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Precisely Targeted Gene Delivery in Human Skin Using Supramolecular Cationic Glycopolymers

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MATERIALS AND METHODS

Materials

Triethylamine (TEA) (BioUltra, $\geq 99.5\%$), 1- Thio- β -D-glucose sodium salt, aluminium oxide (Al_2O_3), methyl iodide (CH_3I) and CuBr_2 were purchased from Sigma Aldrich and used as received. Tris(2-(dimethylamino)ethyl)amine (Me_6TREN) were synthesized according to literature procedures and stored at 4°C prior to use. 2,3,4,5,6-Pentafluorostyrene (PFS), 2-(dimethylamino)ethyl methacrylate and *N,N*-dimethylacrylamide were purchased from Sigma Aldrich and passed over a basic aluminium oxide column to remove inhibitors prior to use. Cu wire was purchased from Sigma Aldrich and wrapped around stir bar. Then it was immersed in hydrochloric acid for 15 mins followed by washing with deionized water and acetone before drying with N_2 . Cu wire was used here to initiate the SET-LRP reaction of DMAEMA. SG1-MAMA (Bloc Builder) was kindly provided by Arkema and used as received. All other reagents and solvents were purchased from Sigma Aldrich or Fisher Scientific at the highest purity available and used without further purification unless stated otherwise.

Instruments and Analysis

Nuclear Magnetic Resonance (NMR)

Hydrogen and Fluorine-19 (^1H and ^{19}F) Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker AVIII 400 using deuterated chloroform (CDCl_3) or deuterated water (D_2O) as the solvent. 2D Nuclear Overhauser Effect Spectroscopy (NOESY) NMR experiments were performed on a 600 MHz Bruker Avance III spectrometer in the aqueous phase at a temperature of 295 K. In details, mixing time was set to 200 μs while the 90° pulse was determined to be 8.7

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μ s. Spectra were recorded with using 20 scans per t1 increment and 16 dummy scans at 295 K. The spectral width was set to 5×5 ppm which leads to a total experiment time of about 6 hours and 30 minutes. After zero filling to $4k \times 2k$ points and apodization, using a 90° -phase shifted squared sine function, the spectra were Fourier transformed.

Gel Permeation Chromatography (GPC)

Gel Permeation Chromatography (GPC) measurements were carried on an Agilent 1260 infinity system operating in dimethylformamide (DMF) with 5 mM Ammonium tetrafluoroborate at 40°C and equipped with refractive index detectors and variable wavelength detectors, 2 PLgel 5 μm mixed-C columns (300×7.5 mm), a PLgel 5 mm guard column (50×7.5 mm) and an autosampler. Standard curves were calibrated with linear narrow poly(methyl methacrylate) standards with a molecular weight ranging from 550 to $46890 \text{ g}\cdot\text{mol}^{-1}$. Samples containing copper ions were passed through the neutral aluminium oxide prior to passing through a $0.22 \mu\text{m}$ Nylon filter before analysis.

Dynamic Light Scattering (DLS)

The hydrodynamic diameters (D_h , the volume weight diameter of the distribution) evolution were determined characterized by a Malvern Zetasizer Nano ZS instrument equipped with a He-Ne laser at 633 nm. DLS measurements were performed by dissolving polymer samples at 1 mg/ml in deionized water and all the samples were passed through a $0.22 \mu\text{m}$ nylon filter before measurement. For complex samples, polymers were dissolved separately in deionized in water and mixed together at a 1:1 CD: Adamantane molar ratio. Then the samples were stirred overnight at room temperature and filtered using a $0.22 \mu\text{m}$ nylon filter before analysis. All the samples were measured at 25°C three times.

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***In Vitro* Transfection and Luciferase Assay**

HEK 293T.17 cells (ATCC, USA) were plated at a density of 50,000 cells well⁻¹ 48 hours prior to transfection. The polyplexes were added to each well in a total volume of 100 μ L and a total dose of 100 ng of DNA in 20 mM HEPES with 5% glucose. The polyplexes were added to cells for 4 hours, and then the media was replaced with 100 μ L complete DMEM (cDMEM) (10% fetal bovine serum (FBS), 5 mg mL⁻¹ L-glutamine, 5 mg mL⁻¹ penicillin streptomycin (ThermoFisher, UK)). After 24 hours, 50 μ L of media was removed and 50 μ L of ONE-Glo™ luciferase substrate (Promega, UK) was added, and the total 100 μ L was transferred to a white 96-well plate and analyzed on a FLUOstar Omega plate reader (BMG LABTECH, UK) with a gain of 3000.

Human Skin Explant Injection, Culture and Digestion

Surgically-resected specimens of human skin tissue were collected at Charing Cross Hospital, Imperial College London, UK. All tissues were collected after receiving signed informed consent from all patients, under protocols approved by the Local Research Ethics Committee. The tissue was obtained from patients undergoing elective abdominoplasty, breast reduction or mastectomy surgeries. Tissue was refrigerated until arrival in the laboratory, where the subcutaneous fat layer was completely removed, and the remaining skin layers were trimmed into 1 cm² sections. Explants were incubated at 37 °C with 5% CO₂ in petri dishes with 10 mL of cDMEM, with daily media replacement. Explants were injected with 2 μ g of DNA intradermally (ID) using a Micro-Fine Demi 0.3 mL syringe (Becton Dickinson, UK) in a volume of 50 μ L. After 3 days, skin was digested. Briefly, skin was minced well with scissors and incubated in 3 mL DMEM supplemented with 1 mg mL⁻¹ collagenase P (Sigma, UK) and 5 mg mL⁻¹ dispase II (Sigma, UK)

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for 4 hours at 37 °C on a rotational shaker. Digests were then filtered through a 70 µm cell strainer and centrifuged at 1750 RPM for 5 minutes. Cells were then resuspended in 100 µL of FACS buffer (PBS + 2.5% FBS) and stained with 100 µL of Aqua Live/Dead Stain (Thermo Fisher, UK) diluted 1:400 in FABS buffer for 20 min on ice. Cells were then washed with 2.5 mL of FACS buffer, centrifuged at 1750 RPM for 5 minutes and stained with a panel of antibodies (**Table S1**) to identify cellular phenotypes for 30 minutes. Cells were then washed with 1 mL of FACS buffer, centrifuged at 1750 RPM for 5 minutes and resuspended in 250 µL of PBS. Cells were fixed with 250 µL of 3% paraformaldehyde for a total final concentration of 1.5% and refrigerated until flow cytometry analysis.

Flow Cytometry

Samples were analyzed on a LSRFortessa™ (BD Biosciences, UK) using FACSDive software (BD Biosciences, UK) with 100,000 acquired events. Gating was performed as previously described.¹ GFP⁺ cells and phenotypes were quantified using FlowJo Version 10 (FlowJo LLC, Oregon, USA).

Statistical Analysis

Graphs and statistical analysis of *in vitro* and *ex vivo* data were prepared in GraphPad Prism, version 8.0. Statistical analysis was performed using a two-tailed t test with $\alpha=0.05$ used to indicate significance.

Synthesis Methods

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Synthesis of 1-adamantyl acrylate (AdaA)

1-Adamantyl acrylate was synthesized according to literature with slight modifications.² 1-Adamantanol (9.13 g, 60 mmol) and triethylamine (14 mL, 100 mmol) were mixed in anhydrous dichloromethane (250 mL). Mixture was kept at 0 °C for 30 mins before dropwise addition of acryloyl chloride (7.31 mL, 90 mmol) under argon atmosphere. Reaction was kept at 0 °C for further 40 mins and then brought up to room temperature overnight while stirring. The mixture was filtered afterwards to remove triethylamine hydrochloride salts. 1-Adamantyl acrylate was purified by brine-acid-base-water wash cycles and extraction. Lastly, DCM was removed under reduced pressure and monomer was dried under vacuum overnight at room temperature. Finally, 1-Adamantyl acrylate was obtained as yellowish solid with 37 % yield.

¹H NMR of 1-adamantyl acrylate (CDCl₃, 400 MHz, ppm, TMS) 6.26 (dd, -CH_aH_b=CHCOOC(CH₂)₃(CH)₃(CH₂)₃, 1H), 6.03 (dd, CH_aH_b=CHCOOC(CH₂)₃(CH)₃(CH₂)₃, 1H), 5.70 (dd, CH_aH_b=CHCOOC(CH₂)₃(CH)₃(CH₂)₃, 1H), 2.15 (br, CH_aH_b=CHCOOC(CH₂)₃(CH)₃(CH₂)₃, 9H), 1.68 (br, CH_aH_b=CHCOOC(CH₂)₃(CH)₃(CH₂)₃, 6H).

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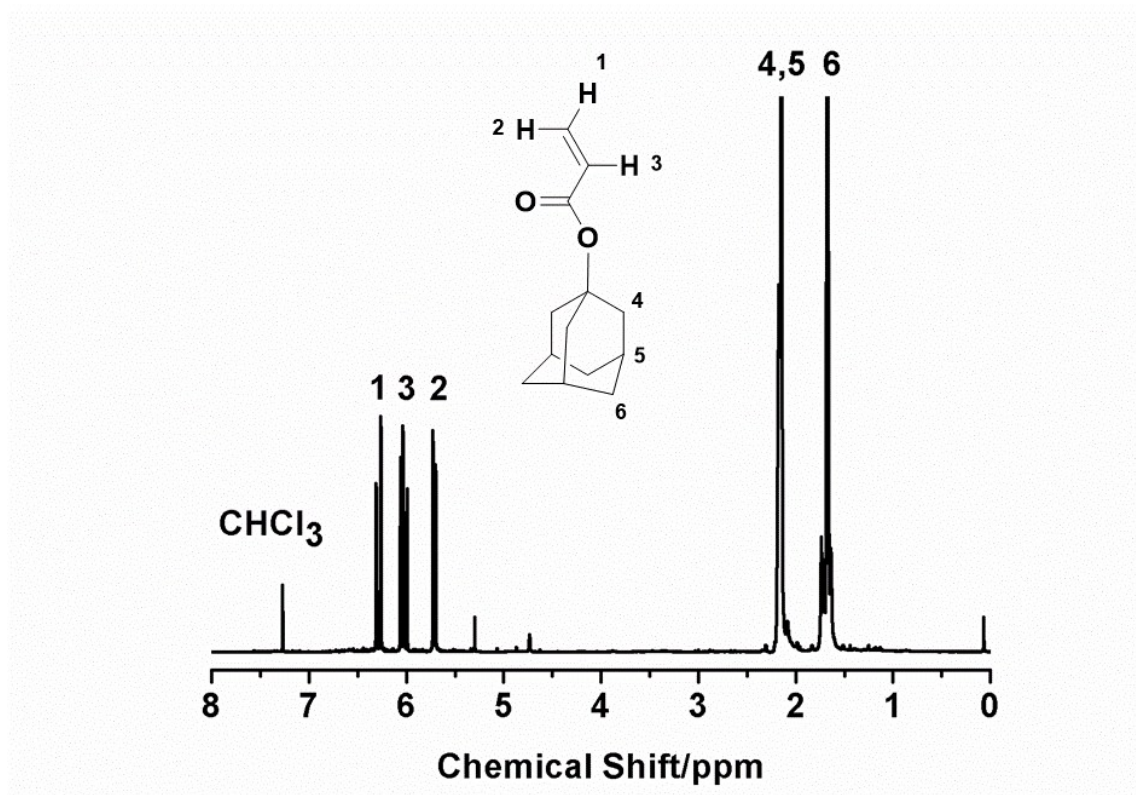


Figure S1. ¹H NMR of 1-adamantyl acrylate.

Synthesis of per-6-deoxy-6-(thiopropyl-2-bromo-2-methylpropanoate)- β -cyclodextrin (CD-Br₇ initiator)

Per-6-thio- β -cyclodextrin (2.5 g, 2 mmol), and dithiothreitol (DTT, 618 mg, 4 mmol) were dissolved in 40 mL anhydrous DMF under Argon and heated to 60 °C. After 60 h the reaction mixture was allowed to cool down to room temperature and allyl 2-bromoisobutyrate (14.53 g, 70 mmol), 2,2-dimethoxy-2-phenylacetophenone (DMPA, 179 mg, 7 mmol) were added to the reaction mixture and stirring was continued for 5 h under UV irradiation (365 nm).

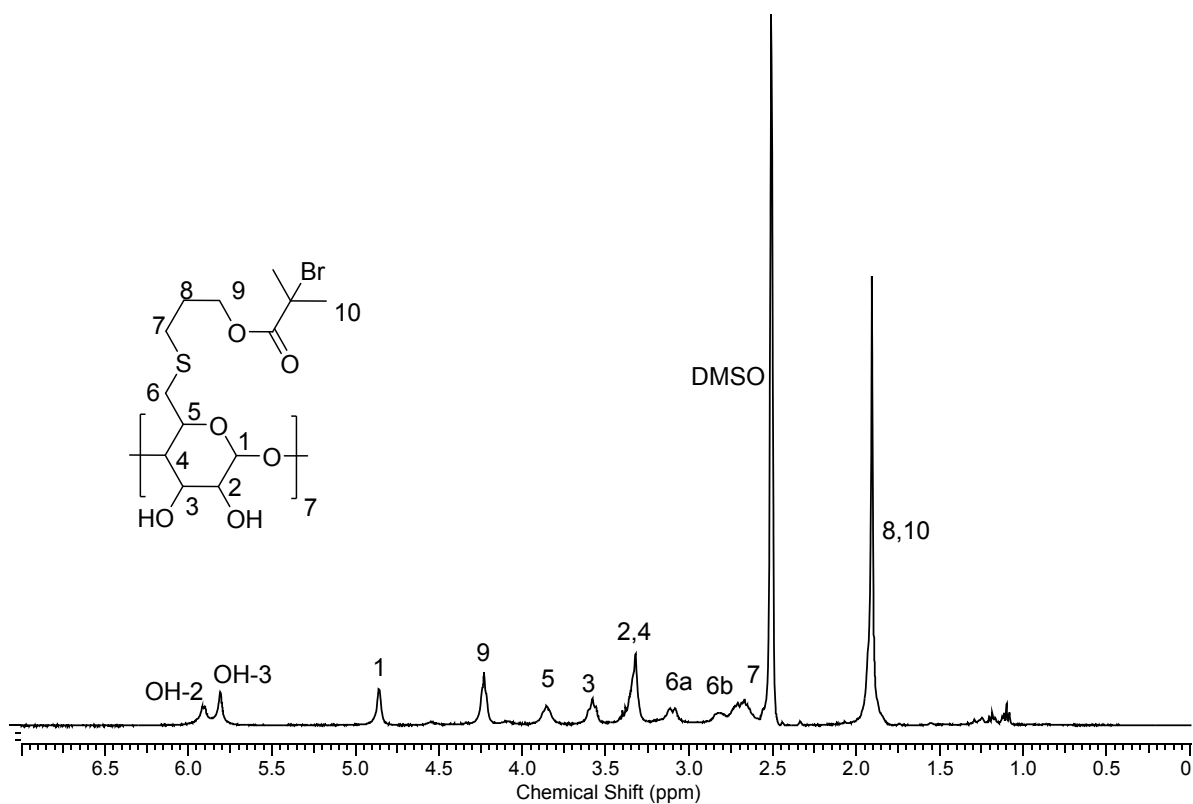
The solution was precipitated in 500 mL of methyl tert-butyl ether (MTBE) in ten 50 mL centrifuge tubes and centrifuged at 8000 rpm for 5 min. The solvent was decanted and all

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precipitated fractions collected in two 50 mL centrifuge tubes and fresh MTBE was added, mixed and centrifuged again. This procedure was repeated 4 times in order to remove DMF and allyl 2-bromoisobutyrate. Subsequently the product was dried under vacuum, yielding a fine beige solid. (3.7 g, yield: 68%).

^1H NMR (400 MHz, $\text{DMSO-}d_6$, 298 K, ppm): δ = 5.90 (*d*, 7 H, 5.6 Hz), 5.8 (*m*, 7 H), 4.85 (*m*, 7 H), 4.22 (*t*, 14 H, 5.2 Hz), 3.85 (*m*, 7 H), 3.57 (*m*, 7 H), 3.33 (*m*, 14 H), 3.09 (*d*, 7 H, 10.6 Hz), 2.82 (*m*, 7 H), 2.69 (*m*, 14 H), 1.90 (*s*, 56 H)

MALDI-TOF MS m/z : calculated for $\text{C}_{91}\text{H}_{147}\text{Br}_7\text{O}_{42}\text{S}_7\text{K}^+$:2733.12; found: 2733.36



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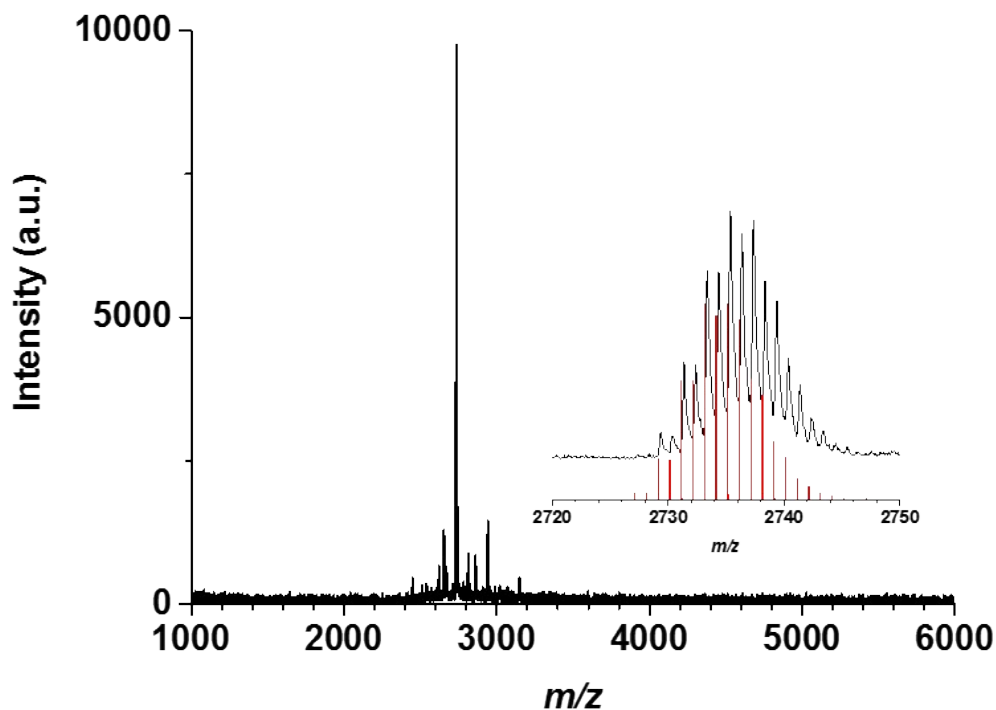


Figure S2. $^1\text{H-NMR}$ spectrum (in DMSO-d_6) (*top*) and MALDI-TOF spectrum with a zoom of the peak of interest (*bottom*) of per-6-deoxy-6-(thiopropyl-2-bromo-2-methylpropanoate)- β -cyclodextrin.

Procedure for Nitroxide-Mediated Polymerization

The synthesis of adamantane-containing copolymers was carried out using nitroxide mediated polymerization (NMP) at $120\text{ }^\circ\text{C}$ for 3 to 5 hours. The composition of the copolymers were adjusted by adjusting the feeding ratio of each monomer. As a representative example, PFS (0.5 mL, 3.61 mmol), DMA (2.48 mL, 24.14 mmol), AdaA (0.50 g, 2.41 mmol), BlocBuilder (92 mg, 0.24 mmol), and anisole (10 mL) were added in an argon-filled Schlenk tube. The mixture was then bubbled with argon while stirring for at least 30 min. The flask was then sealed and the reaction was placed into a preheated oil bath at $120\text{ }^\circ\text{C}$ for 5 hours and stopped by

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cooling down the reaction with liquid nitrogen. NMP of PFS alone was continued for 1 hour to avoid side reactions while NMP of DMA alone was carried out for 3 hours to consume 99 % monomer in the reactions. Monomer conversion and molar mass evolution were monitored by NMR and GPC. All reactions were diluted with 2 mL THF and precipitated into cold diethyl ether twice to remove the residual impurities. The isolated white powder was dried at 40 °C under vacuum for 24 h before further use.

Procedure for Click Reaction of the Block Copolymer and 1-Thio- β -D-glucose

PPFS_{15-*r*}-PAdA_{10-*r*}-PDMA₁₀₀ copolymer (1.00 g, 0.067 mmol) was mixed with thio-glucose (261.84 mg, 1.2 mmol) dissolved in 10 mL of DMSO. TEA (0.50 mL, 1.2 mmol) was then added to the reagent solution. The reaction mixture was stirred for 24 h at 40 °C and the final reaction mixture was analyzed by GPC and ¹⁹F NMR to confirm quantitative substitution of para-fluorine. Subsequently, the mixture was concentrated to 2.5 mL, precipitated into diethyl ether twice, and filtered. The final block glycopolymers were dried in vacuo at 40 °C overnight and obtained as a white powder.

Procedure for Single Electron Transfer Living Radical Polymerization

CD initiated Single Electron Transfer Living Radical Polymerization (SET-LRP) of DMAEMA was carried in DMSO at 25 °C. In brief, Cu(II)Br₂ (0.33 mg, 0.28 eq) was added to a argon purged Schlenk tube and followed by a further 10 minutes of degassing. Meanwhile, Me₆TREN (4.53 mg, 1.33 eq) was mixed with half of the total amount of DMSO and was degassed for 15 minutes. Then the ligand/solvent mixture was transferred with a degassed syringe into Cu(II)Br₂ containing flask. Seven-arm CD initiator (40 mg, 1 eq) together with targeting DP of DMAEMA was dissolved in other half of DMSO and purged for 20 minutes before transferring to the

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Schlenk tube *via* a degassed syringe. The reaction was started by addition of the 5cm Cu(0) wrapped stir bar into the Schlenk tube. The polymerization reaction was followed by ^1H NMR to terminate the polymerization at 40 % conversion by bubbling with air for 5 minutes in order to prevent star-star coupling that is unavoidably in the synthesis of star-shaped polymers. The final reaction mixture was analyzed by GPC and ^1H NMR to determine the molecular properties and monomer conversion. The reaction mixture was dialysis against water for three days with changing the water twice a day. The final star-shape polymers were freeze-dried overnight and recovered as a white powder. PDMAEMA with three different arm length were synthesized in this study.

Procedure for Quaternization of PDMAEMA

CD initiated star-shaped PDMAEMA was quaternized using methyl iodide (CH_3I) as the quaternizing agent. In a typical experiment, PDMAEMA (0.05 g, 0.067 mol of amino groups) is dissolved in THF at 2% w/v concentration and CH_3I (14 g, 0.1 mol) is added to the reaction mixture. The reaction was kept at room temperature for 24 hours under vigorous stirring. Afterward, the reaction mixture was dialyzed for three days against water changed twice a day. Subsequently, the final quaternized polymers were freeze-dried and obtained as a yellow powder. The obtained polymers were characterized using ^1H NMR (Figure S3.).

Plasmid DNA (pDNA) Preparation

Mammalian codon optimize firefly luciferase (fLuc) and enhanced green fluorescent protein (eGFP) were synthesized by GeneArt (Invitrogen, UK) and cloned into pcDNA3.1 (Invitrogen, UK). pDNA was then transformed into *Escherichia coli*, grown in 50 mL LB culture with 1 mg mL^{-1} carbenicillin (Sigma Aldrich, UK) and purified using a Plasmid Plus Maxiprep kit

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(QIAGEN, UK). pDNA concentration and purity were measured on a NanoDrop One (ThermoFisher, UK) prior to complexation. The obtained pDNA has 7047 base pairs.

pDNA Complexation and Characterization

Stock solutions of glycopolymers were prepared at a concentration of 5 mg mL⁻¹ in ultrapure H₂O and purified using a 0.22 µm syringe filter (Millipore, Sigma, UK). pDNA complexes were prepared by diluting the polymer and DNA into 20 mM HEPES buffer (pH 7.4) with 5% glucose, with a ratio of cyclodextrin to DNA of 10:1 (w/w), unless otherwise stated, and a ratio of cyclodextrin to adamantane of 1:1 (w/w) calculated based on the theoretical molecular weight. Complexes were allowed to incubate at room temperature on a rolling shaker for 1 hour prior to use to ensure complex formation. PEI MAX 40k (Polysciences, UK) was used as a positive control and used according to the manufacturer's protocol.

Polyplexes were prepared in a volume of 800 µL 20 mM HEPES with 5% glucose for particle size and charge analysis, and characterized on a Zetasizer Nano ZS (Malvern Instruments, UK) with Zetasizer 7.1 software (Malvern Instruments, UK) in a clear disposable 1 mL cuvette. The polyplexes were analyzed using the following settings: material refractive index of 1.529, absorbance of 0.010, dispersant viscosity of 0.8820 cP, refractive index of 1.330 and dielectric constant of 79. Each sample was analyzed three times, for up to 100 runs or until measurement equilibrated.

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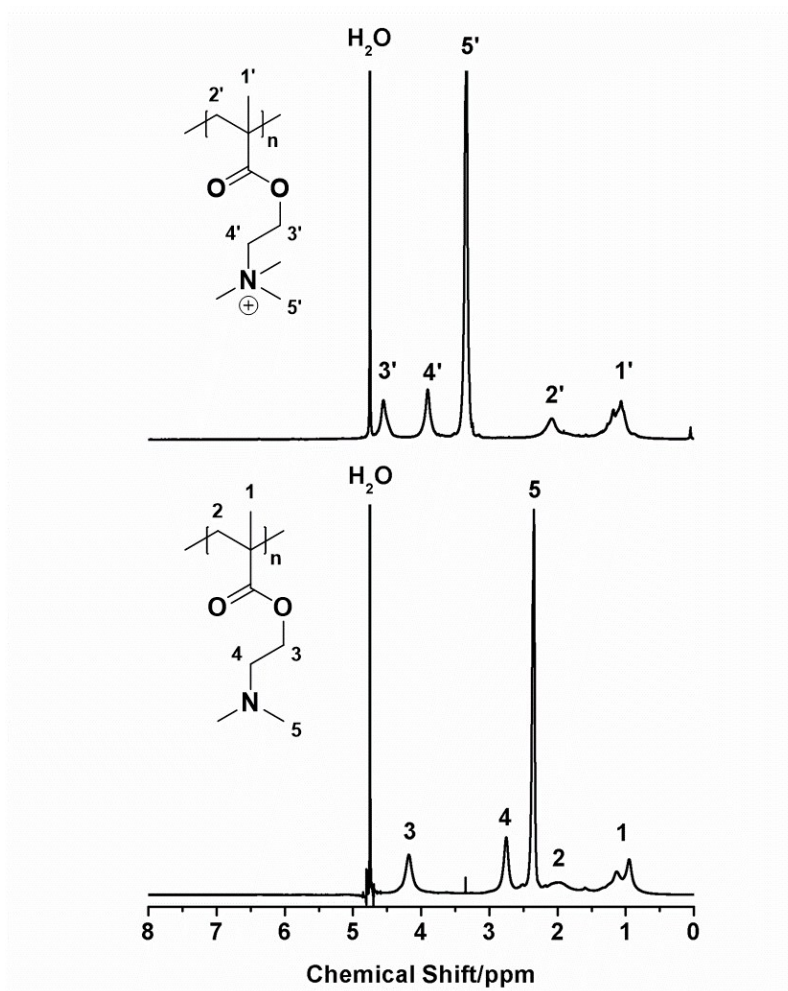


Figure S3. NMR of CD initiated (*bottom*) PDMAEMA and (*top*) quaternized PDMAEMA.

Downfield shift of proton 4 and 5 proved the fully quaternization of PDMAEMA.

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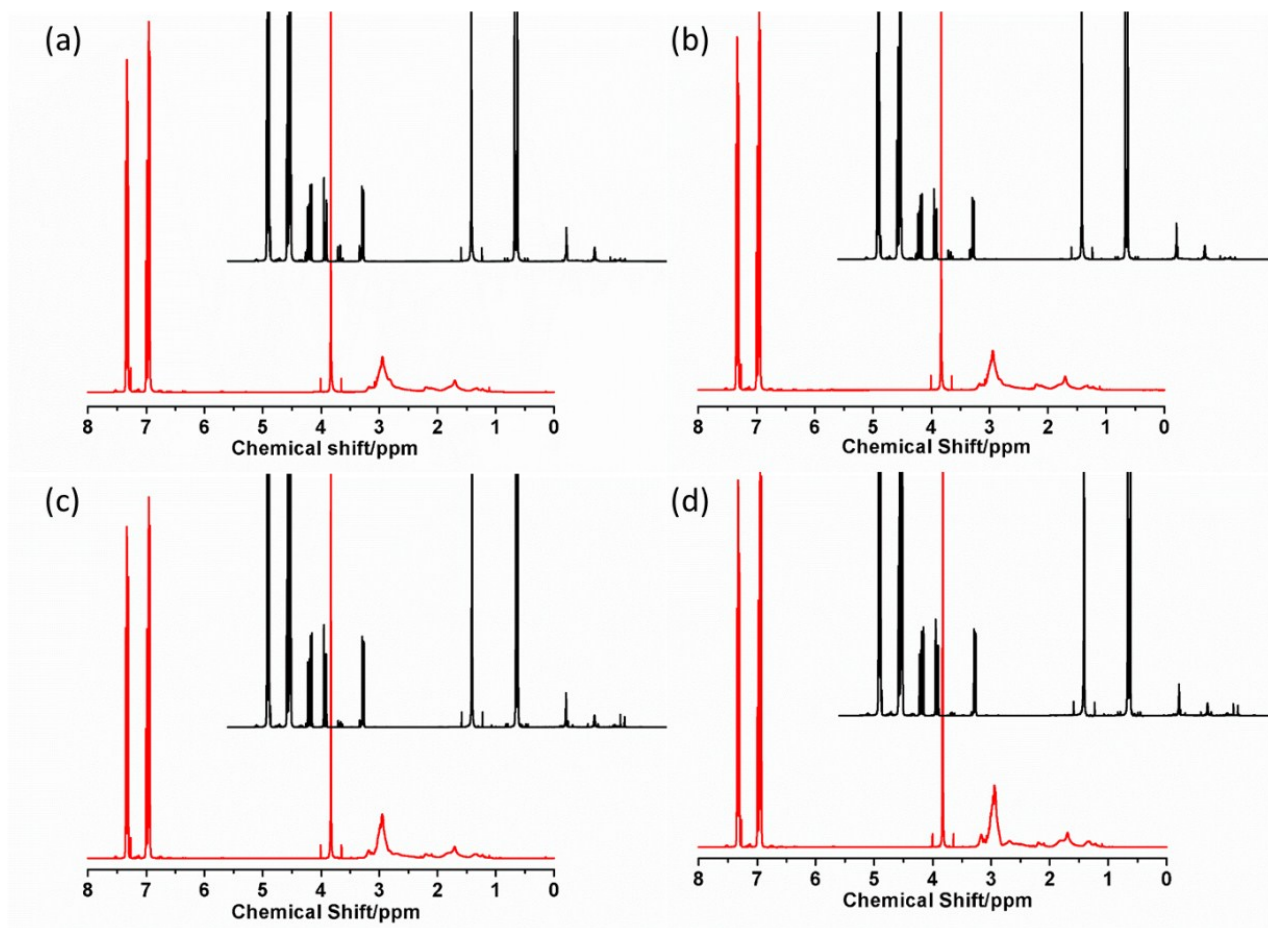


Figure S4. ¹H NMR of t_0 and t_{final} for (a) P1; (b) P2; (c) P3; (d) P4. The disappearance of all the monomer peaks showed the fully consumption of all three types of monomers.

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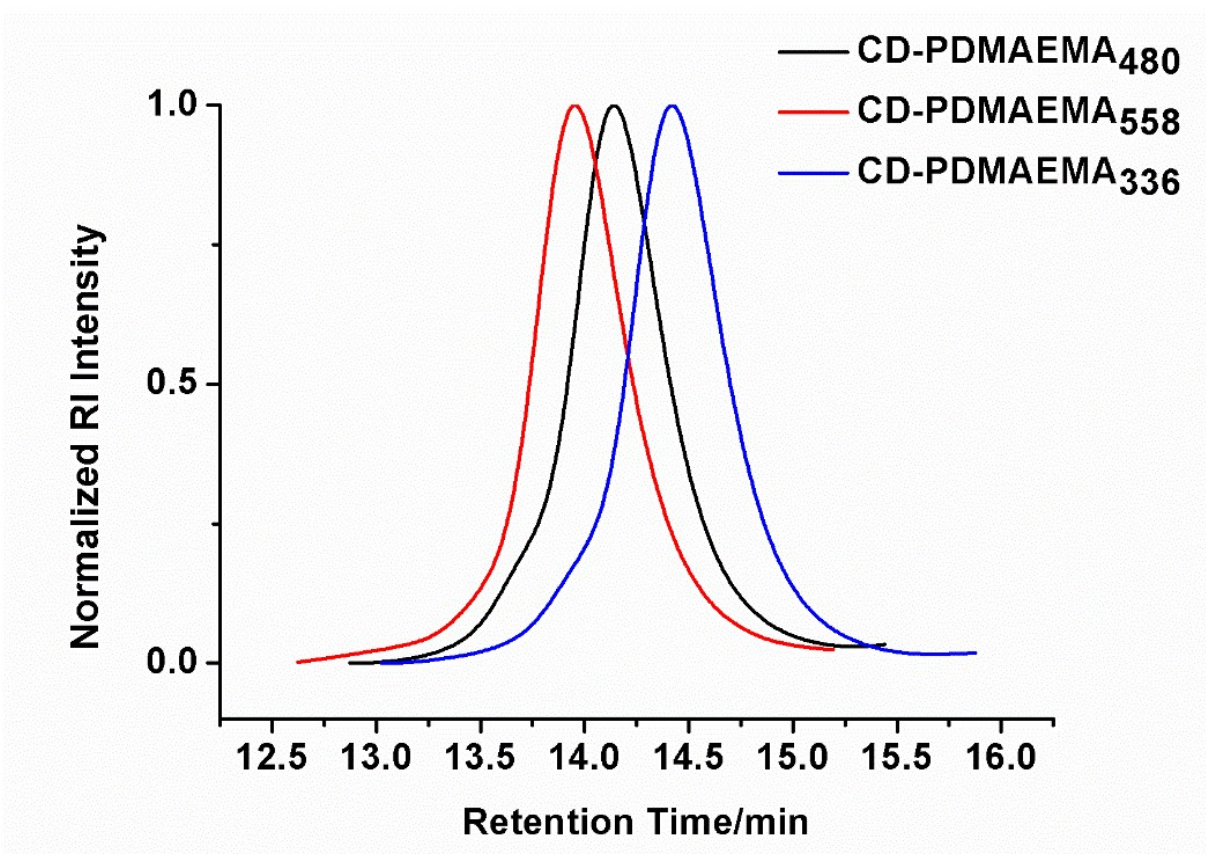


Figure S5. GPC traces of CD initiated PDMAEMA with different chain lengths. The number of monomer on each arm was calculated according to monomer conversion in NMR measurement.

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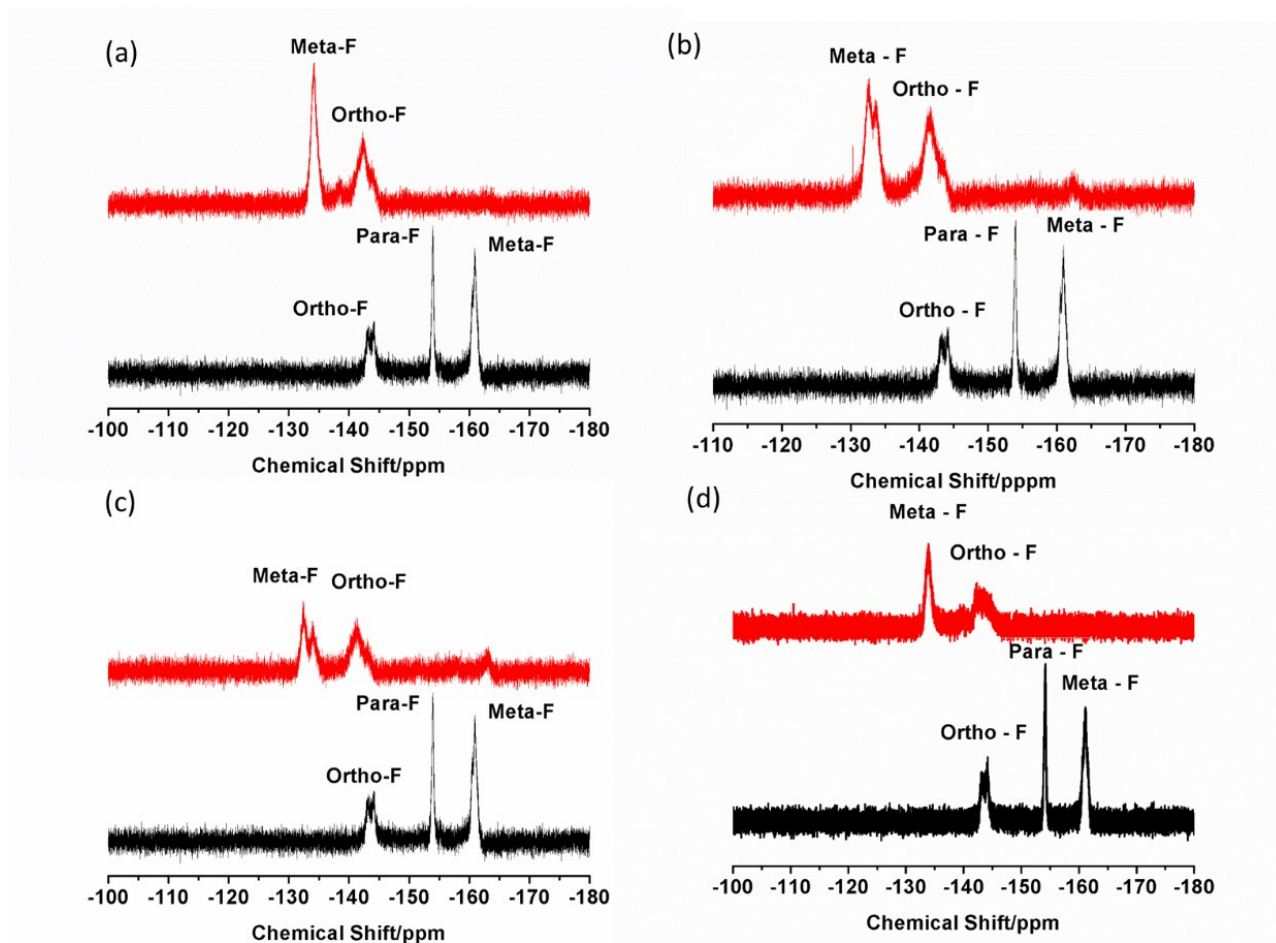


Figure S6. ^{19}F NMR of before and after thio-fluorine for (a)P1; (b)P2; (c)P3; (d) P4. The disappearance of para-fluorine peak confirmed the full substitution with glucose units.

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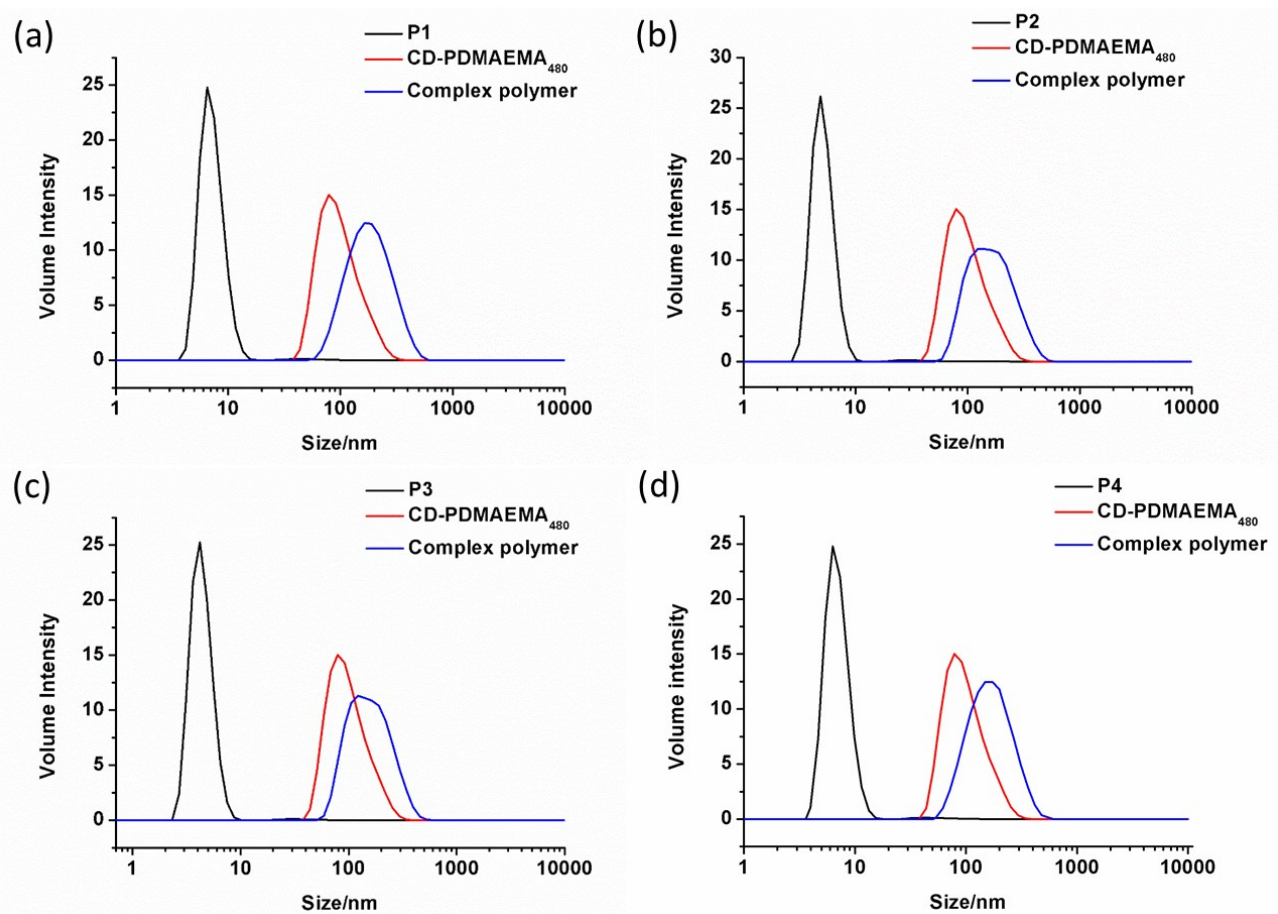


Figure S7. Dynamic light scattering evaluation of complex formation for (a)P1; (b)P2; (c)P3; (d) P4. The increases in particle sizes indicate the formation of larger complexes by mixing two polymers together through adamantane β -cyclodextrin host-guest interaction.

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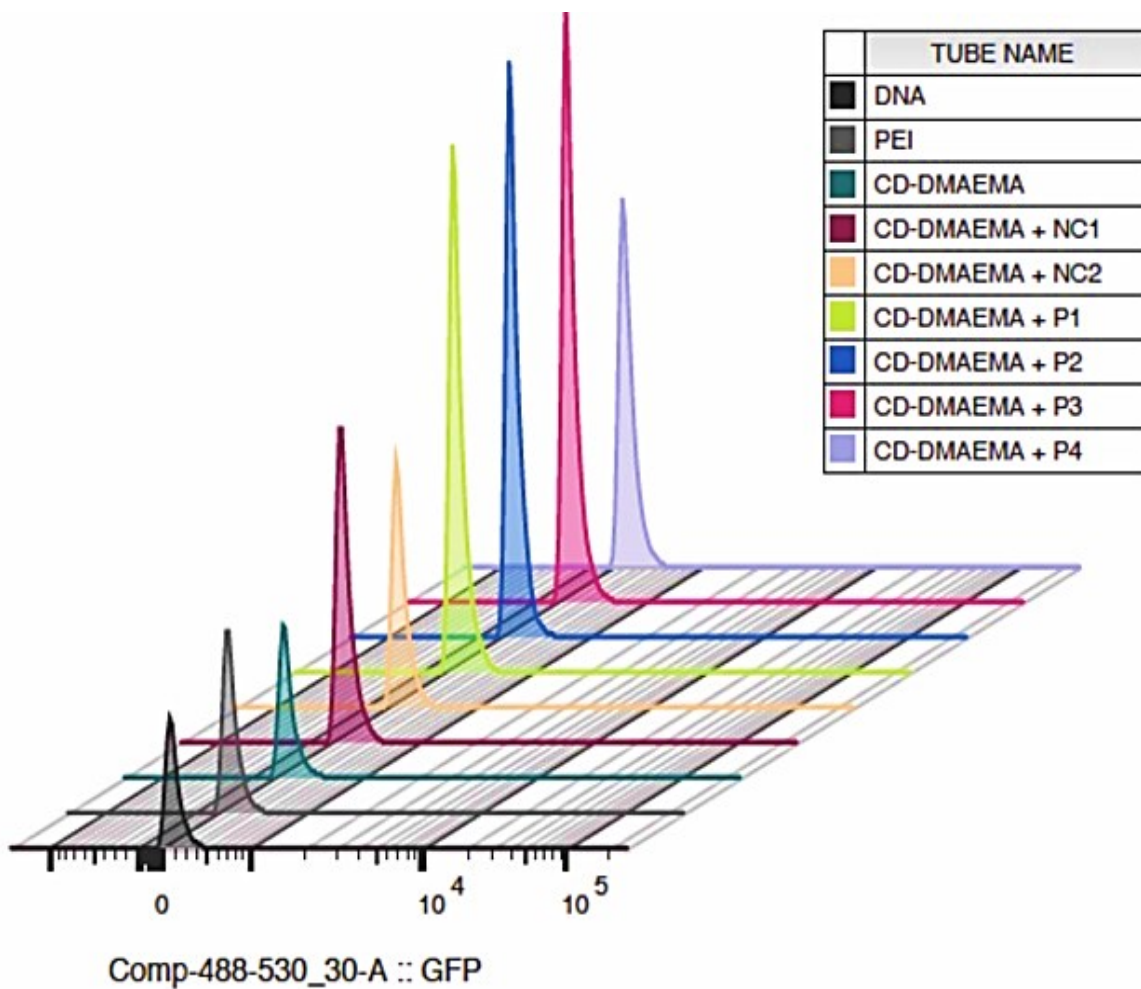


Figure S8. Histogram of number of cells expressing GFP for each formulation in human skin cells after intradermal injection with DMAEMA-CD-Ad-Glu/DNA polyplexes as determined by flow cytometry.

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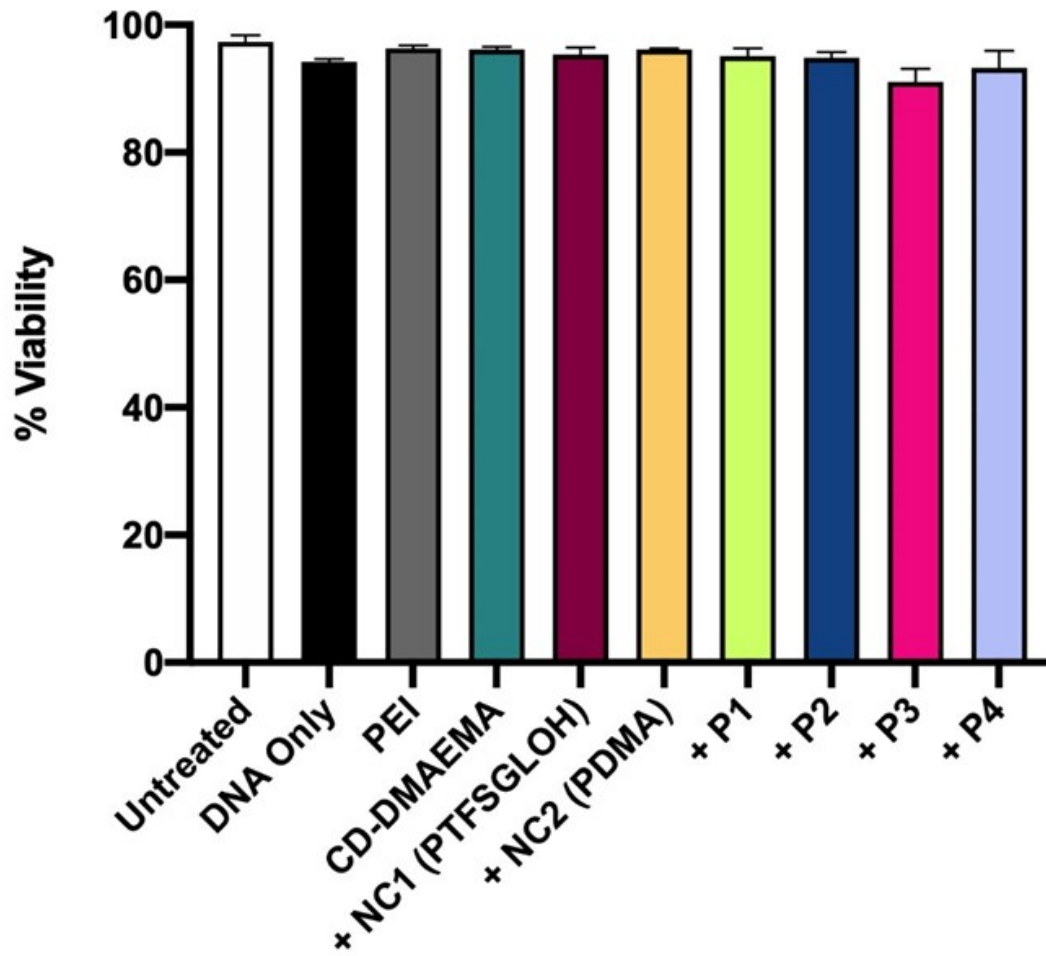


Figure S9. Viabilities of human skin cells after intradermal injection with DMAEMA-CD-Ad-Glu/DNA polyplexes as determined by flow cytometry. Bars represent mean \pm standard deviation for n=3.

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Supplementary Table 1. Antibodies use in flow cytometry.

Antibody	Labelling	Clone	Vendor	Test Volume (μL)
CD1a	PerCP-eFluor 710	HI149	Biolegend	5
CD3	eFluor 450	OKT3	Biolegend	5
CD14	Qdot 605	Tuk4	Biolegend	1
CD11c	PE	3.9	Biolegend	5
CD45	Alexafluor 700	2D1	Biolegend	2
CD56	PE-Cy5	CMSSB	Biolegend	5
CD90	PE-Cy7	5E10	Biolegend	5
CD19	BV 650	HIB19	Biolegend	5

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2. Nakano, Y.; Sato, E.; Matsumoto, A., Synthesis and thermal, optical, and mechanical properties of sequence-controlled poly (1-adamantyl acrylate)-block-poly (n-butyl acrylate) containing polar side group. *Journal of Polymer Science Part A: Polymer Chemistry* 2014, 52 (20), 2899-2910.