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From Spherical to Osmotically Shrunken Paramagnetic Liposomes: An Improved Generation of LIPOCEST MRI Agents with Highly Shifted Water Protons**

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The advent of the molecular imaging era prompts the search for innovative imaging probes to set up novel procedures for pursuing early diagnosis and efficient follow-up of therapeutic treatments.[1] Among magnetic resonance imaging (MRI) agents, those dubbed CEST (chemical exchange saturation transfer)[2] have the unique property of yielding a so-called "frequency-encoded" contrast that may allow, analogous to optical imaging probes, the visualization of different agents in the same region.[3–5] It is expected that such a property can dramatically enhance the diagnostic potential of MRI because many applications, including cancer diagnosis or cell-tracking experiments, may benefit from the simultaneous visualization of different biological targets (or labeled cells).[6]

The frequency-encoded MRI contrast generated by CEST agents is the result of the selective irradiation at the resonance frequency of the labile protons of the probe, whose exchange with the bulk water protons (that has to be smaller than the difference between the resonance frequencies of the two exchanging sites) causes a decrease of the MRI signal. In such a way, a rather small concentration of mobile protons (e.g. in the millimolar range) may be detected in the MR image.[5a] The sensitivity of a CEST agent is primarily dependent on the number of NMR-equivalent mobile protons that operate the saturation transfer (ST) to the bulk water resonance. For this reason, the most sensitive class of CEST agents so far proposed is represented by the so-called LIPOCEST probes, in which the very large number of mobile water protons entrapped in a liposome, and properly shifted from the bulk water by the presence of a lanthanide(III) shift reagent (SR), can be selectively irradiated by the saturation pulse.[7]

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Besides the availability of a high number of exchangeable protons, the potential of a LIPOCEST agent relies on the value of the chemical shift of the intraliposomal water, as larger shifts allow larger exchange rates to be exploited and, very important for in vivo applications, reduce the interference with the magnetization transfer effects associated with the endogenous proteins.[8–9]

Herein, we propose a novel class of LIPOCEST agents properly designed to yield large chemical-shift values of the intraliposomal water resonance thanks to exploitation of the contribution arising from the control of the bulk magnetic susceptibility (BMS) effects.[10–11]

Briefly, the paramagnetic shift induced on intraliposomal water protons by a paramagnetic lanthanide SR is the sum of two contributions [Eq. (1)]:

$$\delta_{wat} = \delta_{wat}^{DIP} + \delta_{wat}^{BMS} \quad (1)$$

At the highest attainable concentrations of a lanthanide SR that contains one fast-exchanging metal-bound water molecule (e.g. [Ln(dotma)], [Ln(dota)], or [Ln(hpdo3a)]),[5] δ_{wat}^{DIP} (DIP = dipolar contribution) can at best reach values close to $\delta = \pm 4$ ppm. δ_{wat}^{BMS} is zero for spherical LIPOCEST agents, but this term can markedly contribute to the water chemical shift when the paramagnetic ions lie in a non-spherical compartment. This term originates from the partial alignment, within the external magnetic field, of the magnetic moments of the paramagnetic centers. In addition to being proportional to the SR concentration, it is directly related to the effective magnetic moment (μ_{eff}) of the Ln^{III} ion and can be strongly influenced by the shape and orientation of the paramagnetic vesicles to the external magnetic field.

Nonspherical liposomes can be prepared by shrinking spherical vesicles through osmotic stress,[12–13] whereas the orientation of the shrunken liposomes in the magnetic field can be modulated by incorporating in the liposome membrane amphiphilic paramagnetic complexes endowed with a proper magnetic anisotropy.[14]

Upon hydrating the thin lipidic film with an ipotonic solution of the SR (osmolarity less than 0.15 Osm), and after dialyzing the liposomes against an isotonic buffer, we found that the resonance of the intraliposomal water protons is considerably downfield shifted. The shift is independent of the magnetic anisotropy of the entrapped SR, which, instead, defines the sign of the shift for spherical LIPOCEST agents (Figure 1).

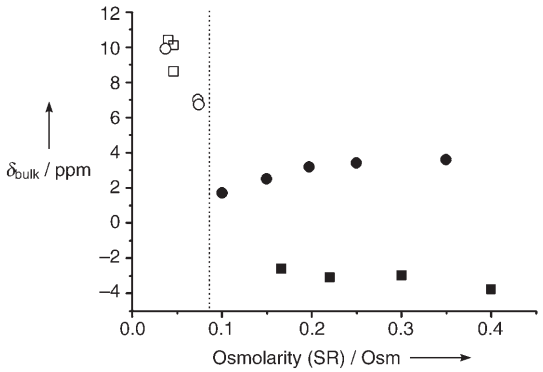


Figure 1. Induced chemical shift, at 298 K, of intraliposomal water protons (referred to as "bulk" water) as a function of the osmolarity of the solution of shift reagent (SR: [Ln(hpdo3a)]; Ln = Tm, Dy) used to hydrate the thin lipidic film (composition: DPPC/DSPE-PEG 95:5 molar ratio; total amount of lipid: 20 mg; see Experimental Section). ■ spherical LIPOCEST entrapping [Dy(hpdo3a)]; □ nonspherical LIPOCEST entrapping [Dy(hpdo3a)]; ● spherical LIPOCEST entrapping [Tm(hpdo3a)]; ○ nonspherical LIPOCEST entrapping [Tm(hpdo3a)].

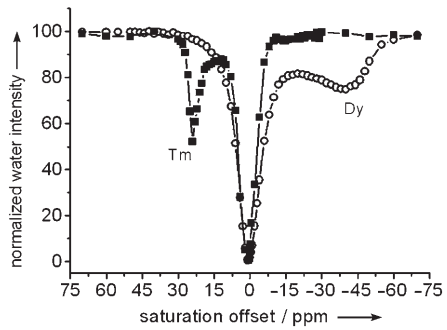
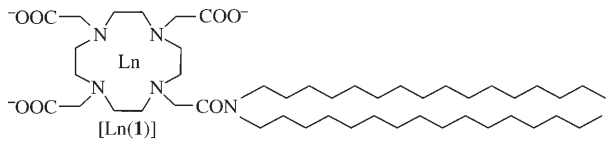


Figure 2. *Z*-spectra (7 T, 312 K; irradiation with 1 × 2 s rectangular pulse, intensity 6 μT) of the two osmotically shrunken LIPOCEST probes encapsulating a hydrophilic SR ([Tm(hpdo3a)] (■) or [Dy(hpdo3a)] (○)) and incorporating an amphiphilic SR ([Tm(**1**)] or [Dy(**1**)]) in the membrane (osmolarity of the hydrating SR solution: 40 mOsm; lipidic film composition: DPPC/DSPE-PEG/[Ln(**1**)] = 75:5:20 molar ratio; total amount of lipid: 20 mg).

This behavior provides clear evidence of the occurrence of a BMS contribution to the observed shift. In fact, for a given shape and orientation of the liposomes, the direction of the BMS shift is the same for any lanthanide(III) ions (all have $\mu_{eff} > 0$). The osmotic shrinkage of the liposomes occurs during the dialysis (carried out against an isotonic 0.3 Osm buffer) necessary to remove the SR that was not entrapped after the hydration of the lipidic film. Though the occurrence of the BMS shift increases considerably the chemical-shift difference between intraliposomal and bulk water protons, a real breakthrough would be achieved if also the orientations of the nonspherical LIPOCEST agents could be properly controlled. It is known that the incorporation of paramagnetic lanthanide(III) centers in non-isotropic phospholipid-based systems (e.g. bicelles (binary bilayered mixed micelles)) can dramatically influence their orientations with respect to the external field depending on the magnetic anisotropy of the Ln^{III} center.[15–16]

To demonstrate that this phenomenon also occurs for shrinked liposomes, we prepared two nonspherical LIPOCEST preparations entrapping a hydrophilic SR, [Ln(hpdo3a)],[5b] and incorporating an amphiphilic metal complex, [Ln(**1**)] in the membrane (20% of the total molar



[Ln(**1**)]

amount of lipids). The two preparations differ only in the magnetic anisotropy of the Ln^{III} ion used: Tm ($C_D > 0$) and Dy ($C_D < 0$), respectively.[17] Figure 2 reports the *Z*-spectra acquired at 7 T and 312 K for the two preparations. The observed shifts for the intraliposomal water resonance are noticeable, with $\delta \approx 18$ ppm (downfield) for the Tm^{III}-containing system and $\delta \approx 45$ ppm (upfield) for the Dy^{III}-containing agent. The absolute shift values for the two shrunken systems are noticeably enhanced if compared with the values observed in the absence of the membrane-incorporated paramagnetic agent. Furthermore, the sign of the induced shift is now different according to the magnetic anisotropy values of the two amphiphilic compounds.

The fundamental role played by the incorporated agent in defining the direction of the induced shift was also confirmed by preparing LIPOCEST agents encapsulating [Dy(hpdo3a)][5a] and incorporating [Tm(**1**)] or encapsulating [Tm(hpdo3a)] and incorporating [Dy(**1**)], respectively. The scrambling of the metal complexes invariantly led to LIPOCEST agents whose shift directions are defined by the magnetic anisotropy of the membrane-incorporated compound (i.e. $\delta_{wat} > 0$ for [Tm(**1**)] and < 0 for [Dy(**1**)]). Note that, besides the osmotic shrinkage and the effect on the shift direction, the presence of the incorporated SR increases the concentration of the paramagnetic centers inside the liposome (about half of the incorporated SR should point inwards), thus contributing to enhancement of both the dipolar and the BMS contributions to the induced shift.

Thanks to this novel generation of LIPOCEST probes, the window of the accessible irradiation frequency values is now considerably extended from $\delta = \pm 4$ ppm (spherical LIPOCESTs) to $\delta = +30$ or -45 ppm. Of course, several benefits may be envisaged for such improved CEST agents. First, it is expected that the increased separation from the resonance of bulk water can drastically reduce the artifacts in the MRI-CEST images generated by the asymmetry of the bulk water signal and/or the inhomogeneity of the imaging coil, mainly responsible for the inhomogeneous distribution of the resonance frequency of the bulk water.[3] Second, but not less important, the extension of the irradiation frequency values will facilitate the setup of imaging protocols aimed at visualizing multiple LIPOCEST probes.

In conclusion, the results reported here represent a substantial step ahead in the field of the MRI contrast agents based on the CEST mechanism. This new generation

of imaging probes couples the outstanding sensitivity displayed by the previous generation of spherical LIPOCEST agents to a significantly extended range of accessible irradiation frequency values, which are approaching those typical for PARACEST agents.[4,18] Interestingly, preliminary results indicated that the stability and the sensitivity of the shrunken LIPOCEST probes are comparable to those of the spherical parent agents.

Experimental Section

Synthesis of [Ln(**1**)] complexes: The Tm and Dy complexes were synthesized following the synthetic pathway previously reported for the analogous Gd chelate.[19]

Preparation of liposomes: Spherical LIPOCEST probes were prepared according to the reported procedure.[7] Osmotically shrunken LIPOCEST probes were prepared by hydrating the thin lipidic film with an aqueous solution of the paramagnetic SR with osmolarity values of less than 0.1 Osm. The composition of the lipidic films is indicated in the figure legends (DPPC = 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPE-PEG = 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000].

NMR measurements: The chemical shifts of the intraliposomal water protons were measured at 298 K on a Bruker Avance 600 spectrometer operating at 14.1 T. *Z*-spectra were acquired at 312 K on a Bruker Avance 300 spectrometer (operating at 7 T) equipped with a microimaging probe (inner diameter 10 mm). A single rectangular saturation pulse (length 2 s, intensity 6 μT) with different saturation offsets (± 70 ppm from bulk water) was applied before a conventional spin-echo RARE sequence (rare factor 8) on a phantom containing the suspension of LIPOCEST agents. The intensity of the water proton signal at a given saturation offset was normalized to the maximum intensity value.

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