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European Journal of Pharmaceutics and Biopharmaceutics 67 (2007) 67–75
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

A study of the formulation design of acoustically active lipospheres as carriers for drug delivery

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Received 12 October 2006; accepted in revised form 17 January 2007
Available online 27 January 2007

Abstract

Acoustically active lipospheres (AALs) were prepared using perfluorocarbons and coconut oil as the cores of inner phase. These AALs were stabilized using coconut oil and phospholipid coatings. A lipophilic antioxidant, resveratrol, was the model drug loaded into the AALs. AALs with various percentages of perfluorocarbons and oil were prepared to examine their physicochemical and drug release properties. Co-emulsifiers such as Brij 98 and Pluronic F68 (PF68) were also incorporated into AALs for evaluation. AALs with high resveratrol encapsulation rates (~90%) were prepared, with a mean droplet size of 250–350 nm. The AALs produced with perfluorohexane as the core material had larger particle sizes than those with perfluoropentane. Resveratrol in these systems exhibited retarded drug release in both the presence and absence of plasma in vitro; the formulations with high oil and perfluorocarbon percentages showed the lowest drug release rates. The addition of PF68 slightly but significantly reduced resveratrol delivery from the AALs. Ultrasound treatment of 1 MHz produced an increase in the drug release from the systems, illustrating the drug-targeting effect of the combination of AALs and ultrasound.

Keywords: Acoustically active lipospheres; Resveratrol; Perfluorocarbons; Drug delivery; Formulation design

1. Introduction

An important prerequisite for the success of applying pharmacologically active drugs is site specificity. Drug encapsulation systems such as liposomes and emulsions have been introduced as parenteral drug carriers offering sustained release and organ targeting [1,2]. Microbubbles represent a new class of parenteral formulations with both diagnostic and therapeutic applications. Microbubbles are comprised of spherical voids or cavities filled by a gas. For medical applications, microbubbles are generally stabilized by a coating material such as phospholipids, surfactants, albumin, or polymers [3]. One prototypical kind of microbubbles using an oily layer around the microbubbles is referred to as acoustically active lipospheres or AALs [4]. Microbubbles can be used to deliver a drug or gene to a specific area of interest, and then ultrasound is used to burst the microbubbles, producing site-specific delivery [5].

Although many studies have investigated the in vivo or clinical advantages of microbubbles or AALs, no investigation of the formulation design has been conducted. The aim of the present study was to explore the effects of perfluorocarbon and oil on the physicochemical characteristics, drug delivery, and safety of AALs. The effect of incorporation of a co-emulsifier was also examined. The model drug used in this study was resveratrol. Resveratrol, a natural product from red wine, can play an important role in the therapy for cardiovascular diseases and cancers [6]. The oral bioavailability and initial half-life (8–14 min) are poor, leading to an irrelevant in vivo effect by oral administration compared to its powerful in vitro efficacy [7]. Hence other routes

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doi:10.1016/j.ejpb.2007.01.008

such as a parenteral injection should be considered in order to obtain better therapeutic benefits. Several phase I clinical trials are currently underway in several locations [8]. Resveratrol, like paclitaxel, is a typical drug with low aqueous solubility. More-extensive clinical use may be somewhat delayed due to a lack of appropriate delivery vehicles.

Lipid-coated microbubbles are potentially interesting delivery systems because of their ability to incorporate drugs for delivery aim [9]. Cavitation of microbubbles with ultrasound can be used to treat vascular thromboses and deliver drugs [3]. Hence it may be feasible to encapsulate resveratrol in AALs so that its pharmacological activity can be delivered to the cardiovascular system. Understanding how the formulation variables influence drug release and targeting may help predict the in vivo behavior of targeted AALs and the design of successful preparations.

2. Materials and methods

2.1. Materials

Perfluorohexane, coconut oil, and Pluronic F68 (PF68) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Perfluoropentane (Fluorinert® Fluid PF-5050) was obtained from Fluka Chemie (Buchs, Germany). Hydrogenated soybean phosphatidylcholine (Phospholipon® 80H) was supplied by American Lecithin Company (Oxford, CT, USA). Brij 98 (polyoxyethylene glycol mono-*n*-dodecyl ether) was from Acros Organics (Geel, Belgium). The cellulose membranes (Cellu-Sep® T3, with a molecular weight cutoff of 3500) were supplied by Membrane Filtration Products (Seguin, TX, USA).

2.2. Preparation of AALs

Soybean phosphatidylcholine (2.8%, w/v in the final product), cholesterol (1.2% in the final product), co-emulsifier (0.8% in the final product), and resveratrol (0.2% in the final product) were dissolved in an appropriate volume of chloroform–methanol (2:1). The organic solvent was evaporated in a rotary evaporator at 50 °C to obtain a thin film, and solvent traces were removed by maintaining the lipid film under a vacuum for 6 h. The film was hydrated with double-distilled water using a probe-type sonicator (Sonics and Materials VCX 600, CT, USA) by a 35-W intensity for 10 min at 60 °C. Then coconut oil (1.8% or 18% in the final product) and perfluorocarbon (16% or 32% in the final product) were added to the system, followed by high-shear homogenization (Pro Scientific Pro250, Monroe, CT, USA) for 5 min and sonication by a probe-type sonicator for 10 min at room temperature.

2.3. Droplet size and zeta potential

The mean particle size (*z*-average) and zeta potential of the AALs were measured by a laser scattering method (Malvern Nano ZS® 90, Worcestershire, UK). The formulations were diluted 100-fold with double-distilled water before the measurement. The determination was repeated three times/sample for three samples.

2.4. Resveratrol encapsulation in AALs

The AALs were centrifuged at 48,000*g* and 4 °C for 30 min in a Beckman Optima MAX® ultracentrifuge (Beckman Coulter, USA) in order to separate the incorporated drug from the free form. The supernatants were analyzed by HPLC for the free form to determine the encapsulation percentage.

2.5. HPLC analysis of resveratrol

The HPLC system for resveratrol included a Hitachi L-7110 pump, a Hitachi L-7200 sample processor, and a Hitachi L-7400 UV/visible detector. A 25-cm-long, 4-mm inner diameter stainless RP-18 column (Merck, Darmstadt, Germany) was used. The mobile phase was a methanol: pH 2.6 aqueous solution adjusted by acetic acid (45:55) at a flow rate of 1.0 ml/min. The UV/visible detector was set at 310 nm.

2.6. Evaporation of AALs

Two milliliters of AALs was pipetted into a cylindrical vial with an opening diameter of 2.5 cm. The sample vial was positioned in an incubator at 37 °C. At determined periods, the vial was weighed and the AAL weight remaining in the vial (%) was calculated. The total duration of the experiment was 12 h. The water and neat perfluorocarbons were also examined as the controls.

2.7. Erythrocyte hemolysis

Blood samples were obtained from a healthy donor by venipuncture and collected into test tubes containing 124 mM sodium citrate (one volume of sodium citrate solution + nine volumes of blood). The erythrocytes were immediately separated by centrifugation at 2000*g* for 5 min and washed three times with four volumes of a normal saline solution. Erythrocytes collected from 1 ml of blood were resuspended in 10 ml of normal saline. Immediately thereafter, 2.5 ml of 2% (w/v) AALs in normal saline was incubated with 0.1 ml of the erythrocyte suspension. Incubations were carried out at 37 °C with gentle tumbling of the test tubes. After 1 h of incubation, the samples were centrifuged for 5 min at 2000*g*. The absorbance of the supernatant was measured at 415 nm to determine the percentage of cells undergoing hemolysis. Hemolysis induced with double-distilled water was taken as 100%.

2.8. In vitro drug release

Resveratrol release from the AALs was measured using a Franz diffusion cell. The cellulose membrane was mounted between the donor and receptor compartments. The

donor medium consisted of 1 ml of vehicle containing resveratrol. The receptor medium consisted of 10 ml of 30% ethanol in pH 7.4 buffer in order to maintain sink conditions during the experiments. The available diffusion area between cells was 1.767 cm^2. The stirring rate and temperature were kept at 600 rpm and 37 °C, respectively. At appropriate intervals, 300-μl aliquots of the receptor medium were withdrawn and immediately replaced with an equal volume of fresh buffer. The amount of drug released was determined by high-performance liquid chromatography (HPLC). The effect of plasma on the release characteristics was investigated by adding 2 ml of human plasma to the donor phase as the release medium.

In the study examining the influence of ultrasound on resveratrol release from the AALs, the donor phase was exposed to ultrasound using a 1-MHz probe (Rich-Mar Sonitron® 2000, Inola, OK, USA) with a 1.5 W/cm^2 intensity and a 20% duty cycle. The head of the transducer was immersed in the mixture of AALs with plasma in the donor. The distance between the probe and the cellulose membrane was 1 cm. Ultrasound was applied for 2 h starting at the beginning of the in vitro release experiment.

2.9. Statistical analysis

The statistical analysis of differences among the various treatments was performed using unpaired Student's *t*-test. A 0.05 level of probability was taken as the level of significance. An ANOVA test was also used if necessary.

3. Results

3.1. Formulation design of AALs with different ratios of oil and perfluorocarbons

Microbubbles were prepared in this study using perfluorocarbons such as perfluoropentane (with a boiling point of 29 °C) and perfluorohexane (with a boiling point of 59 °C). Coconut oil was used as the oil phase because of its superiority in enhancing resveratrol solubility as compared to other oils [10]. Based on the additives and preparation procedure of the AALs formed in this study, they were formulated to load the interior with drug and perfluorocarbon, and the drug was incorporated into a layer of oil that forms a film around the microbubbles, which was then surrounded by a phospholipid membrane [11,12]. On visual inspection, the AALs were white and homogeneous. No precipitation or crystallization was observed, indicating a sufficient loading of AALs for the amount of resveratrol used.

To characterize the physicochemical properties of various AAL formulations, the size and zeta potential of the AAL droplets were examined by means of light scattering. The oil and perfluorocarbon were added to the AALs either in high percentages (18% for oil and 32% for perfluorocarbon) or low percentages (1.8% for oil and 16% for perfluorocarbon). Table 1 depicts AALs prepared with various ratios of oil and perfluoropentane (F1–F4). The perfluoropentane AALs showed droplet sizes of 250–350 nm (Table 1). The small size of the AALs, with substantial populations of submicron-sized bubbles, is an important factor in the efficacy for IV administration. The droplet size increased ($p < 0.05$) as the volume of the oil phase increased from 1.8% to 18% (F1 vs. F3, F2 vs. F4). On the other hand, a smaller size was formed ($p < 0.05$) as the perfluoropentane volume was doubled (F1 vs. F2, F3 vs. F4). Perfluorohexane instead of perfluoropentane was also used for AAL preparation (F5–F8). The size of the AALs with perfluorohexane was greater than that with perfluoropentane as shown in Table 1. The effects of the oil and perfluorocarbon percentages on droplet sizes were similar for both perfluorocarbons.

The absolute zeta potentials of these AALs were −50 to −80 mV as shown in Table 1. The negative zeta potential generally increased as the volume of the oil phase increased or the perfluorocarbon phase decreased. This same trend

Table 1
The composition and characterization of resveratrol AALs determined by vesicle size, zeta potential, and drug encapsulation

Code	Composition[a]	Size (nm)	Zeta potential (mV)	Encapsulation (%)
F1	18% Oil[b] + 32% C_5F_{12}	311.3 ± 49.8	−63.7 ± 1.1	90.0 ± 0.8
F2	18% Oil + 16% C_5F_{12}	358.9 ± 5.3	−81.9 ± 1.7	98.8 ± 0.5
F3	1.8% Oil + 32% C_5F_{12}	264.2 ± 3.1	−43.2 ± 14.1	89.9 ± 0.6
F4	1.8% Oil + 16% C_5F_{12}	338.4 ± 9.6	−77.3 ± 0.5	90.0 ± 0.9
F5	18% Oil + 32% C_6F_{14}	333.2 ± 16.8	−70.2 ± 0.9	92.1 ± 0.6
F6	18% Oil + 16% C_6F_{14}	613.1 ± 21.1	−76.5 ± 1.0	99.0 ± 0.8
F7	1.8% Oil + 32% C_6F_{14}	309.7 ± 17.7	−77.9 ± 1.6	91.1 ± 0.7
F8	1.8% Oil + 16% C_6F_{14}	350.7 ± 18.7	−72.5 ± 0.6	87.6 ± 1.5
F9	18% Oil + 32% C_5F_{12}+0.8% Brij98	611.1 ± 75.4	−58.4 ± 0.9	82.2 ± 0.4
F10	1.8% Oil + 16% C_5F_{12} + 0.8% Brij98	340.7 ± 21.9	−66.2 ± 0.1	75.4 ± 2.1
F11	18% Oil + 32% C_5F_{12} + 0.8% PF68[c]	290.0 ± 21.3	−53.6 ± 1.2	92.1 ± 0.6
F12	1.8% Oil + 16% C_5F_{12} + 0.8% PF68	229.2 ± 2.2	−53.2 ± 1.5	79.2 ± 0.5

Each value represents the mean ± SD ($n = 3$ for size and zeta potential, $n = 4$ for encapsulation).
[a] The ratio of composition is weight/volume (w/v) ratio (%).
[b] Oil, coconut oil.
[c] PF68, Pluronic F-68.

was found for the size distribution. The suitability of microbubbles for clinical investigations depends on several requirements. The first is the development of microbubbles capable of efficiently carrying a payload [12]. All formulations prepared with the different oil and perfluorocarbon percentages (F1–F8) showed resveratrol encapsulation levels exceeding 90% (Table 1). AALs with high oil and low perfluorocarbon levels (F2 and F6) contained the highest drug loading compared to the other systems.

Gas loss represents the primary destructive mechanism of microbubbles [13]. The evaporation of perfluoropentane AALs (F1–F4) by incubating them in a 37 °C oven was thus evaluated. As shown in Fig. 1, the formulation with low oil and high perfluoropentane (F3) demonstrated more-rapid evaporation than the other systems during the period from 2 to 6.5 h ($p < 0.05$). However, no significant difference ($p > 0.05$) in the weight remaining among all AALs was detected at the later stage of the experiment (6.5–12 h). The AALs had retained ~90% of their weight at the end of the experiment (12 h). A similar trend was observed for AALs composed of perfluorohexane (F5–F8, data not shown).

To evaluate the safety of AALs for injection, the hemolytic activity was determined as shown in Fig. 2. For both perfluoropentane and perfluorohexane AALs, the systems with low oil and high perfluorocarbon (F3 and F7) caused more-significant hemolysis compared to the others. The other AALs showed <10% hemolysis of erythrocytes.

The ability of perfluoropentane AALs to deliver resveratrol was examined by determining the drug release. An aqueous solution with 10% glycerol formal as a solubilizer was used for the control group. Glycerol formal is a mixture of 5-hydroxy-1,3-dioxane and 4-hydroxymethyl-1,3-dioxolane (60:40) which are cyclic ether compounds having two oxygen atoms in the ring structure and substituted by alcohol group. Previous studies have shown that glycerol formal is a good solubilizer (solubility >5000 μg/ml) for resveratrol [10,14]. As shown in Fig. 3, resveratrol in an aqueous solution showed the highest release. All AALs retarded the release of resveratrol, with the formulation containing high oil and perfluoropentane (F4) showing the slowest drug delivery rate. The slopes of the resulting plots in Fig. 3 were computed, and the release rates (μg/cm^2/h) were calculated from the slopes as shown in Table 2. There was no significant difference ($p > 0.05$) between the release rates of perfluoropentane and perfluorohexane.

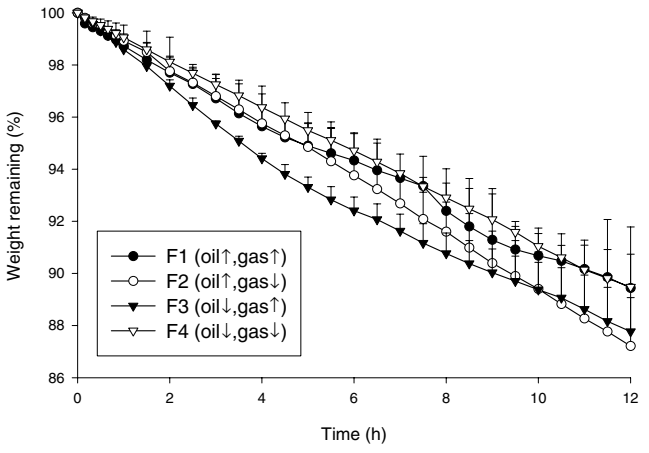

Fig. 1. Percentage weight remaining of perfluoropentane AALs (F1–F4) as a function of time in the evaporation experiment at 37 °C. Each value represents the mean and SD ($n = 3$).

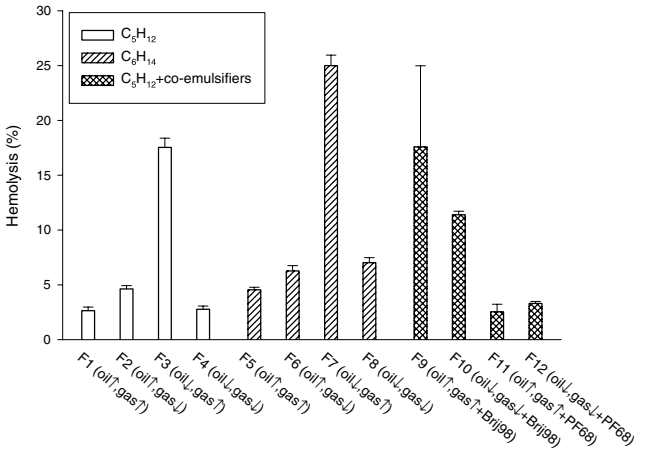

Fig. 2. Hemolysis percentage (%) after 1 h of incubation at 37 °C with various AAL formulations (F1–F12). Each value represents the mean and SD ($n = 3$).

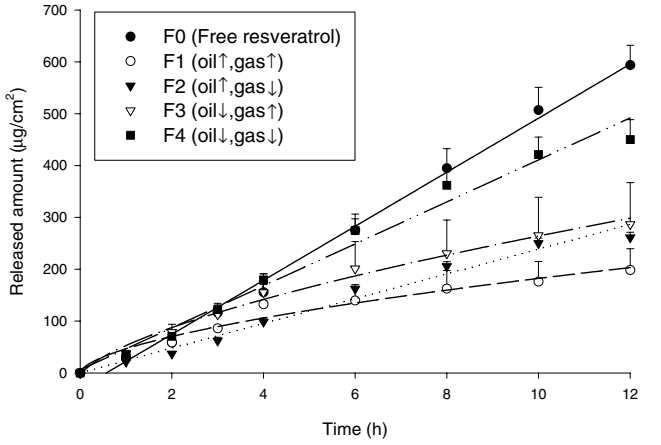

Fig. 3. In vitro release of resveratrol across a cellulose membrane from an aqueous solution with 10% glycerol formal (control, F0) and perfluoropentane AALs (F1–F4). Each value represents the mean and SD ($n = 4$).

3.2. Formulation design of AALs with different co-emulsifiers

Phospholipids alone cannot be used as an emulsifier because they do not produce emulsions over a wide range of oil and water compositions [15]. It is thus necessary to incorporate co-emulsifiers into such systems. This case may also be applicable to the formulation design of AALs. Because of safety considerations based on the hemolysis data, the high oil/perfluoropentane (F1) and low oil/perfluoropentane (F4) formulations were selected for further

Table 2
In vitro release rate of resveratrol (μg/cm^2/h) from AALs with different perfluorocarbons

Composition[a]	Perfluoropentane	Perfluorohexane
18% Oil[b] + 32% perfluorocarbon	15.9 ± 5.2 (F1)	21.8 ± 3.9 (F5)
18% Oil + 16% perfluorocarbon	23.9 ± 0.9 (F2)	27.7 ± 1.0 (F6)
1.8% Oil + 32% perfluorocarbon	23.8 ± 7.2 (F3)	31.5 ± 5.3 (F7)
1.8% Oil + 16% perfluorocarbon	40.5 ± 3.6 (F4)	37.9 ± 2.8 (F8)

Each value represents the mean ± SD ($n = 4$).
[a] The ratio of composition is weight/volume (w/v) ratio (%).
[b] Oil, coconut oil.

investigation of co-emulsifier incorporation. Brij 98 is a non-ionic surfactant with a hydrophile-lipophile balance (HLB) of 15.3 [16]. PF 68 is a hydrophilic non-ionic block copolymer with an HLB of 29.0 [17]. As shown in Table 1, the addition of Brij 98 led to an initial increase ($p < 0.05$) in the droplet size of AALs with high oil and perfluorocarbon (F9). However, it did not increase the droplet size in the system with low oil and perfluoropentane (F10). The addition of PF 68 reduced the size of the AALs (F11 and F12), although there was no significant difference ($p > 0.05$) between the formulations with high oil and perfluorocarbon in the presence or absence of PF 68. The incorporation of co-emulsifiers also led to a decrease in the surface charges of the droplet for all AALs tested (F9–F12). Co-emulsifiers significantly decreased ($p < 0.05$) resveratrol encapsulation in AALs, except for the PF 68-containing formulation with high oil and perfluoropentane (F11, Table 1).

As shown in Fig. 2, Brij 98 further increased the hemolysis of erythrocytes ($p < 0.05$). The incorporation of PF 68 in AALs did not affect the hemolytic activity ($p > 0.05$). Since PF 68 reduced the AAL size and maintained an acceptable level of hemolysis, it was selected as the co-emulsifier for subsequent drug release experiments. The AALs incorporating PF 68 exhibited further retardation of resveratrol release as demonstrated in Table 3. However, this reduction was limited since there was no significant difference ($p > 0.05$) between the release rates of with high oil and perfluorocarbon in the presence or absence of PF 68. In an attempt to simulate the drug release in vivo, a study was performed with plasma as the release medium, as it was anticipated that plasma proteins might have some effects on the release characteristics. As illustrated in Table 3, resveratrol release from the formulation was lower in plasma. The trends of drug release from various formulations in the presence of plasma were similar to those in the absence of plasma. The addition of PF 68 again reduced drug delivery from AALs in plasma.

Table 3
In vitro release rate of resveratrol (μg/cm^2/h) from perfluoropentane AALs with PF 68 in the presence and absence of plasma

Code	Composition[a]	Without plasma	With plasma
F0	Free resveratrol in H_2O	52.1 ± 3.9	30.4 ± 4.0
F1	18% Oil[b] + 32% C_5F_{12}	15.9 ± 5.2	9.8 ± 1.7
F4	1.8% Oil + 16% C_5F_{12}	40.5 ± 3.6	14.1 ± 0.6
F11	18% Oil + 32% C_5F_{12} + PF68[c]	10.1 ± 3.0	6.9 ± 1.6
F12	1.8% Oil + 16% C_5F_{12} + PF68	32.8 ± 3.3	10.7 ± 0.2

Each value represents the mean ± SD ($n = 4$).
[a] The ratio of composition is weight/volume (w/v) ratio (%).
[b] Oil, coconut oil.
[c] PF68, Pluronic F-68.

3.3. Effect of ultrasound on resveratrol release from AALs

AALs with ultrasound have the potential to deliver drugs and genes to specific target areas. To fully explore this new opportunity, it is necessary to understand the influence of ultrasound on drug delivery. Resveratrol release rates in the presence and absence of ultrasound application in plasma are shown in Fig. 4. The 1-MHz ultrasound treatment did not alter resveratrol release ($p > 0.05$) in the free form (F0, Fig. 4a). Resveratrol release significantly increased ($p < 0.05$) after ultrasound application in PF 68-containing AALs (F11 and F12). A larger discrepancy between the drug release with and without ultrasound was observed for the formulation with high oil and perfluoropentane (F11) compared to that with low oil and perfluoropentane (F12).

4. Discussion

It is important to design pharmaceutical formulations with appropriate additive percentages. As shown in Table 1, the droplet size increased as the oil phase increased from 1.8% to 18%. The formulation with oil at a concentration of 1.8% is close to a real microbubble form because of the low or negligible oil phase. On the other hand, the formulation with higher oil concentration (18%) simulates an oil-in-water emulsion or a liposphere form. Such an effect may be explained by a deficiency in phospholipids in the 18% oil formulation. The monolayer shell imparts stability to the AALs, while the emulsifier lowers interfacial tension, adds rigidity, and impedes gas escape and coalescence [18]. Since the emulsifier content in both systems was the same, the emulsifier–oil ratio was smaller in the 18% oil formulation, resulting in a larger droplet size [19,20]. The viscosity of coconut oil should be greater than that of the other additives in the AALs. It is also well established that increasing the viscosity of the emulsion phases leads to an increase in particle size [21]. This effect can also explain the higher particle size produced by perfluorohexane compared to perfluoropentane, since the values of kinematic viscosity measured by capillary viscometer for perfluorohexane and perfluoropentane are 0.66 and 0.40 cSt (centistokes, mm^2/s), respectively.

The increase in the perfluorocarbon percentage in AALs led to a decrease in size. This possibly indicates that the perfluorocarbon extension did not produce instability of the AALs, or 2.8% phospholipids were efficient at stabilizing the entire system. The particle numbers in these systems (i.e., the count rate or particles counted per second) studied were also monitored by a light-scattering method. The

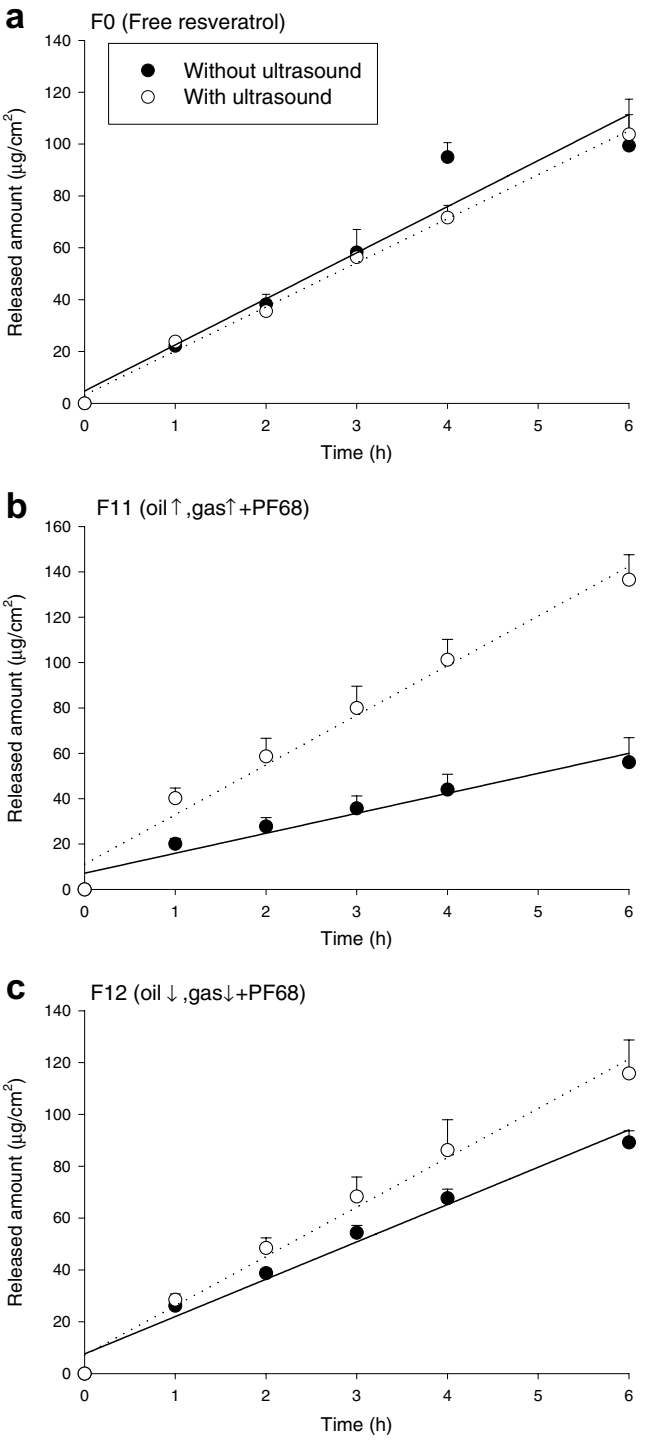

Fig. 4. Effect of a 1-MHz ultrasound at an intensity of 1.5 W/cm^2 for 2 h (with a duty cycle of 20%) on the in vitro release of resveratrol across a cellulose membrane from an aqueous solution with 10% glycerol formal (control, F0) (a), and perfluoropentane AALs with PF 68 (F11 and F12) (b and c). Each value represents the mean and SD ($n = 4$).

count rates of F1–F4 after a 100-fold dilution were 2.5×10^6, 2.1×10^6, 4.8×10^6, and 3.9×10^6, respectively. This result demonstrates that the formulations with a greater perfluorocarbon percentage produced higher particle numbers, indicating a smaller size for each particle. The detailed mechanisms of this phenomenon need to be elucidated in further studies.

The anionic fractions, such as phosphatidylserine and phosphatidylglycerol, in the phospholipids with 80% phosphatidylcholine (Phospholipon® 80H) were responsible for the negative surface charge of the AALs. Phosphatidylcholine exhibits no net charge [22]. The AALs showed a positive correlation between size and the zeta potential. Pearson's correlation coefficient (r) between the size and surface potential for perfluoropentane systems (F1–F4) was 0.99 ($p < 0.001$). The larger surface area of the larger particles may explain this phenomenon. This correlation was reduced but still significant in the perfluorohexane systems ($r = 0.86$, $p < 0.001$).

AALs showed an encapsulation percentage of >90% for resveratrol loading. This means that ~1.8 mg/ml of resveratrol (total dose in AALs = 2 mg/ml) could be entrapped into the lipospheres, which are about 130- and 10-fold higher than the resveratrol solubility in pH 7.4 buffer and coconut oil, respectively [10]. Drugs with an *n*-octanol/water partition coefficient (log *P*) exceeding 9 are required for stable entrapment in oil/water systems such as emulsions [1]. The log *P* of resveratrol is 3.1, which approximates the value of paclitaxel (log *P* of 3.6) [23]. A higher oil ratio generally enables a system to carry a higher drug concentration [21]. However, that was not the case in the present study (Table 1). Coconut oil showed a resveratrol solubility of 180 μg/ml [10]. Since the oil phase alone did not contribute to the sufficient solubility of resveratrol, perfluorocarbons in AALs must have played a role in drug loading. Other possible locations for loading resveratrol are the oil/water interface and aqueous micelles or liposomes formed in the aqueous phase [24]. Phospholipids in the interface and bilayers may offer some affinity to resveratrol. Intraperitoneal resveratrol at a dose of 1 mg/kg was sufficient to inhibit intimal hyperplasia of carotid artery in vivo [10]. Hence the resveratrol dose in AALs is applicable for in vivo status.

Ideal microbubbles should be stable enough in storage and in the vascular system [10]. The weight loss of AALs in the evaporation experiment showed that there were no significant differences ($p > 0.05$) among all AALs tested at the end of the experiment (12 h). Double-distilled water was used as the control. For water, 90.6% of the weight remained after 12 h of evaporation. This value approximated the weight remaining of the AALs, suggesting that perfluorocarbon loss by the AALs was limited. The evaporation rate of neat perfluorocarbons was also examined. Perfluoropentane and perfluorohexane were completely evaporated by a period of 50 min and 3.5 h, respectively. Although 37 °C is above the boiling temperature of perfluoropentane (29 °C), the evaporation rate of perfluoropentane AALs did not exceed that of perfluorohexane AALs. This suggests that the phospholipid monolayer and oil shell may have adequately protected the gas core from evaporation. The higher evaporation of perfluoropentane AALs with low oil and high perfluoropentane

(F3) in the middle stage of the experiment may have been due to an insufficient oil percentage for protecting the large amount of gas in the core.

Safety is also an important prerequisite for injections. The hemolytic potential of injectable forms has generally been found to be correlated to the severity of lesions [25]. Phospholipids are known to cause erythrocyte hemolysis [20]. Hemolysis by phospholipids can be reduced in oil/water emulsions [26]. The extent of hemolysis can also be decreased by increasing the oil concentration in emulsions [20]. This result may explain the low hemolysis of the AALs with high oil contents (F1, F2, F5, and F6). When the oil concentration was reduced, the hemolytic activity may have been enhanced. However, this effect was not observed in formulations with low perfluorocarbon (F4 and F8). This suggests that perfluorocarbons may cause an interaction between erythrocytes and AALs. Another possible reason for the high hemolysis of systems with low oil and high perfluorocarbon is the greater particle numbers increasing the probability of interactions between particles and erythrocytes.

The release of resveratrol from the aqueous solution was rapid with <60% being released over 12 h. The limited amount released may have been due to the use of the in vitro Franz cell. Since a drug is released to the definitive space of the receptor (10 ml in this study), drug loading in the receptor is limited. There may no longer be a concentration gradient between donor and receptor. Nevertheless, this method is still useful for differentiating the release capabilities of various formulations. The resveratrol in AAL formulations had slower drug-release profiles than those in aqueous solution. The sustained release of an incorporated drug in a delivery system is an important characteristic quite often correlated with improved pharmacokinetics and efficacy [27]. It was found that resveratrol release was retarded following an increase in the oil or perfluorocarbon concentration. The release profiles suggest that by altering the composition of the AALs, resveratrol release can be well controlled. This is important for the development of a system as a drug carrier for parenteral use.

The addition of Brij 98 to the AALs generally led to an initial increase in size. An opposite result was obtained with PF 68. One parameter for the surfactant film separating the water and oil domains is the spontaneous mean curvature, H_0. H_0 expresses the natural tendency of a monolayer to bend away from a flat geometry [28]. H_0 is positive for co-emulsifiers with a large polar head group and a small nonpolar group and decreases with the number and size of alkyl chains of the nonpolar group. This may have contributed to the smaller size of AALs with PF 68 (with an HLB of 29.0) than Brij 98 (with an HLB of 15.3). The addition of a hydrophilic co-emulsifier such as PF 68 was required to increase the hydrophilicity of the phospholipids in the films, thus favoring interfacial film curvature. However, Brij 98 incorporation did not increase the size of the AALs with low oil and perfluoropentane (F10). The low oil concentration (1.8%) in this formulation might make the H_0 theory inapplicable. Moreover, Brij 98 adequately stabilized formulations with such a low oil content. This effect may also explain the slight and insignificant size increase of AALs with high oil and perfluoropentane levels after incorporating PF 68 (F11). Increasing the volume of the oil phase led to an increase in the interfacial surface. Hence more co-emulsifiers should be added to stabilize the system [21].

Both Brij 98 and PF 68 shielded the negative charges of the droplets (Table 1). This may have been because the phospholipids were removed from the particles following the addition of copious co-emulsifiers. No significant correlation ($r = 0.29$) was detected between the size and zeta potential of co-emulsifier-containing AALs. This suggests that the relationship between the size and zeta potential is only applicable to plain AALs (F1–F8).

The deposition of co-emulsifiers at the interface of AALs generally reduced resveratrol encapsulation. The phospholipid monolayer may be rearranged in the presence of co-emulsifiers, resulting in some disorder in the monolayer arrangement and drug loss across the interface. Resveratrol encapsulation in PF 68-containing AALs was significantly higher ($p < 0.05$) than in Brij 98-containing systems. This may have been due to the hydrophilic PF 68 stabilizing the entire system based on the droplet size profiles.

PF 68 showed safe characteristics as a co-emulsifier for AALs according to the hemolysis profiles. A contrary result was obtained for Brij 98. A previous study suggested that non-ionic surfactants with structures and HLB values similar to those of Brij 98 (Renex®, polyoxyethylene nonylphenol) can induce hemolysis by affecting the fluidity of erythrocyte membranes [29].

Plasma is a viscous fluid consisting of about 91% water and 9% other substances such as proteins, ions, nutrients, and waste products. In the presence of plasma, drug release may be reduced because of the higher viscosity and longer path lengths for drug diffusion. Another explanation is that resveratrol may strongly bind to albumin and other proteins in plasma [30]. The associated resveratrol and albumin are then unable to pass across the cellulose membrane. A larger reduction in drug release in the presence of plasma was observed for AALs with low oil and perfluoropentane (F4 and F12). Surface-active proteins may destabilize the lipid-containing encapsulated nanoparticles such as emulsions and liposomes and accelerate the release of entrapped compounds. However, albumin may also form interfacial complexes with vesicles forming a rigid layer in the interfacial region, thereby reducing the release of the entrapped drug [25]. This effect may have been more significant in the formulation with PF 68 (F12) since this system showed low drug encapsulation but a relatively large reduction in resveratrol release. This result meshes with the aim of controlled-release dosage forms, in that the drug needs to be retained in the particles even after

administration to regulate the in vivo disposition of the drug.

Drug-filled microbubbles possess the potential to be ''magic bullet'' agents to deliver drugs to precise locations in the body; these precise locations can be determined by focusing ultrasound energy [3]. It is apparent that the use of ultrasound accelerated resveratrol release from AALs but not from the free form (control). As ultrasound pressure waves interact with the microbubbles, the microbubbles begin to oscillate or resonate. This effect results in collapse of the microbubbles [31,32], and abrupt drug release. Upon insonation of sufficient energy from ultrasound, however, they may convert to a gas, in turn increasing the acoustic reactivity and the potential for localized drug release [3]. It is also possible that perfluoropentane in AALs was already in gas form because of its boiling point at 29 °C. Another possible reason is that the rate of heating of AALs increases with high-intensity, focused ultrasound at a 1-MHz frequency [33]. Drug release may be enhanced following an increase in temperature. The temperature of donor medium before and after ultrasound application was monitored. The temperature was increased from 37.1 to 38.2 °C after a 2-h ultrasound treatment. This may suggest that the temperature effect is possible but finite. It can be observed from Fig. 4 that ultrasound showed a more-efficient ability to accelerate resveratrol release from AALs with high oil and perfluoropentane (F11) compared to those with low oil and perfluoropentane (F12). The larger droplet size may have contributed to the increased acoustic reflectivity, thus increasing the ultrasound efficacy [11,34].

5. Conclusions

Due to high lipophilic drug loading, lipid-coated microbubbles can be clinically administered undiluted at a high dose without drug precipitation. AALs of resveratrol have been developed for high drug loading with a mean droplet diameter of ~300 nm. Compared to the in vitro drug release rate from the free form (control), AALs slowed resveratrol delivery from ~50 μg/cm^2/h to 10–40 μg/cm^2/h depending on various AAL systems incorporated. Modulation of the oil and perfluorocarbon percentages can affect the physicochemical characteristics and drug release profiles of the systems. The formulation with high oil (18%) and perfluorocarbon (32%) percentages showed the lowest drug release among all of the AALs tested. The perfluorocarbon composition (perfluoropentane or perfluorohexane) did not largely change the amount of resveratrol released from the AALs, although a larger droplet size was obtained with perfluorohexane. Ultrasound at 1 MHz increased the amount of drug delivered from the AALs, supporting future in vivo application. In general, perfluorocarbons are biologically inert and pose little toxicological risk according to the hemolysis profiles. The perfluoropentane and coconut oil with high ratios in AALs may be a feasible strategy to develop microbubbles because of their small size, acceptable safety, sustained drug release, and high sensitivity to ultrasound treatment. PF 68 as a co-emulsifier was effective to further slow down resveratrol release. The safety evaluated by hemolysis was also maintained after incorporating PF 68. Microbubbles or AALs have potential to evolve into an important, new, widely applicable field with different clinical applications, including diagnostic and therapeutic aims. The results of this study offer preliminary data which can be useful for the future development and formulation design of AALs.

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