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Echogenic Liposome Compositions for Increased Retention of Ultrasound Reflectivity at Physiologic Temperature

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Abstract

Targetable echogenic liposomes (ELIP) for ultrasound enhancement of atheroma have recently been developed; however, their retention of echogenicity at physiological temperature is less than desirable. The purpose of this study was to improve ELIP stability and increase clinical potential. The approach utilized the original procedures but involved manipulation of the lipid composition by reducing the level of unsaturation of the phospholipids components to minimize the rate of loss of echogenicity. Echogenicity was measured using a 20 MHz intravascular ultrasound (IVUS) catheter and quantified (as mean gray scale values) using computer-assisted videoden-sitometry. The optimal preparation for retention of echogenicity stability at physiologic temperature was egg phosphatidylcholine/dipalmitoylphosphatidylcholine/dipalmitoylphos-phatidylethanolamine/ dipalmitoylphosphatidylglycerol/cholesterol (27:42:8:8:15, molar percent). This preparation retained 51 ± 3.5% of its echogenicity after 1 h at 37°C, more than 5× that retained by the previously descried preparation. In this composition nearly 2/3 of the phosphosphatidylcholine is fully saturated. Such an increase in saturation is anticipated to stiffen the lipid acyl chains. The air pockets that are responsible for reflection of ultrasound waves can be assumed to be stabilized by a lipid monolayer at the interface between the air and bulk water. The increased rigidity of that monolayer is presumed to be responsible for reducing the loss of air and extending the duration of echogenic activity. The stability of this improved formulation now appears to be more than adequate for clinical applications.

Keywords

bilayer; ultrasound; stability; freeze drying/lyophilization; formulation; imaging methods; lipids; liposomes; phospholipids; physical stability

INTRODUCTION

Echogenic liposomes (ELIP) originated with the discovery that some compositions of lipid dispersions became echogenic; that is, they reflected diagnostic ultrasound waves; when they were lyophilised.[1–4] It was subsequently recognized that ultrasound reflectivity of such dispersions was due to air associated with the lipid that was probably trapped during

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rehydration of the lyophilized preparation.[5] Gas-liquid interfaces provide a surface at which there is a large discontinuity in density and these surfaces with such characteristics reflect sound very efficiently. Ultrasound reflectivity and ultrasound contrast agents universally contain microscopic bubbles of some kind of gas. The detailed structure of the lipid and air pockets organization in ELIP is unknown, but energetic considerations necessitate that the air be covered with a lipid monolayer. For maximum stability, that lipid layer should have low air permeability. A variety of compositions and preparation procedures have now been found to generate improved ELIP contrast agents[6] that are superior in some respects to Optison™, a commercial clinical product. Those ELIP preparations are highly reflective at room temperature, but they lose reflectivity quite rapidly at 37°C.

While stability for a few minutes may be adequate for a simple contrast agent that highlights blood relative to solid tissues, stability for longer times may be preferable. There are other important potential applications of ELIP for which stability for a longer time is essential. Such applications include targeting to disease sites and drug delivery (which may also be combined with targeting). Targeting can be accomplished by coupling the ELIP surface to a molecule such as an antibody that has a high affinity for diseased cells. In the case of atherosclerosis, this includes vasoactive and pathological endothelium along with atheroma components.[8–10] For example, acoustically active liposomes coupled to antivascular cell adhesion molecule-1 (VCAM-1) and antiintercellular adhesion molecule-1 (ICAM-1), have been used to identify early stages of atherosclerosis.[4,11] More recently, encapsulation by ELIP of tissue plasminogen activator (tPA) has allowed these vesicles to act as both a clinical and a therapeutic agent; highlighting early stages of atherosclerosis, targeted by natural affinity of tPA for fibrin, and also delivering the enzyme cargo to the region where it leads to fibronolysis.[12–14]

It is well known that increased rigidity reduces permeability of lipid monolayers and bilayers to small molecules, and that increased rigidity is conferred by increased saturation of the constituent lipids. In addition, increased rigidity of the wall of ultrasound agents increases their ability to resist the compressive effects of surface tension.[11] Thus, it appeared that liposomes with more highly saturated lipids would retain echogenicity longer than those with unsaturated lipids. Hence, we undertook this project to manipulate the phospholipid composition of ELIP with the goal of identifying a preparation with marked increased echogenic stability.

MATERIALS AND METHODS

Materials

L-α-phosphatidylcholine (chicken egg; EPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-[phosphor-rac-1-glycerol] (DPPG), 1,2-dipalmitoyl-*sn*-glycero-3-phos-phoethanolamine (DPPE), and cholesterol (CH) were all purchased from Avanti Polar Lipids (Alabaster, AL) and stored at −20°C in chloroform. Mannitol and phosphate-buffered saline (1×PBS, pH 7.4) were purchased from Sigma Chemical Co. (St. Louis, MO).

Methods

Preparation of Echogenic Liposomal Dispersions—Echogenic liposomal dispersions were prepared as previously described.[6] The echogenicity and stability of different lipid compositions was determined for a variety of permutations of the molar percents of the phosphatides and cholesterol of the previously published preparation (EPC:DP-PE:DPPG:CH, 69:8:8:15, mol percent).[6] As an initial step, the molar percent of DPPE or DPPG in the standard preparation was increased from 8 to 30, 50, and 70% at the

expense of EPC. DPPC was then added to this standard preparation at 42% (mol percent) at the expense of EPC.

Lipid components for each sample (measured in mole%) were mixed in a 4 mL glass vial as chloroform solutions. The chloroform was then removed by evaporation under argon while the vial was rotated in a 50°C water bath. The resulting lipid film was then placed under vacuum for 4 h at ≤100 mTorr pressure for complete removal of the solvent. The dry lipid film was rehydrated with deionized water at 10 mg of lipid/mL. For all experimental compositions (excluding the standard preparation), the hydrated lipid was then incubated at 55°C for 30 min to insure all lipids were in the liquid crystalline phase during hydration. The mixture was then sonicated in a water bath for 5 min, following which an equal volume of 0.4 M mannitol was then added to give a final mannitol concentration of 0.2 M. Samples of 5 mg were then frozen on dry ice and lyophilized for 24 h. Samples were then hydrated with 5 mL deionized water to give a total lipid concentration 10 mg/mL. The size of liposomes (both standard ELIP and ELIP containing 42% DPPC), rendered nonechogenic by exposure to low pressure, was assessed by dynamic light scattering analysis (Brookhaven Instruments, Holtsville, NY).[15] Diameter distributions were analyzed by intensity.

Ultrasound Imaging, Analysis, and Phantom Control—Imaging of the liposomes was performed with a CVIS 20-MHz high-frequency intravascular ultrasound (IVUS) imaging catheter (3.2F, Boston Scientific Scimed, Inc., Maple Grove, MN) with settings for gain, zoom, compression, and rejection levels constant for all samples. Images were recorded onto super VHS videotape in real time for subsequent playback and image analysis.

IVUS images were digitized to 640 × 480 pixel spatial resolution (approximately 0.045 mm/pixel) and 8-bit (256 gray levels) amplitude resolution. The mean gray scale value (MGSV) of an image is a quantitative measure of the amount of whiteness it exhibits. For an 8-bit bitmap image, an intensity value between 0 and 255 is assigned to each pixel to indicate a linear gray scale where 0 represents complete blackness and 255 represents complete whiteness. Thus, the former corresponds to no ultrasound reflectivity and the latter to maximal reflectivity. The MGSV refers to the average value of these intensity values over a region of interest. In our case, the intensity of ultrasound reflected from a given region in the suspension is measured and represented by MGSV. A MGSV plot is a useful way of representing the spatial distribution of the amount of ultrasound reflected from a given surface. In this study, MGSV were calculated for each digitized image using Image-Pro Plus software (Version 4.1, Media Cybernetics, Silver Spring, MD). The annular area between the vial wall and the imaging catheter was manually outlined (excluding artifact in center from catheter transducer) and designated as the area of interest (AOI). The vial walls and AOI are clearly visible in Figure 1.

To calibrate the catheter, three materials with known reflective properties were imaged biweekly during the experimental period. A low MGSV was obtained by imaging 1× PBS in a 5 mL glass vial. Two intermediate MGSVs were obtained by imaging two sections of an interventional arterial phantom (Model: PVB-1, ATS Laboratories, Inc., Bridgeport, CT). An upper limit MGSV was obtained from imaging the surface of a stainless steel plate (MGSV 255). A predetermined AOI was applied to each image. The mean gray scale values were then graphed with respect to the position (1, 2, 3, or 4) and a linear regression performed. There was no change in catheter response over the course of the 16-week experimental period. These data eliminate aging of the IVUS catheter as a source of experimental error.

Stability Determination—For ELIP stability determination, the MGSV of each sample at 37°C was evaluated up to 7 h. Each ELIP cake was resuspended (10 mg/mL) in room temperature H_2O and an initial image of the sample was analyzed after 400× dilution ([lipid] = 0.025 mg/mL) or 800× dilution ([lipid] = 0.0125 mg/mL) in PBS or human serum at 37°C. The diluted liposomal dispersion was held at 37°C for the desired time periods. Stability in PBS was evaluated up to 2 h. Stability in human serum was evaluated up to 7 h. Echogenicity was determined for each time point.

Stability data are reported both as MGSV and percentage retention. The initial MGSV of each sample was taken to be 100% and percentage retention of echogenicity determined by dividing the MGSV of each time point by the initial MGVS and multiplying by 100%. A background for each time point was calculated by imaging 10 μL of H_2O diluted 400× in 1× PBS (*e.g.*, Fig. 1), analyzing the resulting image, and subtracting the MGSV from experimental liposome results. Unless otherwise noted, all data were corrected in this way for background noise. At each time point, three images were recorded in succession and analyzed, with the catheter being removed and flushed between each image. The MGSVs of the three images were averaged. With this procedure, the standard deviation was consistently less than 5 units (256 units at maximum image gray scale).

Sizing Vesicles—The diameter of nonELIP was assessed according to dynamic light scattering analysis with a BI-200SM goniometer and a BI-9000 digital correlator (Brookhaven Instruments, Holtsville, NY). Measurements were performed at room temperature and delay times of 10 μs to 1 s were examined. Correlation data were fitted with quadratic cumulants, using the algorithm provided with the instrument. Size distributions were calculated on the basis of intensity.

Statistical Analysis—All results are reported and displayed as mean ± standard error of mean. SigmaStat (Version 3.1, Systat Software, Inc., Point Richmond, CA) was utilized to perform these tests of significance with the use of a one-way analysis of variance (ANOVA). Specifically, Holm–Sidak test was utilized for ANOVA with an overall significance level of $p < 0.05$. Data were compared between the different preparation groups at each time point.

RESULTS

Stability of Echogenicity in PBS at 37°C

A clear trend throughout all experiments was the increased retention of echogenicity as the mole percentage of saturated lipid relative to total lipid (and cholesterol) approached 60%. This was most evident with DPPG. As depicted in Figure 2, increasing the DPPG to 50 mole % not only improved stability of this formulation but also increased initial echogenicity compared to other preparations ($p < 0.01$). MGSVs of ELIP with 30 mole% and 70 mole% DPPG showed enhancement compared to those of standard ELIP up to 30 and 120 min, respectively ($p < 0.05$). Retention of echogenicity with ELIP containing 50 mole% DPPG was 45% after 2 h, whereas that of standard ELIP dropped below 20% after 15 min (Fig. 2B). Echogenicity increased as DPPG increased to 50 mole%. However, ELIP with 70 mole % DPPG had lower echogenicity than ELIP with 30 mole% indicating a maximal point in the percentage DPPG-echogenicity relationship. Among these compositions, the optimal amount of DPPG is 50 mole%, with total saturated phospholipid (DPPE and DPPG) comprising 58 mole% of the total lipid.

Increasing DPPE at the expense of EPC produced a similar trend in stability, although not as pronounced as in the case of DPPG (Fig. 3). As the amount of DPPE increased to 30 mole%, ELIP stability increased compared to standard ELIP. MGSV at 50 mole% DPPE

(corresponding to a total saturated lipid content of 58 mole%), was still higher than that of standard ELIP, but slightly lower than 30 mole% DPPE. There was no difference between samples containing 30% DPPE and 50% DPPE. However, stability at 30 mole% and 50 mole% DPPE clearly were different with respect to standard ELIP up to 30 min ($p < 0.05$). When DPPE was incorporated at 70 mole%, the lipid film aggregated upon hydration and revealed little echogenicity.

A composition was also prepared in which the saturated lipid DPPC was included in the standard preparation at the expense of EPC. At a 42 mole% (and total saturated lipid 58 mole%), ELIP stability increased. After 1 h at 37°C, the DPPC-containing composition retained 50.8 ± 3.5% echogenicity (Fig. 4). This is five times more than that of the standard preparation ELIP, which lost 90% of its echogenicity after 15 min at 37°C. Retention of ELIP echogenicity with this optimal formulation was different than our standard ELIP for up to 2 h ($p < 0.001$). IVUS images of the optimal composition and standard ELIP at times 0, 15, and 60 min are shown in Figure 5. There is a clear visual difference between the two groups.

Stability of Echogenicity in Human Serum at 37°C

Stability of both the standard and the optimal ELIP preparations in serum at 37°C is presented in Figure 6. The new composition is quite stable when compared to the standard preparation, with 84% of echogenicity retained at 1 h compared to only 42% for the standard preparation. At 5 h, the standard preparation lost most of its echogenicity while the optimal preparation retained 63% echogenicity. Greater than 50% echogenicity of the optimal preparation was present at 7 h. These results demonstrate a lasting contrast effect of our optimal preparation in more physiological conditions.

Vesicle Size

In general, dynamic light scattering consistently detected two populations of liposomes. In standard ELIP preparations one population was approximately 1.2 μm in diameter and the other approximately 90 nm in diameter. Optimal preparation ELIP (vesicles containing 42 mole% of DPPC) were composed of similar populations, one consisting of vesicles 1.4 μm in diameter and a second population 100 nm in diameter. The majority population in both liposomal preparations consisted of the larger vesicles.

DISCUSSION

The retention of echogenicity of our ELIP can be increased by adjustment of the acyl chain saturation of the component phospholipids. Earlier studies involved manipulation of the parameters in the preparation such as cryoprotectant type and concentration, freezing temperature, and lyophilization conditions, all of which allowed improvement of the echogenicity of this lipid-based ultrasound contrast agent.[6] In order to improve retention of echogenicity by these particles at physiologic temperatures, further investigation was needed. We hypothesized that increasing bilayer rigidity would slow air loss from the vesicle and prolong retention of echogenicity. This hypothesis was based on previous data that indicated that ultrasound reflectivity of these particles is due to an air pocket contained within the bilayer of the liposome. It was also based upon considerable literature showing transport of gasses and other small molecules is slower across monolayers and bilayers, the more highly saturated are the acyl chains.[16–18] To the extent that some echogenicity arises from spherical bubbles covered with a lipid monolayer, a more rigid lipid composition would also reduce the effect of surface tension, which otherwise pressurizes the air and encourages its escape by diffusion.[19]

Indeed, as unsaturated lipid in the ELIP was replaced by any of three different saturated phospholipids (DPPE, DPPG, and DPPC), the retention of ELIP reflectivity increased. For all three sets of composition changes, stability appeared optimal at 58 mole% saturated lipid. At higher saturated lipid concentrations, aggregation occurred with DPPE formulations and phase separation occurred with DPPG formulations. As our previous research indicated that all three phospholipids, along with cholesterol, are important for successful ELIP preparation, formulations without these four components were not evaluated. Specifically, the anionic DPPG contributes to initial echogenicity and prevents aggregation of the liposomes. DPPE is needed for eventual conjugation of a targeting agent. PC provides the basic matrix and CH contributes to a tighter organization of the lipid layer. In the present formulation, EPC and CH probably further contribute to maintaining the lipids, particularly the saturated phospholipids, in the liquid crystalline form, at least at physiological temperatures if not also at room temperature.

Although our mixture is quite complex, there have been some studies of ternary mixtures and it is of interest that, for a mixture of 15 mole%, the molar percent of DPPC to DOPC cannot be higher than about 2:1 if the system is to remain completely in the liquiid phase.[20] Given the unsaturation of the eggPC that we used and that DOPC are fairly similar, this appears to support the suggestion that lipids need to be in a one-phase region (absence of solid phase separation) to have optimal echogenicity.

The composition with the greatest stability was that containing 50% DPPG. However, these liposomes with such high concentrations of DPPG tend to be toxic. Unless modified to reduce the PG exposure, such a contrast agent would not be clinically useful. The DPPE containing preparations were sometimes very highly echogenic, but exhibited high variability in echogenicity (Fig. 3). The DPPC-containing preparation, involving no major change in polar head group, preserved the low toxicity and consistent properties of the original formulation. The inclusion of DPPC at 42% molar percent improves stability of ELIP with little variability.

It should be recognized that although the stability of ELIP has been improved *in vitro*, many factors *in vivo* contribute to the circulation time of liposomes. The present investigation demonstrated that simple compositional variations can increase the utility of ELIP. Our previous ELIP preparation has been compared to standard commercial ultrasonic contrast agents for both echogenicity[7] and stability.[21] The standard ELIP preparation was found to have similar echogenic properties to Optison™ at a concentration of 4× and greater echogenic properties above this.[7] As these ELIP target and aggregate, they tend to aggregate at the target site, making them as or greater specular reflectors than Optison™. Similarly, their stability was found to be greater than that of Optison™ *in vitro*.[21] This study did not directly compare other contrast agents to our optimal ELIP preparation. However, as this optimal preparation is more echogenic and stable than the standard preparation, it makes sense that it should have similar or better characteristics when compared to other contrast agents. We have demonstrated further stability of our standard ELIP under *in vitro* conditions that more closely approximate *in vivo* conditions (ELIP in solutions containing albumin and plasma).[22] The optimal preparation is more stable when compared to the standard preparation in human serum. This further emphasizes its robustness as a clinical contrast agent.

In summary we have found that the optimal ELIP formulation of EPC/DPPC/DPPE/DPPG/CH (27:42:8:8:15, mole%) seems to provide sufficiently high stability without known toxicity effects. This formulation is highly promising for *in vivo* targeted ultrasonic imaging, with potential clinical imaging and therapeutic applications.

Acknowledgments

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Abbreviations

ELIP	echogenic liposome
ICAM-1	anti-intercellular adhesion molecule-1
tPA	tissue plasminogen activator
EPC	L-α-phosphatidylcholine (chicken egg)
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DPPG	1,2-dipalmitoyl-*sn*-glycero-3-[phosphor-rac-1-glycerol]
DPPE	1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine
CH	cholesterol
PBS	phosphate-buffered saline
IVUS	intravascular ultrasound
MGSV	Mean gray scale values
AOI	area of interest

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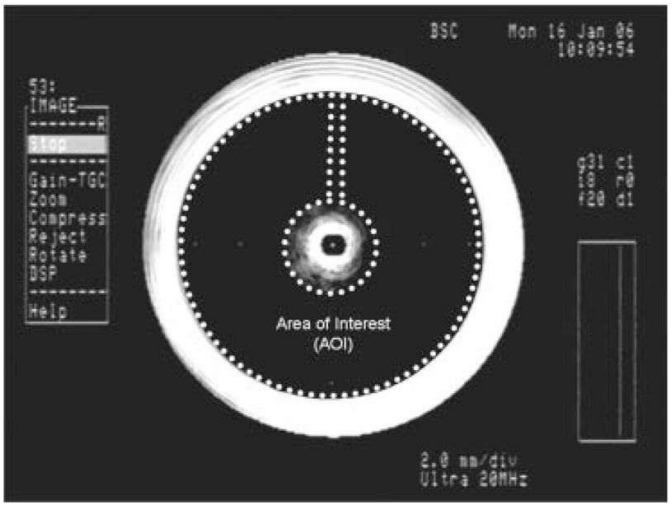

Figure 1.

Background image obtained with a 20-MHz IVUS catheter. This image exemplifies the background MGSV for all experiments. The catheter was inserted into a 5 mL glass vial containing 4 mL of 1× PBS. The AOI is the area that was manually traced and digitally analyzed to obtain the low MGSV, which would range from 5 to 7 units (on a 0–255 unit scale).

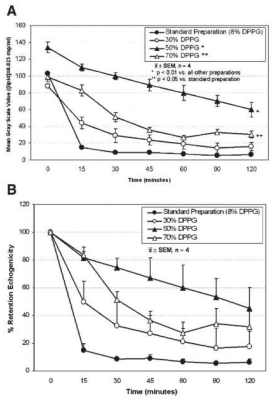

Figure 2.

(A) MGSV of ELIP determined up to 2 h during incubation at 37°C. Lipid concentration was 0.025 mg/mL. This graph depicts stability of the standard preparation and the effect of increasing amounts of DPPG at the expense of EPC. (B) Similar to (A) this graph illustrates percent retention of echogenicity of ELIP. The initial MGSV is taken to be 100%.

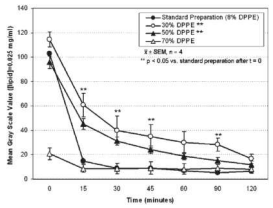

Figure 3.

MGSV of ELIP determined up to 2 h following incubation at 37°C. The lipid concentration was 0.025 mg/mL. This graph depicts stability of the standard preparation and the effect of increasing amounts of DPPE (at the expense of EPC).

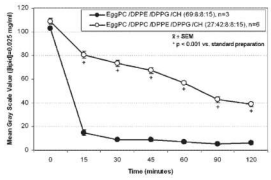

Figure 4.
MGSV of the optimal and standard preparation ELIP in PBS determined up to 2 h during incubation at 37°C. The lipid concentration was 0.025 mg/mL.

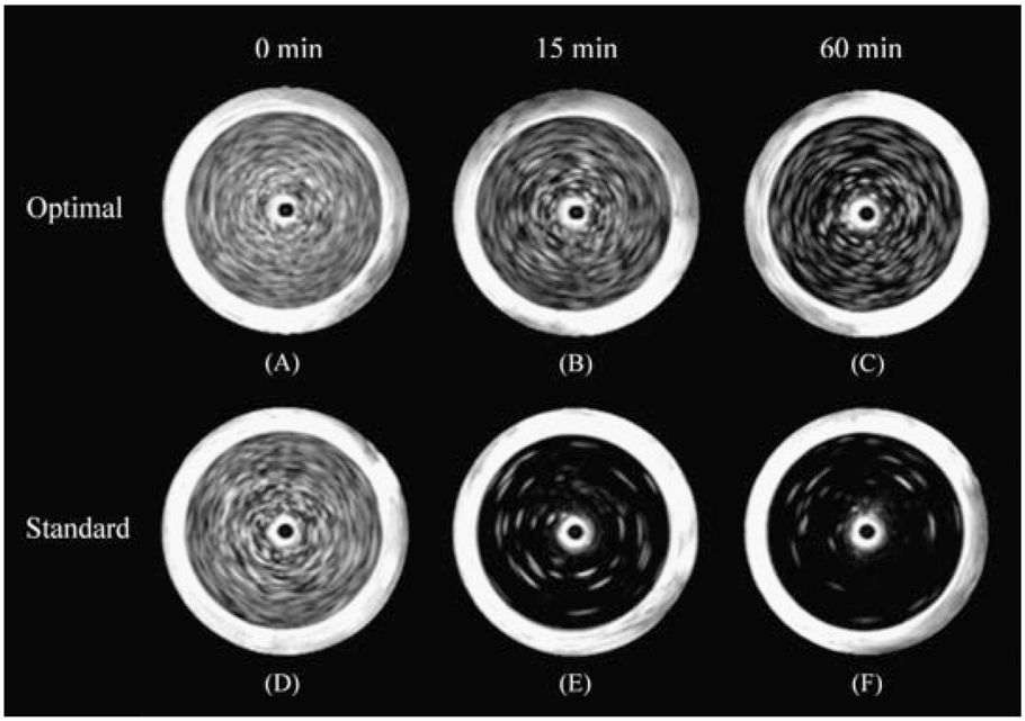

Figure 5.

Top row**-** ELIP images composed of EPC/DPPC/DPPE/DPPG/CH (27:42:8:8:15, mole%). (A) Initial image, (B) image after incubation for 15 min at 37°C, (C) image after incubation for 60 min at 37°C. Bottom row—images of standard preparation ELIP composed of EPC/DPPE/DPPG/CH (69:8:8:15, mol %). (D) Initial image, (E) image after incubation for 15 min at 37°C, (F) image after incubation for 60 min at 37°C. ELIP were diluted to 0.025 mg/mL in 4 mL of PBS. Compare to Figure 1 to illustrate contrast effect of the ELIP compositions.

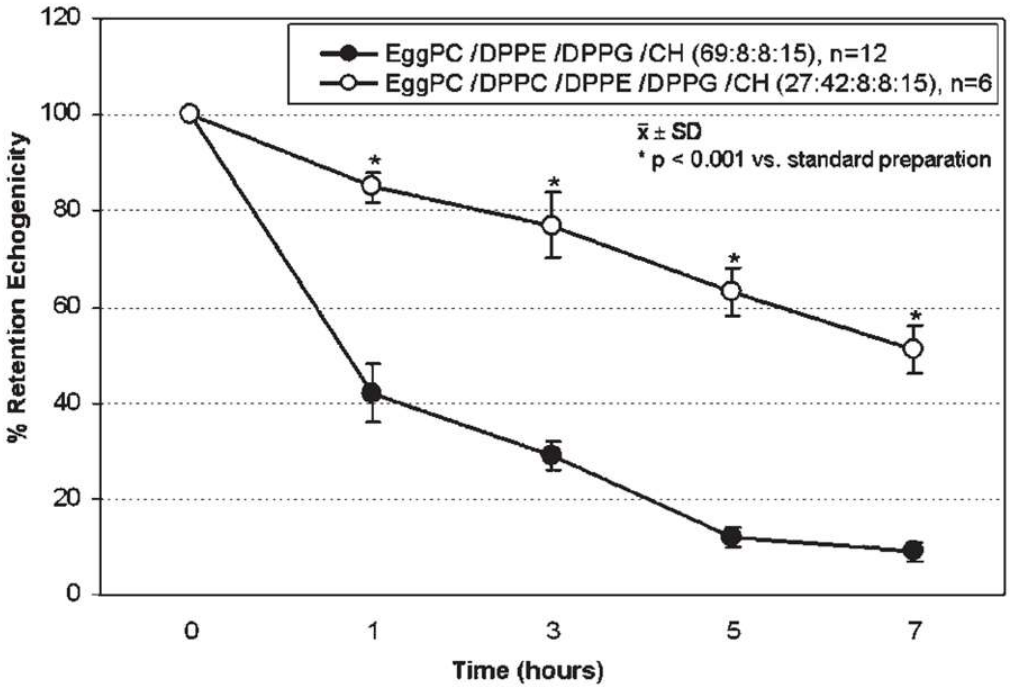

Figure 6.
Percent retention of echogenicity in human serum determined up to 7 h at 37°C. ELIP were diluted to 0.0125 mg/mL in 5 mL of 100% human serum.