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Microfluidic Assembly of Cationic-β-Cyclodextrin:Hyaluronic Acid-Adamantane Host:Guest pDNA Nanoparticles

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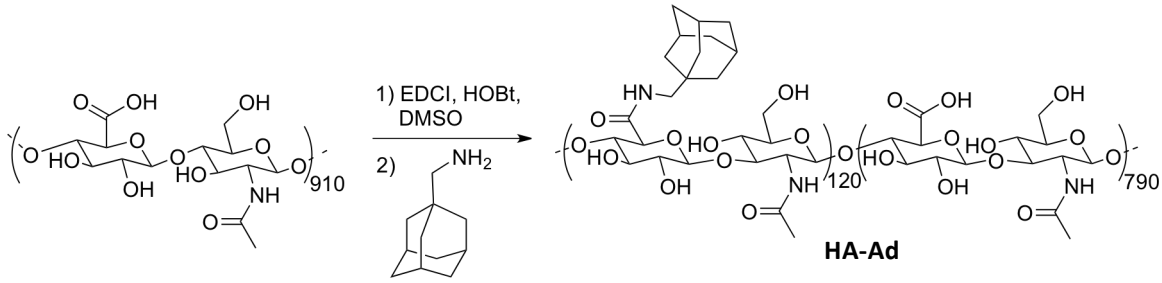
Supporting information

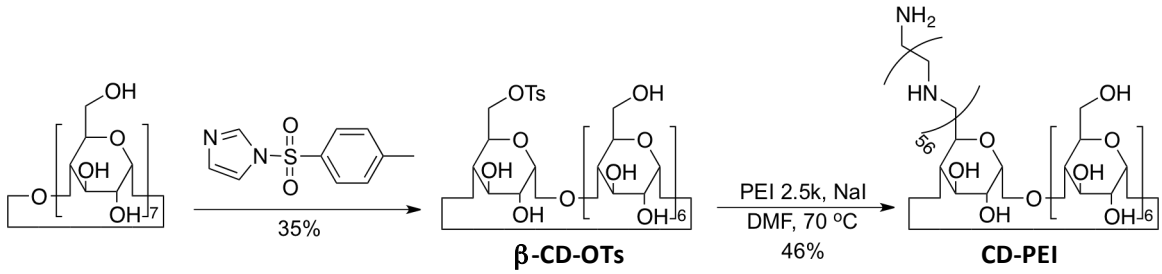
Materials. All solvents were of reagent grade, purchased from commercial sources, and used without further purification, except DMF and toluene, which were dried over CaH_2 under N_2, filtered, and distilled under reduced pressure. β-CD was obtained from Sigma-Aldrich. PEI2.5k was purchased from Polysciences, Inc. Plasmid purification kits were purchased from Qiagen. Dialysis membranes of MWCO = 6000 – 8000 were purchased from Fisher Scientific. ^{1}H NMR spectra were recorded on a 300 MHz VARIAN INOVA 300 NMR spectrometer at 30 °C. Chemical shifts were referenced to the residual protonated solvent peak.

Synthesis of HA-Ad. Hyaluronic acid (Avg MW = 350 kDa) (200 mg, 0.571 μmol) was dissolved in 60 mL dry DMSO at 80 °C. Once dissolved, EDC•HCl (824.6 mg, 4.34 mmol), HOBt (21 mg, 0.156 mmol), and adamantane methylamine (125 uL, 0.751 mmol) were added, and the reaction mixture was stirred at 80 °C overnight. The crude mixture was then loaded into a dialysis bag (FisherBrand, 6000-8000 MWCO), dialyzed against DMSO and H_2O for 2 d each, and then lyophilized. Yield: 222 mg. Adamantane groups: 1 Ad per ~6.76 disaccharides, ~134.66 units, ~14.8% of HA-COOH groups (loading). Approximate MW by NMR: ~365 kDa. ^{1}H NMR (300 MHz, D_2O, δ): 4.63-4.24 (b, C1H acetal), 3.98-2.94 (m, OH and core sugar H's), 2.01-1.72 (m, methamide CH_3, Ad), 1.67-1.48 (b, Ad).



Scheme S1. Synthesis route for the preparation of HA-Ad.

Synthesis of Mono-6-(p-toluenesulfonyl)-6-deoxy-β-cyclodextrin (β-CD-OTs). β-cyclodextrin (35.0 g, 30.8 mmol) was dissolved in 350 mL H_2O with 1-(p-toluenesulfonyl)imidazole (8.9 g, 40.0 mmol) and stirred at room temperature for 4 h. NaOH (50 mL, 20% w/v) was then added to the mixture and stirred for 10 min, inducing a



Scheme S2. Synthesis route for the preparation of CD-PEI.

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precipitate. The precipitate was removed by filtration and the filtrate was collected, then neutralized to pH 7 with ~25 g NH_4Cl to form another precipitate. This precipitate was collected by filtration and washed with 100 mL of H_2O three times and 100 mL of acetone twice and dried overnight under vacuum. Since both mono- and di-tosylate forms existed along with unreacted β-CD, an HP20 column was run. The mixture was loaded in bulk water and eluted with water until no more unmodified β-CD was detected, at which point methanol was used as eluent and fractions were collected. Fractions were confirmed via TLC using 3:2:1:1 isopropanol:H_2O:EtOAc:30% NH_4OH as mobile phase with visualization by acid stain (20% H_2SO_4). Yield: 10.2 g (29.1%). ^{1}H NMR (300 MHz, DMSO-d_6, δ): 7.80-7.66 (d, 2H, S-Ph), 7.50-7.33 (d, 2H, Ph-CH_3), 5.93-5.48 (b, 14H, OH on C2, C3 of CD), 4.87-4.70 (s, 7H, C1H of CD), 4.63-4.08 (b, 6H, OH on C6 of CD), 3.75-3.43 (m, 28H, C2H, C3H, C4H, and C5H of CD, overlap with HDO), 3.43-3.11 (m, 14H, C6H of CD), 2.43-2.34 (s, 3H, CH_3 on OTs).

Synthesis of PEI2.5k-ß-CD. Linear polyethyleneimine (2.5 kDa; 300 mg, 0.12 mmol) was dissolved in 15 mL dry DMSO at 75 °C under Ar. Once dissolved, ß-CD-OTs (1.53 g, 1.187 mmol) and NaI (100 mg, 0.667 mmol) were added and the solution stirred for 2 d. The crude reaction mixture was then loaded into a dialysis bag (FisherBrand, 3500 MWCO) and dialyzed against DMSO and H_2O for 2 d each, followed by lyophilization. Yield: 202 mg (46.5%). ß-CD loading: ~1.0 ß-CD/PEI, 50% loading (primary amines). Approximate MW by NMR: 3.64 kDa. ^{1}H NMR (300 MHz, DMSO-d_6, δ): 4.90-4.76 (s, C1H of ß-CD), 3.79-3.12 (b, C2-6H, PEI NH, ß-CD-OH), 2.87-2.52 (PEI-CH2).

Flow Mixing Protocol. The Chemtrix microreactor was procured from Chemtrix BV, UK. The components were introduced into the microreactor via Labtrix syringe pumps. Gas tight syringes (1 mL volume) were used for the components. The polymer and CD solutions were made at a concentration of 0.1 mM and the pDNA solution was at a concentration of 0.1 µg/µL (solutions were prepared in nanopure sterile filtered water). The components were drawn into the syringes ensuring no air bubbles were formed and used for the experiments. Flow rates of 5 µL/min, 10 µL/min and 20 µL/min for the pDNA were used for the assembly. The flow rates of the CD and HA-Ad streams were adjusted accordingly to afford the N/P ratios. For example 5.3 µL/min was used for an N/P ratio of 10 for the CD-PEI and HA-Ad when the flow rate of the pDNA was 5 µL/min. At this rate, the flow mixing was performed and solution from the output channel was collected for 2 min. This affords particles with 1 µg pDNA at an N/P ratio of 10. A similar protocol was followed for higher flow rates as well, wherein, for 10 µL/min flow rate of pDNA, the solution from the output channel was collected for 1 min and for 20 µL/min flow rate, the solution was collected for 30 sec. The final particle solutions were collected in autoclaved 1.5 mL microcentrifuge tubes. These solutions were then diluted in either nanopure water or media depending on the experimental plan.

Particle Size and Zeta Potential Measurements. The sizes, size distributions and ζ-potentials of the materials were evaluated by dynamic light scattering using a particle size analyzer (Zetasizer Nano S, Malvern Instruments Ltd.) with a scattering angle of 90°. All the samples were diluted in sterile filtered nanopure water up to 1 mL before analysis.

Atomic Force Microscopy Analysis. AFM imaging of the nanoparticles was conducted in tapping mode (MultiMode, Veeco, USA) using dry samples on mica. The AFM tips (PPP-NCH, Nanoscience Instruments, Inc., USA) had a typical radius of 7 nm or less, force constant of 46 N/m and the images were recorded with a scan rate of 0.5 or 1 Hz. Samples were prepared by dropping 2 mL of solution on a mica surface followed by overnight drying at 20 °C.

Gel Retardation Assay. The complexation ability of the flow mixed and bulk mixed complexes were determined by 1% agarose (low melting point) gel electrophoresis. The agarose gels were cast in TBE buffer with GelRed dye at 1:10,000 dilution. Flow mixed and bulk mixed complexes containing 0.2 µg of pDNA at different N/P ratios were loaded onto the gel. A 1:5 dilution of loading dye was added to each well and electrophoresis was carried out at a constant voltage of 55 V for 1 h in TBE buffer. The pDNA bands were then visualized under a UV transilluminator at a wavelength of 365 nm.

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PicoGreen Competitive Binding Assay. The polyplex stability was studied by PicoGreen competition assay. PicoGreen reagent is a fluorophore that selectively binds dsDNA. When bound to dsDNA, fluorescence enhancement of PicoGreen is exceptionally high; little background occurs since the unbound dye has virtually no fluorescence. Increasing amounts of heparin are added to the different polyplexes to study their disassembly in presence of a negatively charged polymer. When challenged with heparin, decomplexed dsDNA binds to PicoGreen. Consequently, the increase of fluorescence serves an indication of the level of decomplexation between DNA and polymers. In other words, highly packed nanocomplexes or highly condensed DNA is expected to have low fluorescence signal. Increasing amounts of heparin is added to the complexes and allowed to incubate for 30 min followed by addition of the Quant-iT PicoGreen reagent (Invitrogen, Carlsbad, CA) and further incubation for 15 min. The fluorescence was measured in a 96-well plate using a platereader under excitation maximum at 480 nm and emission peak at 520 nm. The fluorescence intensity was then corrected by background fluorescence. Results from three independent triplicate experiments were analyzed.

Cell Viability Assay. The cytotoxicity of the HA-Ad and CD using bPEI (25 kDa) as a benchmark was evaluated using the MTS assay in HeLa cells. The relative cell viabilities were measured as a function of amine densities of the CD and bPEI. The cells were cultured in complete DMEM medium supplemented with 10% FBS at 37 °C, 5% CO_2, and 95% relative humidity. The cells were seeded in a 96-well microtiterplate (Nunc, Wiesbaden, Germany) at densities of 7,500 cells/well. After 24 h, culture media were replaced with serum-free culture media containing increasing amine concentrations of bPEI, HA-Ad:CD, and CD and the cells were incubated for 24 h. After 24h, 15µL of MTS reagent was added to each well and incubated for 2 h. Following the incubation period, the absorbance was measured using a microplate reader (Spectra Plus, TECAN) at a wavelength of 492 nm. The relative cell viability (%) related to control cells cultured in media without polymers was calculated with $[A]_{test}/[A]_{control} \times 100\%$, where $[A]_{test}$ is the absorbance of the wells with polymers and $[A]_{control}$ is the absorbance of the control wells. All experiments were conducted for three samples and averaged. The median lethal dose (LD_{50}) is the dose of a toxic material that kills half (50%) of the cells tested. In this study, LD_{50} was the concentration of the carrier at which the relative cell viability decreased to 50%.

In Vitro Transfection/Cell Viability Experiment. HeLa cells were cultured in complete DMEM medium supplemented with 10% FBS at 37 °C, 5% CO_2, and 95% relative humidity respectively at a cell density of 60,000 cells/well in 24 well plates. After 24 h, the culture media was replaced with serum-supplemented media containing the bulk mixed and flow mixed complexes containing 1 µg AcGFP1 pDNA. The cells were incubated with the complexes for 4 h, after which the spent media was aspirated and fresh serum-supplemented media was added. After a further 36 h incubation, the media was aspirated and the cells were washed with PBS, trypsinized and added to FACS analysis tubes. SYTOX 7AAD dead cell stain (1 µL) was added to each sample and incubated on ice for 15 min before analysis by FACS. FL1 channel was used for the GFP fluorescence and FL4 for the 7AAD fluorescence. %GFP mean fluorescence intensity was calculated relative to bPEI, which was considered as 100%.

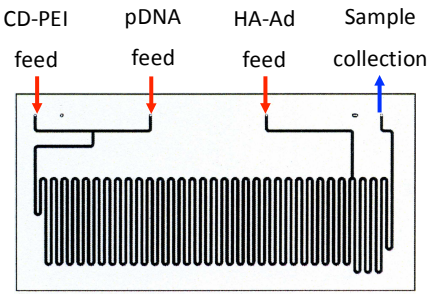

Figure S1. Chemtrix microreactor design used for microfluidic assembly of CD-PEI:pDNA:HA-Ad nanocomplexes. The glass reactor volume is 10 µL and the reactor length is 60 µm.

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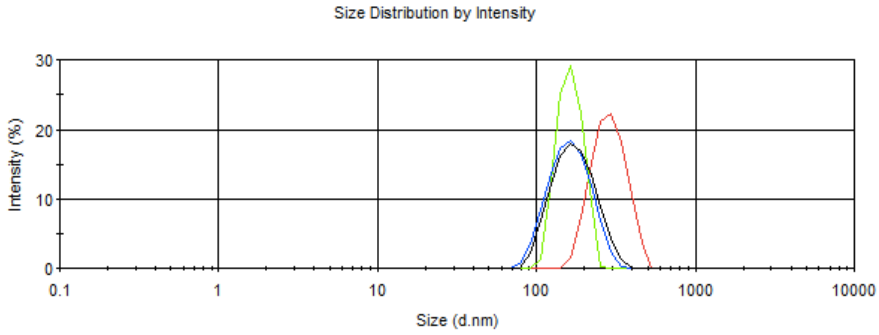

Figure S2. Size distributions by intensity of nanocomplexes produced by (red) bulk mixing and flow mixing at pDNA flow rates of (black) 5, (blue) 10 and (green) 20 µL/min.

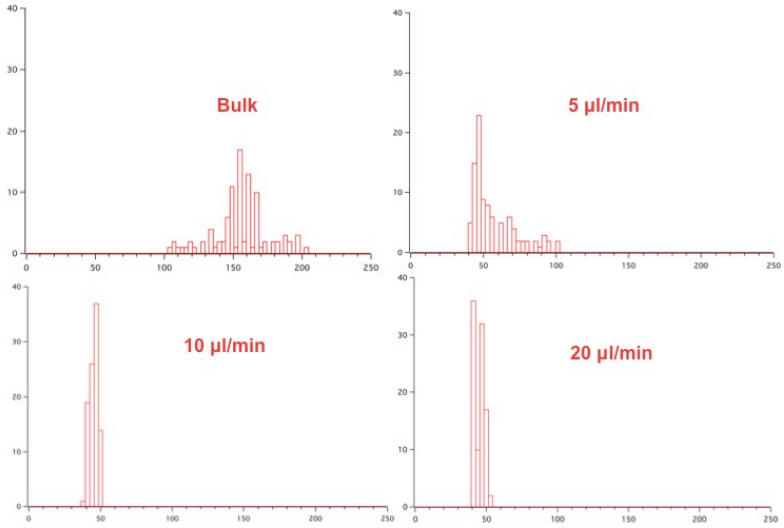

Figure S3. AFM size distributions of nanocomplexes produced by bulk mixing and flow mixing at pDNA flow rates of 5, 10 and 20 µL/min.

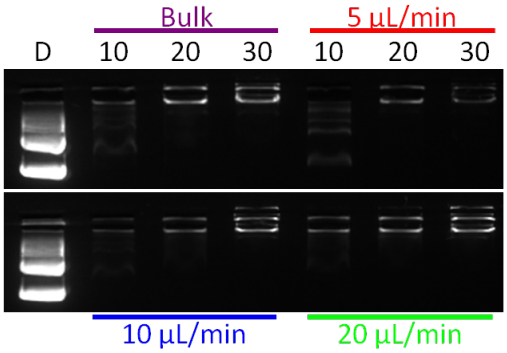

Figure S4. Gel shift assay showing complexation profiles of bulk mixed and flow mixed complexes.

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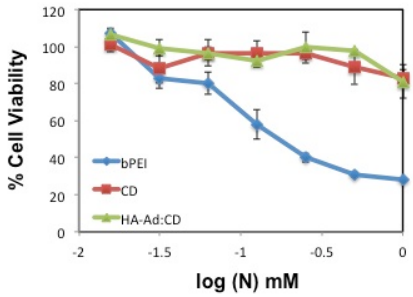

Figure S5. MTS cell viability assay showing relative cell viabilities of CD-PEI, HA-Ad:CD-PEI complexes and bPEI in HeLa cells after a 24h incubation.

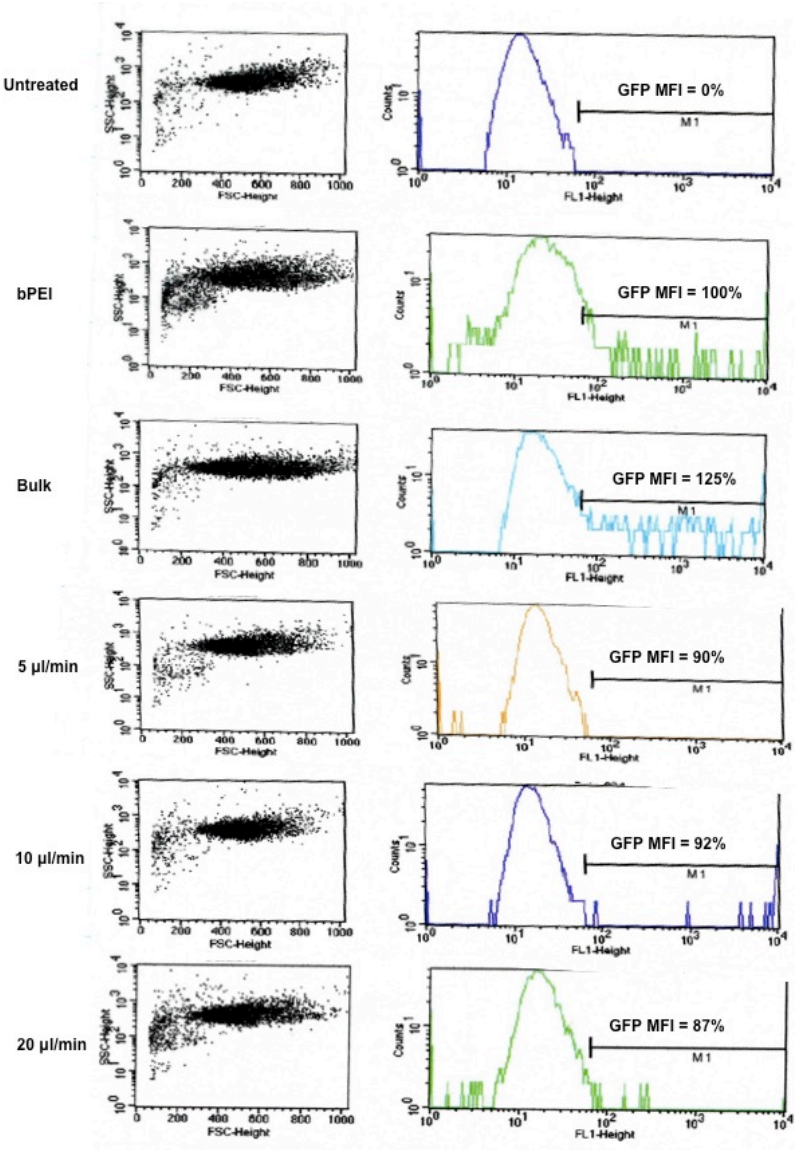

Figure S6. Representative FACS dot and histogram plots of AcGFP1 transfection by bPEI, bulk and flow mixed complexes formulated at N/P = 20. Complexes of 1µg AcGFP1 pDNA were incubated with the cells in 10% serum-supplemented media for 4 h and analyzed after a further 36 h incubation.

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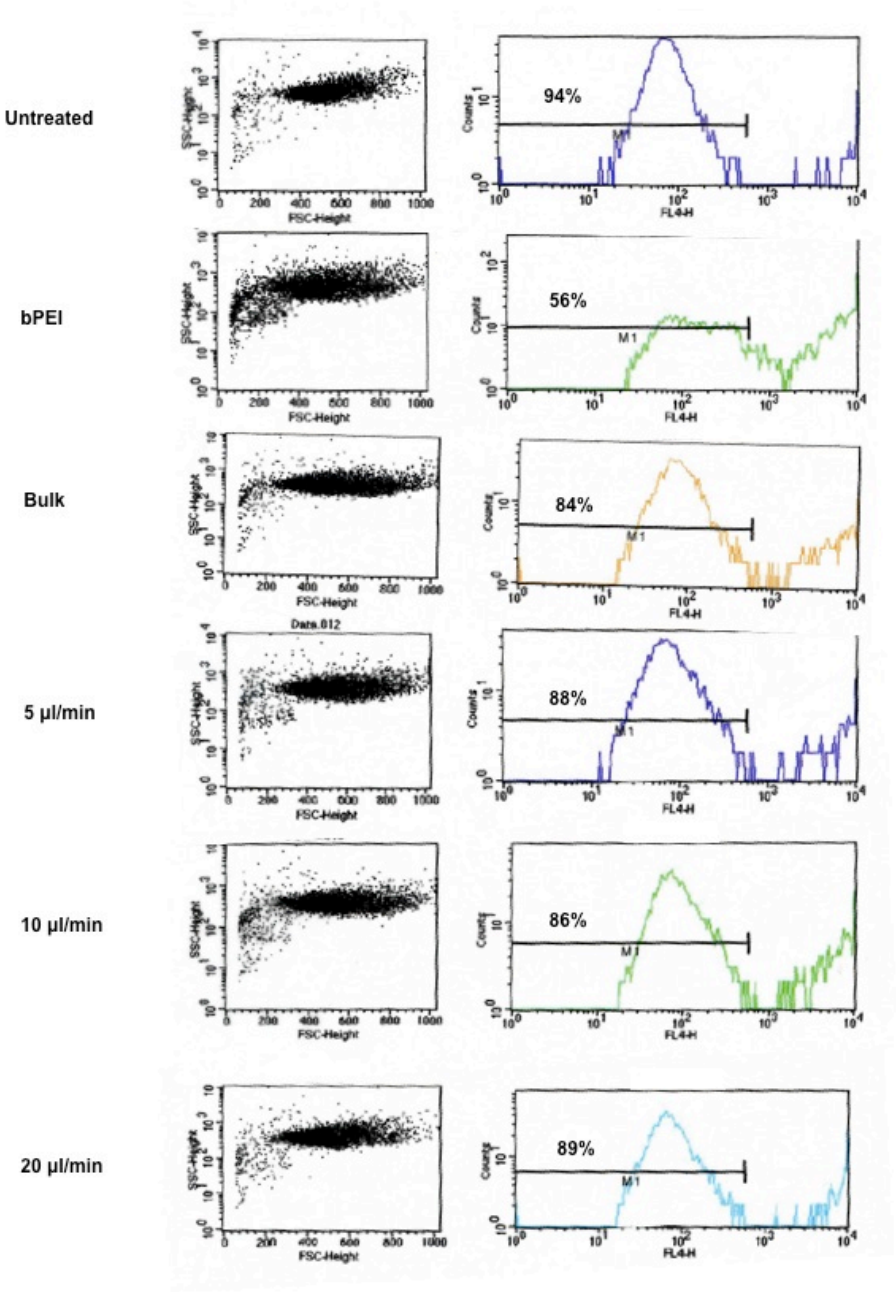

Figure S7. Representative FACS dot and histogram plots of 7AAD labeling of cells treated with bulk and flow mixed complexes formulated at N/P = 20. Complexes of 1 µg AcGFP1 pDNA were incubated with the cells in 10% serum-supplemented media for 4 h and analyzed after a further 36 h incubation.