A Preparation Technique of Fine S/O/W Emulsions for Protein Delivery Formed with Solid Fat

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Abstract: The improvement in the stability of solid-in-oil-in-water (S/O/W) emulsions, which are used as carriers for protein delivery, was investigated. For this purpose, emulsions were prepared using trimyristin, a solid fat, as the oil phase, and using the membrane emulsification and solvent evaporation methods. The samples were made into stable fine emulsions using polyvinyl alcohol, a hydrophilic polymer, as an emulsifier, and by controlling the particle size uniformly. The S/O/W emulsions prepared by this method showed almost no leakage of encapsulated proteins and exhibited controlled release in an intestinal environment.

Key words: S/O/W emulsions, solvent evaporation method, membrane emulsification, solid fat, protein delivery

1 Introduction

In recent years, biopharmaceuticals using proteins as active ingredients have attracted attention. Injections are a common method of administration for biopharmaceuticals; however, the disadvantage of this method is that it causes pain and side effects and requires patient compliance. These problems become more pronounced when repeated administrations are required. Therefore, there is a need for an administration method that is painless, safe, and less burdensome for the patient. The easiest way is to administer the drug orally and allow it to be absorbed through the intestinal mucosal membrane. However, oral administration of proteins often results in poor systemic absorption owing to the degrading action of gastric acid and digestive enzymes and because the hydrophilic surface of proteins has low mucosal permeability. To address this problem, the use of absorption enhancers, chemical modification of proteins, addition of mucoadhesives to improve mucosal retention, and particulate carriers for stable transport have been investigated.

Surfactant nanocoating technology has been used to endow protein surfaces with high affinity for cell membranes without chemical modification. The nanaassemblies prepared by this method can protect the protein from the external environment and retain its biological activity; the nanoparticles formed are lipophilic and have high affinity for cell membranes. The absorption of proteins from the intestine has been reported to improve following the use of these assemblies as carriers. Because these nanaassemblies are not water-dispersible, they are formulated as solid-in-oil-in-water (S/O/W) multiple emulsions in which they are dispersed in the oil phases of O/W emulsions. S/O/W emulsions have been used as oral insulin carriers targeting the intestinal mucosa for delivery, and improved absorption has been observed. However, these S/O/W emulsions exhibited leakage of the encapsulated protein gradually, making long-term storage difficult. To solve this problem, dry formulations that can emulsify upon contact with water have been developed by encapsulating oil droplets in polymer particles. An alternative system has been developed in which the oil phase of the emulsion is encapsulated in porous silica particles and the S/O/W emulsion is formed by spontaneous emulsification in the gastrointestinal tract. However, the inhibition of drug leakage from the S/O/W emulsion form has not yet been achieved.

In this study, we developed S/O/W emulsions with an oil phase that is solid at room temperature, in an attempt to suppress protein leakage from the core compartment. To avoid protein denaturation by heating, we used the solvent evaporation method: the organic solvent was evaporated after forming an O/W emulsion with an aqueous solution and a high volatility organic solvent in which solid fat was dissolved.
2 Materials and Methods

2.1 Materials

Polyoxyethylene sorbitan monolaurate (Tween 20), sodium cholate, polyvinyl alcohol (PVA; polymerization degree: approximately 500), and bovine serum albumin (BSA) were purchased from FUJIFILM Wako Pure Chemical Co. (Japan). Phuronic F-127 and porcine pancreas lipase (≥ 125 units/mg protein using olive oil) was purchased from Sigma-Aldrich (Tokyo, Japan). Sucrose erucic ester (ER-290) was provided by Mitsubishi Chemical Co. (Japan). Trimyristin was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Fluorescein isothiocyanate (FITC) was obtained from Dojin Chemical Co. (Japan). All chemicals were of reagent grade.

2.2 Preparation of S/O/W emulsions using solid fat

FITC-modified BSA was used as the model protein. Surfactant-nanocoated albumin was prepared as previously reported\(^1\). The method is briefly described as follows: 2 mL aqueous 0.1 mg/mL FITC-BSA solution and 4 mL cyclohexane containing 1.3 wt% ER-290 surfactant were stirred with a homogenizer (21,600 rpm, 5 min) to form a W/O emulsion. The emulsion was lyophilized for 2 days to form surfactant-coated albumin. The obtained complex of ER-290 and BSA was dispersed in 2 mL dichloromethane containing 8.5 wt% trimyristin to prepare a solid-in-oil (S/O) suspension. An S/O/W emulsion was prepared by mixing the S/O suspension with 18 mL of 1% emulsifier solution and stirring with a homogenizer (13,500 rpm, 1 min) (IKA, ULTRA-TURAX T25). The oil droplet size was then controlled by pressing (0.2–1.5 MPa at high-pressure nitrogen gas) the mixture through a hydrophilic shirasu porous glass (SPG) membrane (1.1 μm or 0.6 μm pore size). The external pressure type membrane emulsification device (Kiyomoto Iron Works) was used for the high-pressure treatment. Finally, the emulsion was stirred (100 rpm, 2 h) in a thermostatic bath controlled at 40°C to remove dichloromethane, resulting in a solidified oil-phase S/O/W. The liquid S/O/W emulsions used as a comparison was prepared with the exception of the solvent removal process using dichloromethane. Tween 20 and sodium cholate, which are commonly used as emulsifiers. Optical micrographs of the emulsions after membrane emulsification and solvent removal are shown in Fig. 1.

2.3 Observation of S/O/W emulsion morphology

The morphology of the S/O/W emulsions before and after the solvent removal process was examined using an optical microscope (LEICA, DMi1). Observation of the particle surface was performed by scanning electron microscopy (JEOL, JSM-7600F) after lyophilization of the solid fat particles collected by filtering the emulsion through a 0.2 μm pore filter.

2.4 Particle size distribution of emulsions

The particle size distribution of the prepared S/O/W droplets was measured using a laser diffraction particle size analyzer (Shimadzu, SALD-7100) after diluting the emulsions with ultrapure water.

2.5 Evaluation of protein incorporation in emulsions

The S/O/W emulsions were incubated at 4°C, 22°C, and 37°C. The aqueous dispersion was sampled at predetermined times and filtered to remove solid particles, and the FITC-BSA concentration in water was measured using a spectrophotometer (λ excit = 475 nm, λ emission = 515 nm) (Jasco, FP-8300ST).

2.6 Evaluation of protein release from emulsions

The S/O/W emulsion was mixed at a volume ratio of 1:9 with a phosphate buffered saline solution (PBS; pH 7.4) containing 2 mg/mL lipase. The mixture was prepared in a sample tube and shaken (100 rpm) in a thermostatic bath at 37°C. After sampling at predetermined times and filtering to remove solid particles, FITC-BSA concentrations were measured using a spectrophotometer. As a control, the release was evaluated in PBS without lipase.

3 Results and Discussion

3.1 Effect of hydrophilic surfactant on solid fat emulsion formation

To prepare S/O/W emulsions with solid fats as the oil phase, a solvent removal method was used. The solid fats were first dissolved in dichloromethane, and after the formation of the S/O/W emulsions, dichloromethane was removed to precipitate water-insoluble solid particles. Trimyristin, which is highly soluble in dichloromethane, was used as solid fat. The preparation of S/O/W emulsions was first attempted using two low-molecular-weight surfactants, Tween 20 and sodium cholate, which are commonly used as emulsifiers. Optical micrographs of the emulsions after membrane emulsification and solvent removal are shown in Fig. 1.

Here, the emulsion droplet size clearly reflects the diameter of the SPG membrane pores. However, the oil phase underwent coalescence upon solvent removal. When PVA, a hydrophilic polymer, was used as an emulsifier, the S/O/W emulsions retained the initial droplet shape. It has been reported that PVA forms a stable film at the oil-water interface and is stable against changes in pH and temperature\(^1\); these properties allow for the formation of stable emulsions. Conversely, when another polymeric emulsifier, Phuronic, was used, coalescence was observed after solvent removal. This suggests that PVA is suitable for the preparation of solid fat S/O/W emulsions via solvent removal. PVA was also used in the subsequent experiments.
3.2 Size control of S/O/W emulsions with membrane emulsification

The particle size of the emulsions was controlled by selecting SPG membranes with definite pore sizes during the emulsification process. Figure 2 shows the particle size distribution of the solid fat S/O/W emulsions after emulsification and solvent removal.

These results confirm that monodisperse and fine emulsions, with particle sizes reflecting the membrane pore size, were formed by this method. The resulting emulsions were lyophilized, and SEM observations were performed (Fig. 3).

The surfaces of the particles were not smooth, and many folds were observed. This shape is thought to be a characteristic due to the crystallinity of the solid fat. Plate-like materials other than the particles were not observed by
optical microscopy, suggesting that they were formed during the drying process. To evaluate the stability of these emulsions, the variation in particle size at 4°C and 37°C was evaluated over 3 weeks (Fig. 4).

The average particle size of the emulsions prepared using the homogenizer increased with time, and this phenomenon was more pronounced at 37°C. Conversely, there was little change in the particle size of fine emulsions whose particle size was controlled using membrane emulsification. Since the melting point of trimyristin is 55°C, the emulsion is solid at both temperatures. Although the oil phase was solid, it was assumed that Ostwald ripening would proceed if the emulsion was polydisperse. This result indicates that particle size control is important for emulsion stabilization.

3.3 Stability of S/O/W emulsions on protein encapsulation

In this study, solid fats were used as the oil phase of the S/O/W emulsions to prevent protein leakage. To evaluate this effect, protein leakage behavior was measured under...
different temperature conditions. Figure 5 shows the time course of the protein leakage from the emulsion.

For comparison, we used an S/O/W emulsion with soybean oil as the liquid oil phase. In both emulsions, the leakage was larger at higher temperatures, but it could be significantly reduced when the oil phase was solid. The S/O/W emulsions using solid fats showed almost no leakage even after one week of storage at 4°C, suggesting that significant stability was achieved.

3.4 Protein release in intestinal conditions

To enhance the intestinal mucosal absorption of proteins carried in solid S/O/W emulsions, the oil phase must be degraded in the small intestinal tract, and the surfactant-coated proteins must be released. Therefore, the emulsions prepared in this study were evaluated in a gastrointestinal model solution (PBS containing lipolytic enzymes) to confirm their controlled release properties. As shown in Fig. 6, no release was observed in the absence of lipase, whereas a rapid release was observed in the presence of the enzyme. This suggests that controlled release in the gastrointestinal tract is possible even when solid fats are used as the oil phase.

4 Conclusion

In this study, S/O/W emulsions composed of solid fats were prepared using the solvent evaporation method. It was found that the use of low-molecular-weight surfactants as emulsifiers caused the coalescence of oil droplets during the solvent evaporation process, whereas the use of PVA, a hydrophilic polymer, could prevent emulsion destabilization. The emulsions prepared via membrane emulsification showed a controlled particle size and improved stability, as well as high protein encapsulation and effective release properties in the gastrointestinal tract. This emulsion is expected to be used for the efficient transmucosal delivery of biopharmaceuticals.

Author Contributions

KY and ET were involved in research design, research performance and manuscript writing.

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Conflict of Interests
The authors declare that there are no conflicts of interest.

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