Image guided drug release from pH-sensitive ion channel-functionalized stealth liposomes into an in vivo glioblastoma model

Pacheco-Torres, Jesus; Mukherjee, Nobina; Walko, Martin; Lopez-Larrubia, Pilar; Ballesteros, Paloma; Cerdan, Sebastian; Kocer, Armagan

Published in:
Nanomedicine-Nanotechnology biology and medicine

DOI:
10.1016/j.nano.2015.03.014

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Image guided drug release from pH-sensitive Ion channel-functionalized stealth liposomes into an in vivo glioblastoma model

Jesus Pacheco-Torres, PhD\textsuperscript{a,1}, Nobina Mukherjee, PhD\textsuperscript{b,1}, Martin Walko, PhD\textsuperscript{d}, Pilar López-Larrubia, PhD\textsuperscript{a}, Paloma Ballesteros, PhD\textsuperscript{c}, Sebastian Cerdan, PhD\textsuperscript{a}, Armagan Kocer, PhD\textsuperscript{e,*}

\textsuperscript{a}Instituto de Investigaciones Biomédicas “Alberto Sols” CSIC/UAM, c/ Arturo Duperier 4, Madrid, Spain
\textsuperscript{b}Groningen Institute of Biomolecular Sciences and Biotechnology, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
\textsuperscript{c}Laboratory of Organic Synthesis and Molecular Imaging by MRI, Faculty of Sciences UNED, CSIC Associated Unit, Paseo Senda del Rey 9, Madrid, Spain
\textsuperscript{d}Institute of Chemistry, Faculty of Science, P.J. Safarik University in Kosice, Moyzesova 11, Kosice, Slovakia
\textsuperscript{e}University of Groningen, University Medical Center Groningen, Department of Neuroscience, Antonius Deusinglaan 1, 9713AV, Groningen, The Netherlands

Received 9 September 2014; accepted 29 March 2015

Abstract

Liposomal drug delivery vehicles are promising nanomedicine tools for bringing cytotoxic drugs to cancerous tissues selectively. However, the triggered cargo release from liposomes in response to a target-specific stimulus has remained elusive. We report on functionalizing stealth-liposomes with an engineered ion channel and using these liposomes in vivo for releasing an imaging agent into a cerebral glioma rodent model. If the ambient pH drops below a threshold value, the channel generates temporary pores on the liposomes, thus allowing leakage of the intraluminal medicines. By using magnetic resonance spectroscopy and imaging, we show that engineered liposomes can detect the mildly acidic pH of the tumor microenvironment with 0.2 pH unit precision and they release their content into C6 glioma tumors selectively, in vivo. A drug delivery system with this level of sensitivity and selectivity to environmental stimuli may well serve as an optimal tool for environmentally-triggered and image-guided drug release.

From the Clinical Editor: Cancer remains a leading cause of mortality worldwide. With advances in science, delivery systems of anti-cancer drugs have also become sophisticated. In this article, the authors designed and characterized functionalized liposomal vehicles, which would release the drug payload in a highly sensitive manner in response to a change in pH environment in an animal glioma model. The novel data would enable better future designs of drug delivery systems.

© 2015 Elsevier Inc. All rights reserved.

Key words: pH-sensitive liposomes; Mechanosensitive channel of large conductance; Ion channel engineering; Triggered drug delivery; C6 glioma tumors; Magnetic resonance spectroscopy and imaging

Background

Chemotherapy is one of the primary treatments for cancer. Over the last decades, systemic chemotherapeutic interventions have witnessed increased efficacy, decreasing the pathological and socioeconomic repercussions of cancer significantly and improving the outcome of surgically inaccessible tumors and multifocal neoplastic disease. However, these treatments are often shadowed by a plethora of harmful effects mostly derived from the secondary impact of the therapeutic drug on the healthy tissues. Furthermore, limited bioavailability of drugs to cancerous tissues demands larger doses, increasing the risk of multiple drug resistance. On these bases, further improvements in the outcome of chemotherapy will require both, the selection of more robust and specific tumoral targets as well as more efficient drug delivery systems.
The acidic extracellular pH (pH₆) microenvironment of tumors is an aberrant physiopathological circumstance underlying fundamental events of cancer biology including malignancy, invasion or metastasis. It represents a distinct property of tumors, thus providing an inherently robust discriminant for targeted drug delivery to cancers. In fact, the acid extracellular environment of tumors has been previously used to advise the selection of the most appropriate, systemically administered, free chemotherapeutic drugs. However, the use of free chemotherapeutics does not preclude that significant amounts of the administered drug reach the healthy tissues. It does not limit the development of multidrug resistance, and consequently does not restrict satisfactorily the undesired secondary effects of chemotherapy.

The use of liposomal nanocarriers loaded with anticancer drugs has demonstrated more recently, improved sensitivity and specificity when compared to the direct administration of the free drug. These preparations are known to accumulate in the tumoral lesions because of the increased capillary permeability of the tumor vasculature and the reduced clearance capacity of the tumor. Therefore, liposomes decrease the undesired delivery of the cytotoxic drug to healthy tissues significantly. However, the use of liposomes in pH-triggered drug delivery has been limited mainly by the lack of a sensory component in the liposomes that is sufficiently sensitive to detect the mild pH differences between healthy and neoplastic tissues, and in response, trigger efficiently the release of the intraluminal drug.

Recently, we converted one of nature’s sensory ion channels, i.e. mechanosensitive channel of large conductance (MscL) from Escherichia coli, into a pH-sensitive nanovalve. We gained control over its gating mechanism by specifically and selectively modifying its pore region with tailor-made pH-responsive chemical modulators. These changes in the pore of the channel interfere with the gating mechanism of the channel and open its pore. We have shown that these proteoliposomes could sense the ambient pH and, in response to acidic conditions, they could release their intraluminal content in vitro. Furthermore, it was possible to fine-tune the pH sensitivity of these liposomes with an unprecedented precision by manipulating the pKa of the chemical modulators such that the liposomes were able to respond to a physiologically relevant narrow pH range.

Here, we reconstitute pH-sensitive-MscL-based nanovalves into physiologically relevant stealth liposomes, i.e. sterically stabilized liposomes, and describe the pH-triggered in vivo drug delivery into a C6 glioma rodent tumor model. These liposomes were loaded with the paramagnetic chelate Gd-diethyl-diaminopentaacetic acid (Gd-DTPA), the release of which became readily detectable in vivo by Magnetic Resonance Imaging (MRI) methods. After constructing the pH map of individual brain tumors, we delivered intravenously (i.v.) the pH-sensitive liposomes. We could follow by MRI the liposomal release of the intraluminal imaging probe to the in vivo tumor under these conditions. Together, these results show that the pH-sensitized MscL nanovalve may provide a suitable mechanism for pH-triggered drug release from stealth liposomes into tumors in vivo.

Methods

Strains and cell growth

*Escherichia coli* (E. coli) strain PB104 (mscL:CmR; Ara), was used to host the pBAD expression constructs containing *mscl* from *E. coli*, from parental pBAD-myc-his B plasmid (pBAD24derivative; Invitrogen) without myc-his sites. The original Multiple Cloning Site was expanded, and nucleotides encoding for a c-myc epitope and His tag were removed. Chemically (CaCl₂)-competent *E. coli* PB104 cells were transformed with P1BAD G22C-His and were grown in LB medium in the presence of 10 μg/mL chloramphenicol and 100 μg/mL ampicillin. Cells were grown in a bioreactor at pH 7.5, temperature 37 °C, and oxygen control (dissolved oxygen >70%), using a complex medium [12 g/L Bacto-Tryptone (BD), 24 g/L yeast extract (BD), potassium phosphate (17 mM KH₂PO₄ and 72 mM K₂HPO₄) (pH 7)]. It was supplemented with chloramphenicol and ampicillin. At the early and mid-log phases, 10 ml of 40% (vol/vol) glycerol/L medium were added as an additional carbon source. The protein expression was induced in the late logarithmic phase, by adding L-arabinose at 0.1% (wt/vol). Upon induction, another 10 ml 40% (vol/vol) glycerol was added, and cultivation was continued for 90 min.

Membrane vesicle preparation

Cells were harvested by centrifugation and suspended in ice-cold 25 mM Tris · HCl, pH 8.0, to a final OD₆₀₀ of 100-150. Subsequently, DNase (0.5 mg/mL, final concentration), RNase (0.5 mg/mL, final concentration), and 5 mM MgSO₄ were added, and cells were broken using a cell disrupter (Type TS/40; Constant Systems) at 1.7 kbar and 4 °C. Cellular debris was removed by centrifugation for 30 min at 18,460 g and 4 °C. Supernatant was ultra centrifuged at 145,400 g for 90 min at 4 °C. The supernatant was discarded, and the remaining membrane vesicles were re-suspended and homogenized in ice-cold 25 mM Tris · HCl (pH 8.0) to 0.7 g (wt weight)/ml, corresponding to about 30 mg of protein/ml. Membrane vesicles were frozen in liquid nitrogen and stored at −80 °C.

Protein isolation

Mscl-his tagged proteins were purified by nickel-nitriolacetic acid (Ni-NTA) affinity column chromatography, as explained before. Briefly, membrane vesicles corresponding to about 90 mg of total protein were solubilized at 5 mg/ml in 50 mM sodium phosphate (pH 8.0) containing 300 mM NaCl, 1 mM Gd-DTPA, and 100 μg/mL aprotinin. Membrane vesicles sedimenting at 100,000 × g for 60 min were collected, and the soluble fraction was dialyzed against ice-cold 25 mM Tris · HCl, pH 8.0, containing 300 mM NaCl, 1 mM Gd-DTPA, and 100 μg/mL aprotinin. Protein fractions from different molecular weights were collected and precipitated with 30% (vol/vol) ammonium sulfate. After centrifugation at 18,000 × g for 20 min, the protein was redissolved in ice-cold 25 mM Tris · HCl, pH 8.0, containing 300 mM NaCl, 1 mM Gd-DTPA, and 100 μg/mL aprotinin. Then, the protein was dialyzed against ice-cold 25 mM Tris · HCl, pH 8.0, containing 300 mM NaCl, 1 mM Gd-DTPA, and 100 μg/mL aprotinin. Finally, the protein was concentrated to about 1 mg/ml using a SpeedVac (Savant Scientific) at 30 °C and stored at −80 °C.
phosphates (pH 8.0), 300 mM NaCl, 1% (vol/vol) Triton X-100, and 35 mM imidazole (solubilization buffer) for 30 min at 4 °C. After ultracentrifugation at 267,000 g for 20 min at 4 °C, the supernatant was applied to Ni-NTA agarose resin (Qiagen) [30 mg membrane protein/mL per 50% (wt/vol) slurry], which was pre-equilibrated with 10 column volumes (CV) of solubilization buffer, and incubated under mild agitation for 30 min at 4 °C, enabling binding of his-tagged proteins to the matrix. Unbound material was collected as flow through and analyzed when appropriate. The column was washed consecutively with 15 CV wash buffer [50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 0.2% (vol/vol) Triton X-100, 35 mM imidazole] and 10 CV of wash buffer without Imidazol. At this stage, MscL protein was labeled with pH modulator at the 22nd cysteine residue. It was labeled with 1 mg/mL final concentration of the pH modulator in DMSO for 45 min at room temperature. The unbound modulator was washed off the affinity column with 7.5 CV L-histidine wash buffer [50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 0.2% (vol/vol) Triton X-100, 50 mM histidine]. His-tagged proteins were eluted by addition of 15 × 0.5 CV Ni-NTA elution buffer [50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 0.2% (vol/vol) Triton X-100, 235 mM L-histidine]. The protein content of the fractions was checked with the Bradford assay. The total yield of protein was 3-4 mg at an average concentration of ~0.5 mg/mL MscL. Protein was frozen in liquid nitrogen and stored at −80 °C.

**Chemical synthesis**

The pH modulator was synthesized as explained before by esterification of N,N-dimethylglycine with 2-bromoethanol and subsequent exchange of bromine for the methanethiosulfonate group.

**Preparation of drug loaded stealth liposomes**

Chloroform solution of DOPC, Cholesterol, DSPE-PEG2000 (Avanti polar lipids) in the molar ratio 70:20:10 was prepared. After removal of chloroform in rotary evaporator at 25 °C, the lipid mixture was rehydrated for 40 min with 0.5 M Gd-DTPA (Magnevist, Bayer, DE) solution at 50 °C to 20 mg/mL final concentration, after dehydration in rotor evaporator. Subsequently, seven freeze-thaw cycles were performed with rapid freezing in liquid N2 and thawing in a water bath at 60 °C. Aliquots of 1 mL were stored at −80 °C.

**Protein reconstitution in stealth liposomes**

The labeled MscL was reconstituted into Gd-DTPA containing liposomes by a detergent mediated reconstitution method, according to Kocer et al. Briefly, the Gd-DTPA containing liposome mixture was thawed and extruded through a 400 nm filter (11 times). Liposomes were destabilized by the addition of a final concentration of 0.25% Triton X-100. MscL and detergent-saturated liposomes were mixed at a 1:50 weight ratio, respectively, and incubated for 30 min at 50 °C. Subsequently, the appropriate buffer (for fluorescence dequenching assay: 200 mM calcein in 10 mM sodium-phosphate pH 8.0, 150 mM NaCl, 0.125 M Gd-DTPA; for animal experiment formulation: only 0.125 M Gd-DTPA) was added in 1:1 volume ratio and supplemented with 10 mg (wt weight) Biobeads (SM-2 Absorbents) per μL detergent (10 % Triton X-100) used in the sample and lipid preparation. For detergent removal, the sample was incubated overnight (~16 hours) at 4 °C on a rotating plate. Proteoliposomes with an average diameter of 100 nm were formed (as determined by Dynamic Light Scattering). Calcein encapsulated proteoliposomes were used in fluorescence dequenching assay. For animal experiment: proteoliposomes (without calcein) containing 0.1875 M final Gd-DTPA concentration were centrifuged at 202,500 g for 45 min at 4 °C and the supernatant containing external Gd-DTPA was removed by pipetting. Then, the proteoliposomes were resuspended in sterile iso-osmotic buffer containing 10 mM sodium phosphate, 150 mM NaCl at pH 8.0 to be administered to the animal.

**Fluorescence dequenching assay**

In this ensemble measurement, pH-induced release was followed by fluorescence dequenching assay. A self-quenching fluorescent dye, calcein was co-encapsulated together with Gd-DTPA in these proteoliposomes, with the luminal concentrations specified above. The proteoliposomes were applied to a Sephadex G50 size-exclusion column in order to remove the unencapsulated, external calcein dye and Gd-DTPA. Then, the release from proteoliposomes was measured by fluorescence spectrometry (Cary Eclipse Fluorometer) at excitation and emission wavelengths of 497 nm and 516 nm, respectively, under iso-osmotic conditions in buffers with different pH’s. The pH-induced activity of the channels is represented as an increase in the fluorescent signal due to the release of calcein. In a standard assay, 2 μL calcein-filled proteoliposomes were diluted into 2100 μL of isoosmotic efflux buffer at pH 6.15, 6.4, 6.6, 6.8, 7.0, 7.2, and 7.8. The maximum fluorescence of the sample was determined by releasing the proteoliposomes by the addition of 0.5% (v/v) final concentration of Triton X-100. As a control, background release from the control liposomes with unmodified MscL was recorded in identical buffer and experimental conditions.

**In vitro magnetic resonance imaging assay**

pH-sensitive, -insensitive liposomes and liposomes with no embedded ion channels were prepared as described above. After the external Gd-DTPA was removed in a Sephadex G50 size-exclusion column, the 2 mL of liposome samples was divided into four 500 μL aliquots. Per liposome type (i.e. pH-sensitive, -insensitive and liposomes with no ion channels), the pH of the two aliquots were dropped to pH 6.5 by adding 1.2 μL of 20% HCl, whereas the pH of the other two aliquots kept at pH 7.8. After 20 min incubation at room temperature, 100 μL of 10% Triton X-100 was added to the duplicates of each liposome sample, to estimate the maximum amount of Gd-DTPA that was encapsulated. The samples were centrifuged at 202,500 g for 40 min at 4 °C and the supernatant containing the released Gd-DTPA was used for quantification by MRI.

Supernatants containing released Gd-DTPA were placed in 1 mL Eppendorf tubes fixed to a plexiglass device and positioned in the isocenter of the magnet. Longitudinal relaxation times (T₁) were imaged in axial sections and measured at two different pHs (7.8 and 6.5) in samples treated or non-treated with Triton X-100. Series of axial T₁-weighted images were acquired to obtain T₁ maps based on a progressive saturation experiment. The following parameters were used: repetition time (TR) = 6000 to 150 ms,
echo time (TE) = 12.6 ms, 1 average, field of view (FOV) = 32 x 32 mm, acquisition matrix (Mtx) = 128 x 128; 250 x 250 μm² in-plane resolution, slice thickness = 1 mm and 3 slices.

C6 glioma model

The experimental protocols used in these experiments were approved by the appropriate institutional committees; all efforts being made to minimize animal suffering. C57BL6 adult male mice (25-30 g) were housed in a humidity- and temperature-controlled room on a 12 h light/dark cycle, receiving water and food ad libitum. The C6 glioma model was implemented essentially as described previously.37 C6 cells (ATCC CCL-107, LGC Standards, Barcelona, ES) were grown to confluence (10-cm Petri dishes, 37 °C, 5% CO₂/95% O₂) in DMEM supplemented with 5% FBS, 100 μg/mL streptomycin, 25 μg/mL gentamicin, 100 units/mL penicillin and 1 % fungizone, harvested and kept on ice until injection. Animals were anesthetized intraperitoneally with ketamine and xylazine hydrochlorides (8% Ketolar®, 0.5 ml/kg body weight), secured in a stereotaxic holder (Kopf, Tujunga, CA) and administered an intracerebral injection of C6 cells (approx. 10⁶) in the left caudate nucleus. The development of intracranial C6 tumors was followed acquiring consecutive Magnetic Resonance Images; 1, 5, 7, 10 and 15 days after implantation in every animal.

When the tumor reached proper size (approx. 1 cm diameter), animals were anesthetized by inhalation with 3% of isoflurane in 99.9% O₂ (1 L min⁻¹) in an induction chamber and maintained in a humidity- and temperature-controlled room at 37 °C no more than two days before use. The ISUCA solution (Imidazole Succinic Acid sodium salt, SOIREM Res. S.L., Madrid, ES) was prepared in water at 1 M concentration and pH adjusted with sodium bicarbonate solution with yellowish colour. This solution was stored at 4 °C no more than two days before use.

Preparation of solutions for i.v. injection

In order to remove external Gd-DTPA from the preparations, pH-sensitive and insensitive proteoliposomes were purified as follows. The liposome solution (4 ml) was centrifuged (70,000 rpm, 40 min, 4 °C) and the supernatant removed entirely, leaving an almost transparent, small, hard pellet, which is kept at −80 °C until on the day of injection. The liposome pellets were resuspended gently in 0.4 ml of 10 mM sodium phosphate buffer (pH: 8.0) containing 150 mM NaCl, just before the tail vein injection.

The ISUCA solution (imidazole succinic acid sodium salt, SOIREM Res. S.L., Madrid, ES) was prepared in water at 1 M concentration and pH adjusted to 7.4 with sodium bicarbonate and filtered (PALL Corp, Acrodisc Syringe filter, PNN4612, Port Washington, NY 11050, US), yielding a transparent solution with yellowish colour. This solution was stored at 4 °C no more than two days before use.

In vivo magnetic resonance imaging and spectroscopic imaging

MR studies were performed in vivo during tumor development, using a 7.0 T/16 cm Bruker Pharmascan system equipped with a 90 mm diameter gradient insert (360 mT/m). The Transmit/receive arrangements included a ¹H rat brain receive-only surface coil (38 mm, circularly polarized) used as a receiver and an actively detuned transmit-only resonator (Bruker BioSpin MRI GmbH, Rimpar, DE). Data were acquired with a Hewlett-Packard console running Paravision software (Bruker Medical GmbH, Ettlingen, DE) operating on a Linux platform.

Anesthetized animals were fixed with tape to the plexiglass animal-holder to minimize breathing-related motion, and placed in a water-heated probe that kept the body temperature at 37 °C. The physiological state of the animal was monitored throughout the MRI experiment with a Biotrig physiological monitor (Bruker Medical, Ettlingen, DE), recording the respiratory rate and rectal temperature. This setup was then positioned in the isocenter of the magnet.

In the experiments following up tumor development, anatomical T₂ weighted spin-echo images were acquired using Rapid Acquisition Relaxation Enhancement (RARE) sequence.36,39 The following parameters were used: repetition time (TR) = 3000 ms, echo time (TE) = 60 ms, RARE factor = 8, 3 averages, field of view (FOV) = 38 x 38 mm, acquisition matrix (Mtx) = 256 x 256; 148 x 148 μm² in plane resolution, slice thickness = 1 mm and 16 slices.

For the Magnetic Resonance Spectroscopic Images (MRSI) and MRI experiments, animals were prepared as previously described.¹H MRSI was acquired using a PRESS-CSI sequence.35 Briefly, the tumor was localized with a coronal T₂ weighted RARE sequence, and first- and second-order shims adjusted with FASTMAP through a sufficiently large voxel containing tumor and some surrounding healthy tissue. Basal Chemical Shift Imaging (CSI) was performed in two spatial dimensions, exciting a PRESS selected volume of 8 x 8 x 4 mm. Acquisition conditions were: FOV = 22 x 22 mm, CSI matrix = 8 x 8 x 200 μm³ in-plane resolution 687 x 687 μm², TR = 1500 ms, TE = 19.3 ms and 500 transients acquired during 12.7 min. Water suppression was performed with a Variable Pulse Power and Optimized Relaxation Delays (VAPOR) sequence.40

The changes in signal intensity (SI) induced by the injected liposomes were monitored successively in the anesthetized animals using T₁ weighted images. Two types of T₁ weighted images, with the same geometry, were acquired, under “high” and “low” resolution conditions, respectively. The “high” resolution images were acquired using: TR = 350 ms, TE = 10.6 ms, 3 averages, FOV = 22 x 22 mm (the same for MRSI), matrix = 256 x 256, 86 x 86 μm² in-plane resolution, slice thickness = 1 mm and 5 slices, total acquisition time = 4.4 min. In addition, “low” resolution images were acquired to obtain by MRI the same voxel size and voxel grid implemented for the MRSI. Conditions were: TR = 350 ms, TE = 10.6 ms, 3 averages, FOV = 22 x 22 mm (the same for MRSI), matrix = 32 x 32, 687 x 687 μm² in-plane resolution, slice thickness = 1 mm and 5 slices, total acquisition time = 0.5 min.

Image analysis

T₁ values were calculated in a voxel by voxel manner using an in-house written Matlab software (2008a, The Mathworks Inc., Natick, Massachusetts, USA) implementing a non-linear
least squares exponential fitting of the SI to the equation:

\[ SI = SI_0 \left(1-e^{-\frac{pH-pKa}{d_1-pKa}}\right) \]

The relative change in \( T_1 \) values were calculated using the equation:

\[ \Delta T1(\%) = \frac{\overline{T1_{Triton}} - \overline{T1_{pH}}}{\overline{T1_{pH}}} \times 100 + 100 \]

where \( \overline{T1_{Triton}} \) represents the mean \( T_1 \) value from a sample at a certain pH value with no Triton X-100 treatment and \( \overline{T1_{pH}} \) is the mean \( T_1 \) value from the same sample and pH value but treated with Triton X-100. This comparison was done for pH-sensitive liposomes at pH 7.8 and 6.5, pH-insensitive liposomes at pH 7.8 and 6.5 and liposomes with no embedded ion channels at pH 7.8 and 6.5.

MRCSI images were reconstructed using 3DiCSI 1.9.9 software (Hatch Center for MR Research, Columbia University, New York, NY). pH\(_e\) maps were obtained as previously described. Briefly, the basal spectroscopic images, acquired before administration of ISUCA, allowed us to check the spectrometer settings and to verify that there were no endogenous resonances overlapping the aromatic ISUCA peaks. In the presence of ISUCA, Lorentzian line shapes were fitted to the H2 peaks to obtain the H2 chemical shifts using the endogenous creatine resonance as reference (3.05 ppm). Only those voxels with signal-to-noise ratio (SNR) higher than two were considered. Extracellular pH was then determined in each voxel from the chemical shift of the H2 peak using the Henderson-Hasselbalch equation:

\[ pH = pK_a - \log \frac{\delta_1 - \delta}{\delta - \delta_2} \]  

where \( pK_a = 7.07 \), \( \delta \) is the measured ISUCA H2 chemical shift and \( \delta_1 \approx 8.75 \) and \( \delta_2 \approx 7.68 \) are the alkaline and acidic asymptotic values of the ISUCA titration curve.

\( T_1 \) weighted images were analyzed using Image J (W. S. Rasband, U.S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/19972005). Briefly, SI in the initial two \( (i = 1,2)\) \( T_1 \) weighted images were averaged to obtain baseline. Normalized image SI values were then calculated on a voxel-by-voxel basis using the expression:

\[ \Delta SI(\%)_{i,m,n} = \frac{SI_{i,m,n}}{\overline{SI_{i,m,n}}} \times 100 + 100 \]

where \( i \) is the image number, \( m \) and \( n \) are the image matrix sizes, and \( SI \) is the voxel intensity. Mean \( \Delta SI \) values were calculated by averaging the changes occurring in SI during 60 min after liposome administration. \( pH \) and mean \( \Delta SI(\%) \) maps were generated using a MATLAB based home made software.

The spatial distributions of pairs of quantities (e.g. mean \( \Delta SI \) and \( pH \)) were compared by calculating the linear regression and the Pearson correlation coefficient as implemented in the SPSS Statistical Package (18.0, SPSS Inc., Chicago, Illinois, USA).

**Results**

**\( pH \)-induced \( in vitro \) release of an imaging agent from engineered stealth liposomes**

To be able to follow non-invasively the \( in vivo \) \( pH \)-induced release from our stealth liposomes decorated with engineered ion channels, we choose the MR imaging contrast agent Gd-DTPA, as the liposomal payload. Gd-DTPA is commonly used for both clinical and preclinical applications, enhancing the \( T_1 \) weighted MRI signal selectively only in those areas where it accumulates by decreasing the water \( T_1 \) relaxation times.\(^{42}\)

After the encapsulation of Gd-DTPA into the liposomes, we tested whether this imaging agent would affect the operation of the engineered ion channel and whether it would be released. To do so, we measured the \( pH \)-induced release from liposomes \( in vitro \) using both fluorescence dequenching and MRI assays. Briefly, we prepared \( pH \)-sensitive and \( pH \)-insensitive stealth liposomes containing the channel protein MscL.\(^{29}\) In \( pH \)-sensitive liposomes, MscL was covalently modified in its pore region with the \( pH \) modulator (Figure 1A). \( pH \)-insensitive liposomes embedded with the unmodified MscL were used as a control for any nonspecific release either through the lipid bilayer or the MscL channel itself.

The two types of liposomal formulations were loaded with both, the MRI contrast agent (Gd-DTPA), and a self-quenching fluorescent dye (calcein). In the fluorescence dequenching assay, \( pH \)-sensitive and -insensitive liposomes were added to a 2 ml isosmotic bulk solution, and the fluorescence intensity was measured in time. The \( pH \)-triggered release was followed as an increase in the fluorescence intensity due to the dequenching of the fluorescent dye calcein upon its release to the bulk solution. At the end of each experiment, the total liposomal content, i.e. 100% release, was determined by dissolving the liposomes with Triton X-100 and freeing all the calcein. Figure 1B shows representative time course of the \( pH \)-dependent release of calcein for \( pH \)-sensitive and -insensitive liposomes at \( pH \) 6.15 (\( n = 3 \)). The same assay was performed at different \( pH \)'s in the range of 6.15-7.8. The maximum % release from liposomes was shown as a function \( pH \) in Figure 1C. The maximum calcein release from \( pH \)-insensitive liposomes stayed less than 10 %, throughout the investigated \( pH \) range, i.e. \( pH \) 6.15-7.8. However, the \( pH \)-sensitive liposomes showed an apparent release of their intraluminal content in a \( pH \)-dependent manner; the lower the \( pH \), the higher the release (Figure 1C). The differences between \( pH \)-sensitive liposomes at different \( pH \)'s were statistically significant, as determined by Student’s t-test. Furthermore, the calcein release was comparable to that of \( pH \)-sensitive liposomes that are loaded only with calcein.\(^{29}\) indicating that the presence of intraluminal Gd-DTPA does not interfere appreciably with the channel opening mechanism.

Even though fluorescence dequenching assay shows the release of calcein, it cannot detect the release of co-encapsulated Gd-DTPA. Therefore, we developed a simple \( in vitro \) MRI assay to detect Gd-DTPA release from the liposomal preparations. We used three types of liposomes: \( pH \)-sensitive and -insensitive
Figure 1. pH triggered drug release from pH-sensitive and pH-insensitive liposomes in vitro. (A) Schematic representation of the working principle of pH-sensitive liposomes. MscL channels are labeled with a pH-sensitive modulator (left panel) and reconstituted into stealth liposomes (middle panel). Proteoliposomes are loaded with a drug during the reconstitution procedure. The drug release is triggered by the opening of the liposome-embedded MscL channels through the protonation of the pH-responsive chemical modulator on the channel (right panel). (B) Detection of calcein release by a fluorescence dequenching assay. The time course of liposomal calcein release in vitro from pH-sensitive and -insensitive liposomes at pH 6.15. In this assay, as the calcein is released, it dequenches, and the fluorescence signal increases in time. When the release reaches a steady state, Triton X-100 is added (black arrows) to dissolve the liposomes and free the whole drug. The red and blue arrows indicate the maximum % release from pH-sensitive and -insensitive liposomes, respectively (n = 3 and the error bars indicate the standard deviation). (C) The % maximum release obtained from fluorescence dequenching assays from pH-insensitive (gray bars) and pH-sensitive liposomes (black bars) as a function of pH (n ≥ 3 and the error bars indicate the standard deviation). (D) Detection of Gd-DTPA release by in vitro magnetic resonance imaging assay. pH-sensitive and -insensitive liposomes and just liposomes with no MscL channel loaded with calcein and Gd-DTPA. Each liposome sample was divided into four aliquots. The pH of the two aliquots was adjusted to either 6.5 or 7.8. After 20 min, one of the two aliquots at each pH was treated with Triton X-100 in order to release the full liposomal content. All samples were centrifuged, and the supernatants were placed in 1 ml Eppendorf tubes. In each tube, T1 maps were acquired in three axial sections, and T1 values were calculated using a square region of interest (ROI) in the center of the tube. T1 maps were shown over T1 weighted images of an axial section from pH-sensitive liposomes (1-4), pH-insensitive liposomes (5-8) and liposomes with no embedded ion channels (9-12). (E) The mean T1 values and standard deviation were obtained by analyzing all the voxels in each tube. The percentage of change in the mean T1 values of supernatants from liposomal suspensions with respect to their duplicates treated with Triton X-100 was shown. Statistical analysis was performed by using the Student’s t-test.
liposomes and liposomes with no MscL channels. After obtaining the supernatants of liposomal preparations at pH 6.5 and 7.8 as explained in the materials and methods section, we put them in individual Eppendorf tubes. To test the presence of Gd-DTPA, we acquired the T1 maps in three axial sections in each Eppendorf tube. An exemplary T1 map is given in Figure 1D. A sthe Gd-DTPA concentration increases, the T1 relaxation time shortens. As can be seen in Figure 1D, indeed the supernatant of all detergent treated liposomes (tubes 2, 4, 6, 8, 10 and 12), which contain the highest concentration of Gd-DTPA, showed the lower T1 values relative to their only pH-treated duplicates (tubes 1, 3, 5, 7, 9, and 11, respectively). Among the samples, pH-sensitive liposomes (tubes 1 and 3) released more Gd-DTPA as compared to their insensitive counterparts (tubes 5 and 7, respectively) and liposomes with no MscL channels (tubes 9 and 11, respectively) at the same pH value. Moreover, among the pH-sensitive liposomes, the release of Gd-DTPA was higher at pH 6.5 (tube 3) than at pH 7.8 (tube 1).

In order to make a more thorough comparison, we calculated T1 values using a square region of interest in the center of the tube. For each sample, we obtained mean T1 values by analyzing all the voxels. We compared the fractional decrease observed in these T1 values of the medium (revealing Gd-DTPA release) at each pH from each liposomal preparation, with the total release induced by a detergent treatment (Triton X-100) of the corresponding liposome at the same pH. As can be seen in Figure 1E, only pH-sensitive liposomes depicted a significant decrease in T1 values at lower pHs, whereas both, pH insensitive-liposomes and liposomes with no ion channel showed no significant changes in T1 values with pH.

Generating the pH-map of a brain tumor

In order to test whether our ion-channel containing liposomes could sense the pH in a tumor and release its content in vivo, we used the classical C6 glioblastoma model, obtained by implanting C6 cells in the left hemisphere of C57BL6 mice (n = 11). We loaded the pH-sensitive or -insensitive liposomal preparations containing Gd-DTPA and followed its release in vivo by MRI after i.v. administration.

We first generated the pH map of the tumor in vivo using our extracellular pH-sensitive probe (+/-)2-(imidazol-1-yl)succinic acid disodium salt (ISUCA). Briefly, after intraperitoneal (i.p.) injection of ISUCA (Figure 2A), its distribution was followed by Magnetic Resonance Spectroscopic Imaging (MRSI) (Figure 2B) and the measured ISUCA imidazolic H2 chemical shift (Figure 2C) transformed into a pH map (Figure 2D) by using the Henderson-Hasselbach calibration curve.

pH-induced liposomal Gd-DTPA release in the tumor

Once the pH map was obtained, and ISUCA was cleared from the tumor, we recorded the background T1 weighted image of every tumor before injecting i.v. the pH-sensitive (n = 4 mice) or pH-insensitive (n = 7 mice) liposomal preparations in the tail vein (Figure 2E). A representative image of a tumor before injection with pH-sensitive liposomes (corresponding to animal ID 3 in Table 1), is shown in Figures 2E. After injection of the liposomal preparation (Figure 2F), we followed kinetically by MRI the in vivo release of Gd-DTPA from the liposomal lumen, as the local increase in water signal intensities (SI) in T1 weighted MR images (Figure 2G). These images were acquired from the same
voxels where the extracellular pH of the tumor was measured previously by MRSI. Finally, we obtained the statistical correlation between the extracellular pH value, determined by $^1$H MRSI, and the average SI change ($\Delta$SI) detected by T1 weighted MRI, in every voxel of the in vivo tumor (Figure 2).

Both pH-sensitive and -insensitive liposomal formulations were tested in vivo for the release of their intraluminal content in the C6 glioblastoma model.9,37 Figure 3 shows representative results obtained in vivo for the release of intraluminal content of Gd-DTPA from pH-sensitive (top panels) and pH-insensitive liposomes (bottom panels) in two representative mice (corresponding to animal ID 1 and 5 in Table 1, respectively) carrying implanted C6 glioblastomas. The pH$_e$ within these tumors varied between pH 6.6 and 7.0 (Figure 3, A and E). The release of the paramagnetic imaging agent from the pH-sensitive liposomes into the acidic voxels of the tumor in vivo started with the first ten minutes after i.v. injection, lasting up to forty minutes, as followed by the relative mean changes in voxel signal intensity ($\Delta$SI$_{mean}$) (Figure 3B). In the case of pH-insensitive liposomes, there was a slow initial release, which stabilized around 10th minute and did not change for the rest of the measurements (Figure 3F). Panels 3 C and 3 G depict maps of relative $\Delta$SI$_{mean}$ measured in the same voxels during 60 minutes.

**Table 1**

Summary of pH$_e$ dependent release of Gd-DTPA from pH-sensitive and pH-insensitive liposomes into C6 tumors in vivo in the animals investigated.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Type of liposome</th>
<th>pH$_e$ range</th>
<th>Number of voxels</th>
<th>Pearson’s correlation coefficient</th>
<th>Linear regression ($r^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pH sensitive</td>
<td>6.75–6.95</td>
<td>44</td>
<td>-0.7842</td>
<td>0.615</td>
</tr>
<tr>
<td>2</td>
<td>pH sensitive</td>
<td>6.5–6.9</td>
<td>7</td>
<td>-0.9205</td>
<td>0.8479</td>
</tr>
<tr>
<td>3</td>
<td>pH sensitive</td>
<td>6.6–6.8</td>
<td>51</td>
<td>-0.7758</td>
<td>0.6018</td>
</tr>
<tr>
<td>4</td>
<td>pH sensitive</td>
<td>6.6–6.7</td>
<td>15</td>
<td>-0.8315</td>
<td>0.7434</td>
</tr>
<tr>
<td>5</td>
<td>pH insensitive</td>
<td>6.6–6.7</td>
<td>17</td>
<td>0.1971</td>
<td>0.0149</td>
</tr>
<tr>
<td>6</td>
<td>pH insensitive</td>
<td>6.7–6.8</td>
<td>9</td>
<td>-0.2205</td>
<td>0.0293</td>
</tr>
<tr>
<td>7</td>
<td>pH insensitive</td>
<td>6.6–6.8</td>
<td>13</td>
<td>0.1578</td>
<td>0.0559</td>
</tr>
<tr>
<td>8</td>
<td>pH insensitive</td>
<td>6.6–6.7</td>
<td>13</td>
<td>0.3178</td>
<td>0.0865</td>
</tr>
<tr>
<td>9</td>
<td>pH insensitive</td>
<td>6.65–6.8</td>
<td>25</td>
<td>0.4387</td>
<td>0.1924</td>
</tr>
<tr>
<td>10</td>
<td>pH insensitive</td>
<td>6.75–6.85</td>
<td>22</td>
<td>-0.4593</td>
<td>0.211</td>
</tr>
<tr>
<td>11</td>
<td>pH insensitive</td>
<td>6.45–6.55</td>
<td>14</td>
<td>0.1051</td>
<td>0.0111</td>
</tr>
</tbody>
</table>

**Figure 3.** Extracellular pH triggered drug release from pH-sensitive and pH-insensitive liposomes in vivo. The top panels show a representative study with pH-sensitive liposomes whereas the bottom panels depict representative results obtained with pH-insensitive liposomes. (A, E) pH$_e$ maps obtained in vivo with ISUCA. (B, F) The SI evolution over time in different tumor voxels, indicating the pH$_e$ of the corresponding voxel. (C, G) The mean change in signal intensity ($\Delta$SI$_{mean}$) in all tumor voxels due to the contrast agent released from the liposomes. (D, H) The correlation between the pH$_e$ values and the $\Delta$SI$_{mean}$ in a voxel by voxel manner, for all tumor voxels investigated. Inserts: Parameter values from the linear regression analysis.
after the injection. In the case of pH-sensitive liposomes, the voxels with the highest Gd-DTPA release co-localized with the voxels with the lowest pH (Figure 3d and C). The pH-insensitive liposomes, on the other hand, showed significantly less release of the contrast agent, independent of the tumor extracellular pH, consistent with the basal leakage previously detected in vitro in the fluorescence-dequenching and MRI assays. Finally, panels D and H show the relationship between the extracellular pH (pHe) and the relative ΔSI mean values as determined by 1H MRSI and T1 weighted MRI in the same tumor voxels, respectively. A clearly negative correlation between extracellular pH and drug release can be observed when using the pH-sensitive liposomes (Figure 3D), and virtually no correlation can be detected when using the pH-insensitive preparation (Figure 3H).

We repeated this experiment successfully in four animals receiving pH-sensitive liposomes and seven animals receiving pH-insensitive liposomes. The pH range and number of voxels of tumors in all animals are given in Table 1. Despite intrinsic variations between animals and tumors in each animal, the voxels of all animals receiving the pH-sensitive liposomes showed a negative linear regression (r² > 0.6) and high negative Pearson’s correlation coefficients (r ranging from −0.78 to −0.92) between pHe and ΔSI mean (Table 1). This reveals that the release of intraluminal content increases in vivo as the extracellular pH decreases and vice versa. On the other hand, the seven animals receiving pH-insensitive liposomes, showed poor linear regressions between pH and ΔSI (r² < 0.2) and much lower Pearson’s correlation coefficients (r ranging from −0.4 to +0.4), indicating that there is no significant correlation between the extracellular pH of the tumor and the amount of contrast agent released from the pH-insensitive liposomes. Taken together, these results are consistent with those obtained in vitro with the same liposomal preparations (Figure 1C), revealing that, the intraluminal content of pH-sensitive liposomes is effectively released under the mildly acidic pH conditions prevailing in vivo while no significant pHc dependent release occurs from the pH-insensitive preparations.

Discussion

Here, we demonstrate the ability of engineered ion-channels to transform stealth liposomes into very sensitive pH-responsive drug-carriers that can detect mild, physiologically relevant pH differences and release the luminal drug selectively in the low pH areas of the tumor in vivo. The use of an engineered ion channel as a sensory component is advantageous over alternative pH-sensitive liposomal formulations that need to contain high amounts of negatively charged lipids, polymers, or unsaturated lipids, which make liposomes prone to fast clearance from the blood, affect their pharmacokinetic properties and may cause incompatibilities with drug encapsulation.28 Ion channels, conversely, are membrane proteins, which can be sensitized to different triggers, their opening/closing properties can be conveniently manipulated and fine-tuned by small chemical modulators. In addition, ion channels react fast to an external stimulus, and only few ion channels per liposome are sufficient to release the intraluminal content efficiently, thus presenting minimal interference with the physicochemical and physiological properties of hosting liposome.

In recent years, pH-sensitive liposomes have been explored for their use in imaging and intracellular delivery of anticancer drugs, antisense oligonucleotides, ribozymes, plasmids, proteins and peptides in cell cultures.43 These liposomes sense low pH’s that can be found in the late endosomes. Therefore, they are designed to be taken up by the endosomal route into the cells, sense the pH below 5.5 in the late endosome, and in return, destabilize the liposomes and release the content. Similarly, in addition to its ability to sense extracellular pH, our drug delivery system has the potential for intracellular delivery, too. We have shown that the pH sensitivity spectrum of the ion channel in our engineered liposomes can be fine tuned by using different chemical pH-sensing compounds.29

Finally, the engineered ion-channel based pH-sensitive liposomes implemented herein may be useful for improving the performance of chemotherapeutic regimes, helping to decrease undesired systemic toxicity and drug resistance responses.26 Furthermore, they might serve as sensitive image-guided drug delivery devices. However, further investigation is required to test these possibilities. In particular, even though our glioma model depicts high blood-brain barrier permeability, human gliomas may depict a much more complex behavior, with different degrees of blood-brain barrier permeabilities, much enhanced in the high-grade tumors. Thus, improvements in the blood-brain barrier permeability of our engineered liposomes also merit further research.44

Even though the ion channel engineered liposomal drug delivery system offers new opportunities for triggered delivery of drugs and or imaging agents, the potential immunogenicity and scale-up production should be further studied.

In summary, we characterized and evaluated the use of stealth liposomes containing a pH-sensitive nanovalve. We, showed that these preparations reach an intracranial tumor selectively, release their cargo in response to the acidic pHc conditions prevailing in the tumor microenvironment in vivo, and can be visualized non-invasively by MRI/MRSI. These results may help to improve the efficacy of therapeutic interventions in oncologic malignancies and other diseases progressing with alterations in extracellular pH, as ischemic episodes.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSI</td>
<td>Chemical Shift Imaging</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylen Triamino pentaacetic acid</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of View</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravascular</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MRSI</td>
<td>magnetic resonance spectroscopic imaging</td>
</tr>
<tr>
<td>MsCl</td>
<td>mechanosensitive channel of large conductance</td>
</tr>
<tr>
<td>pHc</td>
<td>extracellular pH</td>
</tr>
<tr>
<td>RARE</td>
<td>rapid acquisition relaxation enhancement</td>
</tr>
<tr>
<td>SI</td>
<td>Signal Intensity</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal to Noise Ratio</td>
</tr>
<tr>
<td>TE</td>
<td>Echo Time</td>
</tr>
<tr>
<td>TR</td>
<td>Repetition Time</td>
</tr>
<tr>
<td>VAPOR</td>
<td>Variable Pulse Power and Optimized Relaxation Delays</td>
</tr>
</tbody>
</table>
Acknowledgment

The authors wish to thank Dr. H. Ingólfsson and Dr. Louhivuori for the Graphical abstract image and to Dr. G.T. Robillard for critical reading of the article.

References