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Development of a liposomal delivery system for temperature-triggered release of a tumor targeting agent, Ln(III)-DOTA-phenylboronate

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ABSTRACT

Liposomes, capable of temperature-triggered content release at the site of interest, can be of great importance for imaging and therapy of tumors. The delivery of imaging agents or therapeutics can be improved by application of liposomes with a gel-to-liquid phase-transition temperature suitable for mild hyperthermia (41–43 °C), and by prolonging their circulation time by incorporation of lipids containing polyethyleneglycol moieties. Still, the rapid wash out of the delivered material from the tumor tissue is a major obstacle for both imaging and therapy. In this study, we developed an optimized temperature sensitive liposomal system to be used with mild hyperthermia: highly stable at physiological temperature and with a sharp transition of the bilayer at 41.5 °C, with subsequent rapid release of entrapped compounds such as calcein or tumor cell-targeting contrast agents. Intravital microscopy on calcein/rhodamine containing liposomes was applied to demonstrate the applicability of this system in vivo. The calcein loaded liposomes were injected iv into nude mice with a human BLM melanoma tumor implanted in a dorsal skin-fold window chamber. Arrival of the liposomes at the tumor site and content release after temperature increase were monitored. The results demonstrated not only accumulation of the liposomes at the tumor site, but also a massive release of calcein after increase of the temperature to 41 °C. The versatility of the thermosensitive liposomes was further demonstrated by encapsulation of a tumor cell-targeting DOTA-phenylboronate conjugate and its release at elevated temperatures. The DOTA ligand in this system is able to chelate a variety of metals suitable for both diagnostic and therapeutic applications, whereas the phenylboronate function is able to target specifically to tumor cells through a covalent binding with sialic acid moieties over-expressed on their surface upon heat-triggered release from the liposomal carrier.

1. Introduction

Research in the field of cancer diagnosis and therapy is focused on development of highly sensitive and tumor-selective agents for real-time non-invasive monitoring and drug delivery, as well as quantification of treatment efficacy, which allows tight control and tailoring of the therapy.[1] It is extremely difficult to reach therapeutic levels of drugs at tumor sites without affecting healthy tissues.[2] A promising strategy for improved drug delivery is the application of targeting agents, such as antibodies, aptamers or peptides with affinity to over-expressed epitopes on the cell surface of tumor tissues.[3] Another approach is encapsulation of drugs into carriers such as liposomes, which are nanoscale biocompatible vesicles, composed of a phospholipid bilayer enclosing an aqueous compartment.[4] The success of liposomes for drug delivery to solid tumors is based on their enhanced permeability and retention (EPR) in tumor tissue in combination with their small size[5,6] and long-circulation capacity.[7,8] Modification of the surface of the liposomes by grafting with PEG (poly(ethyleneglycol)) extends their blood circulation half-life time significantly. The PEG chains form of a hydrated shield around the lipids, which inhibits their uptake by macrophages,[9,10] and increases the probability of their extravasation in tumor vascular beds.[11] Even though these conventional liposomes are being used clinically as carriers of therapeutics for a variety of diseases,[12] the biggest challenge today remains to achieve effectively controlled release of the encapsulated drugs from kinetically stable long circulating liposomes.

The concept of thermosensitive liposomes (TSL) first proposed in the late 1970s[13] and studied extensively since then, offers possibilities for controlled drug delivery by means of local hyperthermia. By tuning the lipid composition of the bilayer, it is possible to

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control its transition from gel-to-liquid phase at clinically applicable temperatures.[14] Using 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, T_m = 41.5 °C) as the primary lipid renders the membrane highly permeable to small water-soluble molecules at a convenient phase-transition temperature. To increase the stability of the membrane and to prevent pre-leakage of the drug, a lipid with a longer aliphatic chain and, consequently a higher T_m is often included in the formulation, for example, 1,2-disteraroyl-*sn*-glycero-3-phosphocholine (DSPC, T_m = 56.0 °C).[15,16]

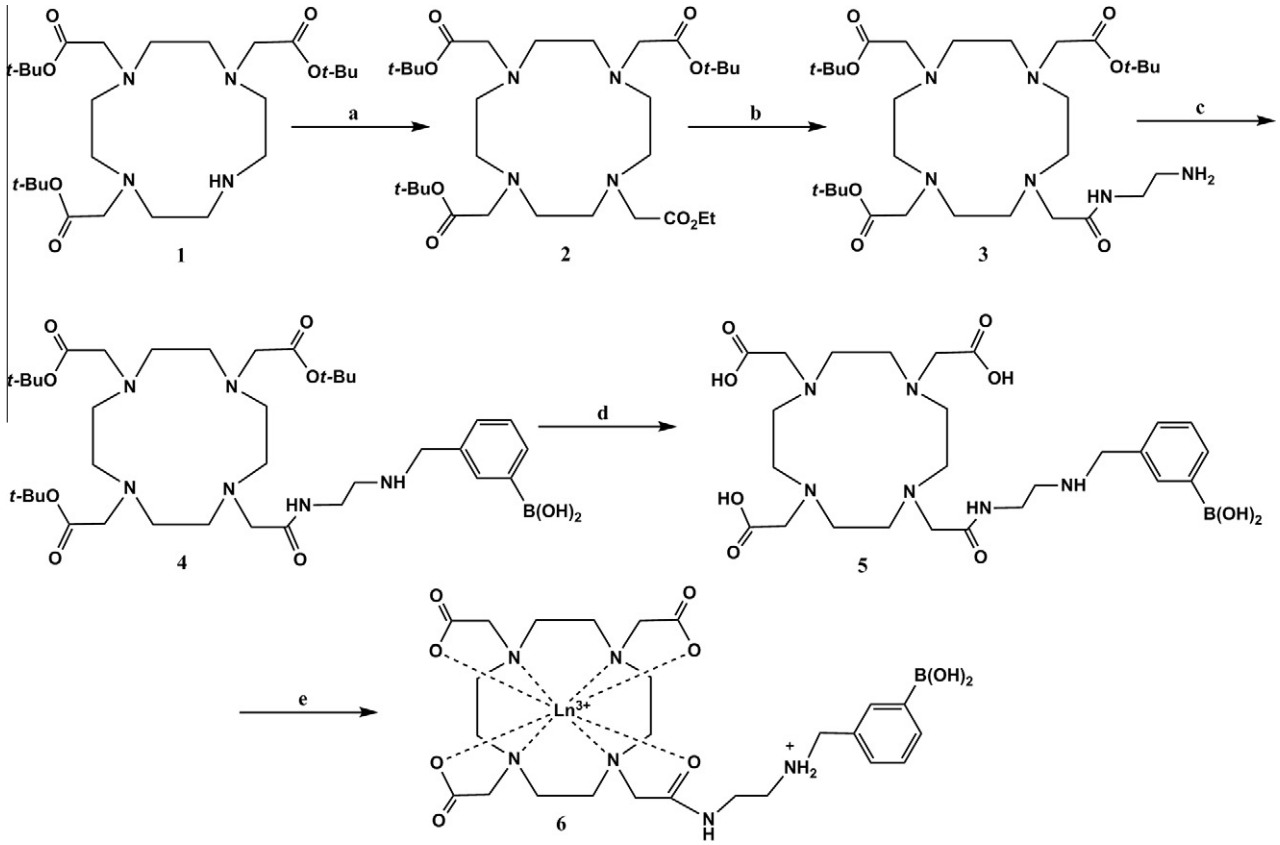
Despite the significant advances that have been made in liposomal drug delivery, the problem that remains to be solved is how to achieve an effective retention of the drug at the tumor site and prevent its rapid wash out and transfer back into the circulation after it has been released from the liposomes.[17]

In our previous work we have demonstrated, in vitro, the recognition of tumor cells by a phenylboronic acid (PBA) targeting vector conjugated to a DTPA-bis-amide chelate (DTPA = diethylenetriamine-*N,N,N′,N″,N″*-pentaacetate) via a positively charged ethylenediamine bridge (EN).[18] The target was sialic acid (Sia), a nine-carbon sugar over-expressed on the surface of tumor cells as the terminal group on glycan chains.[19] Tumor targeting can be ascribed to the formation of a reversible covalent binding between the diol-function of the Sia and the boronate function of PBA. However, two problems were faced during this study: (i) a poor kinetic stability of Ln(III)-DTPA-$(ENPBA)_2$ leading to the leakage of Ln(III) ions, and (ii) competitive binding of the PBA-vector to other sugar moieties in plasma and to erythrocytes, which also contain Sia on their surface. Therefore, in the present study, we have modified the structure of the targeting agent by replacing the DTPA-bis-amide moiety by the kinetically much more stable DOTA analogue (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate). In addition, we have developed thermosensitive liposomal carriers for the efficient delivery of the new Ln(III)-DOTA-ENPBA targeting agent (**6,** Scheme 1) to solid tumors. Encapsulation of this complex into TSL is expected to offer protection against its binding to sugar moieties in the blood-stream and at the same time to result in increased accumulation in tumors. A subsequent application of mild hyperthermia will lead to the release of the targeting agent, which then will bind to the tumor cell surface. The formulation of the lipids was carefully tuned to T_m = 41.5 °C, and used for the encapsulation and subsequent release of the targeting agent Ln(III)-DOTA-ENPBA in the temperature range of 41–43 °C. The liposome in vivo behavior and content release during and after local hyperthermia application were studied by intravital confocal laser scanning microscopy of a xenograft with a highly invasive human melanoma tumor cell line (BLM) grown in a dorsal skin-fold window chamber implanted in nude mice.

2. Results and discussion

2.1. Chemistry

The targeting agent used in this study was prepared following the synthetic route shown in Scheme 1. The targeting PBA function was attached to the DOTA-chelate by a reductive amination reaction of the aldehyde group of 3-formyl phenylboronic acid by the free amino-group of *tert*-butyl protected ethylenediamine-functionalized DOTA (**3**). The formation of the intermediate imine was monitored by the decrease of the intensity of the ^{1}H NMR resonance of the aldehyde function at 9.89 ppm and the increase of the intensity of the imine resonance at 8.24 ppm. After complete conversion, the Schiff base was reduced by addition of $NaBH_4$ to the reaction mixture. The formed boric acid was converted into its tetramethyl ester, which was removed together with the



Scheme 1. Preparation of Ln(III)-DOTA-EN-PBA: Reagents: (a) $BrCH_2CO(O)Et$; (b) neat $NH_2(CH_2)_2NH_2$; (c) $O(H)CPhB(OH)_2$, $NaBH_4$; (d) TFA/CH_2Cl_2; (e) $LnCl_3{\cdot}6H_2O$.

solvents by evaporation under reduced pressure. The resulting phenylboronate-functionalized DOTA derivative **4** was deprotected with trifluoroacetic acid and then purified by cation-exchange chromatography to give the sialic acid targeting agent **5**. La(III) complexation of this ligand was achieved by mixing aqueous solutions of $LaCl_3$ and **5** (molar ratio 1:1.05) at room temperature followed by adjustment of the pH to 7.4.

2.2. Optimization of the formulation of the TSL

Temperature sensitive liposomes encapsulating Ln(III)-DOTA-ENPBA need to be highly stable at 37 °C (body temperature), long circulating due to PEG-coating and have a gel-to-liquid phase transition of the membrane at about 42 °C. At this temperature, the entrapped material should be released from the assembled system as fast as possible.[20] Another important characteristic is the size of the liposomes. Charrois and Allen have shown that the liposome diameter determines their in vivo behavior.[21] The ideal diameter for an efficient tumor uptake was shown to be 100–200 nm. Therefore, for the present study we decided to use liposomes with an effective diameter of 100 (±15) nm, choosing for the benefits of prolonged circulation time and efficient tumor accumulation rather than for a higher amount of encapsulated drug.[22]

It is known that the length of the aliphatic chain and the headgroup of the phosphatidylcholines in the lipid bilayer are determining the thermal properties of a TSL, whereas cholesterol (CHOL) has a stabilizing effect on the fluid-phase.[23] Therefore, a DPPC/DSPC/CHOL/DSPE-PEG formulation was selected, and the ratio of the components was optimized by testing the various compositions using a calcein release assay described in the following section.

2.3. Temperature-induced release of calcein from the TSL

To test the release process, a 90 mM solution of the fluorescent dye calcein was encapsulated into the liposomes. The concentration of the encapsulated dye is above its self-quenching concentration (12 μM) (Fig. S2, Supplementary data). Consequently, the fluorescence is only observable upon dilution of the dye due to its release.[24] This enabled monitoring of the release of entrapped material in situ by measuring the increase of the fluorescence signal intensity (Fig. 1A and B). Different stepwise increasing temperatures were applied to samples of liposomes in HEPES buffered saline (HBS). After each increase the sample was stabilized for 15 min and the fluorescence was measured after the system was quickly cooled down in an ice-bath to stop the release. Initially, a molar ratio of DPPC/DSPC/CHOL/DSPE-PEG of 77:5:15:3 was used in the liposomal formulation. As shown in Figure 1A, calcein release occurs in this case already below 37 °C, which is not suitable for in vivo applications. Therefore, in the next trial the ratio of DPPC/DSPC lipids was changed to 67:15. The increase of DSPC in the formulation significantly improved the thermal behavior of TSL shifting the transitional temperature to 41.5 °C. A massive release of the contents of the liposomes in HBS occurred between 40 and 43 °C, a maximum of 92% was reached at 44 °C. No additional release was found upon further heating to 60 °C. Next, temperature-induced release experiments on the TSL with the optimal formulation (DPPC/DSPC/CHOL/DSPE-PEG = 67:15:15:3, mol %) were performed in the presence of fetal bovine serum (FBS, 50%, v/v) to mimic the in vivo situation. As demonstrated in Figure 1B, a substantially smaller amount of calcein was released under these conditions. This is consistent with the reported hampering of the release of encapsulated material from liposomes due to interaction with the serum proteins caused by insufficient PEGylation of the surface.[25] Therefore, in a final modification of the liposomal formulation, the amounts of cholesterol and DSPE-PEG were decreased to 13 and increased to 5 mol %, respectively, whereas the molar ratio of the lipids DPPC/DSPC was kept the same (67:15). These alterations of the formulation resulted in a dramatic improvement of the performance of the liposomes in the presence of serum. The applied molar ratio of DSPE-PEG is in line with the results of Li et al.,[26] who demonstrated optimal release from cholesterol free DPPC/DSPC/DSPE-PEG thermosensitive liposomes at 5 mol % PEG-DSPE. Differential scanning calorimetry (DSC) measurements confirmed that the thermotropic transition of the composed liposomes takes place at 42 °C (Fig. 2). All further experiments were performed using this optimized liposomal formulation DPPC/DSPC/CHOL/DSPE-PEG = 67:15:13:5 (mol %). With our liposomal system we demonstrate that by adapting the molar ratio of all components of the lipid bilayer an optimal formulation can be prepared with high stability of TSL at physiological temperatures (<40 °C) without affecting the release of content during hyperthermia (41–43 °C).

2.4. Stability of the optimized TSL in vitro

For the applicability of the liposomes it is not only important that they have optimal releasing properties at elevated temperatures, but they also should be sufficiently stable in the blood

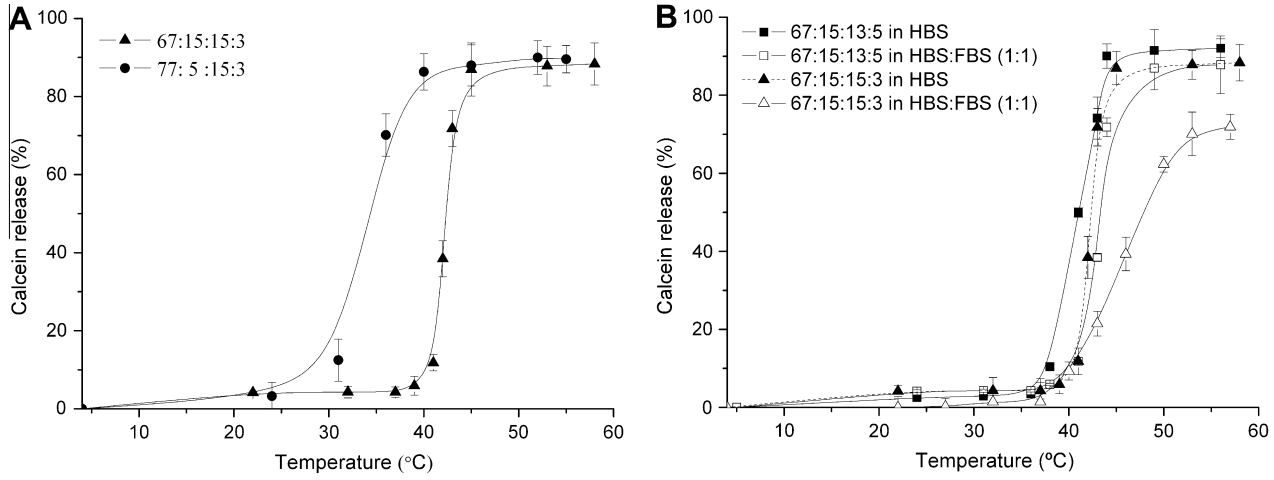


Figure 1. (A) The calcein release from TSL versus temperature after incubating for 15 min in HBS. (B) Effect of an increased amount of DSPE-PEG in the TSL formulation on the calcein release in HBS and in the presence of serum.

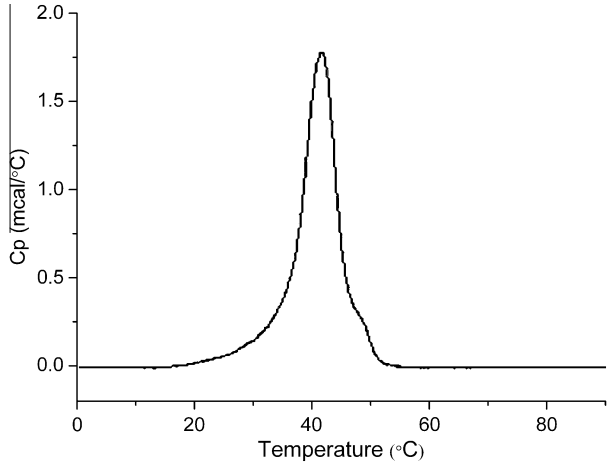


Figure 2. DSC analysis of TSL with lipid composition DPPC/DSPC/CHOL/DSPE-PEG (67:15:13:5, m/m %) in HBS (50%, v/v); heat flow 1 °C/min.

circulation as well as under long-term storage conditions. Serum stability was tested by incubating liposomes in HBS/FBS (1:1) at 37, 39, 41, and 45 °C up to 5 h. The amount of released calcein was monitored by measuring the intensity of the fluorescence signal. The data (Fig. 3) clearly demonstrate that, at 37 and 39 °C, only minor quantities of calcein (<5%) are released during the first 5–10 min. At 41 and 45 °C, the maximum release is also reached within 5–10 min, but then the released amounts are dramatically higher (67% and 83%, respectively). In all cases, almost no further increase in fluorescence intensity was observed during a period of 5 h. The long term stability of the liposome system under storage conditions (at 4 and 20 °C) was evaluated by monitoring the calcein release and the particle size during a period of 30 days. Table 1 compiles the data, from which it is clear that the liposomes are completely stable and do not release their contents during one month of storage under these conditions.

2.5. Release of encapsulated La(III)-DOTA-ENPBA targeting agent from TSL

Liposomes containing the La(III)-complex of DOTA-ENPBA were prepared using a procedure similar to that used for the encapsulation of calcein. A 250 mM aqueous solution (1 mL) of the targeting

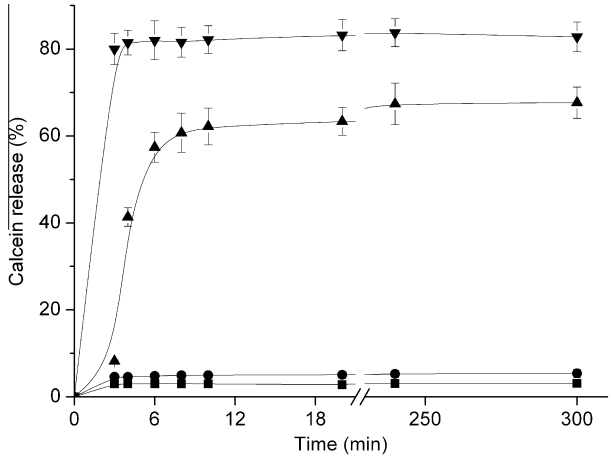


Figure 3. Calcein release from DPPC/DSPC/CHOL/DSPE-PEG liposomes (67:15:13:5, mol/mol) in HBS:FBS (1:1) at different temperatures monitored at different time intervals in the range from 2 to 300 min: 37 °C (■), 39 °C (●), 41 °C (▲), 45 °C (▼). The interrupted *x*-axis is introduced for a better visualization of the start of the release process.

Table 1
Long term stability study on calcein containing TSL

Time (days)	Fluorescence intensity		Mean diameter (nm)	
	4 °C	20 °C	4 °C	20 °C
0	0.107	0.094	108	96
1	0.119	0.083	114	90
5	0.115	0.060	112	100
8	0.105	0.049	114	114
14	0.117	0.048	114	110
30	0.111	0.058	114	104

All values are means of measurements of two TSL batches with an SD of typically 5%.

agent was used for the hydration of the lipid film. ICP-OES measurements of the crude liposomal suspension after destruction by a concentrated Triton-X solution showed that the system contained 2.47×10^{-4} mol La(III)-complex of DOTA-ENPBA, while a phosphate assay indicated the presence of 4×10^{-7} mol of lipids. After extrusion and centrifugation, the liposomal pellet obtained was re-suspended in 1 mL of HBS. The amounts of targeting agent and lipid in the liposomal pellet were measured to be 1.72×10^{-5} and 3.25×10^{-7} mol, respectively. From this it can be concluded that the encapsulation efficiency of DOTA-ENPBA in the liposomes was 8.5%. The release of La(III)-DOTA-ENPBA from these liposomes in HBS/FBS (1:1) medium was evaluated from the amounts of the complex in the liposomal pellet and in the supernatant after heat treatment and separation of entrapped and released compound after centrifugation of the sample. The results of these experiments (see Fig. 4) are in agreement with the thermal behavior observed during the experiments with the TSL with encapsulated calcein. The maximum amount of released compound at temperatures above 40 °C (70%) is only slightly lower than that of calcein. The release at 37 °C (5%) is once again negligible.

2.6. In vivo testing of the calcein loaded TSL by means of fluorescence microscopy

To test the performance of the designed system, liposomes containing 90 mM buffer-solution of calcein in the inner-core and 1 mol % of rhodamine (Rho-PE) in the lipid bilayer were injected in nude mice with a window chamber in which a human BLM melanoma tumor was implanted. Green (calcein) and red (rhodamine) fluorescence were monitored by confocal laser scanning microscopy in the living mouse upon local heating, representing the encapsulated calcein and the liposomal bilayer, respectively. The window chamber bearing mouse was anesthetized, installed under

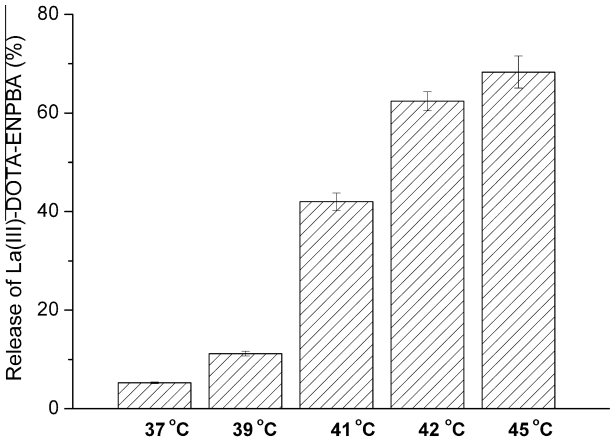


Figure 4. Release of encapsulated La(III)-DOTA-ENPBA targeting agent (1.75×10^{-5} mol per 3.23×10^{-7} mol lipids) in HBS/FBS medium (1:1) from optimized TSL as determined by ICP-OES.

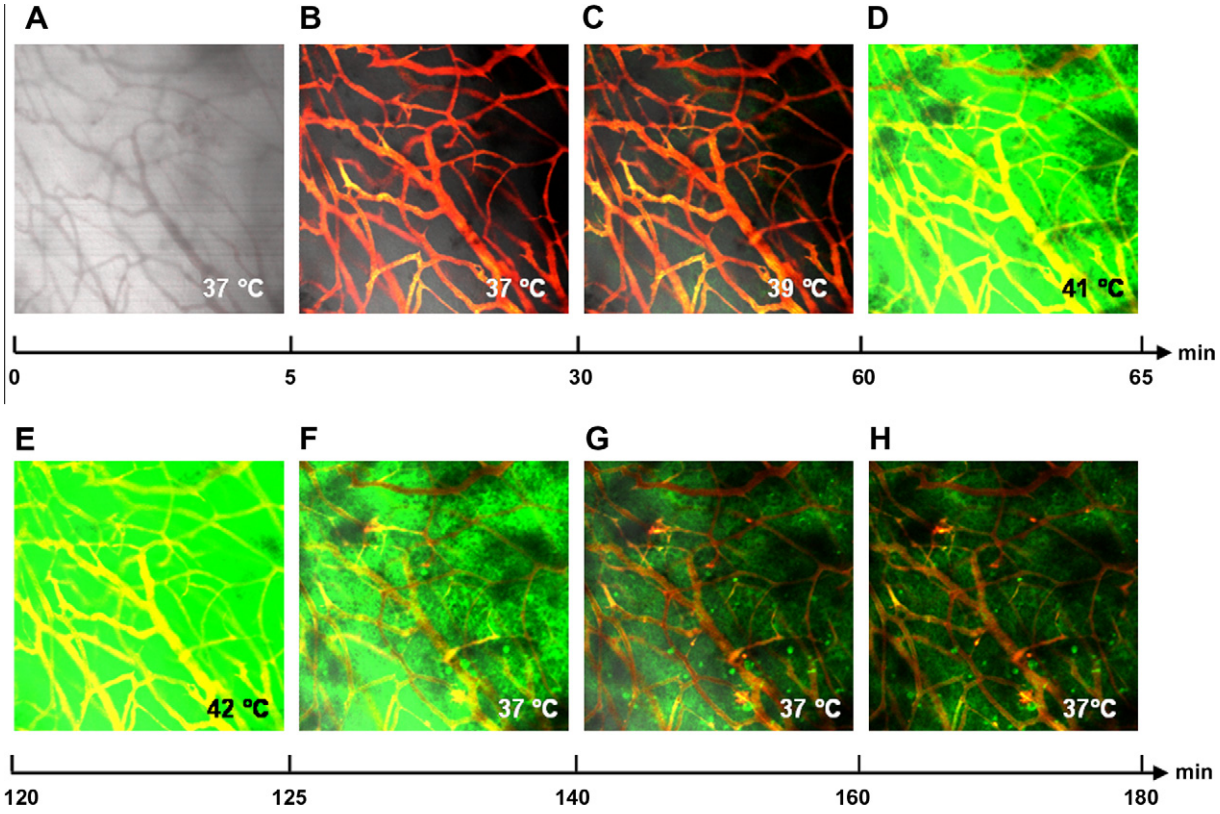


Figure 5. In vivo visualization of calcein release from Rho-PE-grafted TSL by means of invitral fluorescence microscopy (×10 objective lens, overlapped green (calcein) and red (Rho-PE) fluorescence images). The scale represents time path of conducted hyperthermia experiments starting from a pre-injection image (A). After recording of the first post-injection image (B) the temperature was gradually increased to 39 °C (C) and then to 42 °C (D and E), and decreased back to 37 °C (F–H).

a confocal laser scanning microscope and injected intravenously with liposomes. The tumor tissue in the window chamber was heated using an in house developed heating device mounted on top of the window chamber. Online tissue temperature measurements were performed to monitor the local tissue temperature. The dose of injected liposomes used for the intravital microscopy experiments was 4.2 μmol lipids. In 5 min after injection, arrival of liposomes in the tumor vasculature was clearly observed as merely visible red fluorescence originating from the Rho-PE (Fig. 5B). At physiological temperature no calcein release was observed during 30 min of circulation as demonstrated by a lack of green fluorescence (Fig. 5B). Elevating the tissue temperature to 39 °C still did not induce any release of calcein, demonstrating the high stability of TSL. Moreover, during the circulation of liposomes in the system for 1 h no calcein leakage was observed (absence of green fluorescence, Fig. 5C). A mild hyperthermia was started by increasing the tissue temperature initially to 41, and later to 42 °C, resulting in a strong increase of the intensity of the fluorescence indicating a massive release of calcein already after 5 min of treatment (Fig. 5D and E). By subsequent cooling down of the tissue back to 37 °C, the release process was terminated, followed by a slowly washing out of calcein from the tumor tissue in about 1 h (Fig. 5F–H). Exact levels of tumor localized liposomes, as well as their intratumoral and intracellular fate are unknown under current circumstances. However, the findings of high stability during circulation of liposomes at physiological temperature and at a temperature up to 39 °C in combination with a rapid release at 41 and 42 °C suggest that the liposomes, circulating in the blood-stream, will release their content exclusively in the heated region, preventing the undesired delivery of the encapsulated material to other non-target tissues. Optimal delivery strategies will need to be developed, regarding heat application and TSL characteristics to allow for maximal exposure of the tumor to the delivered compound, thereby preventing undesired exposure of healthy tissues.

3. Conclusion

The formulation of a TSL system consisting of DPPC, DSPC, CHOL, and DSPE-PEG, was optimized for efficient performance of temperature-triggered drug release. In vitro tests of this system after encapsulation of calcein as well as of the potential tumor targeting agent Ln(III)-DOTA-ENPBA demonstrated a sharp transition between the gel and the liquid phase at 41–42 °C, with an effective release of the encapsulated material (about 90% for calcein and 70% for Ln(III)-DOTA-ENPBA). Below the transition temperature, the system is essentially stable and the release of the encapsulated material is negligible, both in buffer and in 50% serum. In vivo studies on liposomal delivery with the TSL system to a tumor site in nude mice with implanted human BLM melanoma in temperature-controlled dorsal skin-fold window chambers were performed. The designed liposomes were completely stable at physiological temperatures up to 39 °C and showed a rapid temperature-triggered release of their content during hyperthermia (41 and 42 °C); a massive release of calcein was observed already a few minutes after local increase of the temperature. These results demonstrate high controllability of the designed TSL for the content release in hyperthermia treated tumors. It is expected that, similar to the calcein release observed in vivo, the targeting agent Ln(III)-DOTA-ENPBA will also be released from the liposomes in the heated tumor. In contrast to calcein, temperature-triggered release of the improved targeting agent in the tumor will lead to its

specific delivery due to the binding of the targeting PBA-vector to the sialic acid moieties over-expressed on the tumor cells, as demonstrated previously.[17] Using this approach we will be able to specifically deliver DOTA-ENPBA agent chelated to suitable metal ions for MR and/or nuclear imaging as well as therapeutic radionuclides. Further research along these lines is currently in progress.

4. Experimental

4.1. General methods

The lipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphacholine (DPPC, T_m = 42 °C), 1,2-disteraroyl-*sn*-glycero-3-phosphacholine (DSPC, T_m = 56.0 °C), distearoylphosphatidyl ethanolamine-poly(ethylene glycol)-2000 (DSPE-PEG) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl)-ammonium salt (Rho-PE) were obtained from Avanti Polar Lipids. The solvents used, were of analytical grade. Tert-Bu3DO3A·HBr (**1**) was synthesized according to a published procedure.[27] All other chemicals were purchased from Sigma–Aldrich and were used without further purification. All reactions were monitored by TLC on silica plates detecting the components by UV light or by applying the Dragendorff reagent. Ion-exchange chromatography was carried out on Dowex 50-WX8-200 (H^+). NMR spectroscopy was performed on a Varian Inova-300 spectrometer at 25 °C using 5 mm NMR tubes and operating at 300, 75, and 96 MHz for ^1H, ^{13}C, and ^{11}B, respectively. Fluorescence was measured on a Jasco-J-815 CD spectrometer in Hellma quartz cuvettes, with a sensitivity of 630 V, using emission and excitation wavelengths of 520 and 495 nm, respectively. The liposome extrusions were carried out on a Lipex™ extruder using nucleopore track-etch membranes with pore sizes of 100 and 200 nm (Whatman Schleicher & Schnell). ICP-OES measurements were performed with a Perkin–Elmer Optical Emission Optima 4300 DV Spectrometer. The measurements of the lanthanum and boron contents in the liposomes were calibrated using standard solutions of $La(NO_3)_3$ and H_3BO_3, respectively. Differential scanning calorimetry (DSC) was performed on a Capillary Cell MicroCalorimeter (MicroCal VP-DSC) instrument. The dynamic light scattering (DLS) was studied with a Perkin–Elmer photon counter, equipped with a JDS Uniphase 633 nm 35 mW laser, an ALV sp 125 s/w 93 goniometer, and a fiber detector; 3 mL cylindrical scattering cells were used with an internal diameter of 12 mm at 20 °C. Particle size distributions were calculated using the Contin method.[28]

4.2. Synthesis of the targeting agent

4.2.1. Tris-1,4,7-*tert*-butoxycarbonylmethyl-10-ethoxycarbonyl-1,4,7,10-tetraaza-cyclododecane (2)

Solid K_2CO_3 (4.72 g, 34.15 mmol) was added to a solution of the HBr salt of tris-1,4,7-*tert*-butoxycarbonylmethyl-1,4,7,10-tetraaza-cyclododecane (**1**) (3.05 g, 5.04 mmol) in 28 mL of acetonitrile, and then the mixture was stirred for 1 h at room temperature. After the suspension was cooled down to 0 °C in an ice-water bath, a solution of ethyl bromoacetate (0.95 g, 5.67 mmol) in 7 mL of acetonitrile was added dropwise over 15 min. The reaction mixture was stirred overnight at ambient temperature. The suspended salts were removed by filtration and the solvent was evaporated under reduced pressure. After purification of the residue by column chromatography (silica, CH_2Cl_2/MeOH gradient from 95:5 to 80:20) 2.63 g (88%) of **2** were obtained. ^1H NMR ($CDCl_3$, 25 °C, TMS): δ = 1.16 (t, J = 7.2 Hz, 3H), 1.45 (s, 18H), 1.47 (s, 9H), 1.80–3.80 (broad multiplets, 24H), 4.17 (q, J = 7.2 Hz, 2H). ^{13}C NMR ($CDCl_3$, 25 °C, TMS): δ = 14.14, 30.95, 27.91, 28.02, 51.96, 53.50, 54.71, 54.91, 55.67, 55.73, 62.26, 82.08, 82.16, 172.92, 173.02, 173.60.

4.2.2. Monoamide of ethylendiamine and tris-1,4,7-*tert*-butoxycarbonylmethyl-10-carboxymethyl-1,4,7,10-tetraazacyclododecane (3)

This compound was prepared and characterized according to a published procedure[29] in 63% yield (2.01 g).

4.2.3. Conjugate of 3 and 3-formylphenylboronic acid (4)

To a solution of **3** (2.01 g, 3.27 mmol) in 15 mL of methanol, 3-formylphenylboronic acid (0.53 g, 3.58 mmol) was added. After addition of 8 mL of freshly distilled triethylamine, the reaction mixture was stirred for 2 h at room temperature. The reaction was followed by NMR. After the conversion of the amine to the imine, $NaBH_4$ (1.50 g, 39.61 mmol) was carefully added to the reaction mixture. The resulting solution was stirred at room temperature for 12 h. The solvent was removed under reduced pressure to give a light-yellow foam. The residue was taken up in water and the resulting mixture was extracted three times with dichloromethane. After evaporation of the combined organic phases, 2.34 g of compound **4** (97%) were obtained, which was used in the next reaction step without further purification.

4.2.4. Removal of the *tert*-butyl protective groups from 4

A solution containing **4** (2.34 g, 3.13 mmol) in 5 mL CH_2Cl_2/TFA (3:1) was stirred overnight. The solvent was removed under reduced pressure; the residue was taken up in methanol several times, which was removed each time by evaporation, until a light-yellow foam was formed. The final residue was dissolved in water and the pH was adjusted to 12. This basic solution was brought on the ion exchange column and rinsed with water until the pH of the eluent was neutral. The final product was eluted with a mixture of pyridine (10% in water)/ethanol (1:1). The fractions obtained were analyzed by TLC. Lyophilization of the purified material gave 615 mg (1.06 mmol, 33%) of **5**. ^1H NMR (D_2O, 25 °C, pH 12, internal reference *t*-BuOH at 1.20 ppm): δ = 2.78–3.48 (m, 20H), 3.68–3.71 (m, 8H), 4.70 (s, 2H), 7.49–7.73 (m, 4H). ^{13}C NMR (75 MHz, D_2O, 25 °C, pH 12, internal reference *t*-BuOH at 31.20 ppm): δ = 35.98, 46.59, 48.66, 48.94, 50.64, 51.04, 51.23, 52.23, 56.70, 57.12, 128.96, 130.60, 132.66, 134.91, 135.42, 137.85, 169.81, 173.06, 178.46. ^{11}B NMR (D_2O, 25 °C, pH 12, external reference 0.1 M H_3BO_3 at 0 ppm): δ = −16.79. Complexations were carried out by mixing $LnCl_3{\cdot}6H_2O$ and compound **5** (5 mol % excess) in water, adjusting the pH to 7.4 followed by stirring for several hours. The formation of the complex was identified by ESI-MS as shown in Figure S1 (Supplementary data): m/z = 717.30. The absence of free lanthanide(III) ion was confirmed by a xylenol orange test.[30]

4.3. Preparation of liposomes

Liposomes were prepared from DPPC/DSPC/CHOL/DSPE-PEG using various mol ratios, by film hydration technique followed by extrusion and ultracentrifugation to remove non-encapsulated compounds. The calculated amounts of lipids with a total weight of 90 mg were dissolved in 10 mL of chloroform. The solvent was evaporated under reduced pressure to form a lipid film, which was subsequently dried under a flow of nitrogen gas. Unilamellar liposomes were formed by hydration of the lipid film with a solution of either calcein (90 mM) or of targeting agent (250 mM) in 10 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), containing 135 mM NaCl, pH adjusted to 7.4. The resulting mixture was stirred vigorously followed by extrusion above the transition temperature (50 °C). The crude liposome suspension was extruded under nitrogen pressure (500 psi): three times over a membrane with a cut-off of 200 nm and then three times over a membrane with a cut-off of 100 nm. For the preparation of liposomes for in vivo experiments a filter with 0.2 μm cut-off was used

to sterilize the calcein solution and the final suspension of liposomes. In order to remove non-encapsulated low molecular weight material, the suspension of the liposomes was then centrifuged twice for 1 h at 4 °C and 45,000 rpm (245,000*g*) in a Beckman ultra-centrifuge. The pellet obtained was re-suspended in 500 μl of HBS and stored under nitrogen atmosphere at 4 °C for the following studies. The effective diameter of the liposomes was 100 nm (SD ±5%) as determined by DLS. The phosphate concentration was determined using the colorimetric Rouser assay.[31] To test the transition temperature 500 μl of the liposome suspension was dissolved in 1 mL of 10 mM HBS and, after degassing for 7 min, analyzed by DSC-scanning three times in the temperature range from 10 to 90 °C, 60 °C/h as compared to the reference capillary containing only HBS. For the in vivo experiments, 0.1 mol % of the fluorescent phospholipid Rho-PE was included in the lipid composition.

4.4. Temperature-induced calcein release measurements in vitro

The release of calcein at elevated temperature was determined fluorometrically by measuring the increase of fluorescence signal as a result of de-quenching. The sensitivity of the photomultiplier was optimized on the solution of liposomes in buffer after their destruction with Triton-X (10%) and dilution to a non-quenching concentration (<12 μM). After the dilution degree was determined (Fig. S2, Supplementary data), the liposomes were loaded into a 10 mm cuvette and incubated for 15 min at the desired temperature. After rapidly cooling down in an ice-bath, the fluorescence of the sample was measured at 4 °C. The percentage calcein release was calculated using Eq. 1, where F is the experimental fluorescence value (at $\lambda = 520$ nm) at a given temperature, F_I is the initial fluorescence and F_T is the fluorescence measured after solubilization of the liposomes by addition of 2 μl of Triton-X (10%). For the experiments where the calcein release was studied in the presence of serum, the pellet of the liposomes obtained after centrifugation was suspended in a medium containing 50% heat-inactivated Fetal Bovine Serum (FBS) in HBS.

$$\text{Calcein release } (\%) = \frac{F - F_I}{F_T - F_I} \times 100 \quad (1)$$

4.5. Encapsulation and release of the targeting agent La(III)-DOTA-ENPBA

For the preparation of the TSL containing the targeting agent, the procedure described above was applied, but now using 1 mL of a 250 mM solution of La(III)-DOTA-ENPBA for the hydration of the lipid film. The common characterization procedures were applied after extrusion and centrifugation steps. The amount of encapsulated material was determined by ICP-OES by measuring both the lanthanum and the boron contents per amount of lipids of the Triton-X treated liposomal suspensions: (i) after the hydration step, and (ii) after harvesting of the liposomal pellet after centrifugation. The values are expressed as the molar percentages of La/B per normalized molar amount of lipids. For the temperature-triggered release measurements, the pellet of liposomes with the determined amount of targeting agent was re-dissolved in 1 mL of HBS/FBS medium (1:1), and divided in five vials, each containing 200 μL of suspension. These samples were incubated at 37, 39, 41, 42 and 45 °C, respectively. After 15 min of incubation, the vials were placed into an ice-water bath to stop the release process and then centrifuged. The amount of released compound was determined by ICP-OES measuring samples of supernatant and Triton-X treated suspension. Xylenol orange tests were negative, showing that no free lanthanide was present in the samples.

4.6. Animal model

Specific pathogen-free nude mice (weighing 20 g) were purchased from Harlan-CPB, and fed a standard laboratory diet ad libitum (Hope Farms). A human BLM melanoma xenograft tumor model was used for this study. All animal studies were done in accordance with protocols approved by the committee on Animal Research of the Erasmus MC (Rotterdam, the Netherlands).

4.6.1. Preparation of the dorsal skin-fold window chamber

Preparation of the dorsal skin-fold chamber was performed as described previously.[32] Briefly, mice were anesthetized (100 μL of a 2:1:1 (v/v/v) mixture of saline, ketamine, and xylazine). After dissecting the skin, leaving the fascia, and opposing the skin, the skin-fold of the mouse was sandwiched between two frames, fixed with two light metal bolds and sutures. A small piece of BLM tumor (0.1 mm^3) was transplanted in the fascia, and on both sides, the window was closed with a 12-mm diameter microscopic cover glass of 0.13–0.16 mm thickness. The mice were housed in an incubation room with an ambient temperature of 32 °C and a humidity of 50%. Experiments started 10–14 days after implantation of tumor in the dorsal skin-fold chamber.

4.7. Intravital fluorescence microscopy during hyperthermia

At given time points, mice were anesthetized by isoflurane-based inhalation anesthesia and fixed to the heated microscope stage of a Zeiss LSM 510 META confocal laser scanning microscope. Before examination, a temperature sensor was placed in the tissue in the window chamber for online temperature measurements during hyperthermia. An in house-developed conductive heating device was placed on top of the window for applying hyperthermia. The mice used for this study ($N = 3$) were injected with independently prepared batches of liposomes via the tail vein at a dose of typically 4.2 μmol of lipids. Randomly selected tumor regions were examined with a 10× objective with long working distance. Scans were made with a 488 nm argon laser for detection of calcein (505–550 nm band pass filter) and a 543 nm helium-neon laser for detection of Rho-PE (560–615 nm band pass filter). During hyperthermia of 1 h at 41 °C liposome tumor accumulation and triggered drug release were monitored in the living animal.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.036.

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