SUPPLEMETARY MATERIAL

Materials & Methods

Materials. Branched PEI (25 and 1.8 kDa), PLL hydrobromide (4 and 25 kDa), *N*-hydroxysuccinimide ester of palmitic acid, 5% (w/v) 2,4,6-trinitrobenzosulfonic acid (TNBS), trypsin/EDTA, and Hanks' Balanced Salt Solution (HBSS) were obtained from SIGMA (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM; high glucose with L-glutamine), Penicillin (10,000 U/mL), Streptomycin (10,000 μg/mL) and Lipofectamine-2000^{TM} were from Invitrogen (Carlsbad, CA). FUGENE^{TM} was obtained form Roche Diagnostics (Montreal, QC, Canada) and linear PEIs were from Polysciences (Warrington, PA). Fetal Bovine Serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). A 4.7 kb plasmid incorporating an enhanced green fluorescent protein (pEGFP-N2) and a kanamycin resistance gene was obtained from BD Biosciences (Mountain View, CA), and replicated in kanamycin resistant DH5-□*Ecoli* strain grown in Luria-Bertani medium [11]. The purified plasmid was dissolved in ddH_2O at 0.4 mg/mL. CRL human skin fibroblasts were obtained from a cell bank maintained at the Alberta Cross Cancer Institute (Edmonton, Alberta).

Atomic Force Microscopy (AFM). The MultiMode scanning probe microscope (Digital Instruments Inc., Santa Barbara, CA) was used for all AFM studies. A large-area scanner (J type) with a maximum *xy* scan range of 125 x 125 μm and a *z* vertical range of 5 μm was used, except for imaging naked plasmid DNA, where a small-area scanner (A type) with a maximum *xy* scan range of 0.4 x 0.4 μm and a *z* vertical range of 0.4 μm was used for higher resolution. Single crystal silicon cantilevers were cleaned by exposure to high intensity UV light for 3 minutes before use. The oscillation amplitude of the scanning tip was registered at 0.5 V and the frequency of the

oscillation was in the range of 200-400 kHz. All AFM imaging was conventional ambient tapping mode AFM. The scan rate was typically 1.0-2.0 Hz and the data collection was at 512 x 512 pixels. Images were processed and analyzed using the Nanoscope III software (V5.12).

To visualize pEGFP-N2/polymer complexes, 2 μL of pEGFP-N2 solution (0.02 mg/mL in ddH_2O), 2 μL of polymer solution (0.02 mg/mL in ddH_2O) and 2 μL of 9 mM NaCl solution were incubated for 30 minutes, and 3 μL of this solution was transferred to a freshly cleaved mica surface. After drying at room temperature for 30 minutes, the surface was blow-dried with N_2 (if necessary). Naked plasmid was visualized as above except without addition to any polymer to the sample. The chosen pEGFP-N2:polymer ratios were based on agarose gel-electrophoresis analysis of the complexes, which corresponded to ratios for complete plasmid complexation. The samples were then imaged under tapping mode as described above.

Cellular Uptake of Polymers. The PEI (25 kDa) and PLL (25 kDa) solutions (2 mg/mL) were labeled with 1 mM fluorescein isothiocyanide (FITC) according to the manufacturer's directions (PIERCE; Rockford, IL), dialyzed against 0.1 M phosphate buffer (MW cut-off: 12-14 kDa), and then against ddH_2O to remove the unreacted FITC. TNBS assay was then used to determine the polymer concentrations in the dialyzed samples [11]. FITC-labeled PLL and PEI (1-9 μg/mL) were incubated with fibroblasts grown in 6-well plates (in 2 mL basic medium/well; in triplicate) for ~24 hours. After removing the polymer-containing media, the cells were washed with HBSS, trypsinized, centrifuged, and suspended in HBSS with 3.7% formalin for flow cytometric analysis. The instrument threshold for the negative control sample (i.e., cells incubated with no polymers) was set-up at ~1% level. The percentage of cells exhibiting FITC-fluorescence beyond this threshold value was determined as a function of polymer concentration in the medium. The average

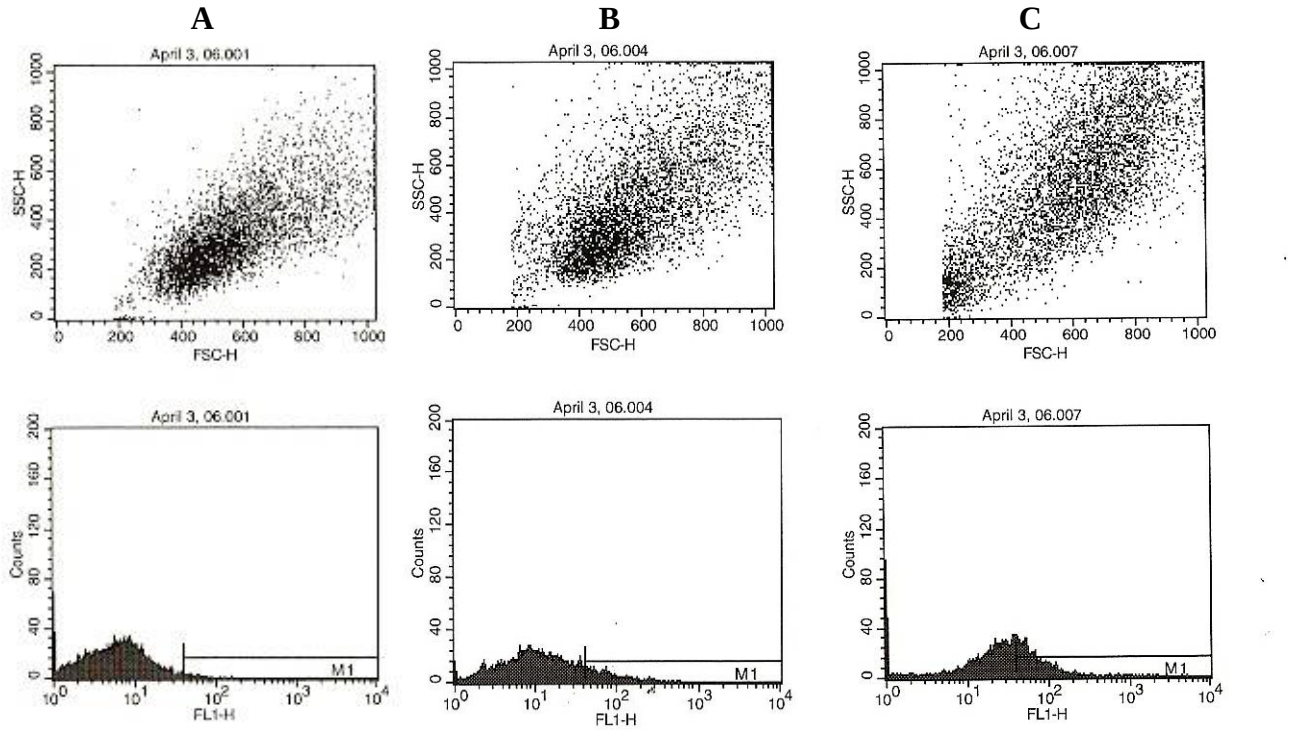
level of fluorescence in this FITC-positive population was used as a relative measure of polymer uptake.

Cellular Uptake of pEGFP-N2. For assessment of pEGFP-N2 uptake, 20 μg of pEGFP-N2 in 300 μL ddH_2O was incubated with 100 μL solution of a succinimide ester of Cy5.5-NHS (1 mg dissolved in 300 μL DMSO; AMERSHAM, St. Laurence, QC), and allowed to react for 2 hours at room temperature. The reaction was stopped by extensive dialysis against TBE buffer. 5 μL of the labeled plasmid solution was mixed with 10 μL of 1 mg/mL polymer solution (PEI, PLL, PLL-PA, and linear PEI) or 6 μL of Lipofecatmine-2000^{TM} or Fugene^{TM}. The solution also contained 150 mM NaCl, and incubated for 30 min at 37 ^{o}C before addition to the cells in 6-well plates (2 mL basic medium/well; in triplicate) for 20 hours. The chosen carrier amounts were based on our previous experience on optimized ratios for each carrier:DNA combination. After removing the pEGFP-N2-containing media, the cells were washed with HBSS, trypsinized, centrifuged, and suspended in HBSS with 3.7% formalin for flow cytometric analysis (λ_{ex}=690 nm, λ_{em}=705 nm). The instrument was calibrated so that the negative control sample (i.e., pEGFP-N2 without any polymeric carrier) gave ~1% cell uptake. The percentage of cells exhibiting Cy5.5-fluorescence beyond this threshold value was determined.

Transfection with pEGFP-N2. The cells used for transfections were grown (in triplicate) in 6-well plates or on glass coverslips to assess transfections by using flow cytometry and epifluorescent microscopy, respectively. The concentrations of all reagents and incubation volumes/times used in this study were optimized based on previously reported studies [10,11]. The polymer/pEGFP-N2 polyplexes used for transfections were prepared by mixing 0.4 mg/mL pEGFP-N2 (in ddH_2O) with

1 mg/mL polymer solutions (in ddH_2O), and bringing the volume to 50 μL with 150 mM NaCl. The polyplexes for each transfection contained 3 μg of pEGFP-N2, and 18 μg of polymer (either PLL or PEI) and added to cells grown in 2 mL medium. After 30 minute of complex formation, the polyplexes were added to the cells grown on 6-well plates. A similar procedure was used with Lipofectamine-2000^{TM}, where the volume of the lipid used was 18 μL. The source/methods for adenoviral transfections were reported previously [11]. The cells were incubated for 24 hours with the transfection reagents, after which the cells were either trypsinized for assessment of EGFP expression, or the medium was replaced with fresh medium for longer time cultures. Flow cytometry was performed on a BD FACscan where the cell fluorescence was detected by □ex= 485 nm and □em = 527 nm for EGFP expression. The instrument settings were calibrated for each run so as to obtain a background level of EGFP expression of ~1% for control samples (i.e., cells incubated with pEGFP-N2 alone without any carrier). An aliquot of the cell suspension used for flow cytometry was manually counted with a hemocytometer to obtain total number of cells recovered from the wells. The cells grown on coverslips were also tranfected in a similar manner, but they were fixed at desired time points, the nucleus stained with Hoecsht 33258 (15 min at 1 □g/mL) and analysed under an epifluorescent microscope. The numbers of GFP-positive cells (based on green cytoplasmic fluorescence) were quantified as a percentage of total number of cells (based on blue nuclear fluorescence) on a coverslip.

Typical Data from Flow Cytometry Analysis



The cytometry data is for cells on day 6 in Figure 4B.

A. Cells incubated with pEGFP-N2 without any carrier (1.0% EGFP-positive cells).

B. Cells incubated with PLL-PA/pEGFP-N2 complexes (12.9% EGFP-positive cells).

C. Cells incubated with PEI/pEGFP-N2 complexes (35.9 % EGFP-positive cells). Note the shift in cell population towards the origin, indicating a larger fraction of smaller, possibly fragmented cells.