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Osmotically Shrunken LIPOCEST Agents: An Innovative Class of Magnetic Resonance Imaging Contrast Media Based on Chemical Exchange Saturation Transfer

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Abstract: The peculiar properties of osmotically shrunken liposomes acting as magnetic resonance imaging–chemical exchange saturation transfer (MRI–CEST) contrast agents have been investigated. Attention has been primarily devoted to assessing the contribution arising from encapsulated and incorporated paramagnetic lanthanide(III)-based shift reagents in determining the chemical shift of the intraliposomal water protons, which is a relevant factor for generating the CEST contrast. It is demonstrated that a highly shifted resonance for the encapsulated water can be attained by increasing the percentage of the amphiphilic shift reagent incorporated in the liposome bilayer. It is also demonstrated that the shift contribution arising from the bulk magnetic susceptibility can be optimized through the modulation of the osmotic shrinkage. In terms of sensitivity, it is shown that the saturation transfer efficiency can be significantly improved by increasing the size of the vesicle, thus allowing a high number of exchangeable protons to be saturated. In addition, the role played by the intensity of the saturating radiofrequency field has also been highlighted.

Keywords: imaging agents · lanthanides · liposomes · magnetic resonance imaging · shift reagents

Introduction

The outstanding achievements in the field of molecular medicine prompt the development of new paradigms in the design of innovative imaging reporters. A very important task deals with detecting more than one imaging reporter in the same anatomical region. As far as magnetic resonance imaging (MRI) modality is concerned, this task can be tackled by encoding the agents with a frequency dependence. This may be easily achieved in the case of heteronuclear detection such as in the MRI of hyperpolarized noble gases[1] or ^{13}C-containing molecules,[2] and ^{19}F-based agents.[3] Though this approach has the great advantage of dealing with images without background signal, there may still be sensitivity problems associated with the detection of heteronuclei resonances.

On this basis, the possibility of designing frequency-encoded MRI protocols based on the detection of ^{1}H water signals is very intriguing. Such compounds were first introduced in 2000 by Balaban and co-workers, who dubbed them CEST (chemical exchange saturation transfer) agents.[4]

Such imaging reporters act by transferring saturated magnetization to the bulk water protons when the absorption frequency of their slow-exchanging protons is properly irradiated by a radiofrequency field. Thus, one may design new protocols in which the MRI contrast is generated "at will" only if the proper frequency, that is, the frequency corresponding to the exchangeable protons of the CEST agent, is used for the irradiation. Importantly, the new approach offers the unique possibility (of the MR agents affecting the ^{1}H water signal) of detecting more than one agent in the same image voxel,[5,6] and, in addition, the pre-contrast

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image can be acquired almost simultaneously to the post-contrast image as their acquisition simply depends on the on/off switch of the irradiation radiofrequency field.

After CEST agents were proposed, it was soon realized that one of the main obstacles for their in vivo use was their low sensitivity. Therefore, since then, many efforts have been devoted to overcoming this limitation.

Among the physicochemical parameters controlling the saturation transfer (ST) efficiency, the number of magnetically pseudoequivalent mobile protons (n) and their exchange rate (k_{ex}), which both directly affect the ST, have received much attention.[7–9] As the route to fast exchange rates is limited by the risk of unsafe in vivo applications (due to the use of highly intense radiofrequency pulses), most of the work has been focused on the design of macromolecular and supramolecular systems containing a large number of exchangeable protons sufficiently shifted from the bulk water resonance.[10–14] Among these systems, the so-called LIPOCEST agents are clearly the most sensitive.[15,16]

LIPOCEST agents are liposomes that encapsulate a paramagnetic lanthanide(III)-based shift reagent (SR) able to affect the resonance frequency of the water protons entrapped in the liposome cavity, which are in slow exchange with the bulk through the phospholipidic bilayer of the vesicle (Figure 1, left). Owing to the extraordinarily high number of water protons that can be entrapped in a single vesicle ($n = 10^7$–10^9), the sensitivity of LIPOCEST agents reaches the sub-nanomolar scale for the most sensitive preparations.

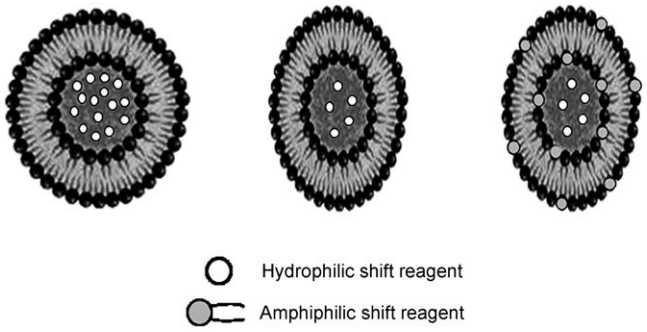


Figure 1. Schematic view of the LIPOCEST agents proposed so far. Left: spherical vesicle encapsulating a hydrophilic shift reagent; middle: nonspherical vesicle encapsulating a hydrophilic shift reagent; right: nonspherical vesicle encapsulating a hydrophilic shift reagent and incorporating an amphiphilic shift reagent.

In addition to sensitivity, another relevant issue for improving the potential of a LIPOCEST agent is the attainment of the largest possible separation between the intraliposomal and bulk water protons ($\Delta_{intralipo}$). For spherical vesicles, typical $\Delta_{intralipo}$ values fall in the $\delta \pm 4$ ppm range (the sign is dependent on the magnetic anisotropy of the encapsulated SR), but more recently, a significant extension of the $\Delta_{intralipo}$ values has been achieved by exploiting the bulk magnetic susceptibility (BMS) shift contribution. The compartmentalization of the paramagnetic SR in nonspherical liposomes required by the BMS contribution was achieved by subjecting the liposomes to osmotic shrinkage (Figure 1, middle).[17] Moreover, an additional increase in the frequency offset of the intraliposomal water protons has been attained by incorporating amphiphilic SRs in the liposomal bilayer[18] (Figure 1, right), and, more recently, by encapsulating multimeric neutral hydrophilic SRs.[19]

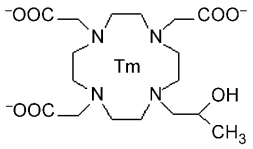
The possibility to explore a wider range of frequency offset values makes these systems excellent candidates for molecular imaging protocols that benefit from multiplex visualization, as recently demonstrated ex vivo on bovine muscle.[6]

To fully exploit the great potential of such innovative systems, it is necessary to optimize all the variables that modulate the ST efficiency. Besides describing the basic properties of osmotically shrunken LIPOCEST agents, this work is primarily aimed at illustrating, for the first time, the relevant role played by the liposome formulation, in particular the amount and the type of encapsulated and incorporated SRs, and at providing some hints for optimizing the ST performance of these very promising MRI probes.

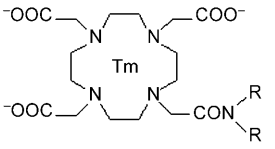
Results and Discussion

From spherical to shrunken liposomes: Osmotically shrunken, large unilamellar liposomes can be obtained by subjecting a suspension of spherical vesicles to hyperosmotic stress. The consequent shrinkage of the vesicles markedly affects the difference in resonance frequency between the intraliposomal and bulk water protons as illustrated in Figure 2.

In this experiment, spherical liposomes encapsulating the neutral SR [Tm(hpdo3a)] (40 mM) and incorporating the amphiphilic complex [Tm(**1**)] (30 mol%) were first suspend-



[Tm-(hpdo3a)]



[Tm(**1**)]; R = $-C_{18}H_{37}$

ed in a buffered medium iso-osmolar with respect to the liposome content (i.e., 40 mOsm) and were then subjected to osmotic stress by adding NaCl to the suspension. The ^{1}H NMR spectrum of the spherical vesicles (Figure 2, trace a) shows a very small offset ($\delta \approx 0.4$ ppm) for the intraliposomal water protons, consistent with the low concentration of SR inside the liposome cavity.

Upon addition of NaCl to the external medium, this resonance started to shift away from the bulk water until reaching a $\Delta_{intralipo}$ value slightly larger than $\delta = 20$ ppm when the liposomes were suspended in an isotonic (300 mOsm) medium. Concomitant with the increased shift, the osmotic stress led to a decrease of the intraliposomal water protons

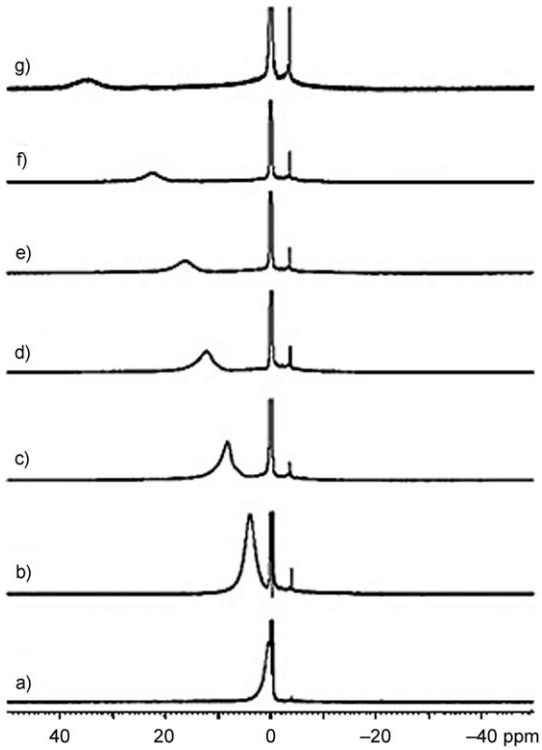


Figure 2. ^{1}H NMR spectra (14 T, 25 °C) of a suspension of liposomes made of DPPC/[Tm(**1**)]/DSPE-PEG2000 (65:30:5 molar ratio) encapsulating [Tm(hpdo3a)] (40 mM) and suspended in a buffered medium (pH 7.4) with increasing osmolarity: a) 40, b) 80, c) 110, d) 160, e) 230, f) 300 (isotonic), and g) 600 mOsm.

signal, which is associated with a progressive line broadening of the resonance. The release of water from the liposome cavity caused by the osmotic shock is quantitatively described in Figure 3, which reports the integral of the shifted resonance (calculated by using as reference the residual HOD signal of the deuterated solvent) as a function of the osmolarity of the external medium.

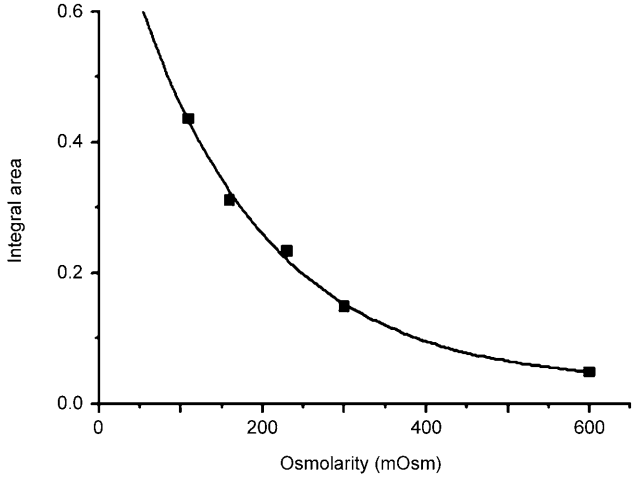


Figure 3. Integral values for the intraliposomal water proton resonance (calculated by using the residual HOD signal of the deuterated solvent as reference) as a function of the osmolarity of the external medium for the LIPOCEST agent described in Figure 2.

The decrease in the signal intensity is the result of the osmotic shrinkage of the vesicle that, besides losing entrapped water, increases the concentration of the paramagnetic species in the aqueous core, thus leading to the shortening of the T_2 relaxation time, which is responsible for the broadening of the resonance. The data reported in Figure 3 were nicely fitted to a simple exponential decay function that allowed an estimation of the intraliposomal water fraction released from the vesicles following the osmotic shock. On the basis of this rough calculation, approximately 75% of the intraliposomal water protons left the vesicles when they were suspended in the isotonic medium. This corresponds to a fourfold increase of the concentration of the paramagnetic species inside the liposome cavity (i.e., from 40 to 160 mM). However, the more relevant result is the significant increase (more than 400%) of the $\Delta_{intralipo}$ values with respect to the highest offsets attainable with the classical spherical LIPOCEST probes, and, interestingly, this result is achieved by encapsulating a much smaller amount of paramagnetic compound (40 mM versus a typical value of 200 mM).

To better characterize the morphology of these nanoparticles, the osmotically shrunken liposomes were subjected to a cryogenic transmission electron microscopy TEM (cryo-TEM) investigation. As revealed by the cryo-TEM image shown in Figure 4, the shrunken liposomes contained lens-shaped vesicles. From a series of TEM images, the structural diversity of the liposomes was judged to be low: in less than 1% of the cases spherical liposomes or another nonlens-shaped structure was observed. The size of the long axis ranges from 50 to 220 nm, with most lenses having a long axis between 120 and 160 nm. Liposomes not subjected to osmotic stress remained spherical (data not shown).

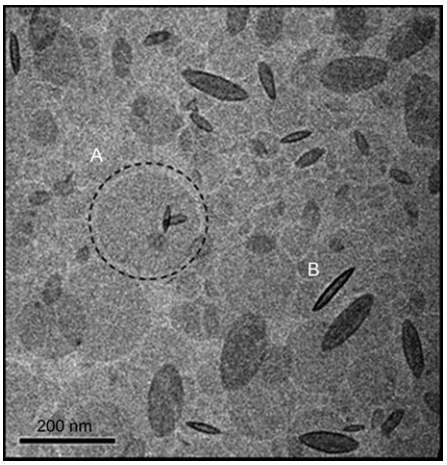


Figure 4. Cryo-TEM image showing a 2D projection of osmotically shrunken LIPOCEST agents encapsulating [Tm(hpdo3a)] (40 mM) and incorporating [Tm(**1**)] (20 mol%). "A" indicates a lens-shaped vesicle with the short axis positioned in plane with the electron beam (which is perpendicular to the image plane) and "B" indicates a lens-shaped vesicle positioned with the short axis perpendicular to the electron beam. The dashed circle is displayed to guide the eye. Shrunken liposomes with all different orientations are observed.

The vesicle size determined by using cryo-TEM is in good agreement with the values obtained by using dynamic light scattering (DLS) measurements. It is important to note that the liposome size obtained from DLS investigations was not sensitive to the osmotic shrinkage. A routine DLS measurement gives details about the diameter of a sphere whose translational motion is equivalent to the particles under examination and, as such, cannot provide details about the shape of the particles studied here. It is likely that the shrinkage of the vesicles does not significantly affect the anisotropic motion of the liposome in the suspension.

$\Delta_{intralipo}$ as a function of the amount of incorporated SR: To illustrate the role played by the incorporated SR in defining the properties of this class of nanosized system, Tm^{III}-based LIPOCEST agents with differing amounts of [Tm(**1**)] complex were prepared and investigated by using ^1H NMR spectroscopy.

Figure 5 shows the $\Delta_{intralipo}$ values measured for osmotically shrunken LIPOCEST agents made of DPPC/DSPE-PEG2000 (the latter phospholipid was incorporated at a fixed amount of 5 mol%), and encapsulating [Tm(hpdo3a)]

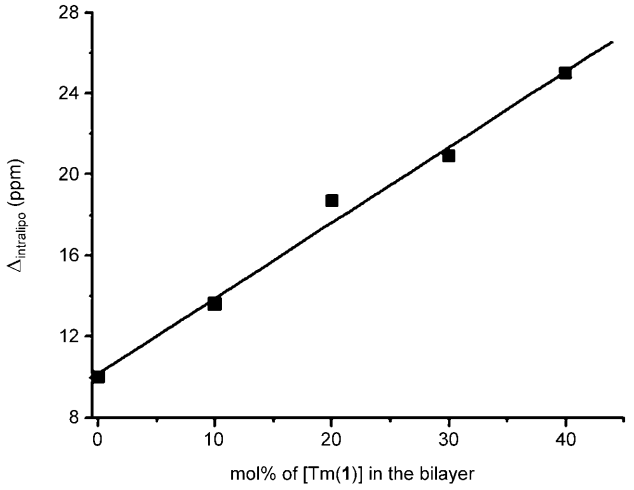


Figure 5. $\Delta_{intralipo}$ values (14 T, 25 °C) for DPPC/DSPE-PEG2000-based liposomes encapsulating [Tm(hpdo3a)] (40 mM) as a function of the amount of incorporated [Tm(**1**)] complex in the bilayer. The amount of the DSPE-PEG2000 component was fixed at 5 mol%.

(40 mM), as a function of the amount of [Tm(**1**)] incorporated in the bilayer. The incorporation of the PEGylated phospholipid is necessary for improving the in vivo stability of the nanovesicles.[20] The mean hydrodynamic diameters of the liposomes were in the 140–145 nm range with a polydispersity index (PDI) lower than 0.1. The data reported in Figure 5 indicate that $\Delta_{intralipo}$ values and the percentage of the paramagnetic amphiphilic SR incorporated in the liposome membrane are linearly correlated.

It is worth remembering that the $\Delta_{intralipo}$ value for such systems is the sum of several contributions as shown in Equation (1):

$$\Delta_{intralipo} = \Delta^{HYP}_{enc} + \Delta^{HYP}_{inc} + \Delta^{BMS}_{(enc+inc)} \quad (1)$$

in which the superscripts HYP and BMS refer to the contributions arising from the chemical hyperfine interaction between the intraliposomal water protons and the Tm^{III} metal centers, and the bulk magnetic susceptibility effects, respectively. The HYP contributions may be different for the encapsulated (enc) and the incorporated (inc) SRs, and, furthermore, they are directly dependent on the intraliposomal concentration of the corresponding agent (with the incorporated SR contributing only for the amount that points into the cavity). On the other hand, the BMS contribution is proportional to the total (enc+inc) concentration of Tm^{III} centers inside the cavity irrespective of their chemical nature. Therefore, the incorporation of higher amounts of the [Tm(**1**)] complex in the liposome bilayer increases both Δ^{HYP}_{inc} and $\Delta^{BMS}_{(enc+inc)}$, thus accounting for the observed linear behavior.

$\Delta_{intralipo}$ as a function of the amount of encapsulated SR: To assess the effect of the amount of the hydrophilic SR encapsulated in the liposome cavity in osmotically shrunken vesicles, LIPOCEST agents incorporating [Tm(**1**)] (20 mol%) and with lipidic films that had been hydrated with different concentrations (in the range 10–250 mM) of [Tm(hpdo3a)] were prepared and characterized. Once extruded, all the liposomes were purified by exhaustive dialysis against an isotonic buffer. The mean hydrodynamic diameter of these samples ranged from 135 to 150 nm (PDI < 0.1).

The measured $\Delta_{intralipo}$ values of these preparations are shown in Figure 6. To account for the obtained result it is important to consider that, contrary to previous experiments dealing with the incorporated compound, the change in the concentration of the encapsulated SR significantly influen-

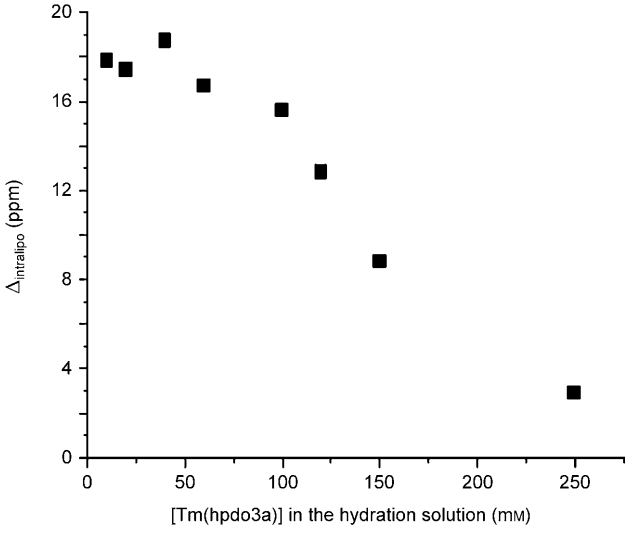


Figure 6. $\Delta_{intralipo}$ values, measured at 14 T and 25 °C, for DPPC/[Tm(**1**)]/DSPE-PEG2000 (75:20:5 molar ratio) liposomes as a function of the concentration of [Tm(hpdo3a)] used for their preparation.

ces the magnitude of the osmotic stress at which the vesicles are exposed. As a consequence, the amount of water leaked from the vesicle core, as well as the shape of the vesicle will be strongly affected and the changes of both these variables have to be carefully evaluated when analyzing the data reported in Figure 6.

Liposomes are usually considered to be ideal osmometers[21] and, according to this behavior, the equilibrium concentration of the encapsulated SR after exposure to the isotonic medium will tend to be equal, regardless of the starting amount of encapsulated [Tm(hpdo3a)]. However, by doing so, the liposomes will shrink to different degrees and, therefore, it is likely that the shapes of the vesicles will not be equal. Upon increasing the concentration of [Tm(hpdo3a)] in the hydration solution, the vesicles shrink less and their shapes will tend to remain spherical, thus reducing the extent of the BMS shift contribution [the term $\Delta^{BMS}_{(enc+inc)}$ in Eq. (1)]. This actually occurred for the liposomes hydrated with 250 mM of [Tm(hpdo3a)], the $\Delta_{intralipo}$ value of which, $\delta \approx 3$ ppm, is typical for a spherical LIPOCEST agent. The increase of the shift values observed as a function of the osmotic stress is likely the result of the shape factor that affects the BMS shift contribution.[22] The leveling of the shift values observed at low concentrations (<50 mM) could suggest that under such conditions the liposomes no longer behave as ideal osmometers, and, therefore, the shape and intraliposomal concentration of the SRs may deviate from the expected trend.

ST efficiency as a function of the radiofrequency saturating field: The intensity of the saturating radiofrequency field plays a role in the MRI–CEST experiment, not only for its significant influence on the magnitude of the CEST contrast, but especially for the important safety implications related to the energy deposited by the saturating field on biological specimens. The Z-spectra (which displays the dependence of the bulk water MR signal on the saturation frequency offset) of a LIPOCEST agent incorporating [Tm(**1**)] (40 mol%) acquired at different radiofrequency intensities (1, 3, and 6 μT) are reported in Figure 7.

The data illustrate the two main effects that the increase of the radiofrequency intensity has on the basic properties of a Z-spectrum: 1) the enhancement of the ST efficiency, witnessed by the marked decrease of the bulk water signal when the resonance of the intraliposomal mobile protons is saturated, and 2) the peak broadening. In particular, the broadening of the central peak, which corresponds to the direct saturation of the bulk water signal, is very relevant to the CEST experiment. Generally, the detection of the CEST contrast of saturating frequency offsets in which the direct saturation is non-negligible may be quite challenging, and this is the main reason why much effort has been made to develop CEST agents with highly shifted mobile protons.

The example reported in Figure 7 indicates that the frequency offset at which the direct saturation is negligible (<5%) increases upon increasing the radiofrequency intensity. However, in the examined samples, the direct saturation

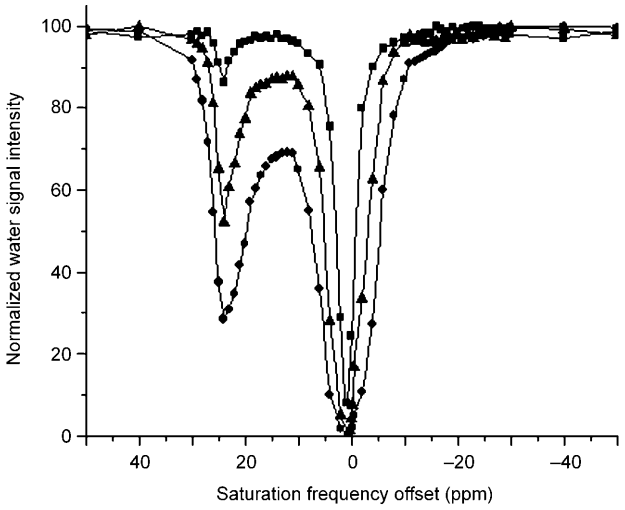


Figure 7. Z-spectra (7 T, 39 °C) registered at different intensities of the saturating radiofrequency field (■ = 1 μT, ▲ = 3 μT, and ● = 6 μT) for a suspension of nonspherical liposomes (DPPC/DSPE-PEG2000 55:5 molar ratio) encapsulating [Tm(hpdo3a)] (40 mM) and incorporating [Tm(**1**)] (40 mol%). The lines interpolating the data were drawn to guide the eye.

can be neglected at offsets larger than $\delta = 20$ ppm, for any intensities in the 1–6 μT range.

The interpolation of the Z-spectra, performed by using smoothing splines, is a very convenient way of calculating the ST efficiency as a function of the saturation offsets (Figure 8).[23] Such curves make even clearer the relevance of the saturating radiofrequency intensity not only in defining the maximum efficiency of the saturation transfer (which is found to be linearly correlated to the radiofrequency intensity), but also in affecting the frequency selectivity of the CEST effect, which is related to the broadening of the CEST peak that characterizes the ST% profile. The profiles shown in Figure 8 indicate that in addition to the ST enhancement, the increase of the radiofrequency intensity

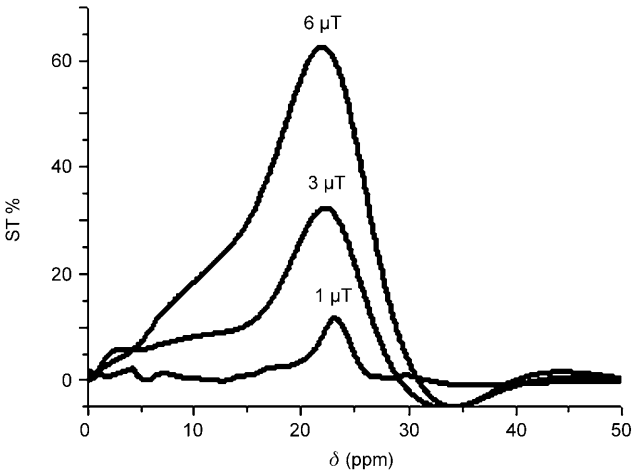


Figure 8. ST% profiles (7 T, 39 °C) obtained from the analysis of the Z-spectra shown in Figure 7.

broadens the CEST peaks, thus reducing the frequency selectivity and making the visualization of more LIPOCEST agents eventually present in the same image voxels more challenging.

The remarkable enhancement of the ST efficiency observed upon increasing the radiofrequency intensity is likely attributable to the more efficient saturation operated by the saturating field on the rather broad resonance of the intraliposomal water protons.

ST efficiency as a function of the amount of incorporated SR: The LIPOCEST agents described in the previous section were also subjected to a study aimed at evaluating their saturation transfer efficiency. Figure 9 displays the ST effects (expressed as a percentage) measured at 7 T and 39 °C as a function of the liposome concentration (see the Experimental Section).

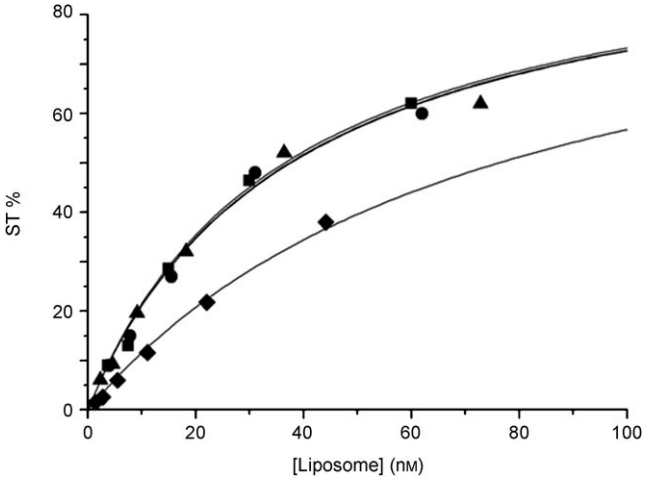


Figure 9. Concentration dependence of the ST % efficiency of osmotically shrunken LIPOCEST agents encapsulating [Tm(hpdo3a)] (40 mM) and incorporating different amounts of [Tm(**1**)]: 10 (◆), 20 (▲), 30 (●), and 40 mol % (■). The ST % values were measured at 7 T and 39 °C using a radiofrequency saturating field of 6 μT. The lines interpolating the data were drawn to guide the eye.

The investigated LIPOCEST agents showed very similar sensitivities with the exception of the preparation incorporating 10 mol % [Tm(**1**)], which displayed a lower efficiency. This preparation was also characterized by the smallest, of the examined samples, $\Delta_{intralipo}$ value ($\delta \approx 7$ ppm at 39 °C) and this characteristic is likely the reason for the observed lower sensitivity. In fact, when the saturation frequency is closer to the resonance of the bulk water protons, the radiofrequency saturating field can directly saturate the latter signal and this process (which is proportional to the magnitude of the applied field) is detrimental to the ST efficiency.

However, the observation that the LIPOCEST agents with larger $\Delta_{intralipo}$ values displayed the same sensitivity suggests that the amount of incorporated SR is not a relevant factor in defining the overall efficiency of this class of LIPOCEST agent. Furthermore, as the ST effect is also dependent on the exchange rate of the intraliposomal water protons, this finding indirectly indicates that the water permeability of the liposome bilayer in these formulations, which is based on saturated phospholipids, is not significantly affected by the presence of the amphiphilic compound, which bears saturated alkylic chains.

To obtain a more quantitative description of the properties of these systems, the Z-spectra acquired for the four samples at the different concentrations were analyzed by using the mathematical model developed by Woessner et al.[24] According to this model, the intensity of the water signal as a function of the saturation frequency offset in a system containing a single pool of "CEST-active" mobile protons is affected by nine variables: four are known because they are related to the experimental setup (the static magnetic field, the length of the saturation field and its intensity) or to the chemical composition of the system (the molar fraction of the pool of mobile protons, here represented by the amount of intraliposomal water). Thus, the remaining five parameters (the exchange rate of the intraliposomal water protons ($k_{intralipo}$), the longitudinal (T_1) and transverse (T_2) relaxation times of the two exchanging proton pools, bulk (*bw*) and the intraliposomal (*intralipo*) water protons) can be obtained by fitting the Z-spectra. The $k_{intralipo}$ values obtained for the examined samples, averaged out for the different concentrations, are reported in Table 1 and confirm that the different sensitivity displayed by the agent incorporating less SR is not due to a different exchange rate.

Table 1. Intraliposomal water exchange rates at 39 °C obtained by fitting the Z-spectra with the model described in ref. [24].

SR incorporated [mol %]	$k_{intralipo}$[a] [s^{-1}]
10	(310.5±55.1)
20	(357.6±52.9)
30	(348.8±49.8)
40	(339.6±51.0)

[a] The reported average values and the corresponding standard deviations refer to the analysis of Z-spectra registered at different concentrations for each sample.

In most cases, the quality of the fitting was unaffected by T_{1w} and $T_{1\_intralipo}$ values, whereas the obtained T_{2w} and $T_{1\_intralipo}$ values ranged from 50 to 200 ms, and from 1 to 5 ms, respectively, depending on the concentration of the LIPOCEST agent.

ST efficiency as a function of the amount of encapsulated SR: As anticipated above, the change in the concentration of the SR during the preparation of nonspherical liposomes yields vesicles that are exposed to different osmotic forces.

As the main variables that can affect the overall ST efficiency for LIPOCEST agents—namely, the exchange rate of the water protons in the liposome cavity (inversely related to the vesicle volume), the number of intraliposomal water

protons (directly related to the vesicle volume), and the phenomena related to the $\Delta_{intralipo}$ value (e.g., direct saturation of the bulk water)—are all strongly influenced by the magnitude of the osmotic shrinkage, the dependence of the ST efficiency on the amount of the encapsulated SR appears, at first glance, to be rather complicated.

However, the data reported in Figure 10 indicate that the ST% (herein normalized to a liposome concentration of 50 nM) displays a sort of "periodic" dependence on the osmotic shrinkage.

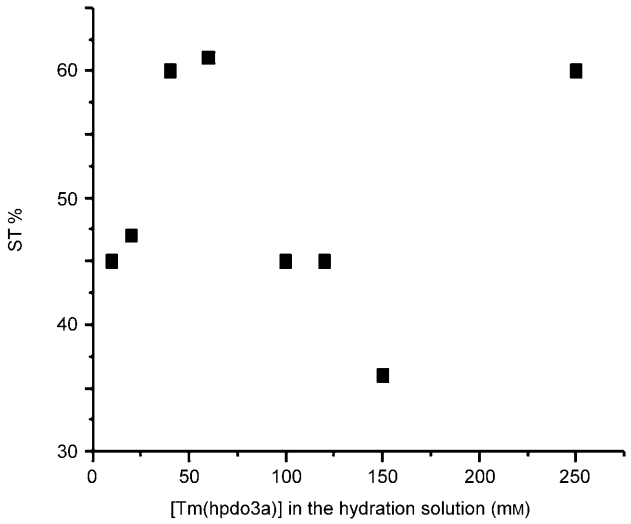


Figure 10. ST% efficiency (7 T, 39°C, normalized to a liposome concentration of 50 nM) for the LIPOCEST agents made of DPPC/[Tm(**1**)]/DSPE-PEG2000 (75:20:5 molar ratio) and prepared by hydrating the lipidic film with different concentrations of [Tm(hpdo3a)].

A possible explanation involves the rather complex interplay between the number of water protons in the liposomal cavity, which decreases upon osmotic shrinkage, the direct saturation of bulk water, which is related to the $\Delta_{intralipo}$ values, and the $k_{intralipo}$ values, which increase upon shrinkage due to the reduction of the cavity volume. Interestingly, the data reported in Figure 10 parallel those displayed in Figure 6 in the range of 50–150 mM of [Tm(hpdo3a)], thus suggesting that in this interval the ST enhancement could be somehow related to the increase of the $\Delta_{intralipo}$ values, for instance, through direct saturation effects. Contrary to the trend displayed in Figure 6, the ST% efficiency increases for the spherical LIPOCEST vesicles (250 mM [Tm(hpdo3a)]), and decreases for liposomes prepared with [Tm(hpdo3a)] concentrations lower than 50 mM. Probably, both these observations might be interpreted in terms of the number of intraliposomal water protons, which increases for spherical liposomes and significantly decreases when the osmotic shrinkage becomes relevant.

ST efficiency as a function of liposome size: The ST efficiency is proportional to the number of exchangeable protons that can be saturated, so it is expected that the liposome size plays an important role in determining the efficiency of a LIPOCEST agent. Figure 11 displays the ST performance of two nonspherical liposomes (both incorporating 30 mol% [Tm(**1**)]), and only differing in their size (280 nm versus 140 nm mean hydrodynamic diameter).

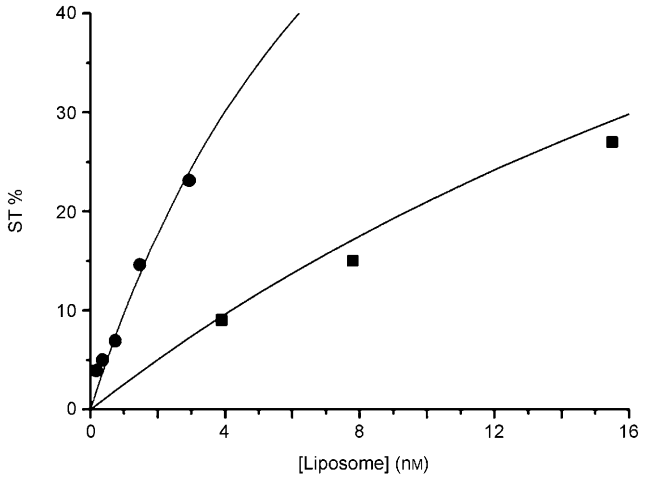


Figure 11. Concentration dependence of the ST% efficiency for osmotically shrunken LIPOCEST agents (DPPC/[Tm(**1**)]/DSPE-PEG2000 65:30:5 molar ratio) encapsulating [Tm(hpdo3a)] (40 mM) differing in their mean diameter: 140 (■) and 280 nm (●). The ST% values were measured at 7 T and 39°C by using a radiofrequency saturating field of 6 μT. The lines interpolating the data were drawn to guide the eye.

When the ST efficiency is analyzed with respect to the vesicle concentration, the larger agent is found to be much more sensitive due to the higher number of intraliposomal water protons that are saturated and transferred to the bulk pool. The ratio between the number of water protons in the liposomal cavity in the two original preparations (when spherical) is about 6 (assuming a bilayer thickness of 5 nm) and it can be reasonably speculated that this difference is maintained after the osmotic shrinkage. By taking as a reasonable threshold for detecting a CEST contrast a ST value of 5%, the larger LIPOCEST agent is fourfold more sensitive (0.5 nM versus 2 nM) than the smaller one. This means that the larger vesicle is slightly less efficient than expected, but this observation can be likely attributed to the size effect on the intraliposomal water exchange. In fact, it is important to remember that k_{ex} is inversely related to the liposome size, and it has been recently demonstrated that smaller LIPOCEST agents transfer saturation more efficiently than larger ones.[16] However, this consideration only applies when the ST performance is normalized to the molar fraction of intraliposomal water. Conversely, when the ST performance is evaluated on the basis of the concentration of nanovesicles (that is preferable in case of targeting experiments), the number of the saturated protons predominates so that a lower concentration of a large LIPOCEST agent works better than the same amount of a smaller system. Of course, when dealing with future in vivo applications of these probes, the choice of the optimal liposome size should

be made by taking into consideration the remarkable influence of this variable on the biodistribution of the vesicle.

Apart from the liposome size, a further sensitivity improvement could be attained by increasing the water permeability of the liposome through modification of the chemical composition of the bilayer. For instance, the substitution of DPPC with unsaturated phospholipids like 1-palmitoyl-2-oleoyl-*sn*-glycerophosphocholine (POPC), even in association with cholesterol, increases the water diffusivity across the membrane.[15,25–27]

Conclusion

In this paper the relevant properties of a new class of osmotically shrunken liposomes acting as MRI–CEST agents have been explored.

We demonstrated that the amount of both the encapsulated and incorporated SR is a crucial factor in defining two of the main features that need to be optimized for the successful in vivo application of such systems, that is, the chemical shift offset, $\Delta_{intralipo}$, of the intraliposomal water protons and the ability of the vesicles to generate CEST contrast. Optimal $\Delta_{intralipo}$ values can be attained by increasing the amount of the incorporated shift reagent and/or inducing a significant shrinkage of the vesicle. In terms of sensitivity, highly efficient vesicles can be obtained by increasing the liposome size to saturate a higher number of exchangeable water protons. In addition, it has been demonstrated that the intensity of the saturating radiofrequency field is a fundamental variable to be considered, especially when a multiplex visualization is sought. More work still needs to be done to further improve the potential of such systems, in particular, it is fundamental to find the proper liposome formulation to optimize the exchange rate of the intravesicular protons, and to improve the overall in vivo stability of the nanovesicles because the generation of CEST contrast from such systems requires the integrity of the vesicles in the biological environment in which they are distributed. Concerning the potential applications of the osmotically shrunken LIPOCEST, it is relevant to mention that we recently demonstrated that the chemical shift of the intraliposomal water protons in such systems is maintained in biological environments.[6]

Experimental Section

Chemicals: All the phospholipids used in the liposome preparation (DPPC = 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPE-PEG2000 = 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000]) were purchased from Avanti Polar Inc. (Alabaster, AL, USA). The [Tm(hpdo3a)] complex was kindly provided by Bracco Imaging S.p.A (Colleretto Giacosa, TO, Italy). The [Tm(**1**)] complex was synthesized according to the procedure reported in the literature for the Gd^{III} analogue.[28]

Preparation of LIPOCEST agents: Large unilamellar LIPOCEST agents were prepared by using the conventional thin lipidic film method. Briefly, the lipids (about 20 mg in total including phospholipids and the amphiphilic [Tm(**1**)] complex) were dissolved in chloroform and the solution was slowly evaporated to remove the solvent until a thin film was formed. The film was then hydrated at 55 °C with an aqueous solution (1 mL) containing a given concentration of [Tm(hpdo3a)]. The resulting suspension of multilamellar vesicles was extruded (Lipex extruder, Northern Lipids Inc., Canada) six times on polycarbonate filters with a pore diameter of 200 nm. The final suspension of liposomes was purified from the nonencapsulated metal complex by exhaustive dialysis (two cycles of 6 h each) carried out at 4 °C against an isotonic (300 mOsm) HEPES/NaCl buffer (HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). The vesicles were investigated by using dynamic light scattering (Zetasizer NanoZS, Malvern, UK) to assess the mean hydrodynamic size and the polydispersity of the system. The concentration of the liposomes was estimated from the ratio between the total surface occupied by the membrane components and the surface area of a single vesicle. The former parameter was roughly calculated from the amount of lipids used in the liposome preparation (adjusted for volume changes occurring during extrusion/dialysis steps) using a surface area of 0.52 nm^2 for DPPC,[29] and 0.92 nm^2 for [Tm(**1**)] (determined in silico), whereas the bilayer area of the single vesicle was calculated from the mean liposome size. The reliability of this approach was checked by using as a reference a spherical LIPOCEST vesicle, for which the concentration can be independently obtained by calculating the actual concentration of the encapsulated SR from the $\Delta_{intralipo}$ value of the preparation.[15]

Cryo-TEM measurements: Cryogenic transmission electron microscopy (cryo-TEM) was performed by using a FEI Tecnai 20 Sphera transmission electron microscope equipped with an LaB6 filament operating at 200 kV. Images were recorded with a bottom-mounted 1 K Gatan charge-coupled device (CCD) camera. A Gatan cryoholder operating at approximately −170 °C was used for the cryo-TEM measurements. The sample vitrification procedure was carried out by using an automated vitrification robot (FEI Vitrobot Mark III). TEM grids (R2/2 Quantifoil Jena grids) were purchased from Aurion. The Quantifoil grids were surface-plasma treated by using a Cressington 208 carbon coater operating at 5 mA for 40 s prior to the vitrification procedure.

NMR measurements: $\Delta_{intralipo}$ values were measured at 298 K on a Bruker Avance 600 spectrometer operating at 14.1 T by taking the shift difference between the intraliposomal and bulk water protons. The liposome suspensions were put in a capillary coaxially inserted in a 5 mm NMR tube containing D_2O as the lock solvent. Z-spectra were acquired at 7 T on a Bruker Avance 300 spectrometer equipped with a microimaging probe (inner coil 10 mm). The sample consisted of several glass capillaries filled up with the given LIPOCEST suspension and put in the probe. A frequency offset range of $\delta \pm 50$ ppm was investigated. A typical RARE spin–echo sequence (rare factor 8) with an echo time of 3.3 ms and a TR value of 3 s was used. An acquisition matrix of 64×64 with a square FOV of 10 mm was used. The whole sequence was preceded by a saturation scheme consisting of a continuous wave pulse 2 s long with a variable radiofrequency intensity (1, 3, or 6 μT). The total acquisition time for each scan of the Z-spectrum was 24 s. The Z-spectra were interpolated by smoothing splines to identify, first, the correct position of the bulk water and, then, the correct ST % value over the entire range of frequency offset investigated. Custom-made software, compiled in the Matlab platform (Mathworks Inc., Natick, MA), was used.[23] The ST % values were calculated by using the following relation [Eq. (2)]:

$$ST\,\% = \left(1 - \frac{I^{\omega}}{I^{-\omega}}\right) \times 100 \qquad (2)$$

in which I^{ω} and $I^{-\omega}$ refer to the bulk water signal intensity at two frequency offsets (ω and $-\omega$) symmetrically distributed with respect to the zero offset of the bulk water resonance. ω is the frequency offset corresponding to the resonance of the intraliposomal water protons (i.e., corresponding to $\Delta_{intralipo}$).

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