Molecular additives significantly enhance glycopolymer-mediated transfection of large plasmids and functional CRISPR-Cas9 transcription activation ex vivo in primary human fibroblasts and induced pluripotent stem cells

William S. Boyle†, Kirk Twaroski‡, Emily C. Woska‡ Jakub Tolar‡, Theresa M. Reineke†*

† Department of Chemistry, University of Minnesota, Minneapolis, Minnesota, 55455, USA

‡ Stem Cell Institute and Division of Pediatric Blood and Marrow Transplantation, University of Minnesota, Minneapolis, Minnesota, 55455, USA

* Corresponding author email: treineke@umn.edu

Supporting Information

Plasmid Sequences. Plasmids encoding green fluorescent protein were purchased from Aldevron (Fargo, ND). To ensure that the large plasmid contained identical promoter and reporter as the small plasmid, a large plasmid based on pszGreen containing a noncoding sequence in the backbone of the plasmid was engineered.

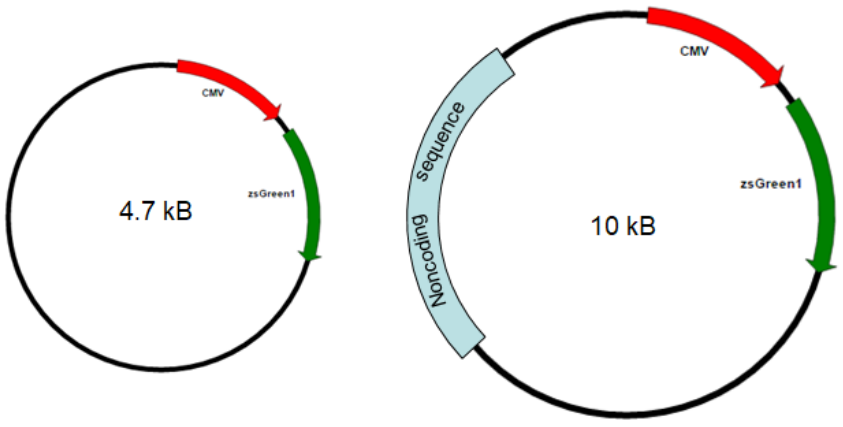

Figure S1: Plasmid maps for pzsGreen (4.7 kbp) and pzsGreen-10k (10 kbp)

Cell Cycle Analysis. Primary fibroblasts were treated with a double thymidine block prior to transfection. To quantify the effect of the double thymidine block, cells were lifted and stained with Vybrant® DyeCycle™ Ruby stain (ThermoFisher Scientific) using the manufacturer's protocol. Three populations of cells with increasing Ruby intensity were identified corresponding to G0/G1, S and G2/M respectively. The thymidine block reduces the number of cells in G0/G1 from 72% to 12% at block release. At block release (when the transfection was started) 66% of the cells are in S or G2/M. This enrichment of S and G2/M phase cells should guarantee that a much larger fraction of cells will undergo division during the transfection compared to a transfection of an unblocked population.

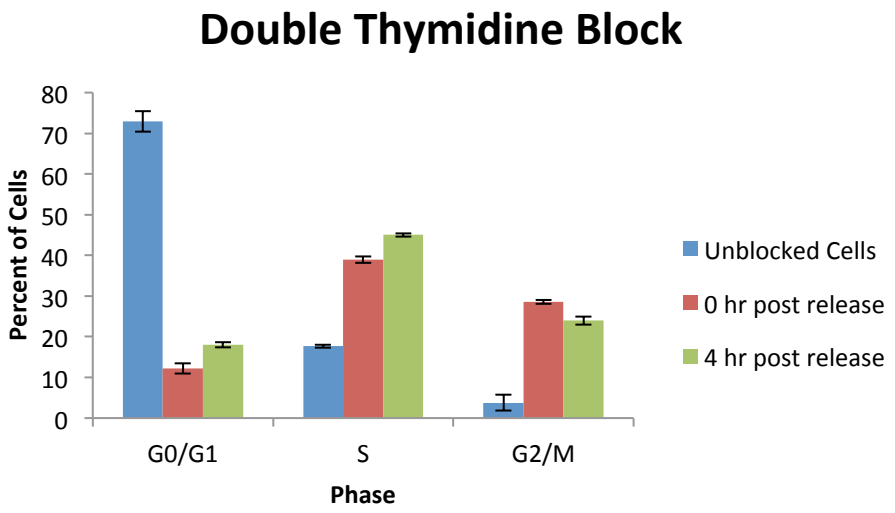

Figure S2: Cell cycle analysis of double thymidine blocked primary fibroblasts. Cell cycle determined by Vybrant® DyeCycle™ Ruby stain. Error bars represent the standard deviation of three replicates.

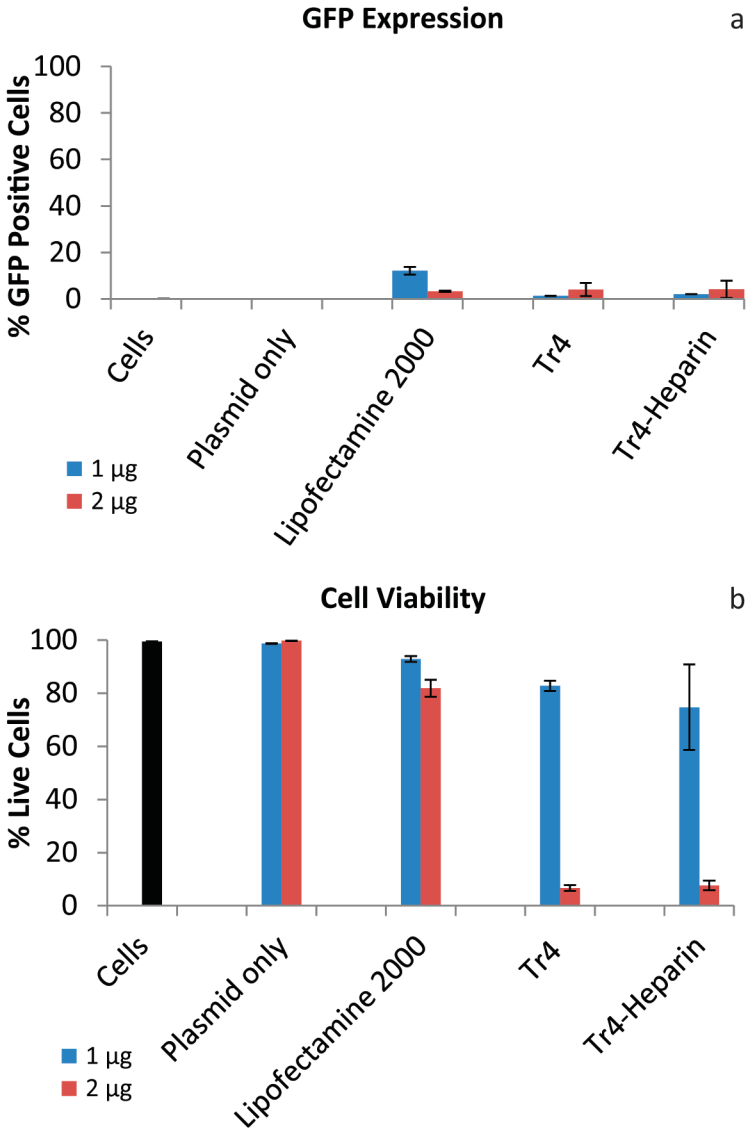

Figure S3: Optimization of large plasmid transfection dose in primary fibroblasts. GFP expression (a) and cell viability (b) as a function of plasmid size.

pDNA Dose Optimization. To ensure that the optimal dose of large plasmid was being used, we investigated the effect of increasing plasmid dose with the large plasmid. Primary fibroblasts were treated with 1 and 2 µg per well of the 10 kbp plasmid. All Tr4 polyplexes were

formed at an N/P of 40. Lipofectamine 2000 transfections were performed using 5 µl/µg DNA. Keeping the N/P ratios consistent lead to the use of twice as much polymer for the 2 µg transfections. Polyplexes were formed in the same final transfection volume (300 µl) for both doses. Plasmid DNA solutions were formed at a concentration of 0.04 µg/µL in 165 µL of DNase/RNase-free water. Tr4 solutions were formed at an N/P ratio of 40 for all experiments in this work (22 µg polymer/µg DNA) in an equal volume of DNase/RNase-free water. The Tr4 solution was then slowly added to the pDNA solution and slowly triturated to mix. After mixing, the polyplex solutions were allowed to incubate at room temperature for 1 hr before addition to the cells. Prior to transfection, Tr4 polyplexes were diluted with 2 volumes of media with or without 10 µg/mL heparin. Diluted polyplexes were then added to each well.

The results showed that 1 µg per well was the optimal dose for the large plasmid. Increasing the dose to 2 µg/well did not provide any increase in transfection efficiency, but dramatically increased toxicity. The cells transfected with 2 µg/well plasmid DNA were almost entirely dead 48 hr post transfection and showed no evidence of GFP fluorescence. While the 2 µg Lipofectamine transfections do not show the same toxicity increase as Tr4-heparin, they do exhibit decreased GFP signal. This could be due to non-optimal particle formulation leading to reduced transfection. In any case, 1 µg DNA per well appears to be the optimal dose for all vehicles regardless of plasmid size.

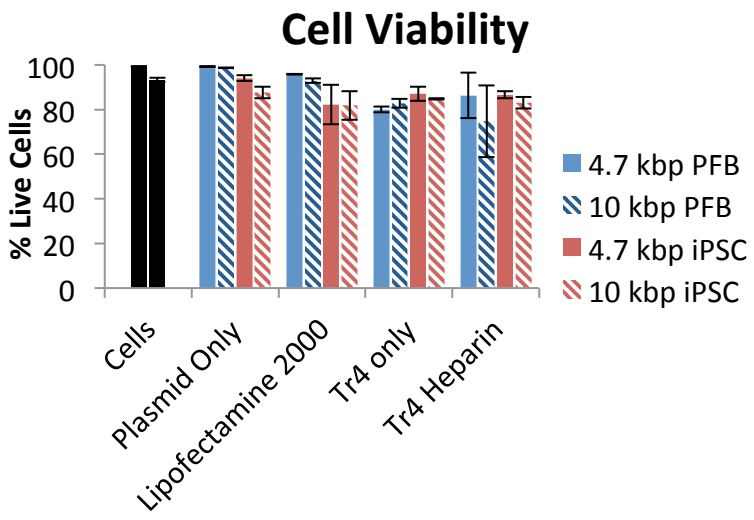

Figure S4: Viability of cells during transfections in primary fibroblasts and iPSCs as quantified by propidium iodide staining. Error bars represent the standard deviation of 3 replicates. Toxicity was quantified by propidium iodide staining during GFP expression analysis.

Toxicity. Primary fibroblasts and iPSCs were transfected with 4.7 and 10 kbp plasmids formulated as described in the experimental section. After transfection, cells were detached with trypsin (300 µL). The resulting cell suspensions were neutralized with cell culture media (700 µL) and transferred to BD falcon round-bottom tubes. The cells were then centrifuged at 1200 rpm for 4 min, and rinsed with PBS. After the rinse, the cells were again centrifuged, and resuspended in 400 µL PBS containing 1% propidium iodide. Cells were then analyzed for GFP gene expression on a BD FACSverse flow cytometer.

Gates were set based on an untransfected control. Based on PI staining, no significant difference in toxicity was observed between large and small plasmid transfections in either cell type. Tr4 and Tr4-heparin transfections show approximately 80-85% cell survival for both cell types. Only live cells were analyzed for gene expression.

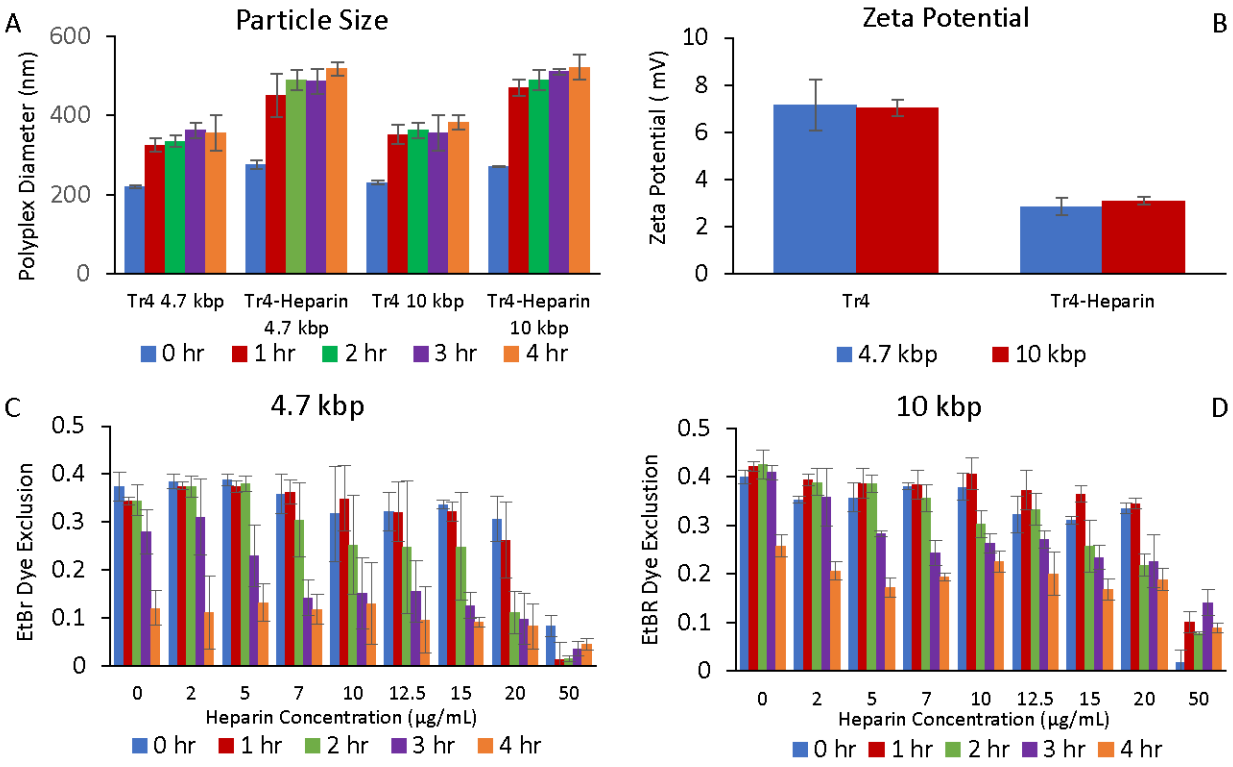

Figure S5: Particle size of Tr4 polyplexes as a function of plasmid size quantified by DLS. Error bars represent the standard deviation of 3 replicates. No significant differences were observed as a function of plasmid size.

Polyplex Stability. Polyplexes were formed as described herein. After polyplex formation, the polyplexes were analyzed by DLS using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) with a 4.0 mW 633 nm HeNe laser and a scattering angle of 173°. Polyplexes were formed in water at an N/P of 40 at a pDNA concentration of 0.01 µg/µl. The polyplexes were then diluted by a factor of 3 with cell culture media (DMEM) containing 10% FBS with or without 10 µg/mL heparin just as they are prior to transfection and analyzed by DLS (Figure S5a). Polyplex size was measured hourly for 4 hours. Particle sizes were reported based on the mean of the most intense peak.

Zeta potential measurements were acquired on a Malvern Zetasizer (Nano ZS, Malvern Instruments, Malvern, U.K.) using the same settings as described for the DLS measurements.

Polyplex solutions were formed in water as described in previous sections. Polyplex solutions were then diluted by a factor of 3 (300 µl diluted with 600 µl) with indicator free DMEM (without FBS) with and without 10 µl/mL heparin. Measurements were acquired in triplicate and reported as an average.

Polyplex stability was assessed using an ethidium bromide dye exclusion assay as previously described.[1] Polyplexes were formed in water at N/P 40 for Tr4 and Tr4-heparin, and with 5 µl lipofectamine/µg DNA. After polyplex formation, polyplex solutions were diluted by a factor of 3 with DMEM containing 10% FBS (with or without 10 µg/mL heparin) containing 4 µM EtBr. Samples were then analyzed for EtBr fluorescence (620 nm) compared to DNA alone and a DNA free blank. Dye exclusion was determined using the formula below.

$$Fractional\ Dye\ Exclusion = 1 - \frac{F_{sample} - F_{blank}}{F_{DNA\ only} - F_{blank}}$$

As shown in Figure S5a, no significant difference can be observed in particle size or aggregation as a function of plasmid size. Immediately after treatment with cell culture media, the particles have a diameter of ~250 nm. Particles formed with small and large plasmids behave similarly regardless of plasmid size over the course of the experimental time period. All polyplex formulations aggregate and/or swell throughout the four-hour measurement period. Polyplexes aggregate and/or swell at a similar rate regardless of plasmid size. This result implies that polyplexes formed with the large plasmid may contain fewer copies of plasmid on average per complex. Due to the high internalization observed with the large and small plasmids, the similar size and colloidal behavior of all formulations, and the stark effects of nuclear permeabilization

on transfection, particle size does not appear to be driving the different transfection efficiencies of large and small plasmid.

Zeta potential measurements in serum free media were used as an analog to study the difference in surface charge in Tr4 and Tr4-Heparin polyplexes as a function of plasmid size. Zeta potential data of the polyplex formulations with large and small plasmid were similar despite a difference in plasmid size. Since both polyplexes were formed with the same amount of pDNA and polycation, it is reasonable that they exhibit the same zeta potential. The Tr4 polyplexes exhibit a slightly positive zeta potential of ~7 mV. After treatment with heparin, the zeta potential decreases to ~2.5 as heparin (a polyanion) likely coats the surface of the polyplexes.

In a solution of free DNA, EtBr can easily intercalate the DNA, leading to increased EtBr fluorescence. In polyplex solutions, complexation of the DNA inhibits EtBr intercalation, and fluorescence is reduced. Hence, EtBr exclusion can be used as a relative indication of polyplex stability as a function of plasmid size for each vehicle. In these experiments, as opposed to the transfections where heparin has been shown to aid transfection, varying concentrations of heparin are used to perturb the polyplexes by competing with the pDNA for binding with the polycation.

As can be seen in Figure S5c-d, there is little difference between polyplexes formed with small and large plasmid, especially at early time points. For Tr4 and Tr4-heparin polyplexes both exhibit ~40% dye exclusion. Over time, and as the heparin concentration is increased, dye exclusion decreases, indicating a loss of polyplex stability (either decomplexation or loosening of inter-polyelectrolyte interactions). Polyplexes formed with the large plasmid polyplexes

appear to be slightly more stable than the polyplex formulation with small plasmid (as a function of both time and heparin concentration). Given the precipitous drop in transfection efficiency observed with large plasmid in transfection experiments with primary fibroblasts, these results indicate that particle instability is not the cause of the reduced transfection efficiency as plasmid size is increased.

Based on the similar physical properties of polyplexes formed from large and small plasmid, and the facts that polyplex formulations formed with both Tr4 and Tr4-heparin exhibit similarly high cellular uptake and do not respond dramatically to treatment with chloroquine and dexamethasone and cell synchronization promote dramatic improvements in transfection, it is unlikely that physical properties of the polyplexes alone are contributing to the dramatic difference between large and small plasmid transfection.

References

(1) Boyle, W. S.,Senger, K.,Tolar, J.,Reineke, T. M. (2017) Heparin Enhances Transfection in Concert with a Trehalose-Based Polycation with Challenging Cell Types. *Biomacromolecules 18,* 56-67.