Development of Freeze-Dried Injectable Formulation for the Novel Lipid A Analog, E5531

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Abstract: A sonicated dispersion of the novel lipid A analog, E5531, was freeze-dried in the presence of various additives such as saccharides and polyalcohols, and their cryoprotective effects were investigated. Fusion of the vesicles was examined by measuring fluorescence energy transfer and size distribution. The ability as cryoprotectants differed among the additive species. The addition of polyalcohols led to considerable fusion. Although monosaccharides, similar to disaccharides, completely prevented the fusion of the vesicles during lyophilization, they showed far less ability to retain the entrapped calcein in the vesicles compared to disaccharides. Differential scanning calorimetry heating profiles of vesicles that had been lyophilized with various additives were obtained. Disaccharides and monosaccharides again resulted in markedly different thermal properties of the vesicles. This variety in cryoprotective ability of saccharide species can be attributed to differences in their interaction with the E5531 head group.

Key words: lipid A, freeze-drying, cryoprotective ability, saccharide, polyalcohol

1 Introduction

Lipid A is a component of bacterial lipopolysaccharide (LPS), and is present in the major amphiphic constituents of the leaflets of Gram-negative bacteria. It is a potent biological active substance (1, 2) and induces the production of prostaglandins, cytokines such as interferon (3), interleukin-1(4) and tumor necrosis factor (TNF) in mammalian cells, such as macrophages and lymphocytes. This compound also induces undesirable toxic effects such as fever and the Schwartzmann bleeding reaction (5).

Numerous attempts have been made to synthesize low toxic lipid A analogs. Recent work in our laboratories has indicated that some lipid A analogs may be potent LPS antagonists (6). While the synthetic disaccharide lipid A analog E5531 (Fig. 1) has a low toxicity, it retains a variety of useful biological activities such as inhibition of the production of TNF (6). This compound has been found to be a specific LPS antagonist, as evidenced by an LPS binding assay, and to inhibit LPS induced TNF production in monocytes/macrophages. This compound can be used as a drug for the treatment of septic shock and, because of this, an injectable formulation would be extremely useful.

Because E5531 is not atable at the conditions used for sterilization, a dispersion of E5531 is usually freeze-dried for parental use. As is the case of liposomes prepared from phospholipids (7, 8), the stabilizers such as saccharides are added to the dispersion in the freeze-drying process. However, in the case of the lipid A analogs, our understanding for the additive effect on the freeze-drying and the stabilization mechanism of the additives is limited.

We report here on and investigation of the cryoprotective ability of various additives such as saccharides

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2 Materials and Methods

2.1 Materials

E5531 was supplied from Eisai Chemical co., Ltd. (Ibaraki, Japan). Calcein (3,3′-bis[N,N-bis(carboxymethyl)aminomethyl]-fluorescein) was purchased from Dojin (Kumamoto, Japan). N-(7-Nitrobenz-2-oxa-1,3-diazol-4yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-PE) and N-(lissamine-rhodamine-B-sulfonyl) dioleoyl-L-α-phosphatidylethanolamine (Rh-PE) were supplied by Sigma Co., Ltd (St. Louis, Mo, USA). Saccarides and polyalcohols were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2 Freeze-Drying

Calcein-entrapped, sonicated particles were prepared as previously described (9). The solution inside the particles was a 5 mM phosphate-NaOH buffer solution containing 70 mM (pH 7.3), and the outside buffer solution contained 365 mM of saccharides or polyalcohols, to maintain an isotonic condition relative to inside solution. The E5531 concentration was 2 mM. The freeze-drying procedure was as follows. Five ml of an E5531 dispersion was placed in 15 ml glass vials. The dispersion in the vials were freeze-dried (Triomaster, model A04 by Kyowa-Shinku Co., Ltd) to a final shelf temperature set at −40°C, and then placed under vacuum at a pressure of 0.075 mbar. The shelf temperature was subsequently set from −40°C to 20°C for 3 h, and thereafter its temperature was set at 32°C for 18 h. The drying chamber was filled with dry nitrogen, and the vials were sealed in the drying chamber. The particle size and leakage of calcein from each sample was measured after rehydration by just adding 5 ml of water.

2.3 Particle Size

E5531 particle size was measured by a dynamic light scattering method (DLS) (10). The DLS apparatus used was a model DLS-7000DL with an Ar laser (λ = 483 nm) manufactured by Ohtsuka Electronics Co., Ltd. The weight averaged diameter ($D_w$) and the distribution of the vesicle size were determined by histogram methods (10).

2.4 Determination of Trapped Volume

The trapped volume inside the particles was determined in order to obtain information on the structure of the E5531 particles. Five milligrams of E5531 was dispersed in 2.5 ml of a 70 mM calcein (pH 7.3) using the above sonication method. The untrapped calcein was removed by gel filtration (Sephadex G-50) at 25°C. The volume of the calcein solution trapped in the particles was determined fluoroermetrically (9) after solubilization of the particles by the addition of 10% Triton X-100, and the aqueous volume trapped per mole of E5531 was evaluated. E5531 in the dispersion was assayed by an HPLC method (detection wavelength: 254 nm).

2.5 Calculation of Percent Retention of Calcein (R%)

The percentage of calcein retained after freeze-drying (R%) was determined by measuring the fluorescence intensity at 520 nm with excitation at 490 nm using model F-4500 fluorophotometer (Hitachi Co., Ltd.). R% was calculated using the method of Crowe et al. (11).

2.6 Fusion

Fusion of the particles induced by freeze-drying was estimated by a probe dilution assay of the fluorescence energy transfer technique as described by Struck et al.
(12). Fluorescently labeled particles containing 1.0 and 0.3 mol% of NDB-PE and Rh-PE in the membrane were prepared in each 365 mM saccharide buffer solution. The labeled particles were mixed with the unlabelled particles at a ratio of 1:1 keeping a total particle concentration of 4 mM. The efficiency of energy transfer \( (E) \) before and after freeze-drying was obtained from the fluorescence emission intensity of NDB-PE at 530 nm (excitation 460 nm) using the following equation,

\[
E' = 1 - \frac{F}{F_0}
\]

Where \( F_0 \) and \( F \) are the fluorescence intensities in the presence and absence of Triton X-100, respectively. Under these experimental conditions, the decrease in the measured energy transfer efficiency indicates that the particles have fused (12), since the energy transfer should be proportional to decrease in the surface density of the energy acceptor (Rh-PE).

### 2.7 Differential Scanning Calorimetry (DSC)

Fifty mg of E5531 was dispersed in 1 ml of a buffer solution (4.25 mM phosphate-NaOH, containing 10% of saccharide) using sonication. After freeze-drying, the samples were immediately weighed in a sample pan in an atmosphere of dry nitrogen, and sample pans were then hermetically sealed. The samples were scanned from 0°C to 90°C at a heating rate of 2°C/min by means of a calorimeter (Model DSC-100, Seiko Co., Ltd.).

### Results and Discussion

#### 3.1 Determination of the Size and Structure of E5531 Particles

Table 1 shows the weight averaged diameter of E5531 vesicle \( \left( D_w \right) \) and the volume of inner space of the particles per mole of E5531. The trapped volumes of small unilamellar vesicles (diameter 20-50 nm), large unilamellar vesicles (diameter: 200-1000 nm) and multilamellar vesicles (diameter: 400-3500 nm) of phosphatidylcholine have been estimated to be 0.2-0.5, 3-4 and 7-10 liter/mol, respectively (13). In Table 1, the trapped volume of E5531 particles were in the range of 0.43-0.49 liter/mole, indicating that the E5531 particles dispersed by sonication were present in the form of small unilamellar vesicles, and this is in consistent with the particle size estimated from DLS experiments.

#### 3.2 Retention and Fusion of E5531 Vesicles during Freeze-Drying

The \( D_w \) and retention of percentage \( (R\%) \) values before and after freeze-drying in the presence of various additives are shown in Table 1. The addition of disaccharides, i.e., lactose and maltose, results in a high \( R\% \) (approximately 88%), and no discernible charge in the \( D_w \) values. The use of monosaccharides results in a much lower \( R\% \) (approximately 28%), but the vesicle size is not increased. Polyalcohols gave a far lower \( R\% \), especially mannitol (0%). A substantial increase in particle size was detected in systems in which inositol and mannitol were added. In order to obtain direct information on the fusion of the particles, the values of energy transfer efficiency \( (E) \) before and after freeze-drying in the presence of glucose, lactose and mannitol were investigated, and the results are shown in Table 2. In the case of glucose and lactose, the value of \( E \) before and after freeze-drying was essentially the same. However, with mannitol, this value decreased by approxi-

### Table 1  Trapped Volume (before Freeze-Drying), \( D_w \) and \( R\% \) before and after Freeze-Drying in the Presence of Various Saccharides and Polyalcohols.

| Additives   | Trapped volume (liter/mol) | \( D_w \) (nm) & \( R\% \) |
|------------|---------------------------|----------------|----------|
|            | Before        | After        | Before  | After  |
| Disaccharide |             |              |         |         |
| Lactose    | 0.45         | 49 ± 6       | 52 ± 5  | 90 ± 5 |
| Maltose    | 0.47         | 53 ± 7       | 54 ± 8  | 86 ± 3 |
| Monosaccharide |         |              |         |         |
| Glucose    | 0.43         | 56 ± 5       | 57 ± 3  | 29 ± 4 |
| Galactose  | 0.44         | 50 ± 8       | 53 ± 6  | 28 ± 7 |
| Polyalcohol |             |              |         |         |
| Inositol   | 0.48         | 54 ± 6       | 1529 ± 285 | 13 ± 2 |
| Mannitol   | 0.49         | 49 ± 7       | 1843 ± 453 | 0       |

### Table 2  Values of Transfer Efficiency \( (E) \) of Various Additive Systems before and after Freeze-Drying.

<table>
<thead>
<tr>
<th>Additives</th>
<th>( E ) Before</th>
<th>( E ) After</th>
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</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>0.45</td>
<td>0.43</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.42</td>
<td>0.41</td>
</tr>
<tr>
<td>Mannitol</td>
<td>0.44</td>
<td>0.20</td>
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</tbody>
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mately 50%, indicating that all of the input particles participate in the fusion events. These findings are consistent with the results presented in Table 1. Inositol and mannitol show very poor cryoprotective ability compared to the other additives. This can be attributed to the formation of an eutectic mixture between these additives and water in the frozen state (7). The fusion observed in these systems is probably caused by mechanical breakage during the crystal formation of ice and these compounds (7).

3.3 DSC Studies

The effect of various additives on the thermal properties of E5531 particles was investigated. The DSC heating thermograms for each system are in Fig. 2. The profile from 0°C to 90°C for the system with no additive shows an endothermic peak (gel to liquid-crystalline phase transition) at around 30.2°C (Fig. 2, curve (a)). The first heating thermogram of the system with lactose shows an endothermic transition peak at around 55.3°C (Fig. 2, curve (b)). On the second scan, the peak shifts to a lower temperature at around 15.4°C (Fig. 2, curve (c)). The temperature on the third scan (Fig. 2, curve (d)) is almost the same as the second one. These results indicate that for a maximal effect, it is necessary for the lactose to interact with E5531 in the liquid-crystalline phase.

The curves (a), (b), and (c) in Fig. 3 show the thermograms for the second scan of the system with added glucose, lactose and mannitol, respectively. The thermogram for the monosaccharide system exhibits higher transition temperature than that for the disaccharide system. The systems with added mannitol shows the highest transition temperature, but the peak temperatures obtained in several repeated scans varied rather widely in the range of 70-80°C.

Hydrogen-bonding between the saccharides and E5531 molecules would be indispensable for the stabilization of E5531 vesicles during freeze-drying like phosphatidylcholine system (11). Hydrogen-bonding significantly reduces the gel-to liquid-crystalline phase transition temperature of anhydrous E5531. Like dipalmitoylphosphatidylcholine (11), in order to achieve the maximal effect on E5531, saccharides must interact with E5531 at a temperature above the phase

![Table 3](image)

<table>
<thead>
<tr>
<th>Additive</th>
<th>Transition Temperature (°C)</th>
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<tbody>
<tr>
<td>First scan</td>
<td>55.3</td>
</tr>
<tr>
<td>Second scan</td>
<td>15.4</td>
</tr>
<tr>
<td>Third scan</td>
<td>16.3</td>
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transition temperature (see Fig. 1). In fact, the transition temperature decreases drastically once the system passes through the liquid-crystalline phase, as shown in Table 3. The phase transition temperature of the second scan can be regarded as representing the thermal property of E5531 when the maximal interaction of saccharides is achieved. According to this interpretation, the DSC heating thermogram on the second scan (Fig. 2) can be used to compare the effect of each saccharide on E5531.

The difference between monosaccharides and disaccharides in their cryoprotective effects would be attributed to the difference in their interaction with the E5531 head group. In spite of the fact that monosaccharides completely prevent the fusion of E5531 particles during freeze-drying, they cause far less retention of entrapped calcein compared with the case of disaccharides.

In conclusion, cryoprotective ability is higher when the interaction between saccharides and E5531 head group is stronger.

Reference