Published in final edited form as:

Anal Chem. 2006 May 1; 78(9): 2939–2947. doi:10.1021/ac051662f.

Optimization of dielectrophoretic DNA stretching in microfabricated devices

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Abstract

We have found that the surface and bulk solution properties in a microfabricated device affect the degree and probability of electrostretching of DNA molecules. Using lambda phage DNA, we found that significantly hydrophilic surfaces between the electrodes decreases the efficiency of stretching. Surfaces treated with higher silane (trimethyl chlorosilane) concentrations performed better presumably due to the decreased non-specific adsorption of DNA on these surfaces compared to their more hydrophilic counterparts. The shape and dimensions of the electrodes also affected the efficiency of stretching. Both lift-off and metal etching methods produced electrodes with random microscopic peaks along the electrode's edge and were poorly suited for stretching. Annealing the electrodes (450°C for 10 min) removed most of these peaks and allowed for more controlled stretching to be obtained. We also found that thin electrodes (65nm) gave close to a 90% success rate of DNA stretching but stretching with thick electrodes (350nm) produced only a 20% success rate.

Keywords

Dielectrophoresis; Single molecule manipulation; Microfluidic

INTRODUCTION

Manipulation of single biomolecules such as DNA, RNA, and proteins facilitated significant advances in biology. For example, an optical trap produced by highly focused laser light can be used to grab and move a molecule as well as exert measurable forces on biomolecules.^{1,2} These manipulations can be used to determine molecular properties such as elasticity both in a natural state and under tensional or torsional stress. Another example is the study of molecular motors by single molecule manipulation.³ By using optical tweezers to follow the progression of a single kinesin molecule on a microtubule rail, it has been found that the two-headed kinesin molecule progresses in steps of 8 nm for each ATP consumed.

Technological advances in micro/nano technology and high resolution visualizing systems facilitate single molecule manipulation. The trapping^{4,5} and stretching^{6,7} of DNA molecules, one of the most widely used molecules in single-molecule experiments, has been achieved using microdevices. Through high-resolution fluorescence microscopy, real-time restriction⁸ and replication⁹ of a single DNA molecule has been observed and, moreover, the transport properties of a single DNA molecule were obtained.¹⁰ In addition, the use of atomic force

microscopy (AFM)¹¹ have affected considerable progress in the arrangement and manipulation of single molecules.

DNA molecules can be stretched by various means including electrostatic, hydrodynamic, or magnetic forces. Washizu used dielectrophoresis and electroosmotic flow to stretch DNA molecules.^{6,7,13} Entropic constriction has also been employed to stretch DNA by electrostatic forces.¹⁴ In order to use a magnetic force, magnetic tweezer¹⁵ that use magnetic fields and beads are necessary. Stretching by hydrodynamic force was performed either with free molecular ends or with one end immobilized to a solid surface.^{16,17,18} In practice, anchoring methods tend to give more consistent stretching than free-end methods. Thus most DNA stretching involves anchoring one end of the DNA molecules onto an electrode, bead, or other solid surface, and stretching the molecule using some type of force.

Dielectrophoretic stretching is one of the most common methods in DNA manipulation.^{6,7} Dielectrophoresis is a non-contact manipulation technique that enables positioning and orientating DNA molecules in micro and nano devices. A Dielectrophoretic force results from the interaction between an induced dipole on the molecule and a non-uniform electric field, and is a function of frequency, electric field and dielectric properties of the molecule and medium. Depending on the conditions, the molecule moves either toward (positive dielectrophoresis) or away from (negative dielectrophoresis) the region of high electric field gradients. If the molecule is more polarizable than its medium, positive dielectrophoresis is observed; conversely negative dielectrophoresis will occur if the molecule is less polarizable. In order to stretch DNA molecules, high frequency (>1MHz) and high voltage (~1 MV/m) AC electric fields are typically used and result in negative dielectrophoresis.

In this work, we have studied the parameters affecting dielectrophoretic stretching of DNA in a microfabricated device and found the stretching is a function of the surface, microelectrode, and bulk solution properties. We have optimized those parameters and been able to achieve more constant DNA stretching.

MATERIALS AND METHODS

Device fabrication

The device for DNA electrostretching was made by bonding two separate sides: channel and electrode (Figure 1). Fluidic channels are made on thick glass (500 μ m thickness, 100-mm diameter, Dow corning Pyrex 7740 in Midland, MI) by hydrofluoric acid etching. 50nm thick chrome and 300nm gold layers were deposited on bare glass surface and photoresist (PR 1827, Shipley company in Newton, MA) was spun, patterned by channel mask layer and developed in MF 319 solution (Rohm and haas electronic materials in Spartanburg, SC). Metal layers on developed area were removed by commercial etchants (Gold etchant, Gold etch-type TFA, Transene Company, Inc. in Danvers, MA; Chrome etchant, CR-14 chromium photomask etchant, Cyantek Corporation in Fremont, CA). The channel shaped glass region was then etched by hydrofluoric acid (J.T. Baker in phillipsburg, NJ). After obtaining the desired depth (20 μ m), the remaining photoresist and metal layers over glass surface were stripped by photoresist removal solution (PRS 2000) and prospective etchants. The wafer was rinsed in DI water, dried by air, and diced into each pieces. Holes were drilled on both ends of channel by electrochemical discharge drilling to inject sample into channel.

Electrodes were fabricated on thin glass wafer (100 μ m thickness, 100-mm diameter, Precision Glass & Optics D263 in Santa Ana, CA). Two different methods were used in this work: Lift-off and Metal etching. For lift-off methods, photoresist was spun, patterned and developed on bare glass wafers. 15~50nm chrome layer and 50~300nm gold layer were deposited over developed photoresist. Then the wafer was immersed in acetone solution (J.T Baker) to lift-

off unwanted parts of the metal and was rinsed in DI water and dried. For metal etching, 50~300nm gold layer and 15~50nm chrome layer were deposited on bare glass wafer and photoresist was spun, patterned and developed. Metal layers were etched (Gold etchant, Gold etch-type TFA, Transene Company, Inc.; Chrome etchant, CR-14 chromium photomask etchant, Cyantek Corporation) and then the remained photoresist was stripped in PRS 2000 solution. The wafer was rinsed, dried and diced by diamond tip glass cutter (Glass cutter diamond, Fisher Scientific). For each process, some wafers were annealed at around 450°C for 10 min to improve the edge of electrode.

The obtained glass channel and electrode sides were bonded by optical adhesive (Type SK-9 Lens bond, Summers optics in Hatfield, PA). The bonded device was wired to printed circuit board (PCB) by gold or aluminum wire bonding for electrical contact. The backside of channel remained opened for accessibility during viewing in an inverted microscope.

Surface treatment

Four different surfaces were prepared in this work: clean glass, piranha cleaned surface, sigma-cote coated surface, and tetramethylchlorosilane (TMCS) coated surface. For the clean glass surface, both channel and electrode pieces were cleaned sequentially with acetone, isopropyl alcohol (2-Propanol, J.T. Baker) and DI water and bonded by UV curing. Piranha cleaned surface was made by soaking in piranha solution (1:3 v/v of hydrogen peroxide (Hydrogen peroxide 30%, J.T. Baker) and sulfuric acid (J.T. Baker)) for 40 min. The glass pieces were washed sequentially with water, warm acetone (120°C), warm isopropyl alcohol (IPA) (120°C) and water, and then dried.

For sigma-cote coated surface, sigma-cote solution (Sigma-Cote, Sigma Aldrich) was injected into channel and left to be dried. To get more lasting surface, it was placed in a 100°C oven for 30 min. To treat surfaces with TMCS (Chlorotrimethylsilane, Sigma Aldrich), the inside of the channel was incubated for 1 hr by TMCS mixture (1:5 v/v solution of TMCS in tetrachloroethane (1,1,2,2-Tetrachloroethane, Sigma Aldrich) solution) and the channel was washed three times with tetrachloroethane (TCE), IPA and DI water, and then air-dried.

To measure surface contact angles, 2 μ l of water was dropped on the treated surface and the drop image was captured horizontally. The image was then analyzed by Image J software (NIH) to measure the angle between the baseline of the drop and the tangent at the drop boundary.

DNA sample preparation

Plain λ -phage DNA and thiol-labeled λ -phage DNA were prepared in this work. For thiol-labeled λ -DNA, λ -phage DNA (New England Bio Lab in Ipswich, MA) with a 12 base-long single stranded “overhang” on each end was obtained and oligonucleotides for “overhang” sequence were synthesized. 5'-termini of λ -DNA was dephosphorylated by shrimp alkaline phosphate. A ligation reaction was done by T4 ligase enzyme and the concentration of thiol-labeled λ -DNA was measured using a spectrometer. The thiol-labeled DNA was labeled again with fluorescent marker YOYO-1 (Molecular Probes in Carlsbad, CA) at a dye-to-base-pair ratio of 1:5.

DI water (pH 8, conductivity 2 μ S/cm) plus 20% β -mercaptoethanol to prevent photobleaching was used as sample buffer and was mixed with 4 wt% of linear polyacrylamide (CEQ Separation gel LPA-1, Beckman Coulter in Fullerton, CA) to enhance molecule stretching. A high concentration of β -mercaptoethanol (20%) was used in this work similar to that used in the work by Namasivayan et al. in 2002 but lower concentrations (4%) have also been used successfully.⁴ Labeled DNA molecules were mixed with that sample solution to make final concentration of DNA sample as 50 pg/ μ l. After mixing, the DNA sample was degassed under the vacuum for 30 min.

Electric field application

Alternating current (AC) voltage was generated from a function generator (MX-9300A, Elenco). The frequency was regulated from 1 Hz to 2.3 MHz and the amplitude was ranged from 2 V_{p-p} to 25 V_{p-p}. Waveforms were monitored by an oscilloscope (TDS 3014B, Tektronix). Figure 2(a) shows the experimental set-up for electric field application. The gradient of electric field around electrode was estimated by electric field simulation (Maxwell software, Ansoft).

Fluorescence microscope

An Inverted fluorescence microscope (TE-200, Nikon) was used to observe the motion of single DNA molecules. High magnification (x100) oil immersion objective was used to get high resolution and an electronic shutter was used to prevent photobleaching. The molecular behavior was visualized using a digital CCD camera (Photometrics Cascade 512B Cooled CCD 16Bit Back Thinned Camera, Roper in Tucson, AZ) and data analysis was done by Metaview software. A 100-W mercury lamp with FITC filter was used as an excitation source. Time-sequenced images were taken with 50~100 ms exposure time per image to obtain time-dependent behavior.

RESULT AND DISCUSSION

Fundamental DNA stretching system

The device we constructed for DNA stretching uses dielectrophoresis to capture and elongate DNA molecules. By using a non-uniform electric field generated by a pointed electrode (high field gradient) and a straight electrode (low field gradient), we can control the movement and extent of stretching, as shown in Figure 2. Generally, DNA molecules are attracted to the higher field gradient region at low frequency (~1 KHz) and stretched by negative dielectrophoresis at high frequency (~1MHz).¹⁹ For immobilization, a thiol-and-gold method was incorporated as described previously.²⁰

The directional movement of the molecules is frequency dependent in accordance with their dielectric properties. That is, the transition frequency at which the dielectrophoretic force reverses from positive to negative will depend on the molecule's dielectric properties. In our system, we applied low frequencies of 500 Hz to 100 kHz, and observed that most molecules moved toward a higher electric field gradient region. Molecules were repelled from high field-gradient regions if frequencies higher than 700 kHz were used. There was a combination of positive and negative dielectrophoresis in the frequency range of 100 kHz to 700 kHz (i.e., some molecules moved toward gradients and some molecules moved in the opposite direction).

DNA elongation is a strong function of solution conditions such as polymer concentration, conductivity, and pH values. Figure 3 shows the effect of polymer concentration on DNA stretching. The highest average (15 μ m) and maximum (22 μ m) stretched length we observed in this polymer-assisted system was with 4 wt% LPA solution. This result strongly supports the previously reported result that approximately 5 times the critical entanglement concentration of this polymer is optimal for stretching.²⁰

A low conductivity, pH 8 buffer is optimal for DNA stretching. Stretching experiments were performed with solutions of pH 4, 8 and 10, but elongation of DNA could only be obtained in the pH 8 solution indicating that dielectrophoretic stretching occurs best near physiological pH values. Low conductivity solutions are also best for dielectrophoretic stretching. High conductivity solutions produce a large amount of joule heating, and resulting molecular movement depends on the temperature increase in the fluid and not on the dielectric force. Moreover, a high level of bubble generation was observed that disrupted the stretching process.

Molecular motion can also be induced by convection. Stretching of DNA molecules and circulation of peripheral molecules occurs at the same time during the dielectrophoresis process when the frequency is higher than 700 kHz. The molecules attracted to the region between the pointed and straight electrodes move linearly, and the molecules immobilized on the pointed electrode are stretched along the field lines. Those molecules that exist outside the gap move in a circular fashion (Figure 4). Minerick et al.²¹ have reported a similar observation during the manipulation of red blood cell in an AC field. They measured both dielectrophoresis linear velocity (either toward or away from high field density region) as a function of AC frequency and angular velocity of electrorotation as a function of frequency. We measured the velocity of negative dielectrophoresis by particle tracking and the value obtained at 2 MHz ($\sim 2.7 \mu\text{m}/\text{sec}$) is in the range of previously reported negative dielectrophoretic velocities. We also observed that the velocity of DNA increased with the increasing frequency, a result consistent with the work of Minerick et al..

Microfabricated electrode characterization

Electrode edge smoothness plays an important role in attracting DNA molecules to the intended stretching area. Rough electrode edges provide many small points along the electrode's edge that generate high electric field gradients resulting in molecular movement. Figure 5(a) shows how DNA molecules are attracted to the rough edges of the electrode, particularly on the straight electrode (vertical white trace on electrode edge), while Figure 5(b) shows how the DNA molecules are attracted to the smooth pointed electrode area only. Pixel intensity measurements of a horizontal line that includes both edges of the electrodes (Figure 5(c,d)) confirm this observation by showing similar intensities on both edges when rough electrodes are used but only one high peak on the pointed electrode side for smooth electrode edges.

We are able to obtain this smooth electrode edge by performing electrode annealing following metal etching. Figure 6 shows scanning electron microscopy (SEM) images of the top and cross-sectional views of a gold electrode after fabrication by lift-off and etching. As seen in the figure, both methods lack a highly smooth and straight edge. Annealing of the 300 nm gold layer at 450°C for 10 min was then performed and the resulting electrode had a smooth edge (Figure 7).

The thickness of the electrode also affects DNA stretching with thinner metal electrodes giving a higher probability of stretching than thicker electrodes. The probability includes both completely and partly stretched molecules (there were always molecules in the gap both in free solution and attached to the surface that did not stretch). Three different thicknesses (350, 135, 65 nm) were used to evaluate the success rate of the stretching. Substantial increase in the probability of observing DNA stretching is obtained when using a thinner metal layer (Figure 8). The cause of this phenomenon is most likely due to the higher vertical field gradient obtained with the thinner electrode.

The electric field properties such as the field gradient, strength, and frequency are adjustable parameters in dielectrophoretic DNA stretching. The tip size of the pointed electrode was varied to investigate how it affects DNA stretching. Figure 9(a) shows the voltage value required to cause positive dielectrophoresis of DNA molecules (i.e., attraction to the high field gradient region). As can be seen from the figure, a smaller voltage value is required to initiate positive dielectrophoresis movement when a sharp electrode tip is used than with a more rounded electrode. A similar phenomenon was observed when higher frequencies were applied to repel DNA from high field-gradient regions (negative dielectrophoresis). As seen in Figure 9(b), the required voltage for a 20 μm electrode tip is approximately 2 times higher than the voltage for a 3 μm electrode tip. The lower voltage for sharper electrodes is a result of the higher electric field gradient generated compared to blunter electrodes at the same voltage.

Changing the gap between the pointed and straight electrodes can also influence DNA stretching. As seen in Figure 10, the effect is more noticeable when the sharp tip is used but negligible with blunt tips. The gap width also affects the formation of DNA “bridges” between electrodes. The average stretched length of λ -DNA in most cases was 15 μm , and DNA bridges were quite often observed when the gap width was narrower than 15 μm . When a gap of 10 μm was used, both ends of the DNA molecule are attracted to the surface of the metal electrodes. When this occurred, the DNA molecule did not shrink after the electric field was turned off.

Electric field properties

DNA molecules can be stretched in our system at frequencies higher than 500 kHz, but steady, stable stretching could not be obtained below 2 MHz. At 700 kHz, the stretched DNA would move away from the electrodes immediately after stretching and would not remain in the gap. Those DNA molecules that were stretched in the fluid flow and failed to remain stationary. At 1 MHz, one end of the DNA molecule would easily attach to the pointed electrode and remain stretched. However, the molecule did not maintain its stretched form for more than 2 seconds, and would recoil back to the pointed electrode. At 2 MHz, the stretched DNA was stable meaning that it did not fluctuate or collapse as long as the field was applied.

Bubble generation occurs easily at low frequencies due to electrolysis. Higher frequencies reduced this reaction with virtually no bubbles generated at frequencies higher than 100 kHz. As seen in Figure 11, bubbles are generated at low voltages when a sharp electrode and low frequency are applied. Moreover, the voltage for bubble generation is quite close to that for molecular attraction, especially in case of a sharp electrode.

Surface properties

The length of stretched DNA molecules is longer on surfaces with a high contact angle than those with a low contact angle. We performed DNA stretching on surfaces with two different contact angles: an untreated glass surface ($\theta=40^\circ$) and a silane coated surface ($\theta=80^\circ$). Figure 12 shows that more than 70% of the molecules on the 80° surface are longer than 15 μm compared to only $\sim 30\%$ on the 40° surface. The fact that a longer stretch length is obtained on more hydrophobic surfaces is consistent with literature data on other stretching techniques.¹⁸

To further investigate the effect of surface hydrophobicity, the surface contact angle was modified by changing the dilution ratio of TMCS in TCE. Figure 13(a) shows the dilution ratio of TMCS in TCE as well as the contact angle of each surface. Figure 13(b) shows the average stretched length of a DNA molecule over the silane-coated surface of several silane concentrations. As seen in these two figures, an increase in TMCS concentration produces a higher contact angle and results in a longer stretched length. The maximum length of the averaged values (15.7 μm) is obtained at the 1:5 ratio of TMCS to TCE, and the longest stretched length of DNA at that point is 25 μm , a length appreciable longer than the fully stretched length of λ -DNA. The stretched length decreases above the 1:5 ratio with the length at high TMCS concentration being shorter than on a clean glass surface with no silane. This result suggests that a surface contact angle of 85° may be optimum for DNA stretching although additional experiments with other surface treatment agents need to be performed to confirm this hypothesis.

Surfaces with high surface contact angles inhibit the molecules' nonspecific adsorption. Figure 14 shows sequential images of DNA stretching (a) over a clean glass surface and (b) over a silanated surface. In Figure 14(a), stretched molecules are observed sticking to the glass surface even after the electric field is turned off. In addition, several coiled DNA molecules stick randomly on the more hydrophilic glass surface. However, the stretched molecules on the hydrophobic surface recoil easily when the electric field is turned off (Figure 14(b)).

Nonspecific adsorption of other DNA molecules was not observed on this surface in any significant degree. In comparing the two, the rate of nonspecific adsorption is almost 4 times lower on silane-coated surfaces than on untreated glass. We have counted the number of DNA molecules in 10 minutes that adhere to a 60 μm^2 surface and found that approximately 11 molecules stick to the silane-coated surface while 40 molecules stick to the clean glass surface.

CONCLUSIONS

In this paper, we have investigated the optimum conditions for robust and controllable DNA electrostretching. By optimizing the solutions conditions, electric field strength and shape, and the surface properties, success of DNA stretching can approach 90%. Also, stretched molecules on more hydrophobic surfaces are less adherent and may be more amenable for additional interactions. For example, certain proteins that bind to DNA may be somewhat inhibited from binding if a stretched molecule is adhered to the surface at a large number of locations.

Molecular manipulation by dielectrophoresis is a relatively common technique but there are still unexplored topics in this area. Additional studies are needed to investigate other parameters such as temperature, surface property and buffer-ion dependence on stretching. With the design of more reliable microfabricated DNA stretching devices, integrated systems may be possible that perform multiple analysis operations on a single DNA molecule extracted from a single cell or other minute samples.

ACKNOWLEDGEMENT

The authors thank Prof. Ronald G. Larson and Prof. David T. Burke for helpful discussions during this work. The authors also thank to Mr. Ji Hoon Kim for helping with thiol labeling of lambda phage DNA. The authors also acknowledge National Institute of Health (P01-HG001984) and National Science Foundation (BES0304316) for funding this project.

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Figure 1. Schematic process of microfabricated electrostretching device. (a) lift-off, (b) metal etching for electrode fabrication.

Figure 2.

Dielectrophoretic stretching of DNA. A non-uniform electric field is generated by a pointed electrode and a straight electrode, and one end of molecule is immobilized at the electrode edge by thiol-and-gold linking. Molecules are attracted at 1KHz, 7 Vp-p (a) and stretched at 1MHz, 23Vp-p (b).

Figure 3.
Effect of LPA concentration to DNA stretching, Approximately 4 wt% of LPA gave the longest stretched DNA length.

a **b** **Figure 4.**

(a) Direction of DNA movement under negative dielectrophoresis condition. Molecules between the electrode gap move linearly and are stretched, and molecules outside of the gap have the motion of co-field rotation. (b) Time sequence of DNA movement under negative dielectrophoresis condition.

Figure 5.

DNA stretching using (a) a rough electrode edge and (b) a smooth electrode edge. Many molecules were attracted to the random peaks of the rough electrode edge while DNA molecules are only gathered around the pointed electrode area in (b). (c),(d) Gray levels obtained through horizontal lines that includes both electrode edges (higher value represents brighter point). Similar degree of gray level was obtained on both electrode edges when rough electrode edge was used (c) while only one high peak was obtained at the pointed tip electrode side when smooth electrode edge was used (d). These graphs were obtained by averaging 6 different lines from different figures.

Figure 6. Scanning electron microscopy (SEM) images of the top and cross sectional view of gold electrode after fabrication by lift-off (a, b) and metal etching (c, d).

Figure 7. SEM image of electrode after annealing at 450°C for 10 min. Annealing was performed after metal etching to make smooth electrode edge. (a) Top view, (b) cross-sectional view.

Figure 8. Effect of electrode thickness on DNA stretching. Substantial increase in the probability of observing DNA stretching was achieved with a 50 nm thick gold layer.

Figure 9.

Voltage required initiating DNA attraction to the pointed tip electrode region (a) and voltage required initiating DNA repulsion from the pointed tip electrode region (b). In both cases, smaller voltages are required to cause molecule movement when sharper electrodes are used.

Figure 10. Effect of different gap sizes on stretching. The effect is more noticeable when a sharp tip is used but negligible with a blunt tip.

Figure 11. Bubble generation. Bubbles are generated at low voltage when a sharp electrode and low frequency are applied.

Figure 12.

The stretched length distribution on both higher contact angle surface (80°) and lower contact angle surface (40°); longer stretched length is obtained on more hydrophobic surface

Figure 13.

Surface contact angles over coated surfaces with different silane concentration (a), and averaged stretched length of DNA molecules over the silane-coated surface of different surface contact angles (b). Higher silane concentration produces higher surface contact angle and longer DNA stretched length.

Figure 14. Sequential image of stretched DNA over (a) clean glass surface and (b) silane-coated surface. Stretched molecules over silane-coated surface did not adhere to the surface and were free in the solution.