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In vivo MRI multicontrast kinetic analysis of the uptake and intracellular trafficking of paramagnetically labeled liposomes

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ABSTRACT

This work aims at developing a MRI method that allows to get more insight into the understanding of the *in vivo* fate of liposomes and their payload. The method relies on the temporal assessment of the contrast changes induced by the presence of a classical relaxation agent versus the effect induced by a CEST (chemical exchange saturation transfer) agent. Liposomes were loaded with the paramagnetic complexes, Gd-HPDO3A and $[Tm\text{-}DOTMA]^{-}$ $[Na]^{+}$, in order to endow the nanovesicles with the characteristic properties of T_1/T_2 and $CEST/T_2$ MRI agents, respectively. The paramagnetically loaded liposomes were injected directly into the tumor (B16 melanoma xenograft in mice) where they generate T_1, T_2, and CEST MR contrasts that were quantitatively monitored over time (0–48 h). The kinetic of each contrast enhancement reports about peculiar properties relative to the fate of the liposomes in the tumor environment. A kinetic model has been set-up to fit the experimental multicontrast data in order to extract the relevant information about the cellular uptake of the liposomes and the release of their payload. Upon comparing conventional stealth liposomes with pH-sensitive ones, it has been shown that the latter ones differ essentially in the step associated with the release of the drug that is likely occurring in the endosomal acidic vesicles.

1. Introduction

Liposomes are nanosized vesicles consisting of a phospholipidic membrane delineating an inner aqueous cavity. They are mainly used as nanocarriers in drug delivery protocols [1,2]. Such use relies on the possibility of loading them either with hydrophilic drugs into their aqueous inner space or lipophilic drugs into the phospholipidic bilayer.

In spite of their large use and proved therapeutic efficacy, there is still a lack of knowledge about their *in vivo* behaviour. The therapeutic efficacy of a drug depends not only on its ability to accumulate at the pathological region, but also on its effective uptake from diseased cells and its intracellular trafficking. So far, most of the information regarding the intracellular fate of liposomes has been extracted from *in vitro* microscopy studies on cultured cells [3–8]; however, the *in vitro* observations are not always reliable to predict the behaviour of the liposomes (and their bioactive payload) in living organisms.

The ability of the liposomes to be loaded with large amounts of hydrophilic and lipophilic molecules, has also been exploited in MRI. The loading of the vesicles with paramagnetic metal complexes has allowed to overcome intrinsic low sensitivity issues of the technique [9–12] and to design new classes of contrast agent [13].

The main aim of this work is to set-up an *in vivo* magnetic resonance imaging (MRI) method to assess distribution, uptake and intracellular behaviour of paramagnetic liposomes in tumoral environment.

To this purpose two types of PEGylated “stealth” [14] paramagnetic liposomes have been considered:

i) liposomes loaded with the clinically approved Gd-HPDO3A complex (Chart 1) which act as T_1 and T_2 MRI contrast agents [9]. The attainable T_1 contrast may be partially or fully “quenched” by the occurrence of slow exchange rates of water molecules through the liposomes' membrane. The effect of the “quenching” effect depends on both the composition of the liposome membrane and the amount of entrapped paramagnetic agent [12]. On this basis, the maximum of T_1 contrast enhancement is expected only when the nanovesicles lose their integrity and release their content. On the other hand, the T_2 contrast is mainly determined by the changes in magnetic susceptibility resulting from the localization of a high amount of paramagnetic complex inside the nanovesicle [10]; therefore, the spread-out of Gd-complex into larger spaces is expected to decrease the T_2 contrast.
ii) liposomes loaded with the paramagnetic shift reagent $[Tm\text{-}DOTMA]^{-}$ $[Na]^{+}$ (Chart 1) that simultaneously act as T_2 and chemical exchange saturation transfer (lipoCEST) [13] agent. In the latter class of agents the intraliposomal water resonance is

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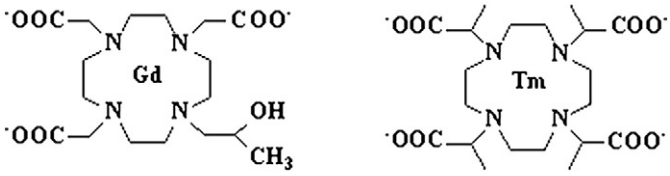

Gd-HPDO3A [Tm-DOTMA]·

Chart 1. Sketched structures of the two paramagnetic complexes used in this work.

shifted from the bulk water signal. Thus, by selectively irradiating the inner water NMR signal, a contrast in the MR image can be generated upon transfer of saturated magnetization [15], the extent of which depends on the water exchange rate between the two water pools. Maximum CEST contrast is observed when intact liposomes are free in the extracellular fluids; it markedly decreases when the lipoCEST agents are endocytosed by cells. This is the consequence of the crossing of additional membranes that, invariantly, results in a drastic decrease of the CEST effect in a given voxel. When the liposome will be eventually degradated, the CEST contrast will be nullified because of the disappearance of the inner water pool. As far as the T_2 contrast is concerned, these nanovesicles behave in an analogous way to the above described Gd-loaded liposomes.

The liposomes used in this work, having the same formulation of the lipophilic membrane, share the same *in vivo* biodistribution.

The MRI responses (CEST, T_1 and T_2) of the liposomal probes depend on their distribution in the tumor micro-environment and on maintenance of their integrity. The elucidation of the *in vivo* cellular uptake and the intracellular behaviour of the liposomes (and their content) will be pursued through a simultaneous kinetic analysis of their CEST, T_1 and T_2 generated contrast.

This multicontrast approach will then be applied to investigate the behaviour of liposomes characterized by different membrane's properties, such as pH-sensitive liposomes, designed to release their content in mild acidic environments [16]. Furthermore, *ex vivo* fluorescence-activated cell sorting (FACS) analysis provides additional information on the cell types involved in the liposome uptake.

2. Materials and methods

2.1. Chemicals

DPPC (1, 2-Dipalmitoyl-sn-glycero-3-phosphocholine), DSPE-PEG-2000 (1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000], ammonium salt), POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine), cholesterol, and THS (α-tocopherol hemisuccinate) were purchased from Avanti Polar Inc (Alabaster, Al USA). Gd-HPDO3A and $[Tm\text{-}DOTMA]^-$ $[Na]^+$complexes were kindly provided by Bracco Imaging (Colleretto Giacosa, TO, Italy). All the other chemicals were purchased from Sigma-Aldrich.

The culture medium DMEM, foetal bovine serum (FBS), glutamine, penicillin–streptomycin mixture and EDTA were purchased from Cambrex (East Rutherford, NJ, USA).

2.2. Liposome preparation

Liposomes were prepared according to the thin film hydration method [17]. The total amount of phospholipids was 40 mg/ml. Two types of liposomes were formulated: i) conventional stealth liposomes composed of DPPC/DSPE-PEG2000 (95/5 molar ratio), or ii) pH-sensitive liposomes composed of POPE/THS/cholesterol (44.5/11/44.5 molar ratio).

The lipid thin films were loaded with Gd-HPDO3A (190 mM solution) or $[Tm\text{-}DOTMA]^-$ (150 mM solution). The liposomes for FACS experiments were prepared by hydrating the lipid thin film with 10 mM solution of carboxyfluorescein. The mean hydrodynamic diameter of the liposomes was determined by dynamic light-scattering measurements carried out on a Malvern ZS Nanosizer (Malvern Instrumentation, UK).

2.3. Animal model

6 to 10-week-old female C57Bl6 mice (Charles River Laboratories, Calco, Italy) were inoculated subcutaneously in the left flank with 0.2 ml of a single suspension containing approximately 1×10^6 B16 murine melanoma cells. B16 cells were grown in a DMEM medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

B16 (murine melanome) cells were obtained from ATCC (Manassas, VA, USA).

2.4. MRI measurements

T_1, T_2, and CEST contrast enhanced MR images were acquired at 7 T on a Bruker Avance 300 (Bruker, Germany) spectrometer equipped with a Micro2.5 microimaging probe. The total concentration of the paramagnetic complexes in the liposomes suspension was determined by measuring the magnetic susceptibility of the suspension [18].

The mice were injected with the liposome suspension ca. 7 days after the cell inoculation, *i.e.* when the tumor mass reached a mean diameter of about 4 mm. The temporal evolution of the different contrast modalities was monitored *in vivo* after intratumor injection of 10 μl of liposomes suspension.

When Gd(III)-loaded liposomes have been injected, a series of T_{1w} (spin-echo sequence, TE = 3.3 ms, TR = 250 ms), and T_2 measurements (multi spin-echo sequence, 50 echoes, TE = 3.3 ms, TR = 5000 ms) were acquired before and after the injection, while a series of Z-spectra and T_2 measurements (sequence and parameters as above) were acquired before and after the intratumor injection of the Tm(III)-based vesicles. The acquisition of Z-spectra has been done from using a RARE sequence (RARE factor 8, effective echo time 4.1 ms) preceded by a square continuous wave pulse (duration 2 s, power 12 μT, frequency range from −20 to 20 ppm in 1 ppm steps).

A total number of 24 mice were examined equally distributed among the four liposome-chelate combinations (*stealth*/Gd; *stealth*/Tm; *pHsens*/Gd; *pHsens*/Tm).

As control, solutions of free Gd-HPDO3A (6 mice) and $(Tm\text{-}DOTMA)^-$ (6 mice) were injected at a dose of lanthanide equal to that used in the administration of the liposomal agents.

The MRI responses were determined as means calculated on the region of interest (roi) drawn in order to contain the whole tumor volume.