Supporting Information - High-Throughput Automation of Endosomolytic Polymers for mRNA Delivery

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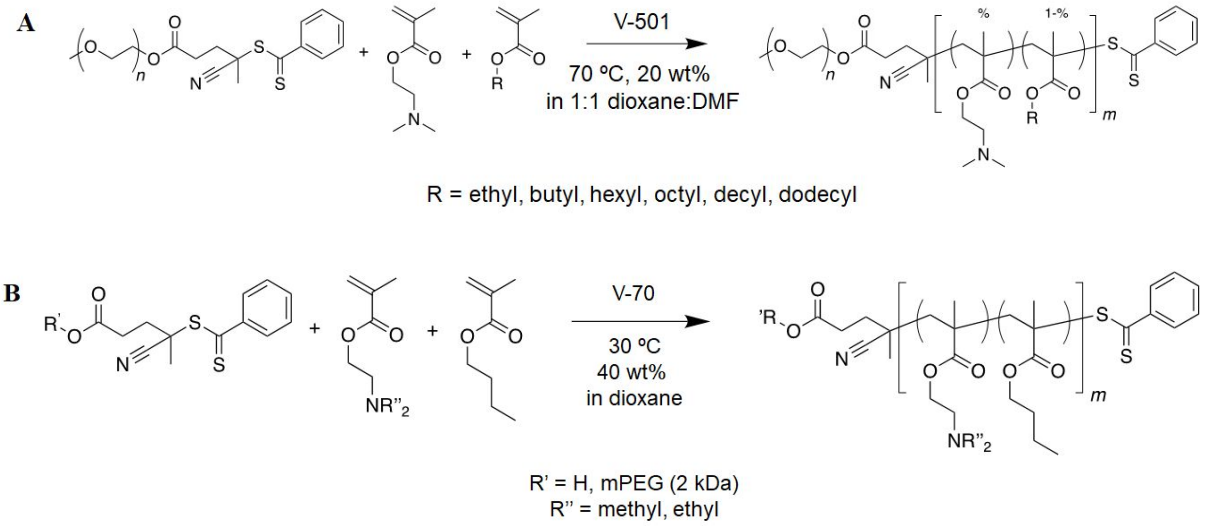


Scheme S1. Generalized Synthetic Scheme for (**A**) PEG-*b*-p(DMAEMA-*co*-AMA) copolymer within in polymer series 1 and (**B**) p(DMAEMA-*co*-AMA) and p(DEAEMA-*co*-AMA) copolymers within polymer series 2.

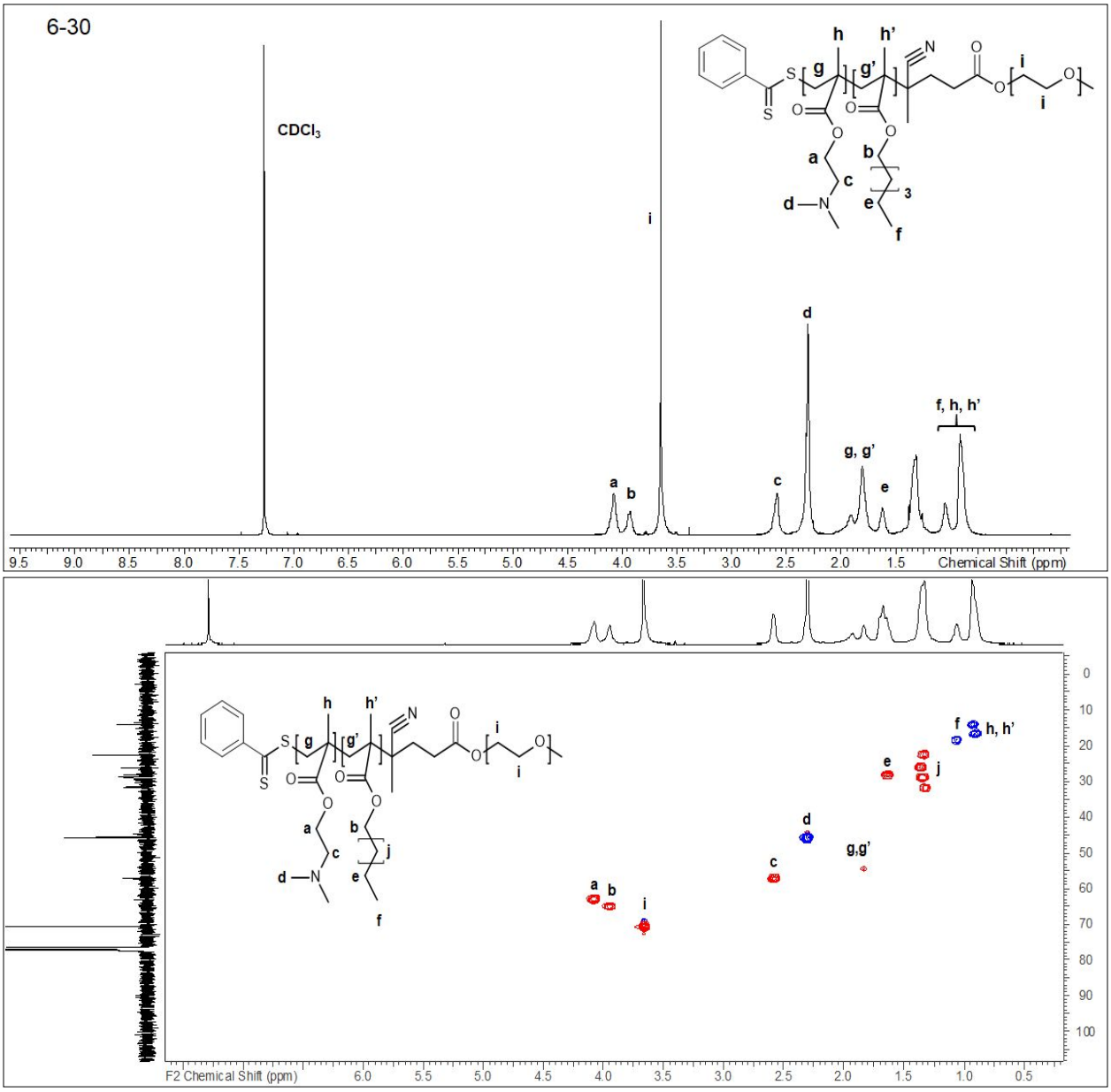


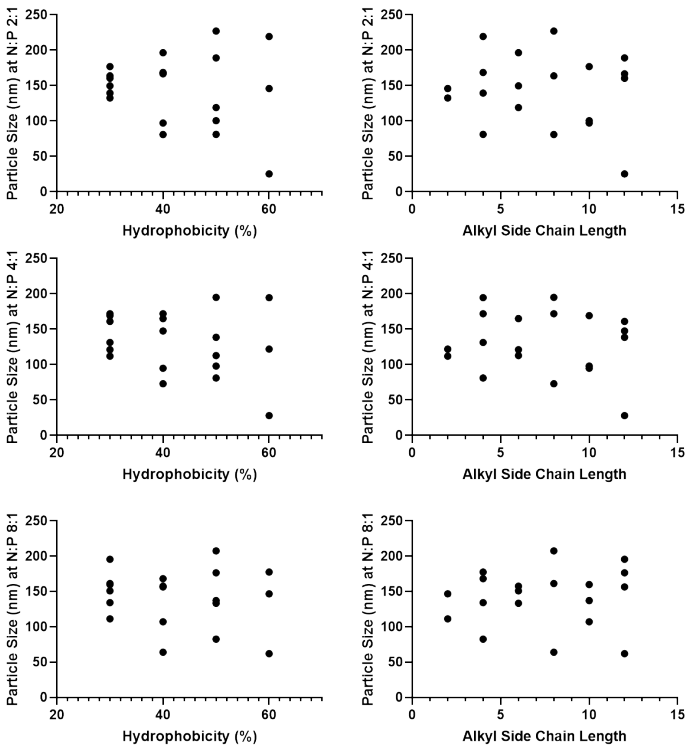
Figure S1. ^{1}H-NMR (top) and HSQC (bottom) spectra in $CDCl_3$ of "6-30" PEG-*b*-p(DMAEMA-*co*-AMA) copolymer from polymer series 1.

Polymer Name	Side chain length [n]	AMA content [%]	Total M_n	M_n 2nd block	DP DMAEMA	DP A_nMA	Fraction A_nMA
2-30	2	30	32274	22274	112.4	45.3	0.287
2-60	2	60	31486	21486	71.4	93.1	0.566
4-30	4	30	32383	22383	105.6	44.4	0.296
4-40	4	40	34203	24203	97.7	65.6	0.402
4-50	4	50	39050	29050	95.3	98.9	0.509
4-60	4	60	34856	24856	71.2	98.6	0.581
6-30	6	30	37061	27061	113.0	58.0	0.339
6-40	6	40	36127	26127	92.2	71.1	0.436
6-50	6	50	33966	23966	71.9	76.6	0.516
8-30	8	30	35295	25295	102.9	48.6	0.321
8-40	8	40	38701	28701	97.9	69.6	0.416
8-50	8	50	38409	28409	78.2	83.3	0.516
10-30	10	30	39043	29043	106.8	56.4	0.346
10-40	10	40	40679	30679	106.5	63.8	0.375
10-50	10	50	46868	36868	99.2	96.0	0.492
12-30	12	30	48079	38079	143.5	63.8	0.308
12-40	12	40	37292	27292	84.7	56.6	0.401
12-50	12	50	41495	31495	77.7	77.4	0.499
12-60	12	60	39954	29954	53.9	85.5	0.613

Table S1. Characteristics in polymer series 1 (composition library) ranging from 30% to 60% (%) alkyl methacrylate (AMA) that contain various lengths of alkyl side chains (n). DP = degree of polymerization (total monomeric units).

Polymer	PEG MW	Total Mn	Total DP	Fraction DMAEMA	Fraction DEAEMA	Fraction BMA	PDI
DB6375	-	6375	38	0.6	0	0.4	1.081
DB7074	-	7074	67.45	0.6	0	0.4	1.326
DB17910	-	17910	119	0.6	0	0.4	1.009
DB25170	-	25170	162.98	0.6	0	0.4	1.032
EB10250	-	10250	35.6	0	0.6	0.4	1.091
EB10970	-	10970	59.5	0	0.6	0.4	1.012
EB17610	-	17610	92.75	0	0.6	0.4	1.038
EB23060	-	23060	127.4	0	0.6	0.4	1.017
PEG-DB9542	2000	9542	33.25	0.6	0	0.4	1.027
PEG-DB12490	2000	12490	63.65	0.6	0	0.4	1.017
PEG-DB22220	2000	22220	88.2	0.6	0	0.4	1.031
PEG-DB21990	2000	21990	120.83	0.6	0	0.4	1.01
PEG-EB9187	2000	9187	28.5	0	0.6	0.4	1.006
PEG-EB12350	2000	12350	47.6	0	0.6	0.4	1.016
PEG-EB20900	2000	20900	99.75	0	0.6	0.4	1.019
PEG-EB18280	2000	18280	109.2	0	0.6	0.4	1.04

Table S2. Characteristics in the polymer series 2 (MW library) with an approximately 60:40 ratio of DMAEMA (D) or DEAEMA (E) to BMA (B) monomers in the absence and presence of an initial PEG block (2,000 g/mol). Mn = molecular weight and DP = degree of polymerization (total monomeric units).



	N:P	Pearson Value	P Value	Significant (alpha = 0.05)?
Hydrophobicity vs. Particle Size	2:1	-0.14	0.28	No
	4:1	-0.24	0.16	No
	8:1	-0.14	0.29	No
Alkyl Side Chain Length vs. Particle Size	2:1	-0.12	0.31	No
	4:1	-0.12	0.32	No
	8:1	0.07	0.38	No

Figure S2. Pearson correlation analysis of hydrophobicity and alkyl chain length vs. particle size within polymer series 1 at 2:1, 4:1, and 8:1 N:P ratios.

Polymer	Dz (nm)		PDI	
	Formulation Method		Formulation Method	
	Vortex	HT	Vortex	HT
4-40	87.7	161.3	0.167	0.218
4-50	98.6	159.9	0.202	0.2305
4-60	122.1	195.8	0.229	0.2245
6-30	61.9	168.3	0.125	0.222
6-40	69.6	157.6	0.228	0.268
6-50	68.1	64.1	0.219	0.2105
8-30	48.0	107.1	0.187	0.23
8-40	58.7	156.6	0.145	0.293
8-50	57.7	82.6	0.172	0.2125
10-30	50.5	133.4	0.106	0.241

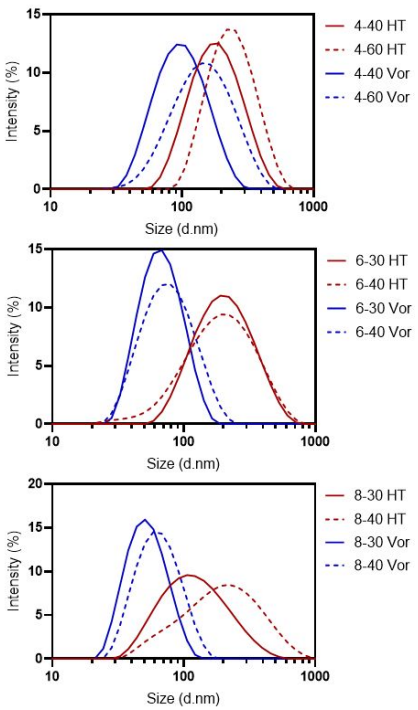


Table S3. Hydrodynamic diameter of polyplexes prepared by conventional preparation (vortexed for 10 sec, incubated for at least 15 min) (Vortex [blue]) and robot pipetting (HT [red]) at 8:1 N:P ratio. DLS were measured with Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) via cuvettes and plates at final mRNA concentration of 25 µg/mL.

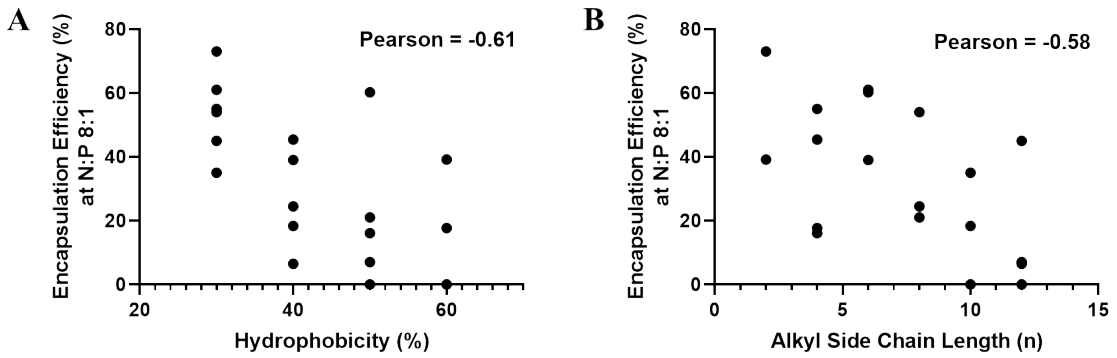


Figure S3. Pearson correlation between encapsulation efficiency of mRNA polymer carriers versus (**A**) hydrophobic percentage and (**B**) alkyl chain length within each copolymer used. $p < 0.005$.

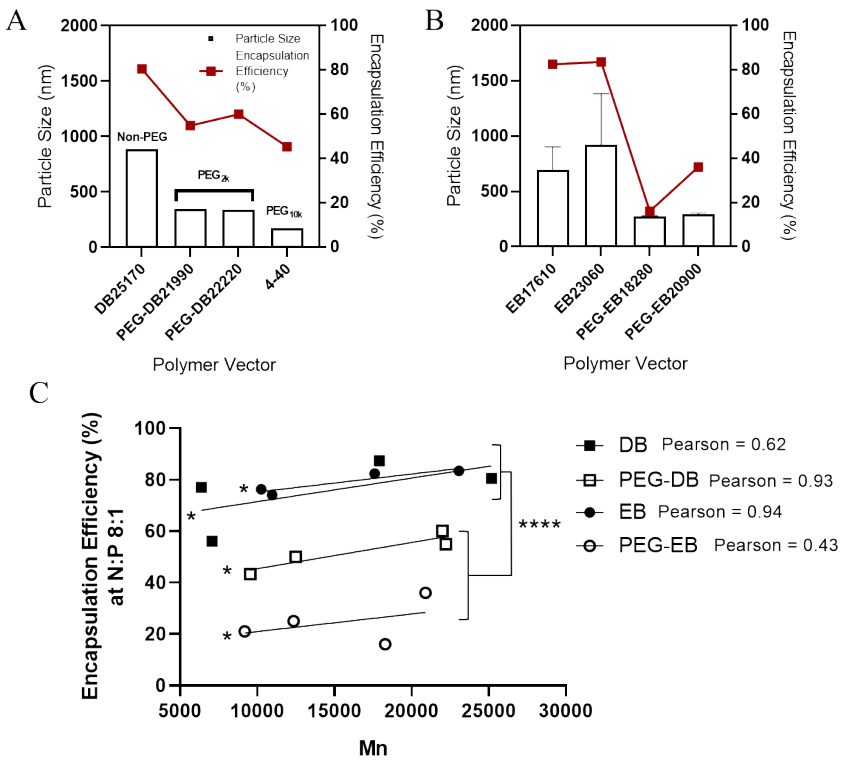


Figure S4. SAR trends of polyplex size and cargo encapsulation depicting influence of PEG addition to (**A**) DMAEMA (DB) and (**B**) DEAEMA (EB) copolymers with comparable amphiphilic block segments at 8:1 N:P ratio. (**C**) Pearson correlation between encapsulation efficiency of mRNA polymer carriers in series 2 versus molecular weight. *= $p < 0.05$ and **** = $p < 0.0001$.

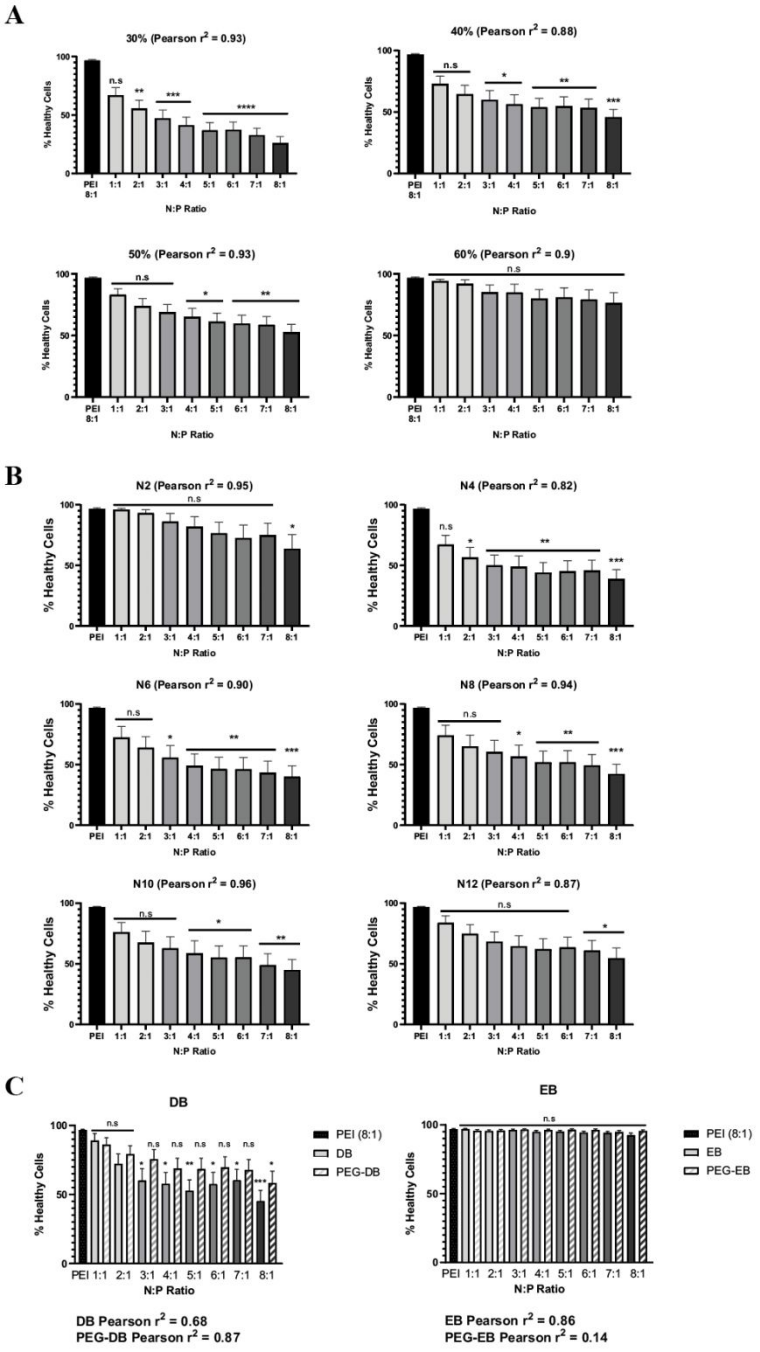


Figure S5. Cytotoxicity of polyplexes with varying amphilicity, alkyl side chain length, and cation type. By increasing N:P ratios, cell health in Huh7 cells was shown to be directly affected by (**A**) the concentration of hydrophobic monomers and (**B**) alkyl side chain lengths. (**C**) Cytotoxicity trends of copolymers (MW = ~9000 to ~22500) containing cationic DMAEMA (DB) and DEAEMA (EB) monomers in the presence and absence of an initial PEG block. Each formulation (1 μg/mL) is loaded with mRNA (4:1 GFP:Cy5) was dosed to Huh7 cells for 24h. Cytotoxicity is represented as the percent healthy cells from all nuclei detected, and graphs show mean values ± SEM from n= 3 independent experiments. Significance was determined in A-C by one-way ANOVA followed by Dunnett's multiple comparisons test to the PEI control where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n.s = not significant.

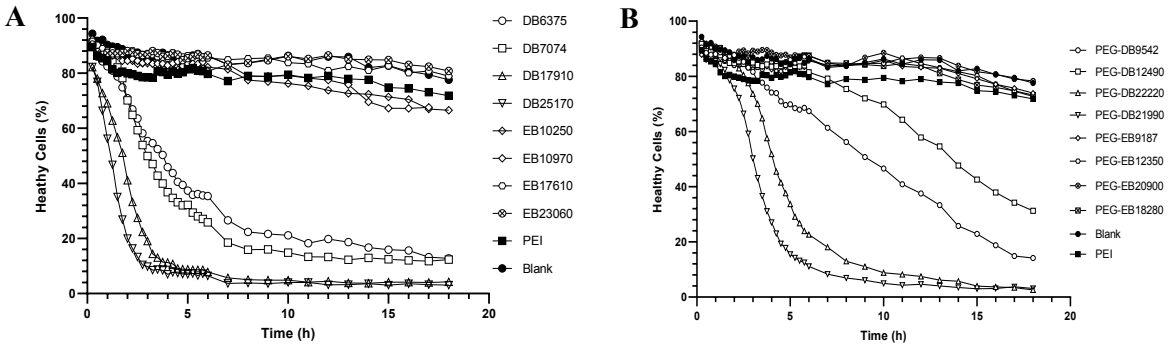


Figure S6. Live-cell cytotoxicity profiles of (**A**) p(DMAEMA/DEAEMA-*co*-BMA) and (**B**) PEG_{2k}-*b*-p(DMAEMA/DEAEMA-*co*-BMA) polyplexes. Cell health was determined through confocal imaging of the nuclei morphology and size.

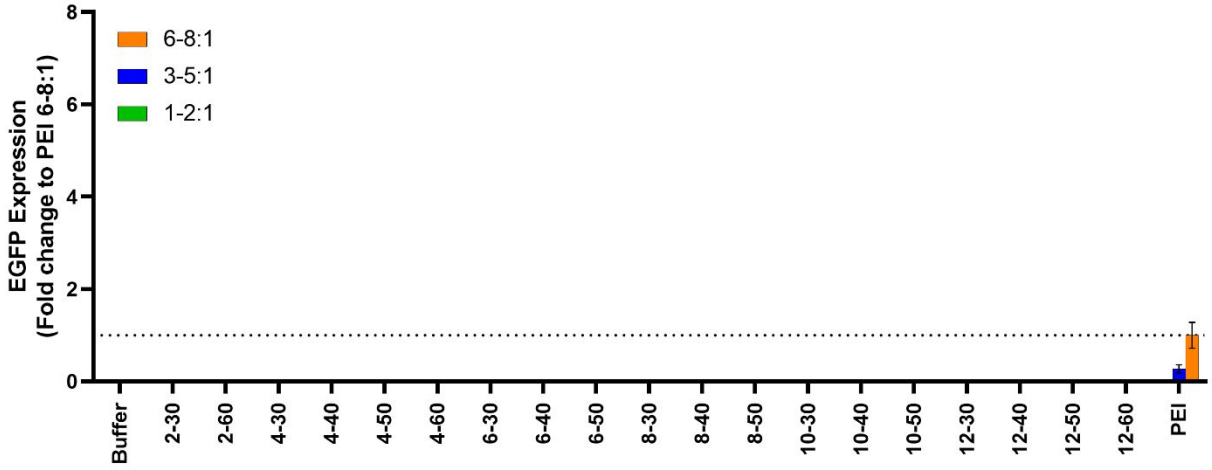
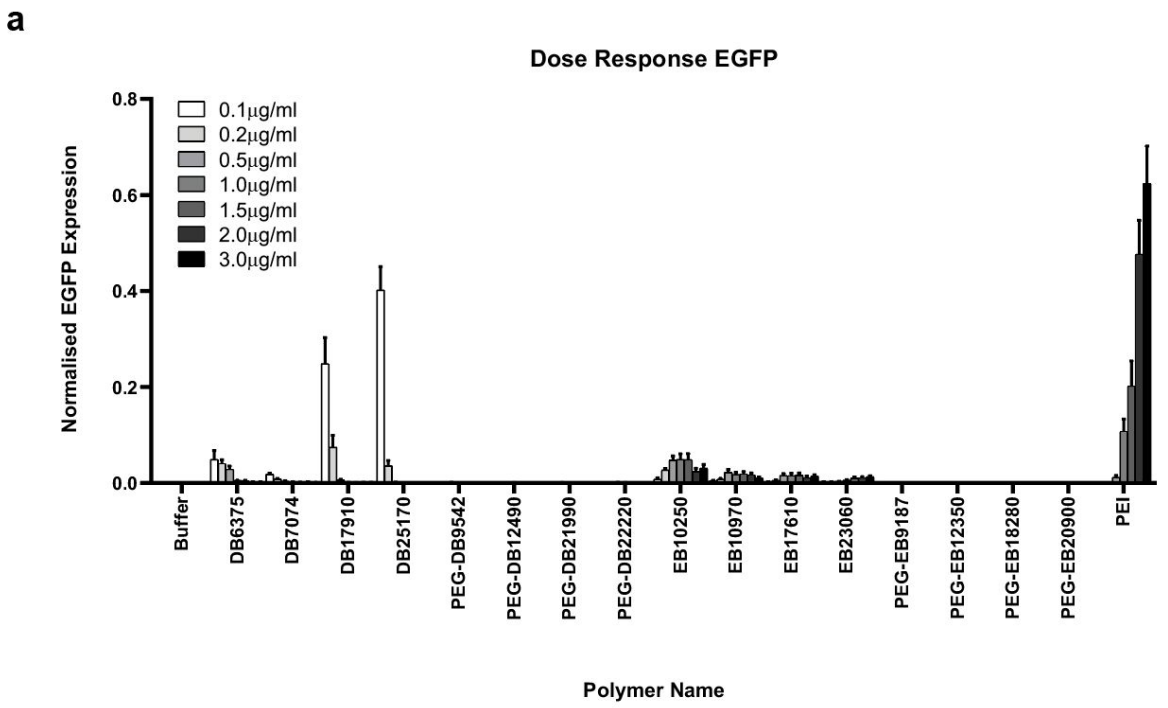
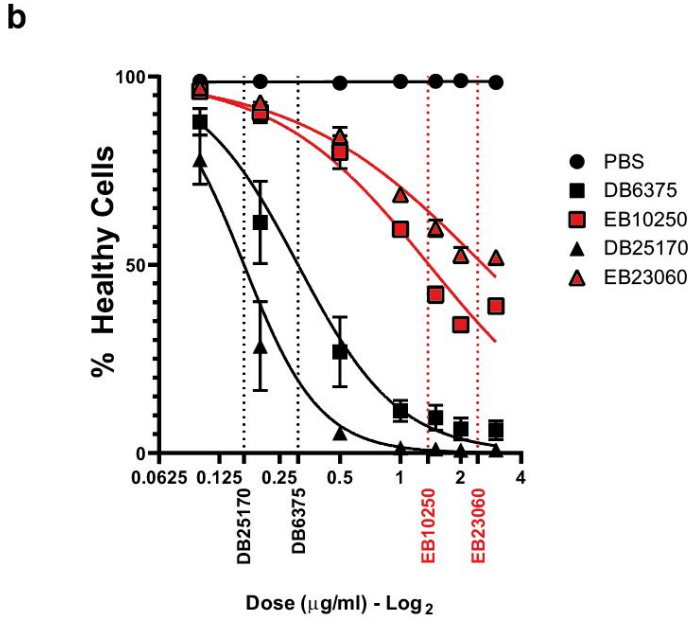


Figure S7. mRNA transfection efficiencies in Huh7 cells at different ranges of N:P ratios of PEG10k-b-p(DMAEMA-co-AMA). Quantitation of polyplex efficiency of polymer set 1 in Huh7 cells following 24 h dosing with 1 µg/mL EGFP mRNA via indicated polyplexes and binned for indicated N:P ratios (determined by percentage of cells positively transfected multiplied by mean cellular EGFP intensity). Bars represent mean values normalized to the PEI control (6-8:1 N:P) from a minimum of n=3 independent experiments ± SEM.





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Polymer	LC50 (µg/ml)
DB6375	0.3087
DB7074	0.2648
DB17910	0.1391
DB25170	0.1659
PEG-DB9542	0.4959
PEG-DB12490	1.115
PEG-DB21990	0.2105
PEG-DB22220	0.2484
EB10250	1.374
EB10970	1.788
EB17610	2.27
EB23060	2.43
PEG-EB9187	>3
PEG-EB12350	>3
PEG-EB18280	>3
PEG-EB20900	>3
PEI	>3
Buffer	>3

Figure S8. Dose dependent studies of the MW polymer library consisting of cationic DMAEMA (DB) and DEAEMA (EB) monomers. EGFP transfection and cell death of indicated polyplexes were examined through microscopy by dosing Huh7 cells at a dose range of 0.1-3µg/mL (all formulated at 8:1 N:P). (**A**) EGFP expression and (**B**) cytotoxicity (LC_{50}) of all polyplexes was examined 24 hrs after initial dosing. (**C**) All polyplexes exhibit LC_{50} values such that percentage of unhealthy/dead cells were determined through microscopy and concentrations were found after line fitting. Bars represent mean values from n=3 independent experiments ± SEM. Significance was determined by two-way ANOVA followed by Dunnett's multiple comparison test to the Buffer control. Significance is denoted by * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, n.s = not significant.