Highly shifted LIPOCEST agents based on the encapsulation of neutral polynuclear paramagnetic shift reagents†

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Received (in Cambridge, UK) 8th October 2007, Accepted 15th November 2007
First published as an Advance Article on the web 26th November 2007
DOI: 10.1039/b715383j

Improved LIPOCEST MRI contrast agents with highly shifted intraliposomal water protons were prepared by entrapping neutral polynuclear Tm(III)-based paramagnetic shift reagents in phospholipidic vesicles.

CEST (chemical exchange saturation transfer) agents are an interesting class of MRI contrast agents that act by transferring saturated magnetization to the bulk water signal when the absorption frequency of their exchangeable protons is properly irradiated.[1,2] Paramagnetic-based systems are excellent candidates for this task, as they induce large shifts to the exchangeable proton resonance. This makes possible the exploitation of faster exchange rates that, in turn, result in a more efficient saturation transfer.[3] Metal-coordinated water protons can be a suitable pool of exchangeable protons, provided the exchange rate is in the slow–intermediate regime on the NMR time scale. We recently showed that all water molecules contained in the inner cavity of a liposome vesicle can be successfully used in saturation transfer experiments, provided their absorption frequency is shifted away from that of the bulk water signal by the presence of a suitable shift reagent (SR).[4] Thus, a pool of the order of 10^6–10^8 water molecules per liposome (depending on vesicle size) may be exploited for creating saturated magnetization to be transferred to the solvent water resonance. Regarding molecular CEST agents, as well as nanosized LIPOCESTs, an important goal is the attainment of systems with the largest possible separation between the resonance of the exchangeable proton pool and the ^{1}H bulk-water signal detected in the MR image. For spherical paramagnetic vesicles, the chemical shift difference between intra- and extraliposomal water protons ($\Delta_{intralipo}$), lies in the ± 4 ppm interval owing to the maximum amount of SR that can be entrapped inside the liposome (this is mainly limited by osmotic processes) and the intrinsic shifting properties of the paramagnetic complex. More recently, a significant extension of the accessible $\Delta_{intralipo}$ values for LIPOCEST agents has been achieved by exploiting the bulk magnetic susceptibility (BMS) shift contribution; this requires the compartmentalization of the paramagnetic SR in non-spherical liposomes.[5,6]

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† Electronic supplementary information (ESI) available: Characterization details. See DOI: 10.1039/b715383j

However, a further enhancement of $\Delta_{intralipo}$ values is an important goal for the improvement of LIPOCEST agents. Larger $\Delta_{intralipo}$ values will allow: (i) the exploitation of faster exchange rates that, in turn, will result in more efficient saturation transfer effects; (ii) the reduction of artifacts arising from field inhomogeneity and spillover effects that are particularly relevant when the signal of the exchanging proton pool lies close to the bulk water signal; (iii) the selective detection of different LIPOCEST probes simultaneously present in the same region of interest, (iv) the negligibility of contributions arising from endogenous proteins or solid-like mobile protons immobilized in tissue (conventional magnetization transfer contrast).

Basically, $\Delta_{intralipo}$ is the sum of two contributions accounting for: (i) the chemical interaction arising from the labile coordination of a water molecule to the metal center of the SR (pseudocontact shift, Δ^{pseudo}), and (ii) the changes in bulk magnetic susceptibility due to the compartmentalization of the paramagnetic SR inside the vesicle (BMS shift, Δ^{BMS}):

$$\Delta_{intralipo} = \Delta^{pseudo} + \Delta^{BMS}$$

The latter term is null for spherical compartments, but it can be largely dominant for asymmetric systems. It is worth remarking that both contributions to $\Delta_{intralipo}$ are directly dependent on the intraliposomal concentration of the SR. This concentration is ruled by osmotic effects for both spherical and non-spherical LIPOCEST probes; basically, the amount of encapsulated SR will depend on the concentration of the paramagnetic agent in the solution that is used for hydrating the thin lipidic film (see experimental procedure below). Successively, the intraliposomal SR concentration can increase or decrease according to whether this solution is hyperosmolar or hypoosmolar with respect to the isotonic buffer used during the dialysis by which liposomes are purified from the shift reagent that has not been entrapped. On this basis, a further enhancement in the $\Delta_{intralipo}$ values can be achieved by encapsulating neutral multimers that, for a given osmolarity value, allowing the entrapment of a higher number of paramagnetic centers. To tackle this task, we synthesized two novel neutral polynuclear Tm(III) complexes: Tm–**1** (binuclear) and Tm–**2** (trinuclear) (Chart 1).

Ligand **1** was obtained by reacting in 2 : 1 molar ratio DO3A-t-butyl tetra ester (prepared according to the published procedure[7]) with 1,12-dibromo-3,10-dioxa-4,9-diazadodecane. The latter compound was synthesized by reacting 1,6-diaminohexane and bromoacetylbromide in 1 : 2 molar ratio; subsequently the t-butyl ester was hydrolyzed with neat trifluoroacetic acid (TFA).

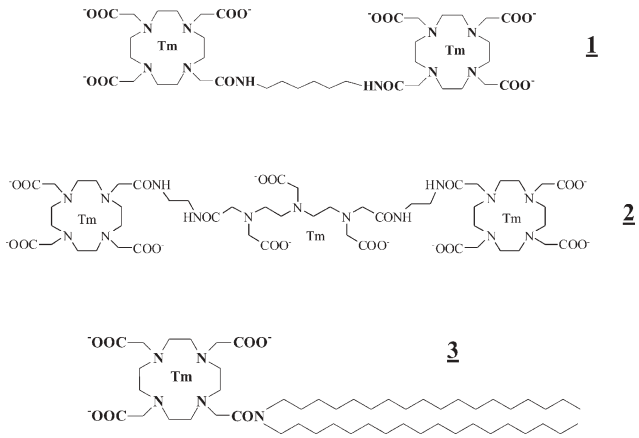

Chart 1 The structures of the Tm(III)-based shift reagents considered in the present study.

Ligand **2** was obtained by reacting DTPA-bis-anhydride with the DOTA-monoamide of ethylenediamine.[8] This reaction followed the general synthetic scheme for DTPA-bisamides and gave a very good yield (up to 70%), provided the DTPA-bis-anhydride was freshly prepared.

The complexation of both ligands was carried out by adding stoichiometric amounts of $TmCl_3$ to a ligand solution at pH = 6.5. The pH was maintained at this value during the reaction by adding NaOH 1 M.

After stirring overnight, the pH was increased to 8 and the precipitate was removed by centrifugation and filtration over a 2 μm syringe filter. Salts were removed by size exclusion chromatography using Sephadex G10 column (5 × 20 cm) and milliQ water as eluent. The osmolarities of solutions containing the Tm–**1** and Tm–**2** complexes were determined with a semi-micro osmometer (Knauer K-7400, Germany).

Fig. 1 compares the dipolar contributions (Δ^{pseudo}) to the bulk water shift of solutions containing either a mononuclear (Tm–HPDO3A), a dinuclear (Tm–**1**) or a trinuclear (Tm–**2**) SR. The HPDO3A ligand is closely related to the DOTA ligand from

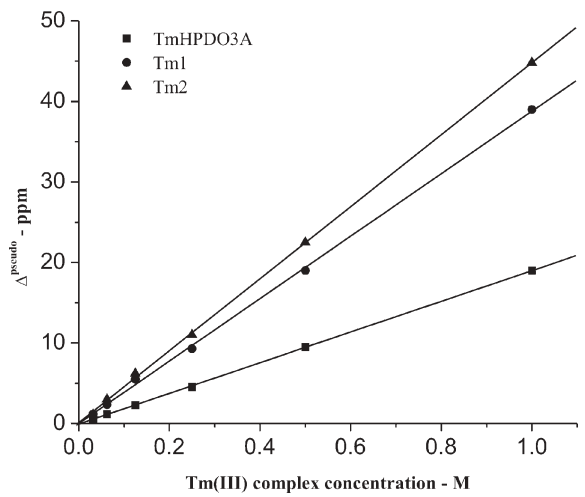

Fig. 1 Δ^{pseudo} values (14.1 T, 298 K) of aqueous solutions containing the Tm-based SRs studied in the present work.

which it is derived by the replacement of an acetic arm with a hydroxypropyl moiety.

The shifting power of the binuclear complex (0.038 ppm $mM_{complex}^{-1}$) was about twice that of the reference mononuclear complex (0.0192 ppm $mM_{complex}^{-1}$). This indicates that the shift induced by each of the two equivalent Tm(III) centers in Tm–**1** (*viz*. 0.038/2 = 0.019 ppm mM_{Tm}^{-1}) equals the effect of the single paramagnetic center of Tm–HPDO3A. Unfortunately, the dipolar shift induced by Tm–**2** was less than three times that induced by Tm–HPDO3A, because in the former compound one metal center lies in a DTPA-like cage, and consequently its ability to shift the water resonance is much lower than when it is lying in a DOTA-like cage owing to the larger angle between the main symmetric axis of the complex and the metal–water proton vector in the macrocyclic coordination environment.[9]

At the maximum concentration of Tm–HPDO3A (*ca*. 0.180 M) that is attainable inside a spherical liposome, the $\Delta_{intralipo}$ value at 298 K is about 3.5 ppm.[5] Interestingly, spherical liposomes with the same membrane formulation, (*i.e.* DPPC : DSPE–PEG2000 in a 95 : 5 molar ratio, where DPPC is dipalmitoylphosphocholine and DSPE–PEG2000 is distearoylphosphoethanolamine–poly ethylene glycol 2000), entrapping Tm–**1** at the same concentration showed a $\Delta_{intralipo}$ value (298 K) of 6.1 ppm, which is almost double with respect to the preparation containing the mononuclear SR.

As mentioned above, a marked increase of $\Delta_{intralipo}$ can also be attained by preparing non-spherical liposomes in order to exploit the additional Δ^{BMS} shift contribution. To further enhance the magnetic susceptibility effect, the incorporation of amphiphilic paramagnetic complexes into the liposome membrane has been proposed.[5] Thus, we prepared and tested osmotically stressed liposomes entrapping Tm–HPDO3A, Tm–**1** and Tm–**2** complexes, also incorporating a Tm–DOTA amphiphilic complex (Tm–**3**, see Chart 1[5]).

The experimental procedure for the preparation of these non-spherical liposomes was as follows:

(1) A lipid mixture containing (in moles) 75% DPPC, 20% Tm–**3** and 5% DSPE–PEG2000, was dissolved in chloroform at room temperature.

(2) The organic solution was dried up by evaporating the solvent so that a thin film was formed.

(3) The thin film was hydrated at 55 °C with a solution that contained either 25 mmol L^{-1} of Tm–HPDO3A (LIPO A) or 25 mmol L^{-1} of Tm–**1** (LIPO B), or 25 mmol L^{-1} of Tm–**2** (LIPO C), respectively. The osmolarities of all solutions were adjusted to a final value of 40 mOsm by adding NaCl.

(4) The resulting multilamellar liposomes were extruded 6 times at 55 °C through polycarbonate filters (pore diameter 200 nm) in order to obtain large unilamellar vesicles (LUV).

(5) LUV suspensions were exhaustively dialyzed against an isotonic buffer (HEPES–NaCl, 300 mOsm, pH 7.4) in order to remove the shift reagent that had not been entrapped.

In Fig. 2, the observed ^{1}H-NMR spectra (14.1 T, 298 K) for all three preparations (A, B, and C, respectively) are reported.

The combination of dipolar and magnetic susceptibility effects significantly increased the shift of the intraliposomal water proton resonance. It is also worth remarking that the shift enhancement observed with the trinuclear SR Tm–**2** (LIPO C) with respect to the binuclear one Tm–**1** (LIPO B) was much larger in the case of

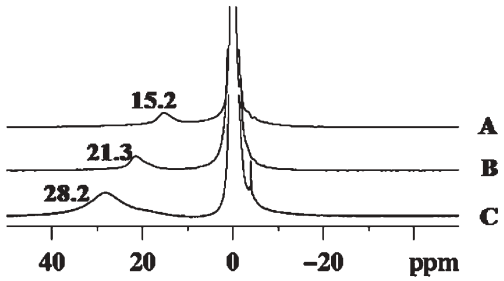

Fig. 2 ^{1}H-NMR spectra (14.1 T, 298 K) of LIPOCEST suspensions. From top to bottom: preparations A, B, and C containing Tm-HPDO3A, Tm–**1** or Tm–**2**, respectively.

non-spherical LIPOCESTs because of the predominant contribution of Δ^{BMS} that, unlike Δ^{pseudo}, is not correlated to the coordination environment of the paramagnetic center.

In summary, in this communication we have shown that the use of neutral polynuclear shift reagents significantly improves the properties of LIPOCEST agents compared to systems containing mononuclear complexes.

Notes and references

1 K. M. Ward, A. H. Aletras and R. S. Balaban, *J. Magn. Reson.*, 2000, **143**, 79.
2 J. Zhou and P. C. M. van Zjil, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2006, **48**, 109.
3 M. Woods, D. E. Woessner and A. D. Sherry, *Chem. Soc. Rev.*, 2006, **35**, 500.
4 S. Aime, D. Delli Castelli and E. Terreno, *Angew. Chem., Int. Ed.*, 2005, **44**, 5513.
5 E. Terreno, C. Cabella, C. Carrera, D. Delli Castelli, R. Mazzon, S. Rollet, J. Stancanello, M. Visigalli and S. Aime, *Angew. Chem., Int. Ed.*, 2007, **46**, 966.
6 S. Aime, D. Delli Castelli, D. Lawson and E. Terreno, *J. Am. Chem. Soc.*, 2007, **129**, 2430.
7 L. Schultze and A. R. Bulls, Patent WO9628433, 1996.
8 S. Aime, F. Fedeli, A. Sanino and E. Terreno, *J. Am. Chem. Soc.*, 2006, **128**, 11326.
9 S. Aime, S. Geninatti Crich, E. Gianolio, G. B. Giovenzana, L. Tei and E. Terreno, *Coord. Chem. Rev.*, 2006, **250**, 1562.

