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Release of a Paramagnetic Magnetic Resonance Imaging Agent from Liposomes Triggered by Low Intensity Non-Focused Ultrasound

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Pulsed Low Intensity Non-Focused Ultrasound (LINFU) was used to trigger the release from liposomes of the clinically approved Magnetic Resonance Imaging (MRI) agent Gadoteridol. The extent of the release was monitored by relaxometric measurements upon changing both ultrasound stimulus (power, application times and mode, duty cycle values) and physico-chemical variables of the theranostic agent (liposomes size, shape, chemical composition, and concentration of the encapsulated agent). The release was not heat-mediated, but promoted by mechanical interactions between the acoustic radiation waves and the soft nanovesicles. The application of pulsed LINFU led to a controlled release detectable by both Nuclear Magnetic Resonance (NMR) relaxometry and MRI. Such promising observations were followed by an *in vivo* proof-of-concept study on a syngeneic B16 melanoma mouse model. The obtained results demonstrated the great potential of LINFU for designing MRI-guided protocols aimed at visualizing the release of drugs from liposomal carriers. This study could bring to the development of a new therapeutic for personalized medicine.

Keywords: Liposomes, Ultrasound, Non-Focused Ultrasound, MRI, Image-Guided Drug-Release, Theranostics.

1. INTRODUCTION

Liposomes are widely used in medicine as drug carriers because they can significantly reduce systemic drug toxicity and improve drug accumulation and bioavailability at the target site.[1,2] Once there, the liposomal shuttles must deliver their cargo to the diseased cells, thereby enabling the drug to exert the desired therapeutic effect. The release of the drug from the nanovesicle is a crucial step that controls the overall success of the therapy. An improvement of the stability of the liposomal formulation associated with the optimization of the pharmacokinetics of the nanomedicine (e.g., use of active targeting approaches) reduces the potentially harmful systemic release of the drug, thereby increasing the accumulation of the carrier at the target site. However, highly stable carriers may jeopardize the release of the drug at the pathological site. For this reason, there is the demand of more sophisticated release modes in which the drug is released upon the occurrence of a proper stimulus. Triggering stimuli can be grouped in endogenous (e.g., pH, enzymatic activity, redox potential) or externally applied (e.g., heat, light, ultrasound).[3] Typically, ultrasound (US) can stimulate the release of material encapsulated in thermo-sensitive liposomes through the temperature rise associated with the application of High-Intensity Focused Ultrasound (HIFU).[4–7] Though the use of HIFU has been demonstrated successful, a mechanic release induced by non-focused US might bring benefits as the reduction of potential heat-associated side effects, and the extension of the US triggered approach also to non thermosensitive carriers. The release of material encapsulated in non-thermo sensitive liposomes using low intensity non focused US has been first reported by Lin and coworkers.[8,9] There is a consensus on the view that the mechanic release occurs through the formation of transient pores in the liposomes bilayer, with a minor contribution from vesicle disruption, likely associated with inertial cavitation effects or intrinsic instability of the carrier.[10–12] However, irrespectively from the specific mechanism involved, several variables either related to the acoustic radiation (e.g., intensity, duration, frequency) or dependent on the liposome characteristics (e.g., bilayer composition, size) can affect the release. The interplay of such variables, combined to the intrinsic complexity of the US set up,

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makes the comparison among literature reports rather difficult. The improvement of the therapeutic outcome using liposomal drugs exposed to non-focused US has been already demonstrated *in vivo* on experimental tumor models[13–16] after the *in vitro* optimization of the release efficiency.[17]

Recently, nanomedicine has received a strong impulse from the emerging field of "theranosis," [18] which aims at developing innovative strategies to provide an imaging[19] support to therapies, including the design of probes for the *in vivo* visualization of drug delivery and release.[20, 21]

Among the available imaging technologies, Magnetic Resonance Imaging (MRI) is certainly well suited for theranostic purposes in virtue of its excellent spatio-temporal resolution and low invasiveness. Typically, MRI contrast generates from chemicals able to affect the relaxation times (T_1 and T_2) of the water protons. Paramagnetic Gd(III) complexes (as T_1 agents) and superparamagnetic iron oxide particles (SPIO, as T_2*/T_2 agents) are the most clinically relevant classes of MRI contrast agents.[22] Importantly, liposomes are excellent carriers for both types of agents, thereby offering valuable options for designing theranostic procedures.[23, 24]

When the task is the visualization of drug release from liposomal carriers, the best approach is to encapsulate a hydrophilic paramagnetic agent in the aqueous nanovesicle core.[26, 27] In fact, in this case, one may go with "MRI silenced" liposomes that can be obtained either by reducing the exchange rate of water molecules across the liposomal membrane or by a proper control of the relaxation rate of the water molecules in the inner aqueous cavity. Then, the release of both the drug and the agent, removes the "quenching" effect, thereby allowing the visualization of the release process through the detection of a contrast enhancement.

Other strategies can be also adopted. For instance, it has been shown that liposomes coated with hydroxyapatite and entrapping nanodots of SPIO in the inorganic shell can release their content upon application of high frequency, low intense, continuous US.[28] In that case, the release of the liposomes payload was associated with changes in negative T_2*/T_2-based MRI contrast.

The primary motivation of this work was to demonstrate, for the first time, the potential of MRI to produce a positive contrast enhancement following the LINFU-induced release of a Gd(III)-based agent from stealth liposomes. The data collected *in vitro* were corroborated by an *in vivo* proof-of-concept that highlighted the ability of MRI to successfully detect the release of the liposome content upon the local application of LINFU to an experimental murine tumor model.

2. MATERIALS AND METHODS

2.1. Chemicals

DPPC (1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine), DSPC (1,2-Distearoyl-sn-Glycero-3-Phosphocholine), DPPG (1,2-Dipalmitoyl-sn-Glycero-3-Phosphoglycerol), POPC (1-Palmitoyl, 2-Oleyl-sn-Glycero-3-Phosphocholine), and DSPE-PEG-2000 (1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-*N*-[Methoxy (Polyethylene Glycol)-2000], Ammonium salt) were purchased from Avanti Polar Inc. (Alabaster, Al, USA). (PolyEthyleneOxy)^{25}-(PolyButadiene)^{40} di-block copolymer was purchased from Polymer Source (Quebec, Canada). Gadoteridol was kindly provided by Bracco Imaging SpA (Colleretto Giacosa, TO, Italy). The dimeric complex Gd-**1** was synthesized according to the procedure reported elsewhere.[29]

All the other chemicals were purchased from Sigma-Aldrich (St. Louise, MO, USA).

The cell culture medium RPMI 1640, fetal bovine serum (FBS), glutamine, penicillin-streptomycin mixture and EDTA were purchased from Cambrex (East Rutherford, NJ, USA).

2.2. Nanovesicle Preparation

Nanovesicles were prepared according to the thin film hydration method.[30] The total amount of membrane components for each preparation was 20 mg/ml. Seven types of vesicles were prepared:

(i) DPPC/DSPE-PEG2000 (95/5 molar ratio),
(ii) DSPC/DSPE-PEG2000 (95/5 molar ratio)
(iii) DPPC/DPPG (95/5 molar ratio),
(iv) DPPC/Chol/DSPE-PEG2000 (55/40/5 molar ratio),
(v) DSPC/Chol/DSPE-PEG2000 (55/40/5 molar ratio),
(vi) POPC/Chol/DSPE-PEG2000 (55/40/5 molar ratio), and
(vii) (PolyEthyleneOxy)^{22}-(PolyButadiene)^{40} (polymersomes).

The thin lipid films were loaded with Gadoteridol (300 mM solution) or Gd-**1** (200 mM solution). The mean hydrodynamic diameter of the liposomes was determined by dynamic light scattering (DLS) measurements carried out on a Malvern ZS Nanosizer (Malvern Instrumentation, UK).

2.3. Release Measurements

The quantitative assessment of probe release after the exposure of the liposomal suspension to US was performed *in vitro* at 0.5 *T* on a fixed frequency relaxometer (Stelar, Mede (PV), Italy). The percentage of Gadoteridol release was calculated using the following expression:[47]

$$\text{Percentage of release}\% = \frac{R_1^{\text{US}} - R_1^{\text{no-US}}}{R_1^{\text{Triton}} - R_1^{\text{no-US}}} \cdot 100 \qquad (1)$$

where R_1^{US} refers to the longitudinal relaxation rate of the liposomal sample after insonation, $R_1^{\text{no-US}}$ is the relaxation rate of the same, non-insonated, sample (where all the Gd-based agent is entrapped in the nanovesicles), and R_1^{Triton} is the relaxation rate of the liposomal suspension in which the encapsulated probe has been fully released upon addition of 10% V/V of Triton-X100.

2.4. Animal Model

6 to 10-week-old female C57Bl6 mice (Charles River Laboratories, Calco, Italy) were subcutaneously inoculated in the left foreleg with 0.2 ml of a suspension containing approximately 1 million of B16 murine melanoma cells. B16 cells were grown in RPMI 1640 medium supplemented with 10 % of FBS, 2 mM of glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. B16 (murine melanoma) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Experiments were performed according to the national regulations and were approved by the local bioethical.

2.5. MRI Measurements

T_1-weighted MR images were acquired at 7.05 *T* on a Bruker Avance 300 (Bruker BioSpin, Milano, Italy) spectrometer equipped with a Micro 2.5 microimaging probe. The measurement of the magnetic susceptibility of the liposomes suspension allowed the determination of the total concentration of the paramagnetic complex according to a previously reported procedure.[31]

When the tumor reached a mean diameter of about 4 mm (ca. 7 days after cell inoculation), 20 μl of the DSPC/DSPE-PEG-2000 liposomes suspension were directly inoculated in the lesion.

A series of T_{1w} images (Spin-Echo sequence, $TE = 3.3$ ms, $TR = 250$ ms), were acquired before and after liposome injection, and then after the tumor exposure to pulsed LINFU. A total number of 3 mice were examined.

MRI T_1 contrast was calculated in the region of interest (ROI) encompassing the whole tumor from the water signal intensity, and expressed as percentage enhancement ($SI^{enh}\%$) according the following formula:[48]

$$SI^{\text{enh}}\% = \frac{SI_{\text{post}} - SI_{\text{pre}}}{SI_{\text{pre}}} \cdot 100$$

where SI_{pre} and SI_{post} are the signal intensity (both normalized with respect to an external reference), before and after the intratumor injection of the liposomes suspension, respectively.

2.6. Ultrasound Apparatus

Pulsed LINFU was generated by the home-built system. The circuit included by a piezoelectric flat transducer (Mastersonic Company, Switzerland, diameter 4.5 cm), with a nominal resonance frequency of $28 \pm 0,4$ kHz and a maximum power of 50 W. US intensity was measured using a calorimetric method, which is commonly used for the measurement of US intensity at low frequency.[32,33,50] The US transducer was immersed in a thermally insulated spherical flask made of latex and filled with water at room temperature (without internal acoustic absorber). With a duty cycle of 50% ($t_{\text{ON}} = t_{\text{OFF}} = 100$ ms) and a cross-section piezoelectric disk area of 15.9 cm^2, an intensity I_{SAPA} (SAPA = Spatial Average Pulse Average) value of 5.8 ± 0.4 W/cm^2 and a spatial-average temporal-average intensity, I_{SATA}, of 2.9 ± 0.3 W/cm^2 were obtained . The acoustic pressure, calculated from the US intensity was 0.21 MPa. On this basis, a Mechanical Index of 1.25 was obtained. The used transducer was a system approved for human use and it was connected to:

(i) a pilot circuit with variable resistance (TEMAT electronic, distributed by C. Quaranta Srl, Chieri (TO), Italy),
(ii) an oscilloscope (Tektronix TDS1001B),
(iii) a multimeter (Fluke 87 V), and
(iv) an attenuated (100×) oscilloscope probe for the connection to the circuit.

The multimeter was inserted in series into the power connection heads in the US transducer to monitor the current absorption during the operation of the piezoelectric component. The oscilloscope was connected in parallel to the feeding circuit to find the frequency and the amplitude of the tension signal.

Working at variable resistance, some variations in transducer power current supply were observed, with a sensibility of ca. 0.1 A. The probe frequency was calibrated at 27.6 ± 0.5 kHz because at this resonant frequency the maximum value of current absorption at the transducer (6.7 ± 0.3 A) and the minimal frequency variability was observed. The oscilloscope allowed the monitoring of the stability of the emitted pulsed US wave during the transducer calibration and during liposome insonation experiments. The experimental setup consisted of the US apparatus, a cylindrical container filled up with an isotonic buffer and internally coated by a polyurethane acoustical foam panels with pyramidal sections (angle sides of 40° to prevent standing wave formation),[34] and a 50 μm thick acoustically transparent latex spherical container for the sample to be insonated.

2.7. Histological Evaluation

All the experiments on mice were conducted in accordance with the Ethical guidelines of the University of Torino.

After US applications, animals from both experimental groups (US treated and controls) were sacrificed under anesthesia by cervical dislocation. Tissue samples from tumor, liver and kidney were immersed overnight in Carnoy's fixative solution at 4 °C and then embedded in paraffin. De-waxed 5 μm sections were stained with hematoxylin-eosin (H&E) and examined under a light microscope.

3. RESULTS

3.1. Comparison Between Continuous and Pulsed US Exposure on Nanovesicles with Different Formulations

Stealth liposomes based on DSPC (mean hydrodynamic size 110 ± 5 nm) and DPPC (mean hydrodynamic size 100 ± 4 nm), both encapsulating ca. 300 mM of the clinically approved T_1 MRI agent Gadoteridol (Chart 1), were exposed to a continuous (i.e., without signal stop) LINFU application for a time ranging from 60 to 300 seconds; each sample was insonated only once and then replaced with a new one (Fig. 1). In the following, all the bars in the graphics will show the standard error calculated by the propagation method, unless otherwise specified.

Differently from literature reports,[20,21] the observed profiles do not have the hyperbole/exponential-like trend, but the release versus time is allometric (logarithmic linear) $Y = A * X^b$, as demonstrated by the analysis reported in Table I.

The liposome formulations used in this experiments have a well-defined gel-to-liquid transition temperature (T_M, DPPC ≈ 41 °C; DSPC ≈ 53 °C), values at which the liposome bilayer become leaky and the entrapped material is released.[49] Therefore, it was crucial to check whether the observed release was or not mediated by a temperature increase caused by the US application. To this purpose, temperature was measured directly in the liposome suspension during the US exposure. The temperature readout after the longest US exposure (5 minutes) indicated a temperature rise of only 2 °C. This result is a robust indication that the release of Gadoteridol from the liposomes was not heat-mediated.

3.2. Kinetics of Gadoteridol Release Upon LINFU Exposure (Continuous Mode)

The release kinetics of Gadoteridol from the two formulations was also monitored after the application of the LINFU stimulus. The objective of this study was to check whether the US-induced release occurs during the US application only, or it lasts longer. Figure 2 shows that the release of the paramagnetic agent stopped just after the US stimulus was switched off. This observation is a clear demonstration that the insonation did not give rise to a long-term destabilization of the vesicles. Furthermore, it emphasizes the high grade of control that can be exerted on the LINFU-triggered release.

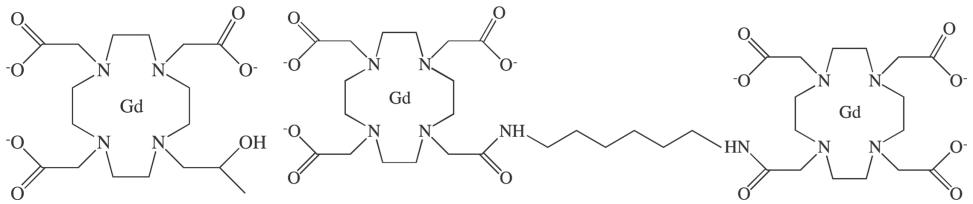

Chart 1. Gadoteridol (top) and Gd-**1** (bottom).

3.3. From Continuous to Duty Cycle (Pulsed) LINFU

The release of Gadoteridol was also investigated by using pulsed acoustic waves. Furthermore, to assess whether the release was affected by the liposomes formulation, samples differing in the chemical composition of the vesicle bilayer (presence of cholesterol, unsaturated alkyl chains, PEG coating, di-block-copolymers[36]) were prepared. Pulsed LINFU exposure consisted of the application of a train of periodic and short (from 10 ms to 1 s) US waves interleaved by periods in which the transducer was switched off. The ratio between ON and OFF periods defines the duty cycle percentage (DC%) that represents the fraction of time in which the transducer is switched on.

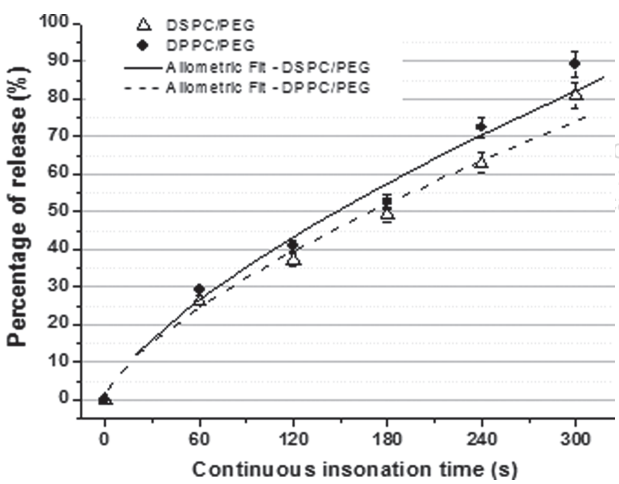

Fig. 1. Release percentage of Gadoteridol as a function of the LINFU exposure in continuous mode, (without duty cycle application) for stealth liposome formulations based on DSPC and DPPC. Reported values are the average of a group of five experiments performed for each insonation time and liposomes type; bars represent the standard error.

Table I. Statistical results of the percentage release of Gadoteridol with US continuous insonation from DPPC and DSPC stealth liposomes. The results indicate a logarithmic linear fit model (ln $Y = A + b * \ln X$) as allometric relation that in both cases can explain over 97% of the data obtained and the correlation coefficient *r* indicates a highly significant relationship between this two variables (insonation time and release of the liposomal content). The *P*-value of the Anova test (< 0.05) indicates a statistically significant relationship. Pearson's Correlation Coefficient values, *r*, strongly correlate insonation time with Gd rel.%.

	Fit model			*ANOVA*	
LIPOSOME	Y = release % X = insonation time	R^2	*Correlation value r*	*F* test	*P*-value
DSPC-PEG	$Y = 1.46^* X^{0.69}$	**0.97**	**0.99**	296,37	**0,0004**
DPPC-PEG	$Y = 1.48^* X^{0.7}$	**0.97**	**0.99**	222,86	**0,0007**

A duty cycle of 50% was used in the first series of experiments, whereas the total US exposure was fixed at 3 min (i.e., 1.5 min US_{ON} and 1.5 min US_{OFF} for each experiment).

Surprisingly, the release profiles are strongly affected by the ton values and, especially, by the physico-chemical characteristics of the vesicles.

3.4. Release as a Function of Duty Cycle

To assess the role of the duty cycle, the release of Gadoteridol from DSPC/DSPE-PEG2000 liposomes and DPPC/DSPE-PEG2000 liposomes was determined as function of DC%, keeping fixed t_{ON} at the values in which the two formulations displayed the maximum release with a duty cycle of 50% (i.e., 250 ms and 100 ms, respectively). The total US exposure was lowered to 1 minute to avoid the possible saturation of the release.

The results shown in Figure 3 indicate that the increase of DC% facilitates the release for both the formulations, with a larger dependence observed for DSPC-based liposomes.

3.5. Effect of Liposome Shape and Payload of the Encapsulated Agent

Some specific applications of liposomes as MRI agents require the preparation of non-spherical vesicles.[40] Furthermore, it has been demonstrated that the shape of nanoparticles can influence their extravascular accumulation.[41] For these reasons, it was deemed of interest to test whether the shape of the vesicles could affect their sonosensitivity. Beside that, it is also noteworthy to evaluate the possible role of the intraliposomal concentration of the entrapped molecule as well as its molecular size.

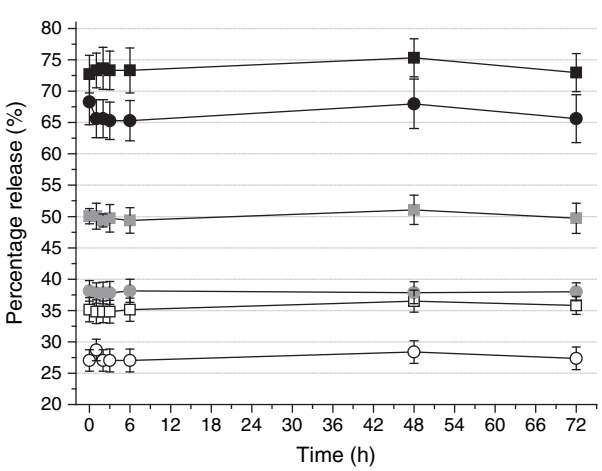

Fig. 2. Time dependence of the release of Gadoteridol from stealth DPPC-(squares) and DSPC-(circles) based liposomes after a single LINFU application (exposure time: 2 min (white), 3 min (grey), and 4 min (black)). During the experiment liposomes were kept at 25 °C.

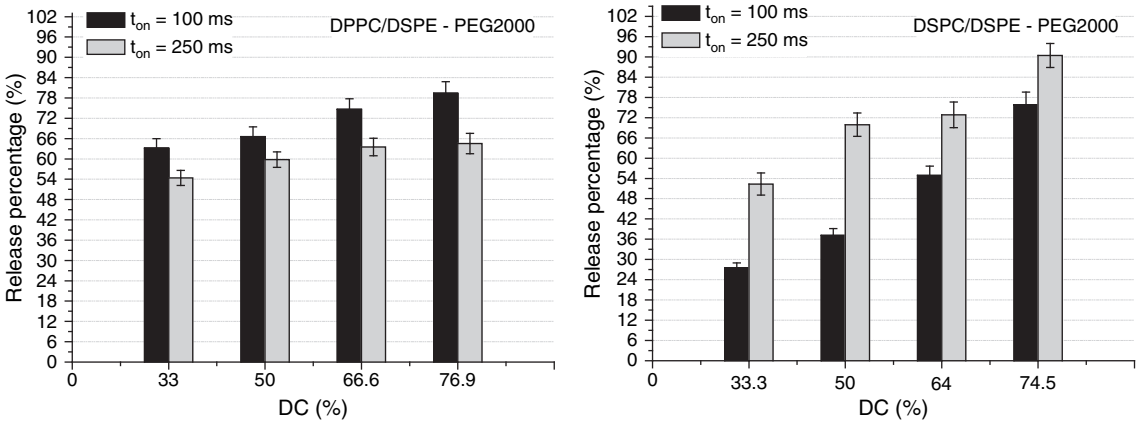

Fig. 3. Dependence of the release of Gadoteridol on the duty cycle upon pulsed LINFU exposure. t_{ON} values were kept fixed to 100 ms and 250 ms. Left: DSPC/DSPE-PEG2000 liposomes, Right: DPPC/DSPE-PEG2000 liposomes. Total insonation time 1 min.

3.6. MR Imaging: *In Vitro* Validation

As first experiment, a phantom containing non stealth DPPC-based and stealth DSPC-based liposomes (previously insonated for different times with a pulsed acoustic waves) was subjected to a MR scan using a conventional T_1-weighted pulse sequence (Fig. 4).

The brightening observed upon increasing the insonation time reflects the enhancement in the longitudinal relaxation rate of the water protons, which is consequent to the release of the paramagnetic agent. Moreover, the longitudinal relaxation rate (R_1) was determined for each sample and the results, subtracted by the diamagnetic contribution (0.4 s^{-1}) are reported in Figure 5.

The data were modeled with an exponential function, whose rate constant is related to the sonosensitivity of the liposomes. On this basis, the release of the DSPC-PEG formulation is ca. three-fold faster than that of DPPC-DPPG, as already predictable from the data reported in Figure 6. However, for insonation time longer than 10 minutes, the relaxation rate measured for the two formulations were almost equal and in agreement with a complete release of Gadoteridol.

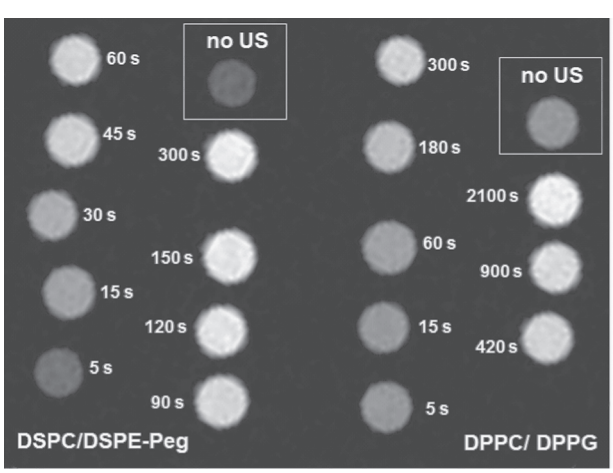

Fig. 4. MRI T_{1w} image (7.05 T, room temperature) of a phantom consisting of several liposomal samples previously insonated with pulsed LINFU for the time indicated close to the sample. DSPC/DSPE-PEG2000 liposomes (left) was insonated using a t_{ON} value of 250 ms, whereas for DPPC/DPPG liposomes (right) was used a t_{ON} value of 100 ms (DC% = 50%). The concentration of Gadoteridol in the suspensions was 0.75 mM and 1.5 mM for DSPC/PEG and DPPC/DPPG, respectively.

3.7. MR Imaging: *In Vivo* Proof-of-Concept

The potential of pulsed LINFU to trigger a MRI detectable release of Gadoteridol from liposomes was also preliminary tested *in vivo* on mice bearing a subcutaneous syngeneic B16 melanoma (average tumor volume: 100 mm^3). This experiment, propaedeutic to future *in vivo* applications, was primarily designed to investigate the release behavior of liposomes in a system containing biological interfaces that can significantly influence the liposome response to the US stimulus. To this purpose, a very small volume (20 μL) of DSPC/DSPE-PEG2000 liposomes loaded with Gadoteridol was injected directly in the tumor mass. Then, the animals ($n = 3$) were subjected to a LINFU exposure directed on the tumor.

Before LINFU exposure, a calibration of the applied US was performed. The variable resistance circuit was adjusted until obtaining a maximum power current of 10 ± 0.6 A, necessary to maintain a stable frequency of 27.6 ± 0.5 kHz. Variable resistance adjustment led to an increase of the acoustic intensity proportional to the increase in the absorption of current monitored using the multimeter connected to the base of the piezoelectric component. An acoustic intensity value (I_{SATA}) of 8.2 ± 0.4 W/cm^2 was measured. This figure was higher than the corresponding value determined for the *in vitro* experiments (2.9 ± 0.3 W/cm^2). This difference was mostly due to the different US attenuation caused by tissue thickness and interface effects occurring *in vivo*.

The animals were placed in a special animal bed that is used for the acquisition of MR images and then immersed in water up to the abdomen with an angle of approximately 45° to minimize reflections and ultrasonic beam attenuation. The US transducer was then positioned in water parallel to the mouse and directed at the sonication point with a distance of about 1.5 cm.

Furthermore, a phono-absorbing crown sponge was positioned around the tumor lesion to border the exposure. The animal bed had a hemi-cylindrical section, with a side opening, which allows the mouse to be positioned correctly for US exposure, and for the subsequent MRI scan, thus avoiding a repositioning procedure.

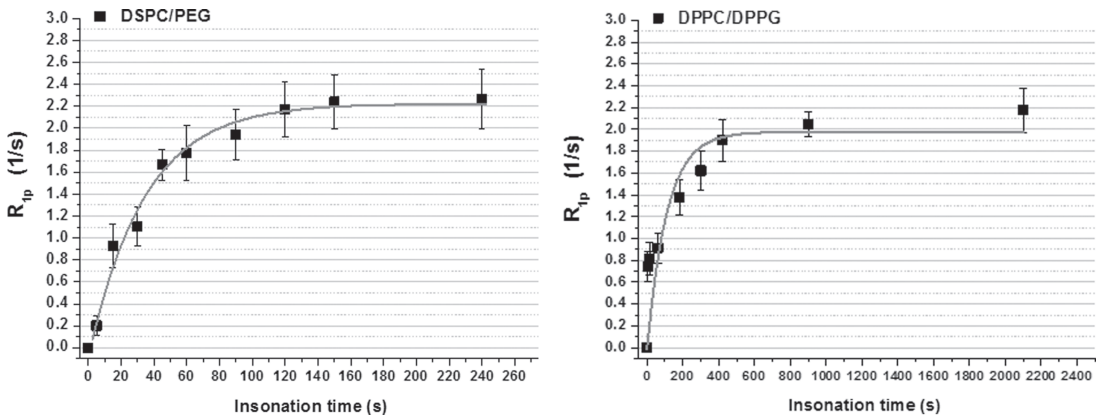

Fig. 5. The graphs show the results obtained on R_1 *p* value; this results were obtained with a concentration normalization versus 1. *Right panel*: DSPC-PEG bilayer liposomes. *Left panel*: DPPC-DPPG bilayer liposomes (the panel within the graph contains the magnification of data in the first 60 seconds of exposure). A different *x*-axis scale was necessary as the membranes showed different release efficiency. The bars represent the standard deviation.

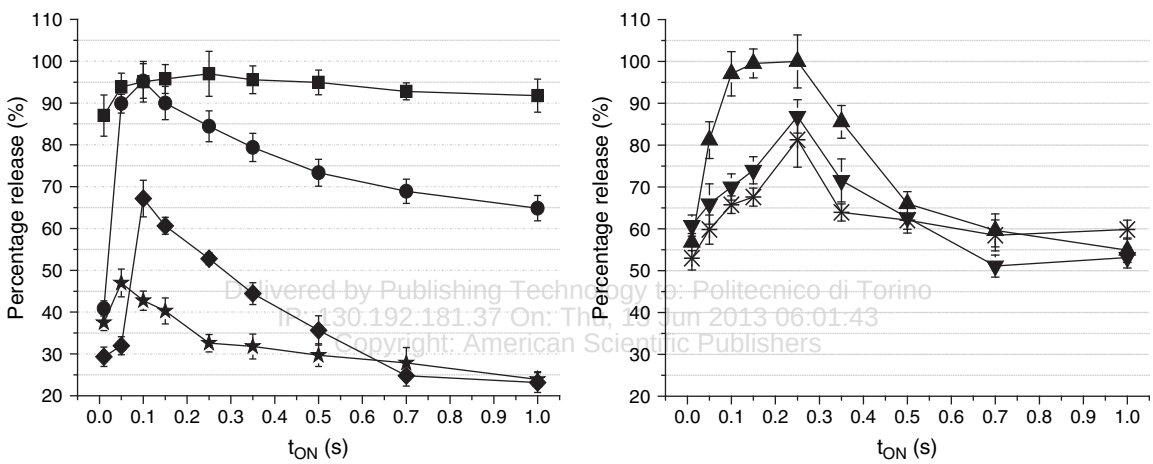

Fig. 6. Release profiles of Gadoteridol as a function of t_{ON} values (DC% = 50%, total US exposure: 3 minutes) from different formulations of nanovesicles: DSPC/DSPE-PEG2000 95/5 120 nm (squares), DPPC/DSPE-PEG2000 95/5 130 nm (circles), DPPC/DPPG 95/5 130 nm (rhombic), polymersomes 190 nm (stars), DSPC/Chol/DSPE-PEG2000 55/40/5 140 nm (upper triangles), DPPC/Chol/DSPE-PEG2000 55/40/5 160 nm (lower triangles), POPC/Chol/DSPE-PEG2000 55/40/5 160 nm (asterisks).

The LINFU application was carried out with a duty cycle of 50%, a t_{ON} value of 250 ms, and a total sonication time of 2 minutes. Immediately after the exposure, the animal bed was repositioned in the MRI scanner and post US images were acquired (Fig. 7).

Table II. R_1 pvalues statistical parameters. Exponential relationship was demonstrated in both cases (R^2 values).

Liposome	Fit model			R^2
DSPC-PEG	$y = A*[1-exp(-k*x)]$		y = release % k = release rate x = insonation time	0.98
	Value	Standard error		
	$A = 2.22$	0.09		
	$k = 0.03$	0.001		
DPPC-DPPG	$y = A*[1-\exp(-k*x)]$		y = release % k = release rate x = insonation time	0.91
	Value	Standard error		
	$A = 1.98$	0.32		
	$k = 0.009$	0.003		

The bright spot observed in the insonated area tumor clearly indicate the effective release of the paramagnetic agent. Furthermore, as Gadoteridol is rapidly excreted by kidneys, a large T_1 contrast was also detected in the bladder just after the LINFU application.

Finally, classical histology exams were performed to check possible cellular damages caused by LINFU exposure. No signs of necrosis and disruption of vascular structures were detected, at least by means of light microscopy examination, either in tumor (Fig. 8) or in distant organs (kidneys and liver).

4. DISCUSSION

This work was mainly aimed at evaluating the potential of MRI to visualize the release of material (e.g., drugs) from nanovesicular carriers stimulated by low frequency non focused US. To achieve this goal, the most straightforward approach was to fill the nanocarrier with a T_1 paramagnetic agent. In fact, when the relaxation time of the water protons of the aqueous core of the vesicle is shorter than the average residence time of the water in the core ($T_1^{intra} < t^{intra}$), then the ability of the paramagnetic complex

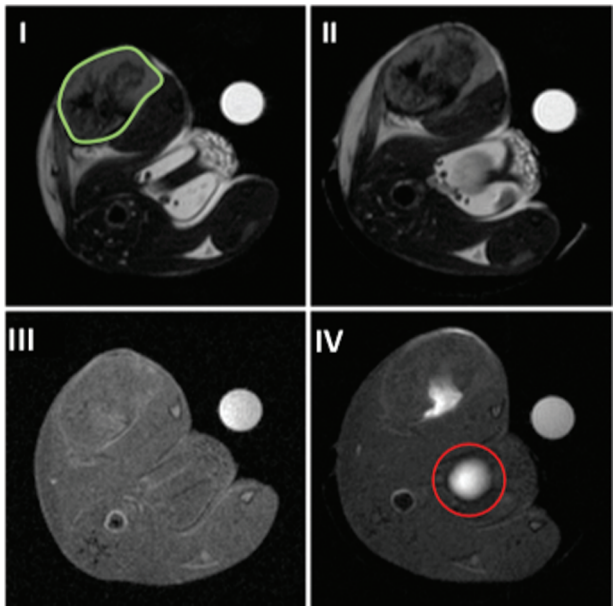

Fig. 7. Axial MR images (7.05 T) of one of the three mice examined bearing a B16 melanoma (indicated by the green line). I and II: T_{2w} images; III and IV: T_{1w} images. I and III: images acquired after liposome injection in the tumor. II and IV: images acquires after local pulsed LINFU application (t_{ON} 100 ms, DC% = 50%, total insonation time 3 minutes). The bright spot outside the tumor (comprised in the red circle) observed in image IV corresponds to the lower portion of the bladder and it arises from the renal excretion of Gadoteridol.

to relax the bulk water protons is "quenched." For this reason, the quenching requires the entrapment of high amounts of the paramagnetic probe (to reduce T_1^{intra}) in a vesicle with a low-permeable membrane (to elongate t^{intra}). When the relaxivity of the entrapped agent is limited by water permeability[35] of the vesicle membrane, the release of the probe is accompanied by a relaxation rate enhancement. Besides quantifying the release efficiency *in vitro*, this phenomenon is the basic concept for the MRI visualization of the release *in vivo*.

As first experiment the release of the clinically approved agent Gadoteridol from conventional DSPC- or DPPC-based stealth liposomes was investigated under the application of continuous low frequency (28 kHz) non focused acoustic waves.

DPPC-based vesicles showed a slightly higher tendency to release their content (ca. 10% difference after 5 min) than DSPC liposomes. It has already been reported that the nature of the phospholipids in the liposome bilayer plays an important role in the sonosensitivity of the vesicle, though an accurate comparison among the published data is not possible due to the differences in the used experimental setup. However, it is noteworthy that the release of Gadoteridol was very high (> 80%) for both formulations after 5 minutes of exposure (Fig. 1).

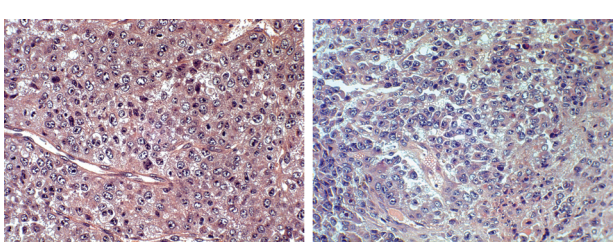

Fig. 8. Histological evaluation. Representative images of tumor tissue from insonated (left) and control (right) mice (×200, hematoxylin-eosin).

Another important finding is illustrated in Figure 2. In fact, the observation that the release occurs only during the US exposure, thereby not affecting the overall stability of the vesicles, allows a high control of the release, and it indicates that the US-mediated mechanism occurs on a very short time-scale without appreciable long-term effects on the vesicle structure.

A relevant result of this study is the assessment of the effects caused by the application of pulsed LINFU, in which a new set of variables (e.g., the length of the pulse and the duty cycle), potentially influencing the sonosensitivity of the vesicles, are introduced in the experimental setup.

For instance, the release profile appeared strongly affected by the t_{ON} values (Fig. 6), as well as by the chemical composition of the bilayer. The results obtained can be summarized as follows:
(i) DSPC-based liposomes generally showed the highest release at any t_{ON} values,
(ii) the incorporation of pegylated phospholipids (stealth liposomes) favored the release,
(iii) polymeric nanovesicles like polymersomes showed the lowest release, and
(iv) the incorporation of cholesterol (Fig. 6 right) caused a general reduction of the release.

Contrarily to what reported in literature,[17] we found that the application of pulsed US promotes the release. As example, the release from DSPC-based formulation increased from 50% (continuous LINFU) to 95% (t_{ON} 250 ms, $DC\% = 50\%$, 3 minutes of sonication).

Unexpectedly, pulsed LINFU induced a differential release even for liposomes differing for only 2 carbon atoms in their principal phospholipid component, as clearly evident by comparing nanovesicles based on DSPC (C18 chains) and DPPC (C16 chains). Using a short t_{ON} value of 10 ms (DC% = 50%, total exposure time 3 min), the release from DSPC-based vesicles was more than 200% higher than DPPC-based liposomes (Fig. 6). Similar, or even higher, differences in the release extent were observed for other formulations like non-stealth liposomes, polymer-based vesicles, and cholesterol-containing liposomes.

The reduced release observed for the non-stealth DPPC/DPPG formulation agreed with data already reported in literature, where it was accounted for in terms of a sort of "antenna effect" played by the PEG coating that can favor the interaction between the acoustic waves and the vesicles.[15]

On the other hand, the lower release efficiency observed for cholesterol-containing liposomes and for polymersomes (which are exclusively made of amphiphilic di-block copolymers), it is likely the consequence of the higher stiffness of the resulting bilayers. Several publications correlated sonosensitivity with vesicles formulation,[37–39] but the different insonation setup, as well as some controversial results, makes difficult to find a general rationale.

Another important observation is the good release obtained with POPC-based liposomes. In fact, this partially unsaturated phospholipid has a low T_M value (−2 °C) that prevents a temperature-mediated release at $T > 0$ °C. Hence, this finding is an additional support to the view that the release is not induced by heating.

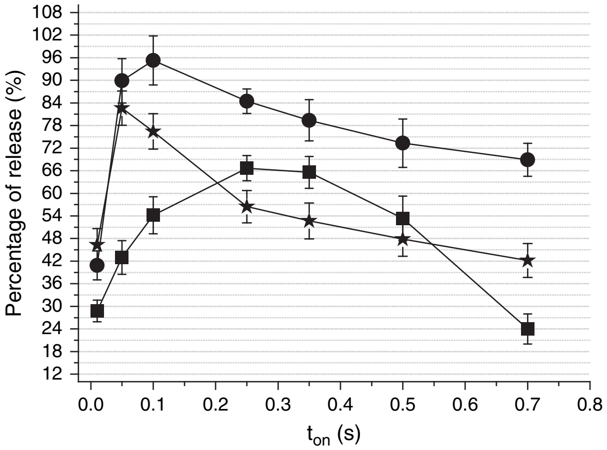

Fig. 9. Release profiles of Gadoteridol from DPPC/DSPE-PEG2000 liposomes upon pulsed LINFU application. Squares: liposomes encapsulating 300 mM of Gadoteridol. Circles: liposomes encapsulating 50 mM of Gadoteridol. Stars: non-spherical liposomes prepared encapsulating 40 mM of Gadoteridol and dialyzed against hyper-osmotic buffer to induce a change in vesicle shape.[39] The vesicles had hydrodynamic diameters comprise in the 120–130 nm interval. Figure 5-clearly highlights the dependence of the sonosensitivity of the liposomes on shape and concentration of the entrapped material.

Taken together, these results not only indicate that the pulse-rate of the US application strongly affects the release efficiency, but also that the relationship between t_{ON} and the composition of the vesicles can be positively exploited to envisage therapeutic protocols in which US can induce a selective, triggered release of a specific drug in spatial- and time-controlled way.

The release was also affected by the duty cycle of the pulsed scheme, with a general enhancement of the release upon increasing DC% (Fig. 3). Furthermore, the release observed for DSPC-based liposomes was more sensitive to DC% than DPPC-based vesicles, thereby outlining once again the great versatility of using a pulsed scheme to control the release.

Other factors, like vesicle shape, and type and concentration of the entrapped agent, were also evaluated. Figure 9 suggests that both shape and intravesicular composition may contribute to the release profile. Particularly interesting was the effect caused by the change in the vesicles shape. In this case, the effective concentration of Gadoteridol in the osmotically shrunken vesicles is higher than the amount used in the hydration of the lipid film (here 40 mM), due to the leakage of water during the shrinkage of the vesicles. However, it has been estimated that the reduction in the inner volume of the vesicles leads to a four-fold increase in the concentration of the entrapped agent.[42] This means that if the liposomes were spherical, the release profile should lie somewhere in the middle between the release observed for the formulations entrapping 50 mM and 300 mM of Gadoteridol. As this was not the case, the conclusion is that the shape of the liposomal carrier is a relevant factor for the LINFU-triggered release.

To assess the role of the size of the encapsulated agent, the release of a paramagnetic agent bigger than Gadoteridol (the dimeric Gd-**1** complex reported in Chart 1)[15] was investigated. The release of Gd-**1** from DSPC/DSPE-PEG2000 liposomes was completely comparable with that one observed for Gadoteridol either at t_{ON} 100 ms or at t_{ON} at 250 ms (data not shown), thereby indicating that, at least for the agents here considered, the size of the encapsulated chemical does not influence the release properties.

Previous studies hypothesized that low frequency non-focused ultrasounds can trigger the release from water-filled nanovesicles primarily by two mechanisms: (i) transient destabilization on the vesicle membrane, and (ii) vesicle disruption induced by inertial cavitation (IC), with a predominance of the former.[12,14,16] However, other mechanisms, likely concomitant, could contribute to explain the results obtained.

In this work, we found that LINFU application did not affect hydrodynamic size and vesicles count (measured by DLS), thereby suggesting that, differently from HIFU,[38,43] IC should not play a relevant role. Moreover, the occurrence of massive inertial cavitation effects can be also excluded by looking at the histology results discussed previously, and it is also important to outline that the inertial cavitation threshold is generally much higher using short pulses, short total insonation time and low duty-cycle values.[51]

Hence, the release mechanism might involve stable cavitation (SC) effects. SC induces an oscillation of the vesicles that do not reach the critical size beyond that IC takes place. The oscillation may lead to a transient and instantaneous destabilization of the membrane structure that results in the fast leakage of the liposomal content.

However, the release model should take into account for other experimental observations herein reported. First, the release is strongly affected by the LINFU application mode: continuous or pulsed. Importantly, in case of the latter mode, the investigated liposome formulations (with the only exception of DSPC/DSPE-PEG2000 liposomes) displayed a specific t_{ON} values (typically in the range 100–300 ms) at which the release was maximal. Therefore, this behavior is somehow correlated with the different physical and mechanical properties of the vesicles (e.g., mass, elasticity, compressibility, membrane compactness and so on). The remarkable dependence of the release on t_{ON} and duty cycle values could be related to the low-frequency component (Hz scale) generated by the pulsed mode that modulates the carrier acoustic wave (28 kHz). Moreover, the release model should also justify the unexpected role played by the shape and the composition of the vesicles cavity (see Fig. 9).

Nevertheless, the development of a reliable physico-chemical model to shed more light on the mechanism that governs the release of liposomal content triggered by pulsed LINFU was beyond the scope of this work, and it will be tackled in future studies.

A first *in vitro* experiment to assess the MRI potential for the visualization of release is reported in Figure 4. A good T_1 contrast enhancement (brightening), dependent on the release extent $R1p$, was observed (Fig. 4); release rate coefficients can be obtained through the fitting of a $R1p$ values as function of insonation time, to quantify the mean release velocity (Fig. 5). Fitting data show two different liposomes k-velocity release values: lowest k DPPC/PEG value indicates release rate lower than the DSPC/PEG liposome.

The data confirmed the possibility to visualize the selective release from a mixture of liposomes having different sonosensitivity trough the selection of an appropriate choice of US exposure and t_{ON} values. As already discussed, DSPC-based liposomes was much more prone to release their content upon LINFU exposure.

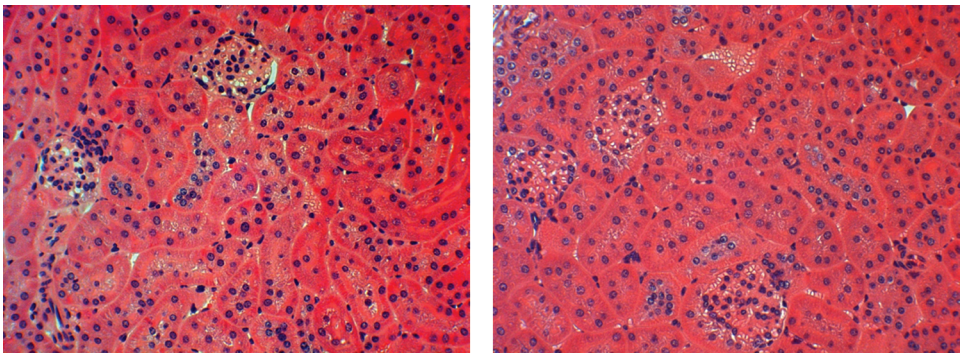

Fig. 10. Left: Histology (×200, hematoxylin-eosin) on a kidney of a mouse subjected to US exposure on the tumor. Right: control (i.e., non insonated mouse).

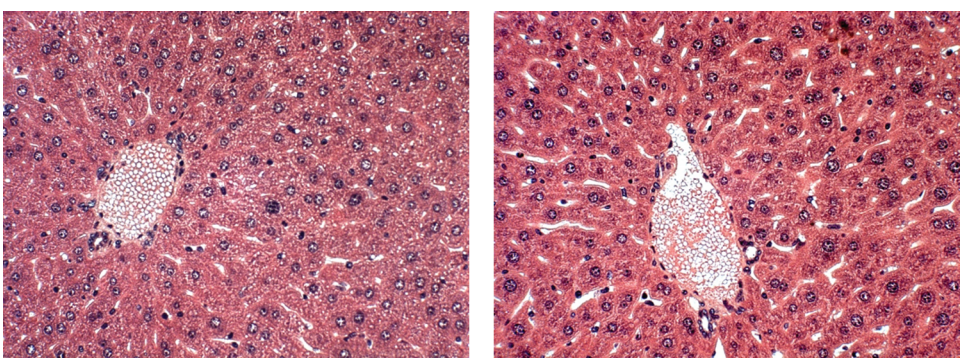

Fig. 11. Left: Histology (×200, hematoxylin-eosin) on a liver of a mouse subjected to US exposure on the tumor. Right: control (i.e., non insonated mouse).

On the basis of these promising results, we deemed of interest to get an *in vivo* proof-of-concept, with the primary aim of developing an insonation setup optimized for *in vivo* experiments. Then, a small volume of a suspension containing DSPC-based liposomes entrapping Gadoteridol was injected in a subcutaneous syngeneic melanoma B16 tumor.

As the result of the local LINFU application, a very good T_1 contrast enhancement[44] ($180 \pm 26\%$) was detected in the lesion, thus paving the way for future preclinical/clinical translations.

To check the bio-safety of the pulsed LINFU setup used for the *in vivo* experiment, a standard histology assessment on the explanted tumor, kidneys, and liver was carried out.

Tumor masses were composed by melanocytes with different grades of nuclear and cytoplasmic anomalies. Neoplastic cells were arranged in nets and trabeculae embedded in a stroma with a high level of angiogenesis (Fig. 8). Ultrasound treatment did not produce any additional alteration on tumor morphology. Scattered tumor necrotic cells were also observed in the specimen not exposed to US. Moreover, the anatomical integrity of the blood vessels walls, independently of their caliber, was also preserved, as no evidence of erythrocytes extravasation was detected. Furthermore, there were not observed elements of damage in the liver and kidney sections belonging from both groups of animals (Figs. 10 and 11).

5. CONCLUSIONS

In this work, the potential of MRI as non-invasive imaging tool for the *in vivo* visualization of LINFU-induced drug release from nanovesicular carrier was successfully demonstrated for the first time. The main potential advantage of the application of pulsed non focused acoustic waves is the possibility of designing new improved therapeutic schemes where different drugs, loaded in proper nanocarriers, could be selectively released in very controlled way by selecting proper values of t_{ON}, duty cycle, and/or total insonation time. The use of this insonation scheme allows the reduction of the exposure times, keeping high the release efficiency, and avoiding cavitation damages. Additionally, as it has been demonstrated that liposomes containing paramagnetic complexes in their inner cavity also act as T2 agents,[22, 45] the herein proposed system could report simultaneously on both drug-delivery[46] (via T2-based imaging) and drug-release (via T1-based imaging). Work is currently on going in our laboratory aimed at:

(i) translating the herein reported results to a preclinical stage in which the paramagnetic nanocarrier (also loaded with a given drug) is administered intravenously,

(ii) developing a theoretical model to accurately describe the release mechanism in order to optimize the overall performance of the theranostic protocol, and

(iii) to explore the use of higher, and more clinical translatable, US frequencies (e.g., 1–3 MHz).

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ABBREVIATIONS

MRI, Magnetic Resonance Imaging; US, Ultrasound; LFUS, Low Frequency Ultrasound; HIFU, High-Intensity Focused Ultrasound; TE, Echo Time; TR, Repetition Time; H&E, Hematoxylin-Eosin; PEG, Poly-Ethylene-Glycole; DC%, Duty cycle percentage; IC, Inertial Cavitation; SC, Stable Cavitation; SPIO, SuperParamagnetic Iron Oxide; DLS, Dynamic Light Scattering; DPPC, 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine; DSPC, 1,2-Distearoyl-sn-Glycero-3-Phosphocholine; DPPG, 1, 2-Dipalmitoyl-sn-Glycero-3-Phosphoglycerol; POPC, 1-Palmitoyl, 2-Oleyl-sn-Glycero-3-Phosphocholine; DSPE-PEG-2000, 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy (Polyethylene Glycol)-2000]; Chol, Cholesterol.

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