Receptor-targeted co-transport of DNA and magnetic resonance contrast agents

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Background: Ligand molecules conjugated to polylysine can be electrostatically bound to DNA and can bind receptors or antigens on the surface of cells, delivering the DNA into specific cells and tissues. Several researchers have used this approach to generate non-viral vehicles for the efficient delivery of DNA to specific cells. We have attempted to adopt this general approach to the cell-specific delivery of magnetic contrast agents for use in magnetic resonance imaging (MRI).

Results: We have synthesized a new class of agents capable of both transfecting genes into cells and enhancing the contrast of the targeted cells for MRI. DNA is used both to encode a marker gene and as a molecular scaffold, which electrostatically binds polylysine conjugated to transferrin, an iron uptake protein, and polylysine modified with gadolinium chelated to diethylenetriaminepetaacetic acid. When cells displaying the transferrin receptor are treated with these particles, high levels of gene expression are observed, higher than with control particles composed only of transferrin, polylysine and DNA. The treated cells show specific MRI contrast enhancement, which did not require expression of the marker gene.

Conclusions: The development of this class of particles permits the use of novel protocols by which genes for genetic therapy and agents for MRI contrast are co-transported. These protocols may allow non-invasive MRI monitoring of DNA delivery for gene therapy in real time.

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Introduction

Magnetic resonance imaging (MRI) has become a powerful tool in clinical and research settings because it is non-invasive and yields an accurate volume rendering of a specimen. Using magnetic field gradients and selective radio frequency (RF) pulses, a one-, two- or three-dimensional image of the specimen is obtained. Typically, the image is based upon the NMR signal from the protons of water where the signal intensity in a given volume element (voxel) is a function of the water concentration and the relaxation times of the spins within the voxel (T_1 and T_2). Local variations in these three parameters provide the vivid contrast observed in MRI images [1]. The relaxation times can be strongly affected by MRI contrast agents, permitting specific fluid compartments or cells to be selectively followed. Unlike light-microscope imaging techniques that are based on the use of organic dyes or fluorochromes, neither MRI nor MRI contrast agents produce toxic by-products from photo-bleaching. Furthermore, MRI is not limited by light scattering or other optical aberrations, permitting high field strength instruments (>7 Tesla) to generate high resolution (10–15 μm) images of developing insects, fish, amphibia and mammals [2].

A major limitation on the use of MRI in basic research settings has been the invasive manipulations and/or microinjections required to label cells or tissues. In clinical settings, limitations resulting from similar constraints together with the potential for using MRI contrast agents to target specific tissues or tumors *in vivo*, have stimulated the syntheses of new paramagnetic agents covalently attached to biological macromolecules [3]. To significantly enhance the observed contrast, however, antibodies or other targeting molecules must bind very large numbers of paramagnetic complexes [4]. Such high ratios of contrast agent to biomolecule cripple the targeting molecule due to ionic and steric effects. Therefore, much of the current research in this field has focused on the use of liposomes as carriers of high concentrations of contrast agents [5], iron oxide particles as high signal strength T_2 contrast agents [6], and more recently, highly modified starburst dendrimers of paramagnetic agents [7].

Here we report a different strategy, based on methods currently under development for the delivery of nucleic acids to specific cells, to deliver both exogenous genes and large numbers of MRI contrast agents to cells. Polypeptides composed exclusively of lysine residues bind electrostatically to DNA and cause it to condense by neutralizing the negative charge. Ligand molecules conjugated to polylysine can thus be bound to DNA. Binding of the ligand molecule within the DNA–ligand complex to receptors or antigens on the cell surface delivers the complex with high efficiency into specific cells and tissues [8–15]. In our modification of this scheme, paramagnetic contrast agents are covalently attached to polylysine for incorporation into a DNA–polycation complex (Fig. 1). For our first test case, we used ternary particles containing the following components: i) DNA encoding the firefly luciferase reporter gene, ii) poly-L-lysine attached to human transferrin, a

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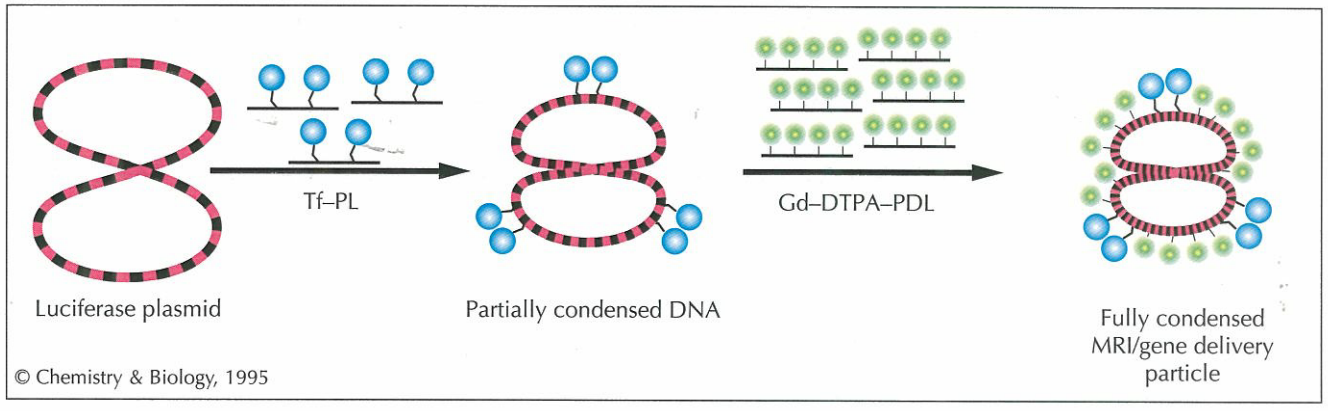

Fig. 1. Summary of the formation of ternary complexes for receptor-mediated uptake of DNA and MRI contrast agents. DNA is partially condensed by the addition of sub-optimal quantities of transferrin–polylysine (Tf–PL). Full condensation to electro-neutrality follows the addition of Gd–DTPA–PDL, producing particles with high transfection efficiency and MRI contrast enhancement.

protein involved in iron uptake, and iii) poly-D-lysine (PDL) attached to varying numbers of the paramagnetic contrast agent, gadolinium diethylenetriaminepetaacetic acid (Gd–DTPA). Uptake of one of these particles by cells expressing the transferrin receptor results in delivery of ~1500 Gd ions per cell, achieving the high ratio of contrast agent per biomolecule required for *in vivo* labeling. After incubation of human T-cell leukemia derived K562 cells with the ternary complexes, detectable MRI contrast enhancement is observed at nearly single-cell resolution. Moreover, the level of gene expression observed in these cells is higher than the levels observed using control particles that do not contain the MRI contrast agent.

Results and discussion

Synthesis and characterization of Gd–DTPA conjugates of PDL

We prepared a series of DTPA-modified PDL derivatives with varying numbers of labeled sites. Simply dissolving PDL in aqueous solutions at high pH, followed by addition of DTPA anhydride, invariably leads to a large distribution of products. At concentrations above 100 μM PDL, the major product isolated from this reaction is a crosslinked material of high molecular weight, as revealed by fast protein liquid chromatography (FPLC). By maintaining the PDL concentration below 100 μM and controlling the rate of anhydride addition, we have modified the PDL used in these experiments with as few as 7 and as many as 60 DTPA chelates with no evidence of crosslinking.

Fluorescence titration experiments were performed to assess the degree of substitution on the 180 residue PDL backbone resulting from various molar input ratios of DTPA and PDL. Europium chelates of DTPA form readily at room temperature and show characteristic and distinct fluorescence with large Stoke's shifts and long fluorescent lifetimes. Titration of the DTPA–PDL conjugates with $EuCl_3$ produced increasing fluorescence intensities followed by inflections in the curves corresponding to complete saturation of the DTPA sites [16]. The ratios of DTPA to PDL determined by fluorescence titration of the various conjugates is shown in Table 1 along with the calculated number of unmodified (and thus positively charged) lysines per DTPA site.

Previous studies on the interaction between DNA and polylysine have suggested that polymers containing seven or more lysine monomers readily form neutral particles with DNA, whereas shorter polymers do not [17]. Therefore, we anticipated that PDL modified with 10 and 22 Gd chelates per PDL chain (16 lysines and 6.7 lysines per Gd chelate, respectively) would interact readily with DNA, whereas PDL modified with 42 and 51 Gd chelates, which have shorter stretches of unmodified lysine residues, would not.

To rapidly test this hypothesis, the effect of PDL conjugates on the secondary structure of DNA in solution was indirectly monitored using a UV hyperchromicity assay. Polycations such as polylysine that interact electrostatically with DNA alter the absorbance and optical rotation characteristics of the nucleic acid [18]. Titration of DNA with unmodified PDL produces a curve with peak UV absorbance corresponding to a 1:1 ratio of lysine monomers to nucleotide residues (Fig. 2). A nearly identical curve is produced when transferrin–polylysine is added to the DNA. The PDL conjugates with ratios of Gd–DTPA per PDL molecule of 10 and 22 show similar effects, suggesting that these modified PDL molecules interact with DNA (Fig. 2). In contrast, PDL conjugates having larger numbers of DTPA chelates (42 and 51) produce no

Table 1. Effect of reagent ratios on modification of poly-D-lysine with DTPA.

DTPA:poly-D-lysine		Unmodified lysine units per DTPA site
Input ratio	Output ratio	
50	10	16.0
100	22	6.7
200	42	3.0
400	51	2.3

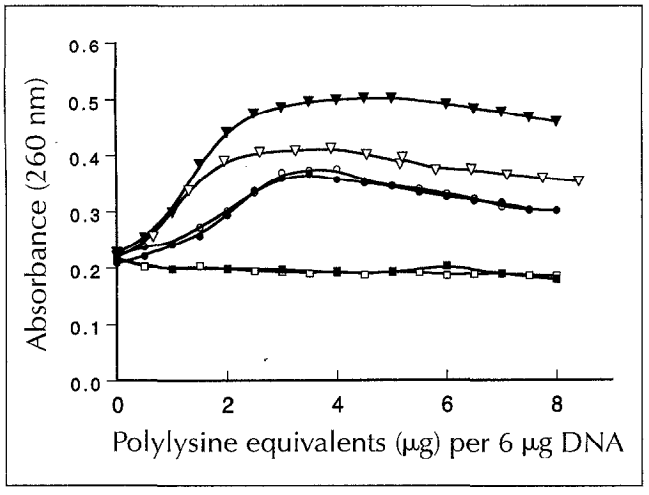

Fig. 2. PDL modified with 10 or 22 Gd chelates per PDL molecule bind to DNA. A UV hyperchromicity assay was used to assess binding of polylysine to DNA. Increasing quantities of PDL or modified polylysine were added to 6 μg plasmid DNA in 1.0 ml HBS while monitoring absorbance at 260 nm. The addition of unmodified PDL (○) or transferrin–polylysine (●) to DNA produces characteristic and nearly identical changes in UV absorbance. Peak absorbance occurs at approximately a 1:1 molar ratio of lysine monomers to nucleotides, corresponding to electro-neutrality and optimal transfection efficiency. Titration of DNA with PDL modified with 10 Gd chelates per PDL molecule (Gd–$DTPA_{(10)}$–PDL; ▽) or 22 Gd chelates (Gd–$DTPA_{(22)}$–PDL; ▼) results in distinct but grossly similar curves. In contrast, Gd–$DTPA_{(42)}$–PDL (□) and Gd–$DTPA_{(51)}$–PDL (■) have minimal effects on DNA absorbance, consistent with a reduced ability of these heavily labeled PDL molecules to interact with DNA.

observable increase in the UV absorbance, suggesting only minimal interaction with the DNA (Fig. 2). Based on these results, the conjugate containing a ratio of 22 Gd–DTPA sites per PDL molecule (Gd–$DTPA_{(22)}$–PDL) was used in subsequent experiments.

Luciferase gene delivery by particles containing DNA, Tf–PL and Gd–DTPA–PDL

Particles were formed by adding varying amounts of transferrin conjugated to poly-L-lysine (Tf–PL) to plasmid DNA encoding the firefly luciferase gene, followed by the addition of a sufficient amount of Gd–$DTPA_{(22)}$–PDL to neutralize the remaining negative charge of the DNA. Tf–PL (3 μg, equivalent to 0.4 μg of unmodified polylysine) was added to 6 μg of DNA to neutralize approximately one tenth of the DNA charge. The remainder of the DNA charge was neutralized by the addition of either 4 μg of unconjugated PDL or an equivalent amount of Gd–$DTPA_{(22)}$–PDL.

High luciferase expression resulted from incubation of the particles containing Gd–DTPA with human erythroleukemic K562 cells in the presence of the lysosomatropic agent, chloroquine (Fig. 3A). It was surprising that the level of gene expression was increased approximately two-fold over that obtained using particles lacking Gd–DTPA–PDL (Fig. 3B). This enhancement of gene expression may be due to neutralization of a portion of the charges on the polylysine backbone by the Gd–DTPA chelates. In the absence of chloroquine, gene expression was not observed (Fig. 3D).

Our data indicate that gene delivery takes place through the transferrin receptor via receptor-mediated endocytosis. Neither unmodified polylysine [8] nor Gd–DTPA–PDL (data not shown) on their own facilitate the transfer of DNA into K562 cells. Furthermore, gene expression is blocked by adding iron-loaded transferrin to the medium during transfection (Fig. 3C) but not by adding apotransferrin, which does not bind to the receptor at neutral pH [19].

Complexes containing DNA, Tf–PL and Gd–DTPA–PDL enhance the MRI contrast of cells

Having demonstrated that DNA complexes containing Tf–PL and Gd–DTPA–PDL are effective gene-delivery vehicles, we then examined the ability of these particles to enhance MRI contrast of transfected K562 cells *in vitro*. Cells treated with these particles were washed extensively in hepes-buffered saline (HBS) and gently loaded into sealed glass capillary tubes, and T_1-weighted images (T_R/T_E = 200 ms/13 ms) were acquired (Fig. 4). MRI contrast enhancement is clearly observed in cells exposed to the Gd-containing particles (Fig. 4a). In contrast, cells exposed to particles lacking Gd–DTPA–PDL show no such enhancement (Fig. 4b). Enhancement of

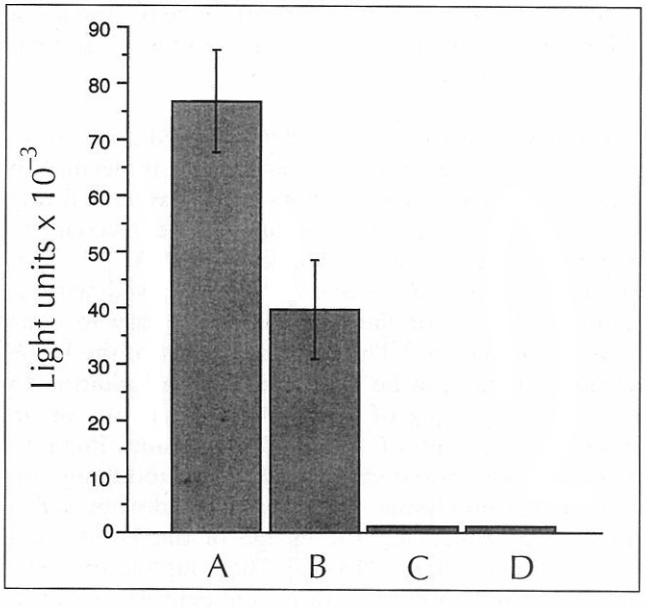

Fig. 3. Transfection of cells is specific and is enhanced using particles containing Gd-modified PDL. Ternary complexes were formed with 6 μg DNA, 3 μg Tf–PL and 4 μg (unmodified lysine equivalents) of either Gd–$DTPA_{(22)}$–PDL (column A) or PDL (column B). Each bar represents the average of values obtained in five independent transfection experiments using K562 cells and normalized to 10^6 cells per experiment. Using Gd–$DTPA_{(22)}$–PDL-containing particles, gene expression was measured in the presence of 20 μg of iron-loaded transferrin to show the effect of competitive uptake (column C) and in the absence of chloroquine to assess the mechanism of cytoplasmic delivery (column D). In these experiments, 1 ng of luciferase produces ~10^5 light units.

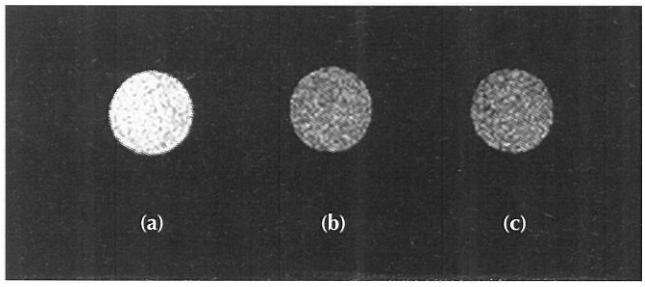

Fig. 4. The ternary complex containing Gd–DTPA–PDL delivers the MRI contrast agent to K562 cells via the transferrin receptor. A two-dimensional horizontal MRI slice is shown through three capillary tubes (2 mm inner diameter) loaded with labeled or control K562 cells. **(a)** Cells exposed to the ternary complex containing 6 μg DNA, 3 μg Tf–PL and 4 μg Gd–$DTPA_{(22)}$–PDL. Bright spots represent aggregates of contrast-agent-containing cells. Mean intensity: 232 ± 25. **(b)** Cells treated with control complexes containing 4 μg unmodified PDL in lieu of the gadolinium-modified PDL. Mean intensity: 121 ± 30. **(c)** Cells treated as in (a) with the addition of 20 μg iron-loaded transferrin to competitively block uptake of the ternary complex. Mean intensity: 126 ± 30. A 10 μg ml^{-1} standard solution of Gd–DTPA in water produces a mean intensity of 142 ± 32, although it should be noted that MRI is extremely sensitive to specific experimental conditions, including solvent composition and viscosity.

the MRI contrast of cells is competitively inhibited by the addition of iron-loaded transferrin, indicating that the observed effect is receptor-specific (Fig. 4c). The magnetic resonance imaging results obtained in these experiments were the same in the presence or absence of chloroquine, suggesting that escape from the lysosome is not necessary for MRI enhancement.

In summary, we have shown that modified polycations can be used to deliver DNA and other molecules to cells at high efficiency. Previous work has shown that ~20 transferrin molecules per complex are necessary to impart receptor-specific uptake of DNA [8]. This number of target molecules can be achieved with as little as 10–15 % of the polylysine necessary to condense a 6 kb plasmid. The remaining 90 % of the DNA negative charge can be neutralized by the addition of approximately 4 μg of unmodified polylysine, or an equivalent amount of modified polylysine. Previous work has demonstrated the utility of modifying this neutralizing polylysine with agents that disrupt endosomes, thus enhancing the escape of the DNA from lysosomal degradation [13–15]. The coupling of adenovirus and influenza virus fusogenic peptides has been especially fruitful in this regard, resulting in high levels of gene expression. In our experiments, the addition of chloroquine during transfection served the purpose of preventing acidification of the endosome and reducing the destruction of the DNA following endocytosis. Thus, most of the polylysine used to neutralize the DNA could be substituted with chelated Gd^{3+}. Inclusion in the complex of another polylysine component that is conjugated to a fusogenic peptide may give high levels of gene expression even in the absence of chloroquine.

Using the Gd–DTPA–PDL which we have synthesized, 22 Gd^{3+} ions are chelated to each PDL molecule, corresponding to ~1200 Gd^{3+} ions per plasmid molecule in the ternary complex. This greatly exceeds the number of contrast agents that can be attached directly to a monoclonal antibody. Moreover, the fast rate of transferrin receptor turnover (2×10^4 transferrin molecules internalized per minute) results in the uptake of extremely large numbers of contrast agent molecules in this system [20]. This uptake is enhanced *in vitro* when the particles are at relatively high concentrations and, prior to transfection, cells are starved for iron, inducing an increase in the number of transferrin receptors on the cell surface [8].

Significance

The delivery of genes to specific cells and tissues for therapeutic and research purposes is of increasing importance. A new method to track the delivery of vectors for gene transfection and genetic therapy is imagined based on the work described in this report. This method combines a magnetic resonance imaging (MRI) contrast agent and a receptor-targeted gene delivery vehicle.

MRI is an ideal tool for non-invasive monitoring of human and experimental subjects. We have synthesized a new class of MRI contrast agents capable of transfecting genes into cells and enhancing the MRI contrast of these targeted cells *in vitro*. The particles are composed of DNA, polylysine attached to transferrin, and polylysine modified with a paramagnetic contrast agent. Modification of this strategy should produce particles capable of functioning *in vivo*. Such particles could be composed of i) DNA encoding a reporter or therapeutic gene, ii) polylysine modified with transferrin or with an alternative cell-targeting molecule, such as a monoclonal antibody, iii) polylysine modified with fusogenic peptides to facilitate release from the endosomal pathway and iv) polylysine (D or L forms) modified with Gd chelates. Co-transport of DNA and MRI contrast agents of this type should result in high levels of gene expression. These particles may prove extremely useful in targeting specific cells *in vivo* as they offer a means other than marker gene expression to monitor construct uptake. With such a tool, clinicians and experimentalists may be able to non-invasively monitor delivery of genetic therapeutic agents in real time.

Materials and methods

Preparation of Tf–PL

Human transferrin (Tf, Sigma) was conjugated to poly-L-lysine (PL) with an average chain length of 220 residues (Sigma) via sialic acid residues on transferrin as described [21], or the Tf–PL conjugate was purchased directly from Sigma. Modified polylysine (Tf–PL) with average molar ratios of transferrin to

polylysine of 2.2–2.5 was dissolved in HBS (20 mm Hepes, 150 mM NaCl, pH 7.4) at a working concentration of 100 μg ml^{-1} for use in subsequent experiments.

Preparation of DTPA–PDL

PDL of average chain length 180 was purified by FPLC size-exclusion chromatography employing a 10 x 30 Pharmacia Superdex 75 column using 0.15 M NaCl, 0.15 M NaPi, pH 7.0 buffer. The purified PDL was desalted by gel filtration using Sephadex G-25 and dried *in vacuo*. The dry PDL was weighed and the total number of lysine monomer units verified spectrophotometrically via a ninhydrin assay [22]. PDL (0.01 M) was placed in a round-bottomed flask and dissolved in 0.05 M HEPES, pH 9.5. Freshly prepared DTPA anhydride was added slowly with stirring. The pH of the solution was monitored and maintained at 9.5 throughout the course of the reaction using a 3 % NaOH solution. The molar ratios of DTPA-anhydride to PDL used in these experiments was 50:1, 100:1, 200:1 and 400:1. The reaction was allowed to proceed at room temperature for 1 h after complete addition of the anhydride, and the crude material was purified by FPLC.

Determination of DTPA content by fluorescence titration

The binding of lanthanide metals such as gadolinium, europium and terbium to chelating agents produces fluorescence with large Stoke's shifts and fluorescence lifetimes. Eu^{3+} (unlike Gd^{3+}) rapidly coordinates with DTPA derivatives, producing a strong fluorescent signal that was monitored to assay DTPA content of the various DTPA–PDL conjugates. Samples of the purified DTPA–PDL were diluted in 0.05 M HEPES, pH 7.4 to a final concentration of 2–4 x 10^{-5} M. Small volumes (<20 μl) of varying molarities of $EuCl_3$ were added to the 1-ml samples to titrate the free DTPA ligand with minimal dilution of the sample. After incubation for 2 min at room temperature, each sample was transferred to a Hitachi Model 2400 spectrofluorimeter. Measurements were made using excitation at 394 nm and monitoring emission at 593 and 616 nm. Molar ratios of DTPA per PDL were calculated using the fluorescence titration data and the polylysine quantification that was based on ninhydrin as an assay for unmodified lysine side-chains [22].

Preparation of Gd–DTPA–PDL

Based on the chelator content determined by Eu titration, a 1.1 molar excess of $GdCl_3$ was added to 1 mg ml^{-1} aliquots of the DTPA–PDL in 0.05 M citrate buffer, pH 6.0. The chelation proceeded for 5 h at 70 °C, and was followed by desalting chromatography with Sephadex G25 to remove unbound Gd. Gd–DTPA–PDL samples were dissolved in HBS at a working concentration of 100 μg ml^{-1}.

Preparation of MRI/gene delivery particles

A 6 kilobase-pair plasmid containing the *Photinus pyralis* luciferase gene under control of the SV40 T-antigen enhancer was purchased from Promega (GeneLight control plasmid). This plasmid was grown in *Escherichia coli*, and DNA was prepared in 100 μg quantities using a commercially available procedure (Qiagen, Midi-100). The supercoiled DNA plasmids were dissolved at a concentration of 100 μg ml^{-1} in 10 mM Tris, 1 mM ethylenediaminetetraacetic acid, pH 7.4 (TE). Plasmid DNA (6 μg) in 430 μl of HBS was mixed with 3 μg of Tf–PL in 30 μl HBS. After 5 min at room temperature, various amounts (generally 4 μg equivalents of polylysine) of Gd–DTPA–PDL with differing degrees of Gd substitution were added to the sample which was brought to 500 μl with HBS and allowed to incubate at room temperature for 30 min.

Transfections

K562 human leukemia cells (ATCC) were grown in suspension in RPMI 1640 medium (Gibco) containing 10 % fetal calf serum (Hyclone) containing 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM glutamine, to a maximum density of 5 x 10^5 cells ml^{-1}. Eighteen to twenty-four hours prior to transfections, cells were placed in fresh medium containing 50 μM deferroxamine (Sigma) to increase surface presentation of transferrin receptors as described [12]. Approximately ten minutes prior to transfection, the cells were washed with fresh medium containing deferroxamine and placed in a 12-well dish at 250 000 cells per ml, 2 ml per well. Chloroquine (10 mM) in H_2O was added to a final concentration of 100 μm.

The ternary particle in HBS was added slowly to the K562 cells, which were incubated at 37 °C for 18–24 h. In some cases, iron-loaded transferrin (Sigma) was added with the ternary complex to competitively inhibit specific uptake of the particles. The cells were then washed twice with fresh medium without chloroquine or deferroxamine and resuspended at 250 000 cells ml^{-1} in fresh medium. After 18 h, the cells were washed twice in HBS and either gently loaded into a glass capillary tube for MR imaging or assayed for luciferase expression. Gene expression was monitored by lysing cells in 100 μl detergent lysis buffer (Clontech). Cell lysate (20 μl) was assayed in a 1 ml standard cell using a luminometer and luciferin substrate from Analytical Luminescence Laboratory. These results were normalized per 10^6 cells and confirmed by parallel measurements using a scintillation counter (Beckman Instruments).

MRI image acquisition

MRI images were acquired using an 11.7 Tesla Bruker AMX 500 MHz NMR spectrometer with microimaging accessory. Glass capillary tubes (2 mm inner diameter) containing ~10^6 cells per tube were immobilized in a plexiglass rack and loaded into the MRI bore. Images were acquired using a multi-slice 3D spin echo protocol where T_R = 200 ms, T_E = 13 ms and the pulse width was set at 9.6 μs. This protocol results in a T_1-weighted image and a 512 by 512 by 32 data array.

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