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A micellar prodrug of paclitaxel conjugated to cyclotriphosphazene

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ABSTRACT

A novel water soluble and biodegradable cyclotriphosphazene–paclitaxel conjugate was prepared by reacting 2′-succinyl paclitaxel with cyclotriphosphazenes bearing equimolar glycyl-L-lysine and methoxy poly(ethylene glycol) as side groups. The macromolecular conjugate was found to self-assemble in aqueous solution to form stable micelles with a mean hydrodynamic diameter of 24.7 nm and a low critical micelle concentration of 10 mg/L. The present conjugate exhibited lower than free paclitaxel but reasonably high in vitro cytotoxicity against selected human tumor cells due to their hydrolytic degradation in PBS solution.

Paclitaxel is one of the most important antitumor agents currently in clinical use, since it exhibits effective antitumor activity against various cancers such as breast, ovarian, and non-small cell lung cancers.[1,2] However, its clinical applications are limited due to its extremely low water solubility (<1 μg/ml)[3] and serious side effects including hypersensitivity and neurotoxicity attributed to its formulating agent, Cremophore EL.[4,5] Therefore, a great deal of efforts has been made to overcome such problems. Intensive studies have been made to replace Cremophore EL for solubilization of paclitaxel using a variety of different carrier systems including polymeric micelles composed of a hydrophilic corona and a hydrophobic core affording to solubilize paclitaxel and controlled-release of the drug from the micelles.[6–14]

There are also numerous studies on the prodrug synthesis of paclitaxel to increase its water solubility through its conjugation to various solubilizing agents including hydrophilic polymers such as poly(ethylene glycol),[15–19] poly(*N*-vinylpyrrolidone) (PVP),[20–22] poly(L-glutamic acid),[23] carboxymethyl dextran,[24] and albumin.[25] Paclitaxel is usually conjugated to the hydrophilic polymers through its 2′-hydroxy group using bifunctional carboxylic acids such as succinic anhydride, since 2′-acyl-paclitaxel derivatives are well known to be hydrolyzed in the blood system.[19]

Recently, we have reported a new type of thermoresponsive micelles self-assembled from amphiphilic cyclotriphosphazenes grafted with equimolar hydrophilic poly(ethylene glycol) and hydrophobic oligopeptide.[26] We have found that these cyclic phosphazene trimers are useful for solubilization of paclitaxel by either physical micellar encapsulation or chemical conjugation to the oligopeptide side group of the trimer. In this paper we report synthesis, characterization and properties of a novel micellar paclitaxel–cyclotriphosphazene conjugate.

The cyclotriphosphazene drug carrier bearing a hydrophilic poly(ethylene glycol) (PEG) and a hydrophobic oligopeptide for conjugation with paclitaxel was prepared by the same procedure as described in our previous work.[26] However, the oligopeptide employed as a spacer group for conjugation with paclitaxel was modified to α-*N*-glycyl-L-lysine with the terminal ε-amine group blocked by benzyloxycarbonyl group (Cbz), which was unprotected for amide coupling reaction with succinyl paclitaxel in the final step. The whole synthetic scheme for the preparation of the cyclotriphosphazene–paclitaxel conjugate is shown in Scheme 1.

The sodium salt of methoxy poly(ethylene glycol) with a molecular weight of 350 (MPEG350) prepared by reaction of MPEG350 (3.32 g, 9.50 mmol) with sodium hydride (0.24 g, 9.98 mmol) in dry tetrahydrofuran (THF) (150 ml) was slowly added under argon atmosphere to a solution of hexachlorocyclotriphosphazene (I) (1.0 g, 2.88 mmol) in dry THF (80 ml) at −65 °C. After stirred reaction for 3 h, the reaction mixture was warmed up and further stirred for 8 h at room temperature to obtain the PEGylated intermediate (II). A dipeptide, glycyl-*N*-(benzyloxycarbonyl)-L-lysine methyl ester (Gly(Cbz)LysMet) (3.57 g, 11.5 mmol) neutralized with triethylamine (3.58 ml, 25.8 mmol) in dry chloroform (100 ml) was added to the reaction solution (II), which was further stirred for 24 h at room temperature. The reaction mixture was filtered to remove precipitated byproducts. After the filtrate was

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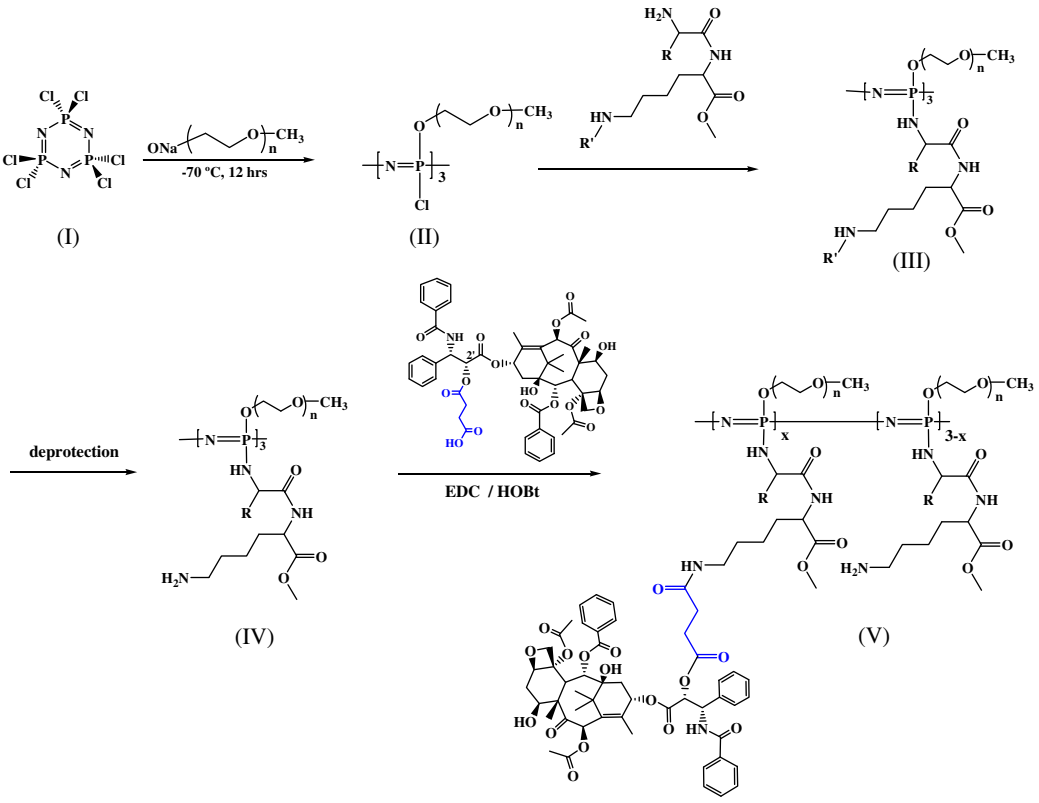

Scheme 1. Synthetic route to the cyclotriphosphazene–paclitaxel conjugate.

evaporated, the concentrate was dissolved in water (30 ml) and dialyzed for 1 day in distilled water using cellulose membrane (MWCO: 1000). The dialyzed solution was freeze-dried to obtain the trimeric derivative (III), which was dissolved in methanol (100 ml) for hydrogenation in the presence of palladium charcoal and 1,3-cyclohexadiene to obtain the unprotected trimeric carrier (IV), $[NP(PEG350)(GlyLysMet)]_3$. In the meantime, a solution of 2′-succinyl paclitaxel (270 mg, 0.29 mmol) prepared according to the literature procedure[27] and triethylamine (0.09 ml, 0.67 mmol) in dry methylene chloride was pretreated successively with amide coupling agents, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) (60 mg, 0.32 mmol) and 1-hydroxy benzotriazole hydrate (HOBt) (40 mg, 0.32 mmol), and then slowly added to the solution of the above trimeric carrier (IV) (500 mg, 0.29 mmol) cooled in dry ice–acetone bath. The reaction mixture was reacted for 6 h and then further stirred for 48 h at room temperature. The reaction solution was subjected to vacuum evaporation, and the resultant product was dissolved in distilled water (20 ml) for dialysis using cellulose membrane (MWCO: 1000) for 24 h. The dialyzed solution was freeze-dried to obtain the final paclitaxel–cyclotriphosphazene conjugate (V), $[N_3P_3(PEG350)_3(GlyLysMet\text{-}2'\text{-}succinylpaclitaxel)(GlyLysMet)_2]$ in 27.0% yield. Also two and three moles of paclitaxel could be reacted per mole of the trimeric carrier (IV), but the resultant products were insoluble in water and will not be discussed in this paper.

The conjugate drug (V) is very soluble in water and polar organic solvents such as alcohols and dimethylsulfoxide and fully characterized by means of multinuclear (^1H, ^{31}P) NMR spectroscopies and elemental analysis[28] along with measurements of its dynamic light scattering (DLS) and critical micelle concentration (CMC). The ^{31}P NMR spectra of the free trimeric carrier (IV) (a) and its paclitaxel conjugate (V) illustrated in Figure 1 show that the phosphorus resonance is shifted by approximately 1 ppm to downfield by paclitaxel conjugation, but cis-non-geminal conformation of both side groups is retained even after unsymmetrical conjugation of only one paclitaxel molecule.

However, it was surprising to note that all the proton resonances both of the hydrophilic methoxy poly(ethylene glycol) (MPEG) and the hydrophobic dipeptide groups of the paclitaxel conjugate appeared with good resolution in dimethylsulfoxide-d_6, but in D_2O most proton resonances of the dipeptide group disappeared or broadened while the MPEG protons remained with almost the same intensity as shown in Figure 2. Such an observation strongly indicates that the conjugate itself forms micelles by self-assembly with orientation of the dipeptide groups including the paclitaxel molecule into the core and the MPEG groups on the outer shell of the micelles.

Therefore, we have performed DLS measurements using Malvern Zetasizer (Nano ZS) for the conjugate in aqueous solution (0.5%) and the resultant particle size distribution of the conjugate with a mean diameter of 24.7 nm was shown in Figure 3. The non-conjugated trimeric carrier (IV) did not form micelles in aqueous solution probably because of low hydrophobicity of the dipeptide group alone, but conjugation with highly hydrophobic paclitaxel seems to drive micelle formation. There are many reports on paclitaxel formulation using polymeric micelles, but to our knowledge there are rare reports on the paclitaxel-conjugated prodrug that forms micelles by self-assembly in aqueous solution. The stability of micelles in aqueous solution is a critical factor for injectable delivery, and therefore, the critical micelle concentration (CMC) of the conjugate was measured by the surface tension method using Tensiometer K100 (Krüss, Germany). The CMC of the conjugate was very low (10 mg/L) as shown in Figure 4, and the particle size distribution remained unchanged even after a thousand times dilution of 0.5% solution of the conjugate.

Another important factor to be considered for conjugate prodrugs is the drug releasing profile. Since conjugate prodrugs contain covalent bonding between the drug and polymeric carrier

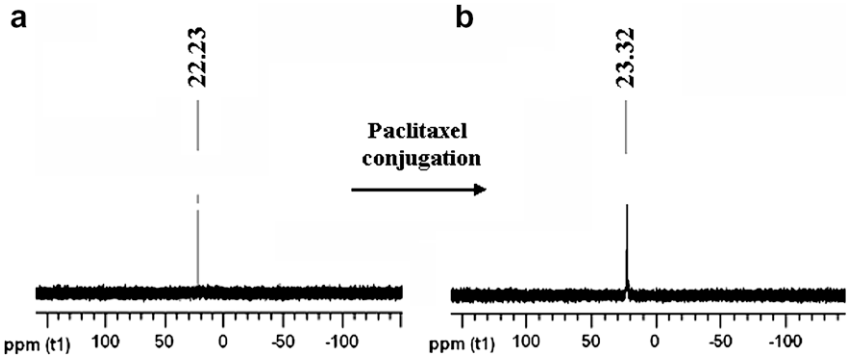

Figure 1. ^{31}P spectra of cyclotriphosphazene (a) and its paclitaxel conjugate (b).

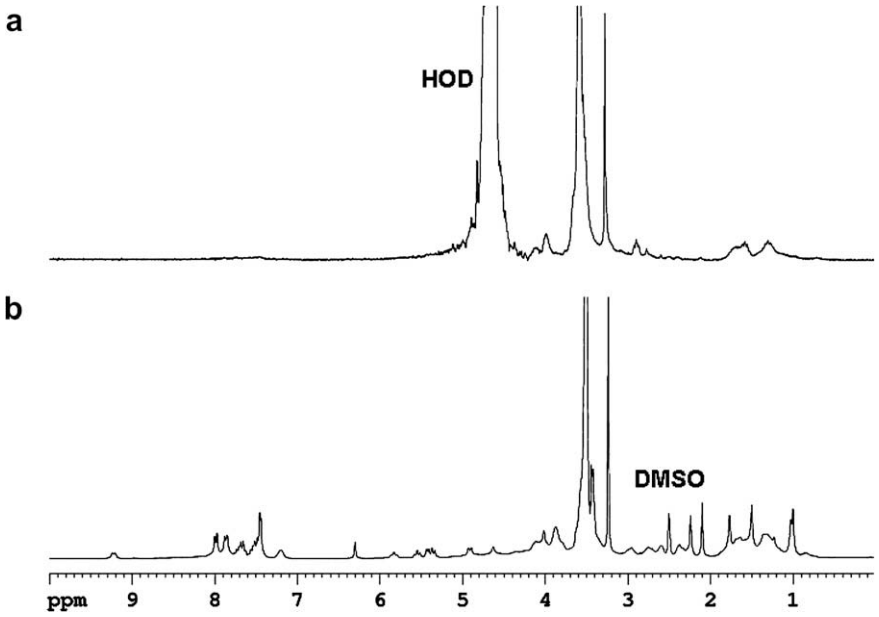

Figure 2. ^{1}H NMR spectra of the cyclotriphosphazene–paclitaxel conjugate in D_2O (a) and DMSO (b).

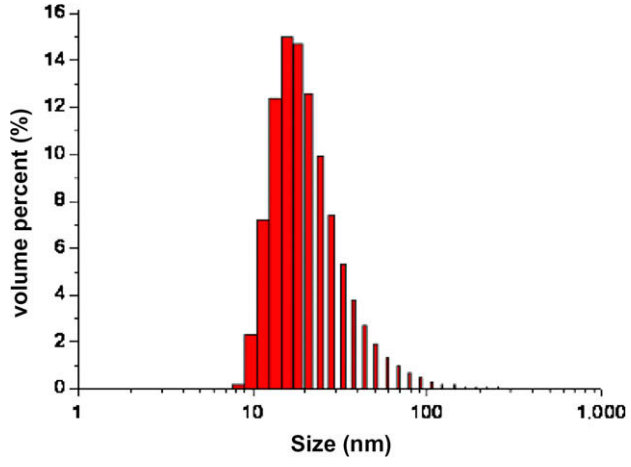

Figure 3. The size distribution of the cyclotriphosphazene–paclitaxel conjugate (mean diameter = 24.7 nm).

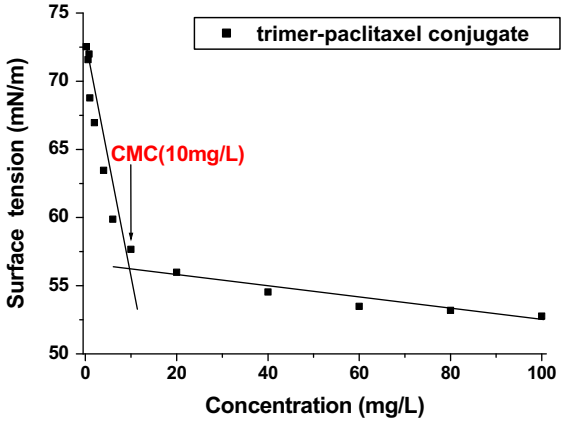

Figure 4. The critical micelle concentration of the cyclotriphosphazene–paclitaxel conjugate measured using the surface tension method.

molecules they should be enzymatically and/or hydrolytically degradable to release the drug molecules in vivo. The present conjugate drug was designed to be at least hydrolytically degradable as above-mentioned.[19] In other words, as shown in Scheme 1, paclitaxel was bonded to one of the two carboxylate ions of succinic acid as an ester group, which is susceptible to hydrolysis in aqueous media. It is well known that hydrolysis of organic acid esters is more susceptible to hydrolysis in ionic solutions due to the anionic nucleophiles to attack the carbonyl group of the acid group than in

pure water. Therefore, we have performed an experiment to examine the releasing profile of paclitaxel from the conjugate drug in buffer solutions of different pHs at 37 °C, and the results were illustrated in Figure 5. It is interesting to observe that the overall releasing rate of paclitaxel in buffer solutions is fairly high, and the conjugate showed the highest degradation rate with more than 50% degradation after 72 h incubation in physiological condition. However, the conjugate drug was not degraded significantly in pure water (data not shown). Such a releasing profile of paclitaxel in the neutral buffer solution is consistent with the results of the following in vitro cytotoxicity test for the conjugate performed in the same buffer solution.

In order to estimate the antitumor activity of the conjugate we have assayed its in vitro cytotoxicity against selected human tumor cell lines of MCF-7 (breast adenocarcinoma), SK-OV3 (ovarian adenocarcinoma), A-431 (vulva squamous carcinoma) and MDA-MB-231 (breast adenocarcinoma) according to our previous modified Sulfur Rhodamine B (SRB) method.[29] The IC_{50} values of the trimer–paclitaxel conjugate measured after 72 h incubation both in the neutral buffer solution and in pure water are listed along with those of free paclitaxel in Table 1. The precursor phosphazene carrier, $[NP(PEG350)(GlyLysMe)]_3$, subjected to cytotoxicity assay against the same cell lines exhibited too low cytotoxicities (IC_{50} > 100 μM), which were not listed in the table. The conjugate exhibited very low cytotoxicity compared with free paclitaxel in pure water but in the buffer solution reasonably high cytotoxicity expectable from its degradability in the same solution as above-mentioned. In addition, the conjugate is expected to release paclitaxel also by lysosomal enzymes in vivo because a dipeptide was employed as a spacer group.[30] Furthermore, the present conjugate forms micelles with an outer shell composed of hydrophilic poly(ethylene glycol), which is known to afford stealthy properties of the micelles,[7,31] and therefore, comprehensive in vivo studies are planned.

In summary, a novel biodegradable cyclotriphosphazene–paclitaxel conjugate was designed and prepared by amide coupling reaction between 2′-succinyl paclitaxel with the terminal ε-amine group of one of the dipeptide lysine side chains of cyclotriphosphazene. The macromolecular conjugate was found to be water soluble and to form stable micelles by self-assembly with a mean hydrodynamic diameter of 24.7 nm and a low critical micelle concentration of 10 mg/L. The present conjugates exhibited lower than free paclitaxel but reasonably high in vitro cytotoxicity against various human tumor cell lines due to their hydrolytic degradation in PBS solution.

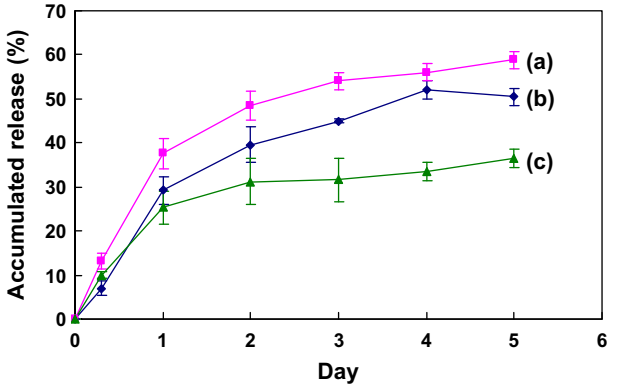

Figure 5. Releasing profiles of paclitaxel from the cyclotriphosphazene–paclitaxel conjugate in buffer solutions of pH of 7.4 (a), 5.4 (b), and 9.4 (c) at 37 °C.

Table 1
In vitro cytotoxicity of the paclitaxel conjugate against selected human tumor cell lines.

Compounds	IC_{50} values (nM) (mean ± SD, n = 3–4)			
	MCF-7	SK-OV3	A-431	MDA-MB-231
Free paclitaxel	39.0 ± 4.9	65.8 ± 30.5	25.1 ± 8.9	47.1 ± 12.2
Trimer–paclitaxel conjugate (water)	447.4 ± 138.2	503.4 ± 114.3	327.2 ± 54.5	379.9 ± 89.2
Trimer–paclitaxel conjugate (PBS)	128.6 ± 37.5	137.5 ± 14.3	53.8 ± 13,9	107.5 ± 10.9

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28. ^{31}P NMR($CDCl_3$, δ): 22.5. ^1H NMR($CDCl_3$, δ): 1.12 (s, 3H, C17-H), 1.26 (t, 3H, C16-H), 1.36 (br, 12H, LysMe γ-CH_2, δ-CH_2), 1.67 (s, 3H, C19-H), 1.79 (br, 6H, LysMe β-CH_2), 1.88 (br, 3H, C18-H), 2.25 (s, 3H, C10-OAc), 2.41 (s, 3H, C4-OAc), 2.49 (br, 2H, succinyl CH_2), 2.74 (br, 2H, succinyl CH_2), 3.05 (br, 6H, LysMe ε-CH_2), 3.39 (s, 9H, MPEG350 OCH_3), 3.66 (br, 72H, MPEG350 OCH_2CH_2), 4.02 (d, 2H, Gly CH_2), 3.79, (d, 1H, C3-H), 3.95 (br, 12H, MPEG350 OCH_2CH_2), 4.13 (m, 2H, C20-H), 4.27 (d, 1H, C7-H), 4.47 (br, 3H, LysMe α-CH), 4.98 (d, 1H, C5-H), 5.43 (s, 1H, C2′-H), 5.66 (d, 1H, C2-H), 5.90 (m, 1H, C3′-H), 6.17 (m, 1H C13-H), 6.29 (s, 1H, C10-H), 7.39 (m, 3′Ph), 7.46 (br, 3′-NBz), 7.50 (m, 2-OBz), 7.80 (d, 3′-OBZ), 8.12 (d, 2-OBz). Elem. Anal. (%) ($C_{123}H_{198}N_{13}O_{50}P_3$). Calcd: C, 54.35; H, 7.17; N, 6.54. Found: C, 54.63; H, 7.17; N, 6.34.
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