Enhancing docetaxel delivery to multidrug-resistant cancer cells with albumin-coated nanocrystals

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Abstract

Intravenous delivery of poorly water-soluble anticancer drugs such as docetaxel (DTX) is challenging due to the low bioavailability and the toxicity related to solubilizing excipients. Colloidal nanoparticles are used as alternative carriers, but low drug loading capacity and circulation instability limit their clinical translation. To address these challenges, DTX nanocrystals (NCs) were prepared using Pluronic F127 as an intermediate stabilizer and albumin as a functional surface modifier, which were previously found to be effective in producing small and stable NCs. We hypothesize that the albumin-coated DTX NCs (DTX-F-alb) will remain stable in serum-containing medium so as to effectively leverage the enhanced permeability and retention effect. In addition, the surface-bound albumin, in its native form, may contribute to cellular transport of NCs through interactions with albumin binding proteins such as Secreted protein acidic and rich in cysteine (SPARC). DTX-F-alb NCs showed sheet-like structure with an average length, width, and thickness of 284 ± 96 nm, 173 ± 56 nm, and 40 ± 8 nm and remained stable in 50% serum solution at a concentration greater than 10 $\mu\text{g/mL}$. Cytotoxicity and cellular uptake of DTX-F-alb and unformulated (free) DTX were compared on three cell lines with different levels of SPARC expression and DTX sensitivity. While the uptake of free DTX was highly dependent on DTX sensitivity, DTX-F-alb treatment resulted in relatively consistent cellular levels of DTX. Free DTX was more efficient in entering drug-sensitive B16F10 and SKOV-3 cells than DTX-F-alb, with consistent cytotoxic effects. In contrast, multidrug-resistant NCI/ADR-RES cells took up DTX-F-alb more than free DTX with time and responded better to the former. This difference was reduced by SPARC knockdown. The high SPARC expression level of NCI/ADR-RES cells, the known affinity of albumin for SPARC and the opposing effect of SPARC knockdown support that DTX-F-alb have exploited the surface-bound albumin-SPARC

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Supporting Information **Available:** Supporting Table and Figures are available free of charge via the Internet at <http://pubs.acs.org>.

interaction in entering NCI/ADR-RES cells. Albumin-coated NC system is a promising formulation for the delivery of hydrophobic anticancer drugs to multidrug-resistant tumors.

Graphical abstract

Keywords

Docetaxel; poorly soluble drugs; albumin; nanocrystals; P-glycoprotein; secreted protein acidic and rich in cysteine (SPARC)

1. Introduction

Development of new anticancer drug products remains challenging because many anticancer drugs are poorly soluble in water and hence require the use of solubilizers for therapeutic applications.¹ Traditionally, organic solvents, co-solvents and surfactants are used to formulate anticancer drugs (e.g., Cremophor EL in Taxol®) for intravenous injection.^{2, 3} However, these excipients often involve dose-limiting toxicities such as severe hypersensitivity reactions, which are not always prevented by antihistamines and corticosteroids.⁴ Colloidal drug carriers, such as nanoemulsions,^{5, 6} liposomes,⁷ nanoparticles⁸ and polymeric micelles⁹ are actively pursued as alternative options; however, low drug loading efficiency, premature drug release in circulation, safety concerns for carrier materials and complicated manufacturing steps limit their clinical translation.¹⁰

Nanocrystals (NCs) have gained increasing interest in the delivery of poorly soluble anticancer drugs. An outstanding advantage of NCs is the high drug content, which minimizes the concerns related to non-drug excipients. Moreover, NCs composed of molecules with high lattice energy can remain stable during the initial phase of circulation and accumulate in tumors via the enhanced permeability and retention (EPR) effect.¹¹ One of the challenges in developing NCs, however, is the control of particle size and surface composition, which are critical to long-term circulation and favorable interaction with target tissues. In our previous work with paclitaxel (PTX), we have demonstrated that Pluronic F127 (F127)-assisted crystallization and surface coating with intact albumin effectively address these challenges.¹² First, F127, a triblock copolymer of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO), forms an amorphous mixture with PTX. During the subsequent hydration step, F127 interferes with the growth of PTX

NCs by binding to the crystal surface.¹³ Albumin then binds to the NC surface with native conformation and reinforces the surface protection keeping the size as small as 200 nm.¹² The surface-bound albumin can also help NCs interact with cancer cells via albumin-binding proteins such as SPARC (secreted protein acidic and rich in cysteine), overexpressed in various cancer cells and their surrounding stroma.^{14, 15} The affinity of albumin for SPARC may facilitate albumin-coated NCs to enter drug-resistant cells that unformulated drug finds difficult to access and/or increase drug accumulation within tumors.^{16, 17}

In this study, we apply the F127-assisted crystallization and albumin coating to docetaxel (DTX), another taxane analog with low water solubility, to examine the extended applicability and utility of this method. DTX is traditionally formulated with Tween 80 and ethanol (Taxotere®). Given the side effects caused by Tween 80 such as hypersensitivity reactions, peripheral neuropathy and hemolytic activity,⁴ reformulating DTX with a minimal amount of excipients is well justified. We expect that albumin-coated DTX NCs will remain stable in serum, satisfying the primary requirement for long-term circulation. On the basis of known interactions between albumin and SPARC, we also hypothesize that the surface-bound albumin will facilitate the transport of DTX to cancer cells and test this hypothesis using three cell lines with different levels of SPARC expression and drug sensitivity. Our results demonstrate that DTX-F-alb NCs can be produced in a small size amenable to cellular uptake and maintain good stability in serum-containing medium. DTX-F-alb NCs improve DTX delivery to SPARC-positive drug resistant cells, demonstrating the contribution of the surface-bound albumin to cellular uptake of DTX.

2. Materials and Methods

2.1. Materials

Docetaxel (DTX) was purchased from LC Laboratories (Woburn, MA, USA). Pluronic F127 (F127) was a gift from BASF (New York, NY, USA). Human serum albumin (96% agarose gel electrophoresis) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad (Hercules, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Hoechst 33342 were purchased from Invitrogen (Eugene, OR, USA). Antibodies against human SPARC (hSPARC) and mouse Sparc (mSparc) were from R&D system (Minneapolis, MN, USA). The antibody against β -actin was from Cell Signaling (Danvers, MA, USA). All other reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Solubility of DTX

Equilibrium solubility of DTX was measured in phosphate buffered saline (PBS, pH 7.4), PBST (PBS containing 0.2% Tween 80), 10% fetal bovine serum (FBS)/PBS and 50% FBS/PBS at room temperature (RT) and 37 °C. An excess amount of DTX (2–2.3 mg) was added to 1 mL of each medium and agitated on a rotary tube shaker for 7 or 24 h. The suspension was centrifuged at 15,700 rcf for 20 min to separate a supernatant, which was filtered with a syringe filter (0.45 μ m pore size) and analyzed with high pressure liquid chromatography (HPLC). DTX in PBS and PBST was analyzed without further treatment.

DTX in 10% FBS/PBS and 50% FBS/PBS was extracted using ethyl acetate.¹⁸ Briefly, 0.5 mL of the filtered sample was doped with 35 µg of carbamazepine (CBZ) as an internal standard, mixed with 1.5 mL of ethyl acetate, and shaken for 1 h. The mixture was then centrifuged at 3,724 rcf for 25 min to separate the ethyl acetate layer, which was transferred to a glass tube and dried under vacuum. The dried samples were dissolved in 50% acetonitrile (ACN), filtered through a 0.45 µm syringe filter, and analyzed with HPLC. DTX standards with known concentrations in 10% FBS/PBS or 50% FBS/PBS were treated and analyzed in the same manner to build a calibration curve.

2.3. HPLC analysis of DTX

DTX was analyzed with reverse phase (RP)-HPLC equipped with a UV detector with the wavelength set at 227 nm (1100 series, Agilent Technologies Palo Alto, CA, USA). The stationary phase was Ascentis C18 analytical column (250 mm × 4.6 mm, particle size 5 µm) (Supelco, St. Louis, MO, USA). Fifty percent ACN was used as an isocratic mobile phase at a flow rate of 1 mL/min. Samples were dissolved in 50% ACN and filtered through syringe filters (0.45 µm pore size) prior to analysis.

2.4. Preparation of albumin-coated DTX nanocrystals (DTX-F-alb, Scheme 1)

Albumin-coated DTX NCs (DTX-F-alb) were prepared as described previously.¹² First, 5 mg of DTX and 20 mg of F127 were dissolved in 3 mL of chloroform. Chloroform was evaporated under vacuum using a rotary evaporator at 40 °C to dryness. The obtained dried film was hydrated with 5 mL of water for 5 sec with bath sonication to form a suspension of F127-coated DTX NCs (DTX-F). The suspension was further sonicated with a SONICS® Vibra-Cell ultrasonic liquid processor (Newtown, CT, USA) at 40% amplitude and a 1:1 duty cycle every 2 sec for 20 min. The suspension was kept cool in an ice bath during sonication.

Albumin coating was performed by simple incubation of DTX-F in 4 mg/mL human serum albumin solution in water. Unless stated otherwise, incubation in albumin solution was performed for 24 h. DTX-F-alb was collected by ultracentrifugation at 58,300 rcf for 15 min at 4 °C. The obtained pellet was resuspended in water by probe sonication at 20% amplitude for a few seconds.

2.5. Characterization of DTX-F-alb

2.5.1. Particle size and zeta potential measurement—Particle size and zeta potential of DTX-F and DTX-F-alb were determined by dynamic light scattering (DLS) using a Zetasizer NanoZS90 (Malvern instruments, Worcestershire, UK). For size measurement, NC suspension was diluted in water or in phosphate buffer (PB, 2.2 mM, pH 7.4). All zeta potential measurements were performed with samples prepared in PB.

2.5.2. Morphology—Nanocrystal shape and surface features were observed with transmission electron microscopy (TEM) and scanning electron microscopy (SEM). For TEM, freshly prepared samples were placed on a 400 mesh formvar-coated carbon grid. Excess sample was removed using a filter paper. The grid was then negatively stained with 1% uranyl acetate, dried in air, and visualized under FEI Tecnai T20 Transmission Electron

Microscope (Hillsboro, OR, USA) at an accelerated voltage of 200 kV. Samples for SEM were sputter coated with platinum for 60 sec and visualized with a FEI NOVA NanoSEM field emission scanning electron microscope (FEI Company, Hillsboro, OR, USA) at an excitation voltage of 5 kV, spot 3, 3 mm of working distance and 30 μ m aperture. The size of DTX-F-alb NCs was analyzed from EM images with the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.5.3. Crystallinity—DTX, albumin, a physical mixture of DTX and albumin, DTX-F, and DTX-F-alb were analyzed with a Rigaku SmartLab™ diffractometer (Rigaku Americas, Texas, USA) equipped with a Cu K α radiation source. The powder x-ray diffraction pattern (PXRD) of each sample was obtained from 5 to 40° (2 θ) at a scanning rate of 4 °/min. The voltage and current were 40 kV and 44 mA, respectively. Differential scanning calorimetry (DSC) thermograms were obtained with a TA Instrument Q2000 (TA Instruments, New Castle, DE, USA) at a heating rate of 10°C/min from 25 °C to 200 °C under a nitrogen purge of 50 mL/min.

2.5.4. Thermogravimetric analysis (TGA)—TGA analysis was performed on TA Instrument Q50 (TA Instruments, New Castle, DE, USA) at a heating rate of 10°C/min from 25 °C to 200 °C under a nitrogen purge of 40 mL/min. A platinum sample pan (Thermal Support, Hayesville, NC, USA) was used for housing lyophilized DTX-F-alb samples.

2.5.5. DTX content—Lyophilized DTX-F-alb was accurately weighed, dissolved in 50% ACN, and analyzed by HPLC. The DTX content was defined as the amount of detected DTX per DTX-F-alb mass.

2.5.6. Content of total and native albumin—The albumin content in DTX-F-alb was determined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A known mass of freeze-dried DTX-F-alb was dispersed in water at approximately 1 mg/mL, mixed with 4 $\times$ Laemmli sample buffer containing 10% 2-mercaptoethanol and 2% SDS and boiled at 95 °C for 5 min. The sample was cooled in ice and resolved by 15% SDS-PAGE. The gel was fixed in aqueous solution containing 40% ethanol and 10% acetic acid for 15 min, stained with QC Colloidal Coomassie Stain overnight, and destained in water for 3 h with repeated exchange. Images of stained gels were obtained with Azure C300 (Dublin, CA, USA) and subjected to densitometric analysis using Azure Spot software (Dublin, CA, USA). A calibration curve was drawn with albumin standards of known concentrations. The total albumin content was defined as the amount of albumin per DTX-F-alb mass.

To evaluate whether the bound albumin on DTX-F-alb maintained its native conformation, DTX-F-alb was analyzed for the esterase-like activity associated with native albumin.¹⁹ Two hundred microliters of albumin solutions with known concentrations or DTX-F-alb suspensions in phosphate buffer (100 mM, pH 7.4) were mixed with 4 μ L of 20 mM *p*-nitrophenyl acetate (pNPA) and incubated at room temperature. Immediately and 1 h after addition of pNPA, samples were centrifuged at 33,900 rcf for 10 min, and the resulting supernatant was used for the measurement of absorbance at 400 nm. The absorbance change, proportional to the rate of pNPA hydrolysis (i.e., *p*-nitrophenol formation), was used to determine the amount of native albumin. The percentage of native albumin was calculated by

dividing the amount of native albumin (determined by the esterase activity) with the amount of total albumin obtained from SDS-PAGE.

2.6. Physical stability of DTX-F-alb in aqueous media

Stability of DTX-F-alb in 10% FBS/PBS, 50% FBS/PBS, and 100% FBS was estimated using DLS according to the literature.²⁰ DTX-F-alb was diluted to concentrations equivalent to DTX 5, 10, 25, 50, 75 and 100 µg/mL in undiluted or diluted FBS/PBS media. The suspensions were separated into 1 mL aliquots and incubated at 37 °C on a rotating shaker. At predetermined time points (0, 1, 4, 7, and 24 h), 3 aliquots from each concentration were taken and analyzed with respect to the size and derived count rate (DCR, kcps) using a Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK). Ten percent, 50% or 100% FBS solutions were treated and analyzed in the same manner. The DCR of 10%, 50%, or 100% FBS was subtracted from the measurements of DTX-F-alb suspensions at the corresponding time point. The net DCR values were plotted against DTX concentrations.

The initial dissolution of DTX-F-alb in FBS and PBS was monitored by continuous measurement of light scattering over the first 50 min after dispersion in each medium at concentrations equivalent to DTX 2.5, 20, and 75 µg/mL. Measurements were made twice every two seconds at the measurement position of 4.65 nm and with an attenuator setting of 10.

2.7. Cell culture

SKOV-3 human ovarian cancer cells, B16F10 mouse melanoma cells (ATCC, Manassas, VA, USA), NCI/ADR-RES human multidrug-resistant ovarian cells (NCI, Fredrick, MD, USA), and 4T1 murine mammary carcinoma cells (Korean Cell Line Bank, Seoul, Korea) were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 IU/mL penicillin and 100 µg/mL streptomycin in humidified atmosphere at 37 °C with 5% CO₂.

2.8. Immunoblotting

Lysates of SKOV-3, B16F10, NCI/ADR-RES, and 4T1 cells (30 µg of total protein) were resolved with 10% SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 5% milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) and probed with antibodies against hSPARC (dilution 1:200, R&D system; AF941), mSparc (dilution 1:1000, R&D system; AF942) and β-actin (dilution 1:1000, Cell Signaling). The membrane was washed with TBST and probed with the corresponding secondary antibodies conjugated with horseradish peroxidase. The bound antibodies were visualized with an enhanced chemiluminescence substrate (Thermo Fisher Scientific, Waltham, MA, USA).

2.9. siRNA-mediated knockdown of hSPARC

NCI-ADR/RES cells were plated in 6 well plates (3×10⁵ cells per well) and cultured overnight. Cells were then transfected with siRNAs targeting hSPARC or control scrambled sequences (25 pmole/well, OriGene, Rockville, MD, USA) using LipofectamineTM RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction. After 24 h incubation with siRNAs, cells were harvested and re-

plated for another 24 and 48 h incubation in fresh medium and probed for the knockdown efficiency of hSPARC via immunoblotting analysis.

2.10. Cytotoxicity of DTX-F-alb

The cytotoxic effects of DTX-F-alb and unformulated DTX (called ‘free DTX’ hereafter) were compared in SKOV-3, B16F10, and NCI/ADR-RES cells. Cells were seeded in 96-well culture plates (10^4 cells per well for SKOV-3 and NCI/ADR-RES; 4×10^3 cells per well for B16F10), incubated overnight and treated with 0.01 – 10,000 nM of free DTX solution or DTX-F-alb suspension in equivalent DTX concentrations. Free DTX solution was prepared by dissolving DTX in DMSO followed by serial dilution with complete culture medium. The DMSO concentration in the medium was < 0.9% and did not affect cell viability. DTX-F-alb suspension was prepared in sterile water at a concentration equivalent to 2.2 mM DTX and diluted with complete culture medium. Cells treated with culture medium were included as a control. After 24 h, the drug-containing medium was replaced with 200 μ L of fresh drug-free medium. Following another 12 – 48 h of incubation, cell viability was measured by the MTT assay, as described previously.¹² The absorbance was measured at 562 nm using a SpectraMax M3 microplate reader (Molecular Devices, CA, USA). After subtracting the absorbance of medium only, each absorbance value was divided by the absorbance of control cells to calculate % cell viability. IC₅₀ values were calculated by fitting the observed data to sigmoidal dose-response curves with a standard slope using GraphPad Prism 7 (La Jolla, CA, USA).

In another set of experiments, cells were exposed to high concentrations of DTX-F-alb (20, 30, and 40 μ g/mL DTX eq. (24.8, 37.1, 49.5 μ M)) or free DTX solution for shorter terms (2 h for SKOV-3 and B16F10; 2, 4, 6 and 9 h for NCI/ADR-RES). After the drug treatment, the cells were rinsed twice with drug-free medium. SKOV-3 and NCI/ADR-RES cells were incubated in drug-free medium for 72 h, and B16F10 cells were incubated for 21 h prior to the MTT assay. The incubation times following drug treatment were adjusted according to the doubling times (Td) of each cell line: B16F10 cells with a Td of 17.2 h,²¹ SKOV-3 cells 35 h,²² and NCI/ADR-RES cells 34 h.²³ This experiment was repeated with hSPARC knocked-down NCI/ADR-RES cells.

2.11. Cellular uptake of DTX-F-alb

2.11.1. Quantitative analysis—Cells were seeded in a 6-well plate at a density of 10^6 cells per well. After overnight, cells were incubated with DTX-F-alb or free DTX for 1 or 3 h at 37 °C at a concentration equivalent to 30 or 40 μ g/mL (37.1 or 49.5 μ M) DTX. After removing the treatments, cells were rinsed twice with complete medium, trypsinized and collected by centrifugation. Cell pellets were suspended in PBS, lysed by three freeze-thaw cycles, and probe-sonicated. The total protein content in cell lysate was analyzed using the micro BCA protein assay and used for normalization. After adding CBZ (an internal standard), each cell lysate was subjected to liquid-liquid extraction using ethyl acetate. After 1 h incubation, the ethyl acetate phase was separated by centrifugation at 3,724 rcf for 25 min, dried under vacuum, reconstituted with 50% ACN, and analyzed with HPLC. Standards with known DTX concentrations in PBS were handled in the same manner. This experiment was repeated with hSPARC knocked-down NCI/ADR-RES cells.

2.11.2. Confocal imaging and flow cytometry—Cellular uptake of DTX-F-alb by NCI/ADR-RES cells was visualized with confocal microscopy. DTX-F-alb was fluorescently labeled by doping rhodamine B at 10 wt% of DTX during the hydration step.¹² The labeled NCs were called DTX*-F-alb. Rhodamine B incorporated in DTX*-F-alb was quantified by dissolving the NCs in 50% ACN and measuring its fluorescence intensity at $\lambda_{\text{ex}}/\lambda_{\text{em}}$ of 540 nm/620 nm. NCI/ADR-RES cells were seeded in a 35 mm dish with a glass bottom (MatTek Corp., Ashland, MA, USA) at a density of 100,000 cells per dish. After overnight, the cells were incubated with DTX*-F-alb corresponding to 30 $\mu\text{g/mL}$ DTX or free rhodamine B at an equivalent concentration for 3 h. Cells were then rinsed twice with PBS and fixed with 4% paraformaldehyde in PBS. Nuclear staining was done with Hoechst 33342 (2 $\mu\text{g/mL}$ for 10 min). After rinsing 2–3 times with PBS, the fixed cells were imaged with Nikon A1R confocal microscope (Nikon America Inc., Melville, NY, USA). Hoechst was detected with $\lambda_{\text{ex}}/\lambda_{\text{em}}$ of 407 nm/425–475 nm. DTX*-F-alb and free rhodamine B were detected with $\lambda_{\text{ex}}/\lambda_{\text{em}}$ of 560 nm/570–620 nm. Another group of NCI/ADR-RES cells was treated in the same way for flow cytometry. After 3 h incubation with DTX*-F-alb or free rhodamine B, the cells were rinsed twice with PBS and analyzed by an Accuri C6 flow cytometer (BD Science, San Jose, CA, USA) with a FL-2 detector ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 488/585$ nm).

2.12. Statistical analysis

All results were expressed as mean $\pm$ standard deviations. Using GraphPad Prism 7 (La Jolla, CA, USA), appropriate statistical analyses were performed, either unpaired t-test with Welch's correction or two-way analysis of variance (ANOVA) followed by Bonferroni's or Tukey's multiple comparisons test. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. Preparation of DTX-F-alb

Based on the low solubility of DTX in PBS (2.31 ± 0.05 $\mu\text{g/mL}$, Supporting Table 1), DTX was deemed a good candidate for NC formation. Albumin-coated DTX NCs (DTX-F-alb) were prepared according to the reported method.¹² Incipient DTX NCs (DTX-F) were made by hydrating a film of DTX and Pluronic F127 (F127) mixture. F127 helped form small NCs (< 300 nm) during the hydration. Without F127, DTX formed precipitates bigger than 1 μm (Supporting Fig. 1). F127 added after the formation of DTX precipitates did not resolve the precipitates, consistent with the report of Liu et al.²⁴ In addition to F127, probe sonication also prevented NCs from growing further. The particle size and the polydispersity index (PDI) were 383.3 ± 2.8 nm and 0.27 ± 0.04 with 5 min sonication but decreased to 248.7 ± 1.67 nm and 0.13 ± 0.05 with a prolonged sonication up to 20 min (Supporting Fig. 2). Sonication for longer than 20 min rather increased the particle size and PDI, likely due to the heat generated during the sonication.

DTX-F was functionalized with human serum albumin. Albumin adsorbed to the NC surface and protected the NCs from aggregation during the subsequent processing steps. Without albumin, DTX-F did not withstand centrifugation or filtration and formed aggregates larger

than 500 nm with the removal of F127 (Fig. 1a, b). On the other hand, albumin-coated DTX-F (DTX-F-alb) maintained its size after the repeated centrifugation and washing (Fig. 1c).

3.2. Characterization of DTX-F-alb

The average albumin content in the purified DTX-F-alb was measured by separating albumin from a known mass of NCs by SDS-PAGE and quantifying the band intensity. The content of volatiles including moisture in DTX-F-alb, measured by TGA, was 0.06% and considered negligible (Supporting Fig. 3). The average albumin content in the purified DTX-F-alb depended on the duration of DTX-F incubation with albumin. The albumin content increased from $4.7 \pm 2.3\%$ with 3 h incubation to $17.7 \pm 4.0\%$ with 24 h (Fig. 2a), accompanied by moderate decrease in surface charge (Fig. 2a). The average DTX content in DTX-F-alb ranged from $79.6 \pm 4.7\text{ wt\%}$ to $76.9 \pm 6.4\text{ wt\%}$, slightly decreasing with the increase of the albumin content. The F127 content was estimated to be no more than 18 wt% of DTX-F-alb based on the mass balance. The esterase-like assay suggested that at least 80% of the surface-bound albumin maintained the intact structure (data not shown), consistent with our previous study with albumin-coated PTX NCs (PTX-F-alb).¹²

DTX-F-alb prepared with 24 h albumin incubation had a z-average of $246.4 \pm 6.9\text{ nm}$ (Supporting Fig. 4) and an average zeta potential of $-16.3 \pm 2.6\text{ mV}$ (Fig. 2a) as measured by DLS. Although longer incubation with albumin increased the albumin content (Fig. 2a), it did not significantly change the particle size of DTX-F-alb (Supporting Fig. 4), suggesting that the albumin layer did not amount to a measurable thickness. SEM and TEM found DTX-F-alb to be a thin rectangular sheet with an average length of $284 \pm 96\text{ nm}$ and a width of $173 \pm 57\text{ nm}$ ($n = 107$) (Fig. 3). The average thickness of the sheet estimated from SEM images was $40 \pm 8\text{ nm}$ ($n = 80$). The size of DTX-F-alb suspended in water remained constant for at least 72 h at 4 °C (Supporting Fig. 5). DTX-F-alb lyophilized with trehalose as a lyoprotectant was reconstituted to the original size (Supporting Fig. 6).

Powder X-ray diffraction (PXRD) patterns of DTX-F-alb and its components were obtained (Fig. 2b). Both DTX powder as-received and DTX-F showed intense sharp peaks typical of crystalline solids. Albumin showed no crystalline peaks, which indicated its amorphous status. A physical mixture of albumin and DTX manifested crystalline peaks of DTX. In contrast, DTX-F-alb showed less intense peaks than DTX-F, indicating the surface coverage with amorphous solid. The PXRD pattern of DTX-F-alb lacked characteristic two peaks of F127^{12, 13}; thus, the weakened peak intensity is attributable to the amorphous albumin. On the other hand, DSC thermograms of DTX-F-alb showed signs of all the ingredients including a sharp melting endotherm at $\sim 170\text{ °C}$, corresponding to the melting point of DTX (Fig. 2c). This indicates that DTX core underneath the amorphous albumin maintained the crystalline structure.

3.3. Physical stability of DTX-F-alb

To predict the stability of DTX-F-alb in cell culture medium and blood, the derived count rates of DTX-F-alb in 10% or 50% FBS/PBS were measured with dynamic light scattering.²⁰ To compare with PTX-F-alb,¹² the analysis was also performed in 100% FBS. Solid nanoparticles scatter light with an intensity (DCR) proportional to the concentration.²⁵

According to this principle, a suspension of stable NCs displays a linear relationship between the concentration and scattering intensity as shown with DTX-F-alb in PBS (Supporting Fig. 7a). Completely dissolved samples show similar intensity to that of blank medium (flat base line). Dissolution of NCs can thus manifest as deviations from the linear relationship. Solubility of the NCs can be determined from the inflection point. DTX-F-alb showed linear relationship with concentration at $> 5 \mu\text{g/mL}$ in 10% FBS (Fig. 4a) and at $> 10 \mu\text{g/mL}$ in 50% and 100% FBS (Fig. 4b, Supporting Fig. 7b) over 24 h. This suggests that DTX-F-alb will remain as NCs at concentrations $> 5 \mu\text{g/mL}$ in cell culture medium (with 10% FBS) and $> 10 \mu\text{g/mL}$ in blood (with 50% serum²⁶). This estimation is consistent with the DTX solubility of 14 and 20 $\mu\text{g/mL}$ in 10% and 50% FBS, respectively (Supporting Table 1). When the dissolution of DTX-F-alb was continuously monitored in 100% FBS, DTX-F-alb maintained its derived count rate over 50 min (duration of observation) at DTX equivalent to 20 and 75 $\mu\text{g/mL}$ (Supporting Fig. 8a). The physical stability of DTX-F-alb in serum-containing medium is comparable to that of PTX-F-alb.¹²

3.4. Cytotoxicity of DTX-F-alb

Cytotoxicity of DTX-F-alb was tested using three cell lines that display different DTX sensitivity and SPARC expression levels. SKOV-3 and B16F10 cells were chosen as DTX-sensitive cells, and multidrug resistant P-glycoprotein (P-gp) expressing NCI/ADR-RES²⁷ as DTX-resistant cells. The expression level of mSparc in B16F10 cells was much higher than that in 4T1 cells (previously reported to be mSparc-positive).¹⁶ The expression level of hSPARC was high in NCI/ADR-RES cells but relatively weak in SKOV-3 cells (Supporting Fig. 9).

With 24 h incubation at concentrations below the solubility of DTX in 10% FBS (14 $\mu\text{g/mL}$, 17 μM), the cytotoxic effects were comparable between DTX-F-alb and free DTX, showing low nanomolar IC_{50} values for SKOV3 and B16F10 cells and $\text{IC}_{50} > 1,000 \text{ nM}$ for NCI/ADR-RES cells (Supporting Fig. 10). This indicates that DTX-F-alb in the concentration range tested (0.01 – 10,000 nM: i.e., 0.008 ng/mL – 8 $\mu\text{g/mL}$) has completely dissolved in culture medium (containing 10% FBS) over 24 h, as predicted from the light scattering and solubility measurements.

With a short-term exposure at high concentrations ($> 20 \mu\text{g/mL}$, above DTX solubility in 10% FBS), the cytotoxic effects of DTX-F-alb differed from those of free DTX in a cell line-dependent manner. At concentrations equivalent to 20–40 $\mu\text{g/mL}$ (24.8–49.5 μM) of DTX, SKOV-3 cells responded similarly to DTX-F-alb and free DTX, both reaching 50% cell viability after 2 h exposure and 70 h follow-up incubation (Fig. 5a). In B16F10 cells, DTX-F-alb was less cytotoxic than free DTX at 30 and 40 $\mu\text{g/mL}$ (Fig. 5b). To the contrary, NCI/ADR-RES cells responded better to DTX-F-alb than to free DTX when exposed to 4 h (Fig. 5c). To rule out the possibility that cytotoxic effects observed in NCI/ADR-RES cells were from F127 itself, NCI/ADR-RES cells were incubated with varying concentrations of F127 (0.001 – 1,000 $\mu\text{g/mL}$, which covers the highest level possible for F127 (40 $\mu\text{g/mL} \times 18\% = 7.2 \mu\text{g/mL}$)). F127 alone did not show any detectable cytotoxic effect against NCI/ADR-RES cells with 4 or 6 h exposure (Supporting Fig. 11). To examine the potential impact of F127 on DTX transport (e.g., P-gp inhibition²⁸) and the resulting cytotoxic effects,

NCI/ADR-RES cells were treated with a physical mixture of DTX (30 or 40 $\mu\text{g/mL}$) and F127 (10 $\mu\text{g/mL}$). Irrespective of the exposure time, the mixture showed no difference from free DTX (Supporting Fig. 12), indicating that F127 was not directly involved in improving cytotoxic effects of DTX-F-alb on NCI/ADR-RES cells.

3.5. Cellular uptake of DTX-F-alb

To understand differential responses to DTX-F-alb with short-term exposure, we compared the levels of DTX taken up in each cell type after 3 h incubation with DTX-F-alb. In both SKOV-3 and B16F10 cells, DTX-F-alb treatment showed lower cellular drug levels than free DTX (Fig. 6a, b). NCI/ADR-RES cells showed an opposing trend. Notably, NCI/ADR-RES cells incubated with DTX-F-alb took up 2.5 times more DTX than those with free DTX in 1 h (Fig. 6c). The fold difference increased to ~ 8 in 3 h (Fig. 6c). NCI/ADR-RES cells took up DTX-F-alb with a lower albumin content (i.e., DTX-F-alb produced by 3 h incubation as compared to 24 h) as efficiently (Supporting Fig. 13).

To complement the measurement of cellular DTX levels, we visualized NCI/ADR-RES cells treated with fluorescently labeled DTX*-F-alb via confocal microscopy and quantified the cellular fluorescence intensity by flow cytometry. DTX*-F-alb particles were indistinguishable from regular DTX-F-alb in the size and shape (Supporting Fig. 14). The cells treated with DTX*-F-alb for 3 h showed bright punctate rhodamine B signals (Fig. 7a) and greater fluorescence intensity than non-treated cells (Fig. 7b, c). Those treated with an equivalent concentration of free rhodamine B did not show detectable fluorescence signals (Fig. 7b, c), indicating that the fluorescence seen with DTX*-F-alb was likely from solid particles, not from a free dye diffusing out of the NCs.

3.6. Effect of SPARC silencing on cellular uptake and cytotoxicity of DTX-F-alb

To examine the contribution of SPARC in cellular uptake and cytotoxicity of DTX-F-alb, NCI/ADR-RES cells were treated with siRNA targeting hSPARC (siSPARC) prior to the DTX-F-alb. Knockdown of hSPARC was confirmed at 48 and 72 h from the siSPARC treatment (Fig. 8a). The siSPARC-treated NCI/ADR-RES cells took up DTX-F-alb significantly less than the wild-type (WT) cells or those treated with negative control siRNA (siNeg) (Fig. 8b). Consistently, the siSPARC-treated NCI/ADR-RES cells were less responsive to DTX-F-alb than WT or siNeg-treated cells (Fig. 8c).

4. Discussion

DTX-F-alb was produced as we described previously. First, DTX and F127 were coprecipitated as an intimate mixture. DTX crystallization then took place during hydration and sonication of the mixture. Both F127 and probe sonication helped limit the size growth. However, the surface protection of F127 is shown to be short-lived and concentration-dependent.^{13, 29} Indeed, DTX-F (NCs coated with F127 alone) did not resist aggregation during the subsequent washing steps with the removal of excess F127. In contrast, albumin adsorbed to the NC surface and maintained the size during the same process, indicating that albumin had higher affinity for NC surface than F127. Although not visually identified on

EM images, the surface-bound albumin attenuated the crystalline PXRD of underlying DTX NCs (Fig. 2b) and allowed them to maintain the size in water at least for 72 h at 4 °C.

DTX-F-alb showed a thin rectangular sheet-like structure with an average length, width, and thickness of 284 ± 96 nm, 173 ± 56 nm, and 40 ± 8 nm, respectively. This size is below the cutoff of hyperpermeable tumor microvessels ($1.2 \mu\text{m}$)³⁰; thus, we expect that DTX-F-alb can reach tumors via the leaky vasculature. It is reported that particles with an average diameter of 100–400 nm can be taken up by non-phagocytic cells via clathrin-mediated endocytosis and >500 nm via macropinocytosis.³¹ Although the size range may also vary with the particle shape, DTX-F-alb with the mentioned dimension is likely conducive to cellular uptake, possibly by macropinocytosis, given that albumin is a known substrate of macropinocytosis in cancer cells.³² The sheet-like structure is consistent with other DTX NCs reported in the literature.^{33–35} It is worthwhile to note that the shape of DTX-F-alb (sheet) is quite distinct from PTX-F-alb (rod) despite the structural similarity between DTX and PTX molecules (Supporting Fig. 15). The apparent difference in crystal structure may be attributable to the orientation of the side chains. According to the literature, *t*-butyl group of DTX is directed outwards, and two phenyl rings stay away from each other allowing neighboring DTX molecules to stack in the same direction.³⁶ On the other hand, PTX molecules arrange in a more compact manner as three phenyl rings fit into the hollow parts of neighboring molecules.³⁶ With this difference, the two taxane analogs may develop distinct intermolecular interactions leading to respective crystal structures.

The light scattering behavior of DTX-F-alb indicated that DTX-F-alb would maintain the particle form in 10%, 50% FBS/PBS, or 100% FBS over 24 h at 37 °C at a concentration above the DTX solubility in each medium ($> 5 \mu\text{g/mL}$ in 10% FBS and $> 10 \mu\text{g/mL}$ in 50% and 100% FBS). When the typical DTX dose of 10–20 mg/kg^{37, 38} is administered to mice with an average blood volume of 1.5–2.5 mL,³⁹ the initial blood concentration of DTX is roughly estimated to be 80–267 $\mu\text{g/mL}$. Given that tumor deposition of circulating nanoparticles generally occurs in the first 24 h,¹¹ we may predict that DTX-F-alb will circulate as nanoparticles and effectively leverage the EPR effect. At a concentration below DTX solubility, DTX-F-alb readily dissolved (Supporting Fig. 8b) to show similar IC_{50} values as free DTX (Supporting Fig. 10). This indicates that DTX-F-alb reaching tumor interstitium and/or intracellular space will dissolve with time to exert the cytotoxic effect.

We next examined cytotoxicity and cellular uptake of DTX-F-alb to find whether the surface-bound albumin facilitates cellular delivery of the NC. As estimated from the esterase-like activity, most of the surface-bound albumin retained its native conformation. Thus, DTX-F-alb was expected to interact with albumin-binding proteins such as SPARC and use it as an alternative pathway to deliver DTX to drug-resistant cells. To test this, we compared the responses of three cell lines with different levels of SPARC expression and DTX sensitivity to the treatment of free DTX and DTX-F-alb (B16F10 cells: drug-sensitive, high mSPARC expression; SKOV-3 cells: drug-sensitive, low hSPARC expression; NCI/ADR-RES cells: drug resistant, high hSPARC expression). The DTX-F-alb concentrations were kept above the solubility in 10% FBS to maintain the particle form. The exposure time was limited to 2 h to reflect dynamic *in-vivo* environment, where the contact of deposited nanoparticles with cancer cells will likely decline or be removed over a short time span. In

SKOV-3 cells, free DTX showed a similar cytotoxic effect as DTX-F-alb (Fig. 5a). B16F10 cells were slightly less responsive to DTX-F-alb than to free DTX, especially at high concentrations (Fig. 5b). In both cell lines, intracellular DTX levels (measured after 3 h incubation) were higher with free DTX than with DTX-F-alb (Fig. 6a and b), indicating more efficient uptake of free DTX. Despite the differential intracellular DTX levels, SKOV-3 cells were similar in their response to free DTX and DTX-F-alb suggesting that the intracellular DTX levels might have been more than enough to achieve maximum cytotoxic effect. This was not the case with B16F10 cells, most likely due to the attenuated dissolution of DTX-F-alb, coupled with the relatively short follow-up incubation time (21 h vs. 72 h). In contrast, NCI/ADR-RES cells showed greater uptake of DTX with DTX-F-alb than free DTX (Fig. 6c). Although neither free DTX nor DTX-F-alb showed significant toxicity with 2 h exposure, NCI/ADR-RES cells responded significantly better to DTX-F-alb than free DTX with a prolonged exposure (4 h) that allowed for greater uptake of DTX-F-alb (Fig. 5c). These results corroborate our working hypothesis that the surface-bound albumin would help DTX-F-alb to enter NCI/ADR-RES cells via albumin-binding proteins including SPARC and provide sustained intracellular DTX exposure, bypassing P-gp efflux pump.⁴⁰ The efficient NC uptake by NCI/ADR-RES cells was observed with DTX-F-alb containing less amount of albumin (Supporting Fig. 13), further supporting the effectiveness of the surface-bound albumin.

The differential uptake behavior was readily noticeable when the intracellular DTX contents of the three cell lines were replotted on the same scale (Supporting Fig. 16). Free DTX uptake varied significantly by cell type reflecting the drug sensitivity, but DTX levels in DTX-F-alb-treated cells was relatively consistent. The cellular level of DTX also suggests potential contribution of SPARC-mediated interactions to cellular entry of DTX (as DTX-F-alb or albumin-bound form of free/dissolved DTX). Due to the species difference, the level of human SPARC (hSPARC) expression in SKOV-3 cells cannot be directly compared to that of mouse Sparc (mSparc) expression in B16F10 cells. Previous reports however showed that SKOV-3 cells express a low level of hSPARC,⁴¹ and that B16F10 cells express a high level of mSparc when compared to other murine cell lines.¹⁶ After DTX-F-alb treatment, cellular DTX levels were higher in B16F10 cells (with high mSparc expression) than in SKOV-3 cells (with low hSPARC expression). Cellular DTX levels in DTX-F-alb-treated SKOV-3 and NCI/ADR-RES cells appeared similar despite differential SPARC expression. However, it needs to be considered that cellular DTX levels in SKOV-3 reflect not only DTX-F-alb but also DTX dissolved in extracellular space during the 3 h incubation (no more than 14 µg/mL (<50% of total NCs) given the equilibrium solubility measured in 10% FBS/PBS, Supporting Table 1) while NCI/ADR-RES cells rely exclusively on DTX-F-alb. The contribution of SPARC to the interaction with DTX-F-alb was also confirmed in the same cell line with differential SPARC expression (Fig. 8). NCI/ADR-RES cells with hSPARC knockdown showed significantly reduced uptake of DTX-F-alb and remained less sensitive to the NCs as compared to WT cells or those treated with negative control siRNA.

Pluronic F127 is known to suppress P-gp pump via depletion of intracellular ATP.^{28, 42} DTX-F-alb contained maximum 18 wt% of F127; however, we did not find the remaining F127 responsible for the increased cytotoxicity of DTX-F-alb in NCI/ADR-RES cells. First, F127 alone showed no detectable cytotoxic effect (Supporting Fig. 11), as expected from the

large body of literature supporting its safety.^{28, 43, 44} Second, a physical mixture of free DTX and F127 (at the highest possible level included in DTX-F-alb) yielded cytotoxic effects no different than free DTX alone (Supporting Fig. 12). Typical concentrations that Pluronic polymers show P-gp inhibitory effect (0.1–10 mg/mL^{33, 42, 45–48}) are much higher than those involved in this study (1–7.2 µg/mL). Therefore, we speculate that the amount of F127 included in DTX-F-alb was not sufficient to negatively impact the P-gp function. Consistently, Liu et al. have reported that PTX NCs stabilized with F127 (i.e., PTX-F with excess F127 unremoved) did not overcome drug resistance in NCI/ADR-RES cells.²⁹ Therefore, we reason that the superior cytotoxicity of DTX-F-alb in NCI/ADR-RES cells is mainly due to the ability to enter the cells, most likely via SPARC-albumin interactions, which is verified by the opposing effect of SPARC knockdown (Fig. 8b, c).

In this study, we focused on the contribution of surface-bound albumin to cellular transport of NCs. However, there are additional potential benefits of albumin in other levels of delivery, expected from diverse roles of serum albumin. A number of studies have demonstrated that albumin is actively taken up by tumor cells by endocytosis or macropinocytosis³² as a major source of energy and nutrients.⁴⁹ Albumin can also bind to endothelial glycoprotein (gp) 60 and/or interstitial SPARC in tumors to preferentially reach the tumor space.^{17, 50} Moreover, as the most abundant serum protein in circulation, albumin serves as a dysopsonin for foreign particles and helps prolong their circulation.^{51–54} Therefore, circulating albumin-coated NCs can exploit tumors' demands for albumin and albumin's transport mechanism in order to reach tumors. Some of these potentials have been demonstrated in our previous and ongoing studies. PTX-F-alb showed greater deposition in B16F10 tumors than Abraxane® (commercial albumin-bound PTX) and outperformed Abraxane® in suppressing tumor growth in a mouse model.¹² In future studies, we will determine whether DTX-F-alb will improve anti-tumor efficacy *in vivo* (in particular, P-gp-overexpressing tumors) by way of the circulation stability, EPR-and/or gp60-mediated extravasation and deposition in tumors, and SPARC-mediated interstitial retention and cellular uptake as compared to a solution form of DTX such as Taxotere.

5. Conclusion

Albumin-coated DTX NCs (DTX-F-alb) were produced with F127 as an intermediate stabilizer. DTX-F-alb showed a small particle size amenable to cellular uptake and remained stable in serum-containing medium suggesting favorable circulation stability. DTX-F-alb improved DTX delivery to multidrug-resistant NCI/ADR-RES cells in a F127-independent manner. The high SPARC expression level of NCI/ADR-RES cells, the known affinity of albumin for SPARC and the opposing effect of SPARC knockdown support that DTX-F-alb may have benefited from the surface-bound albumin-SPARC interaction in entering multidrug-resistant cells. Albumin-coated NC system is a promising formulation for the delivery of poorly water-soluble anticancer drugs to multidrug-resistant tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Effect of centrifugation or filtration on the average size (Z-average, nm) and polydispersity index (PDI) of (a, b) NCs coated with F127 (DTX-F) and (c) albumin-coated NCs (DTX-F-alb). n=3 independent experiments (mean $\pm$ s.d.).

Figure 2.

(a) Representative SDS-PAGE gel image (top) and densitometric quantification (bottom) of albumin adsorbed to the surface of DTX-F-alb (albumin/NC as bars) after 3, 6 and 24 h incubation of DTX-F with albumin. Zeta potentials of the NCs were shown as square symbols. $n = 3$ independent experiments (mean $\pm$ s.d.). (b) Powder X-ray diffraction patterns of DTX as received, albumin, a physical mixture of DTX and albumin, DTX-F and DTX-F-alb. (c) Differential scanning calorimetry thermograms of DTX as received, albumin, F127, and DTX-F-alb.

Figure 3.
(a) SEM and (b) TEM image of DTX-F-alb. Scale bars: 500 nm (a) and 100 nm (b).

Figure 4.

Physical stability of DTX-F-alb in (a) 10% FBS and (b) 50% FBS, estimated by DLS. NCs were incubated at different concentrations in each medium, and their derived count rates were measured over 24 h. The values were obtained by subtracting the derived count rate of each incubation medium (10% or 50% FBS) from those of particle suspensions at each time point. $n = 3$ independent and identical experiments (mean $\pm$ s.d.).

Figure 5.

Cytotoxicity of DTX-F-alb on (a) SKOV-3, (b) B16F10, and (c) NCI/ADR-RES cells treated with free DTX or DTX-F-alb at concentrations equivalent to DTX 20, 30 and 40 µg/mL for a short term (2 h for SKOV-3 and B16F10 cells; 2, 4, 6 and 9 h for NCI/ADR-RES cells).

Cells were incubated for additional 72 h (SKOV-3 and NCI/ADR-RES) or 21 h (B16F10) in drug-free medium prior to MTT assay. n = 6 independent and identical treatments (mean ± s.d.) of a representative experiment (confirmed in 3 independent experiments). **: p<0.01 and ****: p <0.0001 by ANOVA followed by Bonferroni's multiple comparisons test.

Figure 6.

DTX uptake by (a) SKOV-3, (b) B16F10, and (c) NCI/ADR-RES cells after (1 or) 3 h treatment with free DTX and DTX-F-alb at a concentration equivalent to DTX 30 $\mu\text{g/mL}$. Micro BCA assay was performed to determine the total protein content in cell lysate and used for normalization. $n = 2$ independent and identical experiments (mean $\pm$ s.d.). *: $p < 0.05$ and **: $p < 0.01$ by unpaired t-test with Welch's correction.

Figure 7.

(a) Confocal microscopy images of NCI/ADR-RES cells incubated for 3 h with rhodamine B-labeled DTX*-F-alb or free rhodamine B at a concentration equivalent to DTX 30 μ g/mL. Scale bars = 10 μ m. (b) Representative flow cytometry histograms and (c) mean fluorescence intensity of NCI/ADR-RES cells incubated with rhodamine B-labeled DTX*-F-alb or free rhodamine B at a concentration equivalent to DTX 40 μ g/mL for 3 h. $n = 3$ independent and identical experiments (mean $\pm$ s.d.). ***: $p < 0.001$ by one way ANOVA with post-hoc Tukey's multiple comparisons test.

Figure 8.

(a) Immunoblotting results demonstrating the knockdown of hSPARC in NCI/ADR-RES cells. The effect of hSPARC silencing on (b) uptake (after 3 h treatment) and (c) cytotoxicity (after 4 h treatment and 72 h follow-up incubation) of free DTX and DTX-F-alb at a concentration equivalent to 30 μ g/mL DTX. NCI/ADR-RES cells were incubated with siRNA complex for 24 h, harvested, and replated for each treatment. $n = 3$ independent and identical experiments (mean $\pm$ s.d.). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ by two way ANOVA with Sidak's multiple comparisons test.

Scheme 1.

Schematic description of albumin-coated DTX nanocrystal (DTX-F-alb) preparation.