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Design and Evaluation of an Emulsion Vehicle for Paclitaxel. II. Suppression of the Crystallization of Paclitaxel by Freeze-Drying Technique

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Although paclitaxel is soluble in vitamin E up to 40 mg per g, crystallization was detected at loadings higher than 15 mg per g. Water appeared to be an important factor causing the observed crystallization, and therefore, a freeze-drying technique was investigated to produce reconstitutible vitamin E emulsions, to increase drug loading without crystal formation after reconstitution. The emulsion was freeze-dried using a laboratory freeze-drier and the droplet size was measured using dynamic light scattering. The freeze-dried emulsions using sucrose as a cryoprotectant could be easily reconstituted. The loading of paclitaxel in the freeze-dried emulsions could be increased to 25 mg per g of vitamin E without crystal formation, and the mean emulsion droplet size remained smaller than 0.2 μm over 430 days (4 ± 2°C). The previously observed surfactant-enhanced crystallization could also be suppressed using the freeze-drying technique.

Keywords paclitaxel; vitamin E; emulsion; crystallization; freeze-drying; cryoprotection

INTRODUCTION

It has been of great interest to pharmaceutical scientists to develop alternative formulations of paclitaxel due to the potentially serious hypersensitive reactions to the Cremophor EL present in the commercial products (Chao et al., 2005; Soga et al., 2005; Zhang et al., 2005). In a previous paper (Han et al., 2004) we demonstrated that paclitaxel could be loaded into a 20% vitamin E emulsion at 2.5 mg/mL (equivalent to 12.5 mg per g of vitamin E). Although higher loadings of paclitaxel of up to 40 mg per g of vitamin E could be achieved, drug crystals were detected during storage at all loadings higher than 15 mg per g of vitamin E. It was possible that the presence of water in the emulsion reduced the solubility of paclitaxel in the oil phase, as a result of water saturation. It was also likely that the release of paclitaxel into the aqueous phase was an interim step in the crystallization process, since crystals were always observed in the aqueous phase. Therefore, if a reconstitutible "solid" emulsion could be produced by removing the aqueous phase, the crystallization problem could be solved and a higher loading of paclitaxel could be achieved. Freeze-drying was employed in this work.

Although freeze-drying has long been used in the pharmaceutical industry, there are difficulties in using this technique for emulsion systems. It is well known that freezing can destabilize emulsions. In the freezing process, the large number of ice crystals formed may crush the emulsion droplets (Bamba et al., 1995). The formation of ice crystals may also lead to the concentration of the ingredients of the emulsions (Oetjen, 1999), during which preferential precipitation of an ingredient may also occur (Franks, 1998). Such precipitation may result in significant environmental changes, including pH, which in turn may affect emulsion stability. In the drying process, after water is removed, there will simply be no external phase and the oil phase will inevitably coalesce, resulting in the cracking of the emulsion.

Suitable protection therefore is required if one wishes to produce a reconstitutible freeze-dried emulsion. It is well known that saccharides, especially mono-, di-, and oligo- saccharides, can provide satisfactory protection in the freezing-drying process (Franks, 1998; Miyajima, 1997), though the mechanisms remain unclear. One hypothesis is the vitrification theory (Franks, 1998), which suggests that the saccharides promote the formation of glass during the freezing process, which suppresses the formation of ice crystals. Another is the water replacement theory, according to which the cryoprotectants interact with the ingredients being dried, replacing the water molecules when they are removed (Ausborn et al., 1994; Carpenter et al., 1994; Remmele et al., 1997). In both cases, the protectants will function as solid support for oil droplets.

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The overall aim of the present work was to investigate the feasibility of suppressing the crystallization of paclitaxel at 25 mg per g of vitamin E (double the normal loading in a typical paclitaxel-containing liquid emulsion (Han et al., 2004) by the freeze-drying technique. This has involved the investigation of different cryoprotectants, mainly saccharides. It had been found in previous work (Han et al., 2004) that poloxamer 188, a PPO-PEO-PPO block copolymer used to sterically stabilize the emulsion, facilitated the crystallization of paclitaxel, and therefore, the freeze-drying technique was also studied to determine whether the poloxamer 188 facilitated crystallization of paclitaxel could be reduced or eliminated.

MATERIALS AND METHODS

Materials

Egg Lecithin (Lipoid E 80) was obtained from Lipoid KG (Ludwigshafen, Germany). Deoxycholic Acid, sodium deoxycholate, poloxamer 188, (±)-α-tocopherol, glucose, sucrose, trehalose, fructose, and lactose were purchased from Sigma Chemical Company Limited (Poole). Paclitaxel was kindly provided by Bristol-Myers Squibb (New Jersey).

Preparation of Emulsions

Paclitaxel, vitamin E, lecithin, egg lecithin, and deoxycholic acid were dissolved in methanol which was subsequently removed by evaporation under a stream of nitrogen, followed by vacuum desiccation overnight at room temperature. The oily paste that remained was used as the oil phase of the emulsion. Sodium deoxycholate and poloxamer 188 were dissolved in distilled water to provide the aqueous phase of the emulsion. The two phases were emulsified together using a Laboratory Mixer Emulsifier (Serial No 17231, Silverson Machines Ltd., Bucks) to produce a coarse emulsion which was then passed through a Microfluidizer (Model 110T, Serial 7018, Microfluidics Corporation, Newton, Ma.) six times to produce a fine emulsion. Details of the procedures have been given elsewhere (Han et al., 2004). The basic formulation studied was: vitamin E 20%, lecithin Lipoid E 80 1%, deoxycholic acid 0.2%, sodium deoxycholate 0.4%. Emulsions containing different amounts of vitamin E (5 and 10%) were diluted from the 20% vitamin E emulsion with distilled water. Cryoprotectants were added to the samples at the described concentrations and the emulsions were subsequently freeze-dried in an Edwards Modulyo Freeze-Fryer (Edwards Modulyo, Crawley) for 48 h. The samples were then removed from the freeze-dryer, filled with N_2, sealed immediately and stored at 4 ± 2°C. Where poloxamer 188 and paclitaxel were used, the concentrations are indicated separately.

Reconstitution of Freeze-Dried Emulsions

Suitable amounts of water (to form the original composition of the emulsion before lyophilization) were added to the freeze-dried emulsions. The mixtures were then left to hydrate for up to 40 sec, and then hand-shaken gently for a few times unless otherwise indicated.

Dynamic Light Scattering (DLS)

The Z-average diameter (cumulants method) and polydispersity of the emulsion particle size distribution were measured using a Malvern 4700 DLS instrument (Malvern Instruments, Malvern). This instrument is equipped with a Uniphase Model 2213-75SLYV argon laser (San José, Califonia) and is most sensitive to particles smaller than 1 μm. Details of this method were given elsewhere (Han et al., 2001).

Detection of Large Droplets and Paclitaxel Crystals

Since the DLS method is not particularly sensitive to the formation of a small number of large droplets, an Olympus model CHS light microscope (2C0002, Olympus Optical Co. Ltd., Japan) was used to examine the possible formation of large oil droplets (> 1 μm) in the freeze-drying and reconstitution process. The scale bar was generated using a microscopic graticule. All samples were examined in triplicate and the whole sample area under the coverslip was examined field-by-field. Photomicrographs were taken using a video camera attached to the microscope and recorded directly into a computer as digital image files. Care was taken to ensure that areas chosen for the photomicrographs were as representative as possible by a well-trained operator in a consistent manner. The results for the reconstituted emulsions were compared to the original liquid emulsions. The possible formation of paclitaxel crystals was monitored using the same microscope coupled with polarizers.

Although the microscopic method is not quantitative, images obtained after careful examination by a well-trained operator (the same operator throughout) could serve as a qualitative representation of what had been observed. Actually, the microscopic method is more suitable than the conventional light diffraction technique for detecting coalescence in the current situation. While the light diffraction technique is widely used and is generally known to be able to detect particles from submicron to up to 2000 micron, it has been shown by Driscoll et al. (2001) that this technique is not sensitive to a small number of large oil droplets in submicron emulsions. Therefore, microscopy was chosen in preference to light diffraction as a supplement to the DLS for the detection of large oil droplets.

RESULTS AND DISCUSSION

Screening of the Protective Effect of Saccharides in Freeze-Drying

The emulsions that did not contain any saccharides could not be freeze-dried properly and no solid emulsion could be

obtained. Instead, a viscous, almost clear oil phase was produced. Therefore, a cryoprotectant has to be added in order that a solid, reconstitutible emulsion could be made.

The Effect of Glucose

The freeze-dried products containing glucose were self-supporting solids at room temperature. The volume of the freeze-dried emulsions did not appear to be of the same volume as the original liquid emulsions. The surfaces of the solid emulsions were generally higher than the original liquid surfaces. Some were even in the form of a solid "foam." The solid emulsions could be reconstituted with a few gentle shakes, after water was added and left standing for about 40 sec. The reconstituted emulsions had the same appearance as the original emulsion and no creaming was observed within two days (4 ± 2°C and 22 ± 2°C).

Figures 1 A and B shows the particle size and polydispersity of 5, 10, and 20% vitamin E emulsions before freeze-drying and after reconstitution at different glucose concentrations. At a glucose concentration of 5%, the 20% vitamin E emulsion displayed a significant increase in the mean droplet size (*t*-test, $P < 0.05$) while no change was observed with 10 and 5% vitamin E emulsions, which indicates that high vitamin E content (20%) is more susceptible to the stress of freeze-drying. At 10 and 20% glucose content, no significant increase in droplet size and polydispersity was observed; indicating higher concentrations of glucose offered better protective effect. The unchanged mean droplet size and polydispersity for 5 and 10% vitamin E emulsions are good indications that the majority of the emulsion droplets did not coalesce significantly in the freeze-drying and reconstitution process. Although the light scattering is not particularly sensitive for large particles, it has been demonstrated before that this technique could indeed detect changes when the whole population of droplets coalesced substantially during conventional freezing (Han et al., 2001).

Figure 2 shows photomicrographs of the reconstituted emulsions as compared to the original emulsion. Since all original emulsions looked the same at the magnification applied, only one photograph is shown (D1). Emulsions with 20% vitamin E (A1, A2, A3) had more large droplets than the10% (B1, B2, B3) and 5% vitamin E (C1, C2, C3) emulsions. The difference between the emulsions containing different concentrations of

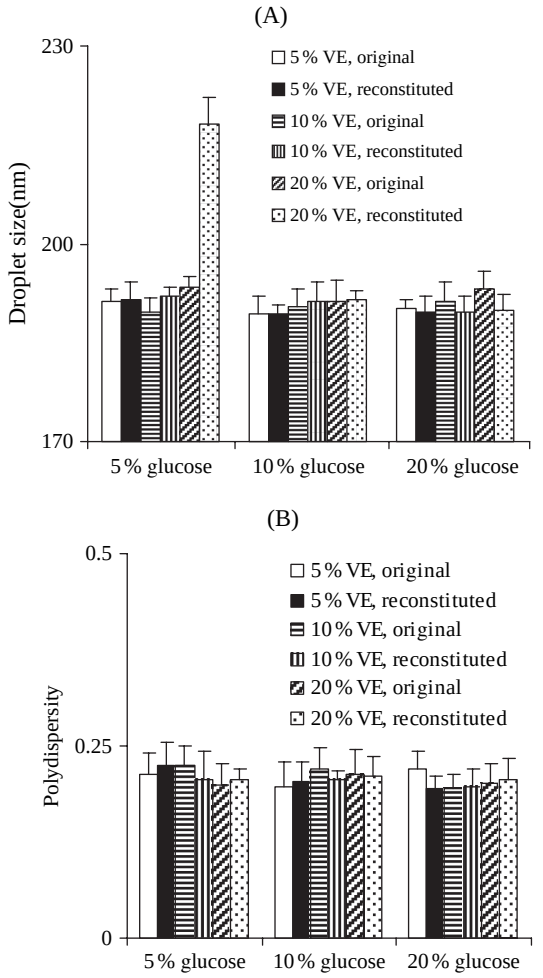


FIGURE 1. Effect of glucose on the freeze-drying of vitamin E emulsions. (A) Mean droplet size, (B) Polydispersity. Formulations: Refer to section 2.2.

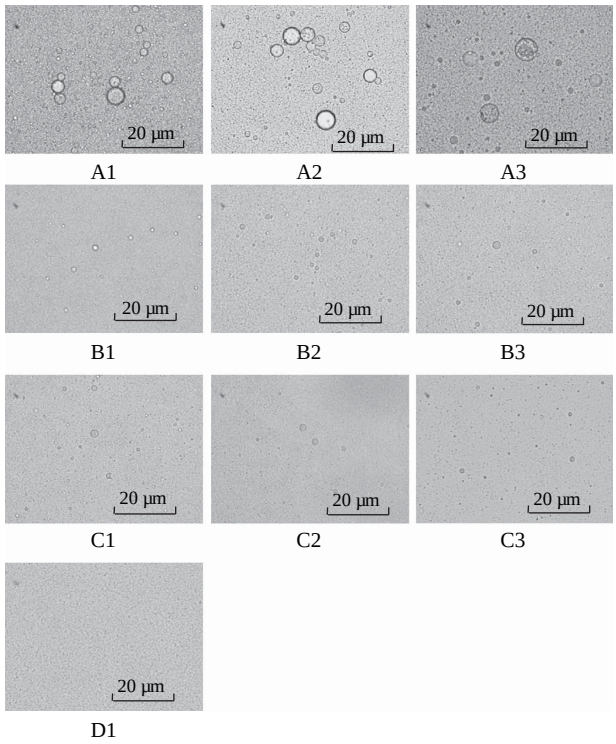


FIGURE 2. Photomicrographs of glucose protected 20% vitamin E (A1, A2, A3), 10% vitamin E (B1, B2, B3) and 5% vitamin E (C1, C2, C3) emulsions. Glucose concentration: A1, B1, and C1, 5%, A2, B2 and C2, 10%, A3, B3, C3, 20%. D1 is the original liquid emulsion (20% vitamin E). Formulations: Refer to section 2.2.

glucose was not as significant as that between different vitamin E contents. Since higher content of vitamin E is an advantage for drug loading, emulsions with 10% vitamin E (B1, B2 and B3) were selected for further evaluation.

The Effect of Sucrose

The freeze-dried products containing sucrose were solid cakes of approximately the same volume as the original liquid emulsions. This is in contrast to those containing glucose, which appeared to have "boiled." The solid emulsions were easily reconstituted with a few gentle shakes after water was added. The reconstituted emulsions had the same appearance as the original liquid emulsions and no creaming was observed within two days (4 ± 2°C and 22 ± 2°C).

Figure 3 shows the droplet size and polydispersity of the original and reconstituted vitamin E emulsions containing different concentrations of sucrose. A significant increase in droplet size was only observed at 5% sucrose for the 20% vitamin E emulsions ($P < 0.05$). No significant change in droplet size and polydispersity were observed in 5 and 10% vitamin E emulsions, indicating that the majority of the droplets did not coalesce significantly.

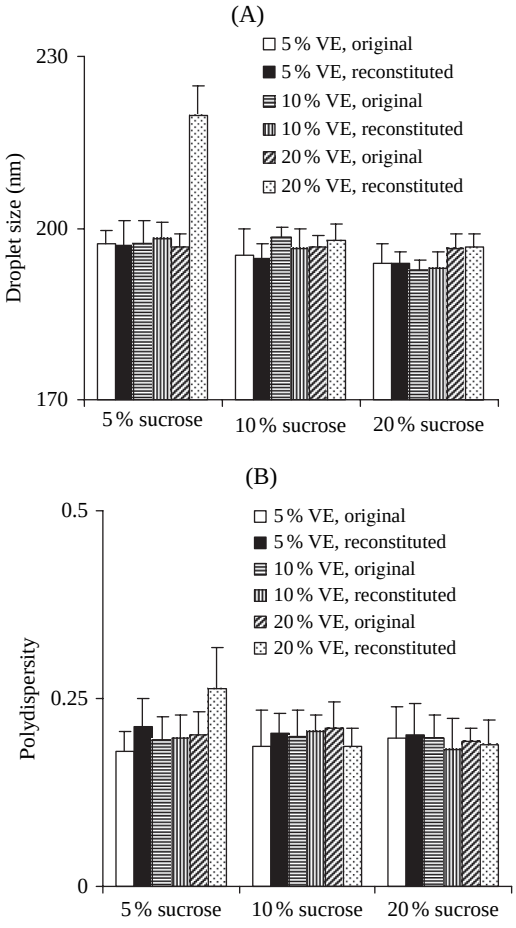


FIGURE 3. The sucrose effect on the freeze-drying of vitamin E emulsions. (A) Mean droplet size, (B) Polydispersity. Formulations: Refer to section 2.2.

Figure 4 (A, B, C) show the micrographs of reconstituted 5, 10, and 20% vitamin E emulsions containing 20% sucrose. One of the original liquid emulsions is shown (Figure 4F) as a comparator. There are more large droplets in C than in A and B, and consequently 20% vitamin E emulsions were not studied further. 10% vitamin E was selected because of the potential higher loading capacity compared to the 5% vitamin E emulsion. Figure 4 (D, E, B) shows the micrographs of the 10% vitamin E emulsions with 5, 10, and 20% sucrose. 20% sucrose had the best protection effect as the least number of large droplets was observed. A combination of 10% vitamin E and 20% sucrose was chosen for further evaluation.

The Effect of Trehalose, Lactose, and Fructose

The freeze-dried emulsions containing trehalose or lactose were self-supporting cakes occupying the same volume as the original liquid emulsions. Emulsions containing fructose appeared to have "boiled" and the freeze-dried product looked like solid foam. The trehalose and fructose protected emulsions were as easily reconstituted as the glucose and sucrose protected emulsions, while the lactose protected emulsions produced visible yellow lumps floating on the surface after the addition of water. The lumps were dispersible with hand shaking but the overall process of reconstitution was slower than with the other protectants.

Figure 5 shows the micrographs of the emulsions protected by trehalose, lactose, and fructose. It is clear that the coalescence in these emulsions was more extensive than that in glucose and sucrose protected emulsions. Due to the poor quality of these emulsions (as indicated by the large number of large droplets), the DLS method was no longer capable to produce consistent data for comparison purpose.

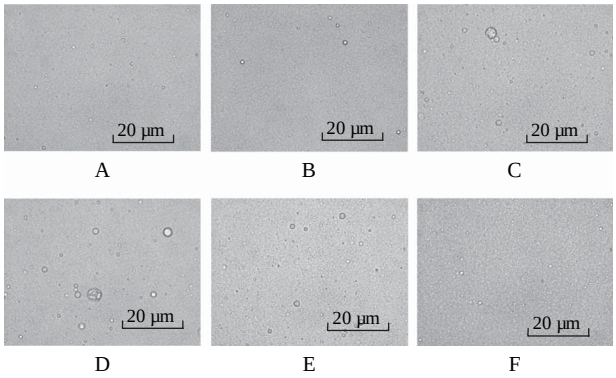


FIGURE 4. Photomicrographs of vitamin E emulsions protected by sucrose. Formulations A, B, and C all contain 20% sucrose. They also contain 5, 10, and 20% vitamin E, respectively. Formulations D and E all contain 10% vitamin E and with variable sucrose concentrations (5, 10%, respectively). Formulations: Refer to section 2.2.

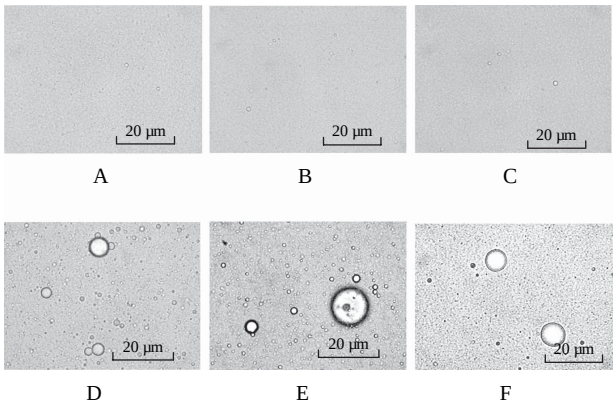


FIGURE 5. Photomicrographs of emulsions containing trehalose, lactose, and trehalose. A, B, C—Original emulsions containing trehalose, lactose, and trehalose, respectively; D, E, and F—Reconstituted emulsions containing trehalose, lactose, and trehalose, respectively. Formulations: Refer to section 2.2.

The Effect of Mixtures of Protectants

Compared to other protectants, glucose is more widely used clinically but was shown to have a poor cake-forming property in this study. An ideal freeze-dried product should be in the form of porous self-supporting cakes so that the desired physical properties essential to reconstitution can be maintained. It was logical, therefore, to try to improve the caking property of glucose by incorporating the better cake-forming protectants sucrose and lactose (3.1.2 and 3.1.3). As expected, in both cases the caking property was greatly improved as compared to using glucose alone. However, microscopic examination revealed that there were more larger droplets in these samples as compared to samples with glucose alone (Figure 6). Therefore, no further studies on mixtures of protectants were performed.

Comparison of the Cryoprotectants Studied

From the photomicrographs (Figure 2 B1, B2, B3, Figure 4 B, Figure 5, and Figure 6), it is clear that the protective effects of trehalose, lactose, and fructose were not as good as those of glucose and sucrose. Mixtures of the protectants at the ratios examined did not improve the protective effect. Therefore, the choice was between glucose and sucrose. Glucose is more frequently used clinically, but sucrose has a better protection effect as judged from the microscopic observation. More importantly, the caking property of the sucrose-based system was superior to that of glucose-based system. Therefore, sucrose was selected for further study.

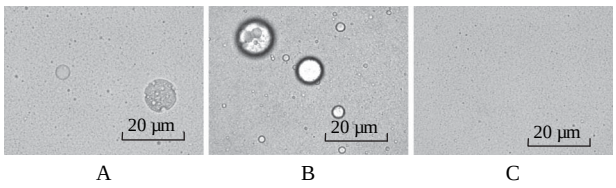


FIGURE 6. Photomicrographs of reconstituted emulsions containing mixtures of saccharides. A—10% glucose and 10% lactose, B—10% glucose and 10% sucrose, C—Original liquid emulsion. Formulations: Refer to section 2.2.

The exact mechanism of the protective effects of these protectants was not clear. The glass forming properties of the saccharides could be part of the reasons for the improved stability in the freeze-drying process. When there are no saccharides, ice crystals will form when the liquid is being frozen. The formation and progression of ice crystal could put mechanical stress on the emulsion droplets and destabilise the emulsions. The formation of crystal will result in the concentrating, sometimes preferential concentrating, of the various ingredients in the emulsion aqueous phase which may in turn result in undesirable environmental changes to the emulsion droplets, including ionic strength and pH (Franks, 1998). Where there are saccharides present, the formation of crystal is suppressed. Instead of forming ice crystals, the external phase of the emulsion forms a glassy state when frozen. Consequently, the destructive effect of the ice crystals is avoided. It has been reported for the preservation of liposomal membranes, the protective effect of the saccharides correlates directly to their glass transition temperature indicating the importance of vitrification (Green and Angell, 1989). However, others argue that although vitrification may be required, it is not in itself sufficient to preserve freeze-dried liposomes and the interaction between the protectants and the molecules being dried is an important factor (Crowe et al., 1994, 1997). In the current study with emulsions, no correlation between the protection effect and glass transition temperature was found. For example, trehalose has a higher glass transition temperature (77°C) than sucrose (56°C) (Franks et al., 1991), but the protective effect was not as good as sucrose.

It is possible that the interaction between the protectants and the phospholipids head groups played an important role in the freeze-drying process of the vitamin E emulsions. The most common feature of the saccharides molecules is the presence of multiple hydroxyl groups. It has been reported that saccharides can interact with the phospholipids head groups through hydrogen bonding (Crowe et al., 1994). After the emulsion is dried, the emulsifier polar head groups will still be in hydrogen bonding with the hydroxyl groups of the saccharides. Therefore the emulsion droplets will still be supported by the solid media after freeze-dried, avoiding coalescence.

The stabilization of emulsions is a complex issue. While there are theories to explain the stability of emulsion systems, which can help the formulation scientist to select possibly good emulsifiers, the final optimization usually has to be based on experimental testing. When there is a combination of emulsifiers, it is especially difficult to ascertain the structure of the interfacial emulsifier layer. Therefore, it is not precisely clear in what structure or configuration do the protectants interact with the interfacial emulsifier layer. Future work is needed to explain why the saccharides have different protective effects in relation to their chemical structures.

Crystal Formation in High Drug Loading Emulsions

A 10% vitamin E emulsion containing 25 mg per g of vitamin E paclitaxel (twice the previously achieved loading of a liquid emulsion) was prepared, half of which was kept at 4 ± 2°C in the liquid form and the other half was freeze-dried immediately (with sucrose as the protectant). The freeze-dried samples were then filled with N_2, sealed and also kept at 4 ± 2°C also. Figure 7A shows that paclitaxel crystals formed within 3 days in the liquid emulsion. This was expected as the loading was above the previously used loading (12.5 mg per g of vitamin E) of a liquid emulsion. Figure 7B shows that no crystals occurred in the freeze-dried emulsion 430 days (4 ± 2°C) after production and 4 h after reconstitution (22 ± 2°C). This could be because the medium for the crystal formation (the aqueous phase) was immediately frozen after emulsion production and subsequently removed.

Table 1 shows that the mean droplet size of the freeze-dried emulsion remained smaller than 0.2 μm within the period studied (up to 430 days) in the freeze-dried state.

In the previous study, paclitaxel crystals could be detected in the emulsion at loadings above 2.5 mg/mL (12.5 mg per g of vitamin E). The lyophilized emulsion produced here has double the loading compared to the previous liquid emulsions (Han et al., 2004) without the crystallisation problem. This would allow the delivery of the same amount of drug using half of the excipients (emulsifiers and vitamin E).

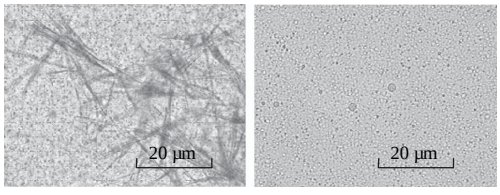


FIGURE 7. Photomicrographs of vitamin E emulsions containing paclitaxel 25 mg per gram of vitamin E (double the previously achieved paclitaxel loading in a liquid emulsion). A—Original liquid emulsion, 3 days after production (4 ± 2°C), B—Freeze-dried emulsion 430 days after production (4 ± 2°C) and 4 h after reconstitution (22 ± 2°C). Formulations: Refer to section 2.2.

Crystal Formation of Emulsions Containing Poloxamer 188

We have demonstrated previously that the addition of 2% poloxamer 188 to the vitamin E emulsion can facilitate the crystallization of paclitaxel at a loading of 12.5 mg per g of vitamin E (Han et al., 2004). In the present study, a 10% vitamin E emulsion containing 12.5 mg paclitaxel per g of vitamin E and 2% poloxamer 188 was prepared, half of which was kept at 4 ± 2°C in the liquid form and the other half was freeze-dried immediately (sucrose as the protectant). The freeze-dried sample was filled with N_2, sealed and stored at 4 ± 2°C. Figure 8A shows that paclitaxel crystals formed within 3 days in the liquid emulsion. This is in agreement with our previous findings (Han et al., 2004). Figure 8B shows that no crystals could be detected in the freeze-dried emulsion 360 days after being freeze-dried (4 ± 2°C) and 4 h after reconstituted (22 ± 2°C). This again could be attributed to the removal of the aqueous phase. The mean droplet size of the reconstituted emulsion was well below 0.2 μm (Table 2). The previously observed crystallisation problem in emulsions containing 2% poloxamer (Han et al., 2004) was avoided in the dry emulsions produced here.

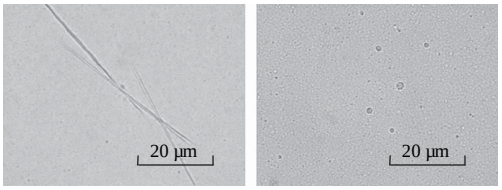
CONCLUSIONS

Reconstitutible solid vitamin E emulsion can be produced using a freeze-drying technique with added cryoprotectants. Sucrose had the best protection effect among various carbohydrates tested, including glucose, trehalose, fructose, and lactose. The freeze-dried emulsions could be easily reconstituted without major coalescence. The loading of paclitaxel in the freeze-dried emulsions could be raised to 25 mg per g of vitamin E (twice the previously achieved loading of equivalent liquid emulsion) without the formation of drug crystals for up to 430 days and the mean droplet size on reconstitution remained smaller than 0.2 μm during the storage period studied. The poloxamer 188 facilitated crystallization of paclitaxel observed in a previous study could also be avoided using this freeze-drying technique.

TABLE 1
Droplet Size Stability of Freeze-Dried 10% Vitamin E Emulsion Containing Paclitaxel* (4 ± 2°C)

Storage Time (day)	0	7	19	430
Droplet size (nm)	178 ± 1	181 ± 1	182 ± 1	191 ± 5
Polydispersity	0.26 ± 0.04	0.23 ± 0.03	0.26 ± 0.04	0.25 ± 0.05

*Loading: 25 mg per g of vitamin E (double the previously achieved loading of a standard liquid emulsion). Formulation: refer to section 2.2.



A B

FIGURE 8. Photomicrographs of vitamin E emulsions containing 12.5 mg paclitaxel per gram of vitamin E and 2% poloxamer 188. A—Original liquid emulsion, 3 days after production (4 ± 2°C), B—Freeze-dried emulsion, 360 days after production (4 ± 2°C) and 4 h after reconstituted (22 ± 2°C). Formulations: Refer to section 2.2.

TABLE 2
Droplet Size of Reconstituted 10% Vitamin E Emulsion Containing 2% Poloxamer 188

Sample	Original Emulsion	Reconstituted
Droplet size (nm)	154 ± 1	165 ± 2
Polydispersity	0.14 ± 0.04	0.21 ± 0.02

*Loading of paxlitaxel: 12.5 mg per g of vitamin E. Formulation: refer to section 2.2.

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