Real-time monitoring and manipulation of single bio-molecules in free solution

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Hung-Wing Li

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Program of Study Committee:
Edward S. Yeung, Major Professor
Mei Hong
Robert S. Houk
Marc D. Porter
Xueyu Song

Iowa State University

Ames, Iowa

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Graduate College
Iowa State University

This is to certify that the doctoral dissertation of
Hung-Wing Li
has met the dissertation requirements of Iowa State University

Major Professor

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ABSTRACT

The observation and manipulation of single biomolecules allow their dynamic behaviors to be studied to provide insight into molecular genetics, biochip assembly, biosensor design, DNA biophysics.

In a PDMS/glass microchannel, a nonuniform electroosmotic flow (EOF) was created. By using a scanning confocal fluorescence microscope and total internal-reflection fluorescence microscope (TIRFM), we demonstrated that negatively charged DNA molecules were focused by the nonuniform EOF into a thin layer at the glass surface. This phenomenon was applied to selectively detect target DNA molecules without requiring the separation of excessive probes and can be applied continuously to achieve high throughput.

A variable-angle-TIRFM was constructed for imaging single DNA molecule dynamics at a solid/liquid interface. Implications we have are that the measured intensities cannot be used directly to determine the distances of molecules from the surface and the experimental counting results depict the distance-dependent dynamics of molecules near the surface. Molecules at low ionic strengths experience electrostatic repulsion at distances much further away from the surface than the calculated thickness of the electrical double layer.

λ -DNA was employed as a nanoprobe for different functionalized surfaces to elucidate adsorption in chromatography. The 12-base unpaired ends of this DNA provide exposed purine and pyrimidine groups for adsorption. Patterns of self-assembled monolayers (SAMs) and patterns of metal oxides are generated. By recording the real-time dynamic motion of DNA molecules at the SAMs/aqueous interface, the various parameters governing the retention of an analyte during chromatographic separation can be studied. Even subtle differences among adsorptive forces can be revealed.

Dynamic conformational changes of the prosthetic group, flavin adenine dinucleotide (FAD), in flavoprotein NADH peroxidase, in thioredoxin reductase, and in free solution were monitored with TIRFM. FAD bound loosely in the proteins changed from the stacked conformation to the unstacked conformation upon laser excitation. FAD in free solution not only underwent conformational changes but also reacted with each other to form a dimer.

Direct measurement of the single-molecule enzymatic cleavage rates of ApaI-DNA complex in the presence of various concentrations of MgCl_2 solution is reported. Results suggest that there exists a distribution of ApaI conformations around the restriction site.

CHAPTER 1. GENERAL INTRODUCTION

Dissertation organization

This dissertation begins with a general introduction to single molecules detection technique. The following chapters are five complete scientific manuscripts. General conclusions summarize the work.

Overview

In the last two decades, single molecules detection and measurement has been performed with a variety of optical experiments¹. The observation and manipulation of single biomolecules allow their dynamic behaviors to be studied to provide insight into molecular genetics²⁻⁴, biochip assembly⁵⁻⁸, biosensor design⁹⁻¹¹, DNA biophysics¹²⁻²⁶, and basic separation theories of capillary electrophoresis (CE) and liquid chromatography (LC)²⁷⁻³⁴. Detection of fluorescence from individual molecules of interest is used for these ultrasensitive analytical and biophysical applications. Measurement at single molecules level can remove the complexity related to ensemble-averaged macroscopic measurements and carry more information than bulk measurements. Since there is no ensemble averaging involved, only single-molecule detection can give an appropriate test for microscopic dynamical theories. Using single-molecule spectroscopy one can, in principle, follow the temporal evolution of any complex reaction path.

Almost all single-molecule imaging methods nowadays rely on lasers to excite fluorescence from the molecules³⁵. Lasers can produce high fluxes of photons so that individual molecules can be forced to absorb and emit up to a million times before they photobleach. Large DNA or protein molecules can be labeled with many highly-fluorescent fluorophors in order to further facilitate detection. Array imaging devices (like CCD) are now widely available with sensitivities capable of recording single photons. This combination is responsible for the success in making single-molecule imaging into a routine laboratory tool. The challenge is to collect as many photons as possible from each molecule and to utilize the signal appropriately.

Basic principles

Basically, single-molecule fluorescent imaging is accomplished by two deceptively simple steps: (i) guaranteeing that only one molecule is in the volume probed by the laser,

and (ii) providing a signal-to-noise ratio from each single-molecule great enough to be distinguishable from the experimental noise.

The first criteria can be easily achieved by a combination of focusing the laser to a small probe volume and having an ultralow concentration of the molecule of interest. However, achieving the second criteria is rather challenging. To have high enough SNR, one requires to pay close attention to maximizing signal while minimizing backgrounds and the laser shot noise. For fluorescence methods, to obtain as large a signal as possible, a molecule of interest or labeling dye with the highest possible fluorescence quantum efficiency and photostability should be selected. In addition, undesirable fluorescent impurities must be excluded, the probed volume is minimized to reduce Raman scattering, and any Rayleigh scattered radiation at the pumping wavelength is rejected.

Signal size. The signal of interest is that arising from the fluorescent photons emitted by the single molecule. In fact, the number of photons emitted per second for one molecule is far smaller than the number of photons per second in the incident laser beam. Fortunately, that fact that the emitted photons are generally red-shifted to longer wavelengths enables detection with reasonable SNR.

Typical fluorescent molecules used for single molecules detection come from the general classes of laser dyes used in biological fluorescent labeling (rhodamines, cyanines, oxazines, etc.)³⁶, rigid aromatic hydrocarbons such as terrylene, or perylene diimides, as well as autofluorescent, such as R-phycoerythrin (RPE), genetically expressed proteins like the green fluorescent protein (GFP) and its relatives³⁷⁻⁴³. Lately, covalently labeling dyes are available from Molecular Probes.

For single DNA molecules imaging experiments covered in this dissertation, a highly fluorescent dye, YOYO-I (Molecular Probes, quantum efficiency = 0.52, excitation and emission wavelengths are 491 nm and 509 nm respectively), is used to label the DNA species. Some proteins are naturally fluorescent due to presence of one or many fluorophores as cofactors, and these have also provided useful single-molecule signals in the cases of flavoprotein (chapter 4). Strong fluorescence single semiconductor quantum dots have been proposed as biological labels. This approach requires methods for attachment of the dots to biomolecules, an area of intense current research (chapter 2).

Background issues. To detect a single molecule, one must simultaneously maximize signal while at the same time scrupulously minimize background photons, noise from unwanted sources, and detector dark counts. Background signals generally scale linearly with the laser power, while detector dark counts is rather low.

Appropriate filters are always essential for suppressing Rayleigh scattering of the excitation radiation. Ideally, filtering for single-molecule experiments must attenuate the Rayleigh scattering while transmitting the desired red-shifted fluorescence from the single molecule by the largest factor. Optical filtering for a specific experiment is usually accomplished by low-fluorescence glass filters, interference filters or by relatively expensive holographic notch attenuation filters.

Due to limitations from photobleaching, one should make the laser power/exposure time/SNR tradeoff carefully. By controlling the laser intensity, the rate at which photons are emitted can be easily varied up to the maximum rate. Observation time scale of the most interest should be chosen for tracking the same bunch of molecules: for fast time information, high laser intensities could be used to give the best possible SNR in short counting intervals before photobleaching. If long-time information is needed, lower intensities would be better. It is also very useful in some experiments to pulse the probing laser at low duty cycle and observe the molecule only briefly with long intervening dark periods.

Another simple but important technique is to minimize the native fluorescent from solvent impurities or the buffer solution, which contribute to the background signal, by photobleaching the buffer solution with a UV lamp overnight.

Microscope configurations. Successful microscopic techniques include scanning methods, such as near-field scanning optical microscopy and confocal microscopy, and wide-field methods, such as total internal reflection and epi-fluorescence microscopy.

Perhaps it is simpler to use wide-field microscopy to observe single-molecule fluorescence. A laser or arc lamp is used to illuminate an area several tens or hundreds microns in diameter, as in a traditional microscope. Proper filtering is used to eliminate excitation light and to pass the single molecule fluorescence to a two-dimensional array detector, usually a CCD. Performing single-molecule imaging in this fashion has two major advantages: many

individual fluorophores can be observed simultaneously, and the position of fluorophores can be monitored at near video rates, allowing experimenter to observe translation of single molecules in real time. However, the primary limitation of wide-field microscopy methods is that the maximum frame acquisition rates for CCD cameras is slower than the response time for single element detectors (like photon multiplier tube), but for experiments requiring maximum temporal resolution on the order of 50 ms, wide-field methods have become a popular and useful single-molecule imaging technique. In this research dissertation, total internal reflection fluorescent microscopy is the main technique we utilized.

Total internal reflection fluorescent microscopy

Total internal reflection fluorescent microscopy (TIRFM) is also known as evanescent wave microscopy. The phenomenon of TIR has been employed to transmit data around the world through fiber-optic networks. Diamond cutters also take advantage of this to enhance sparkling and giving fire to cut diamonds. TIR happens when a beam of light travelling in a medium of high refractive index, such as glass, encounters one of lower refractive index such as the adjoining water or an adherent cell and the incidence angle exceed a certain angle, critical angle, the laser beam can no longer propagate into the second lower refractive index material but reflected back into the first.

Although the beam of light no longer travels through the second material, it creates an electromagnetic field called evanescent field (EF), which has an identical frequency to that of the incident light and extends in the z direction in a few hundred nanometers maximally (as illustrated in figure 1). This field undergoes exponential intensity decay with increasing distance from the surface.

The critical angle θ_c is given by,

$$\theta_c = \sin^{-1} (n_2/n_1)$$

TIRFM uses this electromagnetic field to excite fluorophores. Unlike normal beam of light that travels in a straight line infinitely, the evanescent field propagates parallel to the interface vanishing exponentially with the distance from the interface. So, using the EF, the excitation depth can be limited to a very narrow range in nm range. The decay length (d) of the EF along the depth of the field depends on the incident angle (θ), wavelength of the excitation beam (λ) and the refractive indexes of both the media n_1, n_2 .

$$d = \lambda / 4\pi(n_1^2 \sin^2 \theta - n_2^2)^{-1/2}$$

The angle-dependent decay constant d is typically ~ 150 nm for blue laser excitation. Intensity of the EF decays with depth from the interface (z) is given as:

$$I(z) = I_0 e^{-z/d}$$

where I_0 is the intensity at the interface.

Hence, the primary asset of TIR-based microscopy is that only fluorophores that are sufficiently close to the coverglass–air/water interface will be excited, while those further out in the bulk will not, and the volume of sample which can produce an interfering background is minimized and hence the sample thickness is not an issue. The decay length can be simply regulated with the incidence angle as demonstrated in chapter 3. By comparison, TIRFM provides excitation in an optical section approximately 10 times thinner than what a confocal microscope does. Fluorophor excitation confined to such a thin layer results in extremely high signal to noise ratio because, unlike epi-fluorescence illumination, it does not excite the bulk in a thick specimen. This enhancement in S/N makes TIRFM is good candidate for observing single molecule fluorescence.

Total internal reflection fluorescence is employed to investigate the interaction of molecules with surfaces (chapter 5), an area that is of fundamental importance to a wide spectrum of disciplines in cell and molecular biology. Examples are binding and triggering of cells by hormones, neurotransmitters, and antigens; cell adhesion to surfaces; electron transport in the mitochondrial membrane; cytoskeletal and membrane dynamics; cellular secretion events; polymer translation near an interface; single molecule interactions (chapter 4); and reaction rates (chapter 6).

Microscope objectives. The main function of an objective in fluorescence imaging is to collect the light from the sample as much as possible. Numerical aperture (NA) is the key measure of the ability of an objective to collect light. NA is defined as:

$$NA = n_0 \sin \theta$$

where n_0 is the refractive index of the medium between the lens and the sample and θ is the half angle of light cone collected by the lens through the medium. The amount of light collected is proportional to the square of NA and so small change in NA can cause a great difference. The higher the NA the more light is collected.

Detector. Charge-coupled device (CCD) camera is used for all imaging experiment in this dissertation. The camera detection efficiency can be described by as:

$$\text{Detection efficiency} = \text{QE} \times \text{pixel area} \times \text{time}$$

Hence,

$$\text{Total signal, } S = \text{photon flux density} \times \text{QE} \times \text{pixel area} \times \text{time}$$

Where photon flux density is defined as the number of photos per unit area per unit time arriving the detector. QE is the quantum efficiency of the CCD and it is defined as the fraction of incoming photons that generate a signal in the CCD and it is a function of wavelength of light hitting the CCD.

Once an experimenter has an optimal QE, the signal intensity then will be a function of the CCD pixel area: the larger the pixel, the larger the signal and the higher the dynamic range. However, the trade off is that increasing the pixel size reduces the spatial resolution achieved by the CCD.

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CHAPTER 2. MANIPULATION OF SINGLE DNA MOLECULES VIA LATERAL FOCUSING IN A PDMS/GLASS MICROCHANNEL

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Jinjian Zheng, Hung-Wing Li, and Edward S. Yeung

Abstract

In a hybridized microchannel made of poly(dimethylsiloxane) (PDMS) and glass, nonuniform electroosmotic flow (EOF) was created when an electric field was applied. PDMS and glass pieces were bonded at room temperature after oxidization with oxygen plasma. After oxidization, the PDMS surface has a higher zeta (ζ) potential than that of glass, which results in faster EOF at that surface. When the channel has a large aspect ratio (width $\gg$ depth), the EOF can be considered as a two-dimensional gradient shear flow. By using a scanning confocal fluorescence microscope, we demonstrated that negatively charged DNA molecules were focused by the nonuniform EOF and the electric field into a thin layer at the glass surface of about one-fifth of the channel depth. This phenomenon was applied to selectively detect target DNA molecules using evanescent-field excitation. Streptavidin-conjugated quantum dots (QD) were used to selectively bind biotinylated DNA. After applying an electric field, free QD remained randomly distributed within the channel. Nonbiotinylated DNA molecules were focused onto the glass surface, but they were not labeled and thus nonfluorescent. Therefore, the increase in molecule count after application of the electric field can be solely attributed to QD-DNA complexes which were focused onto the glass surface. This method does not require the separation of excessive probes (QD) and can be operated continuously to achieve high throughput.

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Introduction

It is well-known that early detection of disease can improve the chances for successful treatment. For example, according to the American Cancer Society, the 5-year relative survival rate for all cancers is 62%. However, for cancers that can be detected at their early stages, the 5-year relative survival rate is about 82%.¹ Many diseases like cancer are caused by genetic alterations in certain genes.² Therefore, genetic analysis provides the opportunity to detect disease-associated genes or even predict diseases before the onset of physical changes in the cells. The past decade has seen significant progress in genomics research. Many disease-related mutations or genetic markers have been identified, and numerous genetic analysis methods have been developed.

We have reported several different high-throughput approaches to detect single target DNA molecules in a capillary or a microchannel.³⁻¹⁰ In a recent study, we demonstrated that, by applying a dc field and a Poiseuille flow from cathode to anode, DNA molecules can be focused into a narrow region of several microns around the axis of a capillary.⁸⁻¹⁰ Single molecule detection with an efficiency of $\sim 95\%$ was achieved.⁹ However, since detection only occurs in a narrow strip, the throughput of that method is limited and the overlap of molecules presents problems in single-molecule counting. To solve this problem, the three-dimensional (3D) Poiseuille flow must be replaced with a two-dimensional (2D) gradient shear flow. According to the focusing mechanism discussed in our previous work,⁸ under a gradient shear flow and an electric field, DNA molecules will be focused onto a thin layer instead of a narrow strip. The imaging capacity of the CCD camera can then be fully exploited and the throughput will be substantially improved. A typical 2D gradient shear flow can also be obtained by applying differential pressure between the horizontal walls of a rectangular channel, provided that the channel width is much larger than the channel depth so that viscous effects from the vertical walls can be neglected. During past years, remarkable progress in microfabrication has been made.¹¹⁻¹⁶ Microfabricated devices have been used in electrophoretic separation, chemical analysis, and chemical synthesis.¹⁴⁻¹⁷ Typically, to generate a hydrodynamic flow in a microchannel, a syringe pump or elevated buffer reservoir has to be attached to one end of the microchannel to supply the necessary pressure. In

addition to the need for creating a complicated structure, joint leakage often causes serious problems.

On the other hand, the profile of electroosmotic flow (EOF) in rectangular channels with different zeta (ξ) potentials at the walls has been characterized by Andreev et al.^{18,19} According to their mathematical model, it is possible to obtain a linear gradient in 2D shear flow, provided that the top and bottom walls have different ξ potentials and the width of the channel is much larger than the depth. Our approach is to induce asymmetric shear flow by using electroosmotic flow instead of hydrodynamic flow.

One flexible and cost-effective material for making microfabricated devices is poly(dimethylsiloxane) (PDMS).¹⁵ The PDMS microchannel has been widely used in electrophoresis. The behavior of EOF in this channel has been extensively studied, although the purpose of most studies is to suppress EOF or to obtain a uniform EOF.²⁰⁻²⁴ In this work, we used a PDMS/glass microchannel to obtain a gradient 2D shear flow. The lateral focusing effect of this device will be characterized. The application of this mode of focusing to sensitive immunoassay will be demonstrated by using streptavidin-conjugated quantum dots (QD) to detect single biotinylated DNA molecules.

Experimental Section

Optical Setup. Three different fluorescence-based imaging setups were used in this study. The first setup (Figure 1a) is an epi-fluorescence microscope (Prairie Technologies, Middleton, WI), which can also be used as a laser-scanning confocal microscope. When operated in the confocal mode, an argon ion laser (488 nm, Melles Griot, Carlsbad, CA) was used to provide the excitation. A dry-type Nikon Plan Fluor objective, 40x 0.75 NA, was used both to focus the excitation beam and to collect the fluorescence. This setup was used to obtain the molecular distribution in the microchannel by collecting sectional images in the z-direction.

The second setup (Figure 1b) is a home-built evanescent field fluorescence microscope, where excitation is based on total internal reflection through a quartz prism. A drop of index matching oil (type FF, $n = 1.46$, Cargille, Cedar Grove, NJ) was placed between the microchannel and the prism. An evanescent field ~ 100 nm thick was generated at the bottom surface of the channel, which was made of glass in this study. Only molecules

within the evanescent field can be excited and detected. A CCD camera (Cascade, Roper Scientific) was mounted on top of a standard upright microscope. A water-cooled argon ion laser (488 nm, Innova-90, Coherent, Santa Clara, CA) provided the excitation beam. A mechanical shutter with shutter controller (Uniblitz, Vincent Associates, Rochester, NY) was used to block the laser beam when the camera was off to reduce photobleaching. Fluorescence was collected by an oil-type objective lens (Zeiss 40x Plan-Neofluar 1.3 NA). A sequence of frames were acquired for each sample via V++ software (Roper Scientific).

The third setup (Figure 1c) is similar to that reported previously.⁸ Briefly, an intensified charge-coupled device (ICCD) camera (Pentamax, Roper Scientific, Princeton, NJ) was mounted on a Zeiss Axioskop upright microscope. An air-cooled 488-nm argon ion laser (Uniphase, San Jose, CA) provided the excitation beam. By use of a cylindrical lens, the laser beam was focused normal to the square capillary as a thin sheet. Only molecules in the center plane of the capillary ($\sim 10\ \mu\text{m}$ in thickness) were excited and recorded. A mechanical shutter with shutter controller (Uniblitz) was used to block the laser beam when the camera was off to reduce photobleaching. Fluorescence was collected by a dry-type Zeiss 20x 0.75 NA plan Apochromat microscope objective lens. Two 488-nm holographic notch filters with optical density >6 (Kaiser Optical, Ann Arbor, MI; HNFP) were placed between the objective and the ICCD to remove scattered light from the excitation beam. The camera was operated in the external synchronization mode with the intensifier disabled open. Data were acquired with the WinView software provided by Roper Scientific. This setup was used to record the migration of single molecules in a square capillary.

Microfabrication. The channel pattern used in this study is shown in Figure 1d. A positive photomask was used to make this pattern on glass. The line width is $300\ \mu\text{m}$. The spots at both ends have a diameter of 2 mm. The photoresist coated glass plate (Nanofilm, Valley View, OH) was $76 \times 25\ \text{mm}$ and $0.25\ \text{mm}$ thick. Wet etching technique was employed to create the pattern. The etching time was about 2 min, and the height of the master was about $46\ \mu\text{m}$. A PDMS microchannel was obtained with SYLGARD(R) 184 Silicone Elastomer Kit (Dow Corning, Midland, MI) according to the manufacturer's instructions. The mixture of base and curing agent was poured onto the channel master and cured at $60\ ^\circ\text{C}$ for 2 h. The PDMS film was peeled off from the channel master and bonded to a coverslip

(0.25 mm thick) to form an enclosed microchannel after oxygen plasma treatment. Two through holes were drilled in the PDMS sheet at the ends of the channel and served as buffer reservoirs.

Preparation of Biotinylated DNA. The biotinylated λ -DNA Hind III digest (New England Biolabs, Beverly, MA) was separated in 1% Seakem Gold Agarose gel (Cambrex Bio-Science, Rockland, ME) with 1x TE buffer (pH 8.0). The 23 130 bp band was excised and extracted with QIAEX II Agarose Gel Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The concentration of 23 130 bp biotinylated DNA after extraction was estimated to be 175 pM.

Quantum Dots (QD)-DNA Complex. The 2 μ M stock solution of Qdot 605 nm Streptavidin Conjugate (Quantumdot Corp., Hayward, CA) was diluted to 20 pM (concentration of streptavidin) and mixed with 23 130 bp biotinylated DNA. This solution includes 0.4 pM QD and 8 pM 23 130 bp biotinylated DNA and 112 pM nonbiotinylated 23 130 bp DNA. After vortexing, the mixed solution was left for reaction for 1 h. This solution was used without further dilution in this study.

YOYO-Labeled Lambda DNA. λ -DNA (48 502 bp, New England Biolabs) was labeled with YOYO-1 (Molecular Probes, Eugene, OR) at a ratio of 1 dye molecule per 5 bp according to the manufacturer's instructions. Typically, YOYO-1 labeled DNA samples were first prepared as a 200 pM stock solution, incubated at room temperature for at least 1 h, and diluted with corresponding buffer to the desired concentration just prior to the start of the experiment.

Buffer. The buffers used in this study are designated by the ionic strength. The 5 mM pH 8.0 Gly-Gly (Sigma, St. Louis, MO) buffer was prepared by titrating 5 mM NaOH (Sigma) to pH 8.0 with 50 mM Gly-Gly solution. The buffer was then filtered with 0.22 μ m filter, and left for photobleaching overnight with a mercury lamp.

Other Reagents. The fluorescent polystyrene microsphere (Sigma) has a diameter of $\sim 0.1 \mu$ m, and was diluted to 10 ppm with 1 mM Gly-Gly buffer. Other reagents are of analytical grade.

Data Analysis. The images were analyzed on a PC computer with ImageJ, a Java image-processing program (developed at the U.S. National Institutes of Health and available

on the Internet at <http://rsb.info.nih.gov/ij/>). For WinView files, the specific frames were first selected with the “Threshold and Clipping” function of the WinView software and saved as 8-bit TIFF files. The 8-bit TIFF files can then be opened and analyzed by ImageJ software. Images from the epifluorescence microscope can be opened directly by ImageJ. Before performing the “Image Analysis” function, the “Smooth” function was performed to reduce the noise and improve the accuracy in particle counting. By using the “Particle Analysis” function of ImageJ, we were able to determine the particle number in each frame, particle area, perimeter, center of mass, etc.

Results and Discussion

Profile of EOF in the PDMS/glass Microchannel. As shown by Andreev’s mathematical model,¹⁹ if the top and bottom surface have different ξ potentials, gradient 2D shear flow should be obtained. Before oxidization, a glass surface usually has a higher ξ potential than that of PDMS because of the presence of silanol groups. However, after exposure of both glass and PDMS to oxygen plasma, the ξ potential of PDMS increases significantly while that of glass remains almost constant.²⁰ The actual profile of EOF was obtained by using an epi-fluorescence microscope (Figure 1a) to measure the velocities of 100-nm fluorescent polystyrene microspheres at the glass and at the PDMS surfaces. As discussed in our previous work,⁸ polystyrene microspheres are rigid particles and cannot be deformed and thus cannot migrate laterally. Therefore, they will remain uniformly distributed within the channel and can be tracked at both surfaces. Images of the fluorescent microspheres at the PDMS surface (Figure 2a) and at the glass surface (Figure 2b) were recorded. The streak lengths indicate the migration distances of the microspheres within the exposure time of 850 ms. Since EOF dominates the electrophoretic mobility of the microspheres, the longer streaks imply faster EOF. That is, EOF at the PDMS surface was faster than that at the glass surface.

Lateral Focusing of λ -DNA in PDMS/Glass Channel. As seen in Figure 3, the lateral gradient in EOF velocities (arrows from left to right) will cause nonspherical or deformable particles, such as DNA, to align themselves off axis.⁸⁻¹⁰ Since DNA molecules are driven electrophoretically (EP) from right to left, they experience a lift force due to hydrodynamic drag and will be focused to the bottom (glass) surface. We used YOYO-

labeled λ -DNA to characterize the lateral focusing effect in this dual-surface channel. The channel width was 300 μm , and its depth was about 45 μm . The total length of the channel was about 460 mm, and the detection point was about 20 mm from the cathode. The fluorescence confocal microscope (Figure 1a) was used to scan the microchannel slice by slice, with 2.0 μm per slice, starting from the glass surface toward the PDMS surface. Parts a and b of Figure 4 show the images of DNA molecules at positions of 0, 4.0, 8.0, and 12.0 μm from the glass surface before and after applying electric field, respectively. It is obvious that DNA molecules are brought to the glass surface by lateral focusing.

Figure 5 shows that, before applying electric field, DNA molecules are randomly distributed in the channel, but the molecule numbers at the two surfaces were much lower than that at the middle of the channel. This edge effect can be explained by the electrostatic repulsion at the surfaces, which were both highly negatively charged at pH 8.0.²⁵ After the electric field was applied for 1 min, about 95% of molecules were focused into an 8- μm layer at the glass surface. It is worth noting that there were 339 molecules found at the glass surface after applying electric field (first slice), compared to 1 molecule before applying the electric field. No DNA molecules were found from the 10th slice (18 μm from the glass surface) to the 24th slice (PDMS surface) after applying the electric field for 1 min. We also note that the DNA molecules were always moving axially along the channel. That is, the increase in number was due to focusing rather than adsorption.⁷

For most single-molecule detection methods, one key issue is the reduction of the detection volume in order to improve the signal-to-noise (S/N) ratio.^{9,26} In evanescent-field fluorescence (Figure 1b), only a $\sim 100\text{-nm}$ layer at the surface will be detected.³ Light scattering is almost completely suppressed, and high S/N ratio can be obtained. However, the molecule detection efficiency (MDE), which is defined as the fraction of molecules in a sample that flow through the detector and are actually detected, is relatively low. For example, for a 40- μm deep channel, even if we neglect electrostatic repulsion at the surfaces and assume that DNA molecules are homogeneously distributed within the channel, the MDE is estimated to be only 0.25%. The actual MDE is even lower than 0.25% due to electrostatic repulsion (Figure 5 without field). For disease diagnosis, this is hardly satisfactory.

The lateral focusing method described above provides a promising solution to improve the MDE. As shown in Figure 5, about 24.8% (339 out of 1367) of the molecules were focused onto the glass surface and can be detected. This is a 99-fold increase in MDE. Our previous results showed that the lateral lift force increases with increasing voltage and shear flow rate.⁸ Therefore, the MDE will be further improved if a higher electric field is applied, which will increase the shear flow rate as well.

Application. In immunoassay, usually a dye-labeled antibody is used to bind the antigen. The antibody-antigen complex is then separated from the free antibody and detected. The separation efficiency of bound/free antibody is critical to the success of immunoassay, especially when the antigen concentration is very low. Here, we demonstrate the application of the lateral focusing phenomenon as described above to perform continuous (homogeneous) immunoassay. The streptavidin conjugated quantum dots were used to detect biotinylated DNA molecules in a background of nonbiotinylated DNA molecules.

First, we characterized the migration behavior of DNA-QD complexes under gradient shear flow in an electric field by using a capillary electrophoresis setup (Figure 1c). This method has been well characterized in our previous work.⁸⁻¹⁰ A hydrodynamic (Poiseuille) flow through the capillary can be easily applied by lifting one end of the capillary. A hydrodynamic flow from cathode to anode with a maximum velocity of 0.55 mm/s was applied throughout the experiment, and an electric field of 40 V/cm was applied from the 201st frame in the series of images. The laser power was 15 mW, the exposure time was 40 ms, and the frame rate was 5 Hz. The sample was the 0.4 pM QD-DNA complex solution. The particles from frame 101 to frame 200 (no electric field) and those from frame 401 to frame 500 (after applying electric field for 40 s) were counted using ImageJ software, and the radial distribution of QD-DNA is shown in Figure 6. Since the DNA molecules were not labeled with fluorescent dyes in this experiment, they were not detected unless they were bound to fluorescent QD. As shown in Figure 6, before applying electric field, QD-DNA complexes are randomly distributed within the capillary. However, when an electric field of 40 V/cm was applied for 40 s, the number of DNA-QD complexes at the center region of the capillary increased significantly, implying that the QD-DNA complexes were deformed under shear flow and were thus subjected to lateral lift forces.

As discussed in our previous work, free QD are not subjected to lateral lift force because they are rigid particles and cannot be deformed under shear flow. The fact that QD-DNA complexes and free QD have significantly different migration behavior suggests that we should be able to separate them by using the appropriate shear flow and electric field. We tested the possibility of selective detection of QD-DNA complexes using the PDMS/glass microchannel and evanescent-field fluorescence imaging (Figure 3). For free QD (Figure 7a), the observed particle number was 172 before applying the electric field and 142 after applying an electric field of 30 V/cm for 1 min. There was no major change in particle number after applying electric field in view of molecule counting errors. However, for QD-DNA complexes (Figure 7b), the observed particle number was 35 before applying electric field and 150 after applying an electric field of 30 V/cm for 1 min. The observed particle number increased by more than 4-fold. We then removed the electric field for 5 min and took another image. The observed particle number changed to 37, almost the same as that before applying the electric field. This shows that the density of the QD-DNA complexes equilibrated across the channel during the 5 min period. These results indicate that the increase in particle number could be solely attributed to the existence of biotinylated DNAs, which conjugated with QD and forced them to be focused near the surface. Therefore, the increase in particle number provides quantitative information on biotinylated DNA in the solution without separation of the components.

In immunoassay, usually excess antibody is required to bind the antigen efficiently.⁶ Problems arise when the antigen level is extremely low because the signal from any antibody-antigen complex could be buried in the large background signals from excess antibody. One possible solution is to use the difference in their electrophoretic mobilities for discrimination.⁶ The discrimination based on lateral focusing as shown here should add another factor of 100 (see MDE above) of selectivity toward detecting the rare antigen molecule.

Conclusions

We demonstrated that a simple and cost-effective PDMS/ glass microchannel could be used to produce gradient 2D shear flow and that the resulting lateral focusing could be used for immunoassay without bound/free separation. One advantage of this method is that it

can be operated continuously, which would be essential for high-throughput screening. Although streptavidin-biotin binding was employed here, extension to any immunoaffinity systems should be possible.⁶ In addition, the approach may be useful for various intracellular species from single cells. For the special case of DNA detection, one can easily use the same streptavidin-conjugated QD to detect specific sequences in moderate-sized DNA fragments (those that are deformable) by hybridizing them to biotinylated primers with the complementary sequences. Since EOF is typically faster than the electrophoretic velocity (except at extremely low pH or high ionic strength), continuous transport of samples from anode to cathode can be guaranteed. Since the gradient 2D shear flow is created by the imbalance in EOF and no external pressure is required, implementation is greatly simplified. Adaptation to other areas of microfluidics¹⁷ can be foreseen.

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Figure 1. Optical arrangements used in this study. (a) Fluorescence confocal microscopic imaging; (b) evanescent-field fluorescence microscopic imaging; (c) capillary-electrophoresis-based microscopic imaging; (d) pattern of fabricated microchannel.

Figure 2. Electroosmotic flow velocity at (a) PDMS and (b) glass surfaces. Length of streaks is the integrated trajectory of microspheres over the exposure time.

Figure 3. Mechanism of selective lateral focusing of QD-DNA complexes.

Figure 4. Confocal images of λ -DNA molecules in PDMS/glass microchannel with and without electric field. (a) No electric field: slices at 0, 4, 8, and 12 μm from the glass surface. (b) After applying an electric field of 43.7 V/cm for 1 min: slices at 0, 4, 8, and 12 μm from the glass surface. The sample is 0.3-pM λ -DNA labeled with YOYO-1 at a molecule ratio of 5 bp:1 dye.

Figure 5. Vertical density profile of I-DNA molecules in PDMS/glass microchannel with and without electric field.

Figure 6. Radial focusing of QD-DNA complexes in a square capillary. Electric field: 40 V/cm. Hydrodynamic flow: 0.55 mm/s at the center of the capillary from cathode to anode.

a) QDs only

b) QDs-DNA

Figure 7. Selective focusing of QD-DNA complexes in a PDMS/glass microchannel. (a) Images of QD at the glass surface using evanescent-field fluorescence microscope; (b) images of QD-DNA complexes at the glass surface.

CHAPTER 3. MOTION OF SINGLE DNA MOLECULES AT A LIQUID-SOLID INTERFACE AS REVEALED BY VARIABLE-ANGLE EVANESCENT-FIELD MICROSCOPY

A paper published in Journal of Physical Chemistry¹

Yan He, Hung-Wing Li and Edward S. Yeung*

Abstract

A variable-angle total internal-reflection fluorescence microscope (VATIRFM) capable of providing a large range of incident angles was constructed for imaging single DNA molecule dynamics at a solid/liquid interface. An algorithm using a public-domain image processing program, ImageJ, was developed for single-molecule counting. The experimental counts at various incident angles with different evanescent field layer (EFL) thickness are affected by molecular diffusion. The dynamics of molecules near the surface and the observed counts in VATIRFM are elucidated using a limited one-dimensional random walk diffusion model. The simulation fits well with the experimental counting results. Further analysis using the simulation reveals the details of single-molecule motion. One implication is that the measured intensities cannot be used directly to determine the distances of molecules from the surface, though the majority of fluorescence does come from the EFL. Another implication is that rather than providing molecular concentrations within EFL, the experimental counting results depict the distance-dependent dynamics of molecules near the surface. Thus VATIRFM could be a powerful technique to study the surface repulsion/attraction of molecules within a few hundred nanometers of the surface. Further studies show that molecules at low ionic strengths experience electrostatic repulsion at distances much further away from the surface than the calculated thickness of the electrical double layer.

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Introduction

Understanding the detailed dynamics of molecules near chemically active or modified interfaces is important in many areas of research such as chromatographic retention, biological processes at cell membranes, colloid and soil chemistry, bio-catalysis and bio-recognition, etc. While great advances have been made to elucidate interfacial phenomena both experimentally and theoretically, there remain a number of unsolved questions. For example, the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory^{1,2} has been widely used to describe the interactions between molecules or small particles and a charged surface, and is the basis behind most electrochemical and chromatographic theories. The effective interactive distance is generally believed to be less than 10 nm at typical ionic strengths. In numerous cases, however, researchers observed long-range molecule-surface and particle-surface interactions that cannot be explained using the DLVO theory.³⁻⁸ One way to address this problem is to monitor single-molecule dynamics near the interface at nanometer scale. Direct observation of individual molecule adsorption, desorption and motion at the interface can provide valuable insights into this and other related issues.

Single-molecule spectroscopy (SMS) of individual molecules labeled with fluorescent dyes has been used to investigate molecular behaviors at chemical interfaces.⁹⁻¹² However, wide-field epi-illumination microscopy,¹³⁻¹⁵ confocal laser scanning microscopy,^{16,17} and multiphoton excitation microscopy^{18,19} are all limited in resolution by the diffraction limit of light, which prevents them from monitoring the random motion of individual molecules directly. In contrast, evanescent-field fluorescence (EF) microscopy,²⁰⁻²⁴ or total internal reflection fluorescence (TIRF) microscopy, can overcome the diffraction limit with near-field excitation at a dielectric surface. The intensity of the EF decays exponentially with distance from the surface and its penetration depth typically varies between 40 nm and > 200 nm. As a result of the distance-dependent excitation of EF, scattering or fluorescence intensity changes can be correlated with small positional displacements perpendicular to the surface. This property has been used to measure distance-dependent particle-surface and cell-surface interaction forces,²⁵⁻³¹ monitor unfolding steps of surface-immobilized protein molecules,^{32,33} track motion of slow moving granules near cell membrane before and during exocytosis,³⁴⁻³⁶ etc.

EF microscopy is a non-invasive imaging technique, making it most suitable to study free molecule or particle motion near surfaces. In addition, the thin EF keeps fluorescence background low and makes it easy to define small probe volumes, which is crucial to achieving single-molecule sensitivity.^{3,12,37,38} Using EF excitation, Xu and Yeung have observed restricted motion of single molecules at a fused-silica prism surface. Diffusion of single rhodamine 6G molecules near the surface was found to be slower than in the bulk solution.¹² Single dye-labeled protein molecules were found to be trapped at distances beyond that of the calculated electrical-double layer thickness.³ By combining EF excitation with fluorescence correlation spectroscopy, Starr and Thompson measured local concentrations and diffusion coefficients of fluorescently labeled IgG molecules in the solution adjacent to substrate-supported phospholipid bilayers.³⁹ The local concentrations of IgG molecules were found to be similar to those in bulk, but diffusion coefficients near the membrane were larger at low ionic strength and smaller at high ionic strength. Vigeant and Ford et al. studied behaviors of *Escherichia coli* bacteria close to uncoated and coated quartz surfaces using indirect TIRFM.⁴⁰⁻⁴² They found that some cells were being held near the surface dynamically at a distance too far to be explained by the DLVO theory.^{1,2} The existence of an intermediate layer between bulk fluid and the surface was hypothesized.

Since the exponential decay rate and thus the penetration depth of EF vary with the angle of the incident beam, depth-resolved information can be extracted from variable-angle (VA) TIRF.⁴³⁻⁵⁷ The stack of multi-angle images contains the integrated fluorescence intensity over various thicknesses (from the surface) of the sample. Because the fluorescence response is essentially a Laplace transform of the vertical distribution of the fluorophors, information regarding the z-axial distribution can in principle be derived by inverse Laplace transform, but simple geometries must be assumed to obtain an analytical expression.⁴⁵ VA-TIRF has been implemented either by rotating the EF surface relative to the incident beam^{52,56} or by passing the incident laser beam through a combination of deflecting and positioning optics.^{45,50} Most recently, a compact device with the size of a standard microscope condenser was constructed by Stock and coworkers.⁵⁷

Besides mapping cell-surface contact⁴³⁻⁴⁵ and tracking 3-D granule movement inside cells,⁴⁶⁻⁴⁸ VATIRF has been used to study the z-profile of molecular concentration and other

bulk properties. By measuring spectra and intensities of pyrene solution as a function of incident angles, Rangnekar and coworkers⁵³ noticed that micropolarity near a C18-modified quartz surface increased as the penetration depth of EF decreased from 220 nm to 90 nm. Toriumi et al.⁵⁴ found that the concentration of pyrene monomers show a non-uniform depth profile near thin polymer films. Using time-resolved VATIRF spectroscopy, Byrne and coworkers⁵⁵ found that the distance-dependent fluorescein concentration is uniform at the water-silica interface, but acridine orange (AO) has two populations with distinct z-distribution profiles. Gee et al.⁵⁶ studied conformational changes of bovine serum albumin adsorbed on a silica substrate using time-resolved TIRF anisotropy. Protein molecules appeared to unfold more and experience more rotational restriction as the penetration depth of EF decreased. These results indicated that the behavior of molecules near the surface cannot be explained by traditional DLVO theory and hydrophobic interactions.

Previously, we have studied single-molecule adsorption/desorption events at the solid/liquid interfacial layer. The methodology is based on fluorescence imaging of single DNA molecules labeled with YOYO-1 as a function of time by using a CCD/ICCD camera through an optical microscope.⁵⁸ Double-stranded (ds) DNA is a uniformly charged nanoscale object with dimensions that are readily selectable. It is ideal for mapping out electrostatic effects near a surface while introducing minimal perturbation. Unlike other probes such as small fluorophors, nanoparticles or proteins, intercalator-labeled single DNA molecules provide excellent signal-to-noise ratio even at low labeling ratios and are not prone to photobleaching, blinking or aggregation. In the present work, VATIRF microscopy is used to directly map the z-distribution of single DNA molecule dynamics as a function of distance from the silica/water surface. The behaviors of free, non-adsorbed single molecules are investigated using both measurements and simulation. The long-range effects at low ionic strengths are discussed.

Theory

When total internal reflection occurs at a solid/liquid interface, the intensity of the evanescent field (EF) at distance z from the interface, $I(z)$, is given by

$$I(z) = I(0)e^{-z/d} \quad (1)$$

where $I(0)$ is the laser intensity at the interface, and d is the penetration depth of EF,

$$d = \frac{\lambda}{4\pi\sqrt{n_2^2 \sin^2 \theta - n_1^2}} \quad (2)$$

where λ is wavelength of the laser, θ is the incident angle, and n_1 and n_2 are the refractive indices of the liquid and the solid, respectively. For s-polarized light (used in this work),

$$I_s(0) = |A_s|^2 \frac{4 \cos^2 \theta}{1 - (n_1/n_2)^2} \quad (3)$$

where A_s is the amplitude of the incident light wave. Because a laser beam with constant power will be spread out by a factor of $\cos(\theta)$, the true $I(0)$ is given by

$$I(0) = I_s(0) \cos(\theta) \quad (4)$$

In TIRF, the total collected fluorescence, F , is the integrated fluorescence emission, $F(z)$, over the entire z -axis above the surface,

$$F = \int_0^\infty q(z) F(z) dz \quad (5)$$

where $q(z)$ is the z -dependent collection efficiency of the optics. For bulk measurements, $F(z)$ is given by

$$F(z) = k\varepsilon\phi c(z)I(z) \quad (6)$$

where k is a constant instrumentation factor, ε is the molar extinction coefficient, ϕ is the quantum yield, and $c(z)$ is the concentration of fluorescent molecules. For a single multiply labeled molecule that has a center of mass at z and an equivalent of n fluorophors, the above equation becomes

$$F(z) = kn\sigma\phi I(z) = kn\sigma\phi I(0)e^{-z/d} \quad (7)$$

where σ is the cross section of the fluorophor. In practice, the total fluorescence of a single molecule is collected by a CCD camera with an exposure time of Δt , during which time the molecule is in continuous motion if not otherwise adsorbed to the surface. Therefore, the fluorescence image of a single molecule is actually a projection of the motion of the molecule weighted by the EF excitation intensity decay profile. If the molecule can be considered a dimensionless point and its motion is diffusion controlled, then the mean z displacement of the molecule during Δt is given by,

$$\Delta z = \sqrt{2D\Delta t} \quad (8)$$

where D is the diffusion coefficient. The total fluorescence emission during Δt would be

$$F(\Delta t) = kn\sigma\phi I(0) \int_0^{\Delta t} e^{-z(t)/d} dt \quad (9)$$

Experimental Section

Chemicals and Sample Preparation. Circular DNA vectors pUC19 (2.7 kb) and PhiX174 RF I (5.4 kb) were obtained from New England Biolabs (Beverly, MA). All DNA stock solutions were prepared in 10 mM Gly-Gly buffer at pH 8.2, and were labeled with YOYO-1 (Molecular Probes, Eugene, OR) at a ratio of one dye molecule per five base pairs. The concentrations of stock solutions were 2 nM for the 2.7 kb DNA and 1 nM for the 5.4 kb DNA, respectively. For single-molecule imaging, these stock solutions were further diluted to 5 ~ 12.5 pM with appropriate buffer prior to the start of experiments.

Variable Angle TIR Excitation. The instrumentation setup is shown in Fig. 1. The light source is a 488-nm argon-ion laser (Uniphase Model 2211, San Jose, CA) with maximum power output of 50 mW. The laser beam is directed to a periscope, which consists of a 90° bending prism (CVI Laser, Albuquerque, NM) and a laser line mirror (Newport, Irvine, CA). The periscope rotates the polarization of the laser beam by 90° ensuring s-polarized light at the TIR prism/sample interface. The laser line mirror has > 99% reflectance at 488 nm, and also serves as a bandpass filter to reduce plasma lines. After a mechanical shutter (LS2Z2, Vincent Associates, Rochester, NY) and a beam steering prism, the laser beam is focused by an achromatic doublet convex lens with 300 mm focal length, sent to a 2 mm 90° prism (ALP-RAP-02B, Lambda Research Optics, Costa Mesa, CA) positioned right beside the microscope objective, and steered down to a high precision galvanometer optical scanner (Model 6220, Cambridge Technology, Cambridge, MA). The galvanometer directs the slightly focused laser beam through the TIR prism toward the prism-sample interface at different angles. The TIR prism is an equilateral SF11 prism ($A = H = 25.0$ mm, Thorlabs, Newton, NJ) with $n = 1.78$. It has a surface flatness of $\lambda/10$ and surface quality 40-20. The vertical position of the galvanometer is controlled by a fast high-precision motorized translational stage (SGSP-2020, Sigma Koki Co., Tokyo, Japan) which ensures that the same spot

on the sample area is illuminated when the incident angle changes. Due to space limitations of the microscope, the maximum travel of the translational stage is 18 mm. This allows the angle of the galvanometer to scan from 39° to 90° and the incident angle θ to change from 49° to 76° . The corresponding d ranges from ~ 200 nm to ~ 40 nm. The incident angles are calibrated at two points. One is at $\theta = 90^\circ$, and is set by directing the laser beam parallel to the surface of prism. The other is at $\theta = \theta_c$, the critical angle (48.3° for $n_2/n_1 = 1.78/1.33$), and is set by reducing θ until TIR disappears. All other angles are then calculated based on the specification of the galvanometer. It has been reported that for TIRFM^{45,47} d obtained from calculations matched quite well with those from measurements.

Single-Molecule Fluorescence Imaging. The TIR prism is placed on a homemade prism holder attached to the stage of a Nikon Optiphot-2 upright microscope. The sample compartment is formed by sandwiching 10 μ l sample solution between the prism and a 22×30 mm cover glass (0.17 mm thick), resulting a 22×25 mm rectangular area with solution thickness of ~ 18 μ m. Fluorescence images are collected through a Nikon 40 $\times$ oil Plan Fluor objective (N.A. = 1.30), a 1.25 $\times$ magnifier and filter holder, and projected onto a MicroMAX cooled CCD Camera (NTE/CCD-512-EBFT, Roper Scientific, Princeton, NJ). The CCD has a digitization rate of 1 MHz (16 bits) and a full frame transfer rate of 276 ms, which limits its full-frame sampling frequency to ~ 3 Hz. Each frame consists of 512×512 pixels and each pixel has a dimension of 13×13 μ m. So, in real space, each pixel represents an area of 0.26×0.26 μ m² and the whole chip corresponds to an area of 133×133 μ m². For YOYO-1 labeled DNA single-molecule imaging, a filter block (Chroma Technology, Rockingham, VT) with one 505 nm long pass filter and one 535/50 nm band pass filter are placed between the objective and the CCD.

The CCD camera is operated in the frame-transfer mode and synchronized with a shutter controller (Vincent Associates, Rochester, NY). The shutter on/off duration is provided by a 24-bit timer on a multifunctional data acquisition board (PCI-6036E, National Instruments, Austin, TX) placed in a Pentium4 PC. The timer is operated in the finite-pulse-train mode to control the number of exposures of the CCD. The galvanometer is also controlled by the DAQ board. The motorized translation stage is powered by a 2-axis stage

driver (CSG-602R, Sigma Koki Co., Tokyo, Japan) through the serial port of the PC. All these control operations are performed by a program written in Visual Basic and Measurement Studio (National Instruments). The same program also communicates with the WinView/32 image acquisition software (Roper Scientific, Princeton, NJ) via VB automation to perform image acquisition. If not mentioned otherwise, 20 frames are acquired at each angle, and a total of 12 angles are scanned in each experiment. Table I lists the values of the 12 incident angles relative to the surface normal of the prism, the corresponding surface excitation intensity I_0 , and the penetration depth d . All images were analyzed using ImageJ, an open source image processing and analysis software distributed by NIH. A macro was written to call commands and plug-ins in ImageJ to import WinView/32 files, subtract background at each angle, set threshold according to the uniform reduced background, and count the number of molecules in each frame.

Simulation. To interpret the counting results and elucidate molecular dynamics near the solid/liquid interface, a simulation based on limited one-dimensional random walk diffusion model is performed in Excel. Single molecules are modeled as dimensionless points with a fixed number of fluorescent units inside the EF. They are allowed to start from an arbitrary distance z at time $t = 0$ from the surface, and walk up or down randomly for 1000 steps. Each step has a duration of 0.1 ms, so the total diffusion time is 100 ms, mimicking the 100-ms exposure time of the CCD camera. The length of each step is determined from the diffusion coefficient of the corresponding molecules by using Eq. (8). To consider double-layer repulsion between the surface and the molecules, a boundary $Z_1 \geq 0$ is designated to limit how close molecules can reach the surface. The portion of solution below Z_1 is inaccessible to molecules. For simplification, any time a molecule hits the boundary during one step, it is bounced back. At the end of every step, the fluorescence emission of the molecule is calculated from its z location, d and $I(0)$ according to Eq. (7), with the assumption that all other constants are reduced to unity. The total fluorescence intensity of each molecule during the CCD exposure time is then obtained by integration of corresponding steps. If the total intensity minus a threshold is two times greater than a preset noise level, the molecule is treated as countable. Otherwise, it is omitted. The relative value of the threshold is adjusted according to the reduced background during image analysis, which varies with the power of laser exci-

tation, the number of fluorescence units the molecule carries, and the CCD exposure time. Every trace is calculated 12 times using the incident angles listed in Table I. A large number of traces are calculated and their results are combined to obtain a statistical average. For each set of parameters, 1000+ repetitive runs are needed to obtain a stable output. We confirmed that 1000 steps of 0.1 ms each produced the same simulated curves as 2000 steps of 0.05 ms each.

Results and Discussion

Instrumentation. The purpose of this study is to develop a VATIRF microscope for single molecule quantification near the solid/liquid interface with nanometer resolution. Such implementation requires instrumentation that is not only highly sensitive in single-molecule detection, but also provides both high resolution and large dynamic range in the z-direction. It is necessary to monitor molecular behavior in liquid layers that span the region from a few nm to several hundreds nm above the surface to explore possible differences in microenvironments between the interfacial region and the bulk of the liquid. To achieve this goal, a prism with high refractive index is needed to generate a large range of θ and a wide range of d . While there are several types of materials made from high refractive index materials,⁵⁹ e.g. LiNbO_3 ($n = 2.29$) and Al_2O_3 ($n = 1.78$), silica substrates are preferred because they are commonly used with biological materials. Results obtained on glass/liquid interface are also directly relevant to findings on other chromatographic surfaces such as capillaries and silica-based chromatographic stationary phases. Flint glass SF11 is chosen because it has the highest refractive index ($n = 1.78$) among all glass types.

To generate a large range of $\Delta\theta$, initially we adapted a beam steering design^{49,50} that consisted of a rotatable mirror, two parallel achromatic convex lenses, and a SF11 half-cylindrical prism, where the available $\Delta\theta$ range is determined by the $F/\#$ of the lens adjacent to the prism. For our purpose, a 12.7 mm diameter lens with $F/\# = 1.5$ was used to achieve $\Delta\theta = 24^\circ$. However, the strong focusing effect of the lens caused unwanted angular dispersion of the laser beam and thus uncertainty in d within the probe area. Furthermore, it was very difficult to direct the laser beam to the same spot on the prism surface due to imperfection of curved optical surfaces. In comparison, all optical surfaces in the present setup are flat

except for a long focal length convex lens to reduce the size of the laser spot to become smaller than the viewing area of the CCD. Thus, the angular dispersion is minimized and the optical alignment also becomes much easier. The $\Delta\theta$ range is determined by the maximum separation distance between the objective and the microscope stage and the minimum distance between the TIR prism and the vertical light path above the galvanometer. Currently, we achieve a range of 27° , the largest among all the VATIRFM instruments reported^{49-51,57}.

Image Analysis. In previous solid/liquid interface studies using VA-TIRF, an analytical function such as a step function was assumed and Laplace transform was used to recover unknown parameters from intensity measurements at a series of incident angles⁴⁵. Here, we do not attempt to make any presumptions on what the distribution of molecules should look like. We simply count every molecule in the images acquired at each EF penetration depth and reconstruct the molecular distribution profile from the experimental results. In order to identify and count single molecules in hundreds of frames efficiently at high speed and without human bias, we need an automated procedure that is reliable under all of our experimental conditions. We note that the procedure we developed here using ImageJ is very similar to ones used to count stars in cosmic studies.⁶⁰

Figure 2 depicts a set of single-molecule images from a typical measurement. The sample is 2.7 kb circular DNA, 10 pM in pH 6.5 buffer with ionic strength 5.0 mM. Single DNA molecules appear as bright spots. It can be seen that their number, average brightness and size increase as d increases. The brightness and size of individual molecules in the same frame are different. Nonuniform brightness is due to inhomogeneous illumination of the laser beam as well as differences in residence time in the EF. The size of a molecule spot is not the physical dimension of the DNA but represents x-y motion during the exposure.^{3,12} To count the number of “meaningful” spots or particles in an image, ImageJ uses a standard particle analysis routine. It works by setting an intensity threshold and a range of particle sizes. Only spots that have intensities above the threshold and spot size within the range are counted. To apply this analysis routine to single-molecule counting, the background contribution to the recorded intensity of the molecules must first be eliminated. Background contribution in each image consists of surface scattering and fluorescent impurities from the sample solution. Besides increasing with EF penetration depth, background intensity varies with laser illumina-

tion, focusing, sample concentration, ionic strength, etc. These could lead to complexity in threshold setting and incorrect molecule count. That is, it is not possible to solve this problem “externally” by subtraction of a blank image or by fixing one threshold for each incident angle.

One general way to deal with background variation “internally” is to use ImageJ’s 2D rolling-ball algorithm. It works by moving an imaginary ball across the image, finding the local maximum and background intensity within the radius of the ball, and removing background locally. For single-DNA measurements, a ball radius of 25 pixels was found to be adequate to bring the backgrounds of images taken at 12 different incident angles to the same level. As a result, only one threshold is required for the entire stacks of images (different angles). Another simple method to eliminate background is to take advantage of repetitive measurements, and perform frame-to-frame difference calculations for all the frames taken at the same angle. Because single molecules in solution are moving and never stay at the same location in consecutive frames, their information is preserved after frame-to-frame subtraction while the relatively invariant background contribution is removed. Thus the entire stack is brought to a uniform reduced background level. This simple algorithm also removes molecules that adsorb to the surface, whose count introduces a non-zero offset to the dynamic value and makes comparison among different experiments difficult. In contrast, the rolling-ball algorithm cannot remove adsorbed molecules. In the limit that few molecules are adsorbed onto the surface, the results of both background subtraction algorithms are similar.

After successful background subtraction, the signal-to-noise ratio of images always becomes worse. So, a smoothing filter is applied by replacing each pixel with the average of its 3×3 neighbors. Subsequently, the mean and standard deviation (σ) of the intensity of all 512×512 pixels of the images taken at the largest incident angle are calculated. At that angle, EF penetration depth is the smallest, and only a few molecules can be found. They contribute very little to the average intensity of the frame. Hence the mean and σ are essentially the average intensity and noise level of the reduced background. By making a cut at the mean plus 4σ , the threshold is set “locally” regardless of the different experimental conditions. The size of “meaningful” particles is chosen to be 7 pixels, 2 pixels less than the size of the smoothing filter. This choice is consistent with x-y molecular diffusion over the exposure time. The par-

ticle analysis feature of ImageJ can then be applied to the entire stack of images. The whole procedure is automated using a software macro. The results match quite well with manual counting.

Experimental Counting Results. Figure 3A shows the counting results of the image stack from the 2.7 kb DNA sample solution in Figure 2. The two sets are results from CCD camera exposures of 100 ms and 25 ms, respectively. It can be seen that in both cases, the observed number of molecules increases with d , but not linearly. The slope is large when $d < 60$ nm, and gradually decreases. The counts with 100 ms exposure are always higher than those with 25 ms exposure. Intuitively, the counts at each incident angle reflect the number of molecules in the observation volume whose thickness is defined by d . If that is the case, then at the largest penetration depth $d = 140$ nm, the concentration of molecules must be close to the bulk concentration. From the known bulk concentration, the total viewing area and d , we can calculate the expected count at $d = 140$ nm to be $10 \text{ pM} \times (133 \text{ } \mu\text{m} \times 133 \text{ } \mu\text{m}) \times 140 \text{ nm} \times 6.02 \times 10^{23} \text{ molecules/mole} = 14.9$. In reality, the count for 100 ms exposure is 83.3. The difference between the calculation and measurement is so large that it cannot be accounted for even if we assume the thickness of the observation volume is twice as large as d at high excitation power. In addition, if the CCD camera is probing the same number of molecules during both 100 ms and 25 ms exposures, then the difference in counting results will have to be attributed to a difference in signal-to-noise ratio at different exposure times. Since S/N at large d are higher than that at small d , we expect that for the two exposure times, the difference in counts is smaller or even negligible at larger d . But, as shown in Figure 3A, this difference actually becomes larger as d increases. For $d > 70$ nm, the count for 100 ms exposure is roughly twice of those in 25 ms exposure.

The above observations can only be explained if Brownian motion of DNA molecules (in the z direction) is considered. For 2.7 kb circular DNA, the diffusion coefficient is $3.8 \times 10^{-8} \text{ cm}^2/\text{s}$.⁶¹ During a 100 ms exposure, the mean displacement of a molecule in the z direction is $0.87 \text{ } \mu\text{m}$ according to Eq. (8). This distance is over 6 times larger than the largest d in Figure 3A. Thus, molecules originally far away from the surface can get into the EF during the exposure. The ones that can be identified on the images are those that enter the EF at any point during the exposure, and stay long enough to emit enough fluorescence photons. As a

result, more molecules are seen than are predicted from the bulk concentration. When the exposure time is 25 ms, the mean diffusion distance is half of that during 100 ms, so the number of molecules that enter the EF and stay long enough is also reduced by one half.

This argument is further supported by the relationship between counting results and laser excitation power. In Figure 3B, when the laser output power increases from 10 mW to 30 mW, the counts at all d increase significantly. As the power increases from 30 mW to 50 mW, the change in counts at each d is much smaller. The appearance of a plateau as the power increases cannot be explained if only molecules within the observation volume are countable, because at each incident angle, the S/N and thus the observation volume always increase with the laser power. The number of molecules that can move close enough to the surface is a constant. However, if counting results are related to z -diffusion, at low laser power (< 30 mW), the number of molecules that can be excited and counted is determined by S/N. Therefore, when the laser power exceeds a certain value (30 mW), S/N is high enough that all molecules that enter the EF will be counted.

All experiments discussed so far involve a solution thickness ~ 18 μm , which essentially allows molecules to diffuse freely (mean displacement of 0.87 μm in 100 ms). If the diffusion of molecules is limited, however, the counting results can be different. Figure 4 shows the results of a “fresh” (thick) sample compared to a “near-dry” (thin) sample, where the solution sandwiched between the cover glass and the prism is intentionally drawn out such that the thickness of solution is greatly reduced. The thickness of the “near-dry” sample is estimated to be > 1 μm by comparison with spreading of a small amount of solution on the prism under a cover glass. In the “fresh” sample, molecules appear to pop in and out of the probing volume in the series of images. Any movement of the molecules in the x - y plane cannot be recognized, especially at shallow d , since spots observed from frame to frame cannot be correlated. However, in the “near-dry” sample, most molecules are bright and can be tracked from frame to frame even at the smallest d . Their x - y motion can be clearly identified. There, the molecule count at different d does not show much increase beyond 45 nm. This is consistent with imaging all molecules all the time.

The results from the “near-dry” sample also imply that any distance-dependent variation of molecular counts cannot be attributed to the size of DNA molecules. The radii of gy-

ration of 2.7 kb and 5.2 kb circular DNA molecules have been estimated to be 56 nm and 81 nm, respectively⁶¹. Since the diameters of DNA molecules in solution are comparable to or larger than the d , one may argue that fewer molecules are seen at small d in Figure 2 because their size prevents them from coming close to the surface. If that is the case, we would expect to see molecular counts increase with d regardless of the thickness of the sample solution. But in the “near-dry” situation described above, most molecules are visible even at the smallest d . Therefore, the size of DNA molecules does not affect the counting results. A similar experiment was also performed using the auto-fluorescent protein molecule R-phycoerythrin (RPE), and a similar dependence on d was obtained (data not shown). Because the diameter of a RPE molecule is only 12 nm⁶², the observed exclusion of molecules near the surface is thus confirmed to be unrelated to the size of probe molecules.

The fact that counting results depend on diffusion of molecules and the exposure of the CCD camera implies that single-molecule VATIRF microscopy does not provide direct spatial distribution of molecules in the z direction. Instead of specifying the physical location of molecules from the surface, the “penetration depth” is a parameter that specifies the capability of EF to “snapshot” single molecules. Under a given set of experimental conditions, whether a molecule is counted is determined by how long and how much it is excited by EF. The penetration depth d determines the integration length from the surface that a moving molecule can be effectively excited by the EF. The surface intensity $I(0)$ determines how long a molecule needs to stay in the EF in order to emit enough photons to be detectable. At large incident angles, both $I(0)$ and d are small. A molecule in motion will have less chance to be excited by EF and emits fewer photons. When the incident angle is small, both $I(0)$ and d are large. A molecule with the same trajectory will have more chance to be excited by the EF and emits more photons. In other words, for molecules with different trajectories, whether they are counted at various incident angles depends on their overall closeness to the surface but not their instantaneous location.

Simulation. To further understand the relationship between experimental counting results and molecular behaviors at the solid/liquid interface, a simulation based on limited one-dimensional random walk was performed using Excel. At $\text{pH} > 6$, both the glass prism surface and DNA molecules are negatively charged. There is no strong adsorption of DNA

molecules on the surface.^{37,58} If there is little flow in the bulk solution, the motion of DNA molecules is diffusion controlled and they should assume the shape of a random coil during movement. Because the size of DNA molecules has little effect on the measurements, as discussed above, they can be modeled as a dimensionless point in view of a mean displacement distance of 0.87 μm . Diffusive molecules always undergo 3D Brownian motion. But for TIR imaging, the positional sensitivity in the x-y direction is much worse than in the z-direction. The resolution in the x-y direction is determined by the diffraction limit, which is 0.24 μm , or about the size of one pixel with 40 $\times$ objective. For DNA molecules with a diffusion coefficient of $10^{-8} \text{ cm}^2/\text{s}$, their mean displacement during 100 ms is less than 5 pixels. Except for molecules on the very edge of the viewing area, whether a molecule is counted or not is only determined by its z position relative to the surface. Therefore, one-dimensional diffusion is an adequate approximation to the dynamics of single molecules observed by TIR imaging.

The random walk simulation is performed with 3 assumptions. (1) Each molecule moves independent of other molecules. (2) Steps of a fixed length occur at regular intervals. (3) The direction of each step is equally likely to be + or – independent of previous steps. Also, for DNA molecules at pM concentration and high pH, there is no molecule-molecule interaction. For the length of each step, the mean displacement per time interval of 0.1 ms is calculated from Eq. (8). For 2.7 kb circular DNA with a diffusion coefficient of $3.8 \times 10^{-8} \text{ cm}^2/\text{s}$, its step length is 28 nm. For 48 kb λ -DNA with a diffusion coefficient of $4.7 \times 10^{-9} \text{ cm}^2/\text{s}$, its step length is 10 nm.

While DNA molecules can diffuse freely in most portions of the solution, their motion is affected when they are very close to the surface. The presence of the electric double layer and resulting electrostatic repulsion prevent molecules from contacting the surface directly. According to DLVO theory,^{1,2} the double layer thickness is given by Debye length,

$$\kappa^{-1} = \left(\frac{\epsilon \epsilon_0 k_B T}{2e_0^2 N_A [I]} \right)^{1/2} \quad (10)$$

where ϵ is the dielectric constant ($= 80.0$ for water), ϵ_0 is the permittivity of free space ($= 8.854 \times 10^{-12} \text{ C}^2 \text{ N}^{-1} \text{ m}^{-2}$), k_B is the Boltzman constant ($= 1.38 \times 10^{-23} \text{ JK}^{-1}$), e_0 is the electron charge ($= 1.6 \times 10^{-19} \text{ C}$), N_A is Avogadro's number ($= 6.023 \times 10^{23} \text{ molecules/mole}$)

and $[I]$ is the ionic strength. For a solution with $[I] = 5.0$ mM, $\kappa^{-1} = 4.3$ nm. In the simulation, the thickness of the double layer determines the boundary $Z_1 \geq 0$, which resembles a hard flat surface that bounces back all molecules that hit it. Although this is an oversimplification, it is sufficient to explain the observed molecular dynamics semi-quantitatively. Because of long-range repulsion that will be discussed later, the values of Z_1 obtained from fitting are always larger than the Debye length from calculations according to Eq. (10).

Figure 5 shows the details of the simulation that fits Figure 3A. In Figure 5A, five of the random walk traces are displayed. Figure 5B displays both the simulated and experimental counting result for 25 ms exposure. It can be seen that the simulated counts match quite well with the measurements, both in the trend and in the slope. Figure 6A depicts the effect of CCD camera exposure time through simulation. The results are very similar to Figure 3A in both the trend and the shape of the curves. Figure 6B depicts the effect of laser excitation power through simulation. Compared with Figure 3B, the shapes of the three simulated curves are slightly off from the experimental ones, probably due to oversimplification of the simulation, but the general trend is the same. The counts increase when the power changes from 10 mW to 30 mW, but reach a plateau after that. These comparisons indicate that the molecule count at each penetration depth is controlled by diffusion, and the limited one-dimensional random walk model is a good approximation to real molecular dynamics observed by VATIRFM.

With the time, position and fluorescence intensity at every point of each random-walk trace known, the simulation can provide much more information than just counts. Table II lists some derived information from a typical trace of a molecule for excitation at different angles. It can be seen that the molecule is counted (based on S/N) when $d \geq 46$ nm. Intuitively, as this molecule would be visible at all other angles in VATIRF imaging, one would expect its position to be very close to the surface. In fact, the average z-position of the molecule is 301.3 nm, and the time it stays within EF, whose thickness is defined by d , is not very long. For a total tracing time of 100 ms, the molecule spends only 8.0 ms below 50 nm, 9.5 ms below 69 nm, and 20.7 ms below 140 nm. Moreover, this short stay is not generated in one visit to the EF, but is an accumulation of several rounds of “in-and-out”. Thus, contrary to previous reports by others²², the average z-position of a free molecule cannot be readily

recovered from its fluorescence intensity alone. Furthermore, at every d , the average z -position calculated from the integrated intensity according to Eq. (1) is always smaller than the real (simulated) value and is much larger than d . The calculated values for different d are also all different even though they are for the same trajectory. That indicates that TIRFM and VATIRFM are not capable of providing positional information of fast moving molecules.

On the other hand, although its average z -position is far away from the surface and its residence time in EF is very short, the molecule emits most of its fluorescence during that period. The intensity contribution from the time it stays within EF contributes to over 60% of the total integrated fluorescence intensity at every d . In other words, because of the exponential decay of the EF, TIRFM emphasizes the part of the trajectory of molecules that is close to the surface. Therefore, just being recorded by the CCD camera as one spot on a TIRFM image does not mean the molecule is located inside the EF. It has more likely just "visited" the EF, not once but several times, for short periods during the exposure time of the camera. The low x - y -resolution of the technique makes it impossible to distinguish those separate visits on the imaging plane. Thus, the number of countable spots in an image has little bearing on the molecular concentration (density) within the observation volume. Rather, it is an indication on how frequently the EF has been visited and how easily the molecules are excited.

Because the initial positions of all molecules in the simulation are known, the simulation provides directly the percentage of countable molecules in the sample ($N_c\%$). Table III lists the percentages at the 12 penetration depths for molecules with different range of starting positions. It can be seen that for 100 ms exposure time, even for molecules starting at 700 ~ 800 nm from the surface, 50% are counted at $d = 140$ nm and 33% are counted at $d = 69$ nm. For all molecules starting below 700 nm, 5 times the distance of the largest d , 81% are counted at $d = 140$ nm and 50% are counted at $d = 53$ nm. These results indicate that molecules initially far away from the surface can leave their "signature" on the CCD camera during the exposure time, and the real observation distance of TIRF could be much larger than d .

The results in Figure 7 are derived from an average of 1,600 traces. Figure 7A displays the average time molecules stay in the EF at different initial positions for all molecules (T_a) and for countable molecules (T_c). Their values are listed in columns 2 and 3 of Table IV, respectively. It can be seen that both T_a and T_c are small compared to 100 ms of total expo-

sure time, ranging from 4.3 ms to 15.3 ms for T_a , and 9.8 ms to 18.6 ms for T_c . T_a versus d is linear, indicating that although the trajectory of each molecule is different, their statistical average shows a uniform z -distribution, an expected result from their random diffusion. For countable molecules, T_c is always longer than T_a . Moreover, the difference between T_c and T_a clearly widens as d decreases. That is, molecules must stay within the EF longer than average in order to be counted, and this constraint becomes more serious as d becomes more shallow, due to the repulsive barrier of the double layer.

Figure 7B displays the average integrated fluorescence intensity of all molecules (F_a) and countable molecules (F_c). At each d , F_c is always higher than F_a , indicating that molecules need to fluoresce (be excited) more than average to get counted. Table IV lists the ratios of T_c over T_a and F_c over F_a , respectively. We note that both T_c/T_a and F_c/F_a increase as d decreases. In addition, the two ratios are very close to each other at all d , implying that the two are correlated. A longer stay in the EF results in more overall fluorescence. Furthermore, T_c/T_a and F_c/F_a are quantitatively related to the reciprocal of $N_c\%$ ($1/N_c\%$) at large d . The larger difference between these two values at small d is because of the relatively large step length compared to d . If the step length in the simulation is reduced to 10 from 28, equivalent to changing the probe molecule from 2.7 kb circular DNA to λ -DNA, then $1/N_c\%$ and T_c/T_a are almost identical from $d = 140$ nm down to $d = 46$ nm. Finally, all the simulations in Figure 7 and Table IV have $Z_1 = 4.3$ nm, the theoretical double-layer thickness of a solution with $[I] = 5.0$ mM. If Z_1 changes, the values in the figure and the table would also change, but the correlations between T_c/T_a , F_c/F_a , and $1/N_c\%$ always hold.

In summary, these simulations give a quantitative description of the molecular dynamics observed using VATIRFM. The measured intensities cannot be used directly to determine the z -positions of molecules from the surface. Due to fast diffusion of DNA molecules, the “detection volume” is much larger than the probed volume defined by the penetration depth. TIRFM does emphasize the transit of molecules to the EFL where the majority of fluorescence is recorded. Countable molecules always stay longer inside the EFL and emit more photons than average. Although the counting results do not give molecular concentrations within EFL, they reveal the distance-dependent dynamics of molecules near the surface. Under similar experimental conditions, fewer counts indicate the EFL is difficult

to access by molecules, and more counts indicate that the EFL is accessible to molecules. The percentage of countable molecules among all molecules is correlated to their residence time inside the EFL. Therefore, single-molecule VATIRFM could be used to study the surface repulsion/attraction of molecules with nanometer resolution.

Ionic Strength Effects. Figure 8 shows the effect of ionic strength $[I]$ on the molecule counts. The solutions are 10 pM 2.7 kb circular DNA with pH 6.5 and $[I] = 0.25$ mM, 1.0 mM, 5.0 mM and 10.0 mM, respectively. The CCD exposure time is 25 ms. At $d = 56$ nm, there is a clear increase in the number of molecules when $[I]$ changes from 0.25 mM to 5.0 mM. But at $d = 140$ nm, the number of molecules counted at different ionic strengths are similar. When $d < 50$ nm, S/N is low and the counts at all four ionic strengths are close to zero. Between $d = 53$ nm and 77 nm, the counts at 1.0 mM are higher than those at 0.25 mM, and the ones at 5.0 mM are higher than those at 1.0 mM. In addition, the differences among them increase with d . When $d > 88$ nm, the differences in counts from $[I] = 0.25$ mM to $[I] = 5.0$ mM decrease and eventually disappear. Between $[I] = 5.0$ mM and $[I] = 10.0$ mM, there is little difference in counts even at small d . These results indicate that the dynamics of single molecules in regions far away from the surface is similar, as expected. The probabilities for molecules to reach $z > 100$ nm is the same at all ionic strengths. But at low ionic strengths, the probabilities for molecules to reach $z < 80$ nm are lower than those at higher ionic strengths, indicating that at low ionic strengths it is more difficult for molecules to get close to the surface, i.e., molecules are further away from the surface. This is consistent with the existence of a barrier between molecules and the surface, and that electrostatic forces are responsible for the effect. At $[I] = 1.0$ mM and 0.25 mM, the barrier is thicker than 42 nm but thinner than 100 nm. Hence, its effect is noticeable using VATIRFM. At high ionic strengths, i.e. $[I] = 5.0$ mM and 10.0 mM, either the barrier does not exist or its thickness is thinner than 42 nm. In either case, no differences can be observed in Figure 8.

The effect of ionic strength on the proximity of molecules to the surface can be attributed to double-layer repulsion. For solutions with $[I] = 0.25$ mM, 1.0 mM, 5.0 mM and 10.0 mM, the calculated double layer thickness κ^{-1} are 19.3 nm, 9.6 nm, 4.3 nm and 3.0 nm, respectively, according to Eq. (10). According to DLVO theory, the repulsive potential near the flat prism surface is given by,

$$\Psi(z) = \Psi(0)\exp(-\kappa z) \quad (11)$$

where $\Psi(0)$ is the potential at the surface. Because of the exponential decay nature, DNA molecules should not experience much repulsion beyond the calculated thickness of the double layer. As shown in Figure 8, however, it is obvious that molecules are repelled away from the surface at low ionic strengths, indicating that there exists long-range repulsion between DNA molecules and the surface.

This effect is further elucidated by simulation. For solutions differing only in ionic strength, the diffusion coefficient should be similar, so molecules should have the same step length. The detection threshold should also be constant since it is related only to the reduced background, which is determined by the laser excitation power and the exposure time. That leaves Z_1 as the only variable in the simulation to account for the ionic strength effect. Figure 9A shows the simulation results using the values of the thickness of double layer calculated from Eq. (10) as Z_1 . It can be seen that the differences in the three curves are too small compared to those in Figure 8. Figure 9B shows the best fits to Figure 8, still requiring Z_1 to be inversely proportional to the square root of ionic strength as in Eq. (10). The fitted values of Z_1 in Figure 9B are 80 ± 10 nm, 40 ± 10 nm, 17.9 ± 10 nm, and 12.6 ± 10 nm for $[I] = 0.25$ mM, 1.0 mM, 5.0 mM and 10.0 mM, respectively. Notice the values of Z_1 at $[I] = 0.25$ mM and 1.0 mM are in the resolvable range of VATIRFM. These values are much larger than the calculated thickness of the electrical double layer according to DLVO theory.^{1,2} Therefore, an extended double layer is most likely the reason that causes long-range repulsion between the surface and DNA molecules.

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Table I. Experimental settings for evanescent field microscopy.

Position	θ	$I(0) = I_s \cos \theta$	d
1	65.3	1.00	42
2	62.0	1.43	46
3	59.7	1.76	50
4	58.6	1.94	53
5	57.5	2.14	56
6	56.4	2.34	59
7	55.3	2.55	64
8	54.1	2.77	69
9	53.0	2.99	77
10	51.9	3.23	88
11	50.8	3.47	105
12	49.7	3.71	140

Table II. Details of one trajectory for a single DNA molecule from Figure 6A. Parameters are explained in the text.

d (nm)	z avg (nm)	z cal (nm)	countable	t, z < d (ms)	I%, z < d
42	301.3	115.9	No	8.0	70
46	301.3	122.6	Yes	8.0	67
50	301.3	129.0	Yes	8.0	64
53	301.3	133.6	Yes	9.5	69
56	301.3	138.1	Yes	9.5	67
59	301.3	142.4	Yes	9.5	65
64	301.3	149.1	Yes	9.5	62
69	301.3	155.5	Yes	9.5	59
77	301.3	164.8	Yes	12.1	64
88	301.3	176.1	Yes	13.7	64
105	301.3	190.9	Yes	16.4	64
140	301.3	213.2	Yes	20.7	61

Table III. Percent of molecules that are counted at each EF depth d as a function of their initial positions away from the surface.

d (nm)	100~200 nm	300~400 nm	500~600 nm	700~800 nm	0~700 nm
42	28%	19%	12%	7%	20%
46	46%	35%	24%	15%	36%
50	58%	45%	31%	21%	45%
53	64%	49%	35%	23%	50%
56	68%	53%	38%	26%	53%
59	71%	56%	41%	29%	56%
64	75%	60%	45%	31%	60%
69	78%	63%	47%	33%	63%
77	82%	66%	50%	36%	66%
88	85%	70%	53%	39%	69%
105	90%	75%	56%	43%	74%
140	95%	84%	66%	50%	81%

Table IV. Ratios of residence times T and integrated fluorescence intensities F at various EF penetration depths d compared to the reciprocal of the fraction of molecules counted, $1/N_c\%$.

d (nm)	T_c/T_a	F_c/F_a	$1/N_c\%$
42	2.68	2.60	4.81
46	2.13	2.07	2.67
50	1.88	1.84	2.12
53	1.77	1.74	1.91
58	1.70	1.67	1.80
59	1.62	1.59	1.68
64	1.55	1.53	1.58
69	1.50	1.48	1.52
77	1.44	1.42	1.45
88	1.38	1.37	1.39
105	1.32	1.30	1.32
140	1.21	1.20	1.21

Figure 1

Figure 1. Experimental setup for variable-angle evanescent-field microscopy of single molecules. M, mirror; S, mechanical shutter; PH1, PH2, pinholes.

Figure 2

Figure 2. Single-molecule images at 12 different EF penetration depths. Sample, 2.7 kb DNA at 10 pM; pH 6.5; [I], 5 mM; exposure time, 100 ms.

Figure 3

Figure 3. Molecule counts per image for the conditions in Figure 2 (A) at different exposure times; and (B) molecule counts per image at different laser excitation powers. Sample, 5.4 kb DNA at 10 pM; pH 8.2; [I], 10 mM; exposure time, 100 ms.

Figure 4

Figure 4. Molecule counts for fresh (thick) sample layer versus near-dry (thin) sample layer. Conditions are the same as Figure 3B except that the exposure time is 50 ms.

Figure 5

Figure 5. Simulation of molecular z-motion as a function of time (A) and the resulting molecule counts compared with the experimental data in Figure 3A for 25 ms exposure time (B).

Figure 6

Figure 6. Simulation of normalized molecule count at different exposure times (A) and for different laser excitation powers (B). These are to be compared to the experimental data in Figures 3A and 3B, respectively.

Figure 7

Figure 7. Details of simulated DNA dynamics. Average residence time T of molecules (A) and their integrated fluorescence intensities F (B). a and c refer to all molecules and countable molecules, respectively.

Figure 8

Figure 8. Molecule counts per image at different ionic strengths. Other conditions are identical to those in Figure 3A for 25 ms exposure.

Figure 9

Figure 9. Simulated molecule counts per image for different double-layer thicknesses calculated from (A) DLVO theory and (B) best-fit to Figure 8.

CHAPTER 4. SINGLE DNA MOLECULES AS PROBES OF CHROMATOGRAPHIC SURFACES

A paper published in *Analytical Chemistry*¹

Hung-Wing Li, Hye-young Park, Marc D. Porter and Edward S. Yeung

Abstract

YOYO-I labeled λ -DNA was employed as a nanoprobe for different functionalized surfaces to elucidate adsorption in chromatography. While the negatively charged backbone is not adsorbed, the 12-base unpaired ends of this DNA provide exposed purine and pyrimidine groups for adsorption. Self-assembled monolayers (SAMs) formed on gold substrate provide a wide range of choices of surface with well-defined and well-organized functional groups. Patterns of amino terminated, carboxylic acid terminated and hydroxyl terminated SAMs are generated by lithography. Patterns of metal oxides are generated spontaneously subsequent to deposition of metals. By recording the real-time dynamic motion of DNA molecules at the SAMs/aqueous interface, one can study the various parameters governing the retentivity of an analyte during chromatographic separation. Even subtle differences among adsorptive forces can be revealed.

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Introduction

Single-molecule detection (SMD) techniques have gained increasing attention in the field of life science.¹ Detection of fluorescence from individual molecules of interest is typically used for ultrasensitive analytical and biophysical applications. The observation and manipulation of single biomolecules allow their dynamic behaviors to be studied and provide insight into a wide range of applications like molecular genetics,²⁻⁴ biochip assembly,⁵⁻⁸ biosensor design,⁹⁻¹¹ DNA biophysics,¹²⁻²⁶ and basic separation theories of capillary electrophoresis (CE) and liquid chromatography (LC).²⁷⁻³⁴

Real-time imaging of the motion of individual DNA molecules in free solution,²⁴ particularly adsorption/desorption events at the solid/liquid interfacial layer, provide insights into the fundamental interactions governing the retention and selectivity in chromatographic separation. Double-stranded (ds) DNA is a uniformly charged nanoscale object with dimensions that are readily selectable. It is ideal for mapping out electrostatic effects near a surface while introducing minimal perturbation.^{31, 32, 34} Additionally, certain ds-DNA have ends that are not paired, thereby exposing the purine and pyrimidine groups. These form well-defined regions for hydrophobic and/or hydrogen-bonding interactions. Unlike other probes such as small fluorophors, nanoparticles or proteins, intercalator-labeled single DNA molecules provide excellent signal-to-noise even at low labeling ratios and are not prone to photobleaching or blinking.

Hydrophilic interaction chromatography (HILIC) is the extension of normal-phase chromatography to aqueous eluents. It is a method first described by Alpert for the separation of proteins, peptides, amino acids, oligonucleotides, and carbohydrates.³⁵ This technique employs hydrophilic packings in the presence of mixed aqueous/organic mobile phases. Analytes partition based on their polarity.³⁶ HILIC mobile phases are relatively high in water content (10-50% aqueous). It provides significant advantages with respect to the solubility of many biologically active substances and makes HILIC compatible with electrospray ionization mass spectrometry and evaporative light scattering detection (ELSD). Hence, HILIC was successfully applied for the analysis of polar molecules for drug discovery.³⁷ The popular commercial stationary phases are amino, alumina, diol, carbohydrate, silica, polar polymeric

packing or other polar phases. In this study, we demonstrated the interaction between single DNA molecules in aqueous solution and hydrophilic surfaces with amino, hydroxyl, carboxylic acid and alumina functional groups.

Self-assembled monolayers (SAMs), generated by the adsorption of organic molecules onto gold substrates, have been widely applied in surface science,³⁸⁻⁴⁴ electrochemistry,^{45, 46} biology,⁴⁷⁻⁵¹ biomineralization,^{52, 53} surface engineering,⁵⁴⁻⁵⁷ and sensor development.⁵⁸ SAMs possess the advantages of stable and densely packed structures, controllable and selectable surface functional groups and chemical properties, and simple and rapid preparation. We employed lithography to create patterned functionalized surfaces for our experiments. These well-defined functionalized surfaces can be used as templates for studying the selective and competitive adsorption of DNA molecules.

Experimental Section

Buffer solutions. pH buffer solutions (pH 4.0-7.0) were prepared from 1.0 M solutions of acetic acid, sodium acetate and sodium chloride. A.C.S. grade or higher glacial acetic acid, sodium acetate and sodium chloride (all from Fisher Scientific, Fair Lawn, NJ) were dissolved in ultra-pure 18-M Ω water. Unless specified, the final mass balance of acetate was 25 mM as was the nominal ionic strength as described previously.³¹ All the solutions were photobleached under a mercury lamp overnight and were filtered through a 0.2- μ m filter prior to use.

Preparation of samples. λ -DNA (48,502 bp) was obtained from Life Technologies (Grand Island, NY). All DNA samples were prepared in 10 mM Gly-Gly (Sigma Chemical Co., St. Louis, MO) buffer, pH 8.2. DNA samples were labeled with YOYO-1 (Molecular Probes, Eugene, OR) at a ratio of one dye molecule per five base pairs. DNA samples were prepared at a concentration of 500 pM. For single-molecule imaging, these samples were further diluted to 50 pM with appropriate buffer solutions prior to the start of the experiment.

Substrate preparation. Cover slips of 25 mm by 25 mm were cleaned in an ultrasonic bath for 30 min in detergent and DI water, 30 min in DI water, and 30 min in methanol (twice). Substrates were dried with nitrogen gas in a vacuum evaporator (Edwards). The substrates were deposited with 1 nm of chromium at 0.1 nm/s followed by the deposition of 20

nm of gold (99.99% purity) at 0.1 nm/s under high vacuum (4×10^{-7}). The gold substrates were either used immediately upon removal from the evaporator or stored in a desiccator. The thin coating of gold allows adequate light transmission for fluorescence excitation while maintaining uniform surface coverage.

Monolayer formation and UV photopatterning. Optically transparent gold-coated substrates were immersed in dilute (1-5 mM) ethanolic solutions of the selected thiol compound for ~ 20 h. Thiol compounds of aminoundecanethiol ($-\text{NH}_2$), mercaptoundecanol ($-\text{OH}$), mercaptohexadecanoic acid ($-\text{COOH}$), undec-11-mercapto-1-yl-triethylene glycol methyl ether (EG_3OMe) and octadecanethiol (C_{18}) were used. These samples were then rinsed with ethanol and dried under nitrogen gas. To create the patterns, a transmission electron microscopy (TEM) grid (600, mesh-hole size $30\ \mu\text{m}$, bar size $15\ \mu\text{m}$) was placed between the thiol-coated sample and a quartz plate. A 200-W, medium-pressure mercury lamp (Oriel, Stratford, CT) was used as the light source to selectively remove the monolayer from areas not shielded by the grid. Irradiation times were ~ 20 min. Samples were then rinsed with DI water and with ethanol. After drying under a stream of nitrogen, the samples were immediately immersed into a ethanolic solution of the desired thiol for ~ 15 h. This step fills back in the void areas with a SAM of a second thiol.

Evanescent-wave excitation geometry. The instrumental setup was similar to that previously described.³¹ Each $5\text{-}\mu\text{L}$ of sample solution was sandwiched between a No. 1 ($170\ \mu\text{m}$ thick and $22\ \text{mm}$ square) Corning glass coverslip and another coverslip with the appropriate SAM as shown in Figure 1. The assembly was placed on the prism in contact with index-matching oil. Bulk flow is generated via capillary force by adding solution to the edge of the coverslip. Linear flow rates of $10\text{-}50\ \mu\text{m/s}$ lasting for a minute can be achieved. A laser beam was focused and directed through the prism to the prism-sample interface. The angle of incidence was about 66° . The laser beam was totally internally reflected at the prism/solution interface, and an evanescent field of $\sim 150\ \text{nm}$ thick was created. Fluorescent molecules within this field can be excited and imaged.

Single-molecule detection system. The excitation source was an argon-ion laser (Innova-90, Coherent, Santa Clara, CA) operated at $488\ \text{nm}$. Extraneous light and plasma lines

from the laser were eliminated with an equilateral prism and a pinhole prior to its entrance to the observation region. The total laser power just prior the prism was about 10 mW. The microscope objective was a Zeiss 40× Plan-Neofluar (oil 1.3 NA). The objective was coupled to the coverslip with immersion oil (type FF, $n = 1.48$, Cargille, Cedar Grove, NJ). Images of the irradiated region through the objective were recorded by an intensified CCD camera (Cascade, Roper Scientific, Trenton, NJ). The detector element (camera) was kept at -35°C . A 488-nm holographic notch filter (Kaiser Optical System, Ann Arbor MI) with an optical density of > 6 was placed between the objective and the CCD camera. The digitization rate of the CCD camera was 1 MHz (16 bits). The DAC (digital-analog converter) setting was set to be 3689. The frame-transfer CCD camera was operated in the external synchronization mode. Exposure timing for the CCD camera and laser shutter was synchronized by a shutter driver/timer (Uniblitz ST132, Vincent Associates, Rochester, NY). The CCD exposure frequency was 5 Hz (0.2 s/frame) unless specified. The exposure time for each frame was 10 ms. A sequence of frames were acquired for each sample via V^{++} software (Roper Scientific). All images were analyzed off-line.

Result and discussion

Previously, we have shown how single-molecule adsorption events can be recorded directly.^{31, 33, 59} In water, the diffusion coefficients of small-molecules are large enough that they stay in the evanescent-field layer (EFL) only for short periods of time.^{24, 27} At the frame rates used (< 10 Hz), the molecules can only be recorded in one frame before they diffuse out of the EFL. So, there is no correlation in the locations of molecule spots among a series of images. For large DNA,³¹ each molecule can be tracked over several frames. If adsorption occurs from a flowing sample, one can actually observe immobilization over a period of hundreds of ms. For very large DNA,³¹ adsorption at one of its ends results in stretching of the molecule in the direction of bulk flow and are readily distinguishable from the freely flowing molecules. Therefore, one can determine the fraction of DNA that are adsorbed at any time from the series of single-molecule images. YOYO-I labeled λ -DNA was employed as a nanoprobe for different functionalized surfaces to elucidate adsorption in

chromatography. While the negatively charged backbone is not adsorbed, the 12-base unpaired ends of this DNA provide exposed purine and pyrimidine groups for adsorption.

Homogeneous organic self-assembled monolayer. Under a constant acetic acid/acetate mass balance and constant ionic strength condition (pH 4-7), the pH effect on the fraction of DNA adsorbed on SAMs of carboxylic acid terminated ($-\text{COOH}$), hydroxyl terminated ($-\text{OH}$), C_{18} (classic material for liquid chromatography) and EG_3OMe surfaces was studied and compared as shown in Figure 2. For actual visualization of adsorbed versus free molecules, please refer to the video files in Supporting Information. Additionally, pH 8.2 (10 mM gly-gly), pH 9.0 and pH 10.0 (50 mM phosphate) buffer solutions were used. In general, almost all DNA molecules were randomly diffusing and moving at the liquid/solid interface at $\text{pH} \geq 8.2$ for all types of monolayers studied. They appeared and disappeared at random locations on successive image frames as they moved in and out of the EFL.

The pH effect on adsorption at different surfaces became more obvious when pH decreased. At lower pH, the amount of negative charge on DNA decreased and at the same time the degree of deprotonation of functional groups like $-\text{COOH}$ also decreased. The electrostatic repulsion between DNA and the monolayer was reduced, giving way to attractive interaction between DNA and the functionalized surface. Hydrophobic interaction also governs the adsorption behavior of DNA in slightly acidic solution. When the amount of negative charge on DNA decreases, DNA is able to get closer and interact with the SAM. All DNA within the EFL adsorbed strongly on $-\text{COOH}$ at $\text{pH} \leq 5.0$. This implies that the surface functional groups were nearly neutral. The pK_a of monolayers with carboxylic-acid-terminated alkylthiol has been reported to be within a wide range (pH 5.2-11.5) in the literature.^{46, 60-67} The adsorption events of DNA in this experiment (Figure 2) indicated that the pK_a for the $-\text{COOH}$ SAM samples was around pH 5-6. Exact measurement of the pK_a is not possible due to the presence of competing hydrophobic interactions.

Comparing the fraction of adsorbed DNA on $-\text{OH}$ to that of the alkyl group (C_{18}), there was a relatively higher fraction of DNA adsorbed to the $-\text{OH}$ SAM surface. This result indicates that interaction through hydrogen bonding formation between the hydroxyl group and pyrimidine or purine at the sticky ends of the DNA molecules also plays an important role. Hydrogen bonding formation favors DNA adsorption by adding to the hydrophobic

forces. It should be noted that small differences in Figure 2 translate to large differences in liquid chromatography because of multiple encounters with the surface.

Mixed organic stationary phases. Experiments designed to compare different homogeneous SAM surfaces must be performed with great care. Variations in the underlying fused-silica substrate or metal layers or in the buffer solution can affect the results. Surface coverage here is already much more complete compared to chromatographic packing materials. Still, by building spatially distinct SAM of organic groups on the same surface, one can reveal slight variations among the different adsorptive forces without interference from other effects. Patterns of $\text{-NH}_2/\text{-COOH}$, $\text{EG}_3\text{OMe}/\text{C}_{18}$ and $\text{-OH}/\text{-COOH}$ were generated by lithography. The square islands ($30\ \mu\text{m} \times 30\ \mu\text{m}$) shown in Figure 3a, 3c and 3d were -NH_2 , EG_3OMe and -OH respectively while the grid region were -COOH or C_{18} . The -NH_2 islands in Figure 3b were circular with $3\ \mu\text{m}$ diameter and separated by $10\ \mu\text{m}$.

Even at basic pH where all DNA are highly negatively charged, we were able to see a clear pattern from $\text{-NH}_2/\text{-COOH}$ and $\text{EG}_3\text{OMe}/\text{C}_{18}$. As shown in Figure 3a and 3b (movies M3a and M3b in Supporting Information), all DNA on -NH_2 islands are strongly adsorbed while those on the -COOH grid moved freely. Even when the -NH_2 islands were small as in Figure 3b, DNA was preferentially adsorbed. Another interesting point is that it was possible to observe DNA stretching out (as marked by an arrow in Fig. 3b). Once one of the sticky ends adsorbed onto the surface, the free end of the DNA was stretched out by bulk solution flow. Occasionally, the other sticky end was able to attached to an adjacent -NH_2 island if the bulk flow was high enough. It should thus be possible to stretch DNA to a reproducible and desired length for other types of studies by matching the island separation and the bulk flow rate. When the bulk flow was not high enough, the sticky end of the DNA stayed at an -NH_2 island while the free end of the DNA was partially stretched out and fluctuates in the flow stream as illustrated in movie M3b in Supporting Information. At pH 8.2, all -COOH groups should be deprotonated and in the -COO^- form. So, electrostatic repulsion is too large for DNA to directly interact with the (negative) surface. However, at pH 8.2, -NH_2 groups may be either neutral or positively charged. The amino group provides a site for hydrogen bonding or simply interacts by electrostatic attraction.

For EG₃OMe/C₁₈, Figure 3c (movie M3c in Supporting Information), DNA interacted stronger with C₁₈ than with EG₃OMe because of the higher hydrophobicity of C₁₈. This further confirms that the driving force for interaction on these two organic surfaces is mainly hydrophobic interaction.

For the other pattern composition, -COOH/-OH, distinct differences in adsorption started showing up at pH ~ 5.0. Under this pH, hydrophobic interaction should be the major driving force governing the adsorption of DNA as discussed above. From Figure 3d (movie M3d in Supporting Information), it is obvious that the majority of DNA preferred to adsorb on -COOH than on the -OH surface. That implies that the attraction between -COOH and DNA is stronger than that between -OH and DNA due to the presence of the carbonyl group. The carbonyl group is more polar than the hydroxyl group and so the highly polar DNA has higher preference for -COOH. This illustrates why LC columns with -COOH and alcohol groups work differently upon separating polar analytes. Furthermore, these results also predict that carboxylic acids will be more retained than alcohols on an amine column.

Surface of quartz with organic islands. For chromatography, columns with modified packings are popular for separating sugars, amino acids, pharmaceuticals, etc. The separation efficiency is heavily dependent on the packing (solid) material, particle size and surface coverage of the modifier. Typically, a significant fraction of the silica surface remains exposed and affects chromatography. As a demonstration, we studied the behavior of DNA probe on the surface of quartz and -COOH SAM. At high pH, both the above surfaces are negatively charged (ionization of silanol and carboxylic acid groups). Since DNA also carries negative charge, no adsorption is expected due to electrostatic repulsion. However, the strength of interaction between analytes and these two surfaces can be very different. Real-time imaging of the dynamic motion of DNA on island of SAM with -COOH group on quartz slides can elucidate these interactions. In Figure 4 (movie M4 in Supporting Information), the darker squares were -COOH islands while the brighter region was fused silica. The darker background for the islands was due to the presence of chromium-gold substrate which reduced the transmission of laser light. The fluorescence intensity from DNA on the -COOH island was also dimmer than those on fused silica. Images were recorded at 10 Hz. From the two highlighted molecules in the figure, DNA over quartz traveled faster by 38 pixels com-

pared to DNA over SAM of -COOH over a period of 2 s. This indicates that DNA interacts stronger with -COOH than with silanol, presumably due to the presence of the carbonyl group. Such results provide insights into the relative retention behavior over different stationary phases and why the extent of surface coverage of packings is important for effective separation.

Metal-oxide stationary phases. Mixed oxide surfaces are not only important for fine-tuning hydrogen bonding interactions, but are also relevant to catalytic activity. As a demonstration, we have created islands of gold on top of a grid of chromium. In the optical image in Figure 5a, the bright squares were gold islands ($30\text{ }\mu\text{m} \times 30\text{ }\mu\text{m} \times 20\text{ nm}$) while the dark region was chromium (1-nm thick). After the patterned substrate was exposed to air for a few hours, YOYO-labeled λ -DNA solution at pH 6.0 was pipetted onto the surface and fluorescence images were taken as shown in Figure 5b. The DNA molecules were essentially randomly distributed on the surface. Almost all the DNA moved around from frame to frame and were not adsorbed permanently. Upon further oxidation of the chromium surface, the fluorescence image shown in Figure 5c was obtained. A faint pattern of the preferentially adsorbed DNA molecules is clearly visible. Upon extensive oxidation, adsorption on the chromium layer became even more pronounced as depicted in Figure 5d. In all cases, DNA continued to interact only weakly with the gold surface. This is because gold was not oxidized throughout and thus did not offer sites for hydrogen bonding. Figure 5 shows the oxidation kinetics of chromium in a typical laboratory setting.

Alumina (aluminum oxide) is one of the most common solid adsorbents (stationary phase). It is widely applied to the separation of aromatics and sulfur-containing aromatics because of its Lewis acidity. The strength of adsorption depends on the polarity of the analytes involved. The more polar analytes, the stronger they are adsorbed. We studied the real-time dynamics of adsorption/desorption of DNA on 3-nm aluminum-coated surface (data not shown). Since aluminum was oxidized by oxygen rapidly to form alumina, the surface became highly polar. In contrast to the chromium-coated surface in Figure 5, at pH 8.2, all DNA adsorbed strongly on the aluminum-coated surface which had been exposed to air for about half an hour. Upon further air oxidation, there was no significant increase in the coverage of adsorbed DNA because the oxide layer had already been completely formed in 0.5 h.

Strong adsorption of DNA to alumina was due to the extensive Lewis-base nature of phosphate, pyrimidine and purine on the DNA molecules that interacted strongly with the Lewis acid, alumina. Similar studies can potentially allow probing of mixed oxide systems that are important as surface catalysts.

Conclusion

We demonstrated the manipulation of DNA as a probe of surface functionality. By finely adjusting the pH of the solution, we can observe the subtle differences in the affinity for different surfaces directly and simultaneously. Moreover, real-time imaging of molecule dynamics offers us valuable insights into the fundamental interactions governing separation mechanisms in chromatography.

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Figure 1. Experimental configuration for observing single-molecule interactions with SAM surfaces.

Figure 2. pH effect on the fraction of DNA adsorbed on various SAMs. The ratio of immobilized vs. freely moving DNA in the video files in Supporting Information is plotted.

Figure 3. DNA adsorption on patterns of (a) and (b) -NH_2 (island)/ -COOH (grid) at pH 8.2; (c) EG_3OMe (island)/ C_{18} (grid) at pH 8.2; and (d) -OH (island)/ -COOH (grid), at pH 5.0. The islands are $30\ \mu\text{m} \times 30\ \mu\text{m}$ separated by channels of $10\ \mu\text{m}$, except in (b) where the islands are $3\ \mu\text{m}$ circles. The arrow shows a stretched DNA.

Figure 4. DNA adsorption on $-\text{COOH}$ /quartz pattern. DNA stays longer on $-\text{COOH}$ island (dimmer region) than on quartz grid (brighter region) at pH 8.2. The distance they traveled (upwards) under bulk solution flow from (a) to (b) is different by 38 pixels in 2.0 s.

Figure 5. DNA molecules adsorbed with increased affinity as the surface was oxidized. (a) Optical image of gold islands on a chromium grid; (b) DNA adsorption after 4 h of preparation; (c) DNA adsorption after 1 day; and (d) DNA adsorption after 5 days.

CHAPTER 5. SINGLE-MOLECULE DYNAMICS OF CONFORMATIONAL CHANGES IN FLAVIN ADENINE DINUCLEOTIDE

A paper published in *Journal of Photochemistry and Photobiology A* ¹

Hung-Wing Li and Edward S. Yeung

Abstract

Dynamic conformational changes of the prosthetic group, flavin adenine dinucleotide (FAD), in flavoprotein NADH peroxidase (Npx), in thioredoxin reductase (TrxR), and in free solution were monitored with total internal reflection fluorescence microscopy. FAD bound loosely in the proteins changed from the stacked conformation to the unstacked conformation upon laser excitation. On the other hand, our results show that FAD in free solution not only underwent conformational changes but also reacted with each other to form a dimer.

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Introduction

Flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) are essential cofactors for the biological activity of many proteins. They play an important role in catalysis and electron transfer. Flavoproteins are often critical to electron transport pathways in living systems like photosynthesis, respiration, nitrogen fixation, DNA repair, metabolism, and signal transduction¹⁻¹². They are an interesting family of enzymes for studying the dynamic behavior of biomacromolecules. It is well known that the flavin cofactors make these enzymes suitable for the direct observation of conformational dynamics in catalysis. These prosthetic groups are not only auto-fluorescent, but the redox-active groups are also located in the integral part of the active site. Fluorescence anisotropy and time-resolved fluorescence have been used to study the dynamics of the active site of various flavoproteins^{13,14}.

Flavin cofactors are non-covalently bound to flavoproteins. However, some are bound tightly while some are bound rather loosely to the protein. It is known that one of the flavoprotein called thioredoxin reductase (TrxR) has tightly bound FAD molecules as cofactors¹⁵. In other flavoenzymes like ferredoxin NADPH-reductase (FNR), the prosthetic groups are less tightly held¹⁶. Will the tightness of the binding affect the dynamics of conformational changes? Will the equilibrium of the conformational change shift upon the exposure to laser light? By direct observation of the dynamics of single-protein and single-cofactor molecules, one could address the above questions.

It has been proposed that an intramolecular ground-state complex between the isoalloxazine ring and the adenine moiety exists primarily in aqueous solution, resulting in the formation of a nonfluorescent complex¹⁷. The quantum yield of FAD (0.03) was found to be 9 times lower than that of FMN (0.27) in steady-state fluorescence experiments. However, after enzymatic digestion of the diphosphate bridge of the FAD molecule, the fluorescent intensity of FAD was found to be the same as that of FMN. For this reason, FAD was assumed to exist in two conformations. One of the conformations is a "closed" or stacked conformation in which the isoalloxazine and adenine rings interact through π - π interactions in a stacked geometry that resulted in efficient fluorescence quenching. Another conformation is an "open" or unstacked, extended conformation that is responsible for the FAD fluorescence. In solution, at least 80% of FAD are in the closed conformation. Although it is generally ac-

cepted that the open and closed conformations of the FAD molecules exist, little is known about the conformational dynamics in solution, or the biological implications of such a change.^{18,19}

In this study, we demonstrate a sensitive, fast and simple method for recording the real-time dynamics of conformation changes of the cofactors in free solution and when bound to proteins. We report the dynamics of NADH peroxidase (Npx), TrxR, FMN and FAD in free solution at various pH with the aid of total internal reflection fluorescence microscopy.

Experimental Section

Buffer Solutions. Phosphate buffer solutions of pH 7.5-10.0 were prepared with ultra-pure 18-M Ω water. All the solutions were photobleached under a mercury lamp overnight and were filtered through a 0.2- μ m filter prior to use.

Preparation of Samples. NADH peroxidase (Npx), thioredoxin reductase (TrxR), flavin adenine dinucleotide (FAD, Figure 1), and flavin mononucleotide (FMN) were obtained from Sigma Chemical Co (St. Louis, MO). All samples were used without further purification. Npx and TrxR were derived from *Streptococcus faecalis* ATCC 11700 and *Escherichia coli* respectively. FAD and FMN were of purity not less than 95%. All samples were prepared fresh just prior to the single-molecule imaging experiments. Npx was diluted to 30 nM with appropriate buffer solutions. TrxR, FAD and FMN were diluted to 47 nM, 50 nM and 10 nM respectively with pH 10.0 phosphate buffer solution.

Evanescent Wave Excitation Geometry. The instrumental setup was similar to that previously described²⁰⁻²³. 5 μ L of sample solution was sandwiched between a No. 1 (170 μ m thick and 22 mm square) Corning glass cover slip (Corning, NY) and the hypotenuse face of a right-angle fused-silica prism (Edmund Scientific, Barrington, NJ). A laser beam was focused and directed through the prism to the prism-sample interface. The angle of incidence was about 66°. The laser beam was totally internally reflected at the prism/solution interface, and an evanescent field of $\sim$ 100 nm thick was created. Fluorescent molecules within this field can be excited and imaged.

Single-Molecule Detection System. The excitation source was an argon-ion laser

(Innova-90, Coherent, Santa Clara, CA) operated at 488 nm. Extraneous light and plasma lines from the laser were eliminated with an equilateral prism and a pinhole prior to its entrance to the observation region. The total laser power just before the prism was about 15 mW. The power density at the $80 \times 60 \mu\text{m}^2$ excitation area is 300 W/cm^2 .

Microscope and ICCD Camera. The microscope objective was a Zeiss 40 $\times$ Plan-Neofluar (oil 1.3 NA). The objective was coupled to the cover slip with immersion oil (type FF, $n = 1.46$, Cargille, Cedar Grove, NJ). Images of the irradiated region through the objective were recorded by an electron amplifying CCD camera (Cascade, Roper Scientific, Trenton, NJ). The detector element (camera) was kept at -35°C . A 488-nm holographic notch filter (Kaiser Optical System, Ann Arbor MI) with an optical density of > 6 was placed between the objective and the CCD camera. The digitization rate of the CCD camera was 1 MHz (16 bits). The DAC (digital-analog converter) setting was set at 3689. The frame-transfer CCD camera was operated in the external synchronization mode. Exposure timing for the CCD camera and the laser shutter was synchronized by a shutter driver/timer (Uniblitz ST132, Vincent Associates, Rochester, NY).

Image Acquisition and Analysis. The CCD exposure frequency was 2 Hz (0.5 s/frame) for all the sample images. The exposure time for each frame was 20 ms for Npx and TrxR, and 5 ms and 4 ms for free FAD and FMN respectively, unless otherwise specified. Sequences of frames were acquired for each sample via V^{++} software (Roper Scientific, Trenton, NJ). All frames were analyzed off-line.

Results and Discussion

Npx and TrxR. Like other proteins, Npx tended to adsorb onto the surface of fused silica due to its hydrophobic nature when the pH is close to or below the pI. An image of the adsorbed molecules is shown in Figure 2. The spot sizes do not reflect the physical sizes of the molecules. Rather, they represent the integrated trajectory of x-y motion during exposure. Almost all of the molecules adsorbed onto the fused-silica surface at pH 7.5 while about half of them adsorbed at pH 8.5. However, at higher pH (8.8-10.0), the protein molecules no longer adsorbed to the surface but diffused around. They appeared and disappeared at random locations on successive image frames as they moved in and out of the evanescent field

layer (EFL). At high pH, both the protein molecules and the fused-silica surface are negatively charged due to deprotonation of the protein and the silanol groups on the surface. So, electrostatic repulsion between the protein molecules and the surface minimized the adsorption events.

The number of molecules observed within the evanescent field layer at various pH is shown in Figure 3. Fewer and fewer protein molecules diffused into the EFL as pH increased due to the stronger electrostatic repulsion between the protein molecules and the fused-silica surface. Interestingly, at $\text{pH} > 8.8$, the spot number from the images increased from frame to frame as shown in Figure 4. The increase in spot number leveled off after a few frames. However, this phenomenon did not occur for another flavoprotein, thioredoxin reductase (TrxR). In fact, the number of TrxR molecules decreased from frame to frame due to photobleaching of the protein molecules (Figure 5).

The increase in spot number in the Npx images from frame to frame can be explained by the assertion that the co-factor FAD in the protein molecule underwent a dynamic of conformational change from the "closed" to the "open" conformation when they were exposed to light. Since the "open" conformation is nine times more fluorescent than the closed one, all of the observed spots were attributed to FAD with an open structure. That is, the fluorescent intensity of a closed structure was too weak to be recorded under the same conditions. This phenomenon did not occur in the enzyme TrxR because there should not be any conformational change for FAD in TrxR. The flavin-cofactor is less tightly bound to Npx compared to TrxR. Also, the flavin in TrxR is bound in an almost completely extended conformation, as indicated by x-ray crystallography.

FAD and FMN. Images of the native fluorescent prosthetic groups, FAD and FMN were taken at pH 10.0, where adsorption is negligible. The observed fluorescence intensity of FAD molecules was found to be higher than those of Npx and TrxR molecules. Similar to Npx, the number of FAD molecules in the images increased from frame to frame upon exposure to laser light, as shown in Figure 6. Furthermore, it is evident from Figures 7 and 8 that the rate of increase depended on the laser exposure time and the concentration of the FAD molecules. Under identical concentrations, the longer the exposure time or the higher the laser power, the more the number of molecule spots and the higher the rate of increase (Figure

7). Additionally, under identical exposure times and laser powers, the higher the concentration, the higher the rate of increase in molecule spots (Figure 8). Over the range of conditions studied, the rate of increase in observed molecule number was proportional to the square of the exposure time and the square of the FAD concentration.

Figures 7 and 8 indicate that a photochemical reaction is involved. A second order reaction describes the dependence on the number of supplied photons and the number of FAD molecules. This implies that not only did the opening of the closed FAD conformation require a photon, but also another photoproduct was formed. A possible mechanism is that upon absorbing one photon each, FAD molecules were excited to an open conformation. Then, these excited molecules reacted with other FAD molecules to form a stable dimer that had an even higher quantum yield.

Native fluorescence images of FMN at pH 10.0 were also recorded. Under the same conditions, the fluorescence intensity of FMN molecules was weaker than those of the dimeric product of FAD described above. Also, there was no increase in the molecule numbers monitored from frame to frame. This indicated that there was no conformational change and no photoreaction for FMN. The presence of the adenine ring in FAD was likely responsible for the dimer formation, instead of the isoalloxazine ring.

In order to compare single-molecule fluorescence observations from FAD with ensemble-average measurements, 2 mL solutions of FAD with concentrations of 1-20 μM in a 1-cm cuvette were exposed to a 488-nm laser at $\sim 15 \text{ mW/cm}^2$. The top half of the rectangular cuvette was exposed to the laser while the bottom half was not irradiated. The solution was constantly stirred with a micro magnetic bar to ensure all molecules in the solution were evenly exposed. Emission spectra were taken at regular intervals by a fluorimeter (Perkin Elmer Luminescence Spectrometer LS50B). The relative fluorescence intensity excited at 488 nm at the maximum emission wavelength, 515 nm, was plotted against the total exposure time as shown in Figure 9. We found that the fluorescence intensity from FAD increased with total laser exposure and leveled off at times longer than 15 min. The emission spectrum of FAD did not change throughout the prolonged laser exposure. The fluorescence intensities also did not change upon removing the cuvette from the laser source and storing at room temperature at dark condition. So, the photoproduct was stable physiochemically.

Figure 9 shows that the relative intensity increase did not follow a simple relationship with FAD concentration and total exposure time. From 1 μM to 4 μM , doubling the concentration caused 2.5- to 2.8-fold increases in fluorescence intensity. At higher concentrations, the intensity increased at a lower rate. This did not agree with the results for SMD as discussed above (quadratic dependence). This discrepancy can be attributed to the facts that molecules in bulk solution at concentrations in the μM range (in order to be detectable) underwent self-absorption and that molecules were being photobleached continuously. The absorbance of a 20- μM FAD solution was 0.066 and 0.179 at 488 nm and 440 nm (absorption maximum) respectively. Having such a high value of absorbance, FAD was affected by self-absorption and in turn caused a lower apparent fluorescence intensity.

To evaluate the contribution of self-absorption, a thinner cell (2 mm pathlength) for laser irradiation and spectrometric measurement was used instead of the 10-mm cell. The fluorescence intensity of FAD increased initially linearly over a large range of concentrations, 0.75-30 μM . Based on the above absorption data, self-absorption is negligible with the shorter pathlength. All these solutions were exposed to 488-nm laser for 3 min continuously without stirring. The laser beam was expanded to cover the entire solution simultaneously. The changes in fluorescence emission intensity (as depicted in Table 1) were different from those discussed above. The emission intensity decreased slightly for low concentrations, increased slightly at medium concentrations, and dropped again for higher concentrations. This behavior can be attributed to photobleaching of the photoproduct. Unlike the above experiments in a 1-cm cuvette, the lack of excess solution confined all the molecules in the laser beam region. Even though the highly fluorescent photoproduct was formed, it was not able to move away from the irradiated region and was easily photobleached within the irradiation time of 3 min. The net result is that the intensity increased due to photoproduct formation and decreased from photobleaching, thereby counteracting each other and no obvious changes in emission could be seen. We note that these details about the reaction were not observable in bulk fluorescence measurements. But, in single-molecule measurements, self-absorption is eliminated because of the much lower concentration. Photobleaching is also less of a problem because molecules are recorded and thus counted well before photobleaching. Finally, molecule counting provides proper kinetics information because it is independent of the intensity

of the spots.

Conclusions

We demonstrated a sensitive, fast and simple technique for monitoring the real-time dynamics of the photoproduct formation between FAD molecules. Insight is obtained regarding how tightly the FAD cofactor is bound to a flavoprotein. If FAD is bound tightly, conformational changes are restricted and the number of molecules that could be observed should remain constant. However, if the FAD molecules are loosely bound to the protein, conformational changes from the closed structure to an open structure would be allowed and hence brighter spots could be detected due to the higher quantum yield of the open structure. The observed kinetics point to the formation of a dimer on excitation. Our results indicate that single-molecule experiments are necessary to eliminate artifacts arising from self-absorption or fluorescence quenching.

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Table 1. Relative emission intensity of FAD solution at various concentrations before and after 488 nm laser exposure.

Concentration	0.75 μM	1.5 μM	3 μM	6 μM	12 μM	30 μM
t = 0	3.5	7.3	11.7	23.3	45.8	105.4
t = 3 min	3.4	6.8	11.9	23.5	42.9	100.0

Figure 1. Structure of FADH₂.

Figure 2. Native fluorescence images of Npx in pH 7.5 50 mM phosphate buffer. Excitation was at 488 nm and 300 W/cm². Exposure time was 20 ms at 2 Hz. Each spot registers a fluorescent molecule.

Figure 3. Plot of number of molecules of Npx observed in the first frame at various pH. Other conditions are as in Figure 2.

Figure 4. Native fluorescence images of Npx at pH 10.0 for two irradiation periods. Other conditions are as in Figure 2.

Figure 5. Native fluorescence images of TrxR at pH 10.0 for two irradiation periods. Other conditions are as in Figure 2.

Figure 6. Native fluorescence images of FAD at pH 10.0 for two irradiation periods. Other conditions are as in Figure 2. The number of molecules recorded increased with laser irradiation.

Figure 7. Molecule numbers of FAD (50 nM) at pH 10.0 as a function of irradiation (different exposure times). The excitation wavelength was 488 nm at 300 W/cm². Data was acquired at 3 ms (◆), 5 ms (■) and 7 ms (▲) exposure times at 2 Hz.

Figure 8. Molecule numbers of FAD at various concentrations at pH 10.0 as a function of irradiation. FAD concentration was 50 nM (◆), 25 nM (■) and 12.5 nM (▲). The excitation wavelength was 488 nm at 300 W/cm². Data was acquired at 5 ms exposure time at 2 Hz.

Figure 9. Bulk fluorescence experiments for FAD after irradiation with a 1 cm^2 488-nm laser at 15 mW for various durations. The concentrations ranged from $1\text{ }\mu\text{M}$ to $20\text{ }\mu\text{M}$.

CHAPTER 6. DIRECT OBSERVATION OF ANOMALOUS SINGLE-MOLECULE ENZYME KINETICS

A paper published in *Analytical Chemistry*¹

Hung-Wing Li and Edward S. Yeung

Abstract

We report the direct measurement of the single-molecule enzymatic cleavage rates of Apal-DNA complex in the presence of various concentrations of MgCl_2 solution with total internal reflection fluorescence microscopy (TIRFM). We made use of the native adsorption properties of the two 12-base sticky ends of the DNA molecules to partially immobilize and stretch out the Apal-DNA complex onto a glass surface. Synchronous initiation of reaction was achieved by the influx of Mg^{2+} solution. Once the DNA was cut, the two fragments (38 kb and 10 kb) would either collapse or further stretch out depending on the solution flow. The time required for cleaving each Apal- λ -DNA complex was recorded and analyzed. At low concentrations, the higher the concentration of Mg^{2+} , the faster the DNA was cut. However, Mg^{2+} ion is no longer the limiting factor when its concentration is greater than 5 mM. A surprising result is that at all concentrations the decrease in intact DNA population as a function of time is linear rather than exponential. This suggests that there exists a distribution of Apal conformations around the restriction site.

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Introduction

Direct observation of single molecules in solution has been possible for many years. Time-averaged single-molecule enzyme kinetics has been obtained by incubation to create multiple copies of products.¹⁻³ Temporal correlation of individual turnover events at a single site has been recorded based on fluorescence intensity changes.⁴⁻⁸ However, the measured kinetics is often diffusion limited and thus not particularly informative,^{9, 10} or reflects the convolution of several elementary reaction steps and thus complex.⁷ Furthermore, comparison with ensemble-averaged bulk measurements is not straightforward, as ergodicity may have to be assumed.³ Ideally, one should measure the rates of many single transformations of many single molecules. To do so, one must control the position of every reactant and every product molecule and must synchronize the start of multiple single-molecule reactions.

Single-molecule imaging can provide detailed dynamic information with high throughput.¹¹ One needs to partially immobilized the molecules in order to monitor them over long times. DNA can be extended by solution flow or by spin-coating on 3-aminopropyltriethoxysilinated¹² or poly-lysine-coated glass substrate,^{13, 14} by dielectrophoresis,¹⁵ and by optical trapping or by magnetic tweezers assisted by microspheres conjugated to DNA.¹⁶⁻²³ Schwartz and co-workers^{24, 25} have used restriction enzymes to recognize and cleave single stretched DNA molecules at sequence-specific sites, even when the target DNA was immobilized and intercalated.

Restriction endonucleases are ideal candidates for studying the structural and dynamical details of biochemical reactions. The bulk activity is defined by the Michaelis-Menten model, which gives the average value of an ensemble of enzyme and substrate molecules. *Apal* is a site-specific endonuclease that recognizes the hexanucleotide sequence 5'GGGCCC3' through hydrogen bonds in the major groove of duplex DNA.²⁶⁻²⁸ Cleavage of the sole restriction site in λ -DNA produces two fragments (38 412 bp and 10 090 bp). Site recognition is possible even in the absence of Mg^{2+} , but Mg^{2+} is required as a cofactor to catalyze the cleavage of the phosphodiester bonds.¹³ Here, we monitor the real-time kinetics of the elementary reaction of λ -DNA cleavage by the restriction enzyme *Apal* upon activation by Mg^{2+} .

Experimental Section

Preparation of Samples. λ -DNA (48 502 bp) was obtained from Promega (Madison, WI). Restriction enzyme, ApaI, was obtained from Invitrogen (Carlsbad, CA). The ApaI was fluorescently labeled with Alexa Fluor 532 protein-labeling dye from Molecular Probes (Eugene, OR) and purified according to the manufacturer's instructions. 20 μ L of labeled ApaI was mixed with 0.25 μ g λ -DNA and incubated for 3 h at room temperature. All reaction buffers were free of magnesium ions. Prior to the single-molecule imaging experiments, this ApaI-DNA sample was labeled with YOYO-I (Molecular Probes) at the ratio of one dye molecules per five base pairs and then further diluted by twenty-fold (~ 20 pM) with pH 5.5 25 mM acetate buffer solution.

Evanescent-Wave Excitation Geometry. The instrumental setup was similar to that previously described.²⁹ 4 μ L of sample solution was sandwiched between two No. 1 (~ 170 μ m thick and 22 mm square) Corning glass coverslips and coupled onto the hypotenuse face of a right-angle glass prism via index-matching oil. The excitation source was an argon-ion laser (Innova-90, Coherent, Santa Clara, CA) operated at 488 nm. Extraneous light and plasma lines from the laser were eliminated with an equilateral prism and a pinhole prior to its entrance to the observation region. The total laser power at the prism was about 5 mW. The laser beam was focused and directed through the prism to the prism-sample interface. The angle of incidence was about 66°. The laser beam was totally internally reflected at the glass/solution interface, and an evanescent field of ~ 100 nm thickness was created.

Single-Molecule Detection System. The microscope objective was a Zeiss 40 $\times$ Plan-Neofluar (oil 1.3 NA). Images of the irradiated region through the objective were recorded by an intensified CCD camera (Cascade, Roper Scientific, Trenton, NJ). The detector element (camera) was kept at -35 °C. A 488-nm holographic notch filter (Kaiser Optical System, Ann Arbor, MI) with an optical density of > 6 was placed between the objective and the ICCD camera. The digitization rate of the ICCD camera was 1 MHz (16 bits). The ADC (analog/digital converter) setting was 3689. The frame-transfer camera was operated in the external synchronization mode. Exposure timing for the ICCD camera and the laser shutter was synchronized by a shutter driver/timer (Uniblitz ST132, Vincent Associates, Rochester, NY). For DNA digestion, the camera exposure frequency was 5 Hz (0.2 s/frame) for 25 mM and 5

mM Mg^{2+} , and 2.5 Hz (0.4 s/frame) for 1 mM Mg^{2+} , respectively. The exposure time for each frame was 5 ms. A sequence of frames was acquired for each sample via V⁺⁺ software (Roper Scientific). All frames were analyzed off-line.

Results and Discussion

λ -DNA has 12 unpaired bases at each end. By carefully adjusting the pH and ionic strength to balance the electrostatic and hydrophobic forces, each molecule was stretched out under moderate flow when one or both of its ends adsorbed onto the glass surface²⁹ while the rest of the backbone remained free. Our buffer solution provided suitable conditions for adsorption without polymer coating or other treatment of the surface.

Alexa Fluor 532 (AF) protein-labeling dye binds to primary amines on the enzyme. With dye labeling, this enzyme still shows DNA cutting activity as confirmed by gel electrophoresis. We incubated AF-ApaI and native λ -DNA in Mg^{2+} -free buffer prior to DNA labeling, stretching and imaging. YOYO-I dye was used to label DNA instead of SYBR-green⁹ because of its higher quantum yield and photostability. Not all of the AF-ApaI molecules were visible under the laser power and exposure time employed. Only those that were bound to DNA were readily seen because energy transfer could occur between the two dyes. The effect of YOYO-I on DNA digestion is known.³⁰ Only at very high concentrations of YOYO-I (≥ 1 dye:1 bp) is DNA digestion inhibited due to conformational changes. Here, the dye:bp ratio was 1:5 and no reaction inhibition was observed.

Site-specific binding was observed when AF-ApaI was incubated with λ -DNA (Figure 1). As expected, the position of each bound enzyme molecule was about one-fifth of the length of the stretched DNA. Non-specific binding, multiple binding, or unbound AF-ApaI molecules were not observed. During incubation, the enzyme first binds DNA randomly and then slides along until it reaches a specific sequence.³¹⁻³³ The nonspecifically bound proteins have fast dissociation kinetics.¹³ Having a free DNA backbone and having an untreated surface allow the non-specifically bound enzyme to slide or leave as in bulk solution.

We found that DNA molecules were cleaved nonspecifically into a few fragments upon prolonged exposure at >10 mW laser power, presumably due to photo-generated oxygen. Figure 2 shows such an event. So, one has to spend the "photon budget" from each

molecule wisely. Exposure and image acquisition rates were optimized depending on the kinetics to avoid photobleaching or photocleavage. For reactions that are faster than those here, the laser power can be increased in proportion to the frame rate while the exposure time is decreased, and vice versa.

ApaI-DNA solution was sandwiched between two coverslips and placed on a glass prism mounted on the microscope. Once DNA molecules were immobilized and bulk flow from the initial manipulation has ceased, 2 μ L MgCl_2 solution was added to the edge of the coverslip to infuse Mg^{2+} into the observation region via capillary force (frame 2 of Figure 3, see also movie file in Supporting Information). Bulk flow caused the ApaI-DNA complex to stretch out, marking the start time of the reaction. Since the flow is by capillary action and not hydrodynamic force, the velocity is actually highest at the surface. Besides, we can see the whole DNA starting to stretch and wiggle while being pinned at one end (in the movie after 0.2 s), indicating that the flow rate at the enzyme site is high, not low. In separate experiments, we confirmed with a dye solution that the delivery of Mg^{2+} in this manner was fast (< 1 s) compared to the measured reaction times.

As soon as restriction cleavage took place, one or both of the fragments would collapse and recoil (left fragment in Figure 3) or stretch out (right fragment in Figure 3) depending on the direction of bulk flow and the point of adsorption. Measuring reaction times in this way is more reliable than looking for gap formation within fully immobilized DNA molecules.^{10, 24} The recoil occurs in less than one frame, so the recoil rate does not influence the measured cleavage times. Our movies do occasionally show the cut end adhering to the surface. However, it is more likely that the enzyme adsorbs rather than the cut end. Besides, we only need one fragment to recoil to recognize a cleavage event. Having both cut ends adsorb back on the surface while the strand is wiggling away from the surface is highly improbable. This feature is actually what distinguishes our experiments from those of others,^{{Namasivayam, 2003 #2493}{Wang, 1995 #2481}} where the DNA molecules are at the surface and are readily reattached to the surface after cutting to escape detection of cleavage.

Clearly, the cleavage of DNA molecules was a stochastic process that occurred in seconds. The time taken to cut each DNA molecule with ApaI was recorded (Figure 4) and the fraction of all molecules cleaved as a function of time was plotted in Figure 5. The cleavage

reaction was found to be highly dependent on the concentration of Mg^{2+} . The kinetics for the production of free DNA from the ApaI-DNA complex can be modeled by:

The conversion of enzyme-bound substrate (ES) to enzyme-bound product (EP) is governed by phosphodiester hydrolysis (k_h). Dissociation of the enzyme-bound product yields free enzyme and cleaved DNA (k_r). Since the influx of Mg^{2+} is rapid and the enzyme was pre-attached to the DNA, diffusion rates for the reactants in solution^{9, 10} and the rate for site-specific binding can be neglected. Unique to this system is that recoil of the DNA strand eliminates the reverse reaction entirely. Therefore,

$$k_{\text{cat}} = (k_h k_r) / (k_h + k_r)$$

The times recorded here for the cleavage of single molecules after Mg^{2+} introduction (Figure 4) are equivalent to $1/k_{\text{cat}}$ for population-averaged observations. The apparent rate (slope in Figure 5) for 5 mM Mg^{2+} was about 5 $\times$ of that for 1 mM Mg^{2+} . However, the rate for 25 mM Mg^{2+} was only 1.5 $\times$ that for 5 mM Mg^{2+} . Therefore, the single-molecule reactions here followed the same general trend as ensemble-averaged macroscopic measurements. Initially, the larger the number of Mg^{2+} present, the faster was the rate, reflecting the role of DNA-bound Mg^{2+} in generating the attacking hydroxide ion.³⁴ When DNA became saturated with the cofactor, the overall turnover rate became Mg^{2+} independent.

What is surprising is that the plots in Figure 5 are linear rather than exponential. If all molecules started from the same initial state ES they should have identical probabilities for undergoing cleavage to EP. One would then expect an exponential decay of the population of intact molecules over time.⁶⁻⁸ The absence of exponential behavior indicates the presence of multiple initial states even though specific binding was confirmed prior to the infusion of Mg^{2+} . Such would be the case if the specifically bound enzyme molecule assumed multiple conformations^{1, 4} around the restriction site in the DNA strand. This diversity is independent of Mg^{2+} , as all plots in Figure 5 are linear. Our results thus underscore the value of single-molecule studies, where exquisite control of the reaction led to the revelation of anomalous kinetics.

For these experiments, the DNA molecules are attached only at the unpaired ends to the surface, the rest of the molecule, including the enzyme site, is well away from the surface as can be seen from the wiggling motion in the movie (Supporting Information) under bulk flow. Thus, surface effects are negligible. We have separately performed DNA cleavage studies using unlabeled *ApaI* (data not shown). This is possible because we know that the incubation times used are sufficient to ensure complete and site-specific binding. There was no problem using the native enzyme because we can confirm specific cutting by the relative fragment lengths of the two cut pieces. We obtained the same linear curves as in Figure 5. Thus, labeling of the enzyme did not influence the kinetics. In many ways this is expected, since the labels go on the outside of the protein and not the shielded active site. Furthermore, we labeled the DNA after incubation for site-specific binding with the enzyme. Therefore, the site recognized by the enzyme is shielded from YOYO throughout the cleavage experiments. Besides, the labeling was at a level well below 1:5 bp in all studies. The high affinity of YOYO to DNA also means that there is no exchange or loss of this label to affect the kinetics.

In additional experiments (results not shown), the cleavage rate in pH 7.4 Tris-HCl buffer was found to be about the same as that in pH 5.5 acetate buffer. Also, these experimental results were reproducible with different batches of enzyme, lower laser powers (3 mW), shorter exposure times (3 ms), or lower image acquisition rates (2 Hz).

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Supporting Information Available: Included is a video file (AVI) of Figure 3. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Figure 1. Specific binding of labeled ApaI at the restriction site of single YOYO-labeled λ -DNA molecules. The normalized position is 0.21.

Figure 2. λ -DNA before (a) and after (b) photocleavage with prolonged laser exposure. Arrows show the randomly located cleavage sites.

Figure 3. Time sequence of enzymatic cleavage of single ApaI-DNA complexes. The two DNA molecules were cleaved at different times. The bottom fragment in each case recoiled after cleavage and continued to show up as bright spots. The top fragment on the left was washed away after cleavage. The top fragment on the right continued to adsorb at the cleavage site and was stretched out by bulk flow.

Figure 4. Frequency distribution of reaction times required to cleave each DNA molecule that was already bound to ApaI. Reactions were initiated upon introduction of Mg^{2+} cofactor at concentrations of 25 mM, 5 mM and 1 mM.

Figure 5. Normalized fraction of DNA molecule cleaved as a function of time at different concentrations of magnesium ion ($\diamond$ 5 mM, $\blacktriangle$ 25mM, $\bullet$ 1mM). Data are derived from Figure 4.

CHAPTER 7. GENERAL CONCLUSIONS

The direct observation of individual molecules moving in free solution can provide quantitative measurements of diffusion and interaction times for adsorption/desorption at the liquid/solid interface as well as unexpected forces acting on the molecules. We have been able to use these insights to explain bulk chromatographic processes and to design new separation modes for particles and large molecules. After all, molecules represent the finest nanoscale probe of solution environments.

In the last couple of decades, optical studies on single molecules have raised great hopes, not only in the biosciences, but also in physical chemistry and materials science, because of its unaveraged microscopic information, with minimal contact or perturbation of the system under study.

Different measurements methods have been adopted by researchers over the world in single molecules spectroscopy (SMS) to obtain more insight into the systems with improved detection limits, such as near-field scanning optical microscope, fluorescence correlation spectroscopy, TIRFM, etc.

In this dissertation, only a part of the work of single molecule imaging done in our group is presented. We hope that in the coming years this subject will pave its way to the understanding of different chemical and biological systems at the microscopic level and find a large number of applications in different fields.

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