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Ultrasound-triggered release of materials entrapped in microbubble-liposome constructs: a tool for targeted drug delivery

Alexander L. Klibanov^{a,*}, Talent I. Shevchenko^a, Balasundar I. Raju^b, Ralf Seip^b, and Chien T. Chin^b

^aCardiovascular Division, Department of Medicine, University of Virginia, Charlottesville VA, 22908 USA

^bPhilips Research North America, Briarcliff Manor NY, 10510 USA.

Abstract

We investigated preparation of ultrasound-triggered drug delivery system, based on a pendant complex of microbubble coated with liposomes. Biotinylated decafluorobutane microbubbles were coated with biotinylated liposomes via a streptavidin linker. Liposomes were prepared incorporating calcein and thrombin. Based on initial concentration of calcein, over 1 μm^3 payload volume per each microbubble-liposome particle was achieved, when 100 nm liposomes were used.

Insonation of microbubble-liposome pendants in vitro resulted in the complete destruction of microbubbles and triggered release of a significant fraction of the entrapped material. Treatment with 1 MHz ultrasound (5 pulses, 100 ms, 7 MPa peak negative acoustic pressure) resulted in the release of ~30% of entrapped calcein, as estimated by the fluorescence quenching assay.

Thrombin release from liposomes complexed with microbubbles (11% of entrapped material) due to ultrasound treatment was estimated by a chromogenic substrate study. Prior to insonation, substrate hydrolysis was at background level. Ultrasound-triggered release of thrombin from the pendant complexes caused acceleration of blood clotting.

Keywords

Microbubbles; liposomes; ultrasound; triggered release; thrombin; clotting; calcein

INTRODUCTION

Ultrasound-assisted drug delivery has been investigated as a targeting mechanism that would allow enhancement of drug action in the desired regions. The strategy is to focus ultrasound field and insonate the drug-carrying bubbles that are in the target region, destroy them and release the drug. Until recently, the drug was either incorporated in the microbubble shell (preferred for hydrophobic drugs, such as paclitaxel [1], [2]) or attached to the microbubble shell directly (e.g., plasmid DNA that is bound to microbubble surface by electrostatic interaction [3],[4],[5]). The drug loading capacity for the drug-on-shell approach is limited, especially if a lipid monolayer is used as a microbubble shell, therefore it is desirable to increase the drug payload carried by each bubble.

*Corresponding author. PO Box 800158, Cardiovascular Division, Cardiovascular Research Center, University of Virginia, MR5 Lane Road, Charlottesville VA, 22908-0158 USA. Tel: +1.434.243.9773; fax: +1.434.982.3183. alk6n@virginia.edu; sklib1@gmail.com.

Earlier, we have prepared conjugates of liposomes and gas-filled microbubbles, as pendant-like structures, where liposomes decorated the surface of each microbubble[6],[7] to be applied as drug delivery vehicles. Ultrasound-triggered release of liposome-entrapped dye during insonation of microbubble located near the giant liposome has been reported [8]. Therefore, we can expect that liposomes that are directly attached to microbubble surface may also be ruptured when ultrasound treatment is applied. Recently, such microbubble-liposome complexes loaded with an anticancer antibiotic doxorubicin, [9] were prepared; successful *in vitro* delivery of doxorubicin in a cell culture model in response to ultrasound treatment was reported.

The use of liposome-microbubble complexes for drug delivery, in comparison with plain microbubble preparations, widens the range of substances that could be incorporated, including proteins (e.g., enzymes or antibodies) or other hydrophilic drugs that could not otherwise be associated with the microbubble shell in a stable fashion. In this study we describe preparation of the microbubble-liposome complexes that entrap a model hydrophilic dye, calcein, and an enzyme thrombin. Ultrasound-mediated contents release is also investigated.

MATERIALS AND METHODS

Microbubble preparation and characterization

Microbubbles were prepared from decafluorobutane gas and stabilized with a lipid monolayer that contained biotin binding groups for liposome attachment, in accordance with published procedures [10]. Briefly, 2 mg/ml phosphatidylcholine (Avanti Lipids, Alabaster, AL), 2 mg/ml PEG 150 stearate (Stepan Kessco, Northfield IL) and 0.1 mg/ml biotin-PEG3400-phosphatidylethanolamine (Avanti) were dispersed in 20 ml normal saline with a probe-type sonicator (XL2020, Misonix, Farmingdale NY, with a 1/2" probe, at 40% power setting) to prepare aqueous micellar dispersion. For microbubble preparation, decafluorobutane gas (F2 Chemicals, Lancashire UK) was sparged through the aqueous medium, and sonication performed for 30 sec with the same XL2020 instrument operated at maximum power. Resulting aqueous dispersion of microbubbles was then aliquotted in small vials, sealed under decafluorobutane atmosphere and stored refrigerated until use. Microbubble size distribution was evaluated with Coulter counter equipped with a 50 μ m sensor orifice (Multisizer 3, Beckman Coulter, Miami FL): 10-20 μ l of microbubble dispersion was added to 200 ml normal saline and particle size measured immediately for a 1-30 μ m range.

Liposome preparation and calcein entrapment

Small liposomes (~0.1 μ m) were prepared by the proliposome technique [11]: 10 μ l of ethanol solution containing 4 mg phosphatidylcholine (Avanti), 0.7 mg cholesterol (Sigma) and 5 μ g biotinamidocaproyl-phosphatidylethanolamine (Avanti) was mixed with 10 μ l 0.2 M calcein aqueous solution to produce proliposome gel. The gel was rapidly mixed with 2 ml saline, resulting liposomes immediately vortexed and subjected to repeated centrifugation using either an Eppendorf microfuge (14,000g) or a Sorvall RC5C centrifuge with an angular rotor (40,000g). Liposome size was evaluated with a Nicomp 370 dynamic laser light scattering system (Particle Sizing Systems, Santa Barbara, CA).

Large liposomes were prepared by reverse-phase evaporation technique [12]. Briefly, lipid solution of phosphatidylcholine, cholesterol and biotin-amidocaproyl-phosphatidylethanolamine in chloroform and ethyl ether was mixed with the aqueous medium containing 0.2 M calcein dye (Sigma, St. Louis, MO) at the 3:1 volume ratio between organic and aqueous phase. Water-in-oil emulsion was formed by brief (30 sec)

probe-type sonication of the mixture, and organic solvents were removed on a rotary evaporator under reduced pressure. Unincorporated calcein was removed from liposomes by repeated centrifugal washes in saline, using either an Eppendorf microfuge (14,000g) or a Sorvall RC5C centrifuge with an angular rotor (40,000g).

Thrombin entrapment in liposomes

A similar procedure was applied for thrombin entrapment in liposomes as for calcein. Bovine thrombin (clinical grade, Ethicon, Somerville NJ) was first dialyzed and lyophilized to remove inactive excipients, then dissolved in normal saline prior to the preparation of biotinylated liposomes as described above.

Preparation of the liposome-microbubble complex

Two variants of biotin-streptavidin-mediated attachment of liposome structures to microbubbles were tested.

First technique consisted of preparing biotinylated microbubbles, which were then subjected to multiple centrifugation to remove excess of free biotinylated lipid not incorporated in the microbubble shell (microbubbles floated to the top of the aqueous phase and could be easily separated). Centrifugation was performed in a syringe barrel outfitted with a Luer stopcock valve, so that removal of unwanted infranatant would not disturb the bubble “cake” formed on top of the aqueous medium following centrifugation. IEC NH-SII centrifuge (Thermo Scientific, Waltham, MA) equipped with a bucket rotor ($r=10$ cm, 1000 rpm, 10 min run duration) was used. After removal of free unincorporated lipid from the bubbles, streptavidin was added to the microbubble dispersion (300 ng protein per million bubbles), and subsequent washes were performed to remove excess of streptavidin. Initial excess of streptavidin over the amount of biotin in the bubbles at this step was necessary to avoid formation of microbubble aggregates. Biotin-carrying liposomes (also in excess over available microbubble surface) were then mixed with microbubbles, incubated and washed by centrifugal flotation to remove those liposomes that did not attach to the bubbles and stayed in the infranatant aqueous phase. This technique was acceptable, with the disadvantage of the significant loss of microbubble material due to repeated (up to 10 total) centrifugations. Loss of expensive streptavidin that could not be recovered was also an issue. Use of saline with reduced concentration of dissolved air, kept under decafluorobutane atmosphere, as the flotation buffer solution, somewhat reduced the loss of bubbles, however, an alternative technique described below may be advantageous, because it requires reduced number of microbubble flotations and consequently reduces microbubble loss.

In the alternative approach, the preparation order was reduced: biotinylated liposomes were first incubated with excess streptavidin (the amount of streptavidin is to exceed the amount of biotin located outside of the liposomes by 2-fold, to avoid liposome aggregation via crosslinking). Liposomes were then subjected to repeated centrifugal washes to remove excess of streptavidin that did not attach to the liposome membrane. Resulting streptavidin-liposomes were then added to freshly washed biotinylated bubbles, incubated, to achieve formation of microbubble-liposome complexes, and washed by flotation to remove excess of free liposomes from the microbubble-liposome complexes. Resulting microparticle concentration was determined with Coulter counter.

To determine efficacy of microbubble-liposome pendant complex formation and characterize the entrapped payload volume per complex particle, microbubble-liposome constructs were destroyed by insonation and Triton X-100 detergent treatment to reduce turbidity and release entrapped calcein, and entrapped material subjected to by fluorescence spectroscopy

Calcein fluorescence measurement

Calcein fluorescence was measured in 96-well plates, with a dual monochromator fluorescence microplate reader (Gemini XS, Molecular Devices, Sunnyvale, CA) using 485 nm excitation and 538 emission wavelength, in normal phosphate-buffered saline (PBS, Invitrogen, Carlsbad, CA). To rupture liposome bilayer and achieve complete release of liposomal contents, Triton X-100 (Sigma) was added to the wells (5 μ l 1% aqueous solution per well). Care was taken to ensure lack of internal filter effect during measurements (i.e., linearity of the measurements was confirmed by sample dilution). Released dye fraction was computed in accordance with a formula

$$R\% = (F_{\text{son}} - F_o) / (F_{\text{tx100}} - F_o),$$

where F_o is the initial fluorescence of the non-treated pendant sample, F_{son} is fluorescence of insonated sample, and F_{tx100} is the signal after complete rupture of liposomes by Triton X-100 addition.

Insonation of microbubble-liposome complexes

TIPS system (Philips Research, Briarcliff Manor, NY) operated in the intermittent mode (1Hz) at 1 MHz and 7 MPa nominal peak negative acoustic pressure, was applied for insonation of microbubble-liposome complexes. A thin-walled polypropylene microcentrifuge tube with the microbubble-liposome complex dispersion was placed in the focal zone of the transducer, coupled to the transducer assembly with ultrasound gel stand-off. Ultrasound treatment was performed with 5 pulses using 100,000 cycle pulse length. Depending on the payload (calcein or thrombin) the sample was immediately taken to the calcein fluorescence measurement, or thrombin enzymatic activity measurement, as well as for the *in vitro* blood clotting assay.

Measurement of thrombin enzymatic activity

Enzymatic activity measurement of thrombin for the evaluation of its release from the liposome-microbubble complex was performed with a membrane-impermeable chromogenic substrate (Spectrozyme TH, American Diagnostica, Stamford CT), using repeated 405 nm optical density measurements with a microplate reader (model 3550, Bio-Rad, Hercules, CA) in accordance with manufacturer instructions. Enzymatic activity was estimated as the initial slope of the optical density curve; substrate concentration during the measurement significantly exceeded Michaelis constant ($K_M=3.24 \mu\text{M}$), so that kinetics was studied in the linear range.

Blood clotting study

Samples of EDTA-containing canine blood obtained from a terminal study was replenished to pre-chelation level by adding Ca^{2+} so that clotting process could be initiated, and dispensed in the polyethylene tubes as 1 ml aliquots. Microbubble-liposome pendant preparation aliquots (0.1 ml) containing thrombin were added to the blood samples before and after TIPS insonation, and after Triton X-100 action. Clotting was assessed as clotting time and determined by the inability of the blood sample to flow after tube inversion.

RESULTS

Calcein entrapment and release from microbubble-liposome complex

Microbubble-liposome pendant complex payload volume was estimated using fluorescence spectroscopy, by comparing fluorescence of the initial calcein solution that was used for

liposome loading, with the fluorescence of calcein after release from the purified microbubble-liposome pendant (pendant particle concentration was estimated by Coulter). Based on these fluorescence measurements, each microbubble-liposome pendant prepared with 0.1 μm liposomes, carried amount of calcein equivalent to $\sim 1.1 \mu\text{m}^3$ of the original 0.2 M solution; for larger liposomes, prepared by reverse phase evaporation, this number was tenfold higher.

The concentration of calcein used within the liposome internal aqueous core was high, so the quenching effect was applied for liposome contents leakage monitoring. Upon insonation with TIPS unit, calcein was released from liposome internal aqueous space and diluted into the bulk aqueous phase, and fluorescence increased when compared with non-insonated control (Figure 1, central vs left bar). Quantification of this observation of a significant increase of fluorescence upon insonation confirms that nearly a third of the entrapped dye was released by ultrasound treatment (complete release data obtained following liposome rupture by Triton X-100, Figure 1, right bar).

Thrombin release from microbubble-liposome complex

Spectrozyme TH chromogenic thrombin substrate, H-D-hexahydrotyrosol-alanyl-argininepara-nitroanilide, is a hydrophilic molecule; unlike some other protease substrates, it does not transfer across the lipid bilayers rapidly. Therefore, it was possible to develop an enzymatic assay that would determine if this substrate has come in direct contact with thrombin. Neither thrombin nor Spectrozyme TH can penetrate liposome membrane; therefore, increase of observed substrate hydrolysis rate over the background level could be regarded as a direct indication of the liposome membrane rupture. Such rupture was clearly evident following just five 100 ms 1 MHz intermittent ultrasound pulses delivered to the aqueous dispersion of thrombin-carrying microbubble-liposome pendant complexes with a TIPS device (Figure 2). A major (nearly 17-fold) increase of enzymatic activity after insonation (Figure 2, closed triangles) over the background non-insonated control sample (Figure 2, open triangles) was observed. Approximately 11% of the entrapped thrombin became available to the substrate after insonation. Nearly 89% liposomes in this liposome-microbubble complex were not ruptured by ultrasound and only released thrombin after complete liposome rupture caused by the addition of Triton X-100 detergent (Figure 2, closed squares).

A confirmation of thrombin release from microbubble-liposome pendant was observed in a blood clotting study, when a sample of control blood without thrombin addition clotted in 30 min, blood after the addition of insonated thrombin-liposome-microbubble pendant sample clotted in 17 min, and positive control after thrombin release from pendant by Triton X-100 clotted in 10 min.

DISCUSSION

Ultrasound-triggered gene and drug delivery systems are widely investigated [13]; they are usually designed as ultrasound-sensitive micro- and nanoparticles, that respond to insonation by selective drug deposition in the insonated tissue or by release from the carrier. Ultrasound can cause release of the drug from the particles directly - via mechanical effects, or indirectly, via thermal effects of ultrasound, as is performed for temperature-sensitive liposomes [14,15]. The advantage of ultrasound use for drug carrier activation is obvious: no other externally applied modality allows tight and precise focusing deep within the body; such focusing is now possible even despite the skull interference [16]. An alternative is the application of invasive strategies, such as intravascular catheters, radiofrequency ablation needles, or laparoscopy. Currently, focused ultrasound therapy systems are available in some clinics as thermal ablation devices for the treatment of benign and malignant tumors,

and operated under MRI or ultrasound guidance. Such equipment could be applied for ultrasound-triggered drug delivery with minor modifications.

The overall aim of a successful drug delivery project is to improve therapeutic index, i.e., the ratio between the active dose of the drug and the dose causing toxicity. There are several ways to use focused ultrasound in this setting. First, hyperthermia can be applied to increase tumor blood flow, i.e., drug carrier flux through the target vasculature [17,18]; this has been achieved with doxorubicin-loaded long-circulating liposomes. Second approach is to apply the carrier system that would rapidly release the drug in response to moderate hyperthermia (such as in case of temperature-sensitive liposome) [14]. Third approach is to use superheated liquid fluorocarbon nanodroplets encased in a polymer shell with shell-embedded drug (doxorubicin) [19]: the particles would rupture and release shell fragments into the surrounding tissue for sustained delivery of therapeutic agent. Fourth approach is to use gas-filled microbubbles, complexed with liposomes [6,7,9] or other nanoparticles [20]. Microbubbles are very sensitive to the destruction by ultrasound. In the process of destruction the gas-liquid interface may achieve linear speed ~ 700 m/s [21]. In such conditions, closely located liposomes may be ruptured [8], and their contents released and/or deposited in the surrounding cells and tissues. Additional advantage could be gained by modulation of nearby cell permeability after microbubble destruction, so that intracellular uptake of the delivered drug would increase [22].

The advantage of using microbubble-liposome pendant approach is in the ability to sequester compounds inside the liposome aqueous core in very significant quantities: if we assume liposome size as $0.1\text{ }\mu\text{m}$, then for a bubble with $2.5\text{ }\mu\text{m}$ diameter and fully coated with liposomes ($0.1\times 0.1\text{ }\mu\text{m}$ footprint), we can expect ~ 2000 liposomes per bubble, with a total internal aqueous core volume of $\sim 1\text{ }\mu\text{m}^3$, which is quite similar to our calcein entrapment data (see above). In case of doxorubicin liposomal drug, such as the widely popular Doxil/Caelyx, the load of 2 mg doxorubicin per 10 mg phosphatidylcholine is reported for $\sim 0.1\text{ }\mu\text{m}$ -size liposomes (see Doxil package insert), which translates to $\sim 5\text{ }\mu\text{g}$ drug per 10^8 bubbles, fully coated with liposomes. Such number of particles is routinely used for administration in mice and the drug dose sequestered in such particles is quite similar to the drug dose range ($0.1\text{--}0.2\text{ mg/kg}$) used in the clinic for cancer therapy. If liposomes used for complex preparation are larger, e.g., $0.5\text{ }\mu\text{m}$, their number per bubble will be significantly smaller. Assuming a “square” footprint on the bubble surface for each liposome, equaling its diameter, a simplified geometric calculation reduces liposome number per bubble by 25-fold. Still, with the same geometric assumptions of complete bubble coverage with liposomes, their total entrapped volume should be proportionally higher, $\sim 5\text{ }\mu\text{m}^3$, and we can expect five-fold respective increase of the payload volume per bubble-liposome pendant particle (as observed with calcein-liposomes of different sizes; see Figure 3 for the first approximation of the pendant structure entrapped volume calculation). Accordingly, an order of magnitude improvement of drug load per particle can be achieved by this approach, which will reduce the required number of injected pendant particles, while somewhat increasing resulting particle diameter, from 2.7 to $4.5\text{ }\mu\text{m}$, still capable of capillary passage within the bloodstream.

We may hypothesize that the biodistribution of microbubble-liposome pendant complexes following intravenous injection will be significantly different from the behavior of long-circulating liposomes. The latter, typically $100\text{--}200\text{ nm}$ PEG-coated liposome preparations [23] would circulate for many hours, and eventually accumulate in the liver. Larger particles, even despite PEG coat, while having reduced liver uptake, efficiently accumulate in the spleen [24]. Microbubble-based structures lose gas within minutes after intravenous administration, so within half-hour there will be no circulating microbubbles detectable by ultrasound imaging. It is likely that microbubble-liposome complexes after deflation and

dissolution of the gas core will accumulate in the liver and especially spleen, as regular microbubbles do [25]. Therefore, the use of doxorubicin as a targeted therapeutic might be complicated by the spleen accumulation of the drug carrier, which might be detrimental. The best strategy would be to use drug substances that would be completely inactive unless ultrasound was applied. One example of such drug would be thrombin, to induce targeted clotting of insonated tumor vasculature.

Based on the earlier semi-quantitative calcein fluorescence release data [6,7] we hypothesized that a microbubble-liposome complex that carries thrombin would release it upon ultrasound treatment. This hypothesis was confirmed both with the chromogenic substrate thrombin enzymatic activity study (Figure 2), and in the blood clotting experiment (see Results). Therefore, we may expect selective blood clotting of the targeted vascular bed in response to focused ultrasound treatment after intravenous administration of microbubble-liposome-thrombin pendants. In a preliminary toxicity study (data not shown), no detrimental effects were observed after intravenous administration of thrombin-loaded liposomes in mice, whereas for the positive control, an intravenous injection of the same quantity of thrombin after detergent rupture induced embolism within minutes, demonstrating complete safety of liposome-encapsulated thrombin, yet its high clotting potential after release from liposomes. After these *in vitro* proof-of-principle studies, *in vivo* experiments should be performed on microbubble-liposome pendants, to validate this concept for practicality of application within the vascular bed of interest, such as the tumor-feeding vasculature. Targeted clotting of tumor vasculature has been reported earlier for successful murine tumor therapy with antiVCAM-1-tissue factor fusion protein [26]. The presence of phosphatidylserine on the external leaflet of the plasma membrane of tumor endothelium might be a useful factor of specific clotting in the tumor vasculature [27], minimizing systemic effects of this potentially useful therapeutic application.

CONCLUSION

Liposome-microbubble pendants incorporating calcein, a hydrophilic self-quenching fluorescent dye, or thrombin, a crucial enzyme of the thrombosis cascade, were prepared. We achieved significant payload volume - up to several cubic micrometers of internal liposome aqueous core space per bubble-liposome complex particle. Short medical ultrasound treatment (five pulses of 100 ms duration) resulted in the complete bubble destruction and release of a significant fraction of entrapped contents from the complexes. These microbubble-liposome complexes can be useful as vehicles for ultrasound-triggered drug delivery for a wide variety of pharmaceutical and biopharmaceutical agents, including proteins, with high per-particle drug load.

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Figure 1. Calcein release from liposome-microbubble complexes in response to ultrasound treatment. Calcein fluorescence (Mean $\pm$ Standard Deviation) of untreated pendants (left bar), of ultrasound-treated pendants (center bar), and of completely ruptured liposomes following the addition of Triton X-100 (right bar).

Figure 2. Spectrophotometric evaluation of thrombin release from liposome-microbubble pendants, expressed as degradation of chromogenic substrate Spectrozyme TH. Control untreated pendants (open triangles); pendants following insonation (closed triangles); liposomes ruptured by Triton X-100 (squares).

Figure 3.

Schematic of microbubble-liposome pendant structure and an approximate formula for the calculation of the combined internal volume of liposome per pendant particle. R_{bub} -bubble radius; d_{lip} -liposome diameter; r_{lip} -liposome radius; s_{lip} -surface occupied on the bubble surface by a liposome; v_{lip} -liposome entrapped volume; N_{lip} -number of liposomes attached per microbubble; V_{tot} -total entrapped volume per liposome-microbubble pendant particle.