Supplementary Information

A bioreducible linear poly(β-amino ester) for siRNA delivery

Kristen L. Kozielski,[a] Stephany Y. Tzeng[a] and Jordan J. Green[a]

[a] *Department of Biomedical Engineering, Department of Ophthalmology, the Institute for Nanobiotechnology, and the Translational Tissue Engineering Center*
The Johns Hopkins University School of Medicine
400 N. Broadway/Smith Building Room 5017
Baltimore, Maryland 21231, United States
Tel: 410-614-9113
E-mail: green@jhu.edu

Materials and Methods

Materials

All chemicals for monomer synthesis were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) and used without further purification. Monomers were purchased from Alfa Aesar (Ward Hill, MA). YO-PRO®-1 Iodide was purchased from Life Technologies™ (Carlsbad, CA). Ambion® *Silencer*® eGFP and Ambion® *Silencer*® Negative Control #1 siRNA were purchased from Life Technologies™. Lipofectamine™ 2000 and Opti-MEM™ I were purchased from Invitrogen (Carlsbad, CA) and used according to manufacturer's instructions. CellTiter 96® AQ_{ueous} One MTS assay was purchased from Promega (Fitchburg, WI) and used according to manufacturer's instructions. Cells were grown in 89% GIBCO® DMEM-F12, 1% GIBCO® Antibiotic-Antimycotic (Invitrogen), and 10% Corning Cellgro® Heat-Inactivated FBS.

BR6 synthesis

All chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and used without further purification. Bis(2-hydroxyethyl) disulfide (15.4 g, 10 mmol) and triethylamine (TEA, 37.5 mL, 300 mmol) were dissolved in 450 mL of tetrahydrofuran previously dried with Na_2SO_4 in a 1000 ml round bottom flask. The flask was flushed with N_2 for 10 min and then maintained under a N_2 environment. Acryloyl chloride (24.4 mL, 300 mmol) was dissolved in 50 mL tetrahydrofuran then added to the flask dropwise over 2 hrs while stirring. The reaction was carried out for 24 hrs, then the TEA HCl precipitate was removed by filtration, and the solvent was removed by rotary evaporation. The product was dissolved in 100 mL dichloromethane and washed five times with 200 mL of an aqueous solution of 0.2 M Na_2CO_3 and three times with distilled water. The solution was dried with $NaSO_4$ and the solvent was removed by rotary evaporation. The product 2,2'-disulfanediylbis(ethane-2,1-diyl) (BR6) was confirmed via H^1-NMR: ($CDCl_3$, 400Hz), δ2.95 (2H, *t*, $CH_2CHCOOCH_2CH_2S$), δ3.95 (2H, *t*, $CH_2CHCOOCH_2CH_2S$), δ5.8-5.9 (1H, *d*, $CH_2CHCOOCH_2CH_2S$), δ6.1-6.2 (1H, *dd*, $CH_2CHCOOCH_2CH_2S$), δ6.4-6.5 (1H, *d*, $CH_2CHCOOCH_2CH_2S$).

Polymer Synthesis

Base monomer diacrylate BR6 or hexane-1,6-diyl diacrylate (B6) was polymerized with side chain monomer 4-amino-1-butanol (S4) at a molar base:side chain ratio of 1.01:1 at 500mg/mL in dimethyl sulfoxide at 90ºC for 24 hrs while stirring. For end-capping with 1-(3-aminopropyl)-4-methylpiperazine (E7), base polymer was dissolved in anhydrous dimethyl sulfoxide at 100 mg/mL with 0.2mM E7. The reaction was allowed to proceed for 1 hr at room temperature while shaking. Polymers were characterized via H^1-NMR and GPC. H^1-NMR of

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R647 base polymer: (d_6-DMSO, 400Hz), δ1.3-1.5 (4H, *br*, $NCH_2CH_2CH_2CH_2OH$), δ2.3-2.5 (6H, *br,* $OOCCH_2CH_2N$ and $NCH_2CH_2CH_2CH_2OH$), δ2.6-2.7 (4H, *t*, $OOCCH_2CH_2N$), δ2.9-3.1 (4H, *t*, $COOCH_2CH_2S$), δ4.2-4.4 (4H, *t*, $COOCH_2CH_2S$). H^1-NMR of 647 base polymer: (d_6-DMSO, 400Hz), δ1.25-1.4 (8H, br, $NCH_2CH_2CH_2CH_2OH$ and $COOCH_2CH_2CH_2$), δ1.5-1.65 (4H, br, $COOCH_2CH_2CH_2$), δ2.3-2.4 (6H, br, $NCH_2CH_2CH_2CH_2OH$ and $OOCCH_2CH_2N$), δ2.6-2.7 (4H, br, $OOCCH_2CH_2N$), δ3.3-3.4 (2H, br, obsc, $NCH_2CH_2CH_2CH_2OH$), δ3.95-4.05 (4H, br t, $COOCH_2CH_2CH_2$), δ4.3-4.4 (br, $NCH_2CH_2CH_2CH_2OH$). H^1-NMR of E7 endcap: (d_6-DMSO, 400Hz), δ1.50, (2H, quint, $NHCH_2CH_2CH_2N<(CH_2CH_2)>NCH_3$), δ2.13 (3H, s, $NHCH_2CH_2CH_2N<(CH_2CH_2)>NCH_3$), δ2.3-2.4 (10H, br, obsc, $NHCH_2CH_2CH_2N<(CH_2CH_2)>NCH_3$), δ2.47 (2H, t, $NHCH_2CH_2CH_2N<(CH_2CH_2)>NCH_3$).

Gel Permeation Chromatography

GPC was performed using a Waters GPC system using three Waters Styragel columns in a series (HR 1, HR 3 and HR4) and a Waters 2414 refractive index detector, both maintained at 40ºC throughout all samples, which were loaded using a Waters 717plus autosampler (Waters Corp., Milford, MA). All samples were loaded at 5 mg/mL using 94% THF, 5% DMSO, and 1% piperidine (v/v) as the eluent at a flow rate of 1.0 mL/min for 40 min. Polymer molecular weights were calculated relative to polystyrene standards (Shodex, Japan).

Polymer Degradation Study

Each polymer R647 or 647 was either diluted in PBS or in a solution of 5 mM GSH in PBS to a final polymer concentration of 7 mg/mL. Each was allowed to incubate for 5 min at room temperature and was then frozen at -80°C and lyophilized. GPC was performed on these samples following the GPC protocol above. Excess salts were precipitated out of the GPC solvent and removed by filtration prior to performing GPC.

Gel Retention Assay

Either polymer R647 or 647 was incubated in 25 mM sodium acetate (NaAc) with siRNA at polymer to scRNA weight ratios ranging from 600 wt/wt to 0 wt/wt (siRNA alone), and allowed to form particles at room temperature for 10 min. To compare the effects of a nonreducing and reducing environment on the particles, either PBS or PBS containing L-glutathione (GSH) were added to after the 10min incubation. The final GSH concentration was 5 mM for the reduction experiments. A solution of 30% glycerol was added to the particles in a 1:5 v/v ratio. The particles were loaded into a 1% agarose gel containing 1 μg/mL ethidium bromide and electrophoresed at 100 mV for 20 min. Gels were visualized using UV light exposure.

YO-PRO®-1 Iodide Competition Binding Assay

siRNA was diluted to 1.33 μM in 25 mM NaAc and combined in a 1:1 v/v ratio with 1.33 μM YO-PRO®-1 Iodide in 25mM NaAc in all wells of a black-bottom 96-well plate. Polymers were diluted at concentrations ranging from 512 to 0.5 times these concentrations and combined in quadruplicates in a 1:2 v/v ratio with the siRNA/YO-PRO solution, and allowed to incubate for 15 min at room temperature. 25 mM NaAc without polymer was also added in a 1:2 v/v ratio to four siRNA/YO-PRO solutions to supply background fluorescence values. Fluorescence was measured at 15 min using a BioTek Synergy 2 fluorescence plate reader at 490/510 nm (ex/em).

Particle Size Determination: Nanoparticle Tracking Analysis

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siRNA was diluted to 4.8 μg/mL and polymers were separately diluted to either 450 or 112.5 times this concentration in 25 mM NaAc. The solutions were combined in a 1:1 v/v ratio and allowed to incubate for 10 min at room temperature. Particle solutions were then diluted 1:100 in PBS. Particle size was determined using a NanoSight NS500 and analyzed using NanoSight NTA 2.3 software. Measurements were repeated with two new particle formulations for each condition. The NTA analysis reported the number-average hydrodynamic radius of the particles.

Particle ζ-Potential Determination: Dynamic Light Scattering

Particles were formed as described for determining particle size. Particles were diluted 1:650 v/v in PBS and loaded into a disposable cuvette cell. Particle surface charge was determined via dynamic light scattering (DLS) using a Malvern Zetasizer NanoZS.

Transfection of GBM 319 glioblastoma cells with siRNA against eGFP and Cell Viability

GFP^+ GBM 319 cells were plated at a cell density of 15,000 cells/well in 96-well tissue culture plates and allowed to adhere overnight. siRNA either targeting eGFP or a scrambled, non-targeting control (scRNA) was diluted to 4.8 μg/mL and polymers were separately diluted to either 450 or 112.5 times this concentration in 25 mM NaAc. The solutions were combined in a 1:1 v/v ratio and allowed to form particles for 10 min at room temperature. The cell culture media was replaced with serum-free media, and the particles were diluted in the media in quadruplicates in a 1:6 v/v ratio for a final siRNA dose of 26.7. Cells were incubated with particles for 4 h, after which the particle solutions were removed and replaced with fresh, complete media. Cell viability was assessed 24 hr post transfection using a CellTiter 96® AQ_{ueous} One MTS assay following manufacturers instructions.

Flow Cytometry

At 9 d post-transfection cells were prepared for flow cytometry by trypsinization with 30 μL 0.25% trypsin-EDTA and suspension in 170 μL of a buffer of PBS with 1:50 FBS and 1:200 propidium iodide (PI). The 200 μL suspension of cells was moved to a 96-well round bottom plate and centrifuged at 1000 rpm for 5 min. 170 μL was removed from the supernatant, and the cell pellets were resuspended in the remaining 30 μL. The plate was loaded onto an Intellicyt high-throughput loader and attached to an Accuri C6 flow cytometer. Hypercyt software was used to discriminate events between each well and FlowJo was used to analyze the flow cytometry results. PI signal was used to remove dead or dying cells from analysis. Geometric mean fluorescence FL1 signal was determined for each sample, and GFP knockdown was determined for each condition by the difference in FL1 signal in eGFP siRNA-treated cells to scRNA-treated cells, normalized to scRNA-treated cells.

Statistics

All results are presented as mean ± standard error of the mean. Statistical significance of results for nanoparticle size and ζ-potential were determined using a one-way ANOVA with Tukey's Multiple Comparison post tests. Significance results for gene knockdown and cell viability were deterimined using a one-way ANOVA with Dunnett's post tests using Lipofectamine 2000 as the control. All significance tests with $p < 0.05$ were considered significant.

Electronic Supplementary Material (ESI) for Chemical Communications

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Supporting Data

Gel Permeation Chromatography

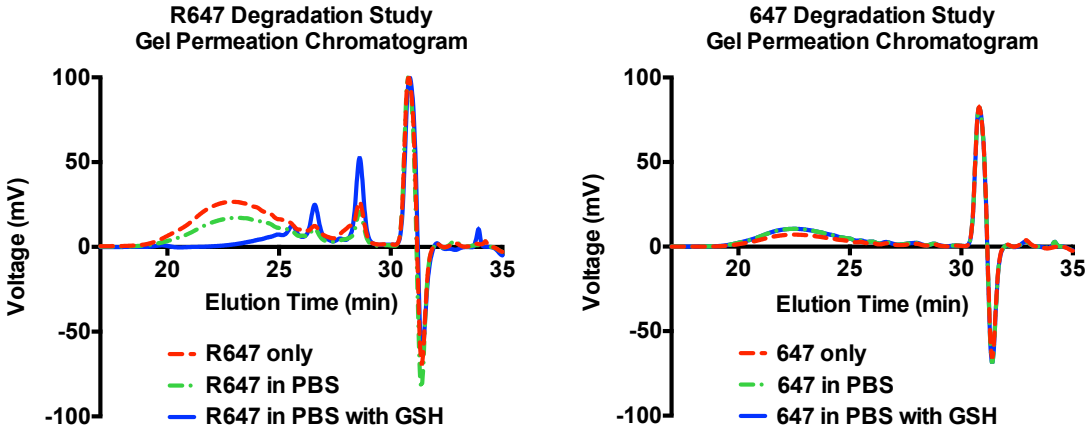

Figure S1. GPC results of R647 (left) and 647 (right) following degradation study. Red dashed lines show GPC chromatogram of untreated polymers, green dashed lines show each polymer following a 5 min incubation in PBS, solid blue lines show each polymer following a 5 min incubation in PBS containing 5 mM GSH. The results demonstrate that a reducing environment comparable to the cytosol is capable of degrading R647 and not 647 within 5 min.