Ultrasound-mediated intracellular drug delivery using microbubbles and temperature-sensitive liposomes

A. Yudina\textsuperscript{a}, M. De Smet\textsuperscript{b}, M. Lepetit-Coiffe\textsuperscript{a}, S. Langereis\textsuperscript{c}, H. Grull\textsuperscript{b,c}, C.T.W. Moonen\textsuperscript{b,*}

\textsuperscript{a} Laboratory IMF UMR 5231 CNRS / University Bordeaux 2, Bordeaux, France
\textsuperscript{b} Eindhoven University of Technology, Dept. of Biomedical NMR, Eindhoven, The Netherlands
\textsuperscript{c} Philips Research Eindhoven, Dept. of Biomolecular Engineering, Eindhoven, The Netherlands

*Correspondence to: C. T. W. Moonen, Laboratory for Molecular and Functional Imaging: from Physiology to Therapy, UMR 5231 CNRS / University Victor Segalen Bordeaux, 146 rue Leo Saignat, Case 117, 33076 Bordeaux, France. Phone: (+33) 5 57 57 45 86. Fax: (++33) 5 57 57 45 97. E-mail address: chr@imf.u-bordeaux2.fr

Abstract:

Keywords:

1. Introduction

Site-directed delivery of drugs remains one of the major challenges in pharmaceutical research. Typical therapeutic agents distribute across tissues according to their pharmacokinetic and pharmacodynamic (pk/pd) properties, leading to a relatively low concentration in the targeted region and an unwanted burden to healthy tissues [1]. Consequently, there is a strong clinical need for drug delivery strategies that enable targeted delivery and facilitate drug uptake. In recent years, a wide variety of multifunctional and stimuli-sensitive particles have been explored for localized drug delivery [2-4]. Despite the promising results yet another challenge remains, as most drug delivery carriers are taken up by endocytic pathways and end up inside the cell within endosomes; a pathway that prohibits efficient drug release into the cytosol. Therefore, the direct delivery of the drug of interest across the cell membrane into the cytosol would be highly beneficial.

Focused ultrasound offers an alternative to complex biological processes for controlling the release, delivery or activation of therapeutic agents [5, 6]. Ultrasound provides two different stimuli depending on the acoustic parameters (\textit{i.e.} frequency, pressure amplitude, and duty cycle) leading to either a strong pressure pulse in the focus area or a temperature increase [7, 8]. Both stimuli can be exploited for drug delivery either using pressure-sensitive or temperature-sensitive delivery vehicles [9]. Ultrasound active microbubbles can be triggered by pressure pulses to oscillate and/or cavitate in the acoustic field. In case of gene delivery, mixing bubbles with nucleotide-based drugs and
exposure to ultrasound can be sufficient to mediate extravasation and cellular uptake leading to an enhanced drug efficacy [10-12]. This effect has been attributed to microscopic fluid motions induced by the oscillating microbubbles in the ultrasound field (referred to as stable cavitation) that drive the therapeutic agents into cells or temporarily open pores in the cell membrane allowing uptake of molecules. Additionally, acoustic radiation forces rather than acoustic cavitation alone may also contribute to enhanced extravasation and interstitial transport [13]. Alternatively, ultrasound-induced perturbation of the plasma membrane or enhanced endocytosis may be responsible for alterations in the permeability [14, 15]. It is also known that cavitation can open the blood-brain barrier, allowing drugs to reach the target cells in the brain [16, 17]. An example of focused ultrasound for DNA targeting has been shown by Huber et al., where naked DNA is transfected into a carotid artery [18]. Alternatively, focused ultrasound allows one to induce local mild hyperthermia (42°C) in a lesion, which can be employed to trigger drug release from temperature sensitive drug carriers [19, 20]. For example, hydrophilic drugs have been encapsulated in the lumen of temperature-sensitive liposomes [21]. At body temperature, the drug remains in the aqueous compartment of the liposomal carrier, but it is rapidly released at the melting phase transition temperature (T_m) of the lipid bilayer [21-24]. The T_m depends strongly on the chemical composition of the lipid bilayer and can be tuned to achieve rapid and quantitative drug release at mild hyperthermia. Temperature-mediated drug enables high concentrations of the drug in the target region, but is less suited to mediate drug uptake into cells.

Here, we postulate a new approach to combine pressure and temperature-mediated drug delivery strategies into one protocol. This two-step protocol may be applied to drugs that benefit from temperature-induced liposomal delivery in terms of protection against plasma degradation leading to increased plasma half life and high targeted concentration, while the pressure-mediated delivery step facilitates drug uptake into the cell. In a proof-of-concept study, we demonstrate a two-step delivery protocol using TO-PRO-3 as a model drug (Figure 1). TO-PRO-3 is a DNA-intercalating agent that displays a strong increase in fluorescence upon interaction with nucleic acids. At physiological conditions, TO-PRO-3 is positively charged and is not able to cross the lipid membrane of viable cells. Therefore, TO-PRO-3 may be employed as a “sensor” for nuclear delivery similar to ‘smart’ contrast agents for imaging [25]. In the first step of our approach, TO-PRO-3 is encapsulated in the aqueous lumen of temperature-sensitive liposomes that are stably entrapping the dye at T=37°C and release the model drug at mild hyperthermia (Figure 1, step 1). In a second step, microbubbles are cavitated by ultrasound inducing permeabilization of the cellular membrane followed by delivery of TO-PRO-3 into the cytosol and subsequently to the nucleus (Figure 1, step 2).
2. Materials and Methods

2.1 Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (MSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) were purchased from Avanti Polar Lipids (USA). Deoxyribonucleic acid (DNA) from herring sperm was purchased from Sigma. Cell-impermeable intercalating dye TO-PRO-3 (MW=671 g/mol, Ex/Em=642/661 nm) was chosen as a model drug on the basis of spectral and chemical properties [26] and purchased from Invitrogen (The Netherlands). Ultrasound contrast agent (Sonovue®) was obtained from Bracco (Italy). Stabilized sulfur hexafluoride microbubbles consist of a lyophilized phospholipids/poly(ethylene glycol)/palmitic acid stored under SF₆ gas. Upon addition of a saline solution (0,91% w/v of NaCl), a suspension of lipid-stabilized microbubbles (2.5 μm mean diameter in the range from 0.7 to 10 μm; 2 ×10⁸ microbubbles/mL) was obtained [27]. C6 murine glioma cells (ATCC) were cultured in humidified incubator at 5% CO₂ in DMEM medium supplemented with 10% fetal calf serum and 1% penicillin / streptomycin (all Invitrogen).

2.2 Preparation of liposomes

The liposomes were prepared by lipid film hydration followed by sequential extrusion. DPPC:MSPC:DSPE-PEG2000 (molar ratio 86:10:4) were dissolved in a solution of chloroform/methanol (4:1 v/v). The organic solvents were removed in vacuo until a thin lipid film was formed, which was further dried overnight under a nitrogen flow. The lipid film was hydrated with a 50 μM TO-PRO-3 solution in HEPES Buffered Saline (HBS), pH 7.4 (20 mM HEPES, 137 mM NaCl) at 60 °C. The suspension was extruded successively at 60 °C through a 400 nm filter (two times), a 200 nm filter (two times), and a 100 nm filter (five times). After extrusion, the extraliposomal TO-PRO-3 was removed by gel filtration through a PD-10 column (GE Healthcare).

2.3 Determination of the phosphorus and TO-PRO-3 concentrations
The TO-PRO-3 concentration of the liposome suspension was determined fluorimetrically (Perkin Elmer LS55, Ex/Em=642/661 nm) in a solution of DNA in HBS (10 mg/mL) after destruction of the liposomes with Triton X-100. The fluorescence intensity was compared with a calibration line of TO-PRO-3 in the same DNA solution. The concentration of phosphorus was determined by means of Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, DRCII, Perkin Elmer) after the destruction of the liposomes with nitric acid and perchloric acid at 180°C.

2.4 Dynamic Light Scattering
The hydrodynamic radius of the liposomes was determined using dynamic light scattering (ALV/CGS-3 Compact Goniometer System, ALV-GmbH, Langen, Germany). Intensity correlation functions were measured at a scattering angle of θ=90° using a wavelength of 632.8 nm. The diffusion coefficient \( D \) was obtained from cumulant fits of the intensity correlation function using ALV software. All reported hydrodynamic radii were calculated using the Stokes–Einstein equation \( r_h = kT/(6\pi\eta D) \), where \( k \) is the Boltzmann constant, \( T \) is the temperature, \( \eta \) is the solvent viscosity, and \( D \) the diffusion coefficient.

2.5 Differential Scanning Calorimetry
The phase transition temperature of the lipid membrane was determined with differential scanning calorimetry (Q2000 differential scanning calorimeter, TA Instruments, USA) during heating from 20 °C to 60 °C at 5 K/min.

2.6 The release of TO-PRO-3
The release of TO-PRO-3 from the temperature-sensitive liposomes was determined by measuring the intensity of fluorescence (Ex/Em=642/661 nm) of liposomes (5 µL) in 2 mL fully supplemented Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen) containing DNA (2 mg/mL) at 37 and 42 °C. At the end of each measurement, 5 µL of a 10% v/v solution of Triton X-100 was added to the solution affording quantitative release of TO-PRO-3 from the liposomes. The TO-PRO-3 release (%) was calculated according to: \( (I_t - I_0)/(I_{100} - I_0) \times 100\% \), in which \( I_t \) is the intensity of the fluorescence at a specific time \( t \), \( I_0 \) is the intensity of the fluorescence at \( t=0 \), \( I_{100} \) is the intensity of the fluorescence after the addition of Triton X-100.

2.7 Cell culture
For the experiments on permeabilized cells 5 × 10⁴ cells were seeded on the cover glasses positioned in the 24-well plate. For the temperature-controlled ultrasound-mediated intracellular delivery experiments 3 ×10⁵ cells were seeded into OptiCell chambers (Thermo Fischer Scientific, Rochester, NY) supplemented with 10 mL of complete medium. The internal space of the OptiCell is formed by two parallel membranes of 50 cm² attached to a rectangular frame with two access ports. The membrane allows free gas exchange as well as micro- and macroscopic imaging and excludes energy loss and standing waves during ultrasound application.

2.8 Temperature-controlled release of TO-PRO-3 on permeabilized cells
C6 cells were fixed with 4% PFA to disrupt the plasma membranes and incubated with 200 μl/well of 2 μM TO-PRO-3 or temperature-sensitive liposomes containing TO-PRO-3 for 20 minutes either at 37 or 42 °C. After two times washing in PBS, cells were counter-stained with Hoechst 33258 for 10 minutes and mounted using SlowFade Gold antifade reagent (Invitrogen).

2.9 Ultrasound setup
A 1.5 MHz mono-element transducer with diameter of 5.8 mm (Imasonic, Lyon, France) was positioned in a water bath. The cell culture monolayer in the OptiCell chamber was positioned in front of the US beam at a distance of 8 mm from the transducer. This distance was chosen on the basis of the maximum acoustic pressure equal to 0.88 MPa p-p (1.0 W electrical effective power) as measured by a 200 μm diameter calibrated hydrophone needle (HNZ-0200; Onda Corp, Sunnyvale, CA). The acoustical parameters were: pulse repetition frequency (PRF) = 1 kHz; electrical effective power = 1W; duty cycle of 20 %; exposure time = 30 s. The choice of the US protocol is based on the preliminary studies [28]. By means of micro- and macroscopic imaging it has been shown that these parameters provide the largest delivery area in combination with minimum cell detachment. At the end of the ultrasound application the local clearance of microbubbles was observed for every area exposed.

2.10 US-mediated intracellular delivery of TO-PRO-3
Fresh complete medium containing 4 × 10⁷ Sonovue microbubbles and 1μM TO-PRO-3 were injected into OptiCell chamber prior to the experiment. 14 mm US exposure zones were drawn on the membrane surface before immersion into the waterbath at 37 °C. At each exposure the transducer was positioned at the center of the corresponding circle and US was applied for 30 s to the 3 circles consecutively. Three other circles served as controls (no US).

2.11 Two-step intracellular delivery protocol
Twelve circles with a diameter of 14 mm were drawn on the surface of the Opticell membrane to indicate the zone of US application. Fresh complete medium (8 mL) with TO-PRO-3 containing temperature-sensitive liposomes (2 mL) was injected into the OptiCell chamber (10 mL total volume). The conditions tested were as following: no microbubbles, no US, 37 °C; no microbubbles, US, 37 °C. After the injection of 4 × 10⁷ microbubbles: microbubbles, no US, 37 °C; microbubbles, US, 37 °C followed by imaging. For the second step, the Opticell was heated to 42 °C for 12 minutes: microbubbles, no US, 42 °C; microbubbles, US, 42 °C. A control experiment was performed consisting of no microbubbles, with/without US at 42°C. The cell viability was determined by means of a Sytox Green staining (Ex/Em = 504/532 nm) performed 26 h post-US.

2.12 Fluorescence microscopy
Live- and fixed cell imaging was performed using the up-right epi-fluorescence microscope Leica DMR (Leica Microsystems, Wetzlar, Germany) equipped with objectives HC PL Fluotar 10X NA 0.3, HCX PL APO 63X oil NA 1.32, HCX Plan Apo
CS 100X oil NA 1.40, appropriate set of filters and CoolSnapHQ camera (Roper Scientific, Evry, France).

3. Results

3.1 Temperature-sensitive liposomes encapsulating TO-PRO-3

Temperature-sensitive liposomes composed of DPPC:MSPC:DSPE-PEG2000 = 86:10:4 (molar ratio) encapsulating TO-PRO-3 were prepared using the lipid film hydration technique followed by sequential extrusion. The mean average hydrodynamic radius of the temperature-sensitive liposomes was 60 nm (polydispersity index < 0.22) as determined by dynamic light scattering (DLS). The melting phase transition temperature ($T_m$) defined as the onset of the phase transition peak was $39.7 \pm 0.2 \degree C$ as evidenced from differential scanning calorimetry (DSC). The phosphorus concentration of the liposomal solution was $34 \pm 1 \text{ mM}$ and the TO-PRO-3 concentration was $7.2 \pm 0.2 \mu \text{M}$.

The release of TO-PRO-3 from the liposomes was measured in fully supplemented medium containing DNA (Figure 2). At $37 \degree C$, no increase in fluorescence was observed up to 1 hour, indicating that the liposomes have no noticeable TO-PRO-3 leakage. At $42 \degree C (T > T_m)$ an increase in fluorescence was detected over time, indicating that the TO-PRO-3 was released from the temperature-sensitive liposomes followed by binding to DNA. At this temperature, approximately 90% of the TO-PRO-3 was released within 10 minutes.

![Figure 2](image.png)

Figure 2. The release of TO-PRO-3 from the lumen of temperature-sensitive liposome in fully supplemented medium containing DNA at $42\degree C (T > T_m)$. At $37\degree C (T < T_m)$, hardly any leakage of TO-PRO-3 within 1 hour was observed.
3.2 Temperature-controlled release of TO-PRO-3 from the liposomes

The first step in our two-step drug delivery strategy is based on the temperature-controlled delivery of encapsulated drugs from the aqueous lumen of a temperature-sensitive liposome. The release of encapsulated TO-PRO-3 at T>Tm was investigated on cells that were permeabilized by 4% paraformaldehyde. In this particular case, the second step in our approach of using microbubbles and ultrasound to induce cellular uptake was not necessary (Figure 1), due to the preceding permeabilization of the cell membrane. The release of TO-PRO-3 from the temperature-sensitive liposomes was investigated at 37 °C and 42 °C (Figure 3, bottom row). Control experiments were performed with TO-PRO-3 at 37 °C and 42 °C (Figure 3, top row). In all the experiments, the nuclei of C6 cells were counterstained with Hoechst 33258 (a cell-permeable dye) that binds specifically to A-T base pairs in dsDNA. The binding of Hoechst 33258 to dsDNA was observed in all wells as evidenced from the fluorescence at 460 nm (Figure 3, blue color). The experiments with TO-PRO-3 showed clear nuclear staining (Figure 3, red color) of the compromised cell membrane regardless of the temperature regimen (37 °C or 42 °C). Once in the cytoplasm, TO-PRO-3 freely diffuses through the nuclear pores and binds to nucleic acids resulting in the nuclear staining. The incubation of permeabilized cells with temperature-sensitive liposomes containing TO-PRO-3 at 37 °C (Figure 3, bottom row) did not afford nuclear staining by TO-PRO-3. This implies that TO-PRO-3 has not reached the nucleus and is still encapsulated by the liposomes, which prevents the DNA intercalation. At 42 °C, nuclear staining by TO-PRO-3 was observed, indicating that TO-PRO-3 was released from the temperature-sensitive liposomes.

**Figure 3. Epifluorescence imaging of step 1: incubation of TO-PRO-3 (upper row) and temperature-sensitive liposomes containing TO-PRO-3 (bottom row) with permeabilized C6 cells at 37 °C (left column, magnification 10x) and 42 °C (middle column, magnification 10x; right column, magnification 100x), counterstained with Hoechst 33258. The TO-PRO-3 fluorescence is shown in red and the Hoechst fluorescence in blue.**
3.3 US-mediated intracellular delivery of TO-PRO-3 in the presence of microbubbles

The results of US-mediated internalization of TO-PRO-3 (Figure 1, step 2) are presented in Figure 4. Using free TO-PRO-3 instead of its liposomal formulation (as in section 3.2) allows testing of ultrasound-mediated internalization (step 2 only). Since cell-impermeable TO-PRO-3 is virtually non-fluorescent in aqueous solution, the areas without US (Figure 4B) showed practically no fluorescence. At the higher magnification (Figure 4D), intracellular TO-PRO-3 (but not nuclear) was noticed. The application of ultrasound in the presence of microbubbles resulted in nuclear staining (Figure 4A and 4D), which was attributed to US-mediated membrane permeabilization. Interestingly, this effect was only present in the areas that were exposed to ultrasound in the presence of microbubbles (data not shown).
Figure 4. Epifluorescence imaging of step 2 only: US-mediated internalization of cell-impermeable TO-PRO-3 (Ex/Em =642/661) in the presence of microbubbles. When internalized and bound to nucleic acids as a result of US application, TO-PRO-3 exhibits nuclear fluorescence (A, C) in contrast to the control areas without US (B, D). Image D was taken with 1s exposure time (as opposed to 100 ms for other images) which explains the elevated background signal.

3.4 Temperature-controlled US-mediated intracellular delivery
The results of the consecutive combination of temperature-controlled release of the TO-PRO-3 and US-mediated internalization are depicted in Figure 5. At 37°C, the application of ultrasound (with or without microbubbles) did not result in the cytosolic delivery of the model drug (Figure 5A-D). Obviously, TO-PRO-3 is stably encapsulated in the aqueous lumen of the liposome and as a result no nuclear staining was observed. At 42°C, the combination of temperature-controlled TO-PRO-3 release from the liposomes (Figure 1, step 1) and sonication in the presence of microbubbles (Figure 1, step 2) resulted in the intracellular delivery of TO-PRO-3 (Figure 5H), whereas the other experiments at 42°C didn’t reveal nuclear staining by TO-PRO-3.
3.5 Cell viability
Since TO-PRO-3 can enter the compromised membrane of the dead cell it is essential to know that the application of the 2-step protocol itself does not result in elevated cell death. Therefore a conventional viability staining with Sytox Green was done 26h post-US to confirm that TO-PRO-3 uptake was not due to the cell death but to the temporal membrane permeability induced by ultrasound. Representative images of this additional “dead cell staining” are shown in Figure 6. The number of cells stained with Sytox Green was similar in both US-treated and control areas and consisted of less than 2% of total cell population.
Figure 6. Conventional viability stain with Sytox Green made 26 h after US application in the experiment (A) and control areas (B). Exposure times are 100 ms for images in TO-PRO-3 channel and 5 ms for images in Sytox Green channel.

4. Discussion

4.1 Optical read-out for evaluation of US-mediated internalization

In the present study, we have used TO-PRO-3 as a cell-impermeant model drug encapsulated in the aqueous lumen of temperature-sensitive liposomes. This dye exhibits a 100 to 1000-fold increase in fluorescence intensity upon binding to nucleic acids, which justifies its application as “smart” sensor for drug internalization [26, 29]. The pharmacokinetic and pharmacodynamic properties of TO-PRO-3, however, may be different from those of actual drugs. Nevertheless, the use of intercalating dyes as a model drug offers the unique advantage that its cellular uptake and localization in the nucleus can be visualized directly, while assessment of real drugs regarding internalization and subsequent localization in cell substructures is indirect and requires more means (e.g. tumor growth curves, apoptosis assays). Our approach allows a straightforward evaluation of a novel two-step delivery protocol involving a successive temperature- and a pressure mediated step.

4.2 Cell viability

The number of cells stained as “dead” by Sytox Green does not differ in US-treated and control areas. The majority of the cells with TO-PRO-3 uptake in the US area does not stain positive for Sytox Green which corresponds to previous findings that US-induced membrane permeabilization for intercalating fluorophores is reversible within 26h [26]. It is worth mentioning that we cannot state that the membrane does not have any slight residual permeability based on Sytox Green staining since its ability to cross the membrane barrier might be somewhat different from that of TO-PRO-3.

4.3 Two-step delivery

In the present study, we have successfully demonstrated a two-step delivery of the cell-impermeable model drug to the nucleus. When TO-PRO-3 is encapsulated in temperature-sensitive liposomes, application of ultrasound with or without microbubbles in the absence of heating is insufficient to mediate the cytosolic uptake of the nuclear dye. The latter conclusion is based on the results obtained with both permeabilized cells (Figure 3) as well as cells treated with US and microbubbles (Figure 5). At these conditions, TO-PRO-3 remained stably encapsulated inside the lumen of the liposomes, e.g. no release of the dye was induced by ultrasound alone or in combination with microbubbles. For delivery of TO-PRO-3 to the nucleus, a heating step of chemically or ultrasonically permeabilized cells (Figure 3 and 5, respectively) appeared necessary to release the dye from the liposome and subsequently induce cellular delivery with uptake in the nucleus. Our approach comprises therefore of two distinct steps: a temperature induced release step of a drug from its carrier and a pressure mediated delivery step to overcome the cellular membrane. In order to combine these two steps into a delivery
protocol, it is important to consider possible in vivo applications concerning choices of drugs, timing and sequence of the steps. As a first point of consideration, drugs can benefit from encapsulation in carriers such as liposomes in two ways: once encapsulated, the pk/pd properties of the liposome determine the biodistribution of the drug, resulting in longer blood circulation times, a change of the excretion pathway and tissue uptake. Due to the enhanced permeability and retention (EPR) effect liposomes can extravasate and accumulate in the tumors leading to a higher drug concentration in the target tissue, while reducing systemic toxicity [30, 31]. Furthermore, the drug carrier protects drugs that suffer from rapid degradation in the blood. For instance, siRNA or pDNA is degraded within minutes if not chemically modified or protected by a carrier [32].

A second aspect to consider is the timing of the individual steps. In our work, the temperature mediated release step was applied first, followed by a pressure mediated delivery step. Here, the drug is released from its carrier but is still outside the cell. The possible time window until the pressure-mediated cell permeabilization needs to be applied is determined by the half life of the drug outside of the cell. The amount of available free drug can rapidly decrease due to degradation, adsorption to other compounds present (e.g. albumin) or – under in vivo conditions – wash-out from the interstitial space. It will be therefore beneficial to apply the pressure mediated step as quickly as possible after the temperature mediated release step. Though the actual in vivo experiment was not performed here, recent studies that probed the time window of ultrasound-induced cell permeability suggest that the order of the two-steps could be reversed [26]. In vitro cell uptake studies with TO-PRO-3 showed that under certain conditions the pressure induced permeability of tumor cells can last for several hours. If these results are translatable to the in vivo situation, tissue and cells could be permeabilized first using ultrasound in combination with microbubbles, before the temperature induced delivery step.

Considering abovementioned points, several variations of the proposed two-step drug delivery approach are possible. For applications in oncology, a protocol could comprise a drug encapsulated in a temperature-sensitive liposome that subsequently accumulates in the tumor utilizing the EPR effect. Next, mild hyperthermia is applied to release the drug followed by intravascular administration of microbubbles followed by ultrasound treatment to mediate cellular uptake. The open question here is whether microbubbles with a diameter of 1-5 µm confined to the vasculature are able to mediate drug uptake in the tumor cells at some distance to the microvessels. There are some indications from pDNA delivery that this is possible [33].

Another protocol may aim for improved extravasation of the drug carrier facilitated by ultrasound, followed by a temperature-controlled release in the tumor tissue. Ultrasound can contribute to the EPR effect by opening the endothelial junctions. Literature studies have shown that the effect of pulsed high intensity focused ultrasound on the extravasation of polystyrene nanospheres persists for more than 24h [34] or even 72h [13] after application of ultrasound. In this case, the pressure mediated step would first open endothelial junctions to mediate extravasation and facilitate long-lived permeability of tumor cells while the subsequent heating releases the drug from the liposomes and makes them bio-available.
5. Conclusion
A novel two-step delivery protocol for intracellular drug delivery comprising a temperature-mediated release step and a pressure-mediated cellular delivery step has been developed. This two-step protocol is potentially beneficial for the intracellular delivery of cell impermeable drugs (e.g. siRNA and pDNA) that commonly suffer from rapid degradation in blood and are not intrinsically taken up by cells. The principle of using two different external triggers for the release and delivery allows one to induce drug delivery in a controlled manner. The proposed two-step delivery protocol can be implemented, for example, on a MR-high intensity focused ultrasound system allowing local heating of target tissue for drug release and pressure mediated delivery under image guidance.

6. Acknowledgements
This study was supported by EC-project FP7-ICT-2007-1-213706 SonoDrugs, Foundation InNaBioSanté-project ULTRAFITT. The microscopy was performed in the Bordeaux Imaging Center of the Neurosciences Institute of the University of Bordeaux II. The authors would like to acknowledge Philippe Legros and Christel Poujol for their assistance with microscopy.

7. References


29. R. Deckers, Yudina, A., Cardoit, L., Moonen, C., A fluorescent chromophore TOTO-3 as a "smart probe" for the assessment of ultrasound-mediated local drug delivery in vivo, Contrast Media and Molecular Imaging, (accepted).


