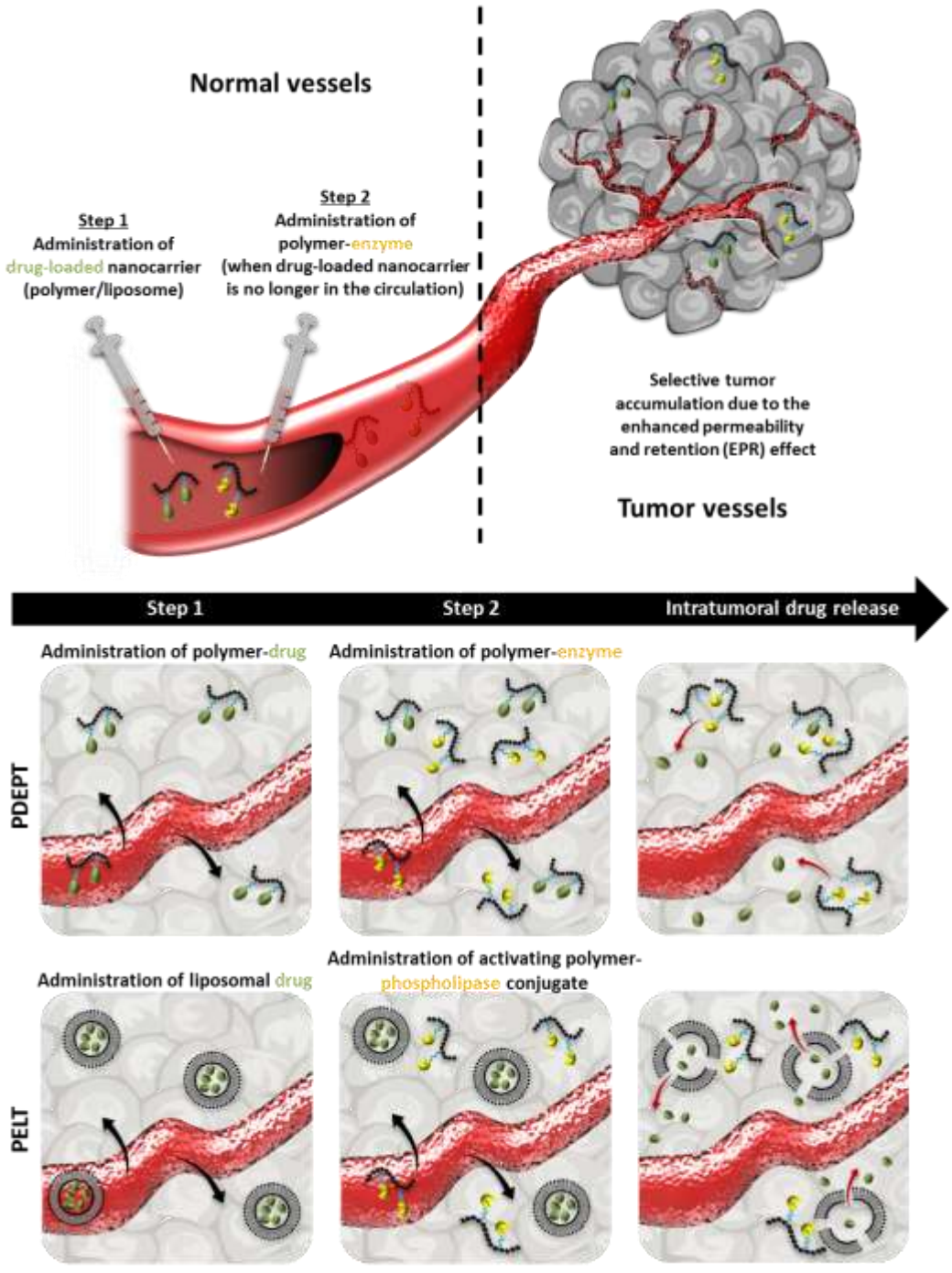


Figure 1. Polymer-directed enzyme prodrug therapy (PDEPT) and polymer enzyme liposome therapy (PELT) mechanisms.

2. PROOF OF CONCEPT

2.1. Polymer-directed enzyme prodrug therapies (PDEPT):

The first PDEPT system developed [3] consists of (I) N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-Doxorubicin conjugate [11] as a polymeric prodrug targeting the tumor via the EPR effect, and (II) HPMA copolymer-Cathepsin B conjugate as the enzymatic trigger system. Doxorubicin was conjugated to the HPMA copolymer via a Gly-Phe-Leu-Gly linker. This peptidic sequence was designed to be cleaved intracellularly by lysosomal cysteine proteases, such as Cathepsin B overexpressed in several cancers [12]. In order to obtain an anticancer activity, the polymeric prodrug needs to reach the tumor site, be internalized into cancer cells via pinocytosis, and be released in the lysosome following a specific enzymatic cleavage. To increase the intracellular availability of Doxorubicin and change its release kinetics, Satchi-Fainaro and Duncan thought of adding a different mechanism for intratumoral extracellular drug release by exogenously administering an enzyme (Cathepsin B) that will accumulate in the cancer tissue via the EPR effect similarly to the way HPMA copolymer-Doxorubicin prodrug conjugate does. To guarantee that Cathepsin B reaches the target site in its active form, the enzyme was conjugated to a polymeric chain of HPMA copolymer, which should prevent immune-recognition and degradation in the bloodstream. After reaching the cancer tissue, the HPMA copolymer-Cathepsin B conjugate cleaves the Gly-Phe-Leu-Gly linker and releases the active drug. To avoid the release of the free drug in the bloodstream, the polymer-enzyme conjugate is injected at a later stage, about 5 hours later, when the polymer-drug conjugate has been cleared from the bloodstream according to its pharmacokinetic profile [13]. The sequential administration of PK1 and HPMA copolymer-Cathepsin B displayed site-selective drug release and superior anticancer activity compared to the polymer-drug conjugate administered alone.

A further evolution of the concept was the development of a PDEPT system involving a non-mammalian enzyme, in order to avoid interference from physiological enzymes and inhibitors

present in the mammalian bloodstream. For this purpose, Satchi-Fainaro and Duncan selected β-lactamase as the triggering non-mammalian enzyme that hydrolyzes β-lactams to substituted β-amino acids [14]. The conjugation of this enzyme to a polymeric chain (HPMA copolymer) becomes in this case of crucial importance to allow not only its selective accumulation at the tumor site, but also to mask the antigenic determinants, i.e. the immunogenicity, of the non-mammalian enzyme. The second component of this two-step therapy is a HPMA copolymer-based prodrug of Doxorubicin, where the active drug is conjugated via a cephalosporin linker, which contains a β-lactam ring, a specific substrate of the β-lactamase. Again, in this case, the two-step therapy was not toxic and improved the anticancer efficacy of the HPMA copolymer-Doxorubicin conjugate [14].

In the direction of developing a polymer-macromolecule conjugate with enzymatic activity and with reduced immunogenicity, a HPMA-catalytic antibody was developed [15]. HPMA copolymer was conjugated to aldolase antibody 38C2, which is one of the most efficient antibodies that catalyze the aldol cleavage using substrates that are not recognized by human enzymes. In addition, the immunogenicity of the antibodies can be easily reduced not only by their conjugation to the polymeric chain, but also by selecting humanized antibodies. Thanks to these features, the polymer-catalytic antibody conjugate was developed as part of the two-step therapy. An Etoposide prodrug was designed to be selectively activated by the catalytic antibody 38C2, and showed superior safety compared to the non-modified Etoposide [16]. Albeit preliminary, this investigation reported the feasibility of a retro-aldol/retro-Michael reaction activation method for the release of an active drug via a catalytic antibody-mediated reaction. In order to become suitable for parenteral administration, the Etoposide prodrug should be further conjugated to a polymer that will guarantee preferential accumulation at the target site. Furthermore, the HPMA copolymer-antibody conjugate should be proven safe for *in vivo* administration.

2.2. Polymer enzyme liposome therapy (PELT)

Albeit the PELT concept has been described by Duncan and co-workers already in 2001 [4], the proof of concept for its activity has been achieved only recently [17]. Ferguson and Satchi-

Fainaro described the preparation of two polymer-phospholipase conjugates to promote the release of anthracycline from commercially-available liposomal formulations. Ferguson *et al.* conjugated HPMA copolymer to phospholipase C [17] . This enzyme was able to promote the degradation of the lipidic membrane of liposomes, increasing the drug release from these formulations [18], and maintaining its enzymatic activity following conjugation to the non-biodegradable HPMA copolymer. Ferguson *et al.* designed and synthesized a conjugate of dextrin-Phospholipase A_2 (PLA_2) [17, 19], an enzyme with proven anticancer activity by targeting the deregulated lipid metabolism in cancer [20]. The general toxicity of the PLA_2 toxin following *in vivo* administration is, nevertheless, the limiting factor for its use as a therapeutic drug. Consequently, conjugation to a polymer is crucial for reducing the non-specific toxicity of the enzyme. Dextrin has the advantage of being biodegradable and able to confer stability to the enzyme during circulation in the bloodstream, while allowing for unmasking of the protein at the target site, according to the Polymer Masked−Unmasked Protein Therapy (PUMPT) model, following treatment with α-amylase [21]. The two polymer-enzyme conjugates described above retained the catalytic activity of the native enzyme, and were able to promote drug release from non-polyethylene glycol (PEG) modified (non-PEGylated) (DaunoXome®) and, to a lesser extent, also from PEGylated liposomes (Caelyx®), probably due to PEG steric hindrance. Furthermore, when dextrin-PLA_2 was exposed to α-amylase the rate of anthracycline release from non-PEGylated liposomes increased.

Although intriguing concepts, only a small number of papers were published on PELT and PDEPT (Table I), and in all of those reported studies, the *in vivo* results were preliminary or missing. Despite the poor appeal of the two-step therapies from the research side, the field of polymer conjugation expanded dramatically over the last 20 years, leading to the development of numerous polymer-enzyme conjugates, polymer-drug conjugates and liposomal systems. Several of these nanomedicines can in principle constitute one of the components of these two-step therapies.

Table I: PDEPT and PELT systems developed

CONJUGATE NAME	POLYMER	ENZYME	DRUG FORMULATION	ENZYME TYPE	STATUS	REF
PDEPT I	HPMA copolymer	Cathepsin B	HPMA-Doxorubicin	Endogenous	POC – *In vivo* Preclinical development	[3]
PDEPT II	HPMA copolymer	β-lactamase	HPMA-Doxorubicin	Exogenous	POC – *In vivo* Preclinical development	[14]
PDEPT III	HPMA copolymer	catalytic antibody 38C2	Etoposide prodrug	Exogenous	POC – *In vitro* Preclinical development	[15]
PELT	HPMA copolymer	Phospholipase C	PEGylated liposomal Doxorubicin (Doxil®) Liposomal Adriamycin (DaunoXome®)	Endogenous	POC – *In vitro* Preclinical development	[17]
PELT	Dextrin	Phopholipase A_2	PEGylated liposomal Doxorubicin (Doxil®) Liposomal Adriamycin (DaunoXome®)	Endogenous	POC – *In vitro* Preclinical development	[17]
PELT+ PUMPT	Dextrin	Phopholipase A_2 + α amylase	PEGylated liposomal Doxorubicin (Doxil®) Liposomal Adriamycin (DaunoXome®)	Endogenous	POC – *In vitro* Preclinical development	[17]

3. POLYMER-ENZYME CONJUGATES

At the moment, there are 20 therapies based on recombinant enzymes approved mainly for the treatment of rare diseases [22]. The problems in enzyme administration, such as immunogenicity, difficulty in targeting and unsatisfactory pharmacokinetics [23], have led to the development of delivery systems to enable their *in vivo* biological therapeutic activity. So far, several polymer-enzyme conjugates have been designed and synthesized (Table II), and those have been extensively reviewed before [24-26]. In addition, the use of biodegradable polymers can be helpful in masking the immunogenic domain during circulation in the bloodstream, while releasing the active enzyme at the site of action, according to the PUMPT mechanism [21].

Over the last few years, conjugation techniques have improved, allowing for site-specific conjugation of cleavable linkers between either linear or branched polymer and the enzyme [24, 27, 28]. Preserving the activity of the catalytic site is a crucial step in the development of polymer-enzyme conjugates, and often a non-specific conjugation of polymers to multiple sites dramatically reduces the enzyme activity [29]. Usually the N-terminal amino acid is not involved in the binding or activity of the enzyme, and therefore it is an attractive binding site for polymers. Furthermore, due to its specific pKa, which differs from the pKa of the side chains of Lys, it is suitable for site selective conjugation [30]. Cys are also attractive binding sites, since they are rarely present in their reactive form in the enzyme. The reactive thiol group can be exposed by selective reduction of the disulfide bond, becoming accessible for conjugation [31]. The conjugation to thiol groups can be achieved via non-reversible thioether covalent bonds, using maleimide reactive groups, or via redox potential cleavable disulfide bond using polymers bearing a thiol group [32]. In addition, besides the traditional chemical conjugation, other strategies have been developed, mainly involving enzymes that catalyze the formation of new peptidic bonds between one amino acid of the protein and the polymer chain. Among the enzymes suitable for conjugation [27], transglutaminase is one of the most common [33, 34], but also sortase and other enzymes are used [27, 29].

3.1. Polymer-Enzyme conjugates in clinical use

Adenosine deaminase. The first polymer-enzyme conjugate to enter the market was the PEGylated Adenosine deaminase ADAGEN® (pegademase bovine), for enzyme replacement therapy in patients with severe combined immunodeficiency disease (SCID) associated with a deficiency of adenosine deaminase (ADA) (Table II) [35]. However, this enzyme is upregulated in cancer cells [36, 37], while its overexpression directly correlates to increased tumor malignancy. Therefore, its inhibition is desired as anticancer treatment [38]. For this reason, this enzyme might not be the best candidate for two-step anticancer therapies.

Asparaginase. An enzyme with intrinsic anticancer activity might be ideal to fully exploit the potential of two-step polymer-derived enzyme prodrug therapy. The cytotoxic properties of Asparaginase, a hydrolytic enzyme, are known for more than 50 years [39], and the recombinant enzyme is currently available in its native form for the therapy of acute lymphoblastic leukemia (ALL) and Non-Hodgkin Lymphoma (NHL) [40]. The enzyme promptly reduces the level of Asparagine in serum, cutting the supply to the cancer cells that are not able to overcome the amino acid shortage [40]. Since 1994, there is also a PEGylated form on the market (Oncaspar®) [41] for the same indications as the native form. As expected, the PEGylated Asparaginase decreases the immunogenicity of the recombinant enzyme and can be used also in cases of patients who developed hypersensitivity against the native Asparaginase [40]. This commercially-available conjugate is based on the recombinant *Escherichia coli* (*E. Coli*) enzyme that reacts with several 5 kDa PEG succinimidyl succinate chains via the amino groups of the enzyme lysine side-chains [42]. Besides Oncaspar®, several other PEG conjugates have been developed, differing in shape and molecular weight (MW) of the polymeric chain (linear or branched), and following distinct conjugation chemistry (random or site specific) [24, 31, 43-45]. In addition, several other polymers have been conjugated to Asparaginase, including biodegradable polypeptides [46] and polysaccharides [47].

Uricase. The third polymer-enzyme conjugate to reach the market was the PEG-Uricase polymer therapeutic, known as KRYSTEXXA® [48]. Uricase is a non-mammalian hepatic enzyme

not present in humans that catabolizes the oxidation of urate into soluble products (allantoin and carbon dioxide) and, for this reason, it is used to treat hyperuricemia [49]. The commercial formulation is based on porcine uricase covalently attached to 10 kDa PEG chains via *p*-nitrophenol chemistry [50]. Also, in the case of uricase, several PEG conjugates have been developed [51, 52], as well as other polymer conjugates [53].

3.2. Other polymer-enzyme conjugates

Trypsin, Chymotrypsin and Papain. Trypsin, chymotrypsin and papain are digestive enzymes responsible for protein degradation via peptide bond cleavage. They have also been tested as support therapy to reduce the adverse-effects associated with chemotherapy in cancer patients, in which they prolonged survival to some extent [54]. They have been extensively investigated as substrates for polymer conjugation [24] and dextrin-trypsin conjugates were the first proof of concept models for PUMPT [21]. Also in this case, the PEG conjugates were among the first to be developed [55-59], but HPMA copolymer conjugates [60-62] and carbohydrate derivatives [61, 63, 64] have also been proposed.

Lysozyme. Lysozyme is an enzyme in the class of the glycosidases and it catalyzes the hydrolysis of the beta-(1-4)-glycosidic bond between N-acetylglucosamine sugar (NAG) and N-acetylmuramic acid sugar (NAM) in peptidoglycan of bacterial and viral cell walls [65]. Lysozyme is mainly used for its antibacterial and immunomodulatory activity [66]. However, anticancer effects have been reported, probably via direct or indirect activation of the immune system [67]. Partially due to its therapeutic effect, but also because it can function as an easily-available model protein, Lysozyme has been conjugated via several chemistries to a wide range of polymers, such as PEG [68, 69], poly (DL-alanine) [70], HPMA copolymers [71], Poly(Glutamic acid) (PGA) [72] and Dextran [73].

Superoxide dismutase (SOD). Superoxide dismutase serves as the main physiological antioxidant system, preventing the effects of reactive oxygen species (ROS). The oxidative stress has been correlated with several pathologies, such as hypertension, atherosclerosis, and vascular remodeling [74]. In addition, SOD has anti-inflammatory properties [75]. For these reasons, SOD has been widely investigated as therapeutic agent for a broad range of diseases

(inflammation, cardiovascular, respiratory), but also as a scavenger of radiation damage and to prevent carcinogenesis [76]. In order to improve the physicochemical properties of the enzyme, SOD was widely conjugated to PEG of different sizes and through different conjugation chemistries [23, 77]. SOD has also been conjugated to amphiphilic polymers, such as Pluronic block copolymers [78], Poly(2-oxazoline) block copolymers [79], HPMA copolymers [80], Dextran [81] and poly(N-vinylpyrrolidone) [82].

***Ribonuclease*.** This hydrolytic enzyme catalyzes the degradation of RNA by cleaving the phosphodiester bonds [83]. It has intrinsic cytotoxic activity and it reached the clinical trials for the treatment of solid tumors, including non-small cell lung, esophageal, and colorectal carcinomas [84]. Ribonuclease has been successfully conjugated to HPMA copolymer [85], PEG [86] and polysaccharides [87].

Table II: Polymer-Enzyme conjugates

POLYMER	ENZYME	ENZYME TYPE	INDICATION	REF
PEG	Adenosine deaminase	Endogenous	Adagen® (Pegademase bovine) Approved (1990) for SCID	[35]
PEG	Asparaginase	Exogenous	Oncaspar® (Pegaspargase) Approved (1994) for ALL.	[31, 41, 44, 45]
Poly(DL-alanine)			Acute lymphoblastic leukemia	[46]
iLevan				[47]
PEG	Uricase	Exogenous	KRYSTEXXA® (PEG–uricase) Approved (2010) for gout and hyperuricemia	[48, 52]
PVP			Gout and hyperuricemia	[53]
PAcM				
HPMA copolymer	α-Chymotrypsin	Endogenous	Relief from the adverse-effects associated with chemotherapy in cancer patients [54]	[60]
PEG				[55, 56]
Dextran				[63]

PEG	Trypsin	Endogenous		[57]
HPMA copolymer				[61]
Dextrin				[21][61]
Dextran				[64]
PEG	Papain	Endogenous		[59]
HPMA copolymer				[62]
PEG	Lysozyme	Exogenous	Antibacterial [66] and anticancer effect [67]	[68]
Poly(DL-alanine)				[70]
PNIPAAm				[69]
PEG	Superoxide dismutase	Endogenous	Anti-inflammatory [75]	[23, 77]
Pluronic				[78]
Poly(2-oxazoline)				[79]
HPMA copolymer				[80]
Poly(N-vinylpyrrolidone)				[82]
Dextran				[81]
PEG	Ribonuclease	Endogenous	Anticancer activity [84]	[86]
HPMA copolymer				[85]
Chitin				[87]
HPMA copolymer	Cathepsin B	Endogenous		[3]
Dextrin	Phospholipase A_2	Endogenous		[19]
HPMA copolymer	Phospholipase C	Endogenous		
HPMA copolymer	β-lactamase	Exogenous		[14]

4. ENZYME-SENSITIVE DRUG CONJUGATES

The polymer-drug conjugate's or liposome's sensitivity to the activity of a specific enzyme is a fundamental feature of polymer-enzyme directed therapies. The pharmacokinetic profile must be known, in order to allow for the second injection of the polymer-enzyme only once the drug-conjugate reaches the target site, thus avoiding drug leakage into the blood circulation. Many of the polymer-drugs developed in the last 20 years rely on enzyme-sensitive drug release profile to enhance intratumoral accumulation of the active molecules. Even though, in other studies the enzyme-cleavable linker conferred stability or enhanced cellular internalization of the supramolecular entity. Nevertheless, several of those nanomedicines have been adequately characterized for their *in vivo* properties to serve as substrates for PELT and PDEPT.

4.1. Cathepsin-sensitive conjugates

Cathepsin B-cleavable conjugates. Cathepsin B is a lysosomal cysteine protease that plays a major role in proteolysis and is upregulated in several pathologies, including cancer [12]. Therefore, Cathepsin B-sensitive linkers have been extensively investigated for site-specific stimuli triggered drug release. In fact, the first two polymer-conjugates (PK1 and PK2) reaching clinical trials were based on the cathepsin-cleavable polypeptide Gly-Phe-Leu-Gly [5, 88]. This linker is stable in plasma [89] and guarantees intracellular lysosomal release of the drugs [90]. This conjugation strategy allows for the formation of polymer-drug conjugates that are significantly more stable in bloodstream than other conjugates, in which the drugs are linked via pH-sensitive ester bonds, such as HPMA copolymer-Paclitaxel [91] and HPMA copolymer-Camptothecin [92] conjugates tested in clinical trials. In addition, PK1 and PK2 pharmacokinetic and biodistribution profiles have been evaluated both at preclinical levels in rodents [11] and under clinical settings [5, 88], making them ideal candidates for two-step therapies [3].

Doxorubicin has served as model drug for the development of several Cathepsin B-cleavable peptide-based conjugates, such as PEG-based [93], dendrimer-based [94, 95] and several HPMA copolymers [96, 97], as well as silica nanoparticles [98] and micelles [99]. However, there were

also interesting works focusing on the development of Camptothecin- [100], Gemcitabine- [101-103] and Paclitaxel- [102, 104-106] based polymers.

A particular attention should be noted on the PGA polymer, which is Cathepsin B-sensitive *per se*, without the need for a peptidic enzyme-sensitive linker. The enzymatic degradation of the PGA backbone promotes the drug release. Several conjugates have been developed, being the most known the Polyglumex, a PGA-Paclitaxel conjugate that reached clinical trials [107], but also the PGA-Paclitaxel-E-[c(RGDfK)$_2$] [108], PGA-Doxorubicin-Paclitaxel [109], PGA-Camptothecin [110], and PGA-oligonucleotides polyplexes [111-113].

Indeed, the Gly-Phe-Leu-Gly cathepsin B sensitive linker was used as a strategy to conjugate short fragments of other non-biodegradable polymers, such as HPMA copolymers, generating a high molecular weight, biodegradable copolymers. Kopecek's group developed several conjugates [114, 115], bearing one drug (paclitaxel [116], gemcitabine [117], and doxorubicin [118]) or two drugs combination (gemcitabine and paclitaxel [119, 120]).

All these polymer therapeutics can be substrates for PDEPT therapy in combination with polymer-cathepsin B conjugates.

Other Capthepsin-cleavable conjugates. Besides Cathepsin B, several cathepsins have been correlated with primary and metastatic cancers [121]. Among them, Cathepsin K is synthesized mainly in osteoclasts, and subsequently secreted to the extracellular matrix where it is involved in bone resorption [122]. Thus, it is a perfect target for stimuli-dependent conjugates. So far, a small number of polymer bearing Paclitaxel and Alendronate have been developed, using the glycine-glycine-proline-norleucine (Gly-Gly-Pro-Nle) peptide, which is cleaved by Cathepsin K [123-126].

4.2. Legumain-sensitive conjugates

Legumain is an asparaginyl endopeptidase overexpressed in several cancers [127], both in the extracellular matrix (ECM) and in the lysosomes. Therefore, linkers presenting asparagine or aspartic acid residues, selectively hydrolysable by this enzyme, have been used to develop enzyme-sensitive prodrugs. To date, both low MW molecules containing legumain-cleavable

linkers [128-130] and polymeric conjugates [131] have been synthesized using peptides containing the alanine-alanine-asparagine sequence (Ala-Ala-Asn). Recently, Lin *et al*. presented hyaluronic acid (HA) legumain-sensitive nanogel, based on the same AAN peptide, for the delivery of Doxorubicin with a high targeting efficiency, both *in vitro* and *in vivo* [132].

4.3. *Matrix metalloproteinases (MMP)-cleavable conjugates*

MMP are mainly involved in collagen degradation and ECM homeostasis. They are also involved in carcinogenesis and metastasis formation by modifying the local ECM that becomes more permissive to cancer cells' migration [133]. As a consequence of their activity on apoptosis, angiogenesis and migration, MMP have been investigated as targets for inhibitors with anticancer activity, but also as substrates for cleavable polymer-drug conjugates.

Albeit limited to *in vitro* characterization, Bruun *et al.* developed PEGylated lipid-based nanoparticles for the delivery of siRNA to the brain that exploited a MMP-sensitive linker to trigger the release of the PEG in the cancer site, releasing the active cargo [134]. The concept of using a PEGylated shield to protect the formulations in the bloodstream and to exploit a linker sensitive to MMP to unmask the formulation in the cancer target site, has been proven effective *in vivo* in 4T1 breast cancer mouse model, with a clear advantage compared to the non-cleavable nanomedicine [135]. Similarly, PEG-based micelles bearing Paclitaxel exploited the MMP sensitive peptide to promote the extracellular release of the PEG layer on the surface and bestow superior anticancer properties [136]. MMP-cleavable linker has been used to conjugate albumin on the surface of silica nanoparticles, to mask their recognition by the mononuclear phagocyte system. At the same time, the albumin coating also masks an arginine-rich cell penetrating peptide, which usually correlates to high cytotoxicity. The obtained enzyme-sensitive supramolecular system showed higher anticancer activity than the controls in the treatment of liver cancer HepG2 xenografts [137].

MMP-sensitive peptidic linkers have been used also for the simple conjugation of drugs to the polymeric backbone, maintaining the *in vivo* anticancer efficacy of the free drug [138]. Interestingly, Peng and Kopecek developed a HPMA copolymer conjugate containing two enzymatically cleavable peptides: Doxorubicin has been conjugated using the conventional Gly-

Phe-Leu-Gly Cathepsin B-sensitive linker to promote lysosomal release of the drug, while the MMP sensitive polypeptide was used to conjugate the HPMA copolymer to iRGD (a cyclic peptide targeting integrins) [139]. This conjugate should accumulate in the tumor thanks to the EPR effect, thus enhancing the chances of the iRGD to bind to the integrins expressed on the tumor endothelium and on several types of tumor cells. Finally, after MMP-mediated cleavage, the free iRGD promotes internalization into the target cells. Indeed, the uptake and *in vitro* activity of the conjugate were superior to the one of the free drug and of the mixture of HMPA-Doxorubicin conjugate co-tested with free iRGD.

In parallel, Ruan and co-workers developed gelatin-based nanoparticles presenting PEGylated gold nanoparticles adsorbed onto their surface. The gelatin is hydrolyzed by the MMP in the tumor ECM, triggering the release of the small gold nanoparticles loaded with Doxorubicin, which led to a slightly enhanced anticancer activity [140].

Table III: Enzyme cleavable polymer-conjugates

ENZYME FOR CLEAVAGE	LINKER ENZYME-SENSITIVE	POLYMER	DRUG	INDICATION	REF
Cathepsin B	Gly-Phe-Leu-Gly	HPMA copolymer	Doxorubicin (PK1)	Solid tumors	[5]
			Doxorubicin (PK2)		[88]
	Gly-Phe-Leu-Gly	Branched HPMA	Doxorubicin		[97]
	Gly-Phe-Leu-Gly	PEG	Doxorubicin		[93]
	Gly-Ile-Val-Arg-Ala-Lys	Silica nanoparticles	Doxorubicin		[98]
	Gly-Phe-Leu-Gly	PEGylated dendrimers	Doxorubicin		[95]
	Val-Cit	PEG-based micelles	Doxorubicin		[99]
	Val-Cit	PEG	Camptothecin		[100]
	Val-Cit	PEG	Paclitaxel		[105]
	Gly-Phe-Leu-Gly	PEG	Gemcitabine		[101]
	Gly-Phe-Leu-Gly	HPMA copolymer	Gemcitabine		[103]
	Gly-Phe-Leu-Gly	HPMA copolymer	Gemcitabine Paclitaxel		[102]

	Gly-Phe-Leu-Gly	PAMAM dendrimers encapsulated in liposomes	Paclitaxel		[104]
	Gly-Phe-Leu-Gly	PEG- Janus dendrimers	Paclitaxel		[106]
	Gly-Phe-Leu-Gly	Dendrimers (NTN1956)	Doxorubicin		[94]
	Peptidic bond in the polymeric backbone	PGA	Paclitaxel (PolyGlumex ®)	Solid tumors	[107]
			Paclitaxel		[108]
			Oligonucleotides		[111, 112]
			Camptothecin		[110]
			Paclitaxel, Doxorubicin		[109]
	Gly-Phe-Leu-Gly	HPMA copolymer	Paclitaxel	Solid tumors	[116]
			Gemcitabine		[117],
			Doxorubicin		[118]
			Gemcitabine Paclitaxel		[119, 120]
Cathepsin K	Gly-Gly-Pro-Nle	HPMA copolymer	Prostaglandin E1	Bone cancer/ metastases	[124]
			TNP-470 Alendronate		[123]
	His-Pro-Gly-Gly-Pro-Gln	PEG	Doxorubicin		[126]
	Gly-Gly-Pro-Nle	Pullulan	Paclitaxel, Alendronate		[125]
Legumain	Ala-Ala-Asn-Leu	PEG	Doxorubicin	Solid tumor	[131]
	Ala-Ala-Asn-Leu	HA	Doxorubicin		[132]
Matrix Metallo - Proteinases	Gly-Trp-Ile-Pro-Val-Ser-Leu-Arg-Ser	PEG-lipoparticles	Oligonucleotides		[134, 135]
	Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln	PEG			

	Gelatin	PEG-gold nanoparticles	Doxorubicin		[140]
	Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln	PEG-micelles	Paclitaxel		[136]
	Pro-Val-Gly-Leu-Ile-Gly	PEG- Janus dendrimers	Paclitaxel		[138]
	Pro-Val-Gly-Leu-Ile-Gly	Silica nanoparticles	Doxorubicin		[137]
	Pro-Leu-Gly-Lys-Ala-Gly	HPMA copolymer	Doxorubicin		[139]

5. ENZYME-SENSITIVE LIPOSOMES

Liposomes are versatile non-toxic, biocompatible and biodegradable vesicle structures, whose lipid composition and consequent amphiphilic nature allow the encapsulation of bioactive molecules with distinct physicochemical properties [141]**.** Different strategies have been devised to increase the half-life circulation of these vesicles and achieve the delivery of payloads to the targeted site, in order to improve their bioavailability while minimizing side-effects. Accordingly, PEG-grafted lipids reduce liposome recognition by cells from the mononuclear phagocytic system, being thus extensively used to improve their stability and blood circulation time [143 ,142]. These PEGylated liposomes extravasate from the altered vasculature at pathological sites by the EPR effect, where subsequently release loaded payloads through diffusion across lipid bilayer [144]. Liposomes were the first drug nanodelivery systems successfully translated into the market and Doxil® was the first PEGylated liposome approved for the treatment of Kaposi's sarcoma and breast and ovarian cancers [145, 146]. However, PEGylated liposomes have limited drug release and interactions with the target cells, which have fostered the development of chemical tools to cleave the PEG chains once the carrier reaches the targeted tissue or cells [147, 148]. In addition, besides successfully modifying the biodistribution and pharmacokinetics of free drugs, additional strategies have been developed to improve the active targeting of liposomes to altered cells in order to impair off-target side effects. Different ligands (e.g. monoclonal antibodies and fragments, peptides, carbohydrates, glycoproteins) have been conjugated at the surface of liposomes to bind to specific receptors expressed on the target cells, enhancing the cell surface binding and/or receptor-mediated internalization of these vesicles [149-151]. The entrapped drugs can then diffuse through the

phospholipid bilayer, in a similar manner to the passive targeting of liposomes via the EPR effect. However, the PEG-based polymeric coating may impair the interaction of these ligands grafted onto the liposome bilayer with the target receptor. Therefore, additional strategies have been devised to remove this polymer coating as soon as the liposome reaches the target site, and to further trigger the release of entrapped bioactive molecules following alterations in the structure of liposomes in response to endogenous (e.g. overexpression of enzymes, reduced pH, temperature, reducing agents) or exogenous (e.g. heat, light, magnetic field) stimuli [152-154]. Later strategies aim to combine long-circulating liposomes, receptor mediated-targeting ability and stimuli-responsive systems in a single multifunctional liposomal formulation. These allow increased intracellular drug levels following enhanced receptor-mediated endocytosis, but also promote the release of drug from liposomes in response to specific stimuli. A particularly promising approach is based on the development of liposomes that are sensitive to extracellular and intracellular enzymes that are increased predominantly at target pathological sites, such as cancer, or tissues affected by inflammation or infection. The structure of these enzyme-responsive liposomes can be altered by multiple enzyme-inducible mechanisms, including the removal of a protective polymer layer at liposomal surface, activation of an entrapped prodrug, destabilization of the phospholipid bilayer, and/or cleavage of a lipopeptide or lipopolymer dispersed within the vesicle bilayer following enzyme digestion **(Fig. 2)**. These enzyme-responsive liposomes have been reviewed recently by Fouladi *et al.* [155]. Here, we will limit the discussion to those formulations relevant for PELT (Table IV).

5.1. PLA_2-sensitive liposomes

Mock *et al.* engineered a liposome responsive to PLA_2, an enzyme with proven anticancer activity (refer to section 2.2) and overexpressed at distinct pathological situations, namely cancer and other inflammatory pathologies, thus constituting promising targets for active drug delivery [156]. Different liposomal formulations have been developed using phospholipids with shorter fatty acid acyl chain at the anionic polar head groups, in order to attain lipid preferential degradation by PLA_2 and consequently improve payload release [156, 157]. The selected PLA_2-targeted liposome candidates showed a superior antitumor activity *in vitro*, and were able to decrease tumor growth at a 2.5-fold greater extent than the PEGylated

conventional formulation tested in a prostate cancer mouse model. Therefore, it can be anticipated that a two-step therapy may additionally increase the efficacy of this engineered formulation upon administration of a polymer-PLA_2 conjugate, such as the dextrin-PLA_2 synthesized by Ferguson and Satchi-Fainaro [17]. In addition, as bacteria secrete PLA_2 as well, and the EPR effect has also been described at sites of bacterial infection[158], these polymer-PLA_2 conjugates may comprise a promising strategy to increase the release of antibiotics at the infection site once delivered by the PLA_2-responsive liposomes developed by Zhu *et al.* [157]. The hydrolytic activity of PLA_2 was additionally improved by the presence of PEG, 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), distearoylphosphatidylglycerol (DSPG) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) in those liposomes [159].

Different PLA_2-sensitive liposomal prodrugs have been prepared by the conjugation of lipophilic drugs to the acyl chain at sn-2 position in phospholipids [160-162] (Table IV). These liposomes showed an increased encapsulation efficacy and subsequent improved release of lipophilic drugs, such as retinoic acid and chlorambucil upon exposure to PLA_2 specifically at the tumor site. Therefore, these lipid-based prodrugs are promising components of a two-stage nanocarrier system.

5.2. MMP-responsive liposomes

Following the rationale explained in section 4.3, different liposomal formulations have been developed using MMP-sensitive peptides, such as the MMP2-cleavable peptide Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln bind to PEG and lipids [163, 164] (Table IV). These PEG-coated MMP-responsive liposomes displayed a prolonged blood circulation time, and the cleavage of the MMP-sensitive peptides at the target site unmasked the liposomal cell surface and therefore improved their cellular internalization, especially if this vesicle was additionally decorated with a targeting moiety specific for target cells. Galactose-coated liposomes presenting a PEG layer attached to a phospholipid anchor via the above-mentioned MMP2-cleavable peptide linker led to increased uptake in HepG2 cells [163].

5.3. Cathepsin B-responsive liposomes

Romberg *et al.* developed a liposome coated by the enzymatic-cleavable poly(amino acid)–lipid conjugate poly(hydroxyethyl l-glutamine)–N-succinyl-dioctadecylamine (PHEG–DODASuc) [165], an alternative to PEG used to transiently sterically stabilize the liposomes and increase their blood circulation time in rats [166]. Model proteases, such as Papain and Pronase E, but also the Papain-like enzyme Cathepsin B successfully degraded this PHEG polymer into 1-2 amino acids *in vitro* [167] (Table IV). The authors took advantage of this polymer coating to stabilize the DOPE-based liposomes to further improve the interaction with targeted cells and enhanced the release of the liposomal contents due to induced liposomal membrane destabilizing phase changes, following cleavage of PHEG-coating [165] **(Fig. 2)**. In fact, the fusogenic lipid DOPE by itself does not lead to the formation of bilayer structures, resulting in lamellar and inverted hexagonal phases under pH above or under 9, respectively [168]. These PHEG-liposomes showed a calcein release rate 30% higher than the one obtained for PEG-liposomes. A clear increase in payload release following PHEG-coating cleavage by Pronase E was shown, and subsequent liposome destabilization, membrane fusion and consequent aggregation were observed **(Fig. 2)**.

As cathepsin B is primordially a lysosomal enzyme, it is a particularly interesting tool to promote nanocarrier endosomal escape and subsequent drug release into the cytoplasm of targeted cells. Zhang *et al.* developed pH- and enzyme-responsive liposomes containing non-bilayer DOPE and the stabilizer cholesterol hemisuccinate (CHEMS) [168]. Under physiological pH, these pH-sensitive liposomes retain their structure. However, at pH 5.5, it occurs the transition from lamellar to hexagonal phase, resulting in lipid destabilization and release of encapsulated drugs. Therefore, these pH-sensitive liposomes constitute promising candidates for the intracellular delivery of drugs, as their entry into the lysosomal and endosomal compartments foster the rapid and extensive release of entrapped payloads at targeted cellular site. DSPE-PEG was used to increase the blood circulation time of the DOPE-based liposomes, being anchored to liposomal surface by a cathepsin B-cleavable peptide (Gly-Phe-Leu-Gly) to prevent the previously observed reduction of pH-sensitivity of liposomes following their PEGylation [169, 170]. Nevertheless, an increase in drug release rate from those pH- and enzyme-responsive liposomes was not observed in the presence of cathepsin B.

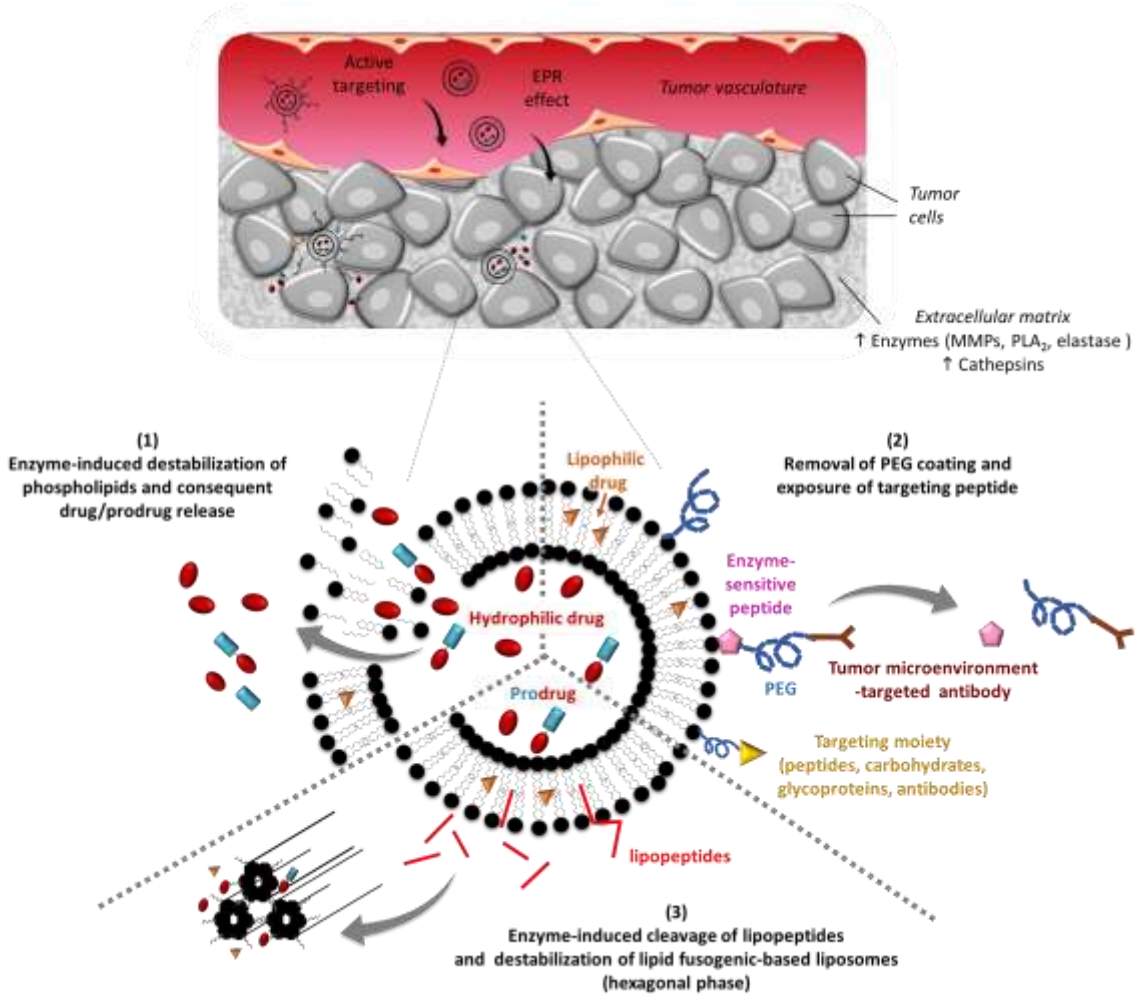


Figure 2. Schematic representation of the enzyme-mediated destabilization mechanisms responsible for an enhanced drug release from liposomes accumulated at tumor microenvironment via active targeting or EPR effect: 1) cleavage of phospholipids by enzymes increased at the extracellular matrix (e.g. Matrix metalloproteinases (MMP), legumain, Phospholipase A2 (PLA_2), elastase) or intracellular level (e.g. cathepsins) leads to an impaired phospholipid bilayer and therefore enhances the release of encapsulated hydrophilic drugs/pro-drugs; 2) removal of enzyme-peptide conjugated PEG layer exposes the targeting moiety at the liposomal surface, enhancing the receptor-mediated endocytosis and subsequent drug release at intracellular level; and 3) cleavage of lipopeptides (e.g poly(hydroxyethyl l-

glutamine) (PHEG)–coating) or lipopolymers dispersed within the lipid bilayer composed by fusogenic lipids triggers liposome fusion, further transition into the hexagonal phase, and consequent release of payloads.

5.4. Other enzymes-responsive liposomes

Elastase and prostate-specific antigen (PSA) are additional enzymes increased at inflamed tissues, such as tumors. Polypeptides sensitive to these enzymes, such as N-acetyl-Ala-Ala [171] and His-Ser-Ser-Lys-Tyr-Gln [172], respectively, have been used in the development of liposomal formulations to enhance payload delivery at those targeted sites. In addition, the conjugation of the fusogenic DOTAP to the above-mentioned elastase-sensitive linker led to a positively charged liposome and, consequently, to an increased fusion of this biphospholipidic vesicle with the lysosomal membrane, resulting in an enhanced intracellular delivery of liposomes and entrapped molecules. Similarly, the polyarginine peptide was conjugated to PSA-sensitive peptide, as well as to a polyanionic peptidic domain [173]. The latter polypeptide establishes electrostatic interactions with the polycationic arginine, which only becomes activated upon the hydrolysis of the PSA-sensitive peptide as a consequence of the high concentrations of this enzyme at tumor tissues. The active targeting of those PSA-sensitive liposomes was further achieved *in vivo* in a prostate cancer mouse model through the use of folate ligands, in addition to the polyarginine peptides, resulting in a prominent reduction of tumor growth [172].

Table IV: Enzyme-cleavable liposomes

ENZYME FOR CLEAVAGE	ENZYME SENSITIVE FUNCTION	DRUG	INDICATION	REF
Phospholipase A_2 (PLA_2)	1,2- distearoyl-*sn*-glycero-3-phosphatidylethanolamine 1,2-distearoyl-*sn*-glycero-3-phosphatidyl- glycerol	Doxorubicin	Cancer, Inflammation	[156]

	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine	Selective retinoic acid receptor β2 (RARβ2) agonist	Cancer	[159]
	C16 and C18 ether chains with phosphatidylcholine or phosphatidylglycerol headgroups	Chlorambucil	Cancer	[160]
		Prostaglandine	Cancer	[162]
		Retinoic acid	Cancer	[161]
Matrix Metalloproteinases	Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln	Cytarabine	Cancer	[163]
		Antinucleosome monoclonal antibody (mAb 2C5)	Cancer	[164]
Elastase	*N*-methoxy-succinyl-Ala-Ala-Pro-Val-DOPE)		Cancer	[171]
Prostate-specific antigen (PSA) prostate-specific membrane antigen (PSMA)	His-Ser-Ser-Lys-Tyr-Gln	siPLK-1	Cancer	[172]
				[173]
Pronase E	poly(amino acid)–lipid conjugate poly(hydroxyethyl l-glutamine)-N-succinyl-dioctadecylamine (PHEG–DODASuc)	Fluorescent calcein		[165]
Cathepsin B	Gly-Phe-Leu-Gly	Doxorubicin	Cancer	[168]

FUTURE PERSPECTIVE

PDEPT and PELT have never reached an advanced investigation stage and remain confined to a few models, partly due to the practical inconvenience of a two-step therapy. However, following recent advances in multiple disciplines, including cancer molecular biology, genomics, proteomics, tumor immunology and chemistry, novel disease markers and related mechanisms have been revealed, suggesting that combinatorial approaches are among the most promising strategies to control multifactorial pathologies, such as cancer. On the other hand, the need for conjugation of drugs to polymers has highly limited the biomedical application of PDEPT. In fact, in the past, this technology was mainly applied to drugs that contain functional groups for covalent conjugation to polymers. Accordingly, paclitaxel, gemcitabine, docetaxel, irinotecan, camptothecin and doxorubicin have been the most used drugs for polymer conjugation [174, 175]. In addition, the use of synthetic routes demands for an extensive physicochemical characterization of polymer-drug conjugates, in order to control end material properties and therefore, obtain reliable and reproducible stability, drug release and subsequent PDEPT/ PELT pharmacokinetics. Even though, significant progress on linker chemistry and materials science have been reported since the first generation of polymer-drug conjugates by Ulbrich and Kopeček [176], thus opening new opportunities for the successful application of PDEPT. More than 25 polymer conjugate-based products have indeed successfully been approved for human use [177]. This demonstrates that different options are already available to overcome those major drawbacks related to the conjugation of bioactive moieties to polymer backbone. One particularly interesting solution is the difluoroalkyl-sulfinate ketone-protected reagent developed by Shabat, Satchi-Fainaro and co-workers, which allows for the direct functionalization of C–H bond in heteroaryl drugs [178]. This is one example among other synthetic approaches (reviewed in [179]) already reported that will most likely expand the possible uses of PDEPT.

This polymer-drug conjugation technology offers as well the opportunity for a selective triggered drug release in the target cells, in contrast to the continuous release of drugs entrapped within nanodelivery systems. The latter can limit the amount of drug available at target site following the indiscriminate release of the drugs while being delivered through circulation. This, in fact, has been underlying the limited clinical translation of nanomedicines

despite the tremendous research and investment in this field, as reviewed by Duncan and Gaspar [180].

Even though, nanomedicines have dramatically changed the efficacy and systemic toxicity of several drugs for distinct medical applications. Liposome-based strategies are the first nanodelivery systems already successfully translated into the clinical use, and many are in different stages of clinical evaluation. In addition to being biocompatible, biodegradable and non-immunogenic, liposomes have well-established metabolism, pharmacokinetic and biodistribution profiles via different routes of administration. Doxil® was the first nanomedicine approved for clinical use by the FDA in 1995 [181] and constitutes an example of enhanced delivery of a drug, doxorubicin in this particular case, to tumor cells following extravasation-dependent ("passive") targeting of PEGylated liposomes. Very recently, the FDA approved a liposome encapsulating a combination of daunorubicin and cytarabine (VYXEOS®, Jazz Pharmaceuticals, Inc.) for the treatment of acute myeloid leukaemia [12]. This is the first nanomedicine entrapping two drugs approved for biomedical applications, opening new avenues for the clinical development and regulatory approval of advanced combinatorial approaches using a single carrier.

As a result, increasing developments of multifunctional nanomedicines responsive to multiple enzymes and/or stimuli may combine the advantages of PDEPT/PELT and nanodelivery tools in a single carrier. The next generation of enzyme-responsive systems developed under a new concept combining nanotechnology-based strategies with advanced conjugation chemistry may, thus, additionally overcome some of the disadvantages of a two-step therapeutic approach, while offering a specific molecular conjugation to increase active targeting and intracellular delivery of payloads. However, despite the development of multiple enzyme-responsive delivery systems, none has been translated into clinical trials. In fact, important challenges still need to be overcome to optimize spatio-temporal release of the active compounds, to achieve a maximum therapeutic index, ensuring high drug concentration at the targeted tissue and reduced effects on the viability of healthy cells. In addition, clear benefit on therapeutic effect and improved safety must be obtained using these two-step systems particularly following the development of complex multifunctional enzyme-responsive

nanodelivery systems. The rational design should consider not only the high cost, but also the challenging translation into clinical use against cancer. Moreover, the treatment of other diseases, which pathologies could facilitate an EPR effect, may benefit from these new tools bridging nanotechnology and enzyme-polymer conjugate technologies. Indeed, recent evidence demonstrates the presence of an EPR effect in bacterial infection and supports the potential application of PELT for the targeted delivery of antibiotics to sites of bacterial infection [158]. Several liposomal antibiotic formulations are already in development or clinical use [182, 183] and may be useful models to combine with a suitable polymer-enzyme conjugate in future studies.

Overall, besides the anticipated long process to bring these systems into clinical practice, significant progress in several complementary areas may change significantly the landscape of these enzyme-responsive systems, allowing for their full potential against multiple pathological situations.

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