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COMMUNICATION

Multilamellar LipoCEST Agents Obtained from Osmotic Shrinkage of Paramagnetically Loaded Giant Unilamellar Vescicles (GUVs)

Martina Tripepi, Giuseppe Ferrauto, Paolo Oronzo Bennardi, Silvio Aime and Daniela Delli Castelli*

Abstract: Moving from nano- to micro-systems may not just be a matter of scale, but it might imply changes in the properties of the systems that can open new routes for the development of efficient MRI contrast agents. This is the case reported in the present paper, where giant liposomes (Giant Unilamellar Vesicles, GUVs) loaded with Ln(III) complexes have been studied as MRI CEST contrast agents. The comparison between nanosized liposomes (Small Unilamellar Vesicles, SUVs) and GUVs sharing the same formulation led to differences that could not be accounted only in term of the mere increase in size (from 100-150 nm to 1-2 μm). Upon osmotic shrinkage GUVs yielded a Saturation Transfer effect of three order of magnitude higher than SUVs consistent with the increase in vesicles volume. Confocal microscopy showed that the shrinkage of GUVs resulted in multilamellar particles whereas SUVs are known to yield asymmetrical, discoidal shape.

MRI takes great advantage from the use of contrast agents as they may add functional information to the outstanding anatomical resolution attainable by this technique.[1] Along the years, most attention has been devoted to relaxation enhancers that affect the relaxation rates of water protons in the region where they distribute.[2] In recent years, much attention has been devoted to chemicals that allow their detection through procedures based on frequency encoding.[3]

In this context the most interesting class is represented by CEST agents that are chemicals that affect the signal intensity of the water proton resonance through the transfer of saturated magnetization from their exchangeable proton pool.[4] A drawback of the CEST agents is represented by their relatively low sensitivity as their detection in a MR image requires the number of exchangeable protons to be in the millimolar range.[5]

An important step ahead along the improvement of the attainable sensitivity was achieved with the introduction of LipoCEST in which the exchangeable pool of protons is represented by the large ensemble of water molecules contained in the liposomal inner aqueous cavity whose NMR resonance is properly shifted by the presence of paramagnetic shift reagents.[5a,6] A further sensitivity enhancement has been achieved on passing from spherical liposomes to osmotically shrunken ones that yield highly shifted values for the intraliposomal water resonance.[7] Up to now these are among the most sensitive CEST agents (hundreds pM for the spherical vesicles to tents pM for the shrunken ones). According to their membrane formulation, the shrunken LipoCEST are able to orient themselves when exposed to a magnetic field providing markedly large effects on the chemical shift of the intravesicular water molecules.[8] Despite the huge potential of these systems, the *in vivo* use has been hampered from macrophagic uptake or cell internalization. In fact, paramagnetic liposomes can work as LipoCEST agents as long as their content remains inside the vesicles whereas it became CEST-invisible when the vesicles undergo a degradation.[9]

In this work we have tried to overcome some of the limitations and to improve the potential shown by the paramagnetic SUVs by increasing their size. Giant Unilamellar Vesicles (GUVs) are liposomes of micron size; they have been known for over half a century and have been used as cell mimicking systems but no use as imaging agents has yet been reported.

These systems have dimensions ranging from 0.8 μm to 2 μm or even higher depending on the methodology of preparation or on the membrane formulation.

GUVs and SUVs bearing the same paramagnetic cargo were prepared to assess their differences when they act as LipoCEST agents.

Giant liposomes were prepared following the so called “natural swelling” method reported in literature with some modifications.[10] Chart 1 summarizes liposomes’ components in the membrane and in the internal cavity. The different samples are summarized in Table 1.

Table 1. Characteristics of the studied liposomes[a]

Name	Formulation	Content	Size
Lipo-1	DPPC/Amphiphilic Tm-complex/Liss Rhod PE/ DSPEmPEG2000 81.95/15/0.05/3	40 mM TmHPDO3A 20 μM 5(6)-carboxyfluorescein	SUV
Lipo-2	DPPC/Amphiphilic Tm-complex/Liss Rhod PE/ DSPEmPEG2000 81.95/15/0.05/3	40 mM TmHPDO3A 20 μM 5(6)-carboxyfluorescein	GUV
Lipo-3	DPPC/Amphiphilic Dy-complex/Liss Rhod PE/ DSPEmPEG2000 81.95/15/0.05/3	40 mM DyHPDO3A 20 μM 5(6)-carboxyfluorescein	GUV

[a] Chemical structures are reported in the Supporting Information.

Because the size of GUVs was expected to range between 1 and 3 μm, the most common techniques used to characterize the particles size (*e.g.* DLS or FACS) could not be used as their

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reliability fall in a lower or higher range of detection, respectively. Therefore, confocal fluorescence microscopy sampling was exploited as the technique for assessing the size of the herein prepared giant liposomes and fluorescent formulations were prepared for this purpose.

Figure 1 shows a representative fluorescence microscopy image of a spherical GUV and the size distribution; the mean size of these giant particles resulted to be 1.22 ± 0.15 μm. As reported in Figure 1B, the phospholipidic membrane can be easily detected by the presence of rhodamine-B-bearing phospholipids (red) and the inner aqueous cavity can be visualized by the presence of the water soluble 5(6)-carboxyfluorescein (green).

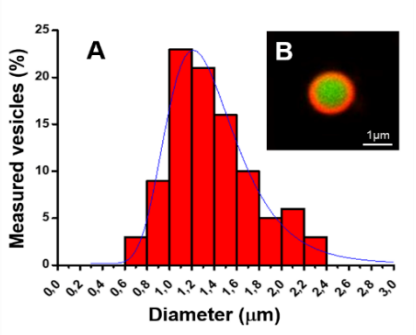

Figure 1. A) Size distribution of fluorescent giant liposomes as measured by acquiring confocal fluorescent microscopy images. B) representative confocal fluorescence microscopy image of a fluorescent spherical giant liposome.

To investigate the potential of giant liposomes as CEST agents (GiantCEST) different formulations were prepared. In particular, small and giant liposomes suspensions containing 40 mM TmHPDO3A (Fig.SI1) in the aqueous cavity and 15% of amphiphilic Tm-complex (Fig.SI2) in the membrane were prepared and characterized. All the samples were resuspended in HEPES/NaCl 0.15 M buffer 300 mOsm/L to induce an osmotic stress (shrunken liposomes).[4a,7]

Z-spectra were acquired at 600 MHz at different presaturation powers. Z-spectra of small and giant liposome suspensions sharing the same formulation are reported in Figure 2A and B, respectively.

The intensity of the irradiating field B_1 that represented the best compromise between maximizing the LipoCEST efficacy and minimizing SAR (Specific Absorption Rate)[11] issues was 5.5 μT. At this B_1 value the small liposomes suspension resulted to have a ST% of 22.15% related to a molar concentration of vesicles of $3.1x10^{-8}$ M whereas the giant liposomes suspension resulted in a ST% of 47.56% related to a molar concentration of vesicles of $2.7x10^{-11}$ M. This means that at the same concentration of vesicles, GiantCEST sensitivity is at least three order of magnitude higher with respect to nanosized LipoCEST, as shown in Figure 2C where the ST% is reported as a function of vesicles concentration. It is worth noting that the sensitivity threshold is about 1.5 pM. Fig.SI3 reports a representative CEST-MR image showing that ???? M of GUVs can be clearly detectable by MRI (ST%>50%) whereas the same concentration of SUVs is not visible.

Figure 2D displays the saturation transfer peaks measured at B_1=5.5 μT. It is possible to observe that even though giant and small vesicles share the same membrane composition and inner core payload, the corresponding intraliposomal water shift is quite

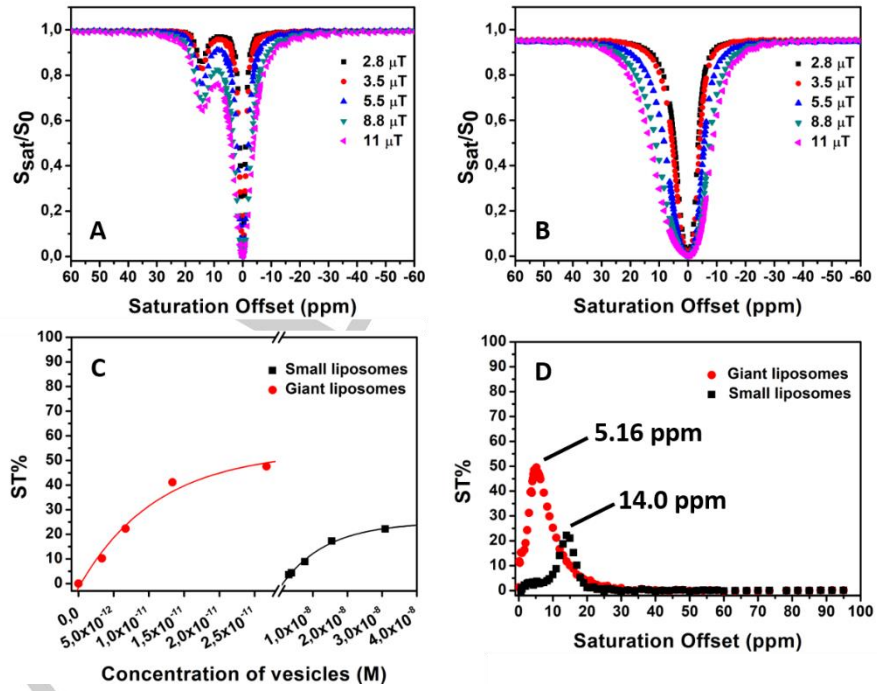

Figure 2. Z-spectra of suspensions containing $3.1x10^{-8}$ M of small Lipo-1 (A) and $2.7x10^{-11}$ M of giant Lipo-2 (B) vesicles acquired at 600 MHz at different presaturation powers. C) ST% in function on the concentration of vesicles (calculated) for a Lipo-1 (black squares) and Lipo-2 (red circles) suspension. D) ST% effect in function of the saturation offset for Lipo-1 (black squares) and Lipo-2 (red circles) suspension.

different, *i.e.* 5.16 ppm and 14.0 ppm, respectively. As reported in literature, osmotically stressed small liposomes have a strongly anisotropic cigar-like shape[8b,7,4a] and this feature allow them to orient in the main magnetic field, hence exploiting the BMS contribution to the intraliposomal water shifts.[8b] The BMS contribution is larger with respect to the dipolar one[12] so for these vesicles it is expected to induce a shift larger than 10 ppm.[7,12b] From the obtained results, giant liposomes intraliposomal shift didn't appear to be affected by the BMS contribution to the same extent as it was observed for the small ones.

To get more insight into the understanding of this unexpected behaviour, a giant liposome containing 40 mM Dy-HPDO3A in the cavity and 15% amphiphilic Dy-complex in membrane was prepared and characterized. Changing the lanthanide metal ion from Tm(III) to Dy(III) of the amphiphilic complex inserted in the membrane, one goes to vary the sign of the magnetic anisotropy of the phospholipidic membrane.[13] In the case of the small shrunken vesicles the analogous Dy/Tm resulted in a dramatic change of their orientation towards the external magnetic field.[7] The evidence that a variation in the orientation has occurred is provided from a change in the sign of the BMS contribution to the shift (from positive to negative). The two formulations of giant vesicles with 15% of amphiphilic Ln-complex in the membrane, where Ln is Tm or Dy, were compared. Figure 3 reports the Z-spectra acquired at 600 MHz at different presaturation powers.

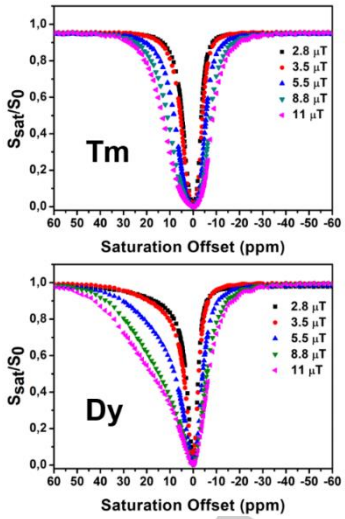

Figure 3. Z-spectra of giant Lipo-2 (top) and giant Lipo-3 (bottom) suspensions acquired at 600 MHz at different presaturation powers in order to evaluate the BMS contribute.

Surprisingly, in case of giant liposomes containing amphiphilic Tm- or Dy- complexes in the membrane, the chemical shift is positive in both cases, thus suggesting that the change in the orientation observed for the analogous SUVs did not take place.[14] This behavior could be explained hypothesizing that the shape of giant liposomes might not be the same as that observed for the smaller ones.

To investigate this possibility, confocal fluorescence microscopy images were acquired. To be visualized by confocal fluorescence microscopy rhodamine-B-carrying giant liposomes entrapping 5(6)-carboxyfluorescein were prepared. Two different aliquots were put against isotonic and hypertonic HEPES/NaCl buffer to generate a spherical and a shrunken liposomes suspension, respectively. Osmotically stressed nanosized liposomes react towards hypertonic medium changing their spherical shape into a cigar-like shape, as reported in literature.[8b]
In Figure 4A an image of an isotonic giant liposome in which it is possible to appreciate the red burden containing the rhodamine-B and the green internal cavity due to 5(6)-carboxyfluorescein is reported; as expected, the vesicle resulted to be spherical. Figures 4B and 4C report to examples of osmotically stressed GUVs either as two-dimensional or three-dimensional section, respectively. It is possible to appreciate the strongly anisotropic but amorphous shape of the vesicle with several invaginations due to the osmotic stress.

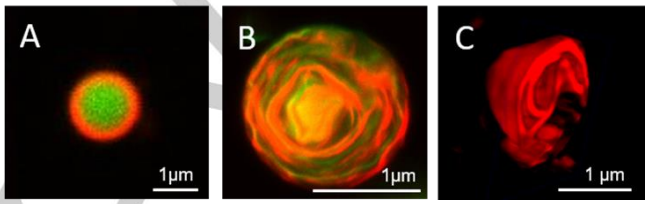

Figure 4. Confocal fluorescence microscopy images of A) a spherical isotonic giant liposome, B) an osmotically stressed globe spiral-like shaped giant liposome C) a 3D section of an osmotically stressed GUV. Both giant formulations contain 0.05% Liss Rhod PE in membrane and 20 μM 5(6)-carboxyfluorescein in the internal cavity.

In order to evaluate the magnetic behavior of giant liposomes toward progressive osmotic stress, a giant liposomes suspension containing 40 mM TmHPDO3A in the aqueous cavity and 15% of amphiphilic Tm-complex in the membrane was prepared and suspended in HEPES/NaCl with increasing osmolarity from an isotonic to a highly hypertonic environment (40÷400 mOsm/L). In Figure 5, it is clearly visible how the peak of intraliposomal water shifts away from the bulk water upon increasing the osmolarity of the external medium. At the highest osmotic stress, an intraliposomal value of about 30 ppm is reached.

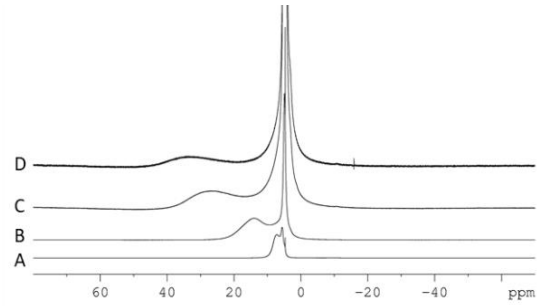

Figure 5. ^{1}H NMR spectra (600 MHz, 298 K) of a suspension of Lipo-2 suspended in HEPES/NaCl (pH 7.4) with increasing osmolarity: A) 40 mOsm/L (isotonic), B) 150 mOsm/L, C) 300 mOsm/L, D) 400 mOsm/L.

Together with the shift, the osmotic stress also resulted in a decrease of the intraliposomal water signal, which is associated with a line broadening of the peak. The decrease in the signal intensity is the consequence of the osmotic shrinkage of the

vesicle that, besides losing entrapped water, increases the concentration of the paramagnetic molecules in the aqueous core, thus leading to the shortening of the T_2 relaxation time, responsible for the broadening of the peak.[8b]
Finally, the advantages of using GUVs in comparison to SUVs for *in vivo* applications has been preliminary tested by using cellular models. In particular, it has been reported that i) internalization of GUVs by macrophages is lower than the one reported for SUVs (Fig.SI4 and Fig.SI5) and cells viability in presence of GUVs is the same reported for SUVs (Fig.SI16). This makes GUVs potential good candidate as CEST MRI CAs for *in vivo* preclinical applications.

In conclusion, the herein reported results show that paramagnetically loaded GUVs display a CEST sensitivity enhancement of three order of magnitude in respect to analogous LipoCEST agents based on nanosized SUVs. Interestingly this expected change does not occur as simple follow-up of the difference in size between SUVs and GUVs because their response to the osmotic changes resulted quite different. Actually the changes induced on liposomes by osmotic shrinkage is a topic extensively investigated over the years as the effects of changes in osmotic pressure on liposomes membrane have been used to mimic the transformation of biological membranes in response to a number of environmental factors. The changes induced on liposomes by osmotic shrinkage have been investigated by means of many techniques (TEM, SAXS, Fluorescent microscopy, etc.). In general, it has been found that, after the initial decrease of the area/lipid ratio, a variety of deformations may occur including the increase of membrane area, phase shrinkage, up to partial solubilization or pore formation and fusion. The osmotic shrinkage is first driven by the water outflow through the bilayer. The decrease in size implies a decrease of the area/lipid ratio which is accommodated with a deformation that for the small SUVs results in a passage from spherical to lens/cigar-shaped ones. Likely in the case of GUVs the deformation results in close contact of opposite bilayers which yield to an extensive rearrangement that, in turn, appears to lead to a multilamellar system. However, we cannot exclude that the multi-lamellar structure is the result of an extensive breaking of the liposome membrane in response to the increased osmotic pressure. It was reported that under osmotic stress, the vesicles are often broken and large holes open without membrane shrinking.[15]
As far as concern the development of new MRI CEST agents, the finding that such paramagnetically labelled multilamellar-structures yields systems analogous to the previously reported LipoCEST agents may open new routes for the design of innovative contrast agents.

Acknowledgements

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Keywords: Giant • LipoCEST • Liposomes • Osmotic stress • Vesicles

References

[1] a) A. S. Merbach, L. Helm, É Tóth in *The chemistry of contrast agents in medical magnetic resonance imaging*. John Wiley & Sons **2013**; b) P. Caravan, *Chem. Soc. Rev.* **2006**, *35*, 512-523.

[2] a) C. F. Geraldes, S. Laurent, *Contrast Media Mol. I.* **2009**, *4*, 1-23; b) L. M. De León-Rodríguez, A. F. Martins, M. C. Pinho, N. M. Rofsky, A. D. Sherry, *J. Magn. Reson. Imaging* **2015**, *42*, 545-565; c) A. D. Sherry, P. Caravan, R. E. Lenkinski, *J. Magn. Reson. Imaging* **2009**, *30*, 1240-1248.

[3] a) S. Lacerda, É. Tóth, *ChemMedChem* **2017**, *12*, 883-894; b) K. Srivastava, G. Ferrauto, V. G. Jr. Young, S. Aime, V. C. Pierre, *Inorg. Chem.* **2017**, *56*, 12206-12213.

[4] a) G. Ferrauto, S. Aime, M. T. McMahon, J. R. Morrow, E. M. Snyder, A. Li, R. Bartha in *Chemical exchange saturation transfer (CEST) contrast agents. Contrast Agents for MRI: Experimental Methods,* Eds.: VC Pierre and M. J Allen, Cambridge, UK: RSC Publishing, **2017**, pp. 243-317; b) P. C. van Zijl, N. N. Yadav, *Magn. Reson. Med.* **2011**, *65*, 927-948; c) K. M. Jones, A. C. Pollard, M. D. Pagel, *J. Magn. Reson. Imaging* **2018**, *47*, 11-27; d) E. Vinogradov, A. D. Sherry, R. E. Lenkiski, *J. Magn. Reson.* **2013**, *229*, 155−172.

[5] a) G. Ferrauto, D. Delli Castelli, E. Di Gregorio, E. Terreno, S. Aime, *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2016**, *8*, 602-618; b) G. Ferrauto, F. Carniato, L. Tei, H. Hu, S. Aime, M. Botta, *Nanoscale* **2014**, *6*, 9604-9607.

[6] a) S. Aime, D. Delli Castelli, E. Terreno, *Angew. Chem. Int. Ed.* **2005**, *44*, 5513-5515; b) B. Chahid, L. Vander Elst, J. Flament, F. Boumezbeur, C. Medina, M. Port, R. N. Muller, S. Lesieur, *Contrast Media Mol. Imaging* **2014**, *9*, 391-399; c) J. M. Zhao, Y. E. Har-el, M. T. McMahon, J. Zhou, A. D. Sherry, G. Sgouros, J. W. Bulte, P. C. van Zijl, *J. Am. Chem. Soc.* **2008**, *130*, 5178-5184; d) K. W. Chan, J. W. Bulte, M. T. McMahon, *Wiley Int. Rev. Nanomed. Nanobiotechnol.* ***2014***, *6,* 111-124.

[7] E. Terreno, C. Cabella, C. Carrera, D. Delli Castelli, R. Mazzon, S. Rollet, J. Stancarello, M. Visigalli, S. Aime, *Angew. Chem. Int. Ed.* **2007***, 46,* 966-968.

[8] a) D. Burdinski, J. A. Pikkemaat, M. Emrullahoglu, F. Costantini, W. Verboom, S. Langereis, H. Grüll, J. Huskens, *Angew. Chem. Int. Ed. Engl.* **2010**, *49*, 2227-2229; b) S. Aime, D. Delli Castelli, E. Terreno, *Methods in enzymology*, **2009**, *464*, 193-210.

[9] V. Deissler, R. Rüger, W. Frank, A. Fahr, W. A. Kaiser, I. Hilger, *Small* **2008**, *4*, 1240-1246.

[10] J. P. Reeves, R. M. Dowben, *J. Cell. Physiol.* **1969**, *73*, 49-60.

[11] V. Roeloffs, C. Meyer, P. Bachert, M. Zaiss, *NMR BIOMED.* **2015**, *28*, 40-53.

[12] a) G. Ferrauto, E. Di Gregorio, S. Baroni, S. Aime, *Nano Lett.* **2014**, *14*, 6857-6862; b) J. A. Peters, J. Huskens, D. J. Raber, *Prog. Nucl. Magn. Reson. Spectrosc.***1996**, *28*, 283-350.

[13] G. Ferrauto, D. Delli Castelli, E. Di Gregorio, S. Langereis, D. Burdinski, H. Grüll, E. Terreno, S. Aime, *J. Am. Chem. Soc.* **2014**, *136*, 638-641.

[14] G. Ferrauto, E. Di Gregorio, M. Ruzza, V. Catanzaro, S. Padovan, S. Aime, *Angew. Chem. Int. Ed. Engl.* **2017**, *56*, 12170-12173.

[15] M. Ohno, T. Hamada, K. Takiguchi, M. Homma, *Langmuir*, **2009**, *25*, 11680-11685.

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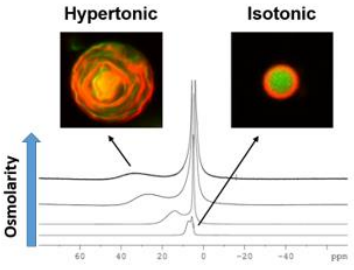
GiantCEST: Giant Unilamellar Vesicles (GUVs) are loaded with Ln(III) complexes to be studied as MRI CEST contrast agents. Upon osmotic shrinkage, GUVs yielded a Saturation Transfer effect of three order of magnitude higher than nanosized liposomes. Confocal microscopy showed that the shrinkage of GUVs resulted in multilamellar particles whereas SUVs are known to yield asymmetrical, discoidal shape.



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