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A novel surface-coated nanocarrier for efficient encapsulation and delivery of Camptothecin to cells

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In the present study, we developed a novel surface-coated nanocarrier (SCN) for efficient and stable encapsulation of a poorly water-soluble anticancer agent, Camptothecin (CPT). Using emulsification and freeze-drying processes in the presence of a hydrophilic surfactant, Pluronic F-68 or F-127, CPT–Pluronic complexes with crystalline features were synthesised. Investigation of the *in vitro* anticancer activities and cellular internalisation of the complexes revealed that the SCN efficiently delivered CPT to cells while maintaining anticancer activity.

Introduction

More than 50% of chemotherapeutic agents developed over the last few decades are poorly water-soluble and their formulation technology that aims to improve solubility and pharmacokinetics is generating increasing attention. Camptothecin (CPT), isolated from the tree *Camptotheca acuminate*, *Nyssaceae* in 1966, is an alkaloid that exhibits antitumour activities on a wide variety of tumours.[1] However, its water-insolubility and chemical instability, owing to the labile lactone ring, have made it challenging for a pharmaceutical application. Therefore, many approaches have been examined to achieve a more efficient and stable delivery of CPT to tumour sites, including physical inclusion in polymer micelles and liposomes and covalent conjugation with hydrophilic polymers.[2–5]

In an attempt to develop a drug carrier for transdermal delivery, we devised a solid-in-oil technique whereby the formation of a water-in-oil emulsion using an aqueous solution of proteins and a cyclohexane solution of hydrophobic surfactants followed by freeze-drying afforded a protein–surfactant complex. Because the water-soluble proteins are coated with hydrophobic surfactants during the process, a nanodispersion of proteins in an oil phase can be achieved.[6,7] No specific interactions are required between the proteins and surfactants because the emulsification and freeze-drying processes drive the inclusion of proteins within the surfactant molecule "layers". Moreover, a high loading efficiency can be achieved. As this technique can be used for altering the water-solubility of drugs, we expected that hydrophobic CPT could be reversely dispersed in water in the presence of hydrophilic surfactants. In this study, we developed a novel surface-coated nanocarrier (SCN) that enabled nanodispersion of CPT in water in the presence of poloxamer surfactant, Pluronic F-68 or F-127, using emulsification and freeze-drying processes. Pluronic F-68 and F-127 are biocompatible nonionic surfactants and are approved by the Food and Drug Administration (FDA) as injectable materials.[8] Our results show that this novel nanocarrier can encapsulate CPT with high efficiency and chemical stability, and most importantly, it exhibits *in vitro* anticancer properties.

Results and discussion

Preparation and characterisation of SCN

CPT is a known poorly water-soluble drug and is only soluble in some organic solvents such as dimethyl sulphoxide (DMSO) and chloroform/methanol. The preparation scheme of SCN is illustrated in Fig. 1. Briefly, a CPT solution in chloroform/methanol was added to cyclohexane and the oil-in-water emulsion was immediately formed using an aqueous solution of either Pluronic F-68 or F-127 by homogenizing the mixture at 26,000 rpm for 90 s. The emulsion was freeze-dried to form a solid CPT–Pluronic complex. The addition of water to the obtained solid resulted in a homogeneous dispersion with a maximum CPT concentration of 400 μg/mL. The encapsulation efficiency was determined by the ultrafiltration method. High loading efficiencies of more than 80% were achieved for both types of complexes (Table 1). Emulsification drives CPT in the oil phase that is surrounded by the aqueous phase containing Pluronic, thereby resulting in efficient encapsulation of CPT. In contrast, in the absence of Pluronic, CPT is barely dispersible in water, forming a white precipitate

even in the presence of 10% DMSO after 1 week (Fig. S1). This indicates that Pluronic is located at the surface of the complex, thus playing an important role in preventing random aggregation of the complex.

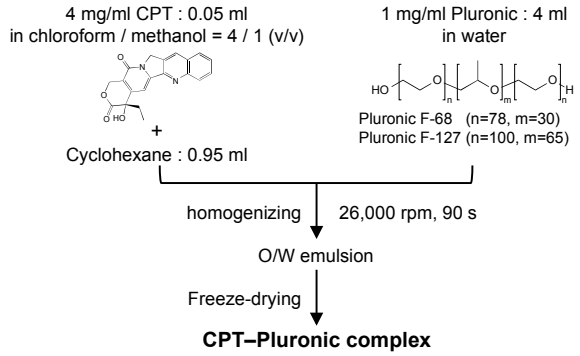

Figure 1. Preparation scheme of CPT–Pluronic complexes.

The size of the complexes was evaluated by dynamic light scattering (DLS). The mean particle sizes of CPT–Pluronic F-68 and CPT–Pluronic F-127 were 464 nm and 504 nm, respectively (Table 1). It has been reported that some homogenizing parameters, such as homogenizing speeds, pressures and number of cycles affect the size of particles formed.[9] Further study on these parameters in our system may allow us to manipulate the size of the complexes. The size distributions barely changed after 1 week, suggesting that the complexes were stable as a water dispersion (Fig. S2). The complexes were subjected to transmission electron microscopy (TEM) analysis. The nanostructures appeared to display crystalline features and layered morphologies (Fig. 2). It has been reported that bare CPT forms lamelliform crystals and nanocrystals formed from CPT and Pluronic feature cubic morphologies.[10,11] The lamellar morphologies observed in the present study clearly stem from the crystalline features of CPT. This may be due to the insolubility of CPT in cyclohexane. CPT is molecularly soluble in chloroform/methanol; however, addition of the CPT solution to cyclohexane for freeze-drying may have caused recrystallisation of CPT. Bare nanocrystals of CPT are known to aggregate into large precipitates.[12] However, herein, Pluronic is believed to coat the nanocrystals through interactions between the hydrophobic propylene oxide (PO) blocks and surface of the CPT crystal. The small difference in sizes between the Pluronic F-68-based and Pluronic F-127-based complexes may be due to formation of the CPT nanocrystals in the early stages of the preparation, presumably before emulsification, thus pre-determining the dimension of the complexes.

Table 1. Size distribution and encapsulation efficacy of CPT by Pluronic surfactants.

Surfactant	Encapsulation efficiency[a] (%)	Loading capacity[b] (%)	Size (nm)	Distribution width (nm)
Pluronic F-68	84.9 ± 0.91	4.04	464 ± 6.6	220 ± 2.4
Pluronic F-127	83.0 ± 0.47	3.95	504 ± 6.3	258 ± 23.0

[a] Percentage of encapsulated CPT from the feed amount.

[b] Weight percentage of loaded CPT in CPT–Pluronic complexes.

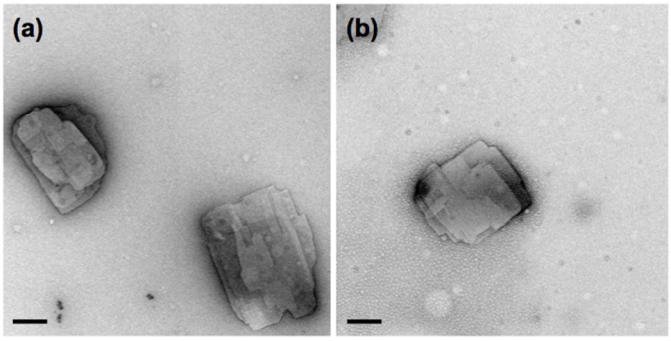

Figure 2. TEM images of CPT–Pluronic complexes: (a) CPT–Pluronic F-68 and (b) CPT–Pluronic F-127. Scale bar: 200 nm.

Release profiles and chemical stability of CPT encapsulated in SCN

In vitro CPT release profiles from CPT–Pluronic complexes were assessed by measuring fluorescence intensities of CPT in the external solution from dialysis. There was no initial burst but a sustained and continuous release of CPT from the complexes was observed (Fig. S3). Approximately 50–60% of the total CPT was released within the first 6 h, and almost 100% of the drug loading content was released within a day. A slower release of CPT can be expected for the Pluronic F-127-based complex when compared with that of the Pluronic F-68-based complex because of the longer hydrophobic blocks of the former surfactant (the average numbers of PO units for Pluronic F-68 and F-127 are 29 and 65, respectively).[8] However, in our CPT–Pluronic complexes, CPT is not molecularly dispersed in the complex and the surface of the aggregated (crystallised) CPT is coated with Pluronic, thereby resulting in a reduced effect of the length of the hydrophobic blocks of the polymer on the release profiles.

CPT is known to hydrolyse easily to form an inactive open carboxylate form at physiological pH.[13] We anticipated that the chemical stability of the CPT lactone form was higher in the CPT–Pluronic complexes because of the crystalline form and the protection by the Pluronic coating. Phosphate-buffered saline (PBS) solutions of the CPT–Pluronic complexes were incubated at 37°C and the hydrolysed CPT with the carboxylate form was quantitatively analysed by high-performance liquid

chromatography (HPLC). Free CPT in 1:9 (v/v) DMSO/water as a control showed rapid conversion into the open carboxylate form, with a conversion efficiency of more than 80% within the first hour. In contrast, slow hydrolysis was observed for the CPT–Pluronic complexes. Approximately 80% of CPT remained as the active lactone form for the first hour (Fig. S4). These results clearly show the beneficial attribute of the formation of CPT–Pluronic complexes on the stability of CPT in the active form.

In vitro anticancer drug activities and intracellular delivery of CPT encapsulated in SCN

To investigate the *in vitro* anticancer efficacy of encapsulated CPT in the CPT–Pluronic complexes, cytotoxicity assays were conducted using mouse melanoma B-16 cell line. Free CPT showed a half maximal inhibitory concentration (IC_{50}) value of ~0.05 μg/mL, which is consistent with those reported previously.[2] For the complexes, the IC_{50} values were slightly higher than that of free CPT *i.e.*, ~0.25 μg/mL and ~0.1 μg/mL for CPT–Pluronic F-68 and CPT–Pluronic F-127, respectively (Fig. 3). The higher cytotoxicity of free CPT may be due to its hydrophobic small molecular weight nature, and it is easily internalised in cells. On the other hand, large-sized complexes may inhibit internalisation by cellular uptake.

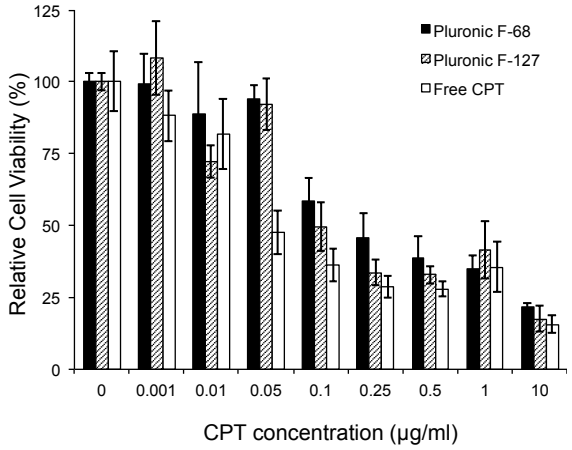

Figure 3. Intracellular delivery of CPT–Pluronic complexes in B16 cells. Data are shown as mean ± standard deviation from representative runs.

A slightly lower IC_{50} value was observed for CPT–Pluronic F-127 complex when compared with that of CPT–Pluronic F-68. This was expected because of the stronger hydrophobic nature of Pluronic F-127 relative to that of F-68.[14] To confirm this hypothesis, the intracellular delivery of the complexes was evaluated using confocal laser scanning microscope (CLSM). Rhodamine-labelled dioleyl phosphatidylcholine (Rho-DOPE) was incorporated into the complexes to afford visualisation of the cells during fluorescence analysis. Similar red fluorescence from Rho-DOPE was observed from the cells for the CPT–Pluronic F-68 complex both after 2 and 6 h incubation. In contrast, the CPT–Pluronic F-127 complex displayed little fluorescence following incubation for 2 h and an intense red fluorescence was observed from the cells following 6 h of incubation (Fig. S5), suggesting a slower cellular uptake of the complex compared with CPT–Pluronic F-68. The overlapping red (complex) and green (endosome/lysosome) fluorescence in the combined image suggests that the CPT–Pluronic complexes are internalised through endocytosis.

The cellular uptake was further confirmed by a flow cytometric analysis. After 6, 12 and 24 h incubation, the gradual cellular uptake of the complexes were observed (Fig. S6). Cellular internalization of CPT–Pluronic F-68 occurred relatively faster than CPT–Pluronic F-127; at 6 h or 12 h incubation, slightly more fluorescence was observed for CPT–Pluronic F-68, whereas at 24 h incubation more CPT–Pluronic F-127 was internalized. However these differences were not significant ($p \approx 0.07$ for 24 h data). It was reported that not only the hydrophobicity of the Pluronic surfactant but the size of the drug–Pluronic complex influenced the cellular uptake efficiency.[14] Although CPT–Pluronic F-127 is composed of more hydrophobic Pluronic surfactant, the slightly larger size compared with CPT–Pluronic F-68 (Table 1) may reduce the efficiency of cellular uptake. Therefore, the cellular internalization was not significantly varied between CPT–Pluronic F-68 and F-127. The difference in IC_{50} values between CPT–Pluronic F-68 and F-127 observed in Figure 3 may stem from the slight difference in the cellular uptake efficiency at 24 h, but the details are unclear at this point.

Conclusion

We demonstrated a novel surface coating method of a poorly water-soluble anticancer drug, CPT. The one-step preparation of CPT–Pluronic complexes using emulsification and freeze-drying enabled efficient and stable encapsulation of CPT. The resulting complexes showed efficient *in vitro* anticancer activities. Our method can be further applied for the preparation of stable aqueous dispersion of varying water-insoluble drugs and hydrophobic functional molecules.

Experimental

Preparation and characterisation of SCN

CPT was dissolved in chloroform/methanol (4:1, v/v) at a concentration of 4 mg/mL. Then, 50 μL of the prepared solution was added to 0.95 mL of cyclohexane. An oil-in-water emulsion was formed in the presence of 4 mL of either Pluronic F-68 or F-127 aqueous solution (1 mg/mL) using a Polytron homogeniser (Kinematica, Bohemia, NY, USA) at 26,000 rpm for 90 s. The emulsion was immediately frozen in liquid nitrogen for 30 min, followed by lyophilisation to afford CPT–Pluronic complexes.

For the DLS studies, the solid complexes were dispersed in 2 mL of water and the size distributions were evaluated on a Zetasizer Nano-ZS (Malvern, Worcestershire, UK). For TEM analysis, 2.5 μL of the complex aqueous dispersion was deposited on a copper TEM grid with a carbon support film, incubated for 2 min, and the excess solution was absorbed by a filter paper. The samples were negatively stained with 2%

uranyl acetate solution in water and imaged on a TEM-2010 (JEOL, Tokyo, Japan), operating at an accelerating voltage of 120 kV.

Determination of *in vitro* drug release profiles of CPT from SCN

CPT–Pluronic complexes containing 0.2 mg of CPT were dispersed in 2 mL of PBS, and 1.5 mL of the solution was placed in a dialysis tube (Spectra/Por 4 dialysis tube, 12–14 k molecular weight cutoff, Spectrum Laboratories). The tube was immersed in 28.5 mL of PBS and incubated at 37°C. After 1, 3, 6, 12 and 24 h of incubation, 0.5 mL of the external solution was withdrawn and the same volume of fresh PBS was added. The amount of CPT released was calculated by measuring the fluorescence intensities at 446 nm (λ_{ex} = 365 nm) using a Perkin Elmer LS55C spectrofluorometer (Waltham, MA, USA). The CPT release (%) after x h was calculated as follows:

CPT release (%) = $[(F_x \times 30\ \text{mL})/(F_o \times 1.5\ \text{mL})] \times 100\%$

where F_x and F_o denote the fluorescence intensities of the external solution at x h and the PBS dispersion before incubation, respectively. In a separate experiment, we confirmed the solubility of CPT in PBS under these conditions.

Stability of CPT encapsulated in SCN

For the chemical stability study, CPT–Pluronic complexes containing 0.2 mg of CPT were dispersed in 4 mL of PBS ([CPT] = 0.05 mg/mL), and the resulting dispersion was incubated at 37°C. As a control, free CPT in 10% DMSO in PBS was incubated under the same conditions. After 0.5, 1, 2, 4, 8 and 12 h of incubation, 0.25 mL of the solution was withdrawn and mixed with 10% DMSO in PBS, and immediately analysed on a Shimadzu HPLC system equipped with a SPD-20A UV–vis detector and a LC-20AT pump (Shimadzu Co., Kyoto, Japan). A Zorbax Eclipse XDB-C18 (4 × 150 mm, Agilent Technologies, Santa Clara, CA, USA) was used for analysis; a mobile phase comprising 0.1% triethylamine acetate in water/acetonitrile (73:27, v/v) and a flow rate of 1 mL/min were employed.[15] Conversion levels of lactone into the carboxylate form were calculated using a calibration curve constructed from a standard solution of the carboxylate prepared by overnight incubation of CPT in a NaOH solution at pH 11.

***In vitro* cytotoxicity studies**

A mouse melanoma B16 cell line was purchased from RIKEN cell bank. B16 cells were cultured in Dulbecco's modified eagle medium (DMEM) (GIBCO, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B (GIBCO). First, 5,000 cells were seeded in wells of a 96-well plate and incubated overnight at 37°C under 5% CO_2 atmosphere. The medium was removed and the CPT–Pluronic complexes dispersed in 100 μL of Opti-MEM (GIBCO) at varying concentrations of 0.001–10 μg/mL were added to the wells. As a control, free CPT solutions in Opti-MEM at the same concentrations were prepared and added to the cells. After 24 h incubation, CPT solution was removed, 110 μL of WST-8 solution (Dojindo, Kumamoto, Japan) in Opti-MEM was added to each well, and the cells were incubated at 37°C. The absorbance at 450 nm derived from WST-8 formazan was recorded on a Power Wave X (BioTek, Winooski, VT, USA). Cytotoxicity was evaluated, using the non-treated cells incubated under the same conditions in the absence of CPT and Pluronic as reference.

Cellular internalisation studies

CPT–Pluronic complexes labelled with Rho-DOPE were prepared as described above, except that 10 μL of 1 mg/mL Rho-DOPE in methanol was added to the Pluronic aqueous solution.

For a CLSM analysis, B16 cells were seeded in a multi-well glass bottom dish (Matsunami Glass Ind., Ltd., Osaka, Japan) at a density of 10,000 cells/well and incubated overnight in DMEM supplemented with 10% FBS at 37°C under 5% CO_2 atmosphere. The medium was removed and the CPT–Pluronic complexes dispersed in 200 μL Opti-MEM at a concentration of 1 μg/mL were added to the wells. After 2 and 6 h of incubation, the samples were removed and the nuclei were stained with Hoechst 33342 (Dojindo, Kumamoto, Japan) for 10 min and the endosomes/lysosomes were stained with LysoTracker® Green DND-26 (Invitrogen, Carlsbad, CA, USA) for 20 min. Confocal images of the cells were obtained using a LSM 700 microscope (Carl Zeiss, Oberkochen, Germany). As a control, physical mixtures of CPT, Pluronic and Rho-DOPE in Opti-MEM were added to the cells and the resulting confocal images were recorded under the same conditions. Although CPT shows a similar fluorescence to that of Hoechst 33342, a control experiment using B16 cells without staining with Hoechst 33342 revealed that the blue emission in Figure S5 was derived from nuclei staining by Hoechst 33342 (data not shown).

For a flow cytometric analysis, B16 cells were seeded in a Nunc cell-culture treated 6-well dish (Thermo Fisher Scientific Inc., Waltham, MA, USA) at a density of 50,000 cells/well and incubated overnight in DMEM supplemented with 10% FBS at 37°C under 5% CO_2 atmosphere. The medium was removed and the CPT–Pluronic complexes dispersed in 2 mL Opti-MEM at a concentration of 0.1 μg/mL were added to the wells. After 6, 12, 24 h of incubation, the samples were removed and cells were washed with PBS. The cells were then tripsinyzed, washed with cold PBS, centrifuged, and dispersed in PBS containing 1% FBS. After filtered through 35 μm cell-strainer, the cells were subjected to a flow cytometric analysis using a SONY ec800 flow cytometer with the excitation wavelength of 488 nm.

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Notes and references

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Graphical abstract