European Journal of Pharmaceutics and Biopharmaceutics 85 (2013) 306–313

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Lyophilization of a triply unsaturated phospholipid: Effects of trace metal contaminants

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ARTICLE INFO

Article history:
Available online 6 April 2013

Keywords:
DLPC
Lyophilization
Liposomes
Chemical stability
Metal contamination
Unsaturated lipids
Oxidation
Freezing
Freeze-drying

ABSTRACT

As liquid liposomal formulations are prone to chemical degradation and aggregation, these formulations often require freeze drying (e.g., lyophilization) to achieve sufficient shelf-life. However, liposomal formulations may undergo oxidation during lyophilization and/or during prolonged storage. The goal of the current study was to characterize the degradation of 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine (DLPC) during lyophilization and to also probe the influence of metal contaminants in promoting the observed degradation. Aqueous sugar formulations containing DLPC (0.01 mg/ml) were lyophilized, and DLPC degradation was monitored using HPLC/UV and GC/MS methods. The effect of ferrous ion and sucrose concentration, as well as lyophilization stage promoting lipid degradation, was investigated. DLPC degradation increased with higher levels of ferrous ion. After lyophilization, 103.1 ± 1.1%, 66.9 ± 0.8%, and 28.7 ± 0.7% DLPC remained in the sucrose samples spiked with 0.0 ppm, 0.2 ppm, and 1.0 ppm ferrous ion, respectively. Lipid degradation predominantly occurs during the freezing stage of lyophilization. Sugar concentration and buffer ionic strength also influence the extent of lipid degradation, and DLPC loss correlated with degradation product formation. We conclude that DLPC oxidation during the freezing stage of lyophilization dramatically compromises the stability of lipid-based formulations. In addition, we demonstrate that metal contaminants in sugars can become highly active when lyophilized in the presence of a reducing agent.

1. Introduction

Liposomal delivery systems have been used to improve the delivery of small molecule pharmaceuticals; there are currently 12 liposomal products on the market [1]. In the case of liposomal formulations designed to deliver siRNA, unsaturated lipids have proven superior to saturated lipids for promoting intracellular nucleic acid delivery [2–5]. Unfortunately, unsaturated lipids are highly susceptible to being oxidized which limits their use in lipid-based commercial products. As liquid liposomal formulations are prone to chemical degradation and aggregation, these formulations are often lyophilized to achieve stability compatible with a marketable product. Furthermore, the increased immunogenicity associated with the use of viral vectors and the resulting safety concerns underscore the need to further develop non-viral delivery systems [6–8]. Liposomal vectors also have the potential to provide a possible avenue for the treatment of diseases that are not responsive to existing therapeutics. Tuberculosis, for example, has become increasingly difficult to treat due to a significant increase in drug resistant strains [9]. While the use of liposomes to deliver small molecule drugs to treat tuberculosis (e.g., Rifampicin) appears promising, the technology has not been fully developed [10]. Hence, additional research efforts to improve stability and efficiency of liposomal delivery systems are needed.

The chemical and physical stability of aqueous liposomal formulations, which have been studied extensively, are limited due to the occurrence of peroxidation, hydrolysis, and aggregation [11,12]. It has been shown that as the number of unsaturated bonds increases, the extent of peroxidation also increases [12]. The decreased bond dissociation energy at the carbon–hydrogen bonds in the bis-allylic position underlies the increased oxidizability of polyunsaturated lipids [13,14]. Previous studies have assessed oxidative lipid degradation by measuring reactive aldehyde products, conjugated dienes, hydroperoxides, and/or oxygen consumption [15,16].

One common approach to improve the overall stability of liposomal formulations is freeze-drying (i.e., lyophilization). Lyophilization of liposomes often incorporates the use of sugars, such as sucrose or trehalose, which form glassy matrices that limit mobility

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http://dx.doi.org/10.1016/j.ejpb.2013.03.028

(vitrification hypothesis), forms hydrogen bonds (water replacement theory), and/or separates the individual liposomes (particle isolation hypothesis) [17–20]. Even in the case of pharmaceutical-grade sugars, the presence of transition metal contaminants is a concern. Transition metals, such as iron, can catalyze the oxidative degradation of unsaturated lipids in the dried state and may also lead to co-degradation of liposomal cargos (e.g., DNA degradation) [21,22]. Saturated and hydrogenated lipids are less prone to oxidative degradation, and therefore, these lipids are often utilized in lipid-based therapeutics (e.g., Doxil®, DaunoXome®, and Ambisome®) [1]. Singly unsaturated lipids such as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) are also less susceptible to oxidative degradation than polyunsaturated lipids and have been used in commercial liposomal products such as DepoCyt® [1]. Although polyunsaturated lipids are significantly more susceptible to oxidative degradation, the use of these lipids in liposomal pharmaceutical preparations is being investigated. Recently, it was demonstrated that lipids with two or more unsaturations were more fusogenic relative to lipids with fewer unsaturated bonds, and this promotes endosomal escape [2]. The requirement for endosomal escape is a major hindrance when delivering nucleic acids to the interior of the cell. Furthermore, the increased endosomal escape has been shown to correlate with increased siRNA delivery and luciferase silencing *in vitro* [2].

In the current study, we have utilized a triply unsaturated lipid, DLPC, and evaluated the acute degradation occurring during lyophilization. While lyophilization is generally a strategy to minimize the degradation of liposome formulations, the lyophilization process exposes liposomal formulations to freezing and drying stresses [23–25]. We investigated the effects of sugar (i.e., sucrose, trehalose, or hydroxyethyl starch) and ferrous ion concentration on DLPC degradation during lyophilization. In an attempt to mimic transition metal contaminants known to be present in pharmaceutical-grade sugars, we spiked ferrous ion into the DLPC samples at iron levels that are commonly found in pharmaceutical-grade sugars [26,27]. Although the effect of transition metals, such as ferric and ferrous ions, on the oxidative degradation of lipids has been studied, a significant proportion of studies which examined iron-catalyzed lipid peroxidation were focused on the stability of consumable foods and the progression of various diseases [28–30]. We believe this to be the first study to address the stability of pharmaceutically relevant unsaturated lipids during lyophilization.

2. Materials and methods

2.1. Materials

1,2-Dilinolenoyl-*sn*-glycero-3-phosphocholine (DLPC; 18:3 (cis) PC) (>99%, Lot # 183-12li) was purchased from Avanti Polar Lipids (Alabaster, AL). Sucrose (Ultrex© ultrapure, Lot # J25H00) was purchased from JT Baker (Center Valley, PA). Trehalose (100%, Lot # 28549A) was purchased from Ferro Pfanstiehl (Waukegan, IL). Hydroxyethyl starch (HES, Batch # 17081421) was purchased from Fresenius Kabi (Linz, Austria). 5-(2-Carboxyphenyl)-5-hydroxy-1-((2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl)methyl)-3-phenyl-2-pyrrolin-4-one potassium salt (proxyl fluorescamine) was obtained from Molecular Probes, Inc. (Eugene, OR). Tris [hydroxymethyl] amino-methane, iron (II; ferrous ion) chloride tetrahydrate, 2-thiobarbituric acid (TBA), 2,6-di-tert-butyl-4-methylphenol (BHT), 1,1,3,3-tetraethoxy propane (MDA), sodium sulfite, and hydrochloric acid were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p, p′-disulfonic acid, disodium salt hydrate (ferroZine iron reagent), and propionic acid were purchased from Acros Organics (Geel, Belgium). Trichloroacetic acid 10% w/v aqueous solution was purchased from Ricca Chemical Company (Arlington, TX). Chloroform, methanol, water, (all of which were HPLC grade), LC/MS grade acetone, ascorbic acid, and Eppendorf tubes (1.5 ml) were procured from Fisher Scientific (Pittsburgh, PA). High grade helium and nitrogen were purchased from Airgas (Radnor, PA). Amber glass vials (5 ml) were procured from West Pharmaceutical Services (Lionville, PA). Phenomenex Strata X solid phase extraction (SPE) tubes (1.0 ml) containing sorbent (30 mg; Lot # S300-139) were used (Torrance, CA).

2.2. DLPC sample preparation

The lipid (DLPC) stock, originally dissolved in chloroform (10.0 mg/ml), was concentrated using a slow stream of nitrogen. The lipid was then further dried under vacuum (559 Torr) for approximately one hour. The lipid (DLPC) was then suspended in Tris buffer (0.5 mM, pH 7.4) to afford a final DLPC concentration of 0.1 mg/ml. The rehydrated lipid (DLPC) was then sonicated (30 s) and vortex mixed (5 s). The resulting DLPC liposome suspension was used immediately after additional dilution with Tris buffer to 0.04 mg/ml. Stock aqueous ferrous ion solutions (400 ppm) were prepared immediately before use in HPLC grade water (Fisher Scientific W5-4); this stock solution was then diluted and added into the study samples. Sucrose, trehalose, and HES were initially prepared as 10% w/v solutions in Tris buffer (0.5 mM, pH 7.4). The sugars were added to the samples such that the final sugar concentration was 2.0%. The final samples (1.0 ml) were prepared in amber glass vials (5 ml) and contained 0.01 mg/ml DLPC, 2% sugar (sucrose, trehalose or HES), and 0.0 ppm, 0.2 ppm or 1.0 ppm ferrous ion. In the experiments evaluating the effect of the oxidation state of the transition metal contaminants, stock sodium sulfite solution was freshly prepared in 0.5 mM Tris buffer and samples prepared as described previously to contain sodium sulfite (2.0 μg/ml). When the effects of buffer ionic strength were tested, samples were also prepared as described above except that the concentrations of buffer, ferrous ion, and DLPC lipid were increased to mimic the concentrations in the frozen state. The volume of the freeze concentrate was calculated to determine to what extent the sample solutes were concentrated in the frozen state [31]. These samples were stored at 4 ± 1 °C overnight.

2.3. Lyophilization protocol

Sample vials were placed directly on the shelf of an FTS Durastop lyophilizer (Stone Ridge, NY). The lyophilization cycle involved freezing samples to −30 °C (8 h, cooled at 0.4 °C/min), and the pressure of the chamber was reduced to 30–60 mTorr. Next, the primary drying occurred at −20 °C (15 h, 2 °C/min) and the secondary drying occurred at 30 °C (30 h, 1 °C/min) and then at 35 °C (1.5 h, 1 °C/min). Sample temperatures were monitored by inserting a thermocouple into a representative sample prior to starting the lyophilization cycle. When samples were removed during freezing, time zero was considered to be 81 min after the shelf temperature had reached −30 °C; at this point, the sample temperature was stable. After sample lyophilization was completed, the vials were stoppered while the chamber remained under vacuum.

2.4. HPLC–UV analysis

To measure DLPC, a Shimadzu analytical HPLC system (LC-20AB, DGU-20A, CTO-20A, Sil-20A HT) equipped with SPD-20A UV–VIS detector was used (Shimadzu Scientific Instruments, Inc.; Columbia, MD). An Aligent zorbax extended-C18 50 × 4.6 mm (5 μm) column equipped with a guard column was used (Santa Clara, CA). The column temperature was maintained at 40 °C with a flow rate of 0.4 ml/min. An isocratic method (20 min run time; 19:1