Manuscript: Gadolinium-Doped LipoCEST Agents: a Potential Novel Class of Dual ^{1}H-MRI Probes

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Supplementary information

Synthesis of $(C18)_2$-PDP

$(C18)_2$-PDP (1(2-pyridyldithio)-3,12,21-trioxo-4,13,22-triaza-7,10,16,19-tetraoxatetracontane, **12**) was prepared according to the seven step synthetic pathway illustrated in Scheme S1.

Briefly, the first step was the synthesis of 2[(Dibenzylamino)etoxy]ethanol (**3**) from 2[(amino)ethoxy]ethanol (**1**) and benzyl bromide (**2**). The reaction was conducted in acetonitrile using potassium carbonate as base and was completed after 2 hours at reflux. The product **3** was obtained with a stechiometric yield.

Compound **3** was transformed in 8[(dibenzylamino)-2,5-dioxa-ottanoic acid (**5**) using bromoacetic acid (**4**). This reaction was performed in THF under reflux and in the presence of sodium hydride (yield: 85 %).

Compound **5** was conjugated with dioctadecylamine (**6**) in chloroform using HATU and DIPEA as dehydrating agents. The product N^1,N^1-dibenzyl-N^9-ottadecyl-3,6-dioxa-8-oxo-9-aza-heptacosylamine (**7**) was obtained stechiometrically.

This compound was reduced to N^9-ottadecyl-3,6-dioxa-8-oxo-9-aza-heptacosylamine (**8**) with hydrogen using palladium supported on carbon as catalyst (solvent THF). The reaction took 14 hours to get a stechiometric yield.

Compounds **8** and **5** were then coupled in chloroform using again HATU and DIPEA as dehydrating agents and the product N^1,N^1-dibenzyl-N^{18}-ottadecyl-3,6,12,15-tetraoxa-8,17-dioxo-9,18-diaza-esatriacontylamine (**9**) was obtained with a yield of 80 %.

The amine **9** was hydrogenated to N^9-ottadecyl-3,6-dioxa-8-oxo-9-aza-heptacosylamine (**10**) using the same procedure described above. After 4 hours the reaction was completed (yield 67.5 %).

The last step was the coupling between compound **10** and the commercially available 3(2-pyridil-dithio)propionic acid (**11**) that was performed in dichloromethane using HATU and DIPEA agents.

The final product 12 was obtained as frothy solid with a yield of 54 % and it was characterized by mass and NMR spectroscopies.

Liposomes preparation and basic characterization

The bilayer components DPPC, PI (both purchased from Avanti Polar Lipids Inc., Alabaster, AL, USA) and $(C18)_2$-PDP, with a molar ratio of 0.75, 0.05, and 0.2, respectively, and a total amount of ca. 20 mg were weighted into a round bottom flask and dissolved in $CHCl_3$. The solvent was removed on a rotary evaporator and its complete removal ensured by leaving the flask attached to a vacuum pump for two hours. The film formed was added with 1 ml of an aqueous solution of [Tm-DOTMA]$^-$ 200 mM and the hydration was accomplished by vortexing the suspension at 60 °C. Next, the suspension containing multilamellar vesicles was extruded six times with 400 (3x) and 200 μm (3x) polycarbonate filters using Lipex Extruder (Northern Lipids Inc., Burnaby, Canada).

The non-entrapped [Tm-DOTMA]$^-$ was removed by exhaustive dialysis performed at 4°C.

The average hydrodynamic diameter of the liposomes was measured by Dynamic Light Scattering (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, UK) and resulted to be of 130 ± 20 nm.

The size of the nanovesicles was controlled after any surface modification (conjugation with Gd(III) complex and successive reductive cleavage) and no significant changes were observed.

Relaxometric determination of the amount of Gd(III) and Tm(III) complexes loaded in the liposomes

The concentration of Gd(III) and Tm(III) complexes in LipoCEST-S-S-Gd sample was determined by mixing a given volume of the liposomal suspension (LipoCEST-S-S-Gd or LipoCEST-SH) with the same volume of HCl 37% and leaving such mixture at 120˚C overnight in a sealed vial. Upon this treatment, the lanthanide ions (Tm^{III} and Gd^{III}) are released from their complexes as free aquo-

ions. By measuring the water proton relaxation rate of these solutions, it is possible to determine their concentration.[1] Relaxation rate measurements were performed at 20 MHz and 25˚C on a Spinmaster spectrometer (Stelar, Mede, Italy), by using a conventional Inversion Recovery pulse sequence.

For LipoCEST-SH, containing only Tm(III) under the form of Tm-DOTMA in the inner cavity of the liposome, the observed relaxation rate R_{1obs} can be related to the concentration of Tm(III) by the formula:

$$R_{1obs} = R_{1W} + [Tm^{III}] \cdot r_{1p}^{Tm(III)} \qquad \textbf{[1]}$$

where R_{1W} is the relaxation rate of pure water (0.50 s^{-1} in 6 M HCl, 25 °C) and $r_{1p}^{Tm(III)}$ is the millimolar relaxivity of the Tm(III) aquo-ion (0.464 $mM^{-1}s^{-1}$ in 6 M HCl, 25 °C).

For LipoCEST-S-S-Gd , containing both Tm(III) and Gd(III):

$$R_{1obs} = R_{1W} + [Gd^{III}] \cdot r_{1p}^{Gd(III)} + [Tm^{III}] \cdot r_{1p}^{Tm(III)} \qquad \textbf{[2]}$$

where $r_{1p}^{Gd(III)}$ the millimolar relaxivity of the Gd(III) aquo-ion (13.5 $mM^{-1}s^{-1}$ in 6 M HCl, 25 °C) and other quantities are as stated above. The content of Tm is obtained by the relaxometric assay applied to LipoCEST-SH (Eq 1); the content of Gd in the liposome containing both metals (LipoCEST-S-S-Gd) is determined by substituting for [Tm(III)] in Eq 2 the value obtained for the LipoCEST-SH sample. The concentrations of Gd(III) and Tm(III) in LipoCEST-S-S-Gd have been estimated to be 0.9 mM and 7.8 mM, respectively. By considering that the total concentration of thiol groups available on the external surface of LipoCEST-SH liposomes is 1.5 mM, the percentage of such groups that have been labelled with Gd-DO3A-PDP turns to be about 60%.

Nuclear Magnetic Resonance Dispersion (NMRD) profiles

A relaxometric characterization of LipoCEST-S-S-Gd was performed by measuring the nuclear magnetic resonance dispersion (NMRD) profiles at 25°C and neutral pH. Figure S1 compares the profile with that one already published for the low molecular size Gd-DO3A-PDP complex.[2] The detailed analysis of these profiles is beyond the scope of the work. However, it appears clearly the different features of the two datasets.

The profile for Gd-DO3A-PDP agent displayed the typical shape for a rapidly tumbling paramagnetic complex (τ_R slightly longer than 100 ps)[2] and, furthermore, the high relaxivity values (r_1 of 9.0 $s^{-1}mM^{-1}$ at 20 MHz) is accounted for the presence of two water molecules coordinated to the Gd(III) centre. Conversely, the profile for the Gd agent linked to the external liposome surface

displayed slightly lower relaxivity values than the free agent except in the 20-100 MHz field interval, where the relaxivity of the liposomal system showed a peak. It is well-established that such relaxivity hump reflects the occurrence of a slow rotational tumbling for the paramagnetic chelate, which is here expected due to the linkage to the nanovesicles. However, such as system reached a maximum relaxivity of 9.8 $s^{-1}mM^{-1}$ at 40 MHz, which was only slightly higher than that of the free reference compound. A possible explanation relies on the ability of Gd(III) centres coordinated in heptadentate DO3A-like cages to bind anions like phosphates or carboxylates that can replace one or both the inner sphere water molecules. Hence, it can be envisaged that the Gd-DO3A group covalently linked to the liposome can fold over the surface of the liposome where a reduction of the hydration state of the Gd(III) centre by the lipid phosphate headgroups can occur.

References

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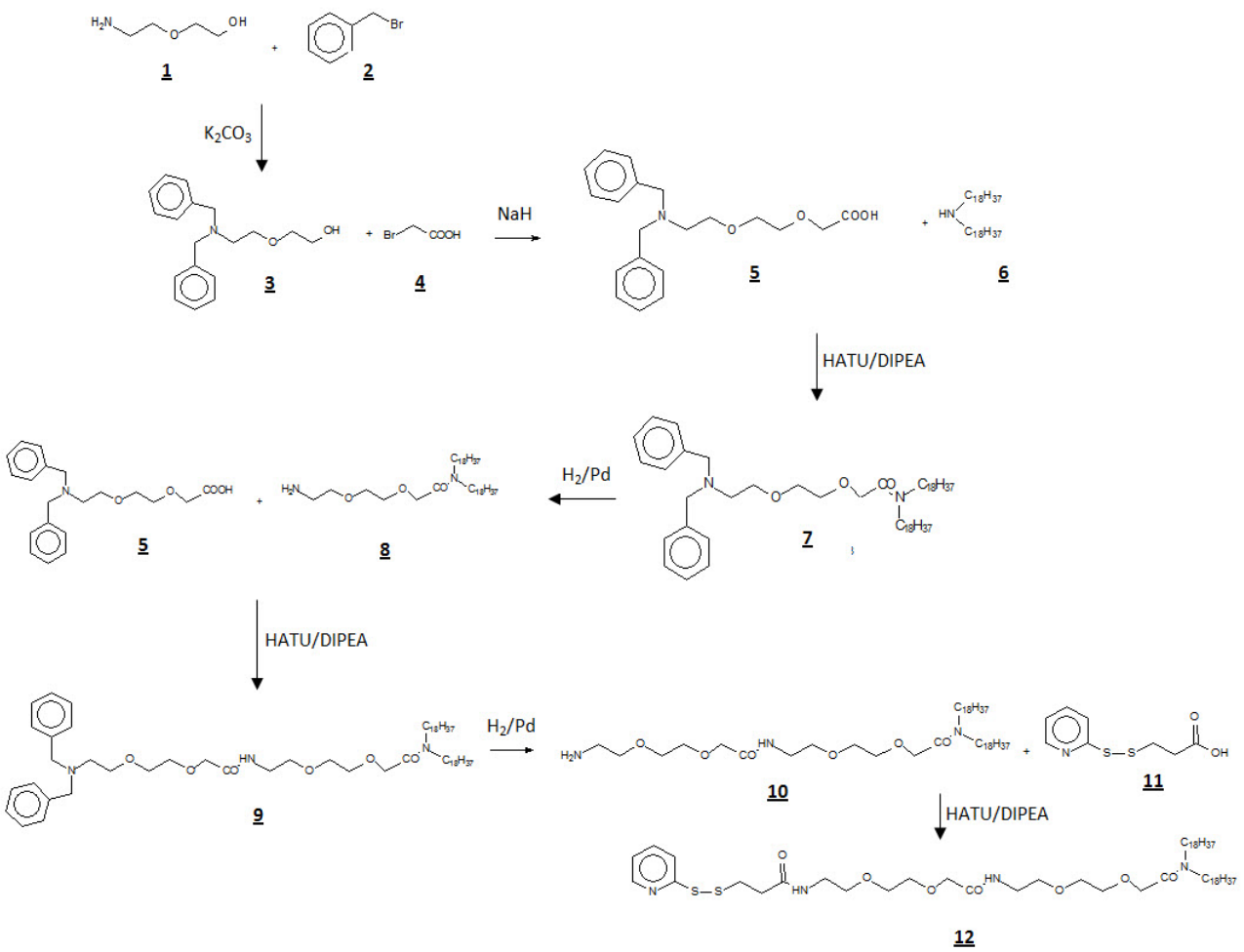
2. Digilio, G.; Menchise, V.; Gianolio, E.; Catanzaro, V.; Carrera, C.; Napolitano, R.; Fedeli, F.; Aime, S. *J. Med. Chem.* **2010**, 53, 4877–4890.



Scheme S1. Synthetic route to $(C18)_2$-PDP (**12**)

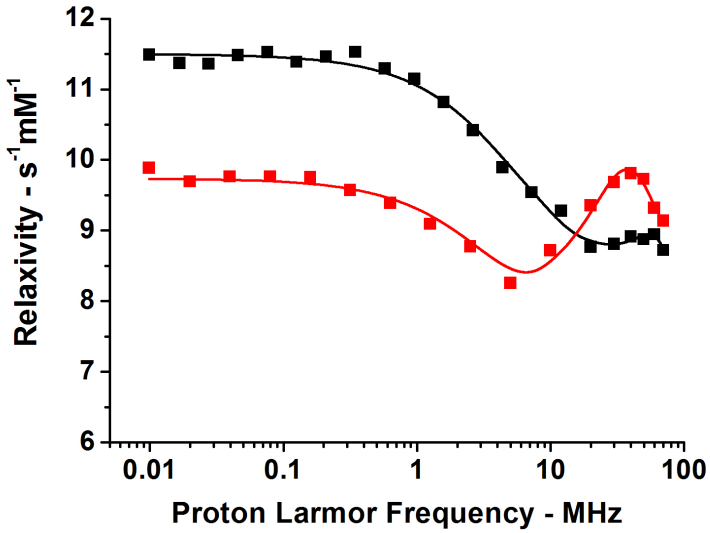


Figure S1. ^{1}H NMRD profiles of LipoCEST-S-S-Gd (red squares) and Gd-DO3A-PDP (black squares) in HEPES buffer pH 7.4, 25°C. The solid lines are interpolated curves for eye-guiding purpose.