Development of Novel Chitosan Derivatives as Micellar Carriers of Taxol

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Purpose. To develop an intravenous injectable carrier composed of chitosan derivatives for taxol.

Methods. A chitosan with lauryl groups attached to amino groups to provide the hydrophobic moieties and, carboxymethyl groups attached to hydroxy groups to provide the hydrophilic moieties (*N*-lauryl-carboxymethyl-chitosan = LCC), was newly synthesized. The solubility of taxol in LCC micelles in aqueous solution was examined. The hemolysis test of LCC and the growth inhibition experiment of taxol-loading micelle using KB cells were also performed as *in vitro* assay.

Results. It was found that LCC solubilized taxol by forming micelles with particle sizes less than 100nm. This particle size was considered effective for passive targeting for tumors. The concentration of taxol in the micellar solution was very high, with a maximum of 2.37mg/mL. This maximum was 1000 times above that in a saturated solution of taxol at pH 7.4. Hemolysis testing as an *in vitro* assay indicated that LCC was safer than Polysorbate 80 (TO-10M) as intravenous surfactant in terms of induction of membrane damage. As judged by cytostatic activity against KB cells, taxol retained activity even when included in LCC micelles. LCC-entrapped taxol was more effective in cytostatic activity than free taxol in low concentrations.

Conclusions. The results of solubilization capacity examination, hemolysis testing, and cytostatic activity suggest that LCC may be useful as a carrier of taxol.

KEY WORDS: taxol; *N*-lauryl-carboxymethyl-chitosan(LCC); chitosan; polymer micelle; solubility; nanoparticle; hemolysis; antitumor activity.

INTRODUCTION

Taxol has promising anticancer activity against most solid tumors, and was approved as an anticancer agent by the U.S. Food and Drug Administration (FDA) in 1992. Since taxol is poorly soluble in water, it is for clinical use formulated in a 50:50 mixture of Cremophore EL (polyethoxylated castor oil) and ethanol, which is diluted with normal saline or dextrose solution (5%) prior to administration (1).

However, serious hypersensitivity reactions have been associated with use of this clinical formulation (2,3), and it is physically incompatible with some intravenous infusion sets, as shown by the leaching plasticizers by the mixture of the surfactant and ethanol (1). A great deal of effort has therefore been directed toward development of aqueous-based formulations for taxol, including soluble semi-synthetic taxol derivatives (4), carriers such as emulsions (5), mixed-micelles (6), liposomes (7), cyclodextrins (8), and polymer-micelles (9).

It has been reported that polymer-micelle is convenient compared with other carriers such as liposomes for use as a passive targeting carrier of anticancer drugs since it is structurally strong and not to be captured by the reticuloendotherial cell system (RES) because of its particle size (20–100nm) (10,11).

The polymer used as a drug carrier needs to be nontoxic, biodegradable, and metabolized in the body. So we aimed chitin-chitosan. In general, chitin-chitosan can not form micelles. Recently, it was reported that the chitosan derivatives with both hydrophobic groups (long acyl groups) and hydrophilic groups (sulfate groups) could form micelles and solubilize hydrophobic compounds (12). However, testing of solubilization of taxol using chitosan derivatives has not been reported.

In this study, we synthesized novel chitosan derivatives with both hydrophobic and hydrophilic groups to solubilize taxol. Several chitosan derivatives with lauryl groups attached to the amino groups to provide hydrophobic moieties, and carboxymethyl groups attached to the hydroxy groups to provide hydrophilic moieties (LCC) were synthesized. The usefulness of LCC as a taxol carrier was evaluated by measuring the loading efficiency of taxol, its hemolysis toxicity, and cytostatic activity of taxol-loading micelles.

MATERIALS AND METHODS

Materials

Taxol was purchased from Chugaiboyeki Co., Ltd. (Japan). Lauryl aldehyde was obtained from Tokyo Kasei Kogyo Co., Ltd. (Japan). Monochloroacetic acid, sodium borohydride and other reagents were obtained from Nacalai Tesque, Inc. (Japan). All organic solvents used were of a reagent or high performance liquid chromatography (HPLC) grade. Polysorbate 80 (TO-10M) was purchased from Nikko Chemicals Co., Ltd. (Japan).

Methods

Synthesis of LCC

The conventional method was used to prepare squid pen chitin (13). Squid pens collected from common squid (*Todarodes pacificus*) were washed and cut into small pieces (less than 1cm^2), and treated with 5% sodium hydroxide at 90°C for 1.5 hours to separate protein. They were washed with distilled water and then treated with 0.7N hydrochloric acid solution for 30 min for demineralization. The residues were washed again, air-dried and ground to powder. Finely powdered squid pen chitin was used to prepare LCC.

Carboxymethylation of chitin was performed using the method of Tokura *et al.* (14). Squid pen chitin (50g) was suspended in 250mL of 55% sodium hydroxide solution and 15mL of 8% sodium dodecylsulfate solution at 4°C, and the slurry was kept in a freezer at −20°C overnight after mechanical

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stirring for 2 hours at 4°C. The obtained frozen alkali-chitin was melted at 4°C and kept in a freezer at −20°C overnight again.

The frozen alkali-chitin was melted at 4°C and then suspended in 500mL of isopropyl alcohol at room temperature, and monochloroacetic acid was added portionwise with mechanical stirring until the reaction mixture was neutralized. The product was filtered and washed with methanol. The obtained solid was extracted with 5 liters of water at room temperature with mechanical stirring overnight. The water extract was centrifuged and the supernatant was concentrated to 2.5 liters on a rotary evaporator. The residue was poured into 8 liters of methanol to precipitate the carboxymethyl-chitin (CM-chitin). The precipitate was filtered and washed with methanol several times. Crude CM-chitin (59g) was obtained in this fashion.

The crude CM-chitin (59g) was suspended in 1 liter of 45% sodium hydroxide solution with mechanical stirring. After dissolution, 600mL of isopropyl alcohol was added to the solution and the mixture was heated at 110°C for 2 hours. The isopropyl alcohol was decanted and the reaction solution was concentrated on the rotary evaporator after neutralization with conc. hydrochloric acid. The residue was dialyzed against deionized water at room temperature and freeze-dried. The carboxymethyl-chitosan (CM-chitosan) (40g) was obtained in this fashion.

CM-chitosan (10g) was suspended in 67mL of water and 67mL of methanol with mechanical stirring at room temperature. Lauryl aldehyde (15g) was added to the solution and the mixture was stirred for 30 min. Hydrogenation was performed with sodium borohydride (5g) dissolved in 50mL of water in small portions for 2 hours with mechanical stirring. The mixture was stirred at room temperature overnight. The reaction mixture was then neutralized with 5N hydrochloric acid solution and the LCC1 was precipitated with methanol. The precipitate was filtered and washed with 90% methanol/water, methanol, hexane and acetone. LCC1 (7g) was obtained in this fashion.

N-Lauryl-carboxymethyl-chitosan derivative with a different degree of substitution (LCC2) was obtained by performing the carboxymethylation reaction twice in the first step, and the lower molecular weight *N*-lauryl-carboxymethyl-chitosan derivative (LCC3) was obtained by using additional hydrolysis of chitin chains.

Analysis of LCC

CM-chitin, CM-chitosan, and LCC well dialyzed against deionized water at room temperature and then freeze-dried were used for experiments.

The degree of substitution of LCCs was estimated by elemental analysis using a Perkin-Elmer CHNS/O 2400 Series II analyzer.

IR spectra were recorded as films with a Jasco FT/IR VALOR III spectrometer.

Viscosity of solutions of CM-chitin was measured with an Ubbelohde type viscometer at 30°C.

Measurement of Particle Size

Micelle solution was diluted with purified water to obtain the optimum intensity for measurement of particle size. Micelle particle size was determined by dynamic light scattering (Otsuka Electronics, DLS-7000). Measurement conditions were as shown below.

Wave length of laser: 632.8nm (He-Ne laser)
Detection angle: 90°
Correlation mode: time interval
Sampling time: 8μsec
Accumulation times: 200 times
Temperature: 23°C

Preparation of LCC Micelle-Entrapped Taxol

For LCC1-TAX (5-3): (LCC1-TAX (X-Y), where X and Y denote the initial concentrations (mg/mL) of LCC1 and taxol, respectively).

LCC1 (50mg) was swollen in 9mL of 0.15M phosphate buffer saline (PBS) (pH7.4) with stirring for 30min at 50°C. Ethanol solution (1mL) including taxol (30mg) was added to the LCC suspension. In order to form micelles, the suspension was sonicated with a probe ultrasonicator (20kHz, 30w) for 30min (Branson, Sonifier 250). The sonication was performed by referring to the method of Akiyoshi *et al.* (15). To remove free taxol and ethanol, the solution was dialyzed (Spectrapor, MWCO3500) against 0.15M PBS (pH7.4) overnight, and the micellar solution was centrifuged at 3000rpm for 30min to remove insoluble taxol. The supernatant was filtered with membrane filter (pore size: 0.8μm), and LCC1-TAX (5-3) micellar solution was obtained. Since a solubility of free taxol is very low compared with that of incorporated taxol as shown in later, the amount of free taxol in this preparation was not taken into account. Other series of micelles were obtained by changing the mixing ratio of LCC derivatives and taxol. Free LCC micelles were prepared by adding ethanol without taxol, using the above method.

Measurement of Taxol Concentration in Micellar Solution

The resulting micellar solution was divided 500μL to a tube, and freeze-dried. A 10mL portion of internal standard solution (20μL/mL dibutylphthalic acid in ethanol) was added to the freeze-dried powder. The mixture was sonicated in a bath sonicator (Branson, 8200) for 5min, and then centrifuged at 3000rpm for 5min. The concentrations of taxol in the supernatant were determined by high-performance liquid chromatography (HPLC). The mobile phase was methanol and water (65:35 v/v). The column was a Tosoh ODS-80TM (4.6mm × 150mm) with 5 μm particles. The flow rate was 1.0mL/min, the detection wavelength was 227nm, and the column temperature was 50°C. Each sample was injected at the volume of 10μL (n=3). Analysis was performed with a Jasco 880-PU pump, a Shimazu CR-4AX integrator, a Jasco 851-AS auto sampler, and a Jasco 875-UV UV-VIS detector.

Solubility of Free Taxol

Excess taxol (2–3mg) was added to 10mL of 0.15M PBS (pH7.4). The suspension was shook (160rpm) for 24 hours at 25°C (Eyera, MMS-24GR) and centrifuged at 3000rpm for 30min, and the supernatant was freeze-dried. Internal standard solution (2mL) (the same as used for taxol-loading micelle) was added to the lyophilized powder. The suspension was sonicated in a bath sonicator (Branson, 8200) and then centrifuged at 3000rpm for 30min. The resulting supernatant was analyzed by HPLC.

Measurement of Zeta Potential

Zeta potential was calculated from the mean particle electrophoretic mobility. Measurements of mean electrophoretic mobility were made by quasi-elastic laser light scattering (Coulter Electronics, Model DELSA 440) in normal saline.

Hemolysis Test

Heparinized blood freshly drawn from a beagle was centrifuged at 3000rpm for 5min to separate the erythrocytes. After removing the buffy coat, the erythrocytes were washed 3 times with 0.15M PBS (pH7.4), and the precipitate was resuspended in PBS. The erythrocyte suspension was adjusted to 2.5 v/v % of the final concentration. The above procedures were performed in an ice box. The PBS used had the following composition: 8.0g NaCl, 0.2g KCl, 2.9g $Na_2HPO_4/12H_2O$, 0.2g KH_2PO_4 in 1L purified water, and the pH of the solution was adjusted at 7.4.

A 0.2mL portion of the erythrocyte suspension was added to 4mL of PBS or sample (TO-10M, LCC1, and LCC1-TAX micelles) which had been adjusted to the appropriate concentration. After the mixture had been incubated at 37°C for 1 hour, it was centrifuged at 3000rpm in 5min to remove unhemolyzed cells. The degree of hemolysis was determined by spectrophotometric determination of the amount of hemoglobin released into the supernatant (λ=416nm) (n=3). Then 0.2mL of the erythrocyte suspension was added to 4mL PBS to obtain 0% hemolysis, and in another tube 0.2mL of the erythrocyte suspension was added to 4mL purified water to obtain 100% hemolysis (same procedure as above). The turbidity of free LCC micelles was compensated for using micellar solution without erythrocytes as the blank.

Cell Growth Inhibition Experiments

The three types stock solution listed below were prepared:

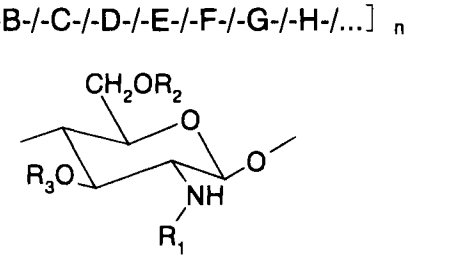
LCC micellar solution of taxol in 0.15M PBS (pH7.4).

LCC micellar solution without taxol in 0.15M PBS (pH7.4).

Free taxol solution in 1% DMSO/MEM. The initial concentration of taxol was 10 μg/mL.

KB human epidermoid carcinoma cells were cultured in minimum essential medium (MEM, Gibco laboratories) supplemented with 10% fetal bovine serum, 1% gultamine and 1% gentamicin at 37°C in a humidified atmosphere of 5% CO_2/95% air.

The antiproliferative activity of test sample was determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) colorimetric assay (16). Exponentially growing KB cells (1000cells/100μL) were transferred to a 96-well flat-bottomed microtiter plate (Corning) and incubated for 24 hours. The stock solution was diluted to graded taxol concentrations (0.0002–0.2μg/mL) by 1% DMSO/MEM. The diluted solution was divided 100μL to a cultured well, and the mixtures were incubated for 72 hours. After drug treatment, 50μL of 1mg/mL solution of MTT in MEM was added to each of the culture wells. Four hours later, the fluid content of each well was removed from the plates and 150μL DMSO was added. The purple formazan product was solubilized by gentle shaking of the plate. The absorbances were measured at 540nm using an automatic microspectrophotometer (Inter Med NJ-2000 immunoreader). Background absorbance was subtracted from each well, and the percentage of control absorbance was considered to be the fraction of cells surviving. The concentrating inhibiting 50% of growth (IC_{50}) was calculated graphically (n=3).



	R_1	R_2	R_3
A	$-(CH_2)_{11}CH_3$	$-CH_2COOH$	$-CH_2COOH$
B	-H	$-CH_2COOH$	$-CH_2COOH$
C	$-(CH_2)_{11}CH_3$	-H	$-CH_2COOH$
D	$-(CH_2)_{11}CH_3$	$-CH_2COOH$	-H
E	$-(CH_2)_{11}CH_3$	-H	-H
F	-H	$-CH_2COOH$	-H
G	-H	-H	$-CH_2COOH$
H	-H	-H	-H

Fig. 1. Chemical structure of LCC.

RESULTS AND DISCUSSION

Characteristics of LCC

Figure 1 shows the chemical structure of LCC. LCC incorporated carboxymethyl groups as hydrophilic moieties and lauryl groups as hydrophobic moieties. As physical properties of the obtained LCC1, the degree of substitution were calculated by comparing the C and N molar ratio obtained from element analysis in each derivative. The increase in the molar ratio indicates the increasing carbon in monosaccharide of chitosan chain, since the monosaccharide include one nitrogen. For example, in the case of carboxymethylation, carboxymethyl group includes 2 carbons, therefore, the degree of carboxymethylation was estimated from the increasing-molar ratio/2. Other substitutions were estimated in the same way. The degrees of carboxymethylation, deacetylation and laurylation were estimated to be 1.4, 1.0, and 0.9, respectively, as shown in Table I. The IR spectra of LCC1 had the absorption due to carbonyl

Table I. Elemental Analyses and the Degree of Substitution (DS) of Chitiosan Derivatives

	Found %			
	C	N	C/N[a]	DS
Squid pen chitin	43.04	6.37	7.88	
CM-chitin	29.84	3.25	10.71	1.4
CM-chitosan	35.97	4.82	8.71	1.0
LCC1	54.33	3.22	19.68	0.9

[a] The molar ratio of carbon to nitrogen.

stretching at 1770cm^{-1}, and that due to the methylene groups at 2910cm^{-1} increased on laurylation (data not shown). The intrinsic viscosity number of CM-chitin was calculated to be 4.0 in a 0.1M NaCl solution at 30°C, from the intercept of the reduced viscosity-concentration plot. The average molecular weight of CM-chitin was estimated to be 5.0×10^4, from the viscosity equation proposed by Inoue *et al.* (17).

Chitosan derivatives were screened for their capacity to form micelles. We found that LCC formed the desired particle size (<100nm) in water for passive targeting. Figure 2 shows the particle size distributions of LCC1 micelles, based on intensity and weight distributions by histogram method. The reason for these different distribution patterns is the scattering intensity by particle size is not revised in intensity distribution. The average particle size based on weight distribution was estimated to be 31nm in the micellar solution obtained using 0.22μm pore size filter. In addition, polydispersity (*dw*/*dn*) defined as the ratio of the average particle size based on weight distribution and number distribution was estimated to be 1.1.

The measurement of particle size distribution by dynamic light scattering, as the first step, autocorrelation function is obtained as the original data. As the next step, autocorrelation function is inverted to intensity distribution by using uni-modal mode (cumulant method) or multi-modal mode (histogram method). And as the last step, intensity distribution is inverted to weight distribution by considering the intensity depending on particle size.

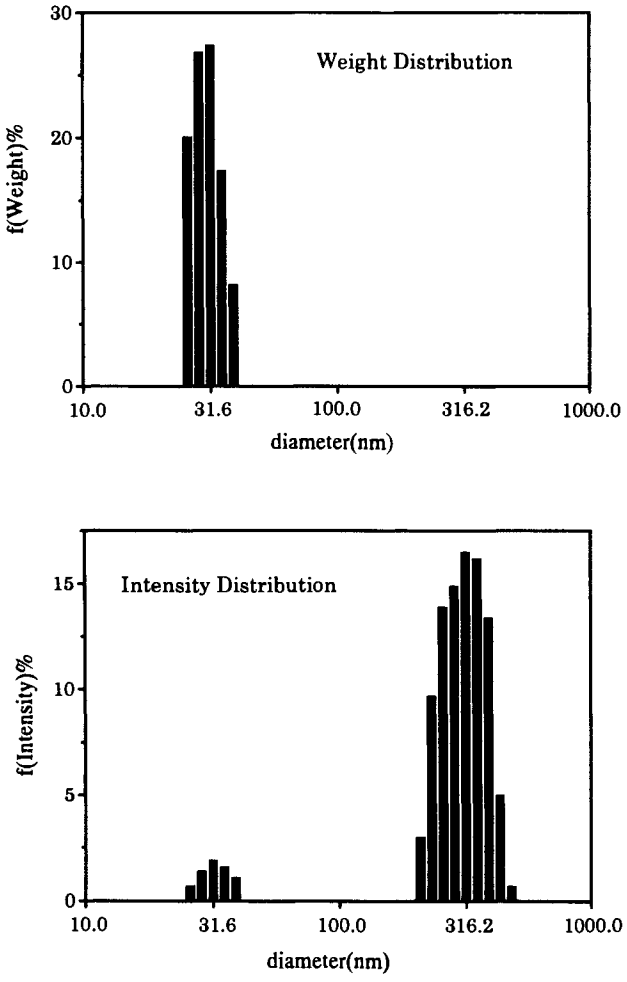


Fig. 2. Particle size distribution based on weight and intensity of LCC1 micelles by histogram method.

In the case of LCC1, it is important to select an analytical method (cumulant or histogram method). The intensity distributions obtained by cumulant and histogram method indicated one peak (314 ± 105nm) and two peaks (32 ± 4nm:314 ± 61nm = 1:9 as peak area), respectively. The different results obtained by each method indicated that a 30nm fraction of low intensity compared with 300nm fraction was not taken into account in cumulant method. On the other hand, the histogram method indicated a good fit between the autocorrelation function and the reverse-autocorrelation function by calculating from intensity distribution and in comparison with the cumulant method. Further, the existence of 30nm fraction was also confirmed by measuring in wide detection angle (ex 150°) the increasing relative intensity of 30nm fraction (data not shown). So we judge the histogram method is adequate to this analysis, and 30nm fraction is the main peak in weight distribution.

The CM-chitin obtained from intermediate syntheses yielded a clear solution in water, and the scattering phenomena detected in the case of micellar solution were not observed in the CM-chitin solution. Thus, the strength of light scattering was insufficient to measure particle size. These findings indicated no particles of desired size existed in the solution.

Based on its molecular structure, LCC was considered to have hydrophobic-hydrophilic balance adequate for formation of micelles. On the other hand, CM-chitin is too hydrophilic to form micelles, since it attached only hydrophilic groups. These considerations suggested hydrophobic groups were required for formation of micelles. Further, to examine the effect on solubility of taxol by micelle formation, LCC2, which has high degree of carboxymethylation, and LCC3, which is of lower molecular weight than the other LCCs, were prepared. Table II summarizes the characteristics of each chitosan derivative.

The Capacity for Solubilization of Taxol in LCC Micelles

The effects of the type of LCC and the addition ratio of LCC and taxol on the capacity for solubilization of taxol are shown in Table III, for LCC-TAX (X-Y), where X and Y denote the initial concentrations (mg/mL) of LCC and taxol, respectively.

Each chitosan derivative increased the solubility of taxol by forming micelles. Run 3 yielded the highest concentration of incorporated taxol. The solubility of taxol in the micellar solution was 2.37mg/mL. This solubility was 1000 times greater than that in a saturated solution of taxol at pH7.4 (<0.001mg/mL). The particle size in the micellar solution obtained was below 100nm, which is considered appropriate for passive targeting carriers for solid tumors.

At present, taxol is used clinically at 30mg/vial, and diluted with agents such as normal saline. For administration of 30mg of taxol using LCC micelles, the solution infusion volume is 13mL/vial, as calculated from the maximum concentration (2.37mg/mL) in run 3. This volume (13mL) is not considered too high for intravenous infusion. The solubility of taxol in LCC micelles is thus sufficient for clinical use.

As compared with LCC1, LCC2 with many hydrophilic moieties indicated lower loading capacity of taxol in the same

Table II. Characteristics of Chitosan Derivatives

Sample	Molecular weight[a]	Degree of substitution		
		Laurylation	Deacetylation	Carboxymethylation
LCC1	50000	0.9	1.0	1.4
LCC2	50000	0.2	1.0	2.0
LCC3	2000	0.25	1.0	1.4
CM-chitin	50000	0	0	1.4

[a] Molecular weight as CM-chitin.

condition (run 4,5). This result suggests the hydrophilic-hydrophobic balance of LCC influences loading capacity of taxol. In addition, the difference of the molecular weight of LCC appeared not to be related to the loading capacity of taxol in this experiment (run 4,5,6).

On the other hand, CM-chitin, which does not include hydrophobic groups, did not form micelles and could not dissolve taxol (data not shown). This suggests that LCC possessing long alkyl chains formed micelle as a result of aggregation of alkyl groups including taxol, but that the hydrophilic portions of LCC orient toward the outer water layer. The introduction of long alkyl chains such as laury groups appears to be required for formation of micelles and entrapment of hydrophobic compounds such as taxol.

In our study, the solubility of taxol was examined using alkyl groups of fixed length (lauryl groups). In another study (12), however, it was reported that chitosan with long acyl groups attached to amino groups, as hydrophobic moieties, and sulfate groups attached to hydroxy groups as hydrophilic moieties (Acyl-S-chitosan) were able to solubilize hydrophobic compounds by forming micelles. And the carbon length in the acyl group affected the solubility of a hydrophobic compound (azobenzene).

The structure of LCC differs from that of Acyl-S-chitosan in type of group substituted, but both have hydrophobic and hydrophilic groups connected to the chitosan chains. The finding that both chitosan derivatives had micellar formation capacity and increased the solubility of hydrophobic compounds, suggested that chitosan derivatives incorporating long alkyl (acyl) groups and hydrophilic groups could form micelles and solubilize hydrophobic drugs. The solubility of hydrophobic compounds was increased with an increase in the carbon length in the acyl group in the case of Acyl-S-chitosan. This finding suggests that the solubility of taxol increase with the use of longer alkyl chains in the same fashion for *N*-alkyl-carboxymethyl-chitosan, such as LCC.

The amount of entrapped taxol appeared to be influenced by the degree of substitution, (lauryl, carboxymethyl, deacetyl) and the carbon length in the alkyl chain of chitosan derivatives, which affected the hydrophilic-hydrophobic balance. Further study may be needed to determine the optimum structure of chitosan derivatives for the solubility of taxol.

Table III. Particle Size and Taxol Loading Capacity of LCC Micelles[a]

Run	Sample	Particle size (nm)[b]	Taxol concentration (mg/mL)	Yield (%)
1	LCC1-TAX(1-0)	82	—	—
2	LCC1-TAX(1-0.5)	34	0.08	16.0
3	LCC1-TAX(5-2)	65	1.03	51.5
4	LCC1-TAX(5-3)	32	2.37	79.0
5	LCC2-TAX(5-3)	69	1.37	45.7
6	LCC3-TAX(5-3)	42	1.54	51.3

[a] LCC-TAX(X-Y) X: The initial concentration of chitosan derivatives (mg/mL) Y: The initial concentration of taxol (mg/mL).
[b] Average particle size based on weight distribution by histogram method.

Hemolysis Studies

Since LCC can form micelles, it might have surfactant activity, suggesting that membrane damage such as hemolysis might occur following the intravenous administration. In order to determine whether LCC induces membrane damage, the degree of hemolysis with LCC1 was compared with that of TO-10M, a typical nonionic surfactant used for intravenous administration. The surfactant was considered safer than Cremophore EL used in clinical taxol formulation, since the maximum tolerable dose per day for intravenous administration of TO-10M was 500mg, compared with 75mg for Cremophore EL. TO-10M was therefore compared with LCC1.

The results are shown in Figure 3. In the case of TO-10M, hemolysis increased as the concentration of surfactant increased above 0.01mg/mL, while for LCC1, the slight increase in hemolysis was noted at 5mg/mL.

TO-10M and LCC1 concentrations of 0.1 and above 5 mg/mL, were required for 50% hemolysis, respectively. LCC1-

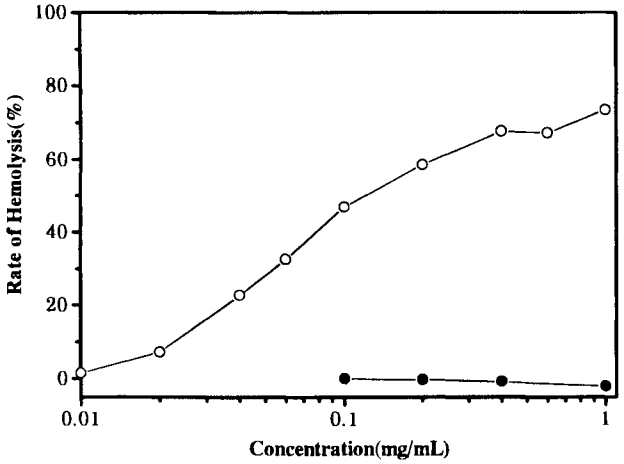


Fig. 3. Hemolytic curves for TO-10M and LCC1. (○) TO-10M; (●) LCC1. Each point represents the mean value from three experiments.

TAX (1-0.5) exhibited the same pattern of hemolysis curve as LCC1 (data not shown). It was thus confirmed that LCC1 is safer than TO-10M as an intravenous surfactant in terms of induction of membrane damage.

In Vitro Assay of Cytostatic Potential

Interaction between drug and polymer often results in loss of pharmacological activity. We therefore examined the cytostatic potential of LCC micelle-entrapped taxol *in vitro*.

This cytostatic assay was performed in culture wells with 0.5% DMSO to prevent precipitation of taxol in samples, in order to correspond with experimental conditions. Figure 4 shows the cytostatic curve obtained from sigmoidal fit of LCC1-TAX (1-0.5) and free taxol. Results are expressed as means and standard errors (SE). The IC_{50}s of LCC1-TAX (1-0.5) and free taxol were 1.92×10^{-3} and 4.57×10^{-3} μg/mL, respectively. It was considered that the cytostatic potential of LCC micelle itself as a blank was negligible (the IC_{50} of LCC1 micelle itself is above 1 mg/mL). The cytostatic potential of taxol was retained even in LCC micellar solution. Furthermore, the cytostatic potential was higher than that of free taxol. To evaluate the significance of difference between two lines confirmed to be parallel by an analysis of covariance, the relative potency against free taxol was calculated. The values on the part of the straight line between 0.001–0.01 μg/mL taxol concentration were calculated. The relative potency (R) of taxol in LCC1 micelles was 0.449 against free taxol. The relative potencies at the lower limit (RL) and upper limit (RU) of the 95% confidence interval were 0.403 and 0.498, respectively. The R was significant of difference from free taxol, since "1" was included in neither RL nor RU. LCC-TAX thus demonstrated greater cytostatic activity than free taxol.

To investigate the effects of LCC-TAX ratio and hydrophilic-hydrophobic balance of LCC on cytostatic activity, in the same way as the above method, LCC1-TAX (4-2) and LCC2-TAX (5-3) were examined. Since IC_{50} varied between culture plates, activity is presented as relative value (R). The

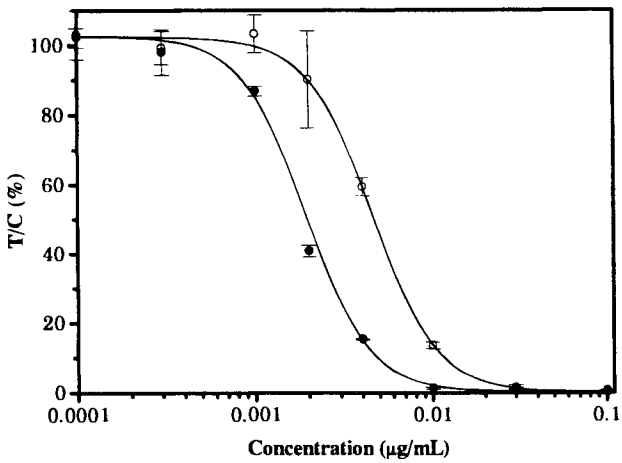


Fig. 4. Growth-inhibitory activity of taxol and LCC1-TAX (1-0.5) against KB cells. (○) taxol; (●) LCC1-TAX(1-0.5). Each point represents the mean value ± SE from three experiments. The taxol concentration and average particle size of LCC1-TAX (1-0.5) are 0.087 mg/mL and 65.8nm, respectively. The particle size based on weight distribution is measured by histogram method.

both Rs were similar values to that of LCC1-TAX (1-0.5). Despite its difference in mixing ratio of LCC-TAX and hydrophilic-hydrophobic balance of LCC, LCC-TAX had higher cytostatic activity than free taxol (Table IV). It is possible that this might be due to the cytostatic effect mediated by the LCC itself even if its a weak effect, in the same way as mixed micellar formation of taxol (6), otherwise due to promote LCC-cell interaction by the negative charge of LCC micellar surface, since, in the case of liposome, higher liposome electrostatic charge promotes liposome-cell interaction, and thereby enhance cellular delivery of liposome-dependent drugs (7,18). The zeta potentials of LCC1 and LCC1-TAX (1-0.5) were −25.5 and −22.2mV in 0.9% NaCl. Anyway, the greatest importance is the antitumor activity of taxol is still retained even when included in LCC micelle.

Generally, it is recognized that the introduction of negative charge into water-soluble polymer molecules prolong their half-life in blood circulation. At the same time, it is reported that water-soluble polymers with strong negative charges are taken up by the liver via scavenger receptor-mediated mechanism (19), in addition, latex particles with strong negative charges are phagocyted by leucocytes (20). In this paper, phagocytosis is enhanced with increasing different in the surface potential between cells and microcapsules. Furthermore, the effects depend on particle size. The phagocytosis reached its maximum when the latex particle size was about 500nm. On the other hand, when the particle size was less than 100nm, phagocytosis effect was very weak for zeta potentials ranging from −20 to −32mV (21).

In the case of LCC, the zeta potential indicated −20 to −25mV in 0.9% NaCl. Although the value might be a strong negative charge in terms of phagocytosis, the particle size suggests that LCC is effective as a passive targeting carrier.

This negative charge might also be evidence of the presence of carboxyl groups at the micellar surface and be induced by the ionized carboxyl group. And, the zeta potentials obtained, even those in normal saline, suggest LCC micelles might have good dispersion stability. Further, in order to achieve optimum stability, the formulation of LCC-TAX should be stored in freeze-dried form, since micellar solution can be prepared by simple dilution with water.

In a previous study (9), it was reported that a certain type of polymer micelle (diblock copolymer of poly (DL-lactide)-*block*-methoxy polyethylene glycol) dissolved taxol by forming polymer micelles. In this substance, poly (DL-lactide) and methoxy polyethylene glycol act respectively as hydrophobic

Table IV. The Relative Potency and Lower and Upper Limits on the 95% Confidence Interval for Several Types of LCC-TAX Against Free Taxol

Sample	Taxol concentration (mg/mL)	Particle size[a] (nm)	R	RL	RU
LCC1-TAX(1-0.5)	0.087	68.6	0.449	0.403	0.498
LCC1-TAX(4-2)	1.23	69.0	0.687	0.647	0.728
LCC2-TAX(5-3)	1.25	70.9	0.418	0.380	0.458

[a] Average particle size based on weight distribution by histogram method.

and hydrophilic moieties. However, the structures of the chitosan derivatives LCC and Acyl-S-chitosan differ from that of diblock copolymer, in that the chitosan derivatives can incorporate hydrophobic and hydrophilic moieties on each chitosan residue. The differences in the types of polymer micelles and in chitosan derivatives may affect the properties such as the loading efficiency of hydrophobic compounds. Our findings suggest, at least, that chitosan derivatives incorporating long alkyl (acyl) groups and hydrophilic groups have micelle formation capacity and the capacity to solubilize hydrophobic compounds.

We found that the newly synthesized chitosan derivatives we tested could solubilize taxol and were safe in terms of membrane toxicity, and micelle-entrapped taxol was more effective than free taxol in an *in vitro* assay. LCC may thus be useful as a carrier to solubilize taxol. The optimum ratio of substitution and the carbon length in the alkyl chain remain to be determined. Furthermore, since LCC micelles formed nanoparticles less than 100nm in size, it may be useful as passive targeting carriers of hydrophobic cancer drugs for solid tumors.

CONCLUSIONS

Newly synthesized LCC dissolved taxol by forming micelles less than 100nm in size. The results of solubilization capacity examination, hemolysis testing, and cytostatic assay suggest that LCC may be useful as a carrier of taxol.

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