Development of nanoemulsions by an emulsification-evaporation technique

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ABSTRACT

The aim of this study was to investigate the preparation of oil-in-water (O/W) nanoemulsions using an emulsification–evaporation process. Nanoemulsions were formed by homogenizing 10 wt% of an organic phase composed by corn oil and hexane mixed at different ratios, and 90 wt% emulsifier solution containing 1.0 wt % of Tween 20 (T20) or β-lactoglobulin (β-Lg). Nanoemulsions were prepared using a two-step homogenization process. First, a coarse O/W emulsion was formed by homogenizing of the organic and aqueous phases with a high-speed blender. Then, the coarse O/W emulsions were passed 5 times through a high pressure homogenizer at 15,000 psi to produce fine O/W emulsions; later these samples were subjected to evaporation to remove their hexane content. Particle sizes and PDI (polidispersity index) were determined by dynamic laser light scattering and nanoemulsions appearance was evaluated using a Turbiscan instrument by means of the area under the backscattering curve (AUC). All measurements were determined before and after hexane evaporation. Before hexane evaporation, the Z-average diameters of nanoemulsions prepared with T20 ranged from 171 to 95 nm, while those using β-Lg ranged from 170 to 125 nm. However, after hexane evaporation, the smallest Z-average diameters obtained using T20 and β-Lg were 60 and 97 nm, respectively. Particle size of nanoemulsions decreased with increasing hexane content both T20 and β-Lg. As the hexane content in organic phase increased, the particle size distribution after hexane evaporation shifted indeed to smaller particles and distribution pattern narrowed. In all cases PDI values were ≤ 0.14, which reflects a good monodispersity of the nanoemulsions. The backscattered light intensity decreased with increasing the particle size. The smaller AUC were obtained after hexane evaporation, and by increasing the hexane content in organic phase. However, the smallest AUC was reached after hexane evaporation using T20 and the highest hexane content in organic phase, so the nanoemulsions looked transparent or translucent.

Keywords: nanoemulsion; hexane; homogeneization; evaporation; droplet size.

INTRODUCTION

Nanotechnology is being used in a vast array of products in several industries, including the food industry where nanoemulsions have been proposed as a delivery system capable of controlling the release of functional ingredients [1-3]. Nanoemulsions are part of a broad class of multiphase colloidal dispersions which are non-equilibrium systems, having unique physicochemical and functional properties (e.g. high encapsulation efficiency, low turbidity, high bioavailability, and high physical stability), which depend not only on composition but also on the preparation method. In particular, nanoemulsions are nanometric-sized emulsions characterized by a great stability in suspension due to their very small droplet size. O/W nanoemulsions have a remarkable small oil droplets size, typically in the range of 10-100 nm, dispersed within a watery continuous phase, with each oil droplet being surrounded by a protective coating of emulsifier molecules [4]. The main particularity of nanoemulsions is their great stability of droplet suspension. A kinetic stability that lasts for months, stability against dilution or even against temperature changes, totally unlike the microemulsions (thermodynamically stable). In fact, nanoemulsions are thermodynamically unstable systems, due to the free energy of emulsion formation greater than zero [5]. Such growth has been fuelled by the potential of harnessing the large surface area to volume ratio of these materials to improve the bioavailability of bioactive ingredients [5].

Two main methods have been used to fabricate nanoemulsions: high energy or low energy. High energy emulsification methods use mechanical energy (e.g. high shear stirring, high-pressure homogenization, ultrasound) capable of generating intense disruptive forces that breakup the oil and water phases and lead to the formation of tiny oil droplets [4, 6]. Low energy emulsification methods are based on the spontaneous formation of tiny oil droplets within mixed oil-water-emulsifier systems when the solution or environmental
conditions are altered (e.g. the phase inversion temperature method and spontaneous emulsification methods) [4].

In the present research the process used to fabricate nanoemulsions was microfluidization, a high energy method, in combination to the solvent displacement technique to produce O/W nanoemulsions containing nano-scaled oil droplet size. Although the combination of these techniques permits to obtain nano-sized oil droplets, fundamental difficulties in this process lie in the selection of the food-grade organic solvents and removal of as much solvent as possible from the final product. However, several works [7, 8] have used homogenization and solvent displacement to produce nanoparticles like nanoemulsions, obtaining optimum results. Appearance, particle size, particle size distribution, and microstructure of nanoemulsions before and after solvent displacement were determined. Ultimately, the information obtained from this study should facilitate the rational design of delivery systems or functional components for utilization within the pharmaceutical, food, and other industries.

MATERIALS & METHODS

Materials
Powdered β-Lg was obtained from Davisco Foods International, Inc. (Lot # JE 002-8-415). Tween 20 was purchased from Acros Organics. Corn oil was acquired from a local supermarket and was used without further purification. Hexane was purchased from Fisher Scientific. Monobasic sodium phosphate, dibasic sodium phosphate and sodium azide were from Sigma-Aldrich. Purified water from a Nanopure water system (Nanopure Infinity, Barnstead International, Dubuque, IA) was used for the preparation of all solutions.

Solution preparation
Emulsions were conformed by two phases: aqueous and organic phases. Aqueous phase corresponded to emulsifier solutions prepared by dispersing 1.0 wt% of β-Lg or T20 or into 5 mM phosphate buffer solution (pH 7.0) containing 0.02 wt% sodium azide and stirring for at least 2 h. The solutions were kept overnight at 4 °C to ensure complete hydration. Organic phases consisting of corn oil and hexane with different ratios (100:0, 90:10, 75:25, 50:50, 25:75, 10:90, 5:95 w/w) were prepared by mixing them using a magnetic stirrer during 60 min at room temperature.

Nanoemulsion preparation
The initial emulsions were prepared by 10% organic phase and 90% aqueous phase. For the organic phase, different proportions of corn oil and hexane were used, while for the aqueous phase the same proportion of emulsifier and phosphate buffer (pH 7) was used (the emulsifier concentration was kept constant at 1.0 wt%). O/W emulsions were prepared using a two-step homogenization process. First, a coarse emulsion was prepared by homogenizing of the organic and aqueous phases with a high-speed blender for 2 min (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland). Second, coarse emulsions were immediately passed 5 times through a high pressure homogenizer (Microfluidics M-110Y, F20Y 75 μm interaction chamber, Newton, MA) at 15,000 psi to produce fine emulsions. To avoid the hexane evaporation during the high-pressure homogenization, the homogenization chamber was cooled using ice. When fine emulsions were obtained, the hexane was evaporated from them using a rotary evaporator (Büchi Rotavapor, RE111, Switzerland) at 55°C. Different evaporation times were used according to the hexane amount in the samples, which were determined by carrying out mass balances before and after hexane evaporation from samples. It is important to highlight that hexane and some of the water could be removed from the samples during evaporation.

Characterization of particle properties
Appearance: The nanoemulsions appearance was evaluated using a Turbiscan instrument (Turbiscan Classic MA 2000, Formulaction, Wynnewood, PA). The emulsion sample was transferred to a cylindrical glass cell (15 mm internal diameter, 150 mm height), and then carefully mixed to ensure they were homogeneous without introducing air bubbles. The sample was analyzed by a light beam emitted in near infrared (850 nm) wavelength which scanned the sample cell from the bottom to the top. Two synchronous optical sensors received respectively the light transmitted through the sample and the light backscattered by the sample. By scanning the sample at preset height intervals, a pattern of the light flux as a function of the sample height was obtained. In this study, the sample in the cell was scanned every 0.4 μm at room temperature while moving along the entire height of the cell (65 mm), and transmission and backscattering profiles as a function
of sample height were collected and analyzed using the instruments software program (Turbisoft version 1.21). To quantify differences in the appearance among samples, the area under the backscattering curve (AUC) was used and calculated by integration using the trapezoidal rule included as a tool in the SigmaPlot 10.0 software (Systat Software, San Jose, CA). Digital photographs were also taken both before and after hexane evaporation using a digital camera (PowerShot SD 1000, Canon, USA).

**Particle size measurements:** The effect of oil/hexane ratio in the organic phase of nanoemulsions on the particle size and particle size distribution was tested. Particle sizes were determined by dynamic laser light scattering instrument (Nano-ZS, Malvern Instruments, Worcestershire, UK) equipped with a 4mW helium/neon laser at a wavelength output of 633 nm. The particle size data is reported as the Z-average mean diameter. To avoid multiple scattering effects, nanoemulsion samples were diluted with 5 mM phosphate buffer (pH 7) at a ratio 1:500 (v/v) and then placed in a capillary test tube that was loaded into the instrument operating with predefined parameters. Samples were equilibrated for 1 min inside the instrument before dynamic light backscattering (detection angle = 173°) data were collected. The Z-average particle diameter was calculated by the instrument using the Stokes-Einstein equation, assuming the emulsion droplets to be spherical. The measurements were done at least in triplicate.

**Statistical analysis:** All experiments were carried out in triplicate using freshly prepared samples. The results were then reported as averages and standard deviations of these measurements. Analyses of variance were carried out when required using Statgraphic Statistical Package (Statistical Graphics Corporation, version 4.0, Rockville, USA) including multiple range tests ($p > 0.05$) for separation of least square means.

**RESULTS & DISCUSSION**

The initial organic phase composition dependence on the particle size of the nanoemulsions was measured using dynamic light scattering (Figure 1). Before and after hexane removal, the oil droplet size decreased with increasing the hexane concentration within the initial organic phase for $\beta$-Lg- or T20-stabilized O/W nanoemulsions. The particle size achieved when the organic phase of the nanoemulsions was conformed only by corn oil was ~ 170 nm. Before hexane evaporation, the particle size obtained for the maximum hexane concentration in the organic phase of $\beta$-Lg-stabilized O/W nanoemulsions was 125 nm, while T20-stabilized O/W nanoemulsions had a particle size of 95 nm; however after hexane removal, the particle sizes obtained were ~ 97 and 60 nm, respectively.

![Figure 1. Effect of the hexane content in the organic phase of O/W nanoemulsions on particle size before and after hexane evaporation. (A) T20-stabilized O/W nanoemulsions; (B) $\beta$-Lg-stabilized O/W nanoemulsions.](image)

The nanoemulsions produced were all monomodal, but the distribution peak was dependent on particle size, hexane concentration in organic phase, type of emulsifier and presence of hexane (Figure 2). T20-stabilized nanoemulsions presented the higher distribution peaks for the smaller particle sizes and higher hexane content in organic phase. However, these values were smaller for the nanoemulsions containing hexane in comparison with the nanoemulsions obtained after hexane removal. Similar behavior was found for $\beta$-Lg-stabilized nanoemulsions, but in this last case the particle size distributions were broader than the corresponding particle size distributions of nanoemulsions containing T20 and they presented peaks of distribution lower than the values obtained for T20-stabilized O/W nanoemulsions. To analyze the quality of the distributions, the polydispersity index (PDI) was calculated. PDI values ≤ 0.1 reflect a very good
monodispersity and quality of the nanoemulsions. For all T20-stabilized O/W nanoemulsions, the PDI values ranged from 0.04 to 0.12, before and after hexane evaporation; however, β-Lg-stabilized O/W nanoemulsions presented PDI values between 0.08 and 0.14.

The reason that the droplets in the nanoemulsions containing hexane in the organic phase were smaller than those in nanoemulsions with only corn oil can be attributed to the initial properties of emulsions previous to homogenization. This behavior was more pronounced for T20-stabilized nanoemulsions. The ability to disrupt droplets within a homogenizer depends on the bulk physicochemical properties of the oil and water phases, such as viscosity and interfacial tension. The viscosity of the disperse phase decreases as the corn oil concentration decreases, thereby decreasing the ratio of dispersed to continuous phase viscosities, which is known to facilitate droplet disruption within a high pressure homogenizer [9]. In addition, hexane has a lower interfacial tension with water than corn oil, and hence the interfacial tension between the dispersed and continuous phases should decrease as the corn oil concentration decreases, which would further favor droplet disruption. In turn, the droplets in the emulsions should shrink when hexane is removed from them by evaporation due to hexane is able to diffuse from organic to aqueous phase, which causes the droplets to shrink in size. Hence, one would expect smaller droplets to be produced for higher hexane contents within the disperse phase after homogenization. This suggests that the influence of hexane amount and hexane removal on oil droplet size is a critical parameter to produce nanoemulsions by using the techniques proposed in this research.

On the other hand, the particle size of nanoemulsions was affected by the type of emulsifier used to stabilize the system. T20-stabilized nanoemulsions showed smaller particle diameters and peaks of particle size distribution than β-Lg-stabilized nanoemulsions when the oil concentration in the organic phase was decreased. These differences could be possibly because of fast-adsorbing behaviour of T20 that is crucial in high-energy emulsification since droplet deformation and disruption, emulsifier adsorption and droplet collisions, all take place in a very short time. T20 is a water-soluble non-ionic surfactant with low molecular weight, while β-Lg is a biopolymer widely used in O/W emulsification. It has been shown that small surfactants such as T20 contain a hydrophilic head group that arranges toward aqueous phase, and one or several hydrophobic tails that tend to go into the oil phase of the emulsions. They are very mobile and can rapidly cover the new oil–water interface during emulsification. On the other hand, high molecular weight emulsifiers such as β-Lg contain a mixture of hydrophobic and hydrophilic groups that makes them very slow at diffusing and adsorbing onto the fresh interface compared with small surfactants [10].

Visually, the T20- or β-Lg-stabilized O/W nanoemulsions containing high hexane concentrations appeared more watery (less opaque) than those containing low hexane concentrations (Figure 3). Nanoemulsions have some interesting physical properties that distinguish them from ordinary microscale emulsions. For instance, microscale emulsions typically exhibit strong multiple scattering of visible light, and, as a result, have a white appearance. By contrast, the structures in nanoemulsions are much smaller than visible wavelengths, so most nanoemulsions appear optically transparent [5]. This above observation was confirmed by measurements of the AUC versus corn oil concentration before and after hexane evaporation (Figure 4). For all emulsions, the AUC increased with increasing corn oil concentration and mean particle size. The backscattered light intensity depends, among others parameters, on the particle size. It increases with increasing particle size. Thus, these results again highlight that smaller droplets can be produced using a
small-molecule surfactant than a protein under similar homogenization conditions. Hence, this study shows that it is difficult to prepare globular protein-stabilized nanoemulsions like β-Lg containing droplets small enough to only scatter light weakly.

**CONCLUSION**

The emulsification/evaporation technique was capable of producing nanoemulsions in almost all of our experiments. Nanoemulsions stabilized by T20 produced much smaller droplet sizes than β-Lg, before and after hexane evaporation. Before hexane evaporation, particle diameters of nanoemulsions prepared with T20 ranged from 171 to 95 nm, while those using β-Lg ranged from 170 to 125 nm. However, after hexane evaporation, the smallest particle diameters obtained using T20 and β-Lg were 60 and 97 nm, respectively. Particle size of nanoemulsions decreased with increasing hexane content both T20 and β-Lg. As the hexane content in organic phase increased, the particle size distribution after hexane evaporation shifted indeed to smaller particles and distribution pattern narrowed. In all cases PDI values were ≤ 0.14, which reflects a good monodispersity of the nanoemulsions. The backscattered light intensity decreased with increasing the particle size. The smaller AUC were obtained after hexane evaporation, and by increasing the hexane content in the organic phase. However, the smallest AUC was reached after hexane evaporation using T20 and the highest hexane content in the initial organic phase, so the nanoemulsions looked transparent or translucent. These results have important implications for the design and production of food-grade delivery systems based on nanoemulsions.
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