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Biochimica et Biophysica Acta 1768 (2007) 669–677
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbamem

Metal contaminants promote degradation of lipid/DNA complexes during lyophilization

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Received 6 September 2006; received in revised form 30 November 2006; accepted 5 December 2006
Available online 8 December 2006

Abstract

Oxidation reactions represent an important degradation pathway of nucleic acid-based pharmaceuticals. To evaluate the role of metal contamination and chelating agents in the formation of reactive oxygen species (ROS) during lyophilization, ROS generation and the stability of lipid/DNA complexes were investigated. Trehalose-containing formulations were lyophilized with different levels of transition metals. ROS generation was examined by adding proxyl fluorescamine to the formulations prior to freeze-drying. Results show that ROS were generated during lyophilization, and both supercoil content and transfection rates decreased as the levels of metal-induced ROS increased. The experiments incorporating chelators demonstrated that some of these agents (e.g., DTPA, desferal) clearly suppress ROS generation, while others (e.g., EDTA) enhance ROS. Surprisingly, there was not a strong correlation of ROS generated in the presence of chelators with the maintenance of supercoil content. In this study, we demonstrated the adverse effects of the presence of metals (especially Fe^{2+}) in nonviral vector formulations. While some chelators attenuate ROS generation and preserve DNA integrity, the effects of these additives on vector stability during lyophilization are difficult to predict. Further study is needed to develop potent formulation strategies that inhibit ROS generation and DNA degradation during lyophilization and storage.

Keywords: Nonviral vectors; Lyophilization; Metal contamination; Reactive oxygen species; Chelating agents; Gene delivery

1. Introduction

There is currently a growing interest in preserving nonviral vectors as dehydrated formulations because they offer the potential for prolonged stability at ambient temperature. The majority of lyophilization studies to date have focused on the acute stability of nonviral vectors and consistently reported that nonviral vectors can be stabilized in the presence of sugars [1–4]. Considering that nucleic acids are highly susceptible to oxidation [5,6], formulation strategies must insure that DNA integrity is maintained during the freeze-drying process.

Free radical oxidation is often considered a major chemical degradation pathway for DNA-based pharmaceuticals [5,7,8]. A significant number of studies have demonstrated the high sensitivity of DNA to reactive oxygen species [ROS] (e.g., hydroxyl radical) that result in damaged bases and/or single and double-strand breaks [9–12]. Indeed, it is well established that exogenous factors such as the presence in excipients of trace amounts of transition metals (e.g., Cu^{2+} and Fe^{2+}) can catalyze the generation of hydroxyl radicals via the Fenton reaction, which can extensively damage DNA [5–7,13,14]. In an effort to circumvent this problem, researchers have utilized strategies such as the addition of chelating agents to their formulations [7,8,15]. Importantly, while these findings suggest that trace metal contaminants can cause DNA degradation and that chelating agents can be used to prevent ROS in aqueous solution, to our knowledge no study has explored the role of the generation of reactive oxygen species (ROS) in the stability of lipid/DNA complexes during drying. Thus, in the present study we evaluated the effects of metal contamination and chelating agents on the formation of ROS during acute lyophilization. Formulations containing the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and L-α-dioleoylphosphatidyl-ethanolamine (DOPE) (1:1 molar ratio) complexed with plasmid DNA were lyophilized in trehalose fortified with different levels of transition metals (Cu^{2+}, Fe^{2+}, Fe^{3+}). We investigated the role of metal ions in the generation of ROS

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doi:10.1016/j.bbamem.2006.12.004

during acute lyophilization by adding a fluorescent probe (proxyl fluorescamine) to the formulations prior to freeze-drying. We also explored the effect of four different chelators on metal-induced ROS generation and the acute stability of lipid/DNA complexes (as indicated by DNA integrity and biological activity upon rehydration). Here, we show that ROS are generated during lyophilization even in the absence of metals, although the addition of metals increased ROS levels. Generally, both supercoil content and transfection rates diminished as the levels of metal-induced ROS increased. The experiments incorporating chelators showed that some of these agents (e.g., diethylenetriaminepentaacetic acid [DTPA], desferrioxamine mesylate [desferal]) clearly suppress ROS generation, while others (e.g., ethylenediaminetetraacetic acid [EDTA]) enhance ROS levels. Surprisingly, there was not a strong correlation of ROS levels (as measured by proxyl fluorescamine) generated in the presence of chelators with the maintenance of supercoil content. The results obtained in this study demonstrate the adverse effects of the presence of metal contaminants, especially iron in the ferrous form, in DNA-containing formulations. In addition, this study underscores the importance of developing formulation strategies that minimize the generation of ROS.

2. Materials and methods

2.1. Chemicals

Trehalose was a gift from Ferro-Pfanstiehl Laboratories (Waukegan, IL). DOTAP and DOPE were obtained in a 1:1 weight ratio from Avanti Polar Lipids (Alabaster, AL). The DNA plasmid encoding the protein reporter luciferase (5.9 kb) was a generous gift from Valentis (Burlingame, CA). DNA was dissolved in sterile 2.5 mM Tris–HCl pH 8.5 and diluted to a concentration of 1 mg/mL prior to use. The luciferase assay kit was obtained from Promega (Madison, WI). Ethidium bromide solution (10 mg/mL) was purchased from Sigma (St. Louis, MO). Proxyl fluorescamine, 5-(2-carboxyphenyl)-5-hydroxy-1-[(2,2,5, 5-tetramethyl-1-oxypyrrolidin-3-yl) methyl]-3-phenyl-2-pyrrolin-4-one potassium salt, was obtained from Molecular Probes Inc. (Eugene, OR). Diethylenetriaminepentaacetic acid (DTPA), bathophenanthroline disulfonate (BPS) and ferrous chloride were obtained from Acros Organics (Fairlawn, NJ). Anhydrous cupric chloride, ferric chloride, ethylenediaminetetraacetic acid (EDTA), and desferrioxamine mesylate (desferal) were purchased from Sigma (St. Louis, MO). Sodium chloride (NaCl), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$) and potassium phosphate monobasic (KH_2PO_4) were purchased from Sigma (St. Louis, MO). All chemicals were of analytical grade and used without further purification.

2.2. Preparation of liposomes

Liposomes containing DOTAP in a 1:1 (w/w) ratio with the zwitterionic lipid DOPE were prepared as previously described [16]. Briefly, the lipid mixture in chloroform was dried under a stream of nitrogen gas and placed under vacuum (10 mTorr) for 1 h to remove residual chloroform. The dried lipid was resuspended in sterile distilled water to a concentration of 2 mg/mL, sonicated to clarity with a Branson Sonifier 250, and stored at 4 °C. The liposomes were freshly sonicated immediately before use.

2.3. Lipoplex preparation

The complexes were prepared with a 3:1 lipid:DNA weight ratio (480 μg DOTAP-DOPE and 160 μg DNA in 2.5 mM Tris–HCl pH 8.5) in polypropylene microcentrifuge tubes by gentle mixing, and incubated for 20 min at room temperature as previously described [17]. This method of preparation results in a heterogeneous suspension of particles with a calculated +/− charge ratio of 0.7 [16]. Depending on the experiment, freshly prepared metal ion solutions (1×10^6 ppb: $CuCl_2$, $FeCl_2$, $FeCl_3$) were added to 2 mL aliquots of the resulting suspension of lipid/DNA complexes to achieve a range of final metal concentrations (0–1000 ppb). Accordingly, freshly prepared chelating agent solutions (DTPA, desferal, BPS and EDTA) were subsequently added to achieve final chelator concentrations ranging from 0 to 800 μM. Proxyl fluorescamine was introduced into these formulations at a final concentration of 0.45 μM. The resulting suspensions were then diluted with an equal volume of an 8% excipient solution (trehalose) in Tris buffer as previously described [18]. Aliquots of 400 μL containing 16 μg of DNA were transferred to clear 1-mL and amber 2-mL flat-bottomed borosilicate lyophilization vials (for samples with and without proxyl fluorescamine dye, respectively) (West Co., Litiz, PA). The stoppers were obtained from West Co. (Litiz, PA), washed with distilled water, and dried overnight in an oven (≈60 °C) prior to use.

2.4. Freeze-drying protocol

Sample vials were placed on the shelf of an FTS Durastop lyophilizer (Stone Ridge, NY). The lyophilization cycle used was performed as follows: shelves were cooled to −40 °C, and held for 2 h (sample temperature ≈−37 °C), the chamber pressure was then reduced to 60 mTorr, primary drying at −40 °C for 30 h, secondary drying at 25 °C for 6 h. After secondary drying, sample vials were stoppered under vaccum and stored at −80 °C until rehydrated. For freezing studies, the same conditions were used to cool the samples in the lyophilizer. Samples were maintained on a −40 °C shelf overnight, and rapidly thawed in a water bath (37 °C) prior to analysis.

2.5. Rehydration protocol

Lyophilized samples containing the appropriate levels of plasmid, cationic agent and excipient (trehalose) were rehydrated to a final volume of 400 μL with filtered distilled water and incubated for 30 min at room temperature. Appropriate volumes of rehydrated samples were used for transfection (7.5 μL) and analysis of DNA structure (50 μL; described below).

2.6. Cell culture

African green monkey kidney cells (COS-7: ATCC No. CRL1651) were obtained from American Type Culture Collection (Rockville, MD). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/mL penicillin G, and 50 μg/mL streptomycin sulfate, and were propagated by reseeding at $1–3\times10^5$ cells/100 mm dish every 2–3 days. In our experiments, cultures were freshly seeded at 2500 cells/well in 96-well plate 24 h before transfection.

2.7. Transfection assay

Aliquots of rehydrated and freshly prepared lipid/DNA complexes containing 0.3 μg DNA in the presence or absence of the metal ions Cu^{2+}, Fe^{2+}, and Fe^{3+}, and the chelating agents DTPA, desferal, BPS and EDTA, were diluted to a final volume of 100 μL with serum-free, antibiotic-free DMEM and distributed into wells of a 96-well plate containing COS-7 cells freshly washed with phosphate buffered saline (PBS). Cells were subsequently incubated with lipoplexes for 4 h before the medium was replaced with 100 μL DMEM containing serum and antibiotics, and allowed to grow for approximately 40 h before the cell culture medium was discarded. Cells were washed and then lysed with 80 μL of lysis buffer (Promega, Madison, WI), as previously described [19]. Twenty microliters of lysate were used to assay luciferase expression according to the manufacturer's protocol (Promega). Luciferase activity was quantified by using a TD-20e Los Alamos Diagnostics 535 Luminometer (Mountain View, CA). Protein concentrations were determined by the Bradford method using a Bio-Rad protein assay (Hercules, CA), according to the directions provided by the manufacturer. Absorbances were measured at