ロールとPEG脂質とオレイン酸からなる。マイクロエマルションを調製後，オレイン酸の先端に，トリプシンを結合させた。遺伝子導入の結果，ヒト膵臓固形癌ヌードマウスにおいて，腫瘍の縮小が観察された[9]。(図8)。

6．経鼻吸収用キトサン被覆マイクロエマルションワクチン製剤

ワクチン製剤の多くは，皮下または筋肉内投与によって，行われている。これらの投与方法は侵襲的であるので，粘膜投与によるワクチン製剤の送達は，粘膜免疫と呼ばれ，近年盛んに研究が行われている。鼻腔に投与された微粒子は，抗原提示細胞(APC)によって，抗原提示が起こると考えられている。従って，APCに取り込まれやすい微粒子として，正電荷で鼻粘膜に付着し，鼻粘膜附属リンパ組織(NALT)のM細胞への取り込みを促進する，キトサン被覆マイクロエマルションワクチン製剤を検討した。0.4～3 μmのキトサン被覆マイクロエマルションは，オブアルブミンの付着率が高く，なおかつ正電荷を有するワクチン製剤である。また，鼻粘膜投与において，腹腔内投与とほぼ同等の高い，IgG抗体とIgA抗体の産生を示した。また，NALTとAPCの取り込みに対する，エマルション製剤の粒子径の影響は，ナノパーティクルほど明確ではなかった[9]。(図9)。

7．おわりに

癌細胞に，特異的な発現をした抗体や受容体と親和性がある，リガンドを用いた精密なターゲティングを可能とするには，まず，体内動態を制御できる，機能性微粒子担体の開発が必須である。機能性脂質マイクロエマルションは，その1つとして有望である。

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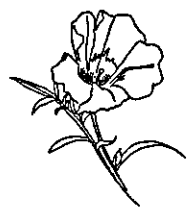

Pharmaceutical Research, Vol. 21, No. 9, September 2004 (© 2004) *Research Paper*

Formulation Optimization of Paclitaxel Carried by PEGylated Emulsions Based on Artificial Neural Network

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Received March 7, 2004; accepted May 16, 2004

Purpose. To develop paclitaxel carried by injectable PEGylated emulsions, an artificial neural network (ANN) was used to optimize the formulation—which has a small particle size, high entrapment efficiency, and good stability—and to investigate the role of each ingredient in the emulsion.

Methods. Paclitaxel emulsions were prepared by a modified ethanol injection method. A computer optimization technique based on a spherical experimental design for three-level, three factors [soybean oil (X1), PEG-DSPE (X2) and polysorbate 80 (X3)] were used to optimize the formulation. The entrapment efficiency of paclitaxel (Y1) was quantified by HPLC; the particle size of the emulsions (Y2) was measured by dynamic laser light scattering and the stability of paclitaxel emulsions was monitored by the changes in drug concentration (Y3) and particle size (Y4) after storage at 4°C.

Results. The entrapment efficiency, particle size and stability of paclitaxel emulsions were influenced by PEG-DSPE, polysorbate 80, and soybean oil. Paclitaxel emulsions of small size (262 nm), high entrapment efficiency (96.7%), and good stability were obtained by the optimization.

Conclusions. A novel formulation for paclitaxel emulsions was optimized with ANN and prepared. The contribution indices of each component suggested that PEG-DSPE mainly contributes to the entrapment efficiency and particle size of paclitaxel emulsions, while polysorbate 80 contributes to stability.

KEY WORDS: artificial neural network; emulsions; optimization; paclitaxel; PEGylated.

INTRODUCTION

Paclitaxel is widely used as an effective anticancer agent for ovarian, colon, and breast cancer. The commercially available product, Taxol (paclitaxel), is currently a formulation of vehicle containing approximately a 1:1 vol/vol mixture of polyoxyethylated castor oil (Cremophor EL) and ethanol due to its extremely poor solubility in water (0.6 mM) and other pharmaceutical agents. Cremophor EL has been associated with hypersensitivity reactions, nephrotoxicity and neurotoxicity (1). Therefore, premedication with corticosteroids and antihistamine as well as long-term infusion of a 5~20-fold dilution of the product is required to reduce the side effects. However, there are a serious problems associated with dilution of the formulation such as compatibility and stability. The stability of diluted paclitaxel was estimated at 12~24 h since its use was recommended within 12 h of dilution in aqueous medium. Thus there is need for a new formulation of paclitaxel that is efficacious and less toxic than the commercial product.

Recently, many alternative formulations have been developed, such as emulsions (2–7), microspheres (8,9), liposomes (10,11), mixed micelles (12,13), cyclodextrins (14,15), and conjugates (16,17). Several compounds are often used in formulations of paclitaxel, such as polysorbate 80, PEG400, poloxamer, PEGylated lipid, and so forth. A common characteristic of the molecular structure of these compounds is the presence of a polyethyleneglycol group of different lengths and shapes. In fact, a polyethyleneglycol group also exists in the molecular structure of Cremophor EL.

The emulsion (o/w) used for the delivery of paclitaxel has attracted much attention (1). Triacetin provides a high level of solubility, 75 mg/ml, and has been used together with lecithin, pluronic F-68, polysorbate 80 and glycerol to produce a paclitaxel-containing emulsion (2). Wheelar *et al.* (3) manufactured a blend of emulsion and liposome with corn oil, EPC, cholesterol, PEG-lipid (polyethylene glycol derivative. mean molecular weight of PEG 2000) and paclitaxel. Lunberg (4) prepared a paclitaxel emulsion made of triolein, dipalmytoylphosphatidylcholine and polysorbate 80, with polyethylene glycol coated on the emulsion surface. Kan *et al.* (5) developed a paclitaxel emulsion with an oil blend of triacylglycerol, EPC and polysorbate 80 in a glycerol solution, while Simamora *et al.* (6) used polysorbate 80 and sorbitan monolaurate, and Constantinides *et al.* (7) used α-tocopherol, α-tocopherylpolyethyleneglycol-1000 succinate (TPGS), Poloxamer 407 and PEG 400. The optimization of these formulations was based on experience, not computer modeling. Also, the role of each ingredient in the formulation was not elucidated.

Recently we have reported injectable PEGylated emulsions composed of vitamin E, cholesterol and PEG-DSPE (polyethylene glycol derivative of distearoylphosphatidylethanolamine, mean molecular weight of PEG 2000) and an anticancer drug prepared by a modified ethanol injection method (18,19). Such PEGylated emulsions also might encapsulate paclitaxel. We tried to develop a Cremophor-free oil-in-water emulsion of paclitaxel using soybean oil as the internal phase, and polysorbate 80, PEG-DSPE and cholesterol as emulsifiers or co-emulsifiers.

Response surface techniques incorporating an artificial neural network (ANN) and the second-order polynomial regression equation (2PE) were used to achieve an optimal emulsion of paclitaxel with a small particle size, high entrapment efficiency and good stability. The application of ANN in the field of pharmaceutical development has gained interest in recent years (20–22). ANN is a learning system based on a computational technique that can simulate the neurologic processing ability of the human brain (23). Using a computor-program, ALCORA, it can also perform a classic opti-

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ABBREVIATIONS: EPC, egg phosphatidylcholine; PEG-DSPE, polyethylene glycol derivative of distearoylphosphatidylethanolamine, mean molecular weight of PEG 2000; PEG 400, polyethylene glycol 400; ANN, artificial neural network; 2PE, the second-order polynomial regression equation.

mization technique based on 2PE. The basic concepts of ALCORA and simultaneous optimization of several responses based on ANN have been described fully (24–27).

The aim of the study was to achieve, by applying an optimization, an optimal PEGylated emulsion containing paclitaxel which has a small size, high entrapment efficiency for paclitaxel and good stability, and to investigate the role of each ingredient in the emulsion using the response surface technique.

MATERIALS AND METHODS

Materials

Paclitaxel was kindly supplied by Bristol Pharmaceuticals K.K. (Tokyo, Japan). PEG-DSPE was purchased from NOF Co. Ltd (Tokyo, Japan). Soybean oil and glucose were obtained from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). Cholesterol and polysorbate 80 were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). PEG 400 was purchased from Kishida Chemical Co. Ltd. (Osaka, Japan). Chemicals for high-pressure liquid chromatography (HPLC) were of HPLC grade and all other chemicals were of analytical grade.

Preparation of Paclitaxel Emulsions

Paclitaxel emulsions were prepared by a modified ethanol injection method (18,19,28). Paclitaxel, soybean oil, PEG-DSPE, cholesterol, PEG 400 and polysorbate 80 were dissolved in 40 ml ethanol, then the ethanol was removed with a rotary evaporator till 1–2 ml was left. Next, a constant volume of 5% glucose solution was added to the ethanol solution. The emulsions formed instantly after further evaporation of the residual ethanol. The concentration of paclitaxel was adjusted to 0.6 mg/ml in the final emulsions containing the 5% glucose solution as external phase with drops of Milli Q water. Then the emulsions were filtrated through 0.45-μm Ekikrodisc filters (Gelman Japan, Tokyo, Japan) to homogenize the droplets of emulsion and stored at 4°C for further detection.

HPLC

The HPLC system was composed of an LC-10AS pump (Shimadzu Co., Ltd., Kyoto, Japan), a SIL-10A auto injector (Shimadzu Co., Ltd.), an SPD-10A UV detector (Shimadzu Co., Ltd.), and a C_{18} 4.6 × 150 mm reverse phase column (Shiseido, Tokyo, Japan; Capcell-park, 3 μm particle size). The mobile phase consisted of acetonitrile-0.1% phosphoric acid (wt/vol) in Milli Q water (55:45, vol/vol), at a flow rate of 1.0 ml/min. Chromatography was performed at ambient temperature (20 ± 2°C). The concentration of paclitaxel in each sample was determined with a constructed calibration curve. The internal standard was n-hexyl p-hydroxyl benzoic acid (Tokyo Kasei Kogyo Co., Tokyo, Japan). For UV detection, the wavelength was set to 227 nm (29).

Particle Size and Entrapment Efficiency

The particle size of paclitaxel emulsions was determined using a laser light scattering instrument (ELS800, Otsuka Electronics, Osaka, Japan) by the dynamic light scattering method.

The entrapment efficiency of paclitaxel emulsions was taken as the percentage of paclitaxel carried by the emulsions and was determined by two methods; Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) column chromatography and filtration. Emulsions were separated from free paclitaxel in the Sephadex G-100 column using a mobile phase of 5% glucose solution. The amounts of paclitaxel in the free fraction and the emulsion fraction were determined by HPLC as described in the "HPLC" section. With the other method, the emulsions were filtrated through 0.45-μm Ekikrodisc and the concentration of paclitaxel in the filtrate was determined by HPLC. It was found that the amount of paclitaxel determined by the two methods was the same in the preliminary experiment. So the simpler method of filtration was selected in this study.

The entrapment efficiency was calculated according to the following equation:

$$\text{Entrapment efficiency (\%)} = (A_e/A_t) \times 100$$

where A_e is the amount of paclitaxel detected in the emulsion form and A_t is the total amount of paclitaxel added.

Stability

After their preparation, paclitaxel emulsions were stored at 4°C in the dark for 10 days. The stability was assessed by monitoring the changes in the particle size and paclitaxel content of emulsions during the storage period, which were calculated using the following equations:

$$\text{Change of particle size (\%)} = (S_{10}/S_0) \times 100$$

where S_{10} was the size on the tenth day and S_0 was the size on the day of preparation;

$$\text{Change of drug concentration (\%)} = (C_{10}/C_0) \times 100$$

where C_{10} was the concentration in the emulsion form on the tenth day and C_0 was the concentration in the emulsion form on the day of preparation. The paclitaxel concentration was determined by the HPLC method.

Experimental Design and Data Analysis

The amounts of soybean oil (X1), PEG-DSPE (X2), and polysorbate 80 (X3) were selected as causal factors in this study. The values listed in Table I in coded form were transformed to physical units as summarized in Table II. A spherical experimental design for three factors was used to prepare the model formulations (Nos. 1–15). The response of the experimental design was adapted to particle size, entrapment efficiency and stability of paclitaxel emulsions on the tenth day.

The software ANN and ALCORA were used to analyze the results of the experiment (25,27). Levels of causal factors were expressed as concentrations (mg/ml).

RESULTS AND DISCUSSION

In this study, a Cremophor-free oil-in-water emulsion of paclitaxel was developed using all excipients, which are less toxic and present in a number of marketed parenteral products. In a preliminary experiment, the particle size and amount of paclitaxel in the emulsion were measured after storage at 4°C for 1 month using oleic acid, vitamin E and soybean oil as an internal phase and polysorbate 80, PEG-

Table I. Spherical (Nos. 1–15) Experimental Design for Three Factors

Formulation no.	X_1	X_2	X_3
1	−1	−1	−1
2	−1	−1	1
3	−1	1	−1
4	−1	1	1
5	1	−1	−1
6	1	−1	1
7	1	1	−1
8	1	1	1
9	−1.73	0	0
10	1.73	0	0
11	0	−1.73	0
12	0	1.73	0
13	0	0	−1.73
14	0	0	1.73
15	0	0	0

DSPE and cholesterol as emulsifiers or co-emulsifiers with a paclitaxel concentration of 0.3 to 1.8 mg/ml. From the results, soybean oil as the oil phase and 0.3 mg/ml of paclitaxel were more stable than the others (data not shown). Therefore, soybean oil was used as the internal phase. Polysorbate 80 with a polyethylene glycol group chain, a surfactant in common use, was used to conduce a paclitaxel formulation. PEG-DSPE was supposed not only to prolong circulation time *in vivo* but also to form an injectable paclitaxel emulsion. Cholesterol was used to stabilize the surface of droplets together with PEG-DSPE (18). Therefore, cholesterol was used in the same amount (weight) as PEG-DSPE. In the formulations of emulsions of paclitaxel, constant amount of paclitaxel, PEG 400 and glucose (constant volume of 5% glucose solution) was added and the final volume of emulsion was adjusted same by drops of Milli Q water.

Spherical Experimental Design

The data of spherical design in the model formulation are listed in Table III, including particle size, entrapment efficiency and stability of paclitaxel emulsions on the tenth day. The data were called responses and marked as Y1 to Y4, respectively. A significant difference in the value of responses can be observed. The entrapment efficiency (Y1) was 79.2%–101.5%, particle size after preparation (Y2) was 256.6–348.5 nm, change of concentration (Y3) was 19.0–99.3% and change of particle size (Y4) was 79.6–106.6%.

ANN was applied to the prediction of responses (Y1–Y4) as a function of causal factors. 2PE was used for comparing the prediction ability.

Three causal factors corresponding to different levels of soybean oil (X1), PEG-DSPE (X2), and polysorbate 80 (X3)

Table II. Levels of Causal Factors* in Physical Form

	Factor level in coded form				
Factor	−1.73	−1	0	1	1.73
Soybean oil (X_1)	4.8	7	10	13	15.2
PEG-DSPE (X_2)	1.3	2	3	4	4.7
Polysorbate 80 (X_3)	15.2	24	36	48	56.8

* Levels of causal factors were expressed as concentrations (mg/ml).

Table III. Results of Experimental Design

	Y1* (%)	Y2† (nm)	Y3‡ (%)	Y4§ (%)
1	90.8 ± 0.5	276.2 ± 3.4	61.9 ± 1.6	99.7 ± 2.1
2	101.5 ± 0.2	277.6 ± 2.3	95.4 ± 2.6	90.4 ± 1.7
3	95.4 ± 1.9	295.3 ± 1.0	35.2 ± 1.3	83.4 ± 1.2
4	86.2 ± 0.9	283.1 ± 2.1	94.2 ± 1.8	94.9 ± 1.1
5	82.1 ± 1.4	328.8 ± 0.5	55.6 ± 2.6	83.9 ± 1.1
6	92.7 ± 1.0	325.0 ± 3.1	99.3 ± 2.9	91.6 ± 1.0
7	90.0 ± 1.0	289.1 ± 3.4	41.7 ± 1.3	79.6 ± 1.3
8	93.4 ± 0.7	335.0 ± 3.1	97.5 ± 2.1	90.2 ± 1.3
9	92.5 ± 1.5	256.6 ± 2.7	88.2 ± 0.1	106.6 ± 1.5
10	85.3 ± 1.6	314.8 ± 2.8	92.2 ± 2.5	93.8 ± 0.7
11	93.7 ± 0.1	348.5 ± 4.1	90.4 ± 1.6	96.4 ± 2.0
12	87.4 ± 2.6	275.0 ± 1.4	74.7 ± 3.6	104.4 ± 0.9
13	90.1 ± 0.4	268.7 ± 4.0	19.0 ± 0.3	93.1 ± 1.4
14	86.2 ± 0.7	291.1 ± 2.5	94.5 ± 2.0	103.4 ± 1.7
15	79.2 ± 0.8	260.5 ± 2.5	58.6 ± 1.2	105.5 ± 1.3

* Entrapment efficiency after preparation.
† Particle size after preparation.
‡ Change of concentration (10 day/0 day).
§ Change of particle size (10 day/0 day).

were used as each nod of the input layer. Reponses were predicted individually with the different sets of ANN. A set of causal factors and responses [45 data pairs; triplicate measurements for 15 formulations (Table I)] was used as tutorial data for ANN. To optimize the structure of ANN, the simulated annealing technique (30) was applied, employing AIC (Akaike's information criterion) as a standard (27). Results are shown in Table IV, suggesting that 4 or 5 nods in the hidden layers were optimal for the prediction of responses, Y1 and Y3, or Y2, and Y4. This means 16 or 20 unknown parameters (3 input nods, 4 or 5 hidden nods, and 1 output nod; i.e., $3\times4 + 4\times1 = 16$ or $3\times5 + 5\times1 = 20$) were required to fit the weights of ANN for the prediction of responses. On the other hand, 2PE requires an estimation of 10 unknown parameters at most as regression coefficients of the polynomial equation [i.e., 3 parameters for the independent term (X1, X2, and X3), 3 for the square term (X1X1, X2X2, and X3X3), 3 for the interaction term (X1X2, X1X3, and X2X3), and 1 for the constant]. To enable an impartial comparison of the predictive ability of ANN and 2PE, we used the coefficient of determination, which was doubly adjusted with degrees of freedom (R**2). As a result, predicted values of

Table IV. Optimal Structures of ANN for Prediction of Responses

Response	Y1	Y2	Y3	Y4
Optimal ANN*	3/4/1	3/5/1	3/4/1	3/5/1
R†	0.984	0.977	0.999	0.991
R**2‡	0.933	0.881	0.996	0.953
AIC§	−80.8	−128	−180	−84.2

* Optimal structure of ANN; input nods/hidden nods/output nods.
† Multiple correlation coefficient between predicted and experimental response values.
‡ Coefficient of determination doubly adjusted with degrees of freedom.
§ Akaike's information criterion. Smaller values of AIC mean a better approximation. The structure of ANN, which gives the smallest value of AIC, was chosen as the optimum.

Table V. Comparisons of Prediction with Results of Experiments

	Y1 (%)	Y2 (nm)	Y3 (%)	Y4 (%)
ANNp*	101	262	96.2	99.7
ANNr†	96.7	262	97.6	98.1
2PEp‡	95.1	267	95.3	97.8
2PEr§	92.6	358	100	88.1

* Predicted by ANN.
† Data of experiment with the optimal formulation predicted by ANN; X1 = 5.50, X2 = 2.18, X3 = 36.0 (mg/ml).
‡ Predicted by 2PE.
§ Data of experiment with the optimal formulation predicted by 2PE, X1 = 5.45, X2 = 2.18, X3 = 42.8 (mg/ml).

responses based on ANN coincided well with the experimental values (Table V). However, approximations of responses based on 2PE were somewhat poorer (R = 0.895 and R**2 = 0.702 for Y1; R = 0.900 and R**2 = 0.714 for Y2; R = 0.977 and R**2 = 0.973 for Y3; R = 0.783 and R**2 = 0.335 for Y4).

Entrapment Efficiency

The contribution indices (27) of the factors in the formulation were calculated by ANN and are summarized in Table VI. The larger the value is the more important the factor. The entrapment efficiency of 0.6 mg/ml paclitaxel after preparation (Y1) was affected in the order PEG-DSPE (X2) > polysorbate 80 (X3) > soybean oil (X1). The soybean oil was relatively less important to entrapment efficiency. The influence of the main factors of PEG-DSPE and polysorbate 80 on the entrapment efficiency is shown in Fig. 1A. It was evident that the entrapment efficiency changed with the amount and ratio of PEG-DSPE and polysorbate 80. The solubility of paclitaxel in soybean oil is not high. Therefore, this finding

Table VI. Contribution Indices (%) of Factors on the Response in ANN Approximation

	Y1	Y2	Y3	Y4
X1	22.2	32.6	25.3	32.1
X2	40.4	44.2	28.4	32.6
X3	37.5	23.2	46.3	36.2

suggested that most of the paclitaxel might be situated in the surface layer of emulsion droplets due to interaction of the drug with the PEG chain of PEG-DSPE and polysorbate 80 (2–7).

Particle Size

The particle size after preparation (Y2) was influenced in the order PEG-DSPE (X2) > soybean oil (X1) > polysorbate 80 (X3) (Table VI). The response of particle size to PEG-DSPE and soybean oil is depicted in Fig. 1B. A medium amount (about 2.4 mg/ml PEG-DSPE and 8.96 mg/ml soybean oil) of PEG-DSPE produced small particles (about 269 nm) in the preparation without any homogenization and extrusion of membranes. This finding corresponded well with the result that PEG-DSPE incorporated into emulsions formed small (less than 150 nm) particles (18,19).

Stability

The change in concentration was affected mainly by polysorbate 80 (X3) rather than PEG-DSPE (X2) and soybean oil (X1). Polysorbate 80 (X3) was also important to the change in particle size (Y4) (Table VI). Figures 2A and 2B show the relationship between the two main factors and respective responses in terms of the experimental design. A large amount (>41 mg/ml) of polysorbate 80 and large (>4.4

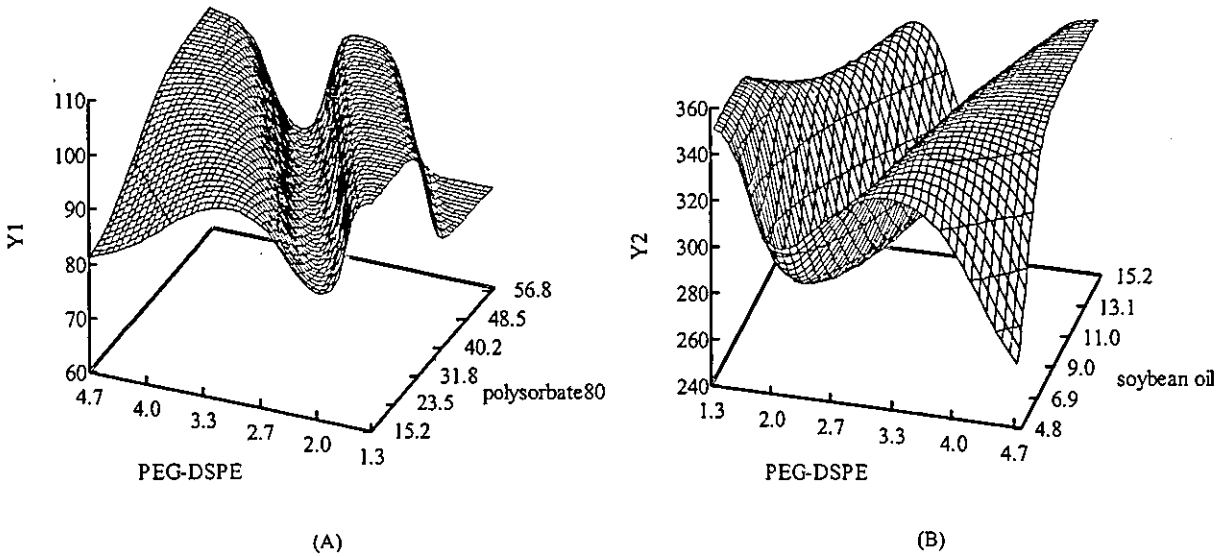

Fig. 1. The effect of PEG-DSPE and polysorbate 80 (mg/ml) on the entrapment efficiency of paclitaxel emulsions (Y1) (A), and the effect of PEG-DSPE and soybean oil (mg/ml) on the particle size of paclitaxel emulsions (Y2) (B).

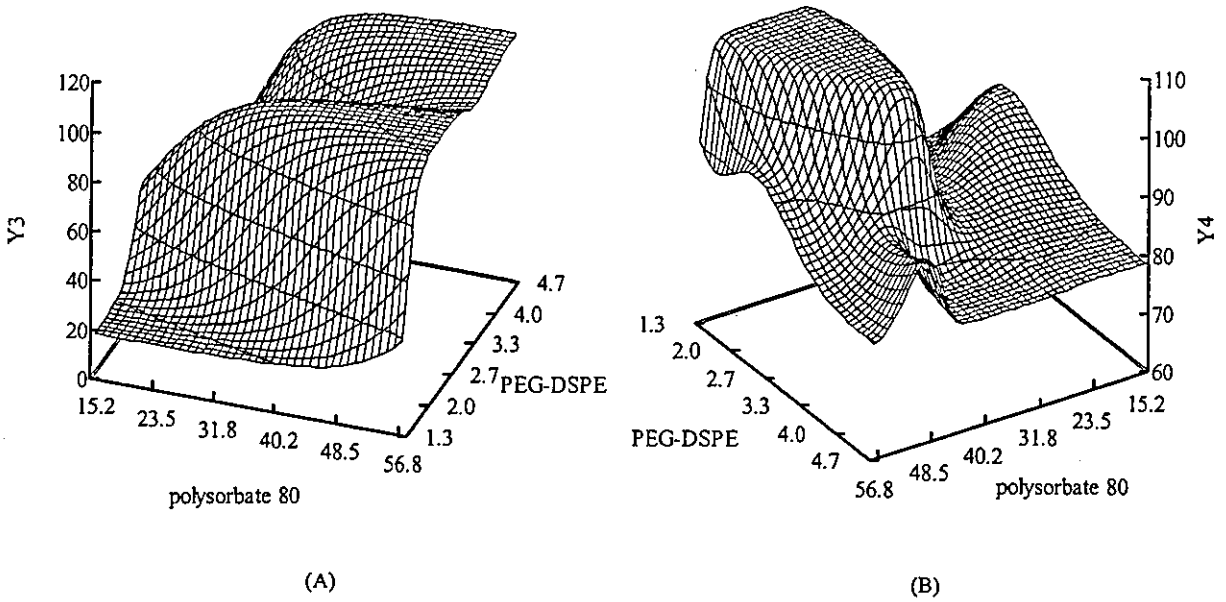

Fig. 2. The effect of polysorbate 80 and PEG-DSPE (mg/ml) on the change in the concentration of paclitaxel emulsions (Y3) (A), and particle size of paclitaxel emulsions (Y4) (B).

mg/ml) or medial (2.1–2.9 mg/ml) amount of PEG-DSPE brought about a more stable concentration of paclitaxel emulsions (Y3) (Fig. 2A), but a large amount (>39 mg/ml) of polysorbate 80 and small amount (<3.3 mg/ml) of PEG-DSPE led to a more stable particle size (Y4) (Fig. 2B). The results indicate that particles modified with a PEG chain proved more stable than those without modification (3).

In some formulations crystals could be observed after ten days and were proved to be paclitaxel by HPLC. And the products of hydrolysis of paclitaxel almost could not be detected by HPLC. Thus it was concluded that the decrease on concentration of paclitaxel was caused mainly by the release of paclitaxel from emulsions and the formation of crystals of paclitaxel in external phase.

Prediction of Optimal Formulation

The software ANN and ALCORA was used to predict the optimal formulation. Two optimal formulations of paclitaxel emulsions were prepared according to the results of predictions by ANN and ALCORA, respectively. The optimal formulations containing 0.6 mg/ml paclitaxel, 30 mg/ml PEG 400, and 50 mg/ml glucose were as follows: by ANN, 5.50 mg/ml soybean oil (X1), 2.18 mg/ml PEG-DSPE (X2), 36.0 mg/ml polysorbate 80 (X3); by 2PE, 5.45 mg/ml soybean oil (X1), 2.18 mg/ml PEG-DSPE (X2), 42.8 mg/ml polysorbate 80 (X3). Then the four parameters of entrapment efficiency after preparation (Y1), particle size after preparation (Y2), change of concentration on the tenth day (Y3) and change of particle size on the tenth day (Y4) were determined. The results are shown in Table V along with the predicted responses. Comparing the predictions and results of experiments, ANN was found to be more suitable for the formulation of paclitaxel emulsions.

Although a number of important formulation parameters remain to be optimized for clinical application, there is indication, at least in paclitaxel PEGylated emulsions, that polysorbate 80 is essential. This information is new with regard to paclitaxel emulsions. PEGylated microemulsions are expected to show long-circulating emulsions *in vivo*. Further research is needed to confirm the efficacy of paclitaxel emulsions *in vivo*.

CONCLUSIONS

We describe here a novel PEGylated emulsion formulation containing paclitaxel that has been optimized by spherical experimental design and ANN for small size (262 nm), high entrapment efficiency (96.7%), and stability. Paclitaxel emulsions were prepared using a very simple procedure and commercialized components for replacement of the toxic Cremophor EL. The contribution indices of each component suggested that PEG-DSPE mainly contributes to the entrapment efficiency and particle size, while polysorbate 80 contributes to the stability of paclitaxel emulsions.

ACKNOWLEDGMENTS

This project was supported in part by a grant from The Promotion and Mutual Aid Corporation for Private Schools of Japan and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. This work was also supported by Hoshi University. There has been a sister-relationship between Hoshi University and the School of Pharmaceutical Sciences, Peking University.

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Journal of Drug Targeting, July 2004 Vol. 12 (6), pp. 373–384

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Polymer Design and Incorporation Methods for Polymeric Micelle Carrier System Containing Water-insoluble Anti-cancer Agent Camptothecin

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A water-insoluble anti-cancer agent, camptothecin (CPT) was incorporated to a polymeric micelle carrier system forming from poly(ethylene glycol)–poly(aspartate) block copolymers. Incorporation efficiency and stability were analyzed in correlation with chemical structures of the inner core-forming hydrophobic blocks as well as with incorporation methods. Among three incorporation methods (dialysis, emulsion and evaporation methods), an evaporation method brought about much higher CPT yields with less aggregation than the other two methods. By the evaporation method, CPT was incorporated to polymeric micelles in considerably high yields and with high stability using block copolymers possessing high contents of benzyl and methylnaphtyl ester groups as hydrophobic moieties. This indicates importance of molecular design of the hydrophobic block chain to obtain targeting using polymeric micelle carriers as well as importance of the drug incorporation method.

Keywords: Polymeric micelle; Camptothecin; Block copolymer; Poly (ethylene glycol); Poly (aspartic acid); Targeting

Abbreviations: PEG, poly(ethylene glycol); P(Asp), poly(aspartic acid); PEG–P(Asp), poly(ethylene glycol)–poly(aspartic acid) block copolymer; PEG–PBLA, poly(ethylene oxide)–poly(β-benzyl L-aspartate) block copolymer; CPT, camptothecin; DMSO, dimethyl sulfoxide; DMF, N,N-dimethylformamide; DBU, 1,8-diazabicyclo [5,4,0] 7-undecene

INTRODUCTION

Polymeric micelles attract much attention as a nano-sized drug carrier system (Kwon and Kataoka, 1995; Kabanov and Alakhov, 1997; Yokoyama, 1998, 2002; Kwon and Okano, 1999; Lavasanifar *et al.*, 2002 and Nishiyama and Kataoka 2003) due to their advantageous characteristics for drug targeting such as very small size in a range of 10–100 nm and high structural stability. Particularly, two advantages are significant for anti-cancer drug targeting to solid tumors; passive targeting ability to solid tumors and applicability to water-insoluble drugs. Polymeric micelles can be delivered selectively to solid tumor sites by a passive targeting mechanism based on the enhanced permeability and retention effect (EPR effect) (Matsumura and Maeda, 1986; Maeda, 2000; Maeda *et al.*, 2002). Long-circulation in the bloodstream is a prerequisite for the EPR effect in order to evade non-selective scavenge at the reticuloendothelial system. Hydrophobic (Illum *et al.*, 1987) and cationic (Takakura and Hashida, 1996) characters of anti-cancer drugs are a promotion factor of this scavenge and can be a serious problem of drug-polymer conjugates because the conjugated drugs are exposed to interact with biocomponents such as proteins and cells. For polymeric micelles, the drug-incorporated inner core is clearly separated from the hydrophilic outer shell that is responsible for interactions with the biocomponents. Therefore, polymeric micelles can attain the long-circulation in the bloodstream (Yokoyama *et al.*, 1991; Kwon *et al.*, 1993) while incorporating a large amount of anti-cancer drugs with cationic and/or hydrophobic characters such as doxorubicin.

The second advantage for solid tumor targeting is excellent applicability to hardly water-soluble or water-insoluble anti-cancer drugs. Since many recently developed potent anticancer drugs such as taxol, camptothecin (CPT) and iressa are water-insoluble, applicability of drug carrier systems to the water-insoluble drug is important. One of the most successful types of drug carriers is a liposome that consists of a lipid bilayer

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ISSN 1061-186X print/ISSN 1029-2330 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10611860412331285251

and an interior aqueous phase which is surrounded by the lipid bilayer. A famous successful example of liposomal carrier systems is Doxil™ that incorporates doxorubicin for tumor targeting. Liposomal carrier systems are favorable for incorporation of hydrophilic and water-soluble drugs in their interior aqueous phase, however, their applications are considerably limited for hydrophobic and water-insoluble drugs because these hydrophobic drugs are loaded in lipid bilayers, not in an interior aqueous phase. It is considered difficult to maintain good targeting properties of the bilayer when this bilayer is loaded with a large amount of the drug. For polymeric micelle systems, a large amount of water-insoluble drug can be incorporated to the hydrophobic inner core with maintaining good properties for targeting owing to the distinctly separated two-phase structure of the hydrophobic inner core and the hydrophilic outer shell.

Anti-cancer drug targeting using polymeric micelle carrier systems was first achieved with doxorubicin (adriamycin) (Yokoyama *et al.*, 1987; Yokoyama *et al.*, 1990; Yokoyama *et al.*, 1991; Yokoyama *et al.*, 1998; Yokoyama *et al.*, 1999). Doxorubicin was conjugated to aspartic acid residues of poly(ethylene glycol)-poly(aspartic acid) block copolymer. This doxorubicin-block copolymer conjugate formed a micellar structure due to its amphiphilic character of the hydrophilic poly(ethylene glycol) block and the hydrophobic doxorubicin-conjugated poly(aspartic acid) block. Furthermore, Doxorubicin was incorporated in the hydrophobic inner core by physical entrapment, and was targeted to solid tumor sites with high selectivity. As a result of this targeting, dramatically enhanced *in vivo* anti-tumor effects were obtained (Yokoyama *et al.*, 1999). This doxorubicin polymeric micelle system is now in the phase II clinical trail starting in autumn of 2003 at the National Cancer Hospital, Japan. In this system, doxorubicin was chemically conjugated to the aspartic acid residue of the block copolymer and this conjugated doxorubicin worked as a hydrophobic species for micelle formation and physical incorporation of further doxorubicin molecules (Yokoyama *et al.*, 1998). This physically incorporated doxorubicin expressed selective anticancer activity by being targeted to solid tumor sites (Yokoyama *et al.*, 1998, 1999). Therefore, the chemically conjugated doxorubicin did not play a role of anticancer effect. It is preferable to design targeting systems without using drug molecules as a biologically inactive hydrophobic species to suppress production cost as well as to allow much wider choice for the hydrophobic species. The wider choice of simpler hydrophobic chemical structures (e.g. phenyl ring and acyl chain) than drug molecules is considered to be very advantageous for optimization of the influencing factors for targeting (e.g. micelle stability, size and drug release rate).

On the other hand, applications of the polymeric micelle carrier systems to other anti-cancer drugs are now actively studied with various drugs such as cisplatin (Nishiyama *et al.*, 1999; Nishiyama and Kataoka, 2003), taxol, methotrexate (Li and Kwon, 2000) and KRN-5500 (Matsumura *et al.*, 1999; Mizumura *et al.*, 2002). For water-insoluble anti-tumor drugs such as taxol and KRN-5500, the drug incorporation is clinically beneficial in evasion of the use of toxic substances that dissolve these water-insoluble drugs for intravenous injection. This is exemplified by cremophor EL and ethanol used to dissolve taxol in an aqueous medium. Since polymeric micelles are generally much less toxic than these substances, the drug solubilization by incorporation to polymeric micelles can decrease toxic side effects that result from these substances. In fact, incorporation of KRN-5500 to polymeric micelles diminished vascular and pulmonary toxicities resulting from organic solvents and surfactants that were used in a conventional formulation (Matsumura *et al.*, 1999; Mizumura *et al.*, 2002). However, targeting of KRN-5500 to tumors has not yet been done. In this paper, block copolymer syntheses and drug incorporation to polymeric micelles are reported for targeting of CPT (Potmesil, 1994; Wall and Wani, 1995a, b) that is a mother compound of topotecan and CPT-11, which are very potent and recently approved as anticancer-drugs.

For tumor targeting with the polymeric micelles, several physical and physico-chemical factors were known to be important such as block length (Kwon *et al.*, 1993; Yokoyama *et al.*, 1993) and drug content (Yokoyama *et al.*, 1998). Even though chemical structures of the inner core-forming block were partially examined for tumor targeting (Yokoyama *et al.*, 1998), no systematic study has been done to establish a strategy of polymeric micelle design for various kinds of anti-cancer drugs without using drug itself as a hydrophobic species for micelle formation and drug incorporation as done in the doxorubicin system (Yokoyama *et al.*, 1998, 1999). For polymeric micelle design, influencing factors on targeting may be hydrophobic strength, rigidity/flexibility, $\pi-\pi$ interactions (cohesive interactions between π electron rich molecules like benzene) of the hydrophobic inner core, block lengths of polymers and drug contents as well as steric space in the hydrophobic block for drug incorporation. This study aims to reveal correlations between these factors and drug targeting. Additionally, only a little study has been done for optimization of drug incorporation procedures to polymeric micelles (Kwon *et al.*, 1994; Kohori *et al.*, 2002). This study also aims to analyze effects of drug incorporation methods.

In this paper, various poly(ethylene glycol)–poly (aspartate ester) block copolymers were synthesized by varying chemical structures of hydrophobic ester groups and their incorporation behaviors (loading efficiency and incorporation stability) of CPT were systemically analyzed by three incorporation methods.

MATERIALS AND METHODS

Chemicals

(s)-(+)-CPT was purchased from Aldrich Chem. Co., Milw., WI, USA. 1,8-Diazabicyclo [5,4,0] 7-undecene

(DBU) was purchased from Wako Pure Chemicals, Tokyo, Japan. N,N-Dimethylformamide (DMF) was dried with molecular sieve 4A, followed by distillation under reduced pressure. Poly(ethylene glycol)–poly(β-benzyl L-aspartate) block copolymer (PEG–PBLA) was synthesized by ring-opening polymerization of benzyl L-aspartate N-carboxy anhydride from a terminal primary amino group of α-methyl-ω-aminopoly(oxyethylene) as described in our previous paper (Yokoyama *et al.*, 1992). Codes of PEG–PBLA were based on molecular weight of the poly(ethylene glycol) chain (PEG) and polymerization degree of BLA units; for example, 5-27 means a block copolymer composed of the PEG block of M.W. of 5000 and the PBLA block possessing 27 units of BLA. PEG–PBLA 5-27 and PEG–PBLA 12-26 were used. Other chemicals were of reagent grade and used as purchased.

Alkaline Hydrolysis of PEG–PBLA Block Copolymer

Poly(ethylene glycol)–poly(aspartic acid) block copolymer (PEG–P(Asp)) was obtained by alkaline hydrolysis of poly(ethylene oxide)–poly(β-benzyl L-aspartate) block copolymer (PEG–PBLA) as reported previously (Yokoyama *et al.*, 1992). Briefly, PEG–PBLA was dispersed in a measured volume of 0.5 N NaOH that contained 1.5 mol equivalents NaOH to the benzyl aspartate residue of PEG–PBLA. With stirring at room temperature, the solution became homogeneous approximately in 15 min. Then, 6 N HCl was added (10 mol equivalents of HCl to the benzyl aspartate residue) to the solution and this solution was dialyzed against 0.1 N HCl, followed by dialysis against distilled water using a Spectrapor 6 dialysis membrane (molecular weight cut-off: 1000). PEG–P(Asp) block copolymer was obtained by freeze-drying of the dialyzed solution. The molecular weight of the PEG block was 5000 and the average number of the benzyl aspartate units were 27. It was known that main chain degradation did not occur by this hydrolysis procedure. It was also known that approximately 75% of the aspartic acid residues of the P(Asp) chain were converted to the β-amide form in this procedure (Yokoyama *et al.*, 1992).

Esterification of Aspartic Acid Residue of PEG–P(Asp)

PEG–(Asp) block copolymer was dissolved in DMF and added by a halogen compound (benzyl bromide, n-butyl bromide, n-butyl iodide, lauryl bromide, or 2-(bromomethyl)naphthalene) and DBU. The reaction mixture was stirred at 50°C for 14.6–18.1 h. Then, the reaction mixture was poured into a ten-fold volume excess diethyl ether and the precipitated polymer was collected by filtration, followed by washing with diethyl ether and drying. In order to remove contaminating DBU in polymer products, polymers were dissolved in DMSO and added by 6 N HCl that was much excess equivalents to the aspartic acid residue of the block copolymer. Then, this solution was dialyzed against distilled water and followed by freeze-drying.

Incorporation of Camptothecin (CPT) to Polymeric Micelles

CPT incorporation to polymeric micelles was examined by three methods; dialysis, emulsion and evaporation method.

(1) Dialysis Method

A block copolymer (5.0 mg) and CPT (0.5 mg) was dissolved in 1.0 ml of dimethyl sulfoxide (DMSO). This solution was dialyzed against distilled water using a dialysis membrane (Spectrapor 4, molecular weight cut-off: 12,000–14,000). After overnight dialysis, the solution in the dialysis membrane was collected and filtrated through a No. 1, 2 or 5A filter (Toyo Roshi, Tokyo, Japan).

(2) Emulsion Method

A block copolymer (5.0 mg) and CPT (0.5 mg) was dissolved in 0.5 ml of methylene chrolide(CH_2Cl_2). This solution was poured into 2.0 ml of distilled water with vigorous stirring using a magnetic stirrer. Methylene chloride was evaporated by overnight stirring at room temperature. The remaining aqueous solution was filtrated through a No. 5A filter.

(3) Evaporation Method

A block copolymer and CPT was dissolved in chloroform in a glass tube. This solution was stirred by a magnetic stirrer in a N_2 gas flow. Chloroform was completely evaporated at room temperature. Distilled water was added in this glass tube, followed by sonication for 2 min using a probe type sonicator model VC 100 (Sonics & Materials Inc., Connecticut, USA) equipped with a standard 6 mm probe in a cycle of sonication for 0.5 s and standby for 0.5 s at 4°C. The obtained solution was treated by the following two ways.

1. The solution was filtrated using a 5A filter paper.
2. The solution was centrifuged at 3900 rpm for 10 min. The supernatant was collected and filtrated through a nylon membrane filter with 1 μm pore (Puradisc 25NYL,6751-2510, Whatman, Clifton, New Jersey, USA).

General Procedure

^{1}H-NMR, UV-VIS, HPLC, and light scattering measurements ^{1}H-NMR were obtained with 1% solutions in 6D-DMSO added by 3% trifluoroacetic acid using a Varian UNITY INOVA NMR spectrometer at 400 MHz. Gel-filtration chromatography was carried out using a Tosoh HPLC system SC-8010 equipped with a Tosoh TSKgel G3000PWXL column. Distilled water was used as eluent

at a flow rate of 1.0 ml/min at 40°C. Fifty microlitter of sample solutions were injected into the column. The detection was performed by absorption at 351 nm using a Tosoh UV-8010 detector.

The CPT amount incorporated to polymeric micelles was determined by UV–VIS absorption spectroscopy. This measurement was done by two ways; in distilled water and in a mixture of DMSO and distilled water (volume ratio of DMSO/water = 9 : 1). In distilled water, the CPT amount was calculated from a value obtained by subtracting absorbance at 600 nm from absorbance at 370 nm to compensate background raise due to the presence of large aggregates. The ε (molar absorptivity) value of 19,900 from Merck Index twelfth edition was used. For determination in the DMSO/water mixture, the CPT amount was calculated from a value obtained by subtracting absorbance at 600 nm from absorbance at 365 nm using $\varepsilon = 20,860$ (This value was obtained with CPT solutions in this mixture.). For Fig. 4, UV–VIS absorption spectra was recorded in absorbance between 0.30 and 0.91 at a peak around 370 nm and their peak heights were normalized in order to compare background between 430 and 600 nm in which CPT or block copolymers have no absorption.

Proportions of a lactone form and an open form of CPT incorporated to polymeric micelles were determined by reversed-phase HPLC (Shenderova *et al.*, 1999), which allows separation of the two CPT forms within a single chromatographic run. CPT was extracted from polymeric micelles by adding DMSO and diluted with an aqueous buffer described below. The HPLC system for this determination consisted of a JASCO HyPer LC-800 system (Tokyo, Japan) at a flow rate of 1.0 ml/min at 40°C. A Waters C_{18} 3.9 × 150 mm reverse phase column was used. The mobile phase was composed of 23% acetonitrile and 77% aqueous buffer (0.1 M KH_2PO_4, 0.5 mM tetrabutylammonium dihydrogen phosphate and 0.4 mM triethyl amine pH = 6). The detection was performed using a fluorescence detector with an excitation wavelength of 360 nm and emission wavelength of 430 nm.

Laser scattering measurements were carried out using a Photal dynamic laser scattering spectrometer DLS-7000 (Otsuka Electronics Co. Ltd., Tokyo, Japan) with a neon laser beam.

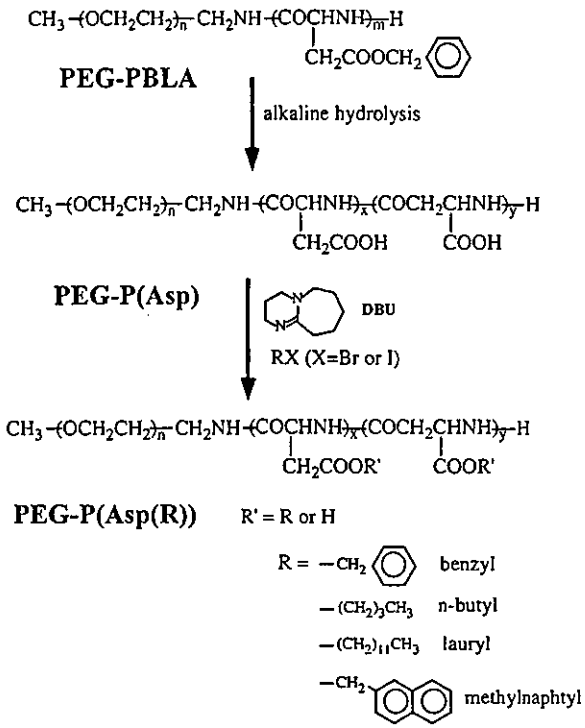

FIGURE 1 Synthetic scheme of block copolymers.

RESULTS

Esterification of Aspartic Acid Residue of PEG–P(Asp)

Various poly(ethylene glycol)–poly(aspartate ester) block copolymers were successfully synthesized from PEG–P(Asp) 5-27 in a synthesis scheme shown in Fig. 1. The obtained block copolymers are coded by a name of hydrophobic group and esterification degree as summarized in Table I. For example, benzyl-44 means 44% of the aspartic acid residue was converted to benzyl

TABLE I Esterification of PEG–P(Asp) block copolymer

		Halogen		DBU				
Code	PEG–p(Asp) 5-27 (mg)	Species	mg (equivalent*)	mg (equivalent*)	DMF (mL)	Reaction† time(h)	Yield (mg)	Esterification‡ (%)
Benzyl-25	198.6	Benzyl-Br	51.0(0.51)	57.0(0.56)	2.5	16.2	144.0	25
Benzyl-44	500.5	Benzyl-Br	236.8(0.83)	212.2(0.83)	5.0	16.0	520.0	44
Benzyl-61	200.0	Benzyl-Br	116.9(1.02)	103.9(1.02)	2.7	14.8	160.2	61
Benzyl-69	200.2	Benzyl-Br	227.0(1.98)	202.8(1.99)	2.0	14.6	154.1	69
n-butyl-47	200.8	n-butyl-Br	91.9(1.00)	101.4(0.99)	2.0	14.8	146.5	47
n-butyl-66	200.0	n-butyl-I	123.3(1.00)	101.4(1.00)	2.0	14.8	157.2	66
Lauryl-45	200.6	Lauryl-Br	165.8(0.99)	102.1(1.00)	2.0	15.6	143.7	45
Methylnaphtyl-53	200.4	Methylnaphtyl-Br	146.6(0.99)	101.2(0.99)	2.0	18.1	188.8	53
Methylnaphtyl-64	1400.1	Methylnaphtyl-Br	1034.7(1.00)	709.6(1.00)	14.0	15.3	1684.7	64

*Mol equivalents to aspartic acid residue of PEG–P(Asp) block copolymer.
†Reaction temperature was 50°C.
‡With respect to aspartic acid residue measured by ^{1}H-NMR spectra.

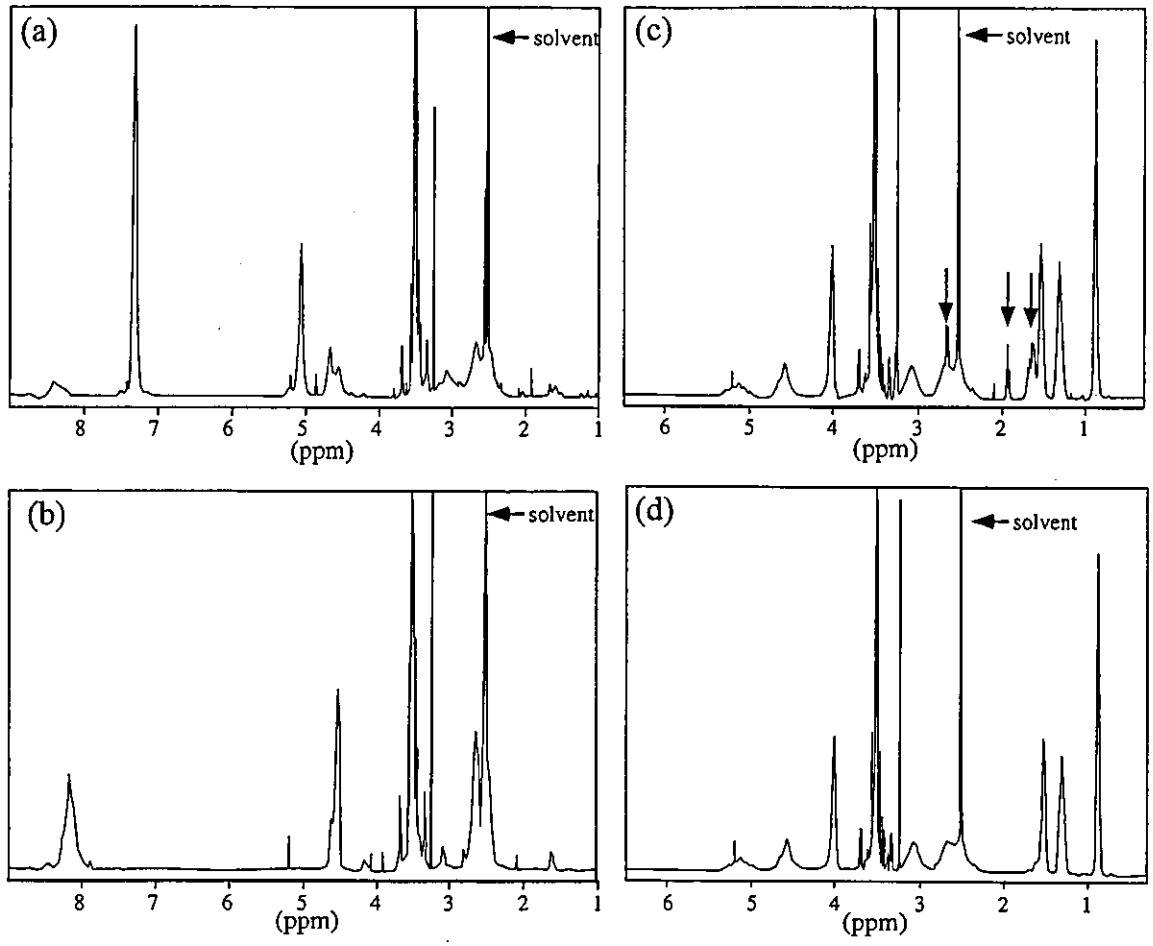

FIGURE 2 Proton NMR spectra of block copolymers. (a) benzyl-44, (b) PEG–P(Asp) 5-27, (c) n-butyl-47 without an acid treatment and (d) n-butyl-47 after an acid treatment. These spectra were obtained with 1% solutions in 6D-DMSO added by 3% trifluoroacetic acid.

aspartate residue. Ester formation at the side chain of the P(Asp) block was confirmed by ^{1}H-NMR spectrum measurements. As shown in Fig. 2(a), benzyl ester formation was observed for benzyl-44 block copolymer by methylene protons (CH_2) and phenyl protons (C_6H_5) of the benzyl ester group at 5.1 and 7.3 ppm, respectively. These protons were absent in a spectrum of PEG–P(Asp) block copolymer as shown in Fig. 2(b). No benzyl bromide was contaminated in the obtained polymer because no peak of methylene protons (CH_2) of benzyl bromide was observed at 4.50 ppm. From a peak area ratio between the methylene protons of the benzyl ester group and methylene protons of the PEG block at 3.50 ppm, esterification degree was determined. As summarized in Table I, the esterification degree was well controlled in a range of 25–69% by adjusting molar ratio of benzyl bromide and DBU to the aspartic acid residue of the PEG–P(Asp). By increasing the molar ratios up to 2.0, a high benzyl ester content such as 69% was obtained.

n-Butyl ester was also successfully introduced to PEG–P(Asp). By using 1.0 equivalent mol of n-butyl bromide, 44% of the aspartic acid residues were converted to n-butyl aspartate. A ^{1}H-NMR spectrum of this butyl case is shown in Fig. 2(c). Butyl ester content was determined by a peak area ratio between the terminal methyl protons (CH_3) at 0.87 ppm of the butyl group and the PEG methylene protons. This ester content was lower than that of the benzyl ester case that provided 61% with 1.0 equivalent mol benzyl bromide. This difference was considered to result from higher reactivity of benzyl bromide than butyl bromide. No contaminated n-butyl bromide was seen in the NMR spectrum. However, a considerable amount of DBU was seen as shown by arrows in the NMR chart. DBU is basic and is expected to be contaminated in the product by interacting with a carboxyl group of the aspartic acid residue of the block copolymer. In order to remove this contaminated DBU, 52.5 mg of the obtained polymer was dissolved in 0.5 ml of DMSO, added by 25 μl of 6 N HCl and dialyzed against distilled water, followed by freeze-drying. After this acid treatment, the contaminated DBU was completely removed from the polymer as shown by disappearance of the DBU peak in Fig. 2(d). No change in the ester content by this acid treatment was confirmed by ^{1}H-NMR spectroscopy. Therefore, this acid treatment was done for all block copolymers to remove

DBU from products. The n-butyl ester content was increased from 47 to 66% by using n-butyl iodide on behalf of benzyl bromide.

Both lauryl and methylnaphtyl ester were also successfully introduced to the aspartic acid residue of the block copolymer using corresponding bromide compounds as well as benzyl and n-butyl cases. Block copolymers were obtained with 45% of lauryl ester and 53 and 64% of methylnaphtyl ester coded as lauryl-45, methylnaphtyl-53 and methylnaphtyl-64, respectively. A considerable difference in the esterification degree was found between methylnaphtyl-53 and methylnaphtyl-64 even though both runs were obtained with the same halogen and DBU molar ratios to the aspartic acid residue of PEG–P(Asp). This is considered to result from a difference in loss amounts during precipitation and purification processes, particularly due to relatively large loss in a small scale of synthesis of methylnaphtyl-53 case. All obtained block copolymers were found to be well dissolved in DMSO, chloroform and methylene chloride and therefore, incorporation of CPT to polymeric micelles forming from these block copolymers were examined by three methods using these three organic solvents.

Incorporation of Camptothecin (CPT) to Polymeric Micelles

CPT incorporation to polymeric micelles was examined by three methods: dialysis, emulsion and evaporation method.

(1) Dialysis Method

Table II summarizes results of CPT incorporation to polymeric micelles by a dialysis method. After dialysis, a precipitate was observed in a transparent solution. After filtration, the precipitate was removed and only very small amounts of CPT was found in the transparent solutions. All examined block copolymer was revealed to provide very small yields of CPT less than 5% by measuring absorbance at 370 and 600 nm ($\varepsilon = 19,900$). Absorbance at 600 nm was subtracted from absorbance at 370 nm to compensate possible background raise due to the presence of large aggregates. This quantification method is considered to a little underestimates incorporated CPT amounts since the ε value was obtained in organic solvent, not in distilled water where measurements in this experiment was done. This underestimation was found to be less than 50% by comparing data with measurements in a mixture of DMSO/water = 9 : 1. Therefore, the fact of the low incorporation yields by the dialysis method was not changed even by this underestimation of CPT.

TABLE II CPT incorporation to polymeric micelles by dialysis method

Run	Polymer	Incorporation conditions	Yield of CPT/μg (%)*
1	PEG–PBLA (5-27)	Standard†,	4 (1)
2	PEG-PBLA (12-27)	0.5 ml DMSO‡	8 (2)
3	Benzyl-25	Standard	5 (1)
4	Benzyl-61	Standard	8 (2)
5	Benzyl-69	Standard	10 (2)
6	n-butyl-47	Standard	6 (1)
7	Lauryl-45	Standard	11 (2)
8	Methylnaphtyl-53	Standard	18 (4)
9	Methylnaphtyl-53	0.5 ml DMSO‡	8 (2)
10	PEG–PBLA (5-27)	0.5 ml DMSO + sonication	228 (45)
11	PEG–PBLA (12-27)	0.5 ml DMSO + sonication	187 (37)
12	Methylnaphtyl-53	0.5 ml DMSO + sonication	37 (7)

*Estimated by (absorbance at 370 nm)—(absorbance at 600 nm) in water using $\varepsilon = 19,900$.
†Polymer 5.0 ± 0.1 mg, CPT 0.50 mg, DMSO 1.0 mL
‡Only volume of DMSO was different from the standard conditions.

Among the examined block copolymers, methylnaphtyl-53 provided the largest CPT yield. By reducing an initial DMSO amount, the CPT yield was lowered from 4 to 2%. PEG–PBLA 12-26 provided the same CPT yield in the reduced DMSO conditions as methylnaphtyl-53. In order to raise the CPT yield, dialysis conditions such as amounts of block copolymer and CPT, amount of DMSO and organic solvent (DMF and N,N-dimethylacetamide) were varied using PEG–PBLA 12-26. No increase in the CPT yield, however, was obtained (data not shown). These low incorporation yields were considered to result from CPT precipitation alone or with block copolymers during the dialysis procedure.

When sonication was applied after the dialysis process, considerable increases in the CPT yield were observed, as shown by runs 10–12 in Table II. The increase was most prominent for PEG–PBLA 5-27 and its CPT yield reached 46%. The obtained solution, however, was cloudy due to the presence of aggregates that were much larger than polymeric micelles with 50–100 nm diameter as shown by UV–VIS absorbance spectrum. Figure 3 shows the spectra whose peak heights around 370 nm were normalized. In Fig. 3(a), considerable absorbance was observed for run 10 of Table II in a range from 430 to 600 nm. Since no chemical species were present for absorption in this range, this absorption resulted from light scattering of large aggregates. It is considered that absorption at 370 nm substantially included scattering phenomenon of the large aggregates due to a significant raise of absorbance from 600 to 430 nm. Therefore, the calculated CPT amount of run 10 in Table II was overestimated considerably.

All these results indicate that CPT incorporation was not obtained with high CPT yields and high solution clarity by the dialysis method.

(2) Emulsion Method

The obtained solution by the emulsion method were all cloudy due to the presence of aggregates larger than polymeric micelles. This cloudiness of the solutions was confirmed by considerably high values of absorbance at 600 nm. Incorporated CPT amount was calculated by subtracting absorbance at 600 nm from absorbance at 370 nm. A spectrum of benzyl-61 (run 5 in Table III) is shown in Fig. 3(b). Since base line from 430 to 600 nm

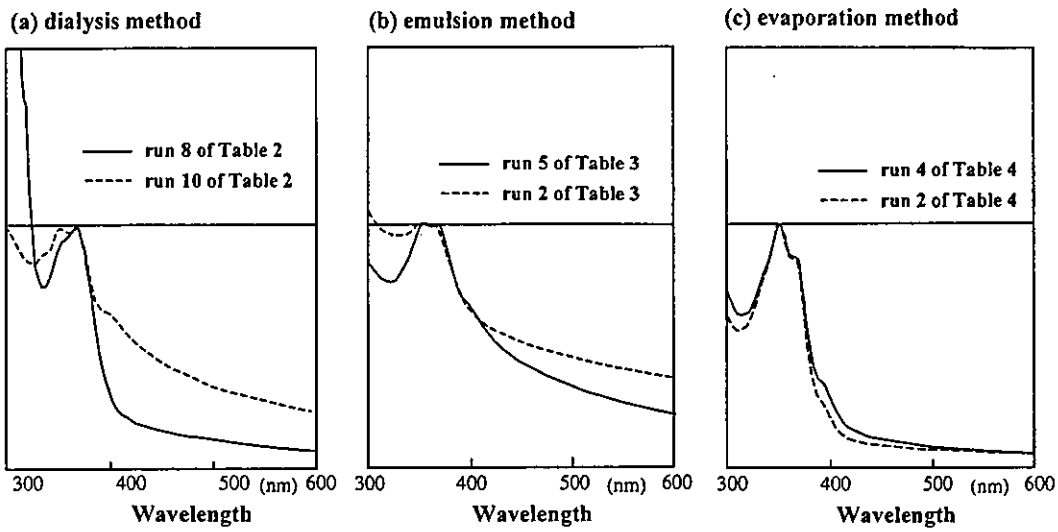

FIGURE 3 UV–VIS absorption spectra of aqueous polymeric micelles containing CPT. Peak heights of around 370 nm were normalized. (a) obtained by dialysis method, solid line: run 8 of Table II, dotted line: run 10 of Table II, (b) obtained by emulsion method, solid line: run 5 of Table III, dotted line: run 2 of Table III and (c) obtained by evaporation method, solid line: run 4 of Table IV, dotted line: run 2 of Table IV.

was not flat (CPT does not possess absorbance between 430 and 600 nm), the calculated CPT amount may be overestimated even after subtracting absorbance at 600 nm. Although the CPT incorporation amounts seem to be higher than those obtained by the dialysis method, incorporation to polymeric micelles was considered unsuccessful due to the presence of the larger aggregates. Sonication did not improve the CPT incorporation in this emulsion method. As shown in Table III, only a small increase in the incorporated CPT amount by sonication was seen for PEG–PBLA 5-27 (comparison between run 1 and 11 in Table III). The large aggregate was also present in the sonicated sample and the base line of UV–VIS spectrum was also high (data not shown). Even when incorporation conditions were varied in CH_2Cl_2 volume (from 0.25 to 1.0 ml), organic solvent ($CHCl_3$), CPT amount (250 μg) and aqueous medium (containing NaCl or acetic acid), the CPT incorporation behavior was not improved (data not shown). As a result, the CPT incorporation to polymeric micelles was not successful by the emulsion method.

TABLE III CPT incorporation to polymeric micelles by emulsion method

Run	Polymer	Incorporation conditions	Yield of CPT/ μg (%)*
1	PEG–PBLA (5-27)	Standard†	130 (26)
2	PEG–PBLA (5-27)	CH_2Cl_2 1.00 ml‡	151 (30)
3	PEG–PBLA (12-27)	Standard	94 (19)
4	Benzyl-25	Standard	81 (16)
5	Benzyl-61	Standard	184 (36)
6	Benzyl-69	Standard	68 (14)
7	n-Butyl-47	CH_2Cl_2 1.00 ml‡	84 (17)
8	n-Butyl-66	Standard	112 (23)
9	Lauryl-45	Standard	85 (17)
10	Methylnaphtyl-53	Standard	113 (23)
11	PEG–PBLA (5-27)	Sonication¶	186 (37)

* Estimated by (absorbance at 370 nm) − (absorbance at 600 nm) in water using $\varepsilon = 19,900$.
† Polymer 5.0 ± 0.1 mg, CPT 0.50 mg, CH_2Cl_2 0.50 ml, distilled water 2.0 ml.
‡ Only volume of CH_2Cl_2 was different from the standard conditions.
¶ Incorporation conditions were the same as those of standard except sonication.

(3) Evaporation Method

CPT incorporation was examined with PEG–PBLA 12-26 by an evaporation method. After compete evaporation of chloroform from the mixed solution of a block copolymer and CPT, 2.0 ml of distilled water was added and the solution was sonicated. The obtained solutions were revealed to contain large amounts of CPT after filtration through a 5A filter. As summarized in Table IV, the CPT yield determined by absorption in water (this assay method was the same as done in Table III) reached approximately 60% for run 1 and 2 with initial 0.25 and 0.50 mg CPT, respectively. These solutions were very transparent as confirmed by low background from 430 to 600 nm in UV–VIS absorption spectrum as shown in Fig. 3(c). An addition of n-hexane or CH3CN to chloroform did not change the CPT yield so much for PEG–PBLA 12-26. In order to measure the incorporated CPT amounts correctly, UV–VIS absorption spectra were measured in a mixture of DMSO and distilled water (DMSO/water = 9 : 1), since CPT was considered to be completely released from the micelle inner core in this mixed solvent. Absorption coefficient of CPT was obtained in this mixture. When the CPT amount was determined in this mixed solvent, it was found that CPT was incorporated quantitatively in polymeric micelles in a range of experimental error. This shows the evaporation method was much better method than the dialysis and emulsion methods for CPT incorporation to the polymeric micelles and that CPT was quantitatively incorporated by this method.

TABLE IV CPT incorporation to PEG–PBLA (12-26) polymeric micelles by evaporation method*

Run	CPT amount (mg)	Solvent	Yield of CPT/ μg (%) Method A†	Method B‡
1	0.25	$CHCl_3$ 0.25 ml	146 (58)	276 (109)
2	0.50	$CHCl_3$ 0.5 ml	297 (60)	544 (109)
3	0.51	$CHCl_3$ 0.5 ml + n-hexane 0.10 ml	350 (69)	540 (107)
4	0.50	$CHCl_3$ 0.5 ml + CH_3CN 0.5 ml	281 (56)	536 (108)

*Polymer 5.0 ± 0.1 mg.
†Method A: measured in distilled water and estimated by (absorbance at 370 nm) − (absorbance at 600 nm) using $\varepsilon = 19,900$.
‡Method B; measured in distilled DMSO/distilled water = 9:1 and estimated by (absorbance at 365 nm) − (absorbance at 600 nm) using $\varepsilon = 20,860$.

Characterization of Polymeric Micelles Incorporating CPT

Camptotecin (CPT) was incorporated to polymeric micelles forming from various block copolymers by the evaporation method and the obtained polymeric micelles were characterized. As summarized in Table V, 11 block copolymers were examined. All the polymers gave clear solutions after sonication. The CPT yields, however, were lower than those in Table IV due to a difference in filtration/centrifugation procedures. All the samples in Table V were measured spectroscopically after centrifugation and filtration through 1 μm filter. This more complete procedure to remove larger aggregates was considered to lower the CPT yield. The CPT yields were relatively low for methylnaphtyl–substituted block copolymer (methylnaphtyl-44) and low substitution contents of benzyl ester cases (benzyl-25, -44 and -61).

These obtained polymeric micelles containing CPT were characterized by two methods; gel-permeation chromatography (GPC) and laser light scattering. Several GPC charts are shown in Fig. 4 and their numerical results are summarized in Table V. All the samples were observed to form polymeric micelle structures by micelle peaks near the gel-exclusion volume that was much smaller than those corresponding molecular weights of the block copolymers (approximately 10,000–20,000). Micelle-forming behavior, however, turned out to be dependent on block copolymer structures by different peak area and elution volume. Peaks at the micelle position were very small for PEG–PBLA 5-27 and 12-26. The peak was detected by absorption at 341 nm, indicating that peak area stands for the incorporated CPT amount. These two PEG–PBLA block copolymers provided very small values of ratios of peak area / [CPT] of the injected sample such as 3.5 and 1.5. This means that most CPT was adsorbed to the GPC column by hydrophobic interactions due to unstable packaging of CPT in the micelles. The benzyl-25 micelles provided a little larger ratio of the peak area/[CPT], 6.1, however, peaks of later elution volume (5.15 and 6.57 ml) were accompanied. It is considered that a low content of benzyl ester resulted in loose packaging of the hydrophobic inner core and that such loosely packed inner core allowed hydrophobic interactions with the column to a considerable degree. By these interactions, elution could be delayed. A weight-average diameter of benzyl-25 was 389 nm, indicating the substantial presence of large aggregates. As the benzyl content increased, micelle peaks became larger and their elution became earlier. For benzyl-61, the elution volume was 3.62 ml and its ratio of peak area/[CPT] was 49.0. This ratio was also high for benzyl-69. Diameter of benzyl-61 and benzyl-69 micelles were 35 and 64 nm, respectively. These diameters

TABLE V CPT incorporation to polymeric micelles by evaporation method

Run	Polymer	CPT amount (mg)	CPT amount (mg)	Solvent†	Yield of CPT/μg (%)‡	GPC analysis* Elution volume (mL)	GPC analysis* Peak area¶ [CPT]	Micelle diameter§ (nm)
1	PEG–PBLA (5-27)	7.6	0.75	$CHCl_3$	507 (68)	3.33	1.5	80
2	PEG–PBLA (12-27)	7.5	0.75	$CHCl_3$	548 (73)	3.38	3.5	n.d.‖
3	Benzyl-25	7.5	0.77	$CHCl_3$	285 (37)	3.30,5.15	6.1,16.9	389
4	Benzyl-44	7.6	0.75	$CHCl_3$	154 (21)	3.60,4.78	23.0,15.8	n.d.‖
5	Benzyl-61	7.6	0.75	$CHCl_3$	275 (37)	3.62,4.30	49.0,38.1	35
6	Benzyl-69	7.5	0.75	$CHCl_3$	420 (56)	3.42	47.6	64
7	n-butyl-47	7.4	0.75	$CHCl_3$	464 (62)	3.30,4.42	14.7,3.6	>1000
8	n-butyl-66	7.6	0.75	$CHCl_3$	498 (66)	3.60,4.35	17.6,5.2	n.d.‖
9	Lauryl-45	7.5	0.75	$CHCl_3$	550 (74)	4.02	11.2	n.d.‖
10	Methylnaphtyl-53	7.6	0.75	$CHCl_3$	338 (45)	3.53	49.9	n.d.‖
11	Methylnaphtyl-64	7.4	0.75	$CHCl_3$	394 (52)	3.63	54.3	39
12	Methylnaphtyl-64	7.5	0.75	$CHCl_3 + CH_3CN$	570 (76)	3.67	59.4	n.d.‖
13	Methylnaphtyl-64	7.5	1.50	$CHCl_3$	734 (49)	3.62	50.7	n.d.‖
14	Methylnaphtyl-64	7.6	1.50	$CHCl_3 + CH_3CN$	1079 (72)	3.68	56.7	n.d.‖

*Column: tosoh TSKgel G3000PWXL, eluent: distilled water, temp.: 40°C
†$CHCl_3$: 1.0 ml per 1.0 mg of CPT, CH_3CN: 0.67 ml per 1.0 mg of CPT
‡Measured in DMSO/distilled water = 9:1 and estimated by (absorbance at 365 nm) − (absorbance at 600 nm) using $\varepsilon = 20,860$.
¶A ratio of peak area/concentration of CPT (μg/ml).
§Weight-average diameter measured by dynamic light scattering.
‖n.d.: not determined.

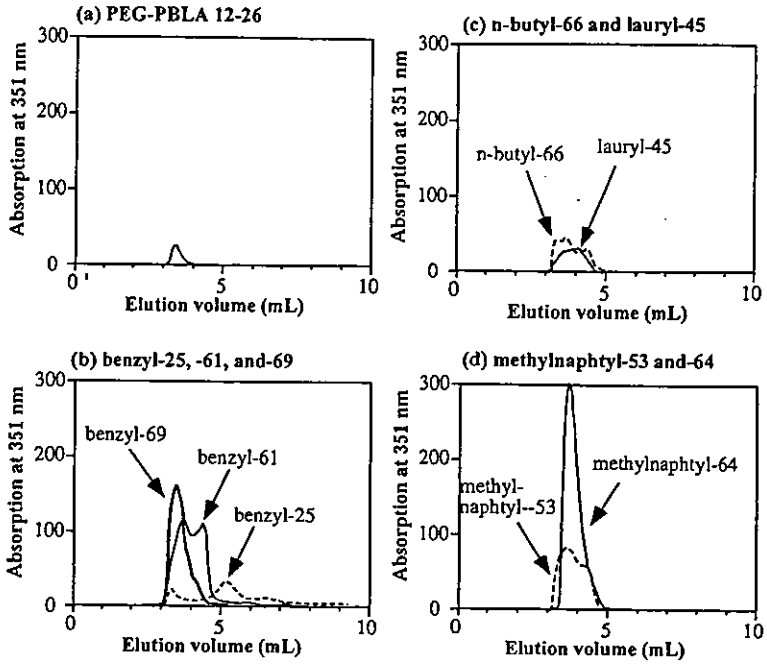

FIGURE 4 Gel-filtration chromatograms of polymeric micelles incorporating CPT. (a) PEG–PBLA 12-26 (run 2 of Table V), (b) benzyl-25, -61 and -69 (run 3, 5 and 6 of Table V, respectively) (c) n-butyl-66 and lauryl-45 (run 8 and 9 of Table V, respectively), (d) metylnaphtyl-53 and-64 (run 10 and 12 of Table V, respectively).

were typical values of polymeric micelles. This shows that a quantity of hydrophobic component was essential for stable CPT incorporation and particularly, benzyl-61 and benzyl-69 block copolymer micelles stably incorporated CPT without forming larger aggregates.

n-Butyl ester substituted block copolymers did not show as stable micelle formation as benzyl ester substituted block copolymers as summarized in Table V. For n-butyl-47, a micelle peak at 3.30 ml was accompanied by the second peak at a later elution volume (4.42 ml) and its peak area/[CPT] ratio value of the first micelle peak was small (14.7). Weight-average diameter was larger than 1000 nm due to large aggregate formation caused by CPT incorporation. This poor micelle stability was not improved by increasing butyl content up to 66% as shown in Table V and Fig. 4(c). Lauryl-45 brought about a high CPT yield such as 73%, however, micelle stability was found to be low by a little delayed elution at 4.02 ml and a small value of the peak area/[CPT] ratio.

In contrast to n-butyl and lauryl cases, methylnaphtyl-substituted block copolymers methylnaphtyl-53 and -64 showed high micelle stability upon the CPT incorporation even though these CPT yields were lower than those of n-butyl-47, n-butyl-66 and lauryl-45. Particle size of run 11 of Table V for metylnaphtyl-64 was 39 nm in diameter that was a typical value of polymeric micelles without larger aggregates. For this polymer, the CPT yield and the peak area/[CPT] ratio was found to increase by adding CH_3CN in the polymer-CPT mixture for both the CPT/polymer ratios (10 and 20 wt.%). Figure 4(d) shows two gel-filtration charts of run 10 and run 12 of Table II. Both these runs brought about typical micelle peaks around 3.6 ml in elution volume. However, run 12 brought about a sharper peak than run 10 which was accompanied by a shoulder around 4.3 ml. In run 14, CPT content was calculated at 13 wt.% in the polymeric micelle assuming no polymer was lost during the incorporation process. Such a high content indicates successful incorporation of water-insoluble drug to polymeric micelles with maintaining good water solubility.

It is known that biological activity of CPT is completely dependent on its forms; the intact lactone form shows cytotoxic activity while the other water-soluble open form does not. It is also known that the lactone forms exists in a pH-dependent equilibrium with the open carboxylate form and that most CPT exists in the inactive open form in human plasma (Burke and Mi, 1994). Therefore, it is preferable to incorporate CPT in the lactone form. By reversed-phase HPLC, 97 and 95% of the incorporated CPT was found to exist in the active lactone form for benzyl-69 (run 6 in Table V) and n-butyl-66 (run 8 in Table V), respectively. The hydrophobic atmosphere of the inner core is considered to contribute to keep CPT in the more hydrophobic lactone form.

DISCUSSION

Poly(ethylene glycol)–poly(aspartate) block copolymers were prepared with corresponding halides and DBU as catalyst. Ono *et al.* (1978) and Rao (1980) reported this esterification reaction for preparations of low molecular weight esters and this method was successfully applied to block polymer syntheses in this paper. For ester syntheses, condensation reaction between carboxyl and

hydroxyl groups with carbodiimides as catalyst is the most common way. However, an anhydride structure forming from two carboxyl groups is the active species reacting with a hydroxyl group to form an ester. Therefore, this condensation reaction is not considered to proceed for obtaining high degree of esterification of polymers because the anhydride formation becomes more difficult due to restricted freedom of the polymeric carboxyl groups as the esterification proceeds. In contrast, the esterification using halides and DBU shown in Fig. 1 is considered preferable for high degree of esterification of polymers, since the active species for esterification is carboxy anion ($-COO^-$) formed by interaction with low molecular weight compound DBU and this anion has nucleophilic attack on a halide to result in ester formation. In this reaction mechanism, intra or inter polymer reaction is not included and therefore, it is considered that high degree of esterification for polymeric carboxyl groups is possible by this synthetic method. In fact, a high esterification value, 69%, was obtained for benzyl ester. By optimizing reaction conditions such as temperature and solvent, a higher esterification degree may be obtained for the benzyl case and it is expected that various hydrophobic groups are conjugated to (PEG–P(Asp)) block copolymer to a considerably high substitution degree. This allows large freedom for block copolymer structures in order to optimize targeting of various kinds of hydrophobic anticancer drugs as well as for establishing strategy of drug incorporation to polymeric micelles.

Incorporation methods are expected to be very influential on incorporation behaviors of hydrophobic drug to polymeric micelles. However, little has been known concerning this influence. For doxorubicin incorporation to a block copolymer micelle with poly(D,L-alctide) inner core, an optimum water/organic solvent ratio was reported to be present as the starting medium of dialysis (Kohori *et al.*, 2002). This study revealed that incorporation method was very influential on incorporation behavior of CPT to polymeric micelles. In fact, choice of the incorporation method was critical for obtaining polymeric micelles containing CPT in a high yield of CPT and with high clarity of the solution. Lavasanifar *et al.* (2001) reported successful incorporation of amphotericin B to polymeric micelles by an evaporation method. By the evaporation method, polymeric micelles containing CPT was successfully obtained with considerably high CPT content such as 13 wt%. More detailed study, however, is required to elucidate the nature of drug incorporation to polymeric micelles and to predict an appropriate method for a new drug case. A hint for this purpose is present in an addition effect of CH_3CN in solvent of the evaporation method. In this incorporation method, solubility of drug and each block as well as interaction between drug and each block is considered to be a crucial role in the incorporation. Since many kinds of solvent can be examined as this additive, there is large freedom for optimization of the drug incorporation and also for analysis of incorporation mechanism.

In our previous report, a slight difference in micelle diameter was observed between two block copolymers with different hydrophobic block for water-insoluble drug incorporation (Yokoyama *et al.*, 1998). In this paper, the CPT incorporation behavior was analyzed with systematically varying both chemical structure and content of hydrophobic ester groups of PEG–poly(aspartate) block copolymers. Substantial effects of the chemical structure and the content on the incorporation stability were found. An interesting finding was that stability of the CPT incorporation was not attained simply by hydrophobicity of the block copolymers. In stability assay by the GPC, PEG–PBLA micelle provided much lower incorporation stability than benzyl-69 block copolymer. Since all the carboxyl groups are benzylated for PEG–PBLA, hydrophobicity of PEG–PBLA is higher than benzyl-69 block copolymer. PEG-PBLA could be handled as benzyl-100, since all the aspartic acid residues were benzylated. However, PEG-PBLA provided much lower CPT incorporation stability than benzyl-69 as judged by the peak/[CPT] ratio. Possible reasons for this inversed relation between benzyl content and the incorporation stability, are the following two.

(1) Difference in polymer main chain structure. Three-quarters of the amino acid units of the benzyl-25, -44, -61, and -69 were β-amide units due to an alkaline hydrolysis procedure in their syntheses, while PEG-PBLA was composed only of α-amide units. A β-amide unit is longer than an α-amide unit. Additionally, configuration of the benzyl-25, -44, -61, and -69 was almost racemic due to the alkaline hydrolysis procedure, while the amino acid units of PEG-PBLA is composed only of L-configuration. The β-amide units in racemic configuration could be advantageous for stronger interactions with CPT molecules.

(2) The aspartic acid residue may work for a good insertion site of CPT molecules. The aspartic acid is hydrophilic, therefore, cannot interact with CPT molecule by hydrophobic interactions as strongly as the benzyl aspartate unit. However, due to the lack of benzyl moiety, CPT molecule may be more accessible to the poly (aspartate) main chain than the benzyl aspartate unit because of the absence of possible steric hindrance of the benzyl group. If this inserted CPT molecule interacts well with adjacent benzyl groups by hydrophobic interactions, the inserted molecule may be more stably interacted with polymers than PEG-PBLA; a case that does not allow CPT to have an access to the main chain.

This fact implies that location of CPT molecules (e.g., deeply inserted to main chain or present between two polymer chain) and other interactions than hydrophobic interactions can contribute to stable CPT incorporation. It is of great interest to analyze detailed factors for stable micelle incorporation of hydrophobic drugs. Furthermore, in comparison between n-butyl-47 and lauryl-45, n-butyl-47 showed higher stability both in earlier elution and

bigger ratio of peak area/CPT concentration ratio. Lauryl-45 block copolymer is more hydrophobic with longer acyl chain (C_{12}) than n-butyl case (C_4). This implies that rigidity of the hydrophobic inner core is a key for stable CPT incorporation since a longer acyl chain is more flexible. The benzyl and methylnaphtyl block copolymers at high esterification degree provided high CPT incorporation stability shown by a sharp and big peak at a small elution volume. This suggests large contribution of $\pi-\pi$ interaction between aromatic groups of CPT molecules and phenyl/naphtyl groups of these block copolymers.

It was reported that the hydrophobic inner core worked as a good reservoir of drug by inhibiting drug inactivation reactions of adriamycin (Yokoyama *et al.*, 1990). Incorporation of CPT in the hydrophobic inner core is a bigger advantage of polymeric micelles for effective CPT delivery, since the hydrophobic atmosphere may work to keep CPT in the active lactone form at a high concentration for a long time period.

In vivo micelle stability, long-circulation properties and drug release behavior are important for CPT tumor targeting. It is also important to obtain correlations between these behaviors and physico-chemical properties evaluated in this paper. This paper opens a strategic way to optimize tumor targeting of anti-cancer drug by systematically changing chemical structures of the hydrophobic blocks for the micelle inner core.

Acknowledgements

The authors thank Dr Yasuo Yamamoto of NanoCarrier Co., Ltd. for his valuable discussion. This work is supported by a Health and Labour Sciences Research Grant for Research on Hepatitis and BSE from the Ministry of Health, Labor and Welfare of Japan and a Grant-in-Aid from the Ministry of Health, Labor and Welfare of Japan (No.15-21).

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Block Copolymer Design for Camptothecin Incorporation into Polymeric Micelles for Passive Tumor Targeting

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Received March 30, 2004; accepted July 20, 2004

Purpose. Polymeric micelles were designed for targeting of a water-insoluble anticancer agent, camptothecin (CPT). Chemical structures of inner core segment were optimized to achieve high incorporation efficiency and stable CPT-loaded micelles.

Methods. Poly(ethylene glycol)-poly(β-benzyl L-aspartate) block copolymer (PEG-PBLA) was synthesized. The PBLA chain was modified by alkaline hydrolysis of its benzyl group followed by esterification with benzyl, *n*-butyl, and lauryl groups. Incorporation of CPT into micelles was carried out by an evaporation method. The stability of drug-loaded micelles was studied by gel-permeation chromatography (GPC), and their *in vitro* release behaviors were analyzed.

Results. CPT was incorporated into polymeric micelles constructed by various block copolymers. Among the esterified groups, block copolymers with high benzyl ester contents showed high CPT loading efficiency and stable CPT-loaded micelles. In chain lengths, 5-27 Bz-69 showed the highest incorporation efficiency. In contrast, 5-52 Bz-67, which had a longer hydrophobic chain, showed low incorporation efficiency. Release of CPT from the micelles was dependent on the benzyl contents and chain lengths. Sustained release was obtained when the benzyl content was high.

Conclusions. CPT was successfully incorporated into polymeric micelles with high efficiency and stability by optimizing chemical structures of the inner core segment.

KEY WORDS: anticancer agent; camptothecin; polymeric micelles; stability.

INTRODUCTION

Camptothecin (CPT) is a potent, anticancer agent acting through the inhibition of topoisomerase I during the S-phase of the cell cycle (1). It exists in two forms depending on the pH value, namely, an active lactone form at pH below 5 and an inactive carboxylate form at basic pH (Fig. 1) (2). At physiologic pH, most CPT molecules exist in the inactive carboxylate form. The stability of the lactone form of CPT is crucial for its anticancer activity. The labile lactone ring and poor aqueous solubility pose many challenges for drug development and drug delivery system (DDS). Various types of DDS have been developed in order to reduce severe systemic toxicities and enhance antitumor effects by improving their pharmacokinetics. Previous studies showed that the lactone ring of CPT was protected upon incorporation of the drug into a lipid bilayer structure like liposomes (3–5) and microspheres (6–8) and upon conjugation to synthetic polymers (9–11) and nanobiohybrids (12).

Recently, polymeric micelles prepared from block copolymers have been proposed to attain effective novel drug delivery for antitumor drugs (13–17), diagnostic reagents (18), DNA (19–20), and enzymes (23). Furthermore, temperature-modulated drug delivery of block copolymer micelles have been studied (21–22). AB-type block copolymers possessing both hydrophilic and hydrophobic segments are known to form micellar structures in aqueous media due to their amphiphilic character (24). Highly hydrated outer shells of the polymeric micelles can inhibit intermicellar aggregation of their hydrophobic inner cores. Consequently, the polymeric micelles maintain a satisfactory aqueous stability irrespective of high contents of hydrophobic drug incorporated into the inner core of micelles. Furthermore, a polymeric micelle in a size range <200 nm reduces nonselective reticuloendothelial system (RES) scavenge and shows enhanced permeability and retention effects (EPR effect) (25) at solid tumor sites for passive targeting.

In our previous reports, an anticancer drug, adriamycin (doxorubicin), was used as our first example for the polymeric micelle drug delivery system, and these adriamycin-containing polymeric micelles showed dramatically higher *in vivo* antitumor activity than free adriamycin and highly selective delivery to a solid tumor by the EPR effect (15).The second example was of KRN 5500 (6-[4-deoxy-4-(2E,4E)-tetradecadienoylglycyl]amino-L-glycero-β-L-mannoheptopyranosylamino-9H-purine). Its successful incorporation into polymeric micelles was achieved by optimizing the block copolymer structure and the incorporation condition (26).

Our previous study for CPT showed that poly(ethylene glycol)-poly(β-benzyl L-aspartate) block copolymer (PEG-PBLA) incorporated CPT into its micelles. However, the stability of these micelles was very poor. These micelles could be used for solubilization of CPT, but not as a stable drug carrier system. We have also successfully synthesized various AB-type block copolymers by varying chemical structures of hydrophobic segment of the poly(ethylene glycol)-poly(aspartate) block copolymers. We found that the CPT incorporation efficiency and stability were improved with the modified hydrophobic segments of block copolymers and the incorporation method (27). In the current work, in order to achieve the more stable CPT-loading micelles for tumor delivery, the factors affecting the incorporation efficiency and the stability of CPT-loading micelles are systematically evaluated by changing the esterified moieties of the hydrophobic segment, the

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ABBREVIATIONS: CMC, critical micelle concentration; CPT, camptothecin; DBU, 1, 8-diazabicyclo[5,4,0]7-undecene; DDS, drug delivery system; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; EPR effect, enhanced permeability and retention effect; FBS, fetal bovine serum; GPC, gel-permeation chromatography; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; PEG-P(Asp), poly(ethylene glycol)-poly(aspartic acid) block copolymer; PEG-PBLA, poly(ethylene glycol)-poly(β-benzyl L-aspartate) block copolymer; RES, reticuloendothelial system.