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Title: **Characterization of Lipid Vesicles Formulation by Multiple Emulsion and Solvent Evaporation Integrated Process for Improved Entrapment of Hydrophilic Bioactive Molecules**

(親水性生理活性分子の内包化の向上のための多相エマルション - 溶媒蒸発プロセスによる脂質ベシクル形成の特性解明)

Abstract

Lipid vesicles are compartments enclosing an aqueous solution with phospholipid membrane(s), formed by the molecular assembly of bilayer-forming lipids. They have been extensively studied as nano/microparticulate carriers for the efficient and controlled delivery of bioactive molecules. However, the preparation of vesicles with homogeneous size entrapping hydrophilic materials with high efficiency is rather difficult. In this study, the main focus has been the characterization of lipid vesicles formulated with integrated process of multiple emulsification and solvent evaporation as a novel technique for the preparation of lipid vesicles with controlled size of few hundred nanometers for optimum and improved entrapment of hydrophilic materials. The commonly known methods such as dry lipid hydration method, reverse-phase evaporation method, etc, possess inherent shortfalls such as low entrapment yield and large size distribution of the vesicle products. Usually, it is difficult to entrap water-soluble material in high yield in lipid vesicles. This is because of their dual dissolution in both the inside the vesicle core and in the outside medium, with low volume ratio.

The main purpose of the research was to characterize lipid vesicle formulation by the integrated process of multiple emulsion and solvent evaporation for improved entrapment of hydrophilic bioactive molecules. Here, $W_1/O/W_2$ multiple emulsions formed by MC emulsification were used as templates for lipid vesicles. To achieve the above purpose, the following objectives were adopted.

In the first part of Chapter 2, the combined process of multiple emulsion and solvent evaporation was employed as a novel method for the formulation of homogeneously dispersed few hundred nanometer-sized lipid vesicles with high entrapment yields of hydrophilic materials. Few hundred nanometer-sized vesicles are important for the applications of vesicles as vehicles for the delivery of pharmaceutics across the vascular

membrane to the diseased sites. The hydrophilic material employed here was the fluorescence marker, calcein (as a model of hydrophilic bioactive molecules).

In the latter part of Chapter 2, the objective was to characterize the lipid vesicle formulation process by varying the process conditions via: the use of various concentrations of different surfactants, each as the continuous phase emulsifier during MC emulsification; and by changing the solvent evaporation conditions. The effects of the changed process conditions were evaluated by determining the entrapment yields of calcein in the final vesicle products. The goal was primarily to ascertain a more friendly non protein surfactant (possibly food grade type) and its best concentration for optimum entrapment of water soluble molecules.

In Chapter 3, the focus was on the entrapment of bioactive molecules in the lipid vesicles and characterization of same, with variation of the process conditions as stated in Chapter 2 objective. The bioactive molecules employed were the pyrimidine anticancer drug, 5-fluorouracil, and the oral hydrophilic anti diabetic agent, metformin hydrochloride. Moreover, the stabilities of the lipid vesicles over storage time were evaluated by the change in size distribution and entrapment yields of the bioactive molecules in the vesicles.

Microchannel (MC) emulsification technique was applied in this study for the formation of $W_1/O/W_2$ multiple emulsions. Two non-ionic surfactants (Tween 80 and Pluronic F68), and two protein surfactants (sodium caseinate and BSA), were used in this study, each as an emulsifier in the W_2 phase.

Summary of results

Chapter 1: In Chapter 1, the historical background of the discovery of lipid vesicles and their potential use as drug delivery materials was reviewed. The different methodologies employed in the formulation of lipid vesicles, their principles, merits and challenges, were highlighted. Integrated process of multiple emulsifications and solvent evaporation, the focused technique in this research was equally introduced. It was recently employed to formulate micrometer sized lipid vesicles by our research group (Kuroiwa *et al.*, 2016). Also reviewed in Chapter 1 were some of the various characterization techniques for lipid vesicles.

Chapter 2: In the first part of Chapter 2, the integrated process of multiple emulsification and solvent evaporation was employed for the formulation of monodispersed lipid vesicles with few hundred nanometer size, using sodium caseinate as the external water phase (W_2)

emulsifier. Homogenization of the water phase with calcein and oil phase containing bilayer-forming lipids via ultra sonication resulted in the formation of the primary water-in-oil (W_1/O) emulsion. This was used as the dispersed phase in the secondary emulsification process by microchannel technique. The uniform-sized large oil droplets containing several internal water droplets were formed and dispersed in the external water phase containing 3.0 wt% sodium caseinate as the emulsifier, resulting in the $W_1/O/W_2$ multiple emulsions. The organic solvent in the large oil droplets was removed by evaporation under ambient condition, leading to the self-assemblage of the lipid components around each water droplet, forming membranes of lipid bilayer(s) resulting in enclosure of each water droplets as lipid vesicle. The entrapment yield of calcein as a model of hydrophilic bioactive molecules inside the lipid vesicles was very high (ca. 89%) compared to common previous methods. The lipid vesicles formed had modal diameter of about 182 nm and were found to be comparable in size to the water droplets of the primary W_1/O emulsion, as evidenced from the DLS and TEM analyses.

Having established the vesicle formulation protocol in section 2.2 using sodium caseinate as the external water phase emulsifier, the second part of Chapter 2 (Section 2.3) focused on the characterization of the integrated process conditions for vesicle formulation. Since sodium caseinate is a protein surfactant and could constitute allergic threat to some individuals, the process characterization sought to explore other surfactants for wider applications of this novel integrated process of lipid vesicle formulation. The effects of various concentrations of non-ionic (Tween 80 and Pluronic F68) and protein (BSA) surfactants on the entrapment yields of calcein in the lipid vesicles were thus investigated. The removal of the organic solvent via evaporation was done under two conditions: facilitated condition (FC) and ambient condition (AC).

The entrapment yields of calcein, as a model of hydrophilic bioactive molecule, by the vesicles prepared using Tween 80 as an emulsifier in the continuous phase increased with decrease in the surfactant concentration, and then it suddenly decreased at the least Tween 80 concentration examined under the AC. For the AC condition, the entrapment yield of calcein increased with decrease in the surfactant concentration at the high concentration region, but decreased with decrease in the concentration at low Tween 80 concentrations. There was a remarkable difference in the entrapment yield of calcein in the lipid vesicles at low Tween 80 concentrations under AC (ca. 75%) when compared with FC (ca. 23 %). This difference was attributed to the effect of destabilization by mechanical agitation and high temperature at low Tween 80 concentrations. For the BSA and Pluronic F68 surfactant systems, the entrapment yields slightly decreased with decrease in their

concentrations. Generally, the observed trends were discussed in relation to three possible mechanisms of leakage of calcein: (1) release of inner water W_1 contents into W_2 phase via the instability/breakage of the W_1/O droplets, (2) reversed micellar transport of W_1 contents from internal to external water phases through the O phase, and (3) destabilization/solubilization of lipid bilayers by surfactants.

Chapter 3: The outcome of Chapter 2 was employed in the entrapment of 5-fluorouracil (5-FU) and metformin hydrochloride (MH), the antineoplatic and antihyperglycemic agents, respectively. The entrapment yields of the drugs were found to be higher than those reported in the literature. The effects of the surfactants on the entrapment yields of each of the drugs in the formulated lipid vesicles were similar to the trend obtained in Chapter 2 section 2.3. However, the entrapment yield of calcein was remarkably higher than those of the drugs. The difference was explained in relation to their differing molecular weights and relative hydrophobicity. The vesicle suspension was equally found to be stable for about one month of storage. During storage, the entrapment yield decreased slightly and was considered to be due to one or combined effects of three possible mechanisms: permeation of the entrapped drug through the lipid bilayers to the continuous phase; destruction of lipid vesicle membranes; and chemical degradation of the entrapped material. Further analysis revealed that the slight decrease in the entrapment yields of the drugs was as a result of minimal leakage of the drugs from vesicle core to the bulk medium through the lipid bilayers.

Conclusion

A novel method for formulating homogeneously dispersed few hundred nanometer-sized lipid vesicles with improved entrapment of hydrophilic bioactive molecules was characterized. The multiple emulsion and solvent evaporation integrated process resulted in the direct conversion of water droplets in primary W_1/O emulsions to lipid vesicles under mild conditions.

i. The achievement of high entrapment yield (ca 89%) of hydrophilic molecules (calcein) using this method is quite beneficial when compared to conventional methods.

ii. The effect of surfactant type and concentration on the entrapment yields of bioactive molecules showed that Tween 80 at low concentration under ambient solvent evaporation condition could give high entrapment yield, and as a food-grade surfactant, is preferable to protein surfactants for broader application of this novel method of lipid vesicle formulation.

iii. As surfactants are not therapeutic agents, less amount of surfactant is preferred for better quality of the vesicle suspension formed. On that regard, Tween 80 at low concentration is considered more suitable than Pluronic F68 for vesicle formulation since the former at low concentrations gave high entrapment yield.

iv. Three possible mechanisms of leakage of calcein were proposed to explain the relative effects of the surfactants on vesicle formulation: (1) destabilization/solubilization of lipid bilayers by surfactants, (2) release of inner water W_1 contents into W_2 phase via the instability/breakage of the W_1/O droplets, and (3) reversed micellar transport of W_1 contents from internal to external water phases through the O phase.

With the outcome of this study, the use of the multiple emulsion and solvent evaporation integrated process offers great potentials for the formulation of lipid vesicles with controlled size and high entrapment yields of hydrophilic bioactive materials. This will be fundamentally significant in providing clues on the design of experimental condition for improved entrapment yields of water-soluble bioactive molecules and thus, would find potential industrial applications in the pharmaceutical, food, cosmetics, and other related fields.