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Design of (Gd-DO3A)_n-Polydiamidopropanoyl-Peptide Nucleic Acid-_D-(Cys-Ser-Lys-Cys) Magnetic Resonance Contrast Agents

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Abstract

We hypothesized that chelating Gd(III) to 1,4,7-tris(carboxymethylaza)cyclododecane-10-azaacetamide (DO3A) on peptide nucleic acid (PNA) hybridization probes would provide a magnetic resonance genetic imaging agent capable of hybridization to a specific mRNA. Because of the low sensitivity of Gd(III) as a magnetic resonance imaging (MRI) contrast agent, a single Gd-DO3A complex per PNA hybridization agent could not provide enough contrast for detection of cancer gene mRNAs, even at thousands of mRNA copies per cell. To increase the Gd(III) shift intensity of MRI genetic imaging agents, we extended a novel DO3A_n-polydiamidopropanoyl (PDAP^m) dendrimer, up to $n = 16$, from the N-terminus of KRAS PNA hybridization agents by solid phase synthesis. A C-terminal _D-(Cys-Ser-Lys-Cys) cyclized peptide analog of insulin-like growth factor 1 (IGF1) was included to enable receptor-mediated cellular uptake. Molecular dynamic simulation of the (Gd-DO3A-AEEA)₁₆-PDAP⁴-AEEA₂-KRAS PNA-AEEA-_D-(Cys-Ser-Lys-Cys) genetic imaging nanoparticles in explicit water yielded a pair correlation function similar to that of PAMAM dendrimers, and a predicted structure in which the PDAP dendron did not sequester the PNA. Thermal melting measurements indicated that the size of the PDAP dendron included in the (DO3A-AEEA)_n-PDAP^m-AEEA₂-KRAS PNA-AEEA-_D-(Cys-Ser-Lys-Cys) probes (up to 16 Gd(III) cations per PNA) did not depress the melting temperatures (T_m) of the complementary PNA/RNA hybrid duplexes. The Gd(III) dendrimer PNA genetic imaging agents in phantom solutions displayed significantly greater T_1 relaxivity per probe ($r_1 = 30.64 \pm 2.68 \text{ mM}^{-1} \text{ s}^{-1}$ for $n = 2$, $r_1 = 153.84 \pm 11.28 \text{ mM}^{-1} \text{ s}^{-1}$ for $n = 8$) than Gd-DTPA ($r_1 = 10.35 \pm 0.37 \text{ mM}^{-1} \text{ s}^{-1}$), but less than that of (Gd-DO3A)₃₂-PAMAM dendrimer ($r_1 = 771.84 \pm 20.48 \text{ mM}^{-1} \text{ s}^{-1}$) ($P < 0.05$). Higher generations of PDAP dendrimers with 32 or more Gd-DO3A residues attached to PNA-_D-(Cys-Ser-Lys-Cys) genetic imaging agents might provide greater contrast for more sensitive detection.

Keywords

chelator; dendrimer; hybridization; molecular imaging; noninvasive; oncogene; receptor; solid phase synthesis

INTRODUCTION

Noninvasive molecular imaging of gene expression by intracellular hybridization of complementary oligonucleotide derivatives to specific messenger RNAs in cells has been attempted by radionuclide imaging or fluorescent imaging to diagnose cancer and other diseases in living systems.¹⁻¹³ Radionuclide imaging is very sensitive, but generally used in human subjects when suspect masses are evident or highly likely. Fluorescent and luminescent imaging are impractical for any suspect mass >2 cm below the surface of the skin. As an alternative, magnetic resonance imaging (MRI) might be effective for noninvasive intracellular hybridization imaging of cancer gene mRNAs in deep-seated malignant foci, due to its capability of 25-100 μ m spatial resolution.⁶ To achieve satisfactory contrast-enhanced imaging of the tumor in a live subject, however, it will be necessary to create a high concentration of Gd-chelated probes in the targeted cancer cells.

Successful intracellular genetic profiling requires multiple steps: probe distribution to tissues, probe permeation into tissue, endocytosis into the cells, hybridization with the target mRNA, and effluxing of unbound probes. To permit a specific genetic image in the targeted cells, nonhybridized probes must efflux from cells that express negligible levels of the target mRNA. Peptide nucleic acids (PNA)¹⁴ have great potential for hybridization applications because PNA is resistant to biological degradation and binds to complementary mRNA with affinity, specificity, and stability exceeding those of corresponding DNA/RNA duplexes.¹⁵ PNA oligomers are uncharged and poorly taken up by cells, which minimizes nonspecific accumulation in cells.¹⁶

Fortunately, delivery of PNA into all cells can be facilitated with basic cytoplasmic transport peptide motifs or nuclear localization peptide motifs conjugated to PNA.^{4,17-19} We previously reported that specific internalization of PNA into malignant cells that overexpress insulin-like growth factor 1 receptors (IGF1R) can be accomplished with a cyclized IGF1 tetrapeptide analog α (Cys-Ser-Lys-Cys) conjugated to PNA.^{7,20} IGF1R internalizes IGF1 into endosomes that acidify, releasing cargo to the cytoplasm, followed by recycling of IGF1R back to the cell surface.^{21,22}

We found that chelating radioactive metal ions [^{99m}Tc for scintigraphic imaging^{7,9} or ⁶⁴Cu for positron emission tomography (PET) imaging^{11,12}] to specific chelator-spacer-PNA-spacer- α (Cys-Ser-Lys-Cys) hybridization probes (see Figure 1) enabled genetic imaging of oncogene mRNAs in cells and living subjects. We imaged *CCND1* mRNA in breast cancer xenografts with specific genetic PET imaging agents, yielding an 8-fold xenograft:contralateral intensity ratio.¹² IGF1 blocking reduced the xenograft signal to background. Similarly, we imaged mutant *KRAS* G12D mRNA in pancreas cancer xenografts with genetic PET imaging agents that exhibited single mismatch specificity, yielding an 8-fold xenograft:contralateral intensity ratio.^{11,23} Control chelator-spacer-PNA-spacer- α (Cys-Ser-Lys-Cys) with 1, 2, or 3 mismatches in the PNA sequence or with Ala replacement peptide sequences showed only background accumulation.¹¹ These results validated noninvasive radioimaging of specific mRNA targets in cancer cells in animal models. Our recent studies of uptake and efflux of the corresponding fluorescent *KRAS* agent by live pancreas cancer cells in real time confirmed the requirement for a correct IGF1 analog, and yielded a mass transfer coefficient.²⁴

However, radiohybridization imaging carries with it the disadvantage of normal tissue exposure to radiation. Therefore, we hypothesized that chelating multiple Gd(III) cations to multiple 1,4,7-tris(carboxymethylaza)cyclododecane-10-azaacetylamine (DO3A) chelators bonded to PNA hybridization probes would provide a magnetic resonance contrast agent capable of hybridization to a specific mRNA. Accumulation of Gd-chelated hybridization probes in cancer cells is limited by the concentration of the specific mRNA targets in the cancer cells, e.g., 20,000 *KRAS* mRNAs in a 1 pL pancreas cancer cell, based on our QRT-PCR analysis,¹¹ or ~30 nM. At one Gd(III) per PNA hybridization probe bound to a single mRNA, 30 nM Gd(III) would not be sufficient for detectable MRI contrast enhancement. Therefore, increasing the number of chelated Gd(III) ions per hybridization probe is necessary, and might be achieved by conjugation of the hybridization probes to polymers or dendrimers with multiple chelator groups.

Polyamidoamine (PAMAM) dendrimers with DTPA chelators to bind multiple Gd(III) ions are also useful MRI contrast agents.²⁵ Fourth-generation PAMAM dendrimers conjugated with 40 Gd(III)-DTPA chelators and a fluorescently labeled anti-EGFR antibody yielded a relaxivity of 323.5 mM⁻¹ s⁻¹.²⁶ A mathematical tissue contrast model was used to estimate a minimum concentration of 300 nM Gd-nanoparticle required for molecular MR visualization in *T*₁-weighted imaging.²⁶ Our preliminary attempts to conjugate HO₂C-spacer-PNA-spacer-D(Cys-Ser-Lys-Cys) activated by 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide to PAMAM dendrimers in water yielded no product (unpublished results), presumably due to side reactions of the activated carboxylates with aromatic amines and lysine amines of HO₂C-spacer-PNA-spacer-D(Cys-Ser-Lys-Cys).

Therefore, we designed a novel family of polydiamidopropanoyl (PDAP) dendrimers, separated from the PNA block by aminoethoxyethoxyacetate (AEEA) spacers, that could be extended from the N-terminus of PNA-AEEA-D(Cys-Ser-Lys-Cys) in a single, continuous, solid phase assembly (Scheme 1). We inserted additional AEEA spacers to protect the PNA probe from interference by the PDAP dendron, and to prevent steric hindrance among DO3A chelators within the dendron. We performed molecular dynamics (MD) simulations of the full imaging nanoparticle to predict its behavior in water, synthesized and purified a series of constructs, and measured the degree of elevation of their *T*₁ relaxivity in phantom solutions.

RESULTS

DO3A, AEEA, and DAP Parameterization

An existing Gd-DO3A parameter set,²⁷ with the gadolinium ion is held in place by four direct bonds to the nitrogen atoms of the DO3A, while the oxygen atoms of the carboxylic acid groups are held in place through electrostatics, was adopted for parameterization of the Gd-DO3A. To provide a more stable structure during charge assignment, the residue in Amber7 was defined as Gd-DO3A-AEEA. The partial charges for each atom of the Gd-DO3A-AEEA residue were calculated as described previously.¹¹ The resulting partial charges are shown in Table S1 (Supp. Info.), while the necessary parameter changes in Amber7 are shown in Table S2. The atom names are shown in Figure 2. A single Gd-DO3A-AEEA was simulated for 3 nsec at 310 K and 1.013 bar in explicit water (TIP3P) to ensure that the parameters did not cause the simulation to crash. The carboxylic acid groups fluctuated, but the expected structure was nonetheless maintained. A snapshot, with the explicit solvent removed, is shown in Figure 3. DAP was parameterized as above. The resulting partial charges are shown in Table S3, while the atom names are shown in Figure 4.

Additional Supporting Information may be found in the online version of this article.

Complete Genetic MRI Nanoparticle Simulation and Analysis

The (Gd-DO3A-AEEA)₁₆-PDAP⁴-AEEA₂-KRAS PNA-AEEA-_D(Cys-Ser-Lys-Cys) genetic imaging nanoparticle was simulated without gadolinium ligated to the DO3A as well as with the gadolinium ion. This was done to evaluate if the presence of the gadolinium ion changes the structure of the MRI imaging nanoparticle. Snapshots of the starting structure and after 3 ns of simulation are shown Figure 5.

The radius of gyration and several pair correlation functions were calculated to evaluate behavior of the equilibrium structure, using the same equations as before.¹¹ The pair correlation functions of water surrounding the peptide, PNA, dendron and Gd-DO3A were calculated. In addition to the distribution of water around the imaging nanoparticle, the pair correlation functions of the PNA, peptide, gadolinium ion, and dendron around the center of the dendron were calculated.

The radius of gyration for both cases studied here (Supp. Info., Figure S1) was extracted from the trajectories obtained as a function of time. The radius of gyration for the case without gadolinium decreased rapidly and steadied out, which agrees with the very compact structure seen in Figure 5C. The average radius of gyration for this case, taking 1.5 ns forward as the equilibrated structure, was $14.61 \text{ \AA} \pm 0.21 \text{ \AA}$. The gadolinium-chelated case started to fluctuate around a steady value after 1500 picoseconds, with a radius of gyration of $16.55 \text{ \AA} \pm 0.53 \text{ \AA}$.

The phenomenon seen here, namely the metal-chelated variant of the molecular imaging nanoparticle exhibiting a larger radius of gyration along with a generally more extended, instead of globular shape, was also seen for the KRAS PET imaging nanoparticle.¹¹ The net charge for the MRI genetic imaging agents was +1 from the positively charged lysine, as the DO3A ligands are neutral when the Gd(III) ion is chelated. The difference in size when comparing the variants with and without Gd(III) present is likely because of the sterically bulkier Gd-DO3A complexes than the DO3A ligands alone. Secondly, the higher local charge densities of the Gd-DO3A ligand complexes repel the different complexes from each other as well as the positively charged peptide analog. These factors combined are likely reasons for the larger radius of gyration for the gadolinium-containing variants of the genetic imaging nanoparticle.

The structure of the molecular imaging nanoparticle can be better understood by investigating its pair correlation function. The pair correlation functions were calculated using only equilibrated time points, which was chosen to be from 1.5 ns forward. Pair correlation functions investigating the behavior of the nanoparticle with respect to itself were calculated from the first carbon atom of the center of the dendron to the PNA, the peptide, and the dendron along with the DO3A ligands of the dendron (Figure S2).

The pair correlation function of the PNA showed a spike 5 Å from the origin and then a steady decay to zero in the gadolinium ligated case, while the nanoparticle without gadolinium has a relatively even increase and decrease. The peptide exhibited a very similar behavior for both the gadolinium-ligated case as well as the gadolinium-free case.

The pair correlation function of the dendron around its center (Figure S2C), is reminiscent of that seen for PAMAM dendrimers.²⁸ Close to the center several spikes are seen, which are the atoms bonded to the center followed by a steady decay. The decay for the gadolinium-free case showed very few minima, indicating that little to no water pockets exist within the dendron. Although the pair correlation function of the dendron includes the DO3A ligands, it was of interest to determine whether the DO3A ligands arrange in any particular way around the center of the dendron (Figure S2D). The case without gadolinium did not show any particular ordering aside from a small spike close to the center followed by a bell-shaped curve decaying to zero.

The gadolinium-ligated case showed a large peak 20 Å from the center and another peak at 25 Å, followed by a cascade of three other smaller peaks.

The pair correlation functions of the different components of the MRI imaging nanoparticle have shown that it forms a compact molecule, which expands upon the ligation of the gadolinium ion. Furthermore, it was revealed that the peptide is not engulfed by the PNA. Solvent accessibility of the different moieties needs to be shown by pair correlation functions of the surrounding solvent.

The pair correlation functions describing the distribution of water around the different parts of the genetic MRI nanoparticle appear in Figure S3. The distribution of water around the PNA (Figure S3A) shows water coming close to the PNA followed by a steady increase in the likelihood of finding water to the bulk density. The fact that the water around the PNA is not at the bulk density is reasonable because the PNA contracted and held the peptide analog and dendron folded closely to it, displacing water in the process. The case without gadolinium exhibited a slower increase to the bulk density in agreement with the more compact structure seen from the radius of gyration. The distribution around the peptide analog (Figure S3B) showed a small spike around 2 Å from the center followed by a steep increase to the bulk density. The spike is likely due to the circular structure of the peptide analog, created by the two cysteines bound to each other. Once again the case without gadolinium bound to its ligands showed a much slower increase to the bulk density due to its much more globular and compact structure. The pair correlation functions of the peptide indicate that in particular the case with gadolinium ligated to it have water coming very close to it without much interference from the moieties to which it is attached. This is further supported by the melting temperatures described below (Table I), which showed virtually no change when compared to a free PNA.

The water distribution around the center of the dendron (Figure S3C) indicates the penetration of water into the dendron. From the pair correlation function it can be seen that water can be present very close to the center of the dendron. For the case without any gadolinium, the pair correlation function showed a quick rise to the density of water close to the center of the dendron followed by a steady increase to bulk density. The gadolinium-chelated nanoparticle exhibited a lower density of water close to the center followed by a steeper increase to the bulk water density. The pair correlation function of water around the gadolinium ion bound to the DO3A ligand (Figure S3D) is of interest to determine whether the complex forces water to arrange around it as seen in experiments reported in the literature.^{29,30} The pair correlation function for gadolinium containing imaging nanoparticle showed two very sharp peaks 3-4 Å from the gadolinium ion, followed by a second peak at 7 Å, and two much smaller peaks at 10 and 15 Å. Comparing the case with gadolinium bound to the case without gadolinium, the effects of the presence of the gadolinium can easily be seen. The gadolinium-free DO3A ligand showed no water close to the center of mass up until 5 Å, where it increased to a plateau much below the bulk density of water. After the plateau around 7 Å, the water density began to increase steadily without exhibiting any of the peaks seen for the cases with gadolinium chelated to the DO3A. This shows that the simulations capture the behavior of the gadolinium-DO3A complex as it induces a degree of order among the water molecules surrounding it. It must be noted that MRI contrast comes from exchange of water molecules in the neighborhood of the gadolinium atoms, so achieving a high water concentration in the vicinity of the gadolinium is valuable.

Synthesis of (Gd-DO3A-AEEA)_n-PDAP^m-PDAP^m-AEEA₂-KRAS PNA-AEEA_n-(Cys-Ser-Lys-Cys) Hybridization Probes

Following solid phase synthesis as in Scheme 1, crude cyclized (DO3A-AEEA)_n-PDAP^m-AEEA₂-KRAS PNA-AEEA_n-(Cys-Ser-Lys-Cys) probes were purified by reversed phase HPLC. As the number of DO3A chelators, *n*, increased, the width of the product peaks increased, implying a distribution of dendrimer populations in the crude products of solid phase

synthesis. Individual peak fractions were analyzed by HPLC, revealing single narrow peaks, then analyzed by electro-spray ionization mass spectroscopy (ESI-MS) (Table I). It is essential to point out that ESI-MS generates multiple peaks, $M+H$, $M+2H$, $M+3H$. . . , with charges of +1, +2, +3. . . , even for a single homogeneous compound. Thus, ESI-MS differs from matrix-assisted laser desorption/ionization mass spectroscopy (MALDI-TOF MS), which generates only an $M + H$ peak (or $M + Na$, $M + K$ peaks) for each component in a mixture. The ESI-MS results were consistent with a single homogeneous compound. Representative results are shown for *KRAS* G12D mutant probes with $n = 2$ (see Figure 6), $n = 8$ (see Figure 7), and $n = 16$ (see Figure 8).

The number of free amino groups available on the growing PDAP dendrimers on the polymer support at each generation was found by measuring the absorbance of the dibenzofulvene-piperidine adduct ($\epsilon_{301} = 7800 M^{-1} \text{ cm}^{-1}$) released upon Fmoc deprotection after each coupling of $(\text{Fmoc})_2\text{-DAP-COOH}$ (Supp. Info. Tables S^4 and S^5). Theoretically, the number of amino groups should double at each coupling. The number of Fmoc deprotection residues released from the growing PDAP dendrimers increased from 1 to 2.00, 3.98, and 7.61 (95.0% yield) for PDAP^3 with eight theoretical amines (Table $S4$). For PDAP^4 dendrimers with 16 theoretical amines, Fmoc deprotection residues released increased from 1 to 1.95, 3.82, 7.58, and 14.16 (88.5% yield) (Table $S5$).

Recognizing the kinetic barriers to Gd(III) chelation to DO3A ,³¹ we determined conditions (80°C for 10 min in $0.10M$ NaOAc , pH 7.5, 10-fold excess GdCl_3) that would yield stoichiometric Gd-DO3A complexes. After chelation of Gd(III) to purified probes, the resulting $(\text{Gd-DO3A-AEEA})_n\text{-PDAP}^m\text{-AEEA}_2\text{-KRAS PNA-AEEA-}_D(\text{Cys-Ser-Lys-Cys})$ complexes were purified again by reversed phase HPLC and characterized by ESI-MS. Representative results are shown for *KRAS* G12D mutant probes with $n = 2$ (see Figure 9) and $n = 8$ (see Figure 10).

The number of active DO3A groups in $(\text{DO3A-AEEA})_n\text{-PDAP}^m\text{-AEEA}_2\text{-KRAS PNA-AEEA-}_D(\text{Cys-Ser-Lys-Cys})$ probes after deprotection and cleavage from the polymer support and HPLC purification was measured and quantified by analytical Gd(III)-DO3A titration (at pH 7.5), followed by spectroscopic measurement of the excess unbound $\text{Gd(III)-arsenazo III}$ complex (at pH 4.0) (Supp. Info. Figures $S^4\text{-}S^6$). The loading ratios of Gd(III) per mole of PNA, as determined by arsenazo III dye titration of free Gd(III) , were 2.0 for WT5503, 7.7 for WT10134, and 14.9 for WT16310 (Supp. Info. Figures S^7 and S^8). These results agreed with magnetic resonance measurements of T_1 relaxivity below.

Influence of Dendrimer Amines, DO3A , and Gd(III) on Thermodynamic Stability of *KRAS* PNA-AEEA- $_D(\text{Cys-Ser-Lys-Cys})$ Hybridization Probes Hybridized to RNA

A representative melting curve is shown in Figure 11 for $m = 1$, $n = 2$. The sigmoid shape of the curve is consistent with a two-state cooperative transition. The melting temperatures in Table I illustrate that increasing numbers of positively charged amines on larger and larger dendrimers increased the T_m s, that increasing numbers of negatively charged DO3A^{-3} chelators lowered the T_m s, and that chelation of positively charged Gd(III) to increasing numbers of negatively charged DO3A^{-3} chelators, neutralizing the charges, yielded constant T_m s. These results agree with standard polyelectrolyte theory.³² The rA:A mismatch in the Val12 sequence consistently lowered the T_m of comparable structures by $11\text{-}12^\circ\text{C}$ relative to the rA:T match in the Asp12 sequence. Hence, the large $(\text{Gd-DO3A})_n\text{-PDAP}^m$ adducts did not perturb hybridization of PNA probes with RNA, in agreement with the results of the MD simulations above. The T_m results were comparable to our observations with smaller chelators.^{11,33}

MRI Phantom Comparison and T_1 Relaxivity

We compared the (Gd-DO3A-AEEA)₂-PDAP¹-AEEA₂-KRAS PNA-AEEA-*D*(Cys-Ser-Lys-Cys), WT5503, and the (Gd-DO3A-AEEA)₈-PDAP³-AEEA₂-KRAS PNA-AEEA-*D*(Cys-Ser-Lys-Cys), WT10134, hybridization probes with the dendrimer agent (Gd-DO3A)₃₂-PAMAM (MW 29595 Da)³⁴, and the standard contrast agent Gd-DTPA (548 Da) (Sigma-Aldrich). Coronal 1-mm slice thickness MR images of 0.4 mL of these compounds at constant 0.6 mM Gd(III) were recorded in 0.5 mL polypropylene centrifuge tubes (see Figure 12). The MRI intensities of (Gd-DO3A-AEEA)₂-PDAP¹-AEEA₂-KRAS PNA-AEEA-*D*(Cys-Ser-Lys-Cys) (1325 pixel/mm²) and (Gd-DO3A-AEEA)₈-PDAP³-AEEA₂-KRAS PNA-AEEA-*D*(Cys-Ser-Lys-Cys) (1296 pixel/mm²) were comparable with the intensity of the control (Gd-DO3A)₃₂-PAMAM dendrimer³⁴ at (1243 pixel/mm²), and about 50% greater than Gd-DTPA (870 pixel/mm²). In terms of molar PNA concentrations, these phantom comparison data mean that the ratios of Gd(III) per PNA were 2.0, 7.8, and 29.8 for the corresponding Gd₂-PNA, Gd₈-PNA, and Gd₃₂-PAMAM probes. These ratios are close to the theoretical ratios: 2, 8, and 32.

The proton T_1 relaxivities, r_1 , of the probes, determined by $(1/T_1)_{\text{obs}} = (1/T_1)_d + r_1[\text{Gd(III)}]$ (see Figure 13), were comparable to the MRI intensities. The measured relaxivities per Gd(III) of $15.32 \pm 1.34 \text{ mM}^{-1} (\text{Gd}) \text{ s}^{-1}$ for (Gd-DO3A)₂-PDAP¹-AEEA₂-KRAS PNA-AEEA-*D*(Cys-Ser-Lys-Cys), $19.23 \pm 1.41 \text{ mM}^{-1} (\text{Gd}) \text{ s}^{-1}$ for (Gd-DO3A)₈-PDAP³-AEEA₂-KRAS PNA-AEEA-*D*(Cys-Ser-Lys-Cys), and $24.12 \pm 0.64 \text{ mM}^{-1} (\text{Gd}) \text{ s}^{-1}$ for (Gd-DO3A)₃₂-PAMAM dendrimer were 1.5-2 times greater per Gd(III) than the relaxivity of $10.35 \pm 0.37 \text{ mM}^{-1} (\text{Gd}) \text{ s}^{-1}$ determined for Gd-DTPA ($P < 0.05$). Hence, the relaxivity per mole of Gd(III) increased with the size of the size of the probe.

Furthermore, the relaxivity per probe rose 75-fold, as the number of Gd(III) per probe rose from 1 to 32 (Table II). As a result, a fully hybridized 30 nM cytoplasmic KRAS mRNA would carry 240 nM Gd(III) at 8 Gd(III) per probe, or 480 nM Gd(III) at 16 Gd(III) per probe, with 15-40-fold elevated relaxivity per Gd(III). These calculated concentrations of intracellular Gd(III) are in accord with the minimum concentration of 300 nM Gd-nanoparticle required for molecular MR visualization in T_1 -weighted imaging.²⁶

DISCUSSION

Molecular Modeling

MD simulations of the (Gd-DO3A-AEEA)₁₆-PDAP⁴-AEEA₂-KRAS PNA-AEEA-*D*(Cys-Ser-Lys-Cys) genetic MRI probe in water (see Figure 5), predicted that it would be able to hybridize unimpeded with KRAS mRNA in an aqueous environment. Incorporation of the metal ion caused the structure to exhibit a larger radius of gyration.

The Gd-DO3A complexation is very strong and has been shown to be unlikely to undergo demetallation in the presence of common serum components,³⁵ yet it has not been shown if very close proximity of Gd-DO3A complexes to each other, or within a larger polymeric matrix, might disrupt the strength of the electrostatic interaction. The pair correlation function of the Gd-DO3A complexes surrounding the center of the dendron (Figure 7d) showed a staggered arrangement, possibly indicating interaction with each other.

The pair correlation functions calculated for the imaging nanoparticle showed that the peptide analog is solvent accessible. Furthermore, it was shown that for the MRI imaging nanoparticle, using the parameters derived previously,²⁷ the behavior of the PNA and peptide are very similar in terms of solvent accessibility and structural arrangement with respect to the N-terminus of the PNA as was seen for the PET imaging nanoparticle.¹¹ This also indicates that the dendron does not interfere with the other two moieties of the imaging nanoparticle. The behavior of the dendron seen from the pair correlation function was also very similar to that

of PAMAM dendrimers,²⁸ further supporting the notion that the dendron does not interfere with the PNA or peptide.

Overall, the MD simulations of the MRI imaging nanoparticle indicated that the peptide analog and PNA are solvent accessible, while the dendron and the peptide, separated from the PNA by AEEA spacers, do not interfere with the PNA. This calculated result implies that the genetic imaging nanoparticle will be able to bind to its intended mRNA target.

Genetic MRI Probe Relaxivities

To create a specific multifunctional PNA-peptide hybridization probe bearing dendron residues which could be internalized by a specific receptor, then bind to a specific mRNA (see Figure 1), and provide sufficient contrast enhancement within cancer cells for MRI, we extended a novel DO3A_n-PDAP^m dendrimer from the N-terminus of a *KRAS* G12D PNA hybridization agent by solid phase synthesis. A C-terminal α (Cys-Ser-Lys-Cys) cyclic peptide analog of insulin-like growth factor 1 (IGF1) was attached to probes to enable receptor-mediated cellular uptake.

In solution phantoms, we observed increasing relaxivities per Gd(III) for the (Gd-DO3A-AEEA)_n-PDAP^m-AEEA₂-*KRAS* PNA-AEEA- α (Cys-Ser-Lys-Cys) hybridization probes as *n* increased from 2 to 8, approaching that of PAMAM dendrimers with *n* = 32. The multi-Gd complexes all displayed greater relaxivities than the small molecule contrast agent Gd-DTPA. This observation correlates with known increases in $1/T_1$ relaxation rates for Gd(III) as the molecular mass of the Gd-carrier increases.^{25,34,36-38} Multiple binding of Gd-chelates to high molecular mass macromolecules, such as serum albumins, polylysine, or dextran, also increases MRI relaxivity of the contrast reagents.^{25,37,38}

From these data we anticipate that (Gd-DO3A-AEEA)_n-PDAP^m-AEEA₂-*KRAS* PNA-AEEA- α (Cys-Ser-Lys-Cys) hybridization probes will exhibit higher relaxivities in live cancer cells upon binding to high molecular mass mRNA/polyribosome complexes, forming large, slow-moving (Gd-DO3A-AEEA)_n-PDAP^m-AEEA₂-*KRAS* PNA-AEEA- α (Cys-Ser-Lys-Cys)/mRNA/polyribosome complexes with few degrees of freedom in viscous cytoplasm. On the basis of the aforementioned theoretical analysis, we postulate that MRI contrast enhancement of hybridization probes bound to mRNA in cancer cells is at least theoretically possible.

We therefore anticipate that elevated relaxivities will yield more intense, possibly discernable MRI contrast in cancer cells than unbound free (Gd-DO3A-AEEA)_n-PDAP^m-AEEA₂-*KRAS* PNA-AEEA- α (Cys-Ser-Lys-Cys) probes in blood and interstitial spaces. In a preliminary evaluation of MRI contrast enhancement in AsPC1 pancreas cancer xenografts at 3 T (Supp. Info. Figures S9-11), we found that 8 Gd(III) per hybridization probe provided detectable contrast at 0.03 mmol Gd kg⁻¹, but two Gd(III) per hybridization probe did not.

Our companion study of tissue distribution and xenograft scintigraphic imaging with ([¹¹¹In] DO3A)₁₆-PDAP⁴-*KRAS* PNA- α (Cys-Ser-Lys-Cys) in the same pancreas cancer model revealed that PDAP dendrimers with up to 16 DO3A chelators attached to PNA-IGF1 analogs did not perturb xenograft uptake of labeled PNA-IGF1 analog probes. The G12D agent displayed the brightest image (xenograft:contralateral >6), while the G12V agent displayed the brightest image in the G12V xenografts.³⁹ Those results and the results in this report validate a preclinical test of multiple-Gd hybridization agents for genetic MRI of pancreatic cancer xenografts. However, higher generations of PDAP dendrimers with 32 or more Gd-DO3A residues attached to AEEA₂-*KRAS* PNA-AEEA- α (Cys-Ser-Lys-Cys) hybridization probes might be necessary to achieve visible MRI contrast enhancement of cancer gene activity in tissues.

MATERIALS AND METHODS

Molecular Dynamics Simulations

The MD simulations of the (Gd-DO3A-AEEA)₁₆-PDAP⁴-AEEA₂-KRAS PNA-AEEA-*D*-(Cys-Ser-Lys-Cys) genetic imaging nanoparticle carried out with Amber7⁴⁰ required the parameterization of several nonstandard residues. The parameters of the *D*-peptides, the PNA, and the aminoethoxyethoxyacetate spacer (AEEA) residues were reported previously.¹¹ The entire molecular imaging nanoparticle was simulated in explicit water (TIP3P). The nonstandard residues were simulated in vacuum using DISCOVER (Insight II, Accelrys) for 1 ns. From this calculation, 10 conformations of the molecule were taken and used to calculate the partial charges using the AM1/BCC method available in the antechamber program of Amber7. The partial charges for each atom were averaged across the 10 conformations.

The genetic imaging nanoparticle built from the nonstandard residues was simulated in explicit water, as described.¹¹ The simulation was run at 310 K, and a pressure of 1.013 bar, and was carried out using a 1 femtosecond time step, with a typical length of 3 ns. The explicit solvent box was taken from the Amber 1999 parameter set,⁴¹ as this force field was designed for general organic and bioorganic molecules. The water model used in these simulations is the TIP3P, with a buffer of at least 13 Å between the molecule and the edge of the periodic simulation box. The cutoff for van der Waals and electrostatic interactions was set to 12 Å.

Assembly of (DO3A-AEEA)_n-PDAP^m-AEEA₂-KRAS PNA-AEEA-*D*-(Cys-Ser-Lys-Cys) Hybridization Probes on Polymer Supports

Hybridization probes were assembled (Scheme 1) by Fmoc coupling, using a four-fold excess of monomers, essentially as described^{42,43} on NovaSyn TG Sieber resin (9-Fmoc-amino-xanthen-3-yloxy TG resin), 0.12-0.17 mmol g⁻¹ (01-64-0092, NovaBiochem, San Diego CA) on a PS3 automated solid phase peptide synthesizer (Protein Technologies, Tucson AZ). Coupling was activated by *N*-[(dimethylamino)-1H-1, 2,3-triazolo[4,5-b]pyridino-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) (GEN076523, Applied Biosystems, Foster City CA) in anhydrous Me₂NCHO (227056-2L, Sigma-Aldrich, Milwaukee, WI) containing 0.4M *N*-methylmorpholine (PS3-MM-L, Protein Technologies). The coupling time for all monomers was 30 min.

First, *D*-(Cys-Ser-Lys-Cys) was extended from the polymer support, starting from the C-terminus, using Fmoc-*D*-Cys(Acm), Fmoc-*D*-Lys(Boc)-OH, and Fmoc-*D*-Ser(tBu)-OH (Novabiochem, San Diego, CA), followed by the linker 8-Fmoc-amino-3,5-dioxo-octanoic acid (Fmoc-aminoethoxyethoxyacetate, Fmoc-AEEA) (Peptides International, Louisville, KY). Next, Fmoc-PNA monomers (Applied Biosystems) were extended sequentially from the N-terminus of the linker-*D*-(Cys-Ser-Lys-Cys)-resin to create the mutant KRAS G12D antisense PNA sequence, N-GCCATCAGCTCC-C, complementary to codons 10-13 of KRAS G12D mRNA.

Finally, the dendrimer was assembled by coupling of two Fmoc-AEEA residues and an (Fmoc)₂-diaminopropanoate (DAP) (B-2265, Bachem Bioscience, King of Prussia, PA), yielding two available amines (generation 1, *m* = 1) (Scheme 1). The dendrimer cycle was repeated to obtain four available amines (Generation 2, *m* = 2), eight available amines, (Generation 3, *m* = 3), and 16 available amines (Generation 4, *m* = 4). 1,4,7,10-tetra (carboxymethylaza)cyclododecane *tert*-butyl ester (DOTA-3tBu) (Macrocyclics, Richardson, TX) was then coupled to samples of generation 1, 3, and 4 resin beads.

After each coupling step unreacted free amino groups were capped by acetylation with 20% Ac₂O in Me₂NCHO containing 0.4M *N*-methylmorpholine, then Fmoc groups were deprotected with 20% piperidine in Me₂NCHO (PS3-PPR-L Protein Technologies), 3 times

for 3 min each. Deprotection and wash solutions were collected and combined after each coupling for quantitation of the released dibenzofulvene-piperidine adduct by absorbance ($\epsilon_{301} = 7800 M^{-1} \text{ cm}^{-1}$). Fmoc yields after each coupling were 95-100% (Suppl. Info. Tables S4 and S5). The typical overall Fmoc yield after 17 coupling steps was 60-70%, which corresponds to 97-98% coupling on average at each step.

Cyclization, Cleavage, and Chromatography of (DO3A-AEEA)_n-PDAP^m-AEEA₂-KRAS PNA-AEEA-_D(Cys-Ser-Lys-Cys) Hybridization Probes

After the final coupling step, the cysteine residues on the polymer support were cyclized to completion on solid phase with 10 equivalents of I₂ in (CH₃)₂NCHO for 4 h at room temperature.³³ Then the hybridization probes were cleaved from the polymer support and deprotected with 80% CF₃CO₂H, 20% m-cresol cocktail, for 4 h at room temperature. The deprotection filtrates plus washes were combined and precipitated in cold Et₂O. The precipitates were washed with Et₂O, dried, and dissolved in water. PNA in hybridization probes was quantitated by absorbance ($\epsilon_{260} = 108,400 M^{-1} \text{ cm}^{-1}$ for *N*-GCCATCAGCTCC-C).⁴⁴

Crude hybridization probes were purified by reversed phase liquid chromatography at 55°C on Alltima C₁₈ columns (Alltech, Deerfield, IL) eluted with linear gradients from 5% CH₃CN in aqueous 0.1% CF₃CO₂H to 30% CH₃CN in aqueous 0.1% CF₃CO₂H. Effluents were monitored at 260, 295, or 300 nm. Peak fractions were lyophilized by centrifugation under vacuum.

Mass Spectroscopy of Purified (DO3A-AEEA)_n-PDAP^m-AEEA₂-KRAS PNA-AEEA-_D(Cys-Ser-Lys-Cys) Hybridization Probes

Electrospray ionization mass spectroscopy (ESI-MS) of positively charged ions was carried out on a API SciEx 3000 spectrometer (Applied Biosystems, Foster City, CA) calibrated with porcine neuropeptide Y (Sigma, St. Louis, MO). A Q1 scan was programmed for 3 s; ~10 scans were averaged to obtain the final spectrum. The spectra of multiply charged ions were deconvoluted by hand (until software was available) and the molecular masses were derived accordingly. Samples were diluted to ~20 pmol μL^{-1} in a diluent of aqueous 0.2% HCO₂H in 50% CH₃CN. Samples were infused at 10 $\mu\text{L min}^{-1}$ into the Ionspray source at a voltage of 5000 V.

Some compounds were also analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry from an α -cyano-hydroxycinnamic acid matrix excited at 338 nm, using a SELDI (Ciphergen, Fremont, CA) calibrated with porcine neuropeptide Y.

Gd(III) Chelation to (DO3A-AEEA)_n-PDAP^m-AEEA₂-KRAS PNA-AEEA-_D(Cys-Ser-Lys-Cys) Hybridization Probes

Spectrophotometric quantitation of unchelated Gd(III) ions was carried out at 23°C by adding 100 μL of a known or unknown Gd(III) sample in 0.10M NaOAc, pH 7.5, to 900 μL of 0.15M NaOAc, pH 4.0, containing 20 nmol of arsenazo III dye. The dye changes from a cherry-red color with a peak at 655 nm to a blue color after forming a specific Gd(arsenazo III)₂ complex in 0.15M NaOAc, pH 4.0, at 23°C^{34,45} (Suppl. Info. Figure S4). Titration spectra (Figure S5) yielded a plot of A₆₅₅ that was linear in the range 0-5 μM Gd(III) (Figure S6).

The number of active DO3A groups in aliquots of (DO3A-AEEA)_n-PDAP^m-AEEA₂-KRAS PNA-AEEA-_D(Cys-Ser-Lys-Cys) hybridization probes (expected to contain 0-10 nmol of DO3A residues) was measured by analytical titration of a known amount of GdCl₃ (10 nmol) in 100 μL of 0.10M NaOAc, pH 7.5, during 10 min incubation at 80°C, followed by mixing

with 20 nmol of arsenazo III dye as above, and measurement of A_{655} (Figures S7 and S8). 90-98% of expected DO3A residues in the hybridization probes were able to bind Gd(III) ions.

Kinetic studies of Gd(III) chelation to (DO3A-AEEA)₂-PDAP¹-AEEA₂-KRAS PNA-AEEA-_D(Cys-Ser-Lys-Cys) over 0-15 min in 0.1 M NaOAc, pH 7.5 at 23, 80, or 90°C revealed stoichiometric chelation reliably after 10 min at 80°C. After the optimal protocol was established, preparative batches of (DO3A-AEEA)_n-PDAP^m-AEEA₂-KRAS PNA-AEEA-_D(Cys-Ser-Lys-Cys) hybridization probes, 0.5 mM in 0.1M NaOAc, pH 7.5, were incubated with at least a 10-fold excess of GdCl₃ at 80°C for 10 min. to maximize chelation. Analytical titration plots of Gd(III) chelation to (DO3A-AEEA)₂-PDAP¹-AEEA₂-KRAS PNA-AEEA-_D(Cys-Ser-Lys-Cys) (Figure S7) and (DO3A-AEEA)₁₆-PDAP⁴-AEEA₂-KRAS PNA-AEEA-_D(Cys-Ser-Lys-Cys) (Figure S8) revealed that 90-98% of the DO3A residues in the hybridization probes were able to bind Gd(III) ions. The final Gd-chelated agents were purified by reversed phase HPLC as above, and then analyzed by ESI-MS.

Thermodynamic Stability of (DO3A-AEEA)_n-PDAP^m-AEEA₂-KRAS PNA-AEEA-_D(Cys-Ser-Lys-Cys) Hybridization Probes Hybridized to RNA

The temperature dependence of light absorbance at 260 nm of KRAS G12D and KRAS G12V (DO3A-AEEA)_n-PDAP^m-AEEA₂-KRAS PNA-AEEA-_D(Cys-Ser-Lys-Cys) hybridization probes hybridized to a KRAS G12D 20-mer RNA, 5'-rAGUUGGAGCUGAUGGCGUAG-3' (Dharmacon, Lafayette, CO) was monitored to determine the relative stabilities of the antisense probe and the mismatch probes essentially as described.^{33,46} To provide the maximum likelihood of a two-state pattern for the duplex association/dissociation transition, the sequence of the RNA was designed to avoid the formation of a hairpin or slipped duplex structure, and to limit the duplex length to 12 bp, leaving a 4 nt overhang on either end of the duplex. Melting curves were recorded at 260 nm in 10 mm path, Teflon-stoppered cuvettes on a Cary 3 spectrophotometer (Varian Associates, Palo Alto, CA) fitted with a thermoelectric temperature-controlled cell holder.

The actual temperature inside the cuvette was monitored using a temperature probe dipped inside one reference cuvette containing buffer. For each temperature ramp, both the KRAS G12D RNA and the (DO3A-AEEA)_n-PDAP^m-AEEA₂-KRAS PNA-AEEA-_D(Cys-Ser-Lys-Cys) hybridization probe were dissolved in 10 mM Na₂HPO₄, pH 7.4, 0.14M NaCl, to a final concentration of 1.0 μM each. The duplexes were annealed by heating at 1°C min⁻¹ to 90°C, and the temperature was held for 3 min at that temperature to dissociate any residual structures before the strands were allowed to re-anneal during cooling to 20°C at 0.5°C min⁻¹.

Thermal melting of the duplexes was carried out by heating at 0.5°C min⁻¹ up to 90-98°C, measuring absorbance at 260 nm, with a spectral bandwidth of 1 nm, every 0.2°C, averaging 10 points collected over 1 s. The melting curves were blanked against the latter buffer alone to account for any changes in buffer absorbance with temperature. The melting temperatures (T_m) were measured in triplicate and calculated automatically as the peak of the first derivative plot.

MRI Phantom Comparison and T_1 Relaxivity

The spin-lattice relaxation times T_1 of test tube phantoms containing the (Gd-DO3A-AEEA)_n-PDAP^m-AEEA₂-KRAS PNA-AEEA-_D(Cys-Ser-Lys-Cys) constructs and the (Gd-DO3A)₃₂-PAMAM dendrimer (29595 Da)³⁴ were measured in 0.15M NaCl, 0.1M NaOAc, pH 7.0 at Gd(III) concentrations of 0.001, 0.01, 0.025, 0.05, 0.1, 0.25, and 0.5 mM. Commercially available Gd-DTPA (548 Da) (Sigma-Aldrich) was measured at 0.005, 0.05, 0.125, 0.25, 0.5, 1.25, and 2.5 mM. The phantoms were imaged using an inversion recovery (IR)-prepared gradient field echo imaging pulse sequence on an Achieva 3 T clinical MRI

system (Philips Medical Systems, Cleveland, OH). The system was fitted with a specially constructed small animal solenoid adapter coil (Philips Medical Systems, Cleveland, OH), with a 38-mm diameter chamber, 80-mm long. The coil was oriented with its long axis perpendicular to the magnetic field B_0 , in the horizontal direction, left-to-right (see Figure 2). Images were collected with an RF flip angle of 15° , a field of view (FOV) of 50 mm, water-fat shift (WFS) = 2.5 pixels, number of signal averages (NSA) = 4, and region of interest (ROI) = 58 pixels/mm².

The acquisition time for imaging 8 slices, with resolution of $0.13 \times 0.13 \times 1$ mm, averaging 4 signals/slice, was ~6 min. Using a series of inversion times (TI) from 22 ms to 900 ms, T_1 was determined for each sample at each concentration by a linear fit of $\ln[M_0 - M(TI)]$ vs. TI, where M_0 is the initial magnetization, and $M(TI)$ is the magnetization after a particular TI.⁴⁷ Since the repetition time (TR) was 150 ms, and the echo time (TE) was 12 ms, M_0 was determined iteratively to optimize the linear fit. Relaxivity, r_1 , was determined based on the equation $(1/T_1)_{\text{obs}} = (1/T_1)_d + r_1[\text{Gd(III)}]$.⁴⁷ Because the gradient field echo sequence generates image intensities that are generally less than $M(TI)$, the linear equation is only a first approximation. Nevertheless, the magnetization was consistent across all samples, including the standard Gd-DTPA, which provided a gold standard.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Schematic of reporter-PNA-peptide hybridization probes for noninvasive magnetic resonance genetic imaging of oncogene expression inside cancer cells. The genetic imaging nanoparticles are designed to bind to the receptor for IGF1, release into the cytoplasm, and hybridize specifically with a mutant *KRAS* mRNA. Multiple chelated Gd(III) ions are intended to provide magnetic resonance contrast enhancement.

FIGURE 2. DO3A with AEEA spacer complexed to gadolinium. Dashed bond represents electrostatic interaction.

FIGURE 3.
Snapshot of molecular dynamics simulation of the Gd-DO3A-AEEA ligand complex after 3 ns in explicit water.

FIGURE 4.
Diaminopropanoyl atom names.

FIGURE 5. Snapshots of molecular dynamics simulations of $(\text{Gd-DO3A-AEEA})_{16}\text{-PDAP}^4\text{-AEEA}_2\text{-KRAS PNA-AEEA-peptide(Cys-Ser-Lys-Cys)}$. **A:** start of the simulation with gadolinium bound using derived Gd-DO3A parameters²⁸. **B:** Same molecule after 3 ns of simulation. **C:** without Gd(III), after 3 ns of simulation. The starting conformation for all three cases looks very similar, so only one example is shown.

FIGURE 6.

Purification of cyclized $(\text{DO3A-AEEA})_2\text{-PDAP}^1\text{-AEEA}_2\text{-KRAS PNA-AEEA-}^{\text{D}}(\text{Cys-Ser-Lys-Cys})$, WT5195. **A:** Reversed phase liquid chromatography of crude product after cleavage from the polymer support (HPLC-crude) on a 4.6×150 mm Altima C_{18} column eluted with a linear 30 min gradient from 5% to 30% CH_3CN in aqueous 0.1% $\text{CF}_3\text{CO}_2\text{H}$, at 1 mL min^{-1} , at 55°C , monitored at 260 nm. **B:** Analytical HPLC of purified peak (HPLC-pure) as in A. **C:** Electrospray ionization mass spectrometry (ESI-MS) of the purified peak in 50% CH_3CN in aqueous 0.2% HCO_2H . Experimental mass: 5195.63 Da; calculated mass: 5194.65 Da.

FIGURE 7.

Purification of cyclized (DO3A-AEEA)₈-PDAP³-AEEA₂-KRAS PNA-AEEA-D(Cys-Ser-Lys-Cys) WT8900. **A:** Reversed phase liquid chromatography of crude product after cleavage from the polymer support (HPLC-crude) as in Figure 6. **B:** Analytical HPLC of purified peak (HPLC-pure) as in A. **C:** Electrospray ionization mass spectroscopy (ESI-MS) of the purified peak as in Figure 6. Experimental mass: 8901.06 Da; calculated mass: 8900.47 Da.

FIGURE 8.

Purification of cyclized (DOTA-AEEA)₁₆-PDAP⁴-(AEEA)₂-KRAS PNA-AEEA-D(Cys-Ser-Lys-Cys) WT13842. **A:** Reversed phase liquid chromatography of crude product after cleavage from the polymer support (HPLC-crude) as in Figure 6. **B:** analytical HPLC of purified peak (HPLC-pure) as in A. **C:** Electrospray ionization mass spectroscopy (ESI-MS) of the purified peak as in Figure 6. Experimental mass: 8901.06 Da; calculated mass: 8900.47 Da.

FIGURE 9. Purification of $(\text{Gd-DO3A-AEEA})_2\text{-PDAP}^1\text{-AEEA}_2\text{-KRAS PNA-AEEA-D(Cys-Ser-Lys-Cys)}$, WT5503. **A:** Analytical reversed phase HPLC profile of crude $(\text{Gd-DO3A-AEEA})_2\text{-PDAP}^1\text{-AEEA}_2\text{-KRAS PNA-AEEA-D(Cys-Ser-Lys-Cys)}$, WT5503, after Gd(III) chelation. **B:** ESI-MS of purified peak fraction of $(\text{Gd-DO3A-AEEA})_2\text{-PDAP}^1\text{-AEEA}_2\text{-KRAS PNA-AEEA-D(Cys-Ser-Lys-Cys)}$, WT5503. Experimental mass: 5503.40 Da; calculated mass: 5503.15 Da.

FIGURE 10. Purification of $(\text{Gd-DO3A-AEEA})_8\text{-PDAP}^3\text{-AEEA}_2\text{-KRAS PNA-AEEA-}\nu(\text{Cys-Ser-Lys-Cys})$ WT10134. **A:** Analytical reversed phase HPLC profile of crude $(\text{Gd-DO3A-AEEA})_8\text{-PDAP}^3\text{-AEEA}_2\text{-KRAS PNA-AEEA-}\nu(\text{Cys-Ser-Lys-Cys})$, WT10134, after Gd(III) chelation. **B:** ESI-MS of the purified $(\text{Gd-DO3A-AEEA})_8\text{-PDAP}^3\text{-AEEA}_2\text{-KRAS PNA-AEEA-}\nu(\text{Cys-Ser-Lys-Cys})$, WT10134. Experimental mass: 10,134.67 Da; calculated mass: 10,134.47 Da.

FIGURE 11.

Absorbance vs. temperature ramp of $1.0 \mu\text{M}$ $(\text{Gd-DO3A-AEEA})_2\text{-PDAP}^1\text{-AEEA}_2\text{-KRAS}$ PNA-AEEA- $\text{D}(\text{Cys-Ser-Lys-Cys})$, WT5503, hybridized to $1.0 \mu\text{M}$ 5'-rAGUUGGAGCUGAUGGCGUAG-3', in $10 \text{ mM Na}_2\text{HPO}_4$, pH 7.4, 0.14 M NaCl .

FIGURE 12.

MRI phantom comparison of Gd contrast agents at 0.6 mM Gd(III) in 0.15M NaCl, 0.1M NaOAc, pH 7.0, with average MRI contrast intensities. **A:** (Gd-DO3A-AEEA)₂-PDAP¹-AEEA₂-KRAS PNA-AEEA-_D(Cys-Ser-Lys-Cys), WT5503, 110 ± 29 . **B:** (Gd-DO3A-AEEA)₈-PDAP³-AEEA₂-KRAS PNA-AEEA-_D(Cys-Ser-Lys-Cys), WT10134, 1325 ± 38 ; **C:** (Gd-DOTA)₃₂-PAMAM, 1296 ± 31 . **D:** (Gd-DOTA)₃₂-PAMAM, 1243 ± 35 . **E:** commercial Gd-DTPA, 0.6 mM, 870 ± 34 . **F:** commercial Gd-DTPA, 0.06 mM, 166 ± 31 . Blank noise was 19 ± 21 .

FIGURE 13.

Plots of $1/T_1$ (obs) vs. $[Gd(III)]$ to determine relaxivity, r_1 , in 0.15M NaCl, 0.1M NaOAc, pH 7.0. ▲: Gd-DTPA; ○: (Gd-DOTA)₃₂-PAMAM; ◆: (Gd-DO3A-AEEA)₂-PDAP¹-AEEA₂-KRAS PNA-AEEA-_v(Cys-Ser-Lys-Cys), WT5503; ■.

SCHEME 1.

Continuous solid phase synthesis of (DO3A-AEEA)_n-PDAP^m-AEEA₂-KRAS PNA-AEEA-D (Cys-Ser-Lys-Cys) hybridization probes, with two DO3A chelators (Generation 1), four DO3A chelators (Generation 2), 8 DO3A chelator groups (Generation 3), or 16 DO3A chelator groups (Generation 4). Gd(III) can be chelated to enable contrast enhancement magnetic resonance imaging (CE-MRI).

Table 1
DO3A-PDAP-dendrimer-KRAS PNA-IGF1 Analogs

Target	Probe	Sequence	Calc. mass (Da)	Obs. mass (Da)	Yield (%)	T_m , °C
Val12 mutant	WT3909	H ₂ N-AEEA-GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	3908.5	3907.8	30.5	69.1 ± 1.0
Val12 mutant	WT4285	(H ₂ N-AEEA) ₂ -PDAP ¹ -AEEA-GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	4285.0	4286.0	23.2	ND
Val12 mutant	WT5818	(H ₂ N-AEEA) ₈ -PDAP ³ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	5817.9	5818.0	16.2	ND
Val12 mutant	WT7668	(H ₂ N-AEEA) ₁₆ -PDAP ⁴ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	7668.2	7666.9	11.3	76.4 ± 1.3
Val12 mutant	WT4295	DOTA-AEEA-GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	4295.5	4295.5	26.5	68.3 ± 0.9
Val12 mutant	WT5204	(DOTA-AEEA) ₂ -PDAP ¹ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	5203.7	5204.6	20.2	68.0 ± 1.2
Val12 mutant	WT8910	(DOTA-AEEA) ₈ -PDAP ³ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	8909.5	8911.9	11.5	66.9 ± 0.5
Val12 mutant	WT13851	(DOTA-AEEA) ₁₆ -PDAP ⁴ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	13850.6	ND	6.8	68.8 ± 0.5
Val12 mutant	WT5512	(Gd-DOTA-AEEA) ₂ -PDAP ¹ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	5512.2	5514.0	16.6	68.0 ± 1.0
Val12 mutant	WT10144	(Gd-DOTA-AEEA) ₈ -PDAP ³ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	10143.5	10143.6	5.8	66.8 ± 0.9
Val12 mutant	WT16319	(Gd-DOTA-AEEA) ₁₆ -PDAP ⁴ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	16319	ND	2.7	66.2 ± 1.0
Asp12 mutant	WT5809	(H ₂ N-AEEA) ₈ -PDAP ³ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	5809.19	5809.70	3.0	ND
Asp12 mutant	WT7659	(H ₂ N-AEEA) ₁₆ -PDAP ⁴ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	7659.01	7659.59	0.71	89.9 ± 2.5
Asp12 mutant	WT5195	(DOTA-AEEA) ₂ -PDAP ¹ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	5194.65	5195.63	12.6	78.7 ± 0.6
Asp12 mutant	WT8900	(DOTA-AEEA) ₈ -PDAP ³ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	8900.47	8901.06	2.4	75.9 ± 0.5
Asp12 mutant	WT13842	(DOTA-AEEA) ₁₆ -PDAP ⁴ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	13841.57	13843.50	0.57	74.1 ± 1.0
Asp12 mutant	WT5503	(Gd-DOTA-AEEA) ₂ -PDAP ¹ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	5503.15	5503.40	10.1	79.7 ± 0.6
Asp12 mutant	WT10134	(Gd-DOTA-AEEA) ₈ -PDAP ³ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	10134.47	10134.67	1.7	78.7 ± 0.6
Asp12 mutant	WT16310	(Gd-DOTA-AEEA) ₁₆ -PDAP ⁴ -AEEA ₂ -GCCAAcAGCTCC-AEEA-d(Cys-Ser-Lys-Cys)	16309.57	ND	0.34	ND

Masses were measured by ESI-MS or MALDI-TOF MS. Yields were calculated with respect to initial amino acid loading of the resin beads. ND: not done.

Table II
Proton T_1 Relaxivities, r_1 , of Gd Contrast Agent Phantoms

Contrast agent	r_1 per mole of probe ($\text{mM}^{-1} \text{s}^{-1}$)	Relative increase in r_1 per mole of probe	r_1 per mole of probe of Gd(III) ($\text{mM}^{-1} \text{s}^{-1}$)	Relative increase in r_1 per mole of Gd(III)
Gd-DTPA	10.35	1.0	10.35	1.0
(Gd-DO3A-AEEA) ₂ -PDAP ¹ -AEEA ₂ -KRAS PNA-AEEA-d(Cys-Ser-Lys-Cys), WT5503	30.64	3.0	15.32	1.5
(Gd-DO3A-AEEA) ₈ -PDAP ³ -AEEA ₂ -KRAS PNA-AEEA-d(Cys-Ser-Lys-Cys), WT10134	153.84	14.9	19.23	1.9
Gd-DO3A) ₃₂ -PAMAM	771.84	74.6	24.12	2.3

Proton T_1 relaxivities, r_1 , were measured in phantoms (Figure 6) at a constant 0.6 mM Gd(III) in 0.15M NaCl, 0.10M NaOAc, pH 7.0.