Electronic Supplementary Material (ESI) for RSC Advances.

A theranostic polycation containing trehalose and lanthanide chelate domains for siRNA delivery and monitoring

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Supporting Information

1. Materials

All chemicals used for monomer and polymer synthesis were purchased from Sigma-Aldrich (St. Louis, MO) at the highest possible purity level. Dry methylene chloride, dimethyl formamide, and methanol were obtained using an MBRAUN MB solvent purification system (M. Braun Inertgas-Systeme GmbH, Garching, Germany) and were loaded with HPLC grade solvents obtained from Fisher Scientific Co. (Pittsburgh, PA). Thin layer chromatography (TLC) was completed using aluminum-backed silica gel plates (silicagel 60, F_{254}) obtained from Merck (Darmstadt, Germany) and visualized using UV light (254 nm) or the following staining reagents: ninhydrin solution in ethanol for the visualization of amines or *p*-anisaldehyde solution in H_2SO_4/acetic acid/ethanol for the visualization of carbohydrates. Preparative scale chromatography purifications were performed using a Buchi Separcore chromatography system (Buchi Labortechnik AG, Switzerland) with Buchi plastic chromatography cartridges or homemade glass columns manually packed with 60-200 mesh Premium

Rf silica gel (Sorbent Technologies Inc., Atlanta, GA). All solvents used for preparative chromatography were HPLC grade obtained from Fisher Scientific Co (Pittsburgh, PA). LC-MS data was obtained with an Agilent Technologies system (Santa Clara, CA) with a time-of-flight (TOF) analyzer coupled to a Thermo Electron TSQ-LC/MS ESI mass spectrometer. NMR spectra were recorded using a Varian MR 400 MHz spectrometer with all compounds in deuterated solvents, namely D_2O, d_4-MeOD, d_6-DMSO, $CDCl_3$, CD_2Cl_2. All deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). ^{1}H-NMR spectra were recorded at 399.7 MHz and ^{13}C-NMR spectra were recorded at 101 MHz. Spectra were analyzed using MNova software (version 7.0.1-8414, Mestrelab Research S.L., Santiago de Compostela, Spain). Spectra/Por® dialysis membranes (MWCO: 1000) were obtained from Spectrum Laboratories, Inc. (Rancho Dominguez, CA).

Gel permeation chromatography (GPC) was conducted using 1.0 wt% acetic acid/0.1 M Na_2SO_4 as the eluent at a flow rate of 0.3 mL/min on size exclusion chromatography columns CATSEC1000 (7μ, 50×4.6), CATSEC100 (5μ, 250×4.6), CATSEC300 (5μ, 250×4.6), and CATSEC1000 (7μ, 250×4.6), which were obtained from Eprogen Inc. (Downers Grove, IL). Peak detection was obtained using a Wyatt HELEOS II static light scattering detector (λ = 662 nm), and an Optilab rEX refractometer (λ = 658 nm). GPC trace analysis was performed using Astra V software (version 5.3.4.18) from Wyatt Technologies (Santa Barbara, CA).

U-87 MG-luc2 (U-87_luc2) cells, human glioblastoma cells genetically engineered to constitutively express luciferase, were obtained from Caliper LifeSciences, Inc. (Mountain View, CA). Luc2 siRNA, which targets luc2 luciferase, and Cy5-labeled Luc2 siRNA were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). The sequence of the sense strand of the Luc2 siRNA is 5'-GGACGAGGACGAGCACUUCUU-3', and the antisense strand sequence is 3'-UUCCUGCUCCUGCUCGUGAAG-5'. The Cy5 fluorophore within the Cy5-labeled Luc2 siRNA was conjugated to the 3' terminus of the sense strand (5'-GGACGAGGACGAGCACUUCUU-Cy5-3'). The TMR fluorophore within the tetramethyl rhodamine (TMR)-labeled Luc siRNA was conjugated covalently to the 3' terminus of the sense strand (5'-GGACGAGGACGAGCACUUCUU-TMR-3') and was purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Scrambled siRNA (siCon) was purchased from Dharmacon, Inc (Lafayette, CO). Bovine serum albumin was purchased from Sigma-Aldrich (St. Louis, MO). DMEM+GlutaMAXTM-I (DMEM), Opti-MEM

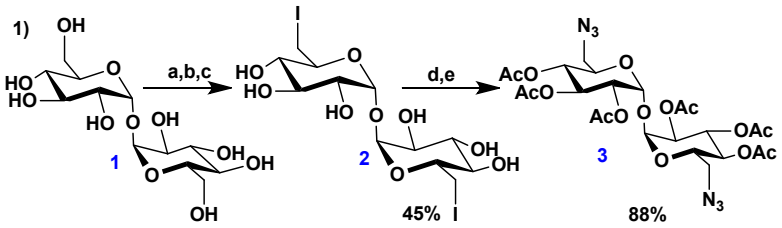
I+GlutaMAXTM-I (Opti-MEM), UltraPureTM Agarose-1000, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), propidium iodide (1.0 mg/mL solution in water), PBS pH=7.4, trypsin, antibiotic-antimycotic and Lipofectamine™2000 (Lipo) were obtained from Invitrogen, Inc. (Carlsbad, CA). DEPC-treated water for the RNA work was obtained from Fisher Scientific (Pittsburgh, PA). INTERFERin™ was a gift from Polyplus-Transfection (Strasbourg, France). jetPEI™ was purchased from Polyplus-Transfection. The Luciferase Assay System was obtained from Promega Corporation (San Luis Obispo, CA). Bio-Rad DC Protein Assay Reagent A, Reagent B, and Reagent S were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). CellScrub™ Buffer was obtained from Genlantin, Inc. (San Diego, CA). Glycofect Transfection Reagent™, having a degree of polymerization of approximately 11, was donated by Techulon, Inc. (Blacksburg, VA). Biological sample fluorescence (for gene expression assays) was measured with a GENios Pro plate reader from TECAN US (Research Triangle Park, NC). Luminescence lifetime measurements were taken on a Synergy™ H1 monochromator-based multi-mode microplate reader in 96 well plates from BioTek (Winooski, VT).

2. Synthetic procedures

2.1. 2, 3, 4, 2', 3', 4'-hexa-O-acetyl-6,6'-diazido-6,6'-dideoxyl-D-trehalose (Compound 3)

Compound **3** was synthesized according to a previously published procedure.[1] The final product was purified on a silica gel column using 10% (v:v) diethyl ether in dichloromethane. Fractions containing the product were collected, the solvent was evaporated, and the product was further recrystallized from 10% (v:v) ethyl acetate in diethyl ether to yield fine white crystals (33% total yield after all steps).[1]

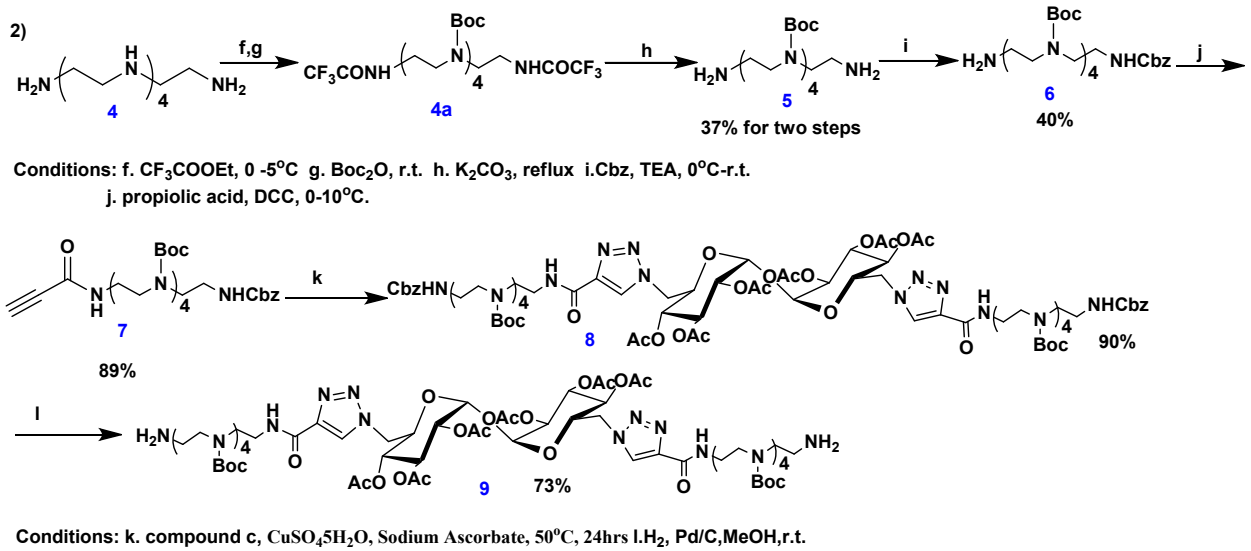
^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 5.49 – 5.40 (overlapping triplets, 2H, 3,3'), 5.39 – 5.29 (d, 2H,1,1'), 5.19 – 5.07 (dd, 2H, 2, 2'), 5.01 – 4.96 (t, 2H, 4,4'), 4.06-4.12 (m,2H, 5,5'), 3.39-3.34, 3.19-3.15(dd, 4H, 6, 6'), 2.12-2.03 (m, 18H, $COCH_3$),
ESI-MS positive ion mode: (expect/found) m/z $[M+Na]^+$ 667.20/667.18, $[M+NH_4]^+$ 662.22/662.21, $[M+H]^+$ 645.19/645.19.



Scheme S1. Synthetic scheme of 2, 3, 4, 2', 3', 4'-hexa-O-acetyl-6,6'-diazido-6,6'-dideoxyl-D-trehalose.

2.2. Synthesis of Tr-N4 macromonomer (Compound 9)

It should be noted that the synthesis of compounds **4**, **4a**, **5**, **6**, and **7** have been previously reported by our group, and the synthetic preparations outlined below (Steps 1-3) represent only slight modifications to our previously reported procedures.[2]



Scheme S2. Synthetic scheme of Tr-N4 macromonmers.[2]

Step 1: 2, 3, 4, 5-tetra (tert-butyloxycarbonyl)-pentaethylenetetraamine (Compound 5)

The starting material **4** was purified via distillation in oxygen free conditions yielding a yellow oil (62.2 g, 268 mmol), which was then dissolved in 400 ml of anhydrous MeOH. Ethyltrifluoroacetate (CF_3COOEt, 84.0g, 591 mmol) was dissolved in 300 ml anhydrous MeOH and was added into the reaction mixture dropwise under N_2 for 24 h. Next, $(Boc)_2O$ (244 g, 1120 mmol) was dissolved in MeOH (300 ml) and added into the solution drop-wise with an addition funnel. The nitrogen inlet was removed and the solution was stirred for another 4 h. Excess $(Boc)_2O$ (10 g) was then added into the solution and stirred for another 2 h. The solid was then removed via filtration, and the solution was purified via flash chromatography. The product fraction was isolated, concentrated, and washed with EtOAc and hexanes. A white solid was collected via filtration. A mixture of ethyl acetate:hexanes (1:1) was used to recrystallize the compound yielding a white powder (**4a**) 55.3 g. Next, **4a** (27.1 g, 32.8 mmol) was dissolved in MeOH (200 mL), H_2O (20 ml) water, 28.9 g of K_2CO_3 was added into the solution, and the mixture was refluxed at 80°C for 12 h. The K_2CO_3 was filtered off, the solution was concentrated, the product was dissolved in chloroform, and then the solid was once again filtered off. The solution was then dried over Na_2SO_4, filtered, and concentrated. The product was recrystallized in ethylacetate:hexanes (3:1). The white solid was collected, dried, and characterized yielding the final product **5** (7.67 g, 12.1 mmol, 37 %).

^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 3.32-3.23 (overlapping d, 20H, -NBocCH_2-), 2.83-2.79 (dd, 4H, NH_2CH_2-), 1.61-1.45 (s, 36H, -NCOO$(CH_3)_3$).

Step 2: 1-carboxylbenzyl-2,3,4,5-tetra(tert-butyloxycarbonyl) pentaethyleneamines (Compound 6)

The 2,3,4,5-tetra (*tert*-butyloxycarbonyl) pentaethyleneamine (**5**) 2.47 g (3.92 mmol) and carboxylbenzyl chloride (CbzCl, 0.650 g, 3.81 mmol) were dissolved in DCM in two separate flasks. The CbzCl solution was then added dropwise into the solution containing **5** at -25 °C (the ice bath was prepared with NaCl) and stirred for 4 h. The solvent was then evaporated, and the product was purified via silica gel chromatography with a solvent system that offered a gradual polarity shift from hexanes:ethylacetate (3:1) to pure ethyl acetate to ethyl acetate:ethanol (5:1). Then the fractions containing the product were concentrated and recrystallized in hexanes:ethyl acetate (1:1) to yield the final product, **6** (1.59 g, 2.08 mmol, 55 % yield).

^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 7.26-7.23 (m, 5H, -C_6H_5), 5.12-5.09 (s, 2H, -$COOCH_2Ph$), 3.38-3.22 (br, 20H, -CH_2CH_2-), 1.53-1.23 (br, 36H, -$OC(CH_3)_3$).

Step 3: 1-alkyne-6-carboxybenzyl-2,3,4,5-tetra(tert-butyloxycarbonyl) pentaethylenetetraamine (Compound 7)

Compound **6** (0.0500 g, 0.0650 mmol) was dissolved in dichloromethane. N,N'-dicyclohexylcarbodiimide (DCC) (0.050g, 0.024 mmol) and propiolic acid (0.015 g, 0.021 mmol) were dissolved together in dichloromethane (in a separate flask than **6**). The solution of **6** was added dropwise into the mixture of DCC and propiolic acid at 0 °C. The ice bath was removed following the addition, and the mixture was allowed to warm to room temperature and then left to stir for 8 h. The mixture was washed (H_2O x 3) and the organic layers were collected and dried with Na_2SO_4. The solvent was then removed via evaporation, and the product was purified via silica gel chromatography using the gradient mobile phase of methanol:dichloromethane (0-0.2). The product factions were collected and concentrated yielding a white powder of **7** (0.042 g, 0.051 mmol, 78 %).

^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 7.32-7.24 (br, 5H, -C_6H_5), 5.07 (s, 2H, -$COOCH_2$-), 3.41-3.30 (br, 20H, -CH_2CH_2-), 2.73 (s, 1H, -CCH), 1.46-1.43 (br, 36H, -$OC(CH_3)_3$).

ESI-MS: calculated mass $[M+H]^+$: 819.48, found [M+H]+ : 819.49

Step 4: di-pentaethylenetetraamine click trehalose macromonomers (Compound 8)

The 1-alkyne-6-carboxybenzyl-2,3,4,5-tetra (tert-butyloxycarbonyl) pentaethylenetetraamine (**7**, 0.213 g, 0.26 mmol), 2,3,4,2',3',4'-hexa-O-acetyl-6,6'-diazido-6,6'-dideoxyl-D-trehalose (**3**, 0.0800 g, 0.124 mmol), and sodium ascorbate (0.0100 g, 0.0500 mmol) were dissolved in t-BuOH (1 mL). $CuSO_4$ (0.0189 g, 0.0750 mmol) was dissolved in H_2O (1 ml) and added into the reaction mixture. The reaction mixture was stirred for 24 h at 50 °C. The mixture was then dissolved in 1 ml DMSO, and 10 ml of H_2O was added to the mixture to precipitate out the compound. The white solid was collected and dried to yield **8** (0.262 g, 0.115 mmol, 90 %).

^{1}H-NMR (400 MHz, CDCl3) δ ppm: 8.02-8.01 (s, 2H, triazole H), 7.20-7.19 (m, 10H, -C_6H_5), 5.34 (t, 2H, -1,1' trehalose H), 5.10-4.92 (s, 4H, -CH_2-Ph), 4.83-4.41 (m, 8H, -2,2',3,3',4,4',5,5'-trehalose H), 3.42-3.21 (s, 40H, -CH_2CH_2-), 2.32-2.08 (br, 18H, -$COCH_3$), 1.39-1.30 (s, 72H, -$OC(CH_3)_3$).

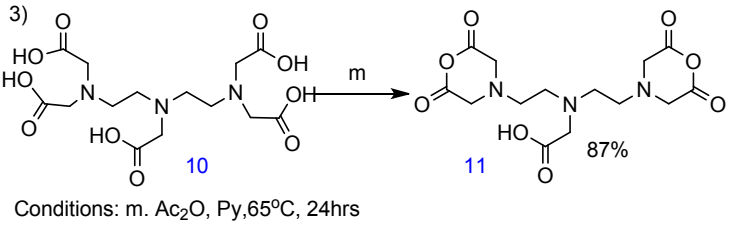
ESI-MS: calculated mass $[M+Na]^+$: 2304.14, found $[M+Na]^+$: 2304.15

To yield compound **9**, the di-carboxylbenzyl-di-pentaethylenetetraamine click trehalose macromonomers (0.208 g, 0.091 mmol) and Pd/C 0.020 g were added into dry CH_2Cl_2, and H_2(g) was bubbled through the solution for 24 h at room temperature. Then the Pd/C was filtered out. The filtrate was concentrated and purified using flash chromatography to yield a purified pale colored powder of **9** (0.150 g, 0.0740 mmol, 79 %).

^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 8.02-8.00 (s, 2H, triazole H, 5.38 (t, 2H, -1,1' trehalose H), 4.78-4.46 (m, 8H, -2,2',3,3',4,4',5,5'-trehalose H), 3.42-3.24 (s, 40H, $-CH_2CH_2-$), 2.34-2.03 (br, 18H, $-COCH_3$), 1.38-1.32 (s, 72H, $-OC(CH_3)_3$).

ESI-MS: calculated mass $[M+H]^+$: 2014.07, found $[M+H]^+$: 2014.15

2.3. Synthesis of DTPA-BA (diethylenetriaminepentaacetic acid bisanhydride) monomer (Compound 11)



Scheme S3. Synthetic scheme of diethylenetriaminepentaacetic acid bisanhydride (DTPA-BA).

The diethylene triamine pentaacetic acid (5.50 g) was added into a dry flask. The pyridine and acetic anhydride were added and stirred at 65 °C for 24 h. Then the solid was filtered out and washed with acetic anhydride and diethyl ether. The product was dried on a vacuum pump to yield a white powder DTPA-BA (4.35 g, 12.2 mmol, 87 %).

^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 3.68 (s, 8H, $-NCH_2COOCO-$), 3.28 (s, 2H,$-NCH_2COOH$), 2.74-2.71 (t, 4H, $-CH_2N-$), 2.59-2.56 (t, 4H,$-NCH_2COOH$).

2.4. Synthesis of trehalose pentaethyleneamine polymers with lanthanide chelation domain (Compound 14)

As shown in Scheme 1 of the main manuscript, the di-pentaethylenetetraamine click trehalose macromonomer (**9**) 201 mg and diethylenetriaminepentaacetic acid bisanhydride (**11**) 0.0360 g were each separately dissolved in 1 ml of dry DMSO. Then the solution containing 11 was added dropwise into the solution of **9** via syringe. The mixture was stirred at 25 ^{o}C for 18 h, purified via exhaustive dialysis against MeOH, and dried to yield a powder (**13**). The protected polymer precursor (**13**) was then added into MeOH, and NaOMe was added (to a pH = 9) and stirred for 3 h to deprotect the acetyl groups. The solution was then exhaustively dialyzed again in MeOH for 24 h and concentrated. This product was then added into a flask containing 5 ml dichloromethane, then 5 ml trifluoroacetic acid was added dropwise over 3 h, and the mixture was stirred for an additional 3 h to remove the Boc groups. The solvent was evaporated and the product was dissolved in water to purify via exhaustive dialysis against ultra pure water for 48 h. The product was then collected and dried to yield a white solid of **14** (0.0170 g, 1.30 μmol, 13 %).

^{1}H-NMR (400 MHz, DMSO) δ ppm: 8.31 (s, 2H, triazole H), 4.65-4.61 (dd, 2H, 1,1'-trehalose), 4.53-4.50 (s, 4H, 6,6'-trehalose), 4.15-4.0, 3.62-3.46 (m, 8H, trehalose H), 3.39-3.31 (s, 4H, triazole $CONHCH_2$), 3.25-3.09 (s, 10H, DTPA-NCH_2COOH), 3.07-2.65 (s, 40H, -NCH_2CH_2N-).

After deprotection, the polymers were chelated with gadolinium and terbium chloride in an aqueous buffer solution at pH = 5 to yield the chelated polymer (**14**) TrN4Gd and TrN4Tb. The final polymer structures were exhaustively dialyzed against ultra pure water to remove free lanthanide ion and lyophilized to dryness to yield white fluffy solids (compound **14**) (TrN4Gd: 0.005 g, 0.38 μmol and TrN4Tb: 0.006 g, 0.46 μmol, 64 %).

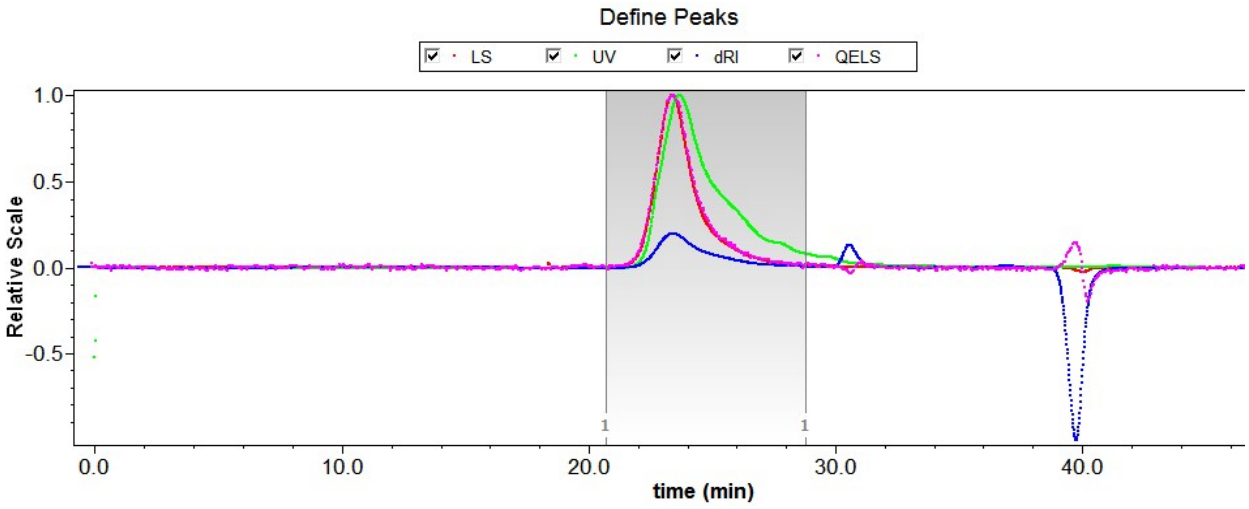


Figure S1. The SEC analysis of the TrN4Gd polymer.

2.5. General synthetic procedure to create the polymer lacking trehalose: N4Gd and N4Tb

Previously, we published the synthesis of the N4Gd and N4Tb materials[3] by polymerizing compound **5** (Scheme S2) with DTPA-BA (compound **12**, Scheme 1) in DMSO followed by removing the Boc protecting groups with trifluoroacetic acid. The polymers were chelated with Gd^{3+} and Tb^{3+} respectively, at pH = 5.5, to yield polymers N4Gd and N4Tb (Figure S2).

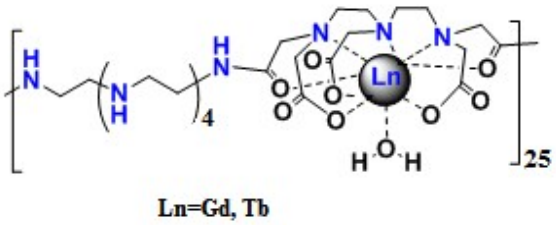


Figure S2. Structures of N4Gd and N4Tb.

3. Polymer Characterization

3.1. Gel permeation chromatography (GPC) analysis of the polymers

The final chelated polymer structures (TrN4Gd, **14**) were characterized using GPC (Figure S1) to determine the weight-average molecular weights (M_w) and polydispersity indices. GPC was performed in an aqueous mobile phase containing 0.45 M sodium acetate in water with 20 % by

volume of acetonitrile at a pH = 7 (adjusted using acetic acid) and at a flow rate of 0.6 mL/min. The columns used for separation were $GMPW_{xL}$ and $G2500PW_{xL}$ (Tosoh Bioscience). This GPC system was equipped with a Waters 2489 UV/Vis detector (Waters Corporation, Milford, MA), a Wyatt Optilab Rex refractometer, and a DAWN HELEOS-II multiangle laser light scattering (MALS) detector (Wyatt, Santa Barbara, CA). The data was recorded and analyzed using the in instrument software package (ASTRA version 6). The dn/dc was also measured using the program embedded in the RI detector of the GPC. The GPC trace of one polymer is shown in Figure S1 (it should be noted that the polymers TrN4Gd and TrN4Tb were chelated from the same batch of compound **13**).

GPC/MALS Analysis:

Compound **14 TrN4Gd**: M_w=1.296×10^4 Da (13.0 kDa), M_n=1.233×10^4 Da (12.0 kDa), Đ=1.08, n_w= 10, n_{eq}=20, dn/dc=0.1350.

Compound N4Gd: M_w=1.6×10^4 Da (16 kDa), Đ=1.2, n_w= 22. Compound N4Tb: M_w=1.83×10^4 , Đ=1.2, n_w= 24.[3]

3.2. ICP-OES analysis of the polymers

Inductively coupled plasma optical emission spectroscopy (ICP-OES) was performed to determine the lanthanide content of the polymers at University of Illinois at Urbana-Champaign using a PerkinElmer 2000DV ICP-OES system.

Table S1. ICP-OES analysis of Gd and Tb content in the trehalose polymers.

Element	*Theoretical*	*Found*
Gd	11.95%	8.65%
Tb	12.02%	8.32%

3.3. Polyplex preparation and gel electrophoresis shift assays

After purification , the TrN4Ln (**14**) polymers were analyzed via gel electrophoresis shift assays to estimate the capability of the polymers to bind siRNA and form polyplexes. The polyplexes were prepared by adding 33 μL of each polymer solution (polymer concentrations calculated according

to the specified N/P ratios) in RNase-free water to formulate a 33 µL solution containing 2 µM siRNA in RNase-free water at room temperature. Agarose gels [2% (w/v)] were prepared by dissolving 1 g agarose in 50 mL TAE buffer (40 mM Tris-acetate, 1 mM EDTA) while heating. Immediately before the solution gelled, 4 µL of ethidium bromide (10 mg/mL) was added, swirled, and the mixture was poured into the gel electrophoresis mold chamber and cooled to ambient temperature. The polyplexes were formed by adding 10 µL of each polymer solution into an Eppendorf tube at various concentrations (N/P ratios) to 10 µL of 2 µM siRNA solution in RNase-free (DEPC-treated) water. The N/P ratio indicates the polymer/nucleic acid ratio for polyplex formulation (the number of secondary amines on the polymer backbone to the number of phosphate groups on the nucleic acid backbone to form the polyplex solutions) as previously described.[1-3] The polyplex solutions were allowed to sit at room temperature for 30 min to promote binding and polyplex formation. Next, BlueJuice™ (Invitrogen) loading buffer (2 µL) was added to each polyplex solution shortly before loading an aliquot into the wells. Electrophoresis was performed at 65 V for 45 min. The complexation of siRNA by the polymer was indicated by the lack of gel migration (or shift) of the siRNA-containing bands.

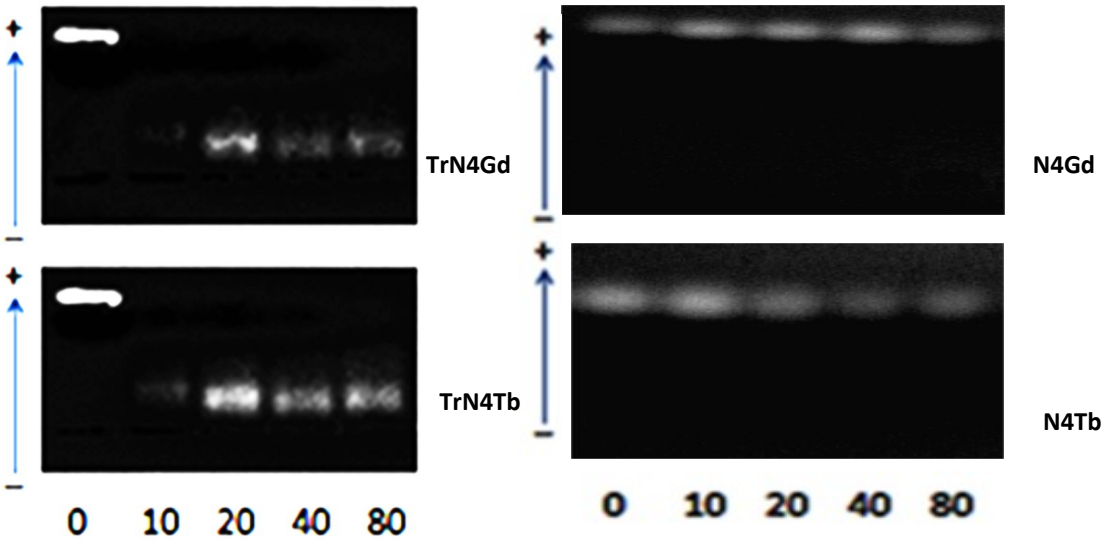


Figure S3. Gel electrophoresis shift analysis of polymers **14** (TrN4Gd and TrN4Tb), N4Gd, and N4Tb formulated with siRNA. siRNA binding and polyplex formation, is qualitatively determined by the N/P binding ratio and observed by the absence of siRNA band migration towards the positive electrode (noticed in the TrN4Gd and TrN4Tb samples at N/P ratios 10-80). The formulation N/P ratios were varied from 0-80, where 0 indicates siRNA only.

3.4. Relaxivity of Gd^{3+} chelated polymers

Water proton T_1 relaxation times of neutral TrN4Gd polymer solutions (**14**, chelated with Gd^{3+}) were studied with a Bruker Minispec (20 mHz, 0.47 T and 60 mHz, 1.41 T) using an inversion recovery pulse sequence (180^{o}- d_t- 90^{o}- acquire). Solutions of the TrN4Gd polymer and Magnevist® (a clinical contrast reagent) were prepared in ultrapure water (5 different concentrations from 2.5 mg/ml to 0.5 mg/ml based on repeat unit molecular weight and on a "per Gd^{3+}" basis analyzed on ICP-OES). It should be noted that due to the dilute nature of the solutions, the pH remained near neutral (pH of the highest concentration polymer solution, 2.5 mg/ml, was pH = 6.49). All the measurements were performed in triplicate at 37 °C (error bars represent the standard deviation of the three measurements).

3.5. Dynamic light scattering study to determine polyplex size

Hydrodynamic diameters and Zeta potentials of the polymer-siRNA polyplex formulations (Figure 2) were determined by dynamic light scattering (DLS) using a ZetaSizer (Nano ZS) instrument from Malvern, Inc. (Worcestershire, United Kingdom) equipped with a 633 nm laser. For each size measurement, polyplexes were formulated by addition of 33 μL of polymer solution in RNase-free water to 33 μL of a 2 μM siRNA solution in RNase-free water at room temperature to form complexes at an N/P ratio of 40 and were allowed to sit at room temperature for 60 min prior to measurement via DLS. Each polyplex solution was then diluted to a final volume of 750 μL with RNase-free water for Zeta potential measurements. Each experiment was performed in triplicate.

3.6. Luminescence lifetime measurements to determine the average number of water coordinate sites on the lanthanide chelates

The number of water coordination sites (q) per chelate was determined by measuring the luminescence lifetimes of the Tb^{3+} chelated polymer TrN4Tb (**14**) in D_2O and H_2O using a Cary Eclipse fluorescence spectrometer (Agilent Technologies, USA). The data was processed with Agilent Cary WinFLR software.

The samples were prepared at a 1 mM concentration (800μL) using D_2O and H_2O and deposited into a small-volume quartz cuvette. Lifetime measurements were performed with the excitation wavelength of 350 nm (slit width 5 nm), emission wavelength of 550 nm (slit width 5 nm), a delay time of 0.1 ms, and a gate time of 0.1 ms. All measurements were performed in triplicate. Data revealed the luminescent lifetime (τ) of TrN4Tb to be $\tau = 1.62$ ms in D_2O and $\tau = 1.27$ ms in H_2O.

The revised Horrocks equation is listed as follows:

$$q_{Tb} = 5\left[\left(\frac{1}{\tau_{H2O}} - \frac{1}{\tau_{D2O}}\right) - 0.06x\right]$$

x = number of N-H oscillators from amide groups coordinated to the Tb^{3+}though the carbonyl (x=2).

3.8. Lanthanide resonance energy transfer (LRET) study of polymer siRNA complexation

A Synergy™ H1 monochromator-based multi-mode microplate reader was applied in this study on a 96 well plate (BioTek, Winooski, VT). The samples were prepared by adding 40 µL of the lanthanide chelated polymer solution into 40 µl of a 2 µM solution of TMR-labeled siRNA and incubated for 60 min. The same volume of RNase free water (20 µl) was added to the control polyplexes to achieve the same concentration of polyplexes at an N/P ratio of 40. To examine the LRET pair (TrN4Tb/TMR-siRNA), the sample was excited at 345 nm. All spectra were collected from 400 nm to 700 nm. The delay time for data collection was varied (20, 40, and 50 µsec) to monitor LRET (Figure 3).

4. Biological Studies

4.1. Flow cytometry study of polyplex uptake into cultured cells

Flow cytometry was performed to examine the cellular uptake of Cy5-labeled siRNA with the various formulations 3 h post-transfection. In general, U-87_luc2 glioblastoma cells were seeded at 300,000 cells/well in 6-well plates 24 h prior to transfection. To transfect, 33 µL of each polymer solution (diluted to yield the proper N/P ratio) was added to 33 µL of a solution containing 2 µM Cy5-labeled siRNA. After 30 min of incubation at room temperature (to form the polyplexes), each polyplex solution was pipetted into 1584 µL of pre-warmed Opti-MEM to yield the final transfection solution. Each well was treated with 500 µL of the obtained transfection solution. After 3 h, the media was removed and cells were washed with 500 µL/well CellScrub™ Buffer for 15 min at room temperature. The CellScrub™ Buffer was then aspirated and cells were exposed to trypsin [0.05% (w/v), 500 µL/well] for 3 min to provide detachment from the plate. Then, complete DMEM (500 µL/well) was applied to inhibit trypsin. The cell suspension was collected and centrifuged at 1000 rpm for 10 min at 4 ^{o}C. The supernatant was removed and cells were washed twice with 0.5 mL PBS and

centrifuged to remove the extracellular polyplexes. Finally, 1 mL PBS was added and the suspensions were kept on ice prior to flow cytometry analysis. Propidium iodide (2.5 μL) was added prior to the analysis to gate out dead cells in flow cytometry analysis. For analysis, a flow cytometer (FACSCalibur and FACSVerse, Becton Dickenson, San Jose, CA) equipped with a helium-neon laser to excite Cy5 at 633 nm was used to count twenty thousand events for each sample. The threshold fluorescence level was defined by manually adjusting the positive region such that <1% of negative control cells were positive for fluorescence. Each experiment was performed and analyzed in triplicate.

4.2. Luciferase and protein assays

Luciferase-expressing glioblastoma cells (U-87_luc2) were seeded at 50,000 cells/well in 24-well plates 24 h prior to transfection. In general, anti-luciferase (Luc2) siRNA, control (siCon) siRNA, and polymer stock solutions were diluted with RNase-free water, and the polyplexes were formed by the addition of 33 μL of polymer solution to 33 μL of siRNA solution, followed by incubation for 30 min at room temperature. The resulting polyplex solutions were then added to pre-warmed Opti-MEM or DMEM to yield the transfection solutions. Cells were washed with PBS before the addition of 200 μL of the transfection solution. The formation of siRNA-containing lipoplexes using Lipofectamine™ 2000 was performed according to the manufacturer's protocol. Polyplexes with the polymers were formulated at various N/P ratios. The cells were incubated with polyplex/lipoplex solutions for 4 h before complete DMEM was added. Forty-eight hours later, the cells were washed with 500 μL PBS and treated with 1x cell lysis buffer (Promega, Madison, WI) for 15 min at room temperature. Aliquots (5 μL) of cell lysate were examined on 96-well plates with a luminometer (GENios Pro, TECAN US, Research Triangle Park, NC) for luciferase activity over 10 s. For each well, 100 μL of luciferase assay substrate (Promega, Madison, WI) was added. The average of duplicate measurements on each individual replicate was utilized for calculation. Three separate sample replicates were performed for each experiment.

The amount of protein (mg) in cell lysates was calculated using a standard curve generated with bovine serum albumin by following the protocol included in a Bio-Rad DC protein assay kit. The relative light unit (RLU)/mg protein was then calculated and averaged across replicate wells. The protein and luciferase levels of non-transfected cells were used for normalizing the data and calculating the extent of gene knockdown. Each treatment was tested in triplicate in 24-well plates. The data was

statistically analyzed by JMP software (SAS Institute Inc., Cary, NC). A student's t-test was used to find significant differences between means of samples at alpha level of 0.05. Significant differences are denoted by "*" in Figure 4.

4.3. Cytotoxicity study via MTT assay

MTT reagent [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was used to estimate the cytotoxicity of the formulations as previously described.[4] U-87_luc2 glioblastoma cells were seeded at 50,000 cells/well in 24-well plates 24 h prior to transfection. The polyplexes were formed following the procedure described above. Transfection media (200 µL) was added to each well; 4 h later, complete DMEM was added at 1 mL/well. Then after 24 h, the media was aspirated and the cells were washed with PBS (500 µL/well). Serum-containing DMEM (1 mL) with 0.5 mg/mL of MTT was added to each well and cells were incubated for 1 h. The media was then replaced with 600 µL of DMSO for 15 min at room temperature. A 200 µL aliquot of the media was transferred to a well of a 96-well plate for analysis by UV-Vis analysis at a wavelength of 570 nm. Samples of non-transfected cells were used for normalization of the data. Each experiment was performed in triplicate.

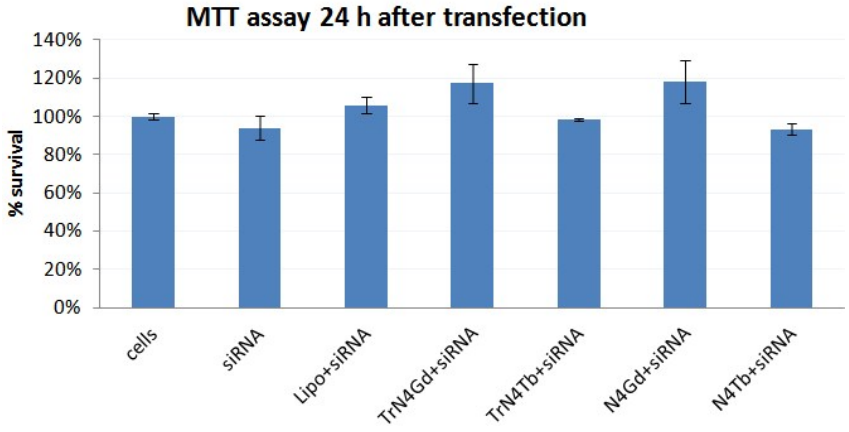


Figure S4. Cytotoxicity examined via MTT assay 24 h after transfection. All the polyplexes were formed at N/P = 40 and the Lipofectamine complexes were formulated at the manufacturer's recommended conditions. The transfection concentration of siRNA was 100 nM. All formulations were examined in triplicate and the error bars represent standard deviations.

References:

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