Video Article

Combining QD-FRET and Microfluidics to Monitor DNA Nanocomplex Self-Assembly in Real-Time

Yi-Ping Ho[1,2], Hunter H. Chen[2,3], Kam W. Leong[2], Tza-Huei Wang[1,3]

[1]Mechanical Engineering, Johns Hopkins University

[2]Biomedical Engineering, Duke University

[3]Biomedical Engineering, Johns Hopkins University

Correspondence to: Tza-Huei Wang at thwang@jhu.edu

URL: https://www.jove.com/video/1432
DOI: doi:10.3791/1432

Keywords: Biomedical Engineering, Issue 30, microfluidics, gene delivery, quantum dots, fluorescence resonance energy transfer, self-assembly, nanocomplexes

Date Published: 8/26/2009

Citation: Ho, Y.P., Chen, H.H., Leong, K.W., Wang, T.H. Combining QD-FRET and Microfluidics to Monitor DNA Nanocomplex Self-Assembly in Real-Time. *J. Vis. Exp.* (30), e1432, doi:10.3791/1432 (2009).

Abstract

Advances in genomics continue to fuel the development of therapeutics that can target pathogenesis at the cellular and molecular level. Typically functional inside the cell, nucleic acid-based therapeutics require an efficient intracellular delivery system. One widely adopted approach is to complex DNA with a gene carrier to form nanocomplexes via electrostatic self-assembly, facilitating cellular uptake of DNA while protecting it against degradation. The challenge lies in the rational design of efficient gene carriers, since premature dissociation or overly stable binding would be detrimental to the cellular uptake and therapeutic efficacy. Nanocomplexes synthesized by bulk mixing showed a diverse range of intracellular unpacking and trafficking behavior, which was attributed to the heterogeneity in size and stability of nanocomplexes. Such heterogeneity hinders the accurate assessment of the self-assembly kinetics and adds to the difficulty in correlating their physical properties to transfection efficiencies or bioactivities. We present a novel convergence of nanophotonics (i.e. QD-FRET) and microfluidics to characterize the real-time kinetics of the nanocomplex self-assembly under laminar flow. QD-FRET provides a highly sensitive indication of the onset of molecular interactions and quantitative measure throughout the synthesis process, whereas microfluidics offers a well-controlled microenvironment to spatially analyze the process with high temporal resolution (~milliseconds). For the model system of polymeric nanocomplexes, two distinct stages in the self-assembly process were captured by this analytic platform. The kinetic aspect of the self-assembly process obtained at the microscale would be particularly valuable for microreactor-based reactions which are relevant to many micro- and nano-scale applications. Further, nanocomplexes may be customized through proper design of microfludic devices, and the resulting QD-FRET polymeric DNA nanocomplexes could be readily applied for establishing structure-function relationships.

Video Link

The video component of this article can be found at https://www.jove.com/video/1432/

Protocol

A. Biotinylation of DNA

Plasmid DNA were covalently biotinylated with guanine-specific biotin labels as described by the manufacturer (Mirus Bio, Madison, WI) but scaled to have ~1-2 biotin labels per DNA. Plasmid DNA (pEGFP-C1, 4.9 kb, Clontech, Mountain View, CA) was labeled in this protocol.

1. Dissolve desired amount of pDNA into TE buffer of DNase-free and RNase-free (molecular biology-grade quality) water to make a 1µg/µL DNA solution.
2. Conduct the labeling reaction using the following reaction mixtures. Add the *Label*IT reagent last.

For 100µg DNA reaction:

DNase-free and RNase-free water	75 µL
10X Labeling Buffer A	20 µL
1µg/µL DNA	100 µL
*Label*IT reagent	5 µL
Total Volume	200 µL

3. Incubate the reaction at 37 °C for 1 hour.

4. Purify the labeled sample by ethanol or isopropanol precipitation following standard protocols.

Note: Gel filtration based columns may lead to high UV absorbance or fluorescence background, which may affect the DNA quantification or fluorescence characterization.
Note: The level of biotinylation may be determined by HABA-based tests.

B. Labeling of the Cy5-Cationic Polymer

Chitosan (390 kDa, 83.5% deacetylated, Vanson, Redmond, WA) was used as a model cationic polymer in this study. The free primary amines on the chitosan polymer backbone were labeled with Cy5-NHS (Amersham Biosciences, Piscataway, NJ).

1. To facilitate complete conjugation of Cy5 dye, calculate the required amount of Cy5-NHS such that the molar ratio of Cy5 : primary amines is 1 : 200.
2. Adjust pH of the chitosan solution (in 25mM acetate buffer) to ~6.5 by addition of NaOH. Note that the NHS reaction is more efficient at basic pH, but the solubility of chitosan here limits the working pH range.
3. While stirring, slowly add the calculated amount of Cy5-NHS (1 mg/ml DMSO) to the chitosan solution in a drop-by-drop manner.
4. Agitate the mixture in the dark at room temperature overnight.
5. To purify, dialyze with 10k MWCO Slide-a-Lyzer (Pierce) for 2 hr against 1% acetate buffer at room temp in the dark.
6. Replace buffer and dialyze another 2 hr at room temp in the dark.
7. Replace buffer and dialyze overnight at 4 °C in the dark.
8. Store purified labeled polymer at -20 °C.

Note: In this study, a standard curve is constructed by measuring the emission intensity of Cy5-NHS ester at 670 nm. Characterize the labeling density by measuring the obtained emission at 670 nm from Cy5-labeled chitosan in the standard curve. Absorbance may also be used to determine the labeling efficiency but was not performed here.

C. Preparation of QD-labeled DNA and Cy5-Polymer

The molar ratio of pDNA to QD was kept in excess (pDNA : QD ≈ 1 : 2) to ensure complete conjugation of QDs to pDNA. The number of QDs labeled onto each pDNA can be estimated through TEM imaging or other equivalent facilities. In our study, the number of QDs per pDNA is estimated to be ~1-3 by TEM and single molecule spectroscopy.[1] Use Millipore Milli-Q gradient water (>18.0 MW, 0.2um filtered) during the preparation.

1. Calculate the required amount of chitosan for 10µg pDNA according to desired N/P ratio, the theoretical ratio of protonated amines in the chitosan solution to the negative phosphates in the DNA solution.
2. Add streptavidin-functionalized 605QDs (Qdot 605 ITK, Invitrogen, Carlsbad, CA) into the biotinylated pDNA solution.
3. Incubate the solution at room temperature in the dark for 15 min.
4. Add the QD-labeled DNA into 50 mM sodium sulfate solution to make the final volume 200µL.
5. Dilute Cy5-chitosan, according to desired N/P ratio, with Milli-Q water to make the final volume 200µL.

Note: Keep the reaction in dark to prevent possible photobleaching.
Important: Be cautious to use the Qdot 605 ITK™ streptavidin conjugate (the ITK series), as Quantum dots in this catalog are designed for the purpose of FRET. The regular Qdot series are conjugated with a PEG layer to prevent non-specific binding, especially for cellular labeling. However, this additional coating enlarges the donor-acceptor distance, resulting in reduced energy transfer efficiency.

D. Fabrication of the SU-8 Masters Using Standard Photolithography

1. Si wafer is piranha cleaned and baked at 200 °C for 5 min.
2. For the designated master thickness of 25µm, spin coat the negative photoresist (SU-8 2025, Microchem, Newton, MA) on Si wafer at 2000 rpm for 30 sec.
3. Soft bake the wafer on a hotplate with a ramp of 65 °C/hr to 95 °C.
4. Expose to UV light (365nm) for 250mJ/cm^2 through a mask film (CAD/Art Services, Bandon, OR) containing the design of microchannels.
5. Post-exposure bake the wafer on a hotplate with a ramp of 65 °C/hr to 95 °C.
6. Develop the wafer using SU-8 photoresist developer.
7. The patterned wafer is hard baked on a hotplate with a ramp of 65 °C/hr to 200 °C. Maintain the wafer at 200 °C for at least 5 hours, then gradually cool the wafer down to room temperature.

Important: Gradual ramping during the SU-8 master baking process is necessary, otherwise the SU-8 structure may detached from the silicon wafer or cracks on the SU-8 structure may be induced by stress-release.

E. Replica Molding of PDMS from the masters and Bonding to the Cover Glass

1. The SU-8 master is placed in a weighing boat.
2. Mix silicone elastomer and curing agent (Poly(dimethylsiloxane), PDMS, Sylgard 184, Dow Corning, Midland, MI) in a 10 : 1 ratio.
3. Pour the PDMS mixture onto the SU-8 master and leave the weighing boat in a vacuum desiccator to remove bubbles.
4. Cure the PDMS at 65 °C for 1-2 hours.
5. Peel the PDMS strip from the Si master mold.
6. Punch channel inlets and outlets of the fluidic device.

7. Clean the PDMS strip and cover glass with ethanol and then air-dry.
8. Treat the cleaned PDMS strip and cover glass with oxygen plasma (20W for 1min).
9. Immediately bond the PDMS strip with cover glass.
10. Leave the bonded microfluidic chip in the oven at 95 °C for overnight.

Important: Plasma treatment and overnight baking are essential to enhanced bonding strength.

F. Monitor the formation of DNA Nanocomplexes In the Microfluidic Device

1. Fill the microfluidic channel with water (to ensure there is no bubbles within the microfluidic channel), before loading the reagents to ensure smooth flow during the experiment.
2. Load the QD-labeled DNA and Cy5-labeled chitosan solutions into two individual glass syringes, through the tubing described in the video.
3. Connect the tubing with the two inlets of microfluidic devices. Be cautious not to introduce any air during the process. Set the flow rate at 20nL/min (PHD-2000 syringe pump, Holliston, MA), under laminar flow conditions.
4. Check the microchannels under the microscope.
5. When the flow is stable (~15 to 20 minutes), QD-mediated FRET should be observed in the center of the channel.
6. Take fluorescence pictures (Cooled CCD, Qimaging, BC, Canada) at different locations along the channel.
7. Analyze the fluorescence images with ImageJ and OriginLab.

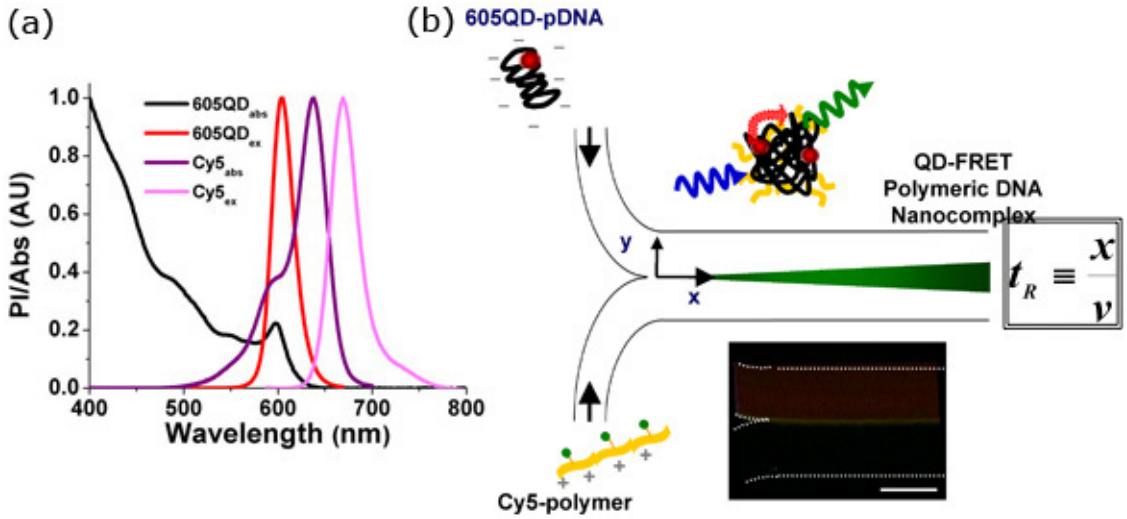

Figure 1. QD-FRET provides a sensitive indication of the onset of DNA Nanocomplexes self-assembly

1. Quantum dot-mediated fluorescence resonance energy transfer (QD-FRET) can provide a quantitative and highly sensitive indication of polyplex stability in either extra- or intra-cellular environments, allowing for unambiguous detection of the onset of interactions between DNA and the gene carrier. The FRET pair, 605QD and Cy5, was chosen based on maximizing spectral overlap between the donor and acceptor and minimizing potential cross-talk. For this pair, the Förster distance is 69.4Å.[3]
2. Self-assembly of the QD-FRET DNA nanocomplexes. Anionic plasmid DNA (pDNA) and the cationic gene carrier were labeled with QD (energy donor) and Cy5 (energy acceptor), respectively. QD-FRET nanocomplexes were formed through electrostatic complex coacervation. Upon excitation at 488 nm, QD-FRET-mediated Cy5 emission indicated formation of a compact and intact nanocomplex. The residence time (t_R) can be calculated according to the distance (x) which measures from where the two streams meet to the position of reaction under investigation, and the mean flow speed (v). Due to the nature of laminar flow, mixing only takes place at the interface (center of each image), allowing precise calculation of mass transport as a function of t_R. Temporal resolution can be adjusted by varying the applied flow rates. (Inset) FRET-mediated signal was observed immediately at the interface when the two streams met, indicating that binding was rapid, occurring within a few milliseconds according to the applied flow rates. Scale Bar: 100µm.

Discussion

- Significance of our work:
  1. This is the first attempt to monitor polymeric DNA nanocomplex self-assembly kinetics in real-time (millisecond resolution) through QD-FRET responses within a simple microfluidic chip.
  2. QD-mediated FRET provides a highly sensitive and quantitative indication of the onset of molecular interactions and throughout the self-assembly process, whereas microfluidics offers a well-controlled microenvironment to spatially analyze the process during the DNA nanocomplex synthesis.
  3. The integration of microfluidics and nanophotonics suggests a new and interesting approach to investigate any type of complexation reactions.
  4. The resulting QD-FRET polymeric DNA nanocomplexes could be readily applied for establishing structure-function relationships.[1,2]

Disclosures

The authors have nothing to disclose.

Acknowledgements

Funding support provided by NIH grant HL89764, NSF grants 0546012, 0730503 and 0725528.

References

1. Ho, Y. P., Chen, H. H., Leong, K. W. & Wang, T. H. Evaluating the intracellular stability and unpacking of DNA nanocomplexes by quantum dots-FRET. *J Control Release* **116**, 83-89 (2006).
2. Chen, H. H. *et al.* Quantitative comparison of intracellular unpacking kinetics of polyplexes by a model constructed from quantum dot-FRET. *Mol. Ther.* **16**, 324-332 (2008).
3. Zhang C. Y., Yeh H. C., Kuroki M., Wang T. H. Single-Quantum-Dot-Based DNA Nanosensor. Nat Mat 4, 826-831 (2005).