Replacement of Extruison by Temperature-Controlled Ultrasonication in Emulsome Production

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Abstract

Emulsomes are lipid-based drug delivery systems comprising of a solid fat core surrounded by phospholipid multi-layers. Stability, prolonged release profile, biocompatibility and high encapsulation capacity for lipophilic compounds are their most prominent features. This study investigates the suitability of a temperature-controlled ultrasonication procedure to replace extrusion step that is widely applied for production and homogenization of emulsomes. In this study, emulsomes homogenized through ultrasonication were evaluated based on physicochemical properties including size, morphology and zeta potential. Temperature-controlled ultrasonication yielded production of tripalmitin-based emulsomes with size 285.6 ± 68.7 nm and zeta potential 31.6 ± 9.3 mV in average. Hexadecylamine in the phospholipid composition conferred the formulation a net positive surface charge. The morphology of emulsomes were investigated with scanning electron microscope. To further investigate whether ultrasonication alters the encapsulation capacity of emulsomes for lipophilic compounds, curcumin was added to the formulation as a model lipophilic substance. The results suggested that the control on temperature at ultrasonication benefited on emulsome production with high reproducibility in size and zeta potential and regarded as the solid feature of the proposed approach. In conclusion, the study provides evidence that the temperature-controlled ultrasonication methodology can safely replace the extrusion step as a robust, reliable approach for production of emulsomes.

Keywords: Emulsome, Ultrasonication, Homogenization, Lipid-based drug delivery systems.

Emülzom Üretimindeki Ekstrüzyon Yügulamasının Sıcaklık Kontrollü Ultrasonikasyon ile Değiştirilmesi

Öz

Emülzomlar, birden çok fosfolipid katmanı ile çevrili kat bir yağ çekirdeğinden oluşan lipit-bazlı ilaç iletim sistemleridir. Stabilite, uzun süreli salım profili, biyoyuvaluluk ve lipofilik bileşikler için yüksek yükleme kapasitesi en belirgin özellikleri. Çalışma, emülzomların üretimi ve homojenizasyonu için yaygın olarak kullanılan ekstrüzyon basamağı yerine sıcaklık kontrolü bir ultrasonikasyon prosedürüne uygulanıla araştırılmaktadır. Bu çalışmada ultrasonikasyon ile homojenize edilen emülzomlar, boyut, morfoloji ve zeta potansiyeli gibi fizyokimyasal özelliklere göre değerlendirilmistir. Sıcaklık kontrollü ultrasonikasyon yöntemi ile, ortalama olarak 285.6 ± 68.7 nm ve zeta potansiyeli 31.6 ± 9.3 mV olan tripalmitin-bazlı emülzomların üretimi sağlanmıştır. Fosfolipid bileşimindeki heksadesilamin, formulasyona net bir pozitif yüzey yükü kazandırmıştır. Emülzomların morfolojisini taramalı elektron mikroskobu ile incelenmiştir. Ultrasonikasyonun emülzomlara uygulanan lipofilik bileşiklerin miktarını değiştirdiği ve deşiftrmediği araştırılmaktadır. Formülasyona lipofilik bileşik olarak kurkumin ilave edilmüşdür. Bu şekilde üretilen emülzomların fizyokimyasal özellikleri, boyut ve zeta potansiyeli açılarından yüksek tekrarlanabilirlik ile emülzom üretimine uygulanan metodun önemli bir özelliği olarak katkah sağladığı görülmüştür. Notice itibariyle elde edilen sonuçlar, sıcaklık kontrollü ultrasonikasyon metodolojisinin, emülzom üretimi için kullanılan ekstrüzyon uygulamasının yerini güvenle alabileceğine dair kanıt sunmaktadır.

Anahtar Kelimeler: Emülzom, Ultrasonikasyon, Homojenizasyon, Lipit-bazlı ilaç iletim sistemleri.
1. Introduction

Composed of an internal solid fat core surrounded by phospholipid (PL) multilayers, emulsome corresponds to a solid lipid nanoparticle formulated with the lack of any surfactant (Amselem et al., 1994, 2018). Emulsome is distinguished from an emulsion by its core that is in solid or liquid crystalline phase rather than oil in a fluid phase (Amselem et al., 1994, 1997, 2018). The solid core, made of fat or triglyceride, offers higher loading capacity for lipophilic compounds compared to the capacity of emulsions made of a fluid core (Amselem et al., 2018; Ucisik et al., 2015a).

The phospholipid layers surround the solid core, thereby making the emulsome similar to a liposome with its surface characteristics (Amselem et al., 1994; Ucisik et al., 2015a). This likeness enables the use of all surface modification strategies that are applicable to liposomes also for emulsomes emulsomes (Ucisik et al., 2013a, 2015a). This allows functionalization of emulsomes for various moieties to confer further abilities, e.g., passive or active cell targeting (Gupta & Vyas, 2007; Ucisik et al., 2013a, 2015b; Vyas et al., 2010).

Drug release from the solid matrix of emulsomes is degradation-dependent and slower than the diffusion-controlled release from emulsions (Amselem et al., 1997; M. Ucisik et al., 2015a). This ensures sustained release of the lipophilic compounds over a prolonged period of time out of the formulation (Bolat et al., 2020; Heiati et al., 1997; Ucisik et al., 2013b).

With a lipid composition lacking surfactants, emulsomes are regarded as safe (Bolat et al., 2020; Pal et al., 2012; Yilmaz et al., 2020). In the absence of surfactants, emulsomes could preserve a high stability profile, which is not only attributed to the solid character of the internal core, but also to the multi-lamellar character of the phospholipids embracing the core (M. Ucisik et al., 2015a). Besides, phospholipid to total lipid ratio (PL:TL) plays also an essential role for the stability and the size of the formulation (Amselem et al., 1994, 1997; Ucisik et al., 2015a). Emulsomes are formulated as a self-assembly, where the temperature regulates the interactions of amphiphilic PL molecules with the hydrophobic lipid molecules during the formation of the assembly. The level of accessibility for PL is critical in determining the final size of the emulsomes (Ucisik et al., 2015a). A high PL:TL ratio may lead to high multi-lamellarity inside the inner structure, while excess amount of PL may lead to formation liposomes as byproducts (Amselem et al., 1994; Ucisik et al., 2015). Therefore, the ratio must be optimized for stability of emulsomes, while preventing the formation of impurities such as liposomes or emulsions.

On the other hand, the size of emulsomes can be additionally adjusted by various homogenization methodologies following the production, among which extrusion is largely preferred (Amselem et al., 1994; Ucisik et al., 2015). This methodology implies multiple passing of the emulsomes through a filter membrane yielding product formation with narrow size distribution. The use of membranes with a variety of pore sizes may enable formation of final emulsome products with various mean sizes from nano scales to micro scales. This provides the researcher the ability to tune the size of the emulsomes depending on the requirements of their research. However, it is also known that a certain amount of the lipid gets stuck on the membrane filter, corresponding to a loss of materials in emulsion production. The material loss may be occasionally non-trivial, in particular if the membrane pore sizes are chosen to be too small than the emulsomes primarily produced. Depending on the size requirements of the application or the presence of high-priced lipids or drug compounds, the extrusion process may become improper for emulsome production.

Addressing these limitations, this study investigates the suitability of temperature-controlled ultrasonication as an alternative to the mostly common extrusion methodology. Accordingly, the extrusion step of emulsome production was replaced with ultrasonication at a controlled temperature above the melting temperature of the lipids. Then the emulsomes were studied for their physicochemical properties such as size, zeta potential, polydispersity index (PDI), particle shape and surface morphology. To further investigate whether ultrasonication alters the encapsulation capacity of emulsomes, curcumin was added to the formulation as a model lipophilic compound. The physicochemical features as well as loading capacity of emulsomes produced with the introduced approach were compared with the values in the literature, where emulsomes were homogenized through an extrusion procedure for their production (Ucisik et al., 2013a, 2013b).

2. Material and Method

2.1. Materials

Glyceryl tripalmitate (tripalmitin, purity ≥99%), 1,2-dipalmitoyl-rac-glycero-3-phosphocholine (DPPC, ≥99%), cholesterol (≥96%), hexadecylamine (HDA, ≥99%), curcumin were purchased from Sigma-Aldrich, Germany. Chloroform (≥99.8%) was obtained from Fluka Chemika, Germany. Dimethyl sulfoxide (DMSO) was purchased from Fisher BioReagents, USA. All chemicals were used as received without further purification.

2.2. Production of Emulsomes

Emulsomes were produced applying the procedure described before with slight modifications (Ucisik et al., 2013a; Ucisik et al., 2013b). Accordingly, 80 mg tripalmitin with or without curcumin (32 mg) was dissolved in 1 ml chloroform. 8 mg DPPC, 4 mg cholesterol and 3.2 mg hexadecylamine with a molar ratio of 10:5:4 were dissolved separately in 1 ml of chloroform. Both lipid solutions were mixed by the help of a vortex and placed in a rotary flask in the rotary evaporator. Both lipid solutions were mixed, and the organic solvent was completely removed using a rotary evaporator (Rotavapor R-215, Büchi, Switzerland) under reduced pressure at 474 mbar and 60 °C. The formed dry film was hydrated with MilliQ water, the temperature was set to 80 °C and the solution was rotated until the pasty lipid film was resuspended. The obtained product was homogenized by ultrasonication bath at between 66-70 °C. Immediately after the ultrasonication, emulsome solution was placed on ice for 10 min. Emulsomes were stored at refrigeration temperature (4 °C) for storage.

2.3. Size and Zeta Potential Analysis

The mean particle size and zeta potential of emulsomes were determined by dynamic light scattering using a Zetasizer instrument (Nano ZS; Malvern Instruments, UK). Accordingly, samples were diluted in 1 mM KCl solution to suitable concentrations. All analyses were performed in the auto-measuring mode at 25°C. The average of triplicate analyses was recorded for each sample.
2.4. Size and Morphology Analysis with Scanning Electron Microscope (SEM)

The particular size of the emulsomes was further investigated with scanning electron microscopy (Zeiss EVO-HD -15; Germany) together with their shape and morphology. The pretreatment procedure comprised a short-term fixation where samples were placed on an aluminum holder and left at 4 °C overnight for drying. Dried samples were then treated with PBS buffer containing 2.5% glutaraldehyde for 15 minutes. Samples were washed three times with distilled water for 10 minutes. After gold sputtering (EM ACE200; Leica, Germany), samples were analyzed under the electron microscope.

2.5. Quantification of Curcumin encapsulated inside Emulsomes

The concentration of curcumin encapsulated inside emulsomes was estimated as described elsewhere (Bolat et al., 2020; Ucisik et al., 2013b; Yilmaz et al., 2020). A 1 mg/mL curcumin stock solution was prepared in DMSO. A standard curve, generated by successive dilution of the stock solution (5, 10, 20, 50, 100 μg/mL) in a 96-well microplate (NEST Scientific, Catalogue no. 701001, China), was used to determine curcumin concentrations in samples prepared by 1:10 dilution of emulsome suspension in DMSO. Sample absorbance was measured at 430 nm on a UV-vis spectrophotometer (Spectramax i3 Multi-Mode Microplate Reader Detection Platform; Molecular Devices, Sunnyvale, CA, USA). A standard curve was prepared from the values of standards. The curcumin concentrations of curcumin-loaded emulsomes were estimated by the readout of the absorbance intensity and corresponding concentration on the standard curve.

Table 1. Average size, zeta potential and PDI values of emulsomes prepared through homogenization with temperature-controlled ultrasound

| Name                      | Average Size (nm) | Average Zeta Potential (mV) | Average PDI |
|---------------------------|-------------------|----------------------------|-------------|
| Emulsome                  | 285.6 ± 68.7      | 31.6 ± 9.3                 | 0.3 ± 0.1   |
| Curcumin-loaded Emulsome  | 278.1 ± 70.4      | 27.2 ± 8.3                 | 0.3 ± 0.1   |

3. Results

Average diameter and zeta potential of distinct emulsome formulations was determined as 285.8 ± 68.7 nm and 31.6 ± 9.3 mV (Table 1), respectively. Polydispersity index was found in average as 0.3 ± 0.1. Size distribution curve showed that the particle sizes vary in a range between 50 and 600 nm (Figure 1), which was also confirmed by SEM analysis (Figure 2). SEM images displayed the spherical shape and smooth surface character of the emulsomes.

Encapsulation of negatively charged curcumin resulted in formation of emulsomes with an average size of 278.1 ± 70.4 nm and zeta potential of 27.2 ± 8.3 nm, where polydispersity index remained in average at 0.3 ± 0.1 (Table 1). The autofluorescence property of curcumin as the model lipophilic load in the system allowed analysis of emulsomes under confocal laser scanning microscope (Figure 3). The fluorescence emission confirmed curcumin’s presence inside the emulsomes.

In aqueous environment, emulsomes were observed to have a dispersed behavior. No aggregation was observed in confocal laser scanning microscopy. Confirming the encapsulation, fluorescence signal of curcumin was visualized at each emulsome particle. Estimated by absorbance measurements, encapsulation of curcumin was achieved in average around 0.07 mg/mL (in the range of 0.5–0.9 mg/mL).
largely influences the shift of the average size underneath the size distribution curve, as previously reported (Ucisik et al., 2013a). Therefore, independent from the PL:TL ratio, the average size of the particles formed are tunable with the membrane’s pore sizes. While smaller pore sizes allow the formation of smaller particles, choice of the pore sizes beneath a certain value may lead to loss of lipids stacked on the membrane in significant amounts. Ultrasonication, on the other hand, may provide a tuning in the particle formation by altering both the ultrasound power and the temperature applied (Siddiqui et al., 2014). In addition, additional sonication while cooling has been shown to increase the stability of lipid nanoparticle systems (Ban et al., 2014), which may further help maintenance for the sizes of emulsomes produced.

For lipid nanoparticles, zeta potential value is a parameter to evaluate the stability of the colloidal system (Shah et al., 2014). In the current study, hexadecylamine was used to confer a net positive surface charge to the emulsomes. The positive zeta potential value of emulsomes produced (31.6 ± 9.3 mV) were expected (Table 1), and a slight difference was apparent from the value for emulsomes of the same composition produced through extrusion earlier (i.e., 32.5 – 41.5 mV) (Ucisik et al., 2013a).

SEM analysis clearly demonstrated the size, shape and morphology of emulsomes (Figure 1). Similar to liposomes, the spherical shape and the smooth surface character displayed in SEM is considered to be a property resulting in as an outcome of having a phospholipid outermost surface (Amselem et al., 2018; Ucisik et al., 2015a). Confirming the DLS data, particle diameters were observed to vary between 100-700 nm. Particles with relatively small sizes appeared dark, while particles with larger sizes appeared brighter on the micrograph (Figure 1). Overall, the particles seem to have uniform character in both structure, shape and morphology, which further indicated that the applied ultrasonication methodology do not harm the particular integrity but eventually help the formation of spherical particles with uniformity. Uniformity has particular importance to obtain emulsomes with high purity. Formation of structurally different lipid particles such as 2D lipid bilayers, liposomes and micelles would be considered as impurities in the emulsome dispersions. This kind of impurities might stem from a non-optimum PL:TL ratio, which favors the assembly of lipids out of the excess PL or lipid (e.g., triglyceride) (Amselem et al., 1994, 2018; Ucisik et al., 2015a). For instance, excess concentrations of phosphatidylcholine as PL was reported to lead to formation of liposomes beside emulsomes as by-products (Paliwal et al., 2009). Besides, the production methodology is also important and should not cause disintegration of the particular structure. According to the SEM images, temperature-controlled ultrasonication procedure appears as a proper approach to homogenize emulsomes without interfering particle’s integrity and purity.

Curcumin was selected as the model lipophilic compound to be incorporated into emulsomes. The choice of curcumin enabled the comparison of the ultrasonication approach with the extrusion technique for emulsome production based upon their loading capacities. Curcumin-loaded emulsome formulations were previously reported and applied for different kind of disease models including cancer (Bolat et al., 2020; Ucisik et al., 2013a, 2013b, 2015a), neurodegenerative diseases (Yilmaz et al., 2020) and parasitic diseases such as Leishmaniasis (unpublished data). A side-by-side comparison of the formulations as product of different production methodologies is, however, for the first time available in the present study. The emulsomes produced in this study have exactly the same constituents with Ucisik et al. (2013),
also with the presence of hexadecylamine in its composition (Ucisik et al., 2013a, 2013b). This resemblance makes the head-by-head comparison of two different homogenization methodologies possible. Accordingly, the concentration of curcumin for emulsomes prepared by ultrasonication was in average around 0.07 mg/mL and the concentration varied between a range of 0.5–0.9 mg/mL for independent samples. Previously, Ucisik et al. (2013) reported that the extrusion methodology yielded encapsulation of curcumin inside emulsomes in average around 0.08 ± 0.02 mg/mL, which seems comparable to the present data (Ucisik et al., 2013b).

The auto-fluorescence properties of curcumin enabled visualization of curcumin inside emulsomes. Confocal microscopy images provided evidence for incorporation of curcumin inside the formulation and displayed that temperature-controlled ultrasonication methodology successfully yields a stable dispersed emulsome formulation in water (Figure 3). Additionally, the confocal microscopy investigations declined any substantial influence of the ultrasonication on dispersity of the emulsomes in water.

5. Conclusions and Recommendations

With its intrinsic features including the biocompatibility, high loading capacity, stability and prolonged release profile, emulsomes are promising drug delivery systems. Emulsomes are studied intensively for clinical use in various applications including viral (Ghosh et al., 2017; Vyas et al., 2010) and fungal/parasitic infections (Gill et al., 2011; Gupta et al., 2007; S. Gupta & Vyasa, 2007; Kretschmar et al., 2001; Pal et al., 2012), dermal therapy (Gupta et al., 2016; Kommana et al., 2016; Raza et al., 2013, 2014), cancer (Alhakamy et al., 2020, 2021; Awan et al., 2020; Bolat et al., 2020; El-Zaafarany et al., 2018; Giri et al., 2017; Li et al., 2011; Rizk et al., 2021; Ucisik et al., 2013b), neurodegenerative diseases (Yilmaz et al., 2020) and autoimmunity (Lowell et al., 1997; VanCott et al., 1998; Wu et al., 2010). The physicochemical features of the formulation such as size, surface morphology, polydispersity and surface potential play essential role in fate of the nanoparticle system in the applications. Purity and uniformity of emulsomes are there particularly important, which necessitates reproducible and robust production methodologies for its production.

As an alternative to extrusion homogenization method, that is widely utilized for emulsome production, temperature-controlled ultrasonication was examined in the presented study for its potential to yield emulsomes with the required characteristics. The presented data declines any significant influence of the ultrasonication on neither the amount of incorporated drug nor the size and dispersity of the emulsomes in water, and hence, provided evidence for suitability of temperature-controlled ultrasonication for emulsome production.

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