

Cytotoxicity study

Differential cell apoptosis between G5 PAMAM-SMTP and buffer control was determined using an Annexin V-FITC Apoptosis Detection Assay. Briefly, HUVEC cells were seeded into 24-well cell culture treated plates at a density of 5×10^4 cells per well and plates were placed in an incubator overnight to allow for cell attachment. After 24 hrs, the cells were washed with PBS, and G5 PAMAM-SMTP/PBS was applied directly to each well. Cultures were incubated in duplicates for 3 hrs or 24 hrs at 37 °C. Positive controls were induced with 6% H₂O₂ for 4 hrs. After incubation, cells were detached from the plate using Trypsin-EDTA, collected into FACS tubes, and washed with PBS three times by resuspending in PBS and centrifuging at 16,000 rpm. Cells were then resuspended in 100 μ L of Annexin V binding buffer and stained with 1 μ L of Annexin V-FITC for 15 min at 37 °C. An extra 400 μ L of Annexin V binding buffer was added to each tube prior to samples being run on a BD LSRII (San Jose, CA) and analyzed with the BD FACSDiva 6.0 software.

We investigated the cytotoxicity of the G5PAMAM-SMTP dendrimer nanocarriers to human cells. The potential toxic effect of the G5PAMAM-SMTP nanocarriers on inducing apoptosis was tested using a HUVEC cell line. Annexin- FITC assay (Figure S1) confirmed a negligible cell toxicity index for the G5PAMAM-SMTP dendrimer nanocarrier compared to controls.

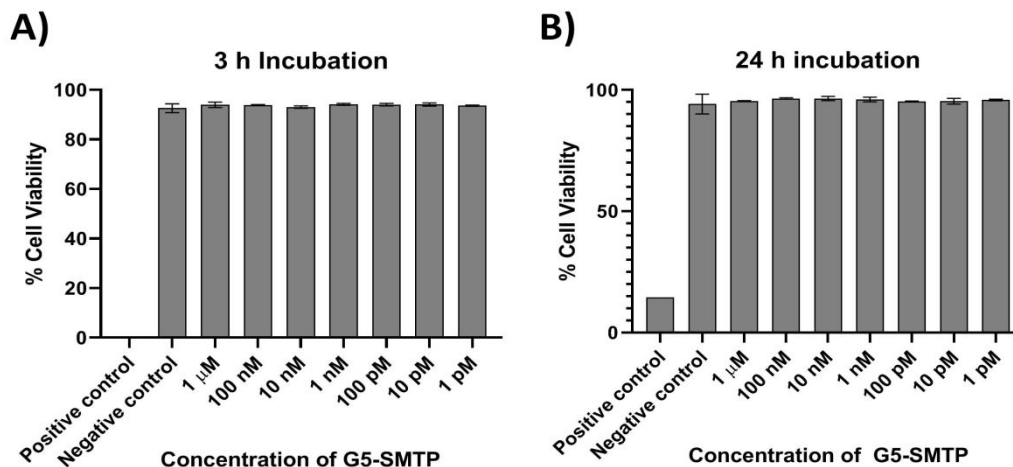


Figure S1. Cell viability upon 3 hr and 24 hr incubation of HUVEC cells with G5-SMTP. Annexin-based apoptosis assay was performed to determine cytotoxicity.

In vitro transfection of muscle cells with EGFP

For this experiment, ~75,000 C2C12 myoblast cells per well were seeded on a 12-well plate 48 hrs prior to transfection. The complex was prepared using 5 μg pEGFP and 17.25 μg G5-SMTP in a total volume of 100 μL . Cells were analyzed by flow cytometry 24 hrs post-transfection. The flow cytometry data (Figures S2) demonstrates a representative set from cells treated with G5-SMTP-pEGFP.

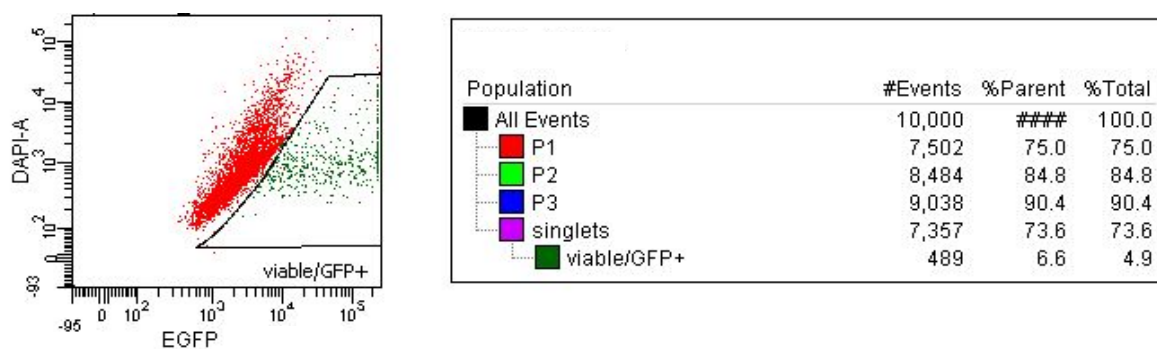


Figure S2. Flow cytometry analysis of cells transfected with G5-SMTP-pEGFP.

UV-vis analysis of polyplex formation

Polyplexes were prepared at varying N/P ratios as previously described, using 5 μg of DNA (0.1 mg/mL) and incubated at room temperature for at least 15 minutes. Samples were then centrifuged at $14,000 \times g$ for 5 minutes to pellet any insoluble aggregates. A volume of 20 μL of the soluble fraction was diluted in HEPES buffer to a final volume of 200 μL and absorbance measurements were made using an Agilent 8453 UV-vis spectrophotometer.

We assessed polyplex formation by UV-visible spectroscopy (Fig. S3-A and S3-B). The data showed two characteristic peaks corresponding to DNA and G5 for all ratios. A decrease in the presence of free DNA at N/P ratios of 0.5 and 1, as indicated by the decrease in absorbance at 260 nm relative to the DNA control was observed. The formation of stable G5 and SMTP polyplexes

was observed for N/P ratio higher than 5. This data indicates a stable binding of plasmid DNA to dendrimer.

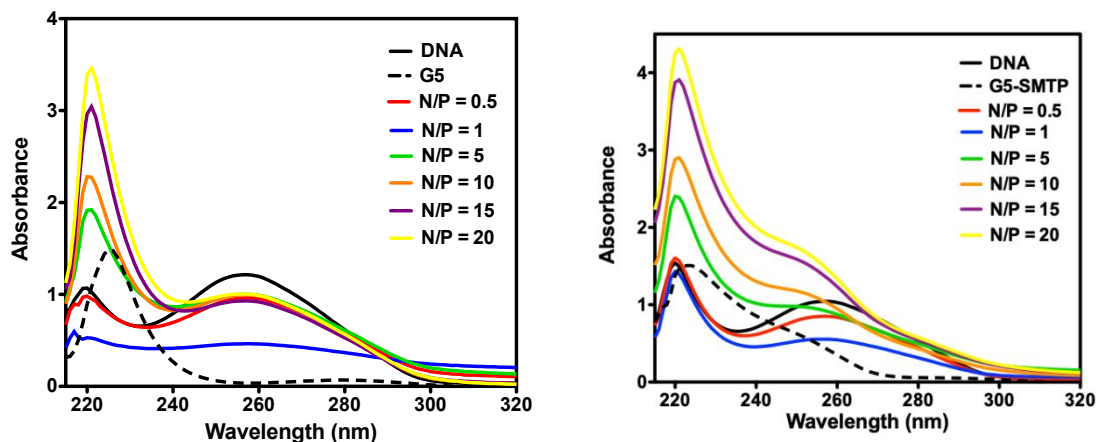


Figure S3. Analysis of polyplex formation. Polyplexes comprising plasmid DNA and G5 (A) or G5-SMTP (B) were analyzed by means of UV-visible spectroscopy.

Protection of plasmid DNA from degradation by serum nucleases

Polyplexes prepared at varying N/P ratios were incubated in complete cell medium containing 10 % fetal bovine serum for 4 h at 37 °C. The nuclease reaction was stopped by addition of EDTA (5 mM final concentration), and polyplexes were dissociated by addition of 1% SDS. Samples were purified by overnight ethanol precipitation at -20 °C. The integrity of the recovered plasmid DNA was then analyzed by gel electrophoresis (Figure S4) in 0.7% agarose at 80 V.

A key feature of any successful gene delivery platform is the ability of the carrier to protect the DNA cargo from degradation by nucleases. This is feasible if the binding of the cargo is internal to the vehicle surface. Therefore, we evaluated the ability of SMTP to confer protection of plasmid DNA in the presence of serum nucleases. At a low N/P of 1, G5 was able to protect the plasmid DNA from degradation, as evidenced by the observed bands (Figure S4A). For SMTP, however, DNA smearing was observed for the polyplex prepared at the same N/P ratio (Figure S4B), which is indicative of DNA degradation. At higher N/P ratios of 5 and above, a greater degree of

protection is observed for both G5 and SMTP. While concentrated smearing is apparent near the loading wells for the SMTP polyplexes (Figure S4B, N/P ratios 5-20), this is indicative of incomplete dissociation of the plasmid from the dendrimer during DNA recovery and not degradation.

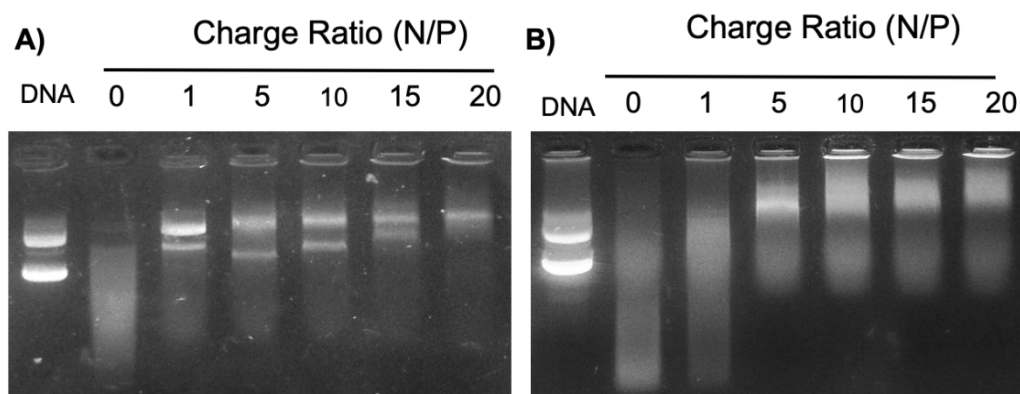


Figure S4. Gel electrophoresis of polyplexes comprising plasmid DNA and G5 (A) or G5-SMTP (B) after incubation with nuclease.

Comparison of *in vitro* transfection using C2C12 and HeLa cells

C2C12/HeLa cells were seeded at a density of 7.5×10^4 cells per well on 24-well plates and incubated in DMEM (supplemented with 10% FBS) at 37 °C, 5% CO₂ until they reached approximately 80-90% confluence. The cells were then washed twice with PBS and replenished with 0.5 mL DMEM prior to transfection. The transfection efficiency of G5-DBP-plasmid and G5-SMTD-DBP-plasmid were compared. 1 µg of pLuc DNA was employed as the plasmid DNA, and polyplexes were added directly to the cells. The cells were then incubated for 24 hrs. After incubation, the cells were washed twice with PBS and lysed by adding 0.4 mL of 1X Promega reporter lysis buffer to each well. One freeze-thaw cycle was performed by freezing the cells at -80 °C and then thawing to complete the lysis. The resultant cell lysates were scraped from the 24-well plates and analyzed for luciferase expression. Data were normalized to the total protein concentration obtained from a BCA assay.

In a comparison of *in vitro* transfection efficiency, Figure S5 shows that DBP-DNA at charge ratio of 1 and G5-SMTP at charge ratio of 2 combined in a single formulation has a significantly higher transfection efficiency in C2C12 cells (Figure S5A). In comparison, *in vitro* transfection efficiency of G5-DBP-DNA in C2C12 was low (Figure S5B). This was mirrored in HeLa cells (Figure S5C-D), where the presence or absence of SMTP did not alter the background levels of transfection. This further confirms that a skeletal muscle targeting peptide serves to selectively improve transfection efficiency within the target cell population.

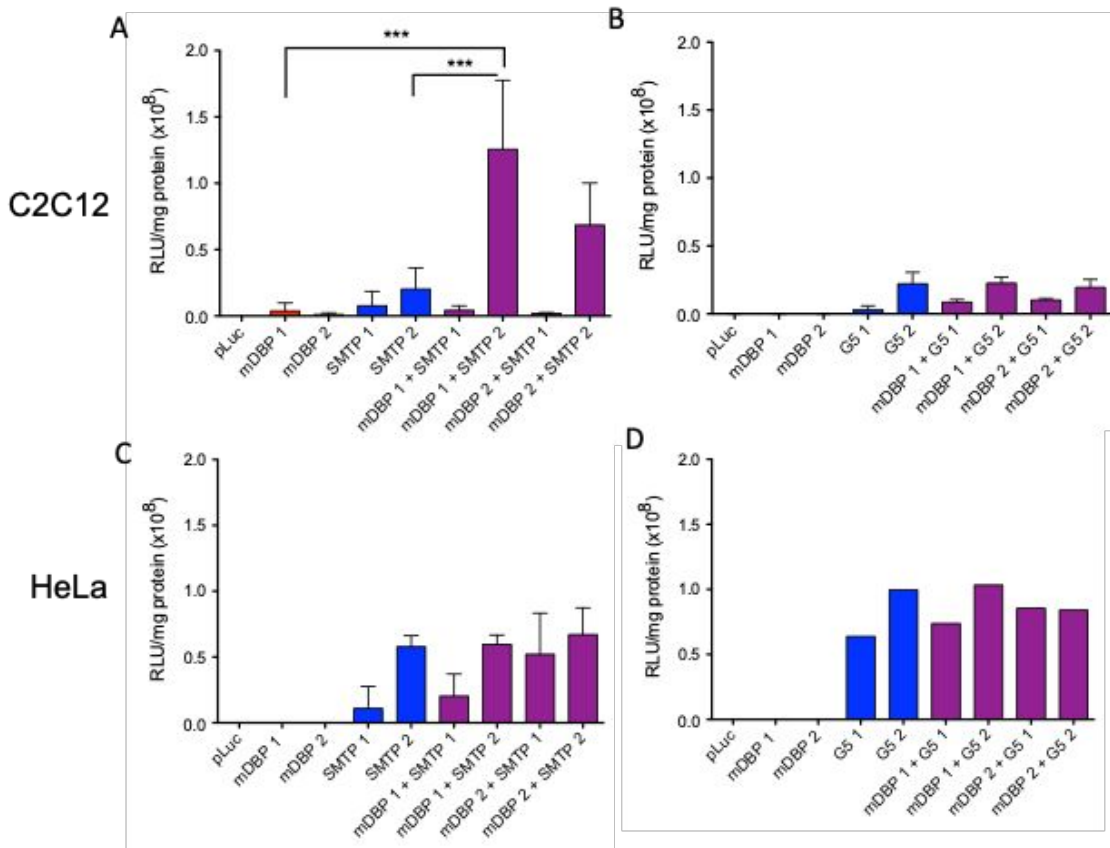


Figure S5. *In vitro* luciferase assays. (A) C2C12 cells were transfected with G5-SMTP-DBP-Rluc polyplexes prepared at varying charge ratios; (B) C2C12 cells were transfected with G5-DBP-Rluc polyplexes prepared at varying charge ratios (C) HeLa cells were transfected with G5-SMTP-DBP-Rluc polyplexes prepared at varying charge ratios; (D) HeLa cells were transfected with G5-DBP-Rluc polyplexes prepared at varying charge ratios. Luciferase expression was monitored in each case 24 hrs post-transfection. Experiments were performed in triplicate, with the data representing the mean and standard error of the mean.

In vitro transfection of muscle cells with EGFP: comparison with Xfect

C2C12 myoblast cells were seeded on a 12-well plate 48 hrs prior to transfection. Cells were transfected with three different polyplexes: pEGFP alone, G5-SMTP-pEGFP, and Xfect-pEGFP. Untreated cells were used as control. Each complex was prepared using 5 μ g pEGFP in a total volume of 100 microliters. Cells were analyzed by flow cytometry 24 hrs post-transfection.

Figure S6 shows the percentage of positive cells in four different groups. The transfection efficiency using pEGFP was negligible and had comparable fluorescence to the untreated cells, indicating the low transfection efficiency of naked plasmid DNA. Conversely, the percentage of positive cells using G5-SMTP as a carrier was comparable to that of Xfect, with the additional benefit of selective *in vivo* targeting.

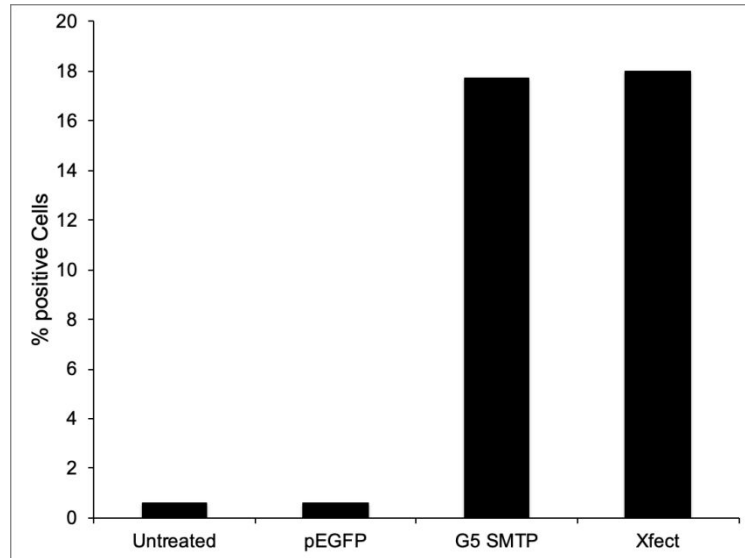


Figure S6. Comparison of transfection efficiency for G5-SMTP and Xfect. Positive cells were identified using flow cytometry analysis and compared with controls.