pH-Dependent Cellular Internalization of Paramagnetic Nanoparticle

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Experimental Procedures

Reagents: All reagents used were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated. NHS-Rhodamine was obtained from Thermo Scientific (Rockford, IL). Generation 5 (G5) of polyamido-amine (PAMAM) dendrimer solution was purchased from Dendritech (Miland, MI). PAMAM dendrimer was first freeze dried under vacuum and resuspended with PBS buffer solution for conjugation reactions. Dendrimeric chelates and their conjugates were purified by repeated ultrafiltration with deionized water using appropriate molecular weight cut-off Millipore's Amicon Ultra centrifugal filters. sulfo-LC-SPDP were purchased from Sigma. Bt-pHLIP (AEQNPIYWARYADWLFTTPLLLLDLALLVDADEGTCG-(dpeg$_4$-biotin) was custom made from New England peptide (Boston, MA).

Conjugation of pHLIP and Rhodamine dye with dendrimer-based paramagnetic nanoparticle: (GdDOTA-4AmP)$_{44}$-G5 was synthesized and characterized according to our recently published method.[1] (GdDOTA-4AmP)$_{44}$-G5 (0.19 g, 2.4 μmol) was reacted with heterobifunctional cross-linker sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate (sulfo-LC-SPDP) (0.005 g, 0.01 mmol) in PBS (pH 7.4) at room temperature for 6 hours. Then pyridinyldisulfide activated (GdDOTA-4AmP)$_{44}$-G5 was repeatedly filtered through a Centricon C-30 diafiltration cell with a 30 kD MWCO until SEC-HPLC revealed that no further low molecular weight material was present. A version of biotinylated pHLIP (0.02 g, 0.42 μmol) with a single cysteine residue at its C terminus (AEQNPIYWARYADWLFTTPLLLLDLALLVDADEGTCG-(dpeg$_4$-biotin) was dissolved in DMSO/H_2O (1:1) and was slowly added to the reaction mixture and further stirred for 6 hours at room temperature. The product was repeatedly filtered through a Centricon C-30 diafiltration cell with a 30 kDa MWCO. The retentate was lyophilized to obtain the final dendrimer-based paramagnetic agent, Gd$_{44}$-G5-ss-Bt-pHLIP. Biotin molecule is attached to the C-terminus of pHLIP in order to quantify the number of pHLIP peptides conjugated with Gd$_{44}$-G5. The number of biotin molecules conjugated with PAMAM Gd$_{44}$-G5 dendrimer was determined using

HABA-avidin assay as instructed by the provider (Pierce Chemical). The HABA assay with biotin and avidin revealed that on average 3.1 molecules of biotin were present in Gd_{44}-G5-ss-Bt-pHLIP dendrimer. Since biotin is attached with pHLIP peptide, therefore, 3.1 pHLIP peptides are also present in Gd_{44}-G5 particle. Finally, rhodamine-NHS dye was conjugated to the remaining amines surface of preloaded Gd_{44}-G5-ss-Bt-$pHLIP_3$ according to our recent published method [2] in order to achieve final conjugate Rho-Gd_{44}-G5-ss-Bt-$pHLIP_3$ as shown in **Figure 1.** The labeling degree of rhodamine was determined by measuring the absorbance of rhodamine (ε_{552} = 80,000 $M^{-1}cm^{-1}$), and 1.2 molecules of rhodamine were conjugated with each dendrimer-based paramagnetic nanoparticle.

Zeta potential measurement: The exterior surface charge of Rho-Gd_{44}-G5-Bt-$pHLIP_3$ particle is assessed by zeta potential measurement (Malvern zetasizer nano series). The surface charges of Gd_{44}-G5-Bt-$pHLIP_3$ at pH 7.4 and 6.5 were -34.21± 1.17 mV, -2.99 mV and -2.41 ± respectively.

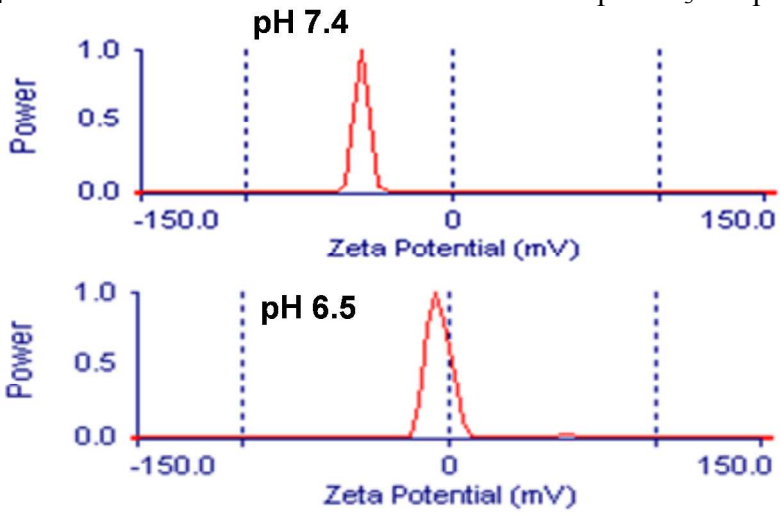

Figure S-1: pH dependant zeta potential of Rho-Gd_{44}-G5-ss-Bt-$pHLIP_3$ nanoparticle.

Fluorescence Imaging: Differentiated cancer cells were incubated in the presence of Rho-Gd_{44}-G5-ss-Bt-$pHLIP_3$. After 3 h of incubation at 37°C, 5% CO2, the cells were washed with probe free media, fixed in 3% paraformaldehyde and analyzed by fluorescent microscopy using rhodamine excitation/emission filters.

Reference

(1) Bhuiyan, M. P.; Aryal, M. P.; Janic, B.; Karki, K.; Varma, N. R.; Ewing, J. R.; Arbab, A. S.; Ali, M. M. Concentration-independent MRI of pH with a dendrimer-based pH-responsive nanoprobe. *Contrast media & molecular imaging* **2015**, *10*, 481-486.

(2) Huang, Y.; Coman, D.; Hyder, F.; Ali, M. M. Dendrimer-Based Responsive MRI Contrast Agents (G1-G4) for Biosensor Imaging of Redundant Deviation in Shifts (BIRDS). *Bioconjugate chemistry* **2015**, *26*, 2315-2323.