Novel cross-linked liposomal Chemical Saturation Transfer or CEST agents

A. Papagiannaros[1], V. Righi[1,2], G. Dai[2], and A. A. Tzika[1,2]

[1]NMR Surgical Laboratory, Department of Surgery, Massachusetts General Hospital and Shriners Burns Institute, Harvard Medical School, Boston, MA, United States, [2]Department of Radiology, Massachusetts General Hospital, Harvard Medical School, Athinoula A. Martinos Center of Biomedical Imaging, Boston, MA, United States

Introduction: Chemical Saturation Transfer (CEST) agents are based in the irradiation at the proper frequency of the labile water protons of the paramagnetic complex and the chemical exchange of magnetization from them to the bulk water signal. The contrast of the image depends mainly on the physiochemical properties of the contrast agents and the extent of the magnetization transfer between the labile protons and the bulk water. In an effort to produce highly shifted CEST agents, that in turn could be turned on or off by the specific RF irradiation of the slowly exchangeable protons, paramagnetic complex metals have been encapsulated into liposomes (1), so that two distinct water pools are created, where the rate of water exchange among them determines the shape of the ^{1}H NMR spectrum, that exhibits two signals, from the intra liposomal and bulk extra liposomal pools. Bulk magnetic susceptibility effects can also be exploited by osmotically deforming the liposomes and thus increasing the chemical shift of the intra-liposomal water protons (2). For these osmotically stressed liposomes, stability is still a major concern, as the membrane tension resulting from their deformation leads to their rupture (3) and thus far their promising contrast characteristics have been demonstrated only *ex vivo* (4). Our previous work was concentrating in using an edge active lipid so as to modulate the water permeability of the liposomal CEST agents in order to produce a highly shifted liposomal agent (4). Here, we present two liposomal agents encapsulating paramagnetic complexes, whose stability was modified through chemical cross-linking of the lipid chains using photo-polymerization in order to increase their stability as it was previously suggested (5).

Materials and Methods: Liposomes were produced using the thin layer hydration method. Briefly, lipids 10^{-6} moles, composed either of the photo-polymerizable 1,2-di-(10Z,12Z-tricosadiynoyl)-*sn*-glycero-3-phosphoethanolamine or a mixture of 80% photo-polymerizable lipid and 20% Gadolinium (Gd^{+3}) containing 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid (gadolinium salt). Chloroformic solutions of the lipids were mixed in a round bottom flask and a film was formed using a flash evaporator (Labonco, USA).

The film was hydrated using either a Thulium (Tm^{+3}) DOTMA ((1*R*,4*R*,7*R*,10*R*)-α,α′,α″,α‴-Tetramethyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid tetrasodium salt) 40 mOsm solution or a hypotonic Phosphate Buffered Saline 40 mOsms. Once hydrated, sonicated unilamellar vehicles were produced using a probe sonicator (Microson, USA). The volume of the liposomes was adjusted to 2 ml and half of the volume of each preparation was placed to dialysis against phosphate buffered saline, cut off value 12400. The second aliquot was photo-polymerized using a standard Hg Arc lamp placed at 8 cm from the sample (5). The absorption of the liposome preparation at 500 nm was used to verify the cross-linking of the particles. The cross-linked nanoparticles were placed to dialysis overnight, before their NMR spectra were acquired. The CEST Z-spectra were acquired in a 4.7 T horizontal magnet (20 cm bore, Bruker Avance console, Bruker Biospin, Billerica, MA) using a custom-built volume coil of 3 cm inner diameter and 10 cm active length. We used a Gd1pulse CEST sequence (6), repetition time TR=2000 ms, N average 4. The main magnetic field (B_0) was shimmed and the RF filed (B_1) was calibrated. The RF frequency offset was +/- 1000 Hz with a frequency interval of 50 Hz and the irradiation RF power was chosen at 14.2 T for the magnetization transfer. The pre-saturated off-resonance pulse ranged from 0 to 10 kHz. The spectra were processed using MesRec lab software (Mestrelab Research) a 7-Hz line-broadening apodization function was applied to Z-spectra FIDs prior to Fourier transformation (FT) and an automated fitting routine based on the Levenberg-Marquardt algorithm was applied.

Results and Discussion: The ^{1}H NMR spectra of the two cross-linked liposomal formulations demonstrated that they can function as a CEST agent. The observed peak at the chemical shift of 0.583ppm for the Tm liposomes and 0.503ppm for the Gd liposomes is as clearly separated from the bulk water peak (0 ppm), as in previously published research (1, 2, 9). Similar spectra were acquired from the non cross-linked liposomes, indicating that the cross-link had no significant effect on the magnetic properties of the liposomes.

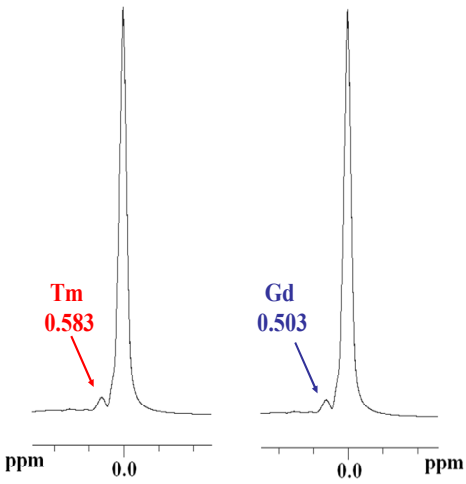

Figure 1: The ^{1}H NMR spectra of the two cross-linked liposomal formulations

Liposomes are a versatile system, which can be used in order to target a number of disease sites. Ligands, such as antibodies, can be easily attached and can direct these contrast agents to a specific site. They can be long-circulating using polymer coating and a variety of ligands, including cell penetrating peptides can be attached to their surface even after their formation, as long as they possess a stable structure (10) Immuno-liposomes represent an established system for the detection of cancer (11) and inflammation (12). Cross-linked liposomes have proven to possess increased stability *in vitro* and *in vivo* that allowed their systematic administration (5), while deformed liposomes have failed to demonstrate similar results in cell culture or *in vivo*.

The most important advantage of our approach is its elegance and simplicity. Photo-polymerization takes place in less than 15 min and used for commercially available lipids and light sources. Our previous work, focused on altering the water permeability of the liposomal membrane in order to increase the signal producing highly shifted CEST liposomes (5). These efforts were hampered again from the lack of stability of the liposomes *in vivo.* Our new approach is directed first in establishing a stable system for *in vivo* application and then to proceed in optimization of their water permeability, so as to produce equally shifted contrast agents that we will target to the specific surface antigens on inflammation sites or tumor nodes so as to perform functional molecular imaging *in vivo.* We have chosen two different paramagnetic complexes and two different encapsulation strategies, one Gd attached to the membrane and one in the water cavity. Although Tm was extensively used for CEST liposomes, Gd can offer greater versatility in the imaging due to the various imaging protocols (12). By targeting these two different liposomal contrast agents to various aspects of the disease, different figures will be imaged by choosing the appropriate imaging protocols in one session, leading to great imaging versatility.

Conclusions: We suggest that the cross-linking of the liposomes renders them with higher stability and optimal magnetic properties and that it is these properties that should allow their successful use in imaging of inflammation and cancer, especially using targeted molecular imaging.

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