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New chemical adaptor unit designed to release a drug from a tumor targeting device by enzymatic triggering

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Abstract—A new controlled drug delivery system for selective chemotherapy was developed. It is based on a chemical adaptor unit, that releases a drug by a spontaneous cyclization mechanism after cleavage of an enzymatic substrate. It also provides a generic linkage of a drug with a targeting device in a manner set to be triggered by defined enzymatic activity. The system is generic and allows using a variety of drugs, targeting devices, and enzymes by introducing the corresponding substrate as a trigger for drug release in the chemical adaptor.

1. Introduction

Lack of selectivity of chemotherapeutic agents is a major problem in cancer therapy and typically is associated with severe side effects. It is obvious that more research is needed to develop more selective and effective anticancer agents. A promising approach to overcome side effects and to achieve a more tumor selective cancer treatment is prodrug therapy. In this concept, a cytotoxic drug is synthetically converted into a nontoxic derivative, teremed prodrug. Upon administration, the prodrug must be selectively activated to regenerate the toxic parent drug at the tumor site.[1] This tumor selective activation should result from properties distinguishing neoplastic from normal cells.[2] We have recently reported on a new concept that combines a tumor targeting device, a prodrug, and a prodrug activation trigger in a single entity.[3] For this, we designed a generic module or chemical adaptor that is based on three chemical functionalities. The first functionality is attached to an active drug and thereby, masks it to yield a prodrug. The second is linked to a targeting moiety, which is responsible for guiding the prodrug to the tumor site, and the third is attached to an enzyme substrate. When the corresponding enzyme cleaves the substrate, it triggers a spontaneous elimination reaction that releases the active drug from the targeting moiety. As a result, prodrug activation will preferentially occur at the tumor site. Here we report on the design, synthesis and proof of concept of a new adaptor unit that utilizes spontaneous cyclization, rather than elimination, to release the active drug.

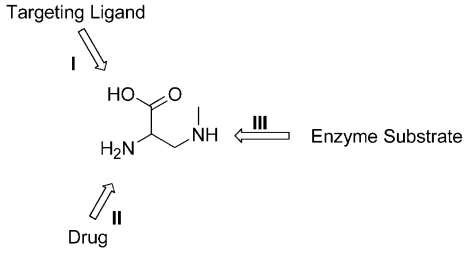

Figure 1. 2-amino-3-methylamino-propionic-acid, the central core of the new chemical adaptor unit.

The central core of our chemical adaptor (Fig. 1) is based on *N*-methyl-diamino-propionic-acid, which has three functional groups suitable for linkage. Group I is a carboxylic acid that is conjugated to a targeting moiety via an amide bond. The drug is linked through the 2-amino group II and the enzyme substrate is attached via

Abbreviations: Boc-*t*-buthyl carbonate, DIPEA; Diisopropyl ethyl amine; DMAP, Dimethyl aminopyridine; DMF, Dimethyl formamide; EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide; EtOAc, Ethyl acetate; Et_3N, Triethyl amine; Fmoc, 9-fluorenylmethoxycarbonyl; He, *n*-Hexane; MeOH, Methanol; NHS, *N*-hydroxy succinimide; PNP, 4-nitrophenyl; THF, Tetrahydrofuran.

Keywords: Prodrug; Self-immolative; Enzyme; Cancer.

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the 3-amino group III by a carbamate bond. Figure 2 illustrates the release mechanism of the drug. Cleaving of the enzyme substrate from complex **1** generates a free amine group (intermediate **2**) that spontaneously cyclizes to form a urea derivative **3** and the active drug.

The design of our generic module allows to potentially link any targeting device to a variety of drugs and to release them with any enzyme by using the corresponding substrate as a trigger. As proof of concept, we designed a pilot system for which we chose *Escherichia coli* Penicillin-G-Amidase (PGA) as the triggering enzyme.[4] As a targeting device we chose the. water-soluble synthetic copolymer; *N*-(2-hydroxypropyl)-methacrylamide (HPMA) which is biocompatible, non-immunogenic and non-toxic.[5] Its in vivo distribution is well characterized[6] and it is known to accumulate selectively at tumor sites due to the enhanced permeability and retention (EPR) effect.[7] Camptothecin (CPT) was selected as the drug in our pilot system.[8] CPT exerts its antitumor activity mainly through inhibition of topoisomerase I. This enzyme, which is found in mammals, binds preferentially to double-stranded DNA, cleaving one strand and forming an enzyme–DNA covalent bond between a tyrosine residue and the 3′ phosphate of the cleaved DNA. Drug-induced accumulation of topoisomerase I-DNA complexes was identified as an essential step, ultimately leading to cell death by apoptosis.[9,10]

The synthesis of the adaptor system was performed straightforward as described in Figure 3. In brief, Nα-Boc-L-2,3-diaminopropionic acid (DAP) was reacted with benzaldehyde and then with sodium borohydride to give secondary amine **4**, which was further reacted with formaldehyde and sodium borohydride to afford tertiary amine **5**. The latter was deprotected by catalytic hydrogenation to generate mono-methylated amine **6** (Fig. 3A). Activated carbonate **7** was then reacted with amine **6** to give compound **8**. The carboxylate of **8** was activated with EDC and NHS and reacted with compound **9** (after removal of the Boc protection group with TFA) to generate amide **10** (Fig. 3B). The Boc protection group was removed from compound **10** to afford free amine, which was further reacted with *p*-nitrophenylcarbonate of camptothecin to give compound **11**. The Fmoc group of **11** was removed with iso-propylamine to generate a free amine, which was mixed with a commercially available form of activated ester of HPMA copolymer to finally afford conjugate **12** (Fig. 3C).

The drug–polymer conjugate was purified by dialysis against water. By measuring its UV spectrum, the number of drug molecules attached to the HPMA-copolymer was determined. Based on the CPT chromophore, which has a λmax at 365 nm, we found that on average three molecules of the drug were linked to one molecule of the HPMA-copolymer. Next we tested whether the CPT drug can be released from conjugate **12** by the catalytic activity of PGA. According to our design, the drug should be spontaneously released after the generation of amine 5 as illustrated in Figure 4. We incubated conjugate **12** with PGA in PBS (pH 7.4) at 37 °C and monitored the appearance of free CPT using an HPLC assay. As Figure 5 shows, CPT was released by the catalytic activity of PGA to form compound **14** and the free drug. No spontaneous CPT release was observed in the absence of the enzyme.

While our previously reported chemical adaptor system[3] utilizes 4-hydroxymandelic-acid as the central core, in this example we have used 2-amino-3-methylamino-propionic-acid. Consequently, the release mechanism of the active drug has changed from elimination to cyclization. Both of these reactions are often used in prodrug chemistry to generate a self-immolative spacer between the drug and the enzymatic substrate. The chemical linkages, which are used to attach the targeting moiety, the drug and the trigger, are relatively stable in physiological conditions, and are constructed from carbamates and amides bonds. Interestingly, another group has already used similar chemical adaptor systems for organic synthesis.[11] An enzyme-labile linker group was attached to a solid support and a third functionality was used for solid-phase synthesis of a target molecule.

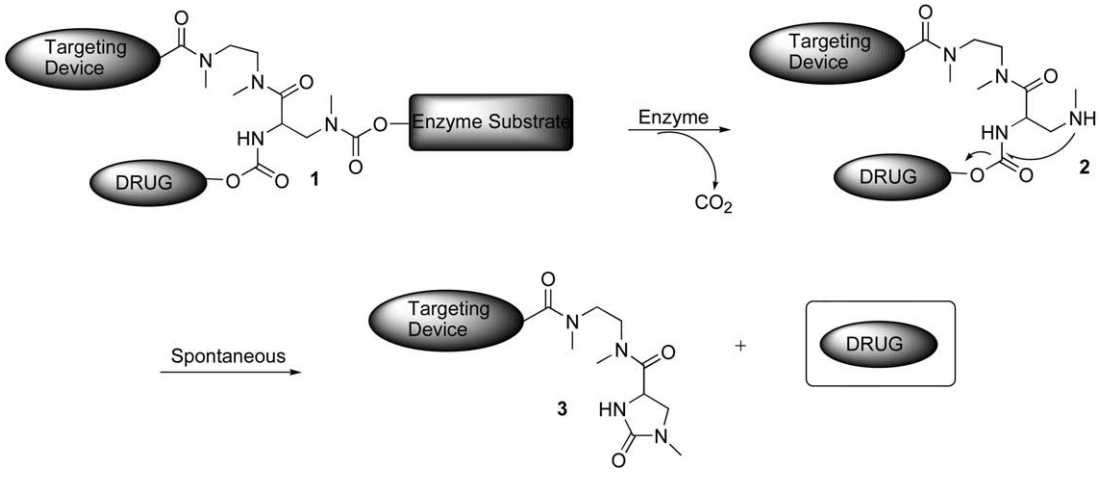

Figure 2. General design of the chemical adaptor system. Cleavage of the enzyme substrate generates an intermediate that spontaneously rearranges to release the drug from the targeting device.

The small molecule was detached from the polymeric support, starting by enzymatic cleavage, leading to generation of an intermediate that spontaneously released the product. The potential of such chemical adaptor systems was very recently highlighted in the literature.[12] The relative large concentration of the enzyme used in this study, could be reduced after optimization of the interaction between the phenylacetamide substrate and the enzyme's binding site. This concept was solved with the use of self-immolative linkers in a study that described in vivo activity in a catalytic antibody-prodrug system.[13]

In conclusion, we have developed a drug delivery system based on a new chemical adaptor unit with the central core 2-amino-3-methylamino-propionic-acid which

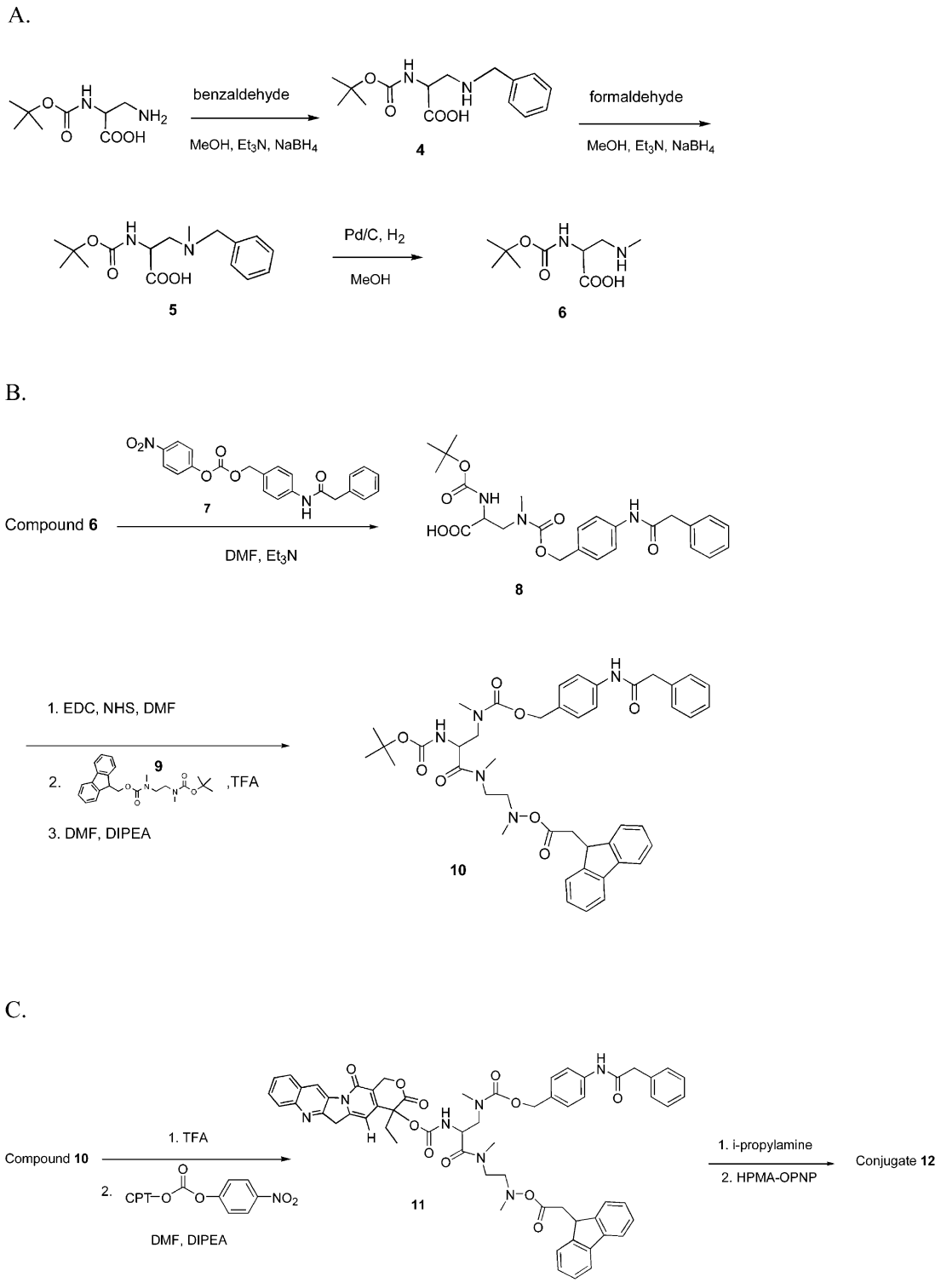

Figure 3. (A–C): Synthesis of a cyclization based chemical adaptor system, using PGA as the activating enzyme, an HPMA copolymer as a targeting device and camptothecin as the drug.

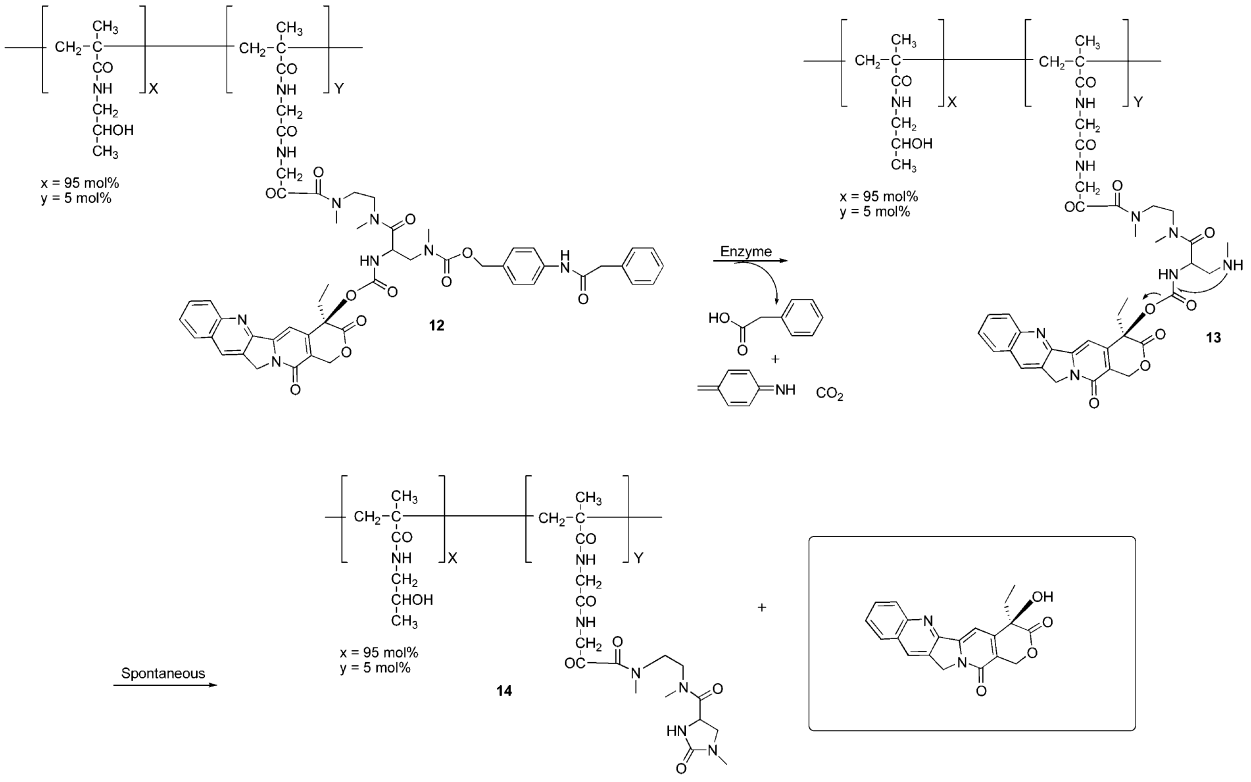

Figure 4. Mechanism of CPT drug release from the HPMA-copolymer, using Penicillin-G-Amidase as the triggering enzyme.

provides a generic linkage of a drug with a targeting device in a manner set to be triggered by defined enzymatic activity. The system is generic and accommodates a variety of drugs, targeting devices, and enzymes by introducing the corresponding substrate as a trigger for drug release in the chemical adaptor. Proof of concept was demonstrated using CPT as the drug, an HPMA-copolymer as the targeting device and PGA as the triggering enzyme. Currently we are synthesizing a variety of chemical adaptor systems using different tumor targeting devices, prodrugs and enzymatic triggers. In vivo studies will be initiated after further optimization of this system.

2. Experimental

2.1. General methods

Thin layer chromatography (TLC): silica gel plates Merck 60 F_{254}: compounds were visualized by irradiation with UV light and/or by treatment with a solution of 25 g phosphomolybdic acid, 10 g $Ce(SO_4)_2 \cdot H_2O$, 60 mL concd H_2SO_4 and 940 mL H_2O followed by heating and/or by staining with a solution of 12 g 2,4-dinitrophenylhydrazine in 60 mL concd H_2SO_4, 80 mL H_2O and 200 mL 95% EtOH followed by heating and/or immersing into an iodine bath (30 g I_2, 2 g KI, in 400 mL EtOH/H_2O (1:1) and warming. Flash chromatography (FC): silica gel Merck 60 (particle size 0.040–0.063 mm), eluent given in parentheses. ^{1}H NMR spectra were measured in $CDCl_3$ using Bruker AMX 200 MHz. The chemical shifts are expressed in δ relative to TMS (δ = ppm) and the coupling constants J in Hz. The spectra were recorded in $CDCl_3$ as solvent at room temperature unless stated otherwise. All general reagents, including salts and solvents, were purchased from Aldrich (Milwaukee, MN). HPMA copolymer-Gly-Gly-ONp (4.4 mole%), (ONp is *p*-nitrophenyl), M.W 30,000D was obtained from Polymer Laboratories (Church Stratton, UK).

Compounds **4–6** were prepared according to procedures described before with some modifications.[14]

2.1.1. Compound 4. To a suspension of the commercially available Nα-Boc-L-2,3-diaminopropionic acid (DAP), (1 g, 4.9 mmol) in MeOH, were added Et_3N (1.48g, 14.7 mmol) and benzaldehyde (1.04 gr, 9.8 mmol) while stirring. After 30 min, the mixture was cooled to 0 °C and $NaBH_4$ (931 mg, 14.7 mmol) was added. After 40 min the solvent was removed under reduced pressure, the mixture was dissolved in NaOH (0.1M) and extracted with ethyl ether. The aqueous phase was acidified with HCl (10% solution) to pH = 6 and extracted with $CHCl_3$.The organic layer was washed with brine, dried over $MgSO_4$ and evaporated. Compound **4** was obtained as a white powder (866 mg, 60%) and was used for the next step without further purification.

^{1}H NMR (200MHz, $CDCl_3$): δ = 7.3 ppm (m, 5H); 4.24-4.13 (brm,1H); 3.75 (1H, bm); 3.5 (1H, m); 3.15 (1H, m); 1.46 (9H, s)

2.1.2. Compound 5. Compound **4** (866 mg, 2.9 mmol) was dissolved in a mixture of Et_3N (1.2 mL, 8.84 mmol) and 10 mL of MeOH. Formaldehyde (35% aqueous solution) (0.76 mL, 8.84 mmol) was added, and the mixture was stirred for 15 min. Then the reaction was

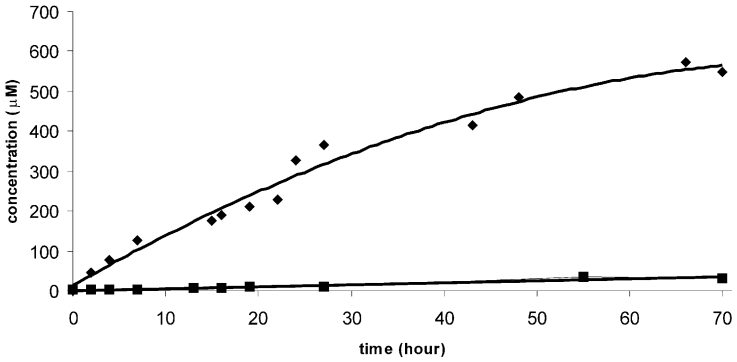

Figure 5. Determination of CPT release from the HPMA-copolymer by PGA versus background reaction in absence of PGA. -◆-?Complex **12** (330 μM) with PGA (4.4 μM in PBS-7.4). -■- Complex **12** (330 μM) in PBS (pH 7.4). Reactions were incubated at 37 °C for the indicated time. The drug release concentration was monitored by HPLC analysis.

cooled to 0 °C, $NaBH_4$ (336 mg, 8.84 mmol) was added and the mixture was stirred for additional 15 min. Similarly, addition of formaldehyde and $NaBH_4$ was repeated twice more. Then the solvent was evaporated, the residue was dissolved in water, acidified to pH = 6 with hydrochloric acid (1M solution) and extracted with $CHCl_3$. The organic layer was washed with brine, dried over $MgSO_4$ and evaporated. Compound **5** was obtained as a white powder (904 mg, 100%) and was used for the next step without further purification.

^{1}H NMR (400MHz,$CDCl_3$): δ = 7.4 ppm (5H, s); 5.75 (1H, s); 4.3 (1H, m); 4.15 (1H, bs); 4.0 (1H, m); 3.4 (1H, m); 3.0 (1H, m); 2.6 (3H, s); 1.5 (9H, s).

2.1.3. Compound 6. A solution of compound **5** (904 mg, 2.9 mmol) in MeOH with catalytic amount of Pd/C was stirred vigorously under H_2 at atmospheric pressure overnight. After filtration and evaporation of the solvent, the residue was precipitated from DCM/He to afford compound **6** (628 mg, 96%). The product was used for the next step without purification.

^{1}H NMR (200 MHz,$CDCl_3$): δ = 4.2 ppm (1H, m); 3.4 (1H, m); 3.2 (1H, m); 2.9 (3H, s); 1.44 (9H, s)

2.1.4. *N*-Fmoc-*N*′-Boc-dimethyl ethylenediamine 9. The mono-Boc-*N*,*N*′-dimethyl ethylene-diamine[3] (500 mg, 2.65 mmol) was dissolved in THF. DIPEA (1.8 mL, 10.6 mmol) was added. The reaction was cooled to 0 °C and 9-fluorenylmethoxy-chloroformate (688 mg, 2.65 mmol) dissolved in THF was added dropwise. The reaction was stirred at room temperature for 15 min and monitored by TLC (EtOAc:He = 1:3). After completion the reaction mixture was diluted with ethylacetate and washed with NH_4Cl. Then the organic layer was washed with brine, dried over $MgSO_4$ and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel (EtOAc: He = 2:3) (360 mg, 55%).

MS (FAB):$C_{24}H_{30}N_2O_4$ $[M+Na]^+$ 433; ^{1}H NMR (200MHz, $CDCl_3$): δ = 7.77 ppm (2H, d, *J* = 7.3); 7.6 (2H, d, *J* = 7.07); 7.4-7.3 (4H, m); 4.59 (1H, d, *J* = 5.3); 4.3 (1H, bs); 4.13 (1H, m); 3.4 (2H, bs); 3.2-2.9 (4H, m); 2.5 (2H, bd); 1.43 (9H, s)

2.1.5. Compound 8. Compound **6** (100 mg, 0.46 mmol) was dissolved in 2 mL DMF and triethylamine (0.193 mL, 1.38 mmol). Compound **7**[3] (149 mg, 0.35 mmol) was added and the mixture was stirred in room temperature for 30 min. The reaction was monitored by TLC (EtOAc:MeOH:AcOH = 9:1:0.1). After completion the DMF was removed under reduced pressure, and the crude product was purified by flash chromatography (ethyl acetate:methanol:acetic acid = 90:9:1) to give pure compound **8** (150 mg, 88%), MS (FAB): $C_{25}H_{31}N_3O_7$ $[M+Na]^+$ 508.1; ^{1}H NMR (200MHz, $CDCl_3$): δ = 7.4–7.2 ppm (9H, m); 5.0 (2H, bs); 3.74 (2H, s); 3.7 (2H,d); 2.98 (3H,s); 1.41 (9H, s).

2.1.6. Compound 10. Compound **8** (150 mg, 0.31 mmol) was dissolved in 2 mL DMF, EDC (72 mg, 0.37 mmol) and NHS (43 mg, 0.37 mmol) were added and the mixture was stirred at room temperature for 1 h and monitored by TLC (EtOAc:MeOH = 9:1) for completion. *N*-Fmoc-*N*′-boc-dimethyl ethylene-diamine **9** (139 mg, 0.34 mmol) was stirred for 5 min in 1 mL TFA, monitored by TLC (EtOAc: He = 1:1) for completion, then TFA was evaporated under reduced pressure. The NHS-ester of **8** was poured into the N-Fmoc-dimethyl ethylene-diamine salt, DIPEA (0.2 mL) was added to the mixture and the reaction was stirred for 1 h. After completion of reaction the solvent was evaporated under reduced pressure, the crude product was purified by column chromatography (EtOAc 100%) to give compound **10** (119 mg, 50%) MS (FAB): $C_{44}H_{51}N_5O_8$ $[M+Na]^+$ 800; ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.8 ppm (2H,m); 7.6 (2H, bs); 7.4–7.3 (9H, m); 5.0 (2H, bs); 4.4 (1H, m); 4.3 (1H, m); 3.74 (2H, s); 3.1–2.87 (10H, m); 1.38 (9H, s).

2.1.7. Compound 11. Compound **10** (55 mg, 0.07 mmol) was cooled to 0 °C and deprotected with 1 mL TFA to remove the Boc group. The excess of the acid was removed under reduced pressure and the residue was dissolved in 2 mL DMF. Camptothecin-PNP-carbonate[3] (39 mg, 0.089 mmol) and 0.030 mL DIPEA were added and the solution was stirred for 1 h. The reaction was monitored by TLC (EtOAc:MeOH = 9:1). After completion the DMF was removed under reduced pressure and the crude product was purified by column chromatography (EtOAc 100%) to give pure compound **11** in the form of white powder (40 mg, 54%).

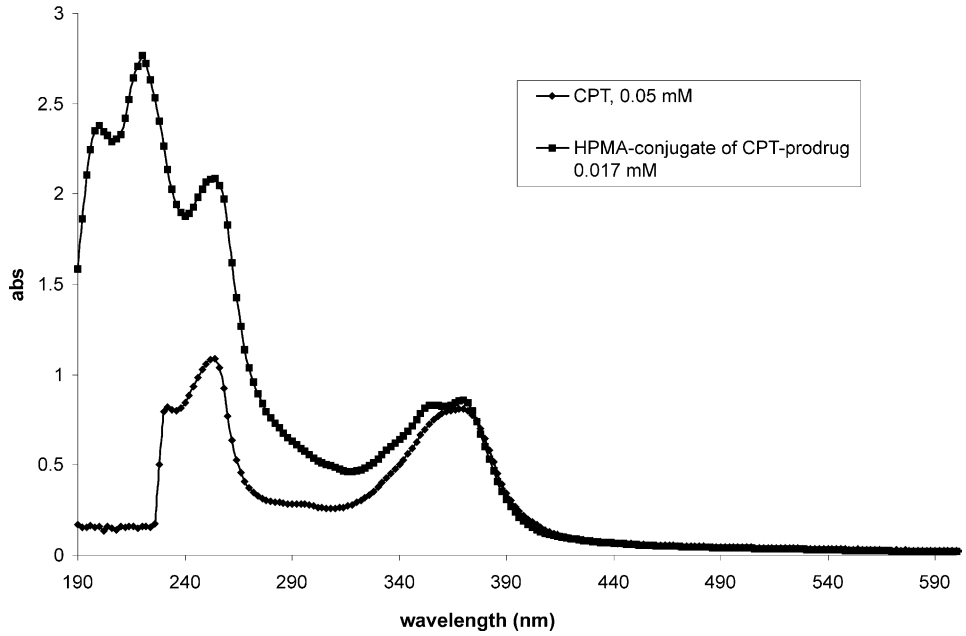

Figure 6. UV spectrum chromatograms of CPT and HPMA conjugate with CPT-prodrug.

HRMS (MALDI): calcd for $C_{54}H_{68}N_4O_{21}$ 1074.4013 $[M+Na]^+$, found 1074.3990.

2.1.8. Conjugate preparation (compound 12). Compound **11** (20 mg, 0.019mmol) was dissolved in DCM (1 mL) iso-propylamine (0.160 mL, 1.9 mmol) was added. The mixture of reaction was stirred for 2 h and monitored by TLC (EtOAc:MeOH = 9:1). After completion of the reaction, the solvent was evaporated, the crude was dissolved in DMF and HPMA co-polymer (25 mg, 0.83 μmol) was added. The mixture of reaction was stirred for 12 h. Then the solvent was removed under reduced pressure. The crude was dissolved in water, centrifuged and dialyzed through MWCO-10,000D membrane against water. The conjugate (21 mg) was obtained after lipholization of the water in the form of pinkish powder.

2.2. UV spectrum measurements

By measuring its UV spectrum, the number of drug molecules attached to the HPMA-copolymer was determined. Based on the camptothecin chromophore, which has a λ_{max} at 360 nm, we concluded that on average three molecules of the drug were linked to one molecule of the HPMA-copolymer. There are approximately ten linkage sites on the HPMA-copolymer. The mild coupling's yield can be probably improved by optimizing the coupling reaction conditions (Fig. 6).

2.3. Drug release analysis

HPMA-drug-conjugate (1 mg, 0.033 μmol) was dissolved in 100 μL solution of PGA (0.35 mg/mL, 4.4 μM) and incubated at 37 °C. The background sample was prepared similarly in PBS-7.4. Drug release was monitored by an HPLC assay using C-18 column, Wavelength; 360 nm, eluent; acetonitrile: water (25:75), flow rate; 1 mL/min.

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