Multi-Color in Vivo MR Imaging of Lymph Nodes using DIACEST Liposomes

G. Liu[1,2], M. M. Moake[3], A. A. Gilad[2,4], M. Jamil[2], Y-E. Har-el[2], C. Long[3], P. Walczak[2,4], J. Zhang[2], A. Cardona[2], M. A. DeLiso[2], G. Sgouros[2], J. W. Bulte[2,4], P. C. van Zijl[1,2], and M. T. McMahon[1,2]

[1]F.M. Kirby Research Center for Functional Brain Imaging, Kennedy Krieger Institute, Baltimore, MD, United States, [2]Department of Radiology, Johns Hopkins University School of Medicine, Baltimore, MD, United States, [3]Department of Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, MD, United States, [4]Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, MD, United States

INTRODUCTION

MR contrast agents are used for visualizing a number of processes, including liposomal delivery of anticancer drugs to their target. However, relaxation-based MR contrast agents are limited as compared to fluorescent contrast agents in that they are not distinguishable from each other. Magnetically labeled liposomes have long been used as MR contrast agents, and are known to accumulate in lymph nodes upon subcutaneous injection.[1] In this study, we demonstrate that we can load liposomes with diamagnetic Chemical Exchange Saturation Transfer (DIACEST) contrast agents and visualize their accumulation in regional lymph nodes in mice. In addition, we demonstrate that multiple types of DIACEST liposomes can be simultaneously detected in-vivo with CEST MRI, dependent on the saturation frequency. As a result, we are able to produce multi-frequency (multi-color) MR images, which has previously been demonstrated only in vitro.[3,4]

METHODS AND MATERIALS DIACEST liposomes containing L-arginine (Larg), poly-L_lysine (PLL), and glycogen (Glyc), were prepared as described previously,[5,6] using a starting solution containing 574 mM Larg, 25 mg/mL PLL(MW=15k-30k) and 100mg/ml Glyc (MW=25k-100k) respectively. Liposomes were rendered fluorescent using rhodamine. The final particle concentration was 30-100 nM and the liposome size ranged from 100-400 nm. C57Bl6 mice were injected with GM-CSF-expressing cancer cell vaccine to produce an immuno-responsive enlargement of popliteal lymph nodes 1 week prior to liposome injection.[7] Delivery of DIACEST liposomes through lymphatic draining was achieved by subcutaneously injecting 30 µl of liposome solution in the hind footpads of mice.[1] Size-dependent pharmacokinetics of radioisotope labeled liposomes (containing $^{111}InCl_3$ in the interior[6]) were determined using small animal µSPECT at four different time points (5, 7, 24 and 48 hours). *In-vivo* MR imaging was performed approximately 24 hours after injection of 100-200 nm liposomes. After completion of MRI, popliteal lymph nodes were removed for histological staining.

All *in-vivo* MR images were acquired on a 9.4T Bruker Avance system equipped with a 25 mm sawtooth RF coil. A modified RARE sequence was used (TR=5.0 sec, effective TE= 21.6 ms, RARE factor =6, slice thickness=0.7 mm, and NA=2) including fat suppression and magnetization transfer (MT) modules. In order to generate multi-color CEST contrast images in this inhomogeneous region around the knee, we developed a new saturation image collection scheme consisting of two parts. The first set of saturation images were used to generate a B_0 map, using the WAter Saturation Shift Reference (WASSR) method[8] modified for use at high field. In brief, this set of images employ a short, weak saturation pulse (t_{sat}= 500 ms, ω_1 =0.5 µT (21.3 Hz)), sweeping from -2ppm to 2 ppm (0.1ppm step), with a reduced TR to keep the imaging time short. A second set of images with the saturation frequency stepped from -6ppm to 6ppm (step=0.3ppm) around the water resonance (0ppm) was acquired with saturation pulse ω_1 = 3.6 µT (150 Hz), t_{sat} = 3sec for CEST weighted images and corrected according to our B_0 map map. This was used to compute MTR_{asym} for our multi-color CEST contrast images. All data processing was performed using custom-written scripts in Matlab. Saturation spectra (z-spectra) were drawn and compared based on the mean water signal of the entire right and left lymph nodes. MTR_{asym}, was computed by $MTR_{asym} = (S^{\Delta\omega} - S^{+\Delta\omega})/ S^{\Delta\omega}$. A contrast-noise-ratio based threshold was used to reduce 'artificial' contrast due to random noise, and background endogenous asymmetric MT effects.

RESULTS AND DISCUSSION Liposomes encapsulating three different DIACEST agents displayed different frequency dependent CEST contrast, thus enabling the assignment of artificial MR colors, i.e. blue for Glyc liposomes at 0.8ppm, red for Larg liposomes at 1.8ppm and green for PLL liposomes at 3.6ppm (Fig. 1A). Using the optimized condition (i.e. liposome size=100-200nm and post-injection time=24hours) for detecting liposome accumulation in the popliteal lymph nodes, we tested the *in-vivo* contrast of these DIACEST liposomes. The mice were injected in two different ways, either in one foot with one "color" of liposome, or in both feet with each foot injected with a different "color" of liposome. One-foot injection allows the direct comparison of MRI signals in both nodes because no significant liposome accumulation was found in the nodes on the non-injected side as confirmed by a SPECT/CT study. In studies on multiple mice (n=5 for PLL and Larg, and n=3 for Glyc), all three types of DIACEST liposomes displayed the ability to increase CEST contrast in the nodes on the injection side, with a mean increase of 11% in ΔMTR_{asym} for PLL liposomes at 3.6ppm, 8% in ΔMTR_{asym} for Larg liposomes at 1.8ppm, and 4% in ΔMTR_{asym} for Glyc liposomes at 0.8ppm, respectively. In Figs. 1B-1D, artificial colors were assigned to these *in vivo* CEST images based on the frequency of the MTR_{asym} map, (3.6ppm (green), 1.8 ppm(red), 0.8ppm(blue)). To demonstrate the ability of simultaneously multi-color imaging on the same subject, a two-foot injection of Larg and PLL liposomes was also performed on multiple mice (n=3). We were able to detect different CEST contrast frequency dependencies (Figures 2C. The CEST image (Fig. 2B) clearly shows that the right popliteal lymph node as a red color (representing Larg type CEST, at 1.8ppm), while the left side shows a green color (representing PLL type CEST, CEST at 3.6ppm). We are currently developing a substitute for PLL, as these liposomes appear to cause damage to the nearby tissues resulting in changes to both CEST contrast (Figs. 1D and 2B) and T2 contrast (Fig. 2A). The majority of these liposomes were found to be extra-cellular as revealed by fluorescence immunohistological staining for anti-CD45 (lymphocytes and macrophages) and rhodamine (*i.e.* liposomes). A good correlation with the CEST signal (MTR_{asym}) was found, confirming that CEST signal came from the presence of liposomes.

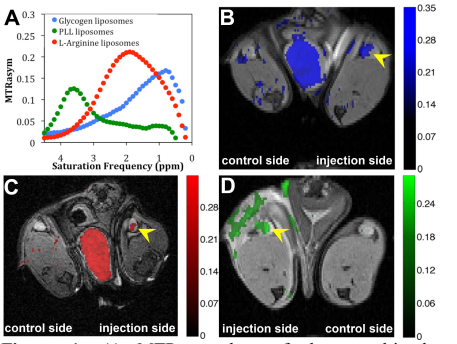

Figure 1. A) MTR_{asym} plots of three multi-color DIACEST liposomes (~30 nM) in *in vitro* (pH 7.3 and 37°C), with blue assigned to Glyc, red assigned to Larg and green assigned to PLL, B) MTRasym image at 0.8ppm of a mouse injected with Glyc liposomes, C) MTRasym image at 1.8ppm with Larg liposomes injected , and D) MTRasym image at 3.6 ppm with PLL liposomes injected.

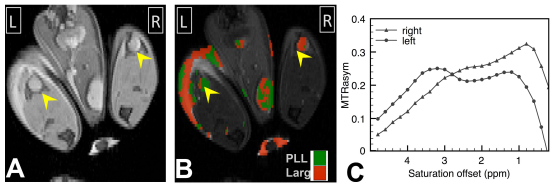

Figure 2. The *in-vivo* demonstration of two-color DIACEST A) Axial T2w image of mouse legs, where the right popliteal lymph nodes, B) a two-color CEST image showing two types of CEST signal corresponding to Larg (red) or PLL (green). The CEST parametric map was produced by the algorithm: if a pixel has a CEST signal higher than the threshold, and this signal is higher at 1.8 ppm than at 3.6ppm, it will be considered as a Larg type pixel and assigned with red color, otherwise it will be considered as a PLL type pixel and assigned with green color. If CEST at both frequencies are less than threshold, no color will be assigned, and C) the MTR_{asym} plots of right and left lymph nodes, showing different CEST signals due to the accumulation of different DIACEST liposomes.

CONCLUSION We acquired the first *in-vivo* multi-color DIACEST MR images for visualizing lymphatic accumulation of liposomes. Three bio-organic molecules, Larg, PLL and Glyc, were adapted to make highly sensitive DIACEST liposomes with distinguishable CEST contrast. We expect this to have applications in MR imaging of liposomal (drug) delivery, as well as a scale of other biological applications where multi-color imaging is beneficial.

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