

POLYPLEX SYNTHESIS BY "MICROFLUIDIC DRIFTING" BASED THREE-DIMENSIONAL HYDRODYNAMIC FOCUSING METHOD

Mengqian Lu,* Yi-Ping Ho,***** Christopher L. Grigsby,** Ahsan Ahmad Nawaz,*
Po-Hsun Huang,* Kam W. Leong** and Tony Jun Huang*

*The Pennsylvania State University, USA

**Duke University, USA

***Interdisciplinary Nanoscience Center (iNANO), Denmark

ABSTRACT

We synthesized polymer-DNA nanocomplexes (polyplexes) using a "microfluidic drifting" based three-dimensional (3D) hydrodynamic focusing in a single-layered, microfluidic device. This synthesis method requires no additional chemical treatment steps or any post processing. Compared with the conventional bulk mixing method [1,2], the polyplexes prepared by our "microfluidic drifting" 3D focusing method [3,4] showed smaller size, slower aggregation rate and higher transfection efficiency. This "microfluidic drifting" 3D focusing method provides a simple, fast, and repeatable method to synthesize high-quality polyplexes, which can be used in nucleic acid therapeutics to reduce the cost and complexity..

KEYWORDS

Microfluidic, Three-dimensional Hydrodynamic focusing, Polyplexes

INTRODUCTION

Gene therapy shows promise in the treatment of many acquired and inherited diseases, but currently requires delivery vectors to facilitate the intracellular uptake of the genetic materials. Polycations have been used to condense nucleic acids to nanocomplexes for transfection applications [5,6]. Usually, the nanocomplexes are prepared by adding polymer solution to DNA solution and then vigorously pipetting or vortex mixing the resulting solution. The nanocomplexes form spontaneously due to the electrostatic interaction between the cationic polymer and negatively charged DNA. Because of the metastable preparation and subsequent aggregation, the nanocomplexes formed by such methods show poor uniformity, batch-to-batch variability, and poor biological reproducibility.

Here we synthesized polymer-DNA nanocomplexes using a "microfluidic drifting" based three-dimensional (3D) hydrodynamic focusing in a single-layered, microfluidic device [3,4]. This synthesis method requires no additional chemical treatment steps or any post processing. Compared with the conventional bulk mixing method [1,2], the polymer-DNA nanocomplexes prepared by our "microfluidic drifting" 3D focusing method showed smaller size, slower aggregation rate and higher transfection efficiency. This "microfluidic drifting" 3D focusing method provides a simple, fast, and repeatable method to synthesize high-quality polymer-DNA nanocomplexes, which can be used in nucleic acid therapeutics to reduce the cost and complexity.

EXPERIMENT

The Plasmid DNA (pDNA) encoding green fluorescent protein (GFP) stock solution and the turbofect transfection reagent were diluted in Opti-MEM reduced-Serum Medium to 13.2 $\mu\text{g}/\text{mL}$ and 13.2 $\mu\text{L}/\text{mL}$, respectively. As shown in Fig. 1, the DNA solution was focused after injection through inlet A. The inset shows the concentration distribution of the DNA solution, which was confined in three dimensions. The polymer solution was injected from inlets B, C and D. Additionally, a very long channel length allowed for a longer diffusion time and ensured that the reaction could be completed within the microfluidic system. The nanocomplexes were collected at the outlet directly without further purification or separation. Several flow rates were carried out for optimization based on the size of the synthesized nanocomplexes, while keeping the ratio of turbofect solution to DNA solution as 2:1.

The DNA and turbofect concentrations were kept the same as the ones used in microfluidic experiments. Later, 1 mL of the turbofect solution was added to 0.5 mL DNA solution, followed by vigorous pipetting.

The polymer-DNA nanocomplex size (Z average diameter, Z_{ave}) and polydispersity indices (PdI) were directly measured by using the Zetasizer NanoS system from Malvern Instrument (Malvern Instruments, Herrenberg, Germany). All measurements were carried out at 25 °C, using the refractive index (1.330) and viscosity (0.8872 cP) of water for data analysis. Each sample was measured in every 3 min delay for a total of 1 hour. The reported

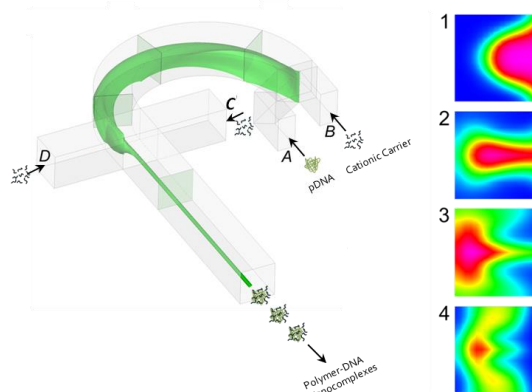


Figure 1: Schematics of the microfluidic device for nanocomplex synthesis by 3D hydrodynamic focusing.

standard deviation was calculated as $\sigma^2 = PDI \times (Z_{ave})^2$ with the assumption of a Gaussian distribution.

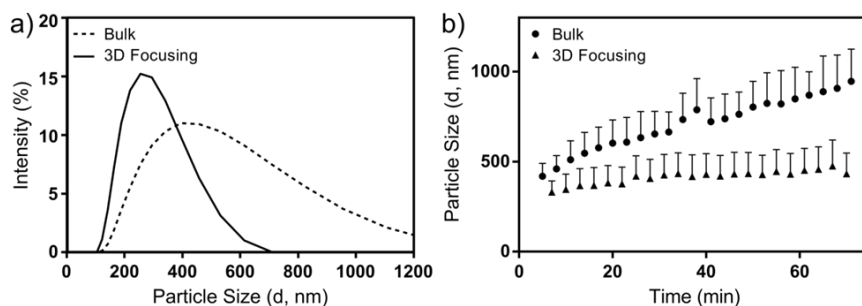


Figure 2: Comparison of nanocomplexes prepared by bulk mixing method and 3D focusing method. (a)

Intensity-based size distribution ($Z_{ave,3D} = 263.0$ nm versus $Z_{ave,bulk} = 419.1$ nm; $PDI_{3D} = 0.131$ versus $PDI_{bulk} = 0.142$); (b) aggregation kinetics.

TRANSFECTION STUDIES

Before transfection human embryonic kidney (HEK293T) cells were seeded at 1×10^5 cells/well in 12-well plates and cultured overnight at 37 °C under 5% CO₂ with 1 mL/well full growth media for 24 hours. Then, the full growth media was replaced with 400 μL Opti-MEM with nanocomplex of 1.5 μg DNA in each well. After 4 hours incubation, the transfection media was replaced with full growth media, and the cells were incubated for 24 hours before characterization.

The transfected cells were studied by optical microscopy and flow cytometry. The apoptosis assay was carried out through flow cytometry after Annexin-V (AV) and Propidium Iodide (PI) staining.

RESULTS AND DISCUSSION

At the optimized flow rate, the 3D focusing method prepares nanocomplexes of smaller size than those prepared by the bulk method ($Z_{ave,3D} = 263.0$ nm versus $Z_{ave,bulk} = 419.1$ nm), as shown in Fig. 2a. The uniformities of nanocomplexes do not show much differences at the beginning ($PDI_{3D} = 0.131$ versus $PDI_{bulk} = 0.142$). We also studied the aggregation kinetics of the nanocomplexes. The bulk mixing method generates heterogeneous nanocomplexes in size and composition. Therefore, the imbalanced surface charges causes the nanocomplexes prepared by the bulk mixing methods to aggregate or flocculate much faster than the ones prepared by the 3D focusing method. Since the 3D focusing method can produce nanocomplexes with higher uniformity, the nanocomplexes prepared in this way should have higher resistance to aggregation. The comparison of the aggregation kinetics of nanocomplexes prepared by bulk mixing and the 3D focusing method are shown in figure 2b, showing that the 3D focusing method can generate nanocomplexes with diminished aggregation, without any additional treatment or adding of stabilizers.

The transfection performance was examined by incubating the nanocomplexes with human embryonic kidney cells (HEK293T). Nanocomplexes prepared by the 3D focusing method showed higher transfection efficiency and similar cell viability compared to the ones prepared by the bulk mixing method. The HEK293T cells were observed by fluorescence microscopy 24 hours after transfection, as shown in Fig. 3. There are significantly more GFP positive cells incubated with nanocomplexes prepared by the 3D focusing method prepared nanocomplexes, showing the higher transfection efficiency.

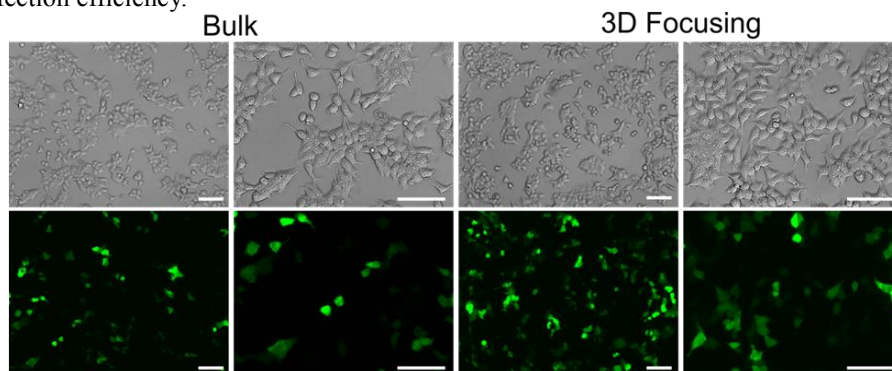


Figure 3: Microscopic observation after 24h post-transfection. The morphologies of cells are similar in both cases, and there are obviously more GFP positive cells in the case of 3D focusing method.

Flow cytometry characterization was carried out after the microscopy observation, and the results are shown in Fig. 4. The FSC/SSC plots are similar in bulk mixing and 3D focusing cases (Fig. 4 a-b). Further cell viability evaluation was carried out by measuring cell death and apoptosis via PI and Annexin V-Cy5 staining, respectively, as shown in Fig. 4 c-d. The results show that nanocomplexes prepared by the bulk mixing method and 3D focusing method induce similar rates of cell death (PI+) and apoptosis (PI-, Annexin V+), which is also shown in Figure 4f. Figure 4e shows the percentage of GFP positive cells. The 3D focusing method induced ~75% GFP expression level, while the bulk mixing method induced ~60%. Therefore, the 3D focusing method can produce nanocomplexes with higher transfection efficiency with similar cytotoxicity compared to the bulk mixing method.

CONCLUSION

In summary, we have synthesized DNA/polymer nanocomplexes using a 3D hydrodynamic focusing method in a single-layered device without additional chemical reagents. The nanocomplexes prepared by the 3D focusing method show smaller size, slower aggregation rate, higher transfection efficiency and induce similar cytotoxicity compared to the ones prepared by the bulk mixing method. The 3D focusing can produce high quality DNA/polymer nanocomplexes in a simple and fast way, while minimizing human factors, which is beneficial for DNA based therapeutics.

ACKNOWLEDGEMENTS

This research was supported by National Institutes of Health (Director's New Innovator Award, 1DP2OD007209-01, HL109442, EB015300, and AI096305), the National Science Foundation, and the Penn State Center for Nanoscale Science (MRSEC). Y. P. Ho is grateful to the support of Danish Research Councils (11-116325/FTP), Karen Elise Jensen Foundation and Carlsberg Foundation. C.L. Grigsby was supported by the Whitaker International Program and the US Fulbright Program. Components of this work were conducted at the Penn State node of the NSF-funded National Nanotechnology Infrastructure Network.

REFERENCES

- [1] Vikas K. Sharma, Mini Thomas, and Alexander M. Klibanov, "Mechanistic studies on aggregation of polyethylenimine-DNA complexes and its prevention," *Biotech. Bioeng.* vol. 80, pp. 614, 2005.
- [2] Yuhong Xu, Sek-Wen Hui, Peter Frederik, and Francis C. Szoka, Jr. "Physicochemical characterization and purification of cationic lipoplexes," *Biophys. J.* vol. 77, pp. 341, 1999.
- [3] Xiaole Mao, John Robert Waldeisen, and Tony Jun Huang, "Microfluidic Drifting"—Implementing three-dimensional hydrodynamic focusing with a single-layer planar microfluidic device, *Lab on a Chip*, 7, pp. 1260-1262 (2007).
- [4] Xiaole Mao, Sz-Chin Steven Lin, Cheng Dong and Tony Jun Huang, *Single-Layer Planar On-Chip Flow Cytometer Using Microfluidic Drifting Based Three-Dimensional (3D) Hydrodynamic Focusing*, *Lab on a Chip*, 9, pp. 1583-1589 (2009).
- [5] Christopher L. Grigsby and Kam W. Leong, "Balancing protection and release of DNA: tools to address a bottleneck of non-viral gene delivery," *J. R. Soc. Interface*, vol. 7, pp. S67, 2010.
- [6] Yi-Ping Ho, Christopher L. Grigsby, Feng Zhao, and Kam W. Leong, "Tuning Physical Properties of Nanocomplexes through Microfluidics-Assisted Confinement," *Nano Lett.* vol. 11, pp. 2178, 2011.

CONTACT

Tony Jun Huang 1-814-863-4209 or junhuang@psu.edu
Kam W. Leong 1-919-660-8466 or kam.leong@duke.edu

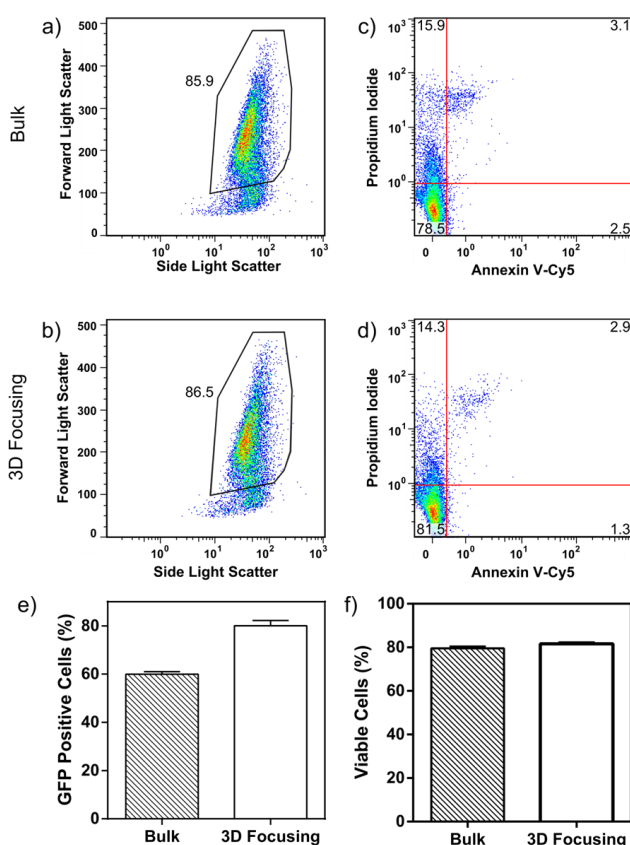


Figure 4: *Quantification of transfection efficiency and toxicity.*