Intracellular uptake of targeted paramagnetic contrast agent leads to quenching of T_1 contrast

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Introduction: Molecular MR imaging, aimed at in vivo imaging of specific biological markers that are upregulated during diseases, is a fast growing field of research. Especially targets that are accessible from the bloodstream are studied extensively. Considerable success has been achieved by the use of targeted paramagnetic emulsions and liposomes.[1, 2] Quantification of the contrast enhancement, however, remains very difficult due to limited information about the dependence of T_1 and T_2 on contrast agent location and concentration at the tissue and cellular level. The local T_1 shortening of bulk water is strongly reduced if contrast agents are internalized by receptor unmediated endosomation, whereas contrast agents that are present freely in the cytoplasm show a higher relaxivity.[3] In this work, we report on in vitro experiments designed to give a better understanding of: (i) the pathways of contrast agent binding and internalization and (ii) changes in T_1 and T_2 as a function of internalized contrast agent concentration and localization. To that aim, human umbilical vein derived endothelial cells (HUVECs) were cultured and incubated with either non-targeted or $\alpha_v\beta_3$-integrin targeted paramagnetic liposomes.[1] The $\alpha_v\beta_3$-integrin is upregulated during a variety of disease processes, including tumor angiogenesis. Internalization kinetics as well as changes in T_1 and T_2 as a function of incubation time were studied.

Materials and Methods:

Cell Culture: HUVECs were grown in EGM-2 medium at 37ºC with 5% CO_2 in a humidified atmosphere. Cells were cultured up to 90% confluency to ensure $\alpha_v\beta_3$-integrin expression.

Contrast Agent: 200 nm liposomes, containing Gd-BSA, DSPC, cholesterol, PEG_{2000}-DSPE and Mal-PEG_{2000}-DSPE and Rhodamine-PE were produced by lipid film hydration and extrusion. [1] A cyclic RGD-peptide, targeted towards $\alpha_v\beta_3$-integrin was coupled to the distal end of Mal-PEG_{2000}-DSPE.

Experiments: HUVECs were incubated with either $\alpha_v\beta_3$ targeted RGD-liposomes (RGD) or non-targeted liposomes (NT) at a concentration of 1 µmol lipid/ml at 37ºC, for different periods of time (0 to 24 hours). After incubation, the cells were washed, trypsinized, pelleted (~$2\cdot10^6$ cells/pellet) and fixed.

Imaging: Both T_1 and T_2 were measured at 6.3T using a fast inversion recovery FLASH and a multi spin-echo sequence, respectively. All CLSM images were obtained confocally. CD31 was imaged using an Alexa Fluor 488 secondary antibody conjugated to an anti hCD31 primary antibody. Liposomes were visualized by imaging the rhodamine present in the bilayer of the contrast agent. Nuclei were visualized using DAPI.

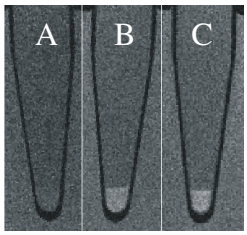

Fig 1: T_1 weighted images of cell pellets: **A)** no incubation, **B)** 2hr incubation with RGD-Liposomes, **C)** 2hr incubation with NT liposomes

Gadolinium content: Gd^{3+} concentration was determined by combining ICP-Mass Spectrometry data (total Gd^{3+} content of cell pellet) and cell pellet volume. Volume of the pellet was determined using a 3D FLASH sequence.

Results & discussion: Figure 1 shows T_1 weighted images of three different pellets, one not incubated (A) and the others incubated with either RGD (B) or NT-liposomes (C). Signal enhancement by the contrast agent was comparable for RGD and NT. This is further illustrated in Figure 2A, which shows the change in relaxation rate R_1 upon incubation time resulting in only slightly higher values for RGD than NT. Remarkably, Figure 2B shows a six times higher uptake (expressed as Gd^{3+} concentration of the cell pellet) of RGD compared to NT. Apparently, the targeted contrast agent was far less effective in T_1 shortening than the non-targeted contrast agent. R_1 versus Gd^{3+} concentration was linear for NT liposomes with an effective $r_1 = 4.6\ mM^{-1}s^{-1}$, close to the r_1 for a liposome suspension at this field strength. For RGD-liposomes, however, the effective r_1 was much lower and not constant, ranging from 1.8 at low Gd^{3+} content to 0.4 $mM^{-1}s^{-1}$ at higher Gd^{3+} content. The R_2 was linear for both NT and RGD-liposomes with r_2=15.3 and 26.9 $mM^{-1}s^{-1}$, respectively. (Figure 2C)

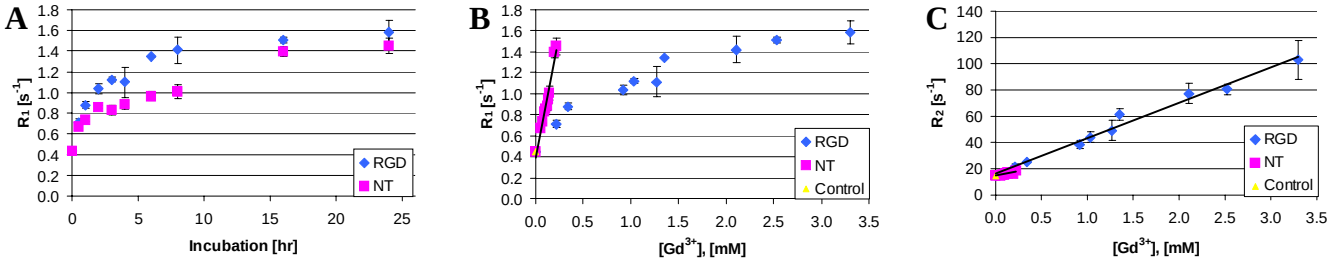

Figure 2. A) R_1 vs Incubation time, B) R_1 vs $[Gd^{3+}]$, C) R_2 vs $[Gd^{3+}]$

Next we studied the liposome internalization pathway. Figure 3 shows confocal laser scanning microscopy (CLSM) images of HUVECs for different incubation times. Both contrast agents seem to be internalized by endosomation and accumulate in the perinuclear recycle center. However, NT-liposomes proved to be mainly present in small intracellular compartments (0.1 to 1 µm in size), whereas RGD-liposomes accumulated in much larger (1 to 5 µm) structures. The efficacy of Gd-based contrast agents depends crucially on water exchange. We postulate that, although the large intracellular compartments are loaded with contrast agent, the low surface to volume ratio of these structures limits water exchange to the remainder of the cell. It can be expected that this process strongly lowers the effective relaxivity.

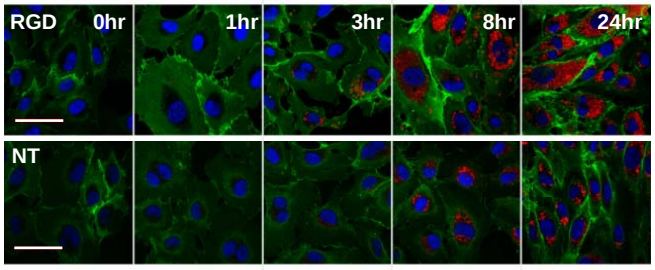

Fig 3, CLSM images of HUVECs incubated with RGD (upper row) and NT liposomes (lower row). Green: CD31, Red: Rhodamine (liposomes), Blue: DAPI. Laser intensity used for NT was eightfold higher than for RGD. Bar = 50µm.

Conclusions: This study showed that the effective relaxation enhancement introduced by an internalized contrast agent depends crucially on the intracellular localization and concentration. The effective relaxivity of internalized $\alpha_v\beta_3$-targeted liposomes was much lower than that of non-targeted liposomes. Although it remains to be demonstrated, this quenching effect may also occur in vivo, which would hamper the detection of a targeted contrast agent with T_1-weighted MRI. We are currently exploiting options to design targeted contrast agents that avoid endosomation and the resulting quenching effect, which would be highly desirable for in vivo applications.

1) Mulder, W.J., et al., Faseb J, 2005. 2) Winter, P.M., et al., Circulation, 2003. 3) Terreno, E., et al., Magn Reson Med, 2006.