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RESEARCH PAPER

Optimization of Acoustic Liposomes for Improved *In Vitro* and *In Vivo* Stability

Nicolas Sax • Tetsuya Kodama

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ABSTRACT

Purpose Liposomes encapsulating perfluoropropane gas, termed acoustic liposomes (ALs), which can serve both for ultrasound (US) imaging and US-mediated gene delivery, have been reported. However, the echogenicity of ALs decreases within minutes *in vivo* due to gas diffusion and leakage, hindering time-consuming procedures such as contrast-enhanced 3D US imaging and raising the need for improvement of their stability.

Methods The stability of ALs preparations incorporating increasing ratios of anionic / unsaturated phospholipids, polyethylene glycol (PEG)ylated phospholipid and cholesterol was investigated by measurement of their reflectivity over time using a high-frequency US imaging system, both *in vitro* and *in vivo*.

Results The retention of echogenicity of ALs *in vitro* is enhanced with increasing molar ratios of PEGylated lipids. Addition of 10 molar percent of an anionic phospholipid resulted in a 31% longer half-life, while cholesterol had the opposite effect. Assessment of the stability of an optimized composition showed a more than 2-fold increase of the detection half-life in mice.

Conclusions Presence of a PEG coating not only serves to provide "stealth" properties *in vivo*, but also contributes to the retention of the encapsulated gas. The optimized ALs reported here can be used as a contrast agent for lengthier imaging procedures.

N. Sax • T. Kodama (✉)
Molecular Delivery System Laboratory
Graduate School of Biomedical Engineering
Tohoku University
4-1 Seiryo-machi, Aoba-ku
Sendai 980-0872, Japan
e-mail: kodama@bme.tohoku.ac.jp

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INTRODUCTION

Acoustic liposomes, liposomes encapsulating perfluoropropane (C_3F_8) gas inside PEGylated liposomes, were previously reported for use as ultrasound contrast agents (UCAs) for ultrasound imaging (1) and for ultrasound-mediated gene delivery, both *in vivo* (2,3) and *in vitro* (4). These liposomes simultaneously encapsulate both liquid and gas (5), but their structure remains unclear and multiple models have been proposed (Reviewed by Huang (6)). While easy to prepare, displaying a low toxicity and a good biocompatibility, these liposomes lose most of their echogenicity within a few minutes after injection. Similarly to some commercially available first generation contrast agents such as Levovist or Albunex (7–9), the encapsulated gas rapidly vanishes due to bubble collapse or diffusion to the outer, gas-undersaturated surrounding solution, i.e. blood (10,11). Given the recent popularity of PEGylated ALs (2,3,12–15), improvement of their stability is a question of interest. ALs with a better stability would allow for a longer imaging time *in vivo* and can be of great use for time-consuming imaging techniques such as 3D imaging of blood vessels. Optimized ALs might also enhance the drug delivery (transfection) efficiency as, in both cases, more ALs would be available for a longer period.

This study aims to develop ALs displaying an enhanced *in vivo* stability. For that purpose, two main strategies can be considered. First, the gas itself can be modified, and replaced with an even less water-soluble gas. The second possibility is the modification of the characteristics of the phospholipid bilayer to decrease its permeability. This can usually be achieved by modification of the lipid composition

or addition of a coating layer either electrostatically or covalently bound to the phospholipid heads. The low solubility and clinical use approval of perfluoropropane as the encapsulated gas make its improvement less pressing than that of the permeability of the shell. Some studies have been reporting optimization of the stability of ALs by modification of their phospholipid composition (16–18), but the ALs reported here differ in that they are coated with a polymer (PEG) and encapsulate low-solubility gas using a simpler, sonication-based method. Also, the relative stability of PEG-coated ALs and comparison with uncoated ones has to our knowledge not been investigated. Furthermore, multiple studies only report lifetimes measured *in vitro*, and whether or not they are good approximations of *in vivo* circulation lifetimes is unclear.

Here, we investigate the effect of surface coating with PEG, especially at the 6 molar % ratio used by multiple groups, including ours (2,3,12–15), and known to improve the circulation time of non-acoustic liposomes (19). The introduction of unsaturations in the hydrophobic tail chains of the lipids leads to a decrease in their phase transition temperature, resulting in either a solid-gel phase or a liquid phase bilayer. The possible relationship between bilayer phase and retention of gas is thus studied as well. Finally, the influence of an anionic phospholipid and that of cholesterol on the stability of gas encapsulation is reported. The present work compares the stability of multiple AL preparations *in vitro*, at first, before assessing that of the most stable candidate in a mouse model *in vivo*.

MATERIALS AND METHODS

Materials

1,2 distearoyl-sn-glycero-3-phosphocholine (DSPC), N-(Carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG2000-OMe / DSPE-PEG2k), 1,2-Distearoyl-sn-glycero-3-phosphoglycerol (DSPG, Sodium salt) and 1-Palmytoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were purchased from NOF Corporation, Tokyo, Japan. Cholesterol was obtained from Sigma, St. Louis, MO. Analytical grade chloroform and the 'phospholipid C' kit were purchased from Wako (Wako Pure Chemicals, Osaka, Japan).

Liposome Preparation

For all liposome samples, DSPC was used as the neutral matrix, and other phospholipids were added at its expense. First, the molar percent of DSPG was increased from 0 to 10, 20, then 30%. The polymer-coated equivalent samples were prepared by using a fixed molar percentage of DSPE-PEG2k (6 mol%), then increasing the molar ratio of DSPG to 40% by 10% increments. A similar approach was used to prepare liposomes incorporating increasing amounts of POPC (0 to 30% or 50% for the uncoated and PEG-coated samples, respectively) or cholesterol (0 - 7 - 15 - 23% for the uncoated samples, 0 - 7 - 15 - 23 - 30% for PEG-coated samples). Finally, the amount of incorporated DSPE-PEG2k was increased from 0% (DSPC-only liposomes) to 3, 6, 9 and 12 mol%.

For all preparations, the adequate amount of each lipid was weighted, deposited in a pear-shaped flask, and chloroform was added until complete dissolution of the lipids. Chloroform was evaporated under reduced pressure with a rotary evaporator (NVC-2100 / N-1000, Eyela, Tokyo, Japan) until a lipid film was obtained. The flask was then placed in a vacuum desiccator overnight for further drying. Next, the dried film was hydrated with 3 mL of phosphate-buffered saline (PBS) (pH 7.2 at room temperature, Sigma, St. Louis, MO) and the resulting multilamellar vesicles underwent 3 freeze-thawing cycles. The size of the liposomes was adjusted to <100 nm using extruding equipment (Northern Lipids Inc., Vancouver, BC, Canada) with three sizing filters (pore sizes: 100, 200 and 600 nm; Nuclepore Track-Etch Membrane, Whatman plc, UK). Lipid concentration was measured using the Wako Phospholipid C enzymatic method according to the manufacturer's protocol.

Preparation of Acoustic Liposomes

The protocol for encapsulation of C_3F_8 derived from that of Suzuki *et al.* (20). For each composition, the stock liposome suspension was diluted in PBS to a lipid concentration of 1 mg/mL, for a final volume of 1 mL. This suspension was then sonicated with a 20 kHz stick sonicator (130 W, Sonics & Materials, Newton, CT) at 50% amplifying strength for 1 min, under a constant flow of C_3F_8. The resulting ALs were stored in closed vials and used within 20 min after preparation.

Stability Measurements *In Vitro*

Echogenicity (sometimes termed reflectivity) of ALs at 37°C was measured using an US imaging system (VEVO770 Imaging System & RMV-708 scanhead, Visualsonics, Toronto, Ontario, Canada). A lipid concentration of 0.025 mg/mL (40× dilution in PBS) was chosen because it was the highest concentration not leading to acoustic shadowing. B-mode images were acquired at t=0, 2, 5, 10, 15, 20 then every 10 min after preparation. A 1 mm^2 region of interest (ROI) was set 1 mm above the well bottom, close to the focal distance of the US probe. The echogenicity of the AL suspension was quantitatively measured as the average grey scale value (GSV) inside the ROI. The experimental setup and calculation method are described in Fig. 1. After subtraction of the background GSV (PBS in absence of ALs), GSVs were then plotted as a function of time, as

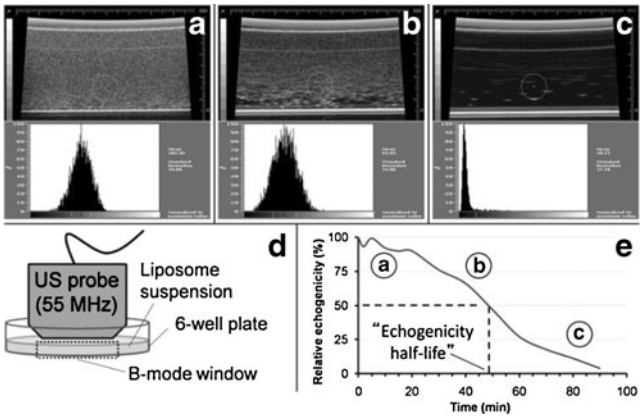

Fig. 1 Loss of echogenicity of ALs with time. US B-mode images were obtained immediately after encapsulation of gas (**a**), 40 min (**b**) and 90 min (**c**) later. Their respective grey scale value (GSV) histograms show a decrease in the US reflectivity of the suspension with time. A schematic representation of the ultrasound imaging experimental system is shown in (**d**), and a typical decrease curve of the 'Standard' composition, used for calculation of its half-life, is represented in (**e**).

percentage of the maximum value obtained for a single sample. The time taken for the GSV to drop to 50% of its maximum was termed 'echogenicity half-life'.

Stability Measurements *In Vivo*

All *in vivo* studies were performed in accordance with the ethical guidelines of the Tohoku University. MXH-10/Mo/*lpr*/*lpr* mice (12–20 weeks) displaying systemic lymphadenopathy with enlarged lymph nodes similar in size to human ones, were used (21). They were anesthetized with O_2/isoflurane (2–2.5% isoflurane) and placed in a supine position on a heating pad at 38°C. For each mouse, skin covering the left inguinal lymph node (LN) was shaved. The cross-section of the center of the LN was imaged using a high frequency US imaging system (VEVO770 Imaging System, Visualsonics, Toronto, Ontario, Canada) and a scanhead with a center frequency of 40 MHz (RMV-704, Visualsonics, Toronto, Ontario, Canada). The US scanhead was placed above the center of the inguinal LN and a ROI corresponding to its largest cross-section was defined. Blood irrigation of LNs was abundant enough to lead to contrast changes in absence/presence of ALs in blood. One hundred microliters of a 1 mg/ml (lipid concentration) suspension of ALs were thus injected into the caudal vein of the mouse. Changes in the average grayscale intensity inside the ROI resulting from the presence of ALs as a function of time were obtained using the built-in VEVO770 analysis software. Intensity values were then plotted against time, and the circulation half-life, defined as the time required for the AL-induced extra intensity to drop to 50% of its maximum, was calculated. For visual confirmation of AL presence, contrast information was also computed using the VEVO770 software and represented in green.

Size and Zeta-Potential Measurements

The size distribution and zeta potential of the acoustic liposomes used *in vivo* were assessed by dynamic and electrophoretic light scattering, respectively (Zeta-potential & Particle size analyzer, ELSZ-2, Otsuka Electronics Co., Ltd., Osaka, Japan). All measurements were performed in triplicate (n=3), in PBS (ionic strength=0.16 M).

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). Statistical significance between half-lives of liposome preparations was determined by unpaired Student's t test (two-tailed) and $P<0.05$ was considered statistically significant. All experiments were conducted at least in triplicate. Statistical calculations were performed with Excel 2007 (Microsoft, WA, USA).

RESULTS

In this study, the "echogenicity half-life" was used to compare the relative stability of ALs suspensions prepared using the same protocol, and encapsulating the same low-solubility gas.

Influence of the Phospholipid Composition on the *In Vitro* Stability of ALs

Liposome preparations incorporating the anionic phospholipid DSPG tend to show an increased stability compared to liposomes lacking DSPG (Fig. 2a). For uncoated liposomes, the increase in echogenicity half-life was only significant for 30 mol% DSPG, while in the case of PEG-coated liposomes (6 mol% DSPE-PEG), as little as 10 mol% DSPG was sufficient to significantly stabilize the ALs, resulting in an echogenicity half-life of 63 min, 31% longer than DSPC/DSPE-PEG2k-only preparation. No further stabilization of the PEGylated ALs was observed for molar ratios of DSPG higher than 10%.

On the other hand, molar ratios of the unsaturation-bearing POPC ranging from 0% to 50% did not significantly affect the stability of ALs coated with PEG (6 mol% DSPE-PEG2k) (Fig. 2b). For uncoated ALs, a slight decrease in stability can be observed for increasing amounts of POPC.

Increasing the molar ratio of cholesterol in DSPC-only ALs led to a decrease of the echogenicity half-life, yet with no statistical significance (Fig. 2c). Statistically significant influence of cholesterol could, however, be observed for PEGylated ALs: their echogenicity half-life went from 48.7 ± 0.8 min (0% cholesterol) to 42.8 ±1.3 min for 15% cholesterol, and further dropped to 25.1 ± 5.8 min for 30% cholesterol. For all samples assayed in Fig. 2a, b, c, the PEG-coated AL

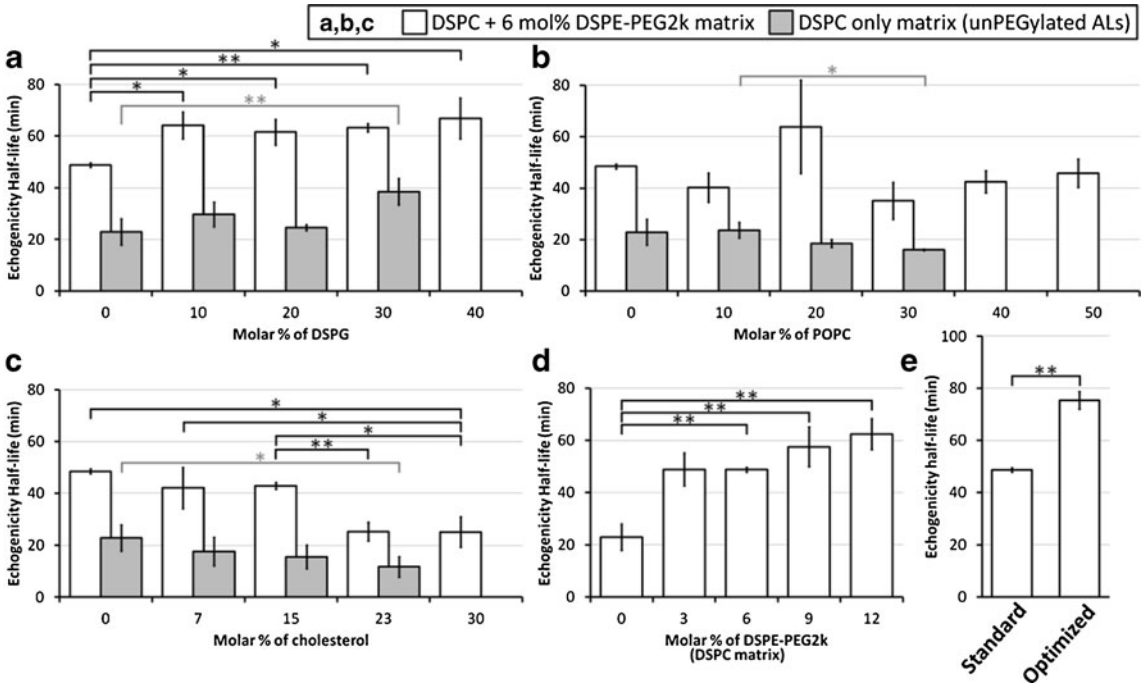

Fig. 2 Effect of increasing molar ratios of DSPG (**a**), POPC (**b**), cholesterol (**c**), DSPE-PEG2k (**d**) on the stability of ALs *in vitro*, and comparison of the Standard composition (DSPC : DSPE-PEG2k / 94:6) with the Optimized composition (DSPC : DSPG : DSPE-PEG2k / 78:10:12) (**e**). (**a, b, c**): Filled bars represent AL samples using only DSPC as a matrix; open bars represent PEGylated AL samples, incorporating 6 mol% of DSPE-PEG2k in the DSPC matrix. (* $P<0.05$, ** $P<0.01$).

suspensions always displayed a significantly higher half-life than their uncoated counterparts.

Figure 2d shows that incorporation of as little as 3 molar percent of DSPE-PEG2k is sufficient to significantly increase the half-life of a DSPC-only liposome (2.1 fold, $P<0.001$), and molar ratios of more than 6% tend to further increase that stability. The best stability inside the investigated range was obtained for 12% of PEGylated lipid, with an average half-life exceeding one hour (62.3±5.8 min, 28% longer than the standard −6 mol%− composition, $P=0.053$).

As both anionic and PEGylated lipids had led to enhanced *in vitro* stability in separate experiments, the effect of their combined presence on AL stability was investigated. An "optimized (OPT)" composition (DSPC : DSPG : DSPE-PEG2k 78:10:12) was thus compared to the "standard (STD)" composition widely reported and routinely used in our laboratory (DSPC : DSPE-PEG2k 94:6). *In vitro*, the optimized preparation displayed a half-life more than 50% longer than the standard one (75.3±3.3 min *versus* 48.7±0.8 min, $P=0.003$) (Fig. 2e).

In Vivo Characterization of the Optimized ALs

Echogenicity of ALs flowing in blood through the LN vasculature was evaluated. Presence of ALs could be detected for more than 6 min after injection (Fig. 3). Similarly to *in vitro* measurements, the brightness induced by the presence of gas inside liposomes in the LN decreased with time in an exponential manner at a slower rate for the optimized ALs. The calculated circulation half-life for the standard composition was 56.5±23.1 s, improving to 132±71 s for the optimized composition (+134%, $P=0.006$) (Fig. 4). The same experiment performed with limited US exposure (1 frame per 30 s) confirmed that the decrease in echogenicity was not induced by the continuous exposure of ALs to US during imaging (data not shown). Table 1 shows no difference in size between the standard and optimized preparations with an average diameter of 0.5 µm, but the optimized preparation incorporating a negatively charged lipid displayed a slightly lower zeta-potential.

DISCUSSION

Influence of the Phospholipid Composition on the *In Vitro* Stability of ALs

In the present results, the retention of echogenicity by ALs with different shell compositions was compared. The factor affecting stability the most was found to be the presence of a PEGylated phospholipid. PEG helps evade detection of the coated particles such as liposomes or gold nanorods by the mononuclear phagocyte system and prolong their plasma half-life (19,22). For this reason, and because of the cost-effectiveness and good initial echogenicity of PEGylated ALs, we successfully used such ALs in the past for imaging and sonoporation, using the

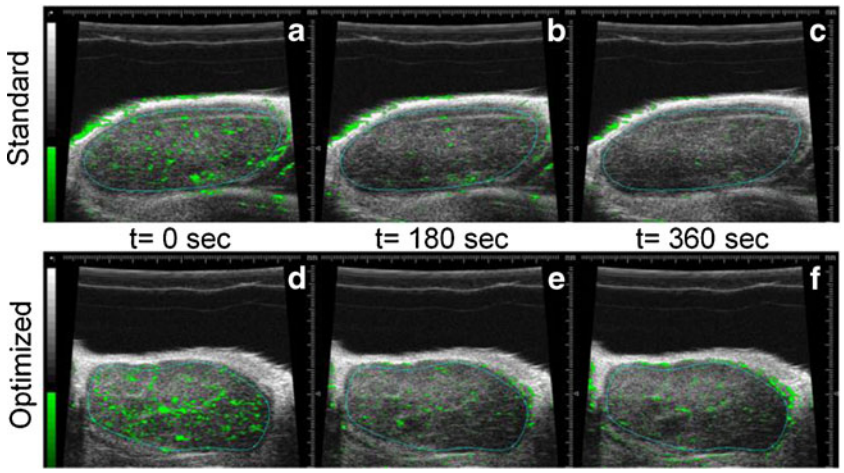

Fig. 3 2D ultrasound images of representative enlarged lymph nodes immediately after injection of ALs, then 180 and 360 sec after injection. Contrast induced by the presence of ALs is represented in green. Both Standard and Optimized preparations lead to a strong contrast immediately after injection (**a, d**, respectively), but a slower decrease can be observed for the optimized preparation (**d, e, f**) compared to the standard preparation (**a, b, c**).

lipid composition found to be most popular in the literature (DSPC – DSPE-PEG2k, 94:6 mol/mol).

The present study shows that PEG also appears to be playing a crucial role in the retention of encapsulated gas inside ALs, despite a resistance reportedly negligible (23). Thus, PEG might provide more than just an additional barrier to the leakage of perfluoropropane gas. As unPEGylated acoustic liposomes with good *in vitro* stability have been reported (17), a possible explanation is that the encapsulation of gas being carried out by sonication rather than by the more gentle freeze-drying method, the fine structure of the resulting ALs could differ, and PEG could help maintaining gas encapsulated between torn lipid monolayers before their resealing. While PEG derivate PEG40-stearate (PEG40S) was shown to negatively affect membrane permeability (24), DSPE-PEG2k, despite a slightly bigger polymer chain length, appears to have the opposite effect probably because its two hydrophobic tails do not disrupt the tight packing of the phospholipid monolayer. To assess the optimal ratio of PEG-bearing phospholipid, molar percentages ranging from 0% to 12% were assayed. Molar ratios higher than 12% were not investigated as, in such compositions, the conversion of some liposomes to micelles could lead to multiple particle populations. Also, for non-acoustic liposomes, 7 ± 2 mol% was shown to be the ratio leading to the best stability (25). As shown in Fig. 2, the longest half-life was achieved with 12% of DSPE-PEG2k. High molar ratios of DSPE-PEG2k probably result in a tighter packing of the polymer brushes further limiting diffusion of the gas through the shell.

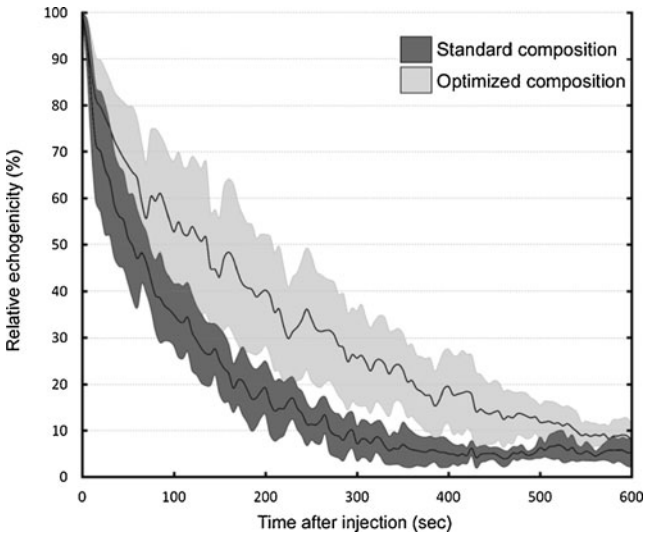

Fig. 4 Decrease kinetics of the echogenicity of the Standard and Optimized preparations in a mouse lymph node *in vivo*. Both follow an exponential decrease pattern, but at a slower rate for the optimized preparation. The resulting half-lives are 56.5 ± 23.1 and 132 ± 71 s, respectively. Filled areas represent one standard deviation (SD) ($n=6$).

Presence of an anionic phospholipid in the composition of acoustic liposomes also significantly increased their half-life. Such results have also been reported for ALs prepared by freeze-drying (17,18) and multiple AL preparations reported since then contain between 8 and 24 molar percent of DPPG (26,27). The mechanism by which anionic phospholipids enhance the stability of gas encapsulation is unclear, as gas is expected only to interact with the hydrophobic tail chains of the phospholipids, which are identical for DSPG and DSPC. The change in surface charge induced by the anionic lipid head might however limit fusion of neighboring liposomes, a process that could account for the leakage of part of the encapsulated gas.

Mechanical properties of lipid bilayers, such as rigidity or ability to quickly reseal small holes, depend on the fluidity (gel phase / liquid phase) of the bilayer (28,29) and the phase of a bilayer is conditioned by the temperature and lipid composition. As encapsulation of gas is performed by sonication of liposomes, at least part of the phospholipid

Table I Comparative Table of Physical Properties of the Standard and Optimized Preparations

			DSPC - DSPE-PEG2k (94:6)	DSPC - DSPG - DSPE-PEG2k (78:10:12)	*P*-value
Particle diameter (nm)			535 ± 189	520 ± 58	0.88
Zeta-potential (mV)		*(n=3)*	−2.1 ± 0.9	−6.1 ± 1.8	0.025
Echogenicity half-life	*In vitro* (min)		48.7 ± 0.8	75.4 ± 3.4	0.0036
	In vivo (sec)	*(n=6)*	56.5 ± 23.1	132.7 ± 71.3	0.0062

Measurements are given as mean ± standard deviation. Both *in vitro* and *in vivo* half-lives of the optimized preparation are significantly longer than those of the standard preparation ($P < 0.01$)

bilayer must be disrupted and behavior of the resulting ALs might differ between solid-gel phase and liquid phase. Increasing the molar ratio of unsaturated phospholipids through incorporation of POPC was thus of particular interest. Interestingly, no clear relationship between the molar ratio of unsaturated lipid and stability could be found. Despite no statistical significance, 20 mol% of POPC increased the echogenicity half-life of PEGylated ALs to over one hour. The effect of unsaturations and bilayer rigidity on the encapsulation stability shall thus be the object of further studies.

Cholesterol is known to rigidify liquid phase bilayers and disrupt solid phase ones, in non-acoustic liposomes (30). The present results show that it affects ALs the same way. As the standard matrix, either with or without PEGylated phospholipid, only contains saturated phospholipids in the solid phase at physiological temperature, cholesterol destabilizes the packing of the hydrophobic tails, thereby increasing the membrane permeability and the gas leakage speed.

The optimized composition developed here incorporated the anionic phospholipid DSPG at 10 mol. %, as higher concentrations did not further improve stability. The molar ratio of DSPE-PEG2k was set to 12%, as it led to the longest half-life in the range investigated. This preparation only differed from the standard preparation by its echogenicity half-life and its zeta-potential, more negative (−6.1 mV) than the standard preparation (−2.1 mV), yet too close to zero to be considered stable. However, no aggregation of the liposomal suspensions could be observed after months of storage. Indeed, PEG coating prevents intervesicle aggregation and shields the real surface potential of the liposomes, more negative than the measured zeta-potential (31).

It should finally be noted that, as the leakage speed of gas depends on the volume of surrounding liquid and the surface area of water-air interface, the echogenicity half-life of a given preparation can differ from that obtained with a different experimental setup. The relative stabilities and order between samples should however be unchanged.

In Vivo Characterization of the Optimized ALs

The intended use of ALs being *in vivo* US contrast imaging, the *in vivo* stability of the optimized preparation was investigated in a murine model. In order to precisely quantify the brightness induced by the presence of ALs and its decrease with time, imaging of an organ or tissue rich in blood vessels is required. Thus, well-irrigated solid tumors are a popular choice. In the present work, we used the enlarged lymph nodes of MXH-10/Mo/*lpr*/*lpr* mice for easy imaging of this organ without the need for inoculation and labor-intensive preparation. High blood irrigation of enlarged LNs led to brightness values similar to those obtained in solid tumors (data not shown). The half-life of the optimized preparation increased two-fold compared to the currently used preparation (See Fig. 4, Table I), and allowed observation of the lymph node vasculature for more than 5 min. However, the constant echogenicity decrease still limits the accuracy of 3D US imaging.

It is likely that the *in vivo* stability of ALs can be further increased by additional optimization of its shell. Also, the encapsulated perfluoropropane gas could be replaced by a perfluorocarbon gas with lower water solubility (32). Other strategies could consist in the optimization of the injection protocol while keeping the current AL composition. In the present study, we injected one single bolus of ALs to assess the stability of the gas encapsulation, but multiple boluses or slower continuous injection of contrast agent are widely adopted methods for easily increasing the imaging window.

CONCLUSION

Empirical optimization of the stability of acoustic liposomes by modification of their lipid composition led to ALs displaying enhanced stability both *in vitro* and *in vivo*. This new composition allowed for a longer imaging period *in vivo* and can be of use for time-consuming imaging procedures. We also showed that coating of the liposomes with a layer of PEG polymer played an important role in the retention of echogenicity *in vitro*. The rapid clearance of both AL preparations suggests that upon surface functionalization with targeting moieties, ALs would notably enhance the reflectivity of the target tissue, as signal from unbound ALs would decrease quickly. Future studies will therefore aim at an active-targeting of cancer or inflammation markers and the usability of the optimized composition for sonoporation and ultrasound-mediated gene delivery.

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