Bioengineering a Reporter System that Combines a Highly Sensitive CEST and Fluorescence Imaging Probe

Amnon Bar-Shir[1,2], Yoshinori Kato[1], Arvind P. Pathak[1], Jeff W.M. Bulte[1,2], and Assaf A. Gilad[1,2]

[1]*Department of Radiology, Johns Hopkins University, Baltimore, MD, United States,* [2]*Institute for Cell Engineering, Johns Hopkins University, Baltimore, MD, United States*

Target Audience: Researchers and clinicians who are interested in molecular and cellular MRI, especially in the non-invasive monitoring of gene expression, gene therapy, and drug delivery and screening with advanced MRI-based methodologies.

Purpose: The overall goal is to bioengineer a new MRI reporter system that allows real-time monitoring of gene expression using CEST-MRI and which encompasses optical capabilities. The specific goals of this study were to overcome the challenges of quantifying the accumulation and sub-cellular distribution of the reporter probe, and minimize contributions from endogenous CEST contrast or direct water saturation.

Methods: Pyrrolo-2'-deoxycytidine (pyrrolo-dC) was dissolved in 10mM PBS at a 20mM concentration. CEST-MRI experiments were performed on an 11.7T Bruker Avance system, as previously described[1]. A modified RARE (TR/TE=6000/9.4ms), including a magnetization transfer module (B_1 = 4.7 μT/4000 ms), was used to acquire CEST-weighted images. The absolute water resonant frequency shift was measured using a modified WASSR method, with the same parameters as in CEST imaging, except for a TR=1.5 sec and a saturation pulse of B_1 =0.5 μT/250 ms. Mean CEST spectra were derived from an ROI for each sample, after B_0 correction for each voxel using Matlab. MTR asymmetry (MTR_{asym})= $100\times(S_{-\Delta\omega} - S_{+\Delta\omega})/S_0$ was computed at different offsets, Δω. 9L rat glioma, engineered to express *Drosophila melanogaster* 2'-deoxynucleoside kinase (Dm-dNK; $9L^{Dm\text{-}dNK}$) and control, non-expressing wild type cells ($9L^{wt}$), 5×10^6 cells per group, were incubated for 4 hours in cell-culture medium containing 2mM pyrrolo-dC. Then, the cells were washed with PBS, lysed, and fluorescence was measured in triplicate using a plate reader (λ_{ex}355nm/λ_{em}460nm). Pyrrolo-dC-loaded liposomes were prepared using a standard lipid hydration method, followed by extrusion.

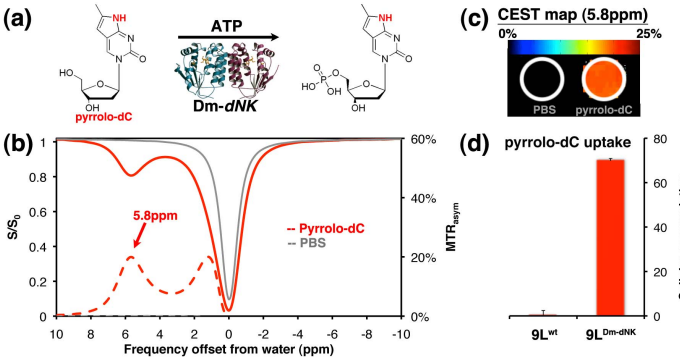

Fig 1. a) Illustration of pyrrolo-dC phosphorylation by *Dm-dNK*. b) CEST spectra (solid lines) and MTR_{asym} plots (dashed lines) for pyrrolo-dC (red) and PBS (gray). c) MTR_{asym} maps of pyrrolo-dC and PBS at Δω=5.8ppm. d) Fluorescence as measured from the cell ($9L^{wt}$ and $9L^{Dm\text{-}dNK}$) lysate after incubation with pyrrolo-dC.

Results: **Fig. 1a** shows a schematic illustration of the phosphorylation of pyrrolo-dC to pyrrolo-dC monophosphate by the enzyme Dm-dNK in the presence of ATP. **Fig. 1b** shows the CEST spectrum and MTR_{asym} plot of pyrrolo-dC (red) compared to that of PBS (gray). The NH proton of the pyrrolo-dC generates a well-defined peak at the 5.8ppm frequency offset from water. The MTR_{asym} maps obtained at Δω=5.8ppm (**Fig. 1c**) demonstrate the high CEST contrast generated by pyrrolo-dC. We capitalized on the optical properties to measure the accumulation of phosphorylated pyrrolo-dC in Dm-dNK-expressing cells. The Dm-dNK gene was cloned into the pcDNA expression vector and was transfected into 9L rat glioma cells ($9L^{Dm\text{-}dNK}$). Next, both $9L^{Dm\text{-}dNK}$ and wild type ($9L^{wt}$) cells were incubated in a medium containing 2mM pyrrolo-dC for 4 hours. As clearly shown in **Fig. 1d**, only the lysate of incubated $9L^{Dm\text{-}dNK}$ cells provides a high fluorescence level, while fluorescence was undetectable for incubated $9L^{wt}$.

As shown in **Fig. 2a**, liposomes loaded with pyrrolo-dC showed higher fluorescence levels compared to empty liposomes. The pyrrolo-dC-containing liposomes generated higher CEST contrast at 5.8ppm, compared to the empty-control liposomes (**Fig. 2b**).

Discussion: Recent advances in molecular MR imaging have revolutionized our ability to monitor gene expression with reporter genes[2-6]. For CEST-based reporter systems, the observed sharp and well-defined NH peak at the 5.8 ppm frequency offset from water is a major advantage, since a large Δω (>3.6ppm) minimizes contributions from endogenous CEST contrast and direct water saturation.

The formation of the pyrrolo-dC monophosphate in the presence of recombinant Dm-dNK (**Fig. 1a**) resulted in accumulation of the fluorophore in the cytoplasm, as its negative charge prevented cellular export. The use of a fluorescent-based CEST-probe, such as pyrrolo-dC, enables its accumulation to be detected only in cells expressing Dm-dNK, which implies that *in vivo* monitoring of Dm-dNK reporter gene expression with CEST-MRI is feasible. Liposomes may be considered as a delivery vehicle to increase pyrrolo-dC circulation time, control its release, and prevent degradation in the circulation.

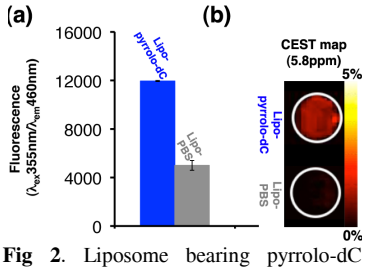

Fig 2. Liposome bearing pyrrolo-dC (Lipo-pyrrolo-dC) compared to empty liposome (Lipo-PBS). a) Fluorescence and b) CEST-MRI.

Conclusion: Synthetic fluorescent nucleosides, such as pyrrolo-dC, can be used for the real-time monitoring of gene expression and tracking liposomal delivery with both optical and MRI modalities. Supported by MSCRFF-0103-00.

References: 1. G. Liu, A. A. Gilad, J. W. Bulte, P. C. van Zijl, M. T. McMahon, *Contrast Media Mol Imaging* **5**, 162 (2010). 2. B. Cohen *et al.*, *Nat Med* **13**, 498 (2007). 3. G. Genove, U. DeMarco, H. Xu, W. F. Goins, E. T. Ahrens, *Nat Med* **11**, 450 (2005). 4. V. D. Kodibagkar, J. Yu, L. Liu, H. P. Hetherington, R. P. Mason, *Magn Reson Imaging* **24**, 959 (2006). 5. O. Zurkiya, A. W. Chan, X. Hu, *Magn Reson Med* **59**, 1225 (2008). 6. A. A. Gilad *et al.*, *Nat Biotechnol* **25**, 217 (2007).