European Journal of Pharmaceutics and Biopharmaceutics 77 (2011) 430–437

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Development and characterization of new nanoscaled ultrasound active lipid dispersions as contrast agents

Elena Eva Julianne Marxer [a,1], Jana Brüßler [a,1], Andreas Becker [b], Joachim Schümmelfeder [d], Rolf Schubert [c], Christopher Nimsky [b], Udo Bakowsky [a,*]

[a] Department of Pharmaceutical Technology and Biopharmaceutics, University of Marburg, Marburg, Germany
[b] Department of Neurosurgery, University of Marburg, Marburg, Germany
[c] Department of Pharmaceutical Technology and Biopharmacy, Albert-Ludwig-University Freiburg, Freiburg i. Brsg., Germany
[d] Department of Cardiovascular Medicine I, St. Georg Hospital Eisenach, Eisenach, Germany

ARTICLE INFO

Article history:
Available online 13 December 2010

Keywords:
Ultrasound
Liposomes
Lipid formulations
Diagnostics
Controlled drug release
Nanoscaled ultrasound contrast agents

ABSTRACT

Ultrasound contrast agents are widely used in clinical diagnosis. In recent years, the use of ultrasound contrast agents as therapeutic agents has gained a lot of attention. Of special interest are ultrasound-enhanced gene delivery in various tissues (e.g. cardiac, vascular, skeletal muscle and tumor tissue), ultrasound-enhanced protein delivery (e.g. insulin delivery) and ultrasound-enhanced delivery of small chemicals (e.g. doxorubicin, vancomycin). Commercially available ultrasound contrast agents such as SonoVue® or Optison® are ranged in a size of 2–8 μm. These micronscaled agents show a good ultrasound contrast enhancement and thus they are used for diagnostic imaging. But they are not suitable for targeted drug delivery to tumor tissues or blood clots because for these applications particles smaller than 700 nm are needed. In the present study, we developed new nanoscaled ultrasound contrast agents with a size between 70 and 300 nm. The lipid formulations show excellent contrast intensities using diagnostic ultrasound of about 1.4 MHz. The negatively charged colloidal dispersions are long-time stable under physiological conditions without loss of ultrasound reflectivity. The adjustable supramolecular organization of the carriers depends on the composition and varies from micellar to liposomal structures. The small size and the circulation stability of these systems make them promising for novel diagnostics and controlled drug release applications.

1. Introduction

Ultrasound has been in use for the last three decades as a modality for diagnostic imaging in medicine. Recently, there have been numerous reports on application of ultrasound energy for targeting or controlling drug release. This new concept of therapeutic ultrasound combined with drugs has induced excitement in various medical applications. Ultrasound energy can enhance effects of thrombolytic agents, transdermal drug delivery, anticancer drugs and gene therapy [1–3]. The addition of ultrasound contrast agents increases cell membrane permeability and enhances the uptake of drugs and genes. The generally accepted mechanism for this enhancement is sonoporation. Juffermans et al. [4,5] demonstrated cell membrane deformation by oscillating microbubbles near the cells surface leading to the formation of transient pores. Additional mechanisms of cellular permeability (i.e. H_2O_2 levels and F-actin cytoskeleton) were studied to understand the effects of ultrasound contrast agents on the uptake of therapeutic compounds.

The first enhancement of ultrasound contrast was reported by Gramiak and Shah [6] in 1968. Supravalvular injections of physiological saline solution lead to clouds of echoes in the aortic rood. This increase in contrast intensity was supposed to be caused by small air bubbles included in the saline solution or produced by the rapid injection rate. A big drawback with those air bubbles was their rapid dissolution in blood. To solve this problem, a stabilization of the gas–liquid interface was necessary. On the one hand, low diffusivity gases (e.g. sulfur hexafluoride, perfluoropentane), and on the other hand, shells were able to increase the bubble lifetime. As shell materials, lipids (e.g. phospholipids), polymers (e.g. alginate) or proteins (e.g. albumin) were used [7]. These newly designed ultrasound contrast agents were usually applied via the parenteral route. The size of contrast agents has to be smaller than 8 μm to enable their penetration through the lung capillaries and thus their remaining in the circulatory system [8]. Commercially available ultrasound contrast agents such as SonoVue® or Optison® are therefore sized between 2 and 8 μm. Different mechanisms

* Corresponding author. Department of Pharmaceutical Technology and Biopharmaceutics, University of Marburg, Ketzerbach 63, D-35037 Marburg, Germany. Tel.: +49 6421 282 5881; fax: +49 6421 282 7016.
E-mail address: ubakowsky@aol.com (U. Bakowsky).
[1] These authors contributed equally to the manuscript.

0939-6411/$ - see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2010.12.007

eliminate the bubbles rapidly from the blood stream. An important elimination route is opsonization where plasma proteins are attached to the bubble surface followed by macrophage uptake. To prolong the circulation time so called Stealth® liposomes were developed in the 1990's. They contain PEGylated lipids with a large hydrophilic part which covers the liposome surface and inhibits the opsonin attachment [9–13].

Recently, different ultrasound-sensitive targeted drug delivery systems were investigated to achieve directed drug delivery of thrombolytic agents or anticancer drugs. Submicron-sized drug delivery systems with diameters less than 700 nm are able to penetrate tumor vessel walls and reach cells outside the blood vessels [8,14,15]. Another field of application for ultrasound active drug delivery systems is sonothrombolysis [16]. This is a new approach to obtain a rapid and effective revascularization by a combination of ultrasound, thrombolytic agents and ultrasound contrast agents. The most common thrombi occur in arterial vessels and are platelet rich, erythrocyte poor and show a dense fibrin mesh. Thrombi of the venous system are platelet poor, erythrocyte rich and show a wide meshed fibrin network [17,18]. It is hypothesized that nanoscaled delivery systems are able to penetrate even arterial blood clots and release their content within the clot to increase thrombolysis.

The aim of our work was the development of novel nanoscaled and long-circulating ultrasound active lipid dispersions as contrast agents.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were purchased from Lipoid GmbH (Ludwigshafen, Germany) and cholesterol (CH) and polyethylene glycol (40) stearate (PEG40S) from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Stock solutions of the lipids (approx. 10 mg/ml) were prepared in a mixture of chloroform:methanol (2:1, v:v) and stored at 4 °C. Chloroform and methanol (both HPLC-grade) were purchased from Fisher Scientific (Loughborough, UK).

2.2. Preparation of lipid formulations

Three lipid dispersions, DPPC:CH 70:30 (mol%), DPPC:PEG40S 98:2 (mol%) and DSPC:PEG40S 98:2 (mol%), were prepared using the thin film hydration method according to [19,20]. Briefly, from a stock solution (chloroform:methanol, 2:1), the lipids were mixed in a round-bottom flask to a total amount of 10 mg lipid. The lipid mixture was dried to a thin film using a rotary evaporator (Heidolph Laborota 4000 efficient, Heidolph Instruments, Schwabach, Germany) under vacuum at 40 °C. The resulting film was rehydrated with 1 ml of phosphate-buffered saline (PBS) pH 7.4 (0.15 mol/l). After vigorous shaking, the lipid dispersions were sonicated in a bath-type sonicator (Bandelin Sonorex RK 100H, Bandelin Electronics, Berlin, Germany, maximal energy) for 20 s at 55 °C (DPPC/CH, DPPC/PEG40S) or at 65 °C (DSPC/PEG40S). Finally, after incubation for 60 min at the above-mentioned temperatures, the dispersions were subsequently sonicated again for 2 min. The lipid formulations were stored at 4 °C.

2.3. Physico-chemical characterization

The physico-chemical properties of the lipid formulations including size, charge, shape and morphology or supramolecular organization were investigated using Dynamic Light Scattering (DLS), Laser Doppler Velocimetry (LDV), cryo-Transmission Electron Microscopy (cryo-TEM), Atomic Force Microscopy (AFM) and Nuclear Magnetic Resonance spectroscopy (^{31}P NMR).

2.3.1. Dynamic Light Scattering

The hydrodynamic diameter of lipid formulations, prepared and stored as described above, was determined by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) equipped with a 10 mW HeNe laser at a wavelength of 633 nm at 25 °C, as described previously [21]. Scattered light was detected at a 173° angle with laser attenuation and measurement position adjusted automatically by the Malvern software. Values given are the means ± standard deviation of ten independent experiments with each experiment comprising three measurements of the same sample with at least 10 runs, as determined by the Zetasizer. The average value was calculated with the number distribution data of ten samples ± standard deviation.

2.3.2. Laser Doppler Velocimetry

The zeta potential was measured with a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) at 25 °C and a scattering angle of 17° by measuring the electrophoretic mobility with Laser Doppler Velocimetry (LDV). Values given are the means ± standard deviation of ten independent experiments with each experiment comprising three measurements of the same sample with at least 10 runs, as determined by the Zetasizer. The average value was calculated with data of ten samples ± standard deviation.

2.3.3. Atomic Force Microscopy

Twenty microliters of the lipid formulations were transferred onto a silicon chip and left to dry. AFM was performed on a vibration-damped Nanoscope IV Bioscope (Veeco Instruments, Mannheim, Germany) as described in detail elsewhere [19,22]. Commercial pyramidal Si_3N_4 tips (NSC16 AlBS, Micromash, Estonia) were mounted onto a cantilever (length 230 μm, resonance frequency 170 kHz and nominal force constant 40 N/m) and measurements were performed in tapping mode™ to minimize damage to the specimens. The scan speed was proportional to the scan size, and the scan frequency was between 0.5 and 1.5 Hz. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction and the height signal in the retrace direction, both signals being simultaneously recorded. The results were visualized either in height or in amplitude mode. The diameter (±standard deviation, SD) of the carrier systems was calculated by measuring 40 individual lipid formulations on a scanned area of 5 μm × 5 μm (repeated on five independent samples).

2.3.4. Cryo-Transmission Electron Microscopy

The lipid formulations were investigated without dilution. Quantifoil® S7/2 Cu 400 mesh, holey carbon film grids (Quantifoil Micro Tools GmbH, Jena, Germany) were prepared according to a standard method [23]. After placing a drop of the sample on the grid, most of the liquid was removed with filter paper, leaving a thin film stretched over the holes. The samples were shock-frozen by dipping into liquid ethane and cooled to 90 K by liquid nitrogen. The samples were transferred to the microscope (Leo 912 Ω-mega, Leo Elektronenmikroskopie GmbH, Oberkochen, Germany) as described elsewhere [24] and then examined at approximately 100 K.

2.3.5. Nuclear Magnetic Resonance spectroscopy

^{31}P NMR measurements were taken in D_2O with 50 ppm spectral width at a resonance frequency of 202.47 MHz (11.7 T) on a JEOL ECA-500 spectrometer (JEOL GmbH, Eching, Germany) using a JEOL broadband observe probe (NM-03520TH5). Thousand scans were accumulated using 4.5 μs pulses. Prior to Fourier transformation, a

line broadening of 30 Hz was applied. All measurements were performed at room temperature (about 25 °C).

2.4. Determination of echogenicity and ultrasound stability

For the characterization of the ultrasound properties of the lipid formulations, a self-made closed-loop flow system according to Kollmann et al. [25] was used (see Fig. 1). The central part of this flow model is a 2.5-l polypropylene beaker filled with agar gel consisting of 3% agar, 85.5% double distilled water, 11% glycerol and 0.5% sodium azide as a biocide. A silicon tube (C-flex®, Cole-Parmer Inc., Illinois, USA) is embedded in this gel in an angle of 30° to mimic the blood vessels in the brain. The agar gel has similar ultrasound properties like human tissue and the C-flex® tube correlates to the blood vessels. A roller pump creates constant flow with the pressure adjusted to fit the mean pressure in the brain artery (120/80 mm Hg). A Tris-albumin buffer pH 7.4 (0.15 mol/l sodium chloride, 0.002 mol/l 2-amino-2-hydroxymethyl-propane-1,3-diol hydrochloric acid (Tris–HCl) and 0.1% bovine serum albumin) reservoir (150 ml) heated up to 37 °C is pumped through the flow model in a circulation system (containing 50 ml buffer in the tubes). Equivalent amounts of the sphere volumes of our nanoscaled lipid formulations and SonoVue® were added to the buffer reservoir. Ultrasound was emitted using a commercial medical diagnostic device (Siemens, SONOLINE Elegra, Erlangen, Germany) equipped with a 2.5 MHz phased array transducer mounted on the agar surface of the ultrasound chamber, and the ultrasound beam was focused on the C-flex® tube. This transducer provides an axial resolution of 0.7 mm. The lateral resolution depends on the width of ultrasonic beam and can be approximated to be 3 mm. The penetration depth was set to 10 cm, and dynamic range was 40 dB.

The lipid formulations were imaged using 1.4 MHz ultrasound frequency. The mechanical index was set to 0.4 to achieve non-linear oscillation of the formulations (e.g. the lipid associates within the dispersions) and therefore harmonics of the fundamental frequency. Phased inversion harmonic imaging was used to receive the harmonic frequency. Images were sampled each 0.5 s for one minute after administration of contrast agents into the buffer reservoir and then stored on a magnetic optical disc as 8-bit greyscale images. The first image was stored as a reference for evaluation of ultrasound contrast intensities which were calculated as mean grey values using ImageJ 1.42q (National Institute of Health, Bethesda (Maryland), USA). The same region of interest (ROI) was evaluated in each picture using a macro. To compare contrast intensities, mean grey values were calculated in percent related to the mean grey value of SonoVue® (set to 100%). For all formulations, the average of at least twenty pictures was analyzed.

For the evaluation of the storage stability, changes in ultrasound contrast and particle size of the lipid dispersions were observed over a period of 14 days. Samples were measured on the day of preparation and after 2, 4, 7 and 14 days.

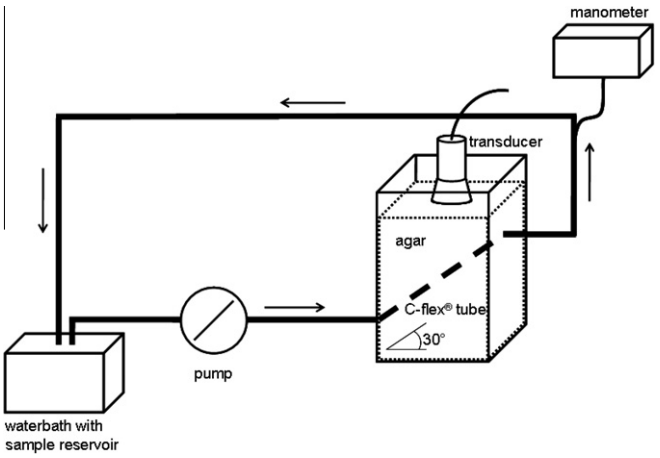

Fig. 1. Schematic diagram of the closed-loop flow system as described in Section 2.

3. Results

The aim of this study was the development of long-circulating ultrasound active and biocompatible lipid formulations with a diameter in a nanoscale range, a defined stability and a high echogenicity. For this purpose, three lipid dispersions (DPPC/CH, DPPC/PEG40S and DSPC/PEG40S) were prepared and evaluated with regard to their physico-chemical properties (size and zeta potential, morphology and echogenicity) and compared to the commercially available ultrasound contrast agent SonoVue®. Finally, the morphology and the supramolecular organization of the lipids within the formulations were analyzed by ^{31}P NMR spectroscopy.

3.1. Dynamic Light Scattering and Laser Doppler Velocimetry

The hydrodynamic diameter of all lipid formulations as determined by DLS is summarized in Table 1. Mean diameters varied from undetectable for SonoVue®, over 173.5 ± 18.2 nm for DPPC/PEG40S (98:2, mol%) and 195.8 ± 25.7 nm for DSPC/PEG40S (98:2, mol%), to 218.7 ± 19.8 nm for DPPC/CH (70:30, mol%) liposomes. All distributions show relatively high polydispersity indices (PDI) between 0.45 and 0.54 (DPPC/CH and DSPC/PEG40S) indicating a non-unimodal size distribution. As expected, all formulations showed a negative zeta potential at pH 7.4 (Table 1).

3.2. Atomic Force Microscopy

Atomic Force Microscopy allows to visualize all formulations under wet conditions and to confirm the particle size measurements performed by DLS. For size determination, all visible particles within a representative scan area were individually evaluated (Table 1, Fig. 2). In principle, the data obtained can deviate substantially from the results of Dynamic Light Scattering measurements. This may be caused by the interaction of soft and flexible lipid formulations with the surface of the silicon support what might lead to artefacts. However, the individual evaluation allowed excluding such artefacts from further analysis. In addition, the visualization and evaluation of such artefacts enables us to judge the stability of the formulations.

The formulations DPPC/CH, DPPC/PEG40S and DSPC/PEG40S showed average particle sizes of 232.4 ± 17.1 nm, 216.7 ± 15.6 nm and 169.6 ± 22.4 nm, while Dynamic Light Scattering produced smaller values for DPPC/CH (220 nm), DPPC/PEG40S (175 nm) and larger values for DSPC/PEG40S (195 nm), respectively. In the case of DPPC/PEG40S and DSPC/PEG40S, two major sub-populations were observed. The major fraction showed an average diameter between 200 and 300 nm and fraction two was smaller with a size of approx. 70 nm. The larger particles were ideally round, while the smaller were of varying morphologies with round, oval and rod-like shapes. Both fractions were relatively resistant to the silicon surface. For the DPPC/CH vesicles, a size distribution between 110 nm and 350 nm without a differentiation into discrete fractions was observed. The vesicle morphology was stable on the silicon support. The average particle size of the commercially available ultrasound contrast agent SonoVue® was 1981 ± 822 nm with a very broad size distribution ranging from 250 nm to 6500 nm.

Table 1
Size and zeta potential measured by DLS, AFM, TEM and LDV.

Lipid formulation	Zeta potential [mV]	Size *d* [nm]			Size range
		DLS ± SD (PDI)	AFM ± SD	TEM ± SD	
DPPC/CH	−5.35 ± 3.24	218.7 ± 19.8 (0.45)	232.4 ± 17.1	201.8 ± 20.2	110–350 nm
DPPC/PEG40S	−1.70 ± 0.82	173.4 ± 18.2 (0.47)	216.7 ± 15.6	133.1 ± 17.7	50–500 nm
DSPC/PEG40S	−3.31 ± 1.03	195.8 ± 25.7 (0.54)	169.6 ± 22.4	199.4 ± 28.3	75–485 nm
SonoVue®	n.d.	n.d.	1981 ± 822	n.d.	250 nm–6.5 μm

3.3. Cryo-Transmission Electron Microscopy

Cryo-TEM was used for visualization of the ultrasound active lipid formulations and representative micrographs are shown in Fig. 3. The images of DPPC/CH show typical micrographs of homogeneously distributed mainly unilamellar liposomes (Fig. 3A) with an average diameter of 201.8 ± 20.2 nm. In the case of DPPC/PEG40S and DSPC/PEG40S, a coexistence of liposomal round shaped and smaller micellar rod-like structures could be visualized (Fig. 3B and C). The liposomal structures (about 250 nm) appear rather angularly and are identical with the large fraction visualized with AFM. The average diameter showed values between 133.1 ± 17.7 nm (DPPC/PEG40S) and 199.6 ± 28.3 nm (DSPC/PEG40S). SonoVue® could not be visualized due to its low stability under vacuum.

3.4. Nuclear Magnetic Resonance spectroscopy

According to Leal et al. [26], ^{31}P NMR can be used to differentiate between liposomal and micellar organizations of lipids within pharmaceutical formulations. The width of the ^{31}P line is sensitive to aggregate size, shape and self-diffusion. Cullis and de Kruijff [27] simulated ^{31}P NMR spectra for different phospholipid arrangements and revealed a low-field shoulder for bilayer structures, whereas micelles showed a symmetric peak. The peak with the low-field shoulder in Fig. 4A leads to the assumption that the

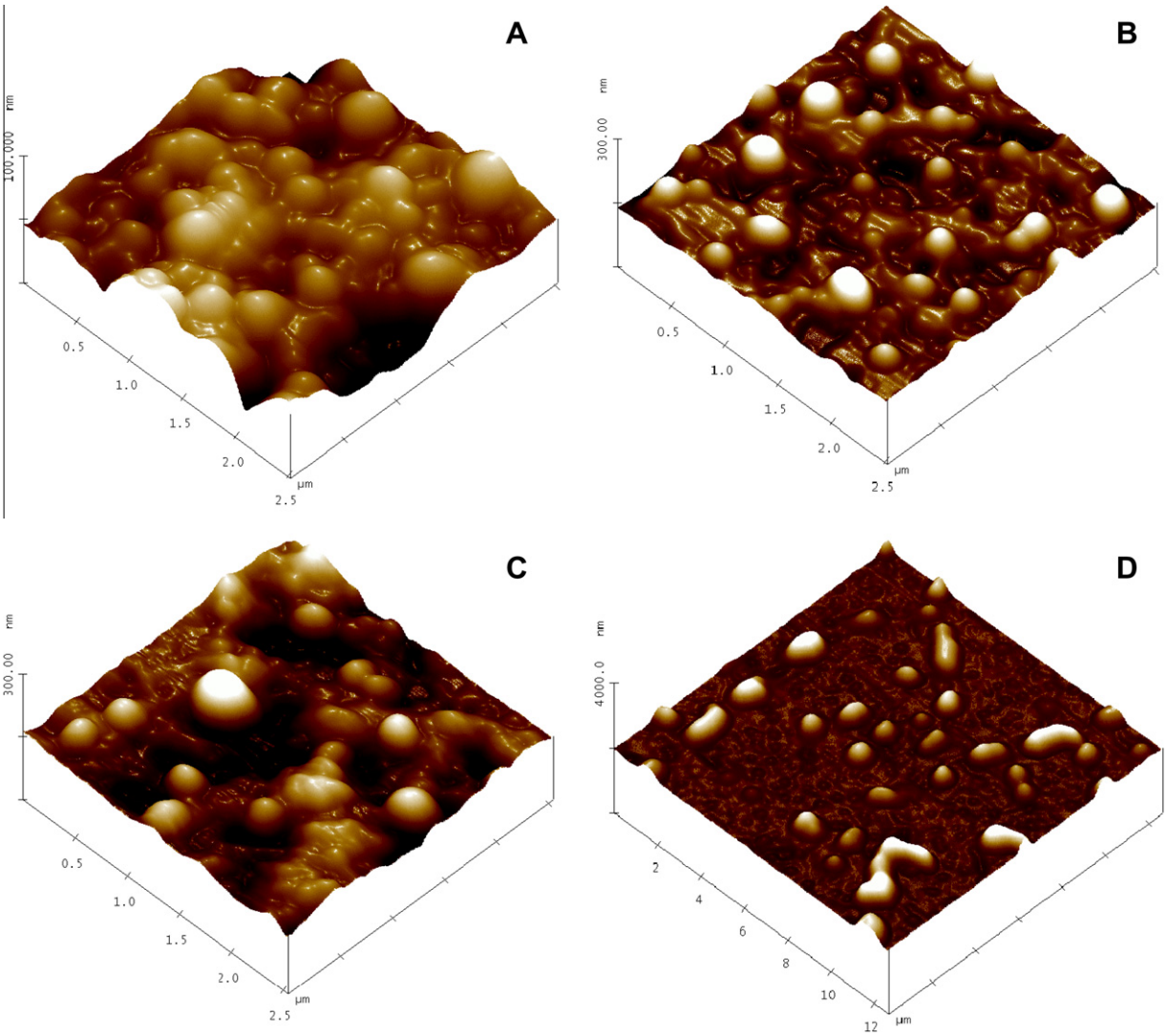

Fig. 2. Visualization of the size distribution and morphology of the lipid formulations by using AFM. (A) DPPC/CH; (B) DPPC/PEG40S; (C) DSPC/PEG40S; (D) SonoVue®. All images are presented in height modus.

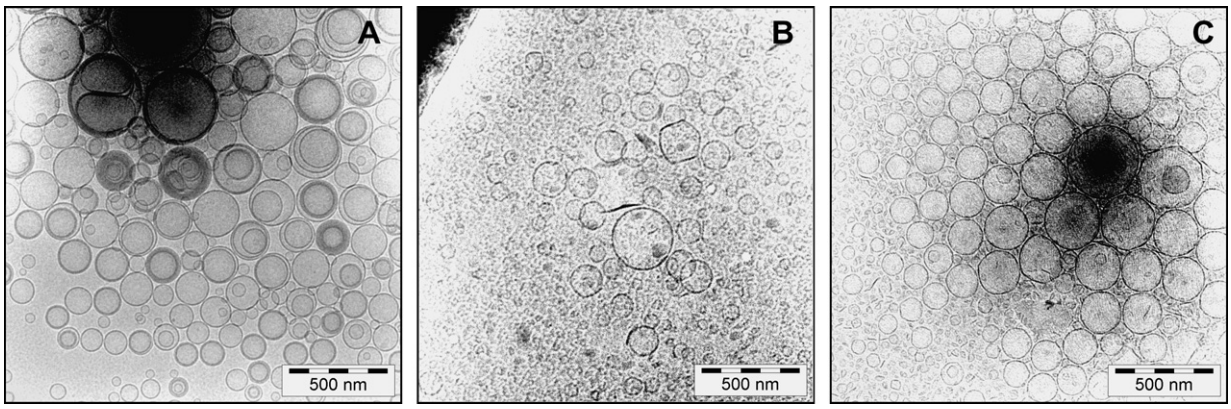

Fig. 3. Visualization of the lipid formulations by using TEM. (A) DPPC/CH; (B) DPPC/PEG40S; (C) DSPC/PEG40S.

DPPC/CH mixture forms mainly liposomes, whereas the symmetric peaks in Fig. 4B and C indicate that micellar structures predominate for DPPC/PEG40S and DSPC/PEG40S mixtures.

3.5. Determination of echogenicity and ultrasound stability

The ultrasound contrast is measured as mean grey value related to the mean grey value of SonoVue® measured on the day of preparation. On the ultrasound images in Fig. 5, contrast intensities of the different formulations can be seen on the second day after preparation. The ultrasound contrast of the commercially available contrast agent SonoVue® is bright and homogeneous with a mean grey value of 170.7%. The DPPC/CH liposomes show a faint but clear contrast (mean grey value 50.9%). Again, a bright ultrasound contrast was determined for the DPPC/PEG40S (mean grey value 119.1%) and DSPC/PEG40S (mean grey value 154.2%) formulations. This contrast is as homogeneous as the contrast of the commercially available ultrasound contrast agent SonoVue®. For a period of 5 min, the ultrasound contrast intensities of the nanoscaled lipid dispersions remained constant whereas the contrast of SonoVue® decreased.

The ultrasound stability during storage at 4 °C was investigated for a period of 14 days (Fig. 6). All formulations revealed the highest value of echogenicity on the second day after preparation. The DPPC/CH mixture showed a distinct decrease in ultrasound reflectivity from 55% on the second day after preparation to about 5% after 4 days. For the PEG40S-containing formulations, a decrease of the ultrasound contrast was observed followed by leveling between 60% and 90%. Only SonoVue® revealed a slow continuous decrease in echogenicity after the second day of preparation.

4. Discussion

The new concept to use therapeutic ultrasound combined with drugs has induced excitement in various medical applications. Ultrasound energy can enhance effects of thrombolytic agents, transdermal drug delivery, anticancer drugs and gene therapy. Therefore, new ultrasound contrast drug carriers with adjustable properties such as small size, biocompatibility, defined ultrasound reflectivity, high drug loading capacity and long circulation effect must be developed.

In the present study, we developed new nanoscaled ultrasound contrast agents based on different lipid formulations (DPPC/CH, DPPC/PEG40S, DSPC/PEG40S) and compared their properties to the commercially available contrast agent SonoVue®.

Several authors described the use of lipids especially DSPC as stabilizers for gas-filled micronsized bubbles or as liposomal drug

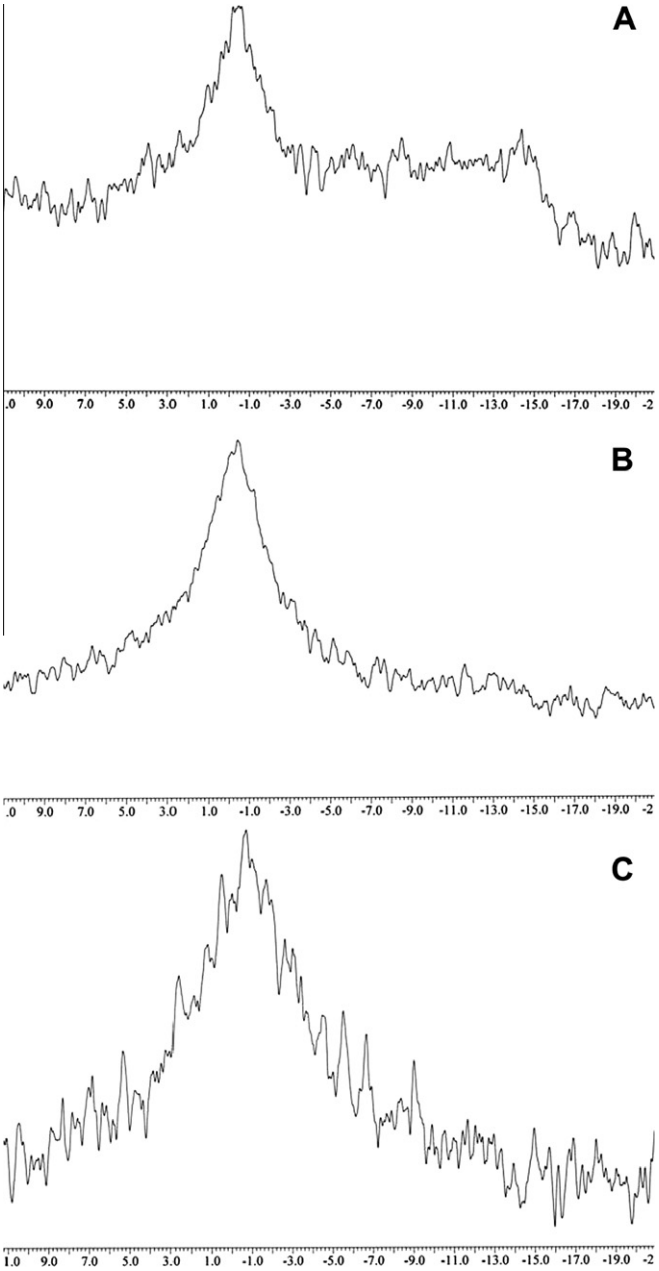

Fig. 4. ^{31}P NMR measurements. (A) DPPC/CH, a peak with a low-field shoulder for liposomes is present; (B) DPPC/PEG40S; (C) DSPC/PEG40S. (B and C) symmetric peaks indicate the presence of micelles.

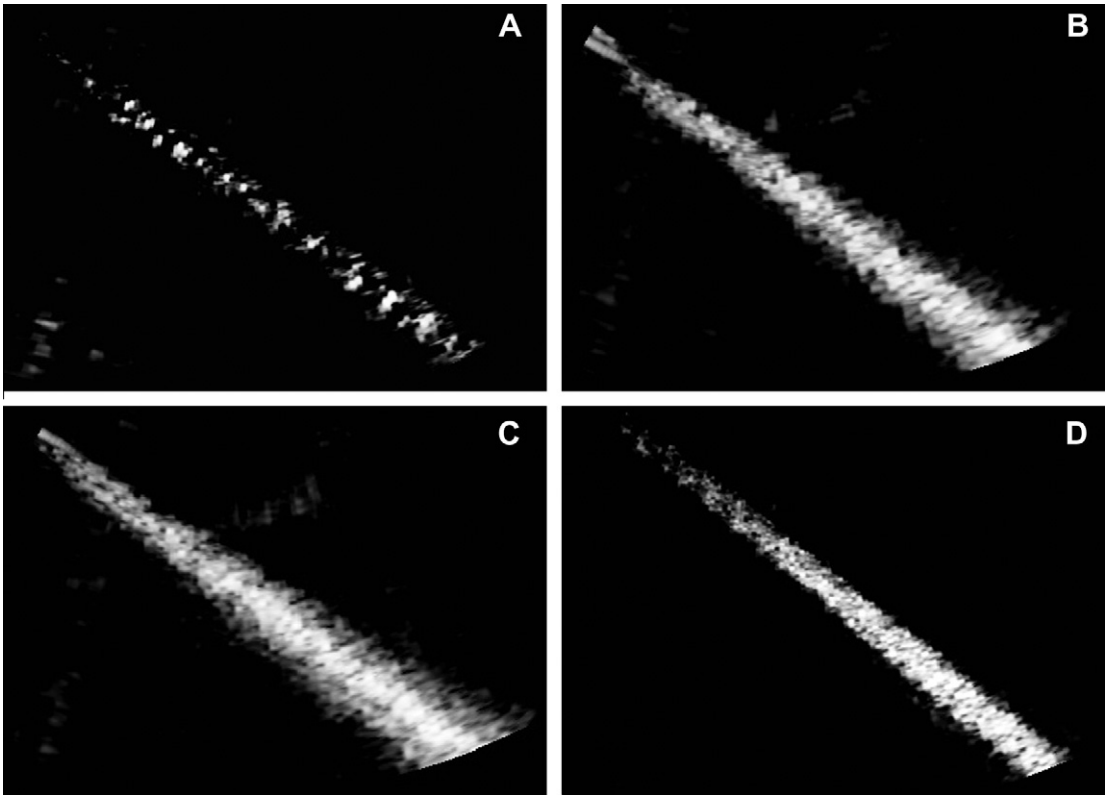

Fig. 5. Visualization of the ultrasound reflectivity of the new lipid formulations and SonoVue®. (A) DPPC/CH; (B) DPPC/PEG40S; (C) DSPC/PEG40S; (D) SonoVue®.

carriers [3andtheliteraturetherein]. Additionally, Talu et al. [28] incorporated (additionally to the DSPC) single-chained PEG-lipids (PEG40S) for the preparation of large microbubbles with an average diameter of 62 μm and a standard deviation of 6 μm. These contrast agents showed a high stability and an excellent echogenicity. The stability was caused by the well-known Stealth® effect induced by the organization of the PEG chains surrounding the liposome surface. Such formulations were also used for the preparation of long-circulating drug carriers. Based on these findings, our formulations consist of classically lipids (DPPC, DSPC, CH) and single-chained PEG40S as stabilizer. Using the common thin film hydration method, nanoscaled lipid formulations with an average size between 70 and 300 nm were prepared. While DPPC/CH showed a homogeneous size distribution with an average size of about 220 nm which is in agreement with previous findings [19,20,29], the PEG40S-containing formulations (DPPC/PEG40S, DSPC/PEG40S) showed a multimodal size distribution with a major fraction between 200 and 300 nm.

To determine the ultrasound contrast intensities of the nanoscaled lipid dispersions, a self-made closed-loop flow model was used. Flow models were also used by Smith et al. [30] and Cintas et al. [31] who used water instead of agar gel and thus paying no attention to the ultrasound properties of human tissue and blood vessels. Improved systems were developed by Schumann et al. [32] using agar gel instead of water and Spengos et al. [33] who placed a human temporal bone between the transducer and the sample. These two systems are flow through models. Our model was set up according to Kollmann et al. [25]. A C-flex® tube and agar gel in which the tube is embedded in an angle of 30° simulate the ultrasound properties of human tissue and blood vessels. The pressure in this closed-loop flow model can be adjusted to human blood pressure. Nanoscaled lipid formulations were added to the buffer reservoir, and the increase of the ultrasound contrast was observed in the C-flex® tube. As can be seen in Fig. 5, the ultrasound contrast enhancement of our nanoscaled lipid dispersions is comparable to SonoVue®. The combination of the small size and the good ultrasound contrast makes our formulations a promising tool for directed drug delivery and simultaneously diagnostic imaging. The investigations concerning storage stability of the ultrasound reflectivity (Fig. 6) revealed a continuous decrease of the ultrasound contrast for SonoVue® probably due to a loss of sulfur hexafluoride during storage. Our PEG40S-containing nanoscaled lipid formulations show only a small decrease in echogenicity. We assume that the ultrasound contrast of our PEG40S-containing formulations is not only caused by incorporated gas. Thus, it remains constant for a period of 14 days.

The physico-chemical characterization of our formulations (DPPC/PEG40S, DSPC/PEG40S) with imaging techniques revealed a coexistence of small-sized particles besides larger particles. The visualized diameters for the PEG40S-containing formulations showed discrete populations of 200–300 nm and about 70 nm. Liposomes are typically sized between 20 nm and several micrometers [13], whereas the size of micelles is below 80 nm [34].

Garbuzenko et al. [35,36] and Schroeder et al. [37] explained the coexistence of different structures in such ultrasound active formulations by the nonideal mixing behavior of liposome-forming lipids such as DPPC with micelle-forming lipids such as PEGylated lipids. It seems possible that under ultrasound induced stress, liposomes undergo structural transformations and a fraction of the liposomes forms smaller non-liposomal molecular assemblies maybe micellar structures. The possible formation of PEG-lipid micellar discs in ultrasonically irradiated liposomal dispersions have been reported by Leal et al. [26], Ickenstein et al. [38] and Schroeder et al. [3,37,39]. We assume that the liposome to micelle ratio is crucial for a good ultrasound contrast which will be further studied. The PEG-lipid content influenced also the absorption of ultrasonic energy by liposome bilayer, because of the organization of PEG moieties extending from the liposome surface [37].

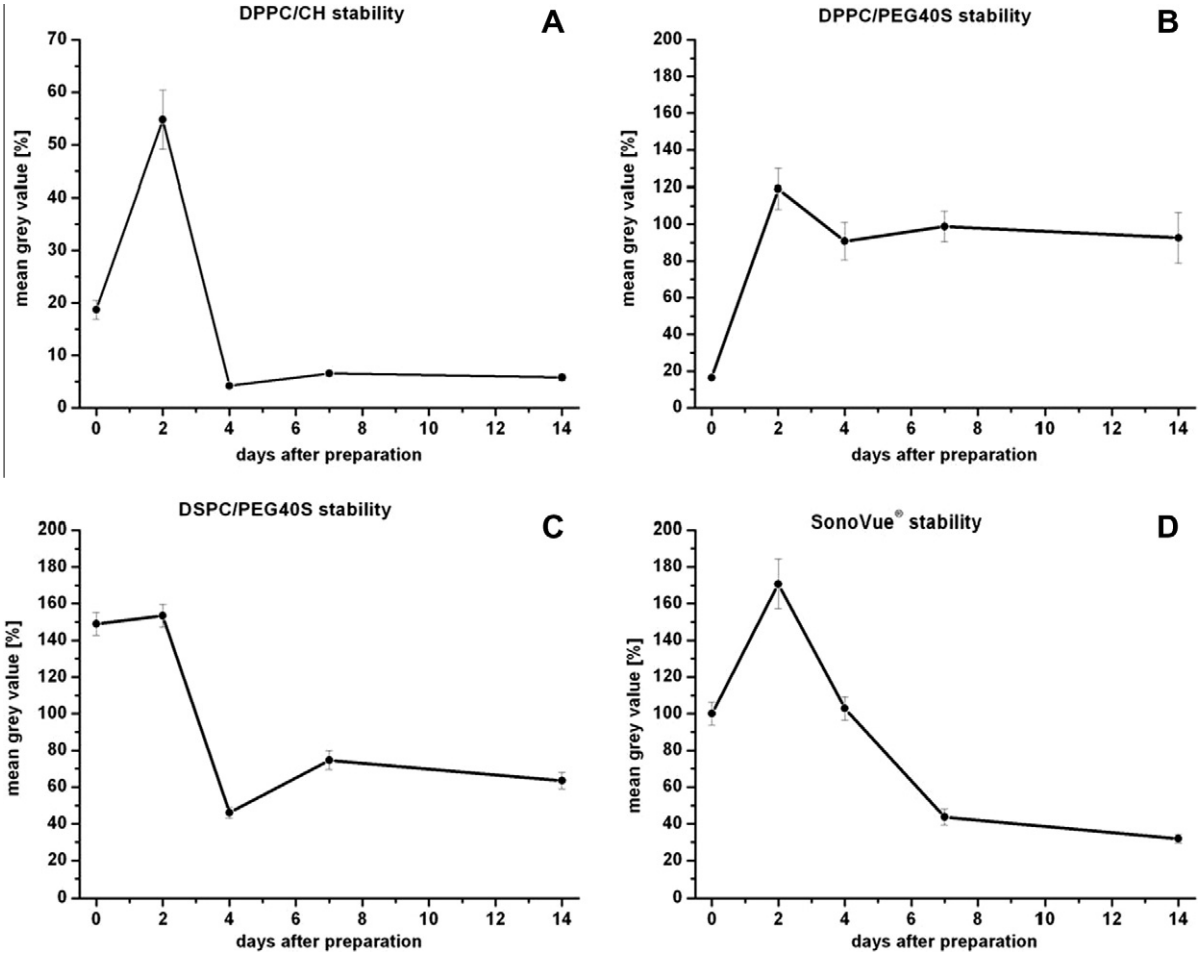

Fig. 6. Stability measurements. The stability of the lipid formulations was measured over a period of 14 days detecting the echogenicity (mean grey values). (A) DPPC/CH; (B) DPPC/PEG40S; (C) DSPC/PEG40S; (D) SonoVue®.

To get a deeper insight to the structural arrangement of the lipids in our formulations, ^{31}P NMR was utilized. Several research groups examined that micellar structures can be differentiated from liposomal structures by interpreting the line shapes of ^{31}P NMR. Schubert et al. [40] discussed that the bilayer structure of a liposomal formulation was reflected in a broad ^{31}P NMR signal, correlated to an anisotropic motion of the lipids within the bilayer.

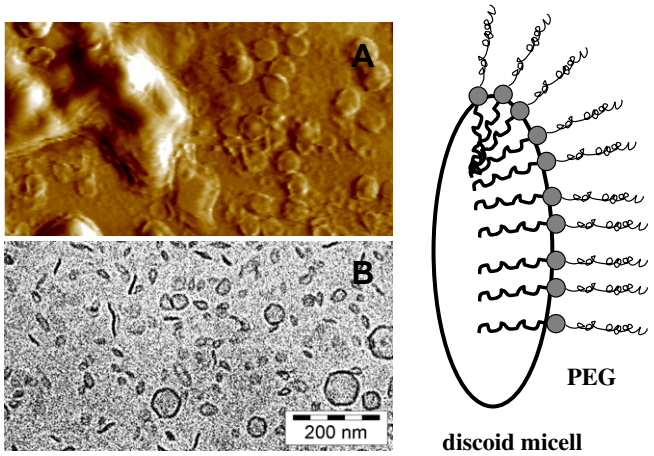

Fig. 7. High resolution AFM. (A) and cryo-TEM (B) images of discoid micelles.

According to Cullis and de Kruijff [27], bilayer structures show a low-field shoulder, whereas micelles reveal a symmetric peak. Leal et al. [26] analyzed the influence of different PEG-lipids and their concentration on the structure of lipid formulations composed of DSPC and CH. Concentrations of more than 11.5 mol% PEG-lipid lead to the formation of micelles and liposomes. Similar results regarding micelles were determined by Soong and Macdonald [41]. In Fig. 4A, a low-field shoulder related to bilayered liposomes can be clearly seen. In high resolution, AFM and TEM images discoidal micelles as proposed by Johnsson and Edwards [42] could be visualized (Fig. 7). In our opinion, the coexistence of liposomes and micellar structures is an essential prerequisite for a nanosized formulation with a high ultrasound activity.

5. Conclusion

Here, we developed and characterized new long-circulating nanoscaled ultrasound contrast agents based on different lipid formulations (DPPC/CH, DPPC/PEG40S, DSPC/PEG40S). It could be shown that small-sized formulations with high long-time stability have a comparable ultrasound reflectivity to the commercially available ultrasound contrast agent SonoVue®. DLS, AFM and cryo-TEM measurements indicate that a size distribution for the formulations between 70 and 300 nm was achieved. All formulations but the classically DPPC/CH liposomes showed a coexistence of liposomal and micellar structures, accompanied by a strong

increase of ultrasound contrast. ^{31}P NMR measurements confirmed this structural model. Ultrasound contrast stability with a homogeneous contrast for up to 2 weeks, the small size, the adjustable properties including long circulation effect make these systems promising for the use as directed drug carriers and diagnostics.

Acknowledgements

The authors thank Sabine Barnert (Freiburg) for cryo-TEM measurements, Sabine (Motzny) for help with the flow model and Dr. Thomas Kämpchen (Marburg) for ^{31}P NMR measurements as well as the group of Prof. Thomas Kissel (Marburg) for technical support. This work was financially supported by the Deutsche Forschergemeinschaft (DFG) (Forschergruppe 629 ''Nanohale'' and 495 ''Biohybridverbindungen'').

References

[1] W.G. Pitt, G.A. Husseini, B.J. Staples, Ultrasonic drug delivery – a general review, Expert Opin. Drug Deliv. 1 (1) (2004) 37–56.
[2] M. Schneider, M. Arditi, M.-B. Barrau, J. Brochot, A. Broillet, R. Ventrone, F. Yan, A new ultrasonographic contrast agent based on sulfur hexafluorid-filled microbubbles, Invest. Radiol. 30 (8) (1995) 451–457.
[3] A. Schroeder, J. Kost, Y. Barenholz, Ultrasound, liposomes, and drug delivery: principles for using ultrasound to control the release of drugs from liposomes, Chem. Phys. Lipids 162 (1–2) (2009) 1–16.
[4] L.J.M. Juffermans, B.D.M. Meijering, A. van Wamel, R.H. Henning, K. Kooiman, M. Emmer, N. de Jong, W.H. van Gilst, R. Musters, W.J. Paulus, A.C. van Rossum, L.E. Deelman, O. Kamp, Ultrasound and microbubble-targeted delivery of therapeutic compounds, Neth. Heart J. 17 (2) (2009) 82–86.
[5] L.J.M. Juffermans, A. van Dijk, C.A.M. Jongenelen, B. Drukarch, A. Reijerkerk, H.E. de Vries, O. Kamp, R.J.P. Musters, Ultrasound and microbubble-induced intra- and intercellular bioeffects in primary endothelial cells, Ultrasound Med. Biol. 35 (11) (2009) 1917–1927.
[6] R. Gramiak, P.M. Shah, Echocardiography of the aortic root, Invest. Radiol 3 (5) (1968) 356–366.
[7] K. Ferrara, R. Pollard, M. Borden, Ultrasound microbubble contrast agents: fundamentals and application to gene and drug delivery, Annu. Rev. Biomed. Eng. 9 (2007) 415–447.
[8] B.E. Oeffinger, M.A. Wheatley, Development and characterization of a nano-scale contrast agent, Ultrasonics 42 (1–9) (2004) 343–347.
[9] G. Blume, G. Cevc, Liposomes for the sustained drug release in vivo, Biochim. Biophys. Acta 1029 (1) (1990) 91–97.
[10] G. Blume, G. Cevc, M.D.J.A. Crommelin, I.A.J.M. Bakker-Woudenberg, C. Kluft, G. Storm, Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times, Biochim. Biophys. Acta 1149 (1) (1993) 180–184.
[11] A.L. Klibanov, K. Maruyama, V.P. Torchilin, L. Huang, Amphipathic polyethylene glycols effectively prolong the circulation time of liposomes, FEBS Lett. 268 (1) (1990) 235–237.
[12] A.L. Klibanov, K. Maruyama, A.M. Beckerleg, V.P. Torchilin, L. Huang, Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target, Biochim. Biophys. Acta 1062 (2) (1991) 142–148.
[13] D.D. Lasic, Liposomes from physics to applications, first ed., Elsevier Science Publishers B.V., Amsterdam, 1993.
[14] N. Rapoport, Z. Gao, A. Kennedy, Multifunctional nanoparticles for combining ultrasonic tumor imaging and targeted chemotherapy, J. Natl. Cancer Inst. 99 (14) (2007) 1095–1106.
[15] S.K. Hobbs, W.L. Monsk, F. Yuan, W.G. Roberts, L. Griffith, V.P. Torchilin, R.K. Jain, Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment, Proc. Natl. Acad. Sci. USA 95 (8) (1998) 4607–4612.
[16] R. Medel, R.W. Crowley, M.S. McKisic, A.S. Dumont, N.F. Kassell, Sonothrombolysis: an emerging modality for the management of stroke, Neurosurgery 65 (5) (2009) 979–993.
[17] A.P. Owens III, N. Mackman, Tissue factor and thrombosis: the clot starts here, Thromb. Haemostasis 104 (3) (2010) 432–439.
[18] N. Wohner, Role of cellular elements in thrombus formation and dissolution, Cardiovasc. Hematol. Agents Med. Chem. 6 (3) (2008) 224–228.
[19] S. Anabousi, M. Laue, CM. Lehr, U. Bakowsky, C. Ehrhardt, Assessing transferrin modification of liposomes by atomic force microscopy and transmission electron microscopy, Eur. J. Pharm. Biopharm. 60 (2) (2005) 295–303.
[20] H. Bakowsky, T. Richter, C. Kneuer, D. Hoekstra, U. Rothe, G. Bendas, C. Ehrhardt, U. Bakowsky, Adhesion characteristics and stability assessment of lectin-modified liposomes for site-specific drug delivery, Biochim. Biophys. Acta 1778 (1) (2008) 242–249.
[21] S. Höbel, R. Prinz, A. Malek, B. Urban-Klein, J. Sitterberg, U. Bakowsky, F. Czubayko, A. Aigner, Polyethylenimine PEI F25-LMW allows the long-term storage of frozen complexes as fully active reagents in siRNA-mediated gene targeting and DNA delivery, Eur. J. Pharm. Biopharm. 70 (1) (2008) 29–41.
[22] V. Oberle, U. Bakowsky, I.S. Zuhorn, D. Hoekstra, Lipoplex formation under equilibrium conditions reveals a three-step mechanism, Biophys. J. 79 (3) (2000) 1447–1454.
[23] A. Fukami, K. Adachi, A new method of preparation of a self-perforated micro plastic grid and its application, J. Electron. Microsc. (Tokyo) 14 (2) (1965) 112–118.
[24] N. Kaiser, A. Kimpfler, U. Massing, A.M. Burger, H.H. Fiebig, M. Brandl, R. Schubert, 5-Fluorouracil in vesicular phospholipid gels for anticancer treatment: entrapment and release properties, Int. J. Pharm. 256 (1–2) (2003) 123–131.
[25] C. Kollmann, R.A. Bezemer, K.E. Fredfeldt, U.G. Schaarschmidt, C.J. Teirlinck, A test object for quality control of the instrument for Doppler (duplex) ultrasonography, Ultraschall. Med. 20 (6) (1999) 248–257.
[26] C. Leal, S. Rögnvaldsson, S. Fossheim, E.A. Nilssen, D. Topgaard, Dynamic and structural aspects of PEGylated liposomes monitored by NMR, J. Colloid. Interface Sci. 325 (2) (2008) 485–493.
[27] P.R. Cullis, B. de Kruijff, Polymorphic phase behaviour of lipid mixtures as detected by ^{31}P NMR. Evidence that cholesterol may destabilize bilayer structure in membrane systems containing phosphatidylethanolamine, Biochim. Biophys. Acta 507 (2) (1978) 207–218.
[28] E. Talu, M.M. Lozano, R.L. Powell, P.A. Dayton, M.L. Longo, Long-term stability by lipid coating monodisperse microbubbles formed by a flow-focusing device, Langmuir 22 (23) (2006) 9487–9490.
[29] K. Tabatt, C. Kneuer, M. Sameti, C. Olbrich, R.H. Muller, C.-M. Lehr, U. Bakowsky, Transfection with different colloidal systems: comparison of solid lipid nanoparticles and liposomes, J. Control. Release 97 (2) (2004) 321–332.
[30] D.A.B. Smith, S.S. Vaidya, J.A. Kopechek, S.-L. Huang, M.E. Klegerman, D.D. McPherson, C.K. Holland, Ultrasound-triggered release of recombinant tissue-type plasminogen activator from echogenic liposomes, Ultrasound Med. Biol. 36 (1) (2010) 145–157.
[31] P. Cintas, F. Nguyen, B.BoneuV. Larrue, Enhancement of enzymatic fibrinolysis with 2-MHz ultrasound and microbubbles, J. Thromb. Haemostasis 2 (7) (2004) 1163–1166.
[32] P.A. Schumann, J.P. Christiansen, R.M. Quigley, T.P. McCreery, R.H. Sweitzer, E.C. Unger, J.R. Lindner, T.O. Matsunaga, Targeted-microbubble binding selectively to GPIIb IIIa receptors of platelet thrombi, Invest. Radiol. 37 (11) (2002) 587–593.
[33] K. Spengos, S. Behrens, M. Daffertshofer, C.E. Dempfle, M. Hennerici, Acceleration of thrombolysis with ultrasound through the cranium in a flow model, Ultrasound Med. Biol. 26 (5) (2000) 889–895.
[34] C. Rupp, H. Steckel, B.W. Müller, Mixed micelle formation with phosphatidylcholines: the influence of surfactants with different molecule structures, Int. J. Pharm. 387 (1–2) (2010) 120–128.
[35] O. Garbuzenko, Y. Barenholz, A. Priev, Effect of grafted PEG on liposome size and on compressibility and packing of lipid bilayer, Chem. Phys. Lipids 135 (2) (2005) 117–129.
[36] O. Garbuzenko, S. Zalipsky, M. Qazen, Y. Barenholz, Electrostatics of PEGylated micelles and liposomes containing charged and neutral lipopolymers, Langmuir 21 (6) (2005) 2560–2568.
[37] A. Schroeder, A. Sigal, K. Turjeman, Y. Barenholz, Using PEGylated nanoliposomes to target tissue invaded by a foreign body, J. Drug Target. 16 (7–8) (2008) 591–595.
[38] L.M. Ickenstein, M.C. Arfvidsson, D. Needham, L.D. Mayer, K. Edwards, Disc formation in cholesterol-free liposomes during phase transition, Biochim. Biophys. Acta 1614 (2) (2003) 135–138.
[39] A. Schroeder, Y. Avnir, S. Weisman, Y. Najajreh, A. Gabizon, Y. Talmon, J. Kost, Y. Barenholz, Controlling liposomal drug release with low frequency ultrasound: mechanism and feasibility, Langmuir 23 (7) (2007) 4019–4025.
[40] R. Schubert, K. Beyer, H. Wolburg, K.H. Schmidt, Structural changes in membranes of large unilamellar vesicles after binding of sodium cholate, Biochemistry 25 (1986) 5263–5269.
[41] R. Soong, P.M. Macdonald, PEG molecular weight and lateral diffusion of PEG-ylated lipids in magnetically aligned bicelles, Biochim. Biophys. Acta 1768 (7) (2007) 1805–1814.
[42] M. Johnsson, K. Edwards, Liposomes, disks, and spherical micelles: aggregate structure in mixtures of gel phase phosphatidylcholines and poly(ethylene glycol)-phospholipids, Biophys. J. 85 (6) (2003) 3839–3847.