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A macromolecular prodrug of doxorubicin conjugated to a biodegradable cyclotriphosphazene bearing a tetrapeptide

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Abstract—A new biodegradable water-soluble phosphazene trimer–doxorubicin conjugate was synthesized, in which equimolar hydrophilic methoxy-poly(ethylene glycol) with a molecular weight of 350 (MPEG350) and a tumor-specific tetrapeptide (Gly-Phe-Leu-Gly) were grafted to cyclotriphosphazene. The present conjugate exhibited cytotoxicity lower than that of free doxorubicin (IC_{50} = 0.10 μM) but a reasonably higher in vitro cytotoxicity (IC_{50} = 1.1 μM) against the leukemia L1210 cell line probably due to its enzymatically controlled release.

Doxorubicin and its derivatives are among the most important antitumor agents and can be used for the treatment of a variety of cancers, such as breast, ovarian, bladder, and lung.[1] However, the clinical value of doxorubicin is limited due to its serious side effects such as cardiomyopathy, which is related to the total dose of the drug.[2] To minimize the dose-related toxic side effects of the drug, various tumor targeting drug delivery systems, such as liposomes, microspheres, nanoparticles, and polymeric micelles, have been developed.[3] Moreover, it has been known that the effectiveness of doxorubicin can be improved by linking a variety of peptides because tumor-associated proteases have served as promising target enzymes for selectively activated prodrug.[4] The development of prodrug, however, often suffers from their poor solubility.[5]

In this context, the amphiphilic tri- and poly(organophosphazenes) bearing a hydrophilic poly(ethylene glycol) and a hydrophobic amino acid as side groups have been shown by the authors to have thermosensitive and biodegradable properties, which offer great potential as new drug delivery systems.[6] These new phosphazene materials offer advantages as drug carriers over the conventional organic polymers in their structural diversity, nontoxicity, biocompatibility, and biodegradability.

In particular, we have recently found that stepwise nucleophilic substitutions of hexachlorocyclotriphosphazene with a hydrophilic methoxy poly(ethylene glycol) with an average molecular weight of 350 (MPEG350) and a hydrophobic tetrapeptide ethyl ester, Gly · Phe · Leu · GlyOEt, resulted in an amphiphilic phosphazene trimer with a lower critical solution temperature (LCST) at 35 °C. This trimer not only can be easily purified to a monodisperse compound using the LCST but also could be functionalized by hydrolysis of the tetrapeptide to conjugate doxorubicin (for structure, see Scheme 1). Usefulness of this conjugate is expected for several reasons: biodegradability of the phosphazene backbone as well as the peptide bond, possible selective delivery of the antitumor doxorubicin due to the tumor-specific degradation of the tetrapeptide spacer, good water solubility, and finally high loading capacity of the drug (>40%). In this paper, we describe the synthesis and cytotoxicity of phosphazene-trimer-based prodrugs containing a lysosomally cleavable tetrapeptide, Gly · Phe · Leu · Gly[7], and doxorubicin.

The cyclotriphosphazene–doxorubicin conjugate was synthesized according to the procedure depicted in Scheme 1. First, the trimeric phosphazene carrier **4** was prepared as follows. The sodium salt of MPEG350 was prepared by the reaction of MPEG350 (3.62 g,

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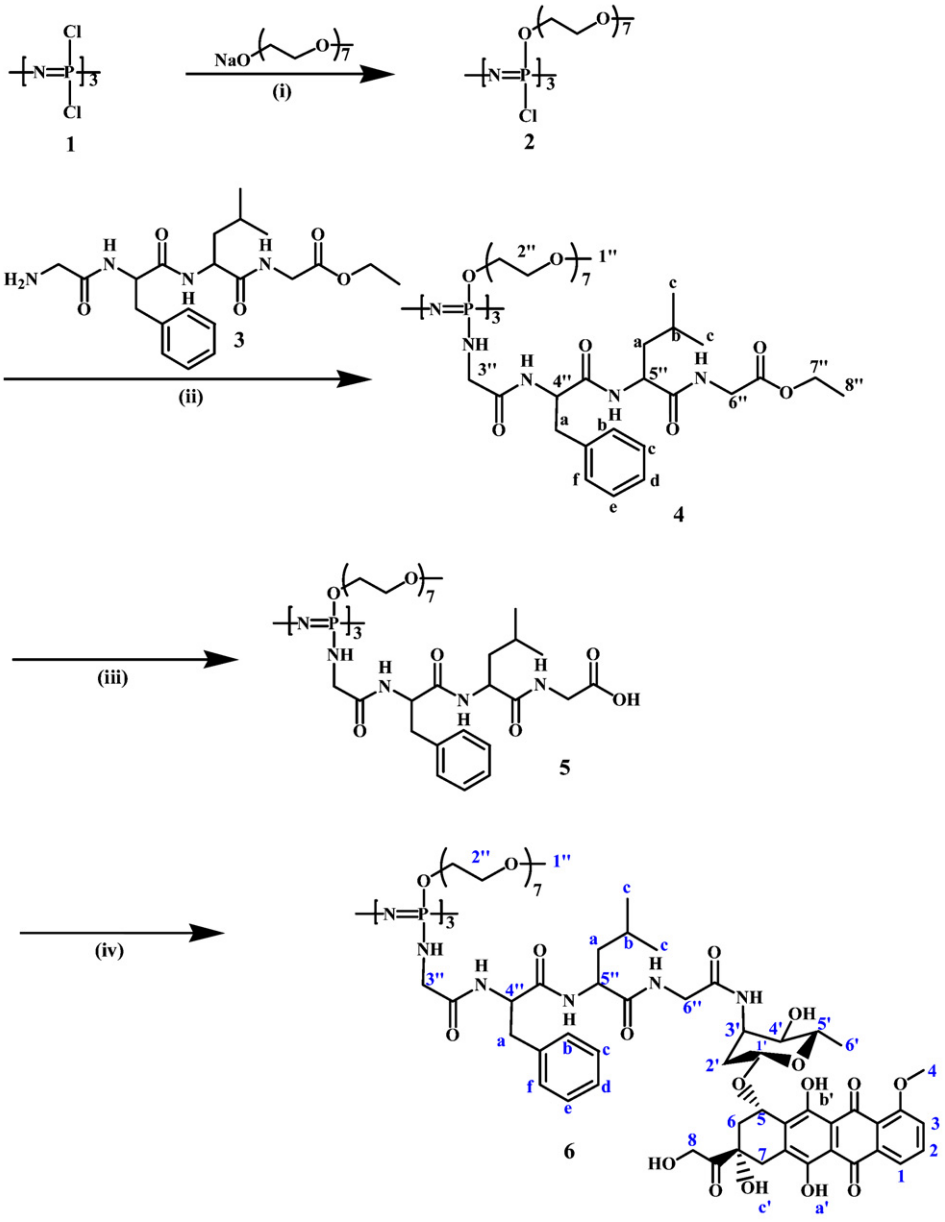

Scheme 1. Synthetic route to the phosphazene trimer-doxorubicin conjugate. Reagents and conditions: (i) −65 °C, 8 h; (ii) NEt_3, 50 °C, 2 days; (iii) NaOH/MeOH, 4 h; (iv) DCC, NHS in THF, rt, 4 h; NEt_3, rt, 12 h.

10.3 mmol) with 1.5 equiv of sodium metal in THF at reflux for 2 days. After the resultant solution was filtered to remove excess sodium metal, the filtrate was dropped slowly to the solution of **1** (1.0 g, 2.88 mmol) dissolved in THF. The reaction mixture was stirred for 8 h at −65 °C to afford *cis*-nongeminally PEGylated trimer **2**.[8] Meanwhile, the tetrapeptide ethyl ester, **3** (5.44 g, 12.94 mmol) synthesized by the known procedure,[9] was dissolved in dry chloroform containing 3 equiv of dry triethylamine. After the solution of **3** was transferred to the trimer solution of **2**, the reaction mixture was stirred at 50 °C for 2 days. It was filtered to remove the resultant salts, and the filtrate was concentrated and reprecipitated twice by a solvent pair of ethyl acetate and *n*-hexane. For further purification, the product dissolved in ultrapure water was subjected to dialysis for 24 h using cellulose membranes (molecular weight cut-off: 1.0×10^3) and then freeze-dried to obtain the drug carrier $[NP(MPEG350)(Gly \cdot Phe \cdot Leu \cdot GlyOEt)]_3$ **(4)**. This compound exhibited a lower critical solution temperature (LCST) at 35 °C, which allowed further purification by LCST.[10] The aqueous solution of **4** (5–10% w/v) in a test tube was heated up to 35–40 °C and the resultant precipitate was separated by centrifugation for 20 min at 4000 rpm to obtain the trimeric carrier derivative[11] **(4)** (yield: 74%) with high purity, which is clearly seen in the ^{31}P NMR spectra before and after purification of **4** in Figure 1.

The final phosphazene trimer–doxorubicin conjugate **6** was synthesized as follows. Sodium hydroxide (0.07 g, 1.87 mmol) was added to the methanolic solution of **4** (1.0 g, 0.41 mmol) and the reaction mixture was stirred for 4 h. The solution was acidified to a pH of 3 and

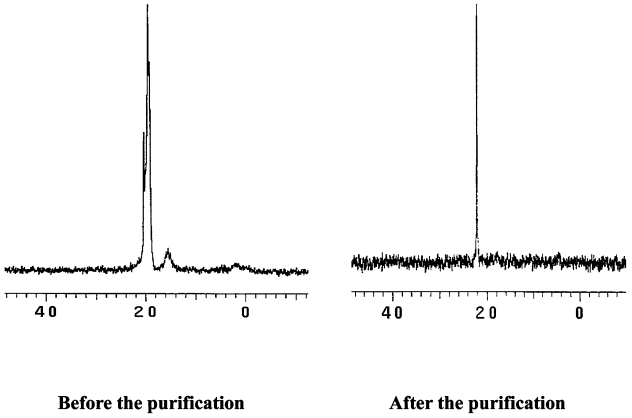

Figure 1. ^{31}P NMR spectra of [NP(MPEG350)(Gly · Phe · Leu · Gly-OEt)]$_3$ before and after purification by using LCST properties.

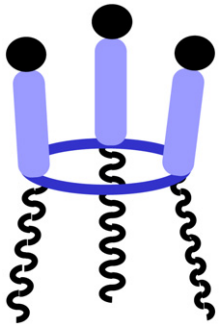

Figure 2. The *cis*-nongeminal structure of [NP(MPEG350)(Gly · Phe · Leu · GlyO-Doxo)]$_3$. Three molecules each of poly(ethylene glycol) (spring) and the tetrapeptide spacer (stick) holding doxorubicin (ball) are oriented in the same direction with respect to the cyclic phosphazene ring (ring).

concentrated. An excess of the solvent mixture of ether and *n*-hexane was added to precipitate the hydrolyzed product, and the precipitate was filtered and vacuum-dried to afford the acid form [NP(MPEG350)(Gly · Phe · Leu · GlyOH)]$_3$ **(5)** (yield: 68%). *N*,*N*′-Dicyclohexylcarbodiimide (0.03 g, 0.13 mmol) and *N*-hydroxysuccinimide (0.02 g, 0.14 mmol), dissolved in THF, were added to a solution of **5** (0.1 g, 0.04 mmol) dissolved in THF, and the reaction mixture was stirred for 4 h at room temperature. Doxorubicin hydrochloride (0.09 g, 0.15 mmol) and 3 equiv of triethylamine were added to the reaction mixture and stirred further for 12 h at room temperature. The reaction mixture was filtered, concentrated, and dialyzed for 24 h against ultrapure water using cellulose dialysis membranes (molecular weight cutoff: 2.0×10^3). The dialyzed solution was freeze-dried and purified by column chromatography (Silica gel, eluent: CH_3OH/CH_2Cl_2(1/4)) to obtain the final phosphazene trimer-doxorubicin conjugate[12] **(6)** (R_f = 0.8, yield: 48%).

The present conjugate was very soluble in water, as well as in most polar organic solvents and was fully characterized by means of multinuclear (^{1}H, ^{31}P) NMR spectroscopies, UV–vis spectroscopy, and elemental analysis. In particular, the cis-nongeminal structure of the conjugate molecule shown in Figure 2 was confirmed from the ^{1}H and ^{31}P NMR spectral data of **6** in acetone-d_6.[12] All the ^{31}P NMR spectra of the intermediate carriers **4** and **5**, and the final conjugate **6** showed a sharp singlet at 22.0, 21.6, and 22.0 ppm, respectively. The UV–vis spectrum of the conjugate drug **6** showed almost the same spectral pattern as free doxorubicin. Therefore, by using its molar absorptivity (ε_{max} = 10,800) at 484 nm, the total amount of doxorubicin conjugated to the trimer could be quantitatively measured, which was completely in accordance with the elemental analysis data. It is also interesting to note that the intermediate carriers **4** and **5** exhibited a LCST at 35 and 55 °C, respectively, but the conjugate **6** did not show a LCST probably due to reduced hydrophobicity of the tetrapeptide side group conjugated to hydrophilic doxorubicin.

The antitumor activity of **6** was assayed in vitro against the leukemia L1210 cell line according to a previous method,[13] and the result is given in Table 1. The present conjugate drug **6** showed an in vitro cytotoxicity (ED_{50} = 1.1 μM) lower than that of free doxorubicin, but activity higher than that of cisplatin. The in vitro activity of the conjugate lower than that of the free drug is probably due to slow release of the drug from the carrier as expected. To test the enhanced tumor-specific biodegradability, the release of free doxorubicin moiety from the tetrapeptide–doxorubicin conjugate in the medium containing plasmin was observed by the HPLC analysis.[4a,14] Incubation of the 4 μM conjugate in the PBS solution with the optimized plasmin concentration of 45 μg/ml for 48 h at 36 °C resulted in the release of 58% free doxorubicin. Since the overexpression of a number of such peptidase enzymes in tumor tissue has been known, the present conjugate containing protease sensitive peptide spacer is expected to exhibit its original anticancer activity with enhanced selectivity toward tumor tissue and now planned to enter comprehensive in vivo tests.

In conclusion, a new thermosensitive biodegradable phosphazene trimer bearing MPEG350 and Gly-Phe-Leu-GlyOEt as side groups has been prepared as a carrier, to which doxorubicin was conjugated through the tetrapeptide spacer. The present conjugate exhibited a cytotoxicity lower than that of free doxorubicin (IC_{50} = 0,10 μM) but reasonably high in vitro cytotoxicity (IC_{50} = 1.1 μM) against the leukemia L1210 cell line probably due to its enzymatic controlled release. Incubation of the tetrapeptide–doxorubicin conjugate with plasmin showed controlled enzymatic release (58%, for 48 h) of the drug.

Table 1. Cytotoxicity of cyclotriphosphazene–doxorubicin conjugate against the leukemia L1210 cell line

Compounds	Cytotoxicity (ED_{50}, μm)
Conjugate **6**	1.1
Doxorubicin	0.10
Cisplatin	1.0

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11. ^{31}P NMR [D_2O, δ]: 22.0. ^{1}H NMR (D_2O, δ): 0.68 (d, 3H, H5″^{c}); 0.87 (d, 3H, H5″^{c}); 1.12 (t, 3H, H8″); 1.2–1.3 (m, 3H, H5″^{a}, H5″^{b}); 2.95 (dd, 2H, H4″^{a}); 3.2 (s, 3H, H1″); 3.25-3.6 (br, 30H, H2″); 3.65 (d, 2H, H6″); 3.83 (d, 2H, H3″); 3.82 (m, 2H, H7″); 4.1 (dd, 1H, H5″); 4.42 (t, 1H, H4″); 7.14 (m, 5H, H4″^{b}, H4″^{c}, H4″^{d}, H4″^{e}, H4″^{f}). LCST: 35 °C..
12. $R_f = 0.8$ (silica gel CH_3OH: CH_2Cl_2 1:4). Yield: 48%. Elem. Anal. (%) Calcd for C, 56.26; H, 6.61; N,6.46. Found: C, 56.13; H, 6.51; N, 6.43. ^{31}P NMR (D_2O, δ): 22.1. ^{1}H NMR (acetone-d_6): 0.68 (d, 3H, H5″^{c}); 0.87 (d, 3H, H5″^{c}); 1.31 (d, 3H, H6′); 1.40 (m, 1H, H5″^{b}); 1.44 (m, 1H, H2′); 1.60 (m, 1H, H2′); 1.67 (m, 2H, H5″^{a}); 2.03 (br, 2H, H7); 2.30 (dd, 1H, H6); 2.75 (dd, 1H, H6); 3.10 (d, 2H, H4″^{a}); 3.30 (s, 3H, H1″); 3.34 (d, 1H, H4′); 3.60 (br, 28H, H2″); 3.95 (m, 1H, H3′); 3.98 (br, 4H, H3″, H6″); 4.06 (br, 3H, H4); 4.17 (d, 1H, H5′); 4.80 (br, 1H, H5″); 4.80 (br, 1H, H4″); 4.80 (br, 1H, H5); 5.10 (s, 1H, Hc′); 5.42 (t, 1H, H1′); 5.45 (dd, 2H, H8); 7.23 (m, 5H, H4″^{b}, H4″^{c}, H4″^{d}, H4″^{e}, H4″^{f}); 7.62 (d, 1H, H1); 7.86 (m, 2H, H2, H3, doxo-arom); 13.2 (s, 1H, Hb′, doxo-OH); 14.0 (s, 1H, Ha′, doxo-OH). UV–vis (λ, ε in H_2O): 498, 10,698 L mol^{-1} cm^{-1}.
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