New Poly(D-glucaramidoamine)s Induce DNA Nanoparticle Formation and Efficient Gene Delivery into Mammalian Cells

*Yemin Liu, Laura Wenning, Matthew Lynch[†], and Theresa M. Reineke**

Department of Chemistry, University of Cincinnati, Cincinnati, OH 45221-0172

[†]Procter and Gamble Company, Corporate Research Division, Cincinnati, OH 45061

Supporting Information

EXPERIMENTAL SECTION

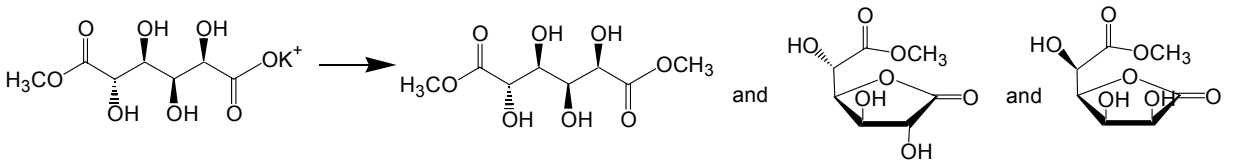
I. Synthesis and purification of comonomers and polymers

General. All reagents used in the synthesis, if not specified, were obtained from Aldrich Chemical Co. (Milwaukee, WI) with purity of more than 98%, and were used without further purification. All polymers were purified by dialysis in ultra-pure water

using Spectra-Por 1000 MWCO membrane. The products were dried by a Flexi-dry MP lyophilizer. The mass spectra were obtained from an IonSpec HiResESI mass spectrometer in positive ion mode. NMR spectra were collected on a Bruker AC-250 MHz spectrometer.

Esterification of D-glucaric acid comonomer.[1] Methanolic HCl was prepared by careful addition of 10 mL acetyl chloride to 12.5 mL methanol at 0 ºC. D-Saccharic acid (monopotassium salt) was refluxed with 50 mL methanol and the solution of methanolic HCl for 4 hours. The solid KCl in the crude reaction mixture was removed by filtration. Triethylamine was added to neutralize HCl. The esterified D-glucaric acid (a mixture of three isomers as shown in Scheme 1) was concentrated and dried under vacuum (8.55 g). Yield: 61.7%. ES/MS: *m/z* 277.0326 $[M+K]^+$ (dimethyl D-glucarate) and 245.0071 (methyl D-glucarate 1,4-lactone and methyl D-glucarate 6,3-lactone) $[M+K]^+$. The dimethyl D-glucarate isomer was estimated to be the major product in the reaction (83%) by the relative intensities of the parent ion peaks in the mass spectrum. Thus, for monomer mole calculation, the molecular weight of the dimethyl D-glucarate isomer was used.

Scheme 1. Structures of esterified D-glucaric acid



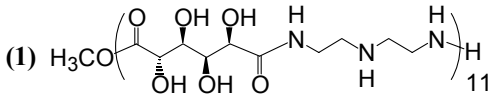
Dimethyl D-glucarate, **83 %**

Methyl D-glucarate 1, 4-lactone

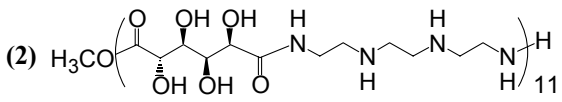
Methyl D-glucarate 6, 3-lactone **17 %**

Purification of pentaethylenehexamine.[2] Crude pentaethylenehexamine was purchased from Acros (Morris Plains, NJ), and was fractionally distilled twice at 50 mTorr. The fraction (50 mL) boiling at 145-155 °C was collected, and then dissolved in methanol (300 mL). Concentrated HCl was added dropwise into this methanol solution with stirring at 0 °C until the pentaethylenehexamine hexahydrochloride precipitated. The solid pentaethylenehexamine hexahydrochloride was collected by filtration, washed with acetone and ethyl ether, then dried under vacuum. ^{1}H-NMR (D_2O with one drop of DCl): δ 3.93 (s, 12H), 3.32 (t, 4H), 3.24 (t, 4H).

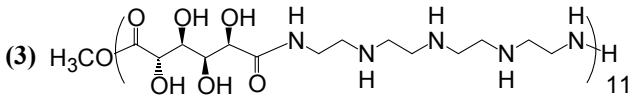
Polymerization.[1] Each polymer was synthesized through condensation polymerization of an amine comonomer with esterified D-glucaric acid in methanol at room temperature. All products obtained were of a white color.



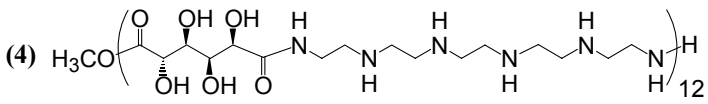
Poly(D-glucaramidodiethyleneamine) (1): Diethylenetriamine (0.09 g, 0.87 mmol) was added to a methanol solution (8.40 mL) of esterified D-glucaric acid (0.20 g, 0.84 mmol). The clear solution became cloudy after approximately 10-15 min while stirring at room temperature. After 48 hours, the polymerization mixture was dissolved in and dialyzed against ultra pure water to purity. Yield: 0.22 g, 0.79 mmol, 91.2%. ^{1}H NMR (DMSO-d_6): δ 7.86 (s, 1H), 7.69 (s, 1H), 4.02 (d, 1H), 3.96 (d, 1H), 3.88 (t, 1H), 3.72 (t, 1H), 3.17 (s, 4H), 2.58 (s, 4H).



Poly(D-glucaramidotriethylenediamine) (2): Triethylenetetramine hydrate (containing 20.42% H_2O, 0.15 g, 0.82 mmol) was added to a methanol solution (8.40 mL) of esterified D-glucaric acid (0.20 g, 0.84 mmol). The clear solution became cloudy after approximately 10-15 min while stirring at room temperature. After 48 hours, the polymerization mixture was dissolved in and dialyzed against ultra pure water to purity. Yield: 0.18 g, 0.56 mmol, 68.6%. ^{1}H NMR (DMSO-d_6): δ 7.89 (s, 1H), 7.70 (s, 1H), 4.05 (d, 1H), 3.97 (d, 1H), 3.89 (t, 1H), 3.73 (t, 1H), 3.20 (s, 4H), 2.60 (s, 8H).



Poly(D-glucaramidotetraethylenetriamine) (3): Esterified D-glucaric acid (0.20 g, 0.84 mmol) was added to a methanol solution (8.40 mL) of triethylamine (0.42 g, 4.15 mmol) and tetraethylenepentamine pentahydrochloride (0.31 g, 0.83 mmol). The clear solution became cloudy after approximately 30 min while stirring at room temperature. After 6 hours, the polymerization mixture was dissolved in and dialyzed against ultra pure water to purity. Yield: 0.11 g, 0.30 mmol, 36.0%. ^{1}H NMR (DMSO-d_6): δ 7.90 (s, 1H), 7.70 (s, 1H), 4.02 (d, 1H), 3.96 (d, 1H), 3.88 (t, 1H), 3.73 (t, 1H), 3.19 (s, 4H), 2.60 (s, 12H).



Poly(D-glucaramidopentaethylenetetramine) (4): Esterified D-glucaric acid (0.20 g, 0.84 mmol) was added to a methanol solution (5.60 mL) of triethylamine (0.51 g, 5.04

mmol) and pentaethylenehexamine hexahydrochloride (0.38 g, 0.84 mmol). The clear solution became cloudy after approximately 30 min while stirring at room temperature. After 8 hours, the polymerization mixture was dissolved in and dialyzed against ultra pure water to purity. Yield: 0.15 g, 0.37 mmol, 44.0%. ^{1}H NMR (DMSO-d_6): δ 7.87 (s, 1H), 7.72 (s, 1H), 4.02 (d, 1H), 3.94 (d, 1H), 3.87 (t, 1H), 3.71 (t, 1H), 3.16 (s, 4H), 2.59 (s, 14H), 2.22 (s, 2H).

II. Polymer and polyplex characterization

Gel permeation chromatography (GPC) experiments. The molecular weight, polydispersity and Mark-Houwink-Sakurada (MHS) parameter for the polymers were analyzed by a Viscotek GPCmax Instrument using a Triple Detection System (static light scattering, viscometry and refractive index). The data were obtained using a ViscoGEL $GMPW_{XL}$ column using a mobile phase of 0.5 M sodium acetate in ultra pure water containing 20% acetonitrile, brought down to a pH = 5.0 with acetic acid. Each sample was prepared at a concentration of 10-15 mg/mL in the eluant previous described, 100 µL of sample was injected onto the column, and the sample was eluted at 1.0 mL/min. All GPC chromatograms are listed on Page 9 and 10. All analytical data for polymers **1-4** are shown in Table 1.

Table 1. The MHS parameter (α), molecular weight (M_w), polydispersity (M_w/M_n), and degree of polymerization (n) data for polymers **1-4** as determined by static light scattering and viscometry.

Polymer	α	M_w (kDa)	M_w/M_n	n
(1)	0.677	3.0	2.0	11
(2)	0.669	3.4	1.4	11
(3)	0.642	3.9	1.4	11
(4)	0.608	4.9	1.6	12

Gel retardation experiments.[3] The DNA-binding ability of **1-4** was examined with gel retardation experiments. Plasmid DNA (gWiz-Luc) was purchased from Aldevron (Fargo, ND) and the DNAse, RNAse free water was purchased from Gibco BRL (Gaithersburg, MD). GWiz-luc (1 μg; 10 μL of a 0.1 μg/μL in DNAse, RNAse free water) was mixed with an equal volume of polymer at N/P ratios (the ratio of polymer nitrogens (N) / phosphate groups (P) on DNA) between 0 and 50. Each solution was incubated for 30 min to allow polymer-DNA binding. An aliquot of 10 μL was run in a 0.6% agarose gel containing 6 μg of ethidium bromide/100 mL TAE buffer (40 mM Tris-acetate, 1 mM EDTA).

Dynamic light scattering. Particle (polyplex) size was measured on a Zetapals dynamic light scattering instrument (Brookhaven Instruments Corporation, Holtsville, NY) at a λ 662.0 nm. GWiz-Luc (3 μg; 150 μL of a 0.02 μg/μL in DNAse, RNAse free water) was complexed with each polymer at N/P ratio 5 and 30 in DNAse, RNAse free water, and then allowed to stand for one hour before diluting to 0.7 mL with DNAse,

RNAse free water. The results of samples are reported as an average of ten measurements: 164 nm (1), 99 nm (2), 81 nm (3), and 54 nm (4) at the N/P ratio 5; 256 nm (1), 441 nm (2), 140 nm (3), and 175 nm (4) at the N/P ratio 30.

Transmission electron microscopy. Polymer-DNA complexes were prepared at an N/P ratio 30 as described above for the dynamic light scattering. After one hour of incubation, 5 μL of sample was applied in duplicate to 400-mesh carbon-coated copper grids (EMS, Fort Washington, PA) for 60 s, after which excess liquid was removed by blotting with filter paper. Samples were then negatively stained with 2% uranyl acetate for 90 s before blotting. Images were recorded using a JEOL JEM-1230 transmission electron microscope operated at 80 kV.

III. Cell culture experiments

Cell transfection and luciferase assay.[3] BHK-21 cells were purchased from ATCC (Rockville, MD) and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 units/mg penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin at 37 ºC and 5% CO_2. Media and supplements were purchased from Gibco BRL (Gaithersburg, MD). BHK-21 cells were plated at 50,000 cells per well in 24-well plates and incubated for 24 hours.

One hundred and fifty microliters of polymer dissolved in DNase free water was added to 150 μL of pDNA (gWiz-Luc, 0.02 μg/μL in DNase free water) at various N/P ratios. The mixtures were then incubated for one hour and diluted to 900 μL with reduced serum

media (pH 7.2, Gibco BRL,Gaithersburg, MD) right before the transfection. Twenty four hours after cell plating, the cells were transfected with 1 μg of pDNA complexed with each of the polymers (**1**-**4**, chitosan, and linear PEI) at the N/P ratios of 5, 10, 15, 20, 25 and 30 (300 μL of each solution above) or with naked pDNA in triplicate in reduced serum media. After 4 hours, 800 μL of DMEM was added to each well. Twenty four hours after transfection, the media was replaced with 1 mL of DMEM. Forty seven hours after transfection, cell lysates were analyzed for luciferase activity with Promega's luciferase assay reagent (Madison, WI) and for vector toxicity as described below. For each sample, light units were integrated over 10 s in duplicate with a luminometer (GENios Pro, TECAN US, Research Triangle Park, NC), and the average was utilized.

Toxicity. Cell viability was measured by the amount of protein in cell lysates obtained 47 hours after transfection with polymers **1-4**, chitosan and linear PEI at various N/P ratios. Protein levels of transfected cells were determined by Bio-Rad's DC protein assay (Hercules, CA) and normalized with protein levels of cells transfected with naked DNA. A protein standard curve was run with various concentrations (0.26 mg/mL - 2.37 mg/mL) of bovine serum albumin (98%, Sigma, St. Louis, MO) in cell culture lysis buffer.

References:

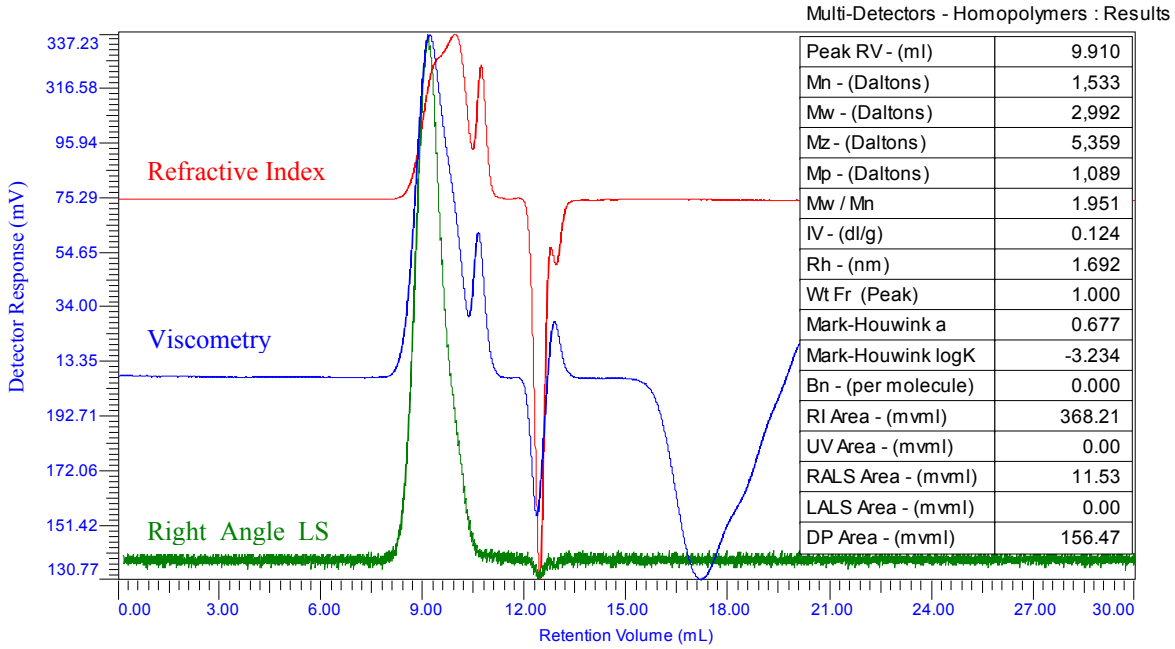
(1) Kiely, D.E.; Chen, L.; Lin, T. H. *J. Am. Chem. Soc.*, **1994**, *116*, 571.

(2) Jonassen, H. B., Bertrand, J.A.; Groves, F.R.; Stearnes, R.I. *J. Am. Chem. Soc.*, **1957**, *79*, 4279.

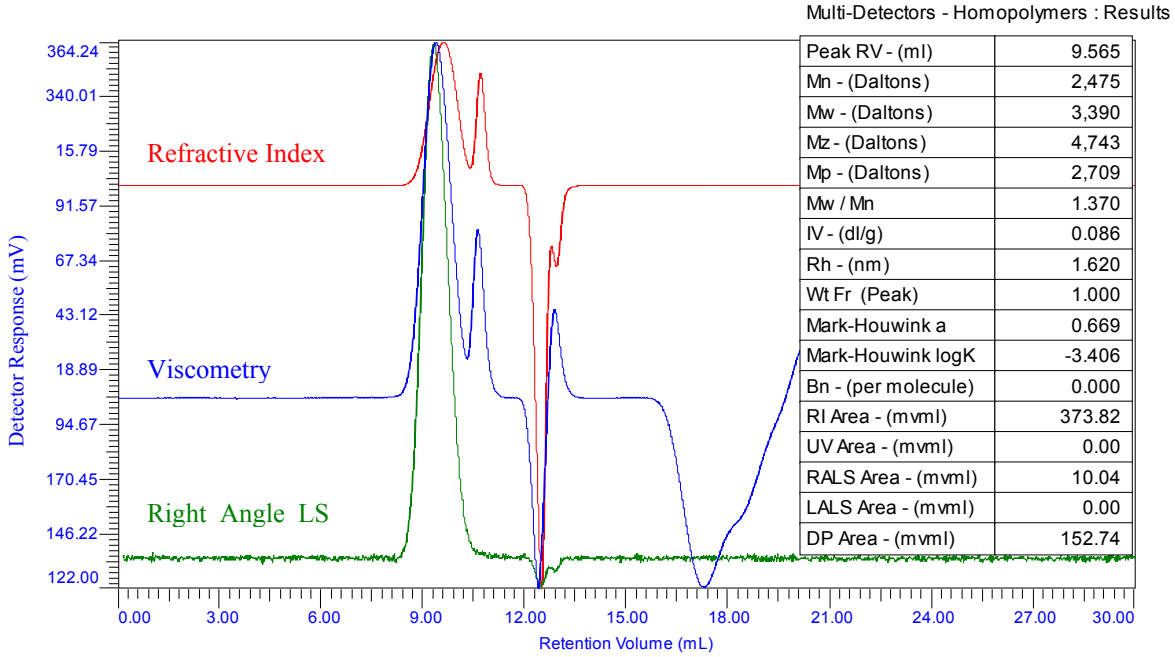
(3) Reineke, T.M.; Davis, M. E.; *Bioconjugate Chem.*, **2003**, *14*, 247

GPC data for polymers 1-4:

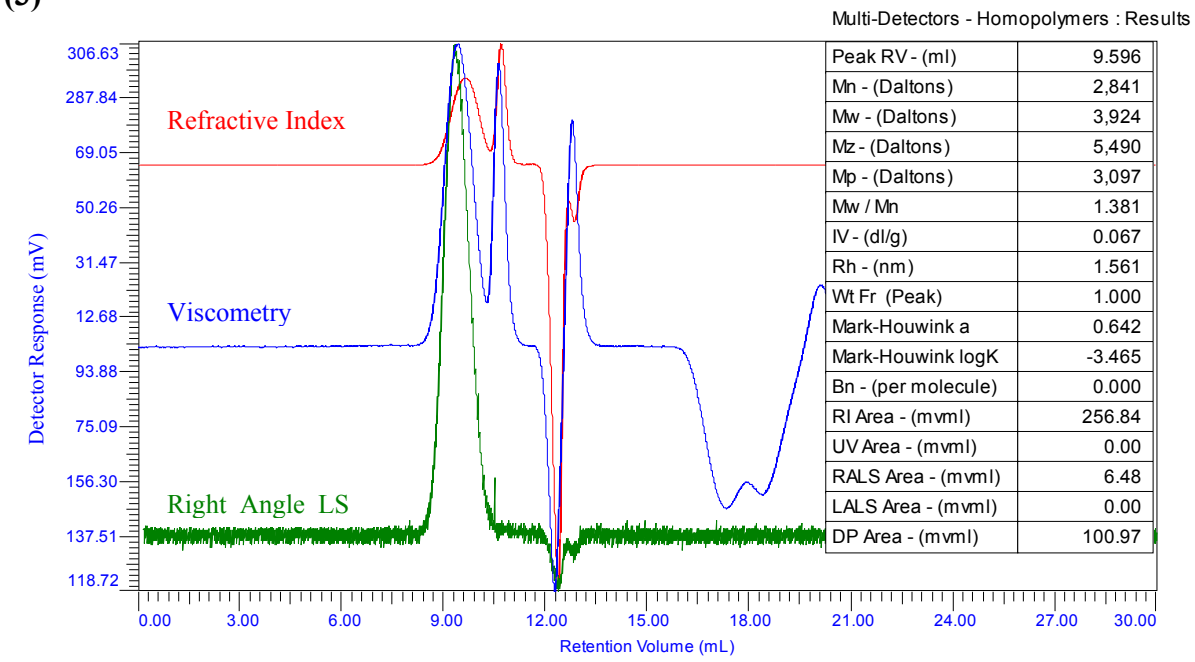
(1)



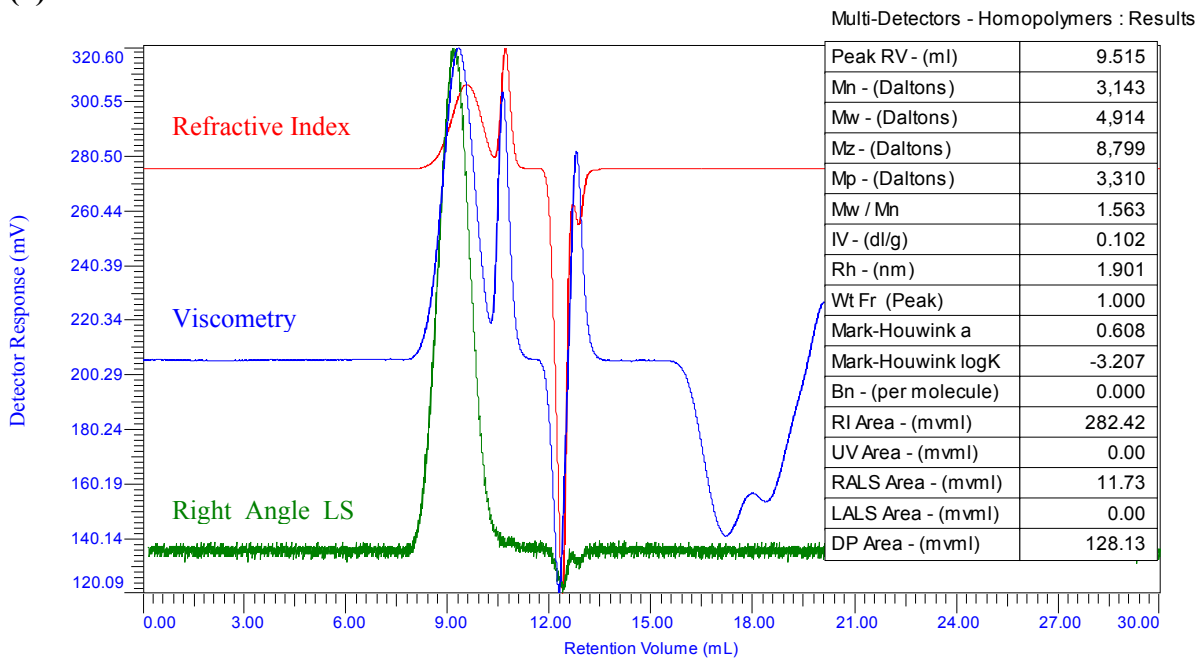
(2)



(3)


Multi-Detectors - Homopolymers : Results
Refractive Index
Viscometry
Right Angle LS
Detector Response (mV)
306.63
287.84
69.05
50.26
31.47
12.68
93.88
75.09
156.30
137.51
118.72
0.00
3.00
6.00
9.00
12.00
15.00
18.00
21.00
24.00
27.00
30.00
Retention Volume (mL)
Peak RV - (ml) 9.596
Mn - (Daltons) 2,841
Mw - (Daltons) 3,924
Mz - (Daltons) 5,490
Mp - (Daltons) 3,097
Mw / Mn 1.381
IV - (dl/g) 0.067
Rh - (nm) 1.561
Wt Fr (Peak) 1.000
Mark-Houwink a 0.642
Mark-Houwink logK -3.465
Bn - (per molecule) 0.000
RI Area - (mvml) 256.84
UV Area - (mvml) 0.00
RALS Area - (mvml) 6.48
LALS Area - (mvml) 0.00
DP Area - (mvml) 100.97

(4)


Multi-Detectors - Homopolymers : Results
Refractive Index
Viscometry
Right Angle LS
Detector Response (mV)
320.60
300.55
280.50
260.44
240.39
220.34
200.29
180.24
160.19
140.14
120.09
0.00
3.00
6.00
9.00
12.00
15.00
18.00
21.00
24.00
27.00
30.00
Retention Volume (mL)
Peak RV - (ml) 9.515
Mn - (Daltons) 3,143
Mw - (Daltons) 4,914
Mz - (Daltons) 8,799
Mp - (Daltons) 3,310
Mw / Mn 1.563
IV - (dl/g) 0.102
Rh - (nm) 1.901
Wt Fr (Peak) 1.000
Mark-Houwink a 0.608
Mark-Houwink logK -3.207
Bn - (per molecule) 0.000
RI Area - (mvml) 282.42
UV Area - (mvml) 0.00
RALS Area - (mvml) 11.73
LALS Area - (mvml) 0.00
DP Area - (mvml) 128.13