

In Vitro and *In Vivo* Study of Two Types of Long-Circulating Solid Lipid Nanoparticles Containing Paclitaxel

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Paclitaxel (Taxol), a diterpenoid isolated from *Taxus brevifolia*, is effective against several murine tumors, and is one of the most exciting anticancer molecules currently available. Due to its low solubility in water, it is clinically administered with polyethoxylated castor oil (Cremophor EL), which causes serious side effects. Inclusion of paclitaxel in solid lipid nanoparticles (SLNs) has proved to be a good approach to eliminate the need for Cremophor EL and improve the drug's antitumor efficacy. This paper describes the development of two types of long-circulating SLNs as colloidal carriers for paclitaxel. SLNs are constituted mainly of bioacceptable and biodegradable lipids. *In vitro* release kinetics showed that the release was very slow, the release of paclitaxel from F₆₈-SLN is linear, and the release of paclitaxel from Brij78-SLN followed the Weibull equation. Pharmacokinetics was evaluated in KM mice after injection of paclitaxel formulated in Cremophor EL or in Brij78-SLN and F₆₈-SLN. Encapsulation of paclitaxel in both SLNs produced marked differences compared with the free drug pharmacokinetics. F₆₈-SLN and Brij78-SLN are long-circulating ($t_{1/2\beta}$, 10.06 and 4.88 h, respectively) compared with paclitaxel injection ($t_{1/2\beta}$, 1.36 h).

Key words stearic acid; solid lipid nanoparticle; Brij78; poloxamer F₆₈; paclitaxel; long-circulating

Paclitaxel, a diterpenoid derived from the needles and bark of the Pacific yew tree, is a potent inhibitor of cell replication, blocking cells in the late G₂-mitotic phase of the cell cycle by stabilizing the microtubule cytoskeleton. Clinical trials have shown that paclitaxel has antineoplastic activity, particularly against primary epithelial ovarian carcinoma, breast cancer, colon, head and neck cancers and non-small cell lung cancer.¹⁾ Paclitaxel is a hydrophobic molecule and poorly soluble in water, for which reason it is solubilized in a 50:50 mixture of Cremophor EL (a polyethoxylated castor oil) and ethanol. Cremophor EL is associated with a number of side effect, including hypersensitivity, nephrotoxicity and neurotoxicity. Furthermore, Cremophor EL dissolves phthalate plastics from commonly used polyvinyl chloride bags and intravenous infusion lines.²⁾ The use of Cremophor EL as a vehicle also appears to alter the biochemical properties of lipoproteins, such as high-density lipoprotein; it has also been shown to mediate partially the cytotoxic activities of paclitaxel in primary cultures of tumor cells from patients.³⁾ Although a premeditation regimen with corticosteroids and antihistamine reduces the incidence of serious hypersensitivity reactions, milder reactions have still been found to occur in 5—30% of treated patients.³⁾

Colloidal drug carriers such as liposomes and nanoparticles can be used to improve the therapeutics index of both established and new drugs by modifying their distribution, thus increasing their efficiency and/or reducing their toxicity. This is because the drug distribution then follows the carrier, rather than depending on the physicochemical properties of the drug.⁴⁾

Despite the promising results achieved with “first-generation” drug carrier systems, their value is limited by their distribution and, in particular, by their recognition by the mononuclear phagocyte system. The major breakthrough, however, was the use of surfactant substituted with poly(ethylene glycol) (PEG) chains with molecular weight of 1000—5000.⁵⁾ This provides a “cloud” of hydrophilic chains at the particle surface, which repels plasma protein. Such “steri-

cally stabilized” particles have been shown to have circulating half lives longer than the conventional solid lipid nanoparticles (SLNs).⁶⁾ This prolongation is almost independent of the injected dose and of particle diameter of 50—300 nm. Thus they can function as reservoir systems and penetrate into accessible sites, such as tumors, other than mononuclear systems.

Stearic acid is a type of native physiologic molecule. It is bioacceptable and biodegradable in the human body, and thus is a good carrier material. Brij78 (polyoxyethylene 20 stearyl ether) is a nonion surfactant. Its hydrophilic end is a chain of PEG; and its hydrophobic end is stearyl alcohol. PEG-DSPE is a phospholipid substituted with PEG. The aim of this study is to prepare surface-modified long-circulating stearic acid nanoparticles containing paclitaxel, and compare two PEGylated nanoparticle formulations, in terms of drug release rate and pharmacokinetics.

Experimental

Materials Paclitaxel injection and paclitaxel were purchased from Beijing Sihuang Pharmaceutical Ltd. (Beijing, China). Brij78, stearic acid, lecithin and poloxamer F₆₈ were from Sigma Chemical Ltd. (St. Louis, MO, U.S.A.), PEG-DSPE was from NOF (Tokyo, Japan); acetonitrile and methyl-*tert* butyl ether were from Fisher corporation (Fair lawn, NJ, U.S.A.). The other chemicals were of analytical reagent grade.

Preparation of SLNs with Brij78 The preparation of SLNs using Brij78 was carried out as follows: 10 ml of acetone solution containing paclitaxel, stearic acid and lecithin was injected into 10 ml of water containing Brij78 at 75 °C under 1000 rpm agitation. The mixed system was evaporated in 75 °C bath to concentrate the system to 5 ml. Then the system was added to an additional 10 ml of water at 0—2 °C under 1000 rpm agitation, and the stearic acid emulsion was fully solidified to form the SLN suspension.

Preparation of SLNs with Poloxamer F₆₈ The preparation of SLN using Poloxamer F₆₈ was the same as above, except that the Brij78 was replaced by poloxamer F₆₈ and PEG-DSPE.

Transmission Electron Microscopy (TEM) TEM analysis was performed using a Hitachi-500 instrument. Before analysis, the SLN dispersions were diluted 1:10 with filtered water, stained with a 5% solution of sodium phosphor tungstic acid, and sprayed on copper grids.

Size Distribution of the Nanoparticle Population The nanoparticle diameter of both SLNs were determined by laser diffraction (LD) at 632.8 nm using Submicron Particle Sizer Model 370 (University of California, Santa

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Barbara, CA, U.S.A.) at a fixed angle and at a temperature of 25 °C. The SLN water dispersions were diluted 1 : 100 with filtered water before analysis.

Percentage of Drug Incorporated into SLNs For the quantitative determination of paclitaxel, a reverse-phase HPLC method was used (HP1100 Binary LC pump liquid chromatograph, RP18 Hypersil column, 250×4.6 mm, 5 μm). The mobile phase was acetonitrile–water (70 : 30, v/v). The analysis was performed at a flow rate of 1 ml/min with the UV detector at 227 nm.

SLN dispersion 0.4 ml was added to the Sephadex G₅₀ column, and then eluted with filtered water. The bluish portion that flowed out was collected, and a final volume of 10.0 ml was obtained by adding filtered water. The amount (M_1) of paclitaxel contained was determined by HPLC. Filtered water was added to another 0.4 ml of SLN dispersions to reach 10.0 ml, and the amount (M_2) of paclitaxel contained was determined by HPLC. The percentage of paclitaxel incorporated into SLNs was calculated by the formula: $p\% = M_1/M_2 \cdot 100$.

In Vitro Release Kinetics of Paclitaxel from dispersion SLNs The *in vitro* release kinetics was determined using a multicompartimental rotating cell system with donor and receptor compartment. A hydrophilic dialysis membrane with a cut-off of 12000, was used. SLN dispersion 0.4 ml was placed in the donor compartment, and the receptor compartment was filled with 30% ethanol. At fixed times, the receptor solution was piped out and replaced with 30% ethanol. To determine the drug concentration, the above HPLC method was applied to analysis the receptor solution.

Pharmacokinetic Studies Pharmacokinetic studies were performed, using male KM mice (20±2 g, Peking University health science center, Beijing, P. R. China). The animals were administered Brij78-SLN or F₆₈-SLN. For comparison, a pharmacokinetic evaluation of the free drug was also performed. The paclitaxel injection (Beijing Sihuang Pharmaceutical Factory, Beijing, P. R. China) was diluted with filtered water to the desired concentration. The different formulations were injected through the tail vein at the paclitaxel dose of 10 mg/kg mouse, and each group consisted of four to five animals.

Blood samples were taken from the retro orbital plexus at various times (0, 30, 60, 120, 240, 480, 840 min). Serum samples were harvested by centrifugation at 4000×g for 10 min and were stored at -20 °C until analysis. To 100 μl of mouse serum was added 50 μl of the internal standard solution (10 μg/ml) and the mixture was extracted with 2 ml of *tert*-butyl methyl ether on a vortex mixer for 60 s. Upon centrifugation at 1500×g for 10 min, the organic layer was transferred to another clean test tube and evaporated under nitrogen at 40 °C, the residue then reconstituted with 200 μl of 20% acetonitrile in deionized water and was mixed in a vortex mixer for 90 s. A portion (50 μl) of the reconstituted sample was injected on the chromatography column. Drug concentrations were determined from the peak area ratios with respect to the internal standard. Pharmacokinetic parameters were determined using a software program (3P87). Elimination, distribution, and disposition were represented by the following parameters: area under the curve (AUC); total body clearance (CL); volume of distribution (V); serum half-life for the distribution and elimination phase ($t_{1/2\alpha}$, $t_{1/2\beta}$).

Results

Characterization of SLNs The diameters of Brij78-SLN and F₆₈-SLN determined by LD were 103.5±29.2 and 220±98 nm, respectively. The transmission electron microphotographs (Figs. 1, 2) show that the SLNs are round or oval.

Percentage of Paclitaxel Incorporated into SLNs The percentages of paclitaxel incorporated into Brij78-SLN and F₆₈-SLN were 58.2±2.5% and 75.4±3.2%, respectively.

Release Kinetics of Paclitaxel from SLNs Figures 3 and 4 show the percentage of paclitaxel released from SLNs. The release of paclitaxel from F₆₈-SLN was very slow and showed a linear trend. After 24 h, paclitaxel release was about 20%. The release of drug from F₆₈-SLN was zero order following the equation: $y = 0.0079x + 0.0077$ ($r = 0.9961$).

The release of paclitaxel from Brij78-SLN was slower than that of F₆₈-SLN. After 24 h, the percentage of drug release was about 7%. The release data were fitted to first-order,

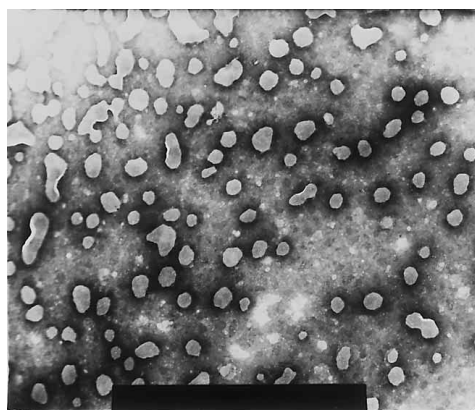


Fig. 1. Transmission Electron Microphotograph of SLNs Prepared with Brij78

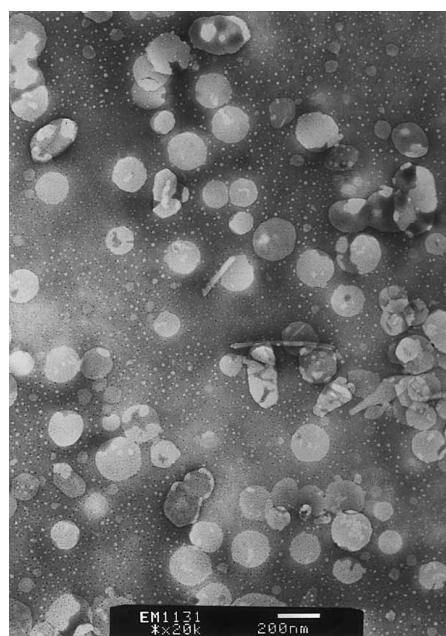


Fig. 2. Transmission Electron Microphotograph of SLNs Prepared with Poloxamer F₆₈

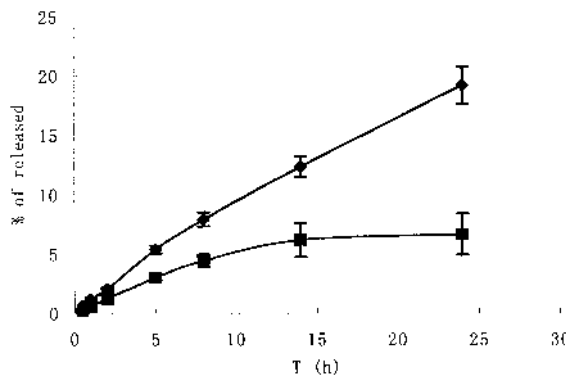


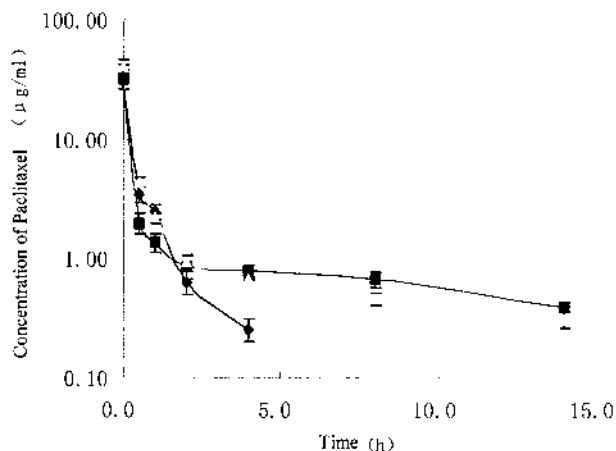
Fig. 3. *In Vitro* Release Profile of Paclitaxel from SLNs Prepared with Brij78 (■) and SLNs Prepared with Poloxamer F₆₈ (◆) in 30% Ethanol at 37 °C (n=4)

Higuchi, and Weibull equations (Table 1). The release of drug from Brij78-SLN followed the Weibull equation best.

Pharmacokinetics The pharmacokinetic behavior of pa-

Table 1. Non-linear Fits of Paclitaxel Released from SLNs Prepared with Brij78 in 30% Ethanol

Model	Equation	<i>r</i>
1st order	$\ln(1-Q)=0.003t+0.0091$	0.9338
Higuchi	$Q=1.1071t^{1/2}-0.9351$	0.9824
Weibull	$\ln \ln[1/(1-Q)]=0.9021 \ln t-5.1766$	0.9842

Fig. 4. Mean Serum Concentration of Paclitaxel after Intravenous Administration of Paclitaxel Injection (◆), SLNs Prepared with Poloxamer F₆₈ (■) and SLNs Prepared with Brij78 (▲) to Mice (*n*=5).Table 2. Mean Pharmacokinetical Parameters of Paclitaxel after Bolus Administration of Paclitaxel Injection, SLNs Prepared with Poloxamer F₆₈ (F₆₈-SLN) and SLNs Prepared with Brij78 (Brij78-SLN) to Mice

	<i>AUC</i> ($\mu\text{g ml}^{-1} \cdot \text{min}$)	<i>t</i> _{1/2} α (min)	<i>t</i> _{1/2} β (min)	<i>CL</i> (ml h^{-1})	<i>V</i> (c) ($\text{ml} \cdot \text{kg}^{-1}$)
Injection	10.64	0.12	1.36	0.94	0.27
Brij78-SLN	18.51	0.14	4.89	0.54	0.23
F ₆₈ -SLN	19.86	0.09	10.06	0.50	0.31

clitaxel incorporated in SLNs was assessed in KM mice after intravenous administration. Brij78-SLN, F₆₈-SLN, and paclitaxel injection were administered at a dose of 10 mg/kg.

The serum paclitaxel concentration-*versus*-time curves are illustrated in Fig. 4. The pharmacokinetic parameters for the two SLNs and paclitaxel injection, based on the total paclitaxel levels in serum measured by HPLC, are listed in Table 2.

After bolus administration, paclitaxel in Cremophor EL follows a biphasic pattern with a rapid terminal elimination phase, and the *t*_{1/2} β was only 1.36 h. Encapsulation of paclitaxel in the SLNs produced a significant change in pharmacokinetic parameters. After bolus administration of Brij78-SLN and F₆₈-SLN, the drug was eliminated rather slowly with *t*_{1/2} β for Brij78-SLN and F₆₈-SLN of 4.89 and 10.06 h, respectively.

Discussion

In recent years, paclitaxel has been used to treat ovarian and breast carcinoma alone or in combination with other antineoplastic agents, such as cisplatin or carboplatin, with positive results. Paclitaxel is commonly administered as a micel-

lar solution to increase its solubility. Cremophor EL is employed to avoid precipitation of the drug when the concentrated solution is diluted. As an alternative carrier, we studied the incorporation of paclitaxel into SLNs.

Paclitaxel-loaded SLNs were prepared by the method of emulsion evaporation-solidification at low temperature. Stearic acid was used as the carrier material, since it is a native physiology molecular and can be easily metabolized in the human body. We first prepared the hot nanoemulsion of stearic acid at 75 °C, because the melting point of stearic acid is 50–60 °C. The organic phase was dispersed to the hot water phase, with evaporation of the organic solvent, and the stearic acid precipitated to form a nanoemulsion, which is stabilized by the surfactant. Paclitaxel has very low solubility in water (0.5–35 $\mu\text{mol/l}$), and thus most part of the drug was partitioned to the stearic acid emulsion drops. Then the nanoemulsion is added to cold water (0–2 °C), and at the low temperature, the stearic acid emulsion drops solidified to form SLNs, into which paclitaxel is incorporated. The cold phase must proceed as quickly as possible, or the emulsion drops will aggregate, which causes the system to be unstable.

To determine the percentage of incorporation, the SLN dispersions were first ultracentrifuged. Because we failed to separate the SLNs from the water, we used a Sephadex column, which is usually employed to separate liposomes from the free drug. The SLNs were eluted at a volume of 9–15 ml, with obvious bluish color. About 75% and 47% of paclitaxel doses were incorporated into F₆₈-SLN and Brij78-SLN, respectively. F₆₈-SLN appeared to incorporate more drug than Brij78-SLN. The reason may be that Brij78 slightly increases the solubility of paclitaxel in water. During the preparation, more drug was dissolved in water and less drug was partitioned to the nanoemulsion, and thus after solidification, less drug was incorporated into the SLNs. Therefore the characteristics of the surfactant may affect the incorporation rate significantly.

The solubility of paclitaxel in water was very low. When we used phosphate buffer, pH 7.4, as the receptor solution, only a trace of paclitaxel was detected in the receptor compartment. When Tween 80 and sodium dodecyl sulfate (SDS) were added to the receptor solution, no change in solubility occurred. However, with the addition of 30% ethanol, paclitaxel was dissolved sufficiently.

From the experimental data, the release kinetics of paclitaxel from F₆₈-SLN appeared to be of zero order ($y=0.0079x+0.0077$, $r=0.9961$), and release of paclitaxel followed the Weibull distribution. The drug encapsulation patterns in these particles, (*i.e.*, matrix type or reservoir type) and surface properties could both affect the release behavior of these SLNs. The amount of paclitaxel released over time is very low, and SLNs could therefore be considered to be sustained-release carrier. This behavior was in accordance with that previously reported.⁷⁾

Brij78-SLN and F₆₈-SLN released 7% and 20% of incorporated drug in 24 h, respectively. The hydrophobic end of Brij78 is the same as that of carrier material, stearic acid. When forming nanoparticles, the Brij78 PEG chain can bind firmly to the nanoparticle surface. Compared with poloxamer F₆₈, it is difficult for Brij78 to disadsorb from the SLN surface.

The slow release of paclitaxel from SLNs suggests that the

paclitaxel might be dispersed in the lipid matrix, and the adsorption of paclitaxel onto the surface of SLNs need not be considered. We can explain this phenomenon by the preparation procedure. After the organic solvent had been evaporated, the paclitaxel was dissolved in the stearic acid nanoemulsion. The rapid quenching of the nanoemulsion might not have allowed the drug to crystallize, and the paclitaxel was trapped in the solid lipid.

The blood paclitaxel concentration–time profiles in mice after intravenous injection, in either Cremophor EL, Brij78-SLN, or F68-SLN formulation, followed a biexponential disposition (Fig. 4, Table 2). Encapsulation of paclitaxel in SLNs showed marked differences in terms of the pharmacokinetic parameters calculated from free paclitaxel, particularly in the $t_{1/2\beta}$ and *AUC*. F₆₈-SLN and Brij78-SLN are long circulating ($t_{1/2\beta}$, 10.06 and 4.88 h, respectively) compared with paclitaxel injection ($t_{1/2\beta}$, 1.36 h). The prolonged $t_{1/2\beta}$ appeared to be related to the reduced clearance rate and hence to the reduced uptake of SLNs by the mononuclear phagocytic system. Compared with Brij78-SLN ($t_{1/2\beta}$, 4.88 h), F₆₈-SLN has a longer circulating time in the blood-

stream ($t_{1/2\beta}$, 10.06 h). The reason may be that PEG-DPSE has a longer PEG chain (MW 2000) than the PEG chain of Brij78 (MW 1200). The chain of PEG can adsorb water molecules, and it provides a thicker “cloud” of hydrophilic chains at the particle surface, which repels plasma proteins. The long chain can adsorb more water and has better flexibility and repels the plasma protein more effectively.⁸⁾

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