Appropriate selection of an aggregation inhibitor of fine particles used for inhalation prepared by emulsion solvent diffusion

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\textbf{ABSTRACT}

\textbf{Context:} Dry powder inhaler (DPI) formulations have been developed to deliver large amounts of drugs to the lungs.

\textbf{Objective:} Fine particles of a poorly water-soluble drug, the model drug ONO-2921, were prepared by the emulsion solvent diffusion (ESD) method for use in a DPI.

\textbf{Methods:} The effects of additives on the fine particle formation of ONO-2921 were estimated when droplets of an ethanolic drug solution were dispersed into aqueous media containing various additives. Subsequently, the suspensions were freeze-dried to create powdered samples to estimate the inhalation properties using a twin impinger and an Andersen cascade impactor.

\textbf{Results:} This simple ESD method produced submicron-sized ONO-2921 particles (approximately 600 nm) in combination with suitable additives. In addition, the freeze-dried powder produced using additives exhibited superior in vitro inhalation properties. Among these methods, the freeze-dried powder produced with 0.50% weight/volume one type of polyvinyl alcohol (PVA-205) displayed the most efficient features in the fine particle fraction (FPF). These results could be explained by the stabilization of the ONO-2921 suspension by PVA-205, indicating that PVA-205 acts as an aggregation inhibitor of fine particles.

\textbf{Conclusions:} The ESD method, in combination with appropriate types and amounts of additives, may be useful for preparing a DPI suitable for delivering drugs directly to the lungs without the assistance of carrier particles.

\textbf{Introduction}

Pulmonary drug delivery is commonly used to treat lung diseases. In addition, the pulmonary administration of drugs may be beneficial for other diseases and conditions. These indications can be divided into the topical treatment of the respiratory tract and the treatment of systemic diseases. The lungs represent an ideal target for drug delivery because of their ability to avoid first-pass metabolism, more rapid onset of therapeutic action, high local drug concentrations within the lungs, and minimization of systemic absorption of drugs, resulting in diminished side effects\textsuperscript{1-2}. Recently, pulmonary drug delivery research has focused on the systemic delivery of poorly absorbable drugs and local delivery of antiasthmatic drugs because the lungs have a large inner surface that occupies an area of approximately 43–102 m\textsuperscript{2}, a thin absorption barrier, and low enzymatic metabolic activity\textsuperscript{3-6}. Pulmonary drug delivery systems comprise four main categories: nebulizers, pressurized metered-dose inhalers (pMDIs), soft-mist inhalers and dry powder inhalers (DPIs). A further differentiation of each system is determined by metering, means of dispersion and design\textsuperscript{7}. Nebulization is cumbersome, expensive, time-consuming and frequently unnecessary even during severe bronchial obstruction\textsuperscript{8}. The application of pMDIs is limited to a small number of drugs because they require chemical and physical stability in the propellant during the storage of the active pharmaceutical ingredient (API)\textsuperscript{9,10}. Although soft-mist inhalers do not contain propellants, it was not breath-actuated\textsuperscript{11}. DPIs, involving the dispersion of powders into aerosols via an inhaler device, are particularly attractive because dry powders generally have greater chemical stability than the liquids used in atomizers\textsuperscript{12,13}. To deliver drugs to the lungs using DPIs, the aerodynamic diameter of drug particles must be within a suitable range (0.5–7 \textmu m)\textsuperscript{14-16}.

The most common method for achieving size reduction is the disruption of larger particles using break-down techniques\textsuperscript{17}, such as jet-milling, milling in a ball-mill, wet-bead milling or high-pressure homogenization\textsuperscript{18}. However, these methods have several disadvantages that result from the mechanical disruption process. The micronization process using mills has disadvantages, one of which is that the high-energy input can alter the surface properties of the particle to create a thermodynamically activated surface\textsuperscript{19-21}. The micronized particles may be highly heterogeneous, charged and cohesive, resulting in a decrement in the product performance. To overcome these problems micronized drugs have been frequently adhered to larger carrier particles, such as lactose and mannitol\textsuperscript{22-24}. The drugs are separated from carrier particles in the airways because of their different entrainment and deposition characteristics\textsuperscript{25}. The strong interactions between carriers and drug particles reduce the separation or dose uniformity; however, this method is extensively employed to produce DPI formulations. High drug-carrier adhesive forces and the consequent inadequate

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\textsuperscript{a} Supplemental data for this article can be accessed here.

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separation (drug redispersion) are among the most important explanations for the poor drug deposition efficiency of DPI formulations\(^{26,27}\).

We prepared drug nanocrystals of a poorly water-soluble drug using the emulsion solvent diffusion (ESD) method\(^{28}\). The ESD method was developed based on the spherical crystallization technique\(^{29}\), which was first successfully applied to prepare controlled release microspheres with acrylic polymers\(^{30}\). This technique has been subsequently applied to the preparation of spherical drug aggregates of several hundred micrometers for improving flowability and compressibility\(^{29,31,32}\). The ESD method has also been applied to prepare nanospheres using biodegradable polymers for encapsulated APIs to be used as drug carriers\(^{33–35}\). Such nanospheres were characterized by the sustained release of encapsulated APIs\(^{36}\). The use of nanospheres as carriers has also been applied in pulmonary drug delivery\(^{37}\). The nanocrystals were directly crystallized using APIs to enhance the dissolution rate\(^{28}\). Another potential application of nanocrystals can be the improvement of inhalation properties for pulmonary delivery\(^{18}\). In this study, we attempted drug nanocrystal preparation by the ESD method without carriers for use in DPIs. The additives used in the ESD method were screened and optimized. The inhalation properties of the powder comprising nanocrystals were improved. In the case of the delivery of large amounts of APIs to the lungs, this approach is promising as particles primarily consist of APIs without carriers, which has not been previously reported. This study aimed to develop submicron-sized drug particles for DPIs using the ESD method. The N-type Ca\(^{2+}\) channel blocker ONO-2921, originally developed for the treatment of neuropathic pain\(^{39,40}\), was used as the model drug substance. ONO-2921 has been modified for pulmonary drug delivery with the aim of systemic absorption. Because a high blood concentration is required, ONO-2921 must have a high drug content (>80%) and inhalation efficiency (>40%) in DPIs. Particle aerosolization may be affected by the interaction of ONO-2921 and additives used in the water phase of the ESD method. To improve the physicochemical and inhalation properties of ONO-2921, additives were screened and the concentrations of these additives were optimized. The change in crystalline form may affect inhalation properties and stability. The mechanisms of submicron-sized particle formation were also considered.

**Materials and methods**

**Materials**

As a poorly water-soluble drug, tert-butyl (4R)-4-(((1R)-2-[[1-benzylpiperidin-4-yl]amino]-1-[[cyclohexylmethyl]thio]methyl)-2-oxoethyl-]amino)carbonyl]-1,3-thiazolidine-3-carboxylate (ONO-2921) (Figure S1) was used as a model drug substance (molecular weight: 604.87, solubility in water: 3.3 µg/mL). This drug substance undergoes first-pass metabolism and has been developed for pulmonary delivery for systemic action.

Four different types of polyvinyl alcohols (PVA-105, polymerization degree = 500, and hydrolyzation degree = 98.5%; PVA-205, polymerization degree = 500, and hydrolyzation degree = 88.0%; PVA-405, polymerization degree = 500, and hydrolyzation degree = 80.0%; and PVA-L-8, polymerization degree = 500, and hydrolyzation degree = 71.0%) (Kuraray Co., Ltd., Tokyo, Japan), Pluronic F-88 (Adeka Co., Ltd., Tokyo, Japan), Tween 20 (Kishida Chemical Co., Ltd., Osaka, Japan), hydroxypropyl cellulose (HPC-L, Nippon Soda Co., Ltd., Tokyo, Japan), mannitol (Mitsubishi Shoji Foodtech Co., Ltd., Tokyo, Japan), and four different types of cyclodextrins (CDs; α-, β-, methyl-β-, and hydroxypropyl-β-CDS) (Ensuiko Sugar Refining Co., Ltd., Tokyo, Japan) were used as additives in the water phase. All other chemicals and solvents were of reagent grade.

**Preparation of ONO-2921 particles**

ONO-2921 particles were prepared by the ESD method in water. ONO-2921 (200 mg), dissolved in ethanol (5 mL), was added to 100 mL of water with additives at a rate of 2.0 mL/min while the solution was stirred at 400 rpm using a propeller-type agitator with three blades (Heidon 600 G; Shinto Scientific Co., Ltd., Tokyo, Japan) at room temperature. Different concentrations of additives [0–2.00%, all weight/volume (w/v)] were used in the water phase of the emulsion. The resultant dispersion was centrifuged for 10 min at 20,000 rpm at 4 °C using a high-speed refrigerated centrifuge (Kubota 7800; Kubota Co., Ltd., Tokyo, Japan) for the separation of the particles and additives. The obtained residue was resuspended in distilled water by sonication. This process was repeated, and the resultant dispersion was subjected to freeze-drying (FD-81TS; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at –120 °C for 72 h.

**Particle size**

The particle size in suspension of the before freeze-dried samples was determined by dynamic light scattering method with a photon correlation spectroscopy (PCS) using Zetasizer 3000HS (Malvern Instruments, Worcestershire, United Kingdom). The measured parameters by PCS were the average particle size diameter. The number of samples is one because of screening additives.

The particle size of the freeze-dried powder was measured by laser diffraction under dry conditions (LDSA-2400A; Tohniichi Computer Applications Co., Ltd., Tokyo, Japan). The deconvolution method was based on Fraunhofer approximation. The optical properties (e.g. refractive index) were not used in this deconvolution. The dispersion of powder samples at an air pressure of 0.294 MPa was evaluated using an LDSA-2400A. The agglomeration was evaluated based on volume distribution span, a measure of the distribution width relative to the median diameter\(^{41,42}\). The span was calculated using the following equation: Span = (D\(_{90}\) − D\(_{10}\))/D\(_{50}\).

**Scanning electron microscopy**

The shape and surface topology of the powders were observed via scanning electron microscopy (SEM) (JSM-330A; Jeol Ltd., Tokyo, Japan). Prior to examination, the samples were mounted onto metal stubs using double-sided adhesive tape, and a thin layer of gold was added under vacuum. The scanning electron microscope was operated at an acceleration voltage of 15 kV.

**Differential scanning calorimetry**

A differential scanning calorimeter (DSC, DSC-6200, Seiko Instruments Inc., Chiba, Japan) with a liquid nitrogen cooling system was used. In the DSC evaluation, 2–3 mg of sample powder was placed in an aluminum pan and measured at a scanning rate of 10 °C/min over the range of 20 °C–220 °C.

**Powder X-ray diffraction**

Powder X-ray diffraction (XRD) analysis was performed using a SmartLab X-ray diffractometer (Rigaku Co., Ltd., Tokyo, Japan). The scanning rate was 10°/min over a 2θ range of 5°–40°. The percent
crystallinity was calculated using the formula of the Hermans–Weidinger method as follows:

\[
\text{The percent crystallinity} = \frac{\text{The intensity derived from the crystal part}}{\text{The intensity derived from the crystal part} + \text{The intensity derived from the amorphous part}} \times 100
\]

The intensities derived from the crystal and amorphous parts were manually separated from the PXRD pattern. Each sample was separated into crystal and amorphous parts under the same conditions.

Measurement of the inhalation properties of ONO-2921 powders

Powder aliquots (6.5 mg) were loaded into size 2 gelatin capsules and placed in the inhalation device. The inhalation properties were evaluated using a twin impinger and an Andersen cascade impactor. A Spinhaler (Astellas Pharma Inc., Tokyo, Japan) was used as an inhalation device. Results were indicated as a percentage of the recovery dose, with total API recovery corresponding to 100%, and drug content was not multiplied.

Twin impinger

Methanol was introduced into the upper (7 mL) and lower (30 mL) stages of a twin impinger. The airflow was adjusted through the apparatus to 60 ± 5 L/min, as measured at the inlet to the throat. The system was subjected to vacuuming for 5 s. After actuation, the remaining powder in the capsule, device, and throat and the deposited particles on the upper and lower stage of the twin impinger, were rinsed with appropriate amounts (25–50 mL) of methanol. The amount of ONO-2921 was measured via high-performance liquid chromatography (HPLC) as described in the section on HPLC analysis.

Andersen cascade impactor

The Andersen cascade impactor (AN-200 system; Tokyo Dylec Corp., Tokyo, Japan) was composed of a throat, stages 0–7, and filter. The pre-separator was not placed in this study. The collection plates were subsequently immersed in 2% weight/weight (w/w) silicon in hexane solution, and the solvent was evaporated to form a thin film of silicon on the surface of the plates to prevent particle bounce. The Andersen cascade impactor was then assembled, and the flow rate through the impactor was set to 28.3 L/min. The capsules were loaded and aerosolized as described previously, and each deposition experiment involved the aerosolization of one capsule. The remaining powders in the capsule, device, throat, and each stage of the Andersen cascade impactor were rinsed with methanol, and methanol was added to produce a final volume of 25 mL. The mass of ONO-2921 deposited in each part of the Andersen cascade impactor was determined using HPLC as described in the section on HPLC analysis. The concentration of these aqueous solutions was fixed at 1.00% w/v in this screening section. Submicron-sized ONO-2921 particles (approximately 600 nm) formed in the presence of PVA-205 in the aqueous phase, although ONO-2921 particles with sizes of 3533.8 nm formed in the absence of additives in the aqueous phase before freeze-drying. This was attributed to crystal growth in the absence of the additives as an aggregation inhibitor. The ONO-2921 particles prepared with other additives were 300–900 nm. Compared to the sample prepared without additives, the ONO-2921 particles prepared with other additives were no major difference in size to the particles prepared with PVA-205 before freeze-drying. (Table S1). Both the additives prevented coalescence and agglomeration of the emulsions or particles and modified the surface of nanospheres in the previous studies. The concentration of these aqueous solutions was fixed at 1.00% w/v in this screening section. Submicron-sized ONO-2921 particles (approximately 600 nm) formed in the presence of PVA-205 in the aqueous phase, although ONO-2921 particles with sizes of 3533.8 nm formed in the absence of additives in the aqueous phase before freeze-drying. This was attributed to crystal growth in the absence of the additives as an aggregation inhibitor. The ONO-2921 particles prepared with other additives were 300–900 nm. Compared to the sample prepared without additives, the ONO-2921 particles prepared with other additives were no major difference in size to the particles prepared with PVA-205 before freeze-drying. (Table S1). Both the additives prevented coalescence and agglomeration of the emulsions or particles and modified the surface of nanospheres in the previous studies. The concentration of these aqueous solutions was fixed at 1.00% w/v in this screening section. Submicron-sized ONO-2921 particles (approximately 600 nm) formed in the presence of PVA-205 in the aqueous phase, although ONO-2921 particles with sizes of 3533.8 nm formed in the absence of additives in the aqueous phase before freeze-drying. This was attributed to crystal growth in the absence of the additives as an aggregation inhibitor. The ONO-2921 particles prepared with other additives were 300–900 nm. Compared to the sample prepared without additives, the ONO-2921 particles prepared with other additives were no major difference in size to the particles prepared with PVA-205 before freeze-drying. (Table S1). Both the additives prevented coalescence and agglomeration of the emulsions or particles and modified the surface of nanospheres in the previous studies.

Screening organic solvent for preparation of particles

Particle size

More than 10 additives (e.g., PVA, surfactants, sugar and CDs), used to prevent the aggregation and fusion of the initial emulsion droplets in the aqueous phase, were screened in this study. These additives were used to prepare nanospheres or nanoparticles or to modify the surface of nanospheres in the previous studies. The concentration of these aqueous solutions was fixed at 1.00% w/v in this screening section. Submicron-sized ONO-2921 particles (approximately 600 nm) formed in the presence of PVA-205 in the aqueous phase, although ONO-2921 particles with sizes of 3533.8 nm formed in the absence of additives in the aqueous phase before freeze-drying. This was attributed to crystal growth in the absence of the additives as an aggregation inhibitor. The ONO-2921 particles prepared with other additives were 300–900 nm. Compared to the sample prepared without additives, the ONO-2921 particles prepared with other additives were no major difference in size to the particles prepared with PVA-205 before freeze-drying. (Table S1). Both the additives prevented coalescence and agglomeration of the emulsions or particles and modified the surface of nanospheres in the previous studies. The concentration of these aqueous solutions was fixed at 1.00% w/v in this screening section. Submicron-sized ONO-2921 particles (approximately 600 nm) formed in the presence of PVA-205 in the aqueous phase, although ONO-2921 particles with sizes of 3533.8 nm formed in the absence of additives in the aqueous phase before freeze-drying. This was attributed to crystal growth in the absence of the additives as an aggregation inhibitor. The ONO-2921 particles prepared with other additives were 300–900 nm. Compared to the sample prepared without additives, the ONO-2921 particles prepared with other additives were no major difference in size to the particles prepared with PVA-205 before freeze-drying. (Table S1). Both the additives prevented coalescence and agglomeration of the emulsions or particles and modified the surface of nanospheres in the previous studies. The concentration of these aqueous solutions was fixed at 1.00% w/v in this screening section. Submicron-sized ONO-2921 particles (approximately 600 nm) formed in the presence of PVA-205 in the aqueous phase, although ONO-2921 particles with sizes of 3533.8 nm formed in the absence of additives in the aqueous phase before freeze-drying. This was attributed to crystal growth in the absence of the additives as an aggregation inhibitor. The ONO-2921 particles prepared with other additives were 300–900 nm. Compared to the sample prepared without additives, the ONO-2921 particles prepared with other additives were no major difference in size to the particles prepared with PVA-205 before freeze-drying. (Table S1). Both the additives prevented coalescence and agglomeration of the emulsions or particles and modified the surface of nanospheres in the previous studies. The concentration of these aqueous solutions was fixed at 1.00% w/v in this screening section. Submicron-sized ONO-2921 particles (approximately 600 nm) formed in the presence of PVA-205 in the aqueous phase, although ONO-2921 particles with sizes of 3533.8 nm formed in the absence of additives in the aqueous phase before freeze-drying. This was attributed to crystal growth in the absence of the additives as an aggregation inhibitor. The ONO-2921 particles prepared with other additives were 300–900 nm. Compared to the sample prepared without additives, the ONO-2921 particles prepared with other additives were no major difference in size to the particles prepared with PVA-205 before freeze-drying. (Table S1). Both the additives prevented coalescence and agglomeration of the emulsions or particles and modified the surface of nanospheres in the previous studies.
the powder properties of samples prepared with methyl-β-CD and hydroxypropyl-β-CD were not evaluated. Table 1 summarizes the drug content of ONO-2921 particles and the particle size of freeze-dried samples dispersed in air using the LDSA-2400A that were processed with different additives using the 1.00% w/v ESD method. The drug content of the freeze-dried samples was >80%, indicating that the obtained particles were mainly composed of ONO-2921. The median particle size ($D_{50}$) of the freeze-dried samples, excluding those processed with methyl-β-CD and hydroxypropyl-β-CD, ranged from 5.0 to 13.7 µm, and the particles were larger than those formed by raw ONO-2921 ($D_{50}$ of raw ONO-2921: 4.2 µm). The result of span in the sample processed with mannitol was 2.1 and the value was wider than the samples processed with other additives. This is attributed to the fact that sugars have been found to be very effective in inhibiting nanoparticle aggregation during freeze-drying. On the other hand, the following case was also reported that mannitol actually accelerated aggregation during freeze-drying. Hollow particle preparation or surface modification was proposed to improve powder aerosolization.

Table 1. Drug content of ONO-2921 and particle size of freeze-dried samples of ESD-processed suspensions using 1.00% w/v of different additives (mean ± SD, n = 3).

| Additive for ESD processing | Drug content (%) | $D_{10}$ | $D_{50}$ | $D_{90}$ | Span |
|----------------------------|------------------|---------|---------|---------|------|
| PVA-105                    | 93.0 ± 0.6       | 2.4 ± 0.0 | 5.7 ± 0.0 | 9.4 ± 0.1 | 1.23 |
| PVA-205                    | 83.6 ± 0.8       | 3.5 ± 0.0 | 8.9 ± 0.2 | 17.3 ± 0.3 | 1.55 |
| PVA-405                    | 87.7 ± 0.4       | 3.8 ± 0.2 | 8.0 ± 0.5 | 13.6 ± 1.3 | 1.23 |
| PVA-L                      | 88.4 ± 1.3       | 4.4 ± 0.5 | 13.7 ± 3.1 | 25.8 ± 3.5 | 1.56 |
| Pluronic F-88              | 92.8 ± 0.3       | 2.0 ± 0.1 | 5.4 ± 0.1 | 9.6 ± 0.4 | 1.41 |
| Tween20                    | 91.2 ± 0.3       | 2.2 ± 0.2 | 6.0 ± 0.4 | 10.6 ± 1.1 | 1.40 |
| HPC-L                      | 96.7 ± 0.4       | 3.0 ± 0.3 | 7.3 ± 0.3 | 12.7 ± 0.4 | 1.33 |
| Mannitol                   | 95.8 ± 1.2       | 1.7 ± 0.1 | 5.3 ± 0.5 | 12.8 ± 5.0 | 2.09 |
| α-CD                       | 96.7 ± 0.6       | 1.8 ± 0.0 | 5.0 ± 0.1 | 8.8 ± 0.1 | 1.40 |
| β-CD                       | 96.9 ± 0.8       | 1.9 ± 0.0 | 5.0 ± 0.0 | 8.7 ± 0.0 | 1.36 |
| Methyl-β-CD                | N/A              | N/A     | N/A     | N/A     | N/A |
| Hydroxypropyl-β-CD         | N/A              | N/A     | N/A     | N/A     | N/A |

Although the freeze-dried samples processed with CDs displayed smaller particle sizes than the other samples when dispersed in air (Table 1), <40% of the freeze-dried samples processed with CDs reached the stage 2 filter, and CDs were not as effective as the additives for producing inhalable particles.

**Particle morphology**

SEM images of the freeze-dried samples processed with PVA-205, Pluronic F-88 and HPC-L, which exhibited better inhalation properties, are shown in Figure 2. The raw ONO-2921 particles consisted of spherical, aggregated particles that were densely packed with the primary particles. The freeze-dried samples processed with PVA-205, PVA-405 and PVA-L also exhibited spherical aggregation (Figure 2(b) and Figure S3 (b) and (c)). The freeze-dried samples processed with Pluronic F-88, HPC-L and the other additives exhibited fibrous aggregation (Figure 2(c,d) and Figure S3 (a, d–g)). The content of the additive was calculated by subtracting the drug content from the freeze-dried samples, although each additive was not directly measured in the freeze-dried samples. The freeze-dried samples processed with PVA-105, Pluronic F-88, Tween 20, HPC-L, mannitol, α-CD, and β-CD, demonstrated approximately 95% of drug content and contained approximately 5% additives. The relatively low additive content could be indicative of little interaction, and the freeze-dried samples exhibited no steric fibrous aggregation. Hollow particle preparation or surface modification was proposed to improve powder aerosolization.

The freeze-dried samples processed with PVA-205, Pluronic F-88 and HPC-L exhibited better aerosolization than other samples. The freeze-dried samples processed with PVA-205, PVA-405 and PVA-L exhibited spherical aggregates. Particle size of the sample processed with PVA-405 was smaller than two different samples. The sample processed with PVA-405 could be dispersed during *in vitro* inhalation and the dispersed particles adhered to the capsule inside. This was caused by the high content of PVA-405 in the sample. It means that particle surface of samples including ONO-2921 with more than 10% may be covered with PVA-405, hydrophilic polymer. The dispersed particles of the sample adhered to the capsule because hydrophilic interaction between capsule surface and particle surface may arise. Particles size dispersed in air of the sample processed with PVA-L8 was bigger than two different particle sizes.
Figure 1. Effect of additives dissolved in the outer phase on the in vitro inhalation properties of the freeze-dried samples determined using a twin impinger: (a) raw ONO-2921 and the samples processed with PVAs and (b) samples processed with surfactants, HPC-L, mannitol and CDs (mean ± SD, n = 3).

Figure 2. SEM images of freeze-dried samples prepared with different types of additives: (a) raw ONO-2921; (b) processed with PVA-205; (c) processed with Pluronic F-88 and (d) processed with HPC-L.
samples. The sample processed with PVA-L8 would not be dispersed sufficiently during in vitro inhalation, as a result mostly deposited on the stage 1 fraction of twin impinger. The balance of dispersibility would be important for fine inhalation property from the result of PVA-405 and PVA-L8. It was proposed that the samples processed with PVA-205 were better result than two spherical aggregated samples due to the balance of dispersibility during in vitro inhalation.

The sample processed with mannitol was wider particle size distribution (span: 2.1), and deposited to the device, the throat and the stage 1 fraction widely. The drug contents in the samples were 97%, while the content of CDs in the samples was low. The particle surface may not be improved by CDs, while the particle surface remained hydrophobicity. As hydrophobic particles generally adhered to the device and the throat, the samples were dispersed too much from the result of particle size and adhered to the device and the throat. There were no major difference among the samples processed with PVA-105, Pluronic F-88, tween20 and HPC-L in terms of particle size. Pluronic F-88 sample was slightly deposited the stage 2 fraction better than the samples processed with PVA-105 and tween20. During in vitro inhalation, if the sample was dispersed too much, the particles adhered to the capsule, the device or the throat. If the sample was not dispersed sufficiently, the aggregation dropped to the stage 1 fraction. We proposed the sample need adequate dispersion. The drug content of HPC-L sample was higher than those four samples and HPC-L sample was dispersed during inhalation easier than those samples. The sample processed with HPC-L deposited the stage 2 fraction better than the samples processed with PVA-105 and tween20.

Optimization of additive concentrations

Inhalation properties

Further concentration optimization of the three additives, PVA-205, Pluronic F-88, tween20 and HPC-L in terms of particle size. Pluronic F-88 sample was slightly smaller particle size of D10 and D50 than three different samples. The sample processed with Pluronic F-88 deposited the stage 2 fraction better than the samples processed with PVA-105 and tween20. During in vitro inhalation, if the sample was dispersed too much, the particles adhered to the capsule, the device or the throat. If the sample was not dispersed sufficiently, the aggregation dropped to the stage 1 fraction. We proposed the sample need adequate dispersion. The drug content of HPC-L sample was higher than those four samples and HPC-L sample was dispersed during inhalation easier than those samples. The sample processed with HPC-L deposited the stage 2 fraction better than the samples processed with PVA-105 and tween20.

Table 2. Drug content of ONO-2921 and particle size of freeze-dried samples of ESD-processed suspensions using different concentrations of additives (mean±SD, n = 3) processed with (a) PVA-205; (b) pluronic F-88 and (c) HPC-L.

| Additives concentration (%) | Drug content (%) | Particle size (µm) |
|----------------------------|-----------------|-------------------|
|                           |                 | D10   | D50   | D90   | Span |
| (a) PVA-205 concentration (%) |                 |       |       |       |      |
| 0                          | 99.1 ± 5.9        | 2.6 ± 0.3 | 7.2 ± 0.6 | 13.9 ± 2.3 | 1.57 |
| 0.10                       | 92.2 ± 0.7        | 3.0 ± 0.2 | 7.5 ± 0.3 | 13.2 ± 0.7 | 1.36 |
| 0.25                       | 88.3 ± 0.6        | 2.9 ± 0.1 | 6.7 ± 0.3 | 12.7 ± 2.0 | 1.46 |
| 0.50                       | 82.6 ± 0.2        | 2.9 ± 0.1 | 6.9 ± 0.0 | 12.4 ± 0.1 | 1.38 |
| 1.00                       | 83.6 ± 0.8        | 3.5 ± 0.0 | 8.9 ± 0.2 | 17.3 ± 0.3 | 1.55 |
| (b) Pluronic F-88 concentration (%) |                 |       |       |       |      |
| 0                          | 99.1 ± 5.9        | 2.6 ± 0.3 | 7.2 ± 0.6 | 13.9 ± 2.3 | 1.57 |
| 0.01                       | 93.1 ± 1.2        | 2.1 ± 0.1 | 5.7 ± 0.2 | 9.6 ± 0.6  | 1.32 |
| 0.05                       | 96.4 ± 2.2        | 2.1 ± 0.0 | 5.8 ± 0.1 | 9.7 ± 0.3  | 1.31 |
| 0.10                       | 92.8 ± 0.3        | 1.7 ± 0.0 | 5.3 ± 0.2 | 9.5 ± 0.8  | 1.47 |
| 0.25                       | 91.2 ± 0.4        | 1.8 ± 0.1 | 5.2 ± 0.2 | 9.1 ± 0.3  | 1.40 |
| 0.50                       | 89.3 ± 0.1        | 1.7 ± 0.0 | 5.0 ± 0.1 | 8.9 ± 0.1  | 1.44 |
| 1.00                       | 92.8 ± 0.3        | 2.0 ± 0.1 | 5.4 ± 0.1 | 9.6 ± 0.4  | 1.41 |
| (c) HPC-L concentration (%) |                 |       |       |       |      |
| 0                          | 99.1 ± 5.9        | 2.6 ± 0.3 | 7.2 ± 0.6 | 13.9 ± 2.3 | 1.57 |
| 0.50                       | 97.3 ± 0.2        | 3.4 ± 0.1 | 9.0 ± 0.7 | 16.5 ± 2.3 | 1.46 |
| 1.00                       | 96.7 ± 0.4        | 3.0 ± 0.3 | 7.2 ± 0.3 | 12.7 ± 0.3 | 1.35 |
| 2.00                       | 94.2 ± 0.2        | 2.6 ± 0.3 | 6.4 ± 0.5 | 11.5 ± 0.9 | 1.39 |

Particle size and morphology

Freeze-dried samples processed with different concentrations of PVA-205 demonstrated little difference in dispersed particle sizes. Similar results were observed in freeze-dried samples processed with different concentrations of Pluronic F-88. Regarding the samples processed with different concentrations of HPC-L, the particle size and the drug content were small with increasing the concentration of HPC-L (Table 2). The content of HPC-L was calculated by subtracting the drug content and increased with increasing processed concentration. The content of HPC-L increased and the samples processed with increasing concentration of HPC-L were difficult to disperse during inhalation, although the particle size decreased. It was proposed that the samples processed with 1.00% w/v HPC-L showed better result in the in vitro inhalation properties.

Figure 4 shows a SEM image of the samples freeze-dried using different concentrations of PVA-205. The freeze-dried sample without PVA-205 (0% w/v PVA-205) exhibited spherical aggregated particles, and the samples freeze-dried with 0.10% and 0.25% PVA-205 (all w/v) exhibited fibrous aggregates. Conversely, the freeze-dried samples processed with 0.50% and 1.00% PVA-205 (all w/v), displayed porous, spherical and aggregated particles. The freeze-dried samples processed with different concentrations of Pluronic F-88 and HPC-L exhibited fibrous aggregations.

The freeze-dried samples with >90% drug content displayed fibrous aggregation. The freeze-dried samples with approximately 80% drug content contained >10% PVA-205. The contained PVA-205 interacted with each other, and these freeze-dried samples exhibited relatively steric spherical aggregation. In general, the particles with a spherical shape exhibited reduced internal friction and better dispersion performance in the air than particles with irregular or needle shapes due to the decreased contact area, which led to higher dispersion. These results indicated that the optimum concentration of PVA-205 was 0.50% w/v. This optimum
Figure 3. Changes in the in vitro inhalation properties of the freeze-dried samples with different concentrations of additives determined using a twin impinger (mean ± SD, n = 3): (a) processed with PVA-205; (b) processed with Pluronic F-88 and (c) processed with HPC-L.
concentration may be attributed to the formation of spherical aggregates, although there was little difference in the dispersed particle sizes.

**Andersen cascade impactor**

Freeze-dried samples processed with 0.50% w/v PVA-205 and 1.00% w/v HPC-L displayed enhanced inhalation properties upon evaluation with a twin impinger. To determine whether the aggregates in the two samples were separated into individual particles, the inhalation properties were measured using an Andersen cascade impactor, and the results are displayed in Figure 5. Each freeze-dried sample exhibited improved inhalation properties with an increased ED and FPF as well as improved results in evaluations using the twin impinger. The ED values of the freeze-dried samples processed with 0.50% w/v PVA-205 and 1.00% w/v HPC-L were 92.6% ± 4.1% and 92.2% ± 3.9%, respectively, and the FPF values were 47.7% ± 5.3% and 41.6% ± 1.5%, respectively (Table 3). The distribution percentages of each fraction of each sample displayed no major differences, and each sample was mainly deposited at stages 0–3 of the Andersen cascade impactor. The samples deposited at each stage of the Andersen cascade impactor were examined using SEM (Figure S4). As 10% of particles were not dispersed by inhalation process and deposited at stage 0, the sample deposited at stage 0 showed over 50 μm of aggregated particle. The samples deposited at stage 1–4 consisted of aggregated particles with diameters in 10 to several micrometers range depending on the stage. The sample size at stage 5 was around 1 μm (Figure S4) and the sample was considered as primary particle considering the result of around 600 nm in suspension (Table S1), although the percentages were small.

**Mechanisms of fine particle formation**

DPI formulations prepared by the ESD method have not been reported previously, although the preparation of fine particles prepared using the ESD method has been generally performed using polymers [e.g. poly(D,L-lactic-co-glycolic acid)]^{34,36,58}. These authors reported that the polymer particles usually exhibited a spherical shape. By contrast, ONO-2921 particles displayed a needle shape (Figure 4(b)). A schematic representation of the formation of ONO-2921 fine particles via the ESD method is illustrated in Figure 6.

To confirm that amorphous ONO-2921 was formed first (Figure 6(3)) and then crystallization and crystal growth of ONO-2921 occurred (Figure 6(4)), crystal properties of the following samples were evaluated: (a) samples freeze-dried immediately after ESD processing without additives; (b) samples freeze-dried after the suspension underwent ESD processing without additives, and the
mixture was centrifuged and resuspended twice, which was the "usual processing" method and (c) raw ONO-2921. The thermal behaviors of those samples in DSC profiles are shown in Figure 7. The crystal properties of those samples in PXRD are also shown in Figure 8. Sample (a) exhibited an exothermic peak at approximately 70°C (Figure 7(a)), indicated by a dotted circle), and the formation of amorphous ONO-2921 was confirmed. Conversely, sample (b) did not display an exothermic peak and ONO-2921 particles consisted of crystalline particles (Figure 7(b)). The per cent crystallinity of sample (a) was 7.7% (Figure 8(a)), while that of sample (b) was 41.1% (Figure 8(b)), and that of sample (c) was 75.8% (Figure 8(c)). These results suggested that, as shown in Figure 6, ESD-processed ONO-2921 particles were formed by crystallization in an amorphous form.

Table 3. ED and FPF determined using an Andersen cascade impactor for freeze-dried powders of ONO-2921 processed with either 0.50% w/v PVA-205 or 1.00% w/v HPC-L solutions (mean ± SD, n = 3).

| Additives  | ED (%)  | FPF (%) |
|------------|---------|---------|
| 0.5% PVA-205 | 92.6 ± 4.1 | 47.7 ± 5.3 |
| 1.0% HPC-L   | 92.2 ± 3.9 | 41.6 ± 1.5 |

Figure 5. In vitro inhalation properties of freeze-dried powders determined by an Andersen cascade impactor for ONO-2921 processed with either 0.50% w/v PVA-205 or 1.00% w/v HPC-L solutions (mean ± SD, n = 3).

Figure 6. Schematic representation of the formation of ONO-2921 fine particles in aqueous media prepared using the ESD method. (1) Spontaneous emulsification of oily solution in the aqueous phase; (2) formation of submicron-sized O/W emulsion adsorbed with additives; (3) formation of amorphous ONO-2921 particles with additives; (4) crystallization and crystal growing of ONO-2921 in the resultant particles.

Figure 7. DSC profiles of ONO-2921 (a) freeze-dried immediately after ESD processing without additives; (b) freeze-dried after the suspension was centrifuged and resuspended twice following ESD processing in the absence of additives and (c) raw ONO-2921.
Raw ONO-2921 exhibited a sharp endothermic peak at 158°C, corresponding to the melting point (Figure 7(c)), and samples (a) and (b) had the same melting point. The patterns of PXRD between sample (a), sample (b), and raw ONO-2921 were not different. The polymorphic change did not occur in the ESD process.

The other freeze-dried samples (e.g., the samples processed with PVA-205 and Pluronic F-88) did not exhibit an exothermic peak, and they had the same melting point as the raw ONO-2921 particles (Figure S5). The polymorphic change also did not occur in the process between raw ONO-2921 and freeze-drying of the ESD-processed samples. The experiments in this manuscript were conducted using the samples immediately after preparation. The stability study remains a future subject, although another group reported that the particles prepared with the ESD method showed no significant changes over 6 months accelerated testing59.

The ESD method was applied to produce ONO-2921 particles for inhalation. A nontoxic solvent, ethanol, was used as an organic phase solution, and ESD-processed particles, which were mainly composed of ONO-2921 and the crystalline form of raw ONO-2921, were obtained.

Conclusions
ONO-2921 particles with >80% drug content for use in DPIs were obtained via the ESD method under the majority of conditions. Although the ESD-processed freeze-dried samples were larger than raw ONO-2921 particles, the inhalation properties of the samples were improved. In particular, the samples processed with 0.50% w/ v PVA-205 exhibited enhanced in vitro inhalation properties. The mechanism of fine particle formation by the ESD method allowed the oil/water (O/W) emulsion of ONO-2921, and submicron-sized amorphous ONO-2921 crystals were formed when an ethanolic solution of ONO-2921 was added to the water phase with additives. To deliver large amounts of APIs to the lungs via a DPI, the preparation of fine API particles by the ESD method is a promising approach for enhancing the drug content and inhalation properties.

Disclosure statement
The author(s) declare(s) that they have no conflicts of interest to disclose.

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