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Understanding the structure and stability of paclitaxel nanocrystals

Jiexin Deng, Leaf Huang, Feng Liu*

Division of Molecular Pharmaceutics, University of North Carolina at Chapel Hill, Eshelman School of Pharmacy, 1318 Kerr Hall, Chapel Hill, NC 27599-7360, USA

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ABSTRACT

Previously, PTX/Pluronic F127 nanocrystals were prepared in our laboratory using the stabilization of nanocrystals (SNC) method. For PTX nanocrystals, dosages could be increased to yield improved antitumor activity over Taxol® without incidence of acute toxicity. The objectives of this current study are to further understand the structure and stability of PTX nanocrystals. More Pluronic F127 surfactant was added in the formulation to attempt to further stabilize the nanocrystals against thermal induced aggregation. However, this resulted in formation of micelles that worsened the stability of nanocrystals. The F127 desorption experiment suggested different surfactant adsorption affinity to nanocrystal surface below and above the CMC. Below the CMC monomers bound to nanocrystal surface with high affinity, but above the CMC low affinity surfactant aggregates readily left the surface upon dilution. At higher temperature the tendency of F127 micellization is enhanced due to drastically lower CMC. Consequently, at 37 °C there was F127 desorption even for nanocrystals prepared with low amounts of F127 (1:5 (w/w) PTX/F127). To improve the stability of nanocrystals, re-nanonization by incubation–sonication procedure was used to disrupt the preferred crystal growth pattern of PTX. Furthermore, we have demonstrated that a higher heating temperature (45 °C vs. 37 °C) used in the incubation–sonication procedure was able to provide even better nanocrystal stability for long periods of incubation time.

1. Introduction

Paclitaxel (PTX), the first of a new class of microtubule stabilizing agents, has demonstrated significant antitumor activity in clinical trials against a broad range of solid tumors, including refractory ovarian cancer, metastatic breast cancer, non-small-cell lung cancer, AIDS-related Kaposi's sarcoma (Singla et al., 2002; Kim et al., 2001). PTX is a highly hydrophobic diterpenoid pseudoalkaloid (of 853 Da, Rowinsky and Donehower, 1995) with poor aqueous solubility of approximately 1 μg/mL (Cheon Lee et al., 2003). Therefore, it is currently formulated as Taxol®, a concentrated solution containing 6 mg PTX/mL of Cremophor EL (polyoxyl 35 castor oil) and dehydrated alcohol (1:1, v/v) (Kim et al., 2001). However, Cremophor EL component of the drug formulation is thought to be responsible for hypersensitivity reactions approaching some 25–30% of patients, since it can induce histamine release by mast and basophil cells (Lasser et al., 1971). Therefore, novel formulations of paclitaxel alternative to the commercial ones are still highly desirable.

Attempts to solubilize poorly water-soluble drugs such as paclitaxel using co-solvents, in micelles, in liposomes, or with cyclodextrin has been of limited success. An alternative formulation is nanosuspensions which are sub-micron colloidal dispersions of pure particles of drug that are stabilized by surfactants (Na et al., 1999; Muller and Peters, 1998; Rabinow, 2004). Techniques such as high-pressure homogenization and wet milling are often used to produce nanosuspensions of small size suitable for pharmaceutical uses, these nanosuspensions are stabilized by surfactant such as Pluronic polymers for storage purposes to prevent aggregation (Ganta et al., 2009; Merisko-Liversidge et al., 1996). According to Noyes–Whitney and Ostwald–Freundlich principles, homogeneous particle sizes of nanosuspension in nanometer range can lead to increased dissolution velocity and saturation solubility, thus increase in bioavailability (Bohm and Muller, 1999; Hintz and Johnson, 1989). Nanosuspensions could be given in various routes of administrations such as oral (Liversidge and Cundy, 1995), parenteral (Peters et al., 2000), ocular (Pignatello et al., 2002), and pulmonary delivery (Jacobs and Muller, 2002). These nanosuspension studies by different routes of administration have revealed benefits such as increased rate and extent of absorption, increased bioavailability, reduced administration volumes, and increased resistance to hydrolysis and oxidation (Rabinow, 2004). Studies have demonstrated that stable nanosuspensions of sparingly water-soluble drugs was comparable or improved over the response elicited by the conventional formulations; dosages could be increased without incidence of acute toxicity, abnormal weight loss, or organ pathology (Liu et al., 2010; Merisko-Liversidge et al., 1996). Currently five products are on the market by the

* Corresponding author. Tel.: +1 919 843 4704; fax: +1 919 966 0197.
E-mail address: fliu@email.unc.edu (F. Liu).

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approval of FDA since the year 2000, all intended for oral delivery; all five products are based on top-down approaches such as media milling and high-pressure homogenization (Van Eerdenbrugh et al., 2008b).

One such nanosuspension formulation is PTX nanocrystals prepared in our lab using simple method of stabilization of nanocrystals (SNC) using Pluronic F127 as the sole excipient (Liu et al., 2010). PTX is relatively complex organic drug having several solid structures such as crystal and amorphous, and the stabilized amorphous aggregate of PTX and Pluronic F127 co-precipitation played an important role in SNC method. The amorphous co-precipitate provided the flexibility such that minimal energy and Pluronic F127 were required in the nanoparticle forming process, and this resulted in simple preparation and high drug loading efficiency (Liu et al., 2010). The resulting nanocrystal formulation has low toxicity due to low PTX to Pluronic F127 ratio, and the nanocrystals can bring higher amount of drug to cancer cells upon delivery, resulting in significant cancer-cell killing both in vitro and in vivo (Liu et al., 2010).

In this study we would like to further understand the structure and stability of PTX/F127 nanocrystals. Our first aim was to understand whether the procedures used in SNC method and the incorporation of Pluronic F127 in the formulation changed the crystallinity of paclitaxel. Our second aim was to elucidate how increasing concentrations of Pluronic F127 surfactant, and subsequent formation of micelles, affected the thermal stability of nanocrystals. Our third aim was to show that nanocrystal stability could be improved by re-nanonization that involves the incubation–sonication procedure. Previously, we reported that PTX nanocrystals is an attractive alternative to the commercially available Taxol® due to its significant anticancer effects and higher maximum tolerated dose (Liu et al., 2010). The novelty of this paper is looking at the structure and stability of the PTX nanocrystals in detail. We report for the first time how the formation of Pluronic F127 micelles affected the stability of our PTX nanocrystals, and the same pattern should be applicable for other nano-colloidal suspension systems with surfactant stabilization. We also report here for the first time that PTX nanocrystal stability could be greatly improved through re-nanonization procedures that disrupted the preferred growth pattern of nanocrystals.

2. Materials and methods

2.1. Materials

Paclitaxel (PTX) was bought from Lc Laboratories (Woburn, MA). Pluronic F127 (F127) and Cremophor-EL were purchased from Sigma, USA.

2.2. Preparation of the PTX samples

The nanocrystals were prepared by the method of stabilization of nanocrystals (SNC), which included three-phase nanoparticle engineering technology (3PNET) (phase 1, amorphous precipitate; phase 2, hydrated amorphous aggregate; and phase 3, stabilized nanocrystal) (Liu et al., 2010). Briefly, paclitaxel (PTX) and F127 were first dissolved in chloroform (in a glass tube) with a weight ratio of 1:5, 1:10, 1:20, or 1:30 (PTX/F127) and then co-precipitated by evaporating the chloroform with a steady stream of nitrogen gas. Traces of chloroform were then removed by keeping the samples under a vacuum with desiccators for 30 min. Following 30 min of hydration (in double deionized water) and vortex, suspensions of the crystal were sonicated for 5 min by a bath-type sonicator (output 80 kC, 80 W) to form nanocrystals. After hydration for 20 min, the samples were sonicated for another 5 min.

The final concentration of PTX in tube was 167.5 μM, and the final concentration of F127 in tube used for weight ratio of 1:5, 1:10, 1:20, or 1:30 (PTX/F127) were 56.75 μM, 113.49 μM, 226.98 μM, and 340.48 μM, respectively. The distribution of cumulative particle size in the nanocrystal formulation was measured using ZetaSizer Nano-ZS of Malvern Instrument (Westborough, MA). Size measurement readings for samples represent average of three measurements. The PDI (Polydispersity index) for size measurements are less than 0.5, which indicates reasonably narrow distribution that allows for meaningful measurements. For the physical mixture of PTX/F127, PTX and F127 were not co-dissolved in chloroform and were not co-precipitated by evaporating the chloroform. Only PTX was dissolved in chloroform, and the chloroform was evaporated with a stream of nitrogen, leaving PTX residues at the bottom of the tube. Then aqueous F127 solution of appropriate concentration was added to the residues of PTX to make the final weight ratio of 1:5 (w/w) PTX/F127. The mixture was then hydrated and sonicated in the same way as described for nanocrystals.

2.3. X-ray powder diffraction

The X-ray powder diffraction (XRPD) patterns for Paclitaxel samples were obtained with a Rigaku Multiflex diffractometer (Rigaku Americas). The X-ray source was Ni filtered CuK-alpha radiation (wavelength 1.5418 A). The X-ray tube was run at a power of 40 kV, 40 mA. The sample size was approximately 60 mg. The 2θ range scanned was 7.5–60° at a rate of 0.25° 2θ/min at increments of 0.02° 2θ.

2.4. Transmission electron microscope (TEM)

Transmission electron microscopy (TEM) images of the resulting nanocrystals were acquired using a Phillips CM12 (FEI, Hillsboro, OR). Freshly prepared nanoparticle samples (5 μl) were dropped onto a 300 mesh carbon-coated copper grid (Ted Pella, Inc., Redding, CA) and allowed a short incubation (5 min) at room temperature. Grids were then stained with 1% uranyl acetate (40 μl) and wicked dry. All images were acquired at an accelerating voltage of 100 kV. Gatan DigitalMicrograph software was used to analyze the images.

2.5. Nanocrystal size increase after dilution

The distribution of cumulative particle size in the nanocrystal formulation was measured using ZetaSizer Nano-ZS of Malvern Instrument (Westborough, MA). To study the effects of dilution on nanocrystal size, 1 mL of undiluted sample (143 μg PTX/mL H_2O, 167.5 μM PTX), 10-fold diluted sample, 50-fold diluted sample were placed in particle size measurement cuvette (SARSTEDT, Nümbrecht, Germany). These samples were left standing at room temperature or incubated in 37 °C, and at various time points their particle size were measured.

2.6. Re-nanonization by incubation–sonication procedure

Re-nanonization involving incubation–sonication procedure was used to improve the stability of nanocrystals. PTX nanocrystal of 1:5 (w/w) PTX/F127 with PTX concentration of 167.5 μM were prepared. The nanocrystal samples were then incubated at either 37 °C or 45 °C for 2 h, and the nanocrystal size was expected to grow dramatically after the incubation. Then, suspensions of nanocrystals were re-nanonized by sonicating for 5 min using a bath-type sonicator (output 80 kC, 80 W). After allowing 20 min for hydration, the samples were further sonicated for another 5 min. The re-nanonized nanocrystals after undergoing sonication–incubation procedure had a much better thermal stability than nanocrystals without re-nanonization. To test this, re-nanonized nanocrystals

were incubated in either 37 °C or 42 °C again to test for thermal stability. The distribution of cumulative particle size in the nanocrystal formulation was followed at various time points during 37 °C or 42 °C incubation using ZetaSizer Nano-ZS of Malvern Instrument (Westborough, MA). At 6 h of incubation at 37 °C or 42 °C, samples were prepared for TEM to allow for visual comparison of nanocrystal size increase of re-nanonized nanocrystal samples and samples without re-nanonization.

3. Results and discussions

3.1. Crystalline structure identification of nanocrystals

Although nanocrystals could be observed under TEM, the crystalline structure of nanocrystals was never evaluated. Therefore, X-ray powder diffraction analysis was carried out for nanocrystals (PTX/F127, 1:5 (w/w)) as well as physical mixture in comparison with pure PTX and F127. Fig. 1 shows the X-ray diffraction peaks for pure paclitaxel, pure F127, nanocrystals, and PTX/F127 physical mixture. As shown in Fig. 1, the X-ray diffraction pattern of the nanocrystals showed the combined peaks of both of the pure components (paclitaxel and F127). F127 is a semicrystalline polymer, with the crystalline phase consisting of PEO layers and amorphous layers formed by PPO and PEO (Shatalova et al., 2008), and the F127 peaks were much more prominent than the peaks of paclitaxel near the base line at 2θ of 10–15°. The X-ray powder diffraction confirmed that in nanocrystal formulation both paclitaxel and F127 components retained their native crystalline structure.

X-ray diffraction peaks for physical mixture were also shown in Fig. 1C. Even though the diffraction peaks for nanocrystal and PTX/F127 physical mixture were highly similar, the crystal formations of the two samples were very different (Supplemental Fig. 1). Nanocrystals were rod-shaped structures stabilized by F127 surfactant on the surface. In the physical mixture, these individual crystals collapsed with each other forming an aggregated mass. Due to the lack of the co-solvation (in chloroform) and co-precipitation step (amorphous precipitate), F127 surfactant in physical mixture could not be adequately coated on the surface of nanocrystals to prevent the aggregation.

Table 1
Nanocrystal size increase after 37 °C incubation for 2 h. Nanocrystals prepared with various amounts of F127 were measured for their sizes before and after 37 °C incubation. Size measurement readings for samples represent average of three measurements. The PDI (polydispersity index) for size measurements are less than 0.5, which means narrow distribution for accurate measurements.

	Measured size (nm)	
	Before 37 °C incubation	After 37 °C incubation
PTX/F127 (1:5, w/w)	176 ± 14	258 ± 15
PTX/F127 (1:10, w/w)	187 ± 11	303 ± 21
PTX/F127 (1:20, w/w)	158 ± 8	359 ± 25
PTX/F127 (1:30, w/w)	181 ± 9	441 ± 35

3.2. Effects of increasing surfactant on thermal stability of nanocrystals

Hydrophobic interactions are negative entropic processes, therefore, the higher the temperature of the suspension, the more thermodynamically unfavorable the system becomes; in hydrophobic suspensions, the tendency of aggregation is enhanced at higher temperature (Lu et al., 2005). As shown in Fig. 2 this is also true for our nanocrystals, and longer crystals were formed after 37 °C incubation for 2 h despite F127 stabilization. The measured size went from 176 ± 14 nm to 258 ± 15 nm after 2 h incubation at 37 °C (Table 1). The nanocrystals were prepared in PTX/F127 weight ratio of 1:5, which is 3:1 in molar ratio. For every 3 molecule of PTX there is only 1 molecule of F127 for stabilization, and maybe the amount of F127 used was not adequate. Also, it has been reported that further increase in surfactant in nanosuspensions could afford thicker coating layer to the nanocrystals, thus providing better long-term stability (Jacobs et al., 2000; Van Eerdenbrugh et al., 2008b). Therefore, to further stabilize the nanocrystals, higher amounts of F127 surfactant were used to prepare PTX/F127 nanocrystals of ratios 1:10, 1:20, and 1:30 (w/w).

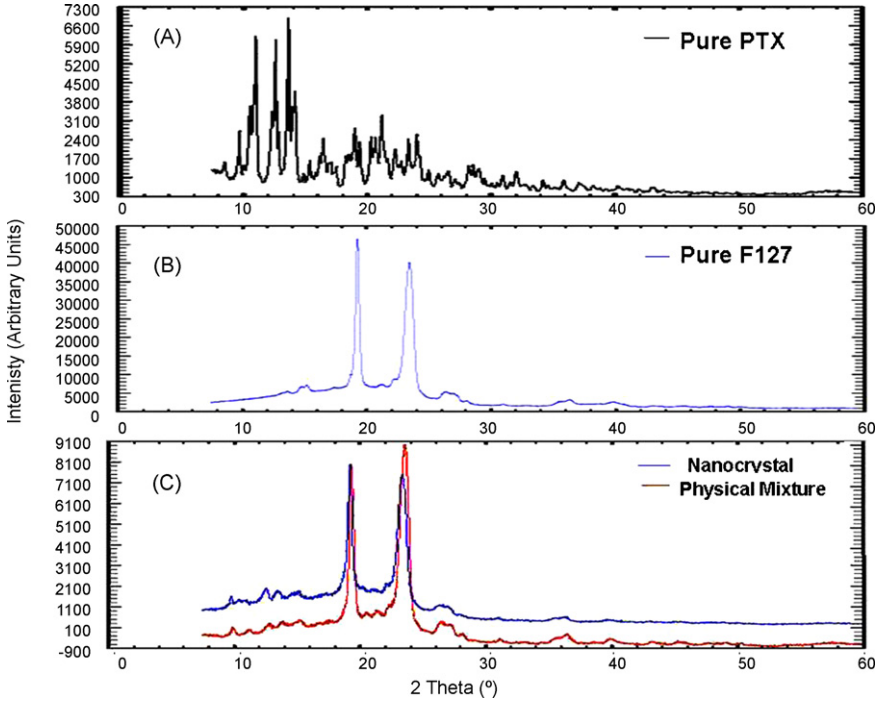

Fig. 1. X-ray powder diffraction peaks for (A) pure paclitaxel, (B) pure F127, and (C) nanocrystals and physical mixture of PTX and F127.

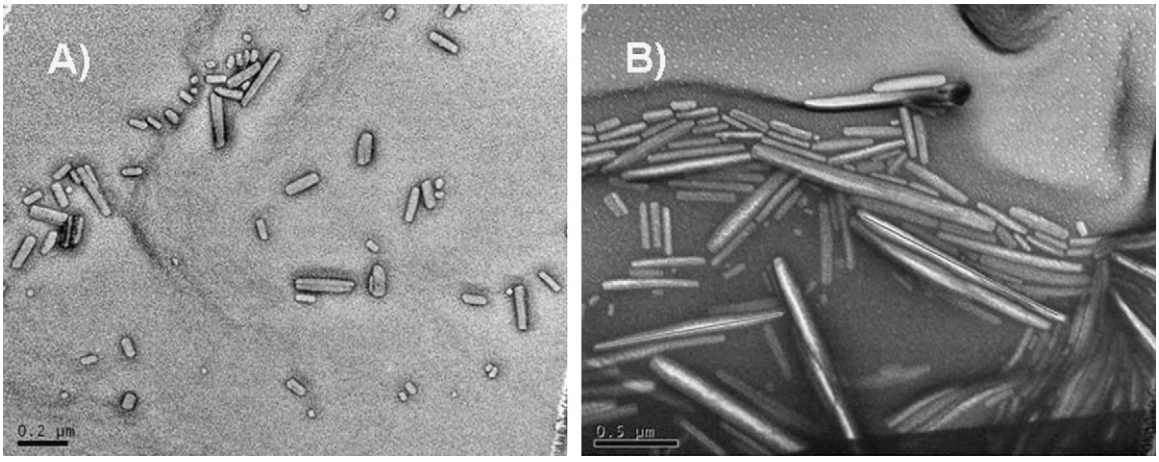

Fig. 2. TEM pictures of nanocrystals for thermal stability studies. (A) Nanocrystals before 37 °C incubation. Measured size: 176 nm (B) longer crystals formed by incubation at 37 °C for 2 h. Measured size: 258 nm.

Contrary to our expectations, the nanocrystals stability was worse as the measured size after 37 °C incubation went even larger with higher amount of F127 surfactant used (Table 1). The measured size for PTX/F127 (1:5, w/w) after 37 °C incubation was 258 ± 15 nm, but for PTX/F127 (1:20, w/w) and (1:30, w/w) the sizes were 359 ± 25 nm and 441 ± 35 nm respectively after 37 °C incubation. This trend was confirmed by manual measurements performed on nanocrystals in TEM (Supplemental Table 1). Adding more F127 surfactant to the preparation did not help to increase the stability of nanocrystals, instead, the nanocrystals size increase was even larger after 2 h 37 °C incubation.

In order to observe the effects of increasing concentrations of F127 on the cytotoxicity of nanocrystals, PTX nanocrystals (100 nM) with different concentrations of F127 surfactants (1:10, 1:20, 1:30 (w/w)) were applied to NCI-H460 cells. It was found that increased amounts of F127 did not alter the cytotoxic effects of nanocrystals on NCI-H460 cells (Supplemental Fig. 2).

The reason why more F127 worsened the stability of nanocrystals became clear in Fig. 3. Instead of forming a thicker layer on the nanocrystal surface for better stabilization, micelles began to form in solution at higher concentration of F127 surfactant. The CMC of Pluronic F127 at 25 °C is 7.19×10^{-5} M (Croy and Kwon, 2004). The concentration of F127 in nanocrystals prepared with 1:5 (w/w) of PTX/F127 is below the CMC of F127. However, for 1:10, 1:20, and 1:30 of PTX/F127, the concentration of surfactant goes beyond the CMC. We hypothesize that the formation of micelles may play a role in thermal instability of the nanocrystals. After the attainment of the CMC, the micelles began to compete for surface adsorption so that the total adsorption at the interface begins to decrease as the micelles become more numerous (Chatterjee and Gupta, 2002). Another study on the adsorption of Pluronic polymers on the adsorption of DDS-glass by radiolabelling also confirmed this effect (Amiji and Park, 1994). Thus, the higher F127 surfactant concentration could actually mean less surfactant adsorption, which would further destabilize the nanocrystals during the 2 h 37 °C incubation, thus contributing to a larger size increase. Croy et al. studied the effects of Pluronic polymers on the aggregation state of poorly soluble nystatin. They found that nystatin could partition into pluronic micelles and the largest contributing factor to the solubilization of nystatin was the number and size of the micelles formed (Croy and Kwon, 2004). However in our case PTX nanocrystals were stabilized by surface adsorption of F127 surfactant, and the formation of micelles would compete with F127 surface adsorption—thus destabilizing the nanocrystals.

3.3. The role of micelles on nanocrystal stability

We believe our nanocrystals are in a meta-stable state stabilized by surface adsorbed F127 surfactant. At low concentration of F127 (1:5 (w/w) PTX/F127) monomers bind with high affinity. At high concentration of F127, monomers aggregated and bind to

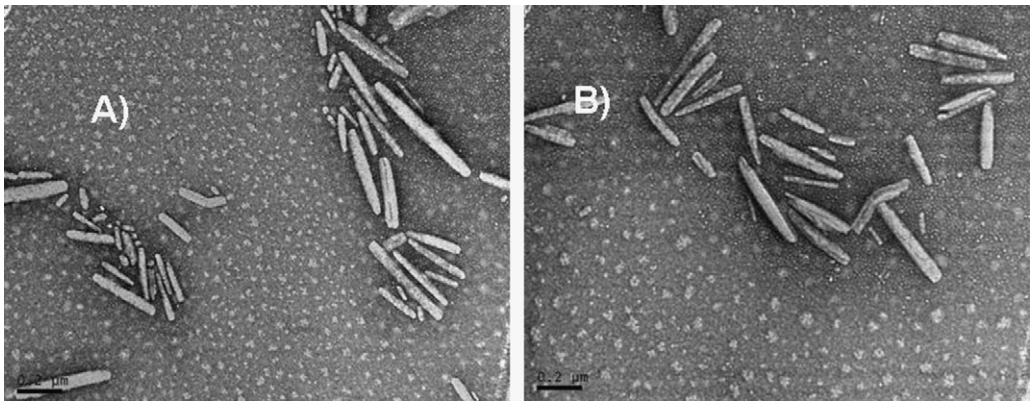

Fig. 3. TEM of nanocrystals of (A) PTX/F127 (1:10, w/w) and (B) PTX/F127 (1:20, w/w). The white spherical structures in the background are micelles formed at high concentration of F127.

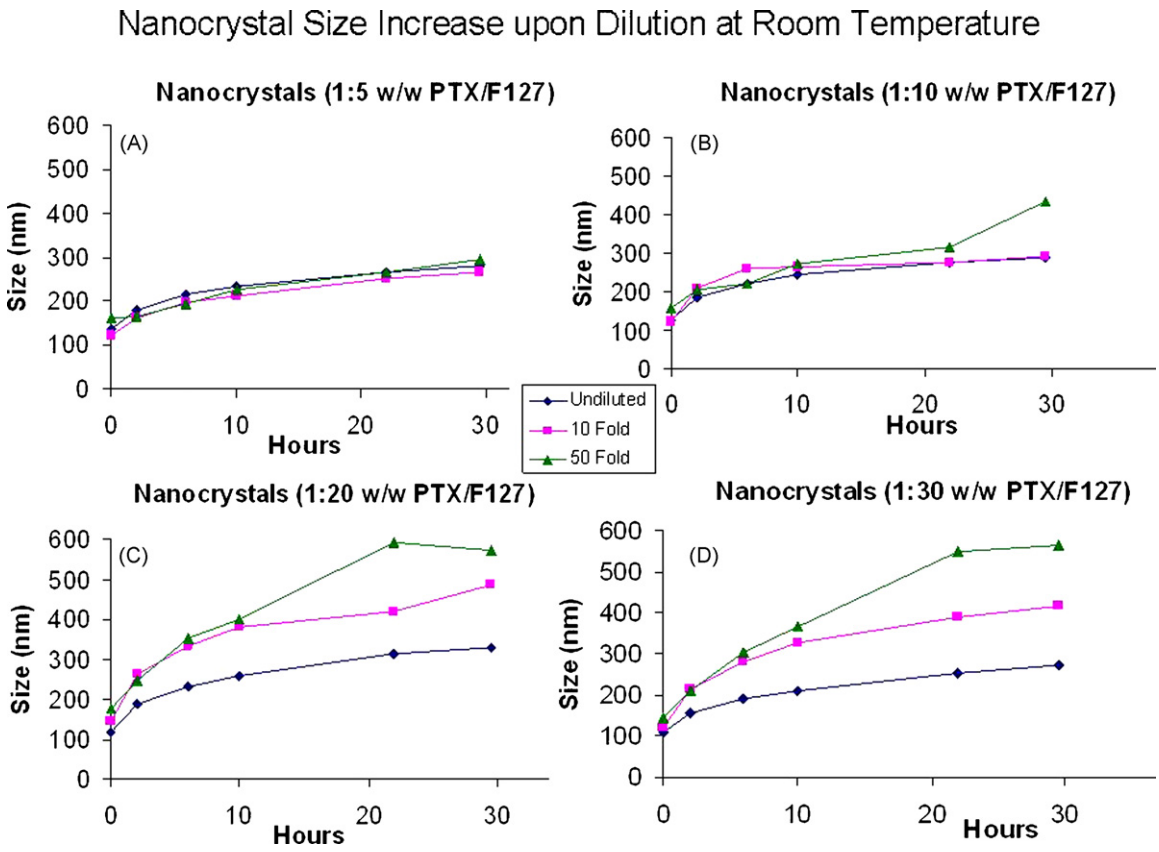

Fig. 4. Nanocrystal Size increase upon dilution at room temperature. (A) Nanocrystals (1:5 (w/w) PTX/F127). (B) Nanocrystals (1:10 (w/w) PTX/F127). (C) Nanocrystals (1:20 (w/w) PTX/F127). (D) Nanocrystals (1:30 (w/w) PTX/F127). Data represents mean value for at least 20 readings for each sample.

surface with low affinity; there is also micelle formation competing for surface adsorption. To prove that surfactant adsorb with different affinities below and above the CMC, nanocrystal samples were diluted 10- or 50-fold to draw the adsorbed surfactant layer into free form. By dilution it is hoped that the equilibrium would be shifted such that the adsorbed surfactant would leave the nanocrystals and go into the free form. If the surfactant does indeed leave, the crystal size increase would be detected.

As seen in Fig. 4A, PTX nanocrystals prepared with 1:5 (w/w) of F127 increased somewhat in size as time went on due to Ostwald Ripening; however, size increase upon dilution was not observed. As shown in Fig. 4C and D, the size of nanocrystals prepared with higher amounts of F127 (1:20, 1:30, w/w) drastically increased upon dilution. Micelle formations were evident at higher F127 concentrations in TEM of undiluted samples at the end of the experiment (Supplemental Fig. 3). These data indicate that indeed the affinity of surfactant binding to the surface of nanocrystals prepared with low amount of F127 (1:5, w/w) was not the same as that of nanocrystals prepared with high amounts of F127 (1:20, 1:30, w/w). At low concentration of F127 (1:5, w/w), no micelles were observed in TEM, and it is likely that monomers bound to nanocrystals with high affinity, and dilution could not pull the surfactant off the surface. At higher concentration of F127 (1:20, 1:30, w/w), micelles started to form that would compete for surface adsorption of F127 (Fig. 5). Also, the size increase upon dilution of the nanocrystals of 1:20 and 1:30 (w/w) PTX/F127 suggests that the surfactant bound with lower affinity, coming off easily upon dilution. It is likely that as F127 concentration increased, monomers started to lose the high affinity to surface of nanocrystals and would instead interact more with each other.

One study by Lin et al. using dynamic light scattering (DLS) and small-angle neutron scattering (SANS) found that below CMC PPO of Pluronic F127 preferred hydrophobic Carbon Black (CB) particle surface to water, but above CMC Pluronic F127 preferred to associate with each other and had decreased affinity to CB surface (Lin and Alexandridis, 2002). Below CMC monolayer of F127 monomers bound to CB surface with high affinity, above CMC adsorbed layer thickness measured by DLS, as well as structure determined by SANS, suggested that the adsorbed layer was in the form of micelles (Lin and Alexandridis, 2002). The observation that at higher bulk surfactant concentration, micelles, or monomer aggregates "hemimicelles", bound to hydrophobic surface with lower affinity is also confirmed by Amiji using radiolabelling methods (Amiji and Park, 1994). This is in line with our observation that at higher F127 concentration surface surfactants readily leave upon dilution, making the crystals increase in size. At higher F127 concentration where micellization occurs, F127 monomers on the surface aggregated with each other and consequently bound the surface with lower affinity.

It is known that the CMC of F127 would decrease drastically from 0.3 wt% at room temperature to 0.025 wt% at 37 °C (Linse and Malmsten, 1992). Therefore, it would be interesting to find out whether the nanocrystal size increases upon dilution at 37 °C where micelle formation would be enhanced. As shown in Fig. 6, for PTX/F127 1:5 (w/w) the nanocrystal size indeed increased upon dilution at 37 °C. At higher temperatures the surfactant adsorption is inhibited more than micellization, meaning that micellization will out-compete surface adsorption (Rosen, 2004). Therefore, monomers on the surface of nanocrystals started to aggregate at higher temperature and were drawn to leave the crystal surface upon dilution, making the crystal prone to aggregate. This size increase upon dilution for nanocrystals of 1:5 (w/w) PTX/F127 was not observed at room temperature, where no micelles were formed.

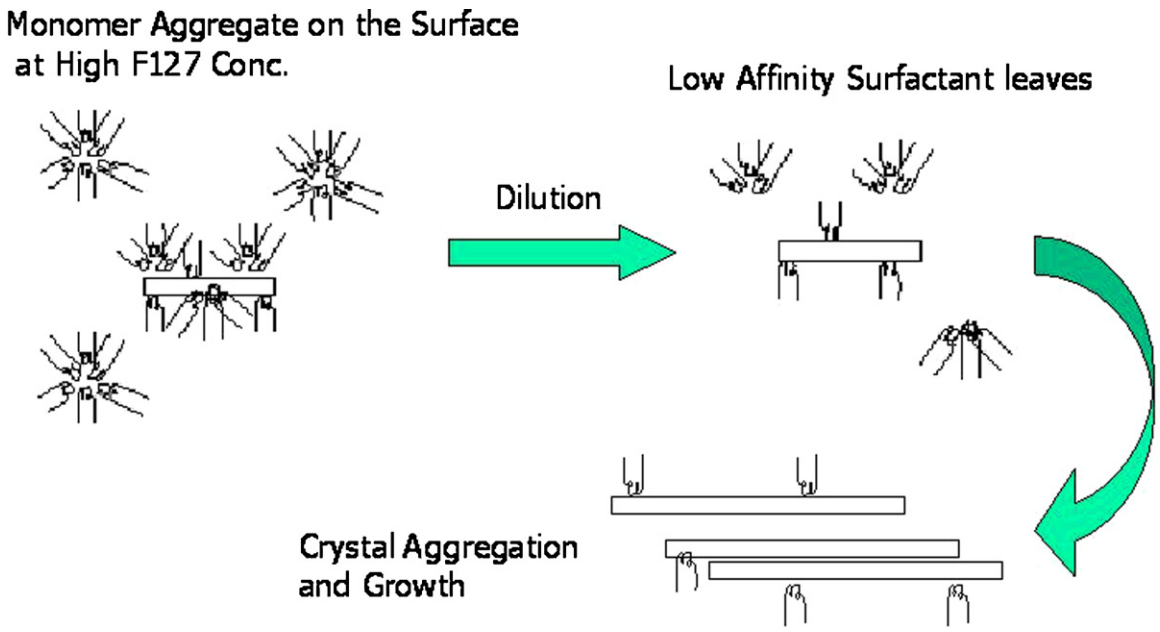

Fig. 5. Size increase of nanocrystals with high F127 concentration upon dilution.

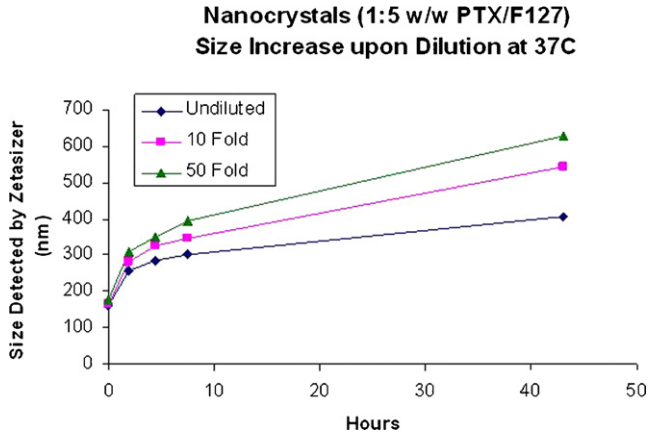

Fig. 6. Nanocrystal (1:5 (w/w) PTX/F127) size increase upon dilution at 37 °C. Data represents mean value for at least 20 readings for each sample.

3.4. Improved nanocrystal stability by re-nanonization

The crystal growth of nanocrystals after 37 °C incubation presents storage problems, because long crystals could also form at room temperature after preparation or reconstitution due to Ostwald ripening (Van Eerdenbrugh et al., 2008a; Arunkumar et al., 2009). This negates important advantages of nanosuspensions, such as the increase in saturation solubility and increase in dissolution rate of compound (Kocbek et al., 2006). Nanosuspensions have shown increased antitumor efficacy by taking advantage of the enhanced permeability and retention (EPR) effect during its prolonged retention phase (Lou et al., 2009). It would be difficult for large crystals to take advantage of the EPR effect. Therefore, re-nanonization by "incubation–sonication" procedure was used to improve the stability of the nanocrystals. After the crystal size increased after the 2 h 37 °C incubation, sonication was used to break down the samples into smaller nanocrystals (139 ± 9.7 nm). As shown in Fig. 7, the "incubation–sonication" procedure effectively decreased the growth of nanocrystals after 2 h 37 °C incubation again (163 ± 8.2 nm) and provided a much better thermal stability for nanocrystals. The nanocrystal growth after re-nanonization from 139 ± 9.7 nm to 163 ± 8.2 nm is much smaller compared to nanocrystals of 1:5 (w/w) PTX/F127 without undergoing re-nanonization (Table 1). It is assumed that the crystal growth pattern would be disturbed by sonicating large nanocrystals after 37 °C incubation, and it would be difficult to re-grow crystals into longer needle-like crystals, thus providing better stability.

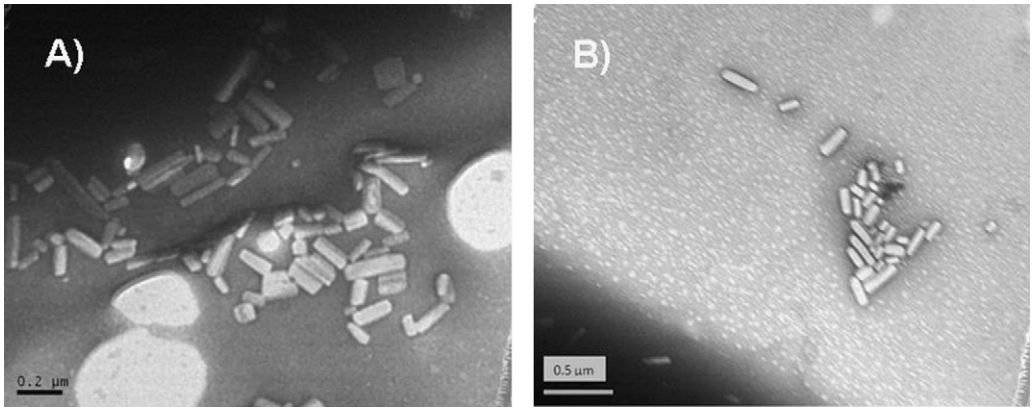

Fig. 7. Nanocrystal TEM pictures after re-nanonization by incubation–sonication procedure. (A) Break down of long crystals into nanocrystals by sonication for 15 min after 2 h 37 °C incubation. Measured size: 139 nm. (B) No observed re-growth into long crystals by incubating again at 37 °C incubation for 2 h. Measured size: 163 nm.

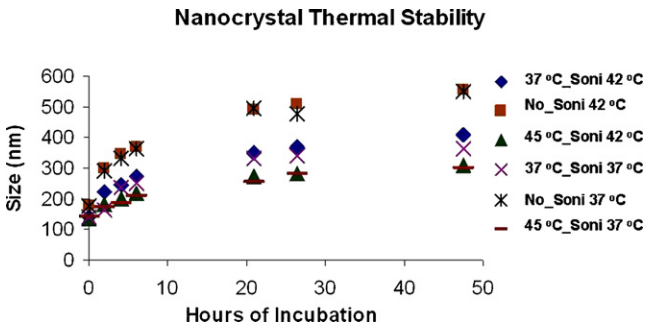

Fig. 8. Nanocrystal thermal stability at different time points during 37 °C and 42 °C incubation. Three types of nanocrystals (167.5 μM PTX) were incubated at either 37 °C or 42 °C: (1) No_Soni (nanocrystals without re-nanonization); (2) 37 °C_Soni (nanocrystal samples were incubated at 37 °C for 2 h, then the larger nanocrystals were re-nanonized using sonication); (3) 45 °C_Soni (nanocrystal samples were incubated at 45 °C for 2 h, then the larger nanocrystals were re-nanonized using sonication). The size increase of nanocrystals during incubation could be very much larger at concentrations higher than 167.5 μM.

The cytotoxic effects of nanocrystal (10 μM) on NCI-H460 cells after the "sonication–incubation" procedure were studied. The different preparative procedures, such as incubation and sonication, did not cause significant difference in cytotoxicity of nanocrystals, and the cell viability were 40–45% across all treatment groups (Supplemental Fig. 4). The crystalline state of nanocrystals after "sonication–incubation" procedure was also evaluated using X-ray powder diffraction analysis. It was found that the nanocrystal crystallinity was unaltered by procedures such as 37 °C incubation and sonication (Supplemental Table 2).

3.5. *Nanocrystal thermal stability after re-nanonization*

We have demonstrated that re-nanonization involving incubation–sonication procedure was able to effectively improve the nanocrystal stability after 2 h 37 °C incubation. We would like to investigate this further by looking at longer incubation periods and a higher temperature of incubation (i.e. 37 °C and 42 °C). We would also like to find out the effect of a different heating temperature used in the incubation–sonication procedure (45 °C vs. 37 °C) can have on the later thermal stability of nanocrystals. Fig. 8 showed that nanocrystals without re-nanonization had the greatest size increase during incubation, but re-nanonization was able to dramatically improve the thermal stability of nanocrystals. Performing incubation–sonication procedure at a higher temperature of 45 °C provided the best improvement in nanocrystal thermal stability at both 37 °C and 42 °C. Furthermore, it seems that nanocrystal size increase profile at 42 °C incubation was not very different from the size increase profile at 37 °C.

At 6 h of incubation, samples were prepared for TEM to visually compare the nanocrystal size increase. From the TEM (Supplemental Fig. 5) it could be observed that nanocrystals without re-nanonization (No_Soni 42 °C) grew dramatically in length to be thin and elongated needle-like crystals at 6 h of 42 °C incubation. Instead, nanocrystals that underwent re-nanonization (45 °C_Soni 42 °C and 37 °C_Soni 42 °C) had a much inhibited growth in length. Nanocrystals that underwent incubation–sonication procedure at 45 °C (45 °C_Soni 42 °C) had a comparatively smaller more homogeneous size than nanocrystals of 37 °C_Soni 42 °C, consistent with the smaller size measurement based on light scattering Zeta-Sizer. Based on these data, we conclude that re-nanonization could dramatically improve the thermal stability of nanocrystals by inhibiting the growth of the nanocrystals in length. Furthermore, the temperature at which nanocrystal growth occurs prior to re-nanonization had a large effect on the later nanocrystal stability, and a higher temperature used in incubation–sonication procedure (45 °C vs. 37 °C) provided even better protection against thermal induced size growth. This is the first demonstration in our knowledge that re-nanonization method (first induce size growth at higher temperature, then re-nanonize by sonication) was able to dramatically improve the thermal stability of nanosuspension systems. The improved stability of re-nanonized nanocrystals was obvious for long periods of time at 37 °C or 42 °C incubation. The good nanocrystal stability would be even more so at room temperature for better storage purposes.

4. Conclusion

In conclusion, the crystalline structure of PTX nanocrystals was unaltered from that of pure PTX based on X-ray powder diffraction analysis. Contrary to our expectations, the addition of more F127 in the formulation resulted in formation of micelles that further destabilized the nanocrystals. The surfactant desorption experiment showed that F127 adsorbed with different affinities to nanocrystal surface below and above the CMC. Below the CMC monomers bound to nanocrystal surface with high affinity, but above the CMC low affinity surfactant aggregates readily left the surface upon dilution. At higher temperature the tendency of F127 micellization is enhanced due to drastically lower CMC. Consequently, at 37 °C there was F127 desorption even for nanocrystals prepared with low amounts of F127 (1:5 (w/w) PTX/F127). Re-nanonization by incubation–sonication procedure effectively improved the stability of nanocrystals by disturbing the preferred crystal growth pattern of PTX. Furthermore, it was found that a higher heating temperature (45 °C vs. 37 °C) used in the incubation–sonication procedure was able to provide even better nanocrystal stability for long periods of incubation time.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2010.02.013.

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