Cellular Delivery of MRI Contrast Agents

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Summary

Magnetic resonance imaging (MRI) is a powerful tool for acquiring images of opaque living animals with the benefit of tracking events over extended periods of time on the same specimen. Contrast agents are used to enhance regions, tissues, and cells that are magnetically similar but histologically distinct. A principal barrier to the development of MRI contrast agents for investigating biological questions is the delivery of agents across cellular membranes. Here, we describe the synthesis and in vitro testing of Gd(III)-based MRI contrast agents containing varying length polyarginine oligomers capable of permeating cell membranes. We examine the effect of the length of oligomer on $T_1$ enhancement and cellular uptake. Furthermore, the effect of incubation time, concentration, and cell type on uptake is explored. Toxicity and washout studies are performed in addition to MRI phantom studies.

Introduction

The field of developmental biology has undergone significant advances using biological molecular imaging techniques such as computer-enhanced light microscopy imaging, positron emission tomography (PET), micro CT, and magnetic resonance imaging (MRI) [1–4]. Of these techniques, MRI has proven to be a particularly powerful tool in clinical and biological settings because of its ability to noninvasively attain internal images. MRI offers a noninvasive means of acquiring three-dimensional images that can map structure and function in vivo. Images are based upon the NMR signal from the protons of water molecules, where the signal intensity in a given volume element is a function of the water concentration and relaxation times ($T_1$ and $T_2$). Intrinsic contrast can be enhanced by using paramagnetic contrast agents. The paramagnetic ion gadolinium Gd(III) is used commonly in MRI contrast agents to provide increased contrast by decreasing the local $T_2$ of nearby water protons [5]. Our group has prepared MRI contrast agents that allow for the observation of developmental events in vivo [6, 7].

A principal barrier to the further development of new contrast agents for the investigation of developmental biological questions is the ability to transport the agents across cellular membranes. The majority of MRI contrast agents are restricted to the extracellular domains, and there are few examples of membrane permeable MR contrast agents reported in the literature [8–15]. As part of our research in preparing contrast agents that cross cell membranes, we are investigating a number of small molecules that facilitate transport of charged and uncharged species.

The recent development of MR contrast agents that can be used to detect biological processes, such as β-galactosidase activity, has provided the means to obtain direct physiological information in the form of a three-dimensional image [16, 17]. A major limitation of these bioactivated agents is their strictly extracellular nature. Our goal in synthesizing contrast agents that permeate cell membranes is to deliver these agents inside the cell. Accomplishing this goal will allow for the detection of biologically important intracellular molecules with MRI, such as enzyme reporters and secondary messengers.

Recently, we have reported a polyarginine-based MRI contrast agent that is able to permeate cell membranes. We demonstrated the use of two-photon laser microscopy and $T_1$ analysis as a means to observe this process [8]. This work expands on that report through the synthesis and thorough testing of a series of membrane-permeable polyarginine oligomers of varying lengths covalently attached to a chelate framework that coordinates a lanthanide ion to the chelate 1,4,7,10-tetraazacyclododecane -N,N',N"-tetraacetic acid (DOTA). The selection of polyarginine (8, 12, and 16 monomer units) is due to published work that demonstrates the ability of these compounds to cross cell membranes [18, 19]. The mechanism of this delivery is not completely understood; however, it is not a result of adsorptive- or receptor-mediated endocytosis [19]. We report the synthesis and thorough cell culture testing of series of lanthanide(III)-based MRI contrast agents conjugated to polyarginine that are able to permeate cell membranes. The synthesis and physical properties of three agents are compared, and their behavior in cell culture is thoroughly examined.

Results

Compounds 1–9 were synthesized as shown in Figure 1. Standard peptide synthesis techniques were used to prepare 8, 12, and 16 amino acid polyarginine oligomers, which were subsequently conjugated to DOTA(tris-f-Bu ester) [20]. The conditions used to cleave the peptides from the resin simultaneously deprotected the t-butyl esters on the ligand. The desired lanthanide ion was added using the appropriate lanthanide hydroxide. Li-
Figure 1. Synthesis of Polyarginine-Containing Lanthanide Chelates

(a) (1) Piperidine, DMF (2) Fmoc-R(Pbf)-OH, HATU, DMF, DIPEA; (b) (1) piperidine, DMF (2) DOTA(tris-t-Bu ester), HATU, DMF, DIPEA (3) 95% TFA, 2.5% H₂O, 2.5% TIS; (b) Eu(OH)₃ or Gd(OH)₃ in water at 80°C for 12 hr.

gands were characterized using ¹H NMR spectroscopy and mass spectrometry, and final products were purified on a Sephadex G-25 column and characterized by mass spectrometry.

Relaxivity measurements were acquired by taking the slope of a plot of T₁ versus concentration. Measurements were taken in 10 mM 3-(N-morpholino)propane sulfonic acid (MOPS), 100 mM sodium chloride, 20 mM sodium bicarbonate, and 4 mM sodium phosphate monobasic at pH 7.41, 59.97 MHz, and 37°C. Since the exact nature of the counter-anion to arginine was not known, gadolinium concentrations for relaxivity measurements were determined by ICP of the measured solutions. There is a high probability that the counter-anions were TFA [21, 22]. Determination of the gadolinium concentration by ICP-MS allows for the calculation of the molar concentration without knowing the exact identity of the counter-anions.

Octanol-water partition coefficients were obtained by dissolving 7–12 mg of 4, 5, or 6 into mixtures of 500 µl water and 500 µl 1-octanol. The resulting mixture was shaken vigorously for 2 hr on a Lab-Line lab rotator (model number 1304). The solvent layers were allowed to separate, and 400 µl of each layer was removed. The solvent was removed under reduced pressure, and the mass of material from each layer was measured. The reported values are for the mass of compound in the 1-octanol layer divided by the mass of compound in the water layer. All octanol-water measurements were repeated in triplicate, and the average value is reported. Relaxivity values and octanol-water partition coefficients are listed in Table 1.

Table 1. Relaxivity Values and Octanol-Water Partition Coefficients Measured for 4, 5, and 6

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relaxivity (mM⁻¹·s⁻¹)</th>
<th>Octanol-Water Partition Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>6.8</td>
<td>0.0233 ± 0.0003</td>
</tr>
<tr>
<td>5</td>
<td>4.8</td>
<td>0.117 ± 0.039</td>
</tr>
<tr>
<td>6</td>
<td>4.4</td>
<td>0.104 ± 0.068</td>
</tr>
</tbody>
</table>

Relaxivity was measured at 59.97 MHz and 37°C in 10 mM MOPS, 100 mM NaCl, 20 mM NaHCO₃, and 4 mM NaH₂PO₄ at pH 7.41. Values for partition coefficients are plus or minus one standard deviation.

All cell experiments described in this paper were repeated in at least triplicate. The gadolinium(III) complexes, 4–6, were synthesized as MRI contrast agents. Because europium(III) is similar in size to gadolinium(III) but with different fluorescent properties, europium(III) complexes can be used for fluorescence microscopy [8]. The europium(III) complexes, 7–9, were synthesized as fluorescence microscopy dyes; however, because a change in the lanthanide ion should not affect the delivery properties of the polyarginine-containing contrast agent, complexes 4–9 were all used in some cell experiments.
To determine the toxicity of 7, 8, and 9, a 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Mouse fibroblast, NIH/3T3 cells were plated at 150,000 cells/ml in a Costar 96-well plate (polystyrene, flat bottom, tissue-culture treated, black with clear bottom) and incubated overnight. Solutions of 7, 8, and 9 in DPBS were added to the wells to give concentrations ranging from 1 mM to 44 mM. The plate was incubated for 1 hr, at which point 10 μl of MTT was added to each well and incubated for 4 hr at 37°C in a 5% carbon dioxide incubator. The detergent reagent supplied with the MTT assay kit (100 μl) was added to each well and incubated in the dark at ambient temperature overnight. The absorbance at 570 nm was measured with background subtraction and used to determine toxicity. None of the compounds affected cell proliferation below 10 mM.

To examine the effect of contrast agent concentration on cellular uptake, ICP-MS was utilized. NIH/3T3 cells were grown in Costar 96-well plate (polystyrene, flat bottom, tissue-culture treated, black with clear bottom) with modified DMEM containing 10% BCS in a 5% carbon dioxide incubator. Cells were incubated with 4, 5, or 6 for 1 hr at 37°C with concentrations of 0.01, 0.1, 0.5, 3, and 10 mM in modified DMEM containing 10% BCS in a 5% carbon dioxide incubator. At the end of the incubation period, the medium was removed, and the cells were rinsed in triplicate with DPBS at ambient temperature. The cells were exposed to 100 μl of 0.25% trypsin and harvested; they were then counted using a hemacytometer. There was an average of 140,000 cells per well. The trypsin/cell suspensions were incubated with concentrated nitric acid at 80°C for 4 hr. The dissolved cells were diluted to 5 ml. The final cell solutions were in 3% nitric acid with 5 ppb of indium as an internal standard. The samples were analyzed by ICP-MS. The amount of gadolinium per cell was calculated, and no significant difference of uptake of gadolinium per cell was observed between any of the incubation times.

In studying the rate at which the polyarginine contrast agents leached from the cells, ICP-MS was used. NIH/3T3 cells, which exhibit contact-inhibited growth, were grown in Corning brand tissue culture flasks (25 cm² with vent cap) with modified DMEM containing 10% BCS in a 5% carbon dioxide incubator. A monolayer of cells was incubated with 4, 5, or 6 (0.3 mM in modified DMEM containing 10% BCS) for 1 hr at 37°C in a 5% carbon dioxide incubator. At the end of the incubation period, the medium was removed and the cells were rinsed in triplicate with DPBS at ambient temperature. Fresh medium (5 ml) was applied to the cells, and they were returned to the incubator. After periods of 24, 48, 72, and 96 hr, the medium was removed and replaced with fresh medium after triplicate rinsing with DPBS. The removed medium was analyzed for gadolinium concentration using ICP-MS. After removal of the medium at 96 hr, the cells were exposed to trypsin and prepared for ICP-MS as described previously. This process was repeated with sampling times of 1, 3, 6, and 10 hr. The results of these experiments are shown in Figure 3. The amount of gadolinium remaining in the cells after 96 hr was $3 \times 10^{-10}$–$5 \times 10^{-10}$ mol/cell for all three compounds.

To determine if the uptake of the polyarginine contrast agents was specific to NIH/3T3 cells, the uptake of 7, 8, and 9 was examined using NIH/3T3, canine kidney epithelial (MDCK), and mouse macrophage (RAW 264.7).
Figure 4. Cell-Type Specificity for 7–9
Cells were incubated with compound for 1 hr and rinsed with DPBS, treated with trypsin, dissolved in nitric acid, and analyzed with ICP-MS. The graph shows uptake per cell for each cell type plotted against complex. Incubation concentration of 7–9 was constant in T<sub>1</sub> from untreated NIH/3T3 cells. Values for GdDO3A are from within each group of cells but was not constant from compound to compound. Error bars represent one standard deviation.

Figure 5. Results of T<sub>1</sub> Study of NIH/3T3 Cells Incubated with 0.3 mM 4, 5, 6, and Gd(III) DO3A (1,4,7-Tris-Carboxymethyl-1,4,7,10-Tetraazacyclodecane)
After incubation, cells were rinsed with DPBS. T<sub>1</sub> values were measured at 60 MHz and 37°C. The graph depicts the percent change in T<sub>1</sub> from untreated NIH/3T3 cells. Values for GdDO3A are from [28]. Error bars represent one standard deviation.

Figure 6. T<sub>1</sub>-Weighted Spin-Echo MR Images of NIH/3T3 Cells at 9.4 T
Images were obtained using a spin-echo pulse sequence with TR 500 ms, echo delay time (TE) 16 ms.
(A) NIH/3T3 cells incubated with 4.
(B) Untreated NIH/3T3 cells.
(C) Deionized water in a capillary tube as a spatial marker. The scale bar represents 1.2 mm.
from the image that the signal intensity of the untreated cells is similar to that of deionized water, which has a long $T_1$. Both untreated cells and deionized water appear dark; in contrast, the intensity of treated cells is higher by a factor of three and they appear bright. Treatment of NIH/3T3 cells with 4 decreases the $T_1$ of the cells and renders them bright in a $T_1$-weighted MR image.

Discussion

The relaxivities and octanol-water partition coefficients of 4–6 are listed in Table 1. The values for relaxivity range from 4.4 mM$^{-1}$s$^{-1}$ to 6.8 mM$^{-1}$s$^{-1}$, with the longer polyarginine oligomers having smaller relaxivities. These values are slightly higher but on the same order of magnitude as Prohance, which has a relaxivity of 3.1 mM$^{-1}$s$^{-1}$ when measured at the same field strength, temperature, and in the same buffer. This result demonstrates that complexes 4–6 can be used as effective MRI contrast agents.

The octanol-water partition coefficients are all less than one, with the value for 4 less than the values of 5 and 6. The partition coefficient is a measure of hydrophilicity, and the values measured show that the smaller (n = 8) polyarginine complex is more hydrophilic than the larger (n = 12, 16) oligomers. This is a fascinating result because the hydrophilicity would be expected to increase with the addition of the hydrophilic amino acid arginine. Interestingly, no direct correlation between the octanol-water partition coefficients of these complexes and membrane permeability was found. This supports previous reports that octanol-water partition coefficients alone are not a reliable indicator of membrane permeability, and other factors must be taken into account when predicting membrane permeability [24–26].

To determine the toxicity of 4–9, MTT and trypsin blue assays were performed. An MTT proliferation assay was performed with 7–9 which demonstrated that all compounds did not affect cell proliferation or viability of NIH/3T3 cells up to 10 mM. Above 10 mM, cells began to show signs of death, both in morphology and through a decrease in absorbance in the MTT assay. Trypan blue viability studies were performed on all experiments discussed in this paper. All cells, regardless of type, incubated with 4–9 at concentrations up to 10 mM, and for all incubation times studied were at least 98% viable.

Since 4–9 were found not to affect cell proliferation or viability up to 10 mM, a range of concentrations from 0.01 mM to 10 mM were tested to determine the amount of material taken up by NIH/3T3 cells as a function of concentration. The trends for compounds 4–6 were very similar (Figure 2). Uptake at low concentrations (0.01–0.1 mM) was minimal and did not appear to change. From 0.1 mM to 3 mM, a dramatic increase in uptake occurred, corresponding to increasing incubation concentration. This trend ceased at 3 mM, at which point no increase in uptake was observed with increasing concentration. ICP-MS was used in this study because of the facile manner with which it allows for extreme accuracy and precision in measuring a large quantity of samples quickly.

Interestingly, the amount of contrast agent taken up per cell was not dependent on the length of incubation when monitored from 0.5–24 hr. However, the washout rate of 4–6 was dependent on time and the number of rinses. This dual dependence is shown in Figure 3, in which the total amount of gadolinium released from an entire plate of cells is plotted against the number of rinses and the duration of the rinses. For both long time medium changes (24, 48, 72, 96 hr) and short time medium changes (1, 3, 6, 10 hr), more compound leached from the cells on the first rinse (24 hr or 1 hr) than on subsequent rinses. Washout from the cells for subsequent rinses remained constant. The difference in the amount released between the first and subsequent rinses was larger for the short times than the short times. Additionally, >99% of the gadolinium was released from the cells by the end of the fourth rinse (calculated using the residual gadolinium content at the end of the experiment and the amount of gadolinium taken up by the cells in Figure 2). These results demonstrate that there is both time dependence and dependence on the number of rinses for the washout of 4–6.

Three cell types (NIH/3T3, RAW 264.7, and MDCK) were incubated with 7–9 to determine if uptake of the agents was cell-type specific (Figure 4). The experiment demonstrated that 7–9 were taken up by all three cell types and that there was a difference in the amount of agent taken up per cell. The highest loading of agent was seen in NIH/3T3 cells, and the lowest loading was seen in RAW 264.7 cells. The smallest amount taken up per cell for the RAW 264.7 cells likely is due, at least in part, to the smaller size of these cells relative to the other two cell types. The uptake trend between cell lines was the same for all of the three compounds tested.

To examine the relative effect on $T_1$ of 4–6, NIH/3T3 cells were analyzed by $T_1$ analysis (Figure 5). Cells treated with 4–6 and rinsed with medium had significantly shorter $T_1$ values than untreated control cells and cells treated with the extracellular contrast agent gadolinium(III) 1,4,7,10-tetraazacyclododecane-1,4,7-trisacetic acid (GdDO3A); however, no significant difference was detected between cells treated with 4–6 (>95% confidence for 5 degrees of freedom in a Student’s t test) [27]. This experiment demonstrates that all three contrast agents should equally enhance contrast in MRI.

MRI studies were performed to examine the ability of 4 to enhance contrast in an MRI image of NIH/3T3 cells (Figure 6). This experiment demonstrated that cells treated with polyarginine-based contrast agents show increased contrast in an MR image. Importantly, the concentration used in this experiment was well below the level toxic to cells. The results of this experiment, together with the results of other experiments, suggest that polyarginine-containing contrast agents are cell-type specific because they label some cell types more heavily than others. Additionally, the time necessary to label cells enough to be visualized by MRI is short (<0.5 hr). This is a critical experiment that demonstrates the ability of these agents to be employed in the study of biological phenomena using MRI.

Significance

The preparation of a reliable intracellular delivery for probes offers the hope for an extremely powerful tool
for the study of in vivo biological activity and is the subject of ongoing work in our laboratory. This work discusses the synthesis and physical characterization of a series of three MRI contrast agents that are able to permeate cell membranes. The behavior of these modified MR agents in cell culture is examined in depth. The cell culture properties of the agents were examined using ICP-MS and T$_1$ analysis, as well as MRI. The properties of the lanthanide complexes described in this paper are well suited for use in the study of biological systems with MRI.

**Experimental Procedures**

All reagents and solvents were the highest commercially available grades and were used without further purification. 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraazacyclododecane.

Wang resin containing an Fmoc protected arginine (3.0 g, 4.6 mmol), O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (1.7 g, 4.4 mmol), and DMF (5 ml) were combined, and diisopropylpropylamine (DIPEA) (1.6 ml, 9.2 mmol) was added. The resulting solution was added to the resin and allowed to react under argon for 3 hr. The resin was washed twice with DMF and then added to a solution of Gd isotopes (1.3 equiv.), aqueous ammonium hydroxide. The mixture was adjusted to 11 with aqueous ammonium hydroxide. The mixture was heated to 80°C and stirred for 12 hr. The solvent was removed and the resin was washed four times with DMF, four times with dichloromethane, four times with methanol, and dried under vacuum. A 30 ml solution of 95% trifluoroacetic acid (TFA), 2.5% trisopropylsilane, and 2.5% water was added to the resin and mixed for 1 hr, then drained. The resin was washed with 15 ml TFA. The filtrate and rinse were combined and reduced in volume to 10 ml. Forty milliliter of 20°C methyl tert-butyl ether (MTBE) was added to precipitate a white solid. The solid was washed three times with cold MTBE, taken up in water, and freeze dried to a white powder. The white powder was exposed to the TFA solution for 7 hr, washed with cold MTBE as above, and freeze dried to yield a white solid. Yield = 1.31 g. H NMR (D$_2$O): δ = 1.63 (m, 16H), 1.80 (m, 16H), 2.6-3.8 (m, 24H), 3.18 (m, 16H), 4.30 (m, 8H); MS calculated for C$_{64}$H$_{124}$N$_{36}$O$_{16}$ [M + H]$^+$: 1654.0, found 1653.8.

**DOTA-(Arginine)$_n$**

Poly-L-lysine-based Wang resin containing an Fmoc protected arginine residue (1.7 g, 0.50 mmol/g) was swelled in dichloromethane for 30 min and washed four times with peptide synthesis grade DMF. The resin was treated twice with a solution of 20% piperidine (20 ml) in DMF for 10 min. The resin was washed four times with DMF. In a separate vial, Fmoc protected arginine (3.0 g, 4.6 mmol), HATU (0.72 g, 1.9 mmol), and DMF (5 ml) were combined, and DIPEA (1.7 ml, 10 mmol) was added. The resulting solution was added to the resin and allowed to react under argon for 12 hr. The solvent was removed and the resin was washed four times with DMF, four times with dichloromethane, four times with methanol, and dried under vacuum. A 30 ml solution of 95% trifluoroacetic acid (TFA), 2.5% trisopropylsilane, and 2.5% water was added to the resin and mixed for 1 hr, then drained. The resin was washed with 15 ml TFA. The filtrate and rinse were combined and reduced in volume to 10 ml. Forty milliliters of 20°C MTBE was added to precipitate a white solid. The solid was washed three times with cold MTBE, taken up in water, and freeze dried to a white powder. The white powder was exposed to the TFA solution for 7 hr, washed with cold MTBE as above, and freeze dried to yield a white solid. Yield = 1.31 g. H NMR (D$_2$O): δ = 1.63 (m, 16H), 1.80 (m, 16H), 2.6-3.8 (m, 24H), 3.18 (m, 16H), 4.30 (m, 8H); MS calculated for C$_{64}$H$_{124}$N$_{36}$O$_{16}$ [M + H]$^+$: 1654.0, found 1653.8.

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**General Lanthanide(III) Complex Synthesis**

To a solution of the free ligand in water (16–32 mM) was added gadolinium(III) hydroxide or europium(III) hydroxide (1.3 equiv.). The reaction mixture was heated to 80°C and stirred for 12 hr. The reaction mixture was then cooled to ambient temperature, and the H was adjusted to 11 with aqueous ammonium hydroxide. The mixture was filtered through a 0.2 μm syringe filter, purified using Sephadex-G25 size exclusion chromatography, and freeze dried to yield a white solid.

**DOTA-(Arginine)$_n$**

Gadolinium(III) (4)

Yield: 0.618 g (86.7%). MS calculated for C$_{24}$H$_{44}$Gd$_2$O$_{12}$ [M + H]$^+$: Gd isotope pattern centered at 1808.1, found Gd isotope pattern centered at 1808.1.
DOTA-(Arginine)$_8$ Gadolinium(III) (5)

Yield: 0.651 g (68.3%). MS calculated for C$_{64}$H$_{120}$EuN$_{36}$O$_{16}$ [M + H]$^+$; Gd isotope pattern centered at 2432.9, found Gd isotope pattern centered at 2432.9.

DOTA-(Arginine)$_{12}$ Europium(III) (7)

Yield: 0.486 g (80.7%). MS calculated for C$_{88}$H$_{168}$EuN$_{52}$O$_{20}$ [M + H]$^+$; Gd isotope pattern centered at 2432.9, found Gd isotope pattern centered at 2432.9.

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REFERENCES


