Controlling Liposomal Drug Release with Low Frequency Ultrasound: Mechanism and Feasibility

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The ability of low-frequency ultrasound (LFUS) to release encapsulated drugs from sterically stabilized liposomes in a controlled manner was demonstrated. Three liposomal formulations having identical lipid bilayer compositions and a similar size (∼100 nm) but differing in their encapsulated drugs and methods of drug loading have been tested. Two of the drugs, doxorubicin and methylpredinisolone hemisuccinate, were remote loaded by transmembrane gradients (ammonium sulfate and calcium acetate, respectively). The third drug, cisplatin, was loaded passively into the liposomes. For all three formulations, a short exposure to LFUS (<3 min) released nearly 80% of the drug. The magnitude of drug release was a function of LFUS amplitude and actual exposure time, irrespective of whether irradiation was pulsed or continuous. Furthermore, no change in liposome size distribution or in the chemical properties of the lipids or of the released drugs occurred due to exposure to LFUS. Based on our results, we propose that the mechanism of release is a transient introduction of porelike defects in the liposome membrane, which occurs only during exposure to LFUS, after which the membrane reseals. This explains the observed uptake of the membrane-impermeable fluorophore pyranine from the extraliposomal medium during exposure to LFUS. The implications of these findings for clinical applications of controlled drug release from liposomes are discussed.

1. Introduction

The anticancer drug Doxil was the first liposomal nanomedicine to be approved by the Food and Drug Administration (FDA, 1995). Doxil is an ∼100 nm sterically stabilized liposome (SSL), remote loaded with doxorubicin by means of an ammonium sulfate gradient.[1−3] Since then, eight other liposomal drugs have been approved by the FDA. These liposomal formulations vary in size, structure, and lipid composition and have different therapeutic aims.

The rationale behind the development of liposome-based drug delivery systems is to improve drug pharmacokinetics and biodistribution and to achieve a controlled drug release rate.[4,5] While the first and second objectives have been met in cases of cancer[1,3,6] and inflammatory[7,8] and infectious diseases,[9,10] the third was not. In general, amphipathic, membrane-residing drugs tend to release too quickly from liposomes, while hydrophobic or nonamphipathic, water-soluble drugs release too slowly for therapeutic efficacy.[6]

An optimal liposomal formulation is expected to accumulate intact at the disease site and then have the drug released at a controlled rate. This can be achieved by formulation features and/or imposed chemical or physical means.[4,11] This work focuses on drug release triggered by physical means, i.e., low-frequency ultrasound (LFUS).

LFUS has been shown to release drugs from other drug delivery systems, such as polymeric micelles[12,13] and polymeric matrices,[14] as well as to enhance the permeability of biological membranes for drug and gene delivery.[15−20] The fact that the bilayer structure as well as many physicochemical properties of the liposome membrane are similar to those of biological membranes[21] led us

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to determine whether LFUS can increase the permeability of liposomes to release an entrapped drug in a controlled manner.

Earlier studies have shown that liposomes release calcein from their intraliposomal aqueous phase in response to 20 kHz ultrasonic irradiation.[22,23] Another study showed that 20 kHz ultrasonic irradiation is more efficient in releasing doxorubicin from liposomes than high-frequency ultrasound (1 and 3 MHz).[24] However, the release mechanism has not been explored yet.

Our research approach was to load therapeutic agents into SSLs, utilizing different loading methods (active versus passive loading), then to ultrasonically irradiate the liposomes in a well-defined manner, and to analyze the kinetics of release, as well as the chemical integrity of the irradiated lipids and drugs. SSLs were selected due to their long circulation time in the blood as well as their long retention in the targeted tissue.[2,25]

Feasibility was demonstrated for two anticancer liposomal formulations, doxorubicin and cisplatin, and one anti-inflammatory liposomal formulation, methylpredinisolone hemisuccinate (MPS).

2. Materials and Methods

2.1. Liposome Preparation. Hydrogenated soybean phosphatidylcholine (HSPC, MW 750, having an iodine value of 3.0; Lipoid, Ludwigshafen, Germany), 51 mol %; polyethylene glycol distearoyl phosphoethanolamine (m^{2000}PEG-DSPE; MW 2774; Genzyme, Liestal, Switzerland), 5 mol %; and cholesterol (Sigma, St. Louis, MO), 44 mol %, were dissolved in absolute ethanol (Gadot, Haifa, Israel) at 62−65 °C (above the gel-to-liquid crystalline phase transition temperature, T_m, of HSPC, 53 °C).[26] This was added to an aqueous solution, at 62−65 °C, of either calcium acetate, ammonium sulfate, or cisplatin to form multilamellar vesicles (MLV).[27] The MLV were downsized to form small unilamellar vesicles (SUV) by stepwise extrusion through polycarbonate membranes (Osmonics, Trevose, PA) using a Lipex extruder (Northern Lipids, Vancouver, Canada) starting at a pore diameter of 400 nm and ending at a pore diameter of 50 nm.

2.2. Drug Loading. *2.2.1. MPS.* Methylprednisolone hemisuccinate (MPS) sodium salt, MW 496.53 (Pharmacia, Puurs, Belgium), a highly potent anti-inflammatory steroid and an amphipathic weak acid (pK_a 4.65), was remote loaded into liposomes using a high intraliposome/low extraliposome (medium) transmembrane calcium acetate gradient, previously developed in our lab[28] and recently adapted for remote loading of MPS by Avnir et al.[29]

For the preparation of SSL-MPS (MPS loaded into sterically stabilized liposomes), the lipids were hydrated in a calcium acetate (200 mM) and dextrose (5%, w/v) aqueous solution (pH 6.5) to form MLV and then downsized to form ∼100 nm SUV by extrusion (see section 2.1). The transmembrane calcium acetate gradient was created by replacing nonliposomal calcium acetate with 5% dextrose (pH 4.0) by dialysis. MPS was then loaded into the liposomes by incubating the liposome dispersion for 1 h at 62−65 °C in a solution of 8 mg/mL MPS in 5% dextrose. The nonloaded MPS was removed by dialysis against 5% dextrose and/or with the anion exchanger Dowex 1X8 (Sigma). The final MPS-SSL had a drug-to-phospholipid mole ratio of ∼0.33.

2.2.2. Doxorubicin. We used the anticancer liposomal drug Doxil, in which the chemotherapeutic agent doxorubicin, an amphipathic weak base, is remote loaded into SSLs utilizing a high intraliposome/low extraliposome ammonium sulfate gradient.[30] The mean liposome diameter was ∼100 nm, and the drug-to-lipid mole ratio was ∼0.3.

Doxil, a gift of ALZA (Mountain View, CA), was supplied as an isotonic suspension containing 2 mg of doxorubicin per milliliter of 10 mM histidine buffer, pH 6.5, with 10% w/v sucrose.

2.2.3. Cisplatin. We used SSLs passively loaded with the anticancer chemotherapeutic agent cisplatin ("Stealth" cisplatin), as described by Peleg-Shulman.[31] The mean liposome diameter was ∼110 nm, and the drug-to-lipid mole ratio was ∼0.032.

Stealth cisplatin, a gift of ALZA, was supplied as an isotonic suspension of 1 mg/mL cisplatin in 10% w/v sucrose, 1 mM sodium chloride, and 10 mM histidine buffer, pH 6.5.

2.3. Ultrasound Apparatus. A 20 kHz low-frequency ultrasonic processor (VC400, Sonics & Materials, Newtown, CT) was used. The ultrasonic probe (13 mm diameter) was immersed in a glass scintillation vial containing 3 mL of the liposome dispersion. Irradiation was conducted at a full duty cycle at varying intensities (from 0 to 7 W/cm^2) and durations (0−180 s). The sample vial was kept in a temperature-controlled water bath, and its temperature was monitored (37 °C) throughout the experiment to prevent heat-induced liposomal drug release.[32−34]

The ultrasonic power density was determined calorimetrically as described by Hill et al.[35]

2.4. Analytical Procedures. *2.4.1. Evaluation of Liposome Lipid Integrity Using Thin Layer Chromatography.* Thin layer gel chromatography (TLC) was used to determine if any chemical changes were induced in the liposome lipids by exposure to ultrasonic irradiation. The lipids of the liposomal dispersions before and after ultrasonic irradiation were extracted by the Bligh and Dyer procedure[36] and analyzed by TLC (silica gel 60, Merck, Darmstadt, Germany), which was developed using a solvent system of chloroform/methanol/water (65:25:4 by volume). Spot detection was performed by spraying the plates with 1.6 M copper sulfate (Sigma) in 6.8% phosphoric acid, v/v (BioLab, Jerusalem, Israel), and then drying the plates with warm air.[37−39]

2.4.2. Liposome Size Distribution Analysis. Liposome size distribution before and after LFUS irradiation was measured by dynamic light scattering (DLS) using an ALV-NIBS/HPPS particle sizer equipped with an ALV-5000/EPP multiple digital correlator, at a scattering angle of 173° (ALV, Langen, Germany). These measurements were confirmed by DLS at three other angles (30°, 90°, and 150°) using the ALV/CGS-3 Compact Goniometer system (ALV). For the latter, the intensity of the DLS signal was also measured.

2.4.3. Determination of Liposomal Cisplatin Levels Using GPC Combined with Atomic Absorption Spectroscopy (AAS). Ultrasonically irradiated and nonirradiated liposomal cisplatin dispersions were chromatographed using GPC on wetted Sephadex G-50-fine (Pharmacia, Uppsala, Sweden) packed in 5 mL polypropylene columns (diameter, 1 cm; Pierce, Rockford, IL), and excess column water was removed by centrifugation at ∼580*g*. Aliquots (150 μL)

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of the liposomal dispersions were applied to the column and then centrifuged at ~580*g*. SSLs were collected at the void volume,[2,40] and SSL phospholipids were quantified using the modified Bartlett method.[2,41] The cisplatin level was determined by AAS (see section 2.4.5.2 below), and the drug-to-lipid mole ratio was calculated.

2.4.4. Cisplatin Biological Activity. The cytotoxicities of SSL-cisplatin, of cisplatin released from SSL by exposure to LFUS, and of free cisplatin were tested on cisplatin-sensitive C26 murine colon adenocarcinoma cells. The cell medium consisted of RPMI 1640 with L-glutamine (90%), fetal calf serum (9%, virus-screened), and a penicillin−streptomycin solution (1%) (all cell medium reagents were from Biological Industries, Beit Haemek, Israel). Aliquots of 800 cells per well were plated in 96-well plates (Nunc, Roskilde, Denmark) and incubated under 5% CO_2 at 37 °C for 24 h. Equal amounts (15 μL) of nonirradiated and LFUS-irradiated SSL-cisplatin dispersions, as well as free nonirradiated cisplatin (0−45 μL of 1 μg/mL cisplatin (Sigma) in saline), were added to separate wells and incubated for 24 h. The surviving cells were then quantified by a methylene blue staining assay at 620 nm, as described by Gorodetsky et al.,[42] using a Synergy HT Multi-Detection microplate reader (BioTek, Winooski, VT).

2.4.5. Drug and Gradient Quantification. Immediately after LFUS irradiation, the released drug was removed, and the drug remaining in the liposomes was quantified.

The released drugs were adsorbed using ion exchange resins: MPS on a Dowex 1X8 anion exchanger (Sigma) and doxorubicin on a Dowex 50W cation exchanger (Sigma).[43] LFUS-released cisplatin was removed by gel exclusion chromatography using Sepharose 6B (Sigma).[44,45] The levels of drugs remaining in the SSLs were quantified using HPLC for MPS, fluorescence for doxorubicin, and AAS for cisplatin (see below).

2.4.5.1. MPS. The MPS concentration and chemical integrity were determined using HPLC (Hewlett-Packard Liquid Chromatograph 1090). ChemStation software (Hewlett-Packard) controlled all modules and was used for the analysis of the chromatography data. The analytical column used was a C18 5 μm Econosphere column (length, 150 mm; inner diameter, 4.6 mm; Alltech, Carnforth, U.K.). The sample injection volume was 20 μL. The eluent was monitored at a wavelength of 245 nm with a 10 nm bandwidth. The mobile phase [acetate buffer, pH 5.8, and acetonitrile (67:33, v/v)] was delivered at a flow rate of 1 mL/min.[46,47] The MPS mean elution time was at ~2.9 min, and the sample run time was ~5 min.

2.4.5.2. Cisplatin. Integrity analysis of ultrasonically released cisplatin was performed using ^{195}Pt NMR spectroscopy. The experiments were performed on an INOVA 500 MHz spectrometer (Varian, Palo Alto, CA) using standard pulse sequences. The Pt chemical shifts were assigned relative to the external reference signal of K_2PtCl_4, set at −1624 ppm. A line broadening of 300 Hz was normally applied, and data were processed using the VNMR software (Varian).[31]

Cisplatin was quantified by AAS of Pt, at 2700 °C (λ = 265.9 nm), using a Zeeman atomic absorption Spectrometer, SpectAA300 (Varian), with a sensitivity of ±1 ng Pt/mL (corresponding to ~ ±1.5 ng cisplatin/mL), and a standard reference Pt solution (BDH Chemicals, Poole, U.K.).

2.4.5.3. Doxorubicin. Doxorubicin was quantified by determining the fluorescence emission intensity at 590 nm (excitation 480 nm), in reference to a doxorubicin standard curve, after disintegrating the liposomes in acidic isopropyl alcohol (0.075 N HCl),[1] using an LS50B luminescence spectrometer (Perkin Elmer, Wellesley, MA) equipped with WinLab PE-FL software (Perkin Elmer).

2.4.5.4. Acetate Gradient. The levels of LFUS-released acetate, as well as intraliposome acetate, were determined enzymatically using the Megazyme acetic acid assay kit (Wicklow, Ireland). The determination of intraliposome acetate required liposome dissolution by ethanol, and therefore the acetic acid standard curve was made with the same amount of ethanol as in the analyte.[29]

2.4.6. Evaluation of the LFUS Effect on Membrane Integrity by Testing Pyranine Uptake. To determine if the liposome permeability was transiently increased during exposure to LFUS, we followed the uptake of the highly hydrophilic, membrane-impermeable fluorescent compound pyranine (Molecular Probes, Eugene, OR)[48] into SSLs. For this, pyranine was added to the external medium of the SSLs. The SSL dispersions were exposed to LFUS (3.3 W/cm^2), and then the anion exchange resin Dowex 1X8 was added to remove nonliposomal pyranine. The determination of intraliposomal pyranine was based on the fluorescence emission intensity at 507 nm, the pH-independent isosbestic point (excitation at 415 nm).[49] Fluorescence measurements were conducted in the presence of the membrane-impermeable fluorescence quencher DPX (*p*-xylene-*bis*-pyridinium bromide, Molecular Probes) to quench the fluorescence of any residual nonliposomal pyranine.[28]

2.4.7. Cryogenic Transmission Electron Microscopy (Cryo-TEM). Cryo-TEM work was performed at the Hannah and George Krumholz Laboratory for Advanced Microscopy (Technion, Haifa, Israel). For each experiment, lipid dispersions at concentrations of 50 and 5 mM in 5% (w/v) dextrose in a total volume of 400 μL were used. Specimens were prepared in a controlled-environment vitrification system at 25 °C and 100% relative humidity and then examined in a Philips CM120 cryo-electron microscope operated at 120 kV. Specimens were equilibrated in the microscope below −178 °C, examined in the low-dose imaging mode to minimize electron beam radiation damage, and then recorded at a nominal underfocus of 4−7 nm to enhance phase contrast.[50] An Oxford CT-3500 cooling holder was used. Images were recorded digitally with a Gatan MultiScan 791 CCD camera using the Digital Micrograph 3.1 software package.

3. Results and Discussion

This study focuses on the ability of LFUS to release drugs from their liposomal formulations and on elucidating the release mechanism.

All liposomal formulations used in this study were sterically stabilized (SSL), were identical in lipid composition (HSPC/cholesterol/mPEG-DSPE) and size distribution (~100 nm), but differed in the encapsulated drug and drug loading method.

A previous study conducted in our lab showed that 20 kHz low-frequency ultrasound is more effective in releasing doxorubicin from liposomes than high-frequency ultrasound (1 and 3 MHz);[24] therefore, this study focused on LFUS.

3.1. Effect of Ultrasound Amplitude on Drug Release. Initial tests verified the dependence of liposomal drug release on the ultrasonic amplitude. Drug-loaded liposome dispersions were irradiated by LFUS for 60 s. The amplitude of irradiation was increased from sample to sample in the range of 0 W/cm^2 (no irradiation, i.e., control) to 7 W/cm^2.

Figure 1 shows that the dependence of liposomal MPS release on the ultrasonic amplitude is biphasic. Both phases are linear but differ in their slopes: a low slope (~3.9 [% release/(W/cm^2)]) up to the amplitude of ~1.3 W/cm^2 and a higher slope (~16.1) above this amplitude. The increase in drug release above ~1.3 W/cm^2 is explained by the initiation of a transient cavitation

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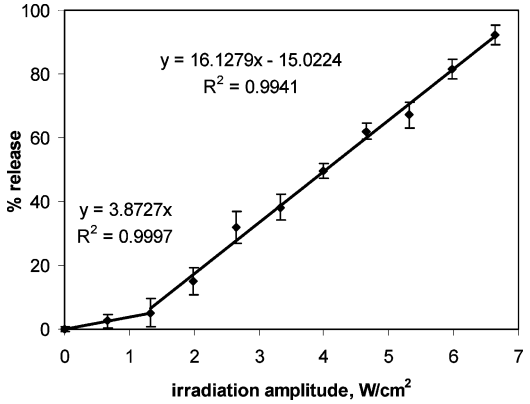

Figure 1. Effect of LFUS (60 s) amplitude on liposomal MPS release.

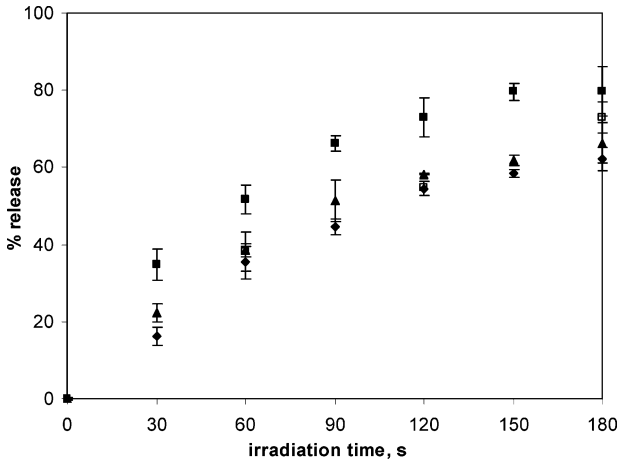

Figure 2. Effect of LFUS (20 kHz, 3.3 W/cm^2) on the release of three different liposomal (SSL) drug formulations [(■) MPS, (▲) doxorubicin (Doxil), (◆) cisplatin (Stealth cisplatin)] and of (□) SSLs with a high intraliposomal/low extraliposomal calcium acetate gradient.

(i.e., the formation, growth, and implosive collapse of bubbles in a liquid) above this energetic threshold.[51]

We suggest that cavitation occurs near the liposome membrane, in the extraliposomal medium and/or by small cavitation nuclei in the intraliposomal aqueous compartment.

Nonirradiated SSLs containing each of the three drugs (doxorubicin, cisplatin, and MPS) released <3% of the loaded drug over the experimental period when kept at 37 °C.

3.2. Effect of Irradiation Time on Level of Release. SSLs containing the drugs doxorubicin, cisplatin, or MPS, or SSLs having a high intraliposome/low extraliposome acetate gradient (the driving force for the remote loading of MPS), were irradiated by LFUS at a constant amplitude (3.3 W/cm^2) for different periods of time, from 0 to 180 s.

For SSLs loaded with MPS, ∼80% of the drug was released within the first 150 s of irradiation, after which drug release plateaus. The other formulations, doxorubicin, cisplatin, and acetate, had similar curve characteristics but slightly lower release levels (Figure 2).

These data show that substantial release of liposomal drugs can be obtained by short-term exposure to LFUS. We define this effect as "dumping", meaning the release of the majority of the encapsulated drug within a short period of time, creating a high concentration of the drug in the vicinity of the irradiated SSLs.

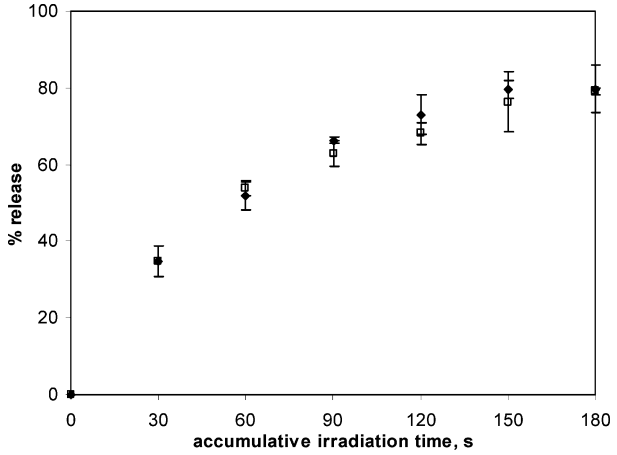

Figure 3. LFUS-triggered (20 kHz, 3.3 W/cm^2) MPS release from liposomes, comparing (◆) continuous and (□) pulsed irradiation modes.

Analysis of drug release data revealed that ultrasonically triggered liposomal drug release (up to ∼150 s), at a fixed ultrasound amplitude, follows first-order kinetics,

$$-\mathrm{d}A/\mathrm{d}t = kA$$

or, in its integrated form, $\log(A_0/A) = kt$, where A_0 is the initial amount of drug loaded in the liposomes and A is the remaining amount of drug in the liposomes after an irradiation time t, indicating that, for a given liposomal drug, release is dependent on irradiation time.

The following first-order rate constants were determined for LFUS-induced release: 0.0053 s^{-1} for MPS, 0.0029 s^{-1} for cisplatin, 0.0033 s^{-1} for doxorubicin, and 0.0031 s^{-1} for acetate (R^2 = 0.994, 0.995, 0.992, and 0.992, respectively).

3.3. Mechanism of Drug Release by LFUS. In an attempt to elucidate the mechanism of drug release, we tested several different LFUS irradiation profiles.

3.3.1. Pulsed Release. Liposomal dispersions containing MPS were irradiated at an amplitude of 3.3 W/cm^2 for different periods of time, comparing the drug release of samples that were irradiated continuously with those irradiated by pulsed mode for the same accumulated irradiation time.

Figure 3 shows that the drug release profiles of continuous and pulsed LFUS modes are almost identical with respect to the actual times of exposure to LFUS, indicating that drug release depends only on the actual irradiation time and that the effect of irradiation on liposomal drug release is cumulative. Therefore, irradiation can be conducted at either the continuous or pulsed mode to obtain the same drug release. These results are important for clinical applications, where several repeated short exposures are usually preferred to one long exposure to prevent heat-related damage to tissue.

3.3.2. Drug Release Occurs Only During Actual LFUS Exposure Time. It is well-established that LFUS is capable of increasing the permeability of biological membranes, and the permeability increase is retained for a long time after irradiation has ended.[16,52,53] We have shown that LFUS is capable of increasing the permeability of the liposome membrane, enabling drug release. We tested whether the permeability increase was prolonged or confined only to the irradiation period.

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(53) Rapoport, N.; Smirnov, A. I.; Timoshin, A.; Pratt, A. M.; Pitt, W. G. *Arch. Biochem. Biophys.* **1997**, *344*, 114−124.

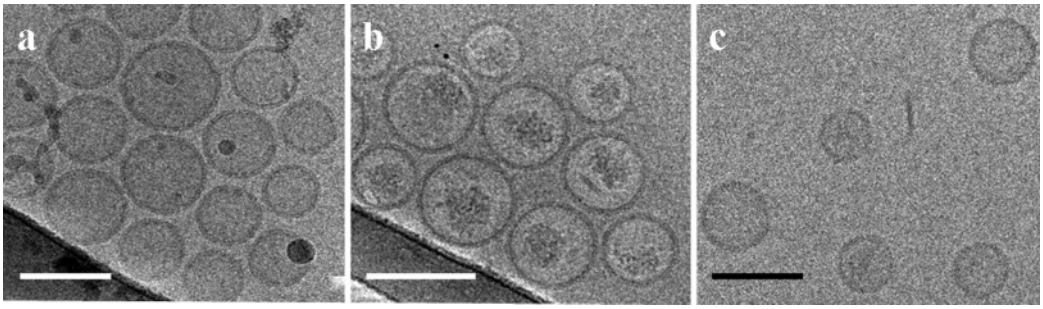

Figure 4. Cryo-transmission electron microscopy images of (a) liposomes before remote loading of MPS, (b) liposomes after remote loading of MPS, and (c) liposomes remote loaded with MPS after being exposed to LFUS (20 kHz, 120 s, 3.3 W/cm^2). The micrographs of (a) empty and (b) drug loaded liposomes are based on work done by Avnir et al.[29] Bar = 100 nm.

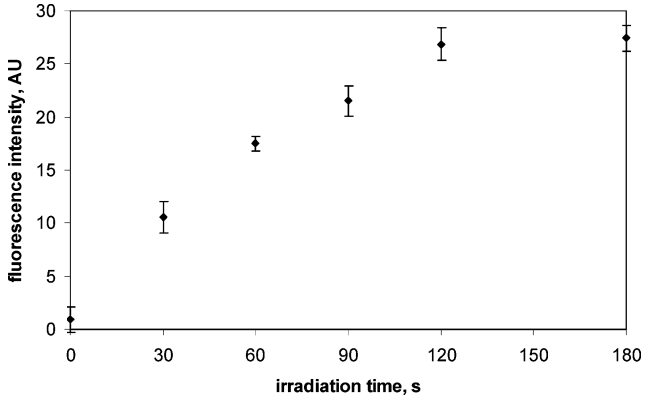

Figure 5. Effect of LFUS (20 kHz, 3.3 W/cm^2) irradiation time on liposome uptake of a membrane-impermeable fluorescent probe, pyranine, from the extraliposomal medium.

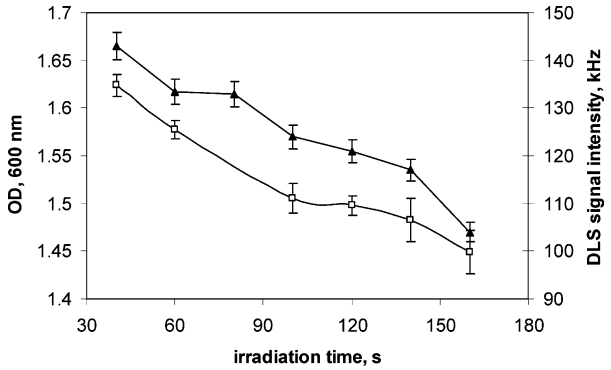

Figure 6. Effect of LFUS (20 kHz, 3.3 W/cm^2) irradiation time on liposomal MPS dispersions: (left axis, □) turbidity and (right axis, ▲) dynamic light scattering (DLS) signal intensity.

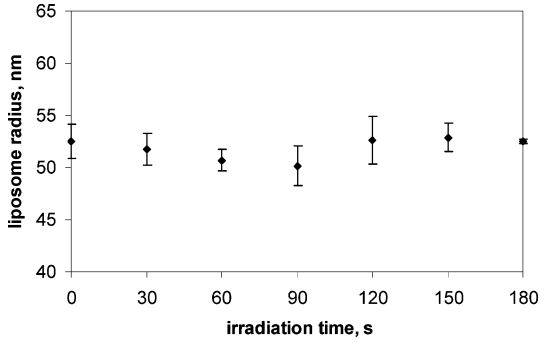

Figure 7. Effect of LFUS (20 kHz, 3.3 W/cm^2) irradiation time on liposomal MPS mean size, as assessed by dynamic light scattering at 90°.

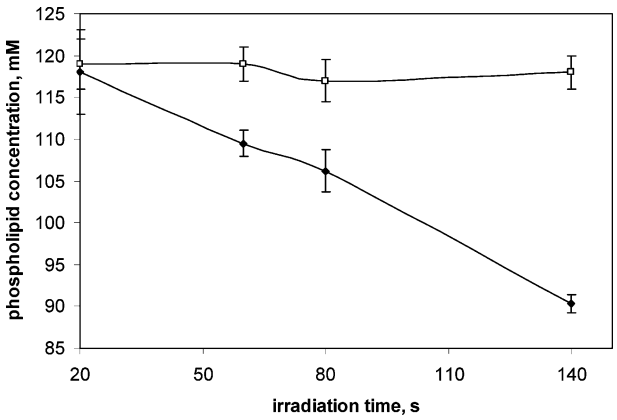

Figure 8. Concentrations of (□) total (liposomal plus nonliposomal) phospholipid and of (◆) liposomal phospholipid in LFUS-irradiated (20 kHz, 3.3 W/cm^2) liposomal dispersions.

For this, liposomal dispersions were irradiated at 3.3 W/cm^2 for periods of 30−180 s, and drug release was determined immediately after irradiation and after 72 h.

The levels of released drug were the same at both times (data points coincide, not shown). This indicates that increased permeability of the liposome membrane is transient and occurs only during exposure to LFUS. It also indicates that, after irradiation is terminated, the liposome membrane becomes impermeable again and drug release stops.

Combining these results with the data shown for pulsed release suggests that LFUS can be used for controlling the level of drug release over prolonged periods of time, which is very important for successful drug delivery.

3.3.3. Cryo-TEM Analysis of Liposome Structure. The structure of liposomes before and after exposure to LFUS was examined by cryo-transmission electron microscopy (cryo-TEM).

Figure 4a presents liposomes before loading with MPS and before exposure to LFUS. The liposome membrane is clearly visible as the slightly darker perimeter of the liposomes surrounding the inner aqueous compartment. Figure 4b presents SSLs remote loaded with MPS by means of a calcium acetate transmembrane gradient. The loaded drug, most likely as a calcium MPS precipitate, appears as the darker area within the SSL aqueous compartment. Figure 4c presents liposomal MPS irradiated for 120 s at 3.3 W/cm^2. No change in the appearance of the liposome membrane or size was visible after irradiation. In all cases, the liposome diameter indicated by cryo-TEM correlates well with the DLS measurements (presented in section 3.3.5).

However, LFUS seems to have a great effect on the presence of the intraliposomal MPS precipitate. While the nonirradiated SSLs show massive amounts of precipitate in the intraliposomal aqueous phase (Figure 4b), the irradiated liposomes (Figure 4c)

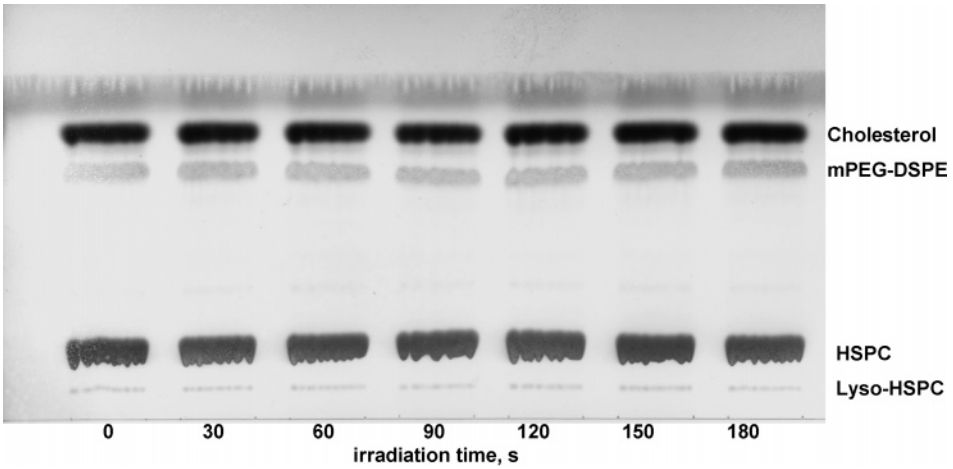

Figure 9. Effect of LFUS (20 kHz, 3.3 W/cm^2) on lipid chemical stability, based on TLC analysis of extracted lipids.

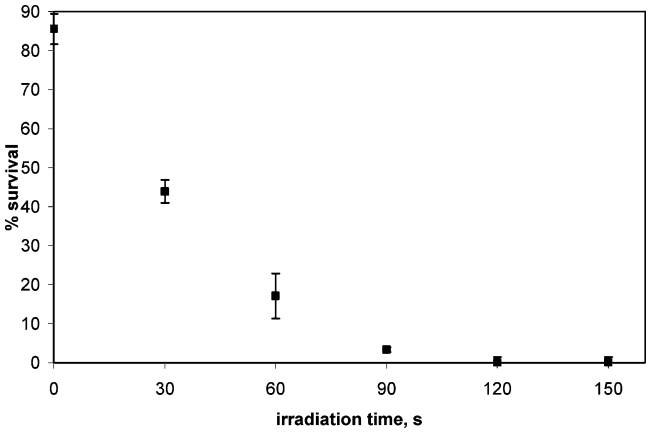

Figure 10. Cytotoxicity of cisplatin released from Stealth cisplatin liposomes by LFUS (20 kHz, 3.3 W/cm^2) to C26 murine colon adenocarcinoma cells in culture.

seem to be either empty or to have much less precipitate, which accords with the release of MPS by exposure to LFUS, as shown in Figure 2.

These findings suggest that LFUS induces transient porous defects in the liposome membrane, enabling drug release, which occurs only during exposure to LFUS, after which membrane integrity is restored.

3.3.4. LFUS Transiently Ruptures the Liposome Membrane. We propose that LFUS induces a transient disruption of the liposome lipid bilayer, releasing the loaded drug. If this is the case, then LFUS may also cause a leakage of extraliposomal medium solutes into the intraliposomal aqueous compartment. This was tested by adding a water-soluble, highly negatively charged, membrane-impermeable fluorophore, pyranine, to the extraliposomal aqueous medium prior to irradiation. The liposomal dispersion was irradiated, and then the level of pyranine in the intraliposomal aqueous compartment was quantified. Figure 5 shows that pyranine is taken up into the liposomal aqueous compartment, having an uptake level proportional to the exposure time of SSLs to LFUS.

These findings support the hypothesis of transient liposome membrane rupture and/or formation of porelike membrane defects as the mechanism of LFUS-induced rapid drug release, followed by rearrangement/resealing of the lipid bilayer. The first-order release kinetics data (see section 3.2) also support such a mechanism.

3.3.5. LFUS Induces Disruption of a Fraction of the Liposomes. We found that the turbidity of the liposomal dispersions decreased as LFUS irradiation time increased. This effect occurred in all three liposomal drug formulations, doxorubicin, cisplatin, and MPS, and also in drug-free liposomes (exemplified in Figure 6 for MPS).

A possible explanation of such a change in turbidity is a decrease in liposome size.[54,55] This assumption was tested by measuring the diameters of LFUS-irradiated liposomes using dynamic light scattering (DLS) at four different angles (30°, 90°, 150°, and 173°; both wide and narrow angles were used to more sensitively test for the presence of smaller or larger liposomes, respectively[56,57]). The results indicated that the liposome diameter remained unaffected, independent of irradiation time (see Figure 7 for DLS data at 90°). Therefore, we propose that the decrease in turbidity is not due to a decrease in SSL diameter or drug release but rather due to a decrease in the number of liposomes in the dispersion as a result of the disassembly of some of the liposomes.

This assumption was tested in two different ways: (i) by recording the DLS signal intensity of the liposomal dispersions irradiated for different times and (ii) by direct evaluation of the liposome phospholipid concentration relative to the total phospholipid concentration (liposomal plus nonliposomal) in dispersions exposed to LFUS for different times.

For liposome preparations of identical size distribution, the DLS signal intensity is proportional to the concentration of liposomes present in each dispersion (number of liposomes per unit volume).[56,57] The decrease in the DLS signal intensity with irradiation time (shown in Figure 6) suggests a decrease in the concentration of liposomes present in the dispersion.

The determination of the amount of total phospholipid, and liposomal phospholipid (liposome peak in GPC), revealed that the amount of liposomal phospholipid decreased, while the total phospholipid remained unchanged with LFUS irradiation time (Figure 8). This further supports our assumption that some of the liposomes are disassembled by LFUS. The fraction of disassembled liposomes after 140 s of LFUS irradiation was ~23%, which is in agreement with the reduction in the DLS signal

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intensity and optical density (OD) at 600 nm (Figure 6). In other experiments, we noticed that longer exposures to LFUS (>30 min) disassembled larger fractions of liposomes, although it must be noted that thermal effects may become dominant in such long exposures to LFUS. However, in the majority of liposomes, short LFUS exposures induced only transient porosity of the membrane rather than complete liposome disassembly. Therefore, the dominant effect is increased liposomal permeability without altering liposome size distribution.

3.4. Chemical Integrity of Ultrasonically Irradiated Phospholipids and Drugs. The chemical integrity of liposomal formulations, including irradiated drugs and lipids, was tested using HPLC for doxorubicin and MPS, NMR for cisplatin, and TLC for lipids.

SSL dispersions containing MPS, doxorubicin, and cisplatin were irradiated for periods of 30−180 s (20 kHz, 3.3 W/cm^2) and then analyzed using nonirradiated liposomal dispersions as reference. The HPLC chromatograms (see section 2.4) of LFUS-irradiated and nonirradiated drugs were identical for both doxorubicin and MPS, as were the NMR spectra for irradiated and nonirradiated cisplatin (data not shown), indicating that LFUS, under the conditions used, does not induce any chemical changes in these three drugs.

Analysis of liposomal lipid extracts of irradiated and nonirradiated SSLs by TLC shows (Figure 9) that no significant chemical changes occurred as a result of exposure to LFUS.

3.5. Cytotoxicity of a Drug Released from SSL by LFUS. The effect of LFUS on the biological potency of released drugs was assessed by determining the cytotoxicity of LFUS-released cisplatin. Stealth cisplatin was selected because no detectable cisplatin is released upon storage without exposure to LFUS. Aliquots of these LFUS-irradiated dispersions were added to cultures of cisplatin-sensitive C26 murine colon adenocarcinoma cells for the evaluation of drug cytotoxicity. As irradiation time increased, more cisplatin was released from the liposomes (Figure 2). Cytotoxicity was found to be proportional to the liposome irradiation time (Figure 10) and similar to that of equal amounts of nonirradiated free cisplatin added to the cells, thus indicating that LFUS-released liposomal cisplatin retained its biological activity.

4. Conclusions

This study demonstrates that low-frequency ultrasound (LFUS) is capable of effectively releasing three different drugs from the intraliposome aqueous compartment within a short period of irradiation (<180 s). Similar release kinetics were measured for all tested liposomal systems, irrespective of the loaded drug or drug loading method, pointing strongly to the effect of LFUS on the liposome membrane as the main cause of drug release and to the feasibility of its application for a wide range of liposomal drugs. Continuous or pulsed drug release can be obtained, depending on the LFUS irradiation mode. The predominant mechanism of drug release is suggested to be the transient formation of porelike defects in the liposome membrane through which the drug is rapidly released. These defects are most likely caused by LFUS-induced cavitation occurring near the liposome membrane in the extraliposomal medium and/or by small cavitation nuclei in the intraliposomal aqueous compartment. The porelike defects in the membrane reseal once LFUS irradiation has stopped. A small portion of the liposomes (~23%) were completely disassembled, suggesting that, in these cases, LFUS-induced defects to the membrane were irreversible.

Exposure to LFUS modified neither the chemical properties of the irradiated liposomal drugs or lipids nor the biological activity of these drugs.

In vivo feasibility was demonstrated in preliminary experiments using LFUS to release cisplatin from Stealth liposomes in a murine lymphoma model (Schroeder et al., unpublished).

The effective release of liposomal drugs by LFUS in such a short period of time is a proof-of-concept pointing to the potential use of LFUS for triggering and controlling liposomal drug release and opens the door to more preclinical and clinical research.

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Abbreviations

AAS	atomic absorption spectroscopy
GPC	gel permeation chromatography
LFUS	low-frequency ultrasound
MLV	multilamellar vesicles
MPS	methylprednisolone hemisuccinate
SSL	sterically stabilized liposome
SUV	small unilamellar vesicles

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