Supporting Information

Tuning Physical Properties of Nanocomplexes Through Microfluidics-Assisted Confinement

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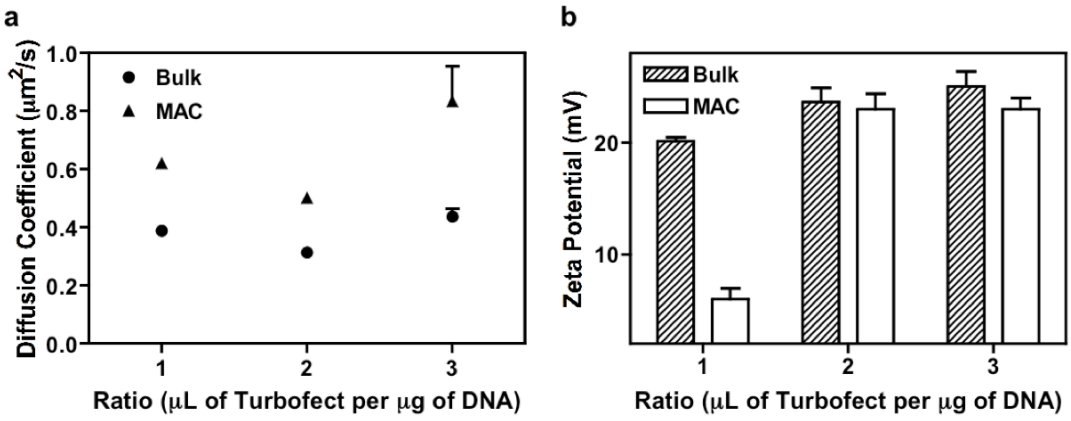
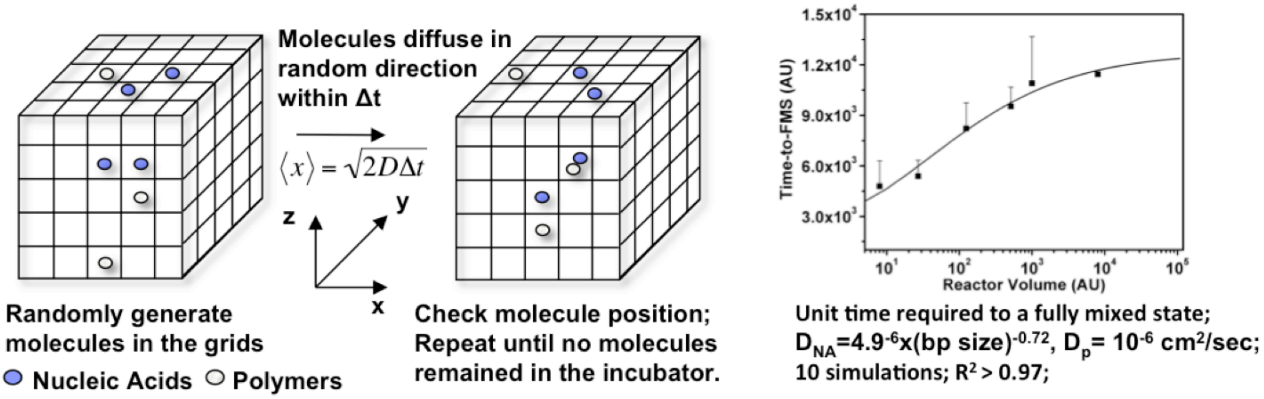
(1) SUPPLEMENTARY FIGURES AND TABLE



Supplementary Figure 1. Characterization of Physical Properties Under Various Turbofect Transfection Reagent to DNA Ratios. (a) MAC-produced nanocomplexes were consistently smaller than bulk preparation. (b) Surface charges of MAC nanocomplexes were lower.



Supplementary Figure 2. Simulation of Confined Diffusion. A coarse grain model was used to simulate the confined diffusion of oppositely charged polyelectrolytes in a micro-incubator. Diffusion in picoliter droplets (300pL in our experiement) is able to fully mix the reagents in a relatively short amount of time, compared to bulk preparation (typically ~ 100 μL). Simulation sets to calculate a unit time required to a Fully Mixed State (FMS); $D_{NA}=4.9^{-6}x(\text{bp size})^{-0.72}$, $D_p= 10^{-6}$ cm^2/sec; Error bar obtained from 10 simulations; Fitted by Hill Equation, $R^2 > 0.97$.

Supplementary Table 1. Hydrodynamic Diameter of Nanocomplexes. Representative size measurement of nanocomplexes prepared at a ratio of 2μL Turbofect transfection reagent per μg of DNA.

Preparations	Time (min)	Z_{ave} (d,nm)	PDI
Bulk	2	406.8	0.161
MAC	2	289.8	0.125
Bulk	30	1036	0.304
MAC	30	390.7	0.272
Bulk	60	1131	0.474
MAC	60	422.7	0.274

(2) EXPERIMENAL METHODS

Materials

Plasmid DNA (pmaxGFP, 3486 bp, Lonza, Switzerland) encoding green fluorescence protein was used as the reporter gene. Commercially available polymeric transfection reagents, Turbofect (poly(2-hydroxypropyleneimine), pHP, Fermentas, Glen Burnie, MD) and jetPEI (linear polyethylenimine, 20kD, Polyplus-Transfection, New York, NY) were obtained from the vendors and used directly. Transfection reagent to plasmid DNA ratio was optimized as suggested in the manufacturers' protocols. For uptake studies, plasmid DNA was labeled with streptavidin-functionalized quantum dots (QDs, Qdot 605 ITK, Invitrogen, Carlsbad, CA) as described previously.[1] Briefly, pDNA was biotinylated as described by the manufacturer (Label IT Biotin, Mirus Bio, Madison, WI) but scaled to have ~1-2 biotin labels per pDNA. Biotinylated pDNA was purified from unreacted reagents by ethanol or isopropanol precipitation and centrifugation following standard protocols.[2] The molar ratio of pDNA to QD was kept in excess to ensure complete conjugation of QDs to pDNA. The number of QDs labeled onto each pDNA is estimated to be ~1-3.[1]

Chip Fabrication and Operation

The droplet generator was fabricated by conventional soft lithography techniques,[3] casting and curing the PDMS prepolymer on a SU-8 3025 (MicroChem, Newton, MA, now acquired by Nippon Kayaku Co., Japan) master (Transparency mask, CAD/Art Services, Bandon, OR) following a standard protocol, which produced a channel height at around 35 μm. PDMS prepolymer (Sylgard 184 Silicone Elastomer Kit, Dow Corning, Midland, MI) was prepared in a 10 : 1 (base : curing agent) ratio and cured at 65°C for 1hr. Cured PDMS strips were cut and punched with through holes (Hole puncher, Technical Innovations, Brazoria, TX) as fluidic connections and bonded with a cover glass through a thin layer of PDMS. The bonded PDMS chip was then left in an oven at 95°C for overnight to enhance the bonding strength.

Prior to nanocomplex synthesis, the channel was filled with oil/surfactant for 30 minutes to ensure the channel was fully wet. Two syringe pumps (PHD2000, Harvard Apparatus, Holliston, MA) were used to control the flow rates of oil/surfactant (7.5 μL/min) and reagents (2.5 μL/min) independently, forming monodisperse water-in-oil droplets at a frequency of around 0.8-1.5 kHz. The droplet volume (~300 pL) and generation frequency were optimized by the flow rate ratio, determined by the competition between continuous phase (carrier fluid) and disperse phase (aqueous reagents). Carrier fluid (FC-40 fluorocarbon oil, 3M, St. Paul, MN) and surfactant (RainDance Technologies, EA Surfactant, Lexington, MA) were selected for their higher density than aqueous solution and neutral charge, respectively. Nanocomplexes synthesized in droplet volume of 100 pL and 300 pL were found to have minimum differences in both size and zeta potential.

Nanocomplexes Preparation and Characterization

Bulk preparation was performed following the protocol stated by the manufacturer or previous studies.[4] Briefly, complexes were formed by adding equal volumes (50μL) of polymer solution (2μL of transfection reagents per μg of DNA) to the DNA solution (0.1μg/mL) and then vigorously mixed by

pipetting or brief vortexing. The mixture was allowed to stand for 10-15 minutes of incubation before transfection. The same reagents were introduced into the picoliter droplets as a comparison, denoted as MAC-prepared nanocomplexes. The MAC-prepared nanocomplexes were collected by breaking the droplets (Droplet Destabilizer, RainDance Technologies) and directly used for subsequent characterization or cellular investigation.

Size and zeta potential were measured with a Zetasizer NanoZS-90 (Malvern Instruments, Southborough, MA). Nanocomplexes were diluted in an ultra-micro cuvette (BrandTech Scientific, Essex, CT) to a final DNA concentration of 10 μg/mL (optimized to a count rate of 100-200 kcps, killo counts per second) for size measurements. Three measurements, each consisting of twelve 10 sec runs, were performed at 25°C at a 90° scattering angle. For the study of aggregation kinetics, 5 measurements were done immediately during the first 15 minutes incubation, and 10 measurements were done subsequently with a 3 min delay. Z average diameter (Z_{ave}), derived from a Cumulants analysis of the measured correlation curve, was reported as the intensity weighted mean hydrodynamic diameter. Polydispersity index (PDI) was calculated from the raw data of the DLS measured intensity autocorrelation function. Both Z_{ave} and PDI were obtained from Zetasizer Software (v.6.12, Malvern Instruments). Reported standard deviation in the size measurement assumed a Gaussian distribution and was defined in the fashion of σ^2=PDI x $(Z_{Ave})^2$ (Malvern Instrument Manual). Zeta potential measurements were performed at a final DNA concentration of 2 μg/mL using a capillary flow cell (Malvern Instruments) at 25ºC. The Smoluchowski model was used to calculate the zeta potential obtained from five measurements, each consisting of twenty runs.

Cell Culture and Flow Cytometry Analysis

At 24 hr prior to transfection, Human Embryonic Kidney 293 cells (ATCC) were seeded (4×10^5 cells/well) in 6-well plates and grown in complete media (MEM supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 U/ml streptomycin, Invitrogen, Carlsbad, CA). Cells were

transfected with either bulk- or MAC-prepared nanocomplexes containing 4 μg DNA in reduced-serum media (Opti-MEM) for 4 h at 37°C. Each preparation was performed in triplicate. Transfection efficiency and cell viability were assayed at 24h post-transfection by flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ). The FSC/SSC was gated with untreated cells to exclude the dead cells or cell debris. Quantitative analysis of cell viability was performed by apoptosis assay with PI and Annexin V-Cy5 (BD Biosciences, Franklin Lakes, NJ). Fluorescence was evaluated with the filter settings of FL1 (GFP), FL3 (PI) and FL4 (Cy5). PI, intercalating in double-stranded nucleic acids, is excluded by viable cells but can penetrate cell membranes of dying or dead cells. Loss of plasma membrane is one of the earliest features in apoptotic program. Annexin V is a Ca^{2+} dependent phospholipid-binding protein that can interact with phosphatidylserine (PS). It is used to distinguish the early apoptotic cells through the exposure of PS on the external cell membrane. Collectively, viable (PI^-, Annexin V^-), dead (or late apoptotic: PI^+, Annexin V^+), and early apoptotic (PI^-, Annexin V^+) cells can thus be differentiated. The fluorescence signals were compensated and gated with negative controls.

Uptake of polyplexes was evaluated in separated experiments using unlabeled polymer and QD-labeled pDNA. After predetermined incubation times at 37 °C, cells were washed once with phosphate buffered saline (PBS) and treated with trypsin–EDTA for 5 minutes. Cells were then fixed by 4% Paraformaldehyde (PFA), washed by heparin (20 units/mL) to remove membrane bound polyplexes,[5] washed again with PBS, then resuspended in PBS for flow cytometry analysis (FACSCanto II, BD Biosciences, Franklin Lakes, NJ). Each time point was performed in duplicate. A 405nm laser served as the excitation and the fluorescence was captured using the P10 channel (dichroric: 502LP, emission filter: 622/36 nm). More than 10,000 cells were measured in each sample to ensure reliable statistics. FlowJo (v. 9.1, Tree Star, Ashland) was used to analyze the flow cytometry data.

Fluorescence Microscopy and Atomic Force Microscopy

Epifluorescent and bright field images were captured with an inverted fluorescence microscope (TE2000U, Nikon Instruments, Melville, NY) equipped with a 100-W mercury arc lamp (X-Cite 120 Fluor system, EXFO, Ontario, Canada) and a cooled CCD (CoolSnap HQ, Roper Scientific, Now Photometrics, Tucson, AZ). Monocolor emission from GFP was collected and filtered through appropriate filters and dichroics. Image processing and analysis was performed with ImageJ (v1.43, http://rsb.info.nih.gov/ij).

Atomic force microscopic imaging was performed in on a multimode NanoScope IIIa (Veeco Instruments, Plainview, NY), using silicon nitride probes (NP-S10, Bruker AFM Probes Nanofabrication Center, Camarillo, CA). Prior to imaging, 5 μL of polyplexes was dropped on freshly cleaved mica (Ted Pella, Redding, CA) and left to adsorb to the surface for 3 minutes. The positively charged polyplexes adsorb readily on negatively charged mica.[6] 30 μL of 1x TAE/Mg^{2+} was then added onto the mica for imaging.[7] Surface area of the nanocomplexes was quantified using the built-in function of particle analysis under ImageJ. More than 1,000 nanocomplexes were investigated for each analysis.

Polyplex Stability and Quantification of Excess Polymer

Polyplex stability was studied by PicoGreen competition assay, which was modified from the generally used EtBr competition assay.[8] PicoGreen reagent contains fluorochrome that selectively binds dsDNA, serving as competitive polycations in the assay. When bound to dsDNA, fluorescence enhancement of PicoGreen is exceptionally high; little background occurs since the unbound dye has virtually no fluorescence. Upon challenged with PicoGreen, 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. Essentially, titrated Quant-iT PicoGreen reagent (Invitrogen, Carlsbad, CA) was added to the nanocomplex solutions and allowed an incubation of 15min. The

fluorescence was measured in a 96-well plate using a platereader (BMG Labtech GmbH, Germany) under excitation maximum at 480 nm and emission peak at 520 nm. The fluorescence intensity was corrected by background fluorescence. Nanocomplex stability was calculated based on the modified definition from previous studies[8]: $[(F_{DNA,PicoGreen}-F_{NC,PicoGreen})/F_{DNA,PicoGreen}]x100\%$, where $F_{DNA,PicoGreen}$ is the fluorescence from the mixture of PicoGreen and DNA in the absence of polymers and $F_{NC,PicoGreen}$ is the fluorescence obtained from the nanocomplexes under the challenge of Picogreen. Results from three independent triplicate experiments were analyzed.

Excess polymer was characterized by trinitrobenzene sulfate (TNBS) assay.[9] Briefly, at least 10μg of nanocomplexes were prepared and centrifuged at 14,000 rpm for 30min. The supernatant was then collected, lyophilized then resuspended in sodium bicarbonate buffer (0.1 M, pH 8.5) to reach an estimated polymer concentration of 20-200 μg/mL. Stock 5% TNBS solution (TNBSA (2,4,6-Trinitrobenzene sulfonic acid, Thermo Fisher Scientific, Rockford, IL) was diluted 500-fold in sodium bicarbonate buffer as a TNBS assay solution. In a 96-well plate, 50 μL TNBS assay solution was added into 100 μL of nanocomplexes solution and mixed well. The mixture was allowed to incubate at 37°C for 1 hr before being assayed by absorbance at 415nm with the platereader. The percentage of excess polymer was determined by a standard curve constructed with titrated polymer solutions.

Numerical Simulation

Monte Carlo simulation[10] was used to model the random diffusion of molecules within an incubator of a given volume (Supplementary Figure 2). The Monte Carlo simulation was implemented in MATLAB. Molecules were first randomly assigned a position based on a given concentration (nominally 1nM). The molecules traveled in all three directions (designated by a random variable) and the traveled distance was determined by the diffusion equation as: $\langle x\rangle=(2D\Delta t)^{0.5}$, where $D_{DNA}=4.9^{-6}x(\text{bp size})^{-0.72}$, $D_{polymer}= 10^{-6}$ cm^2/sec.[11, 12] The simulation was set to stop until no molecule remained unreacted in the incubator and timed as the time-to-FMS (fully mixed state). Ten simulations were conducted and an

averaged value was reported in Supplementary Figure 2. The model assumed that the reaction constant between DNA and polymer is infinitely large and the reaction is thus diffusion dominated.

Statistical Analysis

Results were reported as the mean ± s.e.m. as described for numbers of independently performed experiments. The statistical significance was determined using an unpaired t-test (Prism 4.0, GraphPad Software, La Jolla, CA). Two-tailed P-values were reported in the manuscript unless otherwise stated. Subsequent data processing and fitting were conducted with Origin (OriginPro8, Student Version, OriginLab, Northampton, MA).

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