frontiers in Immunology

Supplementary Material

Sequence 1: **Phosphorothioate-capped 95-BP dsDNA**
A*C*A*CACACACACACACACCATAAAACAACAACAACAACAACACCAACACACACACACACACACCAACAAACAACAACAACAACAA*C*A*A

Secondary Structure Prediction Website: No Structures to Write

Complementary Sequence 1:
T*T*G*TTGTTGTTGTTGTTTGTTGGTGTGTGTGTGTGTGTGTTGGTGTTGTTGTTGTTGTTGTTTTATGGTGTGTGTGTGTGTGTG*T*G*T

Secondary Structure Prediction Website: Delta G = **+8.1** (unlikely to create any secondary structures)

Melting Temperature Prediction Website: GC content = 40 % ; T_M = **74 ºC**
IDT OligoAnalyzer Tool Website: Maximum Delta G (*i.e.* free energy of the oligo sequence binding to its perfect complement) = **-163.94 kcal/mole**

Sequence 2: **Phosphorothioate-capped 70-BP dsDNA**
A*C*A*CACACACACACACACCATAAAACAACAACAACAACAACACCAACACACACACACACACA*C*A*C

Secondary Structure Prediction Website: No Structures to Write

Complementary Sequence 2:
G*T*G*TGTGTGTGTGTGTTGGTGTTGTTGTTGTTGTTGTTTTATGGTGTGTGTGTGTGTG*T*G*T

Secondary Structure Prediction Website: Delta G = **+5.9** (unlikely to create any secondary structures)

Melting Temperature Prediction Website: GC content = 43 % ; T_M = **73 ºC**
IDT OligoAnalyzer Tool Website: Maximum Delta G (*i.e.* free energy of the oligo sequence binding to its perfect complement) = **-118.35 kcal/mole**

Sequence 3: **Phosphorothioate-capped 45-BP dsDNA**
A*C*A*CACACACACACACACACACAAACAACAACAACAACAA*C*A*A

Secondary Structure Prediction Website: No Structures to Write

Complementary Sequence 3:
T*T*G*TTGTTGTTGTTGTTTGTGTGTGTGTGTGTGTGTGTG*T*G*T

Secondary Structure Prediction Website: Delta G = **+3.8** (unlikely to create any secondary structures)

Melting Temperature Prediction Website: GC content = 40 % ; T_M = **66 ºC**
IDT OligoAnalyzer Tool Website: Maximum Delta G (*i.e.* free energy of the oligo sequence binding to its perfect complement) = **-74.88 kcal/mole**

Sequence 4: **Phosphorothioate-capped 20-BP dsDNA**
A*C*A*CACACACACACACA*C*A*C

Secondary Structure Prediction Website: No Structures to Write

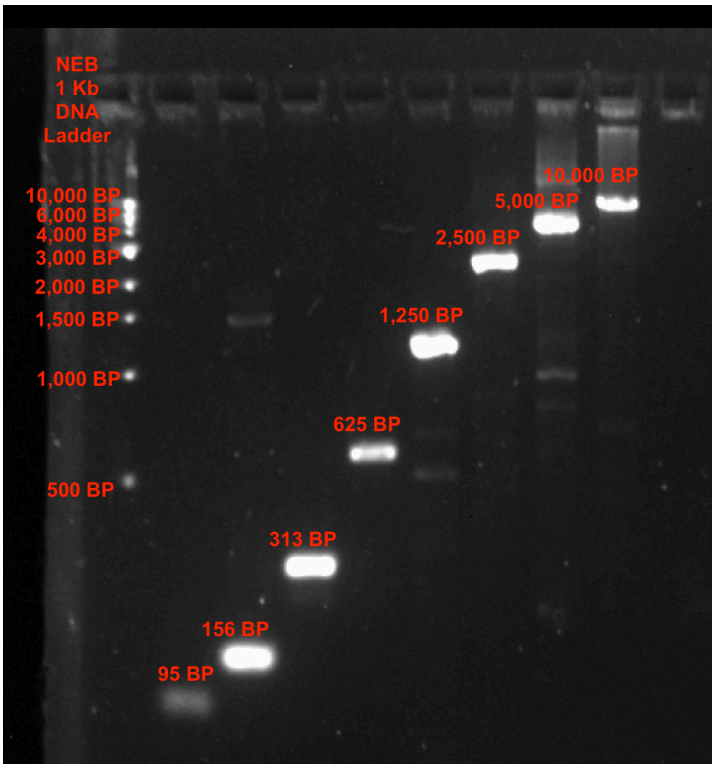
Complementary Sequence 4:
G*T*G*TGTGTGTGTGTG*T*G*T

Secondary Structure Prediction Website: Delta G = **+1.6** (unlikely to create any secondary structures)

Melting Temperature Prediction Website: GC content = 50 % ; T_M = **52 ºC**
IDT OligoAnalyzer Tool Website: Maximum Delta G (*i.e.* free energy of the oligo sequence binding to its perfect complement) = **-31 kcal/mole**

Supplementary Figure 1: The starting ISD library of synthetic dsDNA from IDT.
The star symbol (*i.e.* *) represents a phosphorothioate bond. Theoretical melting temperature values were obtained from the melting temperature prediction website, sciencelauncher.com/oligocalc.html. Theoretical maximum Delta G values were obtained from the IDT OligoAnalyzer Tool website, idtdna.com/calc/analyzer. Theoretical Delta G values for secondary structure formation were obtained from the secondary structure prediction website, rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html.

A



B

95BP TOP: TGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCC

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156BP TOP:
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156BP BOT:
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625BP TOP:
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1250BP TOP:

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1250BP BOT:

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2500BP TOP:

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5000BP TOP:

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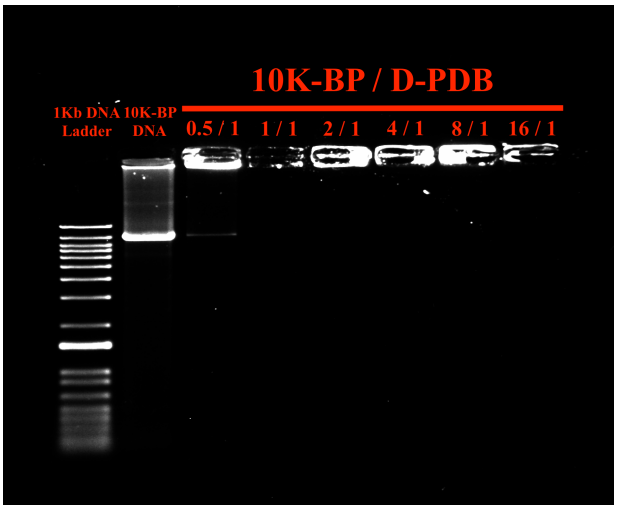
Supplementary Figure 2: The second ISD library of larger PCR-amplified dsDNA.
(A) Gel Electrophoresis of PCR-amplified dsDNA. The NEB 1 kb DNA Ladder was used for reference. **(B)** Sequences for each PCR-amplified dsDNA molecules. Primers used for each PCR-amplification were determined using the NCBI Primer Blast tool website, ncbi.nlm.nih.gov/tools/primer-blast/ and are highlighted in yellow for the forward primers and in red for the reverse primers.

A



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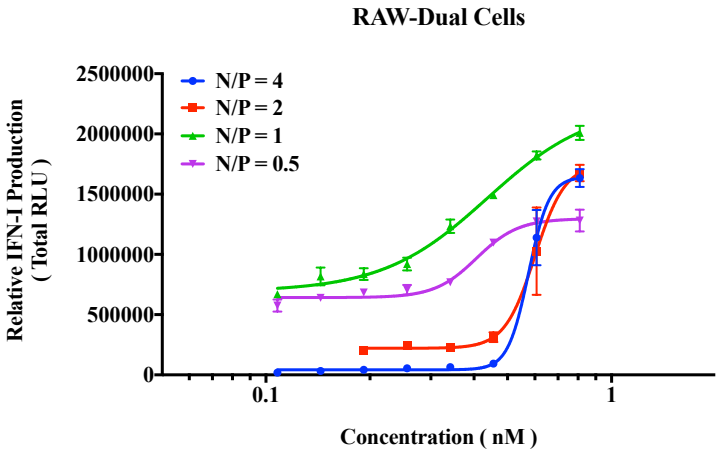

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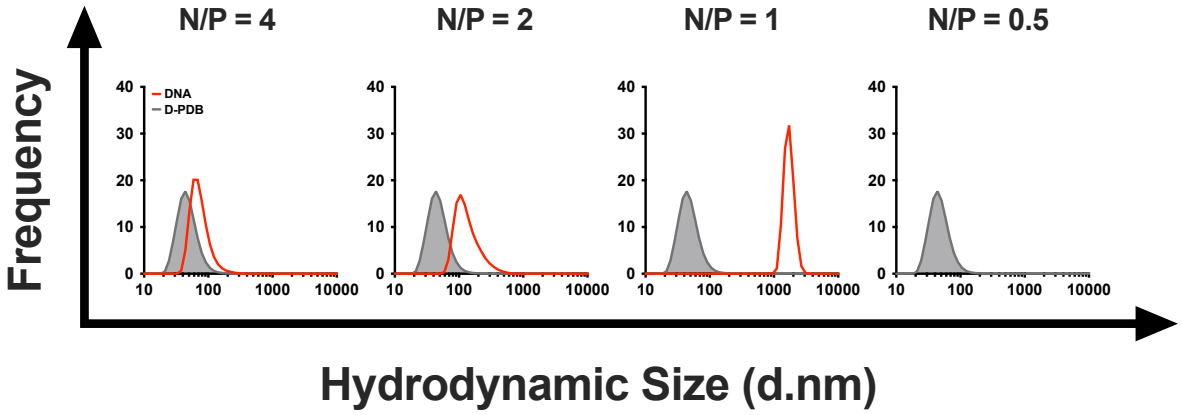
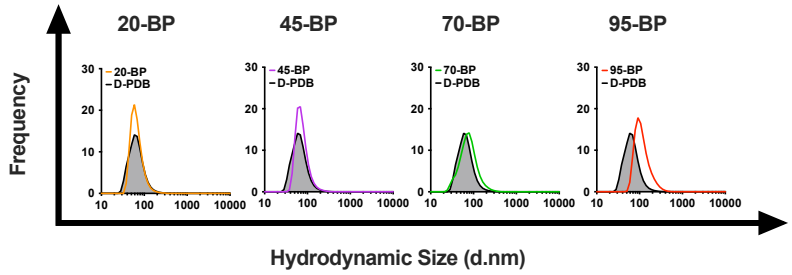


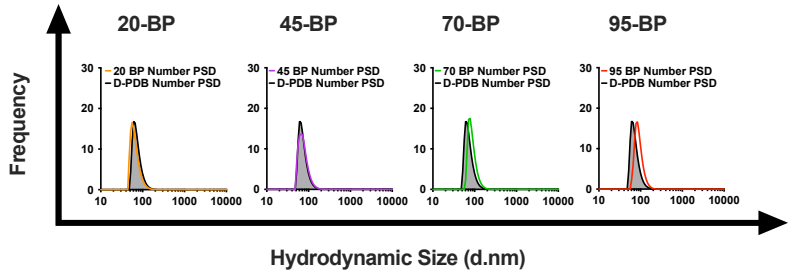
Supplementary Figure 3: Effects of N/P Charge Ratio.
(A) Agarose gel image. Lanes comprise 1 µg DNA mixed with the indicated amount of D-PDB. The TrackIt™ 1 Kb Plus DNA Ladder was used for reference. PCR-amplified 10,000-BP dsDNA / D-PDB at varying N/P charge ratios was tested for DNA loading. **(B)** RAW-Dual reporter cell assay of PCR-amplified 5000-BP dsDNA / D-PDB at varying N/P charge ratios. **(C)** DLS analysis of PCR-amplified 5000-BP dsDNA / D-PDB at varying N/P charge ratios. Frequency indicates the number-based particle size distribution. Hydrodynamic size indicates the particle diameter in nm. N/P = 0.5 was not colloidally stable to collect an accurate size measurement.

A



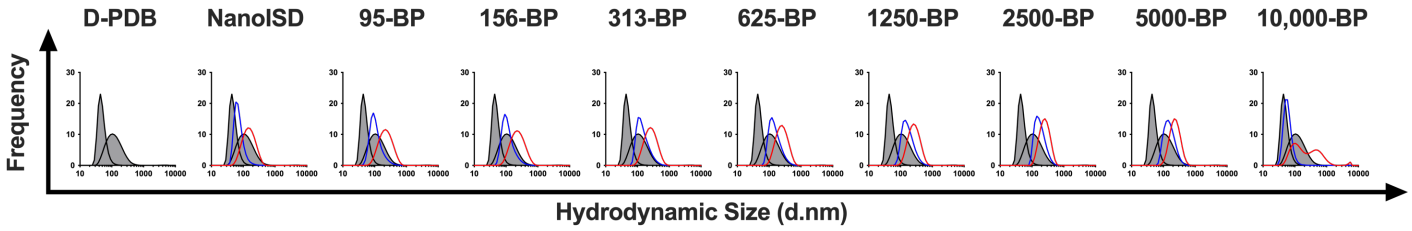
Malvern Zetasizer	Number PSD Peak (Mode)
D-PDB	59 nm
20-BP / D-PDB	59 nm
45-BP / D-PDB	68 nm
70-BP / D-PDB	79 nm
95-BP / D-PDB (NanoISD)	91 nm

B



Anton Paar Litesizer	Number PSD Peak (Mode)
D-PDB	61 nm
20-BP / D-PDB	56 nm
45-BP / D-PDB	66 nm
70-BP / D-PDB	77 nm
95-BP / D-PDB (NanoISD)	84 nm

Supplementary Figure 4: DLS analysis of the synthetic, phosphorothioate-capped dsDNA library.
(A) Synthetic, phosphorothioate-capped dsDNA library complexed to D-PDB at an N/P charge ratio of 4. Data presented as number-based particle size distribution. Malvern Zetasizer used for quantification. **(B)** Synthetic, phosphorothioate-capped ISD library complexed to D-PDB at an N/P charge ratio of 4. Data presented as number-based particle size distribution. DLS using an Anton Paar Litesizer was used as a second form of particle size analysis.



Malvern Zetasizer	Number PSD Peak (Mode)
D-PDB	44 nm
NanoISD	59 nm
95-BP / D-PDB	91 nm
156-BP / D-PDB	91 nm
313-BP / D-PDB	106 nm
625-BP / D-PDB	122 nm
1250-BP / D-PDB	142 nm
2500-BP / D-PDB	142 nm
5000-BP / D-PDB	142 nm
10,000-BP / D-PDB	N/A

Supplementary Figure 5: DLS analysis of the PCR-amplified dsDNA library.
PCR-amplified dsDNA library complexed to D-PDB at an N/P charge ratio of 4. Data presented as both number-based particle size distribution (blue) and intensity-based particle size distribution (red).

A

RAW-Dual Dose Response:

Treatment	Maximum Efficacy (RLU)	EC_{50} Values (nM)
95-BP / D-PDB	396,233	22.4
70-BP / D-PDB	385,838	31.3
45-BP / D-PDB	240,839	43.5
20-BP / D-PDB	84,923	112.9
95-BP	449	N/A
D-PDB	20,651	442.3
cGAMP	522,049	31,579.1

B

THP1-Dual Dose Response:

Treatment	Maximum Efficacy (RLU)	EC_{50} Values (nM)
95-BP / D-PDB	1,648,495	14.1
70-BP / D-PDB	1,089,124	21.8
45-BP / D-PDB	684,302	37.2
20-BP / D-PDB	493,610	115.1
95-BP	1,197	N/A
D-PDB	208,907	537.8
cGAMP	2,514,642	27,759.1

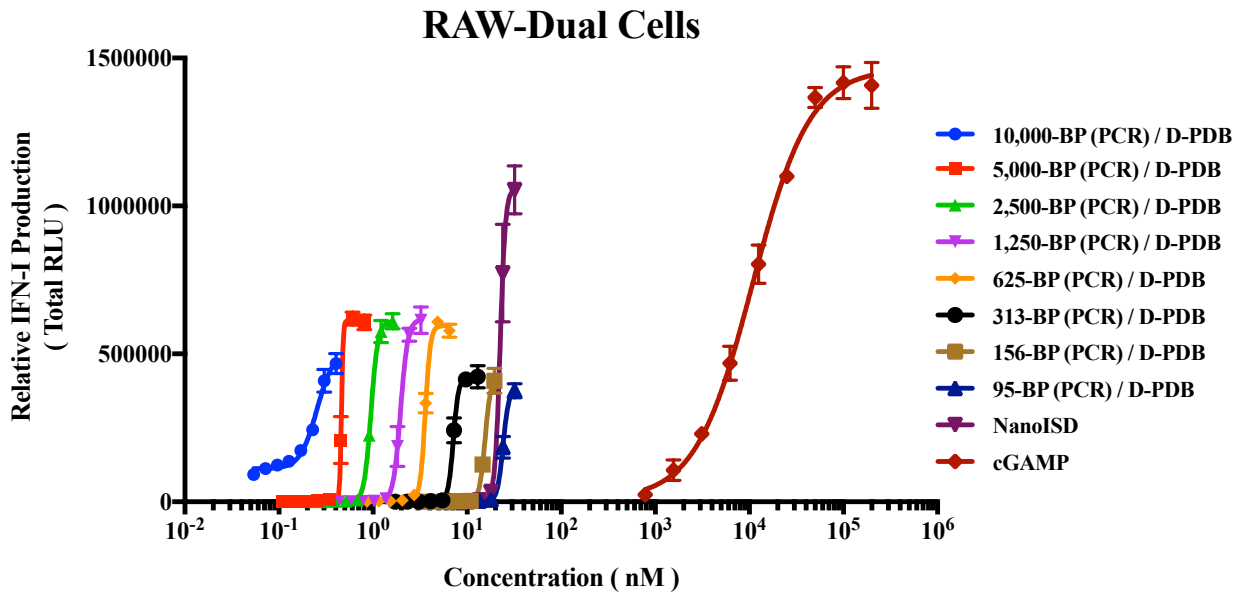
C

A549-Dual Dose Response:

Treatment	Maximum Efficacy (RLU)	EC_{50} Values (nM)
95-BP / D-PDB	1,983,308	19.1
70-BP / D-PDB	1,424,241	24.7
45-BP / D-PDB	749,267	45.0
20-BP / D-PDB	698,816	113.4
95-BP	0	N/A
D-PDB	652,675	535.6
cGAMP	1,695,993	38,503.3

Supplementary Figure 6: Maximum efficacy and EC_{50} values for data presented in *Figure 2.*

A



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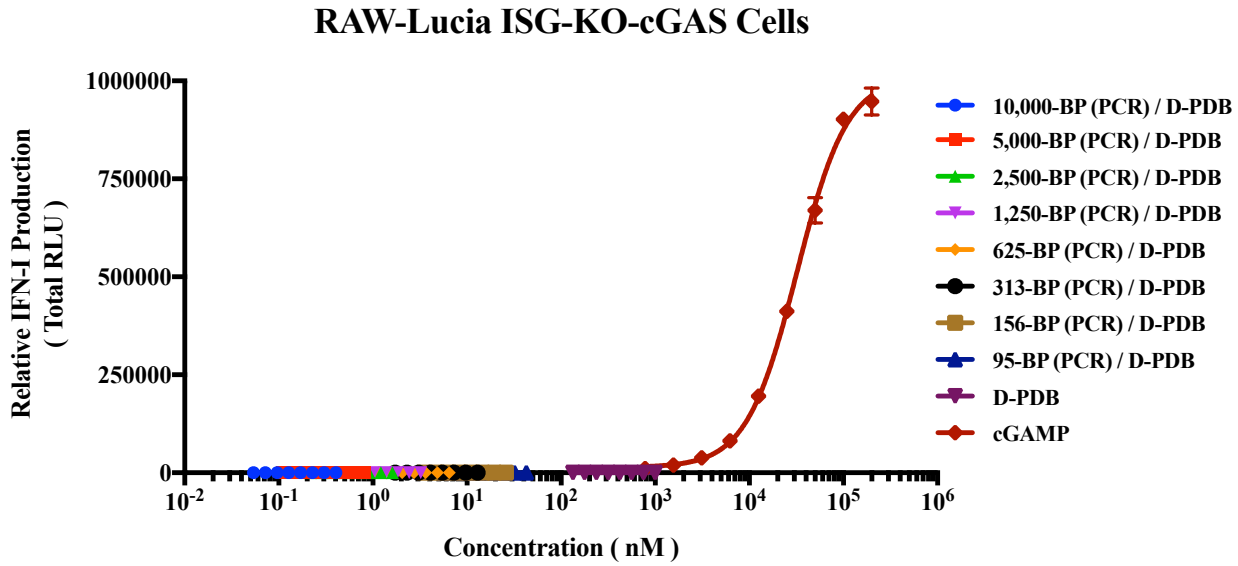
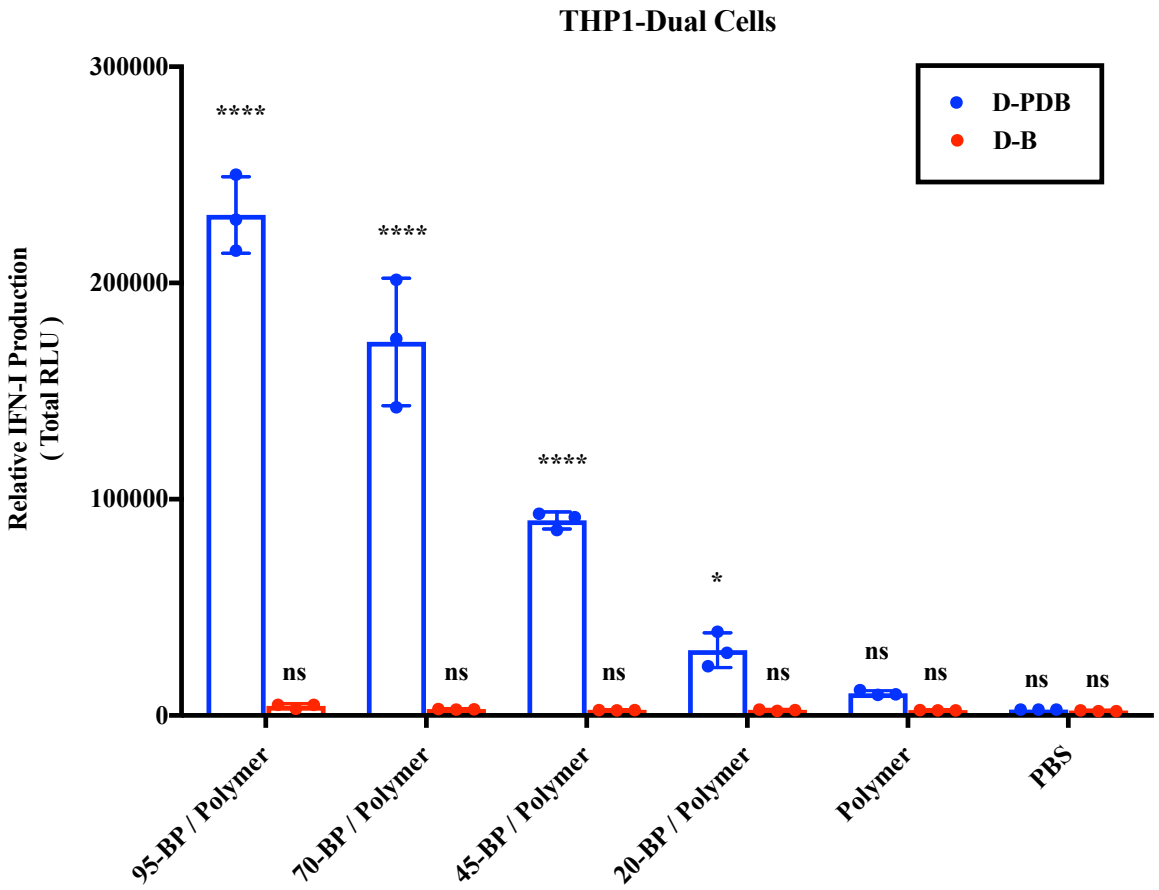


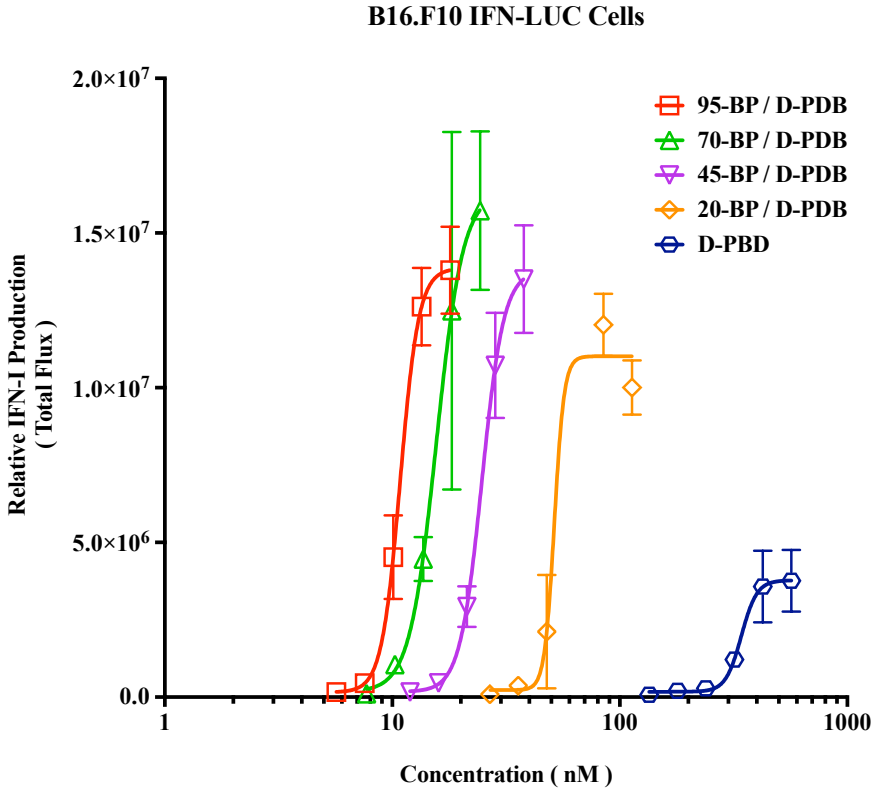
Supplementary Figure 7: Activity of the PCR-amplified dsDNA library with D-PDB.
(A) RAW-Dual IFN-I reporter cell assay of PCR-amplified dsDNA library complexed to D-PDB at an N/P charge ratio of 4. **(B)** RAW-Lucia ISG-KO-cGAS IFN-I reporter cell assay of PCR-amplified dsDNA library complexed to D-PDB at an N/P charge ratio of 4. The dose response curve for free D-PDB is positioned along the x-axis in terms of the molar amount of polymer chains rather than molar amount of loaded dsDNA, and each dose response that utilized the polymer was administered using equivalent D-PDB concentrations.



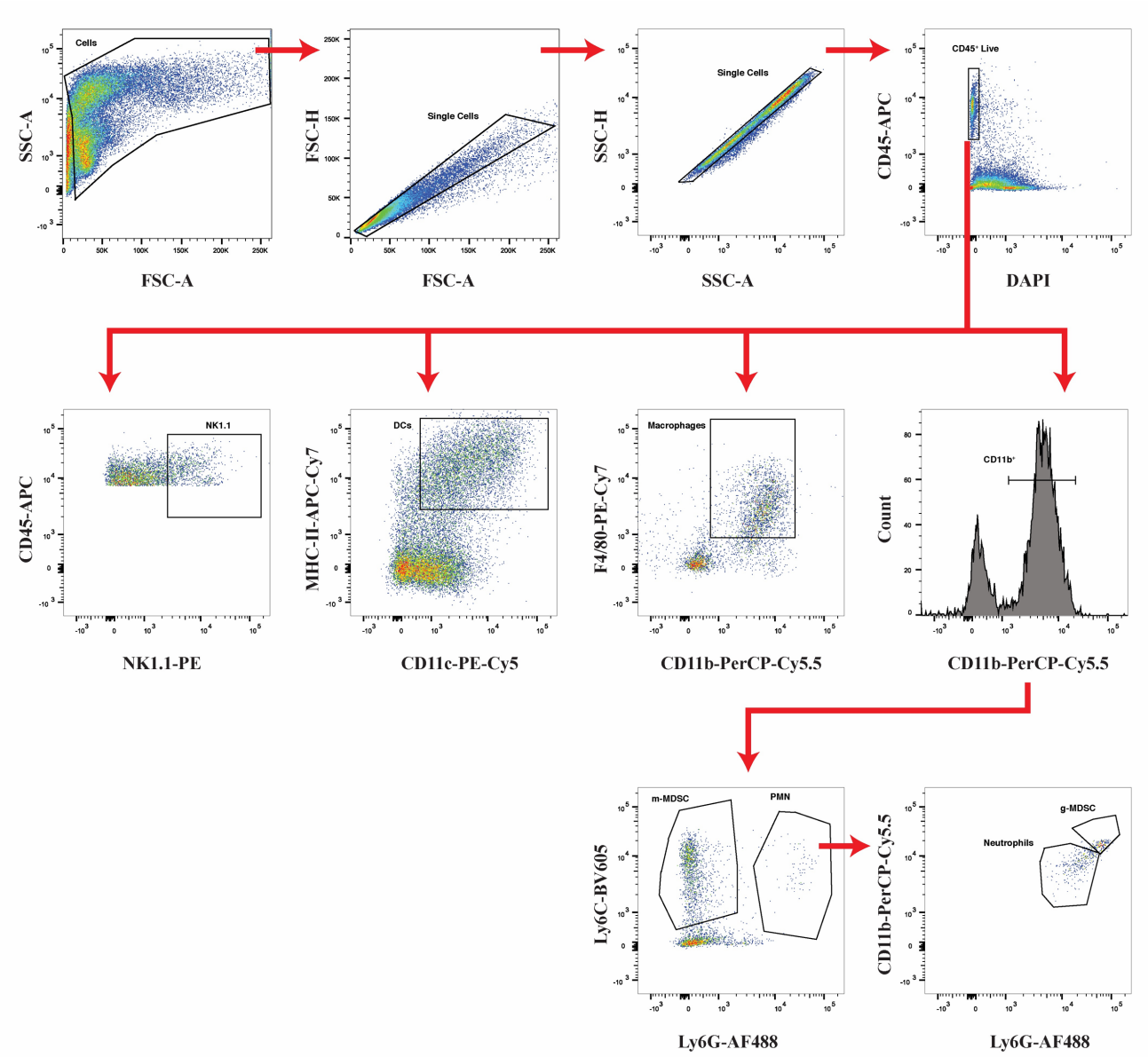
Supplementary Figure 8: Activity of the synthetic ISD library with D-PDB or D-B as carriers. THP1-Dual IFN-I reporter cells were treated with 1.5 µg/mL DNA using an N/P charge ratio of 5. A two-way ANOVA with Sidak test was used for statistical analysis.

Nanoparticle	Zeta Potential (mV)
D-PDB	+ 16.27
20-BP / D-PDB	+ 9.49
45-BP / D-PDB	+ 15.50
70-BP / D-PDB	+ 13.10
95-BP / D-PDB	+ 14.87

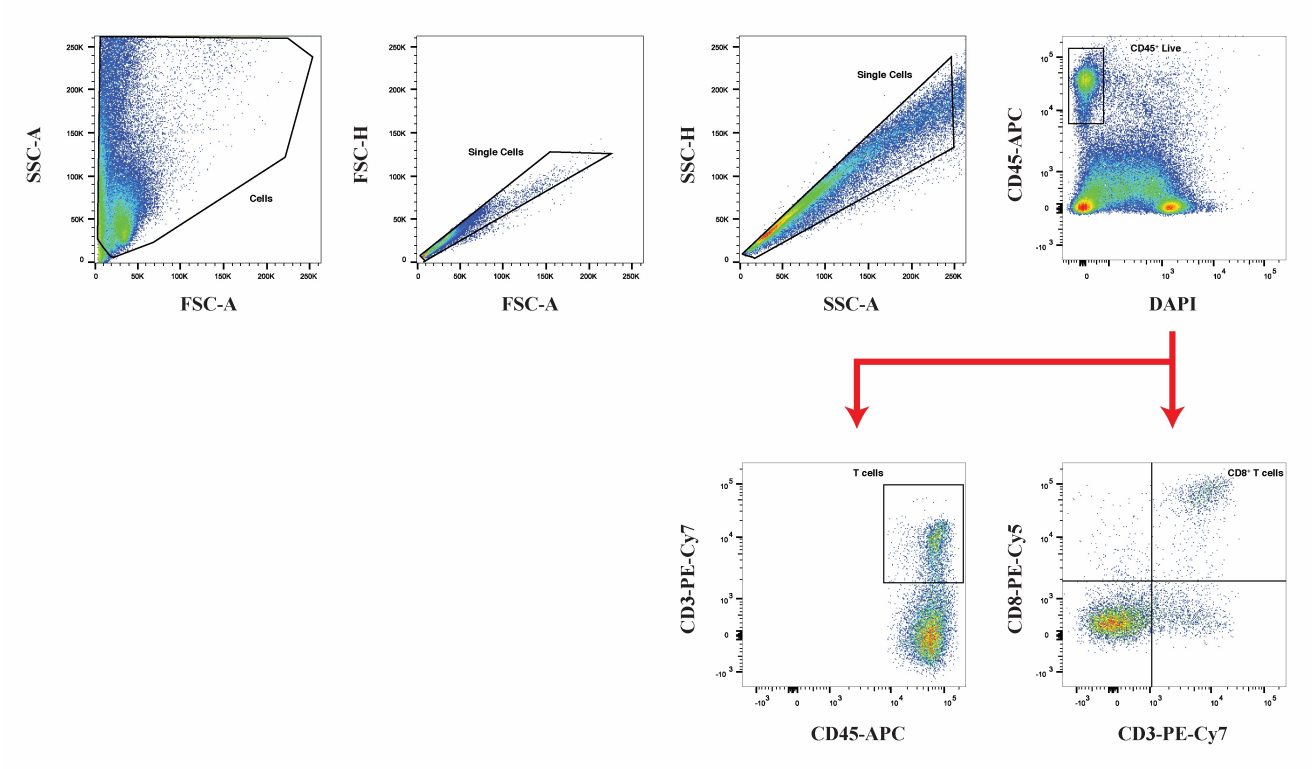
Supplementary Figure 9: Zeta potential of D-PDB and the synthetic ISD library with D-PDB.



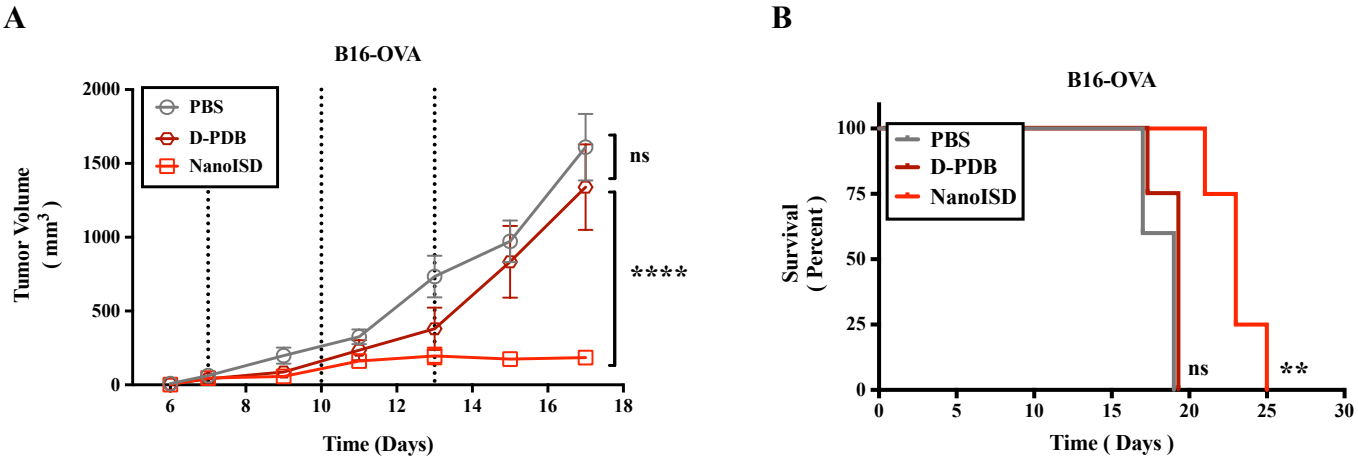
Supplementary Figure 10: Activity of the synthetic ISD library with D-PDB in B16.F10 IFN-LUC cells. B16.F10 IFN-LUC reporter cell assay of synthetic, variable-length ISD library complexed to D-PDB at an N/P charge ratio of 4. EC_{50} values for 95-BP / D-PDB, 70-BP / D-PDB, 45-BP / D-PDB, 20-BP / D-PDB, and D-PDB are 11 nM, 15 nM, 25 nM, 52 nM, and 339 nM, respectively. The dose response curve for free D-PDB is positioned along the x-axis in terms of the molar amount of polymer chains rather than molar amount of loaded dsDNA, and each dose response that utilized the polymer was administered using equivalent D-PDB concentrations.



Supplementary Figure 11: Flow cytometry gating strategy for myeloid cell panel.

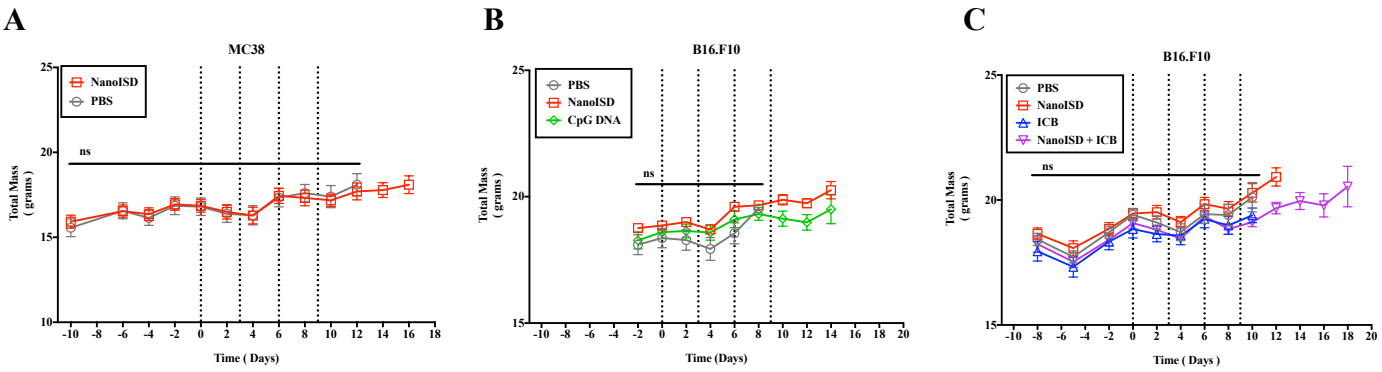


Supplementary Figure 12: Flow cytometry gating strategy for T cell panel.



Supplementary Figure 13: Therapeutic effect of NanoISD in the B16-OVA tumor model.

(A) Tumor growth plot for B16-OVA tumors intratumorally treated with 100 µL of either PBS or NanoISD at a dose corresponding to 20 µg DNA (n = 4 or greater per treatment group). Treatments were administered 3 times q3d as indicated by the dotted lines. Tumor growth curves were truncated to the day that mice began to reach the study endpoint. A two-way ANOVA with Tukey test was used for statistical analysis. Statistics on the graph represent the analysis for the final day shown (*i.e.* day 17). **(B)** Kaplan-Meier Survival Curve for B16-OVA tumors intratumorally treated with 100 µL of either PBS, D-PDB, or NanoISD. Log rank (Mantel-Cox) test was used for statistical analysis.

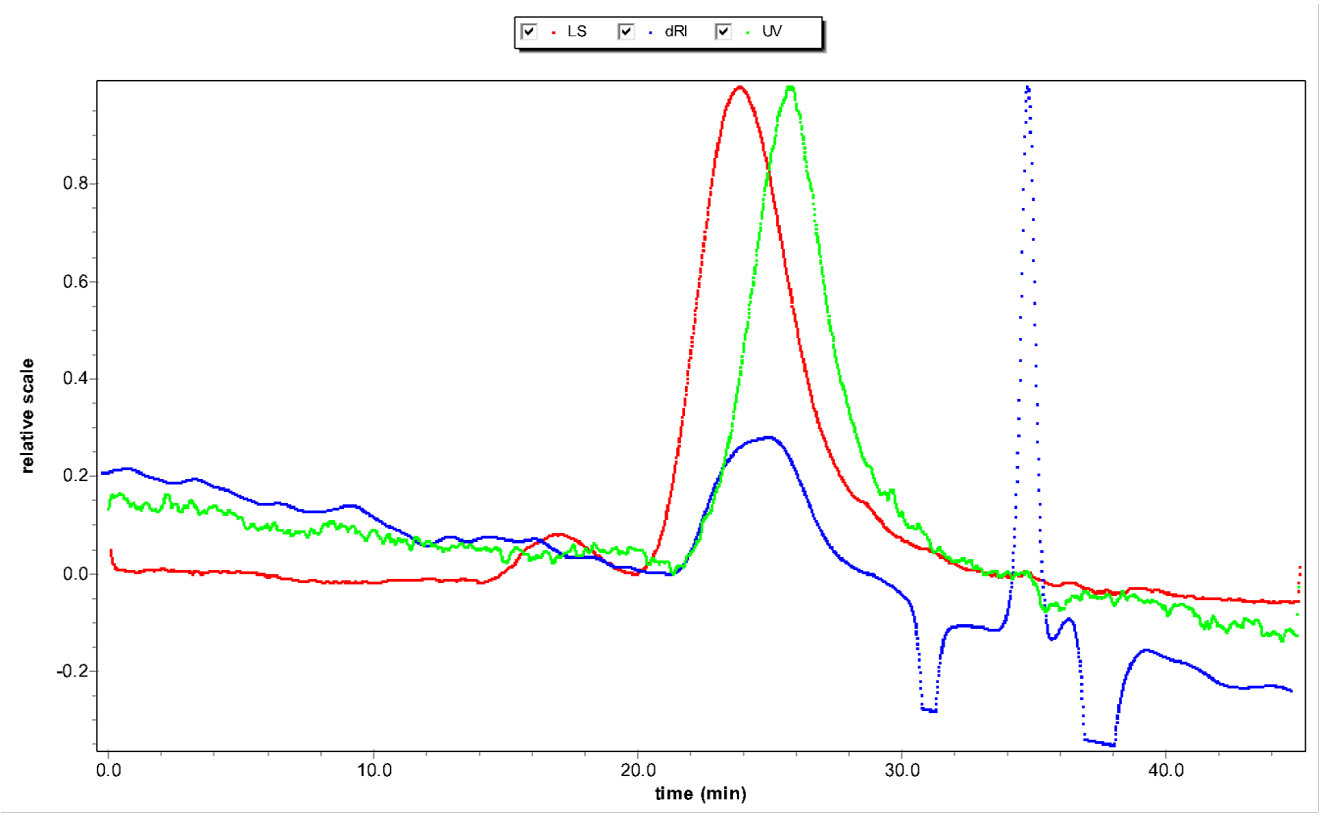


Supplementary Figure 14: Intratumorally injected NanoISD is well-tolerated.

(A) Total mouse weight over time for the mice with MC38 tumors corresponding to studies described in ***Figures 7a and 7b***. Intratumoral treatments were administered 4 times q3d as indicated by the dotted lines. A two-way ANOVA with Sidak test was used for statistical analysis. Statistics on the graph represent the analysis for each of the treatment groups relative to PBS. **(B)** Total mouse weight over time for the mice with B16-F10 tumors corresponding to studies described in ***Figures 7c and 7d***. Intratumoral treatments were administered 4 times q3d as indicated by the dotted lines. A two-way ANOVA with Tukey test was used for statistical analysis. Statistics on the graph represent the analysis for each of the treatment groups relative to PBS. **(C)** Total mouse weight over time for the mice with B16-F10 tumors corresponding to studies described in ***Figures 7e and 7f***. Intratumoral treatments were administered 4 times q3d as indicated by the dotted lines. A two-way ANOVA with Tukey test was used for statistical analysis. Statistics on the graph represent the analysis for each of the treatment groups relative to PBS.

Polymer	PAA (%)	DMAEMA (%)	BMA (%)	Molecular Weight (kDa)
1st Block	0	100	0	9.40
2nd Block	28	35	37	38.69

Supplementary Figure 15: D-PDB composition as determined by ^{1}H NMR analysis.



Supplementary Figure 16: GPC spectrum of D-PDB.
GPC analysis indicated that the total molecular weight of polymer was 41.56 kDa and the PDI was 1.136. Since ^{1}H NMR indicated the total molecular weight of D-PDB was 48.09 kDa, an average molecular weight of 44.8 kDa was used for all calculations regarding polymer concentration.