HPMA Copolymer-phospholipase C and Dextrin-Phospholipase A_2 as Model Triggers for Polymer Enzyme Liposome Therapy (PELT)

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Abstract

'Polymer Enzyme Liposome Therapy' (PELT) is a two-step anticancer approach in which a liposomal drug and polymer-phospholipase conjugate are administered sequentially to target the tumour interstitium by the enhanced permeability and retention effect, and trigger rapid, local, drug release. To date, however, the concept has only been described theoretically. We synthesised two polymer conjugates of phospholipase C (PLC) and A_2 (PLA_2) and evaluated their ability to trigger anthracycline release from the clinically-used liposomes, Caelyx® and DaunoXome®. N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-PLC and a dextrin-PLA_2 were synthesised and their enzymatic activity characterised. Doxorubicin release from polyethyleneglycol-coated (PEGylated) Caelyx® was relatively slow (< 20%, 60 min), whereas daunomycin was rapidly released from non-PEGylated DaunoXome® (~87%) by both enzymes. Incubation with dextrin-PLA_2 triggered significantly less daunomycin release than HPMA copolymer-PLC, but when dextrin-PLA_2 was pre-incubated with α-amylase, the rate of daunomycin release increased. DaunoXome®'s diameter increased in the presence of PLA_2 or dextrin-PLA_2, while Caelyx®'s diameter was unaffected by free or conjugated PLA_2. Dextrin-PLA_2 potentiated the cytotoxicity of DaunoXome® to MCF-7 cells to a greater extent than free PLA_2, while combining dextrin-PLA_2 with Caelyx® resulted in antagonism, even in the presence of α-amylase, presumably due to steric hindrance by PEG. Our findings suggest that *in vivo* studies to evaluate PELT combinations should be further evaluated.

Key Words: PELT, polymer-protein conjugate, phospholipases, polymer therapeutics, liposome

Introduction

Liposomes containing anthracylines (e.g. Caelyx® in Europe, also known as Doxil® in the USA, and Myocet®) have been successfully developed as antitumour agents for the treatment of diseases such as metastatic breast cancer (reviewed in [1]), and such liposomal anthracyclines are routinely used in many combination chemotherapy regimens [2]. Liposomes target human tumours by the enhanced permeability and retention (EPR) effect [3], which prolongs drug circulation time and reduces off-target toxic effects such as anthracycline-related cardiotoxicity [4]. However, despite the clinical advantages of liposomal drugs, they do not lack disadvantages. The main dose-limiting side effect associated with Caelyx® is palmar plantar erythrodysesthesia, also known as 'hand-foot syndrome', which occurs due to its PEGylated coating [5]. Moreover, the relatively slow rate of intratumoural release of the free drug from the liposomal formulations can also limit optimal therapeutic activity [6]. To enable externally-triggered liposomal drug release specifically within tumours, Duncan and Satchi-Fainaro have previously proposed the two-step approach, Polymer-Enzyme Liposome Therapy (PELT) [7], although, to date, *in vitro* proof of principle has not been reported. This concept involves treatment with liposome therapy followed by administration of a polymer-phospholipase conjugate which co-localises in the tumour interstitium by the EPR effect, and triggers rapid, and local, liposomal drug release at the target site [7]. This hypothesis is shown schematically in Figure 1. Clinically, it is expected that in the case of the long-circulating polyethylene glycol-coated (PEGylated) Caelyx®, which encapsulates doxorubicin (Dox), the polymer-phospholipase would likely be administered after several days, since Caelyx® has a serum half-life of 30-90 h [8]. However, in the case of the non-PEGylated DaunoXome®, which encapsulates daunorubicin (Dnm), the second step might be anticipated a few hours after liposome administration, due to its rapid reticuloendothelial system (RES) clearance (serum half-life = ~5 h [9]).

The aim of this study was to develop and test two model polymer-phospholipase conjugates with potential for further development as a clinically suitable 'trigger' for PELT (Figure 1). As several HPMA copolymer-anticancer conjugates have already progressed into clinical trials [10] and the biodegradable polysaccharide, dextrin, which is readily degraded by α–amylase, is used clinically as a peritoneal dialysis solution [11], these were chosen as first model polymers. Moreover, we have already reported methods for synthesis and evaluation of HPMA copolymer-enzyme [7, 12, 13, 14, 15, 16] and dextrin-enzyme conjugates [17, 18]. Phospholipase C (PLC) and phospholipase A_2 (PLA_2) were selected since both have previously demonstrated their ability to trigger drug/probe release from liposomes [19, 20], despite their different mechanisms used to hydrolyse phospholipids. PLC hydrolyses phopholipids on the glycerol side of the phosphodiester bond, causing release of diacylglycerol, while PLA_2 catalyses hydrolysis at the *sn-2* fatty acyl bond, to release free fatty acid and lysophospholipid [21]. Dextrin-PLA_2 conjugates have already been extensively characterised and shown promise as anti-cancer agents in their own right [18, 22], having the added advantage of being able conceal to and reinstate enzymatic activity by the polymer-masked unmasked protein therapy (PELT) concept [17]. Caelyx® and DaunoXome® were selected as model liposomal formulations as they represent the PEGylated and uncoated classes, respectively.

First, methods were optimised for synthesis of HPMA copolymer-PLC and dextrin-PLA_2 conjugates, which were characterised in respect of free and total enzyme content, molecular weight and retention of enzymatic activity. The ability of HPMA copolymer-PLC and dextrin-PLA_2 (± α-amylase) to trigger the release of Dox or Dnm from Caelyx® and DaunoXome®, respectively, was assessed. Given the growing concerns about chronic toxicity associated with non-biodegradable polymers and the benefits of Polymer masked-UnMasked Protein Therapy (PUMPT) using biodegradable polymers, dextrin-PLA_2 emerged as the lead PELT combination for further studies. Therefore, physical stability of Caelyx® and DaunoXome® in the presence

of dextrin-PLA_2 was assessed by dynamic light scattering and *in vitro* cytotoxicity of the combination was performed using MCF-7 cells.

Materials and Methods

Materials

HPMA copolymer-Gly-Gly-p-nitrophenol (ONp) (4.4 mol% ONp, M_w = 34,679 g/mol) was from Polymer Laboratories (Church Stretton, UK). Type 1 dextrin (M_w = 51,000 g/mol) from corn was from ML laboratories (Keele, UK). DaunoXome® (solution for injection; 2 mg/mL) was from Gilead Sciences (Cambridge, UK). Caelyx® (concentrated for injection; 2 mg/mL) was from Janssen-Cilag Ltd (High Wycombe, UK). PLC was from the Defence Evaluation and Research Agency (DERA) (Porton Down, UK). PLA_2 from honey bee venom, 1-Ethyl-3-(3-dimethylaminopropyl carbodiimide hydrochloride) (EDC), sodium metaborate, p-nitrophenyl-phosphorylcholine (pNPPC), copper (II) sulphate pentahydrate (4% w/v solution), bovine serum albumin (BSA), bicinchoninic acid solution (BCA), TRIZMA base, sodium dodecyl sulfate (SDS), TRIZMA hydrochloride (Tris HCl), Triton X-100, ammonium persulfate, acrylamide/bis-acrylamide, tissue culture grade dimethyl sulfoxide (DMSO, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), trypan blue and optical grade DMSO were all from Sigma-Aldrich (Poole, UK). N,N,N,N-tetramethyl-ethylenediamine (TEMED), bromophenol blue, coomassie brilliant blue G-250, 2-mercaptoethanol and pre-stained SDS PAGE standards were from Bio-Rad (Perth, UK). Glycine was from ICN Biomedicals, Inc. 3,3-dimethylglutaric acid (DMG) and calcium lactate were from Merck (Darmstadt, Germany). Sodium acid phosphate, sodium phosphate, sodium chloride and 4-dimethylaminopyridine were from Fisher Scientific (Loughborough, UK). Unless otherwise stated, all chemicals were of analytical grade. All solvents were of general reagent grade (unless stated) and were from Fisher Scientific (Loughborough, UK). The human breast carcinoma cell

line MCF-7 was provided by Tenovus Centre for Cancer Research (Cardiff, UK). Foetal calf serum (FCS), 0.05% w/v trypsin-0.53 mM EDTA and RPMI 1640 with L-glutamine (with and without phenol red as pH indicator) were from Invitrogen Life Technologies (Paisley, UK).

Synthesis of Dextrin-PLA_2 Conjugate

Dextrin-PLA_2 conjugate was synthesised, purified and characterised (including enzymatic activity) using methods previously described [18] and summarised schematically in Figure 2a.

Synthesis of HPMA Copolymer-PLC Conjugate

PLC was conjugated to HPMA copolymer-Gly-Gly-ONp as outlined in Figure 2b. Briefly, PLC (2 mg/mL, 1 mL, 4.3 x 10^{-8} mol) was dissolved under stirring in PBS (pH 7.2) in a round-bottomed flask. Next, HPMA copolymer-Gly-Gly-ONp (2 mg/mL, 2 mL, 5.76 x 10^{-9} mol of ONp), dissolved in double distilled water (ddH_2O), was added dropwise to the solution to avoid crosslinking and the reaction mixture was stirred in the dark at 4°C for 30 min. The pH was then carefully raised to 8.5 by adding saturated tetraborate buffer. The mixture was stirred for another 12 h and the reaction was terminated by adding 1-amino-2-propanol (20 μL) to remove unreacted ONp and avoid further crosslinking. Free ONp, 1-amino-2-propanol, and tetraborate salts were removed from the final yellow solution using Vivaspin tubes (10,000 g/mol Mw cut-off) until no yellow colour remained. The conjugate was purified from the reaction mixture by FPLC (Pharmacia LKB FPLC system; Amersham Pharmacia Biotech, UK) using a pre-packed Superdex 200 HR10/30 column with a UV detector and data analysis using FPLC Director version 1.10 software (Amersham Pharmacia Biotech, UK). The concentrated reaction mixture (200 μL) was injected into a 500 μL loop using PBS (pH 7.4) as a mobile phase and run at 0.5 mL/min. Fractions (1 mL) were collected then the appropriate fractions containing conjugate were pooled and desalted using Vivaspin tubes (10,000 g/mol Mw cut-off). The final conjugate was lyophilised and stored at -20°C.

HPMA copolymer-PLC conjugate was characterised by FPLC and SDS PAGE (12.5% acrylamide gel, 0.75 mm thickness) to assess purity, and the total protein content was determined by the Bradford assay.

Measurement of PLC Activity

PLC activity was measured using pNPPC as the substrate (adapted from [23]). First, pNPPC (40 mM), native PLC (10 μg/mL PLC) and HPMA copolymer-PLC conjugate (10 μg/mL PLC-equivalent) were each dissolved in DMG buffer (10 mM containing 2.5 mM calcium lactate, 0.01 mM zinc sulfate and 0.1% w/v BSA (adjust to pH 7.2 with NaOH)). All samples were prepared fresh before use and placed on ice throughout the experiments. The pNPPC solution (0.5 mL, 2 x 10^{-5} mol) was added to a 1 cm^3 quartz cell incubated at 37°C. To start the assay, free enzyme or conjugate (0.5 mL, 1 x 10^{-10} mol PLC-equivalent) was added to the cuvette. Degradation of phospholipids was monitored by measuring the increase in absorbance at 414 nm for 15 min. DMG buffer (1 mL) was used as a blank. Results were expressed as arbitrary units of absorbance over time. Conjugate activity was calculated as a percentage of the activity of free phospholipase, by comparison of the slope of the linear portion of the curves.

Polymer-Phospholipase Mediated Liposomal Drug Release

To assess the rate and extent of Dox release from Caelyx® and Dnm release from DaunoXome®, liposomes were first diluted in PBS (1:100, pH 7.4). Native PLC and HPMA copolymer-PLC (final experiment concentration of 20 μg/mL PLC-equivalent), and native PLA_2 and dextrin-PLA_2 (final experiment concentration of 50 μg/mL PLA_2-equivalent) were also dissolved in PBS (pH 7.4). All samples were prepared fresh before use and placed on ice throughout the experiments. Next, PLC or HPMA copolymer-PLC (100 μL) was added to each

well of a black 96-well microfluor plate and the plate allowed to equilibrate at 37°C for 30 min. Subsequently, liposome solution was added to the each well (6 replicates) and the plate immediately placed into a fluorescence plate reader at 37°C and fluorescence was measured at various time points (λ_{ex} = 485 nm and λ_{em} = 520 nm). Triton X-100 (1% v/v) was used to release 100% of the drug from the liposome, while PBS was used as a negative control. A blank fluorescence reading was obtained using PBS only. Results are expressed as percentage of total (corrected for background fluorescence of intact liposomes).

The effect of dextrin-PLA_2 on liposomal drug release was also determined following incubation of dextrin-PLA_2 with α-amylase (0.5 mg/mL PLA_2-equivalent incubated with 200 SU/mL α-amylase at 37°C for 16 h in Tris buffer, pH 8.2), and liposomal drug release measured as described above.

Stability Testing of Liposomes

Dynamic light scattering (DLS) (using a Zetasizer Nano ZS (Malvern, UK) and an N4 Plus submicron particle sizer (Beckman Coulter Ltd, UK) for Caelyx® and DaunoXome® measurements, respectively) was used to measure mean diameter and polydispersity of liposome formulations following incubation with dextrin-PLA_2 conjugate, PLA_2, Triton X-100 and in PBS. DaunoXome® or Caelyx® (both 2 mg/mL) was diluted fresh on the day of experiment to 1:100 or 1:200, respectively, in PBS (pH 7.6) and stored at 4°C prior to use. Dextrin-PLA_2 conjugate and PLA_2 were dissolved in PBS (pH 7.6, 100 μg/mL PLA_2-equivalent) and equilibrated at 37°C. Liposome solution (1 mL) was added to a polystyrene cuvette and allowed to equilibrate to 37°C for 30 min. PBS (negative control), dextrin-PLA_2 conjugate, PLA_2 or Triton X-100 (2%) solution (positive control) (1 mL) was added immediately before initiating the first measurement. The stability of the liposomes was followed for 150 min at 37°C (n = 3). Data is presented as mean liposome diameter over time.

Evaluation of In Vitro Cytotoxicity

An MTT assay was used to assess cell viability as previously described [24]. MCF-7 cells were seeded into sterile 96-well microtitre plates (4 x 10^4 cells/ mL) in 0.1 mL/well of RPMI 1640 (without phenol red) with FCS (5% v/v) and incubated at 37 ^oC for 24 h. Next, the medium was removed and various concentrations of DaunoXome® or combinations DaunoXome® and PLA_2 (50 μg/mL) or DaunoXome® and dextrin-PLA_2 (50 μg/mL) (0.2 μm filter-sterilised) were added to the cells. Following a further 67 h incubation, MTT (20 μL of a 5 mg/mL solution in PBS) was added to each well and the cells were incubated for 5 h. The medium was then removed and the precipitated formazan crystals were solubilised for 30 min with the addition of optical grade DMSO (100 μL). Spectrophotometric absorbance of the formazan product was measured at 550 nm. Cell viability was expressed as a percentage of the viability of untreated control cells, and the concentration for 50% inhibition (IC_{50}) value calculated (± SEM).

The multiplicative model was used to evaluate PLA_2 and dextrin-PLA_2's interaction with liposomal drug formulations *in vitro* [25]. This model assumes that each drug acts independently, and therefore predicts that the observed effect of a drug combination is equal to the product of the effect of the individual drugs (i.e. expected cell viability (A+B) = cell viability (A) x cell viability (B)). Synergism is observed when the observed cell viability is lower than the product of each individual drug effect and antagonism is observed when the observed cell viability is higher than the cell viability for the most active drug.

Statistical Analysis

Data were expressed as mean ± the error, calculated as either standard deviation (SD) or standard error of the mean (SEM). Statistical significance was set at $p < 0.05$ (indicated by *). Where only two groups were compared, student's t test for a small sample size was used. Where more than two groups were compared, significance was evaluated using a one-way analysis of

variance (ANOVA) followed by bonferroni post hoc tests to correct for multiple comparisons. All statistical calculations were performed using GraphPad Prism, version 6.0g for Macintosh, 2015.

Results

Characterisation of Conjugates

Polymer-phospholipase conjugates were successfully synthesised and purified (Table 1). SDS PAGE and FPLC analysis confirmed the presence of a high molecular weight conjugate (representative data for dextrin-PLA_2 conjugates are presented in Figure 3a,b). In all cases, conjugates contained < 1% free phospholipase. Two batches of dextrin-PLA_2 conjugates were prepared, which showed good batch-to-batch reproducibility that was comparable to previous studies.

The HPMA copolymer-PLC conjugate retained enzymatic activity, indeed, when evaluated against pNPPC at equivalent protein concentration, slightly greater activity (to ~132.5% ± 4 (SD)) was seen compared to free PLC (Figure 3c). In contrast, conjugation of dextrin to PLA_2 led to reduced activity (to ~67% ± 11.5 (SD)) compared to free enzyme (Figure 3d). However, bioactivity was reinstated (to ~139% ± 15.5 (SD)) when the dextrin-PLA_2 conjugate was pre-incubated with α-amylase.

Triggered Liposomal Drug Release

As expected, both liposome formulations were stable in PBS. Incubation of Caelyx® with free PLC or HPMA copolymer-PLC conjugate led to minimal (<5%) Dox release from the liposomes over the 2.5 h period studied (Figure 4a**)**, and no difference was seen in the rate or extent of Dox release triggered by free and HPMA copolymer-conjugated PLC. However, when the non-PEGylated liposome, DaunoXome®, was incubated with PLC or HPMA copolymer-

PLC conjugate, rapid Dnm release was seen within 5 min in both cases. Release plateaued after ~10 min at ~60-64% total, and was similar for free and HPMA copolymer-conjugated PLC (Figure 4b).

When Caelyx® was incubated with PLA_2, increased Dox release was seen than with PLC (18.4 *vs.* 2.3%, respectively at 60 min), however <2% total drug release was observed in the presence of dextrin-PLA_2 conjugate, even following α-amylase-unmasking (Figure 4c). In contrast, incubation of DaunoXome® with free PLA_2 triggered rapid Dnm release from the liposomes (Figure 4d), with 84.1% of total Dnm released after 60 min. However, dextrin-PLA_2-induced drug release was much slower than that seen for free PLA_2, only reaching 19.5% after 60 min. Pre-incubation of the dextrin-PLA_2 conjugate with α-amylase led to a significantly faster initial rate of Dnm release over this 60 min experiment, but in this case, it was still much slower than seen for both free PLA_2 and the HPMA copolymer-PLC conjugate, only reaching 27.0% total drug release by the end of the experiment.

Liposome Stability

Both liposome formulations showed a classical unimodal size distribution (Figure 5a,c) that was unaltered over a 3 h incubation period. As expected, Caelyx® liposomes were larger than DaunoXome® (mean diameter = 80.1 ± 3.7 (SD) nm *vs.* 50.6 ± 0.2 (SD) nm, respectively).

When Triton X-100 was added to DaunoXome® the vesicle diameter dropped rapidly to 20.1 ± 2.2 (SD) nm within 1 min (Figure 5b). Similarly, addition of Triton X-100 to Caelyx® caused an immediate decrease in liposome diameter (10.7 ± 0.9 (SD) nm within 1 min) (Figure 5d). In contrast, when DaunoXome® was incubated with free PLA_2 the liposomes appeared to get larger over time, reaching a maximum diameter of 122.2 ± 0.1 (SD) nm after 3 h. When liposomes were incubated with the dextrin-PLA_2 conjugate, the mean diameter remained unchanged over the course of the 3 h experiment. However, when the dextrin-PLA_2 conjugate

was unmasked using α-amylase, a slight increase in size of the liposomes was seen (mean diameter = 61.7 ± 0.2 (SD) nm). In contrast, Caelyx® diameter was unaffected by addition of PLA_2, dextrin-PLA_2 or unmasked dextrin-PLA_2.

In Vitro Cytotoxicity of PELT Combination

As expected, DaunoXome® and Caelyx® alone were cytotoxic towards MCF-7 cells in a concentration-dependent manner (Figure 6, Table 2). Addition of PLA_2 or dextrin-PLA_2 (50 μg/mL PLA_2-equivalent) alone reduced MCF-7 cell viability to 92.8% and 57.3%, respectively (results not shown). However, when cells were incubated with liposomal drugs combined with either PLA_2 or dextrin-PLA_2 conjugate, cytotoxicity increased. For both DaunoXome® and Caelyx®, the liposome/ dextrin-PLA_2 PELT combination was more cytotoxic than liposomal drug alone or combined with native PLA_2. However, the Caelyx® and dextrin-PLA_2 conjugate combination did not show the same Dox concentration-dependent decrease in cell viability, as seen with DaunoXome® combinations or Caelyx® with PLA_2. A comparison of observed and expected cell viability (according to the multiplicative model) revealed that, while the observed cell viability was lower than the expected cell viability for the PELT combination using DaunoXome®, the opposite was true for PELT using Caelyx® at <0.005 and <0.01 μg/mL Dox for masked and unmasked dextrin-PLA_2, respectively (Figure 7). In other words, drug combinations with DaunoXome® showed synergism whereas when dextrin-PLA_2 conjugate was combined with Caelyx®, antagonism was observed, even after α-amylase unmasking, especially at the highest concentrations of Caelyx®.

Discussion

Two-step enzyme-prodrug combinations, designed to increase active drug concentration in tumour tissue, were first described in the 1970s (reviewed in [26]), and a number of these

approaches have been tested clinically, however, none are currently in routine clinical use. Typically, they rely on tumour-targeted delivery of a specific drug-activating enzyme (or its gene) using a viral vector (Virus-Directed Enzyme-Prodrug Therapy; VDEPT), or an antibody (Antibody-Directed Enzyme-Prodrug Therapy; ADEPT), combined with administration of a cytotoxic prodrug. A two-step antibody-PLC/ liposome approach, based on an anti-epidermal growth factor receptor (EGFR) antibody-PLC conjugate as a trigger for liposomal drug release, has also been described, which showed synergistic inhibition of cell proliferation *in vitro* and inhibited tumour growth in mice [20]. More recently, Satchi *et al.* reported a two-step polymer conjugate approach, called Polymer-Directed Enzyme-Prodrug Therapy (PDEPT), which utilises a polymer-enzyme conjugate to activate a previously tumour-localised polymeric prodrug [14, 15]. For both PDEPT and PELT approaches described here, the polymer-enzyme conjugate is a key component. Many PEGylated proteins, including those used in cancer (reviewed in [27]), have already entered routine clinical use, and their ability to reduce non-specific protein toxicity, extend the plasma half-life, reduce immunogenicity and enhance passive targeting to tumours by the EPR effect [28] is well established. Given the increasing awareness of chronic toxicity with the use of non-biodegradable polymers, these studies were undertaken to investigate whether a bioresponsive polymer-phospholipase conjugate, using a biodegradable polymer, would be more suitable for PELT than a non-biodegradable polymer.

Liposomal composition, together with phospholipase substrate specificity, will undoubtedly influence the ability of a polymer-phospholipase conjugate to trigger drug release. When DaunoXome® was incubated with free PLC over a range of concentrations, maximal Dnm release occurred at an enzyme concentration of 5 μg/mL (0.12 μM), whereas maximal release was induced at the higher free PLA_2 concentration of 50 μg/mL (3.16 μM) (results not shown). The superior activity of PLC may, in part, be due to its substrate specificity, however, the main phospholipid in DaunoXome® and Caelyx® is phosphatidylcholine (PC) [29], which

is preferentially hydrolysed by both PLC and PLA_2, so PLC's higher potency is more likely to be related to the presence of cholesterol in both formulations, which has been shown to enhance its activity [30].

The results obtained here clearly show that steric hindrance imparted by the PEGylated liposomal surface limits enzyme-induced drug release from Caelyx®. In contrast, both the HPMA copolymer-PLC and dextrin-PLA_2 conjugates were able to induce Dnm release from the non-PEGylated DaunoXome® vesicles. Observation that free PLC and HPMA copolymer-PLC displayed the same rate of liposomal degradation was consistent with observations in the biochemical assay using pNPPC. While free PLA_2 rapidly released Dnm from DaunoXome®, the dextrin-PLA_2-induced release was slower (19.5% release at 1 h), but increased significantly (~1.5-fold) after pre-incubation with α-amylase, as seen previously in the egg yolk bioassay. Dextrin used here had a Mw ~ 51,000 g/mol, that is approximately 3 times higher than PLA_2, which may explain the reduction of enzymatic activity seen compared to free PLA_2, probably due to masking of the enzyme's catalytic site. These conjugate features were chosen to optimise pharmacokinetics *in vivo* (higher molecular weight conjugates display greater EPR-mediated targeting), and also to mask enzymatic activity/toxicity in transit [18]. In contrast, the HPMA copolymer and PLC have similar molecular weights (~40,000 g/mol). This unexpectedly high level of PLC activity seen after conjugation and purification may be due to 'flipping' or unfolding of the catalytic site, a phenomenon that has been described for alpha-toxin PLC and which is reported to enhance enzymatic activity [31]. This suggests that as the conjugate was water-soluble and the PLC was active following conjugation, it is unlikely that crosslinking of the conjugate formed large aggregates that would lead to precipitation and loss of enzymatic activity.

Although the HPMA copolymer-PLC retained greater enzymatic activity than dextrin-PLA_2, resulting in greater liposomal drug release, the fact that the dextrin-PLA_2 conjugate can

capitalise on the benefits of Polymer masked-UnMasked Protein Therapy (PUMPT) [17] makes it a more attractive option for PELT. PUMPT uses conjugation of a biodegradable polymer to mask a protein or peptide's activity (useful to diminish toxicity of a protein like PLA_2 in the bloodstream), and subsequent triggered degradation of the polymer can be used to regenerate bioactivity in a controlled fashion. We have already demonstrated the potential of α-amylase triggered degradation of several dextrin-protein conjugates, including trypsin, melanocyte-stimulating hormone (MSH) [17], PLA_2 [18] and epidermal growth factor (EGF) [32, 33].

Given the clinical advantages of using a biodegradable trigger for PELT, subsequent investigations focused on dextrin-PLA_2 conjugates. Characterisation of liposome diameter showed a significant increase in the size of DaunoXome® in the presence of PLA_2 and, to a lesser extent, unmasked dextrin-PLA_2. This effect was not seen for Caelyx®. A recent study using time-resolved small-angle neutron scattering (SANS) to investigate changes in 1,2-dipalmitoyl-*sn*-glycero-3-15 phosphocholine (DPPC) vesicle structure on exposure to dextrin-PLA_2 (± α-amylase) showed that conjugate unmasking by dextrin degradation results in time-dependent vesicle degradation [34]. These studies also reported time-dependent vesicle swelling following incubation with PLA_2 or unmasked dextrin-PLA_2, which was attributed to membrane permeabilisation and osmotic swelling. This causes membrane stretching, thereby reducing the lateral packing of lipids, which can further modulate its susceptibility to PLA_2 activity [35], and ultimately cause liposome rupture. Although dextrin-PLA_2 conjugate did not have the osmotic effect of free PLA_2, unmasked dextrin-PLA_2 conjugate showed some swelling of liposomes over time, suggesting that activity of PLA_2 had been reinstated (~16 %) following dextrin degradation. Presumably, since Caelyx®'s diameter was unaltered during incubation with PLA_2, the presence of PEG chains on the liposome surface prevented PLA_2 from reaching the phospholipid substrate due to steric hindrance.

Since PLA_2 has previously shown cytotoxicity towards several cancer cell lines (HT29,

MCF-7 and B16F10) [18], but PLC was not toxic towards B16F10, L1210 or CCRF cancer cells (up to 0.5 mg/mL, data not shown), dextrin-PLA_2 was chosen to test in an *in vitro* cytotoxicity model of PELT, since it may offer enhanced anticancer activity. *In vitro* cytotoxicity modeling of the PELT combination is inherently difficult, however, due to the dependence on the EPR effect for co-localisation of liposomes and polymer-enzyme conjugate in the tumour tissues, as well as dextrin-PLA_2's reliance on the intra-tumoural α-amylase concentration for enzyme activation. Nevertheless, preliminary *in vitro* evaluation of cytotoxicity in MCF-7 cells showed that the DaunoXome®/dextrin-PLA_2 PELT combination in the presence of α-amylase resulted in greater cytotoxicity than either DaunoXome® alone or combined with native PLA_2. Enhanced cytotoxicity was not observed when Caelyx® was combined with dextrin-PLA_2, even in the presence of 100 IU/L α-amylase. Although Dnm release from DaunoXome® was shown to be relatively slow over 1 h, these 72 h cytotoxicity assays were conducted in the presence of FCS (containing 7.2 IU/L α-amylase [18],which is expected to slowly degrade dextrin, thus releasing active PLA_2 over time. Moreover, it has been reported that the levels of α-amylase can be up to 85-fold higher in the tumour environment compared to plasma [36, 37], which would provide an opportunity for enhanced dextrin degradation and even more rapid enzyme activation, and thus drug release, selectively within the tumour interstitium. Furthermore, although phospholipases are known to have haemolytic activity, which may be exacerbated *in vivo* in long circulating, polymer-conjugated phospholipase, previous studies showed that concentration-dependent haemolysis was abolished by dextrin conjugation to PLA_2 [18]. We hypothesized that the polymer masks the enzyme while in circulation and then it is unmasked when reaching the tumour site in the presence of amylase present in the tumour.

Data analysis using the multiplicative model of drug interactions confirmed that DaunoXome® acts synergistically with PLA_2 and dextrin-PLA_2, while the PEGylated Caelyx®

was only synergistic with PLA_2 and antagonistic with dextrin-PLA_2, even in the presence of exogenous α-amylase. This result is consistent with the drug release and liposome stability findings, and suggests that dextrin conjugation inhibits PLA_2's interaction with PEGylated phospholipids, which in turn prevents liposomal drug release. Studies have shown that the drug trapped in liposomes in interstitial space remains inactive until it is released in the free form [38, 39], therefore liposomal drug release is essential for Dox's anticancer activity. Previous analysis of PUMPT-PELT by SANS showed that unmasking of dextrin-PLA_2 with α-amylase partially reinstated PLA_2's activity towards DPPC vesicles [34], which is also evident in these studies as a shift of data points towards additivity (dashed line).

Conclusions

These studies demonstrate the feasibility of the PELT approach when using a polymer-phospholipase conjugate to accelerate the release of the drug from non-PEGylated liposomes, such as DaunoXome®. Dextrin-PLA_2 conjugate has several advantages compared to the HPMA copolymer-PLC conjugate, including ease of synthesis/purification and the ability to mask and then reinstate enzymatic activity. In addition, as dextrin is biodegradable, and the HPMA copolymer backbone is not, higher molecular weight conjugates can be used. These have been previously shown to display better extravasation-dependent tumour targeting enabled by the EPR effect. As the dextrin-PLA_2/DaunoXome® combination caused enhanced cytotoxicity in MCF-7 cells, this suggests that further *in vivo* evaluation is warranted. As liposomal Dox is more widely used clinically than DaunoXome®, investigation of other, non-PEGylated, liposomal anticancer agents using the PELT approach would also aid the identification of the best combination for future clinical investigation.

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Disclosure of interest

The authors report no conflicts of interest.

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Table 1 Characteristics of polymer-phospholipase conjugates

Conjugate	Liposome formulation tested	Molecular weight (g/mol)	Protein content (% w/w)	Molar ratio (polymer: phospholipase)
HPMA copolymer-PLC[1]	Caelyx®	80-130,000*	57-72	1:1 to 1:2
Dextrin-PLA_2	DaunoXome®	195,000+	9.4	3:1
Dextrin-PLA_2	Caelyx®	190,000+	9.3	3:1

* measured by SDS PAGE.

+ measured by FPLC, relative to protein molecular weight markers.

[1]values summarise characteristics of 3 batches of HPMA copolymer-PLC used in these studies

Table 2 Cytotoxicity of compounds used in this study.

Combination	**IC_{50} (µg/mL)[a]**	**Combination**	**IC_{50} (µg/mL)[a]**
DaunoXome®	0.082	Caelyx®	0.185
DaunoXome® + PLA_2	0.017	Caelyx® + PLA_2	0.028
DaunoXome® + dextrin-PLA_2	0.0004	Caelyx® + dextrin-PLA_2	ND
		Caelyx + unmasked dextrin-PLA_2	ND

[a] Cell viability at 72 h MTT assay, seeding density 1 x 10^4 cells/mL. Data expressed as mean ± SEM.; n = 18.
ND = not determined

Figure 1 Ferguson et al.

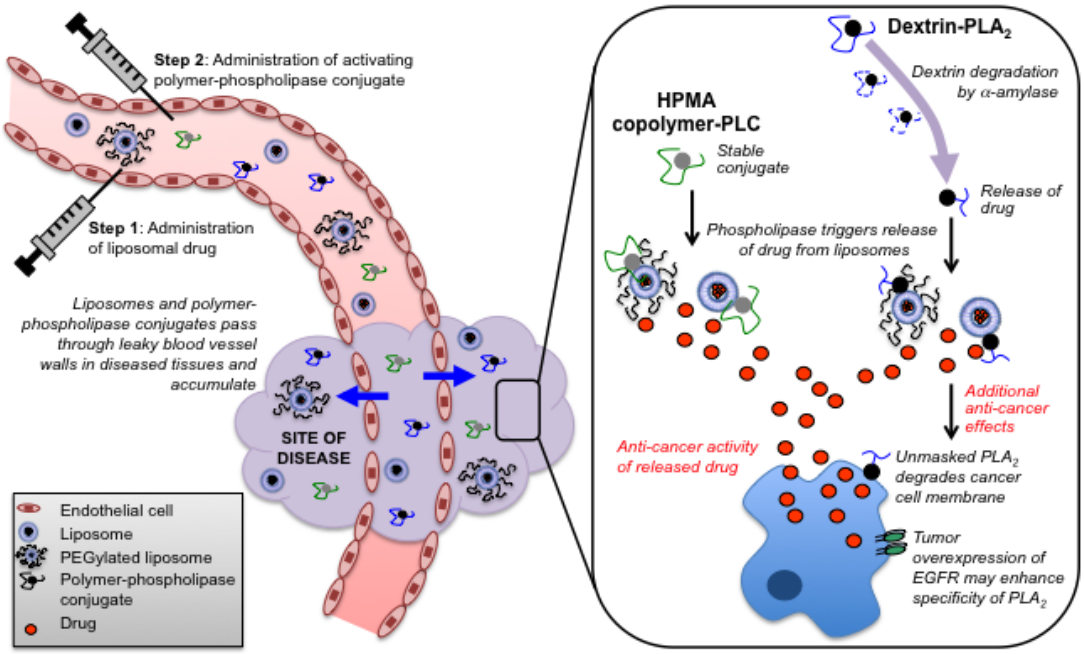
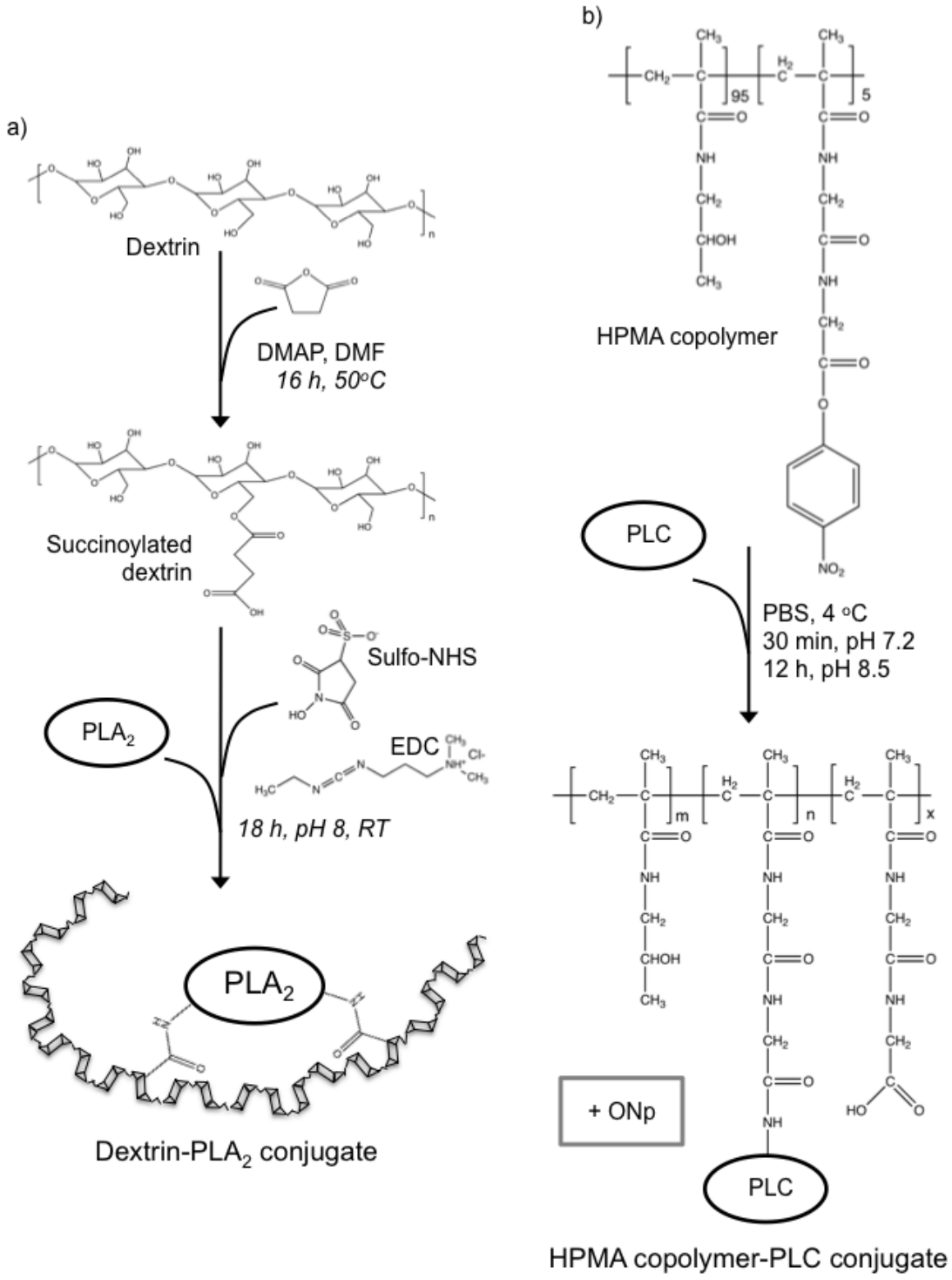


Figure 2 Ferguson et al



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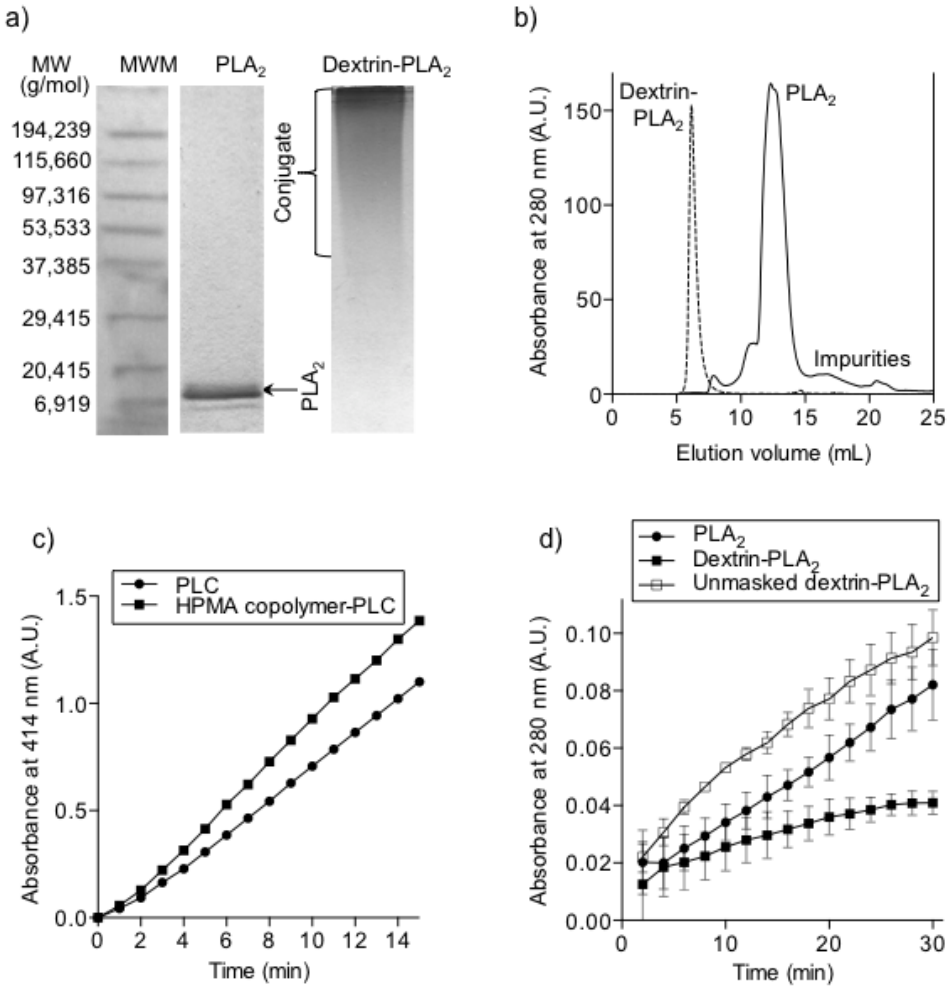

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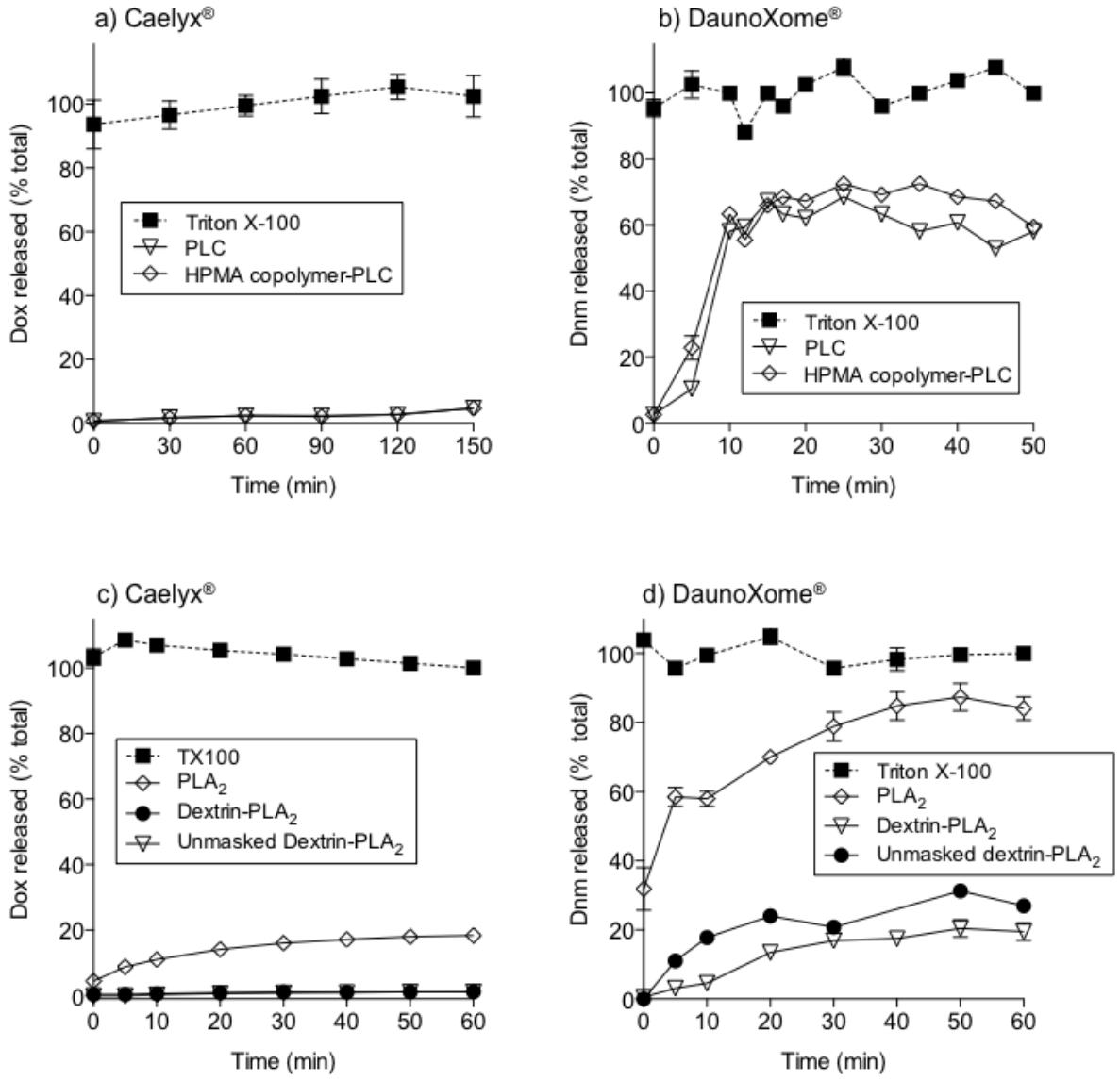

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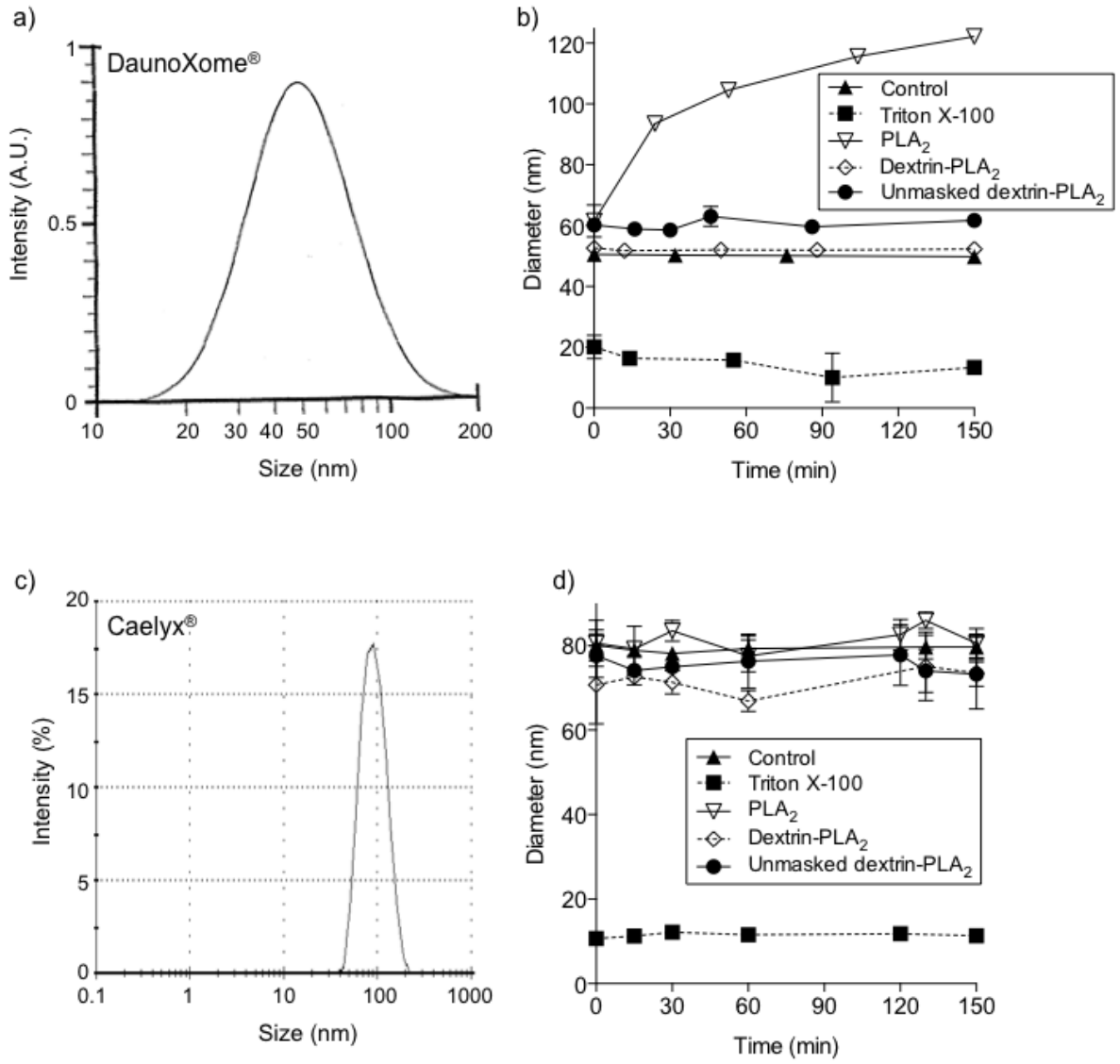

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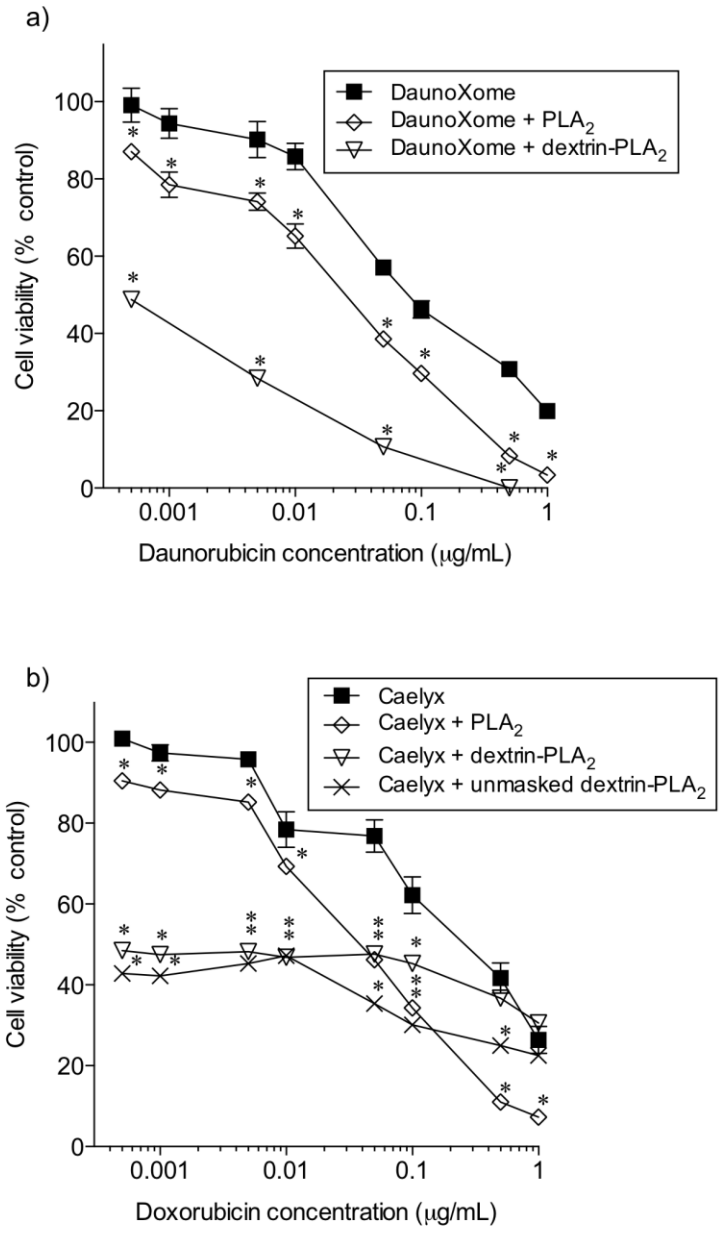

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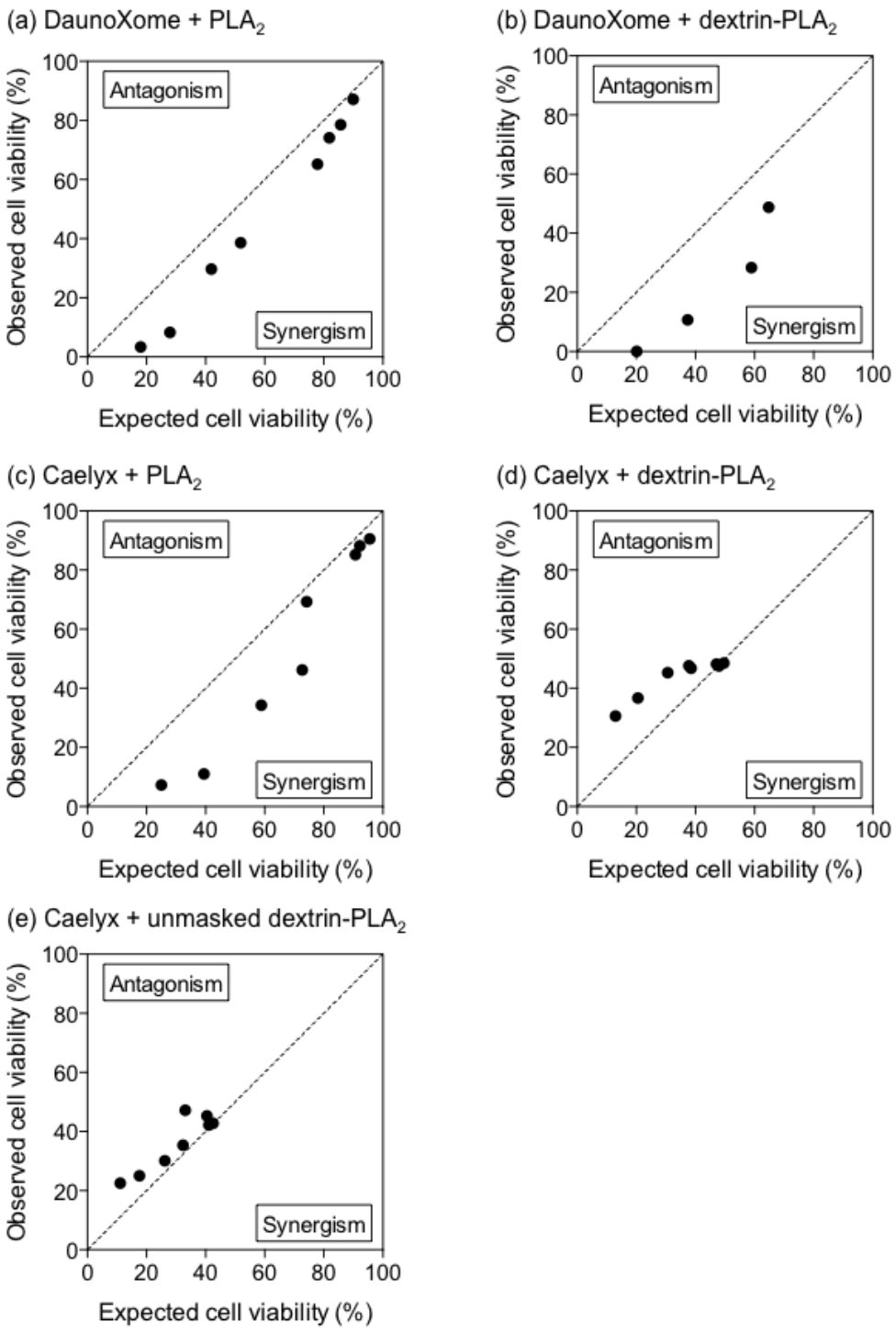

Legend to Figures

Figure 1 Schematic representation of the PELT concept.

Figure 2 Reaction scheme for the synthesis of dextrin- PLA_2 and HPMA copolymer-PLC conjugates.

Figure 3 Characterisation of HPMA copolymer-PLC and dextrin-PLA_2 conjugates. Panel (a) shows SDS PAGE analysis of PLC, HPMA copolymer-PLC, PLA_2 and dextrin-PLA_2 and panel (b) shows characterisation by FPLC of free PLC and HPMA copolymer-PLC conjugates, and free PLA_2 and dextrin-PLA_2 conjugates, respectively. Panels (c) and (d) show measurement of phospholipase activity for PLC and PLA_2, respectively. Data show absorbance as mean ± SD, n = 3.

Figure 4 *In vitro* release of drugs from liposomes. Panels (a) and (c) show Dox release from Caelyx® in the presence of (a) PLC and HPMA copolymer-PLC conjugate (50 μg/mL PLC-equivalent), and (c) PLA_2 and dextrin-PLA_2 conjugate (50 μg/mL PLA_2-equivalent ± α-amylase). Panels (b) and (d) show Dnm release from DaunoXome® in the presence of (b) PLC and HPMA copolymer-PLC conjugate (50 μg/mL PLC-equivalent), and (d) PLA_2 and dextrin-PLA_2 conjugate (50 μg/mL PLA_2-equivalent ± α-amylase). Liposomal drug in the absence of enzyme is included as a control. Triton X-100 (1% v/v) was used for 100% drug release. Data represents % fluorescence compared to Triton X-100 (1% v/v) solution ± SEM, n = 6. Where error bars are invisible, they are within size of data points.

Figure 5 Measurements of liposome diameter for stability testing of PELT combinations. Panels (a) and (c) show typical size distribution curves of DaunoXome® and Caelyx®, respectively. Panels (b) and (d) show variation over time of (b) DaunoXome® and (d) Caelyx® diameter in the absence and presence of PLA_2 and dextrin-PLA_2 conjugate (50 μg/mL PLA_2-equivalent) ± α-amylase. Data represents diameter (nm) ± SD, n =3. Where error bars are invisible they are within size of data points.

Figure 6 Cell viability of MCF-7 cells incubated for 72 h with (a) DaunoXome® or (b) Caelyx® in the absence and presence of PLA_2 and dextrin-PLA_2 conjugate (50 μg/mL PLA_2-equivalent). Data represents % normal growth of control cells ± SEM, n =18. Where error bars are invisible they are within size of data points. * indicates significance compared to DaunoXome® or Caelyx® control, where $p<0.05$.

Figure 7 Comparisons between observed cell viability (%) and expected cell viability (%) for the combination of (a) DaunoXome® + PLA_2, (b) DaunoXome® + dextrin-PLA_2, (c) Caelyx® + PLA_2, (d) Caelyx® + dextrin-PLA_2 and (e) Caelyx® + unmasked dextrin-PLA_2, tested in MCF-7 cells, according to the multiplicative model (expected cell viability (A+B) = cell viability (A) x cell viability (B)). Dotted line indicates additivity.