

## Supplementary Information

Synthesis and Cellular Uptake of a MR Contrast Agent Coupled to an Antisense Peptide Nucleic Acid - Cell Penetrating Peptide Conjugate

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TITLE RUNNING HEAD: Antisense PNA conjugated MR contrast agent

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**Peptide synthesis.** Peptide synthesis was performed by solid-phase Fmoc/tBu-chemistry using a Heidolph Synthesis 1 synthesizer. Wang resin was used as a solid support at a substitution level of 0.30 mmol/g. Fmoc-protected amino acid (four fold excess) were activated in situ by HBTU/HOBt. After 40 min, the Fmoc protecting group was removed by 20% piperidine in DMF twice for 10 min each. The resin was washed with DMF (6X) after each coupling and deprotection step. All chemical steps were followed by a Kaiser test on the resin, and the resulting colorimetric reaction indicated the presence of free primary amines after Fmoc deprotection and the absence of primary amines after the coupling steps.

**PNA synthesis.** Syntheses of dsRed-PNA (tcc gtg aac ggc) and nonsense PNA (ggt cag agt cta) were performed in continuous solid phase synthesis after coupling of an AEEA spacer. Fmoc/Bhoc chemistry was used with the following PNA monomers: Fmoc-A(Bhoc)-OH, Fmoc-C(Bhoc)-OH, Fmoc-G(Bhoc)-OH, and Fmoc-T-OH. Each cycle of elongation consisted of (1) Fmoc deprotection with 20% piperidine in DMF for 2 cycles of 2.5 min at room temperature, (2) washing with DMF, DCM, and twice with NMP for 1min each at room temperature, (3) coupling using a molar ratio of resin/monomer/HATU/DIEA/2,6-lutidine = 1.0:3.0:3.0:3.0:4.5; 3 min on preactivation followed by 30 min coupling at room temperature, (4) capping with 2 mL of 5% acetic anhydride/6% 2,6-lutidine in DMF for 5 min at room temperature, and (5) washing with NMP, DCM, and twice with DMF for 1 min each at room temperature. All chemical steps were followed by a Kaiser test on the resin, and the resulting colorimetric reaction indicated the presence of free primary amines after Fmoc deprotection

and the absence of primary amines after the coupling and capping steps. After 3, 6, 9, and 12 cycles, aliquots of the resin-bound PNAs were cleaved and Boc-deprotected with H<sub>2</sub>O/TIS/TFA (2.5:2.5:95) for 1 h, and ESI-MS analysis was performed to confirm that the observed masses were consistent with the calculated molecular weights of the Fmoc-protected intermediates.

**Synthesis of 4,7,10-tricarboxymethyl-*tert*-butyl ester 1,4,7,10-tetraazacyclododecane-1-acetate (DOTA-(tBu)<sub>3</sub>)** (see reference 28): In brief:

(1) 1,4,7,10-tetraazacyclododecane-1-carboxymethyl-benzylester (**1**): To a stirred solution of 1,4,7,10-tetraazacyclododecane (cyclen) in acetonitrile was added dropwise benzyl bromoacetate (0.5eq.) in acetonitrile. Reaction completed at room temperature for 2h.

(2) 1,4,7,10-tetraazacyclododecane-4,7,10-tricarboxymethyl-*tert*-butylester-1-carboxymethyl-benzylester (**2**): To a stirred solution of **1** in acetonitrile K<sub>2</sub>CO<sub>3</sub> (4eq.) was added followed by the dropwise addition of *tert*-butyl bromoacetate (4eq.) in acetonitrile within 30 min. Reaction completed at room temperature for 48 h.

(3) 4,7,10-tricarboxymethyl-*tert*-butyl ester 1,4,7,10-tetraazacyclododecane-1-acetate (**3**): Pd/C (10% Pd) was added to a solution of **2** in MeOH, and hydrogenation was conducted for 4h at normal pressure. The catalyst was removed by filtration through Celite. The solvent was removed by a rotary evaporator. The resulting yellowish oil was purified by column chromatography (silica gel, 10% MeOH in DCM) to afford the product **3**. ESI-MS (+): calcd C<sub>28</sub>H<sub>52</sub>N<sub>4</sub>O<sub>8</sub>: m/z 572.38; found 573.3 (M+H)<sup>+</sup>; 595.3 (M+Na)<sup>+</sup>.

**Conjugation of DOTA tris(*tert*-butyl) ester (DOTA-(tBu)<sub>3</sub>) with PNA.** The resin-bound PNAs were deprotected with 20% piperidine and coupled with one Fmoc-Lys(Dde)-OH residue. Fmoc was deprotected with 20% piperidine and washed with NMP, DCM, and twice with DMF for 1 min each. Then DOTA tris(*tert*-butyl) ester was coupled on the  $\alpha$ -NH<sub>2</sub> of Lys using a molar ratio of resin: DOTA-(tBu)<sub>3</sub>/HATU/DIEA=1:3:3:6 reacting for 2 h. The complete coupling was demonstrated by Kaiser Test to indicate absence of free amines.

**Labeling of PNA with FITC.** The resin-bound DOTA-(tBu)<sub>3</sub>-Lys(Dde)-PNA was treated twice with 2% hydrazine hydrate in DMF to deprotect the Dde group. Then FITC was coupled on the  $\epsilon$ -NH<sub>2</sub> of Lys using a molar ratio of resin/FITC/DIEA = 1:4:8 reacting for 12 hours. The complete coupling was demonstrated by Kaiser Test to indicate absence of free amines.

The PNA-peptides were cleaved off the resin using TFA/TIS/H<sub>2</sub>O (95:2.5:2.5, v/v/v) for 4 hours. Crude products were precipitated by adding cold diethyl ether (-20°C). The precipitated product was collected by centrifugation and re-suspended in cold diethyl ether. This procedure was repeated twice. Finally products were dissolved in tBuOH/H<sub>2</sub>O (4:1, v/v) and lyophilized.

**Chelating with gadolinium.** PNA conjugate was dissolved in 5 mL H<sub>2</sub>O, one equivalent of GdCl<sub>3</sub>.6H<sub>2</sub>O was added, and the pH was adjusted to 6.5 with Na<sub>2</sub>CO<sub>3</sub>. The reaction mixture was stirred at 60°C for 12 h. The pH was periodically checked and adjusted to 6.0-7.0 using a solution of Na<sub>2</sub>CO<sub>3</sub> and HCl as needed. Afterwards, the mixtures were purified in a first step by HPLC using a water (0.05% TFA) (solvent A)/ACN (0.05% TFA) (solvent B) gradient (from 20% to 40% of solvent B in 40 min) to separate unstable bound Gd<sup>3+</sup>. The

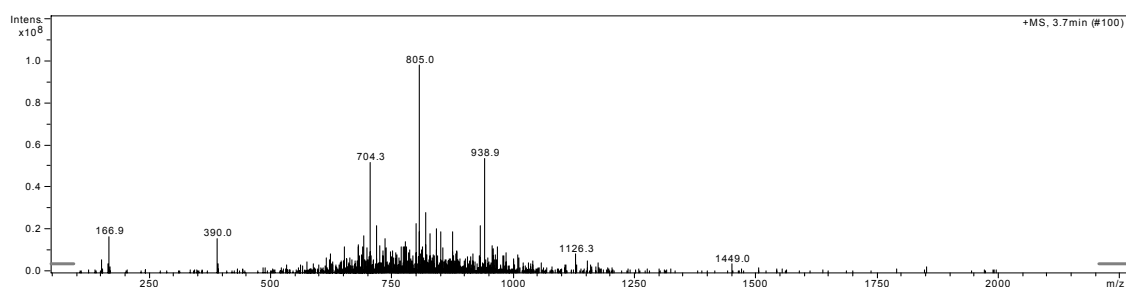
resulting compound was dialyzed (Float-A-Lyzer, cellulose ester membranes, MWCO: 2,000; Spectrum Laboratories, Inc.) to remove other impurities. The solution was lyophilized, and yellow to orange solid was obtained. The absence of free  $Gd^{3+}$  was checked with xylenol orange indicator.

Furthermore, a competitive ligand study was done with DTPA to check the compounds for unstable bound gadolinium. Samples were mixed with excess of DTPA or water, were repurified to remove potential DTPA-chelated gadolinium, and the relaxation rates  $R_1$  were measured at 300 MHz. No significant differences were observed indicating the absence of not-tightly bound Gd in our CA after HPLC purification and dialysis.

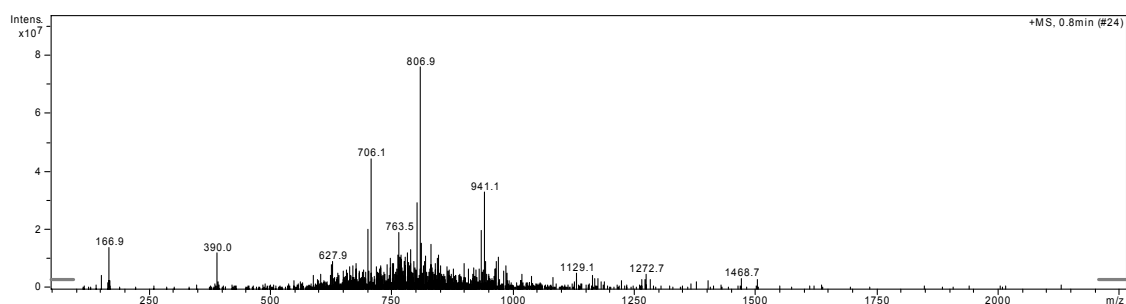
**ESI-MS.** ESI-MS was performed on Agilent 1100 Series LC/MSD Trap system (Agilent, Germany): Nebulizer, 20.0 psi; Dry gas, 5.0 L/min; Dry temperature, 250°C ; Compound stability, 100%; Trap drive level, 100%.

ESI-MS spectra of the compounds:

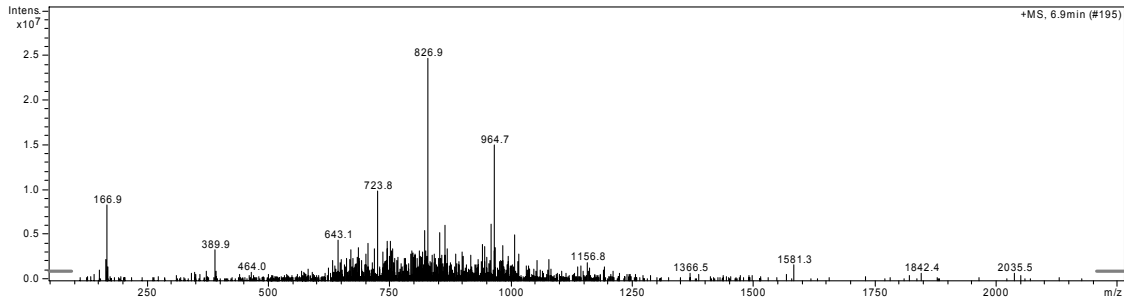
anti-dsRed PNA ligand (MW=5628.2);



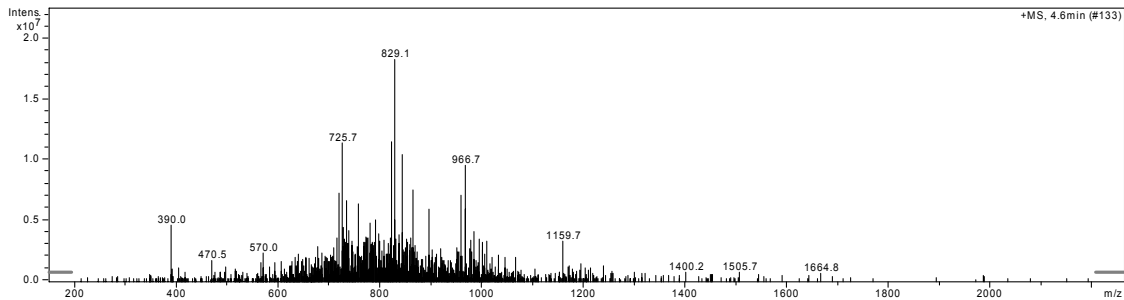
Nonsense PNA ligand (MW=5642.4);



dsRed CA (MW=5782.4);



Nonsense CA (MW=5796.6);



**Data analysis:** The *fitting* to relaxivity curves was performed with self-written routines in MATLAB 6.5 R13 (The Mathworks Inc.). The series of  $T_1$  and  $T_2$  relaxation data were fitted to the following equations: a)  $T_1$  series with varying  $t = TR$ :  $S = S_0 (1 - a \times \exp(-t / T_1))$ . b)  $T_2$  series with varying  $t = TE$ :  $S = S_0 \exp(-t / T_2) + b$ . Nonlinear least-squares fitting of three parameters  $S_0$ ,  $T_1/T_2$ , and  $a/b$  was done for each voxel with the Gauss-Newton method (MATLAB function `nlinf`). For each fitted parameter, the 95% confidence intervals were calculated (MATLAB functions `nlparci`, `nlpredci`) and used as an error estimate of the fitted

relaxation times  $T_1/T_2$  and  $S_0$  (initial signal at  $t = 0$ ). The fit procedure resulted in parameter maps of  $T_1$ ,  $T_2$ ,  $S_0$  and corresponding error maps  $\sigma_{T_1}$ ,  $\sigma_{T_2}$ ,  $\sigma_{S_0}$ .

Image-regions around the tubes were defined as Regions Of Interest (ROIs), and the means and distribution width of the relaxation times of voxels in these regions were calculated: An *iterative* Gaussian fit was used to determine mean and standard deviation (SD) of a distribution with outliers. For this purpose, a distribution histogram was first fitted to a Gaussian to estimate mean and SD. The tails of the distribution were then discarded by using a threshold of three SDs. A repeated fit proved to be robust and converged to the 'true' Gaussian mean and width of the distribution barring the outliers, observed as a result of the non-linear fit of noisy voxels. The processing of the relaxation data thus resulted in specific  $R_{1,2} = 1/T_{1,2}$  values for each tube sample including the standard deviation in the selected ROI ensemble. The ensemble error matched closely the errors of a single-voxel fit, which showed that no further systematic errors were introduced by the image encoding. Finally, the relaxivity  $r_{1,2}$  was calculated from the slope of  $R_{1,2}(c)$  versus the concentration  $c$  of the contrast agent by an error-weighted linear regression.