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Preparation and Characterization of Water/Oil and Water/Oil/Water Emulsions Containing Biopolymer-Gelled Water Droplets

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The purpose of this study was to create water-in-oil (W/O) and water-in-oil-in-water (W/O/W) emulsions containing gelled internal water droplets. Twenty weight percent W/O emulsions stabilized by a nonionic surfactant (6.4 wt % polyglycerol polyricinoleate, PGPR) were prepared that contained either 0 or 15 wt % whey protein isolate (WPI) in the aqueous phase, with the WPI-containing emulsions being either unheated or heated (80 °C for 20 min) to gel the protein. Optical microscopy and sedimentation tests did not indicate any significant changes in droplet characteristics of the W/O emulsions depending on WPI content (0 or 15%), shearing (0−7 min at constant shear), thermal processing (30−90 °C for 30 min), or storage at room temperature (up to 3 weeks). W/O/W emulsions were produced by homogenizing the W/O emulsions with an aqueous Tween 20 solution using either a membrane homogenizer (MH) or a high-pressure valve homogenizer (HPVH). For the MH the mean oil droplet size decreased with increasing number of passes, whereas for the HPVH it decreased with increasing number of passes and increasing homogenization pressure. The HPVH produced smaller droplets than the MH, but the MH produced a narrower particle size distribution. All W/O/W emulsions had a high retention of water droplets (>95%) within the larger oil droplets after homogenization. This study shows that W/O/W emulsions containing oil droplets with gelled water droplets inside can be produced by using MH or HPVH.

KEYWORDS: Membrane homogenization; W/O emulsion; W/O/W emulsion; stability; gelled particles; multiple emulsion

INTRODUCTION

Recently, there has been growing interest in the development of water-in-oil-in-water (W/O/W) emulsions because they have a number of potential benefits over conventional O/W emulsions, such as controlled or triggered release, reduction of fat content, and protection of labile ingredients (*1*−*3*). Nevertheless, there have been many difficulties associated with preparing this type of multiple emulsion for utilization within the food industry due to problems with internal water droplet coalescence and expulsion and diffusion of water molecules from the internal aqueous phase to the bulk aqueous phase (*3*−*5*).

W/O/W emulsions consist of small water droplets contained within larger oil droplets, which are themselves dispersed in a watery continuous phase. They are usually created using conventional homogenization technology in a two-step procedure. First, a W/O emulsion is prepared by homogenizing an oil phase and an aqueous phase together in the presence of a suitable oil-soluble emulsifier (low HLB number). Second, a W/O/W emulsion is prepared by homogenizing this W/O emulsion with another aqueous phase in the presence of a suitable water-soluble emulsifier (high HLB number). However, the intense mechanical stresses that the materials experience during this second homogenization step have the undesirable potential for disrupting the primary water-in-oil (W/O) emulsion droplets, thereby reducing the yield of multiple emulsions (*6*). Because of concern about high-pressure homogenization having a disruptive influence on the primary emulsion droplets, previous workers have often preferred to employ a low-shear mixer for the second stage of emulsification. However, this usually produces highly polydisperse and coarse W/O/W emulsion droplets with poor creaming stability due to the relatively large mean droplet size (*7*).

To produce a stable multiple emulsion it is usually necessary to ensure that the W/O emulsion from which it is prepared is also stable (*3*). The incorporation of a thickening or gelling polymer (e.g., xanthan, alginate, gelatin, agarose, bovine serum albumin, sodium caseinate) within the dispersed aqueous phase of the primary emulsion has been proposed as a way of improving the long-term stability of W/O emulsions, especially

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if the added polymer forms a network throughout the internal water droplet or a gel-like layer at the inner oil−water interface either by cross-linking with enzyme or calcium ions or by thermal processing (*4*, *7*−*14*). This technique offers the possibility of improving water droplet resistance to mechanically induced stresses during homogenization, as well as to droplet leakage or coalescence after homogenization. On the other hand, this technique may be less effective at preventing water diffusion between the oil phases because water molecules can still move through the gel network. In addition, it also offers the possibility of creating novel encapsulation systems with adjustable release properties. An alternative method of improving the stability of W/O and W/O/W emulsions is to form a network of fat crystals either around the water droplets or throughout the oil phase (*15*).

The main objective of the present study was to determine whether stable W/O emulsions could be formed by incorporating whey protein isolate (WPI) into the water phase to form gelled biopolymer particles and, then, to see if these W/O emulsions could be used to form stable W/O/W emulsions. WPI was selected because (i) it is a widely used natural food-grade ingredient; (ii) it has previously been reported to improve the formation and stability of W/O emulsions prepared with lecithin (*12*); and (iii) it forms thermo-irreversible gels upon heating, thereby allowing one to lock in any novel microstructure created (*16*). Finally, we should note that the term "emulsion" is usually used to describe a dispersion of immiscible liquid phases, whereas the inner aqueous phase droplets produced in this study are actually gelled. Nevertheless, we believe that the use of the term "emulsion" is appropriate for these systems because the gelled particles consist predominantly of water and because they are formed from conventional liquid droplets.

MATERIALS AND METHODS

Materials. Polyglycerol polyricinoleate (PGPR 4150, Palsgaard, Denmark) prepared by the esterifcation of condensed castor oil fatty acids with polyglycerol was obtained from Palsgaard Industri de Mexico (St. Louis, MO). As stated by the manufacturer, the polyglycerol moiety of the PGPR was predominantly di-, tri-, and tetraglycerols (minimum of 70%) and contained not more than 10% of polyglycerols equal to or higher than heptaglycerol. WPI (BiPRO lot JE 015-4-420) was obtained from Davisco Foods International Inc. (Le Sueur, MN). As stated by the manufacturer, the powdered WPI had a composition of 97.6 wt % protein, 2.0 wt % ash, and 0.3 wt % fat (dry weight basis) and 4.7 wt % moisture (wet weight basis). Polyoxyethylene-sorbitan monolaurate (Tween 20), sorbitan monostearate (Span 60), sorbitan tristearate (Span 65), sorbitan monooleate (Span 80), analytical grade sodium chloride (NaCl), hydrochloric acid (HCl), sodium hydroxide (NaOH), hexadecane, sodium phosphate (monobasic, anhydrous), and sodium azide (NaN_3) were purchased from the Sigma Chemical Co. (St. Louis, MO). Ethanol, toluene, and sodium phosphate (dibasic, anhydrous) were purchased from Fisher Science (Chicago, IL). Corn oil (Mazola, ACH Food Companies Inc., Memphis, TN) was purchased from a local supermarket and used without further purification. 1,3,6,8-Pyrenetetrasulfonic acid tetrasodium salt (CAS Registry No. 59572-10-0) was purchased from Fisher Scientific International L.L.C. (Hampton, NH). Distilled and deionized water was used for the preparation of all solutions.

Solution Preparation. Emulsifier solution was prepared by dispersing 8 wt % PGPR into corn oil and heating to 50 °C. This PGPR concentration was selected because previous studies have shown that it is capable of forming W/O emulsions containing small water droplets with a narrow size distribution (*7*, *14*). Protein solution was prepared by dispersing the desired amount (15 wt %) of WPI powder into 5 mM phosphate buffer solution at pH 7 containing 0.02 wt % sodium azide (as an antimicrobial agent) and 100 mM NaCl (to facilitate gelation) and stirring for at least 2 h at room temperature to ensure complete dissolution. The pH of the WPI solution was adjusted back to pH 7.0 using 1 M HCl if required, and then the solution was heated to 50 °C before emulsification.

Preparation of W/O Emulsions. Water-in-oil emulsions were prepared by homogenizing 20 wt % aqueous phase with 80 wt % oil phase. The emulsions were prepared at 40−50 °C (rather than at room temperature) because we found that the oil phase was less viscous, and the emulsions produced by homogenization had smaller droplet sizes (see later). The aqueous phase with or without 15 wt % WPI was dispersed gradually into the oil phase under agitation with a magnetic stirrer and then blended together using a high-speed blender (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland) at 50 °C for 2 min. The coarse emulsions were then passed through a two-stage high-pressure valve homogenizer (LAB 1000, APV-Gaulin, Wilmington, MA) three times: 19 MPa (2700 psi) for the first stage and 2.1 MPa (300 psi) for the second stage. Temperatures of the emulsions were 45 ± 1 and 44 ± 1 °C when they were fed into and came out of the homogenizer, respectively. After homogenization, the emulsions were cooled to room temperature (∼23 °C). Then, the emulsion containing water droplets with WPI inside was separated into two portions: (i) one portion was maintained at ambient temperature; (ii) the other portion was heat-treated at 80 °C for 20 min. All emulsions were then stored at ambient temperature for 24 h before being analyzed.

In summary, three different W/O emulsions were prepared:

Emulsion 1 (No-WPI) was prepared by homogenizing 20 wt % aqueous phase (5 mM phosphate buffer, 100 mM NaCl, pH 7) with 80 wt % oil phase (8 wt % PGPR in corn oil).

Emulsion 2 (WPI-no-Gel) was prepared by homogenizing 20 wt % aqueous phase (15 wt % WPI, 5 mM phosphate buffer, 100 mM NaCl, pH 7) with 80 wt % oil phase (8 wt % PGPR in corn oil). This emulsion was not heat-treated after emulsification.

Emulsion 3 (WPI-Gel) was prepared by homogenizing 20 wt % aqueous phase (15 wt % WPI, 5 mM phosphate buffer, 100 mM NaCl, pH 7) with 80 wt % oil phase (8 wt % PGPR in corn oil). This emulsion was heat-treated at 80 °C for 20 min to gel the WPI inside the water droplets. (When an aqueous solution with the same composition was heated at 80 °C for 20 min in a glass test tube, it formed a strong optically opaque gel.)

Influence of Environmental Stresses on W/O Emulsion Stability. The properties and stability of the three different types of W/O emulsions were compared after they were subjected to various environmental stresses:

Shearing. The emulsions were subjected to constant shear for 0−7 min (0, 0.5, 1, 2, 3, 4, 5, and 7 min) using a high-speed blender (M133/1281-0, Biospec Products, Inc.) at room temperature (∼23 °C). The emulsions were then stored at room temperature for 24 h before being analyzed.

Thermal Processing. Emulsion samples (10 g) were transferred into glass test tubes (internal diameter = 15 mm, height = 125 mm), which were then incubated in a water bath for 30 min at different temperatures ranging from 30 to 90 °C. After incubation, the emulsion samples were immediately cooled to ambient temperature in a water bath containing cold tap water. The emulsions were then stored at ambient temperature for 24 h prior to analysis.

Storage. The emulsions were stored at ambient temperature for 1 day, 1 week, 2 weeks, and 3 weeks before being analyzed.

The properties and stability of the W/O emulsions were then characterized by measuring their particle size, microstructure, and sedimentation stability (see below).

Preparation of W/O/W Emulsions. W/O/W emulsions were prepared using the two-stage emulsification method (*7*). First, a 20 wt % W/O emulsion was prepared as described above. Second, 20 wt % of this W/O emulsion was homogenized with 80 wt % of aqueous surfactant solution (0.5 wt % Tween 20, 5 mM phosphate buffer, 100 mM NaCl, 0.02 wt % NaN_3, pH 7) using either a membrane homogenizer or a high-pressure valve homogenizer.

W/O/W Emulsions Prepared Using a Membrane Homogenizer. The W/O emulsions and aqueous surfactant solution were first premixed for several minutes using a stirring bar followed by five passes through a membrane homogenizer at 100 kPa (14.5 psi) (MG-20-5, Kiyomoto Iron Works Ltd., Japan). The pressure vessel was filled with 100 mL of coarse emulsion, and the required driving pressure was built up with

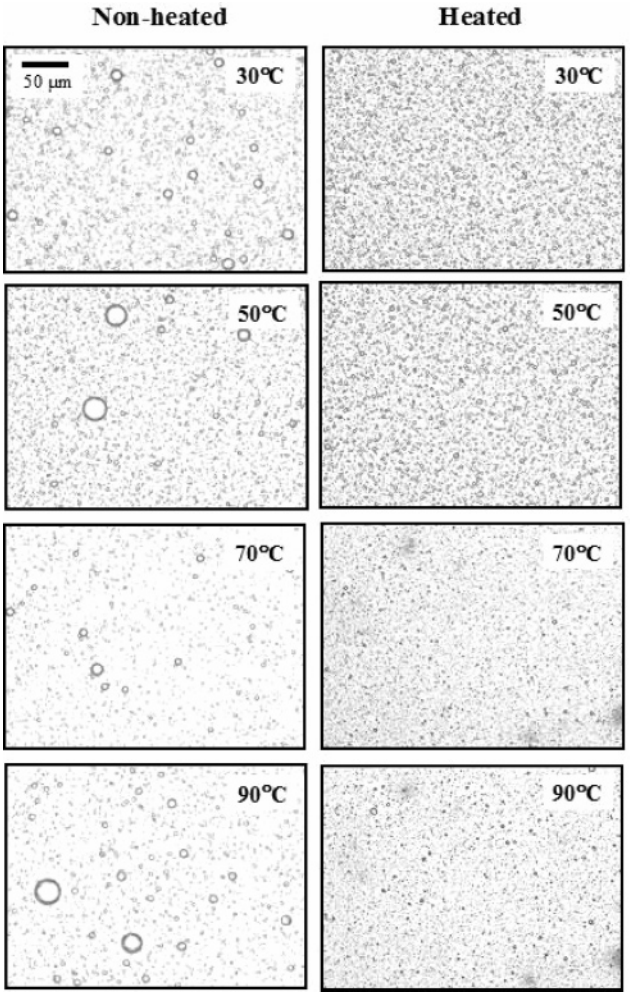

Figure 1. Influence of heat treatment on the microstructure of PGPR-stabilized W/O emulsions (20 wt % aqueous phase, 80 wt % oil phase). Oil and aqueous phases were either heated to 50 °C (heated) or kept at room temperature (nonheated) before emulsification.

compressed air using a pressure regulator (PRG101, Omega, Stamford, CT). The operating pressure was measured with an accuracy of ±1 kPa using a pressure gauge (PG-200-103G-P, Copal Electronics, Tokyo, Japan). When the emulsion had passed through the membrane tube, it was collected into a beaker placed on an electronic balance (Accu-622, Fisher Scientific, Fair Lawn, NJ). The balance was interfaced to a PC computer to collect time and mass data every 2 s using data acquisition software (AccuSeries USB version 1.2, Fisher Scientific, Fair Lawn, NJ). The experiments were carried out at 21 °C. The membrane used was a SPG membrane (8.5 mm inner diameter × 0.8 mm wall thickness) supplied from SPG Technology Co., Ltd. (Sadowara, Japan). The mean pore size of the membrane was 8.0 μm, the effective membrane length was 12 mm, and the effective cross-sectional area was 3.75 cm^2. The membrane tube was cleaned after use by immersing it for 2 days in ethanol plus 2 days in toluene, followed by heating at 500 °C for 30 min in an electric muffle furnace. Measurements of the flux rate after cleaning indicated that the inherent membrane permeability to pure water was completely restored. The emulsions were stored at ambient temperature for 24 h before being analyzed.

W/O/W Emulsions Prepared Using a High-Pressure Homogenizer. Multiple emulsions were prepared by blending 20 wt % W/O emulsion and 80 wt % aqueous surfactant solution (0.5 wt % Tween 20 in buffer solution) together using a high-speed blender (M133/1281-0, Biospec Products, Inc.) for 2 min at room temperature. These coarse emulsions were then passed through a two-stage high-pressure valve homogenizer (LAB 1000, APV-Gaulin, Wilmington, MA) one to three times at either 7 MPa (1000 psi) or 14 MPa (2000 psi): $^9/_{10}$ of the pressure from the first stage, $^1/_{10}$ from the second stage. The emulsions were then stored at ambient temperature for 24 h before being analyzed.

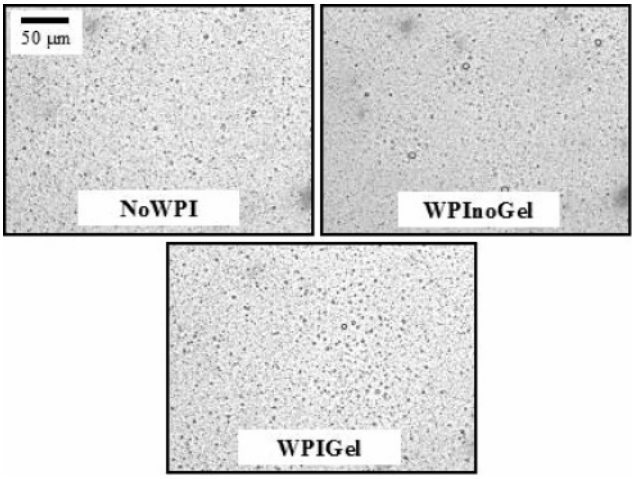

Figure 2. Microstructure of PGPR-stabilized emulsions (20 wt % aqueous phase, 80 wt % oil phase). No-WPI, W/O emulsions that did not contain WPI; WPI-no-Gel, W/O emulsions that contained 15% WPI; WPI-Gel, W/O emulsions that contained 15% WPI and were heat-treated at 80 °C for 20 min after preparation to gel the protein.

Particle Size Measurements. Average droplet sizes of W/O/W emulsions were measured using a static light scattering instrument. To avoid multiple scattering effects, W/O/W emulsions were diluted to a droplet concentration of approximately ~0.005 wt % using buffer solution at the pH and NaCl concentration of the sample and stirred continuously throughout the measurements to ensure the samples were homogeneous. The particle size distribution of the emulsions was then measured using a laser light scattering instrument (Mastersizer, Malvern Instruments, Worcestershire, U.K.). This instrument measures the angular dependence of the intensity of laser light (λ = 632.8 nm) scattered by a dilute emulsion and then finds the particle size distribution that gives the best fit between experimental measurements and predictions based on light scattering theory. Particle size was reported as volume-surface mean diameter, d_{32} ($=\Sigma n_i d_i^3/\Sigma n_i d_i^2$, where n_i is the number of particles with diameter d_i) and volume-weighted mean diameter, d_{43} ($=\Sigma n_i d_i^4/\Sigma n_i d_i^3$).

The mean size of the droplets in the W/O emulsions was determined by dynamic light scattering. The W/O emulsions were diluted to a droplet concentration of ~0.5 wt % with hexadecane (refractive index = 1.434, viscosity = 3.13 mPa s at 25 °C) as a dispersant to avoid multiple scattering effects. The particle size of the emulsions was then measured at 25 °C using a dynamic light scattering instrument (Zetasizer Nano-ZS, Malvern Instruments). This instrument measures the rate of diffusion of particles via intensity fluctuations. Particle size was reported as the scattering intensity-weighted mean diameter, *z*-average.

Optical Microscopy. Emulsions were gently agitated in a glass test tube before analysis to ensure that they were homogeneous. A drop of emulsion was placed on a microscope slide and then covered with a cover slip. The microstructures of the W/O emulsion and W/O/W emulsions were then observed using a conventional optical microscope (Nikon microscope Eclipse E400, Nikon Corp., Japan) equipped with a CCD camera (CCD-300-RC, DAGE-MTI, Michigan City, IN) connected to Digital Image Processing Software (Micro Video Instruments Inc., Avon, MA) and an Olympus Vanox optical microscope with a digital camera (Kodak EasyShare LS443, Japan). More than six pictures were taken for each sample, and a representative one was shown.

Sedimentation Stability Measurement. Sedimentation stability measurements were carried out on the W/O emulsions, where the water droplets tend to move downward because they are heavier than the surrounding oil phase. Ten grams of emulsion was transferred into a test tube (internal diameter = 15 mm, height = 125 mm), tightly sealed with a plastic cap, and then centrifuged at 6500 rpm for 30 min at room temperature (Centric Centrifuge, Fisher Scientific, Indiana, PA).

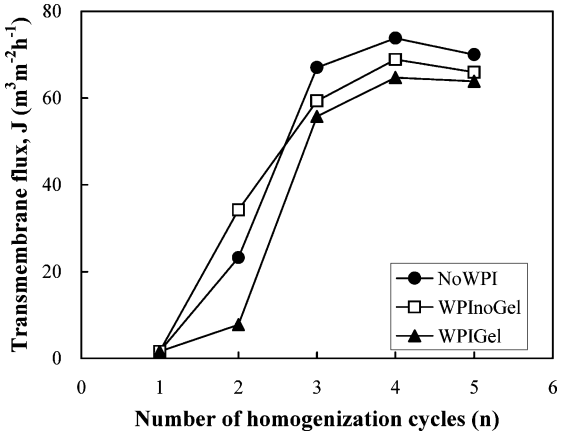

Figure 3. Dependence of transmembrane fluxes on the number of passes through the membrane homogenizer for W/O/W emulsions consisting of 20 wt % disperse phase (W/O emulsions) and 80 wt % aqueous phase (Tween 20 solution).

The extent of sedimentation was determined visually on the basis of the phase separation after the centrifugation step. However, water separation was not visually apparent in any of the systems investigated.

Creaming Stability Measurement. Creaming stability measurements were carried out on the W/O/W emulsions, where the W/O droplets tend to move upward because they are lighter than the surrounding water phase. Ten grams of emulsion were transferred into a test tube (internal diameter = 15 mm, height = 125 mm), tightly sealed with a plastic cap, and then stored for 1 day and 7 days at room temperature. After storage, some emulsions separated into an optically opaque "cream" layer at the top and a transparent (or turbid) "serum" layer at the bottom. We defined the serum layer as the sum of any turbid and transparent layers. The total height of the emulsions (H_E) and the height of the serum layer (H_S) were measured. The extent of creaming was characterized as % serum = $100(H_S/H_E)$. The percent serum provided indirect information about the extent of droplet aggregation in an emulsion. All measurements were made on at least two freshly prepared samples.

Determination of Yield. The "yield" of a W/O/W emulsion was defined as the percentage of water-soluble dye retained within the inner aqueous phase droplets following the homogenization of the W/O emulsion with aqueous phase. Initially, we therefore prepared a standard curve of absorbance versus dye concentration for the water-soluble fluorescent dye used in this study: 1,3,6,8-pyrenetetrasulfonic acid tetrasodium salt (PTSA) (*17*, *18*). A stock dye solution was prepared by dissolving 0.01% (w/v) PTSA in buffer solution (5 mM phosphate, 100 mM NaCl, pH 7). A standard curve was then prepared ($r^2 = 0.996$) by measuring the absorbance of diluted stock dye solutions at 374 nm using a UV−visible spectrophotometer. The dye concentration in the external aqueous phases collected from W/O/W emulsions was then determined using this standard curve.

PTSA (0.2%) was dispersed in the aqueous phase used to prepare the W/O emulsions as described above. W/O/W emulsions were then prepared by homogenizing 20 wt % W/O emulsions with 80 wt % aqueous surfactant solution (0.5 wt % Tween 20 in buffer solution) using either the HPVH (two passes, 14 MPa) or the MH (five passes, 0.1 MPa). Samples of the W/O/W emulsions were then centrifuged for 20 min at 40000 rpm using a centrifuge (Sorvall Centrifuges, DuPont Co., Wilmington, DE) to separate them into a creamed layer and a serum layer. An aliquot (3 mL) of the serum layer from each centrifuged sample was clarified using a syringe-driven filter unit (Millipore Corp., Bedford, MA), and their absorbance was recorded at 374 nm. This procedure was repeated on similar emulsions that had been prepared without dye to obtain blank values, and these were subsequently subtracted from their counterparts with dye. The concentration of dye present in the serum layer was determined from the standard curve.

The entrapment yield (Y) was expressed as the fraction of dye that remained encapsulated within the water droplets after homogenization

$$Y = \frac{M_i - M_e}{M_i} = 1 - \frac{M_e}{M_i} \quad (1)$$

where M_i is the mass of dye initially present in the internal water droplets in the W/O emulsion and M_e is the mass of dye present in the external water phase in the W/O/W emulsion after homogenization. The entrapment yield can be calculated if it is assumed that the amount of dye released from the inner water droplets is proportional to the amount of water released and that the dye is released due to expulsion of the internal water droplets during formation of the W/O/W emulsion. The mass of dye initially present in the internal water droplets in the W/O emulsion is then given by

$$M_i = C_i V_i = C_i \times \phi_{WO} \times \phi_{WOW} \times V_{WOW} \quad (2)$$

The mass of dye present in the external water phase in the W/O/W emulsion after homogenization is then given by

$$M_e = C_e[V_e + (1 - Y) \times V_i] = C_e[(1 - \phi_{WOW}) + (1 - Y) \times \phi_{WO} \times \phi_{WOW}] \times V_{WOW} \quad (3)$$

Here, C_i is the dye concentration in the internal aqueous phase of the W/O emulsion and C_e is the dye concentration measured in the external aqueous phase of the W/O/W emulsion after homogenization. V_i, V_e, and V_{WOW} are the volume of the internal water phase used to prepare the W/O emulsion, the volume of the external water phase used to prepare the W/O/W emulsion, and the volume of the overall emulsion, respectively. In addition, ϕ_{WO} is the volume fraction of water droplets in the W/O emulsion, whereas ϕ_{WOW} is the volume fraction of W/O droplets in the W/O/W emulsion. Substitution of eqs 2 and 3 into eq 1 gives

$$Y = 1 - \frac{C_e}{C_i - C_e}\left(\frac{1 - \phi_{WOW}}{\phi_{WO}\phi_{WOW}}\right) \quad (4)$$

In the remainder of this study, we express the entrapment yield as a percentage: % yield = $100Y$. For the particular system used in this study, $C_i = 0.2\%$ w/v, $\phi_{WO} \approx 0.2$, and $\phi_{WOW} \approx 0.2$. Hence, the yield is given by the following approximate expression: % yield = $100 \times (1 - 100C_e/[1 - 5C_e])$, when C_i and C_e are expressed in % w/v.

Viscosity Measurements. The viscosity of pure oil and pure oil containing 8 wt % PGPR was measured using a dynamic shear rheometer (Constant Stress Rheometer, CS-10, Bohlin Instruments, Cranbury, NJ). Samples were contained in a concentric cylinder cell (the diameter of the rotating inner cylinder was 25 mm, and the diameter of the static outer cylinder was 27.5 mm), and the viscosity of the samples was measured by heating and cooling the samples in a range of temperature from 25 to 90 °C at a shear stress of 0.1 Pa. No influence of the direction of the temperature change (heating versus cooling) on the measured viscosity was observed. Viscosity versus shear rate measurements indicated that both systems were Newtonian fluids; that is, the viscosity was independent of shear rate.

Statistical Analysis. Experiments were performed twice, and the mean and spread of the data were calculated from these duplicate measurements.

RESULTS AND DISCUSSION

Selection of PGPR as a Lipophilic Emulsifier for the Preparation of Water-in-Corn Oil Emulsions. The purpose of this experiment was to identify a suitable lipophilic emulsifier to prepare stable W/O emulsions. A number of nonionic surfactants (8 wt %) with a low hydrophile−lipophile balance (HLB) were therefore tested for their ability to form stable W/O emulsions: Span 60 (HLB = 4.7), Span 65 (HLB = 2.1), Span 80 (HLB = 4.3), and PGPR (HLB = ∼3). Span 60 and Span 65 were insoluble in corn oil at room temperature and so were

not used further. Span 80 was soluble in corn oil at room temperature, but when it was homogenized with water, the resulting W/O rapidly phase-separated. Previous researchers have prepared stable W/O emulsions using Span 80, but they used hydrocarbons (kerosene, $C_{10}H_{22}$ to $C_{16}H_{34}$) as the oil phase rather than corn oil (*6*). The reason for this observed difference might therefore be due to the different properties of the oils used—edible oils tend to be less hydrophobic and contain more surface active impurities than hydrocarbons (*19*, *20*). We found that PGPR was soluble in corn oil and that it could be used to prepare W/O emulsions that appeared to be stable at room temperature (~23 °C). Nevertheless, optical microscopy indicated that these emulsions contained a population of relatively large water droplets (**Figure 1**, nonheated). We observed that the PGPR−corn oil mixture was highly viscous at room temperature and postulated that this might result in inefficient disruption of the water droplets inside the high-pressure homogenizer (*21*). We also noticed that the PGPR−corn oil mixture became much less viscous upon heating, and so we examined the influence of preparation temperature on the formation of the W/O emulsions. To examine the influence of preparation temperature we prepared W/O emulsions under two different conditions: (i) heated emulsion (~40−50 °C), the oil and aqueous phases were heated to 50 °C then homogenized; or (ii) *nonheated emulsion* (~23 °C), the oil and aqueous phases were homogenized at room temperature. The temperature range of 40−50 °C was used for the preparation of the heated emulsions because this was sufficiently high to cause an appreciable decrease in oil phase viscosity while still being appreciably below the thermal denaturation temperature (T_m ~ 74 °C) of whey protein (so no gelation of the aqueous phase would occur prior to homogenization if WPI was present).

The microstructure of the nonheated and heated PGPR emulsions was then characterized by optical microscopy (**Figure 1**). Homogenizing the W/O emulsions at an elevated temperature clearly led to a smaller water droplet size. As mentioned earlier, this was probably because the viscosity of the oil phase decreased appreciably on heating, which made it easier for droplet disruption to occur within the homogenizer (*21*). For example, the viscosity of the oil phase (+PGPR) was 68 and 34 mPa s at 25 and 45 °C, respectively. In addition, there was no evidence of water droplet sedimentation in the W/O emulsions after 1 month of storage at room temperature, which suggested that they were stable to droplet flocculation. The mean droplet diameter (*z*-average) of both emulsions measured by dynamic light scattering was around 300 nm. Nevertheless, these measurements should be treated with caution because dynamic light scattering is not sensitive to the presence of slow-moving particles larger than about 3 μm (*21*), and there were clearly some droplets larger than this in our W/O emulsions.

In subsequent experiments we intended to gel the aqueous phase by incorporating WPI and heating the W/O emulsion above the thermal denaturation temperature of the proteins (see below). It is widely known that temperature can have a pronounced affect on the functional properties of nonionic surfactants; for example, surfactant molecules tend to become dehydrated and more lipophilic with increasing temperature (*21*). We therefore examined the affect of thermal processing (30−90 °C for 30 min) on the PGPR-stabilized emulsions. However, there was no significant difference in the microstructure (**Figure 1**) or mean particle size of the emulsions that had undergone heat treatment (data not shown). This observation is consistent with a previous study that reported that lipophilic surfactants did not change their character upon heating as much as hydrophilic surfactants (*22*).

In light of these results, the W/O emulsions used in the remainder of this study were prepared using PGPR as the emulsifier and were heated to 50 °C prior to homogenization.

Preparation and Characterization of W/O Emulsions. Previous researchers have suggested that the stability of W/O/W emulsions may be improved by encapsulating certain macromolecules in the inner aqueous phase, because this leads to a viscoelastic gel-like structure either throughout the inner aqueous droplet or at the interface between the inner aqueous phase and the intervening oil phase (*5*, *7*, *13*, *14*). In this study, we examined the possibility of improving the stability of W/O/W emulsions by the thermal gelation of whey proteins contained within the inner aqueous phase of the initial W/O emulsions. In this section we examined the influence of WPI gelation on the stability of W/O emulsions, because previous studies have shown that stable W/O/W emulsions can be formed only from stable W/O emulsions (*7*). Initially, we examined the influence of protein concentration (0−20 wt % with 2 wt % increments) on the ability of WPI to form a gel in aqueous solutions (5 mM phosphate buffer, 100 mM NaCl, pH 7) heated at 80 °C for 20 min. We found that optically opaque gels that would not flow when the test tubes containing them were inverted could be formed at WPI concentrations ≥4 wt %. A WPI concentration of 15 wt % was therefore selected for subsequent studies because it was well above this minimum value and it gave optically opaque (white) gels that appeared to be homogeneous and firm.

Three 20 wt % W/O emulsions were prepared by homogenizing aqueous phase (0 or 15 wt % WPI, 100 mM NaCl, pH 7) and oil phase (8 wt % PGPR in corn oil) together as described earlier: (i) 0 wt % WPI (No-WPI); (ii) 15% WPI, without heating (WPI-no-Gel); and (iii) 15% WPI, with heating to 80 °C for 20 min to gel the protein (WPI-Gel). After preparation, all three W/O emulsions contained relatively small water droplets that were evenly dispersed throughout the oil phase (**Figure 2**). Changes in the microstructure and sedimentation stability of these emulsions were then measured after they had been subjected to various environmental stresses, that is, (i) long-term storage (3 weeks at room temperature); (ii) shearing (0.5−7 min in a high-speed blender), and (iii) heating (30−90 °C for 30 min). Optical microscopy measurements indicated that there was no change in the overall microstructure of the three emulsions after storage, shearing or heating (data not shown), with the microstructures appearing similar to those shown in **Figure 2**. In addition, all three emulsions were stable to gravitational separation after they had been subjected to these environmental stresses, there being no evidence of the formation of an oil-rich layer at the top of the emulsion due to downward movement of the water droplets after 3 weeks of storage. These measurements indicated that the presence of gelled or nongelled WPI in the aqueous phase neither improved nor adversely affected the stability of the W/O emulsions. The stability of these emulsions may have been because the relatively high viscosity of the oil phase at room temperature (~68 mPa s) retarded movement (collisions or sedimentation) of the water droplets.

Preparation and Characterization of W/O/W Emulsions. The practical utilization of many W/O/W emulsions has been limited because the relatively large size of the oil droplets they contain makes them highly susceptible to creaming, coalescence, and flocculation (*7*). The oil droplet size in conventional O/W emulsions can usually be reduced by using intense homogenization conditions to disrupt the droplets, such as those found in a

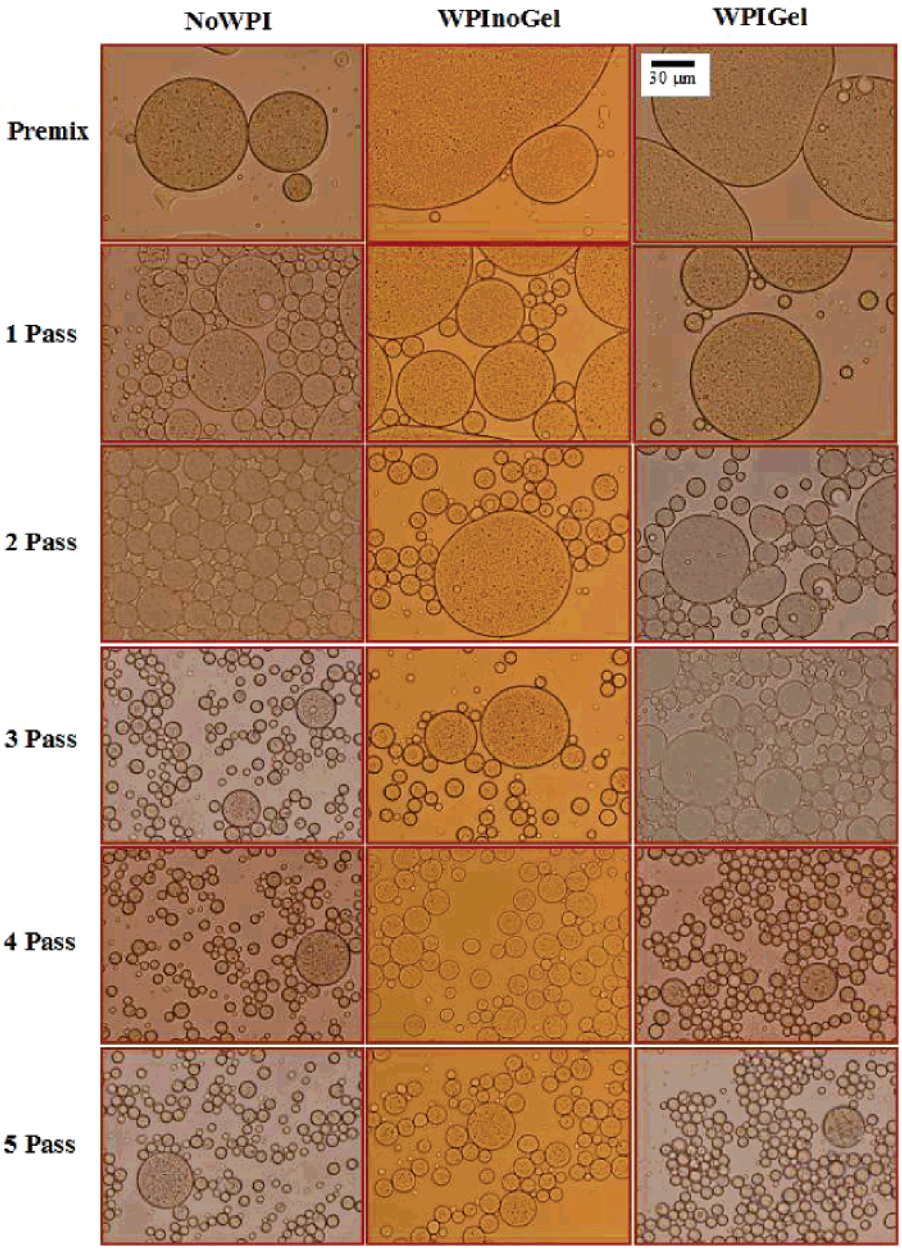

Figure 4. Optical microscopy images of W/O/W emulsions prepared by membrane emulsification using different numbers of passes through the homogenizer.

high-pressure valve homogenizer (*21*). However, this type of homogenizer usually cannot be used to prepare W/O/W emulsions because the intense homogenization conditions required to obtain small oil droplets promotes rupture of the internal water droplets, which leads to loss of water (*23*). We postulated that the gelation of the water droplets within the W/O emulsions used to prepare a W/O/W emulsion would reduce the tendency for water loss to occur during the secondary homogenization stage. Hence, it should be possible to use relatively high-intensity homogenization devices to prepare W/O/W emulsions, thereby creating smaller oil droplet sizes.

In this section, we investigated the effect of mechanical emulsification methods on the droplet characteristics of W/O/W emulsions containing WPI in the internal aqueous phase. W/O/W emulsions were prepared by homogenizing 20 wt % of W/O emulsion and 80 wt % aqueous solution (0.5 wt % Tween 20 in buffer) together using either a low-intensity (membrane homogenizer) or a high-intensity (high-pressure valve homogenizer) mechanical device. For each homogenization device, we prepared W/O/W emulsions using W/O emulsions containing either 0 or 15 wt % gelled (at 80 °C, 20 min) or nongelled WPI in the aqueous phase.

W/O/W Emulsions Prepared by Premix Membrane Emulsification. One of the most important parameters describing the efficient operation of a membrane homogenizer is the transmembrane flux, that is, the volume of material that passes through the membrane per unit of time per unit of surface area. The dependence of the transmembrane flux on emulsion composition and number of homogenization passes is shown in **Figure 3**. For all three W/O/W emulsions, the flux increased as the number of passes increased until it reached a limiting value at four passes, after which it decreased slightly. This indicates that all of the large droplets in the feed emulsion were completely disrupted, and only fine droplets that can easily pass through the pores remained at four passes.

The presence of W/O/W droplets in these emulsions was confirmed by optical microscopy (**Figure 4**). Some coarse water droplets were visible within some of the oil droplets, whereas fine water droplets were visible only as an inhomogeneous "texture" within the oil droplets. The mean diameter of the oil

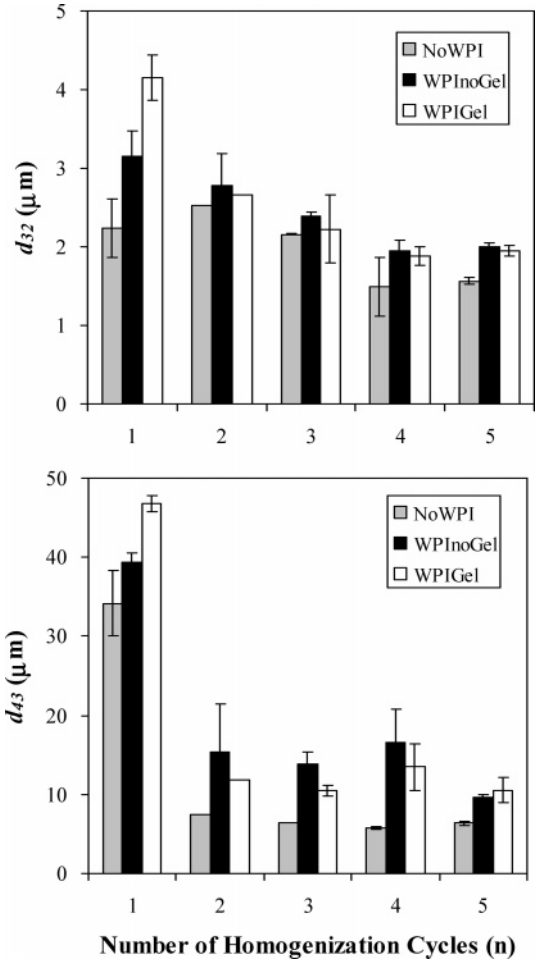

Figure 5. Dependence of mean particle diameters (d_{32} and d_{43}) of W/O/W emulsions on the number of passes through the membrane homogenizer.

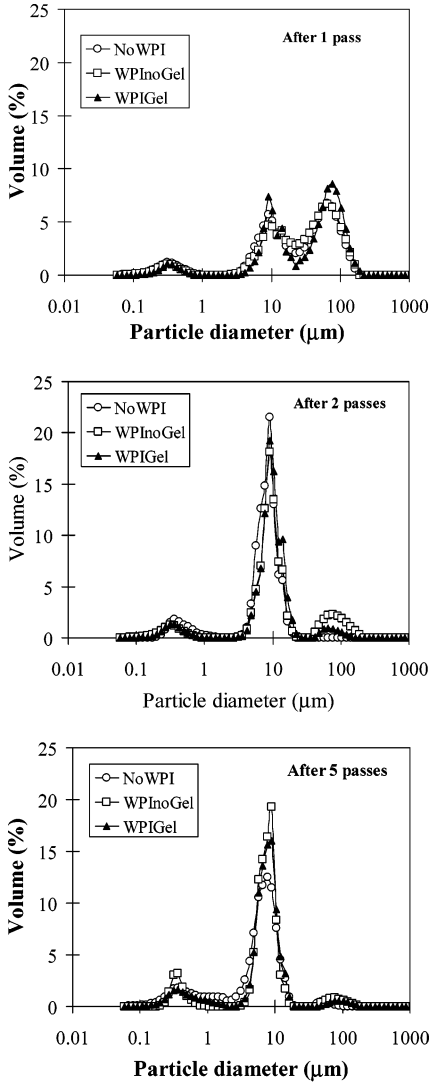

Figure 6. Dependence of particle size distributions of W/O/W emulsions on the number of passes through the membrane homogenizer.

droplets decreased as the number of passes increased, asymptotically approaching a limiting minimum value (**Figure 5**). The volume-surface mean particle diameter (d_{32}), which is more sensitive to the presence of small particles, of the W/O/W emulsions decreased fairly gently as the number of passes increased, eventually reaching values of 1.56 ± 0.04 μm for No-WPI, 2.01 ± 0.05 μm for WPI-no-Gel, and 1.95 ± 0.07 μm for WPI-Gel emulsions after five passes. On the other hand, there was a fairly steep decrease in the volume-weighted mean particle diameter (d_{43}), which is more sensitive to the presence of any large particles, when the number of passes increased from one to two, after which the mean particle diameter reached a fairly constant value: 6.4 ± 0.3 μm for No-WPI, 9.7 ± 0.3 μm for WPI-no-Gel, and 10.5 ± 1.6 μm for WPI-Gel emulsions after five passes. This change could also be seen when the full particle size distributions of the emulsions were examined (**Figure 6**). Although the W/O/W emulsions prepared by membrane emulsification displayed bimodal or trimodal distributions, the majority of droplets fell within a fairly narrow particle size range around 8 μm. For example, the $d < 1$, $1 < d < 10$, and $d > 10$ μm values after five passes were 15, 75, and 10 vol % for No-WPI; 12, 78, and 10 vol % for WPI-no-Gel; and 12, 76, and 13 vol % for WPI-Gel W/O/W emulsions. We always noticed that there was a small population (≤15%) of fine particles ($d < 1$ μm) measured by laser diffraction in the emulsions after membrane homogenization. This would account for the fact that when the emulsions were stored at room temperature for 24 h, they separated into an opaque layer at the top (containing the large droplets) and a turbid layer at the bottom (containing the small droplets): that is, serum percentages after five passes were 66, 54, and 66% for No-WPI, WPI-no-Gel, and WPI-Gel after storage for 1 day, respectively. These measurements suggested that there was not a strong dependence of the oil droplet size in the W/O/W emulsions on the nature of the aqueous phase within the initial W/O emulsion. It seems that the size distributions of droplets produced in the W/O/W emulsions were mainly determined by the homogenizer conditions. Having said this, the emulsions containing WPI (gelled or not gelled) had somewhat larger mean droplet diameters than those containing no WPI (**Figure 5**), suggesting that it may be harder to break up the W/O phase into droplets when the protein is present.

The yield of the W/O/W emulsions prepared by membrane homogenization was determined by measuring the percentage of dye that had been released from the internal water droplets after homogenization. We found that the % yield was greater than 99.8% for the No-WPI, WPI-no-Gel, and WPI-Gel W/O/W emulsions, which indicated that the internal water droplets in all of the original W/O emulsions were not disrupted by the membrane homogenization process. High yields (>90%) have also been reported previously for W/O/W emulsions when they were prepared using >6% PGPR in the oil phase (*14*).

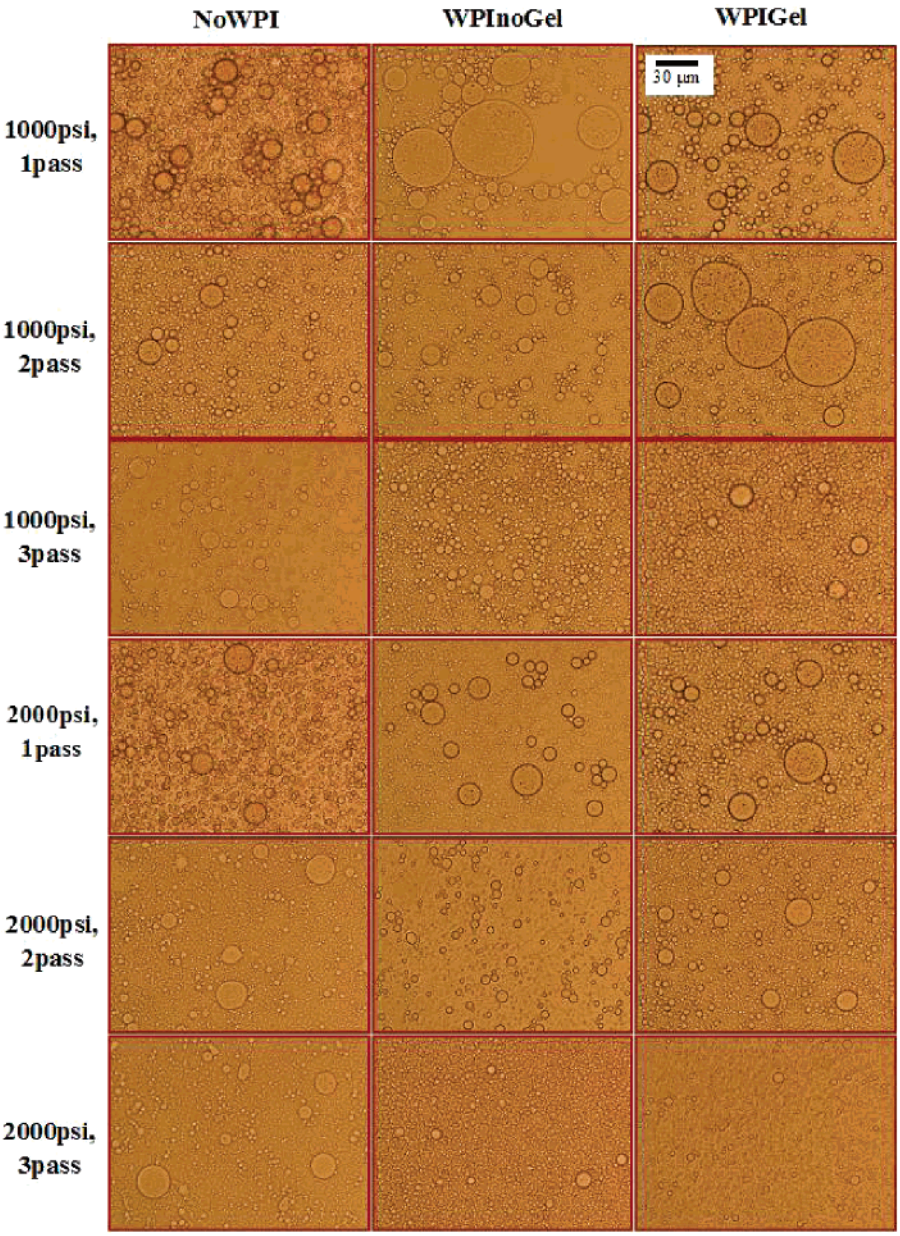

Figure 7. Optical microscopy images of W/O/W emulsions prepared by high-pressure homogenization.

W/O/W Emulsions Prepared by High-Pressure Homogenization. To inhibit creaming by making the outer droplets as small as possible, W/O/W emulsions were prepared by high-pressure valve homogenization using different homogenization conditions: pressure = 1000 psi (7 MPa) or 2000 psi (14 MPa); number of passes = 1−3. The microstructures of W/O/W emulsions produced using this process are shown in **Figure 7**. Emulsions prepared using high-pressure valve homogenization contained smaller droplets than those prepared using membrane emulsification (**Figures 4** and **7**). Small water droplets could be seen entrapped within some of the larger oil droplets produced using relatively mild homogenization conditions (two or fewer passes at 1000 psi; one of fewer passes at 2000 psi). However, it was not possible to see the water droplets when more severe homogenization conditions were used due to the relatively small size of the oil droplets produced. There was no large dependence of the droplet characteristics of the W/O/W emulsions on the presence of WPI and/or on heat gelation (**Figures 8** and **9**). Nevertheless, the W/O/W emulsions containing no WPI had significantly smaller mean droplet diameters (d_{32} and d_{43}) than those containing WPI, especially after three passes at 2000 psi, again suggesting that it may be easier to disrupt the W/O phase in the secondary homogenization stage when no WPI is present. However, the major factor affecting the droplet size distributions produced was the severity of the homogenization conditions, rather than the composition of the inner aqueous phase (**Figures 8** and **9**). The mean particle diameters (d_{32} and d_{43}) of the W/O/W emulsions decreased with an increase in homogenization pressure and number of passes, with the largest droplets having been produced at 1000 psi and one pass (d_{32} = 1.0, 1.2, and 1.3 μm and d_{43} = 4.5, 8.1, and 4.7 μm for No-WPI, WPI-no-Gel, and WPI-Gel, respectively) and the smallest sizes being produced at 2000 psi and three passes (d_{32} = 0.3, 0.4, and 0.5 μm and d_{43} = 0.7, 1.0, and 1.0 μm for No-WPI, WPI-no-Gel, and WPI-Gel, respectively) (**Figure 8**). In general, W/O/W emulsions prepared by high-pressure valve homogenization contained smaller droplets than those prepared using membrane emulsification (**Figure 5**), which could enhance the subsequent stability of W/O/W emulsions to gravitational separation because the velocity at which a droplet

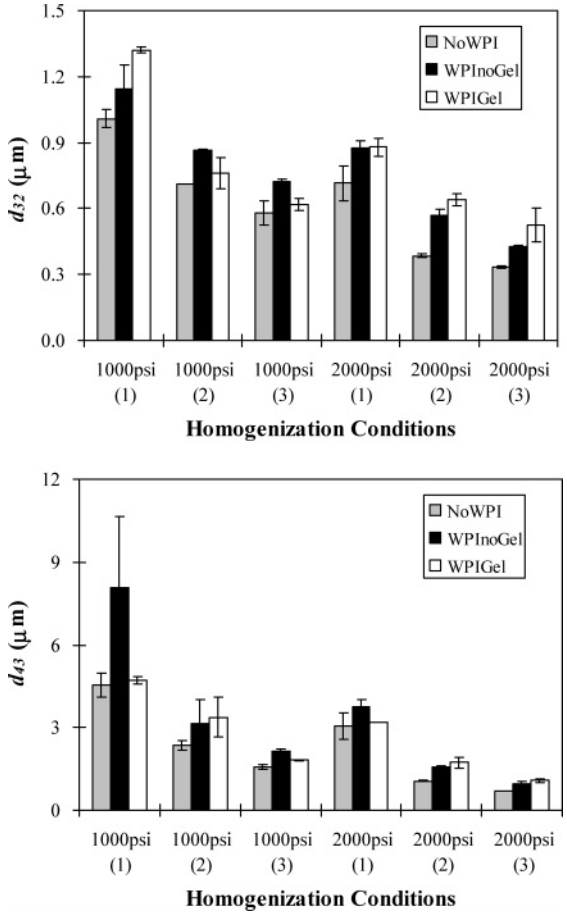

Figure 8. Dependence of mean particle diameters (d_{32} and d_{43}) of W/O/W emulsions prepared using a high-pressure valve homogenizer on the operating conditions: homogenization pressure and number of passes (in parentheses).

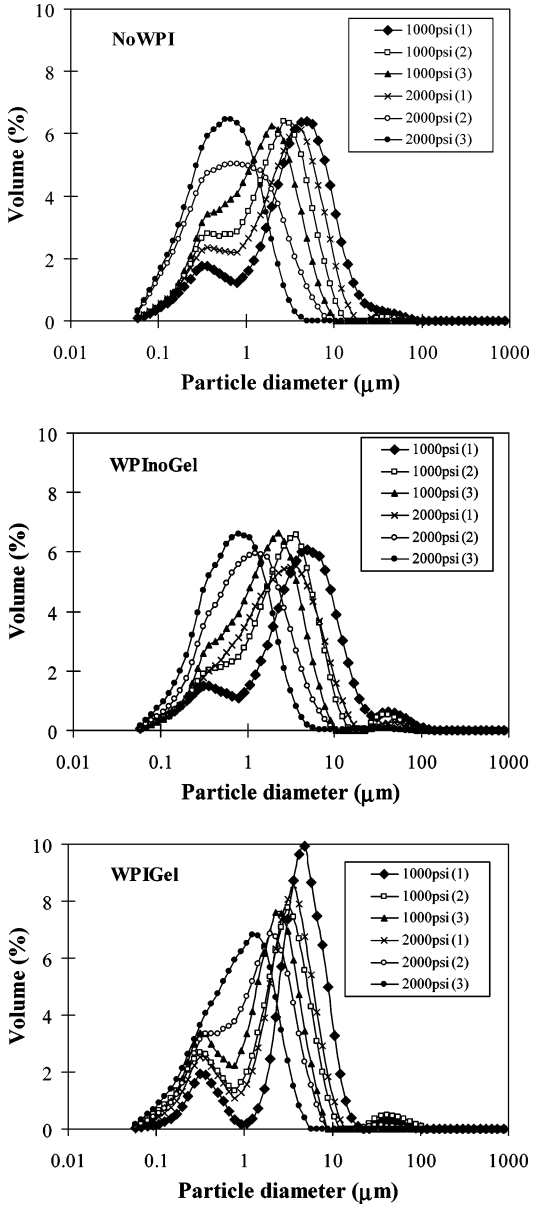

Figure 9. Dependence of particle size distributions of W/O/W emulsions prepared using a high-pressure valve homogenizer on the operating conditions: homogenization pressure and number of passes (in parentheses).

moves is proportional to the square of its radius (*21*). Indeed, no creaming was observed in all W/O/W emulsions after 1 day of storage except those prepared at 1000 psi and one pass (serum = 70, 63, and 71% for No-WPI, WPI-no-Gel, and WPI-Gel, respectively). On the other hand, the particle size distributions prepared by the high-pressure valve homogenizer were appreciably broader than those prepared by the membrane homogenizer (**Figures 6** and **9**). The influence of droplet polydispersity on the long-term stability of multiple emulsions is currently unknown, although this would be an important area for further study.

The yield of the W/O/W emulsions prepared by the high-pressure valve homogenizer was determined by measuring the percentage of dye that had been released from the inner water droplets after homogenization as explained earlier. We found that the % yield was 96.0 ± 2.0, 98.8 ± 0.7 and 98.3 ± 0.3 for the No-WPI, WPI-no-Gel, and WPI-Gel W/O/W emulsions, respectively. As mentioned earlier, similarly high yields (>90%) have also been reported for W/O/W emulsions prepared using >6% PGPR in the oil phase and a high-pressure valve homogenizer (*14*). These results suggest that the internal water droplets in the W/O/W emulsions were highly stable to expulsion during homogenization in all of the systems studied, irrespective of the nature of the internal aqueous phase.

In conclusion, this study has shown that W/O/W emulsions can be produced using either a high-pressure valve homogenizer or a membrane homogenizer that contained gelled internal water droplets. Initially, we hypothesized that W/O/W emulsions containing gelled water droplets would be more stable than those containing nongelled water droplets. Nevertheless, our results indicate that there was little influence of the nature of the internal aqueous phase on the size of the W/O droplets produced in the W/O/W emulsions or on the stability of the internal water droplets during homogenization. Instead, the major factor affecting the mean droplet size in the W/O/W emulsions was the type of homogenizer used to prepare them and the operating conditions. The high-pressure valve homogenizer was capable of producing smaller W/O droplets than the membrane homogenizer, but the particle size distribution was narrower for the membrane homogenizer. The mean W/O droplet size decreased as the number of passes through the membrane homogenizer increased or as the number of passes and homogenization pressure of the high-pressure valve homogenizer were increased. In summary, this study has shown that gelling the internal aqueous phase of W/O emulsions does not facilitate the formation of W/O/W emulsions for the system used in the work.

Nevertheless, the long-term stability of the W/O/W emulsions may be improved by gelling the internal water phase (e.g., by inhibiting coalescence or Ostwald ripening of the internal water droplets), but further research is needed on this subject.

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LITERATURE CITED

(1) Dickinson, E.; McClements, D. J. *Advances in Food Colloids*; Chapman and Hall: London, U.K., 1995.

(2) Auweter, H. Multiple-core encapsulation: double emulsion. In *Microencapsulation of Food Ingredients*; Vilstrup, P., Ed.; Leatherhead Food International: Surrey, U.K., 2001; pp 145−151.

(3) Garti, N.; Benichou, A. Recent developments in double emulsions for food applications. In *Food Emulsions*; Friberg, S. E., Larsson, K., Sjoblom, J., Eds.; Dekker: New York, 2004.

(4) Garti, N.; Aserin, A. Double emulsions stabilized by macromolecular surfactants. *Adv. Colloid Interface Sci.* **1996**, *65*, 37−69.

(5) Benichou, A.; Aserin, A.; Garti, N. Double emulsions stabilized with hybrids of natural polymers for entrapment and slow release of active matters. *Adv. Colloid Interface Sci.* **2004**, *108*−*109*, 29−41.

(6) Dickinson, E.; Evison, J.; Owusu, R. K.; Williams, A. Protein-stabilized water-in-oil-in-water emulsions. In *Gums and Stabilisers for the Food Industry*; Phillips, G. O., Williams, P. A., Wedlock, D. J., Eds.; Oxford University Press: New York, 1994; pp 91−101.

(7) Dickinson, E.; McClements, D. J. Water-in-oil-in-water multiple emulsions. In *Advances in Food Colloids*; Dickinson, E., McClements, D. J., Eds.; Blackie Academic and Professional: Glasgow, U.K., 1996; pp 280−300.

(8) Tanaka, H.; Matsumura, M.; Veliky, I. A. Diffusion characteristics of substrates in Ca-alginate gel beads. *Biotechnol. Bioeng.* **1984**, *26*, 53−58.

(9) Li, R. H.; Altreuter, D. H.; Gentile, F. T. Transport characterisation of hydrogel matrices for cell encapsulation. *Biotechnol. Bioeng.* **1996**, *50*, 365−373.

(10) Nakhare, S.; Vyas, S. P. Preparation and characterization of multiple emulsion based systems for controlled diclofenac sodium release. *J. Microencapsul.* **1996**, *13*, 281−292.

(11) Cho, Y. H.; Shim, H. K.; Park, J. Encapsulation of fish oil by an enzymatic gelation process using transglutaminase cross-linked proteins. *J. Food Sci.* **2003**, *68*, 2717−2723.

(12) Knoth, A.; Scherze, I.; Muschiolik, G. Stability of water-in-oil emulsions containing phosphatidylcholine-depleted lecithin. *Food Hydrocolloids* **2005**, *19*, 635−640.

(13) Weiss, J.; Scherze, I.; Muschiolik, G. Polysaccharide gel with multiple emulsion. *Food Hydrocolloids* **2005**, *19*, 605−615.

(14) Su, J.; Flanagan, J.; Hemar, Y.; Singh, H. Synergistic effects of polyglycerol ester of polyricinoleic acid and sodium caseinate on the stabilisation of water-oil-water emulsions. *Food Hydrocolloids* **2006**, *20*, 261−268.

(15) Garti, N.; Aserin, A.; Tiunova, I.; Binyamin, H. Double emulsions of water-in-oil-in-water stabilized by a-form fat microcrystals. Part 1: Selection of emulsifiers and fat microcrystalline particles. *J. Am. Oil Chem. Soc.* **1999**, *76*, 383−389.

(16) Kitabatake, N.; Fujita, Y.; Kinekawa, Y. Viscous sol and gel formation from process whey protein below 25 °C. *J. Food Sci.* **1996**, *61*, 500−503.

(17) Tokgoz, N. S.; Grossiord, J. L.; Fructus, A.; Seiller, M.; Prognon, P. Evaluation of two fluorescent probes for the characterization of W/O/W emulsions. *Int. J. Pharm.* 1**996**, *141*, 27−37.

(18) Adachi, S.; Imaoka, H.; Hasegawa, Y.; Matsuno, R. Preparation of a water-in-oil-in-water (W/O/W) type microcapsules by a single-droplet-drying method and change in encapsulation efficiency of a hydrophilic substance during storage. *Biosci., Biotechnol., Biochem.* **2003**, *67*, 1376−1381.

(19) Benichou, A.; Aserin, A.; Garti, N. Polyols, high pressure, and refractive indices equalization for improved stability of W/O emulsions for food applications. *J. Dispersion Sci. Technol.* **2001**, *22*, 269−280.

(20) Roberts, D. D.; Pollien, P.; Watzke, B. Experimental and modeling studies showing the effect of lipid type and level on flavor release from milk-based liquid emulsions. *J. Agric. Food Chem.* **2003**, *51*, 189−195.

(21) McClements, D. J. *Food Emulsions: Principles, Practice and Techniques*; CRC Press: Boca Raton, FL, 2005.

(22) Bjerregaard, S.; Vermehren, C.; Soderberg, I.; Frokjaer, S. Accelerated stability testing of a water-in-oil emulsion. *J. Dispersion Sci. Technol.* **2001**, *22*, 23−31.

(23) van der Graaf, S.; Schroen, C. G. P. H.; Boom, R. M. Preparation of double emulsions by membrane emulsification—a review. *J. Membr. Sci.* **2005**, *251*, 7−15.

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