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Delivery of Polyethylenimine/DNA Complexes Assembled in a Microfluidics Device

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Abstract: Polyethylenimine (PEI) and plasmid DNA (pDNA) complexes (PEI/pDNA) are nonviral vectors for gene delivery. The conventional method for producing these complexes involves bulk mixing (BM) of PEI and DNA followed by vortexing which at low N/P ratios results in large particle size distribution, low cytotoxicity, and poor gene transfection, while at high N/P ratios it results in small particle size and better gene transfection but high cytotoxicity. To improve size control, gene transfection efficiency, and cytotoxicity, in this study, we used a microfluidic hydrodynamic focusing (MF) device to prepare PEI/pDNA complexes at N/P = 3.3 and 6.7. We used bulk mixing as control, mouse NIH 3T3 fibroblast cells and mouse embryonic stem (mES) cells as model cell lines, plasmid encoding green fluorescent protein (pGFP) and secreted alkaline phosphatase (pSEAP) as the reporter gene, and commercially available Lipofectamine 2000 as a positive control. The complexes were characterized by atomic force microscopy (AFM), dynamic light scattering (DLS), and zeta potential (ζ) measurement. Confocal laser scanning microscopy (CLSM) and fluorescent labeling techniques were used to visualize the complex size distribution, complexation uniformity, and cellular distribution. The results showed that MF produced complexes were smaller and more uniformly complexed and had higher cell viability and improved exogenous gene expression.

Keywords: Polyethylenimine; plasmid DNA; gene delivery; microfluidic device

1. Introduction

Viral vectors are highly efficient gene delivery systems but suffer from high cytotoxicity and immunogenicity.[1−4] Over the decade, nonviral vectors have received increasing attention as they exhibit low toxicity and immunogenicity.[4,5] Polyethylenimine (PEI), one of the most effective gene delivery polymers studied to date, has been used as a gene delivery vector since 1995[6] to deliver nucleic acids including DNA,[6] siRNA,[7,8] and oligonucleotides.[6,9] When solutions of DNA and PEI are mixed, the complexation by electrostatic

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interactions between the negatively charged phosphate groups of DNA and the positively charged amine groups of PEI condenses the PEI/DNA conjugates into spherical, globular or rodlike nanostructures.[10] The good gene transfection efficiency of PEI is hypothesized to be a result of the proton sponge mechanism.[6] Therefore, PEI can mediate gene delivery efficiently in the absence of any exogenous endosomolytic agent.

The current method of preparing PEI/DNA complexes is a bulk mixing (BM) process which involves the mixing of the two solutions followed by brief vortexing. This results in spontaneous self-assembly into nanostructures. The order of adding reagents greatly influences the resulting particle size and properties: adding the cationic polymer solution to the plasmid solution was 10-fold more efficient than adding plasmid to polymer.[6,11] At N/P (the molar ratio of nitrogen in PEI to phosphate in DNA) ratios near to 3.3, DNA condenses poorly so the particles tend to be large and polydispersed, resulting in poor transfection efficiency but low cytotoxicity. Increasing the N/P ratios generally led to smaller particles and better transfection; however, a high N/P ratio, i.e. N/P > 6, results in a large amount of free PEI (>80%), causing high cytotoxicity.[12,13] Therefore, it is essential to develop better methods to produce complexes at low N/P ratios with uniform size distribution, well-defined structures and compositions, and lower cytotoxicity for more efficient and consistent in vitro and in vivo gene transfection. In this paper, we describe a microfluidic hydrodynamic focusing (MF) device to produce PEI/pDNA complexes.

Microfluidics technology has been used extensively to control and manipulate fluid flows at the micrometer scale. Due to the small channel dimensions and typically low volumetric flow rates, microfluidic devices generally operate at Reynolds number less than one in which the flow is strictly laminar.[14] A microfluidic device which uses the concept of hydrodynamic focusing usually consists of three inlets and one outlet, where the middle inlet (or sample fluid) is squeezed or "hydrodynamically focused" by the other two inlets (or sheath/side fluids) into a narrow stream where molecules between the streams can interdiffuse rapidly thereby reducing mixing time to a few microseconds.[15] Microfluidic hydrodynamic focusing devices have been used for the self-assembly of liposomes without DNA[16,17] and the compaction of calf thymus DNA by cationic lipids[18] and dendrimer.[19] However, none of the above publications have studied the compaction efficiency of pDNA by PEI in addition to evaluating the pDNA structural integrity, transfection efficiency, and cell viability of hydrodynamic focusing assembled PEI/pDNA complexes.

The purposes of this study are to demonstrate that the MF method, at N/P ratios of 3.3 and 6.7, can provide better complexation/condensation of pDNA by PEI and enhanced in vitro gene expression but lower cytotoxicity in NIH 3T3 fibroblast cells and mES cells as compared to BM method.

2. Materials and Methods

2.1. Microfluidic Device Fabrication. Plastic microfluidic devices were fabricated using a modified microfabrication protocol described elsewhere.[20] A high precision computer numerically controlled (CNC) machine (Aerotech, Inc.) was used to fabricate polymeric microfluidic hydrodynamic

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focusing devices on poly(methyl methacrylate) (PMMA) plate. The channel widths were varied by using the appropriate end mill sizes. A PMMA plate was used as the substrate, and a 45 μm thick PMMA film was thermally laminated to form the closed channels by passing the PMMA/film sandwich through a thermal laminator (Catena 35, GBC, Inc.). Prior to thermal bonding, the microchannels were gently brushed to remove any debris and then the PMMA plates were sonicated in isopropyl alcohol (IPA)/DI H_2O (1:10) for 5−10 min to remove grease and then blown dry. Fluidic connectors (Value plastics, Inc.) were bonded onto the PMMA plate by applying a UV curing adhesive around the perimeter of the connectors. The connectors were aligned over the inlet/outlet openings, and the adhesive was cured by exposure to UV irradiation (Novacure 2100, EFXO Corp., Quebec, Canada) for 10 s. The assembled devices were sterilized overnight under UV light in a cell culture hood prior to experimentation.

For flow visualization, the MF device was mounted on an inverted microscope stage (Nikon Eclipse 2000U) with a 10× Nikon Plan Fluoro objective. A programmable syringe pump (Pump 33, Harvard Apparatus, Holliston, MA) was used to control the fluid flow rates independently. The device consists of two inlets and one outlet. The inlets are each connected to PEI and pDNA solution independently. Typically, the PEI streams would be injected first and then the pDNA stream. After the pDNA stream has entered and the hydrodynamic focusing has been established, the products in the outlet stream would be collected after 3−5 min to allow for steady state. The magnitude of the shear stresses applied to the pDNA was controlled by altering the ratio of the flow rate in the side streams to the middle stream.

During this study, the same or similar time points and settings were used for characterization (DLS and zeta potential measurements, AFM, and CLSM), transfection expression studies, and cell viability since the BM and MF samples were prepared separately.

2.2. Plasmids and Polycation. gWiz reporter plasmids encoding the green fluorescent protein (pGFP, 5.7 kb) and secreted alkaline phosphatase (pSEAP, 6.6 kb) were purchased from Aldevron (Fargo, ND) and denoted as pDNA unless otherwise stated. Branched polyethylenimine (PEI) 25 kDa was purchased from Aldrich (St. Louis, MO). A sterile PEI solution (10 mM) was prepared by dissolving PEI in 150 mM NaCl (pH 7) solution and filtered through a 0.2 μm filter then stored at 4 °C until further use.

2.3. PEI-Rhodamine (PEI-Rh) Labeling. The labeling of polyethylenimine (PEI) with rhodamine (Rh) was achieved by the formation of a stable covalent amide bond between the amine-reactive rhodamine dye (excitation and emission wavelengths at 544 and 576 nm, respectively) and primary amines (NH_2) on the PEI. The EZ-Label Rhodamine Protein Labeling Kit (Pierce, Product No. 53002) was used which includes three steps: PEI dissolution, labeling reaction, and removal of excess dye. In a typical preparation, 5.0 mg of PEI (MW 25k) was dissolved in 0.5 mL of borate buffer (pH 8.5) to form a stock solution of 10.0 mg/mL. 100 μL of 10.0 mg/mL PEI solution was transferred into a 1 mL reaction tube. 0.5 mg of *N*-hydroxysuccinimide (NHS)-ester rhodamine (MW 527) was completely dissolved in 100 μL of dimethylformamide (DMF) and added into the PEI solution at a molar ratio of NHS rhodamine:PEI of 5:1. The mixture was allowed to react for 2 h at room temperature and protected from light. After the labeling reaction, the resulting solution was dialyzed in a Slide-A-Lyzer MINI Dialysis unit (molecular weight cutoff, MWCO = 3,500) against 500 mL of 1× PBS buffer (pH 7.4) for a period of 6−8 h with 3 changes of the dialysis buffer, to remove any free rhodamine not conjugated to the PEI.

2.4. Atomic Force Microscopy (AFM). AFM characterization of pGFP is as follows. Eight μg/mL of pGFP in DI water was prepared from a working solution (1 mg/mL). For MF, the pGFP/DI water solution (50 μg/mL) and DI water were injected at flow rates of 10 and 50 μL/min in the middle and side channels, respectively. Three samples—control pGFP, pGFP vortexed for 5 s, and pGFP undergone MF—were pipetted onto freshly cleaved mica separately. After allowing the pGFP to settle onto the mica for 3 min, the mica was blown dry with compressed air or nitrogen. Images were scanned on MFP-3D AFM (Asylum Research, Santa Barbara, CA) in the tapping mode operated in air.

AFM characterization of PEI/pGFP complexes is as follows. The BM method of preparing PEI/pGFP complexes involved the addition of equal volume of PEI (10 mM) in DI water into DNA (16 μg/mL) in DI water followed by vortexing briefly and incubating for 15−20 min. Then 5 μL of the mixture was pipetted onto freshly cleaved mica. After allowing the complexes to settle onto the mica for 3 min, the mica was blown dry with compressed air or nitrogen. For MF, pGFP (50 μg/mL) in DI water and PEI (10 mM) in DI water were injected at flow rates of 10 and 50 μL/min respectively. AFM samples were prepared and scanned as described above.

For BM or MF method of preparing polyplexes, at least four separate samples were prepared and inspected. Also, numerous locations were checked on each sample.

2.5. Dynamic Light Scattering (DLS) and Zeta Potential. The hydrodynamic diameters of the polymer/DNA complexes were determined by dynamic light scattering using a NiComp submicrometer particle analyzer (model 370, Santa Barbara, CA). Scattered light was detected at a 90° angle and at a temperature of 25 °C. The viscosity (0.88 mPa·s) and the refractive index (1.33) of distilled water at 25 °C were used for data analysis.

The zeta potential was determined by ZetaPALS (Brookhaven Instruments Corp., NY). Zeta-potential measurements were carried out in a standard rectangular cuvette where the sampling time was set to automatic and the average values were calculated with the data from five runs. The zeta potential was calculated from the measured electrophoretic mobility using the Smoluchowski approximation.

2.6. Agarose Gel Electrophoresis. Electrophoresis was used to determine the integrity of the plasmid before and after vortexing and MF (i.e., shear stressing). The pGFP

samples were mixed with gel-loading solution (Sigma) and loaded on a 1% ReadyAgarose gel plus ethidium bromide (Bio-Rad Laboratories, Hercules, CA). Electrophoresis was carried out at 120 V for 45 min in a 1× TAE running buffer (Invitrogen). A digital image of the gel was captured under UV light using ChemiDoc XRS system (Bio-Rad).

2.7. Confocal Laser Scanning Microscopy (CLSM) and Fluorescence Labeling. pDNA was labeled with YOYO-1 (Invitrogen) at 1 dye molecule per 100 bp and incubated in the dark for 2 h at room temperature and then stored at −20 °C until further use. PEI (25 kDa) was conjugated with rhodamine (Invitrogen). Polyplexes were formulated by BM and MF, and 50 μL of each was pipetted onto a glass coverslip and observed under a laser scanning confocal microscope (510 Meta CLSM, Zeiss, Germany) with excitation wavelengths of 488 and 568 nm for YOYO-1 and rhodamine, respectively. YOYO-1 was detected with a 515−540 nm band-pass filter, and rhodamine was detected with a 580−620 nm band-pass filter.

To visualize distribution of complexes in mES and NIH 3T3 cells, the cells were seeded on glass coverslips with and without gelatin, respectively, in a 24-well plate at a density of 5×10^3 cells/well 24 h prior to transfection. For mES cells, the glass coverslips were coated with 1% gelatin. Polyplexes were formulated by BM and MF methods before administering to cells. The procedure to fix and stain the cells is described elsewhere.[21] At selected time points after transfection, the cells were fixed with 4% paraformaldehyde−PBS for 1 h, permeabilized with 0.2% Triton X-100−PBS for 15 min with washing in between the steps and stained with 4′-6-diamidino-2-phenylindole (DAPI) to reveal the cells' nuclei. The cells were then sealed in ProLong Gold antifade reagent (Invitrogen). DAPI stained nuclei were detected with a 350 nm filter. CLSM was performed at The Ohio State University's Campus Microscopy & Imaging Facility.

2.8. Cell Culture. NIH 3T3 cells (mouse embryonic fibroblast cell line) were cultured in Dulbecco's modified Eagle's medium: Nutrient Mix F-12 (D-MEM/F-12) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), and 10% (v/v) newborn calf serum (NCS). Cells were maintained in 25 cm^2 T-flasks at 37 °C with 5% CO_2 and subcultured using 0.25% (w/v) trypsin with EDTA 4Na. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Mouse CCE ES cell line was a generous gift from StemCell Technologies, Inc. (Vancouver, Canada).[22,23] The mES cells were grown on gelatin-coated tissue culture flasks in a maintenance medium consisting of Dulbecco's modified Eagle's medium (DMEM with 4.5 g/L D-glucose), supplemented with 15% (v/v) FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.1 mM nonessential amino acids, 10 ng/mL murine recombinant leukemia inhibitory factor (LIF; StemCell Technologies, Vancouver, Canada), 0.1 mM monothioglycerol (Sigma), 2 mM L-glutamine, and 1 mM sodium pyruvate (Invitrogen).

2.9. Polyplex and Lipoplex Preparation. Polyplexes and lipoplexes were prepared by a bulk mixing (BM) method as follows. The desired amount of pDNA (1 μg of DNA is 3 nmol of phosphate), PEI stock solution (10 mM amine nitrogen in 150 mM sodium chloride (NaCl) pH 7), and Lipofectamine, as a positive control, were diluted separately to equal volumes with 150 mM NaCl, pH 7 and gently mixed followed by incubation for 5 min at room temperature. The entire diluted PEI solution and Lipofectamine were then added into the diluted DNA solutions, and the resulting solutions were either vortexed for 5−10 s or gently mixed to yield polyplexes or lipoplexes, respectively. Polyplexes were prepared by a microfluidic focusing (MF) method as follow. The pDNA solution (48 or 120 μg/mL) and PEI solution were injected at flow rates of 10 and 50 μL/min in the middle and side channels, respectively. The concentration of PEI used was based on 1.0 μL of PEI stock solution (10 mM amine nitrogen in NaCl) contains 10 nmol N. That is 10 μL/mL NaCl and 20 μL/mL NaCl multiplied by N/P ratio 3.3 and 6.7, respectively, was used in the side channel. The final pDNA concentrations in polyplexes prepared for NIH 3T3 and mES cells transfection were 8 and 20 μg/mL, respectively.

2.10. Cell Transfection. After incubating polyplexes and lipoplexes for 15−30 min at room temperature, the transfection mixtures were added to the cells. Immediately before transfection, the culture medium was removed and then 1 μg of pDNA in polyplexes or lipoplexes was added into each well. The transfection medium was replaced with 200 μL of fresh growth medium 6 h post transfection and grown at 37 °C and 5% CO_2 until the transfection efficiency was measured.

NIH 3T3 fibroblast cells were seeded in 48-well plates at 10,000 cells per well 24 h before transfection. The cells were grown in an incubator at 37 °C and 5% CO_2.

For mES cells, polyplexes and lipoplexes were mixed with equal volume of ES cell growth medium without serum and 2.5 μg of pDNA in polyplexes or lipoplexes was added into each well. For negative control, cells were treated with growth medium and not treated with complexes for 6 h and replaced fresh growth medium.

The polymer concentrations were calculated from the desired N/P ratio and the amount of pDNA, prepared assuming that 43.1 g/mol corresponded to each repeating unit of PEI containing one nitrogen atom and 330 g/mol corresponded to each repeating unit of DNA containing one phosphorus atom.[24]

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2.11. Detection of Green Fluorescent Protein (GFP) and Secreted Alkaline Phosphatase (SEAP) Expression. The expression of green fluorescent protein (GFP) was examined by visualizing the cells with green fluorescence under an inverted fluorescence microscope (TS100, Nikon) employing an FITC filter at 24, 48, 72, and 96 h post transfection, and similar imaging parameters were used.

The expression of secreted alkaline phosphatase (SEAP) was detected following the suggested protocol by the manufacturer. Briefly, at 2 and 4 days post transfection, endogenous alkaline phosphatase activity was inactivated by heating supernatants from transfected cells at 65 °C for 30 min. The SEAP activity was quantitatively determined by using a colorimetric assay based on hydrolysis of para-nitrophenyl phosphate (PNPP) purchased from Sigma (St. Louis, MO) on a 96-well plate. PNPP was added into each sample well and incubated at room temperature for 30−45 min prior reading at 405 nm on GENios Pro microtiter plate reader (Tecan).

2.12. Cell Viability. The cell viability was determined by MTT assay 48 h (mES cells) or 96 h (NIH 3T3 cells) post transfection as follows. MTT stock solution (5 mg/mL) is added to each well equal one tenth of the original culture volume and incubated for 3 to 4 h at 37 °C, 5% CO_2. Then the medium was removed and replaced with 200 μL of DMSO and further incubated for 15 min. The absorbance at 595 nm of the samples and control, OD595(sample) and OD595(control), respectively, were read on GENios Pro microtiter plate reader (Tecan). The percentage (%) of viable cells is calculated by the quotient of OD595(sample) to OD595(control) multiplied by 100%. Cells as negative control were not treated with complexes.

2.13. Statistical Analysis. Data were represented as mean ± standard deviation (SD) and analyzed by two-tailed Student's t test using JMP software (Cary, NC). $p \leq 0.05$ was considered statistically significant.

3. Results and Discussion

Most previous studies focused on N/P = 6 to 20 or higher. However, the toxicity increases as N/P ratios increase due to excess PEI which limits in vivo applicability. N/P = 3.3 is at equal molar. Some groups reported large polyplexes produced by the BM method. We focused on this low N/P ratio to demonstrate that our method can produce smaller and more uniform polyplexes in addition to lower toxicity. A higher ratio (N/P = 6.7) was also used as comparison.

The microfluidic hydrodynamic focusing (MF) device used in this study is depicted in Figure 1A. It is made of poly(methyl methacrylate) (PMMA) with microchannels 254 μm in width and 70 μm in depth and complexation length of 5 cm. The device consists of two inlets and one outlet. One inlet is connected to a syringe containing branched PEI solution (25 kDa), inlet 1, while the other is connected to the DNA solution, inlet 2. The solutions are in 150 mM NaCl (pH 7). The syringes are mounted on an infuse/withdraw syringe pump which controls the flow rate of the DNA and

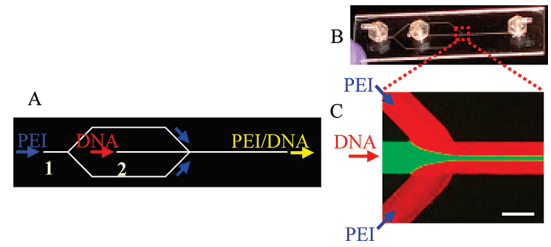

Figure 1. (A) Schematic of MF device. The device consists of two inlets and one outlet. Inlets 1 and 2 are connected to syringes containing branched PEI solution (25 kDa) and DNA solution, respectively. PEI solution is introduced at inlet 1 and split into two side streams while DNA solution is introduced at inlet 2 and flows in the middle channel. The DNA stream is hydrodynamically focused into a narrowly focused stream by the two PEI side streams. (B) An assembled plastic MF chip with inlet/outlet connectors. (C) Fluorescence micrograph of MF flow pattern at a flow rate ratio of 6. Green: fluorescein. Red: PEI labeled rhodamine. (Scale bar = 254 μm).

PEI solutions independently. DNA solution is injected into the middle channel while PEI solution is split into two side channels where the DNA is hydrodynamically focused by the PEI streams from the side channels into a narrowly focused stream. For flow visualization, the MF device was mounted on an inverted microscope stage (Nikon Eclipse 2000U) with a 10× Nikon Plan Fluoro objective. In Figure 1C, fluorescein dye was used to mimic the DNA to demonstrate the hydrodynamic focusing of middle stream in this device. Fluorescein and PEI/Rhodamine solutions were injected at flow rates of 10 and 50 μL/min, respectively, for a flow rate ratio (defined as the total flow rates to the middle flow rate) of 6. This flow rate ratio was used throughout this study because the flow was stable. At a lower flow rate, there will be excessive PEI in the side streams even though a narrower middle stream is obtained. Also, the flow pattern became unstable (data not shown). To vary the extent of focusing of the fluorescein stream, the flow rate ratio can be adjusted easily.

The same time points were used for characterization (DLS and zeta potential measurements, AFM, and CLSM), transfection expression studies, and cell viability since the BM and MF samples were prepared separately.

The magnitude of the shear stresses applied to the pDNA in the MF device may cause irreversible damage to the pDNA structure resulting in loss of gene transfection efficiency. In the MF hydrodynamic region, the DNA will experience a short shearing period (<1 s). This shearing force in the microfluidic might be comparable to or higher than the benchtop vortexing. This is why a 5 s vortex was chosen to study whether this amount of time was sufficient to alter the pDNA structures. Atomic force microscopy (AFM) and gel electrophoresis were used to determine any structural changes to the pDNA with and without vortexing and hydrodynamic focusing. Images were scanned on MFP-3D AFM in the tapping mode operated in air, and the samples

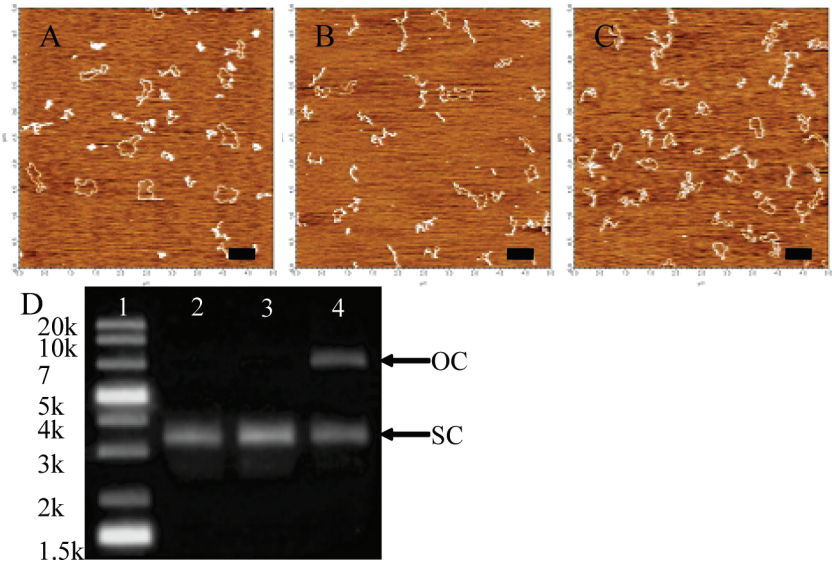

Figure 2. Effect of mixing on topology of the plasmid DNA was assessed by using AFM and agarose gel electrophoresis. AFM images of pGFP control (A), after vortexing for 5 s (B), and after hydrodynamic focusing (C). Agarose gel electrophoresis image (D) where lane 1 is a molecular marker, lane 2 is pGFP control, lane 3 is pGFP after vortexing for 5 s, and lane 4 is pGFP after hydrodynamic focusing. (Scale bars = 500 nm.)

were dry. From Figures 2A−C we can see that supercoiled (SC) and open circular (OC) structures are present. The same samples were also analyzed with agarose gel electrophoresis to distinguish the structure (Figure 2D). From Figure 2D, we can see that SC and OC are present in all the samples and the vortexed sample appears similar to the control. However, the hydrodynamic focused sample has more OC structure as compared to the control, which is probably due to hydrodynamic shear-induced degradation of the pDNA in the focusing region at a flow rate ratio of 6. Although lower velocity ratios can be used and there may be less structural change to the pDNA, the volume produced will be slower and the complexation may be less uniform. The transfection efficiency of the OC form has been shown to be slightly less comparable to the SC.[24,25] In this study we found that even though we have more OC, the expression was still better, indicating that MF complexes were somewhat more effective in transfecting the cells.

Next, the effectiveness of the BM and MF methods for condensing pGFP by PEI was evaluated, and the complexes were characterized by AFM. For BM, PEI/DI water solution (10 mM) was added into pGFP/DI water solution followed by 15 s vortexing. For MF, the pGFP/DI water solution (50 μg/mL) and PEI/DI water solution (10 mM) were injected at flow rates of 10 and 50 μL/min, respectively. Images were scanned on MFP-3D AFM in the tapping mode operated in air, and samples were dry during imaging. However, imaging in 150 mM NaCl was not possible because large NaCl crystals would form during drying. Figures 3A and 3B compare the sizes of dry PEI/pDNA complexes on mica

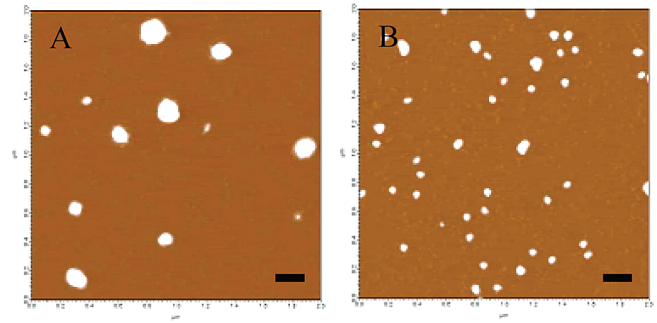

Figure 3. AFM images of PEI/pGFP complexes obtained by BM (A) and MF (B) method at N/P = 3.3. Images were scanned on MFP-3D AFM in the tapping mode operated in air. PEI/pDNA complexes were incubated on mica and dried with compressed air or nitrogen. (Scale bars = 200 nm.)

prepared by bulk mixing and hydrodynamic focusing, respectively, at N/P ratio of 3.3. At N/P of 6.7, the complexes prepared by the BM and MF methods were similar in size distribution (data not shown). Comparing the complex sizes in Figures 3A and 3B, the MF complexes are smaller and the size distribution is more uniform. The average complex size and charge density in buffer at N/P = 3.3 were also determined by a dynamic light scattering (DLS) goniometer and a zeta potential measurement instrument, respectively. The average size and zeta potential of particles formed from the BM and MF methods at N/P = 3.3 were 898 ± 84 nm, +24.8 mV and 494 ± 81 nm, +11.4 mV, respectively, in 150 mM NaCl (pH7). This result indicates that more compact and ordered condensates are formed by MF since the laminar flow condition in microfluidic channels creates a well-defined interfacial region and enhance the diffusional mass-transfer between the species in the two fluids. However, the standard deviation is large possibly due to the time the samples were

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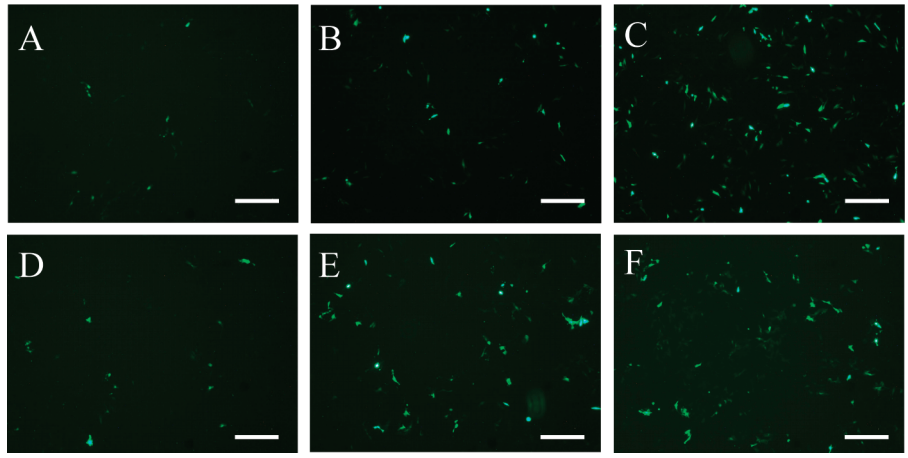

Figure 4. pGFP expression in NIH 3T3 cells at 2 days (A, B, C) and 4 days (D, E, F) post transfection and N/P = 3.3, by BM (A and D); MF (B and E); and positive control (Lipofectamine) (C and F). (Scale bars = 500 μm.

measured since the complex size is dependent on the incubation time after mixing PEI with pDNA. The average size of particles formed from the MF method at N/P = 6.7 was 244.4 ± 11.3 nm. The results demonstrated that the MF method is more potent at low N/P ratios. The MF complexes collected in the vial may experience further mixing. MF can be considered as a premixing step of bulk mixing, i.e. to bring DNA and PEI solutions together in a more ordered way than in bulk mixing. Since the flow in the microfluidics is laminar, microfluidic focusing is used to reduce the diffusion length mixing relies on molecular diffusion.

To assess the transfection efficacy of pGFP after hydrodynamic focusing, i.e. after shear stress had been undergone, pGFP expression in mouse NIH 3T3 fibroblast cells was performed and cell viability was determined. PEI/pDNA complexes at N/P = 3.3 and 6.7 were incubated for 6 h with 1 μg of pGFP; transfection and cell viability were performed in triplicate. pGFP expression at 2 (A−C) and 4 (D−F) days post transfection at N/P = 3.3 are shown in Figure 4. Comparing pGFP expression at 2 days post transfection, we can see that the positive control, pGFP condensed by Lipofectamine (C), yields the highest GFP expression followed by the MF method (B). The conventional method shows very poor gene transfection (A). At 4 days post transfection, the GFP expression from the positive control has slightly diminished, while the GFP expression from the MF method has significantly improved. On the other hand, GFP expression from the BM method shows only slight improvement.

Cell viability is also an important concern in gene delivery. The cell viability percentage (compared to untreated control) at the two N/P ratios and Lipofectamine were determined by MTT assay at 4 days post transfection as shown in Figure 5. As expected, cell viability decreases with increasing N/P ratios,which corresponds to higher amount of free PEI leading to higher cytotoxicity. For both N/P ratios, cells treated with complexes formed by MF have the highest percentage of viable cells (compared to untreated control), which corresponds to the lowest toxicity, followed by BM and Lipofectamine. More cells are alive in the MF method

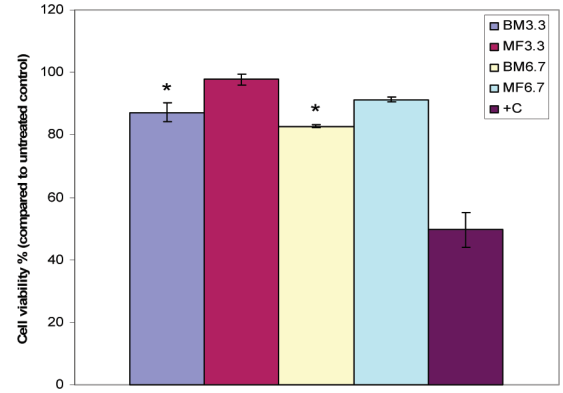

Figure 5. Cell viabilities were determined by MTT assay at N/P = 3.3 and 6.7 and 4 days post transfection. All error bars indicate standard deviations, $n = 5$. The * symbol indicates $p < 0.05$ compared to MF3.3.

probably due to more efficient complexation resulting in a lower amount of free PEI. However, we can see that the positive control has the lowest cell viability 4 days post transfection. This indicates that Lipofectamine is toxic to the cells and most of the cells are dead, hence the GFP expression diminishes after 4 days causing the drop in the positive control's fluorescence as seen in Figure 4F.

Since GFP expression is generally qualitative and a large number of cells would have to be transfected for flow cytometry analysis, plasmid encoding secreted alkaline phosphatase (pSEAP) was used to quantify and compare the activity of secreted alkaline phosphatase (SEAP) by both methods where higher SEAP activity indicates better transfection and expression. As shown in Figure 6, SEAP activity of MF produced complexes is slightly higher at N/P = 3.3 than the BM produced complexes at 2 and 4 days post transfection. The difference is much larger at N/P = 6.7 where the MF produced complexes have 1.2- and 1.7-fold higher SEAP activity at 2 and 4 days post transfection, respectively, compared to BM. Cell viability was similar to pGFP transfection.

To elucidate the difference in pDNA expression at N/P = 3.3 and 6.7, fluorescent labeling and confocal laser scanning

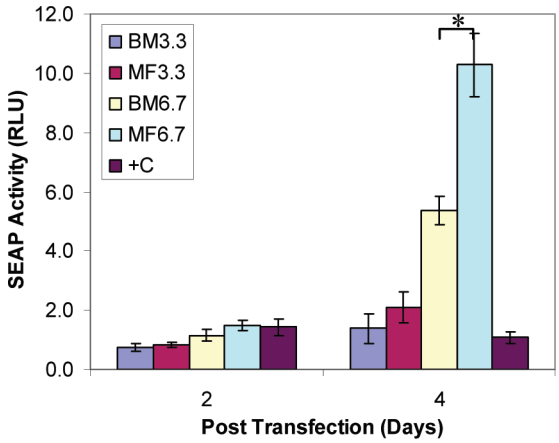

Figure 6. SEAP activity at 2 and 4 days post transfection. RLU stands for relative light units. All error bars indicate standard deviations, $n = 5$. The * symbol indicates $p < 0.05$ compared to MF6.7.

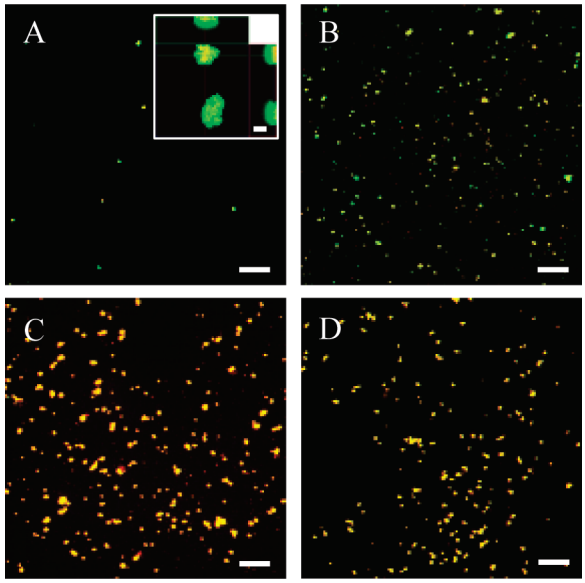

Figure 7. Size distribution and complexation efficiency of PEI/pDNA complexes. Confocal laser scanning microscope images of double-labeled PEI/pDNA complexes prepared by BM (A, C) and MF (B, D) at N/P = 3.3 (A, B) and 6.7 (C, D) in 150 mM NaCl pH 7. (A) Inset shows a stacked confocal image of large aggregates. pDNA was labeled with YOYO-1 dye (green), and PEI was conjugated with rhodamine (red). Yellow spots indicate colocalization of PEI/rhodamine and pDNA/YOYO-1. The images were taken with a Zeiss confocal LSM 510 microscope. (Scale bars = 10 μm, inset = 1 μm.)

microscopy (CLSM) were used to visualize PEI/pDNA complex size distribution and complexation efficiency. In this study, pDNA was labeled with YOYO-1 dye (green) and PEI was conjugated with rhodamine (red) for fluorescence imaging. Yellow spots indicate colocalization of PEI/rhodamine and DNA/YOYO-1. PEI/pDNA complexes depicted in Figures 7A and 7B were formed at N/P = 3.3 by BM and MF, respectively. Since BM is a nonuniform mixing

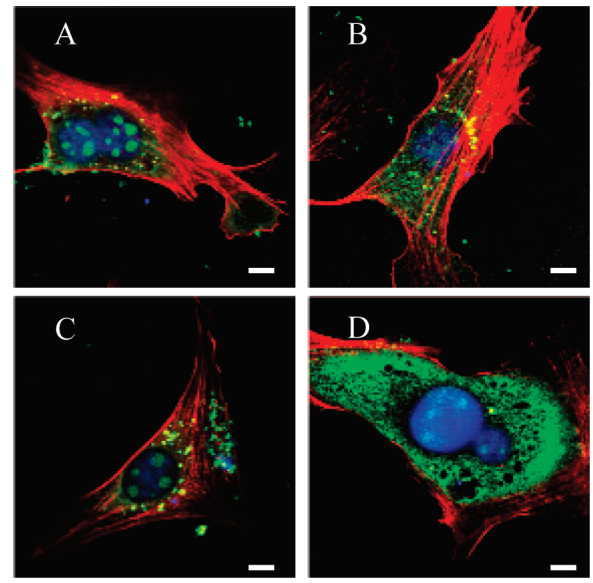

Figure 8. PEI/pDNA complex distribution in NIH 3T3 cells visualized using confocal laser scanning microscopy. NIH 3T3 cells were transfected with double-labeled PEI/pDNA complexes prepared by BM (A, C) and MF (B, D) at N/P = 3.3. Images were taken 2 days (A, B) and 4 days (C, D) post transfection. pDNA was labeled with YOYO-1 dye (green), and PEI was conjugated with rhodamine (red). Yellow spots indicate colocalization of PEI/rhodamine and pDNA/YOYO-1. The cell membrane and nucleus were stained with FM 4-64 (red) and DAPI (blue), respectively. The images were taken with a Zeiss confocal LSM 510 microscope. (Scale bars = 10 μm.)

process, it results in the formation of many large complexes or aggregates even though the remaining few complexes are yellow as shown in Figure 7A. The large complexes or aggregates (see inset) are likely due to random and undefined instantaneous electrostatic interaction of DNA with PEI when mixed. For MF, the mixing process can be precisely controlled by the flow ratio; since the flow inside the microchannel is lamellar, highly defined, and diffusion controlled, no large complexes were observed but complexation was not totally efficient as green (excess pDNA/YOYO-1), red (excess PEI/Rhodamine), and yellow particles are observed in Figure 7B. The particle size distribution was similar to the results obtained by AFM. At a higher N/P of 6.7, the presence of both yellow and red particles and red background in Figure 7C and only yellow particles with uniform color in Figure 7D further confirmed that MF formed PEI/DNA complexes are more uniform and better complexed than BM complexes. Therefore, the higher gene expression may be a result of smaller particle size and more uniform particle construct as characterized by DLS, AFM, and CLSM.

One can observe a slight discrepancy in polyplex sizes imaged by AFM and CLSM as shown in Figures 2 and 7, respectively. The AFM samples were prepared in DI water,

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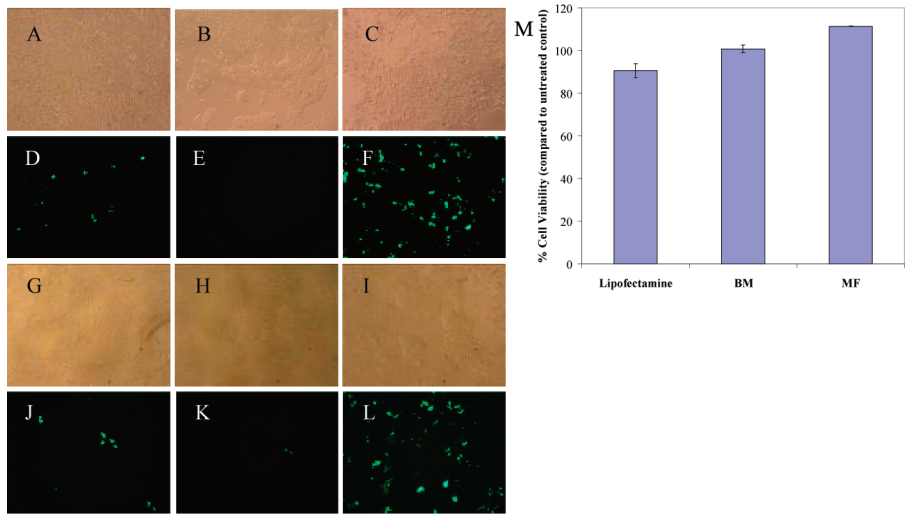

Figure 9. pGFP expression and cell viability in mES cells at N/P = 3.3. Fluorescence images of mES cells transfected by Lipofectamine (column 1), BM (column 2), MF (column 3) formed complexes at N/P = 3.3, 24 h (A−F) and 48 h (G−L) post transfection. 2.5 μg of pGFP/well was used, and transfection was performed in triplicate. (M) Cell viability of mES cells was determined by MTT assay at 48 h post transfection and expressed as percentage to untreated control. All error bars indicate standard deviations, $n = 3$.

and samples were dry during imaging, which resulted in more compact sizes. However, preparing and imaging the sample in 150 mM NaCl was not possible due to the formation of large NaCl crystals during drying, that covered the polyplexes. CLSM samples were prepared in 150 mM NaCl, and the samples were in liquid during imaging. The particles might appear larger because of light scattering.

The fluorescent labeling and confocal laser scanning microscopy (CLSM) techniques have also been used to study cellular uptake and intracellular transport of PEI/pDNA complexes in different cell lines and N/P ratios such as EA.hy 926 endothelial cells at N/P = 7.5,[26] L929 fibroblastic cells at N/P = 10,[27] PaTu 8902 pancreatic carcinoma cells,[28] and COS-7 cells at N/P = 6.[29] In this study, these techniques were utilized to visualize the uptake of PEI/pDNA complexes in NIH 3T3 cells at N/P = 3.3. As shown in Figure 8, we can see that more PEI/pDNA complexes prepared by the MF method are accumulated at the perinuclear region of the cells, i.e. outside the DAPI stained nucleus (blue), as compared to BM (Figure 8A) prepared complexes at 2 days post transfection. At 4 days post transfection, we can see that only a few PEI/pDNA complexes prepared by the MF method are present, which indicates that most of them have disassociated and plasmids have entered the nucleus for transfection.

Gene delivery to embryonic stem cells is of paramount importance to control cell differentiation and function. Embryonic stem (ES) cells are pluripotent cells that can be propagated stably in the undifferentiated state in vitro.[30] ES cells have great potential for gene therapy, cell replacement therapy, tissue engineering, and developmental biology because of their ability to self-renew by unlimited division and differentiate into multiple cell types for treating medical conditions such as heart disease, diabetes, and Parkinson's disease. Efficient gene delivery to these cells is required for genetic alterations,[31] RNAi silencing,[32] and cellular labeling.[33] To further verify this enhanced exogenous gene expression by MF formed PEI/pGFP complexes, mouse embryonic stem (mES) cells which have greater clinical importance were tested. Again, PEI/DNA complexes at N/P = 3.3 and 6.7 were incubated for 6 h with 2.5 μg of pGFP; transfection and cell viability were performed in triplicate. Superior transfection efficiency of MF complexes at N/P = 3.3 is shown in Figure 9. While Lipofectamine (D, J) and BM complexes (E, K) transfected only a few cells, MF complexes (F, L) transfected many more cells. The cell

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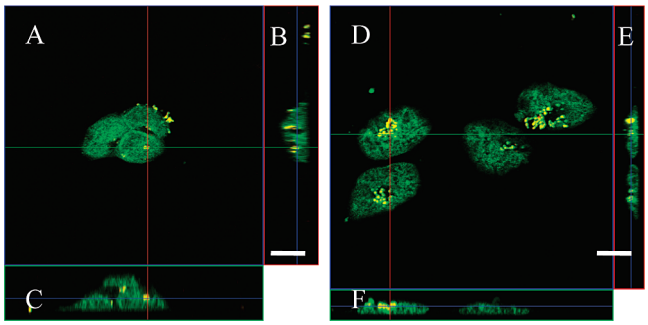

Figure 10. Cellular distribution of double-labeled PEI/pDNA complexes at N/P = 3.3 and 24 h post transfection. Stacked laser scanning confocal microscope images of mES cells transfected with complexes prepared by BM (A−C) and MF (D−F). Orthogonal sections confirm that the complexes are internalized into the cells (B, C) and (E, F). pDNA was labeled with YOYO-1 dye (green), and PEI was conjugated with rhodamine (red). Yellow spots indicate colocalization of PEI/rhodamine and DNA/YOYO-1. The cells are green in color due to the PEI/rhodamine. (Scale bars = 10 μm.)

densities are comparable from phase contrast images. MTT assay was carried out at 48 h post transfection to determine the cytotoxicity of the complexes. As shown in Figure 9M, complexes formed by MF have the highest percentage of viable cells (compared to untreated control), which corresponds to the lowest toxicity, followed by BM and Lipofectamine. The percent cell viability is greater than 100 because mES cells have a fast proliferation rate. For N/P = 6.7, the pGFP expression for both BM and MF nanocomplexes was similar when using 2.5 μg of pGFP/well and the cell viability was higher for the MF (Figure 5). These new results imply that the microfluidic method has great potential to significantly impact stem cell-related research.

Incubation of the BM and MF complexes with mES cells also revealed significant differences in the cellular complexes distribution. In Figure 10(A−C), most accumulated BM complexes are outside the cells and only a few are internalized. In contrast, many more MF complexes are available for transfection, and most are internalized into the cells as shown in Figure 10(D−F). Therefore, the higher gene expression from using the MF method than compared to the BM method can be attributed to more uniform size distribution, more efficient complexation, and more internalized complexes. The more efficient complexation yielded a lower amount of free PEI thus resulting in lower cytotoxicity. Because there are more cells alive, the chance of cells getting transfected also increases. The cells are green in color probably due to the YOYO-1.

4. Conclusion

In conclusion, we have demonstrated that the microfluidic hydrodynamic focusing method could efficiently condense plasmid DNA by PEI to produce more compact and uniform complexes with reduced cytotoxicity at N/P = 3.3 and 6.7 and improved gene transfection at N/P = 6.7. This method is particularly useful for assembling small plasmids and oligonucleotides which are less susceptible to chain breakage at higher flow rate ratios. This technology is now being extended to condense antisense oligonucleotides (AS ONs) and small interfering RNA (siRNA) by various polymers (e.g., chitosan, polylysine, weight linear/branched PEI, etc.), dendrimers, Pluronics, or cationic lipids for application in stem cell development and cancer treatment.

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