Synthesis and Antitumor Activity of Poly(organophosphazene)/ Doxorubicin Conjugates

Soo-Chang Song,* Chong Ok Lee,† and Youn Soo Sohn

Inorganic Chemistry Laboratory, Korea Institute of Science and Technology, Seoul 130-650, Korea
†Korea Research Institute of Chemical Technology, Daejon, Korea
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Doxorubicin shows a wide spectrum of activity for solid tumors such as sarcomas, adenocarcinomas and melanomas, but its strong side effects including myelosuppression, extravasation reaction and cardiotoxicity have been a serious problem.[1] Polymer-anticancer drug conjugates have been studied in order to improve the side effects of the anticancer drugs. In comparison with low-molecular weight anticancer drugs, polymer-anticancer drug conjugates have been found to be accumulated more in tumor tissues than in normal tissues, which is called enhanced permeability and retention (EPR) effect.[2-5] In addition, polymer-anticancer drug conjugates can afford to prolong the antitumor activity by releasing the drug with a controlled rate. Several polymer-doxorubicin conjugates have been studied, and among them, N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer/doxorubicin conjugate is currently under clinical study,[6,7] but there is no report on polyphosphazene/doxorubicin conjugate.

In the previous studies, we have found that poly(organophosphazene)/(diamine)platinum conjugates exhibited high antitumor activity due to controlled release of the platinum moiety from the conjugates.[8,9] We report here the synthesis and antitumor activity of poly(organophosphazene)/doxorubicin conjugates.

Experimental

ε-Caprolacton and 2,2-aminoethoxy ethanol purchased from Aldrich (Milwaukee, USA) were used without further purification. Hexachlorocyclotriphosphazene, D-lactose, and L-glutamic acid purchased from Sigma (St. Louis, USA) were also used as received. The low molecular weight (Mw ≅ 10^4) poly(dichlorophosphazene), 5-Carboxymethyl-1-pentanol (CPA) and [N-(aminobutyl)-O-β-D-galactopyranosyl-(1→4)-D-gluconamide] (AGA) were prepared by the author's procedure.[9,10] L-glutamoyl dibenzyl ester p-toluenesulfonate was used as a spacer for introducing doxorubicin and prepared by the literature method.[11]

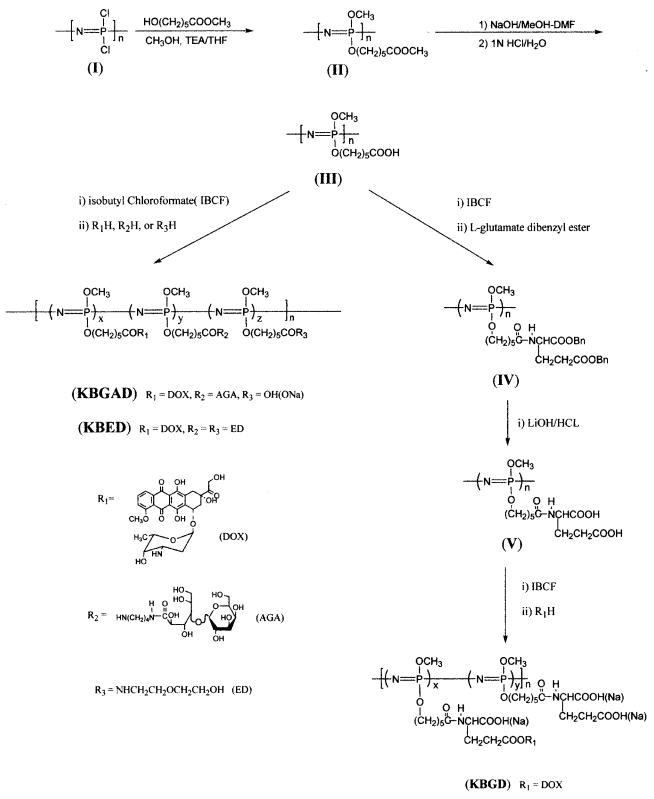
Elemental analysis was carried out with Fisons 1108 CHNS Microanalizer. ^{1}H-NMR measurements were made with a Varian Gemini-300 spectrometer operating at 300 MHz in the Fourier transform mode. Gel-permeation chromatograpy was carried out using a Waters Associates HPLC/GPC 150C unit and two Ultrahydrogel columns (Ultrahydrogel Linear and 250) connected in line at a flow rate of 0.8 ml/min at 35 °C. Polyethylene oxides (MW : 600, 900, 1470, 7100, 12600, 23000, 46000, 95000) were used as standards to calibrate the column. Doxorubicin content in the polyphosphazene/doxorubicin conjugates was caculated by measuring uv-vis absorbance at 495 nm.

For *in vitro* assay against the murine leukemia L1210 cell line, cells maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum were adjusted to 1×10^6 cells/ml and distributed to 24 well tissue culture plates (0.5 mL/well). Test compounds were serially diluted and added to wells (0.5 mL/well). Following 48 h incubation in a 5% CO_2 atmosphere at 37 °C, cell counts were determined with a Coulter Model ZM cell count. Cell growth in the presence of polymeric conjugates was expressed as a percentage of growth in untreated control wells and the concentration of the compound producing 50% inhibition of cell growth was determined (ED_{50}).

In vivo assay was carried out using the ascites cell form of L1210 lymphoid leukemia, which was obtained from BDA/2 donor mice bearing 3- to 5- day tumor growth. L1210 leukemia cells (10^6) were inoculated i.p. in BDF mice (6-8 week old, 20-25 g; 8 mice per group), and 24 h later, compounds were administered i.p. on days 1, 5, and 9. Mortality was recorded and mean survival time was calculated for each group. *In vivo* activity of the polymeric conjugates was expressed as a survival effect (T/C), where T is the mean survival time of the drug treated mice and C is that of control mice.

Synthesis of polyphosphazene/doxorubicin conjugates. The polyphosphazene/doxorubicin conjugate having β-galactosyl moiety (KBGAD) was synthesized according to Scheme 1. Polyphosphazene(**III**) was prepared by the method of the previous report.[9] To the polymer(**III**) (0.1 g, 0.44 mmol) dissolved in DMF (20 mL) isobutyl chloroformate (0.07 mL, 0.52 mmol) and tributyl amine (0.14 mL) were added. After stirring the reaction mixture for 15 min at 4 °C, doxorubicin (42 mg, 0.07 mmol) and AGA (43 mg, 0.1 mmol) were added to the reaction mixture, which was stirred for 10 min at 4 °C and for 3 hours at room temperature. The reaction mixture was then dialyzed with DMF for 2 days and with distilled water for 2 days using a cellulose acetate tubing (mol. wt. cutoff : 3500). The dialyzed solution was freeze-dried to obtain the final polymeric product (KBGAD). Overall yield, 74%.

The polyphosphazene/doxorubicin conjugate having 2-(2-aminoethoxy)ethanol as a solubilizing group (KBED) was synthesized by using the same procedure as for KBGAD



Scheme 1. A synthetic procedure for polyphosphazene/doxorubicin conjugates.

except for using 2-(2-aminoethoxy)ethanol (ED) instead of AGA. Overall yield, 83%.

The polyphosphazene/doxorubicin conjugate having L-glutamate as a spacer for introducing doxorubicin (KBGD) was synthesized also according to Scheme 1. To the polymer(**III**) (0.5 g, 2.2 mmol) dissolved in DMF (40 mL) isobutyl chloroformate (0.38 mL, 2.9 mmol) and tributyl amine (0.7 mL) were added and the reaction mixture was stirred for 15 min at 4 °C. L-Glutamic acid dibenzyl ester p-toluene sulfonate (1.5 g, 3.0 mmol) and tributyl amine (1 mL) were added to the reaction mixture, which was stirred for 10 min at 4 °C and for 3 hours at room temperature. After the reaction mixture was purified by precipitation of polymer(**IV**) using a solvent pair of DMF and diethyl ether, hydrolysis of polymer(IV) by LiOH in methanol and HCl treatment afforded polymer(**V**). To the polymer(**V**) (0.2 g, 0.56 mmol) dissolved in DMF (20 mL) isobutyl chloroformate (0.013 ml, 0.09 mmol) and tributyl amine (0.1 mL) were added. After the reaction mixture was stirred for 15 min at 4 °C, doxorubicin (42 mg, 0.07 mmol) was added to the reaction mixture, which was stirred for 10 min at 4 °C and for 3 hours at room temperature. The reaction mixture was then dialyzed with DMF for 2 days and with distilled water for 2 days using a cellulose acetate tubing (mol. wt. cutoff: 3500). The dialyzed solution was freeze-dried to obtain the final polymeric product (KBGD). Overall yield, 87%.

Results and Disscusion

Poly(organophosphazene)/doxorubicin conjugates were synthesized according to the reaction Scheme 1. The polyphosphazene derivative(**III**) having 5-carboxy-1-pentoxy and methoxy groups was prepared by the two step reaction; poly[(dichloro)phosphazene] was substituted by 5-carboxymethyl-1-pentanol and methanol, and then the methyl ester of polyphosphazene(**II**) was treated with LiOH and acidified with HCl, which was described in the previous report.[9] Quantitative chlorine displacement could be accomplished with 5-carboxymethyl-1-pentoxy and methoxy groups and their mole fractions were 0.55 and 0.45, respectively. An anticancer drug, doxorubicin, and the saccharide as a targeting group could be successfully coupled to the polyphosphazene(**III**) by activation of 5-carboxy-1-pentoxy group of the polymer by the mixed anhydride method.[12] Doxorubicin was also introduced to the polyphosphazene(**III**) by using L-glutamic acid as a spacer in order to examine the effect of the spacer. After L-glutamic acid dibenzyl ester was coupled to the polyphosphazene(**III**) by the mixed anhydride method, the benzyl group of the glutamic acid ester was completely removed by LiOH and then acidified with HCl. Doxorubicin was coupled to polyphosphazene(**VI**) by activation of L-glutamic acid by the mixed anhydride method. After conjugation of doxorubicin to the polymer, the conjugates were extensively dialized with DMF and with distilled water to remove unreacted doxorubicin and other impurities. The characteristics of the polymeric conjugates are listed in Table 1. The content of doxorubicin in the polymeric conjugates could be estimated by measuring the uv-vis absorbance of the conjugates, since doxorubicin exhibits a unique absorbance at 495 nm as shown in Figure 1 and was found to be in the range of 12.3-18.8 mole%. The binding of saccharide derivatives to the polymer was confirmed by ^{1}H NMR: C-4 protons of aminobutyl of [N-(aminobutyl)-O-β-D-galactopyranosyl-(1→4)-D-gluconamide] (AGA) appearing at 2.7 ppm was shifted to 3.2 ppm by consequence of transformation of the free amine to amide and C-1 proton of galactopyranosyl

Table 1. Charaterization data for polyphosphazene/doxorubicin conjugates

Compounds	composition[a]			elem. anal., (%)		M.W.(x10^{-3})[c]
	x	y	z	found	calcd[b]	
KBGAD	0.126	0.262	0.612	C. 40.20	C. 41.66	24.3
				H. 6.83	H. 6.97	
				N. 5.64	N. 5.31	
KBED	0.188	0	0.812	C. 43.83	C. 45.19	28.5
				H. 7.13	H. 7.45	
				N. 6.47	N. 6.82	
KBGD	0.123	0.877	-	C. 39.78	C. 41.55	31.7
				H. 5.64	H. 5.95	
				N. 6.51	N. 6.38	

[a] Molecular structures of the conjugates are shown in scheme 1 and their composition were calculated by UV and ^{1}H-NMR. [b] Calculated from the mole fractions (x,y,z) of substituents of the conjugates. [c] Calculated by GPC based on polyethylene oxide standard.

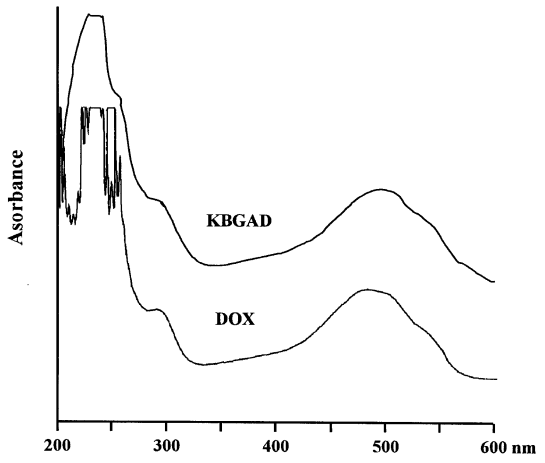


Figure 1. UV-VIS spectra of the polymeric conjugate (KBGAD) and free doxorubicin (DOX) in distilled water

moiety appeared at 4.6 ppm (spectra not shown) which indicate that the saccharide derivative was successfully coupled to the polyphosphazene. The mole fraction of the galactosyl moiety in the polymeric conjugte (KBGAD) was 26.2 mole%. Different kinds of the conjugates were synthesized and their structure are shown in Scheme 1. The conjugates, KBGAD, having a β-galactosyl moiety was designed as a liver targeting polymeric anticancer drug. It was reported that β-galactosyl and galactosylamine moieties can bind to asialoglycoprotein receptors of hepatocyte.[13] Several studies have been reported on the liver targetable polymeric anticancer drugs involving β-galactosyl or galactosylamine moieties, and one compound, N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer/doxorubicin/galactosamine conjugate is currently under clinical study in the United Kingdom.[7] 2,2-Aminoethoxy ethanol was employed as a solubilizing group in KBED. L-glutamic acid was used as a spacer group in KBGD. All the polyphosphazene/doxorubicin conjugates were obtained as red powders. They are soluble in water, DMSO and DMF, but insoluble in most other organic solvents.

Antitumor activity of all the polyphosphazene/doxorubicin conjugates prepared in this study was measured both *in vitro* and *in vivo* using murine leukemia L1210 cell line and the results are listed in Table 2. Growth-inhibitory effect of the polymeric conjugates *in vitro* against murine leukemia cell line was expressed as 50% inhibition of cell growth (ED_{50}). The ED_{50} values of the polymeric conjugates were higher than that of free doxorubicin. The antitumor activity of the polymeric conjugates were expressed as survival effect (T/C) of mice innoculated with murine leukemia L1210 cells. The antitumor activity *in vivo* of the polymeric conjugates seems to be dependent on dose. The T/C values of the polymeric conjugates, KBGAD and KBED increased with increasing dosage. However, the overall antitumor

Table 2. *In vitro* and *in vivo* antitumor activities of polyphosphazene/doxorubicin conjugates against leukemia L1210 cell line

Compounds	*in vitro* ED_{50} (μg/mL)	*in vivo*	
		dose (mg/kg)	T/C (%)
KBGAD	10.9	10	101.4
		20	100.1
		60	120.3
KBED	7.5	6	96.3
		12	94.4
		20	105.6
		40	110.3
KBGD	6.8	6	114.6
		12	114.6
Doxorubicin	0.01	1.8	190.0

activity *in vivo* of the polymeric conjugtes was not higher than that of free doxorubicin. Such a result seems to be due to the stability the of amide bond between polphosphazene back bone and doxorubicin. Therefore, it seems to be necessary to design a new method to conjugate doxorucin to polyphosphazene which can afford more easily to release the drug component *in vivo*.

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