Imaging Agents

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Highly Sensitive MRI Chemical Exchange Saturation Transfer Agents Using Liposomes[**]

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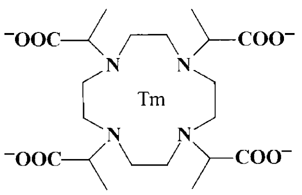
Chemical exchange saturation transfer (CEST) agents represent an emerging class of magnetic resonance imaging (MRI) contrast media that has huge potential. They act as negative agents by reducing the signal intensity of the water protons through a saturation transfer mediated by chemical exchange.[1] Saturation transfer occurs through selective irradiation at the absorption frequency of the exchanging protons of the agent. The great potential of CEST agents lies in the possibility of switching the contrast “on” and “off” at will, since each response is uniquely related to the specific absorption frequency of their mobile protons, thus making it possible to detect several agents in the same sample.[2] Basically, the saturation transfer efficiency is proportional to the exchange rate (k_{ex}) and to the number of mobile protons in the CEST agent. However, the value of k_{ex} must be smaller than the frequency separation ($\Delta\omega$) between the resonances of bulk water and the exchanging protons of the agent if the mobile protons are to be selectively irradiated. For this reason, it might be possible to find efficient CEST agents in the class of paramagnetic metal complexes (the so-called PARACEST agents),[3] in which the presence of unpaired electrons induce large $\Delta\omega$ values. However, the sensitivity of a PARACEST agent is still below that of the conventional relaxing agents based on the Gd^{III} ion, even when k_{ex} and $\Delta\omega$ are optimized.

It has been suggested that an improved sensitivity may be obtained by increasing the number of exchangeable protons. Van Zijl and co-workers suggested the use of polymers, such as polyamino acids, polyamidoamine-based (PAMAM-based) dendrimers, and single-stranded RNA, containing a large number of labile amide protons.[4,5] We have further extended this approach by designing supramolecular adducts between a paramagnetic shift reagent and a substrate containing many, fast-exchanging protons.[6] The latter approach appears promising, but it bears the drawback of showing a severe broadening of the absorption corresponding to the exchangeable protons that, in turn, results in the need to apply a much more intense B_1 field to maximize their saturation.

To design a system with a well-defined paramagnetically shifted resonance that corresponds to a high number of exchangeable protons, we considered the use of liposomes containing a paramagnetic shift reagent for water protons in the aqueous inner cavities. In such systems, which we call LIPOCEST agents, the resonance of the water protons inside the liposomes is shifted from that of the external water to an extent that depends upon the structure and the concentration of the shift reagent entrapped within the liposomes. Thus, the ^1H NMR spectrum of such a suspension will display two signals for water that correspond to water confined in the liposome cavity and to the external solvent. The exchange of water molecules across the liposome membrane is usually slow and can be controlled, to some extent, by varying the composition of the lipidic bilayer. Suitable candidates for the shift reagent should be found among paramagnetic metal complexes containing at least one highly shifted and fast-exchanging water molecule. In principle, several macrocyclic lanthanide(III) chelates derived from 1,4,7,10-tetraaza-1,4,7,10-tetrakis(carboxymethyl)cyclododecane (DOTA), α,α′,α″,α‴-tetramethyl-1,4,7,10-tetraacetic acid (DOTMA), and related ligands display an axially coordinated water that nicely fulfills the latter requirement.[7] Herein we report the results obtained with the $[Tm(dotma)]^-$ complex, which has recently been proposed as a non-invasive MR probe for in vivo thermometry.[8]



$[Tm(dotma)]^-$

$[Tm(dotma)]^-$ has been entrapped within a liposome formed from a blend of unsaturated/saturated phospholipids and cholesterol (palmitoyloleoylphosphatidylcholine (POPC)/dipalmitoylphosphatidylglycerol) sodium salt (DPPG)/cholesterol, 55:5:40 w/w/w). Previous studies on Gd(III) chelates entrapped within liposomes have shown that a mixture of cholesterol and unsaturated phospholipids results in a faster exchange of water across the liposome membrane relative to systems based only on saturated phospholipids.[9–11] When the preparation of the liposomes

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was carried out in the presence of 0.19 M $[Tm(dotma)]^-$, the water signal in the inner compartment resonated (at 312 K) at $\Delta\delta = 3.1$ ppm downfield of the signal of bulk water (Figure 1, top).

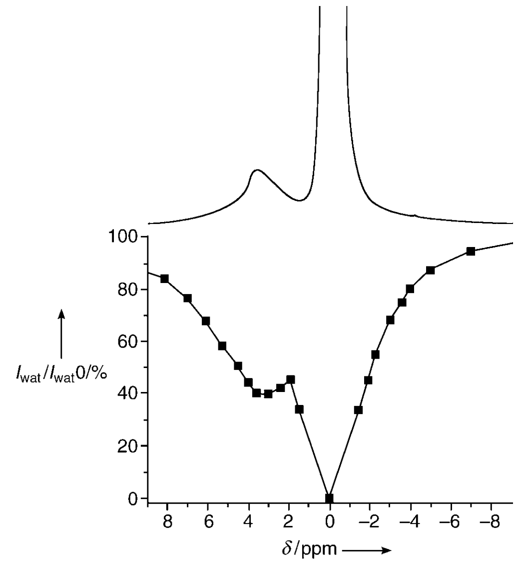

Figure 1. Top: ^1H NMR spectrum, recorded at 14.1 T and 312 K, of a suspension of the LIPOCEST agent entrapping $[Tm(dotma)]^-$. The signal at $\delta = 3.1$ ppm from the bulk water (off-scale signal at 0 ppm) corresponds to the water protons entrapped within the liposomes containing 0.1 M paramagnetic shift reagent, as demonstrated by the Z-spectrum (lower) obtained at 7 T and 312 K (liposome concentration 2.88 nM, B_1 field intensity 12 μT, irradiation time 3 s).

The difference in the chemical shift between the water resonances allows the actual concentration of the shift reagent in the liposome's inner cavity to be assessed, once the dependence of the paramagnetic shift of the bulk water resonance on the concentration of $[Tm(dotma)]^-$ has been determined by an independent experiment.[12] On this basis, it has been possible to determine that the experimental procedure used for the preparation of the liposomes led to an effective intraliposome $[Tm(dotma)]^-$ concentration of 0.1 M. These liposomes showed a mean diameter of 270 ± 2.5 nm (polydispersity index 0.32) which gives a volume of the inner cavity of about 9.2×10^{-18} L. The ratio between the total concentration of the shift reagent in a given liposome suspension and its effective concentration inside the liposomes corresponds to the ratio between the volumes of the two compartments.

In this way the number of liposomes in the suspension, and thus their concentration, can be calculated by the ratio between the inner volume of a single liposome and the total volume of the compartment. The concentration of $[Tm(dotma)]^-$ in the the stock suspension of liposomes entrapping 0.1 M shift reagent was 1.3 mM and the corresponding liposome concentration was 2.8 nM.

The Z-spectrum (7 T, 312 K, B_1 intensity 12 μT, irradiation time 3 s) of such a suspension is reported in Figure 1 (bottom). The profile confirms that the resonance $\Delta\delta = 3.1$ ppm downfield from the bulk water protons corresponds to the pool of mobile water protons encapsulated within the liposomes.

Figure 2 shows the results of the saturation transfer (ST) experiments carried out at 7 T and 312 K upon irradiating the intraliposome water signal as a function of the liposome concentration. The data show the typical pseudohyperbolic behavior[13] that leads to detectable ST effects down to picomolar concentrations of the LIPOCEST agent. If the threshold of the detectable saturation transfer is set at 5 %, in analogy to previous works,[6,13] the agent reported herein can be detected down to about 90 pM!

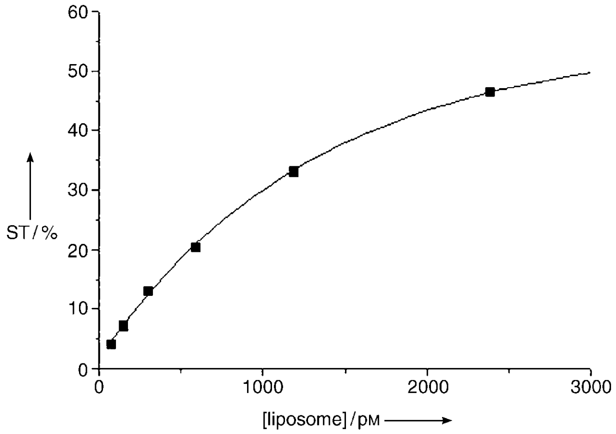

Figure 2. Saturation transfer assessment as a function of the liposome concentration for the LIPOCEST agent entrapping $[Tm(dotma)]^-$. Experimental conditions: 7 T, 312 K, irradiation pulse: train of 120 sinc3 pulses (25 ms each, B_1 field intensity 12 μT); the irradiation frequency was set at 930 Hz from the bulk water. The data points were fitted by using a simple hyperbolic function.

As has been recently pointed out,[14,15] the intensity of the applied B_1 saturation field for attaining the maximum ST effect is directly related to the k_{ex} values of the mobile protons of the CEST agent. It is thus expected that the B_1 field necessary for transferring saturation in LIPOCEST agents is much lower than in other paramagnetic CEST probes containing fast-exchanging protons. In fact, the B_1 field used for liposomes entrapping $[Tm(dotma)]^-$ is about 20 times smaller than that used to saturate the metal-bound water protons in paramagnetic $[Ln(dotamGly)]^-$ complexes (dotam = 1,4,7,10-tetrakis(carbamoylmethyl)-1,4,7,10-tetraazacyclododecane).[13,16]

Figure 3 shows a difference (on/off) CEST image (see the Experimental Section) of a phantom made of eight capillaries containing different liposome concentrations to illustrate the dependence of the saturation transfer efficiency on the liposome concentration. The image clearly indicates that, for this agent, it is still possible to detect contrast at a liposome concentration of about 90 pM. The difference CEST images were acquired in about two minutes (with a total irradiation time of 3 s) by using a modified RARE pulse sequence.

In summary, the results reported here show that LIPOCEST agents represent an innovative class of MR imaging probes of outstanding sensitivity (per liposome). Moreover,

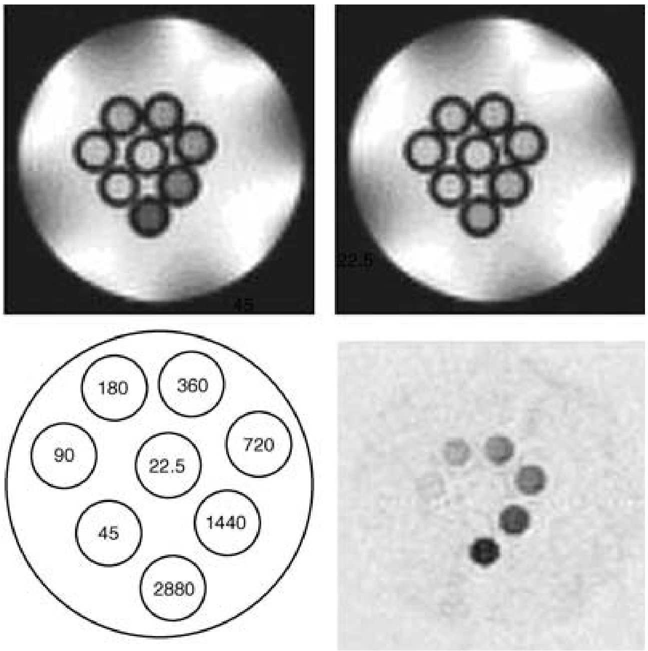

Figure 3. MRI-CEST images (matrix 64×64) of a phantom consisting of eight capillaries containing suspensions of the LIPOCEST agent entrapping [Tm(dotma)]$^-$ in the concentration range 22.5–2880 pM (see bottom left). Experimental conditions: 7 T, 312 K, RARE sequence: TR/TE/NE = 9 s/3.3 ms/1, rare factor = 8, irradiation pulse: train of 120 sinc3 pulses (25 ms each, B_1 field intensity 12 μT). Top left: On-image: irradiation frequency +930 Hz from bulk water. Top right: Off-image: irradiation frequency −930 Hz from bulk water. Bottom right: difference on/off image.

as the chemical shift of water entrapped within the liposomes depends upon the nature and concentration of the shift reagent as well as on the permeability of the liposome membrane, one may envisage facile access to a number of LIPOCEST agents with different absorption frequencies. This approach will allow a dramatic extension of the experiments that can be carried out by having the possibility of irradiating the various LIPOCEST agents present in the same sample specifically.

Experimental Section

The dotma ligand was provided by Bracco Imaging (Milano, Italy) and the corresponding Tm^{III} complex was prepared according to the published procedure, and characterized by ^1H NMR spectroscopy.[7] DPPG, POPC, and cholesterol were purchased from Sigma-Aldrich.

The liposomes were prepared according to the thin-film hydration method.[17]

The size of the liposomes were determined by photon correlation spectroscopy using a 90 Plus instrument (Brookheven, New York, USA) at a fixed scattering angle of 90° and 25 °C.

The total concentration of the Tm^{III} complex in the liposome suspension was determined by using the "bulk magnetic susceptibility" method (*tert*-butyl alcohol was used as the inert reference).[18]

Saturation transfer efficiency (ST%) was calculated by using Equation (1), where I_S and I_0 refer to the intensities of the bulk water signal when the irradiation pulse is set at the resonance frequency of the water protons entrapped within the liposomes (I_S) or on a frequency equally spaced with respect to the bulk water, but in the opposite direction (I_0).[6]

$$ST\% = (1-\frac{I_S}{I_0})100 \qquad (1)$$

ST% values and the MR images were obtained on a Bruker Avance300 instrument operating at 7 T and equipped with a micro-imaging probe (inner diameter 10 mm). The image reported in Figure 3 (bottom right) is the difference between two images (top) obtained by irradiating the resonance frequency of the water protons inside the liposome (on-image, 930 Hz from bulk water) and acquired under off-resonance conditions (off-image, −930 Hz from water protons), respectively. Both images (matrix 64×64) were obtained by using a conventional RARE sequence (TR/TE/NE = 9 s/3.3 ms/1, RARE factor 8) preceded by a train of sinc3 pulses (120 pulses, 25 ms each, irradiation B_1 field 12 μT). The total acquisition time for each image was about 1 minutes.

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