

Preparation of W/O/W Multiple Emulsions with Polymers in the Outer Aqueous Phase

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Conditions for preparing W/O/W emulsions with a polymer in the aqueous phase as a possible entrapping agent to produce powdery multiple emulsions were investigated for food applications of W/O/W emulsions. The stability of these emulsions prepared using various types of emulsifiers was evaluated by the encapsulation efficiency of a hydrophilic marker molecule in the inner aqueous phase. Combinations of hexaglycerin polyricinoleate (Sunsoft 818SX) and soybean lecithin or gum arabic used in the first and second emulsification steps were suitable to prepare stable W/O/W emulsions. Emulsions containing >10% (w/v) polymers in the outer aqueous phase were successfully prepared using these emulsifier pairs by adding the polymers to the emulsions after homogenization. Addition of polymer to the inner aqueous phase did not have a significant effect on emulsion stability in spite of expected alleviation of osmotic pressure difference between the inner and outer aqueous phases.

Keywords: W/O/W emulsion, encapsulation efficiency, surfactant, polymer

In recent years, it has been recognized that many substances among food materials act on human bodies physiologically, and the effective supply of such substances to target organs would be of great benefit. However, the bioavailability of these compounds is often low due to their degradation through the digestive process when ingested orally. To improve the bioavailability, we focused on food emulsions as digestion-proof carriers of bioactive compounds. The bioactive substance would be protected against degradation by incorporating it into the internal dispersed phase of an emulsion. Hydrophilic bioactive substances, which may be degraded through the digestive system or may be prohibited from absorption because the absorption cells of the intestinal epithelium do not have any intrinsic transport systems for these compounds, could safely reach the intestine. In addition, if oils such as medium-chain fatty acids (Sekine *et al.*, 1984; Shima *et al.*, 1997) or their acylglycerols are employed, absorption of the hydrophilic substances may be enhanced.

W/O/W emulsions have been widely explored in pharmacy for decades as potential vehicles for water-soluble drugs in the course of investigations relating to drug delivery systems. Multiple emulsions have been proved to be particularly beneficial in attaining targeting and sustained releasing of drugs. Extensive research studies continue today on W/O/W emulsions entrapping insulin (Matsuzawa *et al.*, 1995; Silva Cunha *et al.*, 1997), water-soluble antibiotics (Nakhare & Vyas, 1994; Okochi & Nakano, 1996), immunosuppressives targeting specific organs (Uno *et al.*, 1997), and so on. Microspheres which consist of biodegradable polymers such as polylactic acid are now attracting a group of researchers to reduce the doses of drugs to prevent undesired side effects which would pose a large restriction in prescription (Yan *et al.*, 1994; Uchida *et al.*, 1997; Giunchedi *et al.*, 1998). In this way, W/O/W emulsions are drawing extensive attention from a wide range of disciplines.

However, the practical use of a W/O/W emulsion as a carrier of a drug or a food constituent is not yet widespread because of its low stability during storage compared to simple W/O or O/W emulsions. One potential strategy to enhance the stability of a multiple emulsion is to dehydrate it. The resultant powdery emulsion would be easy to handle and would have longer shelf life. For production of a powdery W/O/W emulsion, its characterization with entrapping agents in its outer aqueous phase is a prerequisite.

In this study, W/O/W multiple emulsions prepared under various conditions were characterized by the encapsulation efficiency of a hydrophilic substance in the inner aqueous phase using a fluorescent molecule as a model marker. Multiple emulsions with edible polymer molecules in the aqueous phase were then produced, in which the polymers were expected to act as entrapping agents when the emulsions were dehydrated. Preparative conditions suitable for polymer-carrying W/O/W emulsions and their stability were investigated.

Materials and methods

Materials Oleic acid used as the oily phase of W/O/W emulsions, hydrophobic non-ionic emulsifier Span 80 (sorbitan monooleate, HLB 4.3), and hydrophilic non-ionic emulsifier Tween 20 (polyoxyethylene (20) sorbitan monooleate, HLB 16.9) were purchased from Wako Pure Chemical Industries, Osaka. Sunsoft 818SX (hexaglycerin polyricinoleate) was obtained from Taiyo Kagaku (Mie), soybean lecithin was from Wako Pure Chemical Industries, and gum arabic (Mw. 2.2–3 × 10⁵) was from San-ei Sugarochemical (Osaka), respectively. PTSA (1,3,6,8-pyrenetetrasulfonic acid, tetrasodium salt) was obtained from Molecular Probes, OR. Methanol of analytical grade and 40% (w/w) TBAH (tetrabutylammonium hydroxide) were purchased from Wako Pure Chemical Industries and Aldrich Chemical Company, Milwaukee, WI, respectively. The following compounds were used as additives to the aqueous phase of multiple emulsions: dextran T-70 (Mw. ca. 3.77 × 10⁴, Phar-

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macia Fine Chemicals, Uppsala, Sweden), pullulan PI-20 (Mw. ca. 2.0×10^5 , gift from Hayashibara, Okayama), and maltodextrin #100 (Mw. $3.2\text{--}7.5 \times 10^3$, Matsutani Chemicals, Osaka). Gum arabic was also used as an additive to the aqueous phase. All the other chemicals were of analytical grade and were purchased from either Wako Pure Chemical Industries or Nacalai Tesque, Kyoto.

Preparation of the multiple emulsions The multiple emulsions were prepared using a two-step emulsification procedure. PTSA was used as a fluorescent marker molecule at a concentration of 1.0×10^{-4} mol/l in the inner aqueous phase (Tokgoz *et al.*, 1996).

The inner aqueous phase (0.9 ml) and the oily phase (oleic acid containing lypophilic emulsifier, 2.1 ml) were homogenized with a rotor-stator homogenizer (Physoctron NS-50, Nichi-on Irika, Tokyo) at graduation 60 (corresponds to about 22,000 rpm) for 2 min. The outer aqueous phase containing the hydrophilic emulsifier (3 ml) was then added to the resulting dispersion, and homogenization was carried out again at graduation 40 (about 10,000 rpm) for 1 min to obtain W/O/W emulsions. The emulsions were stored without stirring at 37°C, and a portion of them was sampled intermittently for analysis.

Addition of polymers to the aqueous phase(s) of the multiple emulsions W/O/W emulsions with polymers in the outer aqueous phase were prepared in two ways: (1) adding polymer solution to a W/O emulsion before emulsification, in this case pullulan was used as the polymer; (2) adding polymers to the outer aqueous phase after emulsification. An aqueous solution containing hydrophilic emulsifier was used as the outer aqueous phase when homogenization was initially conducted, and after formation of the multiple emulsion, the emulsion was suspended in a polymer solution containing the hydrophilic emulsifier to carry the polymer into the outer aqueous phase. In this method, the PTSA encapsulation efficiency after 3-h of mixing with a polymer solution, rather than that immediately after preparation, was measured to be certain of providing the emulsion with sufficient time to intermingle the outer aqueous phase with the polymer solution completely.

Measurement of PTSA encapsulation efficiency The stability of the multiple emulsions was evaluated by the fraction of PTSA retained within the inner aqueous phase (encapsulation efficiency, β)

$$\beta_t = \frac{M_0 - M_t}{M_0} \times 100 [\%], \quad (1)$$

where M_0 is the total amount of PTSA over the whole system and M_t is the amount of PTSA leaked into the outer aqueous phase at time t .

A multiple emulsion (1 ml) was diluted with distilled water to 40 ml and centrifuged at 7500 rpm for 10 min at 10°C. The lower phase was filtered through a membrane filter (cellulose acetate, pore size = 0.45 μm , Advantec) for complete elimination of W/O emulsions. This sample would contain PTSA derived from the outer aqueous phase of the original multiple emulsion and was subjected to analysis of the PTSA concentration.

The concentration of PTSA was measured using a high performance liquid chromatograph (LC-9A, Shimadzu Corp., Kyoto) equipped with an ODS column (YMC-Pack ODS-AQ, i.d. 4.6 mm \times 150 mm, YMC, Kyoto). A mixture of methanol/dis-

tilled water/40% (w/w) TBAH solution (75/20/5 (v/v/v)) (pH 7.6 adjusted with acetic acid) was used as an eluent at a flow rate of 1 ml/min. The effluent was monitored using a spectrofluorometric detector (RF-550, Shimadzu) at excitation and emission wavelengths of 374 and 404 nm, respectively. The PTSA concentration was, in some cases, measured directly by spectrofluorophotometer (RF-1500, Shimadzu).

Particle size of the emulsion droplets The particle size distribution of the emulsion droplets was analyzed using a centrifugal particle analyzer (SA-CP3L, Shimadzu). Samples were diluted either by *n*-hexane or 1% (w/v) SDS (sodium dodecyl sulfate) solution for the oily and aqueous continuous phases, respectively.

Results and discussion

Droplet size distribution of emulsions The droplet size distributions of the dispersed phase for W/O and O/W emulsions and a W/O/W multiple emulsion are shown in Fig. 1. In the preparation of simple emulsions, homogenization was performed at different rotation speeds for W/O and O/W emulsions, with a slower speed for O/W emulsion simulating the second homogenization step in the preparation of a W/O/W emulsion. Accord-

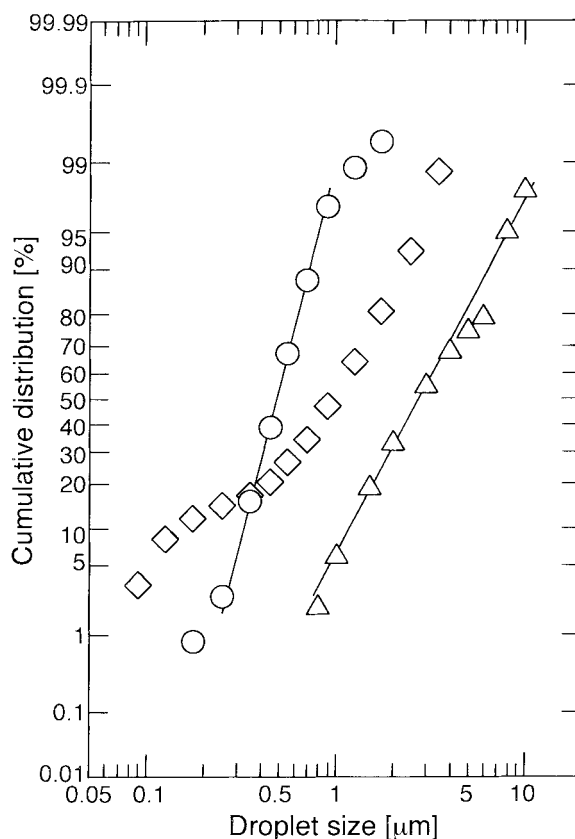


Fig. 1. Droplet size distributions of (○) W/O and (△) O/W emulsions prepared using a homogenizer of the rotor-stator type, Physoctron NS-50. Oleic acid was used as the oily phase. Span 80 and Tween 20 were dissolved in the continuous phase of W/O and O/W emulsions at concentrations of 20 and 1% (w/v), respectively. Symbol \diamond indicates the droplet size distribution of an emulsion prepared through two-step homogenization procedure, using 20% Span 80 and 1% Tween 20 as emulsifiers in the oily and aqueous phases, respectively. The homogenizer was operated at graduations of 60 for 2 min and 40 for 1 min during the first and second emulsification steps, respectively.

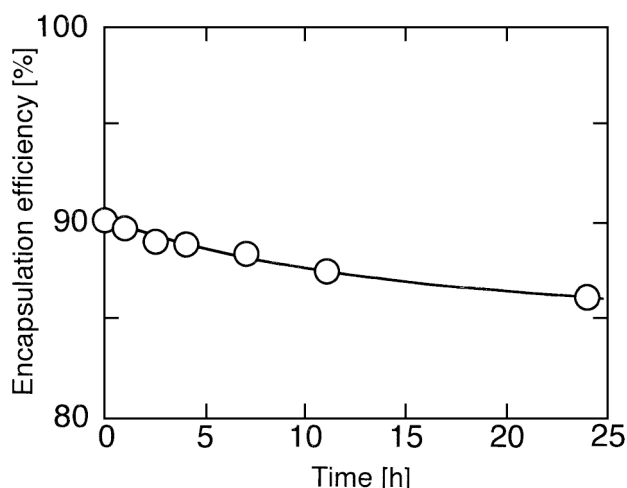


Fig. 2. Time course of PTSA encapsulation efficiency of a W/O/W emulsion during storage at 37°C. The volumetric ratios of the inner aqueous phase (water containing 1.0×10^{-4} mol/l PTSA)/oily phase (oleic acid containing 20% (w/v) Span 80)/outer aqueous phase (water containing 1% (w/v) Tween 20)=3/7/10 (v/v/v).

ingly, the resulting O/W emulsion had larger droplets than the W/O emulsion, but the droplet size distributions for both emulsions showed a monodispersed profile. The W/O/W emulsion was successfully produced using the rotor-stator homogenizer with a PTSA encapsulation efficiency of about 90%, although the size distribution of the emulsion droplet was polydispersed.

Release of PTSA from inner to outer aqueous phase Figure 2 shows an example of the change in the encapsulation efficiency with time during storage of a W/O/W multiple emulsion containing PTSA in the inner aqueous phase. About 10% of the PTSA was detected in the outer aqueous phase immediately after formation of the multiple emulsion, because of insufficient emulsification or adhesion of PTSA to the vessel wall during homogenization. A gradual reduction in PTSA encapsulation efficiency was observed during 24-h storage at 37°C.

The W/O/W emulsion was prone to “cream” to form a W/O emulsion over the outer aqueous phase after *ca.* 3 h of preparation. The phase separation might stem from the density difference between the oily (oleic acid, about 0.9 g/cm^3) and aqueous phases (1.0 g/cm^3). Microscopic observation showed that smaller droplets were adsorbed on the surfaces of larger droplets, although the phase separation observed in this experiment did not lead to droplet coalescence or change in droplet size distribution. The phase-separated multiple emulsions easily recovered their original homogeneity on stirring.

Emulsifier concentration Two kinds of emulsifiers, lipophilic and hydrophilic, are usually used for the preparation of stable multiple emulsions. The effect of the emulsifier concentrations on the emulsion stability was investigated. In preliminary experiments, we examined some combinations of lipophilic and hydrophilic emulsifiers and selected a combination of Span 80 and Tween 20, which has been frequently used in pharmaceutical research, and that of Sunsoft 818SX and soybean lecithin or gum arabic, both of which are edible. Soybean lecithin was employed as a hydrophilic emulsifier in this experiment, in the form of a homogeneous suspension in water.

The PTSA encapsulation efficiencies of multiple emulsions

with Span 80 dissolved in the oily phase as a lipophilic emulsifier and Tween 20 dissolved in the outer aqueous phase as a hydrophilic emulsifier are shown in Fig. 3a. A high encapsulation efficiency was obtained at 20% Span 80 and 1% Tween 20. A lower concentration of Span 80 and a higher concentration of Tween 20 destabilized the emulsions. The reason for the requirement of a relatively high concentration of the lipophilic emulsifier in the oily phase was considered to be the following. The inner aqueous phase-oily phase interfacial area on which the lipophilic emulsifier molecules should be adsorbed to maintain the emulsion structure is much larger compared to the oily phase-outer aqueous phase interfacial area which should be covered with hydrophilic emulsifier molecules. On the other hand, a lower concentration of the hydrophilic emulsifier proved to be suitable for maintaining the emulsion structure because excess hydrophilic emulsifier in the outer aqueous phase may destabilize the emulsion by forming micelles which may enclose some of the oily phase or lipophilic emulsifier.

Multiple emulsions were then prepared using combinations of edible surfactants. Sunsoft 818SX was used as a lipophilic emulsifier and soybean lecithin or gum arabic as a hydrophilic emulsifier. The PTSA encapsulation efficiency of W/O/W emulsions

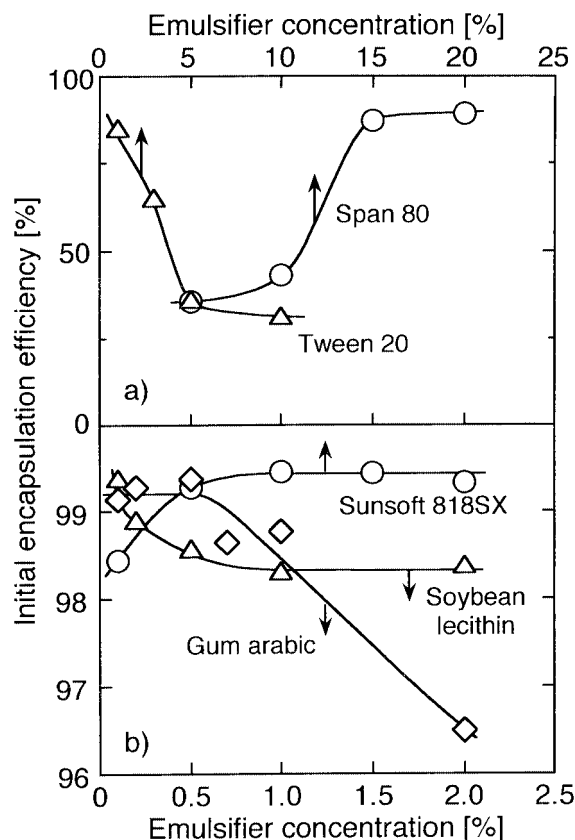


Fig. 3. Influence of emulsifier concentration on PTSA encapsulation efficiency. a) Span 80 and Tween 20 were added to the oily phase (oleic acid) and the outer aqueous phase, respectively. The concentration of Span 80 (○) was varied at a fixed Tween 20 concentration of 1% (w/v), while Tween 20 concentration (Δ) was changed at a fixed Span 80 concentration of 20% (w/v). b) Effect of concentrations of (○) Sunsoft 818SX, (Δ) soybean lecithin, and (◇) gum arabic. Sunsoft 818SX concentration in the oily phase was fixed at 10% (w/v) when soybean lecithin or gum arabic concentration in the outer aqueous phase was varied. Soybean lecithin was added to the outer aqueous phase at a concentration of 0.5% (w/v) when the effect of Sunsoft 818SX concentration was examined.

immediately after preparation is shown in Fig. 3b. As in the case of emulsions where Span 80 and Tween 20 were used as emulsifiers (Fig. 3a), emulsions with a low concentration of Sunsoft 818SX and high concentrations of hydrophilic emulsifiers were prone to destabilize. Sunsoft 818SX concentrations above 5% were adequate for attaining PTSA encapsulation efficiency of as high as about 99%, and soybean lecithin concentration of 0.1 to 0.2% was sufficient for preparation of stable W/O/W emulsion. The same concentration range of the emulsifiers was also found to be satisfactory for the combination of Sunsoft 818SX and gum arabic. Because no addition of hydrophilic emulsifier in the outer aqueous phase may lead to larger droplet size and emulsion instability through droplet coalescence, an emulsifier was added at a low level to the outer aqueous phase throughout this study.

Addition of polymers to the aqueous phase of W/O/W emulsions To obtain powdery W/O/W emulsions, addition of an entrapping agent to the aqueous phase is necessary to maintain the emulsion structure during and after the drying process. As the first step, the formation of stable W/O/W emulsions carrying an entrapping agent in the outer aqueous phase was investigated. Some kinds of polymers were examined for application as entrapping agents. W/O/W emulsions were prepared from W/O emulsion and pullulan solution, and the influence of the pullulan concentration in the outer aqueous phase on PTSA encapsulation efficiency and on the median diameter of the resulting emulsion is illustrated in Fig. 4. When the pullulan concentration in the outer aqueous phase exceeded 10%, phase inversion occurred and the multiple emulsion could not be formed. At above 5%, emulsification by the rotor-stator homogenizer was unsuccessful, probably because of the high viscosity, and leakage of PTSA from the inner to the outer aqueous phase was remarkable. Microscopic observation showed that rather small emulsion droplets were produced at the outer pullulan concentration of 5% and the W/O/W emulsion structure was not identified. The median diameter of the emulsion droplet was reduced along with the increase in pullulan concentration. Stability of the multiple emulsions were improved by adding 1% pullulan, which raised the viscos-

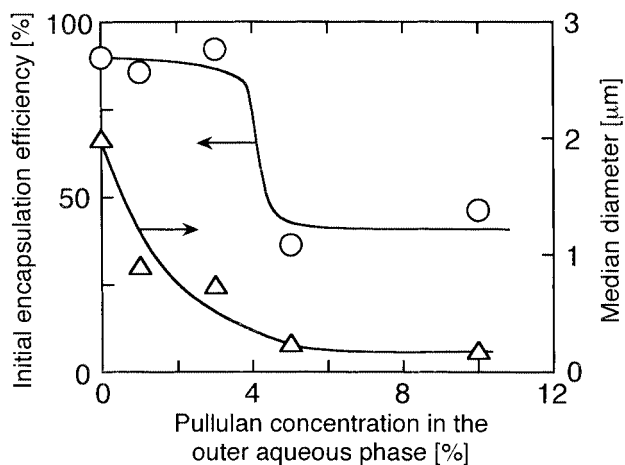


Fig. 4. Relationship between pullulan concentration in the outer aqueous phase and (○) PTSA encapsulation efficiency or (Δ) median diameter of the resulting emulsion droplets. The oily and outer aqueous phases contained 20% (w/v) Span 80 and 1% (w/v) Tween 20 as emulsifiers, respectively. Pullulan was added to the outer aqueous phase before emulsification by a rotor-stator homogenizer.

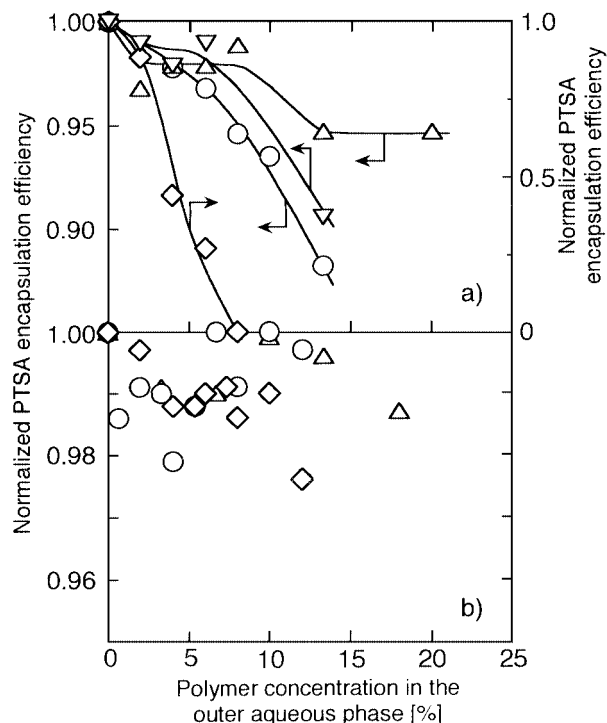


Fig. 5. Effect of polymer concentrations in the outer aqueous phase on PTSA encapsulation efficiency after 3-h storage of W/O/W emulsions prepared using a) 20% (w/v) Span 80 and 1% (w/v) Tween 20 as the lipophilic and hydrophilic emulsifiers. W/O/W emulsions were at first prepared using 1% (w/v) Tween 20 solution as the outer aqueous phase, and polymer solutions were then added to the resultant W/O/W emulsions. Symbols ○, Δ, ◇, and ∇, indicate pullulan, maltodextrin, gum arabic, and dextran, respectively. b) 10% (w/v) Sunsoft 818SX and 0.2% (w/v) soybean lecithin or gum arabic as emulsifiers. (○) Pullulan- or (Δ) maltodextrin-added emulsions prepared using soybean lecithin, and (◇) pullulan-added emulsion prepared using gum arabic. The PTSA encapsulation efficiency was normalized by the value without the addition of polymers.

ity of the outer aqueous phase; droplet size distribution did not change after 4-day storage, although the droplet size increased in the absence of pullulan due to coalescence of the emulsion droplets (data not shown).

Because emulsification tended to fail at a high concentration of pullulan in the outer aqueous phase during the second homogenization step of the W/O/W emulsion preparation, an alternative method of preparing multiple emulsions other than homogenizing a W/O emulsion and pullulan solution should be developed to enable production of stable multiple emulsions carrying a high concentration of the polymer in the outer aqueous phase. In view of the production of powdery multiple emulsions, a higher polymer concentration in the outer aqueous phase (>10%) would be preferable.

To achieve this purpose, a polymer solution of high concentration was mixed with a W/O/W emulsion prepared without the polymer. The effect of the concentration of 4 kinds of polymers in the outer aqueous phase on PTSA encapsulation efficiency of the emulsion after 3-h storage carrying Span 80 and Tween 20 as emulsifiers during the first and second emulsification steps, respectively, is illustrated in Fig. 5a. The ordinate of the figure is normalized by the values of PTSA encapsulation efficiency observed in W/O/W emulsions on adding aqueous emulsifier solution instead of the polymer solution. The encapsulation efficiency was markedly reduced in the emulsion with gum arabic added in

the outer aqueous phase, especially at high concentrations. The contribution of gum arabic to destabilization was assumed to be due to its emulsification ability, i.e., the interaction of gum arabic with the oily phase and the lipophilic emulsifier. Addition of pullulan and dextran lowered the PTSA encapsulation efficiency at concentrations higher than 10%, although the encapsulation efficiency was almost independent of the outer maltodextrin concentration even at a concentration of 20%. Figure 5b shows the PTSA encapsulation efficiency of the emulsion prepared using Sunsoft 818SX and gum arabic or soybean lecithin. In both cases, the PTSA encapsulation efficiency was almost independent of the polymer concentration in the outer aqueous phase. Maltodextrin solution has a low viscosity compared to other polymer solutions such as pullulan, and multiple emulsions containing maltodextrin could be easily prepared even at high polymer concentration, whereas aggregation of the emulsion droplets was likely to occur during storage leading up to phase separation. On the other hand, the high viscosity of the pullulan solution at the outer aqueous phase prevented the resulting emulsions from droplet aggregation and hence phase separation.

Next, the addition of polymers to the inner aqueous phase was examined. Considering the effect of the osmotic pressure difference between the inner and outer aqueous phases, which will unavoidably be encountered during the drying process on the stability of the emulsion, an inner aqueous phase including solute molecules may be favorable to reduce the osmotic pressure difference. Figure 6 shows the relationship between PTSA encapsulation efficiency and polymer concentration in the inner aqueous phase. Addition of gum arabic had an undesirable influence on the PTSA encapsulation efficiency at high concentration as expected from the above experiment in which gum arabic was added to the outer aqueous phase after emulsification (Fig. 5a). In contrast, pullulan and maltodextrin had no significant influence, although a slight increment was perceptible in encapsulation efficiency for these polymers as the inner polymer concentrations increased.

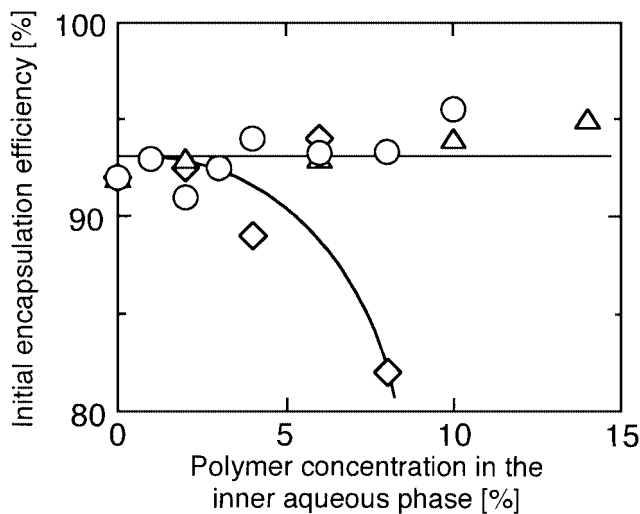


Fig. 6. Influence of the polymer concentration in the inner aqueous phase on PTSA encapsulation efficiency. The W/O/W emulsions contained 20% (w/v) Span 80 and 1% (w/v) Tween 20 in the oily and outer aqueous phases, respectively. The outer aqueous phase did not contain polymers. Symbols ○, △, and ◇, indicate pullulan, maltodextrin, and gum arabic, respectively.

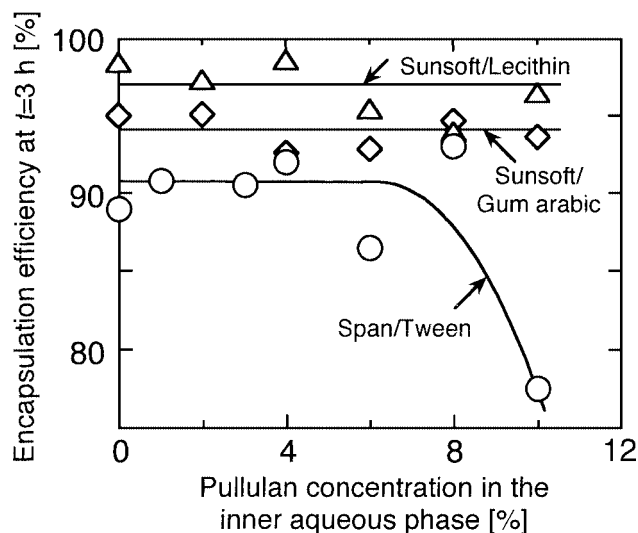


Fig. 7. PTSA encapsulation efficiencies after 3-h storage of W/O/W emulsions carrying pullulan in both aqueous phases. Emulsions were prepared using (○) 20% (w/v) Span 80 as a lipophilic emulsifier and 1% (w/v) Tween 20 as a hydrophilic emulsifier, (△) 10% (w/v) Sunsoft 818SX and 0.5% (w/v) soybean lecithin, and (◇) 10% (w/v) Sunsoft 818SX and 0.5% (w/v) gum arabic. Pullulan was added at a concentration of 13% (w/v) in the outer aqueous phase for the emulsifier combination of Span 80 and Tween 20, and 12% (w/v) for the other combinations.

When both the inner and outer aqueous phases contain a polymer, the difference in osmotic pressure between the phases can be reduced. We expected that the reduction might improve the encapsulation efficiency in a W/O/W emulsion carrying the polymer in the outer aqueous phase. Thus, multiple emulsions including pullulan both in the inner and outer aqueous phases were then prepared. Figure 7 diagrams the PTSA encapsulation efficiency of emulsions carrying three combinations of emulsifiers. When Span 80 and Tween 20 were employed as emulsifiers to prepare a W/O/W emulsion, the stability of the resultant emulsion depended on the pullulan concentration in the inner aqueous phase, and a higher concentration induced emulsion destabilization. On the other hand, a high PTSA encapsulation efficiency was maintained even at an outer pullulan concentration of 10% (w/v) for the emulsions prepared using Sunsoft 818SX and gum arabic or soybean lecithin. As shown here, the addition of a polymer in the inner aqueous phase had no significant influence on the encapsulation efficiency.

On the whole, combinations of lipophilic and hydrophilic emulsifiers employed during the first and second emulsification steps had a great influence on the stability of the resultant emulsions and therefore should be carefully selected to produce stable W/O/W emulsions. An emulsifier pair of Span 80 and Tween 20 resulted in emulsion instability. Span 80 and Tween 20 easily intermingle with each other and are often used together in a mixed form to attain a desired HLB value for production of simple W/O or O/W emulsions, depending on the properties of the oily and aqueous phases. Sunsoft 818SX proved to be a good candidate for lipophilic emulsifier used in the first emulsification step in preparing W/O emulsion. In conjunction with the second emulsification step employing either gum arabic or soybean lecithin as a hydrophilic emulsifier, it could produce highly stable W/O/W emulsions. Addition of a polymer after the second emul-

sification step resulted in multiple emulsions carrying a high concentration of the polymer in the outer aqueous phase. Addition of a polymer to the inner aqueous phase did not have a significant effect on the stability of W/O/W emulsions carrying a polymer in the outer aqueous phase, although the osmotic pressure difference between the inner and outer aqueous phases would be expected to affect the stability of multiple emulsions.

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